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This issue is dedicated to the memory of the late Professor Włodzimierz Mozołowski whose deep concern for people and science will be ever remembered by all who knew him. The papers included were contributed by biochemists from the laboratories headed by Professor's friends and disciples.

ACTA BIOCHIMICA POLONICA
Vol. 23 1976 No. 2—3

J. ŚWIERCZYŃSKI, Z. ALEKSANDROWICZ and M. ŻYDOWO

INHIBITION OF PYRUVATE OXIDATION IN SKELETAL MUSCLE MITOCHONDRIA BY PHENYLPYRUVATE*

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1. Phenylpyruvate inhibits pyruvate *plus* malate oxidation in human and rat skeletal muscle mitochondria in state 3 and in the uncoupled state, it has, however, no effect in state 4.

2. Inhibition by phenylpyruvate of pyruvate oxidation by intact uncoupled rat muscle mitochondria was competitive, with the K_i value about 0.18 mM.

3. It is suggested that the inhibition of pyruvate oxidation is due to the action of phenylpyruvate on muscle pyruvate dehydrogenase, and is the principal cause of the elevated concentration of pyruvate and lactate in blood plasma of phenylketonuric patients.

The primary defect in phenylketonuria, an inborn error of phenylalanine metabolism, is the absence of hepatic phenylalanine hydroxylase (Jervis, 1953). In untreated phenylketonuric patients, this defect results in elevated concentration of phenylalanine and its derivatives: phenylpyruvate, phenyllactate and phenylacetate, in plasma and tissues (Jervis & Drejza, 1966). Sutnick *et al.* (1972) showed that concomitantly in the plasma of patients with untreated phenylketonuria the level of pyruvate and lactate is also raised, with a simultaneous decrease in concentration of glucose. These observations suggest that abnormal pyruvate metabolism is one of the aspects of phenylketonuria. It has been reported previously that phenylpyruvate inhibits pyruvate oxidation by brain homogenates (Bowden & McArthur, 1972) and by brain and heart mitochondria (Gallagher, 1969; Land & Clark, 1974; Halestrap *et al.*, 1974). In these mitochondria, inhibition of pyruvate oxidation is probably due to inhibition of pyruvate transport, as no effect of phenylpyruvate on pyruvate dehydrogenase has been observed. Phenylpyruvate was also shown to inhibit pyruvate carboxylase from rat brain (Patel, 1972) and chicken liver (Scrutton *et al.*, 1969).

* This work was supported by the Polish Academy of Sciences within the project II.1.2.6.

As skeletal muscle is in animal organism the largest organ metabolizing actively carbohydrates, one may suppose that the inhibition of pyruvate metabolism in this tissue is responsible for the elevated concentration of pyruvate and lactate in plasma of phenylketonuric patients. In skeletal muscle the activity of pyruvate carboxylase is very low (Needham, 1973), therefore pyruvate is metabolized mainly by the pyruvate dehydrogenase complex. The effect of phenylpyruvate on this oxidation process in skeletal muscle mitochondria is the subject of the present paper.

MATERIALS AND METHODS

Human muscle was obtained from patients undergoing orthopedic surgery. The patients who had no records of metabolic diseases were anesthetized with brevinarcon and N_2O *plus* O_2.

Rat skeletal muscle was obtained from the hind legs of male Wistar rats immediately after decapitation.

Mitochondria were prepared by the method of Dow (1967), as modified by Świerczyński *et al.* (1975).

Respiration of mitochondria was measured polarographically in the medium containing in a final volume of 2.5 ml: 15 mM-KCl, 50 mM-Tris-HCl, pH 7.4, 6 mM-potassium phosphate, pH 7.4, 5 mM-$MgSO_4$, 2 mM-EDTA, 10 μM-cytochrome *c*, 1 μM-CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), 2 mM-L-malate, 1 mM-pyruvate and, if not otherwise stated, 1.6 mg of mitochondrial protein. For the calculation of initial velocities, the initial linear part of the tracing was taken.

Pyruvate uptake by mitochondria was measured by radioactivity assay as described in the legend to Table 1.

The extract of rat skeletal muscle mitochondria was obtained in the following manner: mitochondria (about 100 mg protein) were washed with 10 ml of 20 mM-potassium phosphate buffer, pH 7.0, containing 10 mM-mercaptoethanol. The washed mitochondrial pellet was suspended in 2 ml of the same buffer and the suspension was frozen at −15° C. After 3 h the frozen mitochondrial preparation was thawed and then diluted to 6 ml with 20 mM-potassium phosphate buffer, pH 7.0, containing 10 mM-mercaptoethanol. The suspension obtained was sonicated for 3×15 sec with 1 min break, at 16 KHz and 0°C in MSE 100 Watt Ultrasonic Disintegrator. After sonication the disrupted mitochondria were centrifuged at 20 000 *g* for 30 min, the supernatant was carefully decanted and used for the enzyme assay.

Pyruvate dehydrogenase activity was determined by monitoring NADH formation at 340 nm with Unicam SP-800 spectrophotometer equipped with Unicam SP-250 Scale Expansion Accessory. The reaction mixture contained in a final volume of 2.5 ml: 60 mM-potassium phosphate buffer, pH 8.0, 5 mM-mercaptoethanol, 2 mM-$MgCl_2$, 0.5 mM-thiamine pyrophosphate, 4 μg of rotenone, 0.5 mM-NAD, 1 mM-sodium pyruvate and 0.4 mg protein of the mitochondrial extract. The reaction was started by the addition of 0.2 mM-CoA (final concentration) and the initial

velocity was taken as the rate of NADH formation during the first 30 sec of the linear increase in absorbance. Reaction rates were corrected for NADH formation in the absence of pyruvate, which constituted not more than 10% of the pyruvate-induced rate.

Protein was estimated as described by Świerczyński & Aleksandrowicz (1974).

Chemicals. Pyruvate (sodium salt), β-phenylpyruvate (sodium salt), rotenone and CoA were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), L-malate from Koch-Light (Colnbrook, Bucks, England), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) from Calbiochem (Los Angeles, Calif., U.S.A.), NAD from Reanal (Budapest, Hungary), [^{14}C]pyruvate from Isocommerz GmbH (Berlin, G.D.R.), [^{14}C]sucrose from the Radiochemical Centre (Amersham, U.K.). All other compounds were of the highest purity available commercially.

RESULTS AND DISCUSSION

The effect of phenylpyruvate on pyruvate *plus* malate oxidation by rat skeletal muscle mitochondria in the uncoupled state is shown in Fig. 1. At 0.8 mM concentration, phenylpyruvate caused an immediate inhibition of pyruvate *plus* malate oxidation. This inhibition could be reversed by 4 mM-pyruvate but not by 4 mM-L-malate. Addition of phenylpyruvate preceding that of pyruvate reduced oxidation of pyruvate by about 70%. The increase of pyruvate concentration to 4 mM restored almost maximal oxygen consumption. Kinetics of the effect of phenylpyruvate on pyruvate *plus* malate oxidation is presented as Lineweaver-Burk plots (Fig. 2a), and reveals competition between pyruvate and phenylpyruvate. The Dixon plot of these results (Fig. 2b) shows that the K_i value for phenylpyruvate is about 0.18 mM.

Inhibition of pyruvate *plus* malate oxidation by phenylpyruvate in intact skeletal muscle mitochondria could be due to the effect either on the transport of

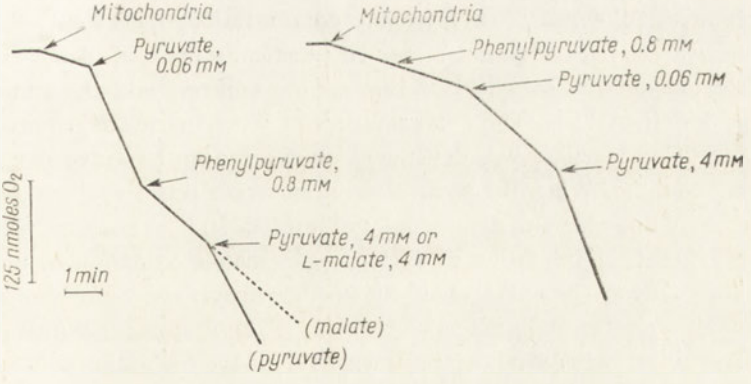

Fig. 1. The effect of phenylpyruvate on pyruvate *plus* malate oxidation in rat skeletal muscle mitochondria. Polarographic tracing of oxygen consumption was taken at 25°C; the experimental conditions were as described in Methods.

pyruvate across the inner mitochondrial membrane; on pyruvate dehydrogenase activity; or on the electron transport chain. Although inhibition of malate dehydrogenase or malate transport across the mitochondrial membrane during pyruvate

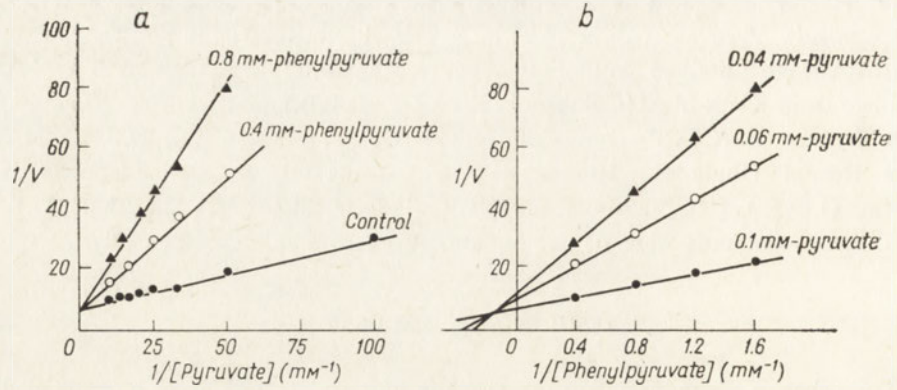

Fig. 2. Inhibition by phenylpyruvate of pyruvate *plus* malate oxidation in rat skeletal muscle mitochondria, presented as double-reciprocal plot (*a*) and as Dixon plot (*b*). Experimental conditions as described in Methods. *V* is expressed as μmoles of O_2/min/mg protein. The points represent mean values from 3 experiments; S. D. did not exceed 10%.

plus malate oxidation could also be taken into consideration, the competition found between pyruvate and phenylpyruvate suggests that pyruvate metabolism is directly affected by phenylpyruvate. Figure 3 illustrates the effect of increasing concentration of phenylpyruvate on the oxidation of pyruvate *plus* malate, pyruvate transport across the mitochondrial membrane, and pyruvate dehydrogenase activity. The results are presented as percentages of the control values. As it may be seen, phenylpyruvate at concentration of 2 mM inhibited pyruvate *plus* malate oxidation in about 70%, pyruvate dehydrogenase activity in about 30%, whereas the transport was hardly inhibited. At 4 mM phenylpyruvate concentration, the extent of inhibition of pyruvate *plus* malate oxidation remained practically unchanged but the activity of pyruvate dehydrogenase was half that of the control, and the transport was reduced by less than 15%. The concentration of phenylpyruvate required to inhibit pyruvate dehydrogenase was dependent on the concentration of pyruvate used in the assay medium. When the pyruvate concentration was 1 to 2 mM, the inhibitory effect of 4 mM-phenylpyruvate did not exceed 30%. The data presented in Fig. 3 suggest that the inhibition of pyruvate *plus* malate oxidation by phenylpyruvate is due mainly to its effect on pyruvate dehydrogenase. Since this enzyme is accessible only in the matrix space (Scott *et al.*, 1972), it seems that phenylpyruvate easily enters this mitochondrial compartment. The above suggestion is in accordance with the results of Paradies & Papa (1973) and Halestrap *et al.* (1974). As shown in Table 1, phenylpyruvate at concentration of 10 and 20 mM inhibited pyruvate accumulation in mitochondria by 20 and 50%, respectively. These data indicate that

phenylpyruvate inhibits pyruvate transport into skeletal muscle mitochondria to a lower extent than into brain and heart mitochondria (Land & Clark, 1974; Halestrap *et al.*, 1974).

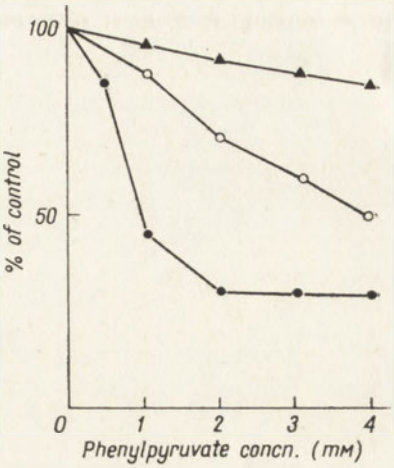

Fig. 3. The effect of phenylpyruvate on: pyruvate *plus* malate oxidation (●), pyruvate accumulation (▲), and pyruvate dehydrogenase activity (○) in rat skeletal muscle mitochondria. Experimental conditions were as described in Methods. The control rates taken as 100 were: oxygen uptake, 125 nmoles/min/mg mitochondrial protein; pyruvate accumulation in the matrix space, 2.3 nmoles/min/mg protein; dehydrogenase activity, 120 nmoles of NADH formed/min/mg protein.

To examine whether the rise in pyruvate and lactate concentration in plasma of untreated phenylketonuric patients is caused by inhibition of pyruvate oxidation in muscle mitochondria, the effect of phenylpyruvate was studied with human skeletal muscle mitochondria in different metabolic states. Pyruvate dehydrogenase is known to be more active in metabolic state 3 and in uncoupled state, and less

Table 1

Effect of phenylpyruvate on pyruvate accumulation in rat skeletal muscle mitochondria

Mitochondria (2.3 mg protein) were preincubated for 30 sec at 5°C in 1 ml of a medium containing: 125 mM-KCl, 20 mM-Tris-HCl (pH 7.4), 4 μg of rotenone, 2 μg of antimycin A, 1 mM-arsenite, and phenylpyruvate at concentration indicated. The reaction was initiated by addition of 1 mM-[^{14}C]pyruvate (300 000 d.p.m.) and stopped at 1 min by a rapid removal of the mitochondria from the medium by centrifugation. The pellets were suspended in 0.5 ml of 10% $HClO_4$, extracted for 30 min and centrifuged at 14 000 *g* for 3 min. The procedure applied for isotopic assay was as described previously (Świerczyński & Aleksandrowicz, 1974; Aleksandrowicz & Świerczyński, 1975). The amount of [^{14}C]pyruvate remaining in the matrix space was calculated from the radioactivity in the $HClO_4$ extract. This was corrected for the [^{14}C]pyruvate present in the sucrose-permeable space *plus* adherent medium, determined from the distribution of [^{14}C]sucrose (320 000 d.p.m.) added to a parallel sample instead of [^{14}C]pyruvate. The results represent means from 3 experiments, S.D. being within ±10%.

	Pyruvate in matrix space (nmoles)	Inhibition (%)
Control	5.20	
+ phenylpyruvate, 10 mM	4.10	21
+ phenylpyruvate, 20 mM	2.40	54

active in state 4 (Wieland & Portenhauser, 1974). The data presented in Fig. 4a,b indicate that phenylpyruvate inhibited pyruvate *plus* malate oxidation both in human and rat mitochondria in state 3 and in uncoupled state but did not affect this process in state 4. This implies that, in phenylketonuria, phenylpyruvate inhibits the pyruvate *plus* malate oxidation by inhibiting the active form of the pyruvate dehydrogenase complex.

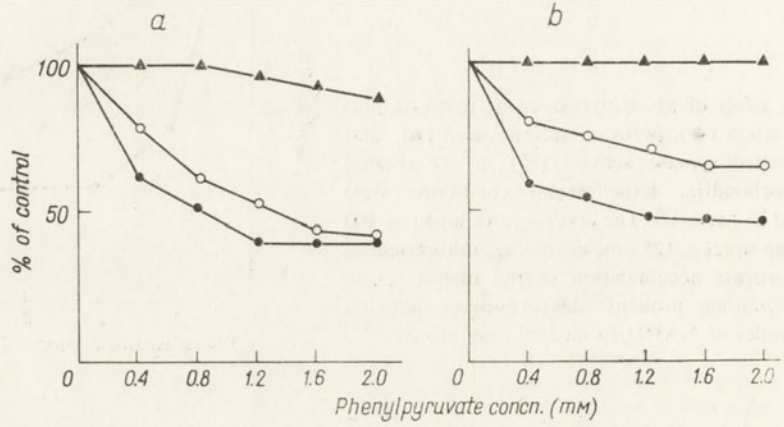

Fig. 4. The effect of phenylpyruvate on pyruvate *plus* malate oxidation in metabolic state 4 (▲) and 3 (○), and in uncoupled state (●) in rat (*a*) and human (*b*) skeletal muscle mitochondria. The experimental conditions were as described in Methods, except that, in state 4, CCCP was omitted and in state 3, 1 mM-ADP was added instead of CCCP. The values for rat muscle mitochondria are the means from 3 experiments, S. D. not exceeding ±10%; the results for human muscle mitochondria represent a single experiment.

Although this assumption is speculative and further studies are needed to confirm it, it explains the results presented in this paper and the results of Clark & Land (1974) who observed that phenylpyruvate inhibits pyruvate *plus* malate oxidation by rat brain mitochondria in metabolic state 3 but not in state 4. This suggestion is also in accordance with the observations of Hoffmann & Hucho (1974) who reported that phenylpyruvate at high concentrations inhibited non-competitively pyruvate dehydrogenase isolated from brain and kidney. However, these authors showed that phenylpyruvate inhibited also pyruvate dehydrogenase kinase, preventing in this way the inactivation of the pyruvate dehydrogenase complex by ATP.

Irrespective of the exact mechanism of phenylpyruvate action on pyruvate *plus* malate oxidation by skeletal muscle mitochondria, the fact that phenylpyruvate greatly impairs the metabolism of pyruvate in the muscle may be of importance in pathological conditions such as phenylketonuria.

It has been reported that phenylpyruvate inhibits hexokinase and 6-phosphogluconate dehydrogenase (Weber *et al.*, 1970) and some enzymes involved in fatty acids synthesis in rat brain (Land & Clark, 1973); however, only citrate synthase and fatty acids synthetase of rat brain (Land & Clark, 1973) and chicken liver pyruvate carboxylase (Scrutton *et al.*, 1969) are inhibited with K_i below 1 mM, whereas K_i values

reported for the inhibition of other enzymes are much higher (see Land & Clark, 1973). Data presented in this paper show that in rat skeletal muscle mitochondria phenylpyruvate inhibits pyruvate *plus* malate oxidation with relatively low K_i. The concentration of phenylpyruvate in the muscle of phenylketonuric patients is not known, the level, however, of this compound in blood of fasting untreated phenylketonuric patients is about 0.1 mM, and increases to 0.5 mM after ingestion of phenylalanine (Patel, 1972). These concentrations of phenylpyruvate seem to be sufficiently high to inhibit pyruvate oxidation in skeletal muscle, and consequently to elevate blood pyruvate and lactate levels, as observed by Sutnick *et al.* (1972) in untreated phenylketonuric patients.

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REFERENCES

Aleksandrowicz, Z. & Świerczyński, J. (1975) *Biochim. Biophys. Acta*, **382**, 92 - 105.
Bowden, J. A. & McArthur, III C. L. (1972) *Nature (London)*, **235**, 230.
Clark, J. B. & Land, J. M. (1974) *Biochem. J.*, **140**, 25 - 29.
Dow, D. S. (1967) *Biochemistry*, **6**, 2915 - 2922.
Gallagher, B. B. (1969) *J. Neurochem.*, **16**, 1071 - 1076.
Halestrap, A. P., Brand, M. D. & Denton, R. M. (1974) *Biochim. Biophys. Acta*, **367**, 102 - 108
Hoffmann B. T. & Hucho, F. (1974) *FEBS Lett.*, **43**, 116 - 119.
Jervis, G. A. (1953) *Proc. Soc. Exp. Biol. Med.*, **81**, 514 - 515.
Jervis, G. A. & Drejza, E. J. (1966) *Clin. Chim. Acta*, **13**, 435 - 441.
Land, J. M. & Clark, J. B. (1973) *Biochem. J.*, **134**, 545 - 555.
Land, J. M. & Clark, J. B. (1974) *FEBS Lett.*, **44**, 348 - 351.
Needham, D. M. (1973) in *The Structure and Function of Muscle* (G. H. Bourne, ed.) vol. 3, pp. 363 - 415. Academic Press, New York and London.
Patel, M. S. (1972) *Biochem. J.*, **128**, 677 - 684.
Paradies, G. & Papa, S. (1973) *9th Int. Congr. Biochem., Stockholm*, Abstr. No. 5 L 5, p. 288.
Scott, K. M., Jurkowitz, M. & Brierley, G. P. (1972) *Arch. Biochem. Biophys.*, **153**, 682 - 694.
Scutton, M. C., Olmsted, M. R. & Utter, M. F. (1969) in *Methods in Enzymology* (S.P. Colowick & N. O. Kaplan, eds.) vol. 13, pp. 235 - 249. Academic Press, New York and London.
Sutnick, M. R., Grower, W. D. & Patel, M. S. (1972) *Pediatr. Res.*, **6**, 432.
Świerczyński, J. & Aleksandrowicz, Z. (1974) *Biochim. Biophys. Acta*, **373**, 66 - 75.
Świerczyński, J., Aleksandrowicz, Z. & Żydowo, M. (1975) *Int. J. Biochem.*, **6**, 757 - 763.
Weber, G., Glazer, R. I. & Ross, R. A. (1970) *Adv. Enzyme Regul.*, **8**, 13 - 36.
Weland, O. H. & Portenhauser, R. (1974) *Eur. J. Biochem.*, **45**, 577 - 588.

HAMUJĄCY WPŁYW FENYLOPIROGRONIANU NA UTLENIANIE PIROGRONIANU W MITOCHONDRIACH MIĘŚNI SZKIELETOWYCH

Streszczenie

1. Fenylopirogronian hamuje utlenianie pirogronianu (w obecności jabłczanu) w mitochondriach w stanie 3 zarówno w mięśniach szczura, jak i w mięśniach człowieka, jest natomiast bez wpływu, gdy mitochondria są w stanie metabolicznym 4.

2. Fenylopirogronian hamuje kompetycyjnie utlenianie pirogronianu (w obecności jabłczanu) w rozkojarzonych mitochondriach szczura. Stała inhibitorowa K_i wynosi 0.18 mM.

3. Sugeruje się, że hamowanie utleniania pirogronianu jest spowodowane głównie działaniem fenylopirogronianu na dehydrogenazę pirogronianową mięśni, co może być zasadniczą przyczyną zwiększonego stężenia pirogronianu i mleczanu w osoczu krwi w przypadkach fenyloketonurii.

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STIMULATION OF CITRATE OXIDATION AND TRANSPORT IN HUMAN PLACENTAL MITOCHONDRIA BY L-MALATE*

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1. Citrate was oxidized by human placental mitochondria at a rate half that for isocitrate, and *cis*-aconitate at a rate by 20% lower as with isocitrate. The apparent K_m values for these substrates were similar.

2. Oxidation of citrate was stimulated by L-malate but the stimulation was abolished by benzene 1,2,3-tricarboxylate and butylmalonate.

3. Citrate uptake by placental mitochondria was inhibited by benzene 1,2,3--tricarboxylate, and raised specifically by L-malate. Stimulation by fumarate and succinate was found to be due to conversion of these substrates to L-malate.

4. It is concluded that human placental mitochondria contain malate-stimulated tricarboxylate carrier system.

It is generally accepted that the transport of tricarboxylic intermediates of the Krebs cycle across the inner membrane of rat liver mitochondria is mediated by a specific carrier system which catalyses an exchange of external tricarboxylate anion or L-malate against an internal tricarboxylate anion (Robinson & Chappell, 1970; Robinson *et al.*, 1971a; Palmieri *et al.*, 1972). The recent results obtained with mitochondria from kidney (Meijer, 1971), white adipose tissue (Halperin *et al.*, 1969), heart (England & Robinson, 1969; Scott *et al.*, 1972), and adrenals (Michejda *et al.*, 1973; Launay *et al.*, 1974) suggest universal occurrence of such transporting system in mitochondria. However, not all experimental data are convergent with this opinion, e.g. the results of Meijer (1971), Meijer *et al.* (1969) and Sluse *et al.* (1971) concerning rat heart mitochondria.

Since the transport of citrate through the mitochondrial membrane seems to be related to such processes as fatty acid synthesis (Sluse *et al.*, 1971), similarly as that of malate to steroidogenesis (Launay *et al.*, 1974), an attempt was undertaken to examine the transport of tricarboxylic acids in human placental mitochondria.

* This work was supported by the Polish Academy of Sciences within the project II.1.1.6.

MATERIALS AND METHODS

Human term placental mitochondria were prepared as described previously (Świerczyński *et al.*, 1976).

Respiration of mitochondria was measured with a Clark oxygen electrode at 25°C in the medium containing in a final volume of 2.5 ml: 15 mM-KCl, 50 mM-Tris-HCl (pH 7.4), 20 mM-potassium phosphate buffer (pH 7.4), 5 mM-$MgSO_4$, 2 mM-EDTA, 1 mM-ADP, 1 mM-arsenite, 10 μM-cytochrome *c*, 0.3% of bovine serum albumin, and mitochondria as indicated. The reaction was started by the addition of substrate. Other additions were as indicated in the Tables and Figures.

The uptake of citrate was measured in mitochondria preincubated for 1 min in the reaction mixture containing in a final volume of 1 ml: 125 mM-KCl, 20 mM-Tris-HCl (pH 7.4) and 2 μg of rotenone. Then 0.1 mM-[^{14}C]citrate (200 000 d.p.m.) was incubated with mitochondria for 5 min, and the reaction was stopped by the addition of 25 mM-benzene 1,2,3-tricarboxylate, followed immediately by centrifugation Further steps of the procedure were performed as described by Świerczyński & Aleksandrowicz (1974) and Aleksandrowicz & Świerczyński (1975). Correction was made for the sucrose-permeable space by subtracting the values obtained when benzene 1,2,3-tricarboxylate — an inhibitor of citrate uptake (Robinson *et al.*, 1971b) — was added to the reaction mixture before citrate. The amount of [^{14}C]citrate present in the sucrose-permeable space *plus* adherent medium was also determined from the distribution of [^{14}C]sucrose (220 000 d.p.m.) added to a parallel sample instead of [^{14}C]citrate (Harris & Van Dam, 1968).

Fumarase activity was measured according to Hill & Bradshaw (1969) by monitoring at 25°C the increase in absorbance at 250 nm with Unicam SP 800 spectrophotometer. The incubation medium contained in a final volume of 2.5 ml: 50 mM-potassium phosphate buffer (pH 7.4), 0.04% Triton X-100 and 1 mg of mitochondrial protein; the reaction was started by the addition of 10 mM-L-malate. Other additions were as indicated.

Each experiment was repeated four times.

Protein concentration was measured as described by Świerczyński & Aleksandrowicz (1974).

Reagents. Citrate, isocitrate, antimycin A, rotenone, fumarate, succinate, 2-oxoglutarate, pyruvate, β-hydroxybutyrate, L-malate and D-malate were from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); malonate and cytochrome *c* from Koch-Light Lab. (Colnbrook, Bucks., England), benzene 1,2,3-tricarboxylic acid from Merck-Schuchardt (Darmstadt, G.F.R.); 1,5-[$^{14}C_2$]citrate, and [^{14}C]sucrose from the Radiochemical Centre (Amersham, England). Butylmalonate was a generous gift from Dr. J. Popinigis. *cis*-Aconitate was a generous gift from Dr. J. Stępiński. All other compounds were of the highest purity available commercially.

RESULTS

Data presented in Table 1 indicate that isocitrate was oxidized more readily by human placental mitochondria than *cis*-aconitate or citrate; the latter was oxidized at a rate about half that for isocitrate. However, the apparent K_m values for

citrate and isocitrate were practically the same, and that for *cis*-aconitate slightly lower. Oxidation of citrate by placental mitochondria was stimulated by L-malate whereas the addition of D-malate was ineffective (Table 2). When the mitochondria were solubilized by 0.04% Triton X-100 and oxidation of citrate was followed under the same conditions as in Table 2, this stimulation was not observed (not shown). Benzene 1,2,3,-tricarboxylate and butylmalonate abolished the oxidation of citrate in the presence of L-malate (Table 3). As shown in Fig. 1, benzene 1,2,3-tricarboxylate

Table 1

V_{max} and K_m values for oxidation of tricarboxylic acids by human placental mitochondria

Mitochondria (10 mg protein) were preincubated for 3 min as described under Methods, then citrate (0.2 - 6 mM), DL-isocitrate (0.2 - 6 mM) or *cis*-aconitate (0.1 - 4 mM) was added.

Tricarboxylic acid	V_{max} (nmoles O_2/mg protein/min)	K_m (M)
Citrate	5.0	6.8×10^{-4}
DL-Isocitrate	10.0	6.2×10^{-4}
cis-Aconitate	8.0	3.0×10^{-4}

Table 2

Effect of malate on the oxidation of citrate in human placental mitochondria

Mitochondria (10 mg protein) were preincubated for 3 min as described under Methods, then citrate (2 mM) and/or malate (1 mM) was added.

Additions	Oxygen uptake (natoms O/10 mg protein/min)
Citrate	30
L-Malate	5
Citrate + L-malate	95
Citrate + D-malate	30

Table 3

Effect of benzene 1,2,3-tricarboxylate and butylmalonate on the L-malate-stimulated oxidation of citrate

Experimental conditions were as described in Table 2. The incubation mixture contained 2 mM-citrate and 1 mM-L-malate. The inhibitors in the indicated concentrations were added prior to the addition of citrate.

Addition	Oxygen uptake (natoms O/10 mg protein/min)	Inhibition (%)
None (control)	95	
Benzene 1,2,3-tricarboxylate, 2 mM	45	53
Benzene 1,2,3-tricarboxylate, 4 mM	13.5	83
Butylmalonate, 5 mM	38	60

inhibited citrate uptake by these mitochondria; half-maximal inhibition was observed at about 2.5 mM concentration of the inhibitor, i.e. at the concentration effective in rat liver mitochondria (Robinson *et al.*, 1971b). This implies that the effect of

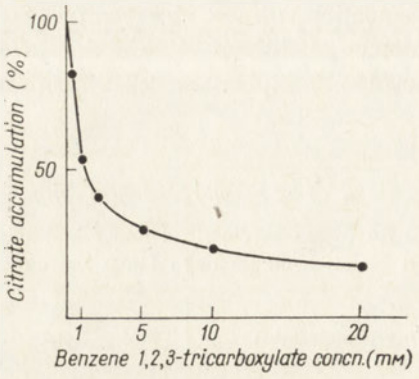

Fig. 1

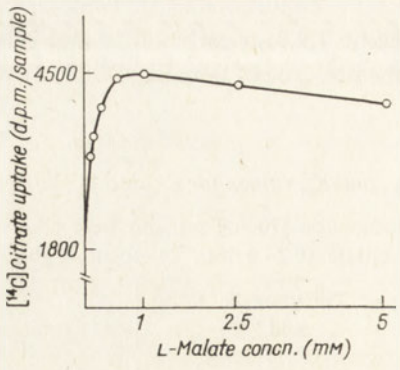

Fig. 2

Fig. 1. Inhibition of citrate accumulation in placental mitochondria by benzene 1,2,3-tricarboxylate. Conditions as described in Methods; the incubation mixture contained 0.5 mM-L-malate and 3.0 mg of mitochondrial protein.

Fig. 2. Effect of L-malate on citrate accumulation by placental mitochondria. Experimental conditions were as described in Methods; the incubation mixture contained 2.2 mg of mitochondrial protein per sample.

benzene 1,2,3-tricarboxylate on citrate oxidation by placental mitochondria is due to the effect of this compound on citrate transport. Data presented in Table 4 show that L-malate stimulated citrate accumulation in placental mitochondria. D-Malate under the same conditions was not effective. Table 4 shows also that butylmalonate abolished the stimulatory effect of L-malate on citrate uptake. In Fig. 2 the dependence of citrate uptake on the concentration of L-malate is presented. Half-maximal stimulation was observed at about 0.2 mM-L-malate.

Table 4

Effect of malate on [^{14}C]*citrate transport to human placental mitochondria*

Experimental conditions as described under Methods; mitochondrial protein, 3.1 mg per sample; malate, 0.5 mM, butylmalonate, 10 mM.

Addition	[^{14}C]Citrate (d.p.m./sample)
None (control)	3100
L-Malate	8300
D-Malate	3000
Butylmalonate	2300
L-Malate + butylmalonate	2400

To check the specificity of L-malate effect, the uptake of [^{14}C]citrate by human placental mitochondria was measured in the presence of L-malate and other mono- and di-carboxylic acids. The results presented in Table 5 show that, in addition to L-malate, also fumarate and succinate stimulated about twofold the citrate uptake. Other monocarboxylate anions: pyruvate, β-hydroxybutyrate and acetate, dicarboxylate anions: malonate and 2-oxoglutarate, as well as phosphate, had no effect on the transport of citrate to the placental mitochondria.

Table 5

Effect of some carboxylate anions and phosphate on [^{14}C]citrate transport to human placental mitochondria

Experimental conditions as described under Methods. Mitochondrial protein (3.3 mg), [^{14}C]citrate (0.1 mM) and other acids (0.5 mM) were added to the incubation mixture.

Addition	[^{14}C]Citrate (d.p.m./sample)
None (control)	3500
L-Malate	7800
Fumarate	7600
Succinate	7600
Malonate	3600
2-Oxoglutarate	3500
Pyruvate	4000
β-Hydroxybutyrate	3500
Phosphate	3300
Acetate	3800

The question arises whether under our experimental conditions stimulation of citrate uptake by succinate and fumarate was caused directly by these metabolites or was due to their conversion to malate. Therefore we studied the effect of inhibitors blocking the conversion of fumarate and succinate to malate. SCN^-, an inhibitor of fumarase activity in heart mitochondria (Massey, 1953), inhibited also this activity in human placental mitochondria (Fig. 3). As shown in Table 6, NH_4SCN was without effect on citrate uptake but abolished stimulation of this

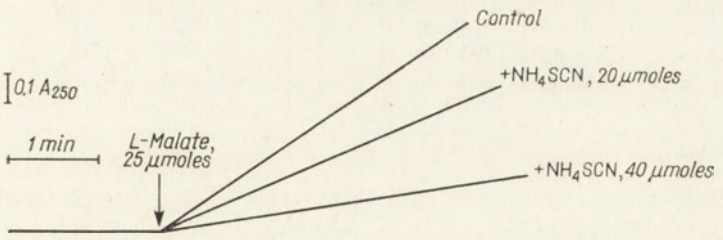

Fig. 3. Effect of NH_4SCN on fumarase activity in placental mitochondria. Experimental conditions were as described in Methods; the incubation mixture contained 1 mg of mitochondrial protein.

process by fumarate. Moreover, butylmalonate also abolished this stimulation. Maleate, an isomer of fumarate, was ineffective. When the conversion of succinate to malate was inhibited by antimycin A or by malonate (Table 7), succinate did not exert the stimulatory effect on citrate uptake. The results presented prove that fumarate and succinate enhanced citrate uptake by human placental mitochondria only after being converted to L-malate.

Table 6

Effect of fumarate on [^{14}C]citrate transport to human placental mitochondria

Experimental conditions as described under Methods. Mitochondrial protein (3.3 mg), [^{14}C]citrate (0.1 mM), and /or NH_4SCN (20 mM), fumarate (0.5 mM), butylmalonate (10 mM), maleate (0.5 mM), were added to the incubation mixture.

Additions	[^{14}C]Citrate (d.p.m./sample)
None (control)	3200
NH_4SCN	3300
Fumarate	6800
Butylmalonate	2500
Fumarate + NH_4SCN	4200
Fumarate + butylmalonate	2600
Maleate	3300

Table 7

Effect of succinate on [^{14}C]citrate transport to human placental mitochondria

Experimental conditions as described under Methods. Mitochondrial protein (3.5 mg), succinate (0.5 mM), malonate (1 mM) and/or antimycin A were added to the incubation mixture.

Additions	[^{14}C]Citrate (d.p.m./sample)
None (control)	2800
Succinate	7000
Succinate + antimycin A, 1 μg	4000
Succinate + antimycin A, 2 μg	3000
Antimycin A, 2 μg	3300
Succinate + malonate	3000
Malonate	2800

The addition of bovine serum albumin, prepared before use according to Lopes-Cardozo & Van Den Bergh (1972), increased citrate uptake 1.5- and 2-fold, respectively, in the presence and in the absence of L-malate (not shown), similarly as it was observed with adrenal cortex mitochondria (Launay *et al.*, 1974).

DISCUSSION

The rate of tricarboxylic acids oxidation by human placental mitochondria is relatively low as compared with other mitochondria. Low oxidation of tricarboxylic acids by placental mitochondria might be due to inhibition of the respiratory chain by endogenous steroids (Yielding *et al.*, 1960; Strittmatter, 1962; Varrichio & Sanadi, 1967; Świerczyński *et al.*, 1975) but not to low permeability of inner mitochondrial membrane, since these substrates are oxidized by placental mitochondria at the same (low) rate as other NAD-linked substrates (Świerczyński *et al.*,1976).

The specific stimulatory effect of L-malate and its precursors on citrate uptake and the inhibition of this uptake by benzene 1,2,3-tricarboxylate, suggest the existence of a tricarboxylic acid transporting system in human placental mitochondria. One possible model for the stimulatory effect of L-malate on the citrate uptake is that malate must first enter into the mitochondrion by the dicarboxylate carrier system before it can exchange with citrate. Butylmalonate inhibits the entry of malate into the mitochondrion and thus prevents the transport of citrate. The stimulatory effect of fumarate and succinate suggests also that these anions must first enter by the dicarboxylate carrier system into the mitochondrion to undergo subsequent conversion to malate, which can then exchange with citrate. The observations presented imply than human placental mitochondria possess the dicarboxylate carrier system. It seems interesting that the transport of fumarate into placental mitochondria is sensitive to butylmalonate (Table 6). Transport of fumarate across inner mitochondrial membrane has been questioned by Chappell & Haarhoff (1967) and Chappell (1968). Other authors, however, have shown that the transport of fumarate into mitochondria is possible (La Noue *et al.*, 1970; Aleksandrowicz & Świerczyński, 1971).

The results described in this paper indicate that L-malate is specific in stimulating citrate uptake by human placental mitochondria. Similar results were obtained by Chappell (1968) with rat liver mitochondria. On the other hand, Kleinke *et al.* (1973) reported that other dicarboxylate anions (D-malate, succinate, fumarate) exchange also with citrate in these mitochondria. Tsang & Johnstone (1973) presented evidence that transport of citrate by adrenal cortex mitochondria was stimulated by malate, succinate and fumarate in the increasing order, but was not stimulated by pyruvate, isocitrate or oxoglutarate. Since, however, in these experiments antimycin A did not abolish the effect of succinate, it seems that this compound is able to activate directly citrate uptake by adrenal cortex mitochondria. In this respect, therefore, human placental mitochondria seem to differ from those of the adrenal cortex.

Meijer (1971) showed that fluoroacetate inhibits pyruvate *plus* malate oxidation in about 30 - 40% in the mitochondria permeable to citrate (rat liver mitochondria), and in more than 80% in those mitochondria which are impermeable to citrate (heart and skeletal muscle mitochondria). He has also shown that fluoroacetate causes accumulation of citrate generated from pyruvate inside heart mitochondria, whereas in liver fluoroacetate does not significantly alter citrate concentration inside

the mitochondria (Meijer, 1971). These results suggest that the rate of citrate influx is related to the rate of efflux of this anion from mitochondria. We did not examine distribution of citrate generated from pyruvate in the presence of fluorocitrate inside the mitochondria. Our preliminary observations showed that inhibition of pyruvate *plus* malate oxidation by fluorocitrate did not exceed 20% when fluorocitrate was used at a concentration which inhibited completely citrate oxidation. This might suggest that citrate readily penetrates the inner membrane from the mitochondrion to cytosol. The data presented above indicate that human placental mitochondria resemble those from liver with regard to citrate transport across the inner membrane and that citrate transport is taking place by a specific transport system.

The physiological significance of the tricarboxylate carrier system in human placental mitochondria is of interest. One of the many functions of citrate in the cell metabolism is to transport acetyl-CoA across the inner mitochondrial membrane, especially in these cells in which fatty acids synthesis takes place in cytosol (Lowenstein, 1968). This is the case with liver where citrate is readily transported across the mitochondrial membrane (Robinson & Chappell, 1970; Robinson *et al.*, 1971a; Palmieri *et al.*, 1972). On the other hand, heart, in which fatty acids are synthesized mainly in mitochondria (Wit-Peeters *et al.*, 1970), shows a low activity of citrate transport system (England & Robinson, 1969; Sluse *et al.*, 1971; Robinson & Oei, 1975). It has been found that in human placenta the activity of fatty acid synthetase and lipogenesis from acetate or citrate are very low (Diamant *et al.*, 1975; Roux *et al.*, 1967). As we have found, the activity of ATP-dependent lyase is also insignificant: about 5 μmoles of citrate cleaved per 100 g tissue per 1 min (unpublished). This suggests that in human placenta citrate is not the carrier for acetyl-CoA across the inner mitochondrial membrane. A possible link between citrate transport and the effect of the Krebs cycle intermediates on steroidogenesis in human placental mitochondria is discussed in the accompanying paper (Klimek *et al.*, 1976).

REFERENCES

Aleksandrowicz, Z. & Świerczyński, J. (1971) *FEBS Lett.*, **15**, 269 - 272.

Aleksandrowicz, Z. & Świerczyński, J. (1975) *Biochim. Biophys. Acta* **382**, 92 - 105.

Chappell, J. B. (1968) *Br. Med. Bull.*, **24**, 150 - 157.

Chappell, J. B. & Haarhoff, K. N. (1967) in *Biochemistry of Mitochondria* (E. C. Slater, Z. Kaniuga & L. Wojtczak, eds.) pp. 29 - 51.

Diamant, Y. Z., Mayorek, N., Neuman, S. & Shafrir, E. (1975) *Am. J. Obstet. Gynecol.*, **121**, 58 - 61.

England, P. J. & Robinson, B. H. (1969) *Biochem. J.*, **112**, 8P.

Halperin, M. L., Robinson, B. H., Martin, B. R. & Denton, R. M. (1969) *Nature (London)*, **223**, 1369 - 1371.

Harris, E. J. & Van Dam, K. (1968) *Biochem. J.*, **106**, 759 - 766.

Hill, L. R. & Bradshaw, R. A. (1969) in *Methods in Enzymology* (J. M. Lowenstein, ed.) vol. 13, pp. 91 - 99. Academic Press, New York and London.

Kleinke, J., Sauer, J. K. W. & Soling, H. D. (1973) *FEBS Lett.* **29**, 82 - 86.
Klimek, J., Bogusławski, W., Tiałowska, B. & Żelewski, L. (1976) *Acta Biochim. Polon.*, **23**, 185 - 192.
LaNoue, K., Nicklas, W. J. & Williamson, J. R. (1970) *J. Biol. Chem.*, **245**, 102 - 111.
Launay, A. N., Michejda, J. W. & Vignais, P. V. (1974) *Biochim. Biophys. Acta*, **347**, 60 - 76.
Lopes-Cardozo, M. & Van Den Bergh, S. G. (1972) *Biochim. Biophys. Acta*, **283**, 1 - 15.
Lowenstein, J. M. (1968) in *Metabolic Roles of Citrate* (T. W. Goodwin, ed.) pp. 61 - 86. Academic Press, London and New York.
Massey, V. (1953) *Biochem. J.*, **53**, 67 - 71.
Meijer, A. J. (1971) *Anion Translocation in Mitochondria*. Ph. D. Thesis, Amsterdam. Academic Service, Amsterdam.
Meijer, A. J., Tager, J. M. & Van Dam, K. (1969) in *The Energy Level and Metabolic Control in Mitochondria* (S. Papa, J. M. Tager, E. Quagliariello & E. C. Slater, eds.) pp. 147 - 164. Adriatica Editrice, Bari.
Michejda, J. W., Launay, A. N. & Vignais, P. V. (1973) *FEBS Lett.*, **32**, 161 - 165.
Palmieri, F., Stipani, I., Quagliariello, E. & Klingenberg, M. (1972) *Eur. J. Biochem.*, **26**, 587 - 594.
Robinson, B. H. & Chappell, J. B. (1970) *Biochim. Biophys. Acta*, **205**, 300 - 303.
Robinson, B. H. & Oei, J. (1975) *Can. J. Biochem.*, **53**, 643 - 647.
Robinson, B. H., Williams, G. R., Halperin, M. L. & Leznoff, C. C. (1971a) *J. Biol. Chem.*, **246**, 5280 - 5286.
Robinson, B. H., Williams, G. R., Halperin, M. L. & Leznoff, C. C. (1971b) *Eur. J. Biochem.*, **20**, 3413 - 3416.
Roux, J. F., Grigorian, A. & Takeda, Y. (1967) *Nature (London)*, **216**, 819 - 820.
Scott, K. M., Jurkowitz, M. & Brierley, G. P. (1972) *Arch. Biochem. Biophys.*, **153**, 682 - 694.
Sluse, F. E., Meijer, A. J. & Tager, J. M. (1971) *FEBS Lett.*, **18**, 149 - 153.
Strittmater, C. F. (1962) *J. Cell. Comp. Physiol.*, **60**, 23 - 32.
Świerczyński, J. & Aleksandrowicz, Z. (1974) *Biochim. Biophys. Acta*, **373**, 66 - 75.
Świerczyński, J., Aleksandrowicz, Z. & Żelewski, L. (1975) *J. Steroid Biochem.*, **6**, 1235 - 1242.
Świerczyński, J., Ścisłowski, P. & Aleksandrowicz, Z. (1976) *Biochim. Biophys. Acta*, **429**, 46 - 54.
Tsang, D. & Johnstone, R. M. (1973) *Biochim. Biophys. Acta*, **318**, 217 - 224.
Varrichio, F. & Sanadi, D. R. (1967) *Arch. Biochem. Biophys.*, **121**, 187 - 193.
Wit-Peeters, E. M., Scholte, H. R. & Elenbaas, H. L. (1970) *Biochim. Biophys. Acta*, **210**, 360 - 370.
Yielding, K. L., Tomkins, G. M., Munday, J. S. & Cowley, I. J. (1960) *J. Biol. Chem.*, **235**, 3413 - 3416.

STYMULUJĄCY WPŁYW L-JABŁCZANU NA UTLENIANIE I TRANSPORT CYTRYNIANU W MITOCHONDRIACH ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Mitochondria łożyska ludzkiego utleniają cytrynian dwukrotnie wolniej niż izocytrynian a *cis*-akonitan z szybkością o 20% mniejszą od szybkości utleniania izocytrynianu. Wartości pozornej stałej Michaelisa dla tych substratów są podobne.

2. Utlenianie cytrynianu jest stymulowane przez L-jabłczan a hamowane przez 1,2,3-trójkarboksybenzen i butylomalonian.

3. Transport cytrynianu przez błonę wewnętrzną mitochondriów łożyska jest hamowany przez 1,2,3-trójkarboksybenzen i specyficznie stymulowany przez L-jabłczan. Fumaran i bursztynian — w warunkach, w których istnieje możliwość ich przemiany do L-jabłczanu — również stymulują transport cytrynianu.

4. Sugeruje się obecność w mitochondriach łożyska translokatora dla kwasów trójkarboksylowych, specyficznie stymulowanego przez L-jabłczan.

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CHANGES IN SIZE AND SHAPE OF CHROMATIN PARTICLES AFTER SUCCESSIVE REMOVAL OF HISTONES*

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The preparations of whole chromatin, chromatin selectively depleted of histone *f1*, depleted of all lysine-rich histones (*f1*, *f2b*, *f2a2*), and DNA were studied by viscosimetric and light scattering methods. The obtained results were used for calculation of the dimensions and packing ratios of DNA for the preparations studied. The packing ratio in whole chromatin is 7.2 and is almost unaffected by selective removal of histone *f1* (6.9), but decreases on successive removal of the remaining four histones, the decrease being dependent more on the quantity than the kind of the dissociated histones.

It has been well established that DNA in chromatin (nucleohistone) has a more compact form than free DNA (Henson & Walker, 1971; Krueger & Allison, 1973). The selective removal of histone *f1* has practically no influence on the conformation of nucleohistone particles, while progressive dissociation of the remaining four histone fractions results in a change to a more extended form (Henson & Walker, 1971; Toczko *et al.*, 1972; Frisman *et al.*, 1974; Toczko & Kaliński, 1974a,b). So far, however, these changes were not interpreted in terms of the effect of particular histone fractions on dimensions of nucleohistone particles. In this paper we present quantitative data on the changes occurring in the dimensions of chromatin particles after removal of particular histones, studied by two independent physical methods: viscosimetry and light scattering.

MATERIALS AND METHODS

Chromatin. This was prepared from calf thymus according to Marushige & Bonner (1966) except that ultracentrifugation through 1.7 M-sucrose was omitted.

Chromatin depleted of histone f1 was prepared from whole chromatin by extraction with 0.6 M-NaCl as described previously (Kaliński & Toczko, 1974).

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Chromatin depleted of all lysine-rich histones. Removal of histones *f1*, *f2a2* and *f2b* was performed in the NaCl - urea system recommended by Bolund & Johns (1973). The chromatin preparation equivalent to 300 E_{260} units was suspended in a mixture of 0.3 M-NaCl - 4 M-urea - 0.05 M-phosphate buffer, pH 7.0, to give a final concentration of 5 - 6 E_{260}, and sheared in the Unipan type 203 homogenizer for 90 s at 120 V. The resulting solution was stirred for 2 h at 0°C and then the depleted chromatin was separated from dissociated histones by chromatography on Sepharose 4B as described previously by Kaliński & Toczko (1974).

Dehistonized chromatin was prepared from whole chromatin by extraction with 2 M-NaCl and separation of the dissociated histones by gel chromatography on Sepharose 4B.

Isolation of DNA from whole chromatin. This was performed according to Stern (1968) as described by Kaliński *et al.* (1972).

Determination of DNA. The concentration of DNA was calculated from the value of E_{260} assuming $E^{1\%}_{1\,cm}$ at 260 nm to be 210 (Subirana, 1973).

Determination of histones and non-histone proteins. Histones were extracted from chromatin preparations twice with 0.3 M-HCl for 30 min at 0°C, then the residue containing non-histone proteins and DNA, was dissolved in 0.1 M-NaOH. Proteins were determined by the method of Lowry *et al.* (1951) with histones from calf thymus as standard.

Polyacrylamide-gel electrophoresis. Electrophoresis of histones extracted from chromatin samples was performed by the method of Johns (1967) as described by Toczko *et al.* (1972). Gels were scanned using an ERJ 65 densitograph (Carl Zeiss, Jena, G.D.R.).

Light scattering. The measurements were made with a photogoniodiffusiometer Sofica 4200 (France) at room temperature using $\lambda = 5461$ Å at angles from 30° to 150°. Solvent (0.05 M-NaCl, pH 7.0) and solutions of chromatin and DNA in this solvent were clarified by centrifugation at 23 000 *g* for 60 min. The results of measurements were evaluated by the double extrapolation method of Zimm (1948). Molecular dimensions were calculated using the following equations:

$$\langle s^2 \rangle^{1/2} = [\langle h^2 \rangle / 6]^{1/2}$$
$$\langle s^2 \rangle^{1/2} = [\langle l^2 \rangle / 6]^{1/2}$$

where: $\langle s^2 \rangle^{1/2}$ is the radius of gyration; $\langle h^2 \rangle^{1/2}$ is the end to end distance for a model of a flexible Gaussian chain; $\langle l^2 \rangle^{1/2}$ is the length of a rigid rod.

Viscosity. Samples of chromatin and DNA were dialysed against 100 vol. (two changes) of 0.7 mM-sodium-phosphate buffer, pH 7.5, at 0 - 4°C. Then relative viscosities were measured at 20°C in rotating cylinder viscometer (Beckman, U.S.A.). Intrinsic viscosities were obtained by extrapolating reduced viscosity (η_{sp}/c) to zero concentration and to zero shear gradient.

Partial specific volumes. The partial specific volumes for DNA and histones were taken as: 0.58 ml/g and 0.75 ml/g, respectively. The partial specific volumes for chromatin samples were calculated as linear combinations of these values using the histone to DNA ratios.

Molecular weight. The molecular weight of DNA was calculated from intrinsic viscosity according to Eigner & Doty (1965). The intrinsic viscosity of DNA determined in SSC (0.15 M-NaCl - 0.015 M-sodium citrate) at 20°C was 67 dl/g which corresponds to a molecular weight of 12.5×10^6. Molecular weights of chromatin and partially histone-depleted chromatin were calculated using molecular weight of DNA and protein to DNA ratios.

Dimensions of DNA and chromatin particles. The axial ratios of molecules were calculated using the equation of Simha (1940) for prolate ellipsoids. The lengths of equivalent ellipsoids were calculated according to the equation:

$$L = 2a = 6.82 \times 10^{-8} ([\eta]\, M)^{1/3} (p^2/\nu)^{1/3}$$

where: $[\eta]$ is intrinsic viscosity; M, molecular weight; $p = a/b$ is axial ratio; ν, a shape factor being a function of p; L is expressed in centimeters.

Packing ratios of DNA. A packing ratio (L_0/L) is defined as contour length of DNA ($L_0 = 6250$ nm) per length of molecule (the length of equivalent ellipsoid).

Reagents. Sepharose 4B was from Pharmacia (Uppsala, Sweden). Reagents for gel electrophoresis were from Serva (Heidelberg, G.F.R.).

RESULTS AND DISCUSSION

The molecular dimensions of particles of whole chromatin, chromatin selectively depleted of histone *f1* and of all lysine-rich histones, as well as fully dehistonized chromatin and DNA, were calculated from viscosimetry and light scattering data.

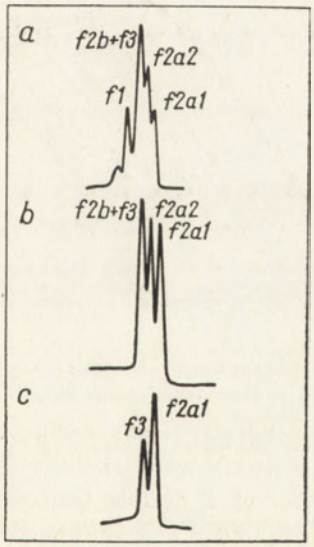

Fig. 1

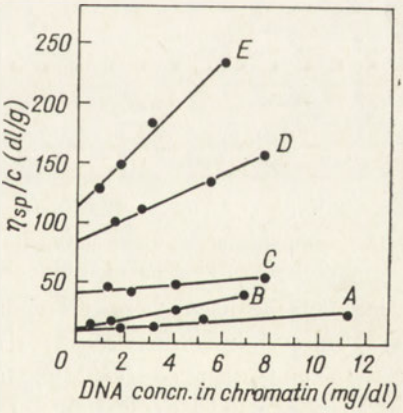

Fig. 2

Fig. 1. Densitometer scans of electrophoretic patterns of acid-extracted histones from: *a*, whole chromatin; *b*, chromatin preparation after 0.6 M-NaCl treatment; *c*, chromatin preparation after treatment with 0.3 M-NaCl -4 M -urea - 0.05 M-sodium phosphate buffer, pH 7.0.

Fig. 2. Variation of reduced viscosity of: *A*, whole chromatin; *B*, chromatin depleted of histone *f1*; *C*, chromatin depleted of histones: *f1*, *f2b* and *f2a2*; *D*, dehistonized chromatin; *E*, DNA isolated from chromatin.

The characteristics and histone composition of the analysed preparations are presented in Table 1 and Fig. 1, respectively. The reduced viscosity and Zimm plots for these preparations are given in Fig. 2 and Fig. 3, respectively. The

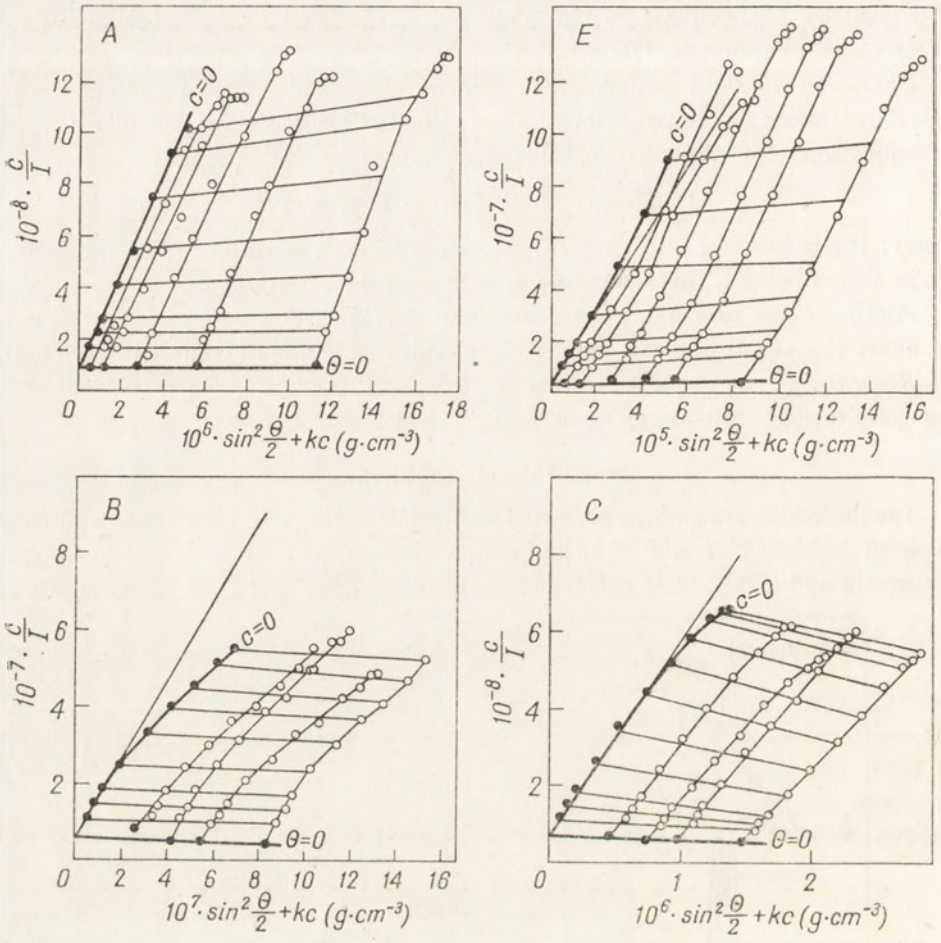

Fig. 3. The Zimm plots of: *A*, whole chromatin; *B*, chromatin depleted of histone *f1*; *C*, chromatin depleted of all lysine-rich histones; *E*, DNA isolated from chromatin.

dimensions of particles were determined independently from radius of gyration ($\langle s^2 \rangle^{1/2}$) and intrinsic viscosity ([η]). From the values of radius of gyration the end to end distance ($\langle h^2 \rangle^{1/2}$) for a model of a flexible Gaussian chain and the length of particles ($\langle l^2 \rangle^{1/2}$) for a model of rigid rod were calculated directly. From the values of intrinsic viscosities the axial ratios (p=a/b) and the lengths of particles were calculated only for a model of prolate ellipsoid, using the values of molecular weight (M) and partial specific volumes ($\bar{V}$) (cf. Table 1). The obtained results are given in Table 2. The comparison of the dimensions of particles shows that very similar results are obtained only for a model of rigid rod (light scattering) and for a model of ellipsoid (viscosimetry). Therefore the packing ratios were calcu-

Table 1

Characterization of chromatin preparations

Molecular weights of chromatin particles were calculated from molecular weight of DNA (determined from viscosity measurements) and protein to DNA weight ratios. Partial specific volumes ($\bar{V}$) for chromatin samples were calculated as described in Materials and Methods.

Preparation	Weight ratios			Mol. wt. $\times 10^{-6}$	$\bar{V}$ (ml/g)
	DNA	histone	non-histone protein		
Whole chromatin	1.00	1.20	0.25	30.6	0.66
Chromatin depleted of histone *f1*	1.00	1.00	0.18	27.3	0.64
Chromatin depleted of histones: *f1*, *f2a2*, *f2b*	1.00	0.42	0.12	23.3	0.61
Dehistonized chromatin	1.00	0.00	0.14	14.7	0.58
DNA isolated from chromatin	1.00	0.00	0.00	12.5	0.58

Table 2

Molecular constants and packing ratios of DNA in chromatin determined from viscosity and light scattering measurements

Conditions of experiments, calculations and symbols as described in Materials and Methods.

Preparation	$[\eta]$ (dl/g)	p=a/b	L (nm)	L_0/L	$\langle s^2 \rangle^{1/2}$ (nm)	$\langle h^2 \rangle^{1/2}$ (nm)	$\langle l^2 \rangle^{1/2}$ (nm)
Whole chromatin	4	100	863	7.2	260	640	900
Chromatin depleted of histone *f1*	5	115	906	6.9	220	540	760
Chromatin depleted of histones *f1*, *f2a2* and *f2b*	30	336	1721	3.6	180	440	620
Dehistonized chromatin	80	609	2156	2.9	320	785	1105
DNA isolated from chromatin	115	690	2220	2.8	360	845	1240

lated from dimensions of equivalent ellipsoids. The value 6.9 of packing ratio of DNA for chromatin particles selectively depleted of histone *f1* was found to be similar to that for whole chromatin. This value decreases to 3.6 for chromatin depleted of all lysine-rich histones and to 2.9 for fully dehistonized preparations.

The presented results indicate that, with the exception of histone *f1*, each of the other four histones (*f2b*, *f2a2*, *f2a1*, *f3*) may participate in maintaining DNA in a compact form in nucleohistone fibres (chromatin solubilized by shearing).

REFERENCES

Bolund, L. A. & Johns, E. W. (1973) *Eur. J. Biochem.*, **35**, 546 - 553.
Eigner, J. & Doty, P. (1965) *J. Mol. Biol.*, **12**, 549 - 580.
Frisman, E. V., Sibileva, N. A., Diakova, E. B., Vorobev, V. I. & Turoverova, L. V. (1974) *Biopolymers*, **13**, 863 - 877.
Henson, P. & Walker, I. O. (1971) *Eur. J. Biochem.*, **22**, 1 - 4.
Johns, E. W. (1967) *Biochem. J.*, **104**, 78 - 82.
Kaliński, A. & Toczko, K. (1974) *Acta Biochim. Polon.*, **21**, 67 - 72.
Kaliński, A., Seyfried, A. & Toczko, K. (1972) *Acta Biochim. Polon.*, **19**, 377 - 382.
Krueger, R. C. & Allison, D. P. (1973) *Biochim. Biophys. Acta*, **312**, 259 - 266.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.
Marushige, K. & Bonner, J. (1966) *J. Mol. Biol.*, **15**, 160 - 174.
Simha, R. (1940) *J. Phys. Chem.*, **44**, 25 - 34.
Stern, H. (1968) in *Methods in Enzymology* (L. Grossman & K. Moldave, eds.) vol. 12, part B, pp. 110 - 111. Academic Press, New York.
Subirana, J.A. (1973) *J. Mol. Biol.*, **74**, 363 - 386.
Toczko, K. & Kaliński, A. (1974a) *Bull. Acad. Polon. Sci., Ser. Sci. Biol.*, **22**, 163 - 165.
Toczko, K. & Kaliński, A. (1974b) *9th FEBS Meeting, Budapest; Abstr.* s3c 17, p. 146.
Toczko, K., Dobrzańska, M. & Chmielewska, I. (1972) *Acta Biochim. Polon.*, **19**, 19 - 24.
Zimm, B. (1948) *J. Chem. Phys.*, **16**, 1093 - 1098.

ZMIANY WIELKOŚCI I KSZTAŁTU CZĄSTECZEK CHROMATYNY WYNIKAJĄCE Z USUWANIA OKREŚLONYCH HISTONÓW

Streszczenie

Wykonano badania metodą wiskozymetryczną i rozpraszania światła dla preparatów chromatyny, chromatyny z selektywnie usuniętym histonem *f1*, chromatyny z usuniętymi histonami lizynowymi (*f1*, *f2b*, *f2a2*) oraz samego DNA. Z uzyskanych pomiarów obliczono wymiary cząsteczek w poszczególnych preparatach oraz stopień upakowania DNA. W chromatynie stopień upakowania DNA wynosi 7.2 i ulega nieznacznej zmianie po usunięciu z chromatyny histonu *f1* (6.9). Sukcesywne usuwanie pozostałych czterech histonów powoduje obniżenie stopnia upakowania DNA proporcjonalnie raczej do ilości a nie rodzaju oddysocjowanych białek.

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THE EFFECT OF CALCIUM RESTRICTION IN THE DIET ON CALCIUM TRANSPORT IN RAT SMALL INTESTINE*

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1. The everted-sac technique has been used to study the time-dependent effect of low-calcium diet on calcium active transport along rat small intestine.

2. In animals maintained on standard diet active translocation of calcium was limited to proximal 10 cm of the intestine.

3. In response to calcium restriction, calcium transport in duodenum was highly stimulated after 3 days, then gradually declined and after 28 days almost disappeared. In proximal jejunum it was the highest between 7 and 21 days. In distal ileum, the transport appeared after 3 days and increased progressively until 21 days, but markedly decreased at the 28-th day. The normal pattern of calcium transport was reestablished on refeeding the animals with standard diet.

In response to restriction in dietary calcium intake, an adaptive increase in the intestinal calcium absorption is known to occur (Fairbanks & Mitchell, 1936; Rottenstein, 1938). On normal diet, the active calcium translocation in the rat is apparently limited to the proximal one-fifth of the small intestine and operates mainly in duodenum (Schachter *et al.*, 1960). On the other hand, the distal part of the small intestine seems to be relatively impermeable to calcium (Schachter *et al.*, 1961), and Wasserman (1964) postulated the existence of a metabolically dependent block to calcium absorption. The appearance of an active mechanism mediating calcium translocation in distal ileum of animals adapted to low-calcium diets is associated with an increase in active transport and enhanced carrier affinity for calcium in the duodenum (Kimberg *et al.*, 1961; Walling & Rothman, 1969, 1970). An increase was also observed in intestinal concentration of the calcium-binding protein, a component of the calcium transport system (Wasserman & Taylor, 1965; Schachter *et al.*, 1967).

In the present work the time-dependent effects of prolonged dietary calcium restriction on the active transport of calcium along rat small intestine were examined.

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MATERIALS AND METHODS

Animals. Wistar rats of either sex were used. The animals were fed on standard laboratory diet (Bacutil, Poland) or low-calcium diet (containing 0.02% Ca) as described by Kenny & Munson (1959) and had access to demineralized water. The calcium-deficient diet was introduced after the weaning at a proper time so that after a desired period of feeding the age of animals was 5 - 6 weeks. Each rat received a supplement of 150 i.u. of vitamin D_3 once a week by stomach intubation. The animals were fasted for 16 hours, then killed by a blow on the head followed by decapitation.

Preparation of segments of the small intestine. The small intestine (from pylorus to the ileocaecal valve) was immediately removed and washed with ice-cold 0.9% NaCl solution. Then the intestine was placed on wet filter paper and eight 5-cm long segments were taken as shown in Fig. 1.

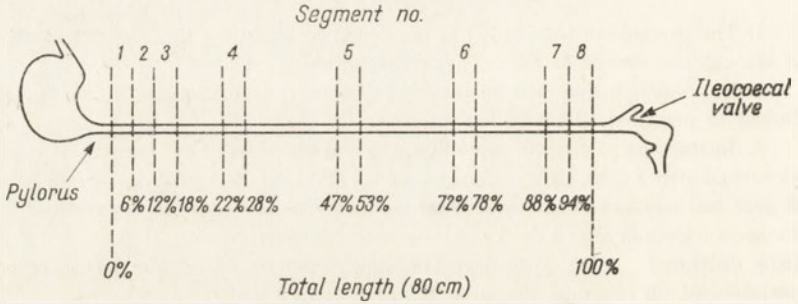

Fig. 1. Diagram of small intestine dissections.

Incubation. The incubation medium (pH 7.4) consisted of 25 mM-Tris-HCl buffer, pH 7.4, 20 mM-fructose, 0.8 mM-NaH_2PO_4, 70 mM-NaCl, 100 mM-mannitol and 0.4 mM-$CaCl_2$ containing ^{45}Ca (20 μCi/100 ml).

The everted intestinal sacs were prepared as described by Wróbel *et al.* (1973), the serosal compartment was filled with 0.5 ml of the incubation medium, the sac was placed in Erlenmeyer flask containing 5 ml of the medium and incubated with shaking for 90 min at 37°C under continuous flow of oxygen. Following incubation, in the internal (serosal) fluid, total and radioactive calcium was determined.

Determination of radioactivity and total calcium. ^{45}Ca radioactivity was measured by the liquid scintillation technique as described previously by Wróbel *et al.* (1973). Total calcium was determined by complexometric titration using murexide as indicator according to Schachter *et al.* (1960).

Presentation of results. Calcium transport was calculated as the difference between the content of ^{45}Ca (or ^{40}Ca) in the serosal fluid before and after incubation, and was expressed as net accumulation of calcium (nmoles/sac/90 min).

Reagents. Vitamin D_3 (sol. aquosa) was from Polfa (Tarchomin, Poland). $^{45}CaCl_2$ (spec. act. 312 mCi/mmole) was supplied by Ośrodek Produkcji i Dystrybucji Izotopów (Warszawa, Poland). All other reagents were analytical grade products.

RESULTS

Calcium transport by everted sacs from different segments of rat small intestine, and the effect of feeding the animals for 3 - 28 days with calcium-deficient diet, is shown in Fig. 2. In animals kept on standard diet (control), the transport of calcium was observed only in the proximal 10 cm of the small intestine, and proceeded mainly in duodenum (segment no. 1). In rats fed on low-calcium diet for 3 days the transport capacity in proximal jejunum (segments no. 1 and 2) was markedly enhanced. Simultaneously, the transport of calcium appeared also in proximal jejunum (segment no. 3) and distal ileum. After 7 days the transport was further enhanced, except in duodenum where it began to decrease; this decrease continued till the 28th day, when the transport in duodenum was abolished almost completely. After 14 days, a decrease was observed in calcium transport in proximal jejunum (segments no. 2 and 3), and a further increase in distal ileum (segments no. 7 and 8); in these segments the activity increased again after 21 days but decreased after 28 days, being then about the same as after 3 days of low-calcium feeding. After 28 days the activity in segments no. 2 and 3 was also quite low. It should

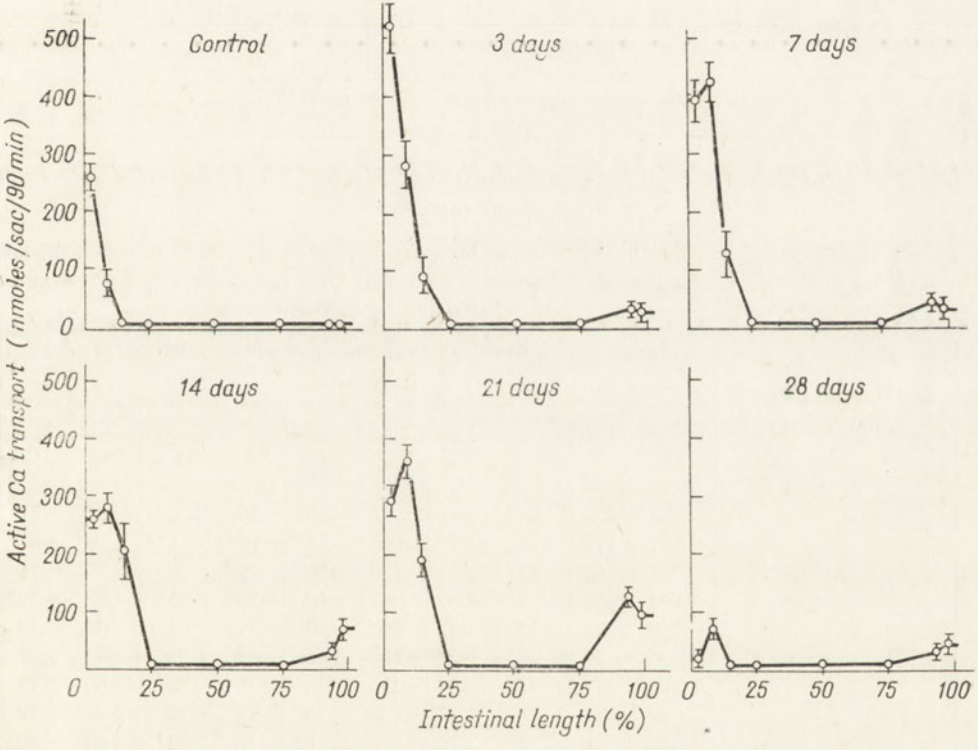

Fig. 2. Active calcium transport by everted sacs from different segments along the small intestine of rats maintained on standard laboratory diet (control) or fed on low-calcium diet for the indicated time. Experimental details as described in Materials and Methods. The results are the means from 6 - 8 animals; vertical lines represent ±S.E.M. Distribution of intestinal segments in percentages of the whole intestine length was calculated assuming the total mean length of 80 cm (cf. Fig. 1).

be noted that throughout the experiment no activity was observed in lower jejunum and proximal ileum.

