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COMPLEXES OF HISTONES WITH DEOXYRIBONUCLEIC ACID

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1. Melting temperature (T_m) and intrinsic viscosity ([η]) of complexes of double-stranded DNA with very lysine-rich (F_1) and arginine-rich (F_3) histones, were studied. 2. In NaCl solutions of low concentration, the formation of complexes was accompanied by an increase in T_m and a decrease in intrinsic viscosity. On raising of NaCl concentration the complexes dissociated, the DNA-F_1 complex at lower ionic strength, and the DNA-F_3 complex at a higher one. 3. At low ionic strength, the DNA-F_1 complex underwent degradation in the presence of 1.0 M-urea, whereas the DNA-F_3 complex was stable even in 3 M-urea. 4. It is suggested that hydrogen bonds are involved in formation of DNA-F_1 complexes, but not of those with histone F_3.

Investigations concerning physico-chemical properties of DNA-histone complexes were mainly carried out on native deoxyribonucleoprotein (DNP) isolated from calf thymus nuclei, and little is known about the properties of the reformed complexes of double-stranded DNA with individual histone fractions.

Roentgenographic studies of Zubay & Doty (1959) showed that the molecule of DNP is less extended and less asymmetrical than that of DNA. Wilkins, Zubay & Wilson (1959) reported that DNP contains double-stranded DNA. Ohba (1966a), by measuring flow birefringence, dichroism and sedimentation constant, demonstrated that histones are able to combine with DNA and that the apparent length of DNA molecule is shortened by this association. Ohba (1966b) showed also that the G-C pairs in nucleohistone, unlike in DNA, are more susceptible to thermal denaturation than the A-T pairs, and he ascribed the stabilization of the A-T pair to the lysine residue in histones.

On the other hand, Giannoni & Peacocke (1963) reported that histone had but little influence on the shape and size of the DNA molecule. Bayley, Preston & Peacocke (1962), who studied the effect of ionic strength on light scattering and intrinsic viscosity of DNP solution, concluded that the DNA molecule forms a core which determines the shape of the complex. Their intrinsic viscosity measurements were in agreement with the results of Eisenberg (1957) but not with those of Jordan & Porter (1954) who reported that the intrinsic viscosity of DNP solutions was dependent on ionic strength.

Bartley & Chalkley (1968) demonstrated that there is a distinct increase in intrinsic viscosity of DNP in urea solution, and suggested that this increase was due mainly to the transition from the supercoil conformation to a more extended rod-like conformation.

Data reported in the literature (Ćvetković & Savić, 1969) indicate that there are differences in firmness of binding between individual histone fractions and single--stranded or double-stranded DNA; histone fraction F_1 has the smallest ability to combine with DNA. Recently Olins (1969) studied the sedimentation constant, circular dichroism and light scattering of reformed complexes of DNA with fraction F_1, and the data obtained offered no indication of the effect of this fraction on the shape and size of DNA; histone F_1 was only found to stabilize the structure of double-stranded DNA, as judged from the observed increase in T_m value.

The aim of the present work was to study the effect of urea on the reformed complexes of DNA with very lysine-rich (F_1) and arginine-rich (F_3) histones, and to compare the results of intrinsic viscosity measurements with the data of Bartley & Chalkley (1968) concerning the effect of urea on thymus DNP. In view of large differences in the structure and amino acid composition of histones F_1 and F_3, differences in the behaviour of DNA-F_1 and DNA-F_3 complexes were anticipated.

MATERIALS AND METHODS

DNA preparations. Sodium salt of DNA was obtained from calf thymus as described by Kay, Simmonds & Dounce (1952), the average yield from 50 g. of thymus being 0.83 g. of Na-DNA. The preparations to be used for viscosity measurements were purified by repeating a few times the last step of the procedure. The preparation contained 18.7% of water (7 hr. at 110°), 13.84% of nitrogen (Kjeldahl method) and 8.35% of phosphorus (Fiske & Subbarow), the N : P ratio being 1.6. The preparation contained no protein detectable by the biuret method. $E^{1\%}_{1\,cm}$ at 260 nm was 176.6; ε_p 6580; E_{260}/E_{230} 2.15; E_{260}/E_{280} 1.7.

Single-stranded DNA was obtained by heating native DNA on a boiling-water bath for 20 min., then cooling rapidly under a stream of tap water. The preparation at 260 nm gave $E^{1\%}_{1\,cm}=224$. The increase in extinction with respect to native DNA was 26%.

Histone fractions F_1 and F_3 were prepared according to Johns (1964), 0.4 and 0.5 g. being obtained, respectively, from 100 g. of thymus. On starch-gel electrophoresis (Hnilica, Edwards & Hey, 1966), the fractions were homogeneous.

Viscosity measurements were carried out using a four-ball Ubbellohde type viscometer placed in a Höppler ultrathermostat. The determinations were carried out at $28\pm0.2°$ at pH 6.7. Kinematic viscosity (v) was determined separately for each ball and plotted according to the equation:

$$A^v/v_0=f(\Delta h) \qquad (1)$$

where v is kinematic viscosity; A, viscometer constant; Δh, difference in the level of fluid. To eliminate the effect of hydrostatic pressure of the solution, Δh was

extrapolated to zero. Intrinsic viscosity ([η]), extrapolated to zero concentration, was determined graphically according to equation:

$$\lim_{c \to 0} \frac{\ln \nu/\nu_0}{c} = [\eta] \qquad (2)$$

where c is concentration in g./100 ml.

The investigated solutions were incubated in a Höppler ultrathermostat at the indicated temperature for 15 min. The solutions of DNA and histones were made in 0.05 M-NaCl. To one volume of DNA, one volume of F_1 or F_3 solution was added, the ratio of histone to DNA being 0.48. The final concentration of DNA was 20.8 µg./ml., and that of histone fraction 10 µg./ml. Higher NaCl concentrations were obtained by adding the appropriate volume of 4 M-NaCl to the mixture of DNA with histone. Relative viscosity was determined for three different concentrations of the DNA-histone complex at a given NaCl concentration. Controls consisted of 0.05 M-NaCl solutions to which the same volume of 4 M-NaCl was added as to the proper sample. Figure 1 presents [η] for DNA solution, and Fig. 2 for the DNA-histone complex.

Spectrophotometric measurements were made in a Zeiss UV-1 spectrophotometer.

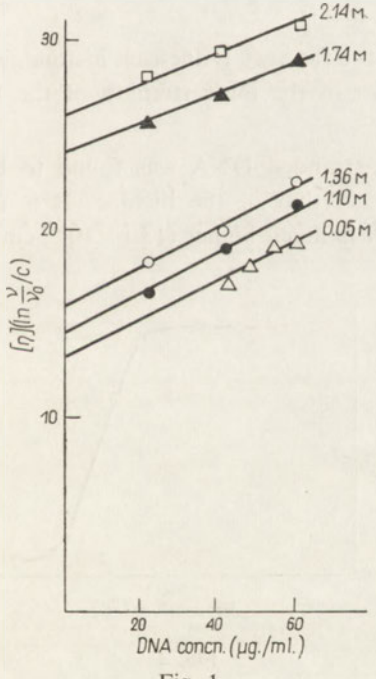

Fig. 1

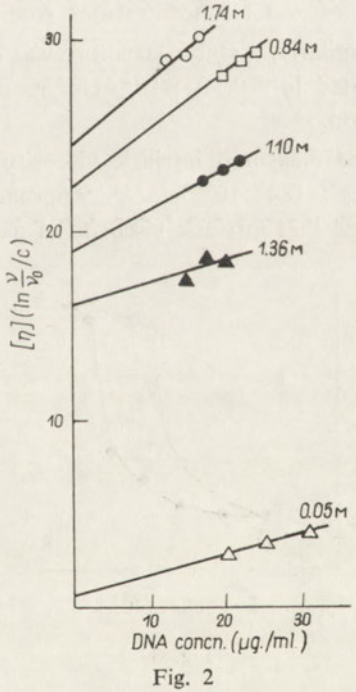

Fig. 2

Fig. 1. Graphical determination of intrinsic viscosity ([η]) of DNA in NaCl solutions at indicated concentrations; temperature 28±0.2°, pH 6.7. For details see Methods.