When the rats which had been kept for 28 days on low-calcium diet, were fed on standard diet for the next 5 days, the normal pattern of calcium transport was reestablished (Table 1).

Table 1

Effect of refeeding with standard diet, after a prolonged period on low-calcium diet, on calcium transport in rat small intestine

The animals were maintained on low-calcium diet from 28 days, then refed with standard diet for 5 days. For details see Materials and Methods. Mean values from 6 - 8 animals ±S.D. are given.

Treatment	Calcium transport (nmoles/sac/90 min)							
	Segment no.							
	1	2	3	4	5	6	7	8
Standard diet	261 ±65	93 ±63	0	0	0	0	0	0
Low-calcium diet	20 ±16	73 ±36	0	0	0	0	25 ±17	40 ±16
Calcium refeeding	175 ±54	88 ±47	0	0	0	0	0	0

Table 2

Effect of low-calcium diet on the accumulation of ^{45}Ca and ^{40}Ca by everted sacs from rat small intestine

Experimental details as described in Materials and Methods. The values are means from 4 - 8 animals ±S.D. The differences between the accumulation of ^{45}Ca and ^{40}Ca calculated with the Student's *t* test show: $^{**}P<0.001$, $^{*}0.1<P<0.05$.

Low-calcium diet (days)	Accumulation of calcium in serosal medium (nmoles/sac/90 min)									
	Segment no.									
	1		2		3		7		8	
	^{45}Ca	Ca	^{45}Ca	Ca	^{45}Ca	Ca	^{45}Ca	Ca	^{45}Ca	Ca
3	519 ±107	470 ±176	284 ±102	308 ±135	92 ±66*	160 ±72*	27 ±16**	126 ±41**	25 ±30*	77 ±61*
7	392 ±93	369 ±50	427 ±95	350 ±40	128 ±67	150 ±40	45 ±33*	76 ±23*	37 ±25*	74 ±29*
14	250 ±37	279 ±76	278 ±75	267 ±97	202 ±100	140 ±68	31 ±9	62 ±21	66 ±30	89 ±27
21	296 ±60	277 ±46	359 ±61	384 ±77	194 ±54	218 ±80	130 ±19	129 ±37	100 ±40	119 ±29
28	20 ±16	20 ±25	73 ±36	78 ±33	0	0	25 ±17	32 ±17	40 ±16	40 ±19
Standard diet (control)	261 ±63	225 ±37	93 ±63	109 ±34	0	0	0	0	0	0

Considering previous evidence (Michalska *et al.*, 1972) which indicated that distal ileum of rat was able to secrete actively endogenous calcium, accumulation of ^{40}Ca and ^{45}Ca by everted sacs from different segments of the small intestine of rats maintained on low-calcium diet was measured. In the present work only the segments showing adaptive response in concentrative calcium transport were examined. The results obtained are summarized in Table 2. Accumulation of ^{40}Ca exceeded markedly accumulation of ^{45}Ca only in segment no. 7 after 3 days of calcium restriction in the diet. Other differences were statistically insignificant.

DISCUSSION

The presented results indicate that adaptive response to restricted calcium intake differs markedly in the intestinal segments investigated and is dependent on the period of feeding with the low-calcium diet. The data obtained suggest possible participation of two mechanisms of adaptation of calcium active transport along small intestine, which differ in site and in time of response.

The first mechanism is involved in the increase in calcium transport against a concentration gradient in the proximal region of small intestine. It is characterized by maximal stimulation of active transport in the duodenum and to a lesser extent in the contiguous part of upper jejunum, occurring after 3 days of dietary calcium deficiency. When calcium restriction is prolonged to 7 - 21 days, duodenal calcium transport declines whereas the transport in upper jejunum is further stimulated. The most efficient calcium active transport occurs under these conditions in the jejunal segment nearest to duodenum.

The second mechanism is involved in the appearance of active transport in distal ileum. The transport of calcium against a concentration gradient in the ileocaecal end becomes significant beginning with the 7th day of dietary calcium deficiency and increases progressively until the 21st day. It should be emphasized that during the treatment no adaptive response was seen in lower jejunum and proximal ileum. When calcium restriction in the diet is continued for 28 days, a marked decrease in concentrative transport of calcium by both the proximal and distal part of small intestine is observed. In the duodenal segment, active transport is abolished almost completely. Thus it seems likely that the adaptation mechanisms affecting active calcium transport in rat small intestine are limited in capacity and do not operate after a prolonged calcium deficiency in the diet. However, the effects of prolonged calcium restriction may be reversed by standard diet.

It should be noted that the response of small intestine to calcium deficiency involves also changes in the ability of distal ileum to release endogenous calcium. This process was found previously to be vitamin D-sensitive and affected by the presence of inorganic phosphate in the incubation medium (Michalska *et al.*, 1972). Active secretion of calcium by rat ileum and jejunum was also reported by Walling & Kimberg (1973). However, the adaptive significance of this phenomenon remains unclear.

It is well documented that adaptation to low-calcium diet depends on the presence of vitamin D (Nicolaysen, 1943), as vitamin-deficient animals show no change in intestinal calcium absorption regardless of the dietary calcium content. Recent findings concerning regulation of vitamin D metabolism indicate that the synthesis of 1,25-dihydroxy-vitamin D_3, a metabolite active in the intestine, is controlled by dietary calcium (Boyle *et al.*, 1971; Omdahl *et al.*, 1972). In our experiments vitamin D_3 was administered to all animals, and the adaptive response in calcium active transport correlated also with the intestinal distribution of 1,25-dihydroxy-vitamin D_3 after repletion with 25-hydroxy-vitamin D_3, which was shown to be highest in duodenum, much lower in ileum and still lower in jejunum of the rat (Walling *et al.*, 1974).

REFERENCES

Boyle, I. T. R., Gray, W. & DeLuca, H. F. (1971) *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 2131 - 2134.
Fairbanks, B. W. & Mitchell, H. K. (1936) *J. Nutr.*, **11**, 551 - 572.
Kenny, A. D. & Munson, P. L. (1959) *Endocrinology*, **64**, 513 - 521.
Kimberg, D. V., Schachter, D. & Schenker, H. (1961) *Am. J. Physiol.*, **200**, 1256 - 1262.
Krawitt, E. L., Stubbert, P. A. & Ennis, P. H. (1973) *Am. J. Physiol.*, **224**, 548 - 551.
Michalska, L., Wróbel, J. & Niemiro, R. (1972) *Acta Biochim. Polon.*, **19**, 333 - 339.
Nicolaysen, R. (1943) *Acta Physiol. Scand.*, **6**, 201 - 209.
Omdahl, J. L., Gray, R. W., Boyle, I. T., Knutson, J. & DeLuca, H. F. (1972) *Nature New Biol.*, **237**, 63 - 64.
Rottenstein, K. V. (1938) *Biochem. J.*, **32**, 1285 - 1292.
Schachter, D., Dowdle, E. B. & Schenker, H. (1960) *Am. J. Physiol.*, **198**, 263 - 268.
Schachter, D., Kimberg, D. V. & Schenker, H. (1961) *Am. J. Physiol.*, **200**, 997 - 999.
Schachter, D., Kowarski, S. & Reid, P. (1967) *J. Clin. Invest.*, **46**, 1113.
Walling, M. W. & Rothman, S. S. (1969) *Am. J. Physiol.*, **217**, 1144 - 1148.
Walling, M. W. & Rothman, S. S. (1970) *J. Biol. Chem.*, **245**, 5007 - 5011.
Walling, M. W. & Kimberg, D. V. (1973) *Am. J. Physiol.*, **225**, 415 - 422.
Walling, M. W., Favus, M. J. & Kimberg, D. V. (1974) *J. Biol. Chem.*, **249**, 1156 - 1161.
Wasserman, R. H. (1964) *Nature (London)*, **201**, 997 - 999.
Wasserman, R. H. & Taylor, A. N. (1965) *J. Biol. Chem.*, **243**, 3987 - 3993.
Wróbel, J., Michalska, L. & Niemiro, R. (1973) *FEBS Lett.*, **29**, 121 - 123.

WPŁYW NIEDOBORU WAPNIA W DIECIE NA TRANSPORT WAPNIA W JELICIE CIENKIM SZCZURA

Streszczenie

1. Metodą odwróconych woreczków jelitowych badano zależny od czasu karmienia wpływ diety niskowapniowej na aktywny transport wapnia w jelicie cienkim szczurów.

2. U zwierząt karmionych dietą standartową aktywna translokacja wapnia ograniczała się do początkowych 10 cm jelita.

3. Niedobór wapnia w diecie silnie stymulował transport wapnia w dwunastnicy po 3 dniach, następnie transport zmniejszał się i po 28 dniach znikał prawie całkowicie. W początkowej części jelita czczego transport był najwyższy w okresie pomiędzy 7 a 21 dniem. W końcowej części jelita krętego aktywny transport pojawiał się po 3 dniach i wzrastał stopniowo do 21 dnia, lecz zmniejszał się wyraźnie w 28 dniu. Ponowne karmienie zwierząt dietą standartową przywracało normalny obraz transportu wapnia.

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ALKALINE RIBONUCLEASE FROM RYE GERM CYTOSOL*

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1. Alkaline ribonuclease (pH optimum 7.6) was isolated from rye (*Secale cereale* L) germ cytosol and partially purified; the preparation was devoid of other nucleolytic activities.

2. The enzyme is a typical endonuclease hydrolysing all phosphodiester bonds in RNA, yielding ultimately purine and pyrimidine nucleoside 2′,3′-cyclic phosphates and the corresponding 3′-phosphates. Upon extensive digestion of synthetic polyribonucleotides, pyrimidine, but not purine, nucleoside 3′-phosphates are formed. The enzyme does not hydrolyse synthetic purine cyclic nucleotides.

3. The enzyme does not depolymerize double-stranded complexes of poly(A) and poly(U).

4. Susceptibility to photooxidation and inhibition by 2-hydroxy-5-nitrobenzyl bromide and *N*-bromosuccinimide implies the involvement of tryptophan residue in the active centre of the enzyme.

Plant ribonucleases are still considerably less known than the analogous enzymes in bacteria and animals, and in comparison with the latter enzymes they exhibit rather low specificity towards RNA. According to Lantero & Klosterman (1973), the partially purified plant ribonuclease preparations obtained so far may contain hardly separable enzymes of various specificities, the joint activity of which may mask the specificity of individual enzymes.

The post-ribosomal supernatant of rye germ exhibits high ribonucleolytic activity both at acid and alkaline pH values (Siwecka & Szarkowski, 1971), while most of the known plant ribonucleases show optimum activity within the acid pH range: 5.0 - 6.2 (Barnard, 1969).

Since no information is available on the occurrence and properties of plant alkaline ribonuclease, isolation of this enzyme from cytosol of rye germs was attempted, and some properties of the obtained preparation were studied. A preliminary account of this work has been presented (Kuligowska *et al.*, 1974).

* This work was supported by the Polish Academy of Sciences within the project 09.3.1.

MATERIALS AND METHODS

Plant material and isolation of embryos. Embryos were isolated from rye grains (*Secale cereale* L. *var.* Włoszanowskie) according to Jonston & Stern (1957) including flotation in 0.2 M-sucrose. For some experiments, rye embryos were purchased from Świebodzin Mills, Poland.

Preparation of 105 000 g supernatant (cytosol). The embryos were homogenized in a cooled porcelain mortar with 5 vol. of 0.13 mM-potassium phosphate buffer containing 0.25 M-sucrose and 10 mM-$MgCl_2$, pH 7.3. The homogenate was filtered through 3 layers of nylon gauze and subjected to three consecutive centrifugations: at 600 *g* (3 min), 12 000 *g* (15 min) and 105 000 *g* (2 h). The 105 000 *g* supernatant was used as a source of the enzyme.

Chemicals. Mononucleotides, cyclic mononucleotides, *p*-nitrophenyl phosphate (disodium salt), bis-*p*-nitrophenyl phosphate (sodium salt) and tRNA were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Highly polymerized wheat germ RNA was obtained from Calbiochem (Los Angeles, Calif., U.S.A.). Sephadex G-50 and G-75 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All the homopolymers were Miles Laboratories (Elkhart, Ind., U.S.A.) products. DEAE-cellulose was obtained from Reanal (Budapest, Hungary). Yeast RNA (Fluka, Buchs, Switzerland) was purified according to Kunitz (1940). DNA from calf thymus was purchased from Merck (Darmstadt, G.F.R.), *N*-bromosuccinimide from Sojuzchimexport (Moscow, U.S.S.R.) and 2-hydroxy-5-nitrobenzyl bromide from Schuchardt (München, G.F.R.).

Assay of enzymatic activities. RNase activity was assayed by the method of Anfinsen *et al.* (1954); 0.1 - 0.3 ml of ribonuclease preparation was added to 1 ml of 0.4% buffered solution of yeast RNA as indicated in the legend to Fig. 1. The sample was made up with buffer to 2 ml and incubated for 1 h at 37°C. The reaction was stopped by the addition of 0.5 ml of 0.75% uranyl acetate solution in 25% perchloric acid. The samples were cooled for 15 min, centrifuged for 10 min at 6000 *g*, 0.2 ml of the supernatant was diluted with 15 vol. of water, and the absorbance was read at 260 nm. The enzyme unit is defined as the amount of activity required to increase the absorbance at 260 nm by 0.01 unit under the conditions of the assay. Specific activity is expressed in units per mg of protein.

DNase activity was assayed in the same way as that of RNase except that DNA was used as a substrate.

Phosphodiesterase and phosphomonoesterase activities were determined by monitoring the increase in absorbance at 400 nm due to liberation of *p*-nitrophenol from bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl phosphate, respectively, as described by Udvardy *et al.* (1969).

Hydrolysis of homopolymers and double-stranded complexes. Hydrolysis of poly(A), poly(C), poly(U) and the mixture of poly(A) and poly(U) at a ratio of 1:2, was carried out as described by Chakraburtty & Burma (1968). The reaction mixture contained in the final volume of 2 ml: 1 mg of substrate and 60 μg of enzyme protein in 0.1 M-Tris-HCl buffer, pH 7.6. The incubation was carried out at 37°C

up to 4 h. After incubation, 0.5 ml of a 0.75% uranyl acetate solution in 25% trichloroacetic acid was added. The mixture was allowed to stand overnight at 4°C and the precipitate was centrifuged off, then 0.2 ml of the supernatant was diluted with 15 vol. of water and the absorbance was read at 260 nm.

Fractionation of RNA hydrolysis products. To determine the mode of action of alkaline RNase, products of partial hydrolysis were analysed by the method of Birnboim (1966) as described by Wyen *et al.* (1969) with some modifications: 2 mg of highly polymerized RNA dissolved in 0.8 ml of 0.1 M-Tris-HCl buffer, pH 7.6, was incubated with 90 μg of enzyme protein at 37°C for 1 and 18 h. The reaction was stopped by the addition of 5 μl of diethylpyrocarbonate, then 0.1 M-Tris-HCl buffer, pH 7.6, was added to each sample to a total volume of 2 ml. The samples were applied to Sephadex G-50 columns (1 × 20 cm) equilibrated with the same buffer. The native nucleic acid and the breakdown products were eluted with 0.1 M-Tris-HCl buffer, pH 7.6; 2 ml fractions were collected and the absorbance was measured at 260 nm. For comparative purposes, RNA hydrolysate obtained with the use of snake venom phosphodiesterase (exonuclease) and pancreatic RNase (endonuclease) was fractionated under the same conditions.

For examination of the final degradation products, 5 mg of highly polymerized RNA was incubated with the enzyme (90 μg of protein) in 5 mM-Tris-HCl buffer, pH 7.6, containing 0.1 M-$MgCl_2$ in a total volume of 3 ml. The mixture was incubated for 48 h at 37°C in the presence of traces of toluene. The reaction was terminated by heating the sample at 100°C for 10 min. After cooling, the sample was applied to a DEAE-cellulose column (1 × 20 cm) equilibrated with 0.01 M-NH_4HCO_3. The breakdown products were eluted with a linear concentration gradient of 0.01 - 0.4 M-NH_4HCO_3, pH 8.6. Fractions of 4 ml were collected and the absorbance was measured at 260 nm.

Identification of degradation products. The appropriate fractions of highly polymerized RNA and synthetic polyribonucleotide hydrolysates were pooled, concentrated and subjected to ascending chromatography on Whatman 3*MM* paper with isopropanol - ammonia - water (7:1:2, by vol.) as a solvent. The separated products were visualized under u.v. light, eluted with 0.1 M-HCl for 24 h, and their absorbance was monitored at 260 nm.

Polyacrylamide-gel electrophoresis. This was carried out according to Davis (1964) in 7.5% gel at pH 8.4; 4.0 mA current was applied per tube for 1-h run. Gels were stained with 0.5% Amido Black in 7% acetic acid. Ribonucleases were detected according to Wilson (1971).

Photooxidation. The enzyme protein, 60 μg, was dissolved in 700 μl of 0.1 M-acetate or Tris-HCl buffer (pH 3.0 - 8.4), and irradiated in the presence of methylene blue (700 μl of 0.05% aq. solution) from a distance of 20 cm with a 100 W incandescent lamp, at 0°C for 1 h.

Inhibition by the tryptophan-modifying compounds. The enzyme protein, 60 μg, was preincubated with 3 mmoles of *N*-bromosuccinimide in 0.5 ml of 0.1 M-acetate buffer, pH 5.0, and RNase activity was assayed under standard conditions.

2-Hydroxy-5-nitrobenzyl bromide, freshly prepared, was added in two portions at a 15-min interval, each time 20 µl of 30% solution in acetone, to 2.5 ml of enzyme solution containing 300 µg of protein in 0.1 M-acetate buffer, pH 5.0. In some experiments 8 M-urea was added to the enzyme solution and kept for 15 min at room temperature prior to the addition of the reagent. The reaction was allowed to proceed for 30 - 60 min at 37°C with magnetic stirring. At intervals, 500 µl portions of the reaction mixture were withdrawn and the enzyme activity was assayed. The control sample consisted of the RNase kept in the same buffer containing 8 M-urea, without the reagent.

Estimation of protein content. The elution of protein from the chromatographic columns was followed by measuring absorbance at 280 nm. In the enzymatic preparations, insoluble protein was determined by the method of Lowry *et al.* (1951) with purified trypsin as a standard.

RESULTS

Isolation and purification of the enzyme. All manipulations were carried out at 4°C. The purification procedure, presented in Table 1, consisted of 5 steps.

Step 1: Acidification. The 105 000 *g* supernatant (250 ml) was brought to pH 4.5 with acetic acid; after 3 h the precipitate formed was removed by centrifugation and the supernatant adjusted to pH 7.0 with 1 M-NaOH.

Table 1

Purification of alkaline RNase from rye germ cytosol

For details see the text.

Step	Volume (ml)	Protein (mg/ml)	Enzyme activity (units/ml)	Specific activity (units/mg protein)	Yield (%)	Purification factor
105 000 *g* supernatant	250	18.5	125 000	25	100	1
1. Acidification to pH 4.5	150	14.0	117 850	55	99	2
2. $(NH_4)_2SO_4$ fractionation	30	9.0	61 360	225	52	9
3. DEAE-cellulose	12	5.5	65 300	980	56	37
4. Sephadex G-75	5	2.2	18 300	1650	15	66
5. Phosphocellulose	10	0.3	7 420	2450	6	98

Step 2: Ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$ was added, with continuous stirring, to the supernatant from Step 1 to 0.55 saturation. After 1 h the sediment was centrifuged off at 12 000 *g* for 30 min and discarded. Solid $(NH_4)_2SO_4$ was added to the clear supernatant to 0.8 saturation and left standing overnight. The precipitate was centrifuged off as previously, dissolved in 20 ml of 0.1 M-citrate-phosphate buffer, pH 7.0, dialysed against the same buffer at concentration of 1 mM, centrifuged again for 15 min at 12 000 *g*, and the supernatant was collected.

Step 3: DEAE-cellulose chromatography. The final supernatant from Step 2 (270 mg of protein) was applied to a DEAE-cellulose column (3 × 40 cm) equilibrated with 0.1 M-citrate-phosphate buffer, pH 7.0. The column was washed with the same buffer and eluted with the buffer containing 1 M-NaCl. The flow rate was 1.5 ml/min and 6-ml fractions were collected.

Step 4: Sephadex G-75 chromatography. RNase-containing fractions from DEAE-cellulose were dialysed, lyophilized, dissolved in 3 ml of 0.1 M-Tris-HCl buffer, pH 7.5 (70 mg of protein) and applied to Sephadex G-75 column (1.5 × 60 cm) equilibrated with 0.1 M-Tris-HCl buffer, pH 7.5; the column was eluted with the equilibrating buffer and 5-ml fractions were collected.

Step 5: Phosphocellulose chromatography. Fractions from step 4 showing RNase activity were pooled (11 mg of protein) and applied to a phosphocellulose column (2.0 × 25 cm) which was equilibrated with 0.1 M-Tris-HCl buffer, pH 7.5, and eluted with a linear gradient of 0.0 to 0.3 M-NaCl in the same buffer; fractions of 5 ml were collected. RNase activity emerged at low NaCl concentration. The most active fractions were combined and dialysed against 1 mM-Tris-HCl buffer, pH 7.6.

The cytosol RNase from rye germs was purified by the described procedure about 100-fold. The preparation obtained was completely devoid of phosphomonoesterase and phosphodiesterase activities, and it did not hydrolyse DNA. Polyacrylamide-gel electrophoresis revealed the presence of a number of protein bands indicating heterogeneity of the preparation.

General properties. As it may be seen in Fig. 1, the purified preparation exhibited a dual pH optimum: a highly active peak at pH 7.6 and a peak of low activity at pH 6.0; the latter constituted about 10% of the activity of the former. This ratio remained constant throughout the whole purification procedure which might imply double pH optima of the same enzymatic activity. However, one cannot exclude definitely the presence of another enzymatic protein of lower pH optimum.

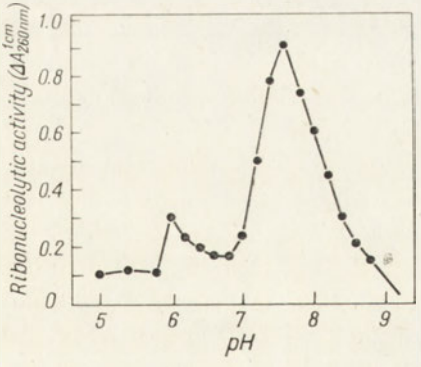

Fig. 1. Effect of pH on the activity of alkaline RNase from rye germ cytosol. Standard assay conditions. Yeast RNA and the following buffers were used: 0.1 M-acetate for pH 4.0 - 5.5; 0.1 M-citrate-phosphate for pH 5.5 - 7.0, and 0.1 M-Tris-HCl for pH 7.0 - 9.0.

Alkaline RNase from rye germ cytosol is a thermolabile enzyme showing the optimum activity at 60°C under the applied assay conditions.

The effect of various metal ions at concentrations of 20 mM on the enzyme activities are shown in Table 2. Cu^{2+}, Zn^{2+}, Mg^{2+} and Hg^{2+} caused a 30 - 80% inhibition. Mn^{2+}, Co^{2+}, Ca^{2+} and Fe^{2+} ions enhanced the activity two- to three-fold, whereas EDTA had no effect.

Table 2

Effect of metal ions and EDTA on alkaline RNase from rye germ cytosol

Standard assay conditions. Yeast RNA was used as a substrate. Preincubation of the enzyme with metal ions at concentration of 20 mM at 37°C for 10 min.

Addition	Relative activity	Addition	Relative activity
None	100	None	100
$ZnCl_2$	50	$CaCl_2$	363
$CuCl_2$	33	$BaCl_2$	124
$MgCl_2$	70	$HgCl_2$	15
$MnCl_2$	202	$CoCl_2$	185
$FeCl_2$	200	EDTA	109

The enzyme was not inhibited by adenosine-, guanosine-, cytidine- and uridine-2′,3′-cyclic phosphates; cytidine- and adenosine-2′,3′-phosphates; adenosine- and cytidine-5′-phosphates; or adenosine-3′,5′-cyclic phosphate, in 1 mM concentration.

Mode of action and substrate specificity. The digestion products of the highly polymerized wheat germ RNA were analysed by chromatography on Sephadex G-50. The chromatographic profile obtained upon 1-h hydrolysis (Fig. 2) showed the appearance of a single peak of oligonucleotides in addition to that corresponding to the initial highly polymerized RNA. The profile of the 18-h RNA digest revealed but a small peak of undegraded RNA and a peak corresponding to mononucleotides. The time-course of hydrolysis implies the endonuclease nature of the investigated enzyme preparation. Identification of the oligo- and mononucleotide fractions was based on the elution profiles obtained with the standard exo- and endonucleases.

To determine the nature of the products formed after exhaustive hydrolysis, RNA was incubated with RNase for 48 h, and the degradation products were separated on a DEAE-cellulose column. As seen in Fig. 3, five peaks were recorded. The respective fractions were identified by paper chromatography. Peak *I* was identified as 2′,3′-AMP and 2′,3′-GMP, peak *II* as a mixture of 2′,3′-CMP and 3′-CMP. Peak *III* contained 2′,3′-UMP and 3′-UMP. Peaks *IV* and *V* consisted of 3′-AMP and 3′-CMP, respectively. These results prove that all the phosphodiester bonds in RNA were hydrolysed by the RNase.

The synthetic polyribonucleotides (Fig. 4) were cleaved by the enzyme at a rate decreasing in the following order: poly(A)>poly(U)>poly(C). A 1:2 mixture

of poly(A) and poly(U) was not attacked by the enzyme. The rate of enzymatic hydrolysis of *Escherichia coli* tRNA was half that found for yeast RNA. In the presence of 100 mM-$MgCl_2$, the enzymatic hydrolysis of the former was shown to be almost completely inhibited.

On 12-h incubation of the enzyme with synthetic polynucleotides (Table 3), the corresponding nucleoside 2′,3′-cyclic phosphates were formed, the amount of which was further increased on 48-h incubation. Differences found in the amount

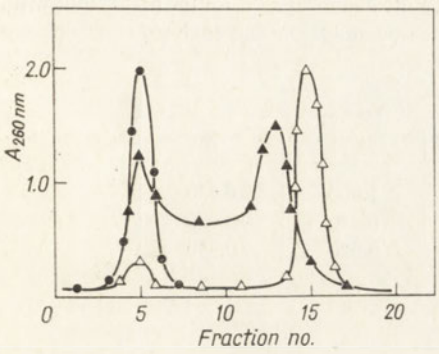

Fig. 2

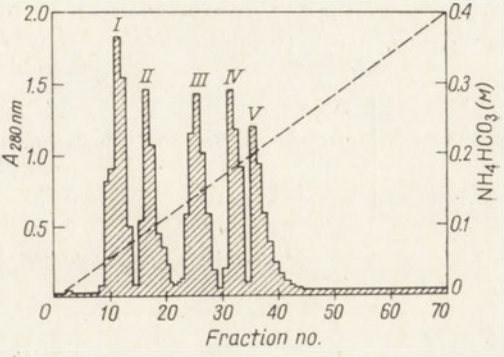

Fig. 3

Fig. 2. Sephadex G-50 gel filtration of breakdown products of yeast RNA with alkaline RNase from rye germ cytosol. Yeast RNA (2 mg) was incubated at pH 7.6 with the enzymatic preparation (90 μg protein) at 37°C. The degradation products were analysed by column chromatography as described in Methods: ●, prior to incubation; ▲, after 1-h incubation; △, after 18-h incubation.

Fig. 3. DEAE-cellulose column chromatography of RNA hydrolysate. Yeast RNA (5 mg) was hydrolysed exhaustively with the rye germ cytosol alkaline RNase (90 μg protein) at 37°C for 48 h, the degradation products were applied to the column and eluted with NH_4HCO_3 gradient as described in Methods.

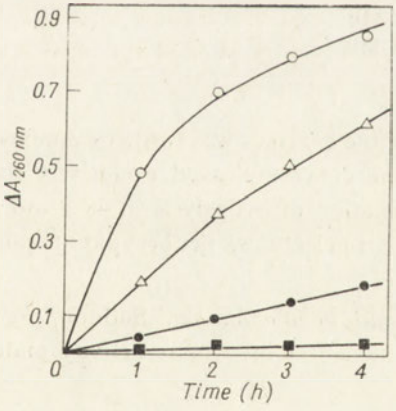

Fig. 4. Hydrolysis of polyribonucleotides by alkaline RNase from rye germ cytosol. ○, Poly(A); △, poly(U); ●, poly(C); and ■, poly(A)+poly(U) hybrid, at concentration of 0.5 mg/ml, were incubated with the enzyme (60 μg protein), and the degradation products were analysed as described in Methods.

of the respective cyclic phosphates are probably due to the fact that 2′,3′-AMP was not cleaved to the respective mononucleotide, while cytidine and uridine 3′-phosphates were formed, and were detected both in the 12-h and 48-h hydrolysate.

Table 3

Degradation of synthetic polyribonucleotides by alkaline RNase from rye germ cytosol

The reaction mixture contained in a final volume of 120 µl: 500 µg of substrate and enzyme solution (60 µg of protein) in 0.1 M-Tris-HCl buffer, pH 7.6. After incubation at 37°C for the period indicated, the digestion products (70 µl) were separated by paper chromatography and analysed spectrophotometrically.

Substrate	$A_{260\ nm}$		
	12 h	48 h	Δ
2′,3′-AMP	0.370	1.000	0.630
2′,3′-CMP	0.390	0.480	0.090
2′,3′-UMP	0.460	0.830	0.370

Table 4

Degradation of nucleoside cyclic phosphates by alkaline RNase from rye germ cytosol

The reaction mixture was as described for Table 3, except that the final volume was 250 µl and the amount of enzyme protein applied, 30 µg. Other conditions as described in Methods. Following incubation, the digestion products were separated by paper chromatography and the absorbance read at 260 nm.

Substrate	Cyclic phosphate hydrolysed (%)
2′,3′-AMP	0.0
2′,3′-GMP	0.0
2′,3′-CMP	21.8
2′,3′-UMP	45.7

Substrate specificity of the enzymes was further examined with cyclic phosphates. As shown in Table 4, the enzyme cleaved exclusively the pyrimidine nucleoside cyclic phosphates. Prolongation of hydrolysis to 48 h did not increase the amount of hydrolysed pyrimidine nucleotides, neither did it cause hydrolysis of purine cyclic nucleosides.

Effect of inhibitors and photooxidation. Sodium *p*-chloromercuribenzoate at 1 mM concentration had no effect on RNase activity under standard assay conditions.

Photooxidation of RNase at 0°C and pH 3.0 resulted in an almost complete inactivation of the enzyme; at pH 4.0 the enzyme retained about 25% of its activity. Illumination at pH 5.0 practically did not affect the activity, whereas at higher pH values the enzyme was inactivated (Fig. 5). The inactivation observed at pH

3.0 - 4.0 may involve oxidation of tryptophan or methionine (Sluyterman, 1962). To specify the target, the reagents reacting with tryptophan were applied. On 30-min reaction with 2-hydroxy-5-nitrobenzyl bromide (Fig. 6), the enzyme lost about

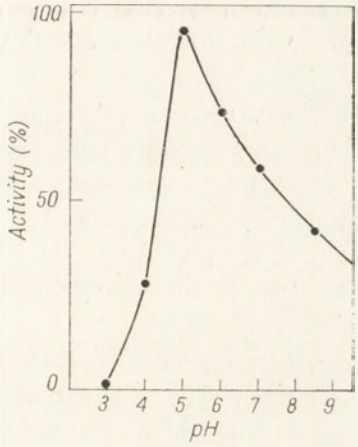

Fig. 5

Fig. 6

Fig. 5. pH-dependence of inactivation of alkaline RNase from rye germ cytosol by the methylene blue-catalysed photooxidation. The enzyme was irradiated as described in Methods and the activity in the irradiated samples (700 µl) was assayed.

Fig. 6. Inactivation of alkaline RNase from rye germ cytosol by 2-hydroxy-5-nitrobenzyl bromide. The enzyme solution (300 µg of protein) was incubated with 8 M-urea and the inhibitor at pH 5.0 as described in Methods. ●, Control (with 8 M-urea); reaction with 2-hydroxy-5-nitrobenzyl bromide: ■, in the presence of 8 M-urea; ▲, in the absence of urea.

70% of its activity both in the presence and absence of 8 M-urea. Preincubation of RNase with *N*-bromosuccinimide for 30 min resulted in a complete loss of enzymic activity.

DISCUSSION

The properties of alkaline RNase from rye germ cytosol are similar to those of other higher plant RNases with respect to susceptibility to metal ions (Merola & Davis, 1962; Friesch-Niggemeyer & Reddi, 1957; Holden & Pirie, 1955; Tang & Maretzke, 1970) and, like all plant RNases, the cytosol RNase from rye germ should be considered a cyclizing phosphotransferase acting on RNA endonucleolytically.

Degradation of RNA and synthetic polynucleotides by the alkaline RNase from rye germ cytosol is in some respects different. Although in both cases nucleoside cyclic phosphates are the intermediate products, adenosine 2′,3′-cyclic phosphate is the final product of poly(A) hydrolysis.

In contrast to the action on synthetic polynucleotides, the exhaustive hydrolysis of highly polymerized native RNA with the enzyme led to both purine and pyri-

midine nucleoside 3′-phosphates. One may assume that purine nucleoside cyclic phosphate linked with the polynucleotide chain through 5′-C is more readily degraded by the enzyme than in the free form. If so, however, a number of 5′-phosphodiester bonds would have to be decomposed during hydrolysis of the polynucleotide chain without passing through the cyclization stage. A similar mechanism of pyrimidine 3′-mononucleotides release has been suggested by Merola & Davis (1962) for soybean RNase.

Reddi (1966) divided RNases of higher plants into two groups according to their ability to hydrolyse nucleoside cyclic phosphates. Group I comprised the enzymes hydrolysing exclusively cyclic purine nucleosides, while group II, the enzymes hydrolysing both purine and pyrimidine cyclic nucleosides. This classification does not apply to the cytosolic RNase from rye germs since the enzyme cleaved only pyrimidine nucleoside cyclic phosphates to the corresponding 3′-nucleotides.

The inability to hydrolyse purine nucleoside cyclic phosphates is characteristic of the alkaline RNase from rye germ cytosol and seems not to be due to contamination with nucleotides (Barnard, 1969) since the addition of nucleotides to the incubation mixture did not change the rate of the reaction.

Bernardi & Bernardi (1966) established with RNases isolated from rye-grass (Schuster, 1957), cucumber (Kado, 1968) and sugar cane leaves (Tang & Maretzke, 1970) that the rate of enzymatic hydrolysis of synthetic polynucleotides depends on their conformation. This observation was not confirmed by our results. Poly(A) was found to be hydrolysed more rapidly than poly(U) and poly(C). Moreover, poly(C) and poly(A), but not poly(U), exhibit a highly-ordered structure at acid pH (Razzel, 1967; Steiner & Beers, 1959). Since degradation of these compounds by the enzymes was studied at pH 7.6, it seems that their secondary structure had but little influence on the rate of hydrolysis.

The photooxidative modification of the cytosolic alkaline RNase of rye germs and the inhibition exerted by *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide imply participation of the indol group of tryptophan residue in the active centre of the enzyme. So far, the nature of the active centre has not been elucidated for any plant RNase. The interaction between *N*-bromosuccinimide and tryptophan has been suggested for the barley RNase (Lantero & Klosterman, 1973). Studies on the active centre of animal, bacterial and fungal RNases are much more advanced. It is known that histidine is indispensable for pancreatic RNase activity (Findlay *et al.*, 1962a,b), whereas for fungal (Yamagata *et al.*, 1962) and bacterial (Nishimura & Ozawa, 1962) RNases both histidine and tryptophan residues proved to be essential. Takahashi (1970) demonstrated that the active centre of RNase T_1 contains not only histidine but also tryptophan. The author claims, however, that the enzyme activity depends mainly on the presence of histidine while tryptophan contributes only to the conformation of the active centre.

Our observations indicate that in alkaline RNase from rye germ cytosol, tryptophan is directly associated with hydrolysis of phosphodiester bonds.

REFERENCES

Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page J. & Caroll, W. R. (1954) *J. Biol. Chem.*, **207**, 201 - 210.
Barnard, E. A. (1969) *Ann. Rev. Biochem.*, **38**, 677 - 732.
Bernardi, A. & Bernardi, G. (1966) *Biochim. Biophys. Acta*, **129**, 23 - 31.
Birnboim, H. (1966) *Biochim. Biophys. Acta*, **119**, 198 - 200.
Chakraburtty, K. & Burma, D. P. (1968) *J. Biol. Chem.*, **243**, 1133 - 1139.
Davis, B. J. (1964) *Ann. N.Y, Acad. Sci.*, **121**, 404 - 427.
Findlay, D., Mathias, A. P. & Rabin, B. R. (1962a) *Biochem. J.*, **85**, 134 - 139.
Findlay, D., Mathias, A. P. & Rabin, B. R. (1962b) *Biochem. J.*, **85**, 139 - 144.
Friesch-Niggemeyer, W. & Reddi, K. K. (1957) *Biochim. Biophys. Acta*, **26**, 40 - 46.
Holden, M. & Pirie, N. W. (1955) *Biochem. J.*, **60**, 53 - 62.
Jonston, F. B. & Stern, H. (1957) *Nature (London)*, **179**, 160 - 161.
Kado, C. J. (1968) *Arch. Biochem. Biophys.*, **125**, 86 - 93.
Kuligowska, E., Szarkowski, J. W. & Klarkowska, D. (1974) *IX FEBS Meet., Budapest. Abstr.* S2 014.
Kunitz, M. (1940) *J. Gen. Physiol.*, **24**, 15 - 32.
Lantero, O. J. & Klosterman, H. J. (1973) *Phytochemistry*, **12**, 775 - 784.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.
Merola, A. J. & Davis, F. F. (1962) *Biochim. Biophys. Acta*, **55**, 431 - 439.
Nishimura, S. & Ozawa, H. (1962) *Biochim. Biophys. Acta*, **55**, 421 - 430.
Razzel, W. E. (1967) *Experientia*, **23**, 321 - 325.
Reddi, K. K. (1966) in *Procedures in Nucleic Acid Research* (G. L. Cantoni & D. R. Davis, eds) pp. 71 - 78. Harper and Row Publ., New York - London.
Schuster, L. (1957) *J. Biol. Chem.*, **229**, 289 - 303.
Siwecka, M. A. & Szarkowski, J. W. (1971) *Bull. Acad. Polon. Sci. Ser. Sci. Biol.*, **19**, 445 - 449.
Sluyterman, L. A. (1962) *Biochim. Biophys. Acta*, **60**, 557 - 561.
Spande, T. F. & Witkop, B. (1967) in *Methods in Enzymology* (C. H. W. Hirs, ed.) vol. 11, pp. 498 - 506. Academic Press, New York.
Steiner, R. F. & Beers, R. F. (1959) *Biochim. Biophys. Acta*, **32**, 166 - 176.
Takahashi, K. (1970) *J. Biochem. (Tokyo)*, **67**, 541 - 547.
Tang, W. J. & Maretzke, A. (1970) *Biochim. Biophys. Acta*, **212**, 300 - 307.
Udvardy, J., Farkas, G. L. & Marre, E. (1969) *Plant Cell Physiol.*, **10**, 375 - 386.
Wilson, C. M. (1971) *Plant Physiol.*, **48**, 64 - 68.
Wyen, N. V., Udvardy, J., Solymosy, F., Marre, E. & Farkas, G. L. (1969) *Biochim. Biophys. Acta*, **191**, 588 - 597.
Yamagata, S., Takahashi, K. & Egami, F. (1962) *J. Biochem. (Tokyo)*. **52**, 261 - 266.

ALKALICZNA RYBONUKLEAZA Z CYTOSOLU ZARODKÓW ŻYTA

Streszczenie

1. Wyizolowano i częściowo oczyszczono alkaliczną rybonukleazę cytosolu zarodków żyta (*Secale cereale* L.) o optimum pH 7,6. Enzym był wolny od zanieczyszczeń innymi aktywnościami nukleolitycznymi.

2. Rybonukleaza jest endonukleazą hydrolizującą wszystkie wiązania fosfodwuestrowe w łańcuchu RNA. Produktami degradacji RNA są cykliczne fosforany nukleozydów purynowych i pirymidynowych oraz odpowiednie 3'-nukleotydy. Rybonukleaza nie hydrolizuje syntetycznych cyklicznych nukleotydów purynowych. Produktami pośrednimi hydrolizy syntetycznych polinukleotydów

są cykliczne fosforany nukleozydów purynowych, jak i pirymidynowych. Natomiast 3′-mononukleotydy purynowe nie powstają podczas hydrolizy polinukleotydów.

3. Rybonukleaza alkaliczna cytosolu zarodków żyta nie depolimeryzuje spiralnego kompleksu poli(A) i poli(U).

4. Wrażliwość na fotooksydację oraz hamowanie przez bromek 2-hydroksy-5-nitrobenzylu i *N*-bromoimid kwasu bursztynylowego wskazują na udział tryptofanu w centrum aktywnym enzymu.

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AMINO ACID SEQUENCE OF HUMAN MUSCLE GLYCERALDEHYDE-3--PHOSPHATE DEHYDROGENASE.

ISOLATION AND AMINO ACID SEQUENCES OF TRYPTIC PEPTIDES

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1. The amino acid composition, N- and C-terminal amino acid sequences, and the subunit molecular weight of glyceraldehyde phosphate dehydrogenase from human muscle, were determined. The obtained results and the maps of tryptic peptides suggest that the enzyme is composed of four identical or very similar polypeptide chains.

2. From the tryptic digest of performic acid-oxidized enzyme, 32 peptides were isolated. The amino acid sequence analysis showed a high degree of homology with the corresponding tryptic peptides of the dehydrogenase from pig muscle, with 9 replacements and probably two additional amino acids in the examined sequences of the human muscle enzyme.

It has been shown that glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) isolated from a number of sources, consists of four polypeptide chains. The amino acid sequences of the enzyme from pig (Harris & Perham, 1968), lobster (Davidson *et al.*, 1967) and yeast (Jones & Harris, 1972) are now available. From human muscle, the enzyme was first isolated by Baranowski & Wolny (1963). However, little is known about its structure and the amino acid composition. The present work concerns the subunit structure of the enzyme and the amino acid sequence of the isolated tryptic peptides. A preliminary account of this work has been published (Nowak *et al.*, 1975).

MATERIALS AND METHODS

Materials. TPCK-treated trypsin and carboxypeptidase A were obtained from Worthington Biochemical Corp. (Freehold, N.Y., U.S.A.). Sephadex G-200 and G-25, DEAE-Sephadex A-50, and CM-Sephadex A-50 were purchased from

Pharmacia (Uppsala, Sweden). Dowex 50 X2 (200 - 400 mesh) was obtained from Serva (Heidelberg, G.F.R.). Silica gel G and GF_{254} were products of Merck A.G. (Darmstadt, G.F.R.). Amberlite IRC-50 (200 - 400 mesh) was purchased from Rohm & Haas Co. (Philadelphia, Pa., U.S.A.). All other chemicals were of analytical grade. Reagents for amino acid sequence analysis were purified as described by Edman & Begg (1967).

The following proteins were used as markers for dodecyl sulphate gel electrophoresis: crystalline glyceraldehyde phosphate dehydrogenase from rabbit and pig skeletal muscles, and rabbit muscle aldolase, prepared in our laboratory; chymotrypsinogen, haemoglobin and bovine serum albumin, products of Sigma Chem.Co. (St.Louis, Mo., U.S.A.).

Purification of glyceraldehyde phosphate dehydrogenase from human muscle. Two preparations of the pure crystalline enzyme were used. One was obtained by the procedure of Baranowski & Wolny (1963), another as described below. The crude extract from human muscle (Baranowski & Wolny, 1963) was fractionated with ammonium sulphate. The precipitate at 0.7 - 0.85 ammonium sulphate saturation was dissolved in 5 mM-Tris-HCl buffer, pH 7.5, containing 1 mM-EDTA and 1 mM-2-mercaptoethanol, and dialysed exhaustively against the same buffer solution. The dialysed preparation was loaded onto a DEAE-Sephadex A-50 column (2 × 30 cm), equilibrated and eluted with the above buffer solution at a flow rate of 30 ml/h. The active fractions were pooled and loaded onto a column of CM-Sephadex A-50 (1.6 × 26 cm) equilibrated with the above buffer solution. The column was first washed with buffer and then eluted with a linear salt gradient obtained by mixing 400 ml of buffer with 400 ml of 0.2 M-ammonium sulphate in the same buffer. The enzyme emerging at 0.17 M-$(NH_4)_2SO_4$ was recrystallized as described previously (Baranowski & Wolny, 1963).

Amino acid analysis of the enzyme. Crystalline enzyme (1 mg) was hydrolysed with 6 M-HCl at 105°C in sealed, evacuated tubes for 22, 48 and 72 h. The hydrolysates were analysed with an automatic amino acid analyser (Locarte, London, England) in a single-column system. Tryptophan was determined according to Spande & Witkop (1967) and cysteine as cysteic acid following oxidation of protein with performic acid (Hirs, 1956).

Amino acid analysis of peptides. The amino acid composition of peptides was determined after 48 h hydrolysis of 0.2 μmoles as described above. With short peptides, only qualitative amino acid analyses were carried out. These peptides after hydrolysis were dansylated according to Gray (1967) and dansyl amino acids were identified by t.l.c. on silica gel. The initial chromatography was performed in chloroform - benzyl alcohol - acetic acid (70:30:3, by vol.), the spots were extracted with butan-1-ol saturated with 0.2 M-NaOH, and the extracts chromatographed with light petroleum (b.p. 40 - 60°C) - *t*-butan-1-ol - acetic acid (5:1:1, by vol.) as solvent.

Tryptic digest. Digestion by trypsin of the enzyme oxidized with performic acid, was performed in 0.5% ammonium bicarbonate buffer, pH 8.9, for 14 h at

37°C at substrate concentration of 5 mg/ml and the substrate to trypsin weight ratio of 50:1. The digest obtained was freeze-dried.

Peptide mapping. The maps of tryptic peptides were done on Whatman *3MM* chromatography paper by high-voltage electrophoresis in pyridine - acetate buffer, pH 5.2, followed by descending chromatography in butan-1-ol - acetic acid - water (12:3:5, by vol.). The peptides were detected with ninhydrin (Heilmann *et al.*, 1957), Pauly reagent, Sakaguchi reagent, or with phenanthrenequinone (Easley *et al.*, 1969).

C-Terminal analyses. Hydrazinolysis was done by the metod of Fraenkel-Conrat & Tsung (1967). The hydrazides formed were removed by absorption on a column of Amberlite IRC-50 (H^+ form) after Llose *et al.* (1964), and the effluent was collected and freeze-dried. The C-terminal amino acids were identified by paper chromatography in butan-1-ol - acetic acid - water (7:2:1, by vol.), or by high-voltage electrophoresis according to Dreyer & Bynum (1967).

Digestion with carboxypeptidase A was performed in 0.2 M-ammonium bicarbonate buffer, pH 8.2, at 37°C, at a dehydrogenase to carboxypeptidase weight ratio 50:1. Samples were withdrawn at 2-h intervals and the amino acids were adsorbed onto Dowex 50 X8 beads. The effluents from the column were dried and examined by paper chromatography and high-voltage electrophoresis.

N-Terminal analyses, and determination of N-terminal sequences of the enzyme. N-Terminal analysis was performed by the dinitrophenyl method of Sanger & Tuppy (1951) with 36 mg of enzyme. After hydrolysis, the ether-extracted amino acid derivatives were separated by two-dimensional t.l.c. on silica gel. The solvent used in the first dimension was toluene - chloroethanol - pyridine - 0.88 M-ammonia (50:30:15:4, by vol.), and in the second dimension, chloroform - benzyl alcohol - acetic acid (70:30:3, by vol.). Acid-soluble amino acid derivatives were examined by one-dimensional t.l.c. with propan-1-ol - 0.88 M-ammonia (70:30, v/v) as solvent.

The N-terminal sequence was determined by the direct phenyl isothiocyanate method of Edman (1949, 1950) on paper strips as described by Fraenkel-Conrat (1954) and Schroeder (1967). The phenylthiohydantoin derivatives of amino acids were identified by t.l.c. on silica gel G or GF_{254} with solvent *E* and *D* of Edman & Sjöquist (1956).

Polyacrylamide-gel electrophoresis. This was carried out in 10% (w/v) gels according to Weber & Osborn (1969). The protein, 10 - 20 μg, was applied to the gel and electrophoresis was run at room temperature for 5 h at 8 mA per gel in Plexiglass tube (0.4 × 12 cm). For staining, Coomassie Blue was used.

Molecular weight of the purified enzyme was estimated by Sephadex G-200 gel filtration on a 1 × 75 cm column, according to Andrews (1965).

Separation of tryptic peptides. The mixture of tryptic peptides was initially fractionated on Sephadex G-25 column. The eluates were monitored at 230 nm and the fractions corresponding to the particular peaks were pooled. The solvent was removed by evaporation under reduced pressure in a rotary evaporator at 40°C. The residues were dissolved in water and lyophilized.

Further purification was performed on Dowex 50 X2 column in the volatile pyridine - acetate buffers at 37°C. For detection of the peptides, samples of 0.5 ml (or less) were withdrawn from each fraction tube, evaporated in vacuum and, following alkaline hydrolysis, analysed by the ninhydrin method (Hirs *et al.*, 1956). The appropriate fractions were combined and evaporated to dryness on a rotary evaporator at 40°C. The residue was taken up in a small volume of water and reevaporated to remove the residual pyridine acetate. The peptides were dissolved in water and stored at −18°C. All the collected fractions were further subjected to high-voltage paper electrophoresis.

High-voltage paper electrophoresis. For analytical electrophoresis, samples of the purified peptide preparations (0.2 or 0.4 ml) were applied to Whatman no. 1 paper and the electrophoresis was run in pyridine - acetate buffer (0.12 M, pH 3.5), at 50 to 75 V/cm for 2 - 4 h in a cooled tank (Michl, 1951). The peptides were detected with ninhydrin, phenanthrenequinone and Pauly reagent (Heilmann *et al.*, 1957; Easley *et al.*, 1969).

For preparative purposes, the electrophoresis was performed under the same conditions but on Whatman no. *3MM* paper. About 0.5 μmole of peptide was applied per 1 cm. After electrophoresis the paper was dried at room temperature and the guide strips were stained with ninhydrin and Pauly reagent. Peptides were eluted from the paper with 10% acetic acid, the solvent evaporated in a rotary evaporator, the residue dissolved in water and reevaporated. The peptide preparations were stored at −18°C.

Paper chromatography. Descending preparative chromatography was performed on Whatman no. 1 paper in the acidic solvent (butan-1-ol - acetic acid - water, 12:3:5, by vol.) of Katz *et al.* (1959) or basic solvent (propan-1-ol - conc.NH_3 - water, 60:6:34, by vol.) of Samuelsson *et al.* (1968). About 0.2 μmole of peptide was applied on the paper. The peptides were eluted and stored as described above.

Edman degradation and N-terminal analysis of peptides. Stepwise degradation of the peptides was carried out by the modification of Edman procedure described by Peterson *et al.* (1972) except that instead of extraction after the coupling reaction, drying in vacuum at 50°C was applied, and after the cleavage reaction the thiazolinones were extracted three times with butyl acetate. The obtained thiazoline derivatives were converted into phenylthiohydantoins by heating in 1 M-HCl at 80°C for 10 min (Edman & Begg, 1967) and identified by t.l.c. on silica gel GF_{254} in solvents V and V + IV of Jeppson & Sjöquist (1967). The amino acid derivatives were detected under u.v. light and by the ninhydrin method (Roseau & Pantel, 1969; Nolan *et at.*, 1973).

After each step of the Edman degradation, a portion of the peptide was taken for N-terminal amino acid determination by the dansyl method of Gray (1967), and the dansylated amino acids were identified by t.l.c. on silica gel as described for amino acid composition analysis of the enzyme.

RESULTS AND DISCUSSION

Molecular weight and subunit structure. Sephadex G-200 gel filtration of the human muscle glyceraldehyde phosphate dehydrogenase gave only one peak, with the same v_e/v_0 as the pig dehydrogenase, indicating that the molecular weight of the human enzyme is similar to that of the pig enzyme, i.e. about 145 000 daltons. Electrophoresis of the reduced human enzyme on sodium dodecyl sulphate - polyacrylamide gel showed only one protein band, and the molecular weight of the dissociated enzyme protein was found to be 35 000 - 37 000 daltons (Fig. 1), which indicated the tetrameric structure of the enzyme.

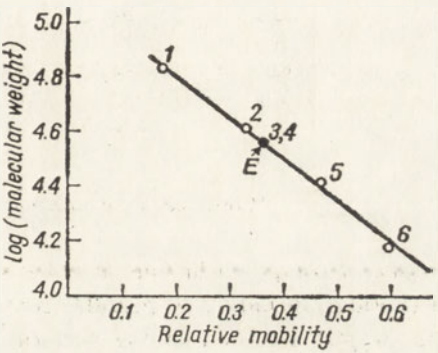

Fig. 1. Molecular weight determination by sodium dodecyl sulphate - polyacrylamide gel electrophoresis of the human muscle glyceraldehyde phosphate dehydrogenase (*E*).

Standard proteins: *1*, bovine serum albumin (mol. wt. 68 000); *2*, rabbit muscle aldolase (40 000); *3,4* rabbit and pig muscle glyceraldehyde phosphate dehydrogenases (36 000); *5*, chymotrypsinogen (25 700); *6*, haemoglobin (15 500). The molecular weights were taken from Weber & Osborn (1969).

Amino acid analysis, and N- and C-terminal sequences. The amino acid composition of human muscle dehydrogenase presented in Table 1, is very similar to the amino acid composition of the enzyme from other sources, especially as concerns the number of basic and aromatic residues; significant differences appear, however, in the number of cysteine residues. The human enzyme has three cysteine residues, similarly as that of monkey (Perham, 1969) and sturgeon (Allison & Kaplan, 1964a,b), as compared with 4 found in pig, badger and rabbit (Harris & Perham, 1968; Perham, 1969; Velick & Furfine, 1963), and with 2 reported for human erythrocytes (Oguchi, 1970) and 5 for the lobster (Davidson *et al.*, 1967).

Glycine was found to be the only N-terminal amino acid of the enzyme, as revealed both by the dinitrophenyl method of Sanger and by the Edman degradation. The next three steps of Edman degradation released successively lysine, valine and probably lysine.

After hydrazinolysis of the human enzyme, only glutamic acid was detected in significant amounts. This amino acid was also found after 2-h treatment of the enzyme with carboxypeptidase A. Prolonged digestion released the next four amino acids: lysine, serine, alanine and methionine. Thus, the C-terminal amino acid sequence of human muscle glyceraldehyde phosphate dehydrogenase was probably the same as in the pig dehydrogenase.

Tryptic digestion. Assuming a complete specificity of trypsin for cleavage of peptide bonds at arginine and lysine residues, a molecule of the human muscle

Table 1

Amino acid composition of human muscle glyceraldehyde-3-phosphate dehydrogenase

All data are average or extrapolated figures from 9 determinations (3 after 22 h, 3 after 48 h, and 3 after 72 h acid hydrolysis). The values are expressed as moles of the amino acid residue per subunit of mol. wt. 36 000. The data for the pig enzyme, taken from Harris & Perham (1968), are included for comparison.

Amino acid	Human muscle	Pig muscle	Amino acid	Human muscle	Pig muscle
Asp	38	38	Ile	19	19
Thr*	20	22	Leu	20	20
Ser*	19	19	Tyr	8	9
Glu	20	17	Phe	14	14
Pro	14	12	Cys	3	3
Gly	34	33	Lys	26	26
Ala	32	32	His	10	11
Val	34	34	Arg	10	10
Met	9	9	Trp	4	3

*Corrected for destruction during acid hydrolysis.

enzyme, if composed of four identical subunits, should yield after trypsin digestion 36 peptides, including 10 peptides containing arginine. The peptide maps obtained after tryptic digestion of the performic acid-oxidized dehydrogenase were reproducible and showed 34 to 40 peptides (Fig. 2), of which 10 - 12 peptides contained arginine, as found by specific staining. These results are in accordance with the assumption that the four subunits of the enzyme are identical. From the comparison of peptide maps of human and pig enzyme it appeared that the two enzymes differ only by 5 - 8 peptides.

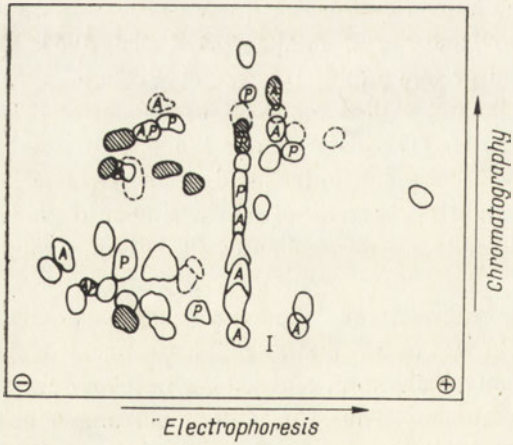

Fig. 2. Combined map of tryptic peptides from human and pig glyceraldehyde phosphate dehydrogenases. The maps were obtained by high-voltage electrophoresis at pH 5.2 followed by chromatography. Solid lines denote the peptides present in both enzymes; dashed lines, peptides from pig dehydrogenase; hatched spots represent peptides found in human muscle only. *P*, Pauly-reagent-positive spots; *A*, arginine-positive spots.

Tryptic peptides. The tryptic digest of the performic acid-oxidized human dehydrogenase, on Sephadex G-25 gel filtration resolved into three major peaks (Fig. 3). Each of the collected fractions corresponding to the first peak was divided

into two equal parts which were pooled, as shown in Fig. 3, giving fraction *I* (solid bar) and fractions *Ia*, *Ib* and *Ic* (open bars). Fractions *II* and *III* were pooled as indicated.

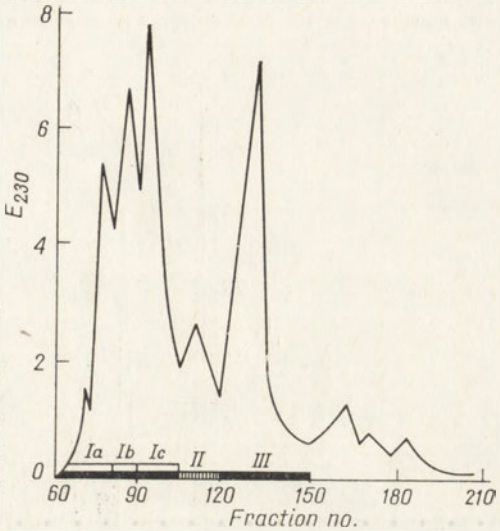

Fig. 3. Fractionation on Sephadex G-25 of the tryptic digest of the human muscle dehydrogenase. To the column (2.5 × 190 cm) equilibrated with 0.05 M-NH_4HCO_3, pH 8.03, the digestion mixture (250 mg protein) was applied and eluted with the equilibration buffer. Fractions of 4 ml were collected at a rate of 22 ml/h. The bars indicate the fractions pooled for further analysis.

On Dowex 50 X2 column chromatography, fraction *I* separated into 13 peaks (Fig. 4a), from which 10 peptides were isolated by high-voltage electrophoresis. From fraction *Ib* (Fig. 4b) only one additional peptide was isolated by chromatography (T16), and it was not further purified. Fraction *II* separated on the Dowex column into 6 peaks; by paper electrophoresis two peptides (T10 and T26) were isolated. Fraction *III* on the Dowex column gave 14 peaks (Fig. 4c), from which 18 peptides were isolated by high voltage electrophoresis or paper chromatography.

Out of 32 isolated peptides, in 17 the amino acid composition and the N-terminal amino acid were quantitatively determined (Table 2). In the remaining peptides, the amino acids were determined only qualitatively.

From the amino acid composition of the tryptic peptides it appears that about 75% of the whole polypeptide chain was isolated as peptides.

The majority of the isolated peptides had the same amino acid composition as the tryptic peptides from the pig muscle dehydrogenase (Harris & Perham, 1968). Only the peptides T10, T15, T19, T23, T29 and T30 differ by one or two amino acid residues from homologous peptides of the pig enzyme, and peptides T1, T31 and T32 have not been detected in the pig muscle dehydrogenase.

Amino acid sequences of tryptic peptides were determined by semimicromodification (Peterson *et al.*, 1972) of the Edman degradation, followed by detection of N-terminal amino acid by the dansylation method of Gray (1967). In a few cases when the amount of the isolated peptides was greater, sequence determination was performed by direct Edman degradation. This method enables simultaneous identification of asparagine and glutamine by ninhydrin staining.

The amino acid sequences were established by the above-described methods for 29 out of 32 isolated tryptic peptides, except that the positions of cysteic acid in peptides T15 and T23 were deduced from the amino acid composition and position of cysteine in the pig muscle dehydrogenase; C-terminal arginine residues

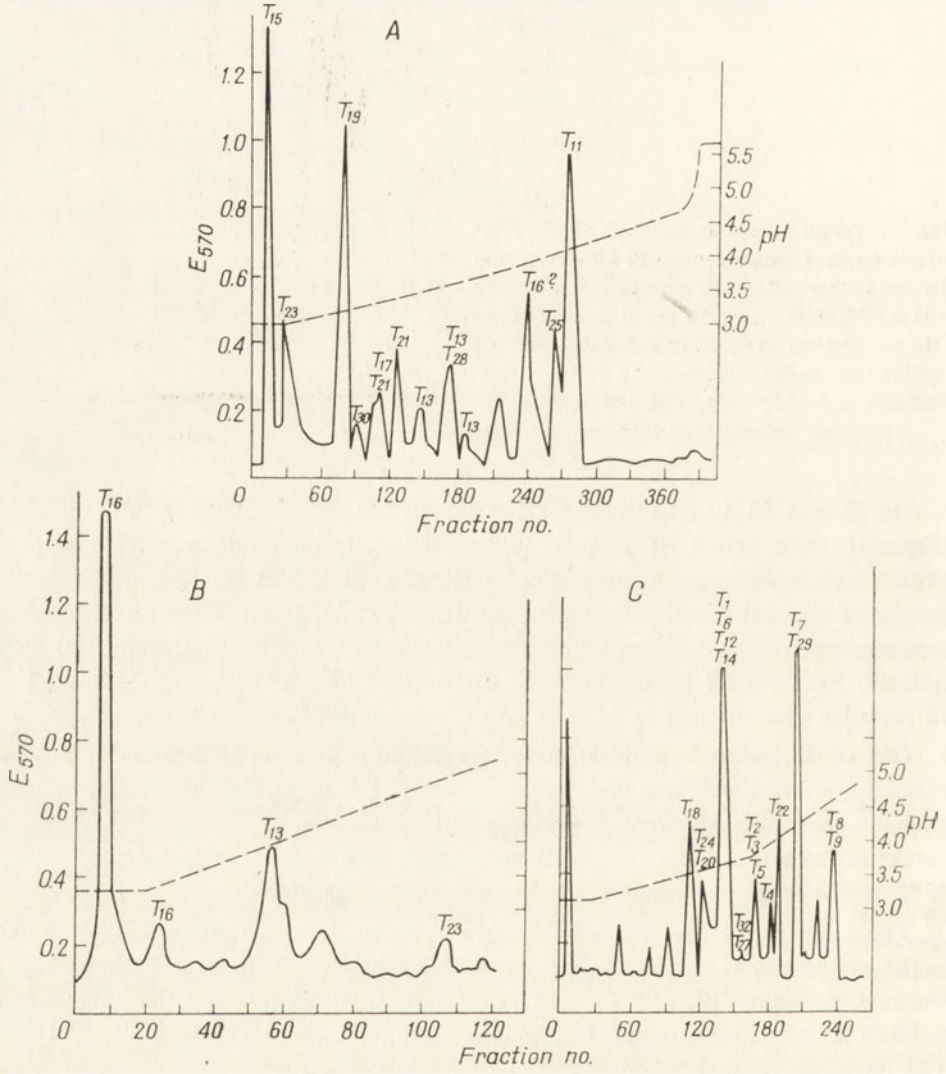

Fig. 4. Dowex 50 X2 chromatography of the tryptic digest fractions separated by Sephadex G-25 filtration (Fig. 3). The columns (0.9 × 100 cm) were equilibrated with 0.2 M-pyridine - acetate buffer, pH 3.1, and eluted with a pH gradient in pyridine acetate; fractions of 4 ml were collected at a rate of 42 ml/h. The linear pH gradient was generated with a hydrostatic apparatus. The mixing chamber contained 0.2 M-pyridine acetate, pH 3.1, and the next three vessels contained the same volume of pyridine acetate buffers: 0.5 M (pH 4.1), 2 M (pH 5.0), and 4 M (pH 5.6). *A*, whole fraction *I*; *B*, fraction *Ib*; *C*, fraction *III*.

Table 2

Amino acid composition of the isolated tryptic peptides

Purification procedures (after gel filtration and ion-exchange chromatography) are indicated as follows: E, high-voltage paper electrophoresis; C, paper chromatography. Cysteine was detected as cysteic acid, methionine as oxide, and tryptophan by fluorescence in u.v. light. The results are expressed as residues per molecule.

	T3	T6	T7	T12	T13	T14	T15	T16	T17	T19	T23	T25	T26	T27	T28	T29	T30
Lys	—	0.7	0.9	1.0	0.9	1.1	0.9	1.2	1.0	0.9	—	2.0	0.9	1.0	0.8	—	1.0
His	—	—	1.0	—	0.8	—	—	2.8	—	—	—	—	—	—	—	—	0.9
Arg	0.9	—	—	—	—	—	—	—	—	—	1.0	—	—	—	—	0.9	—
Cys	—	—	—	—	—	—	1.9	—	—	—	0.9	—	—	—	—	—	—
Asp	1.1	1.2	1.0	—	2.1	2.2	2.0	2.1	1.1	1.0	2.3	—	2.1	—	—	4.2	1.1
Thr	—	—	0.9	—	—	—	1.9	0.8	0.8	1.0	1.9	—	—	—	—	—	—
Ser	—	0.7	0.9	—	1.8	0.8	1.9	1.7	0.7	0.7	0.8	—	—	—	0.8	0.8	0.8
Glu	—	—	1.1	—	0.9	—	—	1.8	—	1.0	—	0.8	—	—	1.9	1.0	—
Pro	—	—	—	—	1.7	—	0.8	—	0.9	0.8	0.6	0.9	—	—	0.9	—	—
Gly	3.3	1.0	1.2	2.1	1.1	—	—	2.2	2.3	1.9	—	—	—	—	1.1	1.2	—
Ala	—	2.1	—	1.0	3.1	—	2.9	2.0	—	4.9	1.0	1.0	—	—	0.9	—	2.0
Val	2.1	—	—	—	2.7	—	—	2.8	0.8	—	2.7	—	—	1.8	—	—	1.7
Ile	—	—	—	—	2.0	—	2.0	2.9	—	0.9	—	—	0.9	—	—	0.9	—
Met	—	—	0.9	—	1.8	—	—	0.8	—	—	—	—	—	—	—	—	2.1
Leu	—	—	—	—	—	1.0	—	1.0	—	1.0	1.9	1.0	—	—	1.0	1.0	1.1
Tyr	—	—	0.8	—	—	1.0	—	—	—	—	—	—	1.0	—	—	1.8	—
Phe	1.0	0.9	1.0	—	1.0	—	—	0.9	—	—	—	—	—	—	—	1.0	—
Trp	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+++	—
Total residues*	8	7	10	4	21	6	17	24	8	15	14	6	5	3	8	14	11
N-terminal residue	Val	Ala	Met	Gly	Ile	Tyr	Ile	Val	Thr	Gly	Val	Leu	Tyr	Val	Glx	Leu	Val
Purification	E	E,C	E	C		E,C	E		E,C	E	E,C	E	C	E,C	E	E	E

* Calculated from the nearest integers.

were located only on the basis of colour spot tests. Three peptides (T13, T16 and T29) were only partially sequenced.

The sequences of peptides from T2 through T30 (Table 3) are, as reported previously (Nowak *et al.*, 1975), similar to, or identical with, the homologous tryptic peptides from the pig enzyme (Harris & Perham, 1968). Different amino acid residues were found only in nine positions: in position 81, Glx instead of Asn (peptide T10); in 116-117, Ile-Val instead of Val-Ile (T13); in 144, Ile instead of Val (T15); in 203 Leu instead of Ile (T19); in 235 Ala instead of Pro and in 240 Leu instead of Val (T23); in 309 Asx instead of Ser (T29), and in position 326, Ala instead of Val (peptide T30).

Of the three peptides which were not found in the tryptic digest of the pig dehydrogenase (Harris & Perham, 1968), T1 would be the N-terminal in the human muscle enzyme as its N-terminal sequence was found to be Gly-Lys-Val-(Lys)-. This sequence has been also determined as N-terminal in the enzyme from human erythrocyte (Tanner & Gray, 1971). The remaining two peptides, T31 and T32, could result from non-specific cleavage of peptide bonds.

The amino acid sequences of peptides T24 and T25 partially overlapped; it seems that the appearance of peptide T25 might be due to resistance of the Lys-Pro bond to the tryptic hydrolysis.

Table 3

Amino acid sequences of the peptides isolated from the tryptic digest of performic acid-oxidized human muscle glyceraldehyde phosphate dehydrogenase

The numbers in the last column indicate positions of amino acid residues in the homologous peptides from the pig muscle enzyme (Harris & Perham, 1968).

Peptide	Sequence	Presumed position
T1	Gly-Lys	
T2	Val-Lys	1—2
T3	Val-Gly-Val-Asx-Gly-Phe-Gly-Arg	3—10
T4	Ile-Gly-Arg	11—13
T5	Leu-Val-Thr-Arg	14—17
T6	Ala-Ala-Phe-Asx-Ser-Gly-Lys	18—24
T7	Met-Phe-Glx-Tyr-Asx-Ser-Thr-His-Gly-Lys	43—52
T8	Phe-His-Gly-Thr-Val-Lys	53—58
T9	Leu-Val-Ile-Asx-Gly-Lys	64—69
T10	Asx-Pro-Ala-Glx-Ile-Lys	78—83
T11	Ala-Gly-Ala-His-Leu-Lys	105—110
T12	Gly-Gly-Ala-Lys	111—114
T13	Ile-Val-Ile-Ser-Ala-Pro-Ser-Ala-Asx-Ala-Pro-Met-Phe-(Val_2,Met,Gly,Asx,His,Glx)-Lys	116—136
T14	Tyr-Asx-Asx-Ser-Leu-Lys	137—142
T15	Ile-Ile-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys	143—159
T16	Val-Ile-His-Asx-His-(Phe,Gly_2,Ile_2,Val_2,Glx_2,Leu,Met,Thr,Ser_2,His,Ala_2,Asx)-Lys	160 - 183
T17	Thr-Val-Asx-Gly-Pro-Ser-Gly-Lys	184—191
T18	Asx-Gly-Arg	195—197
T19	Gly-Ala-Ala-Gln-Asn-Leu-Ile-Pro-Ala-Ser-Thr-Gly-Ala-Ala-Lys	198—212
T20	Ala-Val-Gly-Lys	213—216
T21	Val-Ile-Pro-Glu-Leu-Asp-Gly-Lys	217—224
T22	Leu-Thr-Gly-Met-Ala-Phe-Arg	225—231
T23	Val-Pro-Thr-Ala-Asn-Val-Ser-Val-Leu-Asp-Leu-Thr-Cys-Arg	232—245
T24	Leu-Glx-Lys	246—248
T25	Leu-Glx-Lys-Pro-Ala-Lys	246—251
T26	Tyr-Asx-Asx-Ile-Lys	252—256
T27	Val-Val-Lys	258—260
T28	Glx-Ala-Ser-Glx-Gly-Pro-Leu-Lys	261—268
T29	Leu-Ile-Asx-Trp-Tyr-Asx-(Asx_2,Glx,Phe,Gly,Tyr,Ser)-Arg	307—320
T30	Val-Val-Asp-Leu-Met-Ala-His-Met-Ala-Ser-Lys	321—331
T31	Ser-Lys	
T32	Gly-Val-Lys	

The three cysteine residues present in the human muscle dehydrogenase were found in two of the isolated tryptic peptides: T15 and T23 (Table 4). Peptide T15 contains cysteine-149 and cysteine-153, resembling the peptides from the region of the active centre of all other so far examined glyceraldehyde phosphate dehydrogenases (Perham, 1969). The amino acid sequence of peptide T15 is the same as that of the peptide from human heart enzyme but differs from the corresponding peptides of pig and monkey in having at position 143 isoleucine instead of valine.