Fig. 2. Graphical determination of intrinsic viscosity ([η]) of DNA-F_1 complex in NaCl solutions at indicated concentrations; temperature 28±0.2°, pH 6.7, F_1 : DNA ratio 0.48.

RESULTS

The spectrophotometrically determined melting temperature, T_m=68°, of double-stranded DNA (Fig. 3) agreed well with 69° obtained by the viscometric method (Fig. 4).

The mixture of DNA and the very lysine-rich histone fraction (F_1) exhibited an increase in T_m by as much as 15°, indicative of nucleohistone complex formation (Fig. 3).

The effect of NaCl concentration on intrinsic viscosity of DNA solutions in the presence or absence of urea, is presented in Fig. 5A. The intrinsic viscosities [η] over the range of NaCl concentrations 0.05 - 1.4 M were fairly close indicating that under these conditions the size of DNA molecule did not undergo changes. At higher NaCl concentrations, the intrinsic viscosity increased rather sharply, probably due to formation of the Helmholtz-Stern double layer. Thus, it appears that DNA molecules have a rigid conformation over the NaCl range 0.05 - 1.4 M, in contrast to the behaviour of flexible polyelectrolytes. The obtained results are in agreement with those of Rowen & Norman (1954) who measured the orientation angle of DNA molecules with respect to the flow velocity gradient and demonstrated the rigid conformation of DNA in the range of 0.001 - 10% NaCl concentrations. As shown in Fig. 5A, urea did not affect the intrinsic viscosity of DNA over the whole range of NaCl concentrations studied.

Similarly, a stable structure was observed for the very lysine-rich histone, which exhibited [η]$\approx 0.120 \times 10^{-3}$ cm^3/g. independent of the ionic strength of the NaCl solution.

The measured intrinsic viscosity of single-stranded DNA was found to be as low as 0.184×10^{-3} cm^3/g., approaching closely that of the histone. It is to be stressed that intrinsic viscosity of native DNA is much higher (13.1×10^{-3} cm^3/g.).

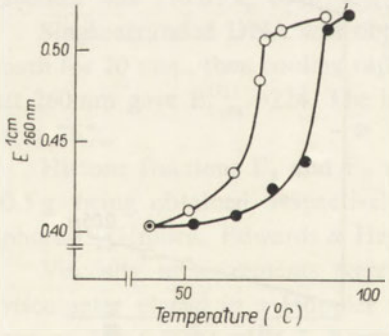

Fig. 3

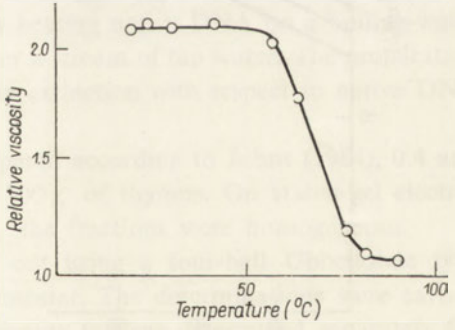

Fig. 4

Fig. 3. Melting profiles of (○), DNA (22.4 µg./ml.) and (●), DNA-F_1 complex, at F_1 : DNA ratio of 0.48. The preparations were dissolved in 0.05 M-NaCl solution, pH 6.7.

Fig. 4. Determination of melting temperature of DNA from relative viscosity measurements; DNA concentration 330 µg./ml. in 0.05 M-NaCl solution. For details see Methods.

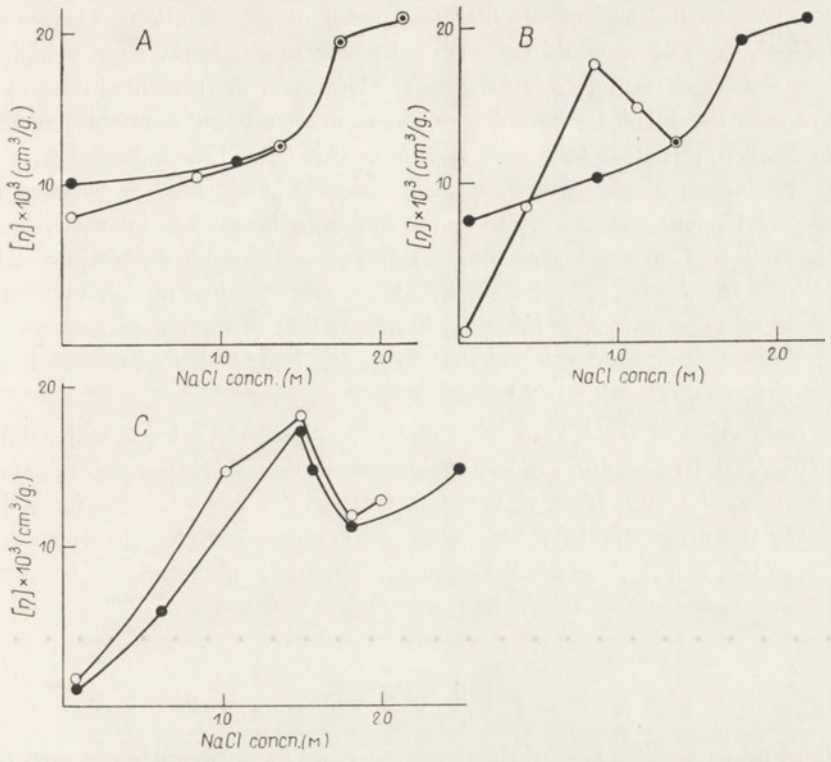

Fig. 5. The effect of NaCl concentration and urea on intrinsic viscosity of: *A*, native DNA; *B*, DNA-F_1 complex (F_1 : DNA ratio of 0.48); *C*, DNA-F_3 complex (F_3 : DNA ratio of 0.48). Incubation at 28 ± 0.2°, pH 6.7, (○), without and (●), with urea. The urea concentrations applied were: *A*, 10.5, 1.33 and 3 M; *B*, 1.05 and 1.33 M; *C*, 3 M.

The results of analogous determinations for DNA complexes with histone fraction F_1, are presented in Fig. 5B. At 0.05 M-NaCl concentration, the intrinsic viscosity of the complex was very low and close to the value for single-stranded DNA. At a ionic strength of 0.85, the intrinsic viscosity was the highest, about 18×10^{-3} cm³/g., whereas at ionic strength exceeding 1.0, the obtained values were somewhat lower and similar to those obtained with solution of native DNA.

According to Kuhn & Kuhn (1945), the low intrinsic viscosity of the DNA--histone complexes is indicative of a decrease in viscosity increment, i.e. of tight packing of particles. The rapid increase in intrinsic viscosity with increasing NaCl concentration, points to a high flexibility of the complex. At NaCl concentrations exceeding 1 M, the intrinsic viscosity decreased so that at 1.36 M-NaCl it attained the value equal to that for DNA.

Some properties of protein molecules may be evaluated from the Fisher plot (Fisher, 1965). This plot represents a relation for globular proteins between the molecule volume and the ratio of polar to non-polar residue volumes. Pticin & Skvorcov (1966) found that all histone fractions give experimental points above the Fisher plot, the value for fraction F_1 being the highest. This indicates that histone F_1

is more asymmetrical and more polar than other histone fractions. Consequently, the DNA-F_1 complexes could be expected to be more stable, have a higher T_m value and dissociate at higher ionic strength. However, the presented data indicate that although the DNA-F_1 complex possessed high melting temperature, histone F_1 was easily released at low ionic strength. This behaviour indicates that other bonds, in addition to electrostatic ones, participate in the binding between DNA and histone F_1. On the assumption that hydrogen bonds are involved, it could be anticipated that urea at higher concentrations would inhibit the binding of DNA with histone F_1. As it appears from the data presented in Fig. 5B, urea at concentrations of 1.05 and 1.33 M inhibited the formation of the complex almost completely. Thus, it may be concluded that hydrogen bonds play an essential role in binding of histone F_1 with DNA.

The interaction of DNA with the arginine-rich histone F_3 led to quite different results (Fig. 5C). In a medium of low ionic strength the DNA-F_3 complex exhibited a low intrinsic viscosity. Urea at 3 M concentration did not affect the intrinsic viscosity over the range of NaCl concentrations studied. Therefore, it appears that, contrary to the very lysine-rich histones, in formation of complexes between the arginine-rich histones and DNA, hydrogen bonds are not essential.

DISCUSSION

According to Phillips (1962), there are ten pairs of nucleotides per turn of the double-stranded DNA helix, and consequently 7000 - 9000 mol. wt. of histone are required for neutralization of phosphate residues. In studies on the interaction between individual histones and DNA, Johns & Butler (1964) applied the precipitation method (Butler & Johns, 1964) and found that the very lysine-rich fraction F_1 precipitates DNA to the greatest extent. According to Johns (1966), the number of basic amino acid residues sterically available for combination with phosphate groups, is greater in fraction F_1 than in other histone fractions. Ćvetković & Savić (1969) demonstrated that in 0.14 M-NaCl at pH 5 and at a weight ratio of histone to DNA of 0.8, fraction F_1 precipitated native thymus DNA almost completely, whereas denatured or renatured DNA was precipitated but partly.

In the present work, the experiments were carried out on very dilute solutions of DNA-F_1 and DNA-F_3 complexes (about 30 µg./ml.) at a weight ratio of histone to DNA of 0.48. In neutral medium at NaCl concentration in the range 0.05 - 2 M no precipitation was observed.

The structure of the reformed DNA-F_1 and DNA-F_3 complexes was dependent on the ionic strength of the medium. At low ionic strength (0.05 M-NaCl) the intrinsic viscosity of both complexes was very low and approached that of solution of histone or denatured DNA. For both complexes, the intrinsic viscosity increased with increasing NaCl concentration; and the complex with fractions F_1 and F_3 approached values characteristic of double-stranded DNA at 1.36 M and 1.7 M-NaCl concentration, respectively. The highest value of intrinsic viscosity was attained

by the DNA-F_1 complex in 0.85 M-NaCl, and by the DNA-F_3 at an almost twice as high salt concentration. Further differences between the two complexes became apparent in the presence of urea. The DNA-F_1 complex probably was not formed at 1 M-urea concentration, whereas the stability of the DNA-F_3 complex was unaltered even at an urea concentration as high as 3 M. In concentrated urea solution double-stranded DNA did not undergo denaturation. Similarly to the results reported by Olins (1969), fraction F_1 was found to stabilize the structure of double-stranded DNA. According to Olins, the melting temperature (T_m) of DNA-F_1 complexes is 86°, and in the present experiments it was determined to be 83°.

The presented results concerning the reformed complex of DNA with histone fraction F_3 in dilute urea solutions are in agreement with the data reported by Bartley & Chalkley (1968). In urea solution, the behaviour of the DNA-F_3 complex differs from that of the DNA-F_1 complex, the latter being dissociated at an urea concentration as low as 1 M.

Bartley & Chalkley (1968) demonstrated that the intrinsic viscosity of deoxyribonucleoprotein is doubled in concentrated (3.5 M) urea solutions. The effect of urea was explained by those authors by a transition from the supercoil conformation prevailing in dilute urea solutions to a more extended conformation in concentrated urea solution. The preparation of native thymus DNP contains only a slight amount of fraction F_1, as may be judged from its ready dissociation and the fact that it forms only 20% of total histone. Thus, the content of fraction F_1 in the native DNP preparation was probably too small to affect its intrinsic viscosity.

On the basis of the presented measurements of intrinsic viscosity of DNA-F_1 and DNA-F_3 complexes it may be assumed that the forces responsible for binding of lysine-rich histones with double-stranded thymus DNA are susceptible to dilute urea solution, at variance with those involved in binding of DNA to arginine-rich histones. In the latter case, some conformational alterations in composite molecules induced by high urea concentrations were not accompanied by dissociation of the complex.

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KOMPLEKSY HISTONÓW Z KWASEM DEZOKSYRYBONUKLEINOWYM

Streszczenie

1. Badano temperaturę topnienia (T_m) i lepkość wewnętrzną ($[\eta]$) kompleksów dwuniciowego DNA z histonami bogatymi w lizynę (F_1) i bogatymi w argininę (F_3).

2. W roztworach o niskiej sile jonowej powstawaniu kompleksów towarzyszył wzrost T_m i spadek lepkości wewnętrznej. Pod wpływem wzrastających stężeń NaCl kompleksy ulegały dysocjacji, przy czym kompleks DNA-F_1 ulegał dysocjacji przy niższej sile jonowej, a kompleks DNA-F_3 przy wyższej.

3. Przy niskiej sile jonowej kompleksy DNA-F_1 ulegają degradacji już pod wpływem 1.0 M-mocznika, natomiast kompleksy DNA-F_3 są trwałe nawet w 3 M-moczniku.

4. Na tej podstawie wnioskuje się, że wiązania wodorowe biorą udział w tworzeniu kompleksów DNA-F_1, natomiast nie biorą udziału w tworzeniu kompleksów DNA-F_3.

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REGULATION OF ATP CITRATE LYASE ACTIVITY *IN VITRO*

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1. Kinetics of the partially purified ATP citrate lyase (EC 4.1.3.8) preparation from rat liver were studied. 2. The Lineweaver-Burk plots of the reaction rate against citrate concentration were biphasic. The K_{m1} (for low citrate concentration) was 0.13 mM, independent of Mg^{2+} and ATP concentrations. On the other hand, K_{m2} (for high citrate concentration) increased with increasing Mg^{2+} and with decreasing ATP concentrations due, probably, to the inhibitory action of Mg^{2+} ion. 3. DL-Isocitrate increased K_{m1} for citrate and lowered K_{m2}; depending on citrate concentration in could act either as a partially competitive inhibitor, or as an activator by removing the excess of Mg^{2+} ion. 4. The values of K_m and V_{max} for ATP increased at higher fixed Mg concentrations. Similarly, K_m and V_{max} for Mg increased at higher fixed ATP concentrations. This suggests that the ATP-Mg^{2-} complex is the true substrate. 5. ADP and P_i are competitive inhibitors with respect to ATP. Lowering of Mg^{2+} concentration as well as addition of isocitrate or EDTA decrease the inhibition of citrate lyase by ADP and P_i, which suggests that their complexes with Mg^{2+} are the active forms of these inhibitors. 6. The possible mechanism of regulation of citrate lyase activity *in vivo* is discussed.

ATP citrate lyase (EC 4.1.3.8) is an extramitochondrial enzyme catalysing the following reaction:

$$\text{Citrate} + \text{ATP} + \text{CoA} \xrightarrow{Mg^{2+}} \text{Acetyl-CoA} + \text{Oxaloacetate} + \text{ADP} + P_i .$$

A number of authors, among them Bhaduri & Srere (1963), D'Adamo & Haft (1965) and Kornacker & Lowenstein (1965a,b) have suggested that the citrate-cleavage reaction may provide extramitochondrial acetyl-CoA for the synthesis of fatty acids from carbohydrates. Daikuhara, Tsunemi & Takeda (1968) have demonstrated that in cytoplasm of the liver cell over 80% of the active acetate may be supplied by this reaction. According to Ballard & Hanson (1967) and Kornacker & Lowenstein (1965a,b) changes in activity of citrate lyase correlate with changes in the rate of fatty acid synthesis. On the basis of these data it has been suggested that this enzyme is a possible control point in lipogenesis. On the other hand, the experiments of Foster & Srere (1968) and Goodridge (1968a,b) indicated that changes in the activity of citrate lyase resulted from, rather than causing, changes in the rate of fatty acid synthesis. Moreover, Foster & Srere (1968) have demonstrated

that under specified conditions there is no correlation between the activity of citrate lyase and lipogenesis. It seems, however, that *in vivo* the activity of the enzyme may be influenced by the concentration of low-molecular-weight metabolites. Atkinson & Walton (1967) and Angielski & Szutowicz (1967b) have suggested that *in vivo* the activity of citrate lyase may be regulated by the energy level of the adenylate system.