Peptide T23 containing cysteine-244 is identical with that isolated from monkey muscle but differs from that of the pig at positions 235 and 240 (Ala being replaced by Pro, and Leu by Val, respectively).

The C-terminal peptide T30 of human enzyme differs from the homologous pig peptide only by one amino acid residue (Ala-326 instead of Val) but is identical with that from human erythrocyte (Tanner & Gray, 1971). The C-terminal sequence of both human and pig enzymes differs largely from those of the lobster and yeast.

Table 4

Amino acid sequences of tryptic peptides containing cysteine residues and C-terminal sequence

For comparison with the glyceraldehyde phosphate dehydrogenase from human muscle, data for the enzyme from human heart and monkey muscle (Perham,1969), pig muscle (Harris & Perham, 1968, lobster (Davidson *et al.*, 1967) and yeast (Jones & Harris, 1972), are included.

Peptide	Sequence
	143 150 152
Human muscle (T15)	Ile-Ile-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys
Human heart	Ile-Ile-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys
Pig muscle	Ile-Val-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys
Monkey muscle	Ile-Val-Ser-Asn-Ala-Ser-Cys-Thr-Thr-Asn-Cys-Leu-Ala-Pro-Leu-Ala-Lys
	232 240 241
Human muscle (T23)	Val-Pro-Thr-Ala-Asn-Val-Ser-Val-Leu-Asp-Leu-Thr-Cys-Arg
Monkey muscle	Val-Pro-Thr-Ala-Asn-Val-Ser-Val-Val-Asp-Leu-Thr-Cys-Arg
Pig muscle	Val-Pro-Thr-Pro-Asn-Val-Ser-Val-Val-Asp-Leu-Thr-Cys-Arg
C-Terminal sequence	321
Human muscle (T30)	Val-Val-Asp-Leu-Met-Ala-His-Met-Ala-Ser-Lys-Glu
Pig muscle	Val-Val-Asp-Leu-Met-Val-His-Met-Ala-Ser-Lys-Glu
Lobster	Val-Ile-Asp-Leu-Leu-Lys-His-Met-Gln-Lys-Val-Asp-Ser-Ala
Yeast	Val-Val-Asp-Leu-Val-Glu-His-Val/Ile-Ala-Lys-Ala

The presented data and the comparison with the pig enzyme permit to conclude that the isolated peptides contain almost the whole amino acid sequence of the human muscle glyceraldehyde phosphate dehydrogenase, except the fragments 25-42; 59-63; 70-77; 84-104; 192-194; 269-306.

REFERENCES

Allison, W. S. & Kaplan, N. O. (1964a) *J. Biol. Chem.*, **239**, 2140 - 2152.

Allison, W. S. & Kaplan, N. O. (1964b) *Biochemistry*, **3**, 1792 - 1801.

Andrews, P. (1965) *Biochem. J.*, **96**, 595 - 606.

Baranowski, T. & Wolny, M. (1963) *Acta Biol. Med. Germ.*, **11**, 651 - 659.

Davidson, B. E., Sajgo, M., Noller, H. F. & Harris, J. I. (1967) *Nature* (*London*), **216**, 1181 - 1185.

Dreyer, W. J. & Bynum, R. (1967) *Methods Enzymol.*, **11**, 32 - 39.

Easley, C. W., Zegers, B. J. M. & DeVijlder, M. (1969) *Biochim. Biophys. Acta*, **175**, 211 - 213.

Edman, P. (1949) *Arch. Biochem. Biophys.*, **22**, 475 - 476.

Edman, P. (1950) *Acta Chem. Scand.*, **4**, 283 - 293.

Edman, P. & Begg, G. (1967) *Eur. J. Biochem.*, **1**, 80 - 91.

Edman, P. & Sjöquist, J. (1956) *Acta Chem. Scand.*, **10**, 1507 - 1509.

Fraenkel-Conrat, H. (1954) *J. Am. Chem. Soc.*, **76**, 3606 - 3607.

Fraenkel-Conrat, H. & Tsung, I. (1967) *Methods Enzymol.*, **2**, 151 - 155.

Gray W. R. (1967) *Methods Enzymol.*, **11**, 139 - 151.

Harris, J. I. & Perham, R. N. (1968) *Nature* (*London*), **219**, 1025 - 1028.

Heilmann, J., Barrollier, J. & Watzke, E. (1957) *Z. Physiol. Chem.*, **309**, 219 - 220.

Hirs, C. H. W. (1956) *J. Biol. Chem.*, **219**, 611 - 625.

Hirs, C. H. W., Moore, S. & Stein, W. H. (1956) *J. Biol. Chem.*, **219**, 623 - 642.

Jeppson, J. O. & Sjöquist, J. (1967) *Anal. Biochem.*, **18**, 264 - 269.

Jones, G. M. T. & Harris, J. I. (1972) *FEBS Lett.*, **22**, 185 - 189.
Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959) *J. Biol. Chem.*, **234**, 2897 - 2900.
Llose, P., de la Tertin, C. & Jutisz, M. (1964) *Experientia*, **20**, 204 - 205.
Michl, H. (1951) *Monatsch. Chem.*, **82**, 489 - 493.
Nolan, C., Fitch, W. M., Utzzell, T., Weiss, L. J. & Margoliash, E. (1973) *Biochemistry*, **12**, 4052 - 4060.
Nowak, K., Kuczek, M., Ostropolska, L., Malarska, A., Wolny, M. & Baranowski, T. (1975) *Z. Physiol. Chem.*, **356**, 1181 - 1183.
Oguchi, M. (1970) *J. Biochem. (Tokyo)*, **68**, 427 - 439.
Perham, R. N. (1969) *Biochem. J.*, **111**, 17 - 21.
Peterson, J. D., Nehrling, S., Oyer, P. E. & Steiner, D. F. (1972) *J. Biol. Chem.*, **247**, 4866 - - 4871.
Roseau, G. & Pantel, P. (1969) *J. Chromatogr.*, **11**, 392 - 395.
Samuelsson, G., Seger, L. & Olson, T. (1968) *Acta Chem. Scand.*, **22**, 2624 - 2642.
Sanger, F. & Tuppy, H. (1951) *Biochem. J.*, **49**, 463 - 481.
Schroeder, W. A. (1967) *Methods Enzymol.*, **11**, 445 - 461.
Spande, T. F. & Witkop, B. (1967) *Methods Enzymol.*, **11**, 498 - 506.
Tanner, M. J. A. & Gray, W. R. (1971) *Biochem. J.*, **125**, 1109 - 1117.
Velick, S. F. & Furfine, C. (1963) *Enzymes*, **7**, 243 - 273.
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.*, **244**, 4406 - 4412.

SEKWENCJA AMINOKWASÓW DEHYDROGENAZY ALDEHYDU 3-FOSFOGLICERYNOWEGO Z MIĘŚNI LUDZKICH

IZOLOWANIE PEPTYDÓW TRYPSYNOWYCH I ICH SEKWENCJA AMINOKWASOWA

Streszczenie

1. Oznaczono skład aminokwasowy, N- i C-końcowe sekwencje aminokwasów i ciężar cząsteczkowy podjednostki dehydrogenazy aldehydu 3-fosfoglicerynowego z mięśni ludzkich. Uzyskane wyniki oraz mapy trypsynowych peptydów wskazują, że enzym składa się z czterech identycznych lub bardzo podobnych łańcuchów polipeptydowych.

2. Z hydrolizatu trypsynowego enzymu utlenionego kwasem nadmrówkowym wyizolowano 32 peptydy i oznaczono ich sekwencje. W porównaniu z trypsynowymi peptydami dehydrogenazy z mięśni wieprza, stwierdzono wysoki stopień homologii (9 aminokwasów odmiennych oraz przypuszczalnie dwa dodatkowe).

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BARBARA KOWALSKA-LOTH, J. ZIELEŃSKI, K. TOCZKO and IRENA CHMIELEWSKA

SIMILARITY IN ACTIVE SITE ARRANGEMENT OF NEUTRAL PROTEASE FROM CALF THYMUS CHROMATIN AND TRYPSIN*

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1. Susceptibility to inhibitors of neutral protease from calf thymus chromatin has been compared with that of trypsin. The chromatin protease reacts stoichiometrically with the inhibitors specific for trypsin (diisopropylfluorophosphate, tosyl-lysyl chloromethane, soybean trypsin inhibitor and Kunitz basic inhibitor from pancreas), but not with the inhibitor specific for chymotrypsin (tosyl-phenylalanyl chloromethane).

2. Chromatin protease, similarly as trypsin, cleaves Lys-X and Arg-X peptide bonds.

3. It is concluded that the structure of active site region of both enzymes is very similar.

Neutral protease is an integral part of chromatin from several animal tissues (Furlan & Jericijo, 1976; Bartley & Chalkley, 1970; Kurecki *et al.*, 1971; Garrels *et al.*, 1972). The enzyme shows a rather narrow specificity. It hydrolyses only *f1* and *f3* histones when they are an integral part of nucleohistone or chromatin complex, but when dissociated from the complex all five histone fractions are digested at the same rate (Bartley & Chalkley, 1970; Kurecki & Toczko, 1974). The enzyme degrades also basic ribosomal proteins and, to a much lower extent, casein and haemoglobin; on the other hand, ribonuclease, lysozyme and albumin are almost completely resistant to the enzyme action (Kurecki & Toczko, 1974).

Neutral protease from calf thymus chromatin was highly purified by Kurecki & Toczko (1974). Its molecular weight was estimated by gel filtration to be about 15 000 and optimum pH 8.5 with total histone as substrate. Kurecki *et al.* (1975) demonstrated that it is a serine-type enzyme, reacting in a typical way with NPGB[1].

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

[1] Abbreviations used: NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; DFP, diisopropylfluorophosphate.

The purpose of the present work was to compare chromatin protease and trypsin with respect to susceptibility to inhibitors and specificity of peptide bond cleavage.

MATERIALS AND METHODS

Isolation and purification of neutral protease. The enzyme was extracted from calf thymus chromatin with cold HCl solution and purified as described by Kurecki *et al.* (1975).

Assay of enzyme activity. Neutral protease activity was determined as described by Kurecki & Toczko (1972) using total histone as substrate. Trypsin activity was determined according to Kunitz (1947) with casein as substrate. The activity of the enzymes was assayed in 0.1 M-Tris-HCl buffer, pH 8.0, the final concentration of substrates being 0.5%.

Assay of active site. The active sites in both enzyme preparations were estimated with NPGB as titrant according to Chase & Shaw (1967).

Assay of enzyme inhibition. Inhibition of the enzymes was determined by two independent procedures:

1. Inhibition of proteolytic activity. The enzyme was preincubated for 10 min at 37°C with the appropriate inhibitor and the residual activity was determined using total histone and casein for chromatin protease and trypsin, respectively. The preincubation with DFP, soybean trypsin inhibitor or basic Kunitz trypsin inhibitor from pancreas was carried out in 0.1 M-Tris-HCl buffer, pH 8.0, and with tosyl-lysyl chloromethane or tosyl-phenylalanyl chloromethane, in 0.1 M--Tris-HCl buffer, pH 7.1.

2. Decrease in active site content. The enzyme was preincubated for 5 min at room temp. with the appropriate inhibitor in 0.1 M-veronal buffer, pH 8.3, and the residual active sites unblocked by the inhibitor were determined by titration with NPGB.

In both procedures, the control sample was incubated with the buffer without inhibitor.

Identification of C-terminal amino acids in histones digested by chromatin protease. Histones were incubated with the enzyme for 6 h at 37°C in 0.1 M-Tris-HCl buffer, pH 8.0, under toluene. The digest was desalted on Sephadex G-25 column and the peptide-containing fractions were dried by evaporation. C-Terminal amino acids were identified after hydrazinolysis (Bailey, 1967) by thin-layer chromatography on Silica Gel G plates in butan-1-ol - acetic acid - water (12:3:5, by vol.) as solvent (Smith, 1958). The staining was performed with ninhydrin, Sakaguchi and Pauly reagents.

Determination of protein. The method of Lowry *et al.* (1951) was used with bovine serum albumin as standard.

Reagents. Sephadex G-25 was from Pharmacia (Uppsala, Sweden), Silica Gel G from Merck (Darmstadt, G.F.R.), trypsin and soybean trypsin inhibitor from Sigma Chemicals (St.Louis, Mo., U.S.A.), DFP from Koch-Light Lab. (Coln-

brook, Bucks., England), tosyl-phenylalanyl chloromethane and tosyl-lysyl chloromethane from Calbiochem AG (Lucerne, Switzerland), NPGB from Instytut Chemii Organicznej PAN (Warszawa, Poland), bovine serum albumin from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Basic trypsin inhibitor from bovine pancreas was a kind gift from Prof. M. Laskowski, Sr. (Laboratory of Enzymology, Roswell Park Memorial Institute, Buffalo, N.Y., U.S.A.). Histones were extracted from calf thymus chromatin with 0.25 M-HCl.

RESULTS AND DISCUSSION

As previously shown by Kurecki *et al.* (1975), neutral protease from calf thymus chromatin is stoichiometrically inhibited by DFP, soybean trypsin inhibitor and basic Kunitz inhibitor from bovine pancreas. This pointed to a similarity in the structure of the active centre region of this enzyme and trypsin.

Table 1

Inhibition of chromatin protease proteolytic activity by low-molecular-weight inhibitors

Inhibitor to protease molar ratio	Tosyl-lysyl chloromethane	Tosyl-phenylalanyl chloromethane
	Inhibition (%)	
0.4	30	5
0.8	55	10
1.2	70	7
1.6	85	12
2.0	100	10
10.0	90	5
20.0	95	8

To obtain further information concerning similarity of chromatin protease and trypsin, the interaction of the chromatin enzyme with low-molecular-weight inhibitors differing in their action towards trypsin and chymotrypsin molecules, was studied (Table 1). Chromatin protease was completely inhibited by a twofold molar excess of the trypsin inhibitor, tosyl-lysyl chloromethane, but its activity was not affected even by a 20-fold excess of tosyl-phenylalanyl chloromethane, an inhibitor of chymotrypsin. These results indicate much higher similarity of the neutral protease from calf thymus chromatin to trypsin than to chymotrypsin.

Since NPGB reacts with chromatin protease stoichiometrically (Kurecki *et al.*, 1975), correlation between proteolytic activity and active site content in the chromatin protease preparation was determined. The results presented in Fig. 1 show

that both the proteolytic activity and the amount of liberated *p*-nitrophenol were dependent on enzyme concentration.

Susceptibility of chromatin protease to soybean trypsin inhibitor was the same as that of trypsin, and the inhibition of either enzyme was proportional to the amount of the inhibitor added (Fig. 2); full inhibition was obtained at 1:1 molar ratio of the inhibitor to the enzyme. The results obtained with DFP and Kunitz trypsin inhibitor from bovine pancreas were practically the same.

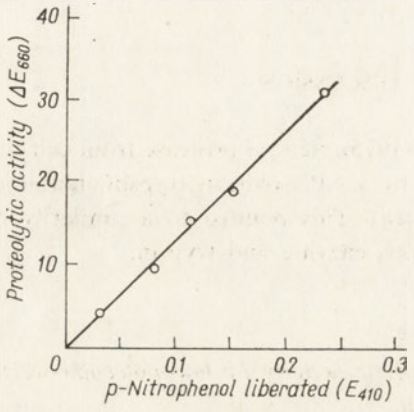

Fig. 1

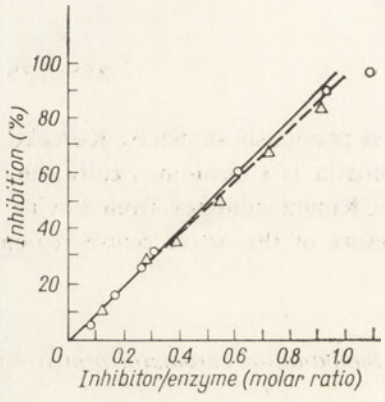

Fig. 2

Fig. 1. Correlation between proteolytic activity and active site titration by NPGB in chromatin protease preparation. Enzyme solution, 1 ml, containing 75 - 500 μg protein in 0.1 M-veronal buffer, pH 8.3, was mixed with 5 μl of 10 mM-NPGB in dimethyl formamide, and after 30 sec E_{410nm} was measured. For proteolytic activity determination the enzyme solutions were diluted 50-fold with 0.1 M-Tris-HCl buffer, pH 8.0 and in 0.5 ml samples the activity was determined. The values have been corrected for 100-fold dilution of the enzyme sample.

Fig. 2. Inhibition of chromatin protease and trypsin proteolytic activities by soybean trypsin inhibitor. The enzyme solution, 0.25 ml (0.13 μM in active site) was preincubated with 0.25 ml of the inhibitor solution (0.01 - 0.14 μM) for 10 min at 37°C, then 0.5 ml of the appropriate substrate was added and the residual proteolytic activity was determined as described in Methods. ○, Chromatin protease; △, trypsin.

The molar ratio of interaction of the inhibitor with chromatin protease and trypsin was confirmed by titration of unblocked active enzyme molecules with NPGB (Fig. 3). One mole of the soybean inhibitor inhibited one mole of either enzyme. The results obtained with DFP and Kunitz trypsin inhibitor were again the same.

The results presented above would suggest that the regions around the active centre of chromatin protease and trypsin are so similar that it proved impossible to detect any differences with the use of the various inhibitors applied.

Chromatin protease was found to resemble trypsin also in specificity of peptide bond cleavage. Lysine and arginine were identified as C-terminal amino acids in peptides of histones digested by this enzyme (Fig. 4). This would indicate that

chromatin protease from calf thymus cleaves bonds Lys-X and Arg-X. The obtained data confirm the similarity in active site arrangement between the neutral protease from calf thymus chromatin and trypsin.

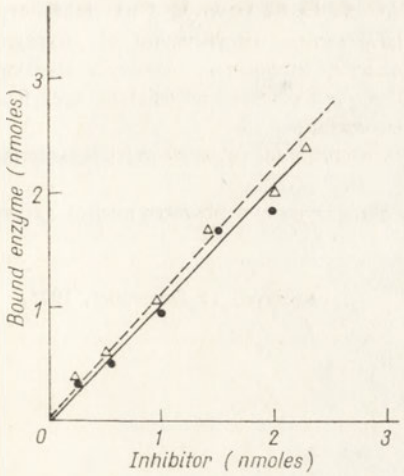

Fig. 3

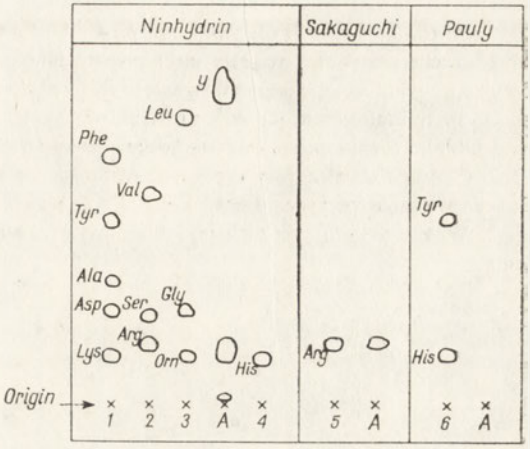

Fig. 4

Fig. 3. Binding of soybean trypsin inhibitor to chromatin protease and trypsin, determined by NPGB titration. The enzyme, 0.5 ml (5 μM in active site), was incubated with 0.5 ml of the inhibitor (0.5 - 5 μM) in 0.1 M-veronal buffer, pH 8.3, for 5 min, then 5 μl of 10 mM-NPGB was added, and liberation of *p*-nitrophenol was monitored at 410 nm. ●, Chromatin protease; △, trypsin.

Fig. 4. Identification of C-terminal amino acids of peptides formed from chromatin protease--treated histones. Ascending t.l.c. on silica gel plate was performed as described in Methods. *1 - 6*, standard amino acids (0.5 μg each in 0.5 μl of 0.1 M-HCl). *A*, The isolated peptides obtained from 2 mg of histones, submitted to hydrazinolysis.

REFERENCES

Bailey, J. L. (1967) in *Techniques in Protein Chemistry* (J. L. Bailey, ed.) pp. 225 - 229. Elsevier Publ. Comp., Amsterdam - London - New York.

Bartley, J. & Chalkley, R. (1970) *J. Biol. Chem.*, **245**, 4286 - 4292.

Chase, T. Jr. & Shaw, E. (1967) *Biochem. Biophys. Res. Commun.*, **29**, 508 - 514.

Furlan, M. & Jericijo, M. (1967) *Biochim. Biophys. Acta*, **147**, 145 - 153.

Garrels, J. I., Elgin, S. C. R. & Bonner, J. (1972) *Biochem. Biophys. Res. Commun.*, **46**, 545--551.

Kunitz, M. (1947) *J. Gen. Physiol.*, **30**, 291 - 296.

Kurecki, T. & Toczko, K. (1972) *Bull. Acad. Polon. Sci., Ser. Sci. Biol.*, **20**, 543 - 546.

Kurecki, T. & Toczko, K. (1974) *Acta Biochim. Polon.*, **21**, 225 - 233.

Kurecki, T., Toczko, K. & Chmielewska, I. (1971) *Bull. Acad. Polon. Sci., Ser. Sci. Biol.*, **19**, 627 - 631.

Kurecki, T., Kowalska-Loth, B., Toczko, K. & Chmielewska , I. (1975) *FEBS Lett.*, **53**, 313 - 315.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.

Smith, I. (1958) in *Chromatographic Techniques* (I. Smith, ed.) pp. 59 - 91. W. Heinemann Medical Books, London.

PODOBIEŃSTWO STRUKTURY WOKÓŁ CENTRUM AKTYWNEGO OBOJĘTNEJ PROTEAZY Z CHROMATYNY GRASICY CIELĘCEJ ORAZ TRYPSYNY

Streszczenie

1. Porównano wrażliwość obojętnej proteazy z grasicy cielęcej i trypsyny na inhibitory. Proteaza chromatynowa reaguje stechiometrycznie z inhibitorami specyficznymi dla trypsyny (dwuizopropylofluorofosforanem, tosylolizylo chlorometanem, inhibitorem sojowym trypsyny i zasadowym inhibitorem Kunitza z trzustki wołowej), ale nie jest wrażliwa na działanie specyficznego inhibitora chymotrypsyny (tosylo-fenyloalanylo chlorometanu).

2. Proteaza chromatynowa przypomina trypsynę w specyficzności substratowej i rozszczepia głównie wiązania peptydowe typu Liz-X i Arg-X.

3. Wyniki wskazują na duże podobieństwo struktury obu enzymów w obszarze miejsca aktywnego.

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INHIBITION BY ALANINE OF AMP-DEAMINASE FROM RABBIT SKELETAL MUSCLE *

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1. Alanine inhibits rabbit muscle AMP-deaminase while aspartate, histidine and glutamate are ineffective.

2. The degree and type of inhibition of AMP-deaminase by alanine depend on pH; at pH 6.5 alanine behaves like an allosteric effector exerting a negative heterotropic effect. At pH 7.0 the inhibition is non-competitive, K_i being as high as 19 mM.

3. The probable significance of the effect of alanine on AMP-deaminase in muscle metabolism is discussed.

AMP-deaminase (EC 3.5.4.6) is an enzyme widely distributed in animal tissues, the highest activity being present in skeletal muscle (Purzycka, 1962; Umiastowski, 1964). The enzyme has an oligomeric structure and is regulated by several low-molecular-weight effectors (Ashman & Atwell, 1972; Boosman *et al.*, 1971). The most potent activator of AMP-deaminase from rabbit skeletal muscle is potassium ion, its optimal concentration being 150 mM in imidazole-HCl buffer, pH 6.5 (Smiley & Suelter, 1967). The most important inhibitors are fluoride and inorganic phosphate (Lee, 1957). Some nucleotides control also AMP-deaminase activity; it has been shown that ATP and ADP activate the enzyme while GTP may inhibit it (Ronca-Testoni *et al.*, 1970). Quite recently it has been reported that IMP — the product of the reaction — may also inhibit the enzyme activity (Melander, 1974).

In this paper the effect of some amino acids on rabbit muscle AMP-deaminase activity is reported. Alanine has been shown to inhibit the enzyme. Kinetics of this inhibition at two different pH values is described and its significance for muscle metabolism is briefly discussed.

* This work was supported by the Polish Academy of Sciences within the project II.1.2.6.

MATERIALS AND METHODS

Enzymatic preparation. AMP-deaminase was purified and crystallized from rabbit skeletal muscle according to the procedure of Smiley *et al.* (1967). The enzyme preparation obtained had a specific activity of 88 μmoles/min/mg of protein at 25°C.

The enzyme assay. The AMP-deaminase activity was measured by following changes in absorbance either at 265 or 285 nm (Kalckar, 1947) using an Unicam SP-800 spectrophotometer fitted with a constant temperature cell housing. The reaction was started by the addition of 20 μl of appropriately diluted enzyme to an incubation medium containing AMP at concentration of 0.07 mM, 150 mM-KCl and 100 mM-imidazole-HCl buffer, pH 6.5. All incubations were carried out at 25°C.

From a 2-min tracing of the change in absorbance the initial linear part was taken for calculation of the initial velocity and the amount of substrate decomposed. The coefficients of Smiley & Suelter (1967) were used for calculation.

Reagents. AMP was obtained from Sigma (St.Louis, Mo., U.S.A.), L-α-alanine from Reanal (Budapest, Hungary) and imidazole from Fluka (Basel, Switzerland). All other reagents of the analytical purity were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

Table 1 illustrates the effect of alanine, aspartate, histidine and glutamine on the activity of AMP-deaminase in rabbit skeletal muscle. As it may be seen, among the amino acids tested only alanine exhibited a distinct inhibitory effect. Figure 1 presents the Michaelis-Menten plots for the reaction catalysed by AMP-deaminase in the presence of alanine at three concentrations, at pH 7.0 (Fig. 1A) and 6.5 (Fig. 1B). The dependence of the reaction rate on the substrate concentration at pH 7.0 was hyperbolic both in the absence and presence of alanine, the inhibitory effect of alanine being dependent on its concentration. However, at pH 6.5 alanine

Table 1

The effect of some amino acids on the activity of AMP-deaminase from rabbit skeletal muscle

The concentration of AMP was 0.07 mM; for experimental conditions see Methods.

Addition	AMP-deaminase activity at amino acid concentration	
	5 mM	10 mM
None	100	100
Alanine	56	28
Aspartate	90	80
Histidine	96	82
Glutamine	100	91

behaved like a negative allosteric effector, as on addition of alanine the curve became sigmoid-shaped.

The Dixon plot of the dependence of reciprocal velocity on the inhibitor concentration at pH 7.0 (Fig. 2) suggests non-competitive inhibition of AMP-deaminase by alanine. The K_i calculated from the plot is about 19 mM.

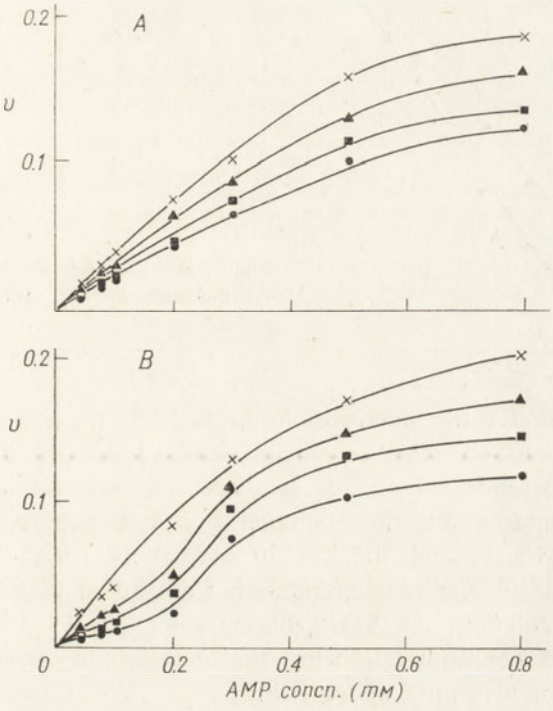

Fig. 1. Michaelis-Menten plots for the reaction catalysed by AMP-deaminase from rabbit skeletal muscle: *A*, at pH 7.0 and *B*, at pH 6.5, without alanine (×), and in the presence of: 5 mM- (▲), 10 mM- (■), or 20 mM- (●) alanine.

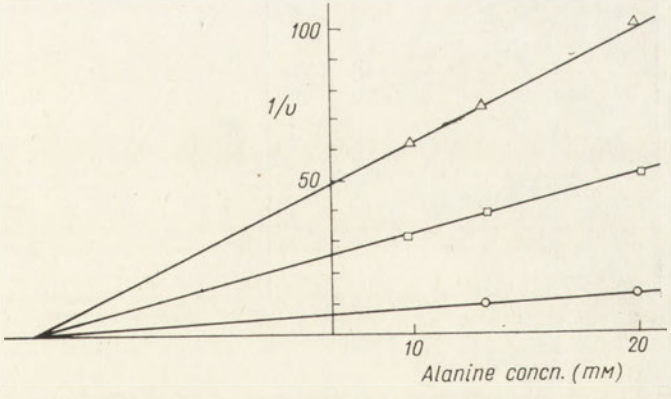

Fig. 2. Dixon plot for the reaction catalysed by AMP-deaminase from rabbit skeletal muscle at pH 7.0 in the presence of alanine. AMP concentration: 0.04 mM (△); 0.10 mM (□); 0.50 mM (○).

From the Hill plot presented in Fig. 3 one may see that the cooperativity coefficient was about 1.0 without alanine, and increased to 1.2 and 1.6 in the presence of 5 mM- and 20 mM-alanine, respectively.

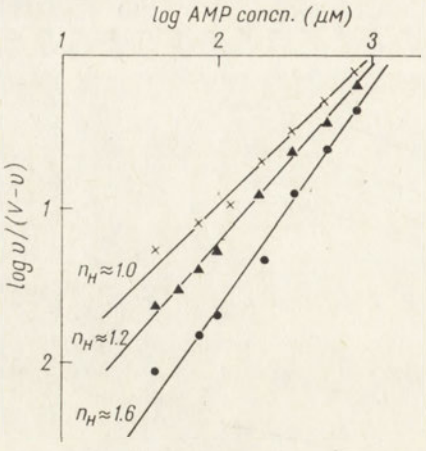

Fig. 3. Hill plot for the reaction catalysed by AMP-deaminase from rabbit skeletal muscle at pH 6.5 in the presence of alanine. Designations as in Fig. 1.

Figure 4 illustrates the interrelationship between the effects of alanine and AMP concentrations on AMP-deaminase activity. At pH 6.5 (Fig. 4B) the inhibition was dependent both on substrate and inhibitor concentration. At low substrate concentration the inhibition was quite appreciable and reached about 40% at 5 mM-, and 70% at 10 mM-alanine (Fig. 4B and Table 1). A further increase in alanine concentration from 10 to 20 mM hardly changed the degree of inhibition. At AMP concentration of 0.5 mM, the enzymatic activity decreased concomitantly with the increase in alanine concentration but the inhibition was only about half that at low substrate concentration.

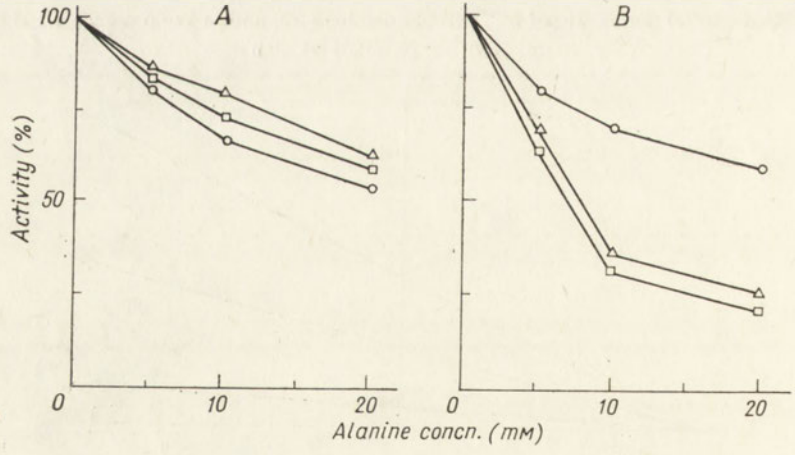

Fig. 4. The dependence of the inhibition of AMP-deaminase by alanine on the inhibitor concentration: *A*, at pH 7.0 and *B*, at pH 6.5. The reaction was carried out as described in Methods, at three AMP concentrations. Designations as in Fig. 2.

This is in contrast to the inhibition observed at pH 7.0 (Fig. 4A), which at this pH value was practically independent of the substrate concentration. The inhibition by alanine at pH 7.0 did not exceed 45%.

DISCUSSION

The inhibition of AMP-deaminase from skeletal muscle by alanine has a complex kinetic characteristics dependent on the pH value, as at pH 7.0 the extent of inhibition depends on the inhibitor concentration, but at pH 6.5 it depends also on substrate concentration.

Alanine is one of the main metabolites in the glucose-alanine cycle (Felig, 1973) involved in energy and nitrogen metabolism of the skeletal muscle (Odessey *et al.*, 1974). In the resting state of the muscle an appreciable part of glucose is converted to alanine. The latter compound is transported to liver where it constitutes one of the main substrates for gluconeogenesis (Felig, 1973). The amino group for alanine formation in the resting muscle is derived mainly from branched-chain amino acids *via* transaminations (Odessey *et al.*, 1974; Ruderman & Berger, 1974). It may be assumed that in the working muscle the main source of amino groups for alanine is AMP (Ruderman, 1972) the deamination of which is a part of the purine nucleotide cycle (Lowenstein, 1972). Although the metabolic significance of this cycle is not entirely clear, its several regulatory functions have been proposed by Lowenstein (1972). It may be therefore concluded that connection between AMP and alanine metabolism in the muscle evidently exists, although the regulatory aspects of this connection are far from being understood. It is worth stressing that ammonia formed during AMP deamination in muscle may cause local changes of pH and thereby influence the activity of several enzymes (Atkinson, 1966), including AMP-deaminase itself, as the inhibitory effect of alanine on this activity was found to depend on the pH value.

REFERENCES

Ashman, L. K. & Atwell, J. L. (1972) *Biochim. Biophys. Acta*, **258**, 618 - 625.
Atkinson, D. (1966) *Ann. Rev. Biochem.*, **35**, 85 - 124.
Boosman, A., Sammons, D. & Chilson, O. (1971) *Biochem. Biophys. Res. Commun.*, **45**, 1025 - 1032.
Felig, P. (1973) *Metabolism*, **22**, 179 - 207.
Kalckar, H. M. (1947) *J. Biol. Chem.*, **167**, 445 - 459.
Lee, Y. P. (1957) *J. Biol. Chem.*, **227**, 999 - 1007.
Lowenstein, J. M. (1972) *Physiol. Rev.*, **52**, 382 - 414.
Melander, W. R. (1974) *Arch. Biochem. Biophys.*, **164**, 90 - 94.
Odessey, R., Khairallah, E. A. & Goldberg, A. L. (1974) *J. Biol. Chem.*, **249**, 7623 - 7629.
Purzycka, J. (1962) *Acta Biochim. Polon.*, **9**, 83 - 93.
Ronca-Testoni, S., Raggi, A. & Ronca, G. (1970) *Biochim. Biophys. Acta*, **198**, 101 - 112.
Ruderman, N. B. (1972) *Isr. J. Med. Sci.*, **8**, 259 - 302.
Ruderman, N. B. & Berger, M. (1974) *J. Biol. Chem.*, **249**, 5500 - 5506.
Smiley, K. L. & Suelter, C. H. (1967) *J. Biol. Chem.*, **242**, 1980 - 1981.
Smiley, K. L., Berry, A. J. & Suelter, C. H. (1967) *J. Biol. Chem.*, **242**, 2502 - 2506.
Umiastowski, J. (1964) *Acta Biochim. Polon.*, **11**, 459 - 464.

ALANINA INHIBITOREM DEZAMINAZY AMP Z MIĘŚNI SZKIELETOWYCH KRÓLIKA

Streszczenie

1. Stwierdzono, że alanina jest inhibitorem AMP-dezaminazy mięśni szkieletowych królika.

2. Typ oraz stopień inhibicji zależy od pH: w pH 6.5 alanina jest silnym inhibitorem enzymu i zachowuje się jak efektor allosteryczny, wykazując negatywny efekt heterotropowy. W pH 7.0 inhibicja ma charakter niekompetycyjny, a K_i wynosi 19 mM.

3. Przedyskutowano znaczenie stwierdzonego faktu dla metabolizmu mięśnia szkieletowego.

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ANNA BARAŃCZYK-KUŹMA, ZOFIA POREMBSKA and IRENA MOCHNACKA

OLIGOMERIC STRUCTURE OF A_1 ARGINASE FROM RAT LIVER AND A_4 FROM KIDNEY. DIFFERENCE IN CHARGE OF SUBUNITS*

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1. The predominant form of rat liver arginase, A_1, and that of kidney, A_4, were isolated and partially purified.

2. It was found that arginase A_4, similarly as A_1, has oligomeric structure. Either of the enzymes on EDTA treatment dissociates into inactive subunits of molecular weight 30 000 daltons. Addition of Mn^{2+} ions restores the activity and causes reassociation of subunits to the native form of 120 000 mol. wt.

3. The subunits of A_4 differ considerably in electrophoretic mobility from subunits of A_4, which probably is the reason why the native forms of the enzyme from kidney and liver differ in electrophoretic behaviour.

The presence of two forms of arginase: A_1 and A_3 in rat liver and A_1 and A_4 in kidney was demonstrated in our laboratory (Gąsiorowska *et al.*, 1970), A_1 being the main form of the enzyme in liver and A_4 in kidney. The two main forms were found to have the same molecular weight of 120 000 daltons, the same pH optimum at 9.2 - 9.5, similar affinity to arginine (K_m 3.9 and 8 mM, respectively), and both were stabilized during storage by Mn^{2+}.

The two forms differed, however, in behaviour on DEAE- and CM-cellulose columns as well as on starch-gel electrophoresis. The liver arginase was adsorbed on CM-cellulose whereas that of kidney, on DEAE-cellulose. The arginase from liver showed on starch-gel electrophoresis at pH 8.3 a positive charge and migrated slowly towards catode, the enzyme from kidney had a negative charge, migrating rather fast towards anode (Porembska *et al.*, 1971). Kaysen & Strecker (1973) also found that arginases from liver and kidney of the rat differ in the behaviour on CM- and DEAE-cellulose. Moreover, they confirmed the non-identity of the two arginases by immunological methods.

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Oligomeric structure of cationic rat liver arginase was repeatedly reported (Hirsch-Kolb & Greenberg, 1968; Hosoyama, 1972; Penninckx *et al.*, 1974) but the molecular characteristics of rat kidney arginase have not so far been studied. It seems possible that different electrophoretic mobility of these two enzymes is related to a difference in their constituent subunits. A preliminary report of this work has been presented (Barańczyk-Kuźma & Porembska, 1975).

MATERIALS AND METHODS

Chemicals. They were from the following sources: L-arginine hydrochloride (Sigma Chem.Co., St.Louis, Mo., U.S.A.), Aquacid II (Calbiochem, Los Angeles, Calif., U.S.A.), ninhydrin (Reanal, Budapest, Hungary), Whatman DEAE-cellulose DE 11 and Whatman CM-cellulose (Whatman Biochemicals, Maidstone, Kent, England), Sephadex G-150 and Blue Dextran 2000 (Pharmacia, Uppsala, Sweden). Marker proteins: bovine serum albumin, chicken ovalbumin, bovine γ-globulin (Cohn fraction II) and horse myoglobin were from Sigma Chem. Co. Other chemicals were of analytical grade (Ciech, Gliwice, Poland).

Arginase assay. The enzyme activity was determined by measuring formation of ornithine from arginine by the method of Chinard (1952). The incubation mixture (1 ml) contained 100 mM-sodium barbitone buffer, pH 9.5, 5 mM-$MnCl_2$, 20 mM-arginine and an appropriate amount of the enzyme preparation. After 10 min at 37°C, 2 ml of conc. acetic acid and 1 ml of acid ninhydrin reagent (125 mg of ninhydrin in a mixture of 6 ml conc. acetic acid and 4 ml of 6 M-H_3PO_4) were added, the mixture heated for 1 h in a boiling-water bath, cooled, and the extinction at 515 nm determined. The enzyme activity is expressed in units (μmoles of ornithine formed/min at 37°C).

Protein was determined as described by Lowry *et al.* (1951) with crystalline bovine serum albumin as standard.

Molecular weight was estimated by gel filtration on Sephadex G-150 column (40 × 2 cm) equilibrated with 100 mM-KCl in 50 mM-Tris buffer, pH 7.5, and eluted with the same buffer. The void volume of the column, as determined with Blue Dextran 2000, was 30 ml. For calibration of the column, marker proteins (5 mg each) were used.

Polyacrylamide disc-gel electrophoresis was carried out in 7.5% gel at pH 9.5 according to Smith et al. (1968) at 4°C and 2 mA/tube for 15 min, and then at 3 mA/tube for 45 min. Protein was stained with 0.5% Amido Black in 7% (v/v) acetic acid. Arginase activity was detected in 2-mm gel slices eluted with barbitone buffer of pH 9.5.

Isolation and purification of the enzyme

In rat liver and kidney, arginase activity is present mainly in the mitochondrial and nuclear fractions (Gąsiorowska *et al.*, 1970). Isolation and purification of liver arginase A_1 and kidney arginase A_4 was performed by a modification of the

method described previously (Gąsiorowska *et al.*, 1970; Dahlig *et al.*, 1975), the procedure being carried out in the presence of 5 mM-$MnCl_2$ to stabilize the enzyme (Greenberg & Mahamed, 1949; Gąsiorowska *et al.*, 1970), and 1 mM-2-mercaptoethanol to prevent possible arginase aggregation (Sakai & Murachi, 1969; Vielle-Breitburd & Orth, 1972).

Animals. Adult male albino rats weighing about 150 g were killed by decapitation, liver and kidney rapidly removed and washed with cold 0.25 M-sucrose. All the subsequent procedure was carried out at 4°C.

Step. 1. Homogenization. For each series of enzyme isolation, about 30 g of liver and 50 g of kidney were homogenized (3 × 1 min at 1900 rev/min) in 5 vol. of 0.25 M-sucrose and centrifuged for 15 min at 11 000 *g*.

Step 2. Extraction. The obtained sediments containing the nuclear and mitochondrial fractions were washed with 0.25 M-sucrose, recentrifuged, suspended in 5 vol. (of the original tissue weight) of a solution containing 5 mM-$MnCl_2$, 100 mM-KCl and 10 mM-Tris-HCl buffer, pH 7.5, and stored at −10°C for 12 h. To extract the enzyme, the suspensions after thawing were kept in an ice-bath for 30 min with gentle stirring and then centrifuged at 6000 rev./min. The supernatants were collected and the sediments were reextracted twice in the same way and combined with the first extracts.

Step 3. First ammonium sulphate fractionation. The fractionation was carried out at pH 7.5 with solid ammonium sulphate. The precipitate at 0.3 - 0.7 sat. for liver and 0.0 - 0.6 sat. for kidney were collected by centrifugation, dissolved in 5 mM-$MnCl_2$ - 1 mM-2-mercaptoethanol - 5 mM-Tris-HCl buffer, pH 8.3, and dialysed for 20 h against 10 litres of the same buffer solution.

Step 4. DEAE-cellulose chromatography. The dialysed solutions were applied to DEAE-cellulose columns (18 × 1 cm) equilibrated with 5 mM-Tris-HCl buffer, pH 8.3, and eluted with a KCl concentration gradient up to 0.3 M in the same buffer. Liver arginase A_1 emerged with the buffer front (Fig. 1), and kidney arginase A_4 at 0.1 - 0.15 M-KCl. The active fractions were pooled, concentrated using Aquacid in the presence of $MnCl_2$ at a final concentration of about 5 mM, and dialysed against 5 litres of 5 mM-$MnCl_2$ - 1 mM-2-mercaptoethanol - 5 mM-Tris-HCl buffer, pH 7.5.

Step 5. Second ammonium sulphate fractionation. The dialysed preparations were cleared by centrifugation and protein precipitated at 0.4 - 0.6 ammonium sulphate sat. for liver and 0.35 - 0.6 sat. for kidney was collected, dissolved in 5 mM-$MnCl_2$ - 1 mM-2-mercaptoethanol - 5 mM-Tris-HCl buffer, pH 7.5, and dialysed overnight against 5 litres of the same solution.

Step 6. CM-cellulose chromatography. The dialysed preparations were applied to CM-cellulose columns (18 × 1 cm) equilibrated with 5 mM-Tris-HCl buffer, pH 7.5, and eluted with a KCl concentration gradient up to 0.3 M in the same buffer. Kidney arginase A_4 emerged with the buffer front (Fig. 2), and liver arginase A_1 was eluted at 0.05 - 0.08 M-KCl. The active fractions of both enzymes were concentrated using Aquacid as described above and dialysed against 5 mM-$MnCl_2$ - 1 mM-2-mercaptoethanol - 50 mM-Tris-HCl buffer, pH 7.5, for 20 h.

Step 7. Sephadex G-150 gel filtration. The obtained preparations were applied to columns (40 × 2 cm) equilibrated with 100 mM-KCl - 50 mM-Tris-HCl buffer, pH 7.5, and eluted with the same buffer. The pooled active fractions were concentrated with Aquacid as above, and dialysed against 2 litres of 5 mM-$MnCl_2$ - 1 mM-2-mercaptoethanol - 50 mM-Tris-HCl buffer, pH 7.5, for 12 h. The dialysed pre-

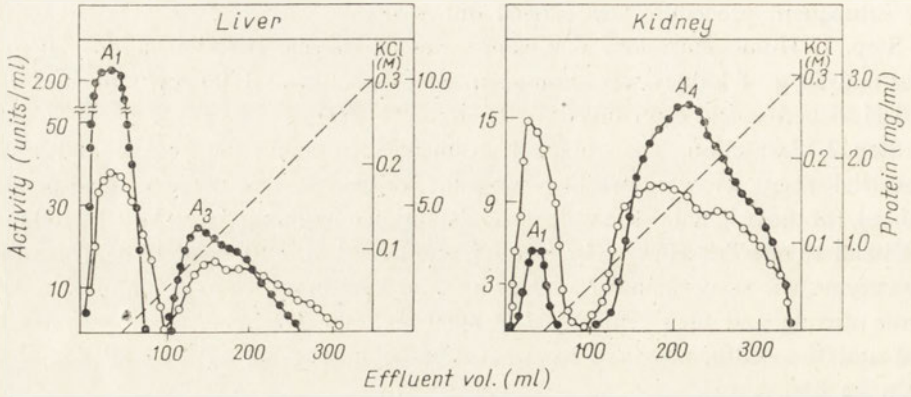

Fig. 1. DEAE-cellulose chromatography of liver (A_1) and kidney (A_4) arginase. About 600 mg of protein from 1st ammonium sulphate fractionation dialysed against 5 mM-$MnCl_2$ - 1 mM-2-mercaptoethanol - 5 mM-Tris-HCl buffer, pH 8.3 (step 3), was applied to the column (18 × 1 cm) equilibrated and eluted with 5 mM-Tris-HCl buffer, pH 8.3, then with a KCl concentration gradient. Fractions of 5 ml were collected and protein and arginase activity determined in a standard assay mixture. ●, Arginase activity; ○, protein; – – –, KCl gradient.

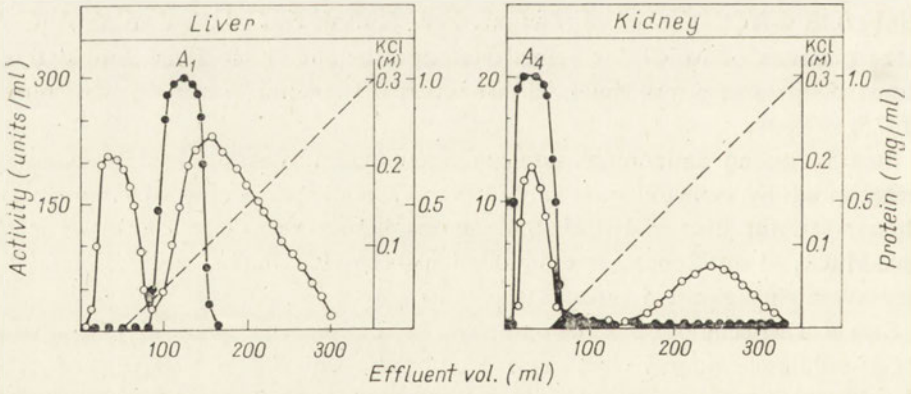

Fig. 2. CM-cellulose chromatography of A_1 and A_4 arginase. About 30 mg of A_1 and 40 mg of A_4 arginase obtained after DEAE-cellulose (step 5) were applied to the column (18 × 1 cm) equilibrated and eluted with 5 mM-Tris-HCl buffer, pH 7.5, then with a KCl concentration gradient. Fractions of 5 ml were collected. Arginase activity was determined in standard assay mixture. ●, Arginase activity; ○, protein; – – –, KCl gradient.

Table 1

Purification of arginase A_1 from rat liver and A_4 from kidney

The values are normalized to a preparation from 100 g of tissue.

Step	Liver				Kidney			
	Activity (units)		Yield (%)	Purification factor	Activity (units)		Yield (%)	Purification factor
	Total	per mg protein			Total	per mg protein		
1. Nuclei and mitochondria homogenate	69 066	3.7	100	—	2080	0.25	100	—
2. Extract	55 060	8	80	2.2	1960	0.7	94	2.8
3. 1st Ammonium sulphate fractionation*	48 000	24	70	6.5	1680	1.4	80	5.6
4. DEAE-cellulose chromatography	26 666	100	39	27	1020	5.1	49	20
5. 2nd Ammonium sulphate fractionation**	22 000	220	32	60	707	8.8	34	35
6. CM-cellulose chromatography	18 888	400	27	108	644	13.3	32	53
7. Sephadex G-150 gel filtration	14 933	1120	21	303	580	29	28	116

* Ppt. at 0.3 - 0.7 sat. for liver, and 0 - 0.6 for kidney.

** Ppt. at 0.4 - 0.6 sat. for liver, and 0.35 - 0.6 for kidney.

parations from liver and kidney, containing 2 and 5 mg of protein/ml, respectively, were stored at $-10°C$.

The purification procedure is outlined in Table 1. The arginase preparation obtained from liver (A_1) was purified about 300-fold, and that from kidney (A_4), about 120-fold; their activity was, respectively, 1120 and 29 μmoles of ornithine/mg protein/min. Higher purification of kidney arginase was not achieved due to instability of the enzyme. However, either of the two obtained enzymes was efficiently separated from the second form of arginase present in the respective tissue, i.e. form A_1 from liver was not contaminated with form A_3, and form A_4 from kidney with form A_1.

RESULTS

The experiments on inactivation and reactivation of the enzyme were carried out with preparations obtained after CM-cellulose chromatography, concentrated in the presence of 5 mM-$MnCl_2$ and dialysed against 5 mM-$MnCl_2$ - 1 mM-2--mercaptoethanol - 50 mM-Tris-HCl buffer, pH 7.5 (step 6). The specific activity of the liver A_1 and kidney A_4 arginase was about 400 and 13 units/mg protein, respectively.

Effect of EDTA and Mn^{2+} on the activity of arginase A_1 and A_4

EDTA inactivated both arginase A_1 and A_4, the degree of inactivation being dependent on the amount of EDTA and the time of treatment. Arginase A_1, 2 μg, incubated at pH 7.5 for 30 min at 37°C with 1 mM-EDTA was inactivated in 50%, and with 14 mM-EDTA the inactivation was complete (Fig. 3a). With 16 mM-EDTA, the inactivation proceeded rapidly during the first minutes of incubation so that after 10 min only 10% of the activity was retained (Fig. 3b).

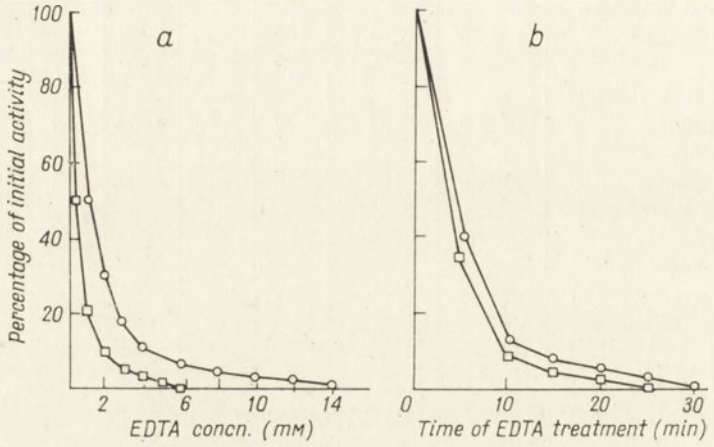

Fig. 3. Effect of EDTA concentration (*a*) and time of treatment (*b*) on the activity of arginase A_1 and A_4. Two mg of protein after step 6 was incubated with EDTA at pH 7.5 and 37°C. Then the residual arginase activity was determined without the addition of Mn^{2+} ions. ○, Arginase A_1 and □, arginase A_4 preparation. In *a*, the time of EDTA action was 30 min; in *b*, 16 mM-EDTA for A_1, and 8 mM-EDTA for A_4 were used.

The preparation of kidney arginase A_4 showed similar susceptibility to EDTA but lower EDTA concentrations were required: 50% inhibition was observed in the presence of 0.4 mM-EDTA, and complete inhibition, with 6 mM-EDTA.

Arginase A_1 and A_4 inactivated by an excess of EDTA (16 mM and 6 mM, respectively), were fully reactivated by the addition to the assay mixture of Mn^{2+} ions in an amount equimolar with respect to EDTA (Table 2). Preincubation of the EDTA-treated enzyme preparations with Mn^{2+} was not required for reactivation. A twofold molar excess of Mn^{2+} ions lowered the reactivation of arginase A_1 and A_4 by about 20%. It should be noted that this amount of Mn^{2+} lowered also the activity of the native enzyme.

Molecular weight of the EDTA-treated arginase

The molecular weight of the liver and kidney arginase present in the preparations after step 6, determined by Sephadex G-150 gel filtration, was 120 000 daltons (Fig. 4) which is an agreement with previous findings (Gąsiorowska *et al.*, 1970).

Table 2

Effect of EDTA and Mn^{2+} on arginase activity

The dialysed and concentrated preparations after step 6 (for details see Methods) were preincubated with EDTA at pH 7.5 at 37°C for 30 min, then arginase activity was determined in the whole mixture, without the addition or with the indicated amount of Mn^{2+}. The activity is expressed as μmoles of ornithine formed per 2 μg of protein per 10 min.

Enzyme preparation	Preincubation with EDTA (μmoles/sample)	Mn^{2+} added (μmoles/sample)	Activity	
			per sample	%
Liver, A_1	0	0	8	100
	0	8	8	100
	0	16	6.4	80
	8	0	0.0	0
	8	5	4.8	60
	8	8	8.0	100
	8	10	8.0	100
	8	12	7.6	97
	8	16	6.0	75
Kidney, A_4	0	0	0.27	100
	0	3	0.27	100
	0	6	0.19	70
	3	0	0.0	0
	3	1.5	0.12	45
	3	3	0.28	101
	3	4.5	0.27	100
	3	6.0	0.21	80

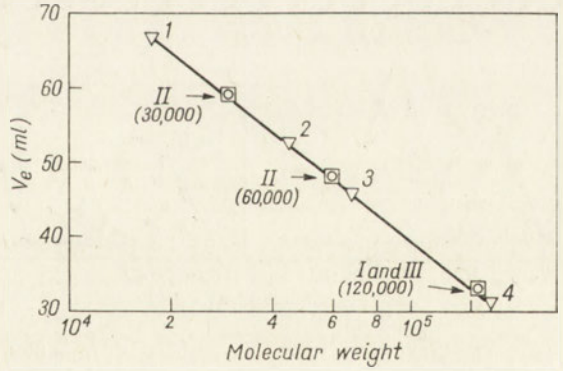

Fig. 4. Molecular weight determination by Sephadex G-150 gel filtration of arginase: ○, from liver (A_1), and □, from kidney (A_4). The column (40 × 2 cm) was equilibrated with 100 mM-KCl in 50 mM-Tris-HCl buffer, pH 7.5, and eluted with the same buffer at a flow rate of 25 ml/h. Fractions of 2 ml were collected. Marker proteins: *1*, horse myoglobin (mol. wt. 17 000); *2*, chicken ovoalbumin (45 000); *3*, bovine serum albumin (69 000); *4*, bovine γ-globulin (150 000). Arginase preparations: *I*, native; *II*, EDTA-treated (inhibited in 90%); *III*, reactivated with Mn^{2+}.

The preparations of A_1 and A_4 completely inactivated by EDTA, on calibrated Sephadex G-150 column gave each a single fraction with a molecular weight of 30 000 daltons, active only after addition of Mn^{2+} (Fig. 5a,c). The enzyme preparations inactivated by EDTA in 85 - 95%, separated into three fractions (Fig. 5b,d). The first fraction, of mol. wt. 120 000, was active without the addition of Mn^{2+} but its activity was enhanced in the presence of manganese. The other two fractions, of mol. wt. 60 000 and 30 000, were inactive without Mn^{2+} added but became active in its presence. In some preparations the 60 000 form was found only in trace amount.

The elution patterns from Sephadex G-150 were dependent on EDTA concentration used for inactivation. With higher EDTA concentration, the 120 000 form did not appear, and the 30 000 form was predominant. With lower EDTA concentration, two or even three forms were simultaneously observed.

There was no evidence of subunit aggregation. The fractions of 30 000 mol.wt. after concentration and dialysis against 50 mM-Tris-HCl buffer, pH 7.5, refiltered on Sephadex G-150 column, emerged at the same elution volume.

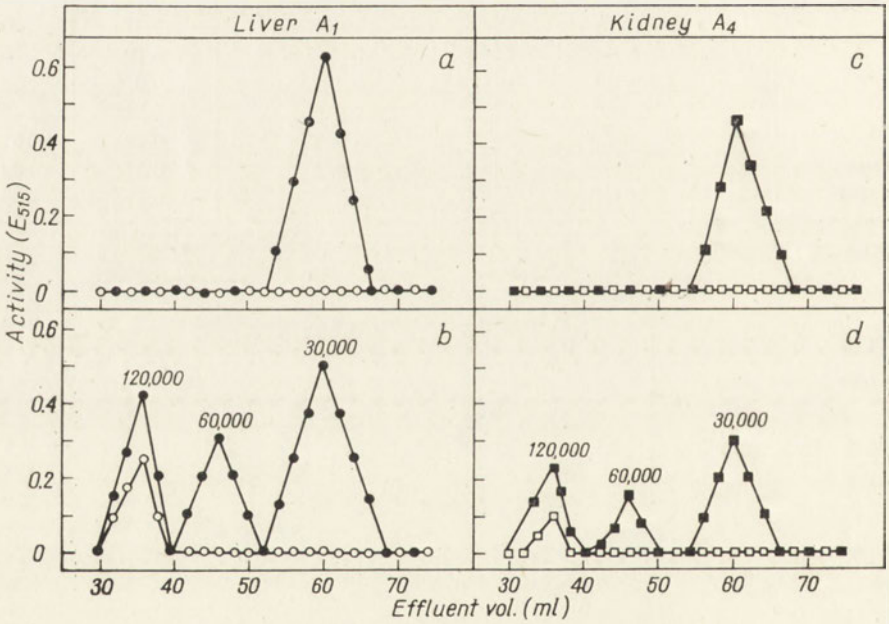

Fig. 5. Sephadex G-150 gel filtration of EDTA-treated arginase A_1 from liver and A_4 from kidney. The enzyme preparations after step 6 were inactivated by treatment with EDTA at pH 7.5 at 37°C. Samples of 2 ml containing 3 mg of protein were loaded on the column (40 × 2 cm) and eluted with 100 mM-KCl - 50 mM-Tris-HCl buffer, pH 7.5. Fractions of 2 ml were collected at a flow rate of 25 ml/h. *a* and *c*, Arginase inactivated completely with EDTA; *b* and *d*, arginase inactivated in 85 - 95%. For activity determination, 2 μg protein of A_1 preparation, and 40 μg of A_4, were used. Arginase activity was measured in the presence of 5 mM-$MnCl_2$ (filled-in symbols) and in the absence of $MnCl_2$ (outlined symbols).

Reassociation of the 30 000 subunits

The fractions of 30 000 mol.wt. of arginase A_1 and A_4, obtained by Sephadex G-150 gel filtration of the EDTA-treated enzyme, were concentrated with Aquacid and dialysed against 5 mM-$MnCl_2$ - 50 mM-Tris-HCl buffer, pH 7.5, at 2 - 4°C for 20 h. To the dialysis residue, fresh dialysis solution was added (to a concentration of 1 - 2 mg protein/ml), and incubated for 30 min at 37°C. On refiltration of these samples on Sephadex G-150, single active fractions were obtained, with a molecular weight corresponding to that of the native enzymes (Fig. 6a,c). In some experiments a second fraction, of about 60 000 daltons, was present.

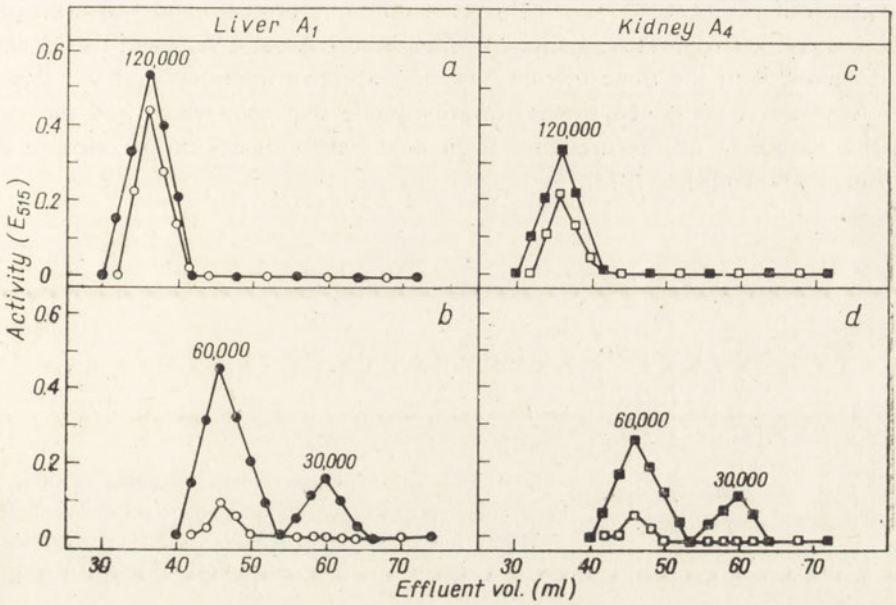

Fig. 6. Sephadex G-150 gel filtration of Mn^{2+}-reactivated 30 000 subunits of arginases A_1 and A_4. The fractions of 30 000 mol. wt. of arginase A_1 and A_4 obtained on gel filtration of the EDTA--treated enzyme (Fig. 5*a* and *c*) were concentrated with Aquacid, and dialysed against 5 mM-$MnCl_2$ - 0.2 M-Tris-HCl buffer, pH 7.5 at 4°C for 24 h. Samples of 2 ml, containing 1 and 2 mg of protein/ml for liver and kidney, respectively, were incubated, loaded on Sephadex G-150 column (40 × 2 cm), and eluted with 100 mM-KCl - 50 mM-Tris-HCl buffer, pH 7.5. Fractions of 2 ml were collected at a flow rate of 25 ml/h. *a* and *c*, Samples incubated, before Sephadex gel filtration, in fresh dialysis mixture for 30 min at 37°C; *b* and *d*, samples with incubation omitted. Arginase activity was measured in the presence of 5 mM-$MnCl_2$ (filled-in symbols) and without Mn^{2+} added (outlined symbols).

When incubation with Mn^{2+} at 37°C was omitted and the dialysed enzyme preparations were directly applied to Sephadex G-150, the form of 120 000 mol.wt. was absent (Fig. 6b,d), only the 60 000 form and traces of 30 000 form being present. The 60 000 subunits showed some activity even without added Mn^{2+}, differing in this respect from the 60 000 subunits obtained by Sephadex G-150 gel filtration, which were completely inactive without manganese added (see Fig. 5b,d). In the

former case the slight activity of the 60 000 subunits was probably due to the presence of trace of free Mn^{2+} ions which resulted in some reassociation into the 120 000 form.

Electrophoretic characteristics of the EDTA-treated and Mn^{2+}*-reactivated arginase*

The liver arginase A_1 (after step 7), non-treated with EDTA, gave on polyacrylamide-gel electrophoresis at pH 9.5 two protein bands and the kidney A_4 arginase, four bands (Fig. 7). In either case only one protein band was associated with arginase activity. The mixture of preparations A_1 and A_4 gave two distinct active bands, with the same mobility as the respective fractions analysed separately. Arginase A_1 migrated slower towards anode than A_4, which had the most negative charge of all the arginases from mammalian tissues investigated in our laboratory (Porembska, 1973).

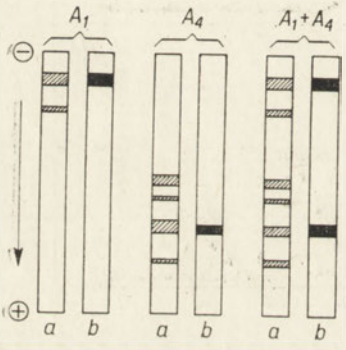

Fig. 7. Diagram of electrophoretic patterns of liver A_1 and kidney A_4 arginase. Polyacrylamide-gel electrophoresis was performed as described in Methods. Enzyme preparations (100 μg protein) after step 7 were used. *a*, Protein; *b*, arginase activity.

Arginase A_1 and A_4 inactivated by EDTA in 85 - 95%, gave on electrophoresis two active fractions each (Fig. 8b). In both cases the slower migrating band had the same mobility as the respective native form (Fig. 8a) and was active without the addition of Mn^{2+}. The second electrophoretic fraction migrating more rapidly than the native enzyme, contained the major part of arginase activity, but in either case it was active only after addition of Mn^{2+}. The enzymes inactivated completely by an excess of EDTA, gave on electrophoresis single fractions which were active only after addition of Mn^{2+} (Fig. 8c). These fractions had the same mobility as those of the respective EDTA-treated preparations separated on Sephadex G-150 columns, which had a mol.wt. of 30 000. In both cases the activity could be demonstrated only after addition of Mn^{2+}.

The 30 000 subunits from kidney showed greater mobility towards anode than those from liver, which points to a considerable difference in their charge.

When the 30 000 subunits from liver and kidney were reactivated by incubation with 5 mM-$MnCl_2$ in 0.05 M-Tris-HCl buffer, pH 7.5, for 30 min at 37°C, they showed on gel electrophoresis single active bands (Fig. 8d) corresponding to those of the native enzyme from the respective tissue (Fig. 8a).

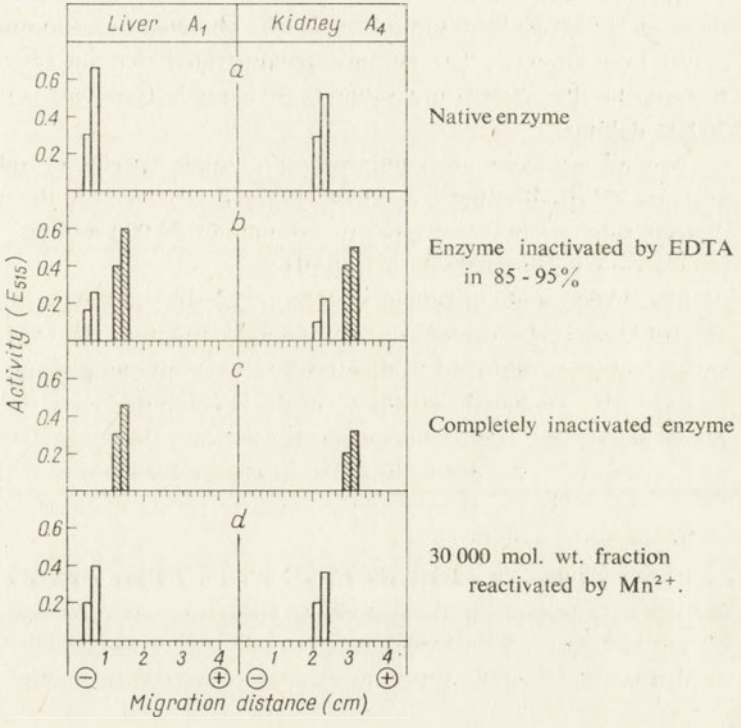

Fig. 8. Polyacrylamide-gel electrophoresis of native and EDTA-treated arginase A_1 and A_4. Preparations after step 6 were used (50 μg protein of A_1 and 100 μg protein of A_4). Outlined bars, the activity determined without the addition of Mn^{2+}; shaded bars, activity determined in the presence of Mn^{2+}.

DISCUSSION

The requirement for Mn^{2+} as a subunit-binding factor seems to be a property common to several reported arginases, both those obtained from various mammalian tissues and from tissues of invertebrates. Human liver arginase (Carvajal *et al.*, 1971), human lung arginase (Dahlig *et al.*, 1975) as well as intestine arginase of polychaete annelid *Pista pacifica* (O'Malley & Terwilliger, 1974) dissociate into subunits after removal of Mn^{2+} by EDTA.

The present report demonstrates the requirement for Mn^{2+} in formation of quarternary structure of both rat liver arginase A_1 and kidney arginase A_4. Removal of Mn^{2+} by EDTA treatment resulted in dissociation of the enzyme into subunits. Depending on EDTA concentration and time of treatment, two kinds of subunits of 60 000 and 30 000 mol.wt., or only one of 30 000 molecular weight were obtained. The subunits were enzymically inactive but addition of Mn^{2+}

restored their activity and caused reassociation of the subunits to the form of 120 000 mol.wt. showing the same electrophoretic mobility as the native enzyme.

The 30 000 subunits of A_1 arginase did not separate on polyacrylamide gel which indicates that the enzyme is composed of a single species of subunits. Although our preparation was not highly purified, these results are in agreement with those of Hirsch-Kolb & Greenberg (1968) obtained on a homogeneous preparation of rat liver arginase. The authors demonstrated that on treatment with 8 M-urea the enzyme dissociated into subunits of a single type with a molecular weight of 30 800 daltons.

Not all arginases are composed of a single species of subunits. Rabbit liver arginase (Vielle-Breitburd & Orth, 1972) dissociated in the presence of sodium dodecyl sulphate into two types of subunits of 32 000 mol.wt., resolved by electrophoresis on polyacrylamide gel at pH 8.3.

The 30 000 mol.wt. subunits of the rat kidney arginase A_4 migrated on polyacrylamide-gel electrophoresis as a single fraction. However, the preparations which had been subjected to dissociation, were inhomogeneous, therefore it cannot be definitely concluded that the form A_4 is composed of a single type of subunits. Nevertheless, our results unequivocally indicate that the 30 000 mol.wt. subunits of liver arginase A_1 distinctly differ in charge from those of kidney arginase A_4. This is probably the reason why the native forms of these two enzymes differ in electrophoretic mobility.

The difference in charge of the liver and kidney arginase subunits might be due to a difference in their primary structure, their biosynthesis being directed by distinct genes. This would support our earlier suggestion (Gąsiorowska *et al.*, 1970) that the multiple forms of arginase observed in mammalian tissues are isoenzymes.