The aim of the present work was to study the effect of substrates and reaction products on the activity of citrate lyase *in vitro* at 37° and at high chloride concentration. The possible mechanism of the regulation of the enzyme activity *in vivo* is discussed.

MATERIALS AND METHODS

Preparation of the enzyme. ATP dependent citrate lyase was isolated as described by Srere (1959) from liver of Wistar rats kept on a standard diet. The last step of the purification procedure involving the use of alumina gel C_γ was omitted and the 50-fold purified enzyme obtained was dissolved in 0.5 M-KCl. The preparation was unstable and, when kept at 0 - 4°, lost 15% of the activity within 24 hr. Therefore it was used for experiments within three days, the enzyme activity being then not lower than 50% of the initial value. In the experiments concerning the effect of 5′-AMP, a crude liver extract was also used; the liver was homogenized with 4 volumes of ice-cold 0.15 M-KCl solution and centrifuged for 45 min. at 30 000 *g* at 0°.

Determination of citrate lyase activity. This was based on the method of Srere (1959) with some modifications. Oxaloacetate formed from citrate, CoA and ATP is measured as the oxidation of NADH by coupling with NAD-malate dehydrogenase (EC 1.1.1.37). The standard incubation mixture contained in a final volume of 0.25 ml.: 20 mM-tris-HCl buffer, pH 7.4, 20 mM-citrate potassium salt, 5 mM-ATP, 10 mM-$MgCl_2$, 0.42 mM-CoA, 10 mM-2-mercaptoethanol, 10 mM-KCl, and partially purified enzyme preparation (20 or 40 μg. of protein). Before mixing, citrate and ATP solutions were brought to pH 7.4. The sample was preincubated without CoA for 5 min. at 37°, then CoA was added, and after 5 min. at 37° the reaction was stopped by adding 1.15 ml. of ice-cold 0.1 M-phosphate buffer, pH 7.4, containing 0.12 mM-NADH and an excess (2 i.u.) of malate dehydrogenase (EC 1.1.1.37). The sample was then kept for 5 min. in an ice-water bath at 0°, then 0.2 ml. of 0.5 M-NaOH was added, and the mixture left for a few hours at 0 - 4°. The sediment formed was centrifuged off, and in the clear supernatant of pH 9.5 the extinction was read at 340 nm in a VSU-2 spectrophotometer (Zeiss, Jena). At room temperature the extinction of samples was unchanged for 6 hr., and at 0 - 4° for 48 hr. Control samples contained no CoA.

The activity of citrate lyase was calculated from the difference in extinction between control and proper samples, and expressed as μmoles of oxidized NADH/ /min./mg. protein.

In preliminary experiments it was found that the reaction catalysed by citrate lyase was inhibited completely when the sample, following incubation, was cooled

to 0° in the presence of phosphate buffer added to 80 mM concentration; on the other hand, these conditions were suitable for quantitative determination of oxaloacetate. Using standard oxaloacetate and 2 i.u. of malate dehydrogenase, it was possible to determine up to 0.1 μmole of oxaloacetate per sample; the determinations were not affected by the applied concentrations of ATP, other substrates and reaction products. The recovery of oxaloacetate from samples subjected to the whole procedure, was complete.

Protein was determined by the biuret method (Gornall, Bardawill & David, 1949).

Calculation of citrate-Mg^- *and ATP*-Mg^{2-} *concentrations.* The approximate concentrations of the complexes were calculated taking the pK_3 for citrate equal to 6.4 (Bates & Pincking, 1949), pK_2 for ATP 6.5 (Martell & Schwarzenbach, 1956; Smith & Alberty, 1956) and the association constants for citrate-Mg^- equal to 0.2 mM^{-1} (Lindenbaum & White, 1959) and for ATP-Mg^{2-}, 20 mM^{-1} (O'Sullivan & Perrin, 1964). As the calculations were made only under conditions when there was a large excess of citrate and ATP with respect to magnesium, it was assumed that the concentration of free Mg^{2+} equalled zero. Likewise, the competition of $citrate^{2-}$ and ATP^{3-} for the enzyme was not taken into account, similarly as the competition of Na^+ and K^+ with Mg^{2+} for $citrate^{3-}$ and ATP^{4-} because of low association constants of their complexes (Lindenbaum & White, 1959; O'Sullivan & Perrin, 1964).

Reagents: ATP, disodium salt, NADH and malate dehydrogenase were from Boehringer (Mannheim, West Germany), ADP, IDP and IMP from Pabst (Milwaukee, Wis., U.S.A.), 5′-AMP from Schuchardt (München, West Germany), 3′,5′-(cyclic)-AMP from Calbiochem. (Los Angeles, Calif., U.S.A.). CoA (Calbiochem., or Sigma Chem. Co., St. Louis, Mo., U.S.A.) was standardized for the content of CoA-SH groups using ATP citrate lyase according to Bergmeyer (1965); tris was from British Drug Houses (Poole, Dorset, England); DL-isocitrate and 2-mercaptoethanol were from Fluka A. G. (Buchs, Switzerland). Other reagents were products of CIECH (Gliwice, Poland).

RESULTS

All experiments were carried out at 0.42 mM-CoA, as it was found that the reaction rate was not affected by changes in CoA concentration from 0.15 to 0.80 mM in the presence of different amounts of the remaining compounds.

Effect of time of incubation and amount of protein. The reaction rate with 20 μg. of enzyme protein and 10 mM-Mg^{2+} was linear with time up to 7.5 min. at different concentrations of ATP from 1 to 10 mM and citrate from 0.5 to 20 mM. In the presence of saturating concentrations of the substrates (20 mM-citrate, 5 mM-ATP, 10 mM--Mg^{2+}), the reaction rate was linear with the enzyme protein from 8 to 30 μg. per sample, and in presence of 0.5 to 2 mM-citrate, from 8 to 64 μg. of protein.

Effect of citrate. In Fig. 1 are presented the Lineweaver-Burk plots showing the reciprocal of velocity against the reciprocal of citrate concentration at three

fixed ATP concentrations (1, 5 and 10 mM) and constant 10 mM-Mg^{2+}. As it can be seen, the plots exhibited two distinct linear regions differing considerably in slope. With 1 mM-ATP the second phase began at 4 mM-citrate concentration, with 5 mM-ATP at 3 mM, and in the presence of 10 mM-ATP, at 2 mM. Similar biphasic plots have been obtained by Le John & Jackson (1968) for glutamate dehydrogenase (EC 1.4.1.2), by Iwatsuki & Okazaki (1967) for thymidine phosphorylase (EC 2.4.2.4), and by Datta & Gest (1965) for homoserine dehydrogenase (EC 1.1.1.3).