The excellent technical assistance of Mrs. Aleksandra Bakuła is gratefully acknowledged.

REFERENCES

Barańczyk-Kuźma, A. & Porembska, Z. (1975) *XIII Meet. Polish Biochem. Soc., Toruń, Abstr. Commun.*, C-19, 111.

Carvajal, N., Venegas, A., Oestreicher, G. & Plaza, M. (1971) *Biochim. Biophys. Acta*, **250**, 337 - 442.

Chinard, F. P. (1952) *J. Biol. Chem.*, **199**, 91 - 95.

Dahlig, E., Porembska, Z. & Mochnacka, I. (1975) *Acta Biochim. Polon.*, **22**, 77 - 85.

Gąsiorowska, I., Porembska, Z., Jachimowicz, J. & Mochnacka, I. (1970) *Acta Biochim. Polon.*, **17**, 19 - 30.

Greenberg, D. M. & Mahamed, M. S. (1949) *Arch. Biochim.*, **8**, 365 - 375.

Hirsch-Kolb, H. & Greenberg, D. M. (1968) *J. Biol. Chem.*, **243**, 6123 - 6129.

Hosoyama, Y. (1972) *Eur. J. Biochem.*, **27**, 48 - 52.

Kaysen, G. A. & Strecker, H. J. (1973) *Biochem. J.*, **133**, 779 - 788.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.
O'Malley, K. L. & Terwilliger, R. C. (1974) *Biochem. J.*, **143**, 591 - 597.
Pennickx, M., Simon J. P. & Wiame, J. M. (1974) *Eur. J. Biochem.*, **49**, 429 - 442.
Porembska, Z., Jachimowicz, J. & Gąsiorowska, I. (1971) *Bull. Acad. Polon. Sci. Ser. Sci. Biol.* **19**, 27 - 30.
Porembska, Z. (1973) *Enzyme*, **15**, 198 - 209.
Sakai, T. & Murachi, T. (1969) *Physiol. Chem. Phys* **1**, 317 - 333.
Smith, J., Lighstone, P. J. & Perry, J. D. (1968) *Clin. Chim. Acta*, **19**, 499 - 505.
Vielle-Breitburd, F. & Orth, G. (1972) *J. Biol. Chem.*, **247**, 1227 - 1235.

OLIGOMERYCZNA STRUKTURA ARGINAZY A_1 Z WĄTROBY I A_4 Z NERKI SZCZURA
RÓŻNICE W ŁADUNKU PODJEDNOSTEK ENZYMU

Streszczenie

1. Wyizolowano i częściowo oczyszczono główne formy arginazy z wątroby (A_1) i nerki (A_4) szczura.

2. Wykazano że, podobnie jak enzym z wątroby, arginaza A_4 jest białkiem oligomerycznym. Oba enzymy w obecności EDTA dysocjują na nieaktywne podjednostki o masie cząsteczkowej 30 000. Dodanie jonów Mn^{2+} przywraca aktywność i powoduje reasocjację podjednostek do natywnej formy enzymu o masie cząsteczkowej 120 000.

3. Podjednostki arginazy A_4 różnią się znacznie szybkością wędrowania podczas elektroforezy na żelu poliakrylamidowym od podjednostek A_1, co prawdopodobnie decyduje o odmiennym ładunku ich form natywnych.

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JADWIGA GRUNDBOECK-JUŚKO

CHEMICAL CHARACTERISTICS OF CHALONES ISOLATED FROM BOVINE SPLEEN

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Isolation of spleen chalones from the postmicrosomal supernatant is described. Two glycoprotein fractions homogeneous on polyacrylamide-gel electrophoresis were obtained. In the biological test on mice, the preparation of 38 000 mol. wt. inhibited mitosis in spleen cells, and the low-molecular fraction (2100 mol. wt.) in thymus cells, showing respectively the properties of chalones B and T.

Chalones are tissue-specific and not species-specific glycoproteins, inhibiting cell divisions (Bullough & Laurence, 1960, 1964). Chalone has been demonstrated in epidermis, liver, lung, kidney, lymph node, spleen, thymus, blood serum, granulocyte and melanocyte (for review see Houck & Hennings, 1973, and Paukovits, 1973).

The lymphocyte chalones were reported to inhibit the proliferation of leukaemic lymphocytes (Houck & Hennings, 1973) and to act as immunosuppressive agent (Kiger *et al.*, 1972). Little, however, is known about their chemical properties. In our previous work (Grundboeck-Juśko, 1975) two of the fractions isolated from bovine spleen were found to inhibit selectively mitosis in spleen or in lymph nodes and thymus cells, of colchicine- and colcemide-treated mice. According to Hermanowicz & Jóźkiewicz-Hermanowicz (1973), 70% of spleen cells belong to the thymus-independent lymphocyte line (B lymphocytes), and 30% to the thymus-dependent one (T lymphocytes). A reversed proportion was found in the lymph nodes. Therefore it was assumed that both chalone T and B are present in spleen cells.

An attempt at chemical characterization of isolated spleen chalones is the subject of the present work.

MATERIALS AND METHODS

Bovine spleen chalone was isolated from supernatants of smooth and rough microsomes as described previously (Grundboeck-Juśko, 1975). The microsome fraction was centrifuged in a CsCl gradient in 1.3 M-sucrose, the separated smooth

and rough microsomes were centrifuged at 140 000 *g* and the supernatants were extracted with butanol. To the extracts 0.5% SDS was added at pH 5.8, and after 24 h the sediments were discarded by centrifugation. At this step of purification, in the supernatant *A* (from smooth microsomes) 4.3 g of protein from 1 kg of fresh spleen was obtained, and in supernatant *B* (from rough microsomes), 76.4 mg of protein. The supernatants were fractionated by QAE-50A Sephadex chromatography, only about 12% of the applied protein being recovered. From supernatant *A*, four protein fractions were obtained, and from supernatant *B*, three fractions (Fig. 1). The fractions were pooled as indicated in the Figure, dialysed against water and lyophilized. The biological activity of the obtained protein fractions was examined in mice as described previously (Grundboeck-Juško, 1975) using at least 5 animals for each of the 2 - 4 tests performed.

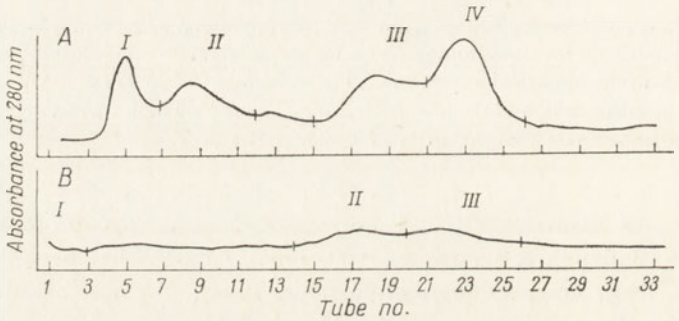

Fig. 1. Fractionation of supernatants *A* and *B* on Sephadex QAE-50A column. To the column (2.2 × 25 cm) equilibrated with 55 mM-NaCl - 8 mM-Tris buffer, pH 8.6, 9 - 10 mg of protein was applied and eluted with the equilibration buffer containing 0.015% sodium azide. Fractions of 3.3 ml were collected at a rate of 13.2 ml/h. Scan speed 2 cm/h = 4 tubes, light path 3 mm.

Analytical methods. Protein was estimated by the method of Wadell (1956) with serum α-globulin as standard. Hexose was determined according to Weimar & Moshin (1953), hexosamine after Rimington (1940), and neuraminic acid according to Warren (1959).

Polyacrylamide-gel electrophoresis was performed according to Gordon (1969).

Ultraviolet absorption spectra were measured in solutions of pH 8.25 with Unicam SP 1700 spectrophotometer (light path 0.1 cm, scan speed 1 nm/sec).

Amino acid composition was estimated with the use of amino acid analyser (Mikrotechma, Praha, type AAA-881, sensitivity 0.005 µl). Hydrolysis was performed in 6 M-HCl at 110°C for 16 h.

Molecular weight was determined by the t.l.c. method (Morris, 1964) on Sephadex G-150 superfine gel, in 5 mM-Tris buffer, pH 7.5.

Reagents. Tris, glucosamine, vitamin B_{12}, ribonuclease A, ovalbumin and bovine γ-globulin were from Koch-Light (Colnbrook, Bucks, England); bovine serum albumin and sodium periodate from British Drug Houses (Poole, Dorset,

England); acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylacrylamide from Fluka A.G. (Buchs, Switzerland); neuraminic acid from Calbiochem (Los Angeles, Calif., U.S.A.), sodium arsenite from E. Merck (Darmstadt, G.F.R.), cytochrome *c*, galactose, mannose and thiobarbituric acid from POCh (Gliwice, Poland), Sephadex from Pharmacia Fine Chemicals (Uppsala, Sweden), α-globulin (bovine) Cohn fraction IV from Mann Research Lab. (New York, U.S.A.).

RESULTS

Three of the seven fractions obtained by QAE-A50 Sephadex chromatography showed antimitotic properties. They were: fractions *A-I*, *A-IV* and *B-III*.[1] Fraction *A-I* had the highest content of protein (89%), a small amount of hexose and traces of neuraminic acid. Fractions *A-IV* and *B-III* contained, respectively, 40 and 20% of hexose, and no neuraminic acid (Table 1). The amino acid composition of fractions *A-I* and *A-IV* is presented in Table 2. Histidine, tyrosine and tryptophan were not determined, the peak at 205 nm (Fig. 2) pointed, however, to the high content of aromatic amino acids in these fractions. The specific extinction coefficients were estimated at 215, 225 and 280 nm (Table 3).

The molecular weight determined by thin-layer gel chromatography on Sephadex G-150 (Fig. 3) showed a large difference between the fraction *A-I* (38 000) and the two other fractions, *A-IV* (2100) and *B-III* (2500) (Table 3). The fractions

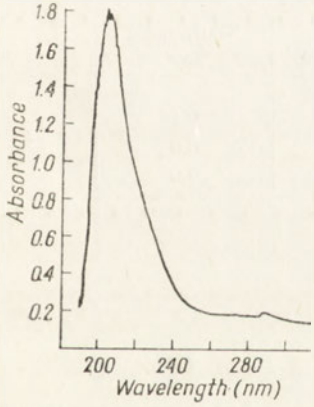

Fig. 2

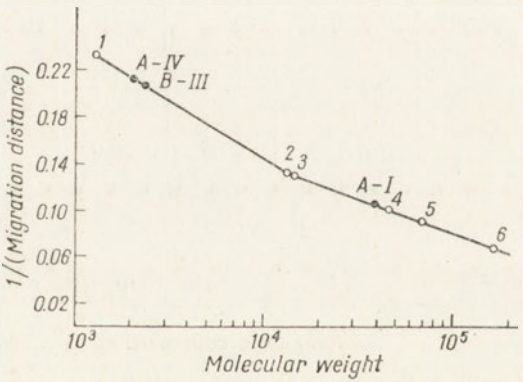

Fig. 3

Fig. 2. Ultraviolet absorption spectrum of fraction *A-I*. Protein concentration 1 mg/ml, light path 0.1 cm, scan speed 1 nm/sec. For fractions *A-IV* and *B-III* similar absorption spectra were obtained.

Fig. 3. Molecular weight determination by t.l.c. on Sephadex G-150 gel. Plate 16×25 cm, layer thickness 0.5 mm, pH 7.5, time 3 h, angle 30°. To the gel, samples of 0.5 µl were applied: standards (20 - 25 µg); isolated fractions (10 - 15 µg protein); and vitamin B_{12} (20 µg). *1*, Vitamin B_{12} (mol. wt. 1355); *2*, cytochrome *c* (13 600); *3*, ribonuclease A (13 700); *4*, ovalbumin (45 000); *5*, bovine serum albumin (67 000); *6*, bovine serum γ-globulin (160 000).

[1] In the previous paper (Grundboeck-Juśko, 1975) these fractions were designated I-A_1, sup. II, IV-A_1, sup. II and III-B_1, sup. II, respectively.

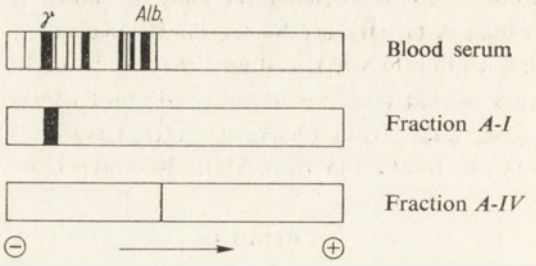

Fig. 4. Diagrams of polyacrylamide-gel electrophoresis at pH 9.2 of bovine blood serum and the isolated fractions *A-I* and *A-IV*. The electrophoresis was run in 0.05 M-borate buffer for 3 h at 2.5 mA per gel (9×0.5 cm). The amount of protein applied was 20 - 50 μg in 50 μl. For staining 0.1% Amido Black was used.

Table 1

Percentage distribution of protein and carbohydrates in the fractions isolated from bovine spleen

The pooled and lyophilized fractions separated by Sephadex QAE-A50 chromatography (cf. Fig. 1) were used.

Fraction	Protein	Hexose	Hexosamine	Neuraminic acid
A-I	88.7	11.1	0.08	0.1
A-IV	59.1	40.7	0.21	0.0
B-III	79.0	20.0	0.22	0.0

Table 2

Amino acid composition of fractions A-I and A-IV

The results are expressed in μmoles per 100 mg of protein. Histidine, tyrosine and tryptophan were not estimated.

Amino acid	*A-I*	*A-IV*	Amino acid	*A-I*	*A-IV*
Lysine	2.962	1.345	Alanine	4.460	1.616
Arginine	2.832	0.806	Cysteine	1.628	0.539
Aspartic acid	6.968	3.123	Valine	3.680	0.913
Threonine	4.493	1.506	Methionine	0.000	1.184
Serine	5.308	1.506	Isoleucine	1.985	0.645
Glutamic acid	5.470	2.639	Leucine	5.340	1.400
Proline	3.418	1.345	Phenylalanine	0.423	0.000
Glycine	4.460	1.777			

Table 3

Specific extinction coefficient and molecular weight of the fractions A-I, A-IV and B-III

Fraction	$E^{1\%}_{1\ cm}$			Mol. wt.
	$\lambda = 280$ nm	$\lambda = 225$ nm	$\lambda = 215$ nm	
A-I	37.0	192.0	230.4	38 000
A-IV	26.0	96.0	195.6	2 100
B-III	—	—	—	2 500

A-I and *A-IV* were homogeneous on polyacrylamide-gel electrophoresis (Fig. 4). Fraction *A-I* had the same mobility as γ-globulins, whereas fraction *A-IV* migrated much faster resembling prealbumin. Fraction *B-III* was not visualized by Amido Black.

In the biological test on mice (Table 4), fraction *A-I* caused a 50% decrease in mitotic index of spleen cells in all experimental animals, and had no effect on lymph node and thymus cells. On the other hand, fraction *A-IV* inhibited cell divisions only in thymus, and *B-III* inhibited mitosis in thymus and in lymph node.

Table 4

The effect of the fractions isolated from bovine spleen on the mitotic index in lymphatic organs of mice

The mice were injected intraperitoneally with colcemide (10 μg) and then with the particular fractions (400 μg of protein) as described by Grundboeck-Juško (1975).

Fraction	Lymph node	Spleen	Thymus
A-I	no reaction	42 - 67% decrease (in all animals)	no reaction
A-IV	no reaction	no reaction	38 - 48% decrease (in 67% of animals)
B-III	40 - 60% decrease (in 50% of animals)	no reaction	83 - 87% decrease (in all animals)

DISCUSSION

The obtained protein fractions showing chalone activity were found to be glycoproteins, similarly as all so far investigated chalones. Their specific absorption spectra (Tombs *et al.*, 1959) pointed to a high content of aromatic amino acids.

The presented results clearly indicate that the two fractions obtained from spleen smooth microsome supernatant, homogeneous on polyacrylamide-gel electrophoresis, were distinct proteins. These proteins differ in carbohydrate content, molecular weight and biological properties. We assume that the fraction *A-I* with mol.wt. of 38 000, corresponds to chalone B, and fraction *A-IV*, of mol.wt. 2100,

to chalone T. So far, the existence of these two kinds of lymphocyte chalone was only postulated.

Differences in molecular weight of chalones have been reported by several authors. The molecular weight of erythrocyte chalone varies in the range 2000 - - 4000 (Kiviliakso & Rytömma, 1971), that from epidermis 20 000 - 40 000 (Boldingh & Laurence, 1968) and from lymphocyte 20 000 - 70 000 (Fraser *et al.*, 1974).

The isolation of pure chalone B and T may prove to be of importance in therapy: they could be used to overcome graft rejection, to inhibit formation of antibodies, and to control the pathological proliferation of lymphocytic tissue.

REFERENCES

Boldingh, W. H. & Laurence, E. B. (1968) *Eur. J. Biochem.*, **5**, 191 - 198.
Bullough, W. & Laurence, E. (1960) *Proc. Roy. Soc. B.*, **151**, 517 - 536.
Bullough, W. & Laurence, E. (1964) *Exp. Cell. Res.*, **33**, 176 - 194.
Fraser, L. B. Munro, C. S. & Cater, D. B. (1974) *Brit. J. Exp. Path.*, **55**, 541 - 553.
Gordon, A. H. (1969) *Electrophoresis of protein in polyacrylamide and starch gels.* North Holland Publ. Comp., Amsterdam - London.
Grundboeck-Juśko, J. (1975) *Bull. Vet. Inst. Pulawy*, **19**, 41 - 48.
Hermanowicz, A. & Jóźkiewicz-Hermanowicz, M. (1973) *Fol. Arch. Med. Wew.*, **50**, 687.
Houck, J. G. & Hennings, H. (1973) *FEBS Lett.*, **32**, 1 - 8.
Kiger, N., Florentin, I. & Mathe, G. (1972) *Transplantation*, **14**, 448 - 454.
Kiviliakso, E. & Rytömma, T. (1971) *Cell Tissue Kinet.*, **4**, 1 - 9.
Morris, C. J. O. R. (1964) *J. Chromatogr.*, **16**, 167 - 175.
Paukovits, W. R. (1973) *Blut*, **27**, 217 - 222.
Rimington, G. (1940) *Biochem. J.*, **34**, 931 - 940.
Tombs, M. P., Souter, F. & MacLagan, N. F. (1959) *Biochem. J.*, **73**, 167 - 171.
Wadell, W. J. (1956). *J. Lab. Clin. Med.*, **48**, 311 - 318.
Warren, L. (1959) *J. Biol. Chem.*, **234**, 1971 - 1975.
Weimar, H. E. & Moshin, J. R. (1953) *Ann. Rev. Tubercul.*, **68**, 594 - 602.

CHARAKTERYSTYKA CHEMICZNA CHALONE UZYSKANEGO ZE ŚLEDZIONY BYDLĘCEJ

Streszczenie

Izolując chalone z supernatantów postmikrosomalnych śledziony, otrzymano dwie glikoproteidowe frakcje, homogenne w elektroforezie na żelu poliakrylamidowym. W teście biologicznym na myszach frakcja o ciężarze cząsteczkowym 38 000 hamowała mitozy komórek śledziony, a frakcja niskocząsteczkowa (c.mol. 2100) mitozy komórek grasicy. Frakcje te wykazywały więc odpowiednio właściwości chalone B i chalone T.

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PREPARATION AND PROPERTIES OF tRNA FROM HUMAN PLACENTA

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1. The tRNA preparation obtained from human placenta by phenol extraction and DEAE-cellulose chromatography was homogeneous on ultracentrifugation and showed high amino acid acceptor activity.

2. The analysis of the isoaccepting tRNA by reversed-phase chromatography (RPC-5 system) showed the presence of 5 fractions for glycine and leucine, 3 for tyrosine, alanine and valine, 2 for arginine and 1 for phenylalanine.

Transfer RNA of animal, plant and bacterial origin can be readily isolated with a high yield; on the other hand, isolation of tRNA from human tissues is more difficult to achieve as the material is usually obtained *post mortem* and partially decomposed by ribonucleases (Roe, 1975). Placenta is the only fresh human tissue available for experiments, and moreover the processes of protein biosynthesis occur in this tissue with great intensity.

The isolation of tRNA from human placenta by the methods of Roe (1975), Abadom & Elson (1970) and Matthaei & Schoech (1967) in our hands was not wholly successful as the preparations obtained were not sufficiently pure and had rather low acceptor activity. Therefore we have undertaken an attempt to elaborate such a method which, yielding a comparatively pure tRNA, would permit the use of a quite small amount of the material.

MATERIALS AND METHODS

Reagents. Tris, Triton X-100 and dithiothreitol were from Koch-Light Lab. (Colnbrook, Bucks., England); DEAE-cellulose DE-11, polychlorotrifluoroethylene, 100 - 200 μ (Plascon PTFE-2300) and trioctyl methylammonium chloride from Serva (Heidelberg, G.F.R.); PPO and POPOP from Reanal (Budapest, Hungary) and Fluka A.G. (Buchs, Switzerland), respectively; L-[U-^{14}C]cysteine, spec. act. 26.9 Ci/mole, was from the Radiochemical Centre (Amersham, England). Other uniformly ^{14}C-labelled amino acids were from UVVVR (Praha, Czechoslovakia):

phenylalanine (spec.act. 315 Ci/mole), alanine (105 Ci/mole), valine (175 Ci/mole), glycine (70 Ci/mole), glutamic acid (175 Ci/mole), arginine (210 Ci/mole), proline (175 Ci/mole), leucine (210 Ci/mole) and tyrosine (315 Ci/mole). Other reagents were supplied by POCH (Gliwice, Poland).

Material. Human term placentae were obtained immediately after normal deliveries from the Institute of Gynaecology and Obstetrics of the Medical School in Wrocław and transferred in ice. The placenta was washed with cold water to remove blood and blood clots, separated from foetal membranes, and disintegrated. All the procedure including disintegration of the placenta and preparation of tRNA, aminoacyl-tRNA and aminoacyl-tRNA synthetases, was carried out at 4°C.

Preparation of tRNA. Placenta (300 g) was homogenized for 3 min with 500 ml of a solution containing 0.2 M-sodium acetate buffer at pH 5.0, 0.02% polyvinyl sulphate, 200 ml of 1 M-sucrose and 500 ml of water-saturated phenol containing 0.1% (w/v) of 8-hydroxyquinoline (Anandaraj & Cheraiyl, 1974; Ortwerth, 1971). Then the homogenate was shaken for 1 h and centrifuged for 1 h at 2500 *g*. The upper aqueous layer was removed, added with 250 ml of water-saturated phenol containing 0.1% of 8-hydroxyquinoline, shaken for 30 min and centrifuged as above. The upper layer was collected and mixed with 0.1 vol. of 1 M-sodium acetate buffer, pH 5.0, and 2 vol. of ethanol. After overnight sedimentation, the supernatant was decanted, the precipitate spun down and dried over P_2O_5.

The dried preparation (about 0.3 g) was shaken for 1 h with 50 ml of a solution containing 10 mM-magnesium acetate, 1 mM-EDTA and 10 mM-sodium acetate buffer, pH 5.0. After centrifugation for 15 min at 6000 *g*, the supernatant was applied to the DEAE-cellulose column (1.6 × 20 cm) and eluted with a linear NaCl gradient up to 1.2 M in the same buffer solution. Fractions of 5 ml were collected, monitored at 260 nm and acceptor activity determined. The active fractions were pooled, tRNA was precipitated by adding 2 vol. of ethanol, left standing overnight at −20°C, then the supernatant was decanted. The sediment was centrifuged for 15 min at 6000 *g*, washed with cold ethanol, centrifuged and dried over P_2O_5.

Aminoacyl-tRNA synthetase was prepared by the method of Penneys & Muench (1974) with some modifications: about 100 g of placenta was washed with buffer *A* (10 mM-phosphate buffer, pH 6.9, 60 mM-2-mercaptoethanol and 15% glycerol) and homogenized with 250 ml of the same buffer. After centrifugation for 20 min at 6000 *g* the supernatant was centrifuged for 1 h at 105 000 *g*; to the obtained supernatant, ammonium sulphate was added to 0.6 saturation, stirred for 1 h and centrifuged for 30 min at 6000 *g*. The precipitate was extracted successively with buffer *A* solution containing ammonium sulphate at saturation of 0.5, 0.45, 0.4 and 0.35; after 10 min with stirring, the successive mixtures were centrifuged at 6000 *g* for 30 min. The supernatants were combined, solid ammonium sulphate was added to 0.6 saturation, stirred for 30 min and centrifuged as above. The sediment was dissolved in 10 ml of buffer *A* and dialysed overnight against 3 litres of this buffer. The dialysis residue was clarified by centrifugation for 30 min at 6000 *g* and stored at −20°C.

The tRNA acceptor activity was determined according to Yang & Novelli (1971). The reaction mixture contained in a volume of 1 ml: 50 mM-Tris-HCl buffer, pH 7.5, 50 mM-KCl, 2 mM-dithiothreitol, 4 mM-ATP and 3 - 30 mM-magnesium acetate (the optimum concentration of Mg^{2+} for particular amino acids is given in Table 1), 10 nM-[^{14}C]amino acid, 1 - 5 E_{260} units of tRNA and 2.5 mg protein of aminoacyl-tRNA synthetase preparation. The reaction was carried out at 30°C. After 15 and 20 min, 50 µl portions were applied on Whatman 3 *MM* paper discs, washed three times with 5% trichloroacetic acid, then once with ethanol, and dried; the radioactivity was then determined with a scintillation counter with an efficiency of 30%.

Reversed-phase chromatography was performed according to Pearson *et al.* (1971) on a column of polychlorotrifluoroethylene coated with trioctyl methylammonium chloride (system RPC-5).

The preparations of particular [^{14}C]aminoacyl-tRNA's were obtained by the method of Yang & Novelli (1971) using concentrations of Mg^{2+} given in Table 1. The preparations were precipitated with ethanol at −20°C, left standing for a few hours, then centrifuged at 6000 *g* for 15 min; the sediment was dissolved in buffer *B* (10 mM-sodium acetate buffer, pH 4.5, 6 mM-2-mercaptoethanol, 10 mM-magnesium acetate and 1 mM-EDTA), and 30 E_{260} units applied to the RPC-5 column (0.7 × 110 cm). The column was eluted with a linear NaCl concentration gradient (0.45 - 0.9 M) in 500 ml of buffer *B*. Fractions of 2 ml were collected, in which E_{260} and radioactivity were determined. For radioactivity determination, 1 ml of the eluate was mixed with 5 ml of the scintillator "tritosol" (Friche, 1975) and measured with a scintillation counter with an efficiency of 26%.

RESULTS

The tRNA preparation obtained by phenol extraction showed on DEAE-cellulose chromatography three distinct peaks of absorption at 260 nm (Fig. 1). Amino acid acceptor activity appeared only in peak *III*, emerging from the column at 0.55 - 0.8 M-NaCl concentration. One milligram of dry tRNA obtained from this peak,

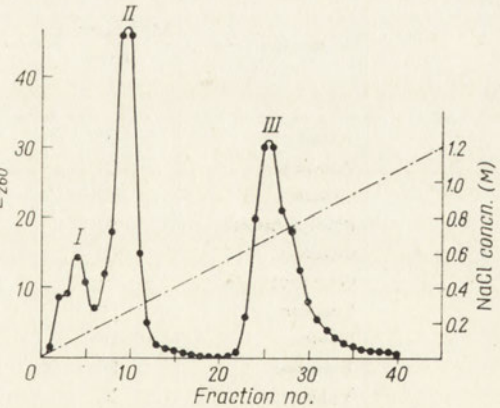

Fig. 1. DEAE-cellulose column chromatography of the tRNA preparation from human placenta extracted with phenol at pH 5.0. A solution containing about 2700 E_{260} units of tRNA was applied to the column (1.6 × 20 cm) and eluted with the linear NaCl concentration gradient in 500 ml of a buffer consisting of 10 mM-sodium acetate, pH 5.0, 10 mM-magnesium acetate, and 1 mM-EDTA. Fractions of 10 ml were collected at a rate of 150 ml/hour.

in aqueous solution corresponded to 18.2 E_{260} units, and the yield from 100 g of fresh placenta was 8.5 mg. This preparation, when submitted to analytical ultracentrifugation, sedimented as a single symmetrical peak with $s_{20,w}$ of 4.1 (Fig. 2).

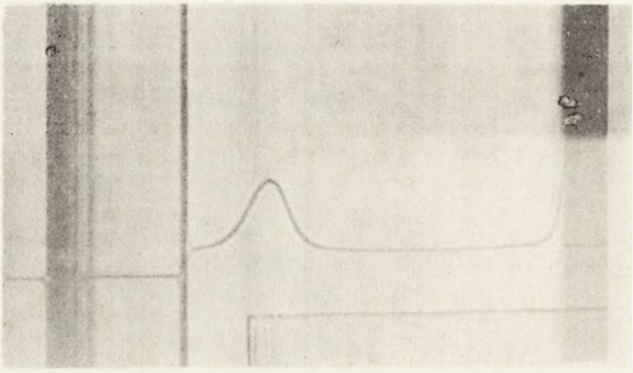

Fig. 2. Sedimentation diagram of the tRNA preparation after DEAE-cellulose chromatography (peak *III*, Fig. 1). A 1% solution of tRNA in 0.2 M-NaCl was centrifuged at 20°C in MOM 3170 ultracentrifuge. The pattern was photographed after 35 min at 60 000 rev./min.

To check whether the active tRNA fractions did not contain partially degraded particles, the procedure of Nishimura & Novelli (1965) was applied. The preparation was heated at 85°C for 5 min, quickly cooled in ice and aminoacylated. The results showed that the activity was practically the same as in the non-heated control sample.

The acceptor activity of the tRNA preparation towards 10 amino acids was assayed in a homologous system (Table 1), in the presence of optimum concentra-

Table 1

Amino acid acceptor activity of the tRNA preparation from human placenta

The conditions of aminoacylation were as described under Methods. The acceptor activity towards particular amino acids was assayed at the experimentally determined optimum Mg^{2+} concentrations. The results are mean values from 2 - 4 determinations.

Amino acid	Mg^{2+} concn. (mM)	Activity (pmoles of amino acid/1 E_{260} unit of tRNA)
Alanine	20	55.8
Arginine	20	27.3
Cysteine	20	24.8
Phenylalanine	30	37.8
Glycine	10	25.3
Glutamic acid	10	3.9
Leucine	3	51.0
Proline	15	8.9
Tyrosine	15	18.4
Valine	20	23.3

tion of Mg^{2+}, which had been experimentally determined for each amino acid. The placental tRNA showed an especially high acceptor activity towards leucine and alanine, and a rather low activity towards glutamic acid and proline.

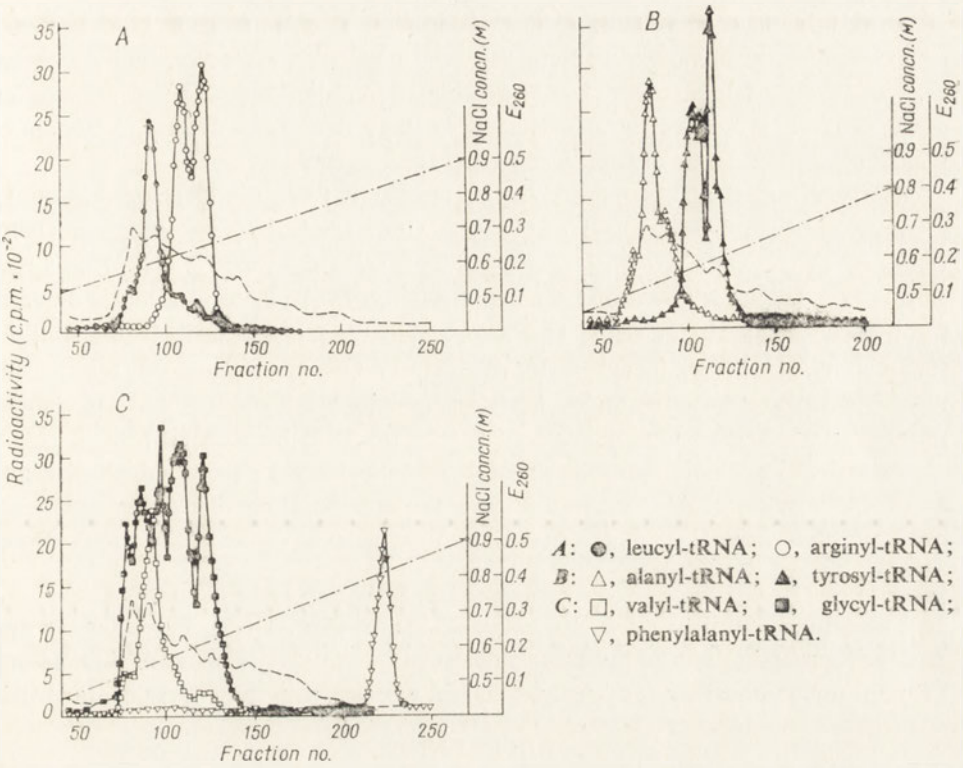

Fig. 3. RPC-5 chromatography of [^{14}C]aminoacyl-tRNA's from human placenta. About 30 E_{260} units of the particular aminoacyl-tRNA was applied to the column (0.7 × 110 cm) and eluted with a 0.45 - 0.9 M-NaCl linear concentration gradient in 500 ml of a buffer consisting of 10 mM-sodium acetate, pH 4.5, 6 mM-2-mercaptoethanol, 10 mM-magnesium acetate and 1 mM-EDTA. Fractions of 2 ml were collected at a rate of 10 ml/h, and (— — —), E_{260} and (——), radioactivity were determined. (—·—), NaCl concentration.

The [^{14}C]aminoacyl-tRNA's synthesized *in vitro* were subjected to separation on RPC-5 columns, and the elution profiles are presented in Fig. 3; $tRNA^{Leu}$ and $tRNA^{Gly}$ separated each into 5 fractions, $tRNA^{Val}$, $tRNA^{Ala}$ and $tRNA^{Tyr}$ into 3 fractions, and $tRNA^{Arg}$ into 2 fractions. $tRNA^{Phe}$ emerged last from the column as a symmetrical peak at about 0.86 M-NaCl concentration.

DISCUSSION

In the present work tRNA was isolated from fresh human placenta by extraction with water-saturated phenol at pH 5.0, and DEAE-cellulose chromatography in a NaCl concentration gradient. By this procedure a preparation free of rRNA and polysaccharides was obtained. The phenol extraction at acidic pH did not

result in a loss of the amino acid acceptor activity, and the addition of 0.02% polyvinyl sulphate (Ortwerth, 1971) reduced the ribonuclease activity by 74%, which permitted to obtain a rather high yield of the preparation: about 8.5 mg of tRNA from 100 g of placenta.

DEAE-cellulose chromatographic pattern of tRNA indicates the presence of a component which could contain degraded molecules. However, the experiment with aminoacylation carried out after heating and cooling the obtained preparation of tRNA, did not reveal any molecules partially decomposed by the action of nucleases, or denatured by the chromatographic technique.

The determinations of amino acid acceptor activity of the tRNA from human placenta reported by Matthaei & Schoech (1967) and Abadom & Elson (1970) were carried out at 10 mM-Mg^{2+} concentration, and those of Roe (1975) at 5 mM. In our experiments it was found that the optimum Mg^{2+} concentration is different for the particular amino acids, and a "standard" concentration of 10 mM-Mg^{2+} strongly inhibited e.g. leucyl-tRNA synthetase. On application of appropriate magnesium concentrations, higher acylation activity was demonstrated for alanine, phenylalanine, leucine and tyrosine than that reported by Roe (1975).

The RPC-5 gave a high resolution of isoaccepting tRNA's from human placenta, as $tRNA^{Gly}$ and $tRNA^{Leu}$ separated each into five fractions. Thus the emergence of $tRNA^{Phe}$ as a single symmetrical peak may be taken as evidence for a high degree of purification of this tRNA species.

The authors wish to express their gratitude to Prof. Dr. J. Pawełkiewicz and Dr. W. Kędzierski (both of the Department of Biochemistry, College of Agriculture, Poznań) for practical suggestions and helpful discussion in the course of this work.

REFERENCES

Abadom, P. N. & Elson, D. (1970) *Biochim. Biophys. Acta*, **199**, 528 - 531.
Anandaraj, M. P. J. S. & Cherayil, J. D. (1974) *Anal. Biochem.*, **58**, 190 - 194.
Friche, U. (1975) *Anal. Biochem.*, **63**, 555 - 558.
Matthaei, J. U. & Schoech, G. K. (1967) *Biochem. Biophys. Res. Commun.*, **27**, 638 - 643.
Nishimura, S. & Novelli, G. D. (1965) *Proc. Natl. Acad. Sci. U.S.A.*, **53**, 178 - 184.
Ortwerth, B. J. (1971) *Biochemistry*, **10**, 4190 - 4197.
Pearson, R. L., Weiss, J. F. & Kelmers, A. D. (1971) *Biochim. Biophys. Acta*, **228**, 770 - 774.
Penneys, N. S. & Muench, K. H. (1974) *Biochemistry*, **13**, 560 - 564.
Roe, B. A. (1975) *Nucl. Acids Res.*, **2**, 21 - 42.
Yang, W. K. & Novelli, G. D. (1971) in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 20C, pp. 47 - 48. Academic Press, New York - London.

PREPARACJA I WŁAŚCIWOŚCI tRNA Z ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Drogą ekstrakcji fenolowej i chromatografii na DEAE-celulozie wyizolowano z łożyska ludzkiego tRNA wykazujący jednorodność w trakcie ultrawirowania oraz wysoką aktywność akceptorową.

2. Analiza izoakceptorowego tRNA w układzie RPC-5 wykazała obecność 5 frakcji dla glicyny i leucyny, 3 dla tyrozyny, alaniny i waliny, 2 dla argininy i 1 dla fenyloalaniny.

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NON-HISTONE CHROMATIN PROTEINS DURING MATURATION OF AVIAN ERYTHROID CELLS *

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1. In chicken erythroid cells (erythroblast, reticulocyte and erythrocyte) maturation was accompanied by a decrease in the content of non-histone chromatin proteins.

2. Phenol-soluble non-histone chromatin proteins (phosphoproteins) from the three cell populations studied, showed differences in the behaviour on sodium dodecyl sulphate polyacrylamide-gel electrophoresis, and isoelectric focusing. Phosphoprotein of immature cells had a higher content of fractions of about 86 000 and 23 000 daltons than the phosphoproteins of erythrocytes.

Avian erythroid cells are an attractive model for investigations on behaviour of chromatin components during cell maturation. In the course of this process, the erythroid cells first lose the ability to synthetize DNA (Williams, 1972). Then their chromatin undergoes progressive condensation which is accompanied by an increase in the content of histone *f2c* (Dick & Johns, 1969; Appels *et al.*, 1972; Billett & Hindley, 1972; Sotirov & Johns, 1972) as well as progressive cessation of RNA and protein synthesis (Kabat & Attardi, 1967; Sadgopal & Kabat, 1969; Sanders *et al.*, 1973). It has also been found that non-histone chromatin proteins differ in the final stages of erythroid cell maturation (Shelton & Neelin, 1971; Sanders, 1974).

In view of the above-mentioned findings it was of interest to compare the non-histone chromatin proteins from mature avian erythrocytes with the same proteins from the earlier stages of development of erythroid cells, i.e. reticulocytes, as well as erythroblasts from anemic bone marrow.

* This work was supported by the Polish Academy of Sciences within the project 09.3.1.

MATERIALS AND METHODS

Induction of haemolytic anemia. Adult chickens (Rhode Island Red) were made anemic by subcutaneous injections of 10 mg phenylhydrazine (2% solution in 0.9% NaCl) per 1 kg body weight on five consecutive days. Blood and bone marrow were taken 24 h after the last injection.

Isolation of cells. Erythrocytes and reticulocytes (chiefly mid and late polychromatic erythrocytes) were obtained from blood of normal and anemic hens, respectively. The immaturity of blood cells was monitored with Brilliant Cresyl Blue stain. Reticulocytes formed 83 - 98% of red cells in the case of regenerating blood, whereas in normal blood they did not exceed 2%.

The bone marrow cells were obtained from leg bones of anemic chicken according to Billett & Hindley (1972), and to increase the concentration of erythroblasts, the heavier cells were removed by centrifugation (6000 *g*, 20 min) through ficoll at a density of 1.07 g/cm^3.

The three types of the isolated cells are presented in Fig. 1.

Isolation of nuclei. Nuclei of all the studied cells were isolated by the sucrose method (pH 6.7) according to the procedure described by Wierzbicki *et al.* (1973).

Preparation of chromatin and chromatin proteins. Chromatin preparations were obtained according to Spelsberg & Hnilica (1971). Histones were extracted from chromatin with 0.2 M-H_2SO_4 at 4°C, and non-histone proteins were obtained after extraction of chromatin residue with 1 M-NaOH.

Preparation of nuclear phosphoproteins. Phosphoproteins (phenol-soluble non-histone chromatin proteins) were extracted from nuclei or chromatin preparations by the method of Teng *et al.* (1971).

Polyacrylamide-gel electrophoresis. Phosphoproteins were characterized by electrophoretic analysis in 10% polyacrylamide-gel containing 0.1% SDS and 0.14 M-2-mercaptoethanol, pH 7.4 (Teng *et al.*, 1971). Approximate molecular weight was determined against the relative migrations of α-phosphorylase (94 000), bovine serum albumin (67 000), catalase (60 000), ovalbumin (45 000), deoxyribonuclease I (31 000), chymotrypsinogen (25 000), myoglobin (17 800) and cytochrome *c* (12 000). Gels were scanned at 560 nm using an ERJ 65 densitograph (Carl Zeiss, Jena, G.D.R.).

Isoelectric focusing. This was performed in 4% acrylamide rods (0.5 × 9 cm) containing 2.4% ampholine, pH range 3 - 10, and 8 M-urea (Gronow & Griffiths, 1971). The anode solution was 0.2% H_2SO_4 and the catode solution 0.4% triethanolamine. Electrophoresis was carried out at 4°C and 100 V for 21 h. The gels were stained with 0.1% Coomassie Brilliant Blue (Vesterberg, 1971). To measure the pH gradient, blank gels were cut into 2-mm slices, suspended in 1 ml of deaerated 0.01 M-KCl, left overnight at room temperature, then the pH of each solution was determined.

Other analytical procedures. RNA was measured by the orcinol method (Schneider, 1957) and DNA according to Burton (1956) with, respectively, yeast RNA and calf thymus DNA as standards. Protein was determined by the microbiuret

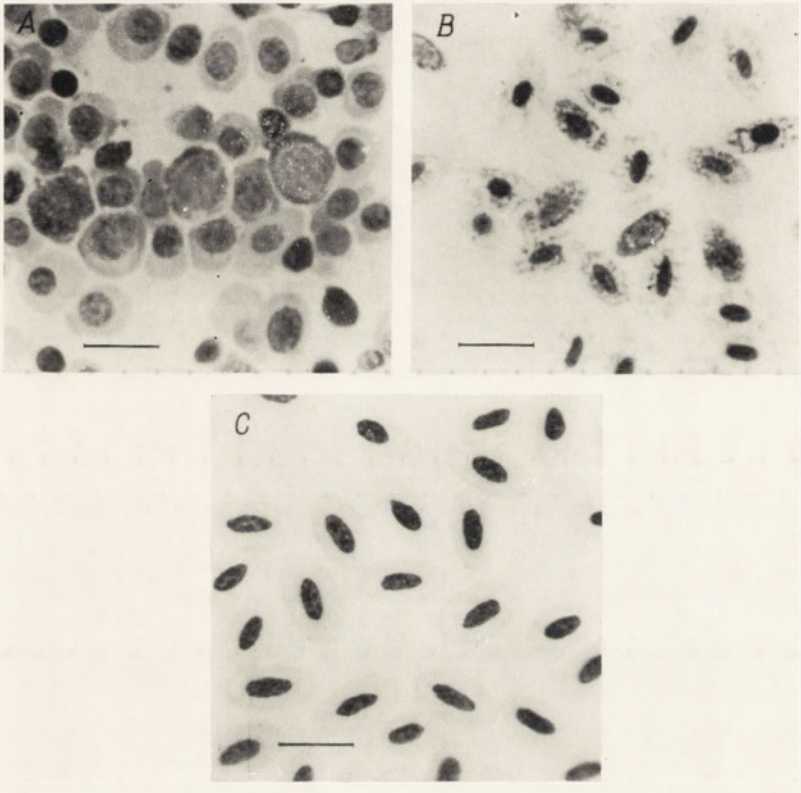

Fig. 1. Chicken erythroid cells at different stages of maturation. *A*, Erythroblast-enriched forms from anemic bone marrow stained by Poppenheim method; *B*, reticulocytes and *C*, erythrocytes stained with Brilliant Cresyl Blue. The bars represent 10 μm.

method (Itzhaki & Gill, 1964) with bovine serum albumin as a standard. Phosphorus was estimated colorimetrically (Krajewski & Urbanek, 1961) after hydrolysis of phosphoproteins with 1 M-NaOH.

Reagents. Ficoll was from Pharmacia (Uppsala, Sweden). α-Phosphorylase and deoxyribonuclease I were purchased from Worthington Biochemicals Corp. (Freehold, N.J., U.S.A.); bovine serum albumin, catalase, ovalbumin, chymotrypsinogen, myoglobin and cytochrome *c* were products of Serva Feinbiochemica (Heidelberg, G.F.R.). Sodium dodecyl sulphate (SDS) specially pure, calf thymus DNA and yeast RNA were supplied by B.D.H. Chemicals Ltd (Poole, Dorset, England).

Acrylamide was a product of Koch-Light Lab. Ltd (Colnbrook, Bucks, England); *N,N'*-bisacrylamide was from Fluka AG (Buchs S.G., Switzerland), and 1,4-tetramethylenediamine (TEMED) was from K & K Lab. Inc (Hollywood, Calif., U.S.A.). Ampholine was from LKB - Producter AB (Bromma, Sweden) and Coomassie Brilliant Blue R from Sigma Chem. Co. (St. Louis, Mo., U.S.A.). Other reagents were analytical grade products supplied by POCh (Gliwice, Poland).

RESULTS AND DISCUSSION

The chromatin preparations obtained from the investigated types of cells showed similar ultraviolet absorption spectra. The increase of A_{260}/A_{280} and A_{260}/A_{230} ratios observed with chromatins from older cells (Table 1), as well as the results of the chemical analysis (Table 2) indicate that the content of total protein in chromatin decreases considerably in the process of cell maturation. This seems to be caused by a gradual decrease of the amount of non-histone protein in chromatin. These results differ from those of Sanders (1974) who found a higher content of the total chromatin protein in duck erythrocytes than in reticulocytes.

No significant differences were found between phosphoprotein preparations isolated either from the chromatins or directly from the nuclei. The content of phosphorus was higher in the phosphoprotein preparations obtained from the

Table 1

Ultraviolet absorption spectra of chromatins from chicken erythroid cells at different stages of maturation

Limit values from 12 experiments are given.

Spectral ratios	Erythroblasts	Reticulocytes	Erythrocytes
A_{320}/A_{260}	0.06 - 0.09	0.06 - 0.09	0.03 - 0.06
A_{280}/A_{260}	0.62 - 0.65	0.60 - 0.62	0.58 - 0.60
A_{260}/A_{230}	1.00 - 1.16	1.10 - 1.13	1.30 - 1.36
A_{260}/A_{270}	1.13 - 1.20	1.13 - 1.17	1.17 - 1.20
A_{260}/A_{280}	1.55 - 1.62	1.61 - 1.65	1.67 - 1.73
A_{260}/A_{290}	2.83 - 3.20	3.13 - 3.32	3.40 - 3.80

Table 2

Composition of chromatin from chicken erythroid cells at different stages of maturation

The results are expressed as mg/mg DNA, ±S.D.

	Erythroblasts $n=4$	Reticulocytes $n=4$	Erythrocytes $n=8$
Total protein	1.90±0.07	1.76±0.06	1.42±0.08
Histones	0.85±0.03	1.00±0.10	1.02±0.12
Non-histone protein	0.95±0.12	0.65±0.07	0.36±0.08
RNA	0.11±0.01	0.09±0.03	<0.01

Table 3

Phosphorus content in nuclear phosphoproteins from chicken erythroid cells at different stages of maturation

Mean values of 3 experiments ±S.D. are given.

	Alkali-labile phosphorus (mg/100 mg protein)
Erythroblasts	0.94±0.09
Reticulocytes	0.79±0.05
Erythrocytes	0.61±0.04

younger cell populations than in those from mature cells (Table 3). Thus, in agreement with Gershey & Kleinsmith (1969) and Shelton *et al.* (1972), we may expect a higher biological activity of non-histone chromatin proteins in younger cells than in mature ones.

On polyacrylamide-gel electrophoresis the phosphoproteins of erythroid cells separated into 15 - 18 distinct bands of molecular weight from 12 000 to 140 000 daltons (Fig. 2). The phosphoproteins from immature cells differed both qualitatively and quantitatively from those of erythrocytes. As it appears from densitometric tracings, several fractions decreased or even completely disappeared during maturation of the cells, e.g. the fractions of 86 000 and 23 000 daltons, which form distinct peaks in the case of erythroblasts, and are quite low for reticulocytes and erythrocytes. The fraction of 56 000 daltons showed a less pronounced decrease, whereas fractions of 51 000 and 47 000 daltons did not appear at all in the mature erythrocytes. It seems that the changes observed in nuclear phosphoproteins are mainly quantitative. According to Ruiz-Carrillo *et al.* (1974) quantitative rather than qualitative changes in non-histone proteins predominate as maturation of duck erythroid cells proceeds. Shelton & Neelin (1971) and Sanders (1974) have also reported some significant quantitative differences in gel electrophoretic patterns of the non-histone chromosomal proteins from goose and duck reticulocytes and erythrocytes.

Isoelectric focusing of nuclear phosphoproteins demonstrated that all three types of chicken erythroid cells contain the same three main groups of fractions with isoelectric points at pH range 8.5 - 8.0; 7.7 - 7.0 and 6.0 - 5.8 (Fig. 3). During the process of maturation the content of fractions with pI between 8.5 - 8.0 increases

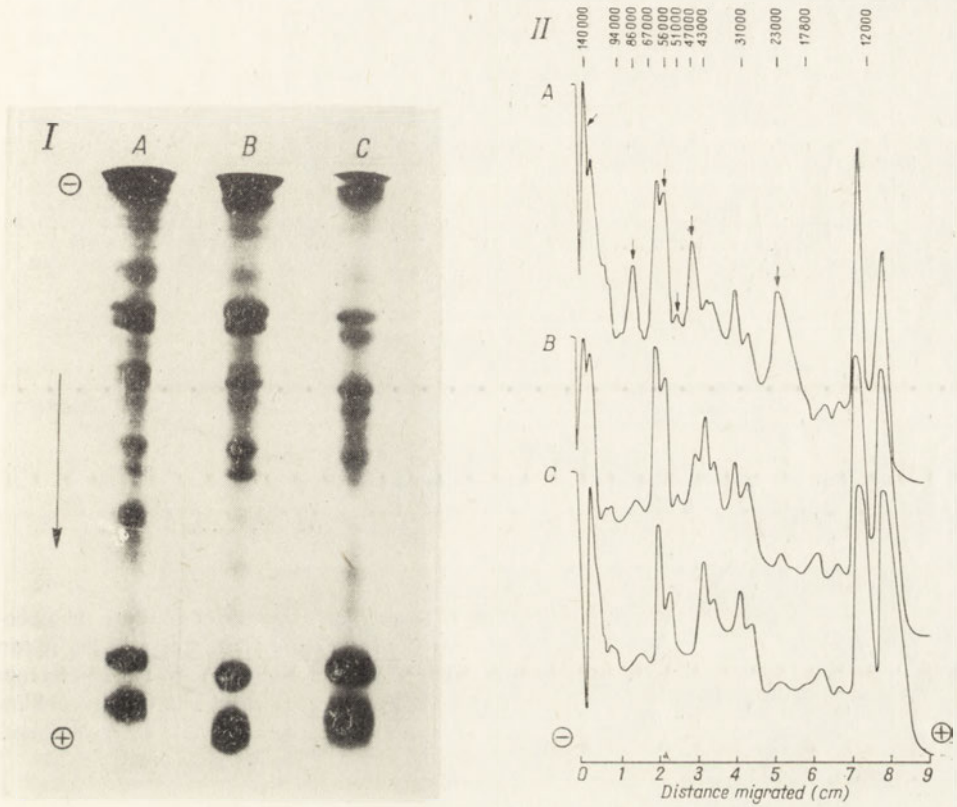

Fig. 2. Electrophoretic patterns (*I*) and densitometric tracings (*II*) of nuclear phosphoproteins from chicken erythroid cells after sodium dodecyl sulphate gel electrophoresis at pH 7.4 according to Teng *et al.* (1971). About 120 µg of protein were applied per gel. The protein was stained with 0.02% Coomassie Blue in 12.5% trichloroacetic acid. *A*, Erythroblasts; *B*, reticulocytes; *C*, erythrocytes.

while the content of fractions with pI at pH range 7.7 - 7.0 decreases. It seems possible that the changes in non-histone chromatin proteins accompanying the process of cell maturation are connected with a gradual loss of ability to synthetize protein by older cells.

There are also two interesting dominant fractions of low molecular weight about 12 000 daltons (cf. Fig. 2). They occur in all investigated cell populations

showing a significant quantitative increase in older cells. These fractions seem to be characteristic for erythroid cells and have been also noticed by Vidali *et al.*

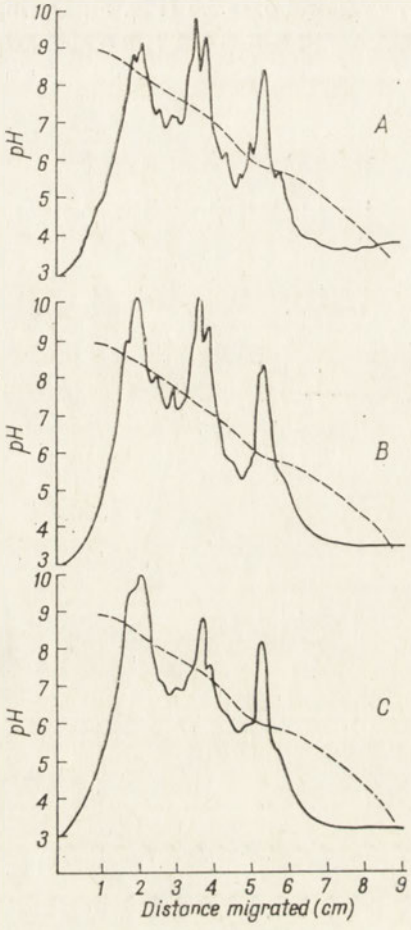

Fig. 3. Isoelectric focusing of nuclear phosphoproteins from chicken erythroid cells in the system of Gronow & Griffiths (1971) over the pH range 3 - 10. The protein was stained with Coomassie Blue. *A*, Erythroblasts; *B*, reticulocytes; *C*, erythrocytes; — — —, pH gradient.

(1973) in duck erythrocytes and by Seligy & Miyagi (1974) in chicken erythrocytes. However, on the basis of experimental data obtained so far we cannot exclude the possibility that these fractions result from contamination by globin.

REFERENCES

Appels, R. Wells, J. R. E. & Williams, A. F. (1972) *J. Cell. Sci.*, **10**, 47 - 59.
Billett, M. A. & Hindley, J. (1972) *Eur. J. Biochem.*, **28**, 451 - 462.
Burton, K. (1956) *Biochem. J.*, **62**, 315 - 322.
Dick, C. & Johns, E. W. (1969) *Biochim. Biophys. Acta*, **175**, 414 - 418.
Gershey, E. L. & Kleinsmith, L. J. (1969) *Biochim. Biophys. Acta*, **194**, 519 - 525.
Gronow, M. & Griffiths, G. (1971) *FEBS Lett.*, **15**, 340 - 344.
Itzhaki, R. F. & Gill, D. M. (1964) *Anal. Biochem.*, **9**, 401 - 410.

Kabat, D. & Attardi, G. (1967) *Biochim. Biophys. Acta*, **138**, 382 - 399.
Krajewski, T. & Urbanek, H. (1961) *Zesz. Nauk. U.Ł.*, **10**, 31 - 36.
Ruiz-Carrillo, A., Wangh, L. J., Littau, V. C. & Allfrey, V. G. (1974) *J. Biol. Chem.*, **249**, 7358 - 7368.
Sadgopal, A. & Kabat, D. (1969) *Biochim. Biophys. Acta*, **190**, 486 - 495.
Sanders, L. A. (1974) *Biochemistry*, **13**, 527 - 534.
Sanders, L. A., Schechter, N. M. & McCarty, K. S. (1973) *Biochemistry*, **12**, 783 - 791.
Schneider, W. C. (1957) in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, pp. 680 - 684. Academic Press, New York.
Seligy, V. L. & Miyagi, M. (1974) *Eur. J. Biochem.*, **46**, 259 - 269.
Shelton, K. R. & Neelin, J. M. (1971) *Biochemistry*, **10**, 2342 - 2348.
Shelton, K. R., Seligy, V. L. & Neelin, J. M. (1972) *Arch. Biochem. Biophys.*, **153**, 375 - 383.
Sotirov, N. & Johns, E. W. (1972) *Exp. Cell Res.*, **73**, 13 - 16.
Spelsberg, T. C. & Hnilica, L. S. (1971) *Biochim. Biophys. Acta*, **228**, 202 - 211.
Teng, C. S., Teng, C. T. & Allfrey, V. G. (1971) *J. Biol. Chem.*, **246**, 3597 - 3609.
Vesterberg, O. (1971) *Biochim. Biophys. Acta*, **243**, 345 - 348.
Vidali, G., Boffa, L. C., Littau, V. C., Allfrey, K. M. & Allfrey, V. G. (1973) *J. Biol. Chem.*, **248**, 4065 - 4068.
Wierzbicki, R., Orlando, M., Tentori, L. & Vivaldi, G. (1973) *Ann. Ist. Super. Sanitá*, **9**, 176 - 179.
Williams, A. F. (1972) J. *Cell. Sci.*, **10**, 27 - 46.

BIAŁKA NIEHISTONOWE CHROMATYNY W PROCESIE DOJRZEWANIA PTASICH KOMÓREK ERYTROIDALNYCH

Streszczenie

1. Procesowi dojrzewania kurzych komórek erytroidalnych (erytroblast, retikulocyt, erytrocyt) towarzyszy spadek zawartości białka niehistonowego chromatyny.

2. Analiza rozpuszczalnych w fenolu białek niehistonowych (fosfoproteidów), przeprowadzona metodą elektroforezy w żelu poliakrylamidowym zawierającym siarczan dodecylu sodu oraz metodą ogniskowania izoelektrycznego, wykazała występowanie różnic między tymi białkami izolowanymi z badanych populacji komórek. Fosfoproteidy niedojrzałych form charakteryzują się wyższą zawartością frakcji o masach cząsteczkowych około 86 000 i 23 000 daltonów niż fosfoproteidy erytrocytów.

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REGULATION OF PROGESTERONE BIOSYNTHESIS IN HUMAN PLACENTAL MITOCHONDRIA BY KREBS CYCLE METABOLITES*

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1. 2-Oxoglutarate, succinate, fumarate, malate and citrate, *cis*-aconitate and isocitrate stimulate conversion of cholesterol to progesterone in human placental mitochondria.

2. The stimulatory effect of dicarboxylic and tricarboxylic acids depends on the activity of malate dehydrogenase (decarboxylating) ($NADP^+$) (EC 1.1.1.40) and isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42), respectively.

Mitochondria from steroidogenic tissues have an additional electron transporting chain, involved in the steroid hydroxylations. NADPH is a highly specific electron donor in this system (Toren *et al.*, 1964; Simpson & Boyd, 1967; Robinson & Stevenson, 1971a), and regeneration of NADPH pool plays an important role in the regulation of steroid biosynthesis in these tissues.

It is well known that in mitochondria from adrenals (Koritz, 1966; Hall, 1972), ovaries (Robinson & Stevenson, 1971a; Užgiris *et al.*, 1971) and testis (Menon *et al.*, 1967) the Krebs cycle metabolites are the most important source of electrons for mixed oxidases systems. In mitochondria from the tissues producing steroid hormones, different substrates serve as a source of reducing equivalents for the cholesterol side chain cleavage reaction. In adrenals and ovaries, NADPH is generated either directly by NADP-dependent dehydrogenases (Robinson & Stevenson, 1971a) or indirectly either by NAD-dependent dehydrogenases associated with transhydrogenase (EC 1.6.1.1., Robinson & Stevenson, 1971a; Hall, 1972) or by succina e and fatty acids oxidation *via* reversed electron flow and transhydrogenation (Koritz, 1966; Robinson & Stevenson, 1971b; 1972).

The regulatory mechanism of progesterone biosynthesis in placenta is largely unexplored. The conversion of cholesterol into pregnenolone and progesterone in this tissue is catalysed by a NADPH-dependent enzyme system (Morrison *et al.*, 1965; Ryan *et al.*, 1966; Mason & Boyd, 1971). Our previous results (Bogusławski

* This work was supported by the Ministry of Higher Education, Science and Technology within the project 1/16 - 02. 02.

et al., 1972; 1974) pointed to the role of malate and NADP-dependent "malic enzyme" in the regulation of placental steroidogenesis.

In the present work, the dependence of progesterone biosynthesis on other mitochondrial metabolites engaged in regeneration of NADPH pool, was investigated.

MATERIALS AND METHODS

Reagents. Progesterone, pregnenolone, L-malate, fumarate and malonate were purchased from Koch-Light Lab. (Colnbrook, Bucks., England); citrate, DL--isocitrate, *cis*-aconitate, 2-oxoglutarate, succinate and malate dehydrogenase were obtained from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); NAD^+, NADH, $NADP^+$, NADPH and citrate lyase from Boehringer Corp. (London, England); [4-^{14}C]cholesterol (spec. act. 58 mCi/mmole), [^{3}H]pregnenolone (spec. act. 6.9 Ci/mmole) and [^{3}H]progesterone (spec. act. 225 mCi/mmole) from the Radiochemical Centre (Amersham, England). All other chemicals were analytical grade products of Polish origin.

Preparation of mitochondrial fraction from human term placenta was performed as described by Bogusławski *et al.* (1972).

Mitochondria suspended in 1 vol. of cold water were sonicated for 5 min, with intervals for cooling. The sonicate was then centrifuged at 20 000 *g* for 10 min, the pellet was discarded, and the supernatant obtained was recentrifuged at 105 000 *g* for 30 min.

The incubations were carried out in duplicate at 37°C in the air with constant shaking for 1 h. The incubation mixture contained in a final volume of 2.5 ml: 20 mM-potassium phosphate buffer, pH 7.4, 10 mM-magnesium sulphate, 1 mM--$NADP^+$ and 15 mg of mitochondrial protein suspended in 0.154 M-KCl, or 5 mg of supernatant protein (105 000 *g*) of sonicated mitochondria. The reaction was initiated by the addition of [^{14}C]cholesterol (0.15 μCi) and terminated by freezing instantaneously the sample at −20°C.

Isolation of ^{14}C-labelled steroids and the radioactivity measurements were performed as described by Bogusławski *et al.* (1974).

Citrate and isocitrate were determined according to Williamson & Corkey (1969).

Aconitase activity was measured in the supernatant (105 000 *g*) of sonicated placental mitochondria (1.1 mg of protein) in the incubation medium without $NADP^+$ and Mg^{2+}, using citrate as a substrate. Changes in absorbance at 240 nm accompanying *cis*-aconitate formation were monitored at 25°C. The millimolar absorption coefficient of 3.414 was used for calculation of the rate of *cis*-aconitate formation.

NADP-dependent isocitrate dehydrogenase in the supernatant (105 000 *g*) of sonicated mitochondria (0.1 mg of protein) was measured following the reduction of NADP, at 340 nm and 25°C.

Oxygen consumption by the mitochondrial preparation (15 mg of protein) was measured in Warburg apparatus. Incubation conditions were the same as for measurements of steroid biosynthesis.

Protein was determined by the biuret method (Layne, 1957).

RESULTS

The results presented in Table 1 proved the stimulatory effect of dicarboxylates on [^{14}C]cholesterol to [^{14}C]progesterone conversion in human placental mitochondria. The addition of succinate or fumarate to the mitochondrial suspension increased the conversion, similarly as it was observed previously with malate (Bogusławski *et al.*, 1974). The effect of 2-oxoglutarate was less pronounced, but malonate caused a significant inhibition of the 2-oxoglutarate-dependent progesterone biosynthesis. A marked increase of progesterone synthesis was also noted on addition of Mn^{2+}. In the absence of Krebs cycle metabolites, Mn^{2+} was without any effect on the conversion of cholesterol to progesterone (Bogusławski *et al.*, 1974).

Table 1

Effect of dicarboxylic Krebs cycle metabolites on progesterone biosynthesis in human placental mitochondria

The incubation mixture was as described in Methods; dicarboxylic acids and manganese were added at concentration of 10 mM and 1 mM, respectively. Progesterone synthesis was measured by determination of ^{14}C incorporation from cholesterol to progesterone.

Addition	Incorporation of ^{14}C (d.p.m./sample)	Conversion (%)
None (control)	0	0
Malate	3560	1.10
Malate + Mn^{2+}	7200	2.30
Fumarate	3250	1.10
Fumarate + Mn^{2+}	6330	2.00
Succinate	2780	0.89
Succinate + Mn^{2+}	5850	1.90
2-Oxoglutarate	2060	0.66
2-Oxoglutarate + Mn^{2+}	2530	0.81
2-Oxoglutarate + malonate	870	0.28
Malonate	0	0

Conversion of cholesterol to progesterone, as measured by ^{14}C incorporation, was more efficient in the presence of citrate or isocitrate (Table 2) than in the presence of dicarboxylic acids. The conversion of [^{14}C]cholesterol to [^{14}C]progesterone in the presence of NADP and citrate or isocitrate exceeded that caused by NADPH added at the same concentration as NADP. Nucleotides added separately: $NADP^+$, NAD^+, as well as NADH even in the presence of $NADP^+$, had no effect on progesterone biosynthesis, and addition of NAD^+ did not affect stimulation caused by NADPH.

Conversion of [^{14}C]cholesterol to [^{14}C]progesterone increased with the concentration of citrate added to the mitochondrial suspension (Fig. 1) whereas DL-isocitrate or *cis*-aconitate at concentrations exceeding 4 mM inhibited progesterone formation.

Table 2

Effect of pyridine nucleotides, citrate and isocitrate on progesterone biosynthesis in human placental mitochondria

The incubation mixture was as described in Methods except that $NADP^+$ was omitted. Progesterone synthesis was measured by determination of ^{14}C incorporation from cholesterol to progesterone.

Addition	Incorporation of ^{14}C (d.p.m./sample)	Conversion (%)
None (control)	0	0
NADPH, 1 mM	17 900	5.60
NADPH, 1 mM + NAD^+, 0.2 mM	14 200	4.40
$NADP^+$, 1 mM	0	0
Citrate, 10 mM	3 500	1.10
Citrate, 10 mM + $NADP^+$, 1mM	21 000	6.60
DL-Isocitrate, 4 mM	11 360	3.50
DL-Isocitrate, 4 mM + $NADP^+$, 1 mM	31 900	10.00

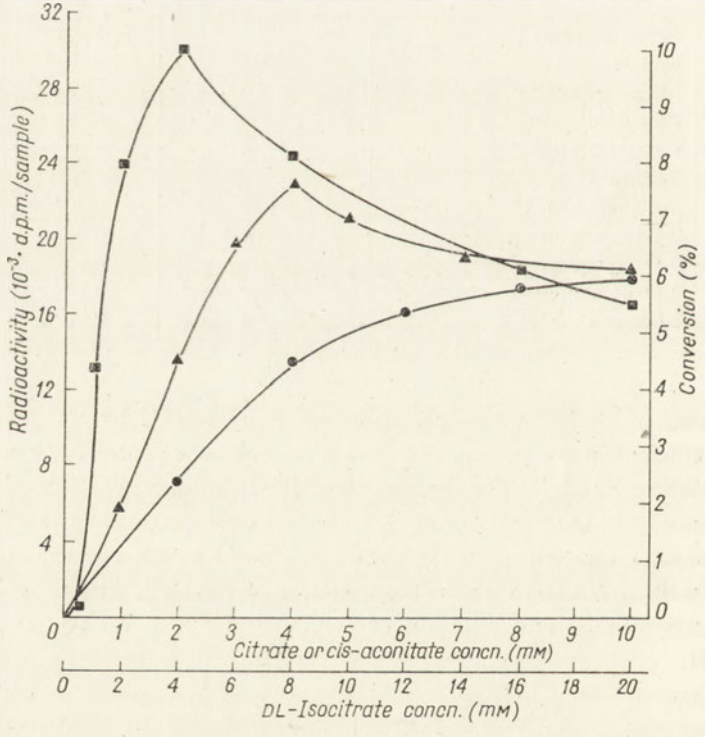

Fig. 1. Tricarboxylic-acid-dependent conversion of cholesterol to progesterone. Effect of isocitrate (■), *cis*-aconitate (▲) and citrate (●). The tricarboxylic acids were added to the suspension of mitochondria in the incubation mixture, as described in Methods.

Comparison of the citrate- and isocitrate-dependent progesterone formation with the amount of citrate and isocitrate metabolized (Fig. 2) under the experimental conditions used, proved that the less pronounced effect of citrate on progesterone biosynthesis was due to lower utilization of this metabolite. On the other hand, no correlation was found between the effect of isocitrate and the rate of its metabolism. No inhibition of isocitrate utilization or oxygen consumption was observed in the range of substrate concentrations examined.

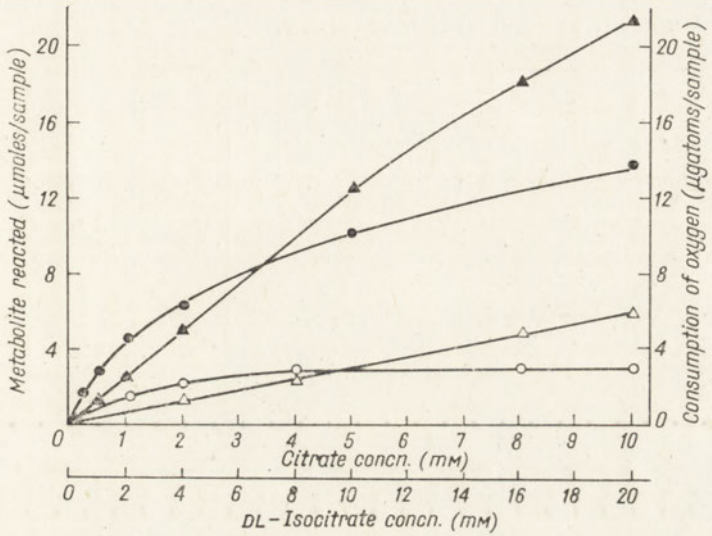

Fig. 2. Uptake of citrate (△) and isocitrate (▲); and oxygen consumption by placental mitochondria in the presence of: citrate (○) and isocitrate (●). For details see Methods.

The stimulatory effect of citrate and isocitrate on progesterone and pregnenolone formation was also observed with the supernatant fraction (105 000 *g*) of sonicated

Table 3

Effect of citrate and isocitrate on progesterone and pregnenolone biosynthesis in intact mitochondria and supernatant (105 000 g) of sonicated mitochondria

The incubation mixture was as described in Methods. Biosynthesis of progesterone and pregnenolone was measured by determination of ^{14}C incorporation.

Addition	Mitochondrial preparation	^{14}C incorporation (d.p.m./sample)			Conversion (%)
		progesterone	pregnenolone	total	
DL-Isocitrate (4 mM)	Intact mitochondria	26 480	0	26 480	9.10
	Supernatant (105 000 *g*)	8 380	43 240	51 620	18.10
Citrate (2 mM)	Intact mitochondria	7 120	0	7 120	2.40
	Supernatant (105 000 *g*)	13 800	25 100	38 900	13.70

mitochondria (Table 3). These results show that the stimulatory effect of citrate and isocitrate is not dependent on the intactness of the mitochondrial membrane.

Lineweaver-Burks plots presented in Figs. 3 and 4 illustrate the substrate affinity of aconitase and NADP-dependent isocitrate dehydrogenase in the mitochondrial supernatant fraction (105 000 *g*) at the concentration range at which tricarboxylic acids affect progesterone synthesis. Under these conditions lower substrate affinity of aconitase limited isocitrate formation and NADPH generation in the course of pregnenolone and progesterone biosynthesis.

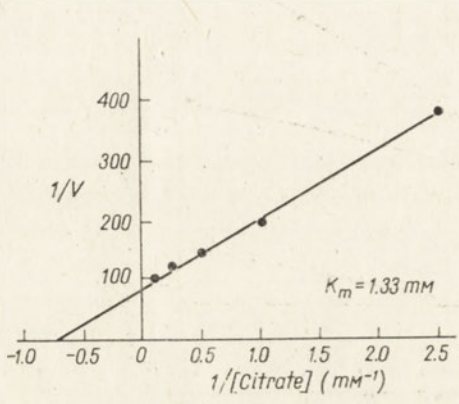

Fig. 3

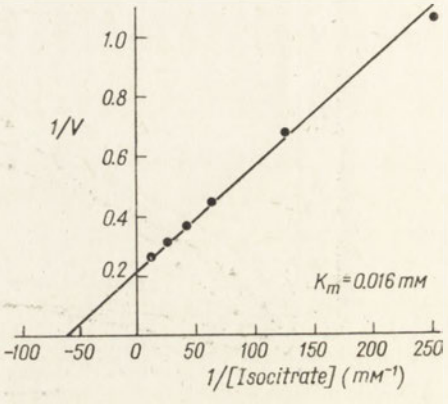

Fig. 4

Fig. 3. Lineweaver-Burk plots for aconitase activity. *V* is expressed as μmoles of *cis*-aconitate/min/mg protein. For details see Methods.

Fig. 4. Lineweaver-Burk plots for NADP-dependent isocitrate dehydrogenase activity. Concentration of isocitrate was calculated for D-isocitrate only. *V* is expressed as μmoles of NADPH/min/mg protein. For details see Methods.

In intact mitochondria accessible to citrate, the observed higher K_m values for aconitase may explain the limited metabolism of citrate to isocitrate. A low K_m value obtained for NADP-dependent isocitrate dehydrogenase may also explain the high conversion of cholesterol to progesterone even at low isocitrate concentration (cf. Fig. 1).

DISCUSSION

The results presented provide evidence for an important role of dicarboxylic and tricarboxylic Krebs cycle intermediates in the conversion of cholesterol to progesterone in human placental mitochondria.

In contrast to adrenals and ovaries in which NADH was found to be a possible electron donor in the cholesterol side chain cleavage reaction (Hall, 1972; Robinson & Stevenson, 1971a), placental mitochondria used exclusively NADPH as a source of reducing equivalents. The observed stimulatory effect of the Krebs cycle metabo-

lites may be explained as the effect on NADPH generation in the NADP-dependent dehydrogenase reactions.

The inhibitory effect of malonate on 2-oxoglutarate-dependent progesterone formation indicates that conversion to malate is a step necessary for the effect of 2-oxoglutarate. This result is at variance with the lack of inhibition by malonate of the 2-oxoglutarate-supported conversion of cholesterol into pregnenolone and progesterone in ovarian mitochondria (Robinson & Stevenson, 1971a). The effect of succinate and fumarate observed in the present work suggests that both these intermediates may act as precursors of malate — a substrate for NADP-dependent "malic enzyme". According to this suggestion one could expect that the stimulatory effect of Mn^{2+} obtained in the presence of these three substrates, would be the same. We assume therefore that "malic enzyme" is responsible for the effect of 2-oxoglutarate, succinate, malate and fumarate.

Comparison of the effects of malate and its precursors with those of citrate, *cis*-aconitate or isocitrate shows that 10% of cholesterol was converted to progesterone in the presence of 4 mM-DL-isocitrate, and about 2% conversion was found in the presence of 10 mM-malate. The difference in the isocitrate and malate effect is due to the higher activity of NADP-dependent isocitrate dehydrogenase as compared with NADP-dependent "malic enzyme" (Aleksandrowicz *et al.*, 1971). It may be assumed that citrate and *cis*-aconitate influence biosynthesis of progesterone from cholesterol after being converted to isocitrate. A low activity of aconitase in mitochondria is responsible for the less pronounced effect of citrate and *cis*-aconitate as compared with isocitrate.

In contrast to adrenal mitochondria (Hall, 1972), placental mitochondria are capable of synthesizing progesterone from cholesterol. Pregnenolone, an assumed product of cholesterol side chain cleavage in other steroidogenic tissues (Robinson & Stevenson, 1971a; Užgiris *et al.*, 1971) was not detectable under our experimental conditions. It is well documented that the effect of malate involves conversion of cholesterol to pregnenolone but not the transformation of pregnenolone to progesterone (Bogusławski *et al.*, 1974). The results of experiments performed with supernatant (105 000 *g*) of sonicated mitochondria show that pregnenolone, but not progesterone, accumulates in the presence of either citrate or isocitrate. This proves that the stimulatory action of the latter metabolites is connected with the conversion of cholesterol to pregnenolone.

There are two possible sources of NADPH for cholesterol side chain cleavage by the placental mitochondria preparation: i.e. NADPH is either generated directly by NADP-dependent mitochondrial "malic enzyme" which is also responsible for the effect of fumarate, succinate and 2-oxoglutarate, or by a much more effective NADP-dependent isocitrate dehydrogenase responsible for the effect of isocitrate, *cis*-aconitate and citrate.

The experiments presented suggest that concentration of tricarboxylic acids within the human placental mitochondrion plays an important role in progesterone formation.

REFERENCES

Aleksandrowicz, Z., Świerczyński, J., Bogusławski, W., Klimek, J. & Żelewski, L. (1971) *9th Meet. Polish Biochem. Soc., Abstr. Commun.*, p. 122.

Bogusławski, W., Klimek, J. & Żelewski, L. (1972) *FEBS Lett.*, **27**, 181 - 183.

Bogusławski, W., Klimek, J. & Żelewski, L. (1974) *Acta Biochim. Polon.*, **21**, 119 - 124.

Hall, P. F. (1972) *Biochemistry*, **11**, 2891 - 2897.

Koritz, S. B. (1966) *Biochem. Biophys. Res. Commun.*, **23**, 485 - 489.

Layne, E. (1957) in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, pp. 447 - 466. Academic Press, New York.

Mason, J. I. & Boyd, G. S. (1971) *Eur. J. Biochem.*, **21**, 308 - 321.

Menon, K. M. J., Dorfman, R. I. & Forchielli, E. (1967) *Biochim. Biophys. Acta*, **148**, 486 - - 494.

Morrison, G., Meigs, R. A. & Ryan, K. J. (1965) *Steroids*, **6**, Suppl. 2, 177 - 188.

Robinson, J. & Stevenson, P. M. (1971a) *Eur. J. Biochem.*, **24**, 18 - 30.

Robinson, J. & Stevenson, P. M. (1971b) *FEBS Lett.*, **17**, 53 - 57.

Robinson, J. & Stevenson, P. M. (1972) *FEBS Lett.*, **23**, 327 - 331.

Ryan, K. J., Meigs, R. & Petro, Z. (1966) *Amer. J. Obstet. Gynecol.*, **96**, 676 - 682.

Simpson, E. R. & Boyd, G. S. (1967) *Biochem. Biophys. Res. Commun.*, **28**, 945 - 950.

Toren, D.,. Menon, K. M. J., Forchielli, E. & Dorfman, R. I. (1964) *Steroids*, **3**, 381 - 390.

Użgiris, V. I., McIntosh, E. N., Alonso, C. & Salhanick, H. A. (1971) *Biochemistry*, **10**, 2916 - 2923.

Williamson, J. R. & Corkey, B. E. (1969) in *Methods in Enzymology* (J. M. Lowenstein, ed.) vol.13, pp. 434 - 513. Academic Press, New York and London.

REGULACJA BIOSYNTEZY PROGESTERONU W MITOCHONDRIACH ŁOŻYSKA LUDZKIEGO PRZEZ METABOLITY CYKLU KREBSA

Streszczenie

1. Stwierdzono, że α-ketoglutaran, bursztynian, fumaran, jabłczan i cytrynian, *cis*-akonitan i izocytrynian stymulują konwersję cholesterolu do progesteronu w mitochondrialnej frakcji łożyska ludzkiego.

2. Stymulujący efekt dwukarboksylowych kwasów jest zależny od aktywności NADP-zależnego enzymu jabłczanowego a trójkarboksylowych kwasów od NADP-zależnej dehydrogenazy izocytrynianowej.

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POTASSIUM-DEPENDENT THERMAL SENSIBILITY OF AMP-DEAMINASE FROM RABBIT SKELETAL MUSCLE*

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1. AMP-deaminase (AMP-aminohydrolase, EC 3.5.4.6) from rabbit skeletal muscle showed sigmoid-shaped plots of velocity *versus* substrate concentration at four temperatures tested between 15° and 35°C. In the presence of 20 mM-KCl, the plot was sigmoid only at 30°C and became hyperbolic at the other temperatures tested. In the presence of 150 mM-KCl the plots were hyperbolic at all the temperatures applied.

2. The K_m value depended on temperature and concentration of KCl, whereas V_{max} was the same for the 20 mM- and 150 mM-KCl-activated enzyme.

3. The value of enthalpy of the enzyme-substrate complex formation was the same for both the 20 mM- and 150 mM-KCl-activated enzyme at lower temperature range (< 38°C), whereas at higher temperatures (> 38°C) this value was much more negative in the presence of 20 mM-KCl than of 150 mM-KCl.

It is generally accepted now that skeletal muscle AMP-deaminase of rabbit, rat, chicken and probably other animals is an oligomeric protein showing distinct regulatory properties (Boosman *et al.*, 1971; Coffee & Kofke, 1975). Potassium and sodium ions have been shown to be the most important activators and inorganic phosphate the most important inhibitor of the activity of this enzyme (Lee, 1957; Smiley & Suelter, 1967; Ronca *et al.*, 1972). In addition, some nucleotides like ATP, ADP and GTP regulate the muscle AMP-deaminase activity, to the extent depending on salt composition of the reaction mixture and the temperature at which the reaction is carried out (Ronca *et al.*, 1972; Kaletha, 1976).

The results of many investigations point to the complex kinetics of the reaction catalysed by rabbit muscle AMP-deaminase. In the presence of 150 mM-KCl the plot of reaction velocity *versus* substrate concentration was hyperbolic, whereas in the absence of KCl the plot was sigmoid-shaped (Smiley & Suelter, 1967).

It has been found in our laboratory ten years ago that AMP-deaminase shows two types of response to temperature at low ($5 - 8 \times 10^{-5}$ M) substrate concentration:

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the enzyme from poikilothermic animals like carp, trout, frog shows no well-defined optimum over a wide temperature range, whereas the rate of reaction catalysed by AMP-deaminase from homeotherms like hen, rat and rabbit changes substantially with the increase in temperature, showing a distinct optimum at 35 - 40°C (Żydowo *et al.*, 1965). It has been shown also that thermal susceptibility of muscle AMP-deaminase does not change upon purification of the enzyme, but differences in this susceptibility have been observed during ontogenesis (Kaletha & Żydowo, 1971; Kaletha, 1975).

The aim of the present paper was to investigate the effect of temperature on the activity of crystalline rabbit skeletal muscle AMP-deaminase in the presence and absence of potassium. The experiments were carried out in a wide range of temperatures (15 - 45°C) at different concentrations of the substrate and salts.

MATERIALS AND METHODS

Preparation of the enzyme. AMP-deaminase was prepared from rabbit back and leg muscles by the procedure described by Smiley *et al.* (1967). The enzyme adsorbed on phosphocellulose column was eluted with 1 M-KCl. The crystalline enzyme obtained was purified about 200 times and its specific activity was 88 μmoles per minute per mg of protein as measured according to Smiley & Suelter (1967).

The enzyme assay. AMP-deaminase activity was estimated spectrophotometrically according to Kalckar (1947) by monitoring for 2 min either the decrease in absorbance at 265 nm or the increase in absorbance at 285 nm. The initial velocity (μmoles of AMP/min) of the reaction was calculated using the molar coefficients according to Smiley & Suelter (1967). The rate of adenylic acid deamination was estimated at six different temperatures ranging from 15 to 45°C, with the aid of Unicam SP-800 spectrophotometer fitted with a constant-temperature cell housing. The reaction mixtures contained in the final volume of 3 ml: AMP at concentrations 0.04, 0.07, 0.1, 0.2, 0.3, 0.5 and 0.8 mM, and 100 mM-imidazole-HCl buffer, pH 6.5; besides, the mixture contained either 20 or 150 mM-KCl or, in the absence of KCl, 150 mM-tetramethylammonium chloride. After equilibration of temperature, 20 μl of appropriately diluted enzyme (0.6 μg protein in the case of the KCl-activated enzyme, and 45 μg protein in the case of the non-activated enzyme) was added to start the reaction. In the presence of 150 mM-KCl no thermal denaturation was detected irrespective of the temperature applied. In the absence of KCl, at higher temperature (40 and 45°C) the time-course of the reaction was no more linear with time, therefore these results were not included.

Protein concentration was estimated either spectrophotometrically according to Warburg & Christian (Layne, 1957) or by the biuret method of Gornall *et al.* (1949).

Reagents. Adenosine monophosphate (AMP) was purchased from Sigma (St. Louis, Mo., U.S.A.); cellulose phosphate was from Whatman (Maidstone, England); imidazole from Koch - Light (Colnbrook, England); all the other chemicals were purchased from P.O.Ch. (Gliwice, Poland).

RESULTS

Figure 1 presents Michaelis-Menten plots of the reaction catalysed by the rabbit skeletal muscle AMP-deaminase activated with 150 mM-KCl (Fig. 1a) or 20 mM-KCl (Fig. 1b), and non-activated (Fig. 1c), at four temperatures: 15, 25, 30 and 35°C. As it may be seen, the shape of the plots depended on the composition of the incubation medium. In the presence of 150 mM-KCl the plots were hyperbolic at all temperatures tested. Similarly, when the reaction was catalysed by the 20 mM-KCl--activated enzyme, the plots were hyperbolic although at 30°C an irregularity in the hyperbolic relationship was observed. When KCl was omitted from the reaction

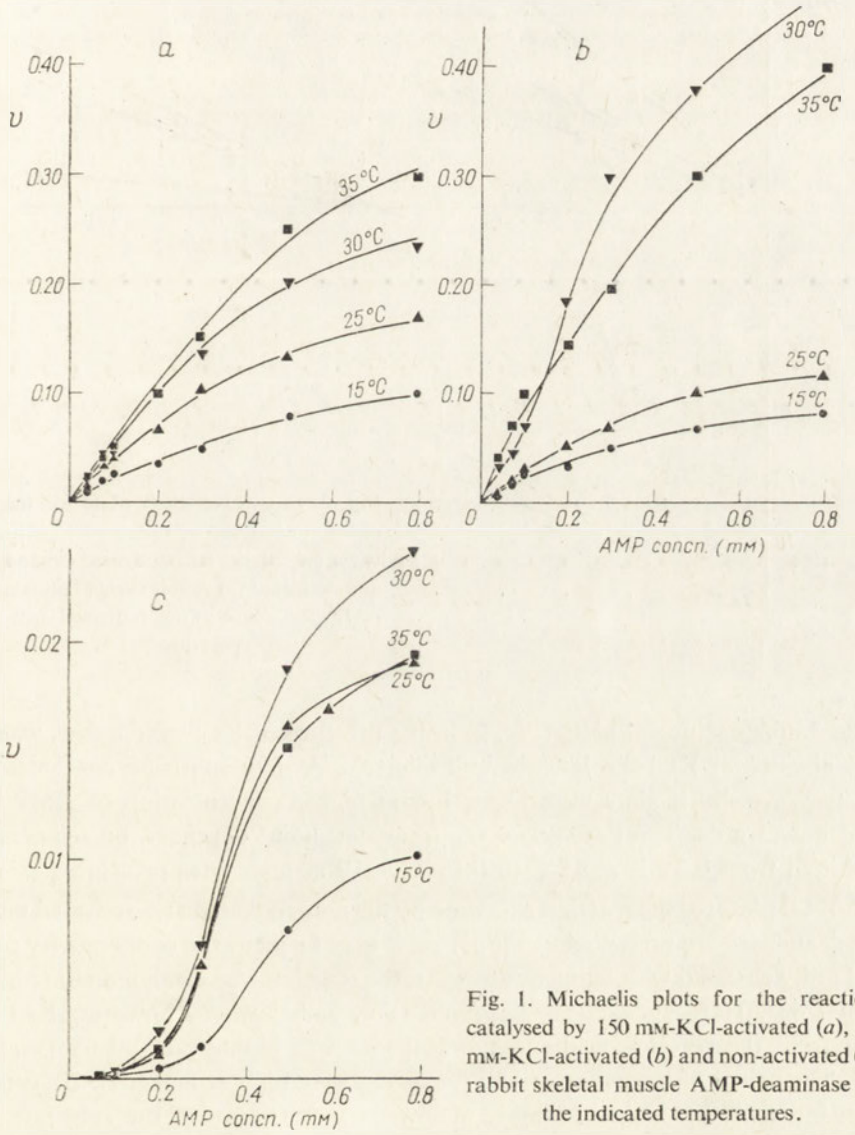

Fig. 1. Michaelis plots for the reaction catalysed by 150 mM-KCl-activated (*a*), 20 mM-KCl-activated (*b*) and non-activated (*c*) rabbit skeletal muscle AMP-deaminase at the indicated temperatures.

medium, the Michaelis-Menten plot was sigmoid-shaped at all temperatures tested, which seems to indicate cooperativity in the action of AMP-deaminase under these conditions. The velocity of the reaction increased in all experiments with the increase in temperature except when the reaction was catalysed by the non-activated enzyme; in this case, at higher substrate concentration the reaction rate was lower at 35°C than at 30°C (Fig. 1c).

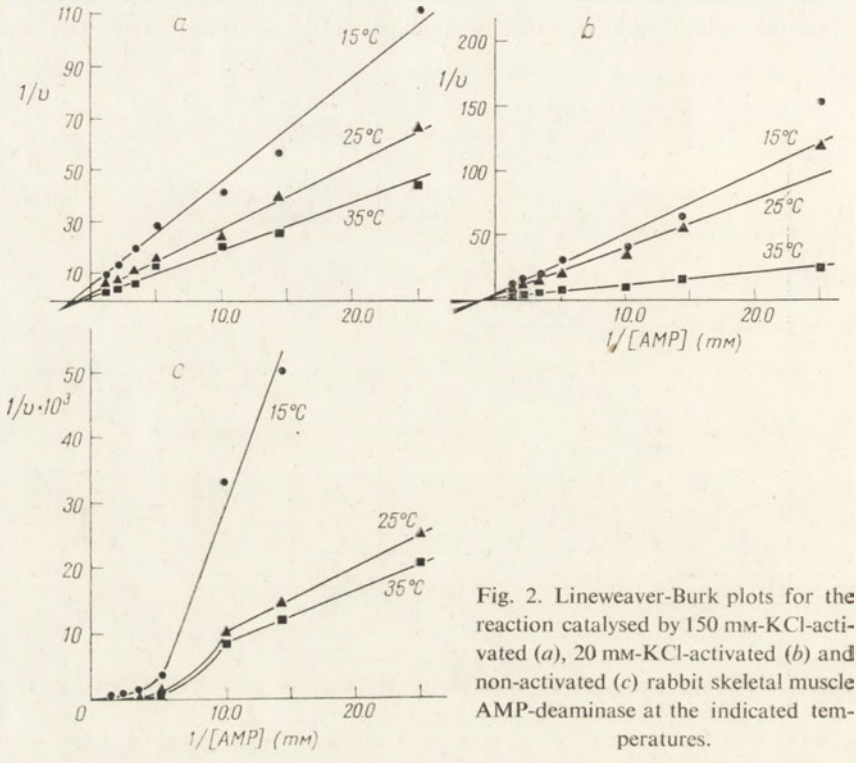

Fig. 2. Lineweaver-Burk plots for the reaction catalysed by 150 mM-KCl-activated (*a*), 20 mM-KCl-activated (*b*) and non-activated (*c*) rabbit skeletal muscle AMP-deaminase at the indicated temperatures.

The double-reciprocal plots (Fig. 2) in the presence of KCl were linear, whereas in the absence of KCl the kinetics was biphasic. At low substrate concentration, the Lineweaver-Burk plots were linear but at higher concentration of AMP they curved downwards. The position of the transition point depended on temperature and shifted from 0.2 mM at 15°C to 0.1 mM-AMP at higher temperatures.

The Hill plots for the reaction catalysed by the non-activated and activated muscle AMP-deaminase are presented in Fig. 3. As it may be seen, the cooperativity coefficient hardly changed with temperature when the reaction was catalysed either by the 150 mM- or 20 mM-KCl-activated enzyme (Fig. 3a,b); when, however, KCl was absent from the reaction medium, the Hill plot was biphasic at all temperatures tested. The values of the cooperativity coefficient were high at higher AMP concentration but they diminished abruptly at lower concentration of the substrate.

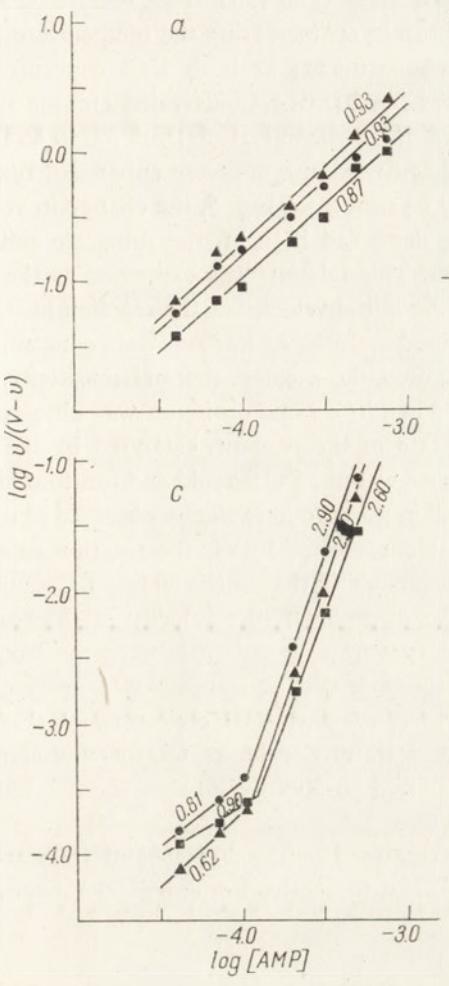

Fig. 3. Hill plots for the reaction catalysed by 150 mM-KCl-activated (*a*), 20 mM-KCl-activated (*b*) and non-activated (*c*) rabbit skeletal muscle AMP-deaminase at: (▲) 15°C; (●) 25°C; (■) 35°C. Cooperativity coefficient (n_H) was calculated from the plot.

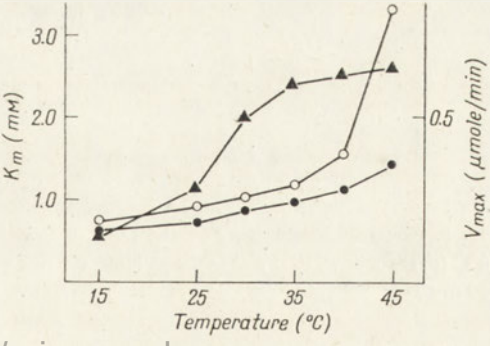

Fig. 4. Effect of temperature on V_{max} (▲), and on K_m of the 150 mM-KCl (○) and 20 mM-KCl (●) activated rabbit skeletal muscle AMP-deaminase. K_m and V_{max} were determined graphically from Lineweaver-Burk plots at each temperature tested. Each point represents a mean from 3 experiments.

The value of V_{max} was the same for the 20 mM- and 150 mM-KCl-activated AMP--deaminase (Fig. 4), and it increased abruptly on increasing the temperature up to 35°C. K_m values were also temperature-dependent; at both KCl concentrations the K_m increased with temperature but for the 20 mM-KCl-activated enzyme the increase was especially pronounced: on raising the temperature from 35° to 45°C, K_m values for the 150 mM- and 20 mM-KCl-activated enzyme were enhanced, respectively, about 1.5 and 2.8 times. As it may be seen from Fig. 5, the change in velocity of AMP deamination with temperature depended both on the substrate and KCl concentration in the incubation medium. This relation was expressed as the ratio v_t/v_{15} which indicates how many times the initial velocity of the reaction at a given temperature was greater than that measured at 15°C. At low substrate concentration (0.07 mM) the non-activated enzyme showed the greatest thermal sensitivity, with a well-defined temperature optimum at 35°C. The same optimum was observed for the 20 mM-KCl-activated enzyme. The rate of the reaction catalysed by the non--activated and 20 mM-KCl-activated enzyme was at 35°C fivefold and fourfold higher, respectively, than at 15°C. The abrupt fall in the reaction velocity observed at temperatures exceeding 35°C, is of considerable interest. At 45°C the reaction catalysed by the non-activated enzyme was undetectable, and that of the 20 mM-KCl-activated enzyme was lower than at 15°C. On the other hand, the velocity of the reaction catalysed by the 150 mM-KCl-activated enzyme changed little over the range of temperatures tested, and no distinct optimum could be seen at low substrate concentration. This optimum appeared, however, at higher AMP concentration (Fig. 5b). In the presence of 0.5 mM-AMP, the temperature optimum for the non-activated and 20 mM-KCl-activated enzyme was shifted to 30°C while that for the 150 mM--KCl-activated enzyme remained at 35°C.

The Arrhenius plot for the reaction catalysed by the 20 mM- and 150 mM-KCl--activated enzyme (Fig. 6) was biphasic, and the activation energy (E) determined

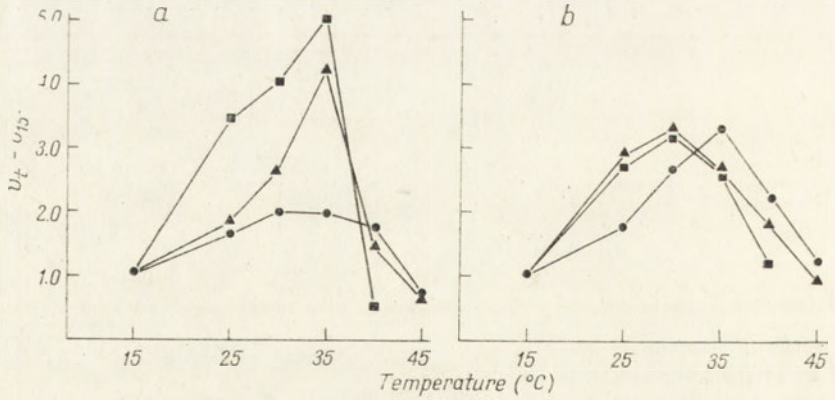

Fig. 5. Effect of temperature on the ratio of initial velocity at a given temperature (v_t) to that at 15°C (v_{15}) for low, 0.07 mM (*a*), and high, 0.5 mM (*b*) AMP concentrations. Reaction catalysed by: 150 mM-KCl activated (●), 20 mM-KCl-activated (▲), and the non-activated (■) rabbit skeletal muscle AMP-deaminase.

from the slope of this plot was about 4260 and 800 cal per mole of substrate at, respectively, <35°C and >35°C. The transition point lied at about 35°C.

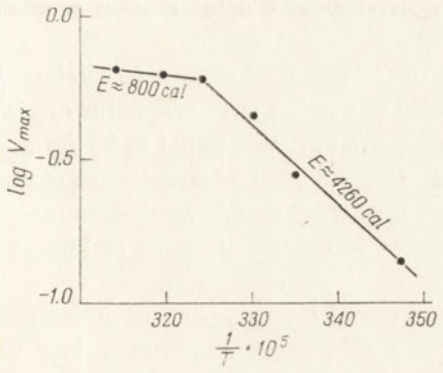

Fig. 6

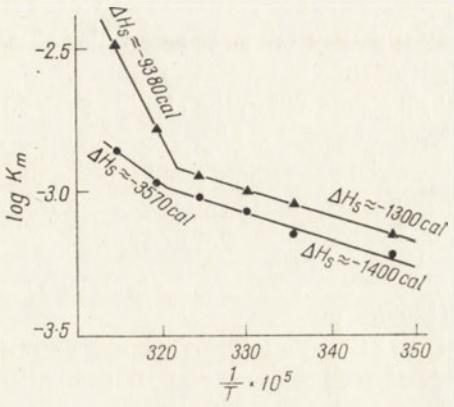

Fig. 7

Fig. 6. Arrhenius plots for the reaction catalysed by KCl-activated rabbit skeletal muscle AMP-deaminase. The values of energy of activation (E) were calculated from the plots.

Fig. 7. Effect of temperature on log K_m for the 150 mM-KCl (●) and 20 mM-KCl (▲) activated rabbit skeletal muscle AMP-deaminase. Enthalpy values (ΔH_s) were calculated from the plots.

As it may be seen from Fig. 7, illustrating log K_m as a function of temperature, the slope of the plots changed at about 38 - 40°C. At temperatures below the transition point, the values of ΔH_s were negative and amounted to about −1300 and −1400 cal, respectively, for the 20 mM- and 150 mM-KCl-activated enzyme, whereas at higher temperature (>38°C) these values were −9380 and −3570 cal, respectively, for the enzyme activated by KCl at two concentrations.

DISCUSSION

In considering the allosteric properties of rabbit skeletal muscle AMP-deaminase, it seems of interest that KCl concentration as low as 20 mM was sufficient to cause a complete disappearance of the cooperativity among the subunits of this enzyme, and to change kinetics of the reaction from sigmoid-shaped in the absence of KCl to hyperbolic in the presence of 20 mM-KCl. It has recently been shown that in the presence of 7 mM-KCl in the incubation medium, the kinetics of rat muscle AMP-deaminase is still sigmoidal (Ronca *et al.*, 1972; Kaletha, 1976). It may be supposed, therefore, that KCl concentration of 20 mM is sufficient to promote change of the quarternary structure of the enzyme molecule.

The biphasic type of the Lineweaver-Burk and Hill plots implies possible existence of two active forms of AMP-deaminase. Each of these forms may operate at a different substrate concentration and exhibit different kinetics, characterized by different cooperativity coefficients. A similar biphasic Hill plot was also observed for the

7 mM-KCl-activated rat muscle AMP-deaminase (Kaletha, 1976). One may also conclude that the temperature sensitivity of AMP-deaminase depends on the concentration of both KCl and the substrate in the incubation medium. Our results suggest that in the presence of KCl the enzyme molecule is much more resistant to thermal denaturation. It seems also that higher substrate concentration promotes this resistance.

The abrupt fall in the ability of the 20 mM-KCl-activated enzyme to catalyse the reaction, observed at higher temperatures, could be due in a large part to the decrease in the enzyme-substrate affinity (assuming $K_m \approx K_s$). V_{max} was found to be the same for the 20 mM- and 150 mM-KCl-activated enzyme, and for the non-activated enzyme (Smiley & Suelter, 1967), which suggests that the rabbit muscle AMP-deaminase is an allosteric enzyme of the "K_m type", according to the nomenclature of Monod *et al.* (1965).

Heat of formation of the enzyme-substrate complex was dependent on temperature and KCl concentration. It seems that at higher temperatures more heat was evolved during formation of the complex than at temperatures below 38°C. In the temperature range from 15 to 38°C formation of the adenylate - AMP-deaminase complex evolved about 1300 and 1400 cal when catalysed by the 20 mM- and 150 mM-KCl-activated enzyme, respectively, whereas the activation of this complex ($\Delta H^* = E - RT$) in this range of temperatures required about $4260 - 600 = 3660$ cal, the total net effect being about 2400 and 2300 cal per mole of substrate. At temperatures above the critical temperature of 38 - 40°C, formation of the adenylate - AMP--deaminase complex resulted in evolution of about 9380 and 3570 cal with the 20 mM- and 150 mM-KCl-activated enzyme; absorption for the subsequent activation was about $800 - 600 = 200$ cal, which gives, respectively, about -9180 and -3370 cal per mole of substrate.

One cannot exclude, however, that differences observed in kinetics of the reaction catalysed by the KCl-activated and non-activated enzyme were partially due to the differences in dilution of enzymatic protein in the assay mixtures. This possibility cannot be excluded in the case of the non-activated enzyme, as the reaction rate was measurable only at a very high enzyme concentration. Dilution of the 150 mM--KCl-activated enzyme had no effect on the kinetics of the reaction.

The author wishes to thank Professor Dr. M. Żydowo for his interest in this work.

REFERENCES

Bladergroen, W. (1955) *Einführung in die Energetik und Kinetik biologischer Vorgänge*, pp. 219 - 222, Wepf, Basel.

Boosman, A., Sammons, D. & Chilson, O. (1971) *Biochem. Biophys. Res. Commun.*, **45**, 1025 - - 1032.

Coffee, C. J. & Kofke, W. A. (1975) *J. Biol. Chem.*, **250**, 6653 - 6658.

Dixon, M. & Webb, E. C. (1971) *Enzymes*, pp. 150 - 158. Longman, London.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.*, **177**, 751 - 766.

Kalckar, H. M. (1947) *J. Biol. Chem.*, **167**, 461 - 475.
Kaletha, K. (1975) *Int. J. Biochem.*, **6**, 471 - 474.
Kaletha, K. (1976) *J. Thermal Biol.*, **1**, 157-161.
Kaletha, K. & Żydowo, M. (1971) *Int. J. Biochem.*, **2**, 20 - 26.
Layne, E. (1957) in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 451. Academic Press, New York.
Lee, Y. P. (1957) *J. Biol. Chem.*, **227**, 999 - 1008.
Monod, J., Wyman, J. & Changeux, J. P. (1965) *J. Mol. Biol.*, **12**, 88 - 118.
Ronca, G., Raggi, A. & Ronca-Testoni, S. (1972) *Ital. J. Biochem.*, **21**, 305 - 321.
Smiley, K. L. & Suelter, C. H. (1967) *J. Biol. Chem.*, **242**, 1980 - 1981.
Smiley, K. L., Berry, A. J. & Suelter, C. H. (1967) *J. Biol. Chem.*, **242**, 2502 - 2506.
Żydowo, M., Makarewicz, W., Umiastowski, J. & Purzycka, J. (1965) *Acta Biochim. Polon.*, **12**, 319 - 325.

ZALEŻNA OD POTASU WRAŻLIWOŚĆ CIEPLNA AMP-DEZAMINAZY Z MIĘŚNIA SZKIELETOWEGO KRÓLIKA

Streszczenie

1. Wykresy zależności szybkości reakcji od stężenia substratu dla AMP-dezaminazy z mięśni szkieletowych królika w czterech badanych temperaturach od 15° do 35° C były sigmoidalne. W obecności 20 mM-KCl wykres był sigmoidalny tylko przy 30°C, natomiast przy innych temperaturach był hiperboliczny. W obecności 150 mM-KCl wykres był hiperboliczny w całym badanym zakresie temperatur.

2. Wartość K_m była zależna od temperatury i stężenia KCl, podczas gdy wartości V_{max} dla enzymu aktywowanego przez 20 mM- i 150 mM-KCl były jednakowe.

3. Wartości ciepła tworzenia (ΔH_s) kompleksu enzym-substrat były jednakowe dla enzymu aktywowanego 20 mM- i 150 mM-KCl w niższych temperaturach (< 38°C), podczas gdy w temperaturach wyższych (> 38°C) wytworzeniu tego kompleksu towarzyszyło wydzielenie znacznie większej ilości energii w obecności 20 mM-KCl niż w obecności 150 mM-KCl.

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REGULATION OF ACONITATE HYDRATASE ACTIVITY FROM RAT KIDNEY CORTEX BY BICARBONATE

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1. The increase in pH value and bicarbonate concentration stimulated citrate synthesis from pyruvate and malate, inhibiting simultaneously conversion of isocitrate to citrate.

2. Bicarbonate inhibited competitively the activity of aconitate hydratase, probably binding with the two active sites of the enzyme. The K_i values for the cytoplasmic and mitochondrial enzymes were, respectively, 27 and 38 mM. The pH optimum for both forms of the enzyme in Tris-HCl buffer was in the range 7.8 - 8.6, and in bicarbonate buffer varied from 7.2 to 8.0, depending on the form of the enzyme and the substrate used.

3. Only free, completely dissociated citrate anion acts as a substrate for aconitate hydratase.

4. The role of aconitate hydratase as a factor controlling the rate of citrate metabolism in kidney in metabolic alkalosis is discussed.

The increase in citrate concentration in kidney and its excretion in urine in metabolic alkalosis is a result of the reduced intracellular metabolism and the increased synthesis of this compound (Stern *et al.*, 1952; Crawford, 1963; Balagura-Baruch *et al.*, 1973a,b). It is believed that the level of citrate in the cells of proximal kidney tubules is dependent on hydrogen ion concentration in this tissue (Crawford, 1963). It seems, however, that this pH-dependence is not due to a general regulation of metabolic process and mitochondrial transport of citrate but results from the direct effect of bicarbonate, the main component of physiological buffer systems, on the activity of some enzymes (Zeylemaker *et al.*, 1970; Horne & Nordlie, 1971; McDaniel & Longmore, 1971; Łysiak *et al.*, 1975). It has also been found that bicarbonate inhibits oxidation *in vitro* of citrate in slices and mitochondria of kidney cortex (Simpson, 1967; Adler *et al.*, 1971). Moreover, bicarbonate decreases the rate of citrate transport into the mitochondria, stimulating at the same time its outflow from this subcellular fraction (Simpson & Angielski, 1973). The observations of Robinson & Chappell (1970) and Brand *et al.* (1973)

imply the regulatory role of aconitate hydratase in oxidation of citrate in liver mitochondria.

In the present work it has been demonstrated that bicarbonate stimulates in rat kidney cortex the mitochondrial synthesis of citrate from pyruvate and malate, and inhibits the activity of mitochondrial and cytoplasmic aconitate hydratase. Kinetics of this inhibition was also studied. The obtained results were discussed with respect to the physiological role of aconitate hydratase in the regulation of citrate metabolism in kidney. The preliminary report of this work has been presented (Stępiński & Angielski, 1975).

MATERIALS AND METHODS

Animals. Wistar rats of either sex weighing 200 - 300 g were used. The animals were killed by decapitation, kidneys immediately removed and immersed in cold 0.25 M-sucrose solution containing 5 mM-Tris-HCl and 1 mM-EDTA, pH 7.0. All further steps of the preparation procedure were carried out at 0 - 4°C.

Isolation of subcellular and submitochondrial fractions from kidney cortex. The kidney cortex separated from the medulla was homogenized with 9 vol. of 0.25 M-sucrose solution containing 5 mM-Tris-HCl and 0.2 mM-EGTA, pH 7.0, and centrifuged at 600 *g* for 10 min. From the supernatant, the mitochondrial, microsomal and cytoplasmic fractions were obtained by successive centrifugations at 10 000 *g* for 20 min and at 150 000 *g* for 1 h. The submitochondrial fractions were prepared as described by Sottocasa *et al.* (1967). To separate the inner membranes from the matrix, mitoplasts were disintegrated with Lubrol WX (0.16 mg/mg protein) and centrifuged at 150 000 *g* for 1 h. Protein content in the subcellular fractions was calculated on the basis of absorbance at 280 nm using for each fraction the empirical coefficient expressing the relation between $A_{280\ nm}$ and protein content as measured by the biuret method (Gornall *et al.*, 1949).

Incubation of kidney cortex mitochondria. The mitochondria isolated by the method of Johnson & Lardy (1967) were incubated for 3 min at 37°C in the media containing the appropriate substrates (see Table 1), and equilibrated with the mixture of 5% CO_2 and 95% O_2. After incubation, mitochondria were rapidly separated by centrifugation through a layer of silicone oil as described by Harris & van Dam (1968). In the deproteinized extracts of mitochondria and supernatant, malate and isocitrate were determined fluorimetrically (Goldberg *et al.*, 1966), and citrate with the use of ATP-citrate lyase (EC 4.1.3.8), malate dehydrogenase (EC 1.1.1.37) and NADH.

Preparation of cytoplasmic and mitochondrial aconitate hydratases. A 20% kidney cortex homogenate in 0.25 M-sucrose containing 5 mM-Tris-HCl, pH 7.0, was centrifuged at 30 000 *g* for 20 min. The supernatant was fractionated with ammonium sulphate as described by Henson & Cleland (1967), the obtained pellet was dissolved in 0.1 M-sucrose and dialysed for 2 h against 0.1 M-sucrose. The obtained preparation is further referred to as the "cytoplasmic aconitate hydratase".

The kidney cortex mitochondria isolated according to Johnson & Lardy (1967) were suspended in 0.1 M-sucrose, disintegrated by ultrasonication (1 min in a 60 W sonicator; MSE, London, England) and centrifuged at 30 000 *g* for 20 min. The supernatant collected is further referred to as the "mitochondrial aconitate hydratase".

Both enzymic preparations were stored at −15°C under nitrogen.

The enzyme assay. The activities of the marker enzymes of the submitochondrial fractions: NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42), glutamate dehydrogenase (EC 1.4.1.2), rotenone-insensitive NADH - cytochrome *c* reductase, and succinate - cytochrome *c* reductase were assayed according to Sottocasa *et al.* (1967). The activity of lactate dehydrogenase (EC 1.1.1.27) was determined in the medium composed of 50 mM-Tris-HCl, pH 7.4, 100 mM-KCl, 0.1 mM-NADH, 2 μg of antimycin A and 2 mM-potassium pyruvate. All determinations were performed at 37°C in the presence of 0.2% Triton X-100.

Aconitate hydratase was determined at 37°C in the presence of, alternatively: citrate (3 mM), *cis*-aconitate (0.1 mM) or DL-isocitrate (1 mM), i.e. at a concentration exceeding 10 - 15 times the respective K_m values (Henson & Cleland, 1967; Villafranca & Mildvan, 1971; Eanes & Kun, 1974). The incubation medium containing 2 mM-$MgCl_2$ was buffered alternatively with 50 mM-Tris-HCl, 30 mM-potassium phosphate or 5 - 100 mM-bicarbonate buffer. The pH value of the bicarbonate buffer was adjusted by changing concentration of bicarbonate or CO_2 pressure. Osmomolarity of the medium was adjusted to about 300 mOsM by adding potassium chloride. The reaction was started by the addition of the enzyme (0.02 - 0.2 mg protein/sample). The rate of citrate conversion to isocitrate was determined in the presence of 0.3 mM-NADP and 0.2 unit of NADP-dependent isocitrate dehydrogenase. Conversion of citrate to *cis*-aconitate was carried out at 37°C in 50 mM--Tris-HCl, pH 7.4, containing 100 mM-KCl and 0.67 - 2 mM-potassium citrate. The amount of *cis*-aconitate formed was determined spectrophotometrically at 240 nm (ε=3.41; Henson & Cleland, 1967).

With *cis*-aconitate or isocitrate as substrates, the reaction was stopped after 3 - 5 min incubation by the addition of $HClO_4$ to a final concentration of 3%. In the deproteinized samples the amount of citrate and D-isocitrate was determined by the methods described by Bergmeyer (1965). pH of the reaction mixture was checked in the parallel samples, using a PHM 27 pH-meter with an E 5021 capillary electrode (Radiometer, Copenhagen, Denmark). Protein was determined by the biuret method (Gornall *et al.*, 1949) with bovine serum albumin as a standard.

Concentration of citrate anion ($citrate^{3-}$) was calculated from the citrate dissociation constant ($pK_3 = 5.53$) and the complex formation constants of citrate--Mg^{1-} ($K = 2.2 \times 10^3$), citrate-Mn^{1-} ($K = 4.2 \times 10^3$) and citrate-Ca^{1-} ($K = 1.9 \times \times 10^3$) (Sillen & Martell, 1971; Meyer, 1974).

Reagents. cis-Aconitate and Tris were from Fluka A.G. (Buchs, Switzerland); NADP from Reanal (Budapest, Hungary); Lubrol WX from ICI Organics (Providence, R.I., U.S.A.); EGTA, enzymes, and the corresponding substrates from Sigma Chem. Corp. (St.Louis, Mo., U.S.A.); sucrose from Reachim (Moscow,

U.S.S.R.), cytochrome *c* from Biomed (Kraków, Poland), bovine serum albumin, fraction V, from B.D.H. (Poole, Dorset, England), citric acid, $KHCO_3$ and EDTA from P.O.Ch. (Gliwice, Poland).

RESULTS

Effect of pH and bicarbonate on citrate formation in the kidney cortex mitochondria. From the results presented in Table 1 it may be seen that an increase in bicarbonate concentration, parallelled by the increase in pH, resulted in an enhanced formation of citrate and malate from pyruvate. On addition of malate to pyruvate, the level of citrate was further increased with a concomitant enhancement of malate consumption. On the other hand, when isocitrate served as a substrate, citrate level in mitochondria and in the incubation medium distinctly decreased with the increase in pH and bicarbonate concentration. The decrease was related to the lower isocitrate consumption as the sum of citrate and isocitrate in the incubation medium remained almost constant.

Table 1

Effect of pH on concentration of citrate formed from pyruvate and isocitrate in kidney cortex mitochondria

Mitochondria (3.8 - 4.0 mg) were suspended in 25 mM-sucrose containing 100 mM-KCl and 2 mM-$MgSO_4$. To the incubation mixture with pyruvate, 2.5 mM-phosphate was added, and to the mixture with isocitrate, 10 mM-phosphate *plus* 2 mM-ATP. The buffer containing 10 mM-bicarbonate, pH 7.05, or 30 mM-bicarbonate, pH 7.55, at constant CO_2 pressure of 36 mm Hg, was used.

Substrate	HCO_3^- (mM)	pH	Citrate		Malate		Isocitrate	
			mitochondria (nmoles/mg protein)	medium (mM)	mitochondria (nmoles/mg protein)	medium (mM)	mitochondria (nmoles/mg protein)	medium (mM)
Pyruvate (1 mM)	10	7.05	1.0	0.016	1.3	0.022		
	30	7.55	4.4	0.037	1.8	0.043		
Pyruvate (1 mM) + malate (1 mM)	10	7.05	3.4	0.123	4.7	0.726		
	30	7.55	6.0	0.227	3.9	0.671		
Isocitrate (3 mM)	10	7.05	6.5	0.74			1.0	1.04
	30	7.55	4.5	0.51			1.6	1.54
Isocitrate (3 mM) + malate (1 mM)	10	7.05	9.1	1.02			1.0	1.04
	30	7.55	6.5	0.94			1.3	0.92

The relation of the above-mentioned overall effect of bicarbonate on citrate metabolism to the activity of aconitate hydratase was subsequently investigated both in mitochondrial and cytoplasmic compartments of the kidney cortex cell.

Distribution of aconitate hydratase activity in the kidney cortex. As it is shown in Fig. 1, the activity of the cytoplasmic enzyme constituted about 70% of the total

activity of aconitate hydratase in kidney cortex; the remaining 30% was found in the mitochondrial fraction. Distribution of this activity in mitochondria corresponds to that of glutamate dehydrogenase and NADP-dependent isocitrate dehydrogenase, but it differs significantly from the distribution of succinate - cytochrome *c* reductase and rotenone-insensitive NADH - cytochrome *c* reductase (Fig. 1B). These data suggest that mitochondrial aconitate hydratase is located exclusively in the matrix. The presence of lactate dehydrogenase (3% of total activity) and glutamate dehydrogenase (20% of total activity) in the top layer fraction indicated contamination of this fraction by cytoplasm and matrix of the disrupted mitochondria.

pH and buffer dependence of cytoplasmic and mitochondrial aconitate hydratase activity. In the preliminary experiments it has been established that changes in

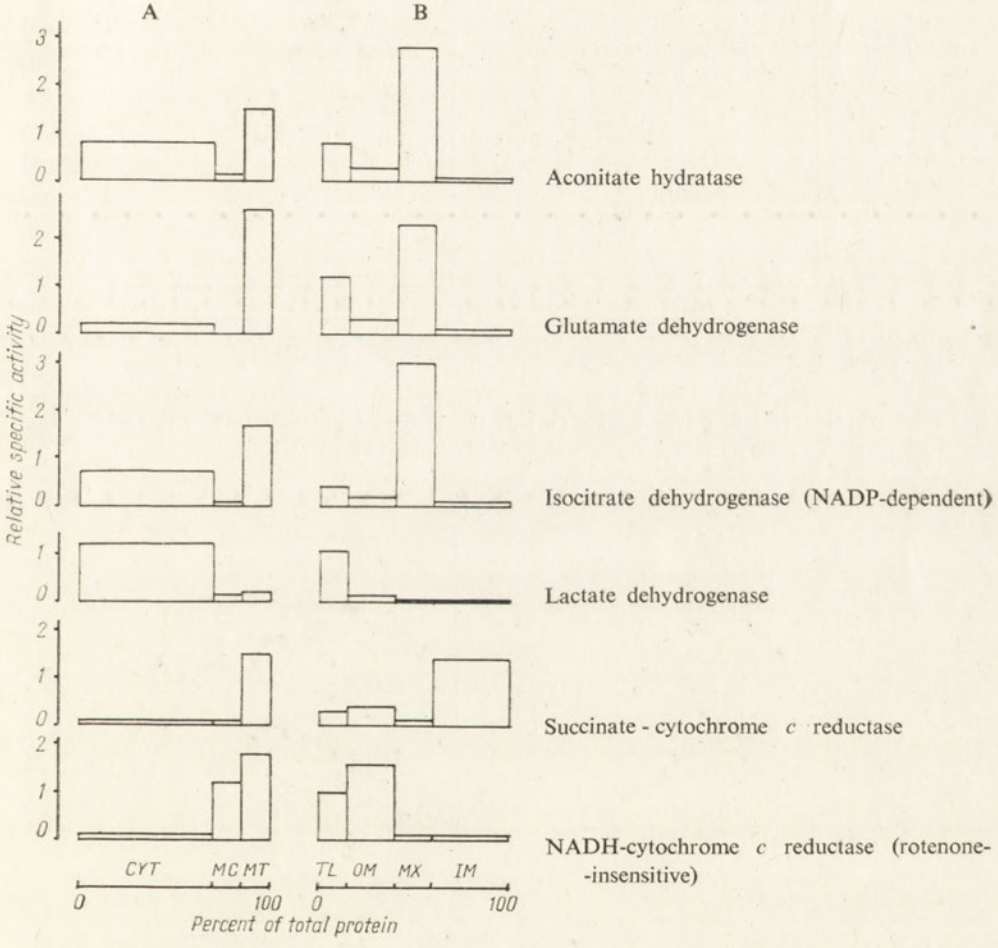

Fig. 1. Distribution of aconitate hydratase in subcellular (*A*) and submitochondrial (*B*) fractions of rat kidney cortex. *A*: *CYT*, cytoplasm; *MC*, microsomes; *MT*, mitochondria; *B*: *TL*, top layer; *OM*, outer membranes; *MX*, matrix; *IM*, internal membranes.

the osmolarity from 200 to 400 mosM and in KCl and Tris concentration from 20 to 100 mM, have no effect on the response of the enzyme kinetics to bicarbonate (unpublished). In Tris-HCl buffer, the mitochondrial and cytoplasmic enzymes showed pH optima at 7.8 - 8.6, irrespective of the substrate used (Figs. 2,3). In bicarbonate buffer, in which the CO_2 pressure was maintained at a constant level (36 mm Hg) and concentration of bicarbonate varied, the optimum for the citrate to isocitrate conversion was observed at pH 7.2 - 7.4 (Fig. 2A) for the cytoplasmic, and at pH 7.5 - 7.6 for the mitochondrial enzyme (Fig. 2B). A further increase

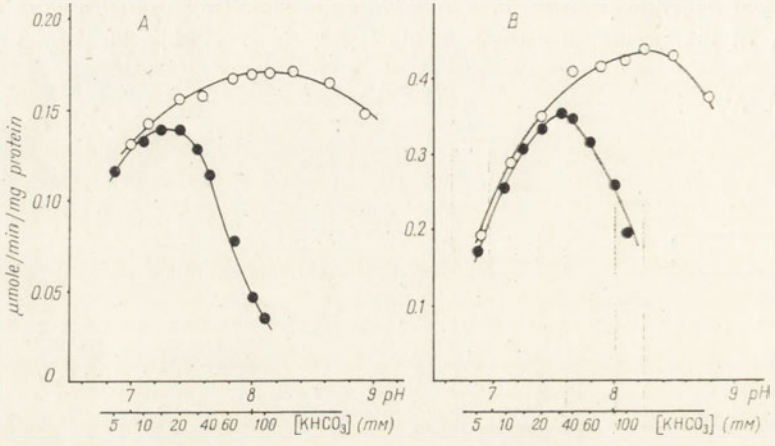

Fig. 2. Effect of pH on the activity of cytoplasmic (*A*) and mitochondrial (*B*) aconitate hydratase in 50 mM-Tris-HCl (○) and bicarbonate (●) at the indicated concentration. The activity was assayed by measuring conversion of citrate (3 mM) to isocitrate.

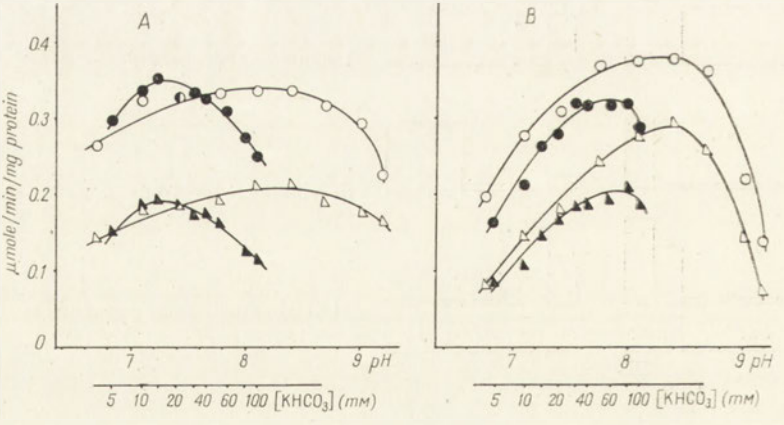

Fig. 3. Effect of pH on the activity of cytoplasmic (*A*) and mitochondrial (*B*) aconitate hydratase in 50 mM-Tris-HCl (○, △) and bicarbonate (●, ▲) at the indicated concentration. The activity was assayed by measuring conversion of *cis*-aconitate to citrate (△, ▲) or isocitrate (○, ●).

in pH, and a concomitant increase in bicarbonate concentration, resulted in a rapid decrease of activity. At pH 8.1 the cytoplasmic enzyme showed only 20% of the activity observed at this pH value in Tris-HCl buffer, whereas the activity of the mitochondrial enzyme was still half of the maximum activity.

The activity of aconitate hydratase measured with *cis*-aconitate (Fig. 3) and isocitrate as substrates was inhibited by bicarbonate to a smaller extent: at pH 8.1 the activity of the cytoplasmic enzyme decreased, respectively, to 60 and 40%, while the activity of the mitochondrial aconitate hydratase was not inhibited at this pH value.

The results presented prove that the effect of bicarbonate buffer depended on changes in bicarbonate concentration and not on changes in pCO_2 value. This is in agreement with the data presented in Fig. 4, illustrating the effect of pH and bicarbonate on the activity of cytoplasmic aconitate hydratase at two concentrations of citrate. The pH values of the buffer depended, at a fixed CO_2 pressure, on concentration of bicarbonate ion (Fig. 4A). As it may be seen, the reaction with 4 mM-citrate was inhibited to a much smaller extent than that with 2 mM-citrate. One should also remember that the increase in pH and concentration of bicarbonate ion was accompanied by an increase in concentration of carbonate ion (CO_3^{2-}). It seems, however, that neither carbon dioxide nor carbonic acid affected the enzymatic activity. The increase in CO_2 pressure even prevented inhibition by 40 mM-bicarbonate with 2 mM-citrate (Fig. 4B). This could be due to decreased concentration of carbonate resulting from the lowered pH of the medium. Thus, it cannot be excluded that both bicarbonate and carbonate ions inhibit aconitate hydratase.

Preincubation of cytoplasmic and mitochondrial aconitate hydratase for 1 min in 50 mM-Tris-HCl buffer, pH 7.4, had practically no effect on the enzyme activity, whereas on 1 min preincubation in 20 mM-bicarbonate buffer, pH 7.4, the cyto-

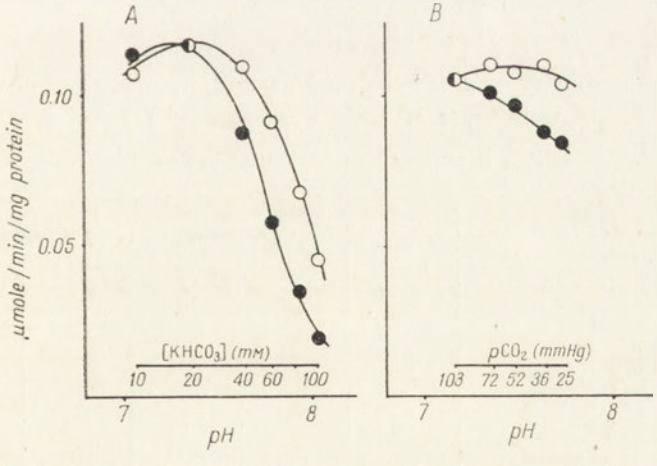

Fig. 4. Effect of pH and varying concentrations of bicarbonate ion (*A*) and CO_2 pressure (*B*) on the activity of cytoplasmic aconitate hydratase. Citrate concentration: ●, 2 mM; ○, 4 mM.

plasmic enzyme retained about 70%, and the mitochondrial one, about 30% of the initial activity. Preincubation prolonged to 3 min decreased the activity of either enzyme to about 20%.

Mechanism of action of bicarbonate on the aconitate hydratase activity. V_{max} of the reaction catalysed by cytoplasmic aconitate hydratase with citrate as a substrate was practically the same in 50 mM-Tris-HCl buffer, 30 mM-phosphate buffer or 30 mM-bicarbonate buffer (Table 2); the differences found were statistically insignificant. The maximum rate of the reaction was not affected by the increase in bicarbonate buffer concentration (Fig. 5A).

The K_m value for citrate in 50 mM-Tris-HCl buffer was 0.34 mM (Table 2), in 30 mM-phosphate buffer 0.81 mM, and in 30 mM-bicarbonate buffer, 1.63 mM. A further increase in bicarbonate buffer concentration to 60 mM led to an increase in K_m to 5.1 mM.

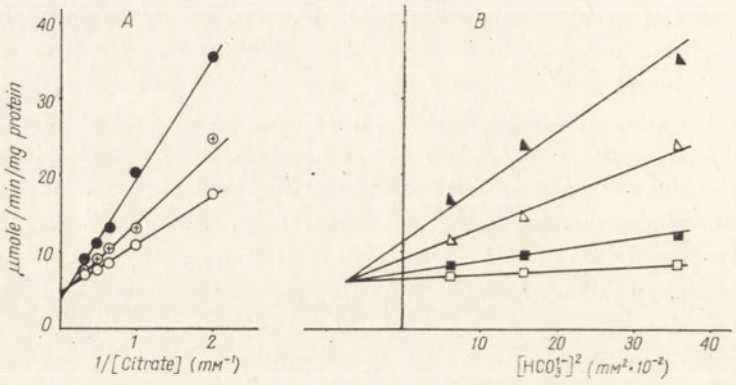

Fig. 5. Inhibition of cytoplasmic aconitate hydratase by bicarbonate buffer, pH 7.5. *A*, Lineweaver-Burk plots; bicarbonate buffer concentration: ○, 25 mM; ⊕, 40 mM; ●, 60 mM. *B*, Dixon plots; citrate concentration: ▲, 0.5 mM; △, 1 mM; ■, 1.5 mM; □, 3 mM.

Table 2

V_{max} and K_m values for cytoplasmic and mitochondrial aconitate hydratase with citrate as a substrate in various buffers of pH 7.4 - 7.5

V_{max} is expressed as nmoles/mg protein/min.

Buffer	Conc. (mM)	Enzyme			
		cytoplasmic		mitochondrial	
		V_{max}	K_m (mM)	V_{max}	K_m (mM)
Tris-HCl	50	96	0.34	378	0.81
Phosphate	30	104	0.81	—	—
Bicarbonate	20	90	1.05	414	0.81
	30	98	1.63	354	1.20
	40	108	2.70	375	1.38
	60	122	5.10	330	2.20

Somewhat different values were obtained for the mitochondrial enzyme. K_m in 50 mM-Tris-HCl buffer was 0.81 mM and in 30 mM-bicarbonate buffer, 1.20 mM. On increasing bicarbonate concentration to 60 mM, the K_m value increased to 2.2 mM.

These results point to a competitive, with respect to citrate, type of inhibition of aconitate hydratase by bicarbonate.

The kind of buffer had no effect on the cooperativity coefficient n (Atkinson, 1966), which varied from 0.95 to 1.13. This indicates that aconitate hydratase does not show the characteristics of an allosteric enzyme.

The K_i was found to be 27 and 38 mM for the cytoplasmic and mitochondrial enzyme, respectively (Fig. 5). K_m values for citrate were also linearly related to the square of bicarbonate concentration (Fig. 6); this suggests binding of bicarbonate ion to two active sites of the enzyme. The kinetics of this reaction follows the equation of Zeylemaker *et al.* (1970):

$$K_m\, i = K_m \left(1 + \frac{I}{K_i}\right)^2$$

Where $K_m\, i$ is the K_m in the presence of a defined concentration of the inhibitor, I. The K_m value for citrate calculated from this equation is 0.41 mM for the cytoplasmic, and 0.36 mM for the mitochondrial aconitate hydratase. The values obtained are closely similar to those obtained in Tris-HCl buffer.

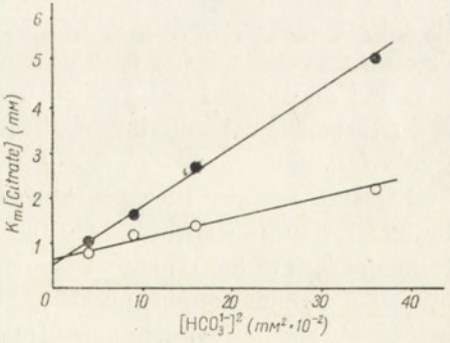

Fig. 6. Effect of concentration of bicarbonate buffer on K_m values with citrate as a substrate for the cytoplasmic (●) and mitochondrial (○) aconitate hydratase.

Effect of Mg^{2+}, Mn^{2+} *and* Ca^{2+} *on the aconitate hydratase activity.* Divalent cations inhibited competitively (with respect to citrate) the enzyme activity measured by conversion of citrate to *cis*-aconitate (Fig. 7A). However, when the correction was introduced for the concentration of citrate-metal^{1-} complexes, the double reciprocal plot of the reaction rate *versus* citrate anion concentration

(Fig. 7B) gave a single curve. These results confirm the observations of Eanes & Kun (1974) that the completely dissociated citrate anion serves as substrate for aconitate hydratase.

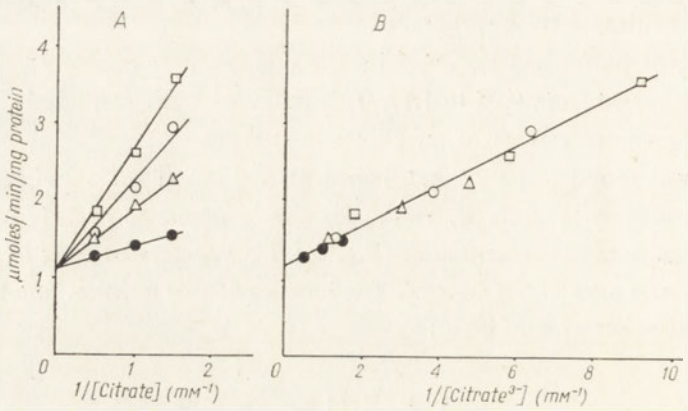

Fig. 7. Lineweaver-Burk plots for the effect of divalent cations on the activity of cytoplasmic aconitate hydratase. The activity was measured by conversion of citrate to *cis*-aconitate and expressed as a function: *A*, of citrate concentration, *B*, of citrate anion concentration. ●, Control; and in the presence of: ○, 2 mM-$MgCl_2$; □, 2 mM-$MnCl_2$; △, 2 mM-$CaCl_2$.

DISCUSSION

The results presented confirm the earlier observations of Simpson (1967), Adler *et al.* (1971) and Simpson & Angielski (1973) that, under physiological conditions, the intracellular metabolism of citrate is regulated by changes in pH and bicarbonate concentration. It has been demonstrated that synthesis of citrate from pyruvate and malate in kidney cortex mitochondria is stimulated on increasing pH and bicarbonate concentration, with simultaneous reduction of isocitrate to citrate conversion.

Evidence has also been provided that direct inhibition of aconitate hydratase by bicarbonate is responsible for the lower rate of citrate metabolism at higher concentrations of bicarbonate. Since 1954 it is known that the activity of aconitate hydratase depends not only on pH but also on the kind and concentration of buffer in the assay medium (Morrison, 1954; Dickman & Speyer, 1954). Our results obtained with Tris-HCl, phosphate and bicarbonate buffers are in agreement with these earlier observations: differences in the activity of aconitate hydratase from rat kidney mitochondria were found to be due to different affinities of the enzyme toward substrate in various buffers. These differences were manifested in K_m values, irrespective which direction of the reaction catalysed by the hydratase was studied. V_{max} values remained unchanged.

The K_m value for the enzyme from kidney cortex with citrate as a substrate, calculated from the results of our experiments, was in Tris-HCl practically the

same as that calculated from the equation of Zeylemaker *et al.* (1970), and corresponded closely to those reported for pig liver (Eanes & Kun, 1974), rat liver (Brand *et al.*, 1973), ox liver (Henson & Cleland, 1967) and pig heart (Villafranca & Mildvan, 1971).

Bicarbonate inhibited to a greater extent the cytoplasmic than the mitochondrial aconitate hydratase, shifting at the same time the pH optimum of either enzyme to lower values. With both enzymes, the inhibitory effect of bicarbonate was more pronounced on citrate conversion than when *cis*-aconitate or isocitrate were used as substrates for aconitate hydratase. Our results suggest that bicarbonate acts as a competitive inhibitor, as the extent of inhibition depended on concentration of citrate, and the inhibition could be reversed by the addition of citrate to the reaction mixture. These observations are consistent with the results of Balagura-Baruch *et al.* (1973a) who demonstrated that on intravenous administration of citrate, consumption of this compound in kidney increased rapidly. Moreover, preincubation of kidney cortex mitochondria with tricarballylic acid protected aconitate hydratase against inhibition by bicarbonate (McDaniel & Longmore, 1971).

Our results suggest also that bicarbonate ion binds with the kidney cortex enzyme at two different active sites. This type of active centre in the molecule of aconitate hydratase was postulated by Villafranca & Mildvan (1971) for the enzyme from pig heart. The mechanism of action of bicarbonate ion is thought to consist in blocking the cation group which is responsible for binding the carboxyl group of the substrate (Zeylemaker *et al.*, 1970). According to Glusker (1968) and Villafranca & Mildvan (1972) the activity of aconitate hydratase is based on participation of such a cation group in the active centre of the enzymatic protein.

Inhibition of the cytoplasmic and mitochondrial aconitate hydratase by bicarbonate is evidenced by the K_i values 27 and 38 mM, respectively. These concentrations, although high, do not exceed the concentration which may be expected to occur *in vivo*.

The level of bicarbonate in cytoplasm of kidney cell is estimated to be 15 - 20 mM under normal CO_2 pressure (about 40 mm Hg) and pH 7.32 (Struyvenberg *et al.*, 1968). At this concentration, partial inhibition of cytoplasmic aconitate hydratase could be expected. An increase in pH to 7.5, and that in bicarbonate concentration up to 40 mM — which occurs in metabolic alkalosis — might exert a profound effect on the activity of the enzyme.

It is difficult to evaluate concentration of hydrogen ion and bicarbonate in the mitochondrial matrix *in vivo*. It is believed that both values are higher than in cytoplasm (Waddell & Bates, 1969). On the other hand, one should remember that the activity of the mitochondrial enzyme is affected by bicarbonate to a smaller extent than that of the cytoplasmic enzyme.

Concentration of citrate in the cytoplasm of kidney cell is about 0.15 mM (Angielski, 1971). However, the level of free citrate anion would be much lower. Assuming that magnesium alone forms complexes with about 70% of the total citrate present in kidney (Veloso *et al.*, 1973), concentration of citrate anion would not exceed 0.05 mM. On the other hand, in the presence of 20 mM-bicarbonate, the

K_m value for the cytoplasmic aconitate hydratase, calculated with respect to free citrate anion, is 0.32 mM. A comparison of these two values would indicate that the activity of the cytoplasmic enzyme *in vivo* does not exceed about 10% of the maximum activity.

On the other hand, the increase in pH and bicarbonate concentration, observed under conditions of metabolic alkalosis, should lead to the increased synthesis of citrate in mitochondria (Stern *et al.*, 1952; and the present paper), and reduction of its further metabolism (Simpson, 1967; and the present paper). This would, in turn, increase transport of citrate from mitochondria to cytoplasm (Simpson & Angielski, 1973). Thus it seems that the increase in citrate concentration in renal tissue, observed in alkalosis (Crawford, 1963; Adler *et al.*, 1971) concerns mainly the cytoplasmic compartment. The increase in citrate concentration in cytoplasm could partially reverse inhibition of cytoplasmic aconitate hydratase by bicarbonate. In this way, a part of citrate could be converted to 2-oxoglutarate. The increase in the intracellular concentration of this compound in kidney, and its increased urinary excretion in alkalosis (Crawford, 1963) suggest inhibition of a further step in oxidative metabolism, possibly of the activity of succinate dehydrogenase (EC 1.3.99.1). In view of this, it should be remembered that bicarbonate is a strong ($K_i = 12$ mM) inhibitor of this enzyme, competitive with respect to succinate (Zeylemaker *et al.*, 1970).

The results presented prove that the activity of aconitate hydratase in kidney may be regulated to a large extent by physiological concentrations of bicarbonate ion, citrate, and divalent cations, and may respond *in vivo* to any disturbance in the acid-base equilibrium.

REFERENCES

Adler, S., Anderson, B. & Zemotel, L. (1971) *Am. J. Physiol.*, **220**, 986 - 992.
Angielski, S. (1971) *Monografie Biochemiczne*, **24**, 29 - 45.
Atkinson, D. E. (1966) *Ann. Rev. Biochem.*, **35**, 85 - 124.
Balagura-Baruch, S., Burich, L. & King, V. F. (1973a) *Am. J. Physiol.*, **225**, 385 - 388.
Balagura-Baruch, S., Burich, L. & King, V. F. (1973b) *Am. J. Physiol.*, **225**, 389 - 393.
Bergmeyer, H. U. (1965) in *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) pp. 313 - 323. Verlag Chemie, Weinheim - Academic Press, New York.
Brand, M. D., Evans, S. M., Mendes-Mourao, J. & Chappell, J. B. (1973) *Biochem. J.*, **134**, 217 - 224.
Crawford, M. A. (1963) *Biochem. J.*, **88**, 115 - 120.
Dickman, S. R. & Speyer, J. F. (1954) *J. Biol. Chem.*, **206**, 67 - 75.
Eanes, R. Z. & Kun, E. (1974) *Mol. Pharmacol.*, **10**, 130 - 139.
Glusker, J. P. (1968) *J. Mol. Biol.*, **38**, 149 - 162.
Goldberg, N. D., Passonneau, J. V. & Lowry, O. H. (1966) *J. Biol. Chem.*, **241**, 3997 - 4003.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.*, **177**, 751 - 766.
Harris, E. J. & van Dam, K. (1968) *Biochem. J.*, **106**, 759 - 766.
Henson, C. P. & Cleland, W. W. (1967) *J. Biol. Chem.*, **242**, 3833 - 3838.
Horne, R. N. & Nordlie, R. C. (1971) *Biochim. Biophys. Acta*, **242**, 1 - 13.
Johnson, D. & Lardy, H. (1967) in *Methods in Enzymology* (R. Estabrook & M. E. Pullman, eds.) vol. 10, pp. 94 - 96. Academic Press, New York.