The Michaelis constants for low citrate concentrations (K_{m1}) and with three fixed ATP concentrations were 0.13 mM (Fig. 1A). At higher citrate concentrations, the K_m values (designated K_{m2}) were dependent on ATP concentration (Fig. 1B). The lowering of ATP from 10 to 5 mM resulted in an increase in K_{m2} for citrate from 1.7 to 5.3 mM, and at 1 mM-ATP the K_{m2} was still higher. On the other hand, a decrease in Mg^{2+} concentration from 10 to 2 mM, in the presence of 5 mM-ATP (Fig. 2) decreased the K_{m2} for citrate from 5.3 to 0.8 mM but had no effect on K_{m1}. The presented data seem to indicate that the biphasic course of the citrate lyase activity with respect to citrate concentration could be due to the inhibitory effect of an excess of Mg^{2+} ion and to an allosteric activation of the enzyme by higher citrate concentrations. A lowering of Mg^{2+}, or its elimination, by higher ATP concentration, would facilitate the activation of the enzyme by citrate resulting in lower K_{m2} value. On this assumption, the first portion of the plot would correspond to gradual saturation of the active site of the enzyme with citrate. The same K_{m1} values obtained for citrate both in the presence of an excess of Mg^{2+} and its relative deficiency, seem to indicate that both the citrate-Mg^{-} complex and free citrate can act as equivalent substrates for citrate lyase.

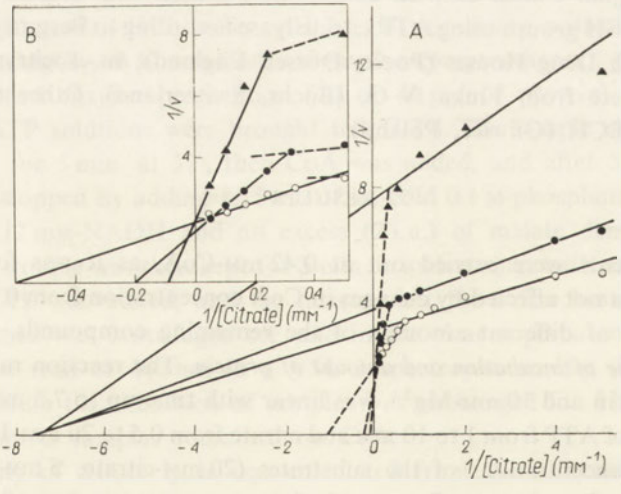

Fig. 1. Double-reciprocal plots of initial velocity against citrate concentration at 10 mM-Mg^{2+} and three values of ATP: (▲), 1 mM; (●), 5 mM; and (○), 10 mM. Protein 20 µg. Citrate concentration: *A*, from 0.2 to 20 mM; *B*, from 2 to 20 mM. In this and subsequent figures, *v* is expressed in µmoles of oxidized NADH/min./mg. protein.

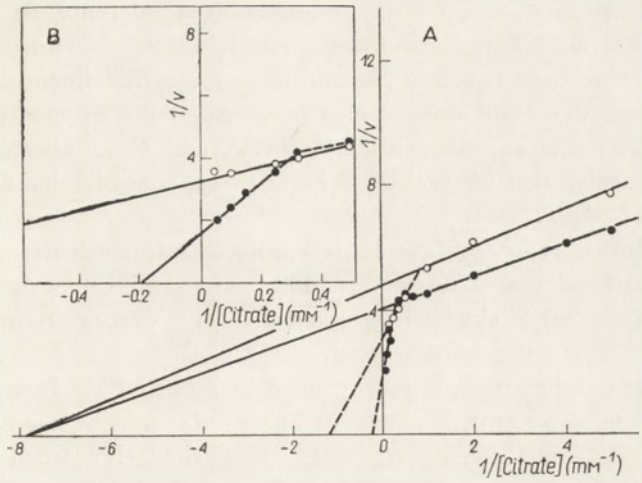

Fig. 2. Double-reciprocal plots of initial velocity against citrate concentration at 5 mM-ATP and two values of Mg^{2+}: (●), 10 mM, and (○), 2 mM. Other conditions as in Fig. 1.

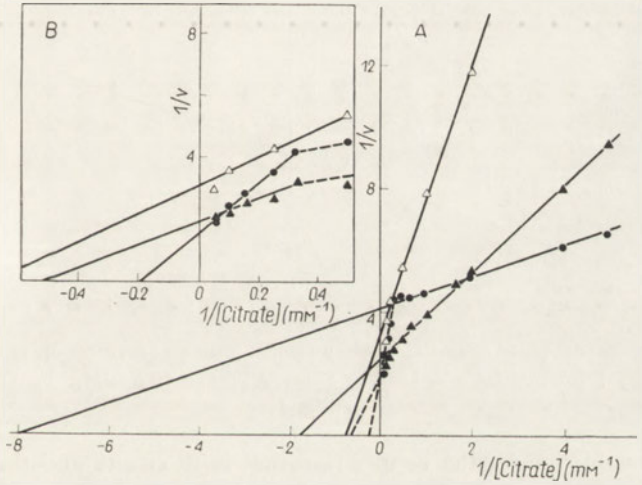

Fig. 3. Double-reciprocal plots of initial velocity against citrate concentration and the effect of 10 mM-DL-isocitrate, at 10 mM-Mg^{2+}. (●), Control, 5 mM-ATP; (▲), with isocitrate and 5 mM-ATP; (△), with isocitrate and 1 mM-ATP. Other conditions as in Fig. 1.

As the excess of Mg^{2+} seems to be largely responsible for the biphasic course of the plot, the effect of DL-isocitrate was studied. Isocitrate possesses a structure similar to that of citrate and the capacity to chelate with bivalent cations (Schubert & Lindenbaum, 1952; Li, Westfall, Lindenbaum, White & Schubert, 1957). At 10 mM-Mg^{2+} concentration and with 5 mM-ATP, in the presence of 10 mM-DL-isocitrate the plot remained biphasic (Fig. 3) but the K_{m1} for citrate increased from 0.13 to 0.6 mM, and K_{m2} decreased from 5.3 to 1.7 mM; the enzyme activity was somewhat inhibited at lower citrate concentrations and enhanced at higher

ones. On the other hand, at 1 mM-ATP on addition of isocitrate the biphasic character of the plot disappeared and the K_m was 1.34 mM.

From the above data it seems possible to suppose that isocitrate, having an affinity for the substrate site and the regulatory site of the enzyme, may act both as a competitive inhibitor with respect to citrate, and as an allosteric activator. It seems also possible that the activation by isocitrate could be due to the binding of the excess of Mg^{2+}.

In the presented experiments, with increasing citrate concentration there was a decline in the ratio of [Mg] to the sum of [ATP] and [citrate]. Under these conditions, especially when this ratio became lower than unity, the concentration of the ATP-Mg^{2-} complex could also be decreased.

The effect of citrate concentration on the activity of citrate lyase at a [Mg] : : [ATP] + [citrate] ratio of 1, is shown in Fig. 4; also under these conditions the Lineweaver-Burk plot was biphasic; K_{m1} was 0.13 mM, similarly as when the ratio was

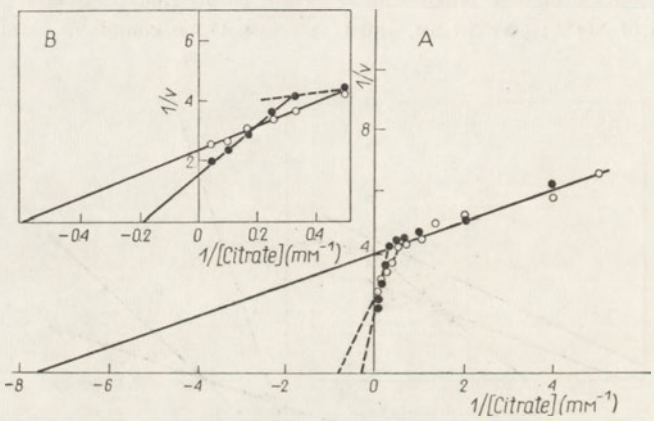

Fig. 4. Double-reciprocal plots of initial velocity against citrate concentration at (○), 5 mM-ATP and a [Mg] : [ATP] + [citrate] ratio = 1; (●), 5 mM-ATP and 10 mM-Mg^{2+}. Other conditions as in Fig. 1.

varying. This would confirm the supposition that both citrate and the citrate-Mg^{-} complex may act as equivalent substrates for citrate lyase. At the ratio [Mg] : : [ATP] + [citrate] = 1, the maximum velocity at higher citrate concentrations (V_{max2}) was lower than at constant Mg^{2+} concentration, this being possibly due either to lower amount of the non-complexed citrate, or to the inhibition by free Mg^{2+} ions.