Łysiak, W., Pieńkowska-Vogel, M., Szutowicz, A. & Angielski, S. (1975) *Acta Biochim. Polon.*, **22**, 211 - 220.
McDaniel, M. L. & Longmore, W. J. (1971) *J. Biol. Chem.*, **246**, 4818 - 4824.
Meyer, J. L. (1974) *Anal. Biochem.*, **62**, 295 - 300.
Morrison, J. F. (1954) *Aust. J. Exp. Biol. Med. Sci.*, **32**, 877 - 883.
Robinson, B. H. & Chappell, J. B. (1970) *Biochim. Biophys. Acta*, **220**, 569 - 573.
Sillen, L. G. & Martell, A. E. (1971) *Stability Constants of Metal-Ion Complexes* (Special Publication No. 25) Supplement No. 1 to Special Publication No. 17, 2nd ed.; Chemical Society, London.
Simpson, D. P. (1967) *J. Clin. Invest.*, **46**, 225 - 238.
Simpson, D. P. & Angielski, S. (1973) *Biochim. Biophys. Acta*, **298**, 115 - 123.
Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967) in *Methods in Enzymology* (R. Estabrook & M. E. Pullman, eds.) vol. 10, pp. 448 - 463. Academic Press, New York.
Stern, J. R., Ochoa, S. & Lynen, F. (1952) *J. Biol. Chem.*, **198**, 313 - 321.
Stępiński, J. & Angielski, S. (1975) in *Biochemical Aspects of Renal Function* (S. Angielski & U. C. Dubach, eds.) pp. 65 - 69. Hans Huber Publ., Bern - Stuttgart - Vienna.
Struyvenberg, H., Morrison, R. B. & Relman, A. S. (1968) *Am. J. Physiol.*, **214**, 1155 - 1162.
Veloso, D., Guynn, R. W., Oscarsson, M. & Veech, R. L. (1973) *J. Biol. Chem.*, **248**, 4811 - - 4819.
Villafranca, J. J. & Mildvan, A. S. (1971) *J. Biol. Chem.*, **246**, 5791 - 5798.
Villafranca, J. J. & Mildvan, A. S. (1972) *J. Biol. Chem.*, **247**, 3454 - 3463.
Waddell, W. J. & Bates, R. G. (1969) *Physiol. Rev.*, **49**, 285 - 329.
Zeylemaker, W. P., Klaasse, A.D.M., Slater, E. C. & Veeger, C. (1970) *Biochim. Biophys. Acta*, **198**, 415 - 422.

REGULACJA AKTYWNOŚCI HYDRATAZY AKONITANOWEJ Z KORY NERKI SZCZURA PRZEZ DWUWĘGLAN

Streszczenie

1. W mitochondriach kory nerki szczura wzrost pH i stężenia dwuwęglanu stymuluje syntezę cytrynianu z pirogronianu i jabłczanu, hamując jednocześnie przemianę izocytrynianu do cytrynianu.

2. Dwuwęglan jest kompetycyjnym w stosunku do cytrynianu inhibitorem hydratazy akonitanu (EC 4.2.1.3), wiążąc się prawdopodobnie z dwoma miejscami aktywnymi enzymu. Wartości K_i dla cytoplazmatycznej i mitochondrialnej hydratazy akonitanu wynosiły odpowiednio 27 i 38 mM. Optimum pH obu enzymów w buforze Tris-HCl występuje pomiędzy 7,8 a 8,6, natomiast w buforze dwuwęglanowym waha się od 7,2 do 8,0, zależnie od formy enzymu oraz użytego substratu reakcji.

3. Substratem hydratazy akonitanu był tylko wolny, całkowicie zdysocjowany anion cytrynianowy.

4. Dyskutowana jest rola hydratazy akonitanu jako czynnika kontrolującego szybkość przemiany cytrynianu w nerce, szczególnie w stanach alkalozy metabolicznej.

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GLUTAMATE METABOLISM IN RAT KIDNEY MITOCHONDRIA*

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1. Kidney cortex mitochondria did not swell in ammonium or potassium salts of glutamate even in the presence of valinomycin or 2,4-dinitrophenol. Aminooxyacetate diminished the reduction of nicotinamide nucleotides in intact mitochondria in the presence of glutamate.

2. Transamination with oxaloacetate appeared to be the main pathway of glutamate metabolism in isolated rat kidney cortex mitochondria under various metabolic conditions. Ammonia formation was negligible. The γ-aminobutyrate pathway was found to be of almost no importance.

It is known from studies *in vivo* that plasma glutamine contributes in about 90% to renal ammoniagenesis. About two-thirds of urinary ammonia comes from amide nitrogen of glutamine, whereas amine nitrogen of glutamine is a source of additional one-third of ammonia excreted in the urine (Pitts *et al.*, 1963; Lotspeich, 1967). It is, therefore, generally assumed that glutamate dehydrogenase plays an important role in renal ammoniagenesis (Goldstein & Schooler, 1967; Preuss, 1969; Hems & Brosnan, 1971; Simpson, 1972). It is known, however, that even in liver, the tissue showing the highest activity of glutamate dehydrogenase (Williamson *et al.*, 1967), glutamate is metabolized mainly by transamination with oxaloacetate (Borst & Slater, 1960; Krebs & Bellamy, 1960; De Haan *et al.*, 1967). The present work was performed to elucidate contribution of various pathways in glutamate metabolism by intact rat kidney cortex mitochondria.

MATERIALS AND METHODS

Wistar rats weighing 180 - 250 g were stunned and killed by decapitation. The kidneys were quickly removed and demedullated. Mitochondria from kidney cortex were prepared as described by Klingenberg & Slenczka (1959). Only mitochondria

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showing good respiratory control and the appropriate ADP/O ratio were used for the experiments.

Measurement of respiratory control and ADP/O ratio. Mitochondria (about 3 mg of protein) were incubated in a medium containing 16 mM-Tris-HCl buffer, pH 7.4, 125 mM-KCl, 5 mM-K_2HPO_4, 1 mM-EDTA, 5 mM-2-oxoglutarate or succinate und 0.175 mM-ADP in a final volume of 2.5 ml. Oxygen was measured polarographically at 25°C using the Radiometer instrument provided with water-jacketed chamber and magnetic stirrer.

Conditions of incubation. Mitochondria (4.8 - 6.5 mg of protein) were incubated in 25 ml Erlenmayer flask in metabolic bath-shaker at 30°C with 6.5 ml of one of the two following media. The standard Tris-phosphate medium contained: 130 mM-KCl, 18 mM-Tris-HCl, 10 mM-K_2HPO_4, 1 mM-EDTA and 10 mM-glucose (pH 7.4). The bicarbonate-phosphate medium contained: 130 mM-KCl, 10 mM-K_2HPO_4, 23 mM-$KHCO_3$, 10 mM-glucose, 4.8 mM-$MgCl_2$, 1 mM-EDTA, 0.4 mM-ADP and 5 units/ml of hexokinase; the medium was equilibrated with a gas mixture (CO_2:N_2:O_2, 5:76:19, by vol.) at 30°C for 30 min to obtain pH 7.4.

Different respiratory states of mitochondria in Tris-phosphate medium were produced by the following additions: a) uncoupled state: 0.15 mM-2,4-dinitrophenol (DNP); b) oligomycin-inhibited state: 4.8 mM-$MgCl_2$, 0.4 mM-ADP, 5 units/ml of yeast hexokinase (Sigma type III) and 1 µg/ml of oligomycin.

At time intervals indicated, 0.5 ml aliquots of the mitochondrial suspension were withdrawn and added to 0.05 ml of $HClO_4$ (33%) for the assay of metabolic intermediates, or to 0.05 ml of trichloroacetic acid (50%) for NH_4^+ determination. The protein pellet was centrifuged off, the $HClO_4$ supernatant was neutralized to pH 6.5 with 6 M-K_2HPO_4 and recentrifuged to remove $KClO_4$.

Analytical methods. Oxygen was measured polarographically using Clark type oxygen electrode. Reduction of intramitochondrial $NAD(P)^+$ was measured by recording changes in fluorescence of mitochondrial suspension. The assay was performed in the cuvette containing in a final volume of 2 ml: 16 mM-Tris-HCl buffer, pH 7.4, 5 mM-K_2HPO_4, 150 mM-sucrose, 10 mM-KCl, 3 mM-$MgCl_2$, 0.05 mM-EDTA and mitochondrial suspension (1.1 mg of protein). The mitochondria were first depleted of endogenous substrates by treatment with 0.05 mM-DNP for 10 - 15 min at 25°C, then the respiration was blocked with antimycin (2 µg) and 3 min later the substrate (5 mM) was added; 0.2 mM-aminooxyacetate was added just after antimycin addition. The measurements were carried out on Eppendorf photometer with fluorimetric equipment at 313 - 366 and 430 - 470 nm at 25°C.

2-Oxoglutarate and glutamate were determined by the enzymatic methods as described by Bergmeyer & Bernt (1965) and aspartate according to Pfleiderer (1965). NH_4^+ was measured by the method of Okuda *et al.* (1965). Spectrophotometric measurements were carried out with the use of Eppendorf or Vitatron photometer.

γ-Aminobutyrate was estimated as follows: the deproteinized mitochondrial extract (2.0 ml), was introduced to Amberlite IR-120 (100 - 200 mesh) column (0.9 × 9 cm) and washed with 17 ml of water. Amino acids were eluted with

2 M-NH_4OH and evaporated to dryness at 50°C. The residue was dissolved in 0.30 ml of water and a 5 µl sample was submitted to thin-layer chromatography on Cellulose-MN ECTEOLA plate (3.5 × 17 cm). Chromatograms were developed in isopropanol - acetic acid - water (40:2:10, by vol.) solvent system, dried under a gentle stream of hot air and stained overnight with 0.3% solution of ninhydrin in butanol - acetic acid mixture (100:3, v/v) (Arx & Noher, 1963). Amino acids were identified by comparison with the appropriate standards and estimated using a densitometer connected with Vitatron photometer provided with U-12 and U-4 filters.

The protein content of mitochondrial preparation was determined by the biuret reaction (Gornall *et al.*, 1949) with bovine serum albumin as a standard.

Materials. 2-Oxoglutarate was prepared according to Krebs *et al.* (1961). Acetoacetate was prepared from ethylacetoacetate according to Krebs & Eggleston (1945) and standardized by the enzymatic method (Mellanby & Williamson, 1965). γ-Aminobutyrate, succinate and NAD were obtained from Reanal (Hungary); antimycin, oligomycin, aminooxyacetic acid, β-hydroxybutyrate and malate dehydrogenases from Sigma Chem.Co (U.S.A.); glutamate dehydrogenase (in ammonium or glycerol solution), NADH and glutamate-oxaloacetate aminotransferase from Boehringer GmbH (G.F.R.); 2,4-dinitrophenol and Tris from B.D.H. (England). Cellulose for thin-layer chromatography was MN-300 G/ECTEOLA (Marcherey & Nagel Co, England). Other reagents, of analytical grade, were from P.O.Ch. (Gliwice, Poland).

RESULTS

Swelling of rat kidney cortex mitochondria. Figure 1 shows the optical density readings at 546 nm of mitochondrial suspensions in 125 mM-ammonium salts of:

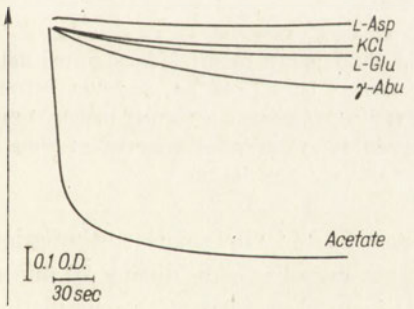

Fig. 1

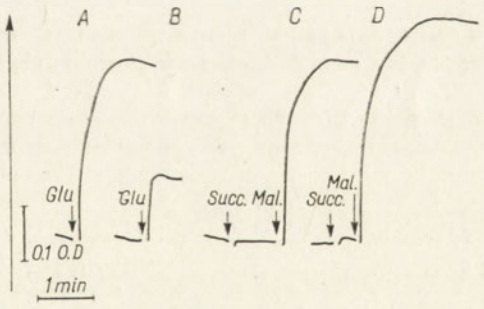

Fig. 2

Fig. 1. Swelling of rat kidney cortex mitochondria. Mitochondria (0.85 mg of protein) were suspended in 2.0 ml of 125 mM-KCl or 125 mM-L-glutamate (L-Glu), L-aspartate (L-Asp), γ-aminobutyrate (γ-Abu) or acetate (ammonium salts) containing 2 µg antimycin and buffered with 5 mM-Tris-HCl to pH 7.4. Optical density was followed at 546 nm.

Fig. 2. Changes in reduction of intramitochondrial NAD(P)$^+$ upon addition of glutamate (*A*, *B*) or succinate *plus* malate (*C*, *D*). Changes in fluorescence were monitored as described in Methods. Aminooxyacetate (0.2 mM) was added to the medium containing glutamate (*B*) and succinate *plus* malate (*D*).

acetate, L-glutamate, L-aspartate or γ-aminobutyrate, buffered to pH 7.4 with 5 mM-Tris-HCl. The mitochondria in glutamate or aspartate solution showed only a slight decrease in optical density, equal to the changes observed in KCl solution. The response in γ-aminobutyrate solution was clearly visible but it was much lower than in the acetate solution. The addition of valinomycin or 2,4-DNP and valinomycin to the K-glutamate medium did not affect the rate of mitochondrial swelling (not shown).

Reduction of intramitochondrial NAD(P)$^+$. The addition of glutamate or malate to the suspension of rat kidney mitochondria in buffered sucrose produced a significant increase in fluorescence indicating a rise in reduction of nicotinamide adenine nucleotides. Succinate was without any effect (Fig. 2 *A*,*C*). Aminooxyacetate, an inhibitor of transaminases (Hopper & Segal, 1962), greatly diminished the effect of glutamate but not that of malate (Fig. 2 *B*,*D*).

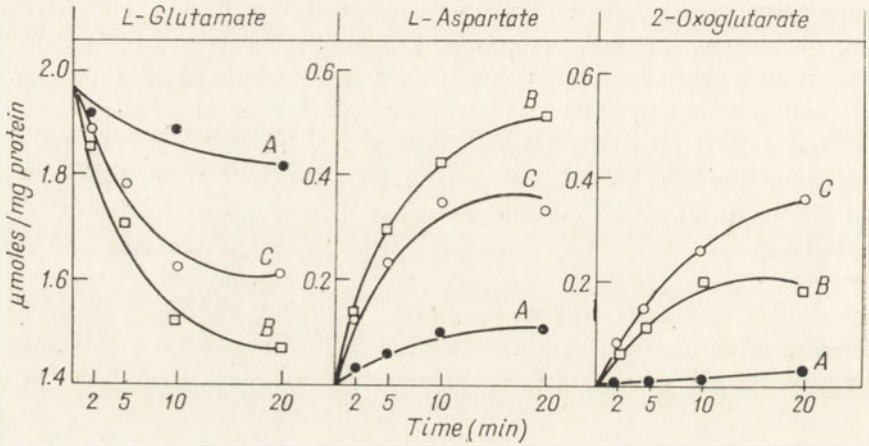

Fig. 3. Effect of malate and acetoacetate on glutamate metabolism by rat kidney cortex mitochondria. Each vessel contained the standard Tris-phosphate buffer, pH 7.4, and alternatively: *A*, L-glutamate (2 mM): *B*, glutamate (2 mM) and malate (1 mM); *C*, glutamate (2 mM), malate (1 mM) and acetoacetate (0.5 mM). Reactions were started by addition of mitochondria (1.0 - 1.3 mg of protein/ml). The values are the means of four experiments.

Utilization of glutamate by kidney cortex mitochondria. Glutamate consumption, as shown in Fig. 3, did not exceed 0.1 μmole per mg of protein during 20 min of incubation and was accounted for, quantitatively, by aspartate accumulation. Addition of malate increased almost fivefold both glutamate utilization and aspartate formation. Significant accumulation of 2-oxoglutarate was also observed. Addition of acetoacetate, as a source of acetyl-CoA, together with malate clearly depressed glutamate utilization and aspartate formation, and enhanced 2-oxoglutarate accumulation, as compared with malate alone.

Uncoupling of kidney cortex mitochondria with DNP increased slightly glutamate consumption, which was accompanied by increased accumulation of aspartate and 2-oxoglutarate (Fig. 4). Oligomycin, on the other hand, clearly diminished

glutamate utilization and 2-oxoglutarate accumulation. Aspartate formation, however, was increased almost twofold as compared with the control value. Similar effects of oligomycin on glutamate metabolism in the presence of malate

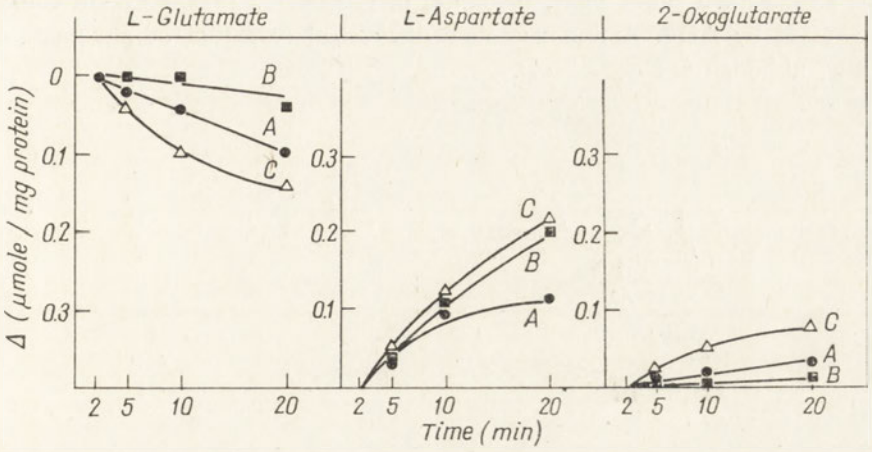

Fig. 4. Effect of oligomycin and 2,4-dinitrophenol on glutamate metabolism by rat kidney cortex mitochondria. Experimental conditions and the basic incubation medium containing 2 mM-glutamate were as in Fig. 3. Other additions: *A*, none (control); *B*, 4.8 mM-$MgCl_2$, 0.4 mM-ADP, 5 u./ml hexokinase and 1 μg/ml of oligomycin; *C*, 0.15 mM-DNP.

and acetoacetate are seen in Fig. 5. In both above experiments the amount of aspartate formed exceeded twice the amount of glutamate consumed. This effect as well as substantial decrease in 2-oxoglutarate accumulation caused by oligomycin

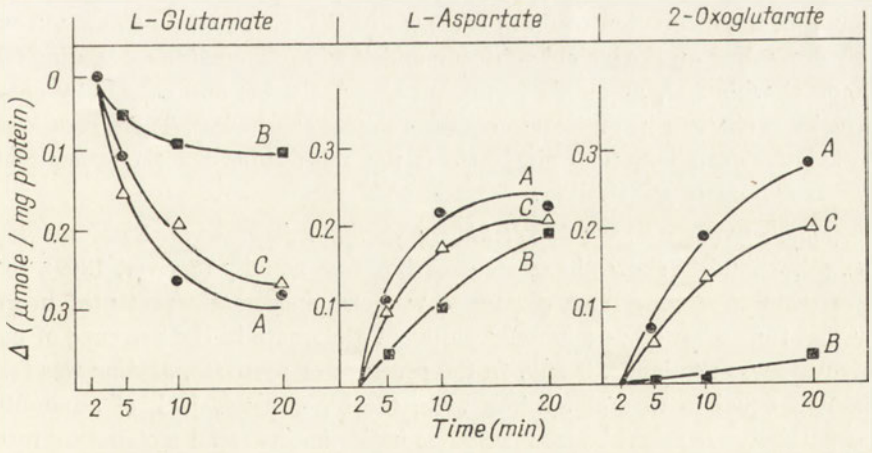

Fig. 5. Effect of oligomycin and 2,4-dinitrophenol on metabolism of glutamate (2 mM), in the presence of malate (1 mM) and acetoacetate (0.5 mM). Other conditions and designations *A*, *B*, *C* as in Fig. 4.

can be explained by anaerobic dismutation of 2-oxoglutarate to glutamate. The concentration of endogenous ammonia reached in these samples 0.2 mM.

Oxidation of glutamate. Tracing of respiration of kidney cortex mitochondria (Fig. 6) shows that arsenite, an effective inhibitor of 2-oxoglutarate oxidation, inhibited also, with a small delay, oxidation of glutamate. The inhibition could not be reversed by ADP. Aminooxyacetate diminished oxidation of glutamate significantly, although not completely.

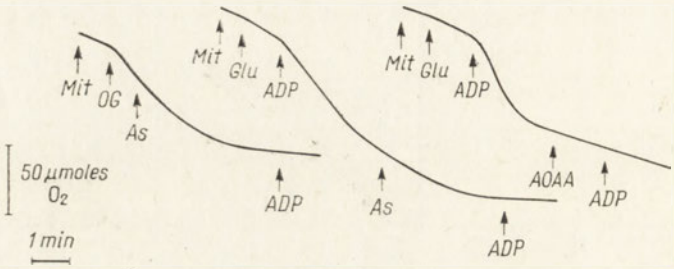

Fig. 6. Polarographic records of mitochondrial respiration with glutamate. Rat kidney cortex mitochondria were incubated in a medium containing 16 mM-Tris-KCl buffer, pH 7.4, 5 mM-K_2HPO_4, 125 mM-KCl, 1 mM-EDTA in a final volume of 2.0 ml. To 1.1 mg of mitochondrial protein (*Mit*), 5 mM-2-oxoglutarate (*OG*), 5 mM-glutamate, 0.175 mM-ADP, 1 mM-arsenite and 0.2 mM-aminooxyacetate (*AOAA*) were added as indicated.

Ammonia formation from glutamate. Mitochondria of rat kidney cortex in active metabolic state induced by ADP and phosphate acceptor were incubated with glutamate in bicarbonate-phosphate medium. Accumulation of aspartate and ammonia in the incubation medium is shown in Fig. 7. Aspartate formation varied greatly depending on the second substrate added with glutamate as a sparing factor. In contrast with that, ammonia formation was negligible and essentially constant despite large changes in glutamate consumption (non shown). These results prove that oxidative deamination of glutamate is not responsible for the severalfold increase in glutamate metabolism.

γ-Aminobutyrate pathway in rat kidney cortex mitochondria. Since rat kidney cortex mitochondria show glutamate decarboxylase activity (Scriver, 1969), formation of γ-aminobutyrate from glutamate was examined. Data presented in Fig. 8 show that formation of γ-aminobutyrate from glutamate in the presence of malate or pyruvate was negligible, if any. In the presence of pyruvate, alanine was formed in amount equal to that of aspartate. On the other hand, lack of accumulation of γ-aminobutyrate might be interpreted as indicating its rapid metabolism through the γ-aminobutyrate transaminase and succinic semialdehyde dehydrogenase pathway. It was shown, however, that rat kidney mitochondria do not respire in the presence of γ-aminobutyrate (Fig. 9) despite their permeability to this substrate.

These data differ from the results of Lancaster *et al.* (1973) suggesting that about one-fourth of glutamate disposal in rat kidney cortex slices may be accounted for by the γ-aminobutyrate pathway.

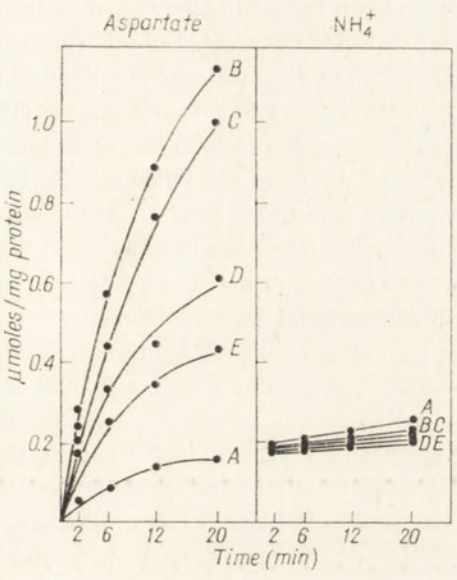

Fig. 7

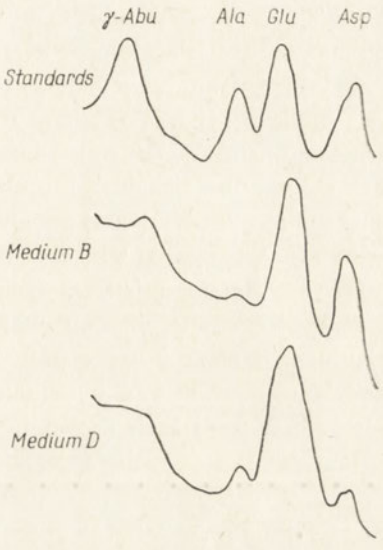

Fig. 8

Fig. 7. Aspartate and ammonia formation from glutamate by kidney cortex mitochondria. Mitochondria (0.75 - 1.0 mg of protein/ml) were incubated in bicarbonate-phosphate medium. The reaction was started by addition of mitochondria. Other additions: *A*, none; *B*, 0.75 mM-malate; *C*, 0.75 mM-malate *plus* 0.3 mM-acetoacetate; *D*, 0.75 mM-pyruvate; *E*, 0.75 mM-citrate. Values are means from 4 - 5 experiments.

Fig. 8. Densitometric tracings of thin-layer chromatograms of glutamate metabolites. The incubation was performed as described for Fig. 7, and samples from media *B* and *D* were deproteinized, chromatographed and recorded as described in Methods.

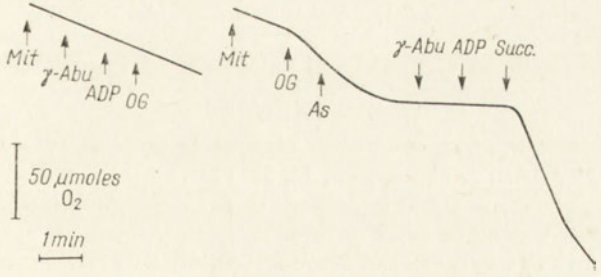

Fig. 9. Mitochondrial respiration in the presence of γ-aminobutyrate. The incubation medium and experimental conditions were as described in Methods and Fig. 6, except that 2-oxoglutarate concentration was 10 μM.

DISCUSSION

The data presented indicate that glutamate dehydrogenase plays a negligible role in glutamate metabolism in isolated rat kidney cortex mitochondria. Under various metabolic conditions, aspartate appears to be the sole product of glutamate metabolism. The rate of this process greatly depends on the availability of oxaloacetate. Ammonia production is almost nil and does not respond to great changes in glutamate metabolism. On the other hand, inhibition of glutamate-oxaloacetate aminotransferase markedly reduces glutamate consumption. According to Kovaćević (1971) the predominance of the transamination pathway in the metabolism of exogenous glutamate may be explained by restricted permeability of the inner mitochondrial membrane to glutamate, and possibly by a more favourable location of the aminotransferase as compared with that of the dehydrogenase. This interpretation would suggest that glutamate is transported into mitochondrial matrix as 2-oxoglutarate, the product of transamination with intramitochondrial oxaloacetate.

Another possible interpretation is that glutamate enters rat kidney cortex mitochondria through the anion-anion exchange mechanism. Rat brain and heart mitochondria have been shown to transport glutamate across the mitochondrial membrane in exchange for aspartate (Chappell, 1968; Brandt & Chappell, 1974). If this is so, then the data presented provide evidence that direct oxidation of glutamate is of little importance in glutamate metabolism in kidney cortex mitochondria. It seems also that under appropriate conditions (high redox state of the mitochondrial nicotinamide nucleotides) glutamate dehydrogenase might be involved in synthesis rather than in oxidation of glutamate.

If ammonia is not formed from glutamate in kidney cortex, one may assume that glutamate-oxaloacetate transamination is coupled with the purine nucleotide cycle (Setlow *et al.*, 1966). Thus AMP would be a source of urinary ammonia, and reamination of IMP by aspartate would then direct the glutamate nitrogen into this pathway.

The authors wish to express their gratitude to Prof. Dr. S. Angielski for his interest, advice and helpful discussion. Skilful technical assistance of Miss Ewa Major is gratefully acknowledged.

REFERENCES

Arx, E. & Noher, R. (1963) *J. Chromatogr.*, **12**, 329 - 341.
Bergmeyer, H. U. & Bernt, E. (1965) in *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer) pp. 315 and 384. Verlag Chemie, Weinheim.
Borst, P. & Slater, E. C. (1960) *Biochim. Biophys. Acta*, **41**, 170 - 177.
Chappell, J. B. (1968) *Brit. Med. Bull.*, **24**, 150 - 157.
Brandt, M. D. & Chappell, J. B. (1974) *Biochem. J.*, **140**, 205 - 210.
De Haan, E. J., Tager, J. M. & Slater, E. C. (1967) *Biochim. Biophys. Acta*, **131**, 1 - 13.
Goldstein, L. & Schooler, J. M. (1967) *Adv. Enzyme Regul.*, **5**, 71 - 86.

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.*, **177**, 751 - 766.
Hems, D. A. & Brosnan, J. T. (1971) *Biochem. J.*, **123**, 391 - 397.
Hopper, S. & Segal, H. L. (1962) *J. Biol. Chem.*, **237**, 3189 - 3195.
Klingenberg, M. & Slenczka, W. (1959) *Biochem. Z.*, **331**, 486 - 517.
Kovaćević, Z. (1971) *Biochem. J.*, **125**, 757 - 763.
Krebs, H. A. & Eggleston, L. H. (1945) *Biochem. J.*, **39**, 408 - 419.
Krebs, H. A. & Bellamy, D. (1960) *Biochem. J.*, **75**, 523 - 529.
Krebs., H. A., Eggleston, K. V. & D'Alessandro, A. (1961) *Biochem. J.*, **79**, 537 - 549.
Lancaster, G., Mohyddin, F., Scriver, Ch. S. & Whelan, D. T. (1973) *Biochim. Biophys. Acta*, **293**, 229 - 240.
Lotspeich, W. D. (1967) *Science*, **155**, 1066 - 1075.
Mellanby, J. & Williamson, D. H. (1965) in *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer) pp. 454 - 458. Verlag Chemie, Weinheim.
Okuda, H., Fujii, S. & Kawashiwa, Y. (1965) *Tokushima J. Exp. Med.*, **12**, 11 - 23.
Pfleiderer, G. (1965) in *Methods of Enzymatic Analysis* (ed. H. U. Bergmeyer) pp. 381 - 383. Verlag Chemie, Weinheim.
Pitts, R. F., de Haas, J. & Klein, J. (1963) *Am. J. Physiol.*, **204**, 187 - 191.
Preuss, H. G. (1969) *Nephron*, **6**, 235 - 241.
Scriver, Ch. S. (1969) *Ann. N.Y. Acad. Sci.*, **166**, 83 - 96.
Setlow, B., Burger, R. & Lowenstein, J. M. (1966) *J. Biol. Chem.*, **241**, 1244 - 1245.
Simpson D. P. (1972) *J. Clin. Invest.*, **51**, 1969 - 1978.
Williamson, D. H., Lund, P. & Krebs, H. A. (1967) *Biochem. J.*, **103**, 515 - 527.

METABOLIZM GLUTAMINIANU W MITOCHONDRIACH NERKI SZCZURA

Streszczenie

1. Mitochondria kory nerki szczura nie pęcznieją w soli amonowej ani potasowej glutaminianu nawet w obecności walinomycyny lub 2,4-DNP. Aminooksyoctan obniża stopień redukcji nukleotydów nikotynamido-adeninowych w nieuszkodzonych mitochondriach w obecności glutaminianu.

2. Transaminacja przy udziale szczawiooctanu jest główną drogą przemiany glutaminianu w mitochondriach kory nerki w różnych stanach metabolicznych. Synteza amoniaku jest znikoma i nie wiąże się z dużymi zmianami zachodzącymi w metabolizmie glutaminianu. Przemiana glutaminianu poprzez γ-aminomaślan jest znikoma.

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EFFECT OF (—)HYDROXYCITRATE ON THE ACTIVITIES OF ATP CITRATE LYASE AND THE ENZYMES OF ACETYL-CoA METABOLISM IN RAT BRAIN

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1. (—)Hydroxycitrate is a potent inhibitor of ATP citrate (*pro*-3S)lyase (EC 4.1.3.8) from rat brain, the inhibition being uncompetitive with respect to $MgATP^{2-}$ and competitive with citrate (K_i 0.8 μM).

2. The rate of oxygen consumption by rat brain synaptosomes and the activities of fatty acid synthetase, carnitine acetyltransferase, glucose-6-phosphate dehydrogenase and acetyl-CoA-synthetase are not affected by (—)hydroxycitrate.

3. (—)Hydroxycitrate inhibits the activities of isocitrate dehydrogenase, malate dehydrogenase (decarboxylating) and aconitate hydratase at millimolar concentrations.

The regulatory role of ATP citrate (*pro*-3S)lyase (EC 4.1.3.8) in nervous system is still controversial. During development of this system, changes in the activity of citrate lyase are parallel to those in the rate of fatty acid synthesis (D'Adamo & D'Adamo, 1968; Szutowicz *et al.*, 1974b; Patel & Tonkonow, 1974). This implies participation of citrate lyase in transport of acetyl-CoA from the intramitochondrial pool. However, changes in the activity of citrate lyase are not correlated with the profile of changes in the activity of choline acetyltransferase and biosynthesis of acetylcholine (Szutowicz *et al.*, 1975a; Marchisio & Giacobini, 1969). According to Tuček (1967) the activity of citrate lyase is too low in brain of adult rabbit and guinea pig to cover high demand for acetyl-CoA in biosynthesis of acetylcholine. On the other hand, Szutowicz *et al.* (1975a) reported that at all stages of development of nervous system in chicken and rat the activity of citrate lyase exceeded that of choline acetyltransferase.

It seems that application of a specific inhibitor of citrate lyase might be conclusive in elucidating the physiological role of this enzyme in brain. The presented results suggest that (—)hydroxycitrate, an inhibitor of liver citrate lyase (Lowenstein, 1971) may accomplish this task.

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MATERIALS AND METHODS

Material. White Wistar rats of either sex, weighing 220 - 260 g, were used for the experiments. Synaptosomes were isolated by differential centrifugation using a Ficoll density gradient according to Verity (1972). The cytosol fraction was obtained by centrifugation at 100 000 *g* for 60 min at 2 - 4°C.

Analytical methods. ATP citrate lyase from rat brain was purified as described previously (Szutowicz *et al.*, 1975b).

The activities of the enzymes were determined at 37°C by the following methods: ATP citrate (*pro*-3S)lyase (EC 4.1.3.8) by the method of Srere (1959) as described by Szutowicz *et al.* (1975b); fatty acid synthetase according to Alberts *et al.* (1974); choline acetyltransferase (EC 2.3.1.6) according to Fonnum (1969); phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) after Glock & McLean (1953); isocitrate dehydrogenase ($NADP^+$) (EC 1.1.1.42) according to Plaut (1962); malate dehydrogenase (decarboxylating) ($NADP^+$) (EC 1.1.1.40) as described by Ballard & Hanson (1967), and aconitate hydratase (EC 4.2.1.3) by the method of Villafranca (1974). Acetyl-CoA synthetase (EC 6.2.1.1) was determined by the modified methods of Buckley & Williamson (1973) and Stern (1971). The assay medium in a final volume of 0.25 ml contained: 50 mM-Tris-HCl buffer (pH 8.2), 5 mM-potassium acetate, 5 mM-potassium ATP, 0.4 mM-lithium CoA, 10 mM-$MgCl_2$, 1 mM-dithiothreitol, 0.5 i.u. of citrate synthase, 10 mM-potassium oxaloacetate, and the enzymatic protein (0.2 - 0.4 mg). The reaction was started by the addition of the enzyme and was stopped after 30 min with $HClO_4$. Citrate formed was determined in the deproteinized samples by the method of Dagely (1965). The results were corrected for citrate formation in the absence of CoA. Carnitine acetyltransferase (EC 2.3.1.7) was determined in the medium containing in a final volume of 0.25 ml: 100 mM-Tris-HCl buffer (pH 7.4), 30 mM-DL-carnitine, 0.8 mM-acetyl-CoA, and the enzyme protein (0.01 - 0.02 mg). The reaction was started by the addition of acetyl-CoA and stopped after 15 min with trichloroacetic acid. Samples were neutralized with 1 M-Tris containing 0.05 M-EDTA, and -SH groups were determined with 5,5′-dithio-*bis*-(2-nitrobenzoic acid). The results were corrected for -SH groups formed in the absence of DL-carnitine.

The rate of oxygen consumption by synaptosomes was measured by means of Clark electrode in the medium described in the legend to Fig. 3.

Concentration of free Mg^{2+} and its complexes with citrate and ATP was calculated using the stability constants of the following complexes: $MgATP^{2-}$, 20 000 M^{-1} (O'Sullivan & Perrin, 1964); Mg-$citrate^{1-}$, 1950 M^{-1} (Li *et al.*, 1959). It was assumed that the reactants were in the fully ionized form (Schneider *et al.*, 1964; Li *et al.*, 1959).

Reagents. NADH, NADPH, DL-isocitrate (sodium salt), L-malate, pyruvate (potassium salt), gluconate-6-phosphate (sodium salt), bovine serum albumin (fraction V), ADP, malate dehydrogenase, lactate dehydrogenase, citrate synthase, citrate lyase (of bacterial origin), and tetraphenylboron were from Sigma Chem. Comp. (St.Louis, Mo., U.S.A.), malonyl-CoA from PL-Biochemicals (Milwaukee,

Wis., U.S.A.), acetyl-CoA from Calbiochem (Los Angeles, Calif., U.S.A.), glucose-6-phosphate (sodium salt) from Reanal (Budapest, Hungary), Ficoll and Sephadex G-200 from Pharmacia (Uppsala, Sweden). Other chemicals were from P.O.Ch. (Gliwice, Poland).

Lactone of (—)hydroxycitric acid was a generous gift from Dr.Y.S.Lewis (Mysore, India). The lactone was converted into tripotassium salt by hydrolysis with potassium hydroxide according to Lowenstein (1971).

[1-^{14}C]Acetyl-CoA was from the Radiochemical Centre (Amersham, England).

RESULTS

Effect of (—)hydroxycitrate on ATP citrate lyase

Inhibition of brain citrate lyase by (—)hydroxycitrate (Fig. 1A) was found to be uncompetitive with respect to $MgATP^{2-}$ complex: the concentration of free Mg^{2+} and the $[ATP^{4-}]/[MgATP^{2-}]$ ratio remained constant. The same type of inhibition held also when concentration of $MgCl_2$ in the medium was raised and that of ATP (0.5 or 5.0 mM) remained constant (Fig. 1B). In this case, the increase in Mg^{2+} concentration raised the level of $MgATP^{2-}$ and lowered the $[ATP^{4-}]/[MgATP^{2-}]$ ratio.

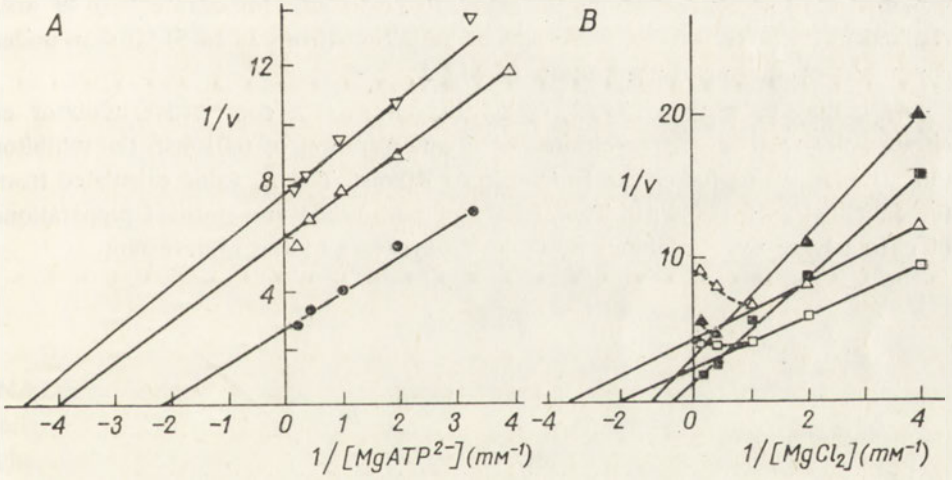

Fig. 1. Effect of (—)hydroxycitrate on the activity of purified ATP citrate lyase from rat brain. Double-reciprocal plots of the specific activity *versus*: *A*, $MgATP^{2-}$ concentration, in the presence of 1 mM-citrate, 0.5 mM-Mg^{2+} and: △, 0.005 mM- or ▽, 0.01 mM-(—)hydroxycitrate; ●, control; and *B*, $MgCl_2$ concentration, in the presence of 1 mM-citrate and 5.0 mM-ATP (full symbols) or 0.5 mM-ATP (open symbols); △, ▲, with 0.005 mM-(—)hydroxycitrate; □, ■, control. Protein, 0.05 mg/sample.

Data presented in Table 1 show that at low concentration of ATP and with Mg^{2+} concentration increasing from 0.5 to 10.0 mM the extent of inhibition of citrate lyase by (—)hydroxycitrate was raised from 23 to 54%, although the level of $MgATP^{2-}$ complex remained practically unchanged. The same response in the

Table 1

Effect of (—)hydroxycitrate on ATP citrate lyase from rat brain at different concentrations of $MgCl_2$ *and ATP*

The millimolar concentrations of Mg complexes and that of free Mg^{2+} were calculated as described in Methods. (—)Hydroxycitrate concentration was 0.005 mM.

$MgCl_2$ added (mM)	ATP added at concentration: 0.5 mM				5.0 mM			
	Mg^{2+}	Mg-$citr^{1-}$	$MgATP^{2-}$	Inhibition (%)	Mg^{2+}	Mg-$citr^{1-}$	$MgATP^{2-}$	Inhibition (%)
0.25	0.02	0.04	0.19	23	0.01	0.01	0.23	22
0.50	0.04	0.08	0.38	27	0.01	0.01	0.48	21
1.00	0.25	0.33	0.42	38	0.01	0.03	0.96	45
5.00	3.63	0.88	0.49	46	0.31	0.38	4.31	50
10.00	8.56	0.94	0.50	54	4.17	0.89	4.94	53

activity of citrate lyase to (—)hydroxycitrate was observed at high ATP concentration, when an increase in $MgCl_2$ level was accompanied by a concomitant increase of $MgATP^{2-}$ complex. The increase in $MgATP^{2-}$ concentration from 0.45 to 4.54 mM at fixed concentrations of Mg^{2+} (0.5 mM) and Mg-$citrate^{1-}$ (0.49 mM) resulted in a slightly smaller increment of inhibition (from 38 to 51%) than under conditions of varying concentration of Mg^{2+}.

As it may be seen in Fig. 2, (—)hydroxycitrate is a competitive inhibitor of citrate lyase with respect to citrate. At a concentration of 0.01 mM, the inhibitor raises the K_m value for citrate from 0.15 to 2.0 mM. The K_i value calculated from the data in Fig. 2 is 0.8 μM. The activity of both crude and purified preparations of citrate lyase were inhibited by (—)hydroxycitrate to the same extent.

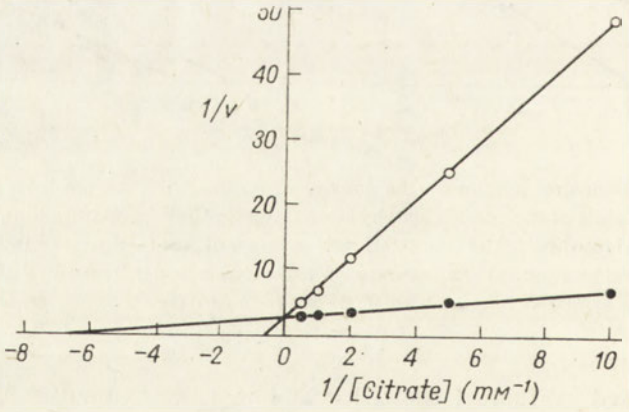

Fig. 2. Double-reciprocal plots of specific activity of ATP citrate lyase *versus* citrate concentration, at fixed concentrations of ATP (5 mM), $MgCl_2$ (10 mM) and (—)hydroxycitrate (0.01 mM). Citrate concentration from 0.1 to 2.0 mM, protein 0.05 mg/sample. ●, Control; ○, with (—)hydroxycitrate.

Effect of (—)hydroxycitrate on the enzymes involved in fatty acid and acetylcholine biosynthesis

(—)Hydroxycitrate at a concentration of 4 mM did not affect the activities of cytoplasmic acetyl-CoA synthetase and mitochondrial carnitine acetyltransferase (Table 2), the enzymes which, along with citrate lyase, generate cytoplasmic acetyl-CoA. Moreover, (—)hydroxycitrate was without any effect on fatty acid synthetase and choline acetyltransferase.

Table 2

Effect of (—)hydroxycitrate on the enzymes of acetyl-CoA metabolism and NADPH dehydrogenases

Enzyme	(—)Hydroxycitrate concentration	
	1 mM	4 mM
	Inhibition (%)	
ATP citrate lyase	100	—
Acetyl-CoA synthetase	—	0
Carnitine acetyltransferase	—	4
Choline acetyltransferase	+2	3
Fatty acid synthetase	0	10
Aconitate hydratase		
with citrate	20	55
with aconitate	27	51
Isocitrate dehydrogenase ($NADP^+$)	—	17
Phosphogluconate dehydrogenase (decarboxylating)	16	18
Glucose-6-phosphate dehydrogenase	10	+1
Malate dehydrogenase (decarboxylating) ($NADP^+$)	28	80

The effect of (—)hydroxycitrate on the enzymes involved in regeneration of NADPH, required in fatty acid synthesis, was also examined. Glucose-6-phosphate dehydrogenase was not inhibited by 4 mM-(—)hydroxycitrate, while phosphogluconate and isocitrate (NADP) dehydrogenases were inhibited by 17%, "malic enzyme" by 80% and aconitase by 50%. However, the value of $I_{0.5}$ obtained with (—)hydroxycitrate for "malic enzyme" and aconitase was about 400 times higher than that obtained for citrate lyase (Table 3).

Effect of (—)hydroxycitrate on respiration of synaptosomes

Preincubation of synaptosomes for 5 min with 0.5 mM-(—)hydroxycitrate, or the addition of (—)hydroxycitrate at the same concentration to the incubation medium, did not affect the rate of their respiration in state 4 with 1 mM-malate and 1 mM-pyruvate as substrates, or in state 3 following the addition of 1 mM-ADP (Fig. 3a,b).

Table 3

$I_{0.5}$ values for the inhibitory effect of (—)hydroxycitrate

The activity of ATP citrate lyase was determined with 0.5 mM-citrate as a substrate. The amount of cytosol protein was 0.3 mg for determination of citrate lyase and aconitate hydratase, and 0.06 mg for determination of malate and isocitrate ($NADP^+$) dehydrogenases.

Enzyme	$I_{0.5}$ (mM)
ATP citrate lyase	0.0038
Malate dehydrogenase ($NADP^+$)	1.5
Aconitate hydratase	
with aconitate	2.2
with citrate	3.7
Isocitrate dehydrogenase ($NADP^+$)	>10

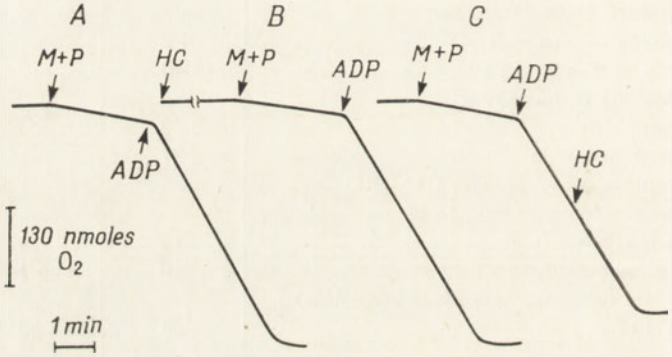

Fig. 3. Effect of (—)hydroxycitrate (*HC*) at 1 mM concentration on oxygen consumption by synaptosomes from rat brain, oxidizing 1.0 mM-pyruvate (*P*) and 1 mM-malate (*M*) in the presence and absence of ADP (1 mM); the bar denotes the rate of oxygen consumption (nmoles/min/mg protein). The incubation medium contained in a final volume of 3 ml: 100 mM-KCl, 5 mM-KH_2PO_4, 25 mM-Tris-HCl buffer, pH 7.4, 1 mM-EGTA (potassium salt), and 0.2 ml of the synaptosome suspension (about 3 mg protein) in 0.32 M-sucrose containing 10 mM-Tris-HCl (pH 7.4), 0.5 mM-EGTA, and bovine serum albumin (1 mg/ml). The incubation was carried out at 37°C.

DISCUSSION

(—)Hydroxycitrate was found to be an inhibitor of ATP citrate lyase from rat brain. Its K_i value resembled closely that for the enzyme from rat liver, 0.57 μM (Watson *et al.*, 1969). Since the inhibition is uncompetitive with respect to $MgATP^{2-}$ and competitive with citrate, it may be assumed that (—)hydroxycitrate reacts with the phosphorylated form of the enzyme. Moreover, the inhibition was

directly correlated with the increase in $MgCl_2$ concentration. This might imply that Mg-hydroxycitrate complex is a more potent inhibitor of citrate lyase than free (—)hydroxycitrate, or even may be the exclusive form of the inhibitor.

(—)Hydroxycitrate does not affect the activity of acetyl-CoA synthetase and carnitine acetyltransferase, and therefore permits to study participation of these two enzymes in biosynthesis of acetyl-CoA groups in brain tissue under conditions of inhibited citrate lyase activity. (—)Hydroxycitrate is not expected to affect formation of NADPH, as it does not inhibit the reaction catalysed by glucose-6-phosphate dehydrogenase which is considered the main source of the reduced form of this nucleotide in cell cytoplasm. Other NADP-dependent dehydrogenases are affected to a small only extent. The respiration rate of synaptosomes also remained unaffected. It seems, therefore, that (—)hydroxycitrate may serve as a usefu tool for studying the metabolism of acetyl residues in this subcellular fraction of the brain. $MgADP^{1-}$ and glutamate, the competitive inhibitors of brain citrate lyase, are both less specific and several hundred times less effective (Szutowicz *et al.*, 1974a, 1975b). The usefulness of (—)hydroxycitrate is still more evident since the analysis of the two molecular forms of citrate lyase showed that their ratio remains unchanged during development of the central nervous system (Szutowicz, 1974b).

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REFERENCES

Alberts, A. W., Ferguson, K., Hennessy, S. & Vagelos, P. R. (1974) *J. Biol. Chem.*, **249**, 5241 - 5249.

Ballard, F. J. & Hanson, H. W. (1967) *Biochem. J.*, **102**, 952 - 958.

Buckley, B. M. & Williamson, D. H. (1973) *Biochem. J.*, **132**, 653 - 656.

D'Adamo, A. F. Jr. & D'Adamo, A. P. (1968) *J. Neurochem.*, **15**, 315 - 323.

Dagely, S. (1965) in *Methods of Enzymatic Analysis* (H. U. Bergmeyer, ed.) pp. 313 - 319. Verlag Chemie Weinheim, Academic Press, New York.

Fonnum, F. (1969) *Biochem. J.*, **113**, 291 - 298.

Glock, G. E. & McLean, P. (1953) *Biochem. J.*, **55**, 400 - 405.

Li, N. C., Lindenbaum, A. E. & White, J. M. (1959) *J. Inorg. Nucl. Chem.*, **12**, 122 - 128.

Lowenstein, J. M. (1971) *J. Biol. Chem.*, **246**, 629 - 632.

Marchisio, P. C. & Giacobini, G. (1969) *Brain Res.*, **15**, 301 - 304.

O'Sullivan, W. J. & Perrin, D. D. (1964) *Biochemistry*, **3**, 18 - 26.

Patel, M. S. & Tonkonow, B. L. (1974) *J. Neurochem.*, **23**, 309 - 313.

Plaut, G. W. E. (1962) in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 5, pp. 645 - 651. Academic Press, New York.

Schneider, P. W., Brintzinger, H. & Erlenmeyer, H. (1964) *Helv. Chim. Acta.* **47**, 992 - 1002.

Srere, P. A. (1959) *J. Biol. Chem.*, **234**, 2544 - 2547.

Stern, J. R. (1971) *Biochem. Biophys. Res. Commun.*, **44**, 1001 - 1007.

Szutowicz, A., Stępień, M. & Angielski, S. (1974a) *J. Neurochem.*, **22**, 85 - 91.

Szutowicz, A., Stępień, M., Łysiak, W. & Angielski S. (1974b) *Acta Biochim. Polon.*, **21**, 331 - 338.

Szutowicz, A., Frazier, W.A. & Bradshaw, R. A. (1975a) *J. Biol. Chem.*, **251**, 1516 - 1523.

Szutowicz, A., Stępień, M., Łysiak, W. & Angielski, S. (1975b) *J. Neurochem.*, **25**, 11 - 20.
Verity, M. A. (1972) *J. Neurochem.*, **19**, 1305 - 1317.
Tuček, S. (1967) *Biochem. J.*, **104**, 749 - 756.
Villafranca, J. J. (1974) *J. Biol. Chem.*, **249**, 6149 - 6155.
Watson, J. A., Fang, M. & Lowenstein, J. M. (1969) *Arch. Biochem. Biophys.*, **135**, 209 - 217.

WPŁYW (—)HYDROKSYCYTRYNIANU NA AKTYWNOŚĆ LIAZY CYTRYNIANOWEJ ZALEŻNEJ OD ATP ORAZ ENZYMÓW PRZEMIANY ACETYLO-CoA W MÓZGU SZCZURA

Streszczenie

1. Kwas (—)hydroksycytrynowy jest silnym inhibitorem liazy ATP cytrynianowej (EC 4.1.3.8.) z mózgu szczura (K_i=0,8 μM), akompetycyjnym w stosunku do $MgATP^{2-}$ i kompetycyjnym w stosunku do cytrynianu.

2. Związek ten nie wpływa na szybkość zużycia tlenu przez synaptozomy mózgu szczura i nie hamuje aktywności syntetazy kwasów tłuszczowych, acetylotransferazy karnitynowej, dehydrogenazy glukozo-6-fosforanowej i syntetazy acetylo-CoA.

3. Milimolowe stężenia (—)hydroksycytrynianu hamują aktywność dehydrogenazy izocytrynianowej, dehydrogenazy jabłczanowej dekarboksylującej i hydratazy akonitanowej.

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TRANSFER OF CHOLESTEROL AND LECITHIN BETWEEN ERYTHROCYTES AND SERUM *IN VITRO*

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1. In human blood incubated *in vitro*, the transfer of free cholesterol and lecithin from erythrocytes to serum was not related to glycolytic activity in erythrocytes or esterification of cholesterol in serum.

2. The stability of free cholesterol concentration in serum was dependent on the activity of lecithin : cholesterol acyltransferase (EC 2.3.1.43), and the stability of lecithin concentration in erythrocytes, on glycolytic activity.

The lipid structures formed by phospholipids and free cholesterol in cell membranes and serum lipoproteins show some similarities (Lenaz, 1974). Transfer of lipids from one structure to another has been repeatedly reported, among others in blood (Cooper, 1970; Nelson, 1972). Changes in the distribution of lipids between erythrocytes and serum have been observed in hereditary spherocytosis (Langley & Felderhof, 1968; Cooper & Jandl, 1969a,b), inborn deficiency of lecithin : cholesterol acyltransferase (LCAT[1]) (EC 2.3.1.43) (Gjone *et al.*, 1968), and acute and chronic liver diseases (Gjone & Norum, 1970; Simon, 1971).

Murphy (1962) demonstrated that also during blood incubation *in vitro*, free cholesterol passes from erythrocytes to serum, but he observed no transfer of phospholipids. This was confirmed by Cooper & Jandl (1969b) who showed that cholesterol is translocated simultaneously with phospholipids only when the cell membranes are injured by prolonged incubation of erythrocytes depleted of glucose.

In blood, lecithin plays a special role among phospholipids, as it is involved in the reaction of cholesterol esterification catalysed by serum LCAT. The lysolecithin formed in this reaction is transported by albumin to erythrocyte membranes (Switzer & Eder, 1965). Mulder *et al.* (1965) demonstrated by isotopic methods that lysolecithin is esterified in erythrocyte membranes into lecithin, in the reaction with acyl-CoA catalysed by acyltransferase. In the absence of ATP or acyl-CoA,

[1] Abbreviation used: LCAT, lecithin : cholesterol acyltransferase.

lysolecithin can be esterified into lecithin by dismutation reaction between two molecules of lysolecithin.

The aim of the present work was to study the effect of serum LCAT activity, and energy metabolism in erythrocytes on displacement of cholesterol and lecithin between erythrocyte membrane and serum lipoproteins.

MATERIALS AND METHODS

Incubation of blood. The blood obtained from young healthy donors (women) was defibrinated, and erythrocytes were separated. Clear serum was diluted with 0.5 vol. of 160 mM-bicarbonate buffer of pH 7.55. To the diluted serum, erythrocytes were added to a haematocrit value of 25 - 30%, and the reconstituted blood was added with: streptomycin (2.5 mg/ml), penicillin (500 units/ml) and glucose (to 4 mM concentration) as energy source. The incubation was carried out at 37°C for 12 h as described previously (Solecki *et al.*, 1973), with gentle stirring, CO_2 being removed through a semipermeable membrane. During incubation, changes in pH value of the medium did not exceed ± 0.05 unit. Stability of the erythrocyte membranes was assessed after incubation by haematocrit value determination, cell count, diameter and volume of erythrocytes, and haemoglobin content in the medium, as described previously (Solecki *et al.*, 1973). Changes in the haematocrit value after incubation did not exceed $\pm 2\%$, and changes in cell count ± 1 million/cm^3. With the glucose-metabolizing erythrocytes, no changes in haemoglobin or sodium and potassium ion concentration in serum were observed. On the other hand, with erythrocytes depleted of glucose, ATP and 2,3-diphosphoglycerate, an increase in haemoglobin content in serum (up to 300 mg/100 ml) was found, with simultaneous changes in potassium and sodium ion concentration.

Removal of glucose, ATP and 2,3-diphosphoglycerate from erythrocytes. The erythrocytes were washed three times according to Whittam (1958), then incubated in tightly closed teflon vessels for 8 h at 37°C in a medium of pH 7.7, composed of 90 mM-NaCl, 50 mM-$NaHCO_3$, 8 mM-Na_2HPO_4, 1 mM-KH_2PO_4, 4 mM-KCl, 1 mM-$MgCl_2$, and containing 0.5% of polyvinylpyrrolidone; the osmomolarity of the medium, determined in an osmometer, was 330 mOsM. After this treatment, the amount of glucose in erythrocytes decreased below 0.34 mmole per 1 litre of packed erythrocytes, and the amount of ATP and 2,3-diphosphoglycerate decreased below 10% of the initial values. In erythrocytes, glucose was determined by the *o*-toluidine method of Ek & Hultman (1959), ATP by the hexokinase method according to Lamprecht & Trautschold (1965), and 2,3-diphosphoglycerate by the method of Krimsky (1965).

Inhibition of LCAT activity. The serum was heated at 57°C for 30 min, or 0.5 mM-iodoacetate, the SH group inhibitor, was added.

Determination of lipids. From erythrocytes, lipids were extracted according to Dodge & Phillips (1967) and from serum according to Folch *et al.* (1957). Prior to extraction, serum was centrifuged for 30 min at 16 000 *g*.

Total lipid phosphorus and phosphorus of lysolecithin and lecithin, isolated by thin-layer chromatography (Parker & Peterson, 1965), were estimated according to Bartlett (1959). To calculate the amount of phospholipids, the phosphorus value was multiplied by 25. The content of lysolecithin in erythrocytes corresponded to less than 4% of total phospholipids, and it was not determined due to technical difficulties.

Total cholesterol, and free cholesterol separated from the esterified one by the digitonin method (Dryer, 1970) were assayed by the colour reaction with $FeCl_3$ (Badzio, 1964).

The concentration of lipids in serum and erythrocytes was expressed in micrograms per 1 ml of reconstituted blood at the haematocrit value given in the respective legends to Tables.

RESULTS

The incubation of glucose-metabolizing erythrocytes in serum with LCAT activity (Table 1) resulted in a decrease in free cholesterol content in erythrocytes, whereas the amount of total phospholipids and lecithin was unchanged. At the same time, in serum the amount of lecithin decreased and that of lysolecithin became almost twofold higher. Total cholesterol increased due to the increase in esterified cholesterol, which corresponded to the decrease of cholesterol in erythrocytes.

Table 1

Net transfer of lipids between erythrocytes and serum during incubation of reconstituted blood composed of glycolysing erythrocytes and cholesterol-esterifying serum

The incubation conditions were as described in Methods. The haematocrit value of the reconstituted blood was 29%. The results, expressed as μg/ml of reconstituted blood, represent mean values from 6 samples, ±S.D. Data for phospholipids were calculated from lipid phosphorus using the factor 25.

	Serum			Erythrocytes			Net displacement	
	Incubation (h)		Δ	Incubation (h)		Δ	found	expected*
	0	12		0	12			
Cholesterol								
total	792±6	880±14						
free	309±13	290±15	−19	395±6	320±19	−75	−94	−107
esterified	483±5	590±7	+107					
Phospholipids								
total	862±20	865±26		876±20	882±16			
lysolecithin	51±6	99±5	+48				+48	+140
lecithin	592±12	474±17	−118	242±6	235±8	−7	−118	−218

* Calculated on the basis of cholesterol esterification.

According to Glomset (1968), cholesterol in serum is esterified by the transacylation reaction with lecithin, leading to formation of lysolecithin. On the assumption that in lecithin molecule the 1-position is occupied by stearic acid and the 2-position by linoleic acid, for esterification of 1 g of cholesterol (mol. wt. 387),

2.03 g of lecithin (mol. wt. 786) is required, and 1.3 g of lysolecithin (mol. wt. 504) is formed. However, the observed decrease of lecithin in serum was half of the amount corresponding to the cholesterol esterified, and the increase in lysolecithin corresponded only to about one-third of the calculated value. It may be supposed that a part of lysolecithin formed in the reaction catalysed by LCAT was esterified into lecithin on erythrocyte membranes.

The results of incubation of the reconstituted blood in which erythrocytes had been deprived of glucose, ATP and 2,3-diphosphoglycerate (non-glycolysing erythrocytes), and in serum LCAT activity had been inhibited, are presented in Table 2. Inhibition of cholesterol esterification in serum and limited energy supply in erythrocytes caused significant changes in distribution of lipids. Both free cholesterol and lecithin passed from erythrocytes to serum, and their decrease in erythrocytes corresponded to the increase in serum. The decrease of lysolecithin in serum may indicate that also in this case it was converted in erythrocytes into lecithin, probably by the dismutation reaction (Mulder *et al.*, 1965).

Table 2

Net transfer of lipids during incubation of non-glycolysing erythrocytes in serum with inhibited LCAT activity

From erythrocytes, glucose, ATP and 2,3-diphosphoglycerate were removed as described in Methods. The incubation conditions as described in Methods except that pH was 7.8 and glucose was omitted. Haematocrit value 25%. The results, expressed as μg/ml of reconstituted blood, represent mean values from 6 samples, ± S.D.

	Serum			Erythrocytes		
	Incubation (h)		Δ	Incubation (h)		Δ
	0	12		0	12	
Cholesterol						
total	760±8	853±13				
free	263±3	354±10	+91	315±8	226±7	−89
esterified	497±7	499±11				
Phospholipids						
total	1152±11	1198±28		752±13	705±26	
lysolecithin	66±5	31±3	−35			
lecithin	525±8	630±8	+105	232±5	150±5	−82

In another series of experiments, the blood was deprived of either LCAT or glycolytic activity. In these experiments, to obtain higher initial lysolecithin concentration, fresh serum was preincubated for 12 h at 37°C.

The incubation of glycolysing erythrocytes in serum with inhibited LCAT activity (Table 3) resulted in accumulation in serum of free cholesterol and lecithin, and a decrease in lysolecithin content, whereas the amount of esterified cholesterol was unchanged. The increase of lecithin in serum was not accompanied by an equivalent decrease in erythrocytes. Thus it seems possible to suppose that also in this case lysolecithin passed from serum to erythrocytes, and lecithin from erythrocytes to serum. This would indicate that, with blocked LCAT activity, there is also a

Table 3

Net transfer of lipids between erythrocytes and serum on incubation of reconstituted blood composed of glycolysing erythrocytes and serum with inhibited LCAT activity

Serum was preincubated for 12 h in 160 mM-bicarbonate buffer, pH 7.55, in the presence of antibiotics, then LCAT was inactivated by heating for 30 min at 57°C. During the preincubation of serum, erythrocytes were stored at 4°C in 110 mM-phosphate buffer, pH 7.40, containing the antibiotics and glucose. The serum and erythrocytes were combined and incubated as described in Methods. Haematocrit value 25%. The results, expressed as μg/ml of reconstituted blood, represent mean values from 6 samples, ±S.D.

	Serum			Erythrocytes		
	Incubation (h)		Δ	Incubation (h)		Δ
	0	12		0	12	
Cholesterol						
total	1036±38	1092±68				
free	272±8	337±16	+65	328±4	269±5	−59
esterified	764±5	754±11	−10			
Phospholipids						
total	1152±22	1163±15		782±22	698±16	
lysolecithin	140±8	46±8	−94			
lecithin	630±11	727±7	+97	225±7	200±7	−25

transfer of lipids from erythrocytes, but the loss of lecithin is compensated by its resynthesis in erythrocytes.

The incubation of non-glycolysing erythrocytes in serum with LCAT activity (Table 4) resulted in a marked decrease of cholesterol in the erythrocytes, accompanied by an equivalent increase in esterified cholesterol in serum. There was also

Table 4

Net transfer of lipids during incubation of reconstituted blood composed of non-glycolysing erythrocytes and serum with LCAT activity

From erythrocytes, glucose, ATP and 2,3-diphosphoglycerate were removed as described in Methods. Serum was preincubated as described in the legend to Table 3. The reconstituted blood was incubated as described in Methods, except that glucose was omitted and pH was 7.8. Haematocrit value 30%. The results, expressed as μg/ml of reconstituted blood, represent mean values from 6 samples, ±S.D.

	Serum			Erythrocytes			Net displacement	
	Incubation (h)		Δ	Incubation (h)		Δ	found	expected*
	0	12		0	12			
Cholesterol								
total	624±6	724±7						
free	169±6	187±5	+18	366±8	266±11	−100	−82	−92
esterified	455±6	537±7	+92					
Phospholipids								
total	648±13	729±10		990±13	897±10			
lysolecithin	91±7	118±7	+27				+27	+121
lecithin	356±5	310±6	−40	315±5	270±6	−45	−85	−186

* Calculated on the basis of cholesterol esterification.

an increase of lysolecithin in serum but it was much lower than what could be expected for the cholesterol esterification reaction. Thus lysolecithin appeared to be esterified on erythrocytes also in this experimental system. Both in erythrocytes and serum, lecithin decreased but the total decrease was only half that expected for the LCAT-catalysed reaction. This may point to lysolecithin being esterified under these conditions mainly by the dismutation reaction.

DISCUSSION

In the reconstituted blood incubated *in vitro*, the displacement of free cholesterol and lecithin was observed to occur in one direction only, i.e. from erythrocytes to serum. This direction was not dependent either on energy metabolism in erythrocytes, or on LCAT activity in serum; however, in serum with LCAT activity the concentration of free cholesterol did not undergo greater changes (Tables 1 and 4), and the glycolytic activity favoured the stabilization of lecithin concentration in erythrocytes (Tables 1 and 3).

The lack of distinct changes of free cholesterol in serum despite the increase of esterified cholesterol, may be explained by the shift of cholesterol from erythrocytes. d'Hollander & Chevallier (1972), basing on experiments with rat blood, supposed that the esterified cholesterol present in serum is esterified on erythrocyte membranes, and they suggested that LCAT acts directly on the surface of erythrocytes.

A shift of lecithin between erythrocytes and serum has a more complex character, and the following observations should be considered: (1), in the system with LCAT and glycolytic activities, the decrease of lecithin was found to be stoichiometrically lower than the increase in esterified cholesterol; (2), in erythrocytes, the content of lecithin decreased only when they were deprived of glycolytic activity; (3), lysolecithin in erythrocytes is esterified to lecithin (Mulder *et al.*, 1965; Tarlov, 1966); (4), the esterification of lecithin in serum has not been observed (Lichtman *et al.*, 1974).

These facts point to the occurrence in the extravasated blood of lecithin and lysolecithin circulation between serum and erythrocytes. It may be suggested that lecithin from the erythrocyte membrane passes to serum where it reacts with cholesterol, and the lysolecithin formed is transported by albumin to erythrocytes (Switzer & Eder, 1965; Mulder *et al.*, 1965). Simultaneously, free fatty acids are transported to erythrocytes. In erythrocyte membrane, lysolecithin is esterified to lecithin (Mulder *et al.*, 1965; Tarlov, 1966), which then passes to serum.

Although so far there is no direct proof, it seems possible to assume that cholesterol leaves the erythrocyte membrane together with lecithin. However, in erythrocyte membrane the ratio of free cholesterol to lecithin diminished due to continuous recirculation of lecithin. If such a process occurs also *in vivo*, it seems pertinent to ask about the source of renewal of cholesterol in erythrocytes. Angel & Farkas (1974) suggested that erythrocytes are involved in the transport of free cholesterol

released from adipose tissue. Glomset (1968), considering the physiological role of cholesterol esterification in serum, assumed that the decrease in the content of free cholesterol in lipoproteins increases their ability for cholesterol uptake from cell membranes.

The results of the experiments described in the present paper indicate that, in considering the role of lecithin : cholesterol acyltransferase, the displacement of lipids between lipoproteins of cell membranes and lipoproteins of blood serum should also be taken into account.

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REFERENCES

Angel, A. & Farkas, J. (1974) *J. Lipid Res.*, **15**, 491 - 499.
Badzio, T. (1964) *Chem. Anal.*, **9**, 823 - 830.
Bartlett, G. R. (1959) *J. Biol. Chem.*, **234**, 466 - 468.
Cooper, R. A. (1970) *Semin. Hematol.*, **7**, 296 - 322.
Cooper, R. A. & Jandl, J. H. (1969a) *J. Clin. Invest.*, **48**, 736 - 744.
Cooper, R. A. & Jandl, J. H. (1969b) *J. Clin. Invest.* **48**, 906 - 914.
d'Hollander, F. D. & Chevallier, F. (1972) *J. Lipid Res.*, **13**, 733 - 744.
Dodge, J. T. & Phillips, G. B. (1967) *J. Lipid Res.*, **8**, 667 - 675.
Dryer, H. L. (1970) in *Fundamentals of clinical chemistry* (N. W. Tietz, ed.) pp. 359 - 361. Saunders Co., Philadelphia.
Ek, J. & Hultman E. (1959) *Nature* (*London*), **183**, 108 - 109.
Folch, J. F., Lees, M. & Sloane, S. G. H. (1957) *J. Biol. Chem.*, **226**, 497 - 509.
Gjone, E. & Norum, K. R. (1970) *Acta Med. Scand.*, **187**, 153 - 161.
Gjone, E., Torsvik, H. & Norum, K. R. (1968) *Scand. J. Clin. Lab. Inevst.*, **21**, 327 - 332.
Glomset, J. A. (1968) *J. Lipid Res.*, **9**, 155 - 167.
Krimsky, J. (1965) in *Methods of enzymatic analysis* (H. U. Bergmeyer, ed.) pp. 238 - 243. Academic Press, New York.
Lamprecht, W. & Trautschold, J. (1965) in *Methods of enzymatic analysis* (H. U. Bergmeyer, ed.) pp. 543 - 551. Academic Press, New York.
Langley, G. R. & Felderhof, C. H. (1968) *Blood*, **32**, 569 - 585.
Lenaz, G. (1974) *Sub-Cell Biochem.*, **3**, 167 - 248.
Lichtman, M. A., Marinetti, G. V. & Gordesky, S. E. (1974) *Nouv. Rev. Franc. d'Hematol.*, **14**, 5 - 24.
Mulder, E., Van Den Berg, J. W. O. & Van Deenen, L. L. M. (1965) *Biochim. Biophys. Acta*, **106**, 118 - 127.
Murphy, J. R. (1962) *J. Lab. Clin. Med.*, **60**, 86 - 109.
Nelson, G. L. (1972) in *Blood lipids and lipoproteins: quantitation, composition and metabolism* (G. L. Nelson, ed.) pp. 317 - 386. J. Wiley and Sons Inc., New York.
Parker, F. & Peterson, N. F. (1965) *J. Lipid Res.*, **6**, 455 - 460.
Simon, J. B. (1971) *J. Lab. Clin. Med.*, **77**, 891 - 900.
Solecki, A., Badzio, T. & Rogulski, J. (1973) *Acta Biochim. Polon.*, **20**, 55 - 62.
Switzer, S. & Eder, H. A. (1965) *J. Lipid Res.*, **6**, 506 - 511.
Tarlov, A. (1966) *Blood*, **28**, 990 - 991.
Whittam, R. (1958) *J. Physiol.*, **140**, 479 - 497.

PRZESUNIĘCIE *IN VITRO* CHOLESTEROLU I LECYTYNY POMIĘDZY ERYTROCYTAMI I SUROWICĄ

Streszczenie

1. W czasie inkubacji krwi ludzkiej *in vitro* przesunięcie cholesterolu wolnego i lecytyny z erytrocytów do surowicy jest niezależne od aktywności glikolitycznej w erytrocytach i od estryfikacji cholesterolu w surowicy.

2. Aktywność acylotransferazy lecytyna : cholesterol (EC 2.3.1.43) podtrzymuje stałe stężenie cholesterolu wolnego w surowicy, a aktywność glikolityczna stabilizuje stężenie lecytyny w erytrocytach.

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ACTIVITY OF METHOXYAMINE-MODIFIED f2 RNA IN INITIATION AND ELONGATION STEPS OF PROTEIN SYNTHESIS

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1. Messenger activity of phage f2 RNA modified with methoxyamine under non-denaturing conditions was studied in *E. coli* cell-free system. The incorporation of amino acids into phage polypeptides was decreased, and the synthesis of phage-specific proteins was diminished. The RNA replicase synthesis was more affected than synthesis of coat protein. The impaired messenger activity of the methoxyamine-modified f2 RNA was due to the blocking of elongation process by modified cytosines present in RNA chain.

2. Specificity of f2 RNA to stimulate ribosomal binding predominantly at the coat protein initiation site was not affected by methoxyamine-treatment, as demonstrated by unchanged binding of f[^{3}H]Met-tRNA and [^{14}C]alanyl-tRNA to ribosomes.

3. Unfolding of f2 RNA molecule on treatment with methoxyamine in the presence of guanidine·HCl resulted in a significant increase of RNA capacity to direct fMet-tRNA binding to ribosomes. Sucrose-density gradient profiles revealed the formation of polysome-like initiation complexes indicating that ribosomes were able to bind at many hitherto inaccessible initiation codons in RNA molecules. fMet-tRNA bound to ribosomes in the presence of unfolded RNA was found to be fully reactive with puromycin.

The ordered and primary structure of RNA of *E. coli* small phages are important factors regulating its translation. Discovery of long non-translated RNA regions between the cistrons and at the ends of phage RNA chain suggested that these regions could contain specific nucleotide sequences or other structural features recognized by ribosomes or protein factors involved in initiation of polypeptide synthesis (Steitz, 1969; Weissmann *et al.*, 1973). Studies of Lodish (1970a) with formaldehyde-treated bacteriophage f2 RNA proved also the involvement of RNA

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secondary and tertiary structure in regulation of translation of RNA bacteriophage genes. Similar conclusions were reached from the studies of nuclease-cleaved phage RNA (Lodish, 1968; 1970a; Voorma *et al.*, 1971) or RNA heated under various ionic conditions (Fukami & Imahori, 1971).

Although much is known about the mechanisms which regulate the translation of phage mRNA (for reviews see Kozak & Nathans, 1972; Weissmann *et al.*, 1973), there are some steps in phage polypeptide synthesis which still remain incompletely understood. This refers mainly to the mechanism of recognition of initiation sites in mRNA by ribosomes and initiation factors. The molecular basis of this process in one of the most intriguing questions still to be solved in the field of protein synthesis. The use of different types of chemically modified messengers containing either specifically modified bases or changed three-dimensional structure may represent one of the possible approaches to this problem.

In the preceding paper (Wodnar-Filipowicz *et al.*, 1975) preparation of two different forms of methoxyamine-modified f2 RNA was described. It was shown that in f2 RNA modified extensively with methoxyamine under non-denaturing conditions only those cytosines which are not involved in formation of secondary and tertiary structure were susceptible to methoxyamine attack. This type of modified RNA retained the three-dimensional structure characteristic for native f2 RNA molecule. When the modification was performed in the presence of a denaturing agent, i.e. 6 M-guanidine·HCl, practically all cytosine residues became reactive towards methoxyamine and the resulting modified RNA lost irreversibly its ordered structure. The activity of both types of modified f2 RNA in initiation and elongation steps of polypeptide synthesis is the subject of the present paper.

MATERIALS AND METHODS

Chemicals. Sources of *O*-methylhydroxylamine (methoxyamine), guanidine hydrochloride, Tris, PPO and POPOP were as described in the preceding paper (Wodnar-Filipowicz *et al.*, 1975), 2-mercaptoethanol was from Koch-Light Lab. (Colnbrook, Bucks, England), *E. coli* stripped tRNA from General Biochemicals (Chagrin Falls, Ohio, U.S.A.), ATP, GTP, phosphoenolpyruvate, phosphoenolpyruvate kinase (EC 2.7. 1.40) and ribonuclease A (EC 2.7.7.17) from Calbiochem (Los Angeles, Calif., U.S.A.), sucrose from Schwarz-Mann (Orangeburg, N. J., U.S.A.). Puromycin dihydrochloride was obtained from Nutritional Biochemicals Co. (Cleveland, Ohio, U.S.A.), leukovorin from Lederle (München, G.F.R.), poly(G,C) from Miles Laboratories (Elkhart, Ind., U.S.A.), Bio-Solv solubilizer BBS-3 from Beckman Instruments (Fullerton, Calif., U.S.A.). Fusidic acid was a gift from Dr. Godtfredsen, Leo Pharmaceutical Company (Ballerup, Denmark). Sephadex G-25 was a product of Pharmacia (Uppsala, Sweden), DEAE-cellulose, Whatman *3MM* papers and fiber glass filters (Whatman GF 83) were from W. and R. Balston Ltd (Maidstone, Kent, England), Millipore filters (HAWP 02500) from Millipore GmbH (Neu-Isenburg, G.F.R.).

O-[^{14}C]Methylhydroxylamine·HCl preparations (spec. act. 0.05 mCi/1.7 mg or 40 μCi/0.1 mg) were, respectively, from New England Nuclear Corp. (Boston, Mass., U.S.A.) or from Isotope, Leningrad (U.S.S.R.); [U-^{14}C]serine (160 mCi/mmole), [*Me*-^{3}H]methionine (5 Ci/mmole), and [2,5-^{3}H]histidine (500 mCi/mmole) were obtained from the Radiochemical Centre (Amersham, Bucks., England); [U-^{14}C]arginine (105 mCi/mmole) was from C.E.A. (Gif-sur-Yvette, France); [U-^{14}C]alanine (72.6 mCi/mmole) and [2-^{14}C]uracil (1.0 mCi/2.11 mg) from the Institute of Radioisotopes (Praha, Czechoslovakia).

Isolation of f2 RNA. The f2 RNA was isolated by phenol extraction of purified phage f2 as described by Zagórski *et al.* (1972).

Labelling and isolation of f2 [^{14}C]*RNA.* The phage growing culture was incubated with [^{14}C]uracil (0.6 mCi per 500 ml of the growing culture) according to Steitz (1968) and the labelled f2 RNA was isolated as described by Zagórski *et al.* (1972). The specific activity of ^{14}C-labelled f2 RNA was about 7000 counts min^{-1} μg^{-1}. f2 RNA labelled with radioactive methoxyamine was prepared by addition of 100 - 200 μCi of [^{14}C]methoxyamine to 1 ml of the modification mixture. The specific activity of the [^{14}C]methoxyamine-labelled RNA was 7500 counts min^{-1} μg^{-1}.

Modification of f2 RNA and poly(G,C) with methoxyamine. Both unlabelled and ^{14}C-labelled phage f2 RNA was modified with 1 M-methoxyamine as described previously (Wodnar-Filipowicz *et al.*, 1975), modification in the presence of 10 mM--magnesium acetate or 6 M-guanidine·HCl being further referred to as modification, respectively, under non-denaturing and denaturing conditions. Time and temperature of modification are given in legends to Tables and Figures. In control samples, methoxyamine was replaced by 1 M-NaCl - 10 mM-sodium phosphate buffer, pH 5.5.

Poly(G,C) was modified in 1 M-methoxyamine - 6 M-guanidine·HCl, pH 5.5, at the concentration 250 μg/ml at 37°C for 5 days. The T_m values for the unmodified and methoxyamine-modified poly(G,C) in SSC (0.15 M-NaCl - 0.015 M-sodium citrate, pH 7.2) were found to be 74 and 53°C, respectively. Although the number of modified cytosines in poly(G,C) treated with methoxyamine was not determined, the observed 21°C shift in T_m indicates a high degree of modification leading to pronounced changes in base-pair interactions.

Preparation of E. coli 30 000 g supernatant, ribosomes, initiation factors and labelled aminoacyl-tRNAs. E. coli Qβ supernatant S-30, *E. coli* MRE 600 ribosomes (1 M-NH_4Cl-washed) and crude initiation factors were prepared as described by Zagórski *et al.* (1972).

As a source of aminoacyl-tRNA synthetases and formyltransferase, the *E. coli* 105 000 *g* supernatant freed of nucleic acids by chromatography on DEAE-cellulose, was used. f[^{3}H]Met-tRNA (45 pmoles/A_{260} unit), 11 220 counts min^{-1} $pmole^{-1}$, was prepared according to Lelong *et al.* (1970) by charging *E. coli* tRNA with [^{3}H]methionine and formylation using calcium leucovorin as a formyl donor. The remaining non-formylated methionyl-tRNA was subsequently discharged enzymatically and f[^{3}H]Met-tRNA purified by chromatography on Sephadex G-25. High-voltage electrophoresis (in 50 mM-ammonium formate buffer, pH 5.5, 1 h, 60 V/cm, Whatman *3MM* paper) of f[^{3}H]Met-tRNA subjected to RNase A digestion

showed that 89% of the radioactivity corresponded to the spot of formyl-methionyl--adenosine.

[^{14}C]Alanyl-tRNA (62 pmoles/l A_{260} unit), 176 counts min^{-1} $pmole^{-1}$; [^{14}C]seryl-tRNA (43 pmoles/l A_{260} unit), 353 counts min^{-1} $pmole^{-1}$; and [^{14}C]arginyl-tRNA (62 pmoles/l A_{260} unit), 232 counts min^{-1} $pmole^{-1}$, were prepared by charging *E. coli* tRNA with the respective ^{14}C-labelled amino acid in 2 ml of a mixture containing: 0.1 M-Tris-HCl, pH 7.6, 0.03 M-KCl, 0.01 M-$MgCl_2$, 9 mM-mercaptoethanol, 3 mM-ATP, 6 mM-phosphoenolpyruvate, 10 µl of phosphoenolpyruvate kinase, 5 mg of tRNA, 1.25 mg of 105 000 *g* supernatant protein purified on DEAE--cellulose, and 0.05 - 0.10 µmole of radioactive amino acid. The resulting aminoacyl--tRNAs were purified by phenol method followed by Sephadex G-25 filtration.

Binding of labelled aminoacyl-tRNAs to ribosomes. The binding of f[^{3}H]Met--tRNA to ribosomes was performed usually in 50 µl mixtures containing: 50 mM--Tris-HCl, pH 7.5, 5 mM-Mg acetate, 80 mM-NH_4Cl, 12 mM-mercaptoethanol, 1 mM-GTP; ribosomes washed with 1 M-NH_4Cl, 2 A_{260} units; crude initiation factors, 35 µg; and f[^{3}H]Met-tRNA, 0.6 A_{260} unit. When binding of [^{14}C]aminoacyl-tRNAs was simultaneously studied, incubation mixtures contained additionally 0.5 mM-fusidic acid and one of the following [^{14}C]aminoacyl-tRNAs: [^{14}C]alanyl--tRNA (0.78 A_{260} unit), [^{14}C]seryl-tRNA (0.62 A_{260} unit), or [^{14}C]arginyl-tRNA (0.46 A_{260} unit). Crude initiation factors contained enough elongation factor EF-T to stimulate aminoacyl-tRNA binding to A site of ribosomes (see also Zagórska & Szafrański, 1973). The amounts of f2 RNA added are given in legends to Tables and Figures.

Incubations were performed at 25°C for 20 min except when otherwise indicated. The amount of aminoacyl-tRNA bound to ribosomes was measured by millipore filter technique (Nirenberg & Leder, 1964).

To investigate the reaction of puromycin with fMet-tRNA bound to ribosomes, 40 µg of puromycin was added to the incubation mixture (50 µl) after completion of the binding. The mixture was subsequently incubated for 15 min at 25°C and formylmethionylpuromycin extracted with ethylacetate at pH 5.5 according to Leder & Bursztyn (1966). The radioactivity was determined in Bray's solution (Bray, 1960).

Synthesis and polyacrylamide-gel electrophoresis of phage proteins. Incorporation of [^{14}C]alanine and [^{3}H]histidine in the presence of different f2 RNA preparations (60 µg of RNA/100 µl) carried out with the use of *E. coli* S-30 supernatant was performed as described previously (Zagórski *et al.*, 1972), except that incubation mixtures were not supplied with additional exogenous tRNA. The radioactivity was measured according to Mans & Novelli (1961).

The preparation and polyacrylamide-gel electrophoresis of [^{14}C]alanine and [^{3}H]histidine doubly-labelled polypeptides were performed as described by Zagórski *et al.* (1972) except that the gel slices were solubilized in alkaline H_2O_2 and counted according to the method of Goodman & Matzura (1971) with the use of Beckman BBS-3 solubilizer.

Sucrose-density-gradient centrifugation was carried out in SW 50.1 rotor of L-50 Beckman Spinco ultracentrifuge at 4°C. A cooled incubation mixture (100 µl)

was layered on top of 5 ml of 10 - 40% linear sucrose gradient in 50 mM-Tris-HCl, pH 7.5, 70 mM-NH_4Cl, 10 mM-magnesium acetate. In experiments described in Figs. 2 and 5, fractions after centrifugation were collected from the bottom of the tubes directly on Whatman *3MM* paper discs, dried, then immersed for 15 min in cold 10% trichloroacetic acid (TCA), washed once with 5% TCA, once with ethanol - ether (1 : 2, v/v), once with ether, dried and counted in toluene-based scintillator. In experiments described in Fig. 3 and Table 2, 2-drop fractions were first diluted with 1 ml of water and A_{260} was measured. Albumin was added as a carrier and proteins were precipitated by addition of 0.2 ml of 50% TCA containing 50 mM non-radioactive alanine. Precipitates were collected on Whatman fiber glass filters, GF 83, washed twice with 5 ml of 50% TCA containing 50 mM-alanine, dried and counted as described above.

Radioactivity present in fractions from control experiments (incubation without added f2 RNA) was subtracted from respective fractions of every gradient.

Radioactivity was measured in Packard Tricarb or Nuclear Chicago Isocap 300 liquid scintillation counters.

RESULTS

Messenger activity of f2 RNA modified with methoxyamine under non-denaturing conditions

The effect of modification of cytosines in f2 RNA on its messenger activity was estimated by studying the incorporation of [^{14}C]alanine and [^{3}H]histidine in *E. coli* cell-free system into phage-specific polypeptides. Incorporation of histidine was taken as a measure of synthesis of RNA replicase as this amino acid is not present in phage coat protein (Weber *et al.*, 1966); and the incorporation of alanine, the amino acid present in both polypeptides, reflects mainly synthesis of coat protein which is the predominant product of bacteriophage RNA translation (Nathans *et al.*, 1962).

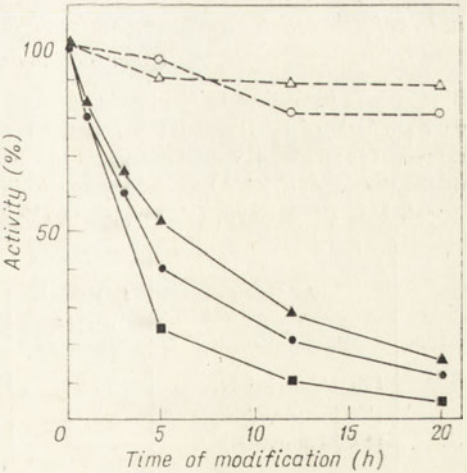

Fig. 1. Messenger activity of methoxyamine-modified f2 RNA measured by its ability to stimulate the incorporation of [^{14}C]alanine and [^{3}H]histidine in *E. coli* cell-free system. Samples of f2 RNA were modified under non-denaturing conditions for the time indicated. Incorporation directed by f2 RNA modified at 21°C: ●, histidine; ▲, alanine; and by f2 RNA modified at 37°C: ■, alanine. Control: incorporation of ○, histidine and △, alanine directed by f2 RNA incubated at 21°C with 1 M-NaCl. For details see Methods. Messenger activity of f2 RNA treated with methoxyamine for zero time, was taken as 100.

Modification of f2 RNA with methoxyamine under non-denaturing conditions strongly affected its messenger activity (Fig. 1). RNA treated with the mutagen for 20 h at 21°C retained 16% and 11% of native f2 RNA activity measured, respectively, by incorporation of alanine and histidine. Modification at 37°C resulted in faster inactivation of f2 RNA. Unmodified f2 RNA incubated parallelly under control conditions did not show a substantial loss in activity. It should be noted that the f2 RNA samples modified for 20 h were found to contain as much of high molecular (about 29S) undegraded f2 RNA as the starting material. This is in agreement with the results reported in the preceding paper (Wodnar-Filipowicz *et al.*, 1975). It can therefore be assumed that the decrease in the messenger activity of methoxyaminated f2 RNA was a result of modification of cytosine residues to *N*-4-methoxycytosine and *N*-4-methoxy-6-methoxyamino-5,6-dihydrocytosine derivatives.

In order to identify the step of protein synthesis at which translation of modified RNA was affected, the capacity of methoxyamine-treated f2 RNA to form the initiation complexes with *E. coli* ribosomes and formyl-[^{3}H]methionyl-tRNA was studied. The f2 RNA contains three initiation sites at which the synthesis of three different polypeptides is initiated (Steitz. 1969; Lodish & Robertson, 1969). The initiation codon AUG in coat protein cistron is followed by alanine codon, that in A protein cistron by arginine codon, while that in the cistron of RNA replicase by serine codon. It is therefore possible to distinguish between initiation complexes formed at the three binding sites by simultaneous binding of f[^{3}H]Met-tRNA and ^{14}C-labelled alanyl-, seryl- or arginyl-tRNAs. This approach as well as a similar method involving formation of initial dipeptides corresponding to different phage proteins, are widely used in studies on the mechanism of phage RNA translation (Lodish & Robertson, 1969; Roufa & Leder, 1971; Voorma *et al.*, 1971; Ota *et al.* 1972).

Table 1

Binding of f[^{3}H]*Met-tRNA and* [^{14}C]*alanyl-,* [^{14}C]*seryl- and* [^{14}C]*arginyl-tRNAs to E. coli ribosomes directed by native f2 RNA and f2 RNA modified with methoxyamine under non-denaturing conditions*

Binding was performed in 50 µl assay mixture as described in Methods, with 30 µg of f2 RNA, 0.6 A_{260} unit of f[^{3}H]Met-tRNA and the indicated ^{14}C-labelled aminoacyl-tRNA. Results are expressed as pmoles of bound aminoacyl-tRNA per sample. Values obtained in control experiments (in the absence of f2 RNA) corresponding to 0.54, 0.50, 0.41 and 0.94 pmoles in the case of f[^{3}H]Met--tRNA, [^{14}C]alanyl-, [^{14}C]seryl- and [^{14}C]arginyl-tRNAs, respectively, were subtracted.

Addition	Native f2 RNA		Modified f2 RNA	
	f[^{3}H]Met--tRNA	[^{14}C]AA-tRNA	f[^{3}H]Met--tRNA	[^{14}C]AA-tRNA
[^{14}C]Alanyl-tRNA	1.96	1.79	2.37	1.63
[^{14}C]Seryl-tRNA	2.12	0.33	2.66	0.20
[^{14}C]Arginyl-tRNA	2.05	0.18	2.83	0.20

The results presented in Table 1 show that native f2 RNA stimulated binding of almost equimolar amounts of f[^{3}H]Met-tRNA and [^{14}C]alanyl-tRNA to *E. coli* ribosomes. Binding of [^{14}C]seryl-tRNA and [^{14}C]arginyl-tRNA was very low. These data are in agreement with the results of the authors cited above, indicating that in the case of native f2 RNA the initiation complex was efficiently formed only at the coat protein cistron binding site. Initiation sites of both A protein and RNA replicase cistrons are much less accessible to ribosomes. Replicase cistron binding site is rendered more accessible to ribosomes only after translation of a part of coat protein gene (Capecchi, 1967; Lodish & Robertson, 1969) but in our experiments this was prevented by addition of fusidic acid to the incubation mixture. This compound blocks the translocation process mediated by elongation factor EF-G (Haenni & Lucas-Lenard, 1968; Tanaka *et al.*, 1968) which could be present in small amounts in the crude preparation of initiation factors used in our experiments. The treatment of f2 RNA with methoxyamine did not abolish its capacity to stimulate binding of ribosomes at the coat protein initiation site, as binding of [^{14}C]alanyl-tRNA was found to be practically unchanged. Binding of [^{14}C]seryl--tRNA and [^{14}C]arginyl-tRNA to ribosomes was low both in the presence of modified and unmodified f2 RNA.

It can be, therefore, concluded that modification of f2 RNA did not impair the initiation step of phage coat protein synthesis. This conclusion is also supported by experiments in which initiation complexes labelled with f[^{3}H]Met-tRNA formed in the presence of both unmodified and methoxyamine-modified f2 RNAs, were analysed by sucrose-gradient sedimentation (Fig. 2). In either case, the complexes formed sedimented as sharp peaks with sedimentation velocity little higher than that of 70S ribosomes (this is readily explained by the fact that initiation complexes consist of 70S ribosomes and 29S f2 RNA). The results showing that methoxyamine-modified f2 RNA formed a monosome-like initiation complex can be also considered as an additional proof that its ordered structure was not changed.

The decrease found in messenger activity of the methoxyamine-modified f2 RNA seemed to be due to the blocking of the elongation process by modified cytosine molecules (Figs. 3 and 4, Table 2). Figure 3 shows the results of experiments in which amino acids incorporating mixtures directed by native or metoxyamine-modified (4 h at 37°C) f2 RNAs were centrifuged in sucrose-density gradients. The amount of radioactivity attached to monosomes and polysomes and of radioactivity released to supernatant in systems directed by these RNAs as well as by RNAs modified for 8 and 12 h, are calculated in Table 2. It was found that in the case of translation of unmodified f2 RNA, after 30 min of incubation 86% of [^{14}C]alanine-labelled polypeptides was released from polysomes and found either at the top of the gradient (Fig. 3a) or in 30S region which corresponds presumably to the specific complex of phage RNA and coat protein synthesized *in vitro* (Capecchi, 1966; Sugyiama *et al.*, 1967). The observed release occurred apparently when ribosomes translating RNA genes reached the termination signals located at the end of each cistron, although it is known that small amounts of uncompleted polypeptides can become

detached unspecifically from ribosomes in the form of peptidyl-tRNA even in the absence of termination codons (Bretscher *et al.*, 1965; Takanami & Yan, 1965).

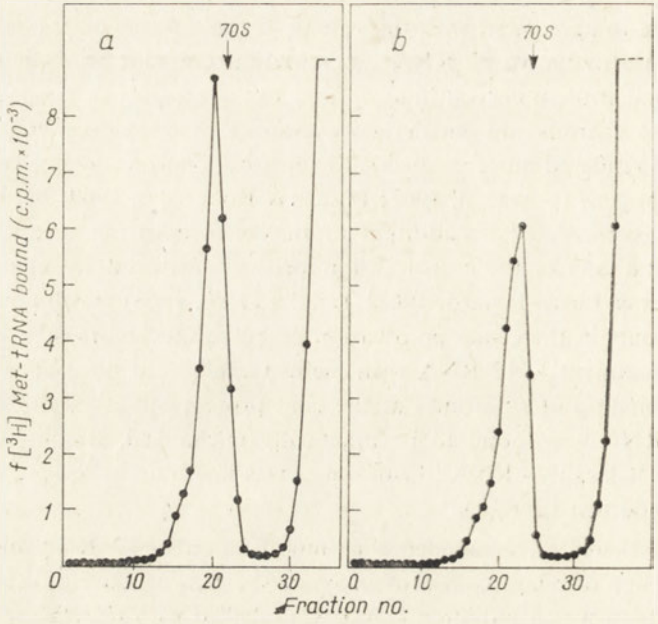

Fig.2. Sucrose-gradient profiles of initiation complexes formed in the presence of f[^{3}H]Met-tRNA and (*a*), native f2 RNA, (*b*), f2 RNA modified with methoxyamine under non-denaturing conditions for 3 days at 37°C. Binding of f[^{3}H]Met-tRNA to ribosomes was performed as described in Methods with RNA concentration 120 µg/100 µl of the incubation mixture. Centrifugation was carried out at 40 000 r.p.m. for 80 min at 4°C. For other details see Methods. Arrows indicate position of 70S ribosomes run in a parallel tube.

Table 2

Estimation of polysome-attached and released [^{14}C]*alanine-labelled polypeptides directed by native f2 RNA and f2 RNA modified with methoxyamine under non-denaturing conditions*

f2 RNA was modified under non-denaturing conditions at 37°C for the time indicated. Details of [^{14}C]alanine incorporation and sucrose-gradient centrifugation are described in Methods and text.

f2 RNA	[^{14}C]Alanine incorporation (%)	Distribution of [^{14}C] alanine radioactivity (%)	
		Polysome- and monosome-attached polypeptides	Released polypeptides
Native	100	14.0	86.0
Modified for 4 h	34	40.5	59.5
Modified for 8 h	17	49.5	50.5
Modified for 12 h	10	67.5	32.5

When RNA modified for 4 h at 37°C was used as messenger, less radioactive polypeptides were found to be released from polysomes as compared with the system containing unmodified f2 RNA. Only a small amount of radioactivity was found in 30S region (Fig. 3b). This could be due to a low affinity of the methoxyamine--modified f2 RNA for coat protein in specific complex formation (Chroboczek

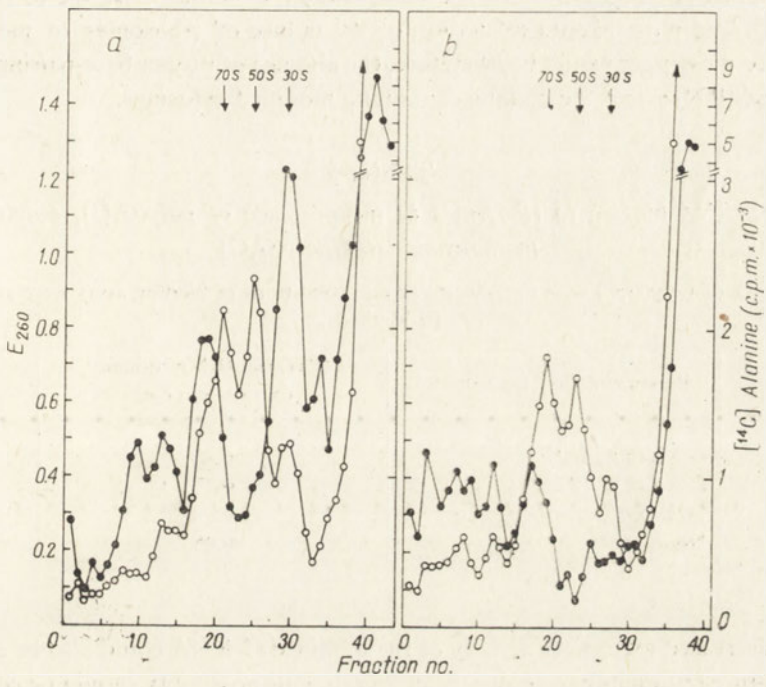

Fig. 3. Sucrose-gradient profiles of incorporating mixtures synthesizing [^{14}C]alanine-labelled polypeptides in the presence of (*a*), native f2 RNA and (*b*), f2 RNA modified with methoxyamine under non-denaturing conditions. RNA in experiment (*b*) was modified for 4 h at 37°C. Incorporation of [^{14}C]alanine was performed in 0.25 ml incubates for 30 min at 37°C. Mixtures were centrifuged at 39 000 r.p.m. for 2 h at 4°C. For other details see Methods. (○), E_{260}, (●), [^{14}C]alanine incorporation. Arrows indicate positions of 70S, 50S and 30S ribosomes centrifuged in a separate gradient.

et al., 1973). With prolonged time of RNA modification the relative amount of radioactivity corresponding to monosome and polysome fractions increased. In the case of the f2 RNA modified for 12 h as much as 67.5% of [^{14}C]alanine label remained attached to polysomes (Table 2).

The above presented results indicate that modified cytosine residues formed a block which prevented ribosomes from translating RNA message. Genetic data as well as the activity of nucleotides containing *N*-4-methoxy- or *N*-4-hydroxycytosine in replicating systems *in vitro* indicate that modified cytosines show base--pairing properties of both C and U (Phillips & Brown, 1967; Kochetkov & Budowsky, 1969; Singer & Fraenkel-Conrat, 1970; Banks *et al.*, 1971; Budowsky *et al.*, 1971).

However, the results of experiments of Singer & Fraenkel-Conrat (1970) on translation of methoxyamine-modified poly(C) and our data about the activity of methoxyamine-modified poly(G,C) (Table 3) indicate that at the level of translation this is not the case. As codon GUG in known to act as an initiator, f[^{3}H]Met-tRNA binding in the presence of methoxyaminated poly(G,C) could be expected to occur if modified cytosines had the base-pairing properties of uracil. It was found, however, that modified poly(G,C) did not stimulate binding of initiator tRNA to ribosomes (Table 3). The most likely explanation of the failure of ribosomes to move along a modified messenger would be, therefore, the absence of proper base-pairing between aminoacyl-tRNAs and the codons containing modified cytosines.

Table 3

f[^{3}H]*Met-tRNA binding to ribosomes in the presence of poly(G,U), poly(G,C) and methoxyaminated poly(G,C)*

Modification of poly(G,C) with methoxyamine and conditions of binding assay were as described in Methods.

Polymer added (μg/sample)	f[^{3}H]Met-tRNA bound (pmoles/sample)
Poly(G,U), 10.8	6.40
Poly(G,C), 13.2	0.77
Modified poly(G,C), 24.0	0.86
None	0.95

The decreased messenger activity of the modified f2 RNA could also be explained by premature termination of polypeptide chains.This possibility should be considered because methoxyamine is known as a mutagenic agent leading to C→T or C→U transitions (Phillips & Brown, 1967; Kochetkov & Budowsky, 1969). One could expect that modification of cytosine residues in codons such as CCA, CAG, CGA might result in generation of codons resembling functionally the termination codons UAA, UAG, UGA. This, however, would result in a decrease, rather than an increase, in the amount of ribosome-bound polypeptides in the systems programmed by modified RNA. The progressive increase in polysome- and monosome-bound radioactivity observed with time of f2 RNA modification (Table 2) and the results of experiments in which addition of suppressor tRNA preparations isolated from different suppressor strains of *E. coli* did not increase translation of modified f2 RNA (unpublished), make this possibility very unlikely.

SDS-polyacrylamide-gel electrophoresis of polypeptides synthesized *in vitro* in the presence of modified or unmodified f2 RNA also seems to indicate that modified cytosine residues blocked the elongation of growing polypeptides (Fig. 4). f2 RNA at zero time of modification by methoxyamine and unmodified f2 RNA after 20 h of incubation under control conditions directed the synthesis of both RNA replicase and coat polypeptides. Electrophoretic separation of polypeptides

coded by f2 RNAs modified for different time periods showed that synthesis of RNA replicase was damaged to a much greater extent than that of coat protein. After 5 h of modification only traces of completed replicase were produced while synthesis of coat polypeptide was still quite significant. After 12 h of modification, the synthesis of complete replicase was not observed while coat polypeptide was still produced. Histidine counts present in the coat protein region correspond most likely to some degraded or unfinished replicase polypeptides. The most likely explanation of these results is that the first methoxyamine hits would damage replicase cistron which is about 4 times as long as that of the coat protein (Capecchi, 1966; Weber *et al.*, 1966), preventing the synthesis of complete replicase.

It should be mentioned, however, that the possibility that methoxyamine might have affected the ability of f2 RNA to initiate RNA replicase synthesis, has not been excluded.

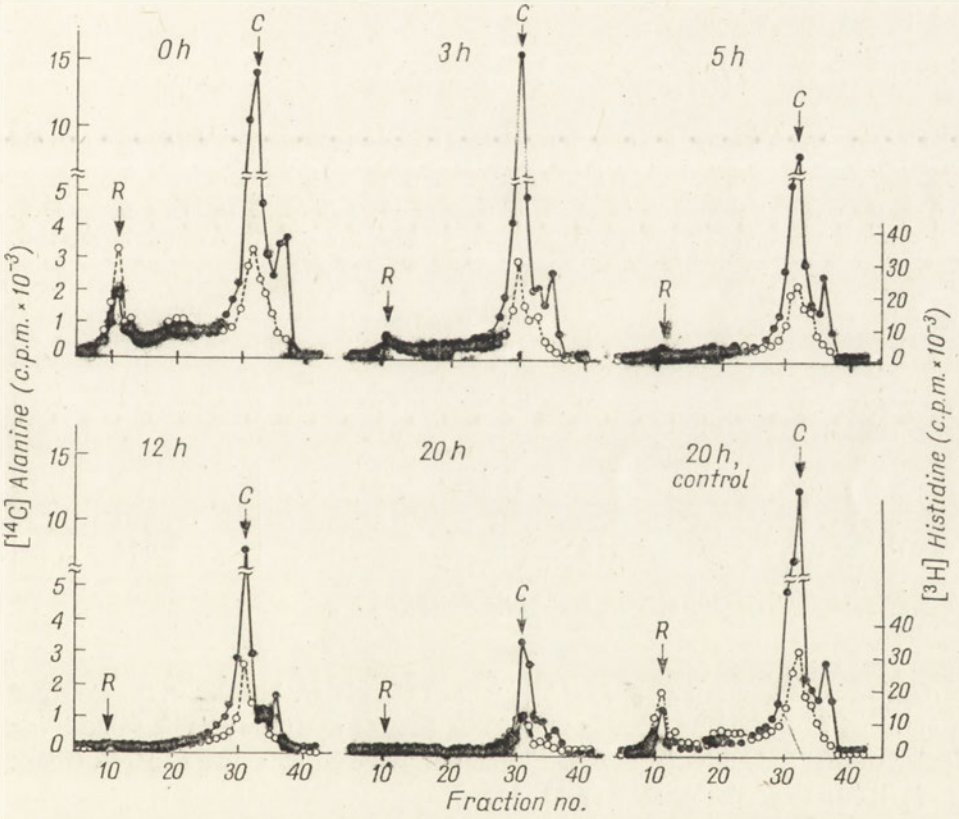

Fig. 4. Polyacrylamide-gel electrophoresis of [^{3}H]histidine- and [^{14}C]alanine-labelled polypeptides coded by f2 RNA modified with methoxyamine under non-denaturing conditions. The modification was performed for the time indicated. In control experiments, unmodified f2 RNA after incubation for 20 h at 21°C was used. For details see Methods. Arrows refer to the positions of RNA replicase (R) and coat protein (C).

Activity of methoxyamine-unfolded f2 RNA in initiation of polypeptide synthesis

We have reported previously (Filipowicz *et al.*, 1972) that f2 RNA modified with methoxyamine under denaturing conditions (in the presence of 6 M-guanidine·HCl) loses its ordered structure and is a very efficient template in stimulating f[^{3}H]Met-tRNA binding to ribosomes. The activity of f2 RNA extensively modified under these conditions exceeds about 50 times the activity of native f2 RNA when assayed for stimulation of initiation complex formation.

Table 4 shows some characteristics of f[^{3}H]Met-tRNA binding to ribosomes directed by methoxyamine-unfolded f2 RNA. Similarly to the binding directed by native RNA, it was dependent upon the addition of GTP and crude initiation factors. fMet-tRNA attached to ribosomes in the presence of methoxyamine-unfolded f2 RNA reacted readily with puromycin to form formylmethionylpuromycin, which indicates that specific initiation complexes were formed with fMet-tRNA located at P site on ribosome. After reaction with puromycin little f[^{3}H]Met-tRNA remained bound to ribosomes.

Table 4

f[^{3}H]Met-tRNA binding to ribosomes and f[^{3}H]Met-puromycin formation in the presence of methoxyamine-unfolded f2 RNA

f2 RNA was modified with methoxyamine in the presence of 6 M-guanidine·HCl for 5 days at 37°C. Conditions of binding assay and f[^{3}H]Met-puromycin formation were as described in Methods. RNA concentration was 6 μg/50 μl.

Incubation mixture	f[^{3}H]Met-tRNA bound to ribosomes (pmoles)	f[^{3}H]Met-puromycin formed (pmoles)
Complete	8.50	
GTP omitted	0.89	
Crude initiation factors omitted	0.65	
f2 RNA omitted	0.83	
Complete + puromycin added*	1.43	8.00
f2 RNA omitted, puromycin added*		0.57

* Puromycin was added after 20 min of incubation.

Optimum concentration of Mg^{2+} ions for fMet-tRNA binding in the presence of unfolded f2 RNA was found to be 5 mM (results not shown), this value being similar to that for initiation complex formation directed by native f2 RNA (Clark *et al.*, 1970; Zagórska & Szafrański, 1973).

Sucrose-density-gradient profiles of initiation complexes formed in the presence of methoxyamine-unfolded f2 RNA (Fig. 5) indicate that many new initiation codons in RNA molecule became accessible to ribosomes after its ordered structure had been destroyed. Initiation complexes were labelled with f[^{3}H]Met-tRNA and either with methoxyamine-unfolded f2 [^{14}C]RNA (Fig. 5a) or with f2 RNA modified

under denaturing conditions with radioactive [^{14}C]methoxyamine (Fig. 5b). In both cases polysome-like structures could be observed. This is in contrast with the results presented in Fig. 2 where only monosomes were observed when initiation

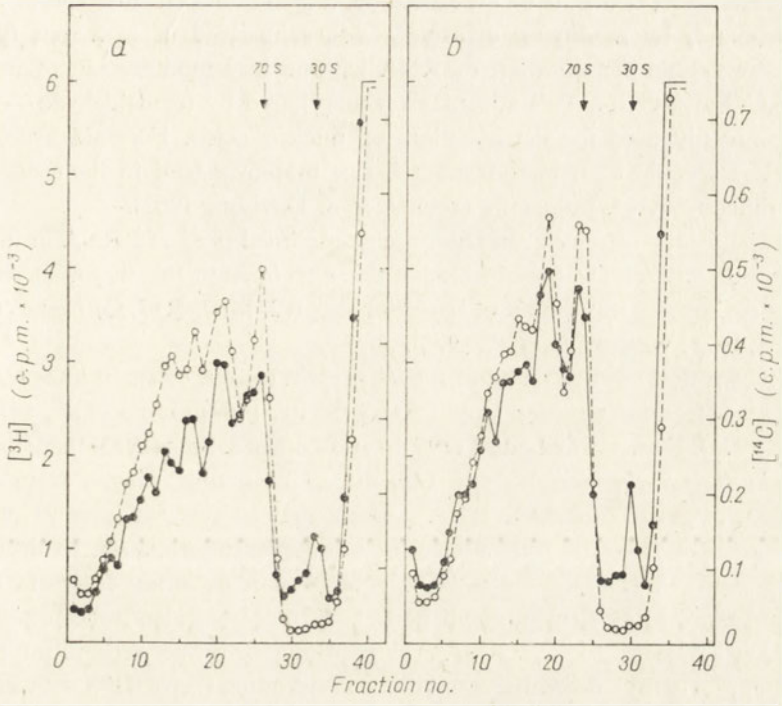

Fig. 5. Sucrose-gradient profiles of initiation complexes formed in the presence of f[^{3}H]Met-tRNA and f2 RNA modified with methoxyamine under denaturing conditions (in the presence of 6 M-guanidine·HCl). (*a*), f2 [^{14}C]RNA was modified with unlabelled methoxyamine; (*b*), unlabelled f2 RNA was modified with radioactive [^{14}C]methoxyamine. f2 RNA was modified for 5 days at 37°C. Binding of f[^{3}H]Met-tRNA to ribosomes was performed for 15 min at 25°C as described in Methods using 6 μg of f2 RNA/100 μl of the incubation mixture. Centrifugation of mixtures was carried out at 43 000 r.p.m. for 65 min at 4°C. For other details see Methods. Arrows indicate the positions of 70S and 30S ribosomes. (○), ^{3}H radioactivity; (●), ^{14}C radioactivity.

complex was formed in the presence of either native f2 RNA or RNA modified under non-denaturing conditions. From a comparison of the results shown in Figs. 2 and 5 it can be concluded that RNA ordered structure is a major factor preventing the binding of ribosomes at normally unfunctional initiation codons hidden in RNA secondary and/or tertiary structure. Destruction of this structure results in formation of initiation complexes at several sites within one f2 RNA molecule.

DISCUSSION

Specific modification of RNA proved to be a very valuable approach to elucidation of structural features of ribonucleic acids and molecular basis of RNA-protein interactions. This method was broadly used in studies of structure of different tRNAs

and their interactions with cognate aminoacyl-tRNA synthetases, as well as in structural studies of 5S ribosomal RNA (Cashmore *et al.*, 1971; Bellemare *et al.*, 1972; Goddard & Schulman, 1972; Litt & Greenspan, 1972; Chang, 1973).

Methoxyamine proved to be an especially valuable tool in this type of studies. This compound, by modifying cytosine residues involved in hydrogen bonding, leads to irreversible denaturation of RNA without any modification of initiation codons AUG and GUG. This advantage is not shared by formaldehyde — a compound commonly used for denaturation of nucleic acids. Formaldehyde, which is much less specific than methoxyamine, can moreover lead to the cross-linking between different RNA fragments (Freifelder & Davison, 1963).

In this paper the activity of methoxyamine-modified phage f2 RNA in initiation of polypeptide synthesis was studied, as at this step operate the major mechanisms regulating differential translation of bacteriophage (f2, MS2, R17, Qβ) genes (Kozak & Nathans, 1972; Weissmann *et al.*, 1973).

It is known that the beginning of a coat protein cistron exists in a form of loop exposing at its top the initiation codon AUG (Steitz, 1969; Gupta *et al.*, 1970; Min Jou *et al.*, 1972). Findings of Lodish (1970b) and Leffler & Szer (1973) that ribosomes from *Bacillus stearothermophilus* and *Caulobacter crescentus* cannot translate the coat protein gene of f2 or MS2 RNA indicate that sterical exposure of initiation codon is probably not the only prerequisite for efficient initiation to occur. The results of Steitz (1973) that the isolated coat protein initiation fragment of R17 RNA is no more so efficiently bound by *E. coli* ribosomes also indicate that some additional structural features of RNA are important for proper recognition process.

Revel *et al.* (1972) studied the interaction of initiation factor IF-3 with initiation site of coat protein cistron. They presented evidence that the *E. coli* IF-3, which is supposed to be responsible for recognition of initiation sites, may interact with cytosine-rich sequence situated at 5′ side of AUG codon initiating the coat protein synthesis. Data shown in the present work indicate that modification of all RNA cytosines accessible for methoxyamine attack under non-denaturing conditions did not affect the process of initiation (Table 1, Fig. 2). Thus it seems possible that the cytosine residues are not involved in the recognition of initiation site for coat protein synthesis.

Results of experiments with methoxyamine-unfolded f2 RNA prove that the ordered structure is a decisive factor preventing the binding of ribosomes at the AUG or GUG sequences other than those functioning normally as the initiation codons. Initiation complexes formed in the presence of methoxyamine-unfolded f2 RNA (Fig. 5) were of polysome type indicating that, under these conditions, initiation can occur at several distinct places in one RNA molecule. It has been shown recently that ribosomes freed of initiation factors can recognize AUG or GUG codons in methoxyamine-unfolded f2 RNA (Szkopińska *et al.*, 1975). In contrast to this, initiation complexes formed in the presence of native f2 RNA and initiation factors consisted only of monosomes (Fig. 2).

Lodish (1970a) using formaldehyde-treated f2 RNA has shown that destruction of RNA ordered structure permits a very efficient and independent initiation of

translation of all three RNA cistrons. Also studies with nuclease-degraded phage RNA (Lodish, 1968; Voorma *et al.*, 1971) or RNA heated under various ionic conditions (Fukami & Imahori, 1971) have led to the conclusion that RNA ordered structure is a factor both regulating expression of three phage genes and preventing the initiation of polypeptide synthesis at non-functional AUG or GUG codons.

It is worth mentioning that even RNA which does not normally exhibit messenger activity, e.g. ribosomal RNA or tRNA, becomes active as template in protein synthesis when its ordered structure is loosened (Mc Carthy *et al.*, 1966; Dunlap & Rottman, 1972; Filipowicz *et al.*, 1972).

Although initiation of translation of f2 RNA treated with methoxyamine under non-denaturing conditions was not impaired, modification of RNA affected strongly the synthesis of complete phage polypeptides (Figs. 1 and 4). Experiments in which release of nascent polypeptides synthesized in the presence of native and modified f2 RNA was compared (Fig. 3, Table 2) indicate that failure of methoxyaminated RNA to direct synthesis of complete proteins was probably due to the blocking of elongation process by modified cytosines. This is in agreement with the results of Singer & Fraenkel-Conrat (1970) who found that modification of poly(C) with methoxyamine or hydroxylamine leads to a substantial decrease of its messenger activity. Therefore one can assume that at the level of codon - anticodon interaction, base pairing of modified cytosine with adenine in tRNA is not efficient enough to allow translation.

The block in elongation process observed in our experiments could be also due to modified cytosines that contained saturated 5,6 double bond (*N*-4-methoxy-6--methoxyamino-5,6-dihydrocytosine). It is known that irradiated poly(U) containing saturated 5,6 double bond loses its activity as mRNA (Rottman & Cerutti, 1966). Similar results have been obtained with u.v.-irradiated R17 RNA containing uridine photohydrates (Remsen & Cerutti, 1972).

The results presented in this paper show that the *E. coli* ribosomes formed polysomes with methoxyamino-unfolded f2 RNA, whereas in the presence of either native f2 RNA or RNA modified under non-denaturing conditions only monosomes were observed. The puromycin reaction indicates that in the initiation complexes formed in the presence of unfolded f2 RNA, f[^{3}H]Met-tRNA occupies the P site on ribosomes. Direct evidence is presented that ordered structure of f2 RNA is a decisive factor preventing initiation of translation at non-functional AUG or GUG codons.

Modification of f2 RNA with methoxyamine strongly inhibits the ability of RNA to code for phage proteins. This is probably due to the blocking of elongation process by modified cytosine residues present in f2 RNA.

REFERENCES

Banks, G. R., Brown, D. M., Streeter, D. G. & Grossman, L. (1971) *J. Mol. Biol.*, **60**, 425 - - 439.

Bellemare, G., Jordan, B. R., Rocca-Serra, J. & Monier, R. (1972) *Biochimie*, **54**, 1453 - 1466.

Bray, A. G. (1960) *Anal. Biochem.*, **1**, 279 - 285.
Bretscher, M. S., Goodman, H. M., Menninger, J. P. & Smith J. D. (1965) *J. Mol. Biol.*, **14**, 634 - 639.
Budowsky, E. I., Sverdlov, E. D. & Spasokukotskaya, T. N. (1971) *FEBS Lett.*, **17**, 336 - 338.
Capecchi, M. R. (1966) *J. Mol. Biol.*, **21**, 173 - 193.
Capecchi, M. R. (1967) *J. Mol. Biol.*, **30**, 213 - 217.
Cashmore, A. R., Brown, D. M. & Smith, J. F. (1971) *J. Mol. Biol.*, **59**, 359 - 367.
Chang, S. E. (1973) *J. Mol. Biol.*, **75**, 533 - 547.
Chroboczek, J., Pietrzak, M. & Zagórski, W. (1973) *J. Virol.*, **12**, 230 - 240.
Clark, B. F. C., Marcker, K. A., Rudland, P. S., Anderson, J. S. & Dube, S. K. (1970) in *Macromolecules, Biosynthesis and Function, Proc. 6th Meet. Fed. Eur. Biochem. Soc.* (S. Ochoa, C. Asensio, C. F. Heredia & D. Nachmansohn, eds.) pp. 73 - 84. Academic Press, London.
Dunlap, B. E. & Rottman, F. (1972) *Biochim. Biophys. Acta*, **281**, 371 - 380.
Filipowicz, W., Wodnar, A., Zagórska, L. & Szafrański, P. (1972) *Biochem. Biophys. Res. Commun.*, **49**, 1272 - 1279.
Freifelder, D. & Davison, P. F. (1963) *Biophys. J.*, **3**, 49 - 56.
Fukami, H. & Imahori, K. (1971) *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 570 - 573.
Goddard, J. P. & Schulman, L. H. (1972) *J. Biol. Chem.*, **247**, 3864 - 3867.
Goodman, D. & Matzura, H. (1971) *Anal. Biochem.*, **42**, 481 - 486.
Gupta, S. L., Chen, J., Schaefer, L., Lengyel, P. & Weissmann, S. M. (1970) *Biochem. Biophys. Res. Commun.*, **39**, 883 - 889.
Haenni, A. L., & Lucas-Lenard, J. (1968) *Proc. Natl. Acad. Sci. U.S.A.*, **61**, 1363 - 1369.
Kochetkov, N. K. & Budowsky, E. I. (1969) *Progr. Nucleic Acid Res. Mol. Biol.*, **9**, 403 - 438.
Kozak, M. & Nathans, D. (1972) *Bacteriol. Rev.*, **36**, 109 - 134.
Leder, P. & Bursztyn, H. (1966) *Biochem. Biophys. Res. Commun.*, **25**, 233 - 238.
Leffler, S. & Szer, W. (1973) *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2364 - 2368.
Lelong, J. C., Grunberg-Manago, M., Dondon, J., Gros, D. & Gros, F. (1970) *Nature (London)*, **226**, 505 - 510.
Litt, M. & Greenspan, C. M. (1972) *Biochemistry*, **11**, 1437 - 1442.
Lodish, H. F. (1968) *J. Mol. Biol.*, **32**, 681 - 685.
Lodish, H. F. (1970a) *J. Mol. Biol.*, **56**, 627 - 632.
Lodish, H. F. (1970b) *Nature (London)*, **226**, 705 - 707.
Lodish, H. F. & Robertson, H. D. (1969) *Cold Spring Harbor Symp. Quant. Biol.*, **34**, 655 - 673.
Mans, R. J. & Novelli, G. D. (1961) *Arch. Biochem. Biophys.*, **94**, 48 - 53.
McCarthy, B. J., Holland, J. J. & Buck, C. A. (1966) *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 683 - 691.
Min Jou, W., Haegeman, G., Ysebaert, M. & Fiers, W. (1972) *Nature (London)*, **237**, 82 - 87.
Nathans, D., Notani, G., Schwartz, J. H. & Zinder, N. D. (1962) *Proc. Natl. Acad. Sci. U.S.A.*, **48**, 1424 - 1431.
Nirenberg, M. & Leder, P. (1964) *Science*, **145**, 1399 - 1407.
Ota, Y., Ohta, T. & Imahori, K. (1972) *J. Biochem. (Tokyo)*, **71**, 743 - 746.
Phillips, J. H. & Brown, D. M. (1967) *Progr. Nucleic Acid Res. Mol. Biol.*, **7**, 349 - 368.
Remsen, J. F. & Cerutti, P. A. (1972) *Biochem. Biophys. Res. Commun.*, **48**, 430 - 436.
Revel, M., Groner, Y., Pollack, Y., Berissi, H. & Herzberg, M. (1972) *FEBS Symposium*, **23**, 237 - 249.
Rottman, F. & Cerutti, P. A. (1966) *Proc. Natl. Acad. Sci. U.S.A.*, **55**, 960 - 966.
Roufa, D. J. & Leder, P. (1971) *J. Biol. Chem.*, **246**, 3160 - 3167.
Singer, B. & Fraenkel-Conrat, H. (1970) *Biochemistry*, **9**, 3694 - 3701.
Steitz, J. A. (1968) *J. Mol. Biol.*, **33**, 923 - 936.
Steitz, J. A. (1969) *Nature (London)*, **224**, 957 - 964.
Steitz, J. A. (1973) *Proc. Natl. Acad. Sci. U.S.A.*, **70**, 2605 - 2609.
Sugyiama, T., Herbert, R. R. & Hartman, K. A. (1967) *J. Mol. Biol.*, **25**, 455 - 463.

Szkopińska, A., Zagórski, W., Zagórska, L. & Szafrański, P. (1975) *Eur. J. Biochem.*, **60**, 289 - 294.
Takanami, M. & Yan, Y. (1965) *Proc. Natl. Acad. Sci. U.S.A.*, **54**, 1450 - 1458.
Tanaka, N., Kinoshita, T. & Masukawa, M. (1968) *Biochem. Biophys. Res. Commun.*, **30**, 278 - 283.
Weber, K., Notani, G., Winkler, N. & Konigsberg, W. (1966) *J. Mol. Biol.*, **20**, 423 - 425.
Weissmann, C., Billeter, M. A., Goddman, H. M., Hindley, J. K. & Weber, H. (1973) *Ann. Rev. Biochem.*, **42**, 303 - 328.
Wodnar-Filipowicz, A., Filipowicz, W. & Szafrański, P. (1975) *Acta Biochim. Polon.*, **22**, 11 - 24.
Voorma, H. O., Benne, R. & den Hertog, T. J. A. (1971) *Eur. J. Biochem.* , **18**, 451 - 462.
Zagórska, L. & Szafrański, P. (1973) *Acta Biochim. Polon.*, **20**, 101 - 111.
Zagórski, W., Filipowicz, W., Wodnar, A., Leonowicz, A., Zagórska, L. & Szafrański, P. (1972) *Eur. J. Biochem.*, **25**, 315 - 322.

AKTYWNOŚĆ RNA FAGA f2 MODYFIKOWANEGO DZIAŁANIEM METOKSYAMINY W PROCESACH INICJACJI I ELONGACJI SYNTEZY BIAŁEK FAGOWYCH

Streszczenie

1. W układzie bezkomórkowym z *Escherichia coli* badano aktywność matrycową RNA faga f2 modyfikowanego metoksyaminą w warunkach niedenaturujących. Stwierdzono, że modyfikacja f2 RNA obniża jego zdolność do stymulacji włączania aminokwasów oraz syntezy białek fagowych. Synteza replikazy RNA jest silniej uszkadzana niż synteza białka kapsydu. Ustalono, że przyczyną obniżenia aktywności matrycowej f2 RNA jest blokowanie procesu elongacji przez zmodyfikowane cytozyny znajdujące się w łańcuchu RNA.

2. Przyłączanie rybosomów do miejsca inicjacyjnego cistronu białka kapsydu f2 RNA nie ulega zakłóceniu po modyfikacji RNA metoksyaminą w warunkach niedenaturujących. Świadczy o tym niezmienione przyłączanie f[^{3}H]Met-tRNA i [^{14}C]alanylo-tRNA do rybosomów w obecności zmodyfikowanego f2 RNA.

3. Rozplecenie struktury przestrzennej f2 RNA w wyniku modyfikacji metoksyaminą w obecności guanidyny prowadzi do znacznego wzrostu jego aktywności w stymulowaniu przyłączania fMet-tRNA do rybosomów. W gradiencie sacharozy obserwuje się kompleksy inicjacyjne złożone z wielu rybosomów przyłączonych do jednej cząsteczki f2 RNA. Świadczy to o przyłączaniu się rybosomów do kodonów inicjujących, które normalnie ukryte są w strukturze przestrzennej natywnego f2 RNA. fMet-tRNA przyłączony do rybosomów w obecności rozplecionego f2 RNA reaguje wydajnie z puromycyną, co przemawia za jego lokalizacją w peptydylowym miejscu P na rybosomach.

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RNA POLYMERASES I AND II IN GERMINATING WHEAT EMBRYO*

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1. The RNA polymerase I was practically absent in the resting embryos and appeared several hours after the beginning of imbibition, whereas the level of polymerase II was high in the resting embryos and did not increase significantly during the imbibition phase.

2. Incorporation *in vivo* of [^{14}C]valine into polymerase I and II indicated that the synthesis of RNA polymerase I is initiated in germinating embryos much earlier than that of RNA polymerase II.

3. It is suggested that RNA polymerase II is stored in resting wheat embryos to support mRNA synthesis at the onset of germination, whereas the RNA polymerase I activity appears at a further stage of germination.

Multiple forms of RNA polymerase (ribonucleoside triphosphate : RNA nucleotidyl transferase, EC 2.7.7.6) were isolated from a variety of eukaryotes, including higher plants (Horgen & Key, 1973; Jendrisak & Becker, 1973; Gore & Ingle, 1974; Fabisz-Kijowska *et al.*, 1975). The characteristics of RNA polymerases I, II and III and their participation in the transcription of ribosomal, messenger and transfer RNA sequences were described (Chambon *et al.*, 1970; Roeder & Rutter, 1970; Sklar *et al.*, 1975). Little is, however, known about changes in the proportions of the multiple enzymes during development. In plant tissues, the ratio of RNA polymerase II to RNA polymerase I decreases during cell differentiation (Rizzo & Cherry, 1975; Mehta *et al.*, 1975). The level of RNA polymerase II in isolated wheat embryos was found to drop with the germination time (Mazuś, 1973). The present work was undertaken to compare changes in RNA polymerase I and II in the embryos of germinating wheat grains.

MATERIALS AND METHODS

Plant material and germination. Wheat grains (*Triticum aestivum, ssp. vulgare, var. lutescens Al., cv. Kutnowska*) were soaked for 5 min in 1% sodium hypochlorite solution and rinsed thoroughly with sterile water. Intact seeds were incubated

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at 22°C in the dark either in water or [1-^{14}C]valine solution (1 μCi/ml). At appropriate times of germination (0 to 36 h), embryos were excised and used for extraction of RNA polymerase.

Extraction and fractionation of RNA polymerase. The procedure was similar to that described by Roeder (1974). A sample of 200 embryos, weighing 0.4 to 2.9 g (depending on the age), was frozen and ground in a mortar kept in a solid CO_2--ethanol bath. Further steps were performed at 0 - 4°C. The powdered tissue was extracted with 10 - 12 ml of extraction buffer (50 mM-Tris-HCl, pH 8.1, 5% glycerol, 5 mM-$MgCl_2$, 0.1 mM-EDTA, 0.1 mM-mercaptoethanol) and filtered through cheese--cloth to remove cell debris. The crude extract was used directly for assays or was further purified. To solubilize the enzymes, saturated $(NH_4)_2SO_4$ was added to a final concentration of 0.3 M and the viscous mixture was homogenized in an Ultra--Turrax homogenizer until the lysate was no longer viscous. The homogenate was centrifuged at 20 000 *g* for 20 min. RNA polymerase was precipitated from the supernatant with $(NH_4)_2SO_4$ at 0.5 saturation and, after being desalted on Sephadex G-25, the sample containing approximately 10 mg of protein was applied to a DEAE--Sephadex (A-25) column (1.2 × 10 cm). The column was washed with 10 ml of the extraction buffer containing 0.05 M-$(NH_4)_2SO_4$, and the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ (0.05 - 0.50 M) in 100 ml of the same buffer, fractions of 1 ml being collected. The final preparations of RNA polymerases I and II were purified 30- and 50-fold, respectively. Protein content and ^{14}C radioactivity were measured in the successive fractions. For the enzyme assay, bovine serum albumin was added to all fractions to a concentration of 1 mg per 1 ml; the activity was measured immediately either in the presence or in absence of α-amanitin.

Assay of RNA polymerase. The standard reaction mixture contained, in a final volume of 0.1 ml, 12 μmoles of Tris-HCl (pH 8.0), 25 μg of heat-denatured calf thymus DNA, 0.05 μmole each of ATP, CTP, GTP, 2.3 μCi of [5-^{3}H]UTP; 0.05 μmole of $MnCl_2$, 0.3 μmole of $MgCl_2$, 0.25 μmole of spermidine, and 0.05 ml of the enzyme solution. α-Amanitin was added to a concentration of 1 μg per 1 ml. The mixture was incubated at 30°C for 10 min. The reaction was started by the addition of the enzyme solution and terminated by pipetting onto filter paper disc followed by immersion in the ice-cold 5% trichloroacetic acid solution containing 1% $Na_4P_2O_7$. The disc was washed five times for 5 min each in this solution, twice in 99.8% ethanol, air-dried, and used for the measurement of radioactivity in a Packard liquid scintillation spectrometer.

The activity of α-amanitin-insensitive RNA polymerase I (Chambon, 1974) was determined directly in the presence of the inhibitor in the assay mixture and that of α-amanitin-sensitive RNA polymerase II was calculated as the difference between the total and the α-amanitin-insensitive activities.

Other methods. Protein was estimated according to Lowry *et al.* (1951). Incorporation of [^{14}C]valine to RNA polymerases was measured in a Packard counter, using 500 μl fractions from the DEAE-Sephadex A-25 columns, and 5 ml of Bray scintillator.

Reagents. Sephadex G-25 and DEAE-Sephadex (A-25) were obtained from Pharmacia (Uppsala, Sweden). Calf thymus DNA, spermidine hydrochloride, ATP, CTP, GTP as well as 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]--benzene were supplied by Sigma Chem., Co. (St. Louis, Mo., U.S.A.). Bovine albumin, crystallized, was from B.D.H. (Poole, Dorset, England), α-amanitin from C.H. Boehringer Sohn (Ingelheim am Rhein, G.F.R.) and Tris from Fluka AG (Buchs SG, Switzerland). [5-^{3}H]Uridine-5′-triphosphate, ammonium salt (spec. act. 19.8 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, Bucks, England), and [1-^{14}C]DL-valine (spec. act. 2.7 mCi/mmole) from the Institute for Nuclear Research (Świerk, Poland). Other reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

RESULTS

Crude extracts from wheat embryos germinated for different time periods were found to contain RNA polymerases I and II in varying proportions, as judged by α-amanitin sensitivity (Fig. 1). The activity of polymerase II (α-amanitin-sensitive) was high in the quiescent embryo, remained at an almost unchanged level for the first 24 hours, an increased sharply thereafter. On the other hand, the activity of polymerase I (α-amanitin-insensitive) was very low in the resting embryo and, after a short lag period, increased gradually over the investigated time period.

The two polymerase activities were separated from each other on DEAE-Sephadex column (Fig. 2). The enzymatic activity of the fraction constituting peak II was found

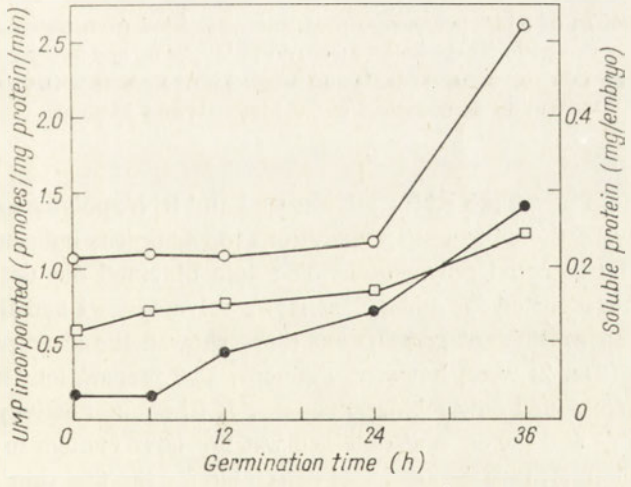

Fig. 1. Changes in the activities of RNA polymerases I and II in wheat embryo during germination. At the indicated times of germination, embryos were harvested and extracted as described in Materials and Methods. The crude extract was assayed for protein content (□). The activity of α--amanitin-insensitive RNA polymerase I (●) was determined directly, and that of α-amanitin-sensitive RNA polymerase II (○) was calculated as the difference between total activity and α-amanitin--insensitive activity.

to be sensitive to α-amanitin, while the second fraction (peak I) was insensitive to this compound. The separated activities were further characterized with respect to the requirements for template, divalent cation and ionic strength, and were found

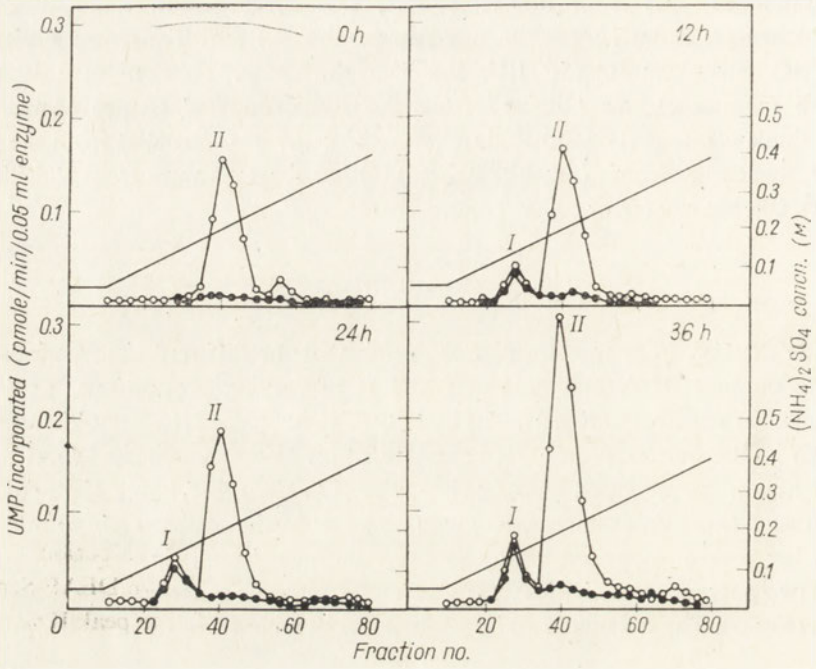

Fig. 2. Elution profiles of RNA polymerase preparations isolated from resting and germinating wheat embryos. The preparations were isolated from dry, 12 h-, 24 h-, and 36 h-germinated embryos, chromatographed on DEAE-Sephadex (A-25) and assayed in the presence (●) or absence (○) of α-amanitin as described under Materials and Methods.

to correspond, respectively, to RNA polymerase I and RNA polymerase II (Table 1). The insensitivity of the enzymatic preparations to rifampicin indicates the lack of contamination by bacterial polymerases. The data obtained did not differ significantly from those reported for other plant RNA polymerases I and II, and the preparations isolated at different germination times showed the same properties. The elution profiles (Fig. 2) were, however, different. The preparations from ungerminated embryos contained only RNA polymerase II (the minor activity in the second peak was unstable and the amount obtained was not large enough to make its characterization possible); polymerase I was detectable for the first time 12 h after the beginning of imbibition, and then its activity continued to increase.

The delayed appearance of polymerase I activity raised the possibility that the enzyme was absent in the resting embryos and was synthesized *de novo* when germination started. To get more information, incorporation of [^{14}C]valine *in vivo* into both RNA polymerases was measured (Fig. 3). The data presented suggest that RNA

Table 1

Characteristics of RNA polymerases of germinating wheat embryos following separation on DEAE-Sephadex

Fractions of peak I (no. 20 - 30) containing the polymerase I, and fractions of peak II (no. 35 - 50) containing the polymerase II (cf. Fig. 2, 36 h) were pooled and tested for the activity under the indicated conditions. The „complete" assay system corresponded to the standard incubation conditions. The enzymatic activities found in this system for peaks I and II were, respectively, 306 and 38 pmoles of UMP incorporated/mg protein/min.

Assay system	RNA polymerase	
	Peak I	Peak II
"Complete"	100	100
−DNA	10	8
Native DNA used	50	50
−ATP, CTP, GTP	2	3
$+(NH_4)_2SO_4$, 0.1 M	51	108
+α-amanitin, 0.1 μg/ml	96	8
+α-amanitin, 10 μg/ml	95	6
+Rifampicin, 10 μg/ml	114	110
$-Mg^{2+}$, $-Mn^{2+}$	5	6
$+Mg^{2+}$, 1 mM	48	30
$+Mg^{2+}$, 5 mM	100	64
$+Mg^{2+}$, 10 mM	103	100
$+Mn^{2+}$, 0.5 mM	100	94
$+Mn^{2+}$, 2.5 mM	85	24
$+Mn^{2+}$, 10 mM	47	5

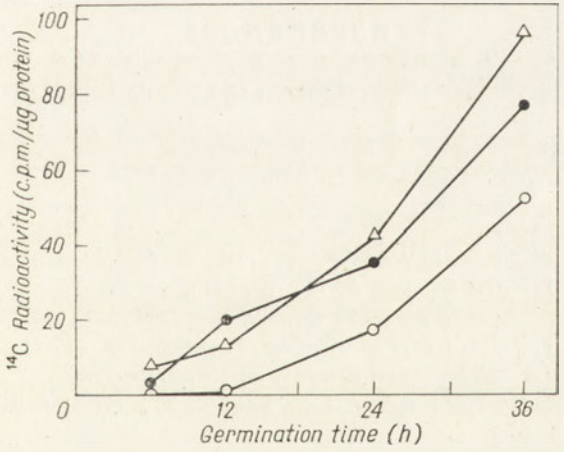

Fig. 3. [^{14}C]Valine incorporation *in vivo* into wheat embryo RNA polymerases I (●) and II (○), and into total soluble protein (△). Specific radioactivities of RNA polymerases I and II were calculated from the protein content and ^{14}C radioactivity found in the eluted fractions corresponding to the enzyme activity peaks I and II, respectively (see Fig. 2). Specific radioactivity of total soluble protein was calculated from the protein content and acid-precipitable ^{14}C radioactivity found in the crude enzyme extract.

polymerase I is synthesized as early as at the sixth hour of germination, whereas RNA polymerase II, present in the quiescent embryo, was not synthesized *de novo* until the 12th hour of germination.

DISCUSSION

Three independent lines of evidence: (a) changes in total α-amanitin-sensitive and α-amanitin-insensitive activities, (b) DEAE-Sephadex elution profiles, and (c) labelling experiments, consistently indicate that RNA polymerases I and II behaved in germinating wheat embryo in different ways. The activity of RNA polymerase I appears to be very low or absent in quiescent wheat embryo and arises, probably by *de novo* synthesis, after germination starts. On the other hand, RNA polymerase II is present in the resting embryo at a rather high level and its activity does not increase until later stages of germination. It seems reasonable to assume that the enzyme stored in the ungerminating embryo is necessary to catalyse the RNA synthesis that is induced in immediate response to the environmental stimuli for germination, whereas RNA polymerase I does not seem to be indispensable for this process. In view of the known functions of RNA polymerases I and II (Chambon *et al.*, 1970; Roeder & Rutter, 1970; Sklar *et al.*, 1975), the synthesis of mRNA, rather than rRNA, may be expected to occur in embryos at the onset of germination. Preliminary data on [^{14}C]uridine incorporation *in vivo* (Dobrzańska *et al.*, 1973) indicate that this assumption may be true for germinating wheat embryos.

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REFERENCES

Chambon, P. (1974) in *The Enzymes* (P. D. Boyer, ed.) vol. 10, pp. 295 - 297. Academic Press, New York.

Chambon, P., Gissinger, F., Mandel, J. L., Kedinger, C., Gniazdowski, M. & Meihlac, M. (1970) *Cold Spring Harbor Symp. Quant. Biol.*, **35**, 693 - 707.

Dobrzańska, M., Tomaszewski, M., Grzelczak, Z., Rejman, E. & Buchowicz, J. (1973) *Nature (London)*, **244**, 507 - 509.

Fabisz-Kijowska, A., Dullin, P. & Walerych, W. (1975) *Biochim. Biophys. Acta*, **390**, 105 - 116.

Gore, J. R. & Ingle, J. (1974) *Biochem. J.*, **143**, 107 - 113.

Horgen, P. A. & Key, J. L. (1973) *Biochim. Biophys. Acta*, **294**, 227 - 235.

Jendrisak, J. & Becker, W. M. (1973) *Biochim. Biophys. Acta*, **319**, 48 - 54.

Jendrisak, J. J. & Becker, W. M. (1974) *Biochem. J.*, **139**, 771 - 777.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265 - 275.

Mazuś, B. (1973) *Phytochemistry*, **12**, 2809 - 2813.

Mehta, S. L., Lodha, M. L., Naik, M. S. & Sigh, J. (1975) *Phytochemistry*, **14**, 2145 - 2146.

Rizzo, P. J. & Cherry, J. H. (1975) *Plant Physiol.*, **55**, 574 - 577.