For estimation of the possible co-operativity of citrate with the enzyme, the interaction coefficient n was determined from the slope of Hill plots: $\log \frac{v}{V_{max} - v}$ *versus* log of molar citrate concentration (Atkinson, 1966). As it may be seen from Table 1, both at low and high citrate concentrations n was close to unity and was independent of the applied concentrations of ATP and Mg^{2+}, and of the presence of isocitrate, indicating no co-operativity of citrate with the enzyme under these

Table 1

K_m, V_{max} *and interaction coefficients (n) for citrate at different fixed concentrations of ATP and* Mg^{2+}, *and the effect of isocitrate*

V_{max} is expressed in μmoles/mg. protein/min. Protein content 20 μg./sample.

ATP (mM)	Mg^{2+} (mM)	Isocitrate (mM)	K_{m1} (mM)	V_{max1}	n	K_{m2} (mM)	V_{max2}	n
10	10	—	0.13	0.31	0.89	1.68	0.51	0.95
5	10	—	0.13	0.25	0.96	5.25	0.65	1.00
1	10	—	0.13	0.15	0.83	10.70	0.50	1.11
5	2	—	0.13	0.21	0.93	0.80	0.40	0.97
5	*	—	0.13	0.25	0.93	1.74	0.44	0.90
5	10	10	0.60	0.45	0.95	1.68	0.51	1.08
1	10	10	1.34	0.37	1.02	—	—	—

* [Mg] : [ATP] + [citrate] ratio = 1.

conditions. It seems possible that the enzyme has two independent active and regulatory sites.

Effect of ATP. The K_m value for ATP at 20 mM-citrate was 0.55 mM, independent of Mg^{2+} concentration over the range from 1 to 10 mM (Fig. 5). The lowering of Mg^{2+} resulted in a decrease of V_{max}, and the inhibition by the substrate appeared at lower ATP concentration (Fig. 6). However, from the reciprocal plots for the ATP-Mg^{2-} complex it appeared that the lowering of Mg^{2+} decreased the K_m values for the complex from 0.44 mM at 10 mM-Mg^{2+} to 0.17 at 1 mM-Mg^{2+}. At the same time the inhibition by substrate appeared at lower ATP-Mg^{2-} concentration. It may be assumed that, under the above conditions, the amount of free Mg^{2+} was negligible due to the presence of an excess of complexing compounds, i.e. citrate and ATP.

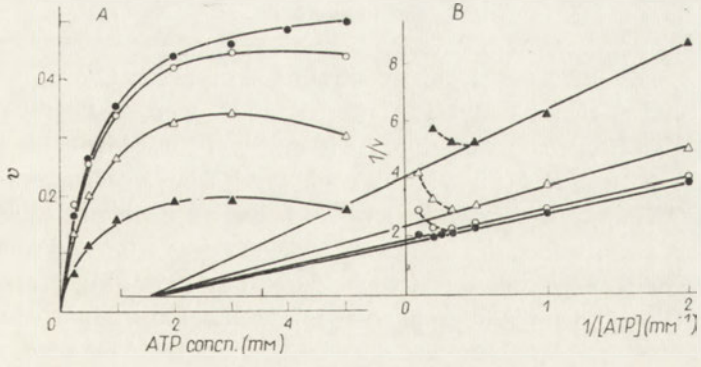

Fig. 5. *A*, The effect of ATP concentration on the initial velocity at 20 mM-citrate and four values of Mg^{2+}: (●), 10 mM; (○), 5 mM; (△), 2 mM; and (▲), 1 mM. *B*, Lineweaver-Burk plots of the same data.

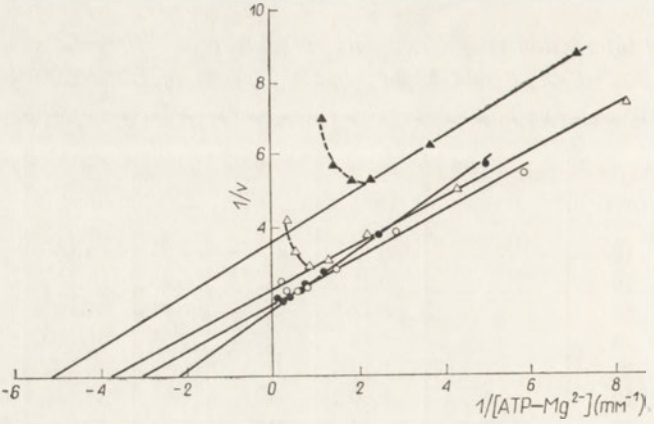

Fig. 6. Double-reciprocal plots of initial velocity against ATP-Mg^{2-} concentration at 20 mM-citrate and four values of Mg^{2+}: (●), 10 mM; (○), 5 mM; (△), 2 mM; and (▲), 1 mM. Protein 20 µg. Concentration of the ATP-Mg^{2-} complex from 0.08 to 6.35 mM.

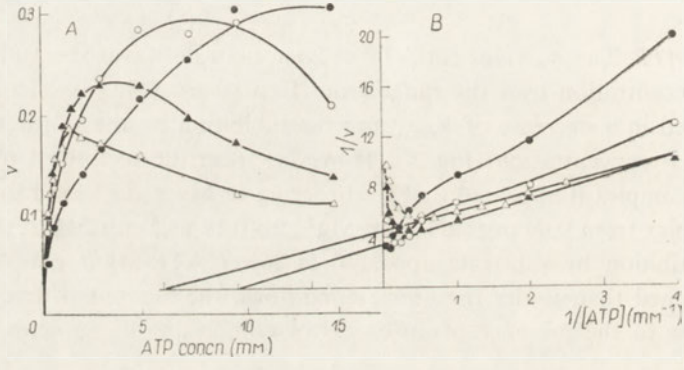

Fig. 7. *A*, The effect of ATP concentration on the initial velocity at 2 mM-citrate and four values of Mg^{2+}: (●), 10 mM; (○), 5 mM; (▲), 2 mM; and (△), 1 mM. Protein 40 µg. *B*, Lineweaver-Burk plots of the same data.

In the presence of a tenfold lower citrate concentration (2 mM), with ATP as variable substrate, changes in Mg^{2+} affected much more distinctly the reaction rate than at high citrate concentration. At 10 mM- and 5 mM-Mg^{2+}, the Lineweaver-Burk plots were sigmoidal (Fig. 7). This could result from a decline, with increasing amounts of ATP, of the free Mg^{2+} ion which has an inhibitory effect on citrate lyase. In these experiments, the $[S]_{0.5}$ value for ATP was estimated from Hill plots (Atkinson, 1966) and it was found to be 3.0 mM at 10 mM-Mg^{2+} and 1.1 mM at 5 mM-Mg^{2+} (Fig. 8). On the other hand at low, 2 and 1 mM, Mg^{2+} concentrations, the Lineweaver-Burk plots were linear, and K_m values for ATP were, respectively, 0.43 and 0.33 mM. Similarly as in the presence of 20 mM-citrate, at lower Mg^{2+} concentrations V_{max} decreased and the substrate inhibition appeared at lower ATP concentrations.