Roeder, R. G. (1974) *J. Biol. Chem.*, **249**, 241 - 248.

Roeder, R. G. & Rutter, W. J. (1970) *Proc. Natl. Acad. Sci. U.S.A.*, **65**, 675 - 682.

Sklar, V. E. F., Schwartz, L. B. & Roeder, R. G. (1975) *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 348 - 352.

RNA POLIMERAZY I i II W ZARODKU KIEŁKUJĄCEJ PSZENICY

Streszczenie

1. Aktywność polimerazy I była praktycznie nieobecna w suchych zarodkach i pojawiała się dopiero po kilku godzinach kiełkowania. Poziom polimerazy II był wysoki w suchych zarodkach i nie ulegał większemu wzrostowi w czasie imbibicji.

2. Wcielanie *in vivo* [^{14}C]waliny do polimerazy I i II wskazuje, że synteza polimerazy I rozpoczyna się w kiełkujących zarodkach znacznie wcześniej niż synteza polimerazy II.

3. Sugeruje się, że polimeraza II jest przechowywana w suchych zarodkach pszenicy dla potrzeb syntezy mRNA w najwcześniejszej fazie kiełkowania, podczas gdy aktywność polimerazy I powstaje w późniejszej fazie kiełkowania.

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TRYPSIN INHIBITORS IN BOVINE LUNG AND PANCREAS DURING DEVELOPMENT

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1. The activity of trypsin inhibitors in lung and pancreas increased with age of the animals.

2. In foetal lung the activity of the basic Kunitz-type inhibitor was 1% that, and in calf lung 5 - 30% that, found in adult animals.

3. In foetal pancreas the basic Kunitz-type inhibitor was not detected, and in calf pancreas its activity was much lower than in adult animals. The activity of the acidic Kazal-type inhibitor in foetal pancreas was significantly lower than in calf or adult animal.

Among the polypeptide inhibitors of proteases, bovine basic trypsin inhibitor of the Kunitz type (Kunitz & Northrop, 1936) and acidic trypsin inhibitor of the Kazal type (Kazal *et al.*, 1948) have been the most extensively studied. Either of these inhibitors consists of a single polypeptide chain with three disulphide bonds and their molecular weight is closely similar. The trypsin-specific Kazal inhibitor has been found to occur in pancreas of all the animal species so far studied, whereas the basic inhibitor of the Kunitz type which, in addition to trypsin, inhibits chymotrypsin, kallikrein, plasmin and acrosine, was found only in ruminants — both in pancreas and other tissues. This inhibitor was isolated, among others, from bovine lung (Anderer & Hörnle, 1966), ovary (Chauvet & Acher, 1972), heart, thyroid gland, pituitary posterior lobe, kidney and rumen mucosa (Wilusz *et al.*, 1973), and from spleen and spleen capsula (Łomako & Wilusz, 1974).

It is assumed that in pancreas the acidic Kazal inhibitor protects trypsinogen against premature activation, there are, however, no data concerning the role of the basic Kunitz inhibitor. Vogel *et al.* (1968), taking into account the occurrence of this inhibitor only in ruminants and its appearance only after the birth of the animals, suggested that it is closely connected with the presence in the alimentary tract of microorganisms or their metabolism products.

The aim of the present work was to examine the activity of the two trypsin inhibitors in pancreas, and of the Kunitz type inhibitor in lung, during bovine development.

MATERIALS AND METHODS

Reagents. *N*-Benzoyl-L-arginine ethyl ester hydrochloride (BAEE) was from Fluka AG. (Buchs S.G., Switzerland). Other reagents were from the same sources as in the previous work (Wilusz *et al.*, 1973).

Material. Pancreas and lung of adult animals, 3 - 4-month-old calves and foetuses at 9 and 6 months of gestation, were obtained from the slaughterhouse immediately after killing of the animals, brought to the laboratory in ice and immediately used for isolation of the inhibitor(s).

Isolation of the inhibitors. From pancreas, the basic and acidic inhibitors were extracted together with trichloroacetic acid and separated by CM-cellulose column chromatography by the method elaborated by Wilimowska-Pelc *et al.* (1973).

From lung, the basic inhibitor was extracted with 5% NaCl in 0.2 M-HCl and purified as described by Wilusz *et al.* (1973). Following chromatography on CM--cellulose, the inhibitor was further purified by affinity chromatography on Sepharose 4B bound with trypsin after Kassell & Marciniszyn (1971) except that 0.01 M-HCl (not containing either NaCl or $CaCl_2$) was applied for elution.

Polyacrylamide-gel electrophoresis was performed after Reisfeld *et al.* (1962) in 15% gel at pH 4.5. The protein was stained with 1% Amido Black in 7% acetic acid.

Inhibitor activity toward trypsin and chymotrypsin was determined after Kunitz (1947) as described previously (Wilusz *et al.*, 1973) and expressed in units, 1 unit corresponding to 1 mg of inhibited enzyme. The calculations were based on 50% inhibition. The activity toward kallikrein was estimated according to Brown (1960) after 15 min preincubation of the inhibitor with enzyme.

Protein determination. Trypsin, chymotrypsin and basic trypsin inhibitor were determined at 280 nm; for calculations, the following specific extinction coefficients were used: 0.67 for trypsin (Laskowski & Laskowski, 1954), 0.495 for chymotrypsin (Wu & Laskowski, 1955), and 1.2 for the basic inhibitor (Kassell *et al.*, 1963).

RESULTS AND DISCUSSION

In the crude preparations from adult bovine pancreas the total trypsin inhibitor activity was almost twice as high as in calf pancreas, and about five times as high as in foetal pancreas (Table 1). To determine the contribution in these changes of the basic and acidic inhibitors, they were separated by CM-cellulose chromatography (Fig. 1). The acidic inhibitor emerged at 0.05 M and the basic one at 0.5 M acetate buffer concentration. In adult pancreas, the activity of the two inhibitors calculated per 100 g of fresh tissue, was similar (Table 1). In calf pancreas, the activity of the basic inhibitor was much lower than in adult animals, and in

Table 1

Activity of trypsin inhibitors in bovine pancreas during development

The inhibitor activity is expressed in units/100 g of fresh tissue. Results of one representative experiment are given.

Step of preparation	Foetal		Calf 3 - 4 months	Adult
	6 months	9 months		
5% trichloroacetic acid extract	1.4	2.1	4.0	7.0
Ppt. at 0.8 $(NH_4)_2SO_4$ sat. (dialysed against 0.01 M-acetate buffer, pH 5)	0.97	1.5	2.8	4.8
CM-cellulose chromatography				
acidic (Kazal) inhibitor	0.0	0.85	1.6	1.7
basic (Kunitz) inhibitor	0.0	0.0	0.55	1.5

pancreas of 9- and 6-month foetuses it was not detected at all. Since the amount of the material available was only 20 and 5 g, respectively, the presence of the basic inhibitor in foetal pancreas cannot be excluded.

The activity of the acidic Kazal inhibitor varied to a much smaller extent. In pancreas of adult animals and that of calf, the activity was similar, and in the 9-month foetus it was smaller by nearly a half.

The lung does not contain the acidic trypsin inhibitor, thus the activity found in the crude extract corresponded solely to that of the basic inhibitor. In the crude extract from calf lung (Table 2) the inhibitor activity varied largely but was consistent-

Table 2

Activity of basic Kunitz trypsin inhibitor in bovine lung during development

The results of single experiments are given. The inhibitor activity is expressed in units/100 g of fresh tissue.

Step of preparation	Foetal, 9 months	Calf, 3 - 4 months	Adult
NaCl extract (5% NaCl in 0.2 M-HCl)	0.0	5.0; 7.5; 13.5; 16.2; 25.4; 28.0	95.3; 100.0; 105.0
Trichloroacetic acid extract of ppt. at 0.85 $(NH_4)_2SO_4$ sat., after heating*	1.1	5.4	65.0
CM-cellulose chromatography	0.5	3.5	54.0

* From the NaCl extract, protein was precipitated at 0.85 ammonium sulphate sat., extracted with 4% trichloroacetic acid, the extract heated for 15 min at 80°C, the soluble protein precipitated at 1.0 ammonium sulphate sat., dissolved in water and dialysed against 0.05 M-acetate buffer, pH 4.4.

ly much lower than in adult animals, whereas in the extract from foetal lung it was undetectable. However, concentration of the protein with ammonium sulphate permitted to demonstrate the presence of the basic trypsin inhibitor in this material. The CM-cellulose chromatography (Fig. 2) showed that the inhibitor activity in

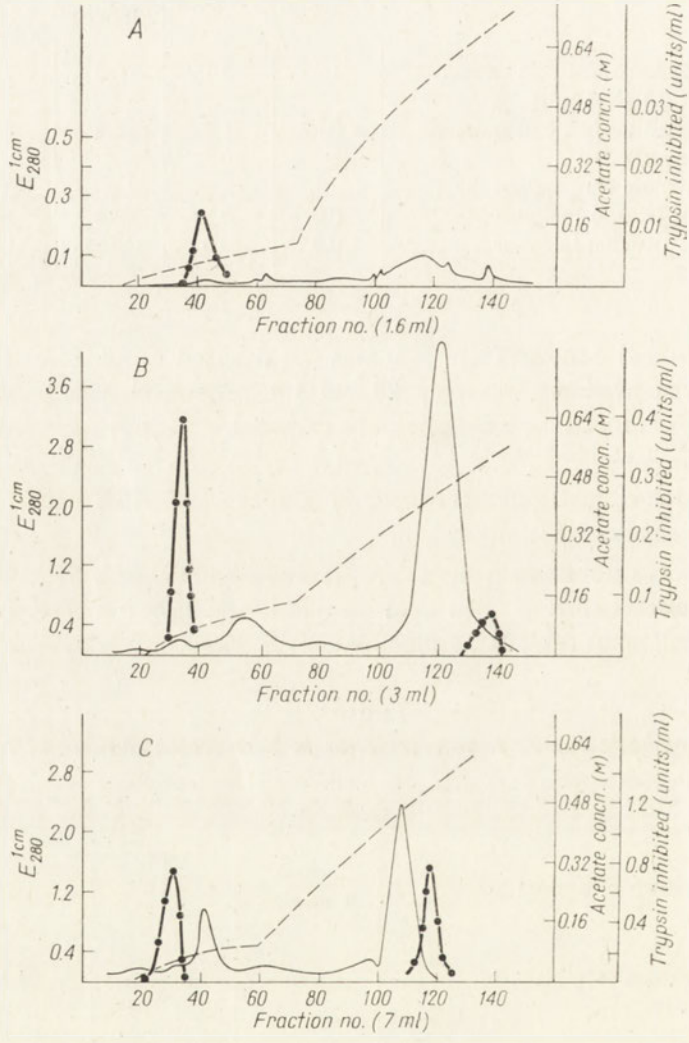

Fig. 1. CM-cellulose column chromatography of bovine pancreas preparations. To the column equilibrated with 0.01 M-acetate buffer, pH 5, the protein precipitated at 0.8 ammonium sulphate saturation was applied and eluted with a convex concentration gradient of acetate buffer, pH 5. The flow rate was 60 ml/h. ——, E_{280}; ●, trypsin inhibitor activity; — — —, acetate buffer gradient.

A, Foetal, 9 months (protein from one pancreas weighing 20 g; column 2 × 7.5 cm);

B, Calf (protein from 300 g of tissue; column 1.8 × 23 cm);

C, Adult (protein from 200 g of tissue; column 2.5 × 30 cm).

foetal lung corresponded to about 1% of that found in adult animals, and the inhibitor was also effective against chymotrypsin and kallikrein. This is in agreement with the behaviour of the Kunitz inhibitor isolated from adult and calf lung and other tissues.

The basic inhibitor isolated by CM-cellulose chromatography from calf and foetal lungs was subjected to further purification. From the active fraction of the calf lung preparation, the protein was precipitated with ammonium sulphate, dissolved in water and separated on Bio-Gel P 10 column (Fig. 3). The active fractions were pooled and the inhibitor crystallized in the presence of ammonium sulphate at pH 4.4. The obtained crystals had the same shape as those of the inhibitor isolated from adult animals. The inhibitor from foetal lung was purified by affinity chromatography on trypsin bound to Sepharose 4B (Fig. 4). From 100 g of foetal lungs about 70 μg of the inhibitor was obtained. On 15% polyacrylamide-gel electrophoresis at pH 4.5 the inhibitor, like that from calf lung, migrated as

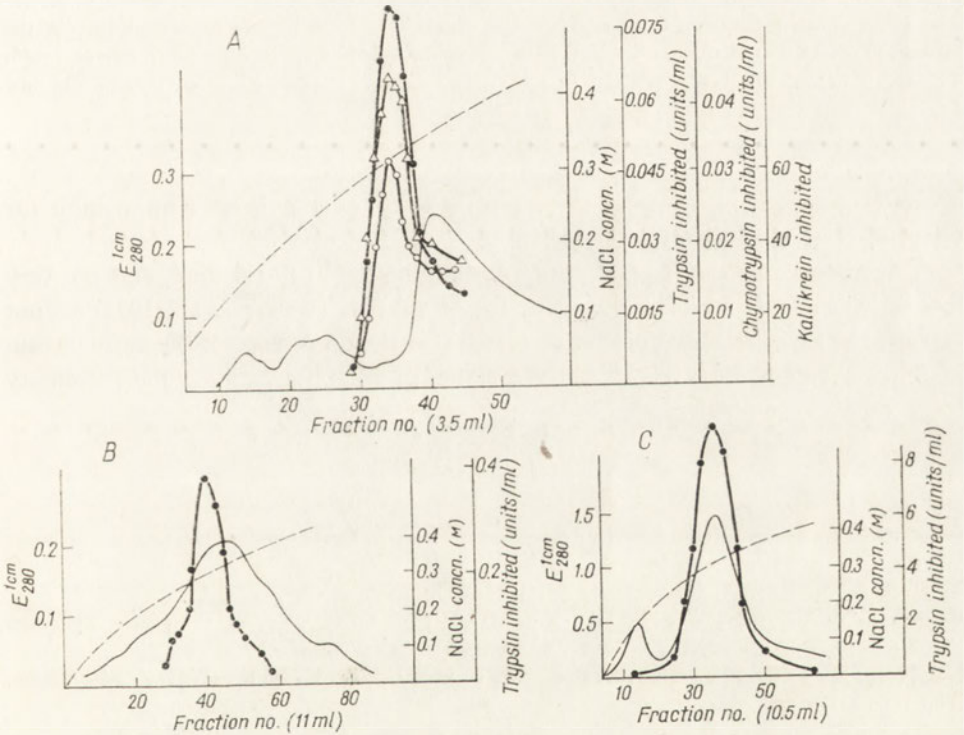

Fig. 2. CM-cellulose column chromatography of bovine lung preparations. To the column equilibrated with 0.05 M-acetate buffer, pH 4.4, the protein soluble in trichloroacetic acid was applied and eluted with a convex NaCl concentration gradient in the equilibration buffer. ——, E_{280}; ●, trypsin inhibitor activity; △, chymotrypsin inhibitor activity; ○, kallikrein inhibitor activity (expressed in kallikrein biological units/ml); — — —, NaCl concentration gradient.

A, Foetal, 6 months (protein from 300 g of lung; column 1.3 × 22 cm; elution rate 30 ml/h);

B, Calf (protein from 1.8 kg of lung; column 2 × 32 cm; elution rate 60 ml/h);

C, Adult (protein from 4.1 kg of tissue; column 2.8 × 25 cm; elution rate 60 ml/h).

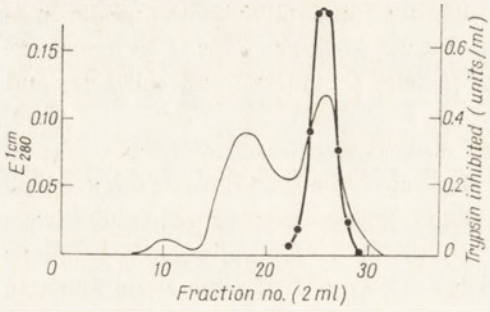

Fig. 3

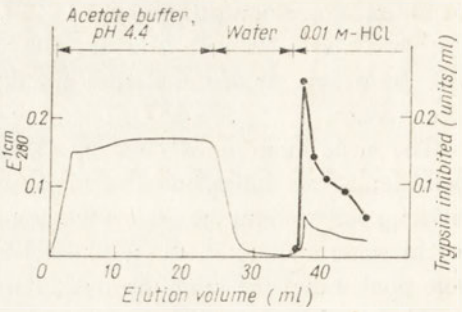

Fig. 4

Fig. 3. Bio-Gel P 10 column chromatography of the basic trypsin inhibitor from calf lung. The eluate from CM-cellulose column (cf. Fig. 2B), 2 ml, was applied to the column (1 × 63 cm) equilibrated with 0.01 M-HCl, and eluted with the same solution at a rate of 12 ml/h. ——, E_{280}; ●, trypsin inhibitor activity.

Fig. 4. Chromatography of the basic trypsin inhibitor from foetal lung on trypsin bound to Sepharose 4B. The active fractions from CM-cellulose (nos. 30 - 44, cf. Fig. 2A) were applied to the column (0.4 × 0.8 cm) equilibrated with 0.05 M-acetate buffer, pH 4.4, and eluted at a rate of 5 ml/h with the indicated solutions. Fractions of 1 ml were collected. ———, E_{280}; ●, trypsin inhibitor activity.

a single protein band showing the same mobility as the basic Kunitz inhibitor obtained from the lung of adult animals.

The presence of the basic Kunitz trypsin inhibitor in foetal lung, and the very low activity of this inhibitor in bovine rumen mucosa (Wilusz *et al.*, 1973) do not support the suggestion of Vogel *et al.* (1968) that the occurrence of the basic trypsin inhibitor in ruminants is related to the presence of microorganisms in the alimentary tract of these animals.

REFERENCES

Anderer, F. A. & Hörnle, S. (1966) *J. Biol. Chem.*, **241**, 1568 - 1572.

Brown, M. E. (1960) *J. Lab. Clin. Med.* **55**, 616 - 624.

Chauvet, J. & Acher, R. (1972) *FEBS Lett.*, **23**, 317 - 320.

Kassell, B. & Marciniszyn, M. B. (1971) in *Proc. Int. Res. Conf. on Proteinase Inhibitors* (H. Fritz & H. Tschesche, eds.) pp. 43 - 46. de Gruyter, Berlin - New York.

Kassell, B., Radicevic, M., Berlow, S., Peanasky, R. J. & Laskowski, M. Sr. (1963) *J. Biol. Chem.*, **238**, 3274 - 3279.

Kazal, A., Spicer, B. S. & Brahinsky, R. N. (1948) *J. Am. Chem. Soc.*, **70**, 3034 - 3040.

Kunitz, M. (1947) *J. Gen. Physiol.*, **30**, 291 - 310.

Kunitz, M. & Northrop, J. H. (1936) *J. Gen. Physiol.*, **19**, 991 - 997.

Laskowski, M. & Laskowski, M. Jr. (1954) in *Advances in Protein Chemistry* (M. L. Anson, K. Bailey & J. T. Edsall, eds.) vol. 2, p. 216. Academic Press, New York.

Łomako, J. & Wilusz T. (1974) *Arch. Immunol. Ther. Exptl.*, **20**, 383 - 391.

Reisfeld, R. A., Lewis, V. J. & Williams, D. E. (1962) *Nature (London)*, **195**, 281 - 283.

Vogel, R., Trautschold, J. & Verle, E. (1968) *Natural Proteinase Inhibitors*, p. 95. Academic Press, New York.

Wilimowska-Pelc, A., Malicka-Błaszkiewicz, M. & Mejbaum-Katzenellenbogen, W. (1973) *Acta Biochim. Polon.*, **20**, 355 - 363.

Wilusz, T., Łomako, J. & Mejbaum-Katzenellenbogen, W. (1973) *Acta Biochim. Polon.*, **20**, 25 - 31.

Wu, F. C. & Laskowski, M. (1955) *J. Biol. Chem.*, **213**, 609 - 619.

INHIBITORY TRYPSYNY W PŁUCACH I TRZUSTKACH BYDLĘCYCH W ROZWOJU

Streszczenie

1. Stwierdzono, że aktywność inhibitorów trypsyny w płucach i trzustkach wzrasta wraz z wiekiem zwierząt.

2. Płuca płodu zawierają 1%, a płuca cieląt 5 - 30% tej ilości zasadowego inhibitora typu Kunitza, jaką wykazuje się w płucach osobników dorosłych.

3. W trzustkach płodu nie wykazuje się inhibitora typu Kunitza, a w trzustkach cieląt aktywność tego inhibitora jest znacznie niższa niż u osobników dorosłych. Aktywność kwaśnego inhibitora trypsyny typu Kazala w trzustkach płodu jest wyraźnie niższa niż w trzustkach cieląt i osobników dorosłych.

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RECENZJE KSIĄŻEK

Peter Langen, ANTIMETABOLITES OF NUCLEIC ACID METABOLISM. Gordon & Breach, Science Publishers, Inc., New York and London 1975. XII+273 pp. Price, £ 4.80 or $ 12.00.

This is an English translation of the 2nd edition of this volume, originally published in German under the title "Antimetabolite des Nucleinsäure-Stoffwechsels" by Akademie-Verlag, Berlin (German Democratic Republic). The first edition appeared in 1967, and the present edition, prepared in 1974, has been brought up to date. This English translation of the 2nd German edition was further revised, with addition of new material, so that the contents cover numerous references for 1974. In large part this was done by addition of a Supplement at the end of the volume.

The author, Head of the Department of Cell Kinetics in the Institute of Molecular Biology of the Academy of Sciences of the German Democratic Republic, is himself a well-known worker in the field of antimetabolites of nucleic acid metabolism. The English translation, by Dr. Thomas A. Scott of the Department of Biochemistry, University of Leeds, England, is indeed competently done.

The volume starts off with a general outline of the mode of action of antimetabolites, their intracellular metabolism, and such problems as the development of resistance. This is followed by an extensive compilation of known antimetabolites, many of them synthesized in the laboratory, and what is known about the mode of action of each. The emphasis throughout is largely on the increasing employment of some of these compounds in cancer chemotherapy, but the material presented is equally of value to those interested in the development of anti-viral agents.

This English translation of the 2nd German edition has been further expanded and revised to include new additions on „active site directed irreversible enzyme inhibitors" (a field extensively developed by the late B. R. Baker), selective inhibition of differentiation processes by 5-halogenated thymine analogues, and the cell cycle and kinetics of cell proliferation.

In a field which has been growing at such a rapid rate during the past few years, because of the increasing attention being devoted to the development of anti-tumour and anti-viral agents, the sections on the properties and chemotherapeutic effectiveness of various analogues should be particularly valuable. The magnitude of the task involved for the author is illustrated by the fact that there are 1940 literature references, apart from those included in the Supplement at the end of the volume.

The volume is well printed and nicely illustrated. It should be of value not only to those interested in fundamental studies on the mechanism of action of antimetabolites, and in chemotherapeutical applications, but also to biochemists and to synthetic organic chemists, particularly those engaged in the synthesis of analogues of purines and pyrimidines and their nucleosides and nucleotides.

David Shugar

D. Beyersmann, NUKLEINSÄUREN. VEB Deutscher Verlag der Wissenschaft, Berlin 1975; str. IX+177, cena M 24,—

Książka D. Beyersmanna omawia w zwięzły sposób bardzo szeroki wachlarz zagadnień związanych z kwasami nukleinowymi. Począwszy od elementów chemicznej budowy nukleotydów, poprzez strukturę chemiczną DNA i RNA, wyznaczanie masy cząsteczkowej i konformację kwasów nukleinowych, aż do biosyntezy nukleotydów, replikacji, transkrypcji, translacji i regulacji tych

procesów; mowa jest także w książce o mechanizmach mutacji, czynnikach mutagennych, o transformacji, konjugacji i transdukcji.

Wszystko to zawarte jest zaledwie na 177 stronach tekstu i opatrzone dość bogatym materiałem ilustracji, wzorów i schematów. Zrozumiałe więc jest, że sposób przedstawienia jest nader zwięzły; jednakże jest on przystępny, jasny i umożliwiający korzystanie z książki także czytelnikowi nie posiadającemu przygotowania biochemicznego. Autor zrezygnował z przedstawienia dróg eksperymentalnych, jakimi biologia molekularna doszła do zawartego w książce, przyjętego dzisiaj powszechnie, opisu zjawisk. Każdy jednak z sześciu rozdziałów zakończony jest wykazem kilku lub kilkunastu pozycji literatury, która stanowi propozycję uzupełniającej lektury dla czytelnika.

Jest więc omawiana książka dobrym kompendium o kwasach nukleinowych, z którego mogą korzystać studenci zarówno kierunków biologicznych, jak i chemicznych. Można ją polecić także jako pomoc w nauczaniu oraz jako źródło podstawowych informacji dla wszystkich tych, którzy, nie będąc specjalistami w tej dziedzinie, chcieliby poznać chemię i biologię kwasów nukleinowych.

Mariusz Żydowo

W. W. Shreeve, PHYSIOLOGICAL CHEMISTRY OF CARBOHYDRATES IN MAMMALS. W. B. Saunders Company, Philadelphia, London, Toronto 1974; str. XIV+330, cena £ 5.25.

W ciągu ostatnich 30 lat biochemia stała się jedną z najżywiej rozwijających się dyscyplin biologicznych i chociaż pochodzenie swoje wywodzi z fizjologii ssaków, to jednak stała się tak dalece odrębną dziedziną, że zaawansowani nawet studenci mają trudności w znalezieniu właściwych relacji pomiędzy biochemią a zjawiskami fizjologicznymi i patologicznymi. Omawiana książka jest szóstym tomem serii, która także i nazwą swoją „chemia fizjologiczna" nawiązuje do zrywających się więzów pomiędzy biochemią a fizjologią.

Chemia fizjologiczna węglowodanów u ssaków ma bardzo długą tradycję w badaniach metabolicznych i przedstawienie tego zagadnienia w sposób interesujący, współczesny i zwięzły z pewnością nie było łatwe. Jednakże autor osiągnął to, przedstawiając na 330 stronach współczesne wiadomości o podstawowych cechach budowy chemicznej węglowodanów występujących u zwierząt, o zasadniczych ciągach metabolizmu cukrów, ich regulacji, metabolicznej specyfice narządowej oraz o patologicznych aspektach przemiany węglowodanów. Ponadto każdy rozdział jest opatrzony wyborem odsyłaczy do oryginalnych prac doświadczalnych o kluczowym znaczeniu dla poznania doświadczalnych podstaw omawianych zagadnień.

Rozdziałów w omawianej książce jest osiem. Noszą one kolejno tytuły: Chemia węglowodanów; Trawienie i absorpcja węglowodanów; Komórkowa translokacja węglowodanów; Wewnątrzkomórkowe spożytkowanie monosacharydów; Biosynteza monosacharydów; Metabolizm polisacharydów; Chemia i metabolizm węglowodanów w glikolipidach; Obrót i losy węglowodanów w krążeniu. Tekst książki uzupełniony jest słownikiem skrótów oraz alfabetycznym skorowidzem rzeczowym.

Jest to więc podręcznik adresowany do studentów, którzy już posiedli podstawowe wiadomości zarówno z biochemii, jak i z fizjologii; omawia głównie powiązania pomiędzy metabolizmem węglowodanów a zjawiskami fizjologicznymi, ale dzięki temu, że zawiera także wiadomości o podstawowych ciągach przemian węglowodanów i ich regulacji, może być z korzyścią czytany przez nieprzygotowanego czytelnika. Książkę można szczególnie polecić biochemikom nauczającym na wydziałach lekarskich i weterynaryjnych, lekarzom zainteresowanym zaburzeniami metabolizmu oraz wszystkim, którzy chcieliby zyskać szerszy pogląd na fizjologiczne i patologiczne aspekty biochemii cukrów.

Mariusz Żydowo

Tibor Zsolnai, DIE CHEMOTHERAPEUTISCHEN UND PESTICIDEN WIRKUNG DER THIOLREAGENZIEN. Akadémiai Kiadó, Budapest 1975, str. 415.

Odczynniki sulfhydrylowe reagujące w sposób bezpośredni lub pośredni ze związkami zawierającymi grupy-SH, stanowią istotną grupę związków zaliczanych do chemoterapeutyków lub pestycydów.

W omawianej książce zebrano dane dotyczące budowy chemicznej odczynników sulfhydrylowych, ich reagowania na poziomie molekularnym, efektów terapeutycznych w różnych stanach patologicznych oraz toksycznego działania jako pestycydów i herbicydów. W oparciu o schematycznie podane podstawowe ciągi metaboliczne przedstawiono ogólny metabolizm ustrojowy. W kolejnych 25 tablicach zestawiono działanie na poszczególne enzymy następujących odczynników sulfhydrylowych: jonów metali ciężkich, soli fenylo-rtęciowych, *p*-chlororteciobenzoesanu, *p*-chlororteciosulfonianu, salyrganu i innych semiorganicznych związków rtęciowych, arseninów i innych semiorganicznych połączeń arsenu, *o*-jodobenzoesanu, jodoacetamidu, jodooctanu i innych związków alkilujących, chinonów, kwasu maleinowego i jego *N*-etyloimidu. Z danych zawartych w tablicach można w łatwy sposób dowiedzieć się o wielkości działania inhibującego odczynnika sulfhydrylowego w podanym zakresie stężeń na określony enzym. Na tej podstawie czytelnik może wyrobić sobie pogląd o konsekwentnym ograniczeniu określonych procesów metabolicznych w komórce w wyniku działania stosowanego odczynnika sulfhydrylowego. Pozwala to ująć w aspektach biochemicznych działanie odczynników sulfhydrylowych na procesy katalityczne przebiegające w ustroju z udziałem enzymów, których prawidłowa funkcja zależna jest od obecności reaktywnych grup sulfhydrylowych. Porównanie efektu działania jednego odczynnika sulfhydrylowego na różne enzymy względnie różnych odczynników sulfhydrylowych na aktywność tego samego enzymu umożliwia ocenę współzależności istniejącej między funkcją enzymu zawierającego grupy sulfhydrylowe a budową chemiczną odczynnika sulfhydrylowego. Takie obszerne wprowadzenie jest niezwykle cenne dla dalszego studiowania kolejnych rozdziałów książki, traktujących o selektywnym działaniu odczynników sulfhydrylowych jako chemoterapeutyków lub pestycydów.

Poszczególne rozdziały książki usystematyzowane są według sposobu reagowania odczynników sulfhydrylowych z grupami -SH związków wielkocząsteczkowych lub drobnocząsteczkowych. Szeroki przegląd odczynników sulfhydrylowych tworzących merkaptany, alkilujących lub arylujących, względnie tworzących związki addycyjne, pozwala czytelnikowi zapoznać się z ich budową chemiczną, chemoterapeutycznym działaniem w wielu stanach patologicznych oraz ochronnym działaniem w stosunku do świata zwierzęcego i roślinnego jako związków bakteriostatycznych i mykostatycznych.

Książka zawiera bardzo bogatą literaturę przedmiotu, obejmującą 1700 pozycji. Stanowi ona pożyteczne studium dla chemików leku, toksykologów, biochemików i lekarzy.

Leon Żelewski

Peter W. Hochachka and George N. Somero, STRATEGIES OF BIOCHEMICAL ADAPTATION. W. B. Saunders Company, Philadelphia, London, Toronto 1973; str. IV+358.

Omawiana książka stanowi bardzo interesującą i udaną próbę opisania językiem biologii molekularnej mechanizmów adaptacji ustrojów żywych do warunków środowiska. Wypełnia ona dotkliwą lukę istniejącą pomiędzy najnowszymi osiągnięciami biochemii i biologii molekularnej z jednej strony, a ugruntowanymi już dobrze obserwacjami fizjologów porównawczych dotyczącymi procesów przystosowania żywych ustrojów do zmieniających się warunków środowiskowych.

Autorom udało się uniknąć w tej książce encyklopedycznego nagromadzenia faktów. W oparciu o liczne dane eksperymentalne przedstawiają ogólne mechanizmy i „strategie" przystosowania na poziomie podstawowych procesów metabolicznych, starając się przy tym wskazać, w jakich okolicznościach i przez jakie organizmy „strategie" te są wykorzystywane.

Całość ujęta jest w 10 rozdziałów, Pierwszy rozdział stanowi wprowadzenie w zagadnienie, precyzując jednocześnie szereg pojęć i objaśniając krótko najbardziej fundamentalne procesy niezbędne dla utrzymania życia. Rozdział drugi omawia sposoby przystosowania metabolizmu różnych organizmów i tkanek do zaopatrzenia w tlen. Przedstawione są początki i ewolucja metabolizmu anaerobowego, aerobowego i fotosyntezy. Autorzy rozważają różne aspekty anaerobowego metabolizmu mięśni szkieletowych kręgowców oraz wzajemną współzależność metabolizmu węglowodanów i lipidów. W rozdziale tym przedstawiono także charakterystyczne cechy metabolizmu mięśni skrzydłowych owadów. Rozdział trzeci poświęcony jest dwutlenkowi węgla, a głównie fotosyntezie jako podstawowemu procesowi wiązania dwutlenku węgla.

W rozdziale czwartym autorzy zajmują się problemem wody, jej dostępności i przystosowaniem procesów metabolicznych do różnych stężeń soli w środowisku. Omówione są charakterystyczne cechy metabolizmu organizmów pozbawionych osmoregulacji, jak np. bakterii, oraz dwie „strategie" przystosowania do zmiennego zasolenia: regulacja wewnątrzkomórkowej osmolarności i dążność do uniezależnienia płynów wewnątrz i zewnątrzkomórkowych od wpływów zmiennego stężenia soli w środowisku zewnętrznym.

Rozdziały 5 i 6 przedstawiają mechanizmy adaptacyjne decydujące o rodzaju wydalanych końcowych produktów przemian azotowych u kręgowców i bezkręgowców. Rodzaj wydalanych z ustroju końcowych produktów przemian azotowych pozostaje w ścisłym związku z ilością dostępnej wody w środowisku.

Kolejny rozdział opisuje wpływ temperatury na składniki chemiczne, struktury i metabolizm organizmów żywych. Omówiona jest regulacja temperatury ciała i mechanizmy kompensacyjne, dzięki którym szybkość procesów metabolicznych pozostaje względnie stała pomimo znacznych wahań temperatury. Szczególną uwagę poświęcają autorzy znaczeniu oddziaływań enzym-substrat dla utrzymania stałej szybkości procesów metabolicznych. Obniżenie temperatury zazwyczaj prowadzi do zwiększenia powinowactwa enzymów do substratów, co z kolei pozwala na utrzymanie w przybliżeniu niezmienionej szybkości procesów enzymatycznych. Ten mechanizm kompensacyjny porównywany jest do wpływu dodatnich efektorów allosterycznych na enzymy i określany obrazowo jako „pozytywna modulacja termiczna".

Następne rozdziały przedstawiają zależność procesów metabolicznych i struktur komórkowych od ciśnienia oraz przystosowanie organizmów wodnych do przebywania na różnych głębokościach. Dla organizmów tych korzystna jest sytuacja, gdy gęstość ich ciała jest w przybliżeniu równa gęstości otaczającego środowiska, co pozwala na pozostawanie na żądanej głębokości bez nakładu energii. Możliwości adaptacyjne organizmów wodnych mogą być w tym względzie dwojakie: zmiana zawartości stosunkowo ciężkich składników ustroju bądź zastąpienie składników o wysokiej gęstości substancjami o mniejszej gęstości.

Ostatni rozdział poświęcony jest hemoglobinie. Autorzy dokonują w nim analizy mechanizmów, dzięki którym hemoglobiny kręgowców spełniają sprawnie swoje funkcje w różnych warunkach środowiskowych.

Na zakończenie każdego z rozdziałów podano najważniejsze pozycje bibliograficzne zalecane jako lektura uzupełniająca, z podziałem na wydawnictwa książkowe i opracowania monograficzne oraz artykuły w czasopismach archiwalnych. Całość uzupełnia obszerny indeks przedmiotowy.

Recenzowana książka posiada liczne walory dydaktyczne. Poszczególne zagadnienia przedstawione są w formie logicznego, systematycznego wykładu. Uwzględniając najnowsze zdobycze biochemii i biologii molekularnej autorzy nie rezygnują ze zwięzłego przypomnienia podstawowych pojęć, dzięki czemu książka nie wymaga od czytelnika głębszego przygotowania biochemicznego. Walory dydaktyczne omawianego wydawnictwa podnosi bardzo staranna szata graficzna oraz liczne bardzo przejrzyste ilustracje i schematy.

Książka stanie się niewątpliwie ogromną pomocą w nauce dla studentów biologii, a także znajdzie czytelników spośród pracowników naukowych różnych dyscyplin przyrodniczych. Staraniem Państwowego Wydawnictwa Naukowego pozycja ta ma się ukazać w przekładzie na język polski.

Wiesław Makarewicz

STRUCTURE AND BONDING, vol. 20. J. D. Dunitz, P. Hemmerich, R. H. Holm, J. A. Ibers, C. K. Jørgensen, J. B. Neilands, D. Reinen and R. J. P. Williams, eds. Springer-Verlag, Berlin, Heidelberg, New York 1974; str. 167, cena DM. 66.-

Structure and Bonding jest seryjnym wydawnictwem, ukazującym się nieregularnie, publikującym artykuły monograficzne z zakresu chemii ze szczególnym uwzględnieniem fizykochemicznych własności cząsteczek i wiązań chemicznych o znaczeniu biologicznym. Omawiany 20 tom tej serii zawiera cztery pozycje, wszystkie poruszające zagadnienia interesujące dla biochemika.

Pierwszy artykuł (str. 1 - 21) przedstawia poglądy na temat roli metali dwuwartościowych w mechanizmie enzymatycznych reakcji przenoszenia grup fosforanowych niepodstawionych i podstawionych, jak np. reszty nukleotydowe. Reakcje tego typu są ogromnie rozpowszechnione w organizmach żywych; należą tu wszystkie reakcje przenoszenia reszt fosforanowych na ADP i z ATP na inne akceptory oraz reakcje związane z syntezą, naprawą i degradacją kwasów nukleinowych, które polegają na przenoszeniu reszt nukleotydowych na polinukleotydy lub wodę. Enzymy katalizujące tego typu reakcje wymagają metali dwuwartościowych jako kofaktorów lub zawierają metal dwuwartościowy trwale związany z białkiem w postaci metaloenzymu. W swoich rozważaniach autorzy opierają się głównie na wynikach uzyskanych badaniem jądrowego rezonansu magnetycznego (NMR).

W drugim artykule na str. 32 - 58 omówiona jest enzymatyczna redukcja rybonukleotydów. Reakcja ta budzi zrozumiałe zainteresowanie jako dostarczająca substratów niezbędnych do syntezy DNA. Scharakteryzowane są dwa typy reduktaz rybonukleotydów, jeden występujący w *E. coli* i w tkankach zwierzęcych, zawierający niezbędne dla aktywności żelazo związane niehemowy, i drugi typ reduktaz, wymagający bezwzględnie dla aktywności obecności adenozylokobalamine jako koenzymu i występujący, jak się wydaje, wyłącznie u mikroorganizmów. Autorzy opisują także własności systemu tioredoksyna-reduktaza tioredoksynowa, który pośredniczy w przenoszeniu wodorów z NADPH.

Ostatnie dwa artykuły omawiają na podstawie wyników uzyskanych techniką elektronowego rezonansu paramagnetycznego (EPR) i spektroskopii Mössbauera, stan elektronowy żelaza w niektórych naturalnie występujących związkach zawierających żelazo (str. 59 - 99) oraz zastosowanie spektroskopii Mössbauera do badania hemoproteidów (str. 101 - 167). Autorami tych opracowań są fizycy i zrozumienie ich wymaga od czytelnika dobrej znajomości teorii i interpretacji wyrafinowanej fizycznej techniki badawczej, jaką jest spektroskopia Mössbauera.

Na końcu tomu umieszczono w układzie alfabetycznym według nazwisk pierwszych autorów indeks artykułów opublikowanych w dotychczas wydanych tomach 1 - 20 *Structure and Bonding*. Pozwala to na łatwe zorientowanie się w całokształcie zagadnień przedstawianych w tej interesującej serii wydawniczej.

Wiesław Makarewicz

C. A. Knight, CHEMISTRY OF VIRUSES, wyd. 2. Springer-Verlag, Berlin - Nowy York, 1975; stron X+325, rys 47; cena DM 48.—

Przed dwunastu laty C. A. Knight, wybitny biochemik, którego prace stały się jednym z fundamentów współczesnej wirusologii, podjął ambitne zadanie przedstawienia chemii wirusów w ujęciu całościowym w jednym zwięźle napisanym tomie. Książka ta była wydarzeniem na rynku księgarskim, a obecnie czytelnik dostaje do rąk jej drugie uzupełnione i zaktualizowane wydanie.

Tytuł książki, *Chemia wirusów*, brzmiący nieco staroświecko w czasach, gdy moda zaleca dodawać słowo „molekularny" kiedykolwiek jest mowa o budowie wirusów, dobrze odzwierciedla sposób prezentacji materiału. Jeszcze zaś lepiej ilustruje punkt widzenia autora krótki wstęp historyczny, skromnie zatytułowany „Niektóre zdarzenia, prowadzące do chemicznej ery wirusologii". Obejmuje on okres od wczesnych obserwacji Iwanowskiego (1892) i Beijerinck'a (1898) nad chorobą mozaikową tytoniu, do identyfikacji przez W. M. Stanley'a w r. 1934 wirusa tej choroby jako krystalizowalnej molekuły. Sens bowiem tego wstępu jest oczywisty: coprawda wnikliwa obserwacja jest

dobrą podstawą do sformułowania intuicyjnie słusznej hipotezy, to jednak dopiero rygorystyczna analiza biochemiczna czyni z niej fakt naukowy.

Takie *credo* autora sprawia, że najciekawszą i najbardziej wartościową częścią książki, obejmującą około 1/3 całego tekstu, jest rozdział pt. „Skład wirusów". Omówiono w nim kolejno: białka, kwasy nukleinowe, lipidy, węglowodany i poliaminy występujące w cząsteczce wirusa, kładąc zresztą nieco większy nacisk na wirusy roślinne niż zwierzęce. Metody służące do izolowania i analizy poszczególnych składników opisane są zwięźle, ale jasno i kompetentnie. Czytelnik, uprzednio z nimi nie zaznajomiony, może je naogół wykorzystać w swej pracy bez uciekania się do podręczników techniki laboratoryjnej czy oryginalnych publikacji. Wady i zalety poszczególnych metod ilustrowane są licznymi przykładami z publikacji oryginalnych.

Bezpośrednio powiązany z rozdziałem o składzie wirusów jest rozdział pt. „Wpływ czynników chemicznych i fizycznych na wirusy". Tutaj jednak metodologia jest uwzględniona w stosunkowo małym zakresie, natomiast szczegółowo przedstawiono kinetykę inaktywacji oraz mechanizmy inaktywacji i mutagenezy przez różne czynniki. Zagadnienia budowy wirusów uzupełnia krótki rozdział, poświęcony ich morfologii. Zawiera on obszerne tabelaryczne zestawienie cech morfologicznych wszystkich ważniejszych wirusów, w podziale według typowych dla nich gospodarzy (zwierzęce, roślinne, owadzie, bakteryjne). Omówiono również wspólne dla wirusów ich podstawowe formy krystalograficzne, heliks i ikozahedron. Szczegółowy jednak opis został oparty o obrazy uzyskiwane w mikroskopie elektronowym po barwieniu negatywnym, a więc w kategoriach wirusów kulistych, wydłużonych, otoczkowych i bezotoczkowych itd. W rezultacie otrzymuje się pełny obraz stanu obecnej wiedzy o morfologii wirusów, pomimo że pominięte zostały RNA-wirusy określane jako A, B i C, zapewne ze względu na ich ciągle raczej enigmatyczny charakter wirusów „w poszukiwaniu chorób".

W odrębnym rozdziale omówiono metody izolowania i oczyszczania wirionów. Główną jego zaletą jest to, że zawarto w nim opis wielu klasycznych metod, powszechnie do dzisiaj stosowanych ale tak rutynowych, że odnośniki do nich są często pomijane w bieżących publikacjach. W tym też zakresie rozdział ten może wielu, nawet doświadczonym, wirusologom znacznie ułatwić pracę w laboratorium. Jednakże nowsze metody albo są ujęte w nadmiernym skrócie, jak rozdział między fazy rozpuszczalników, czy frakcjonowanie polimerami hydrofilnymi, albo pominięte. Zwłaszcza odczuwa się tutaj brak omówienia rotorów zonalnych, zamkniętych czy najnowszych — przepływowych. Na szczęście dane o tych urządzeniach są łatwo dostępne w licznych czy nawet — zbyt licznych artykułach przeglądowych, a także — w coraz bardziej kompetentnie napisanych materiałach reklamowych i instrukcjach różnych producentów aparatury.

Chemia wirusów C. A. Knight'a jest jedną z tych książek, które pod skromnym tytułem kryją ogromne bogactwo informacji. Jest ona przede wszystkim przeznaczona dla tych, którzy dopiero zamierzają zająć się wirusologią, ale nawet doświadczony wirusolog znajdzie w niej wiele pożytecznych danych. Jej wielką zaletą jest to, że zarówno opisywana metodologia jak i przytoczone wyniki omawiane są w szerszym kontekście wniosków o charakterze ogólno-biologicznym. „*Chemia wirusów*" jest więc nie tylko podręcznikiem ale również monografią, z której korzyść wyciągną i wirusolodzy i specjaliści z innych dyscyplin biologii.

Kazimierz Zakrzewski

Lelio Orci and Alain Perrelet, FREEZE-ETCH HISTOLOGY. Comparison between Thin Section and Freeze-Etch Replicas. Springer-Verlag Berlin - Heidelberg - New York 1975; stron 168, cena 145 DM.

Począwszy od lat 50-ych, kiedy to wprowadzono mikroskopię elektronową do badań cytologicznych, jednym z ważniejszych problemów stała się sprawa prawdziwości wykrywanych przy jej pomocy faktów. Postawiono zatem pytanie, w jakim stopniu zdjęcia preparatów otrzymywane przy kilkasetkrotnie większej niż w mikroskopie świetlnym zdolności rozdzielczej, a ujawniające skomplikowane układy strukturalne — odzwierciedlają stan faktyczny organizacji żywej komórki. Jedynym możliwym sposobem odpowiedzi na to pytanie była i jest nadal dokładna analiza poszczególnych

etapów procesu przygotowania materiału biologicznego, aż do momentu uzyskania ultracienkich (o grubości kilkuset Angstromów) skrawków. Na proces ten składa się, jak wiadomo, utrwalenie komórki działaniem czynników chemicznych, następnie odwodnienie jej i przesycanie mieszaniną syntetycznych żywic, które po spolimeryzowaniu nadają jej twardość bliską twardości szkła. Jest rzeczą oczywistą, że każdy z wymienionych powyżej etapów musi w jakiś sposób naruszać stan pierwotny materii komórkowej. Jedynym osiągalnym optimum jest takie dobranie warunków (i użytych odczynników), że wiadomym był sposób zachodzenia tych zmian i żeby zmiany te albo w ogóle nie wpływały, albo wpływały w sposób znany na kształt struktur wykrywalnych w mikroskopie elektronowym. Analizę tych zjawisk można przeprowadzić tylko stosując wymiennie różne techniki przygotowania materiału.

Ogólny obraz organizacji ultrastrukturalnej komórki został odkryty i zaakceptowany powszechnie już w latach 50-ych. Trzeba jednak przyznać, że dopiero zastosowanie zupełnie innej techniki przygotowania materiału, mianowicie rozwiniętej w latach 60-ych techniki kriorytowania, dostarczyło najbardziej przekonywających dowodów jego prawdziwości. W technice tej materiał — po utrwaleniu chemicznym, lub nieutrwalony — zostaje w bardzo niskiej temperaturze gwałtownie zamrożony, po czym w warunkach wysokiej próżni przełupany a następnie pozostawiony w warunkach, w których zachodzi częściowa sublimacja lodu. Ten moment jest właśnie momentem „rytowania" płaszczyzny przełupu i powoduje on lepsze odsłonięcie jej rzeźby. Tak spreparowaną płaszczyznę napyla się cienką warstewką platyny i węgla, tworząc w ten sposób cieniutką, ściśle przylegającą do materiału błonkę. Błonka ta daje się łatwo oddzielić i stanowi wierną replikę powierzchni badanej próbki, gotową do badań w konwencjonalnym transmisyjnym mikroskopie elektronowym. Otóż na tak uzyskanych elektronogramach zasadniczy obraz ultrastruktury komórki, poszczególnych jej organelli odpowiada znanym już obrazom otrzymywanym z materiału skrawanego, lecz przedstawia je w ujęciu trójwymiarowym. Co więcej, a jest to najważniejszym atutem tej techniki, pozwala ona na badania morfologiczne niedostępnego dotychczas wnętrza rozłupanych różnych struktur błonowych.

Pięknie wydana przez znane wydawnictwo Springera (Springer-Verlag, Berlin, Heidelberg, New York 1975) książka Lelio Orci i Alain Perrelet pt. *Freeze-Etch Histology* jest pierwszą, która w sposób metodyczny i niesłychanie przekonywający przedstawia czytelnikowi omówione powyżej zalety tej techniki, a także uczy korzystania z jej osiągnięć. Zgodnie z tytułem — jest to ogólny zarys histologii, ilustrowany porównawczo elektronogramami z ultracienkich skrawków i z replik otrzymywanych z materiału poddanego kriorytowaniu. Zestawienia takie, każdorazowo zaopatrzone w bardzo zwięzłe i treściwe objaśnienia (wraz z najważniejszymi pozycjami z literatury) dostarczają, obok wspaniałych efektów wizualnych, bardzo interesujących i ważnych informacji dotyczących ultrastruktury komórki w różnych jej wyspecjalizowanych formach, jakie występują w poszczególnych narządach.

Prezentując najpierw obraz budowy błony plazmatycznej autorzy prowadzą czytelnika od obrazu jej wnętrza, stosunkowo często spotykanego w literaturze, do form wysoce zróżnicowanych, jak np. pełne kanalików mikropinocytotycznych wnętrze błony komórek śródbłonka, czy błony mięśni gładkich wykazujące obecność podobnych otworów uszeregowanych w charakterystyczne pasma wzdłuż mięśnia, lub błony rzęsek, które są jakby ozdobione u nasady paroma równoległymi rzędami regularnie ułożonych szeregów makromolekuł.

Następne strony poświęcone są jądru komórkowemu, gdzie przede wszystkim wyeksponowana została błona jądrowa z charakterystycznymi dla niej porami. Pozostałe strony książki zajmują obrazy replik komórek wchodzących w skład takich narządów jak trzustka, jelito, wątroba, nerki, tchawica, płuca, mięśnie, tkanka nerwowa, tłuszczowa i krew — zawsze porównywane z elektronogramami otrzymywanymi według techniki konwencjonalnej. Trudno jest wymienić wszystkie szczegóły, które można zobaczyć i których można się nauczyć studiując tę książkę. Jakkolwiek jej szata zewnętrzna — duży format i znaczna przewaga powierzchni zajętej przez ilustracje nad powierzchnią zajętą przez tekst — może sprawiać wrażenie albumu, to jednak w istocie jest to poważna książka naukowa, której przestudiowanie można polecić każdemu, kto interesuje się ultrastrukturą komórki.

Aleksandra Przełęcka

STRUCTURE AND BONDING, vol. 23. J. D. Dunitz, P. Hemmerich, R. H. Holm, J. A. Ibers, C. K. Jørgensen, J. B. Neilands, D. Reinen and R. J. P. Williams, eds. Springer-Verlag, Berlin - - Heidelberg - New York 1975; stron 193, rys. 50; cena DM 74.—

Dwudziesty trzeci tom aperiodycznego wydawnictwa pt. *Structure and Bonding* zawiera cztery artykuły poświęcone wybranym zagadnieniom dotyczącym niektórych metaloproteidów, metaloporfiryn oraz mechanizmu wymiany H → D w związkach aromatycznych.

W pierwszym artykule J. A. Fee omawia własności fizykochemiczne oraz zależność struktury i funkcji azuryn, stellacyanin, plastocyanin, jak również niektórych oksydaz zawierających w swojej cząsteczce miedź. Wspólną cechą pierwszej grupy kuproproteidów wyizolowanych z różnych szczepów bakterii, roślin niefotosyntetyzujących i chloroplastów jest niski ciężar cząsteczkowy (do 30 000 daltonów), zawartość 1 lub 2 atomów Cu^{2+}, punkt izoelektryczny poniżej pH 7,0 i potencjał redox ok. 300 mV. Białka te są w większości glikoproteidami, posiadają zwartą konformację i znaczną zawartość struktury α-helikalnej. W komórkach bakterii i roślin spełniają najprawdopodobniej funkcję przenośników elektronów w reakcjach jednoelektronowych.

W grupie oksydaz multimiedziowych omówiono lakkazy, ceruloplazminę i oksydazę kwasu askorbinowego. Każdy z powyższych enzymów katalizuje ogólną reakcję:

$$2\,AH_2 + O_2 \rightarrow 2\,A + 2H_2O$$

której mechanizm jest niezwykle złożony, zależny od struktury enzymu, który ją katalizuje, zawartości i rodzaju jonów miedziowych, potencjału redox, etc. Ciężar cząsteczkowy enzymów „niebieskich" zawiera się w granicach od ok. 60 000 do 160 000, posiadają one 4 - 10 jonów Cu^{2+} w cząsteczce i wykazują trzy możliwe rodzaje połączeń kompleksowych z jonami miedzi: obecność Cu^{+} i Cu^{2+} — silna absorpcja przy 600 nm (typ 1); Cu^{2+} częściowo skoordynowany z atomami N białka — słaba absorpcja przy 600 nm (typ 2); pary jonów Cu^{2+}—Cu^{2+} — intensywna absorpcja przy 330 nm (typ 3). Stąd też enzymy zawierające miedź w swojej cząsteczce wykazują znaczne zróżnicowanie wartości potencjału redox (200 - 800 mV), własności spektralnych, magnetycznych i konformacji. Posiadają też, jak już wspomniano, niezwykle złożony mechanizm katalitycznej funkcji.

W drugim rozdziale M. F. Dunn omawia rolę cynku w procesie katalizy zarówno modelowych układów metaloorganicznych, jak i metaloenzymów. We wszystkich połączeniach Zn^{2+} tworzy aktywne centrum z czterema wiązaniami koordynacyjnymi, przy czym trzy miejsca koordynacyjne zajęte są przez wiązania z resztami aminokwasowymi polipeptydu, natomiast czwarte miejsce jest ekskluzywnie zajęte przez cząsteczkę wody lub jon OH^{-}. Taka tetrahedryczna struktura centrum wiązania metalu tworzy charakterystyczne zagłębienie w cząsteczce białka, będące miejscem interakcji substratu na powierzchni enzymu. Najczęstszymi ligandami dla jonu cynku w cynkoproteidach jest histydyna i kwas glutaminowy. Z opisanych przez autora wyników badań nasuwa się konkluzja, że ogólnie w procesie katalitycznym cynk zawarty w polipetydzie aktywuje skoordynowaną cząsteczkę wody dla nukleofilnego ataku podczas chemicznej transformacji stosownego substratu.

W trzecim artykule W. Schneider omawia dotychczasowy dorobek związany z procesem inkorporacji jonu metalu do układu porfirynowego przy syntezie metaloporfiryn. Badanie kinetyki tej reakcji w roztworach wodnych i w rozpuszczalnikach organicznych dla różnych porfiryn i jonów metali (Fe^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+} i Zn^{2+}) pozwala na wyciągnięcie ogólnych zależności charakterystycznych dla tego procesu. Wprowadzenie metalu do pierścienia porfirynowego jest związane z wyparciem dwóch protonów należących do azotów układów pyrolowych. Z kolei jon metalu, będący zawsze w stanie uwodnionym $[Me(H_2O)_n^{z+}]$, ulega desolwacji podczas reakcji. Taki mechanizm wiązania jonów metali przez układ porfirynowy jest zdecydowanie różny niż to ma miejsce w przypadku tworzenia kompleksów z ligandami o otwartym łańcuchu.

Użycie ciężkiego wodoru, głównie D_2, do badania struktury związków organicznych stało się ostatnio potężnym narzędziem w studiach nad mechanizmem reakcji chemicznych i biochemicznych. Przez śledzenie wymiany H→D uzyskano cenne informacje o stanie podstawowym i wzbudzonym wielu cząsteczek. Na przykładzie kilku związków aromatycznych, M. Ordin i D.M. Bollinger wskazu-

ją różnorodne podejścia, głównie dla badania mechanizmu elektrofilnej substytucji w określonych warunkach kwasowo-zasadowych, w obecności i nieobecności stosownych katalizatorów. Powyższe badania, głównie w odniesieniu do organometalicznych kompleksów, mają znaczenie podstawowe w wyjaśnianiu mechanizmu reakcji charakteryzujących życie na naszej planecie, jak oksygenacja hemoglobiny, wiązanie azotu, zjawisko fotosyntezy.

W sumie książka zawiera duży ładunek najnowszych osiągnięć współczesnej chemii organicznej i biochemii na temat udziału kompleksów metaloorganicznych i metaloproteidowych w fundamentalnych procesach życiowych. Ponad 500 pozycji najnowszego piśmiennictwa ułatwi zainteresowanym głębsze poznanie każdego z omówionych problemów.

Włodzimierz Ostrowski

H. T. Banks: MODELING AND CONTROL IN THE BIOMEDICAL SCIENCES. Springer-Verlag, Berlin - Heidelberg - New York 1975; stron IV+114, cena DM 18.—

Niewielka ta książka, wydana techniką fotolitograficzną z maszynopisu, jest szóstym tomem z serii: *Lecture Notes in Biomathematics* i zawiera materiały wykładów autora na 14 Seminarium Kanadyjskiego Kongresu Matematycznego w University of Western Ontario (Kanada) w r. 1973, oraz materiały niektórych jego wykładów w Brown University w Providence, Rhode Island (U.S.A.).

Książka ta jest przeglądem najnowszych metod i modeli matematycznych stosowanych do opisu różnorodnych zjawisk biologicznych i procesów biochemicznych. Autor potraktował w niej dość obszernie następujące zagadnienia: kinetykę reakcji enzymatycznych w różnych układach, rozprzestrzenianie się epidemii, homeostazę poziomu glukozy w organizmie oraz wzrost i radioterapię nowotworów. W ostatnim rozdziale autor dał krótki przegląd szeregu innych zagadnień — niektóre z nich naszkicowane są tylko pobieżnie — nadających się do przedstawienia w formie modeli matematycznych: poruszanie się istot dwunożnych, dializę przeciwprądową, dawkowanie leków dla utrzymania odpowiedniego ich poziomu w organizmie, oddychanie owadów przez przetchlinki, optymalizację diagnostyki medycznej i kilka innych.

W większości przypadków autor stosuje modele deterministyczne; jedynie dla opisów rozwoju epidemii i wzrostu nowotworów wprowadza modele probabilistyczne. Stosowane metody matematyczne obejmują: równania różniczkowe, analizę systemów, metody aproksymacji, modele symulacyjne, teorię prawdopodobieństwa, procesy stochastyczne i teorię niezawodności.

Opisy procesów biologicznych, służące jako podstawa do modelowania matematycznego, są zgodne z aktualnym stanem wiedzy.

Modele reakcji enzymatycznych opiera autor na równaniach Michaelisa-Menten i Briggsa-Haldane'a, i rozpatruje przebiegi reakcji przy różnych stosunkach stężeń substratu i enzymu, aproksymacje dla warunków specjalnych, hamowanie i aktywację enzymów oraz stany stacjonarne reakcji.

W następnym rozdziale omówione są reakcje enzymatyczne w układach, w których enzym jest związany z błoną półprzepuszczalną dla substratów i produktów. Dalszym rozwinięciem tego zagadnienia jest układ, w którym błona składa się z dwóch warstw zawierających dwa enzymy prowadzące następujące po sobie reakcje (produkt pierwszej jest substratem drugiej reakcji). Odpowiednio skonstruowane układy tego typu mogą prowadzić aktywny transport substancji wbrew gradientowi stężeń. Autor podaje tu przykład realnego układu prowadzącego reakcje:

$$\text{Glukoza} + \text{ATP} \xrightarrow[\text{błona 1}]{\text{heksokinaza}} \text{ADP} + \text{Glc-6-P} \xrightarrow[\text{błona 2}]{\text{fosfataza}} \text{ADP} + P_i + \text{glukoza}.$$

Sumarycznie proces ten daje przeniesienie glukozy z jednej strony błony na drugą, sprzężone z rozkładem ATP. Zjawiska te dają się łatwo wymodelować matematycznie, a uzyskane wyniki są zgodne z danymi doświadczalnymi.

Autor rozważa też przypadki kolumny wypełnionej enzymem unieruchomionym w nośniku oraz urządzenie przepływowe, w którym produkt reakcji jest usuwany ze środowiska na drodze

dializy. Systemy takie stwarzają szereg możliwości regulacji przebiegu reakcji, i kilka takich przykładów przedstawia autor w formie wyrażeń matematycznych.

Następny rozdział poświęcony jest omówieniu reakcji „kaskadowych", w których produkt wcześniejszej reakcji jest enzymatycznym katalizatorem reakcji następnej. Układy takie charakteryzują się znacznym „wzmocnieniem", tj. słaby bodziec początkowy daje w rezultacie szybko silny efekt końcowy. Przykładami „kaskad" są: procesy krzepnienia krwi, glikogenoliza, aktywacja komplementu w reakcjach immunologicznych, niektóre procesy skurczu mięśnia itd. Autor przedstawił próby matematycznego modelowania glikogenolizy — procesu najlepiej poznanego od strony chemicznej.

W rozdziale czwartym przedstawiono modelowanie szerzenia się epidemii w populacjach osobników wrażliwych lub częściowo wrażliwych. Już stosunkowo prosty model prowadzi do wykazania „efektu progowego": aby epidemia rozprzestrzeniała się, do populacji musi być wprowadzona co najmniej pewna określona liczba osobników zakażonych. Poniżej tej liczby ognisko epidemii wygasa samoistnie. Uwzględnienie licznych czynników w rzeczywistości wpływających na rozwój epidemii prowadzi do bardzo złożonych modeli. W rozwiązanie tych zagadnień liczni autorzy włożyli wiele pracy, bowiem znalezienie adekwatnego modelu tych zjawisk pozwoliłoby na wybór optymalnych — z punktu widzenia efektywności i kosztów — metod zwalczania epidemii. Zagadnienie to nadal pozostaje otwarte; brak jest jeszcze dostatecznie dokładnych wartości liczbowych parametrów mających tu istotne znaczenie.

Z taką sytuacją spotykamy się w wielu innych przypadkach tam, gdzie chcemy modelować naturalne procesy biologiczne z dostatecznie dużą wiernością; większość dotychczas opracowanych modeli wykazuje pewne braki i zbyt daleko idące uproszczenia, i wymaga dalszych udoskonaleń.

Zagadnienie nowotworów autor rozpatruje w dwóch aspektach. Pierwszy — to probabilistyczne modele przedstawiające rozwój komórek nowotworowych w oparciu o stochastyczny opis zachowania się indywidualnych komórek populacji. Modele te — pod względem formalnym — zbliżone są do modeli rozprzestrzeniania się epidemii. Aspekt drugi — to model przeżywalności komórek napromieniowanych promieniowaniem jonizującym, potraktowany deterministycznie w oparciu o teorię pojedynczych trafień wielu celów. Ujęcie to prowadzi do opracowania odpowiednich strategii radioterapii nowotworów.

Jak widać, modelowanie matematyczne może w niektórych przypadkach przynosić bardzo duże korzyści praktyczne.

Ostatni rozdział książki, wskazując szereg zagadnień z bardzo różnych dziedzin nadających się do matematycznego modelowania, może stymulować do podejmowania prac w tym kierunku.

Bibliografia przedmiotu, obejmująca 185 pozycji, sięga po rok 1974.

Książka stanowi dobre wprowadzenie do zagadnień matematycznego modelowania procesów biologicznych dla matematyków zainteresowanych tą problematyką, lub dla biologów z odpowiednim przygotowaniem matematycznym.

Andrzej Morawiecki
Stanisław Gnot

MOLECULAR ASPECTS OF MEMBRANE PHENOMENA. H. R. Kaback, H. Neurath, G. K. Radda, R. Schwyzer and W. R. Wiley, eds. Springer-Verlag, Berlin - Heidelberg - New York 1975; stron 238, cena DM 64.—

Omawiana książka jest zebraniem referatów przedstawionych na międzynarodowym sympozjum, które odbyło się w listopadzie 1974 roku w ośrodku Battelle Research Center w Seattle (Waszyngton).

Tematami wiodącymi konferencji były trzy kluczowe własności błon:

1. Dynamika procesów zachodzących w błonach na poziomie molekularnym
2. Mechanizmy właściwego rozpoznawania i przekazywania przez błony informacji
3. Udział błon w procesach sprzężenia energetycznego (wiązania energii).

Skład chemiczny błon, ich budowa w różnym stanie pobudzenia, molekularna budowa ATPazy i jej udział w procesach przenoszenia energii — to główne tematy pierwszej części książki.

Analiza struktury ATPazy bakteryjnej i mitochondrialnej u zwierząt wyższych była tematem referatu S. Fergusona, W. Lloyda i G. Radda z Uniwersytetu z Oksfordu. Autorzy wykazali udział tyrozyny w centrum aktywnym enzymu oraz katalityczną funkcję podjednostki β. Stwierdzili ponadto, że udział ATPazy w procesie przenoszenia energii jest ściśle związany z jednoczesną interreakcją pięciu podjednostek enzymu, a symetria błony warunkuje kolejność etapów. Zmienność struktury części lipidowej błony jest ważnym czynnikiem modelującym aktywność ATPazy bakteryjnej.

Budowie ATPazy poświęcony był również referat D. Gutnicka z Uniwersytetu w Tel Aviv. Autor badał mutanty z defektami procesu syntezy ATP. Wykazał, że u *E. coli* warunkiem fosforylacji oksydatywnej jest odpowiednia konformacja conajmniej pięciu podjednostek, umiejscowionych w specjalnym odcinku błony. Wydaje się, że prezentowany przez autora model badań na selektywnych mutantach będzie skuteczną drogą do wyjaśnienia roli poszczególnych podjednostek, ich biosyntezy oraz regulacji aktywności enzymatycznej.

E. Shechter, L. Letellier i T. Gulik-Krzywicki z Uniwersytetu w Paryżu oraz z Centrum Genetyki Molekularnej C.N.R.S. w Gif-sur-Yvette, badając u *E. coli* zależność struktury warstwy lipidowej błony od transportu, wykazali jej odmienne stany konformacyjne podczas przechodzenia różnych związków. Transportowi proliny towarzyszy „nieuporządkowany" stan lipidów, co warunkuje przesuwanie się nośnika białkowego obarczonego aminokwasem. Natomiast podczas transportu glukozy lipidy pozostają w stanie „uporządkowanym", co wskazuje na udział innych mechanizmów niż przy transporcie aminokwasów.

R. Post, K. Taniguchi i G. Toda (Vanderbilt University, Nashville) przedstawili wyniki badań nad udziałem Na,K-ATPazy w transporcie jonów i powstawaniu ATP. Autorzy, badając kolejne etapy procesu, sugerowali powstawanie, w obecności nieorganicznego fosforanu, fosforowej pochodnej enzymu zależnego od potasu a następnie powstawanie z ADP cząsteczki ATP, czemu towarzyszyło przeniesienie jednego mola sodu.

W drugiej części sympozjum, poświęconej mechanizmom przekazywania informacji, przedstawiono referaty dotyczące badań nad receptorami hormonów polipeptydowych i acetylocholiny.

R. Schwyzer z Instytutu Molekularnej Biologii i Biofizyki z Zurychu zaprezentował model przekazywania informacji przez kortykotropinę oraz α-melanotropinę. Autor zwrócił uwagę na dehydrogenazę 6-fosfoglukozy, która wiążąc specyficznie wspomniane wyżej hormony, może stanowić dogodny model dla badania mechanizmu łączenia hormon - receptor.

L. Kohn i R. Winand (National Institute of Health, Bethesda i Institut de Medicine, Liège) przedstawili badania nad receptorami tyreotropiny w tarczycy i ich znaczeniem w egzaftalmii.

A. Karlin, M. McNamee i C. Weill (Columbia University) dyskutowali swoje wyniki nad receptorami acetylocholiny w elektrycznych organach ryb.

W trzeciej części sympozjum prezentowano wyniki badań nad kontrolą transportu elektronów, mechanizmem wiązania energii oraz nad rolą pęcherzyków izolowanych z błon bakteryjnych w procesie aktywnego transportu.

Wśród wielu interesujących informacji na szczególną uwagę zasługuje proponowany przez P. Dutton, K. Petty, R. Prince i R. Cogdell (University of Pennsylvania, Philadelphia) model łańcucha oddechowego. Autorzy na przykładzie błony bakterii *Rhodopseudomonas spheroides* przedstawili schemat transportu elektronów i protonów z dokładnym omówieniem roli poszczególnych przenośników, ich rozmieszczeniem w błonie oraz wzajemnym współoddziaływaniem. Jest on pięknym przykładem ilustrującym, w jaki sposób oddzielne cząstki związków, tworząc odpowiednie substruktury, mogą spełniać określoną funkcję fizjologiczną. Jest też jeszcze jednym dowodem, że w komórkach żywych organizmów możliwość zmiany konformacji określonej struktury jest podstawą regulacji wielu procesów biologicznych.

Omawiana książka dostarcza wielu informacji o budowie i funkcji błon. Wartość jej podnosi dodatkowo obszerny wykaz piśmiennictwa załączony do każdego rozdziału.

Zofia Porembska

A. M. Mayer and A. Poljakoff-Mayber, THE GERMINATION OF SEEDS. Pergamon Press, Oxford 1975; stron 192, cena $11.50.

Opracowanie to stanowi piąty tom *Plant Physiology Division* z *International Series of Monographs in Pure and Applied Biology*. Jest ono drugim, poprawionym i uzupełnionym wydaniem z 1963 roku.

Treść książki zawarto w siedmiu rozdziałach które obejmują: strukturę nasion i siewek; chemiczny skład nasion; czynniki warunkujące kiełkowanie; spoczynek oraz stymulację i hamowanie kiełkowania; metabolizm kiełkujących nasion; wpływ inhibitorów i stymulatorów kiełkowania oraz ich przypuszczalną rolę w tym procesie; ekologię kiełkowania; ekologiczną rolę czynników zewnętrznych.

W aktualnej literaturze brak jest opracowania monograficznego obejmującego w sposób zwięzły tak szeroki wachlarz zagadnień związanych z kiełkowaniem nasion, o mechanizmie którego wiemy jeszcze tak mało. Dlatego też książka ta może być bardzo wartościowym kompendium dla rozpoczynających pracę badawczą fizjologów roślin, botaników i agronomów. Przystępne i interesujące ujęcie opracowania czyni jego treść zrozumiałą dla wszystkich biologów interesujących się hodowlą roślin. Każdy rozdział kończy obszerna bibliografia obejmująca prace źródłowe, jak również i monograficzne.

Ponieważ jest to wznowienie wydania z 1963 roku, ze zrozumiałych względów przeważa w nim bibliografia obejmująca ten okres. Autorami opracowania są znani botanicy z Uniwersytetu w Jerozalem, interesujący się głównie fizjologią procesów kiełkowania nasion, stąd najbardziej wyczerpująco omówiono czynniki warunkujące kiełkowanie nasion, natomiast mniej uwagi poświęcono zagadnieniom mechanizmów regulacji tych procesów.

Kazimierz Kleczkowski

EXPANSION OF THE USE OF IMMUNIZATION IN DEVELOPING COUNTRIES. First WHO Seminar, Kumasi, Ghana, 12 – 19 November 1974. WHO, Geneva 1975; str. 32, cena fr. szw. 12.—

Światowa Organizacja Zdrowia wydała sprawozdanie z seminarium dotyczącego zagadnień związanych z rozwojem szczepień ochronnych w krajach rozwijających się. Sprawozdanie przedstawia obecną sytuację w zakresie szczepień ochronnych w krajach afrykańskich, problemy związane z upowszechnieniem szczepień oraz sugestie dotyczące sposobów przezwyciężenia istniejących trudności — m.in. dane dotyczące najbardziej efektywnych form ew. pomocy ze strony krajów rozwiniętych.

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