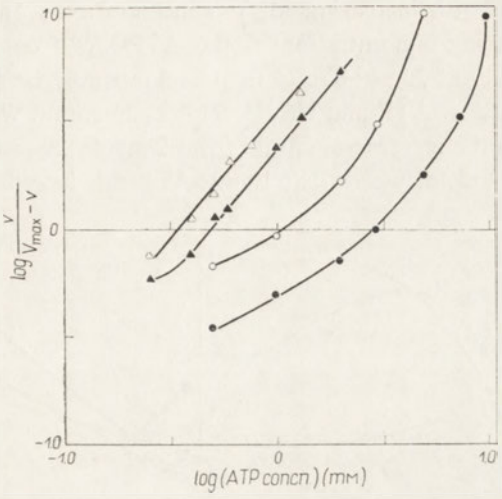

Fig. 8. Plots of log $v/V_{max} - v$ versus log ATP concentration, at 2 mM-citrate and different fixed Mg^{2+} concentrations: (●), 10 mM; (○), 5 mM; (▲), 2 mM; (△), 1 mM. ATP and protein as in Fig. 7.

Effect of magnesium. From the above data it follows that the reaction rate as a function of ATP concentration was considerably influenced by Mg^{2+} concentration. A similar effect of ATP was observed when Mg^{2+} was the variable substrate (Figs. 9 and 10). With 20 mM-citrate and at 10 mM-ATP, the K_m for Mg^{2+} was 4.15 mM, and at 1 mM-ATP the K_m decreased to 2 mM; with 2 mM-citrate, the respective values

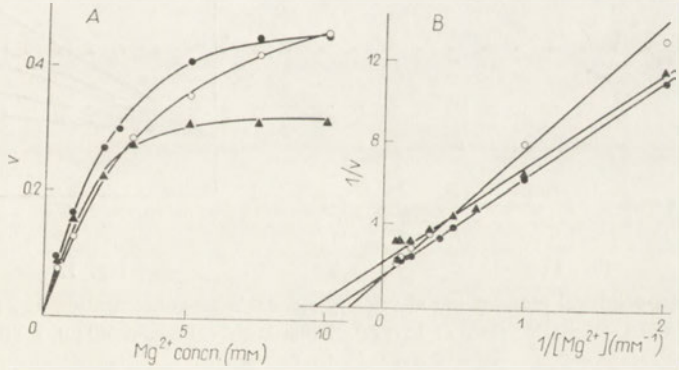

Fig. 9. *A*, The effect of Mg^{2+} concentration on the initial velocity at 20 mM-citrate and three values of ATP: (○), 10 mM; (●), 5 mM; and (▲), 1 mM. Protein 20 µg. *B*, Lineweaver-Burk plots of the same data.

were 3.6 and 0.6 mM. At lower ATP concentration (1 mM), there was also a decrease in V_{max}, which was more pronounced with 2 mM-citrate; also the inhibition by Mg^{2+} appeared at much lower Mg^{2+} concentration. The presented data indicate that the ATP-Mg^{2-} complex is the true substrate for citrate lyase. Thus the increase

in the reaction rate with increasing Mg^{2+} concentration would be dependent on the parallelly increasing concentration of the ATP-Mg^{2-} complex. The fact that K_m for Mg^{2+} is higher at 20 mM-citrate than at 2 mM may be explained by competition for Mg^{2+} between ATP and citrate. This competition was most pronounced at 1 mM-ATP, when the increase of citrate from 2 mM to 20 mM raised the K_m value for Mg^{2+} almost fourfold, whereas at 10 mM-ATP this increase was only 1.2-fold.

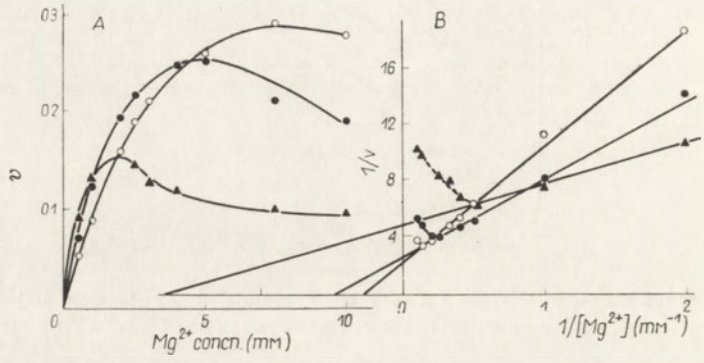

Fig. 10. *A*, The effect of Mg^{2+} concentration on the initial velocity at 2 mM-citrate and three values of ATP: (○), 10 mM; (●), 5 mM; and (▲), 1 mM. Protein 40 μg. *B*, Lineweaver-Burk plots of the same data.

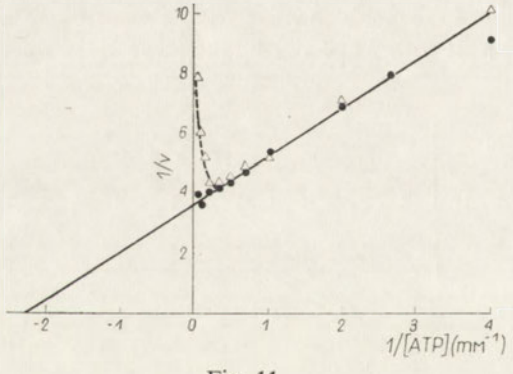

Fig. 11

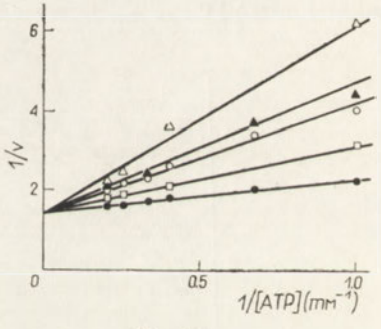

Fig. 12

Fig. 11. Double-reciprocal plots of initial velocity against ATP concentration at 2 mM-citrate and (●), [Mg] : [ATP] + [citrate] ratio = 1; (△), 2 mM-Mg^{2+}. Protein 40 μg. ATP concentration from 0.25 to 10 mM.

Fig. 12. The effect of ADP and P_i on the activity of citrate lyase in the presence of 20 mM-citrate and 10 mM-Mg^{2+}. Double-reciprocal plots of initial velocity against ATP concentration: (●), control, (□), with 0.5 mM-ADP; (○), with 1.0 mM-ADP; (△), with 2.0 mM-ADP; (▲), with 5.0 mM-P_i. Protein 20 μg. ATP concentration from 1 to 5 mM.

In the above experiments, the ratio [Mg] : [ATP] + [citrate] was varying. To check the influence of this factor, the effect of ATP concentration on the reaction rate was examined at 2 mM-citrate under conditions where this ratio equalled unity, and compared with the results obtained with fixed 2 mM-Mg^{2+} content (Fig. 11).

The same values of K_m for ATP (0.45 mM) and the same V_{max} were obtained but the inhibition by an excess of ATP was observed only at the varying ratio (i.e. fixed Mg^{2+} concentration).

Effect of ADP and P_i. These two reaction products are known to inhibit citrate lyase. In the presence of 20 mM-citrate, ADP, up to a concentration of 2 mM, and 5 mM-P_i inhibited the enzyme activity, the inhibition being competitive with respect to ATP (Fig. 12). K_m for ATP in the presence of 1 mM-ADP was increased from 0.55 to 1.5 mM, and with 5 mM-P_i to 1.75 mM. K_i for ADP determined at different concentrations of citrate and ATP (Fig. 13) was 0.45 mM and was of the same order as the K_m for ATP. The inhibition by ADP and P_i was dependent not only on the concentration of ATP but also on that of citrate. At fixed 10 mM-Mg^{2+} concentration, the decrease of citrate from 20 mM to 2 mM in the presence of 5 mM-ATP enhanced the inhibition by 1 mM-ADP or 5 mM-P_i, and at 2 mM-citrate and with the concentration of ATP decreased to 1 mM the inhibition was still greater (Table 2). The inhibitory effect of ADP and P_i was additive, and when these compounds were added together to the reaction mixture containing the substrates at concentrations corresponding approximately to those found in liver (0.5 mM-citrate, 3 mM-ATP, 0.42 mM-CoA), the inhibition was 80%.

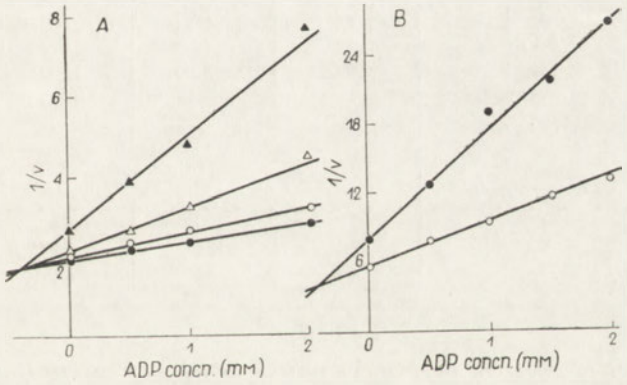

Fig. 13. Dixon plots on the inhibitory effect of ADP: *A*, at 20 mM-citrate, 10 mM-Mg^{2+} and four values of ATP: (●), 5 mM; (○), 4 mM; (△), 2.5 mM; and (▲), 1 mM; *B*, at 2 mM-citrate, 3 mM-ATP and (●), 10 mM-Mg^{2+}, (○), 5 mM-Mg^{2+}. Protein 20 µg.

The inhibition by ADP and P_i was lower when the concentration of Mg^{2+} was lowered or in the presence of complexing compounds, such as EDTA and isocitrate (Table 3). This seems to indicate that ADP-Mg^- and P_i-Mg complexes are the active, or the most active forms.

Effect of other nucleotides. 5′-AMP up to a concentration of 2 mM did not affect the activity of purified preparation of citrate lyase, nor had it any effect on the inhibition by ADP and P_i. 2 mM-IMP had no influence on the enzyme activity, IDP

Table 2

The effect of citrate and ATP concentrations on the inhibition of citrate lyase by ADP and orthophosphate

With 20 mM citrate, 20 µg. of protein was used, and with lower citrate concentrations, 40 µg. of protein/sample.

	Concentration of substrates (mM)				
Citrate	20.0	2.0	2.0	2.0	0.5
ATP	5.0	5.0	3.0	1.0	3.0
Mg^{2+}	10.0	10.0	10.0	10.0	10.0
Addition	Inhibition (%)				
ADP, 1 mM	24	37	55	66	54
P_i, 5 mM	23	35	38	59	33
ADP, 1 mM + P_i, 5 mM	43	69	80	98	81

Table 3

The effect of Mg^{2+} concentration, EDTA and isocitrate on the inhibition of citrate lyase by ADP and orthophosphate

Protein content, 40 µg./sample.

	Concentration of substrates (mM)			
Citrate	2.0	2.0	2.0	2.0
ATP	3.0	3.0	3.0	3.0
Mg^{2+}	10.0	5.0	10.0	10.0
DL-Isocitrate	—	—	10.0	—
EDTA	—	—	—	2.5
Addition	Inhibition (%)			
ADP, 1 mM	55	36	42	35
P_i, 5 mM	38	31	21	23
ADP, 1 mM + P_i, 5 mM	80	66	64	66

Table 4

Effect of different nucleotides on the activity of citrate lyase

The nucleotides were added to 2 mM concentration.

	Concentration of substrates (mM)	
Citrate	2.0	2.0
ATP	5.0	1.0
Addition	Relative reaction rate (%)	
None, control	100	100
ADP	39	24
5′-AMP	102	94
3′,5′-(cyclic)-AMP	91	69
IDP	55	31
IMP	94	101

and 3′,5′-(cyclic)-AMP were inhibitory, but their effect was smaller than that of ADP (Table 4).

On the other hand, in crude liver extract in the presence of 10 mM-Mg^{2+} and 1.75 mM-citrate the citrate lyase activity was 25% inhibited by AMP added at a ratio of 1 : 2 with respect to ATP. This could be explained by the presence in the crude extract of adenylate kinase (EC 2.7.4.3) catalysing the reaction:

$$AMP + ATP \xleftrightarrow{Mg^{2+}} 2\,ADP.$$

DISCUSSION

The kinetics of ATP citrate lyase was studied at 37°, pH 7.4, high chloride concentration and in the presence of 0.42 mM-coenzyme A. Biphasic double-reciprocal plots of the reaction rate against citrate concentration were consistently obtained at different fixed concentrations of ATP and Mg^{2+} (Figs. 1 and 2). This could be explained by citrate acting not only as substrate but also as activator of the enzyme. As an activator, citrate could act on the regulatory site either directly, or indirectly by complexing the excess of Mg^{2+} ion. It seems that both those mechanisms could be involved. In our experiments, the effect of citrate was observed at high, 120 - - 160 mM, concentration of Cl^- ion. According to Plowman & Cleland (1967) this amount of Cl^- overcomes the activation by citrate. However, it should be noted that the activation by citrate observed by us appeared at much higher citrate concentration than in the experiments of Plowman & Cleland (1967) and Inoue *et al.* (1966). Consequently, it may be supposed that the mechanism of activation of citrate lyase by citrate under the conditions applied in the present work, consists in competition with chloride for the regulatory site of the enzyme. In such a case citrate should be a more effective activator than Cl^- ion.

Another possibility is the occurrence of two forms or two isoenzymes of citrate lyase, one having a high affinity for citrate (K_{m1} 0.13 mM), and another a low one (high K_{m2}). Both free citrate, and the complex with Mg^{2+}, could act as equivalent substrates for the first isoenzyme. In this case free Mg^{2+} ion would act as a non--competitive inhibitor with respect to citrate, and isocitrate as a competitive one. For the second isoenzyme only free citrate could be the substrate. Such an assumption explains the lowering of K_{m2} value observed on decreasing Mg^{2+} concentration, increasing the content of ATP, or on addition of isocitrate, as all those factors would lead to an increase in the amount of free citrate. It seems that isocitrate acts unspecifically since other weakly complexing organic anions, such as malonate or maleate, also enhance the activity of the enzyme in the presence of an excess of Mg^{2+} ion, decrease the K_{m2} and increase V_{max1} (Angielski & Szutowicz, 1967b). On the other hand, at low citrate concentrations isocitrate seems to act as a specific inhibitor of the enzyme.

An increase in citrate concentration from 0.2 to 20 mM at various fixed concentrations of ATP and Mg^{2+} over the range from 1 to 10 mM (Figs. 1 and 2) results in a decrease in the ATP-Mg^{2-} complex by 15 to 35%. However, as the resultant

decrease in the reaction rate would not exceed 2 - 10% (calculated from Fig. 11), this could not cause the biphasic course of the plots of the reaction rate against citrate concentration. It could even be a factor decreasing the biphasic character of the plots.

The sigmoidal kinetics of the reaction rate against ATP concentration (Fig. 7B) at 2 mM-citrate and 5 or 10 mM-Mg^{2+} may be explained by the synergistic effect of the increasing concentration of the substrate, ATP-Mg^{2-}, and a decrease of the inhibitory effect of Mg^{2+} due to lowering of its concentration. This interpretation is supported by the linearity of the reciprocal plots at low fixed Mg^{2+} concentrations (1 and 2 mM). It is also confirmed by the linear reciprocal plot of the reaction rate against Mg^{2+} concentration at fixed ATP concentration over the range from 1 to 10 mM, and 2 mM-citrate (Fig. 10).

Atkinson & Walton (1967) and Angielski & Szutowicz (1967b) suggested that *in vivo* the activity of citrate lyase could be regulated by changes in the concentration and ratio of the adenine nucleotides. This suggestion is substantiated by the presented results: the lowering of citrate concentration from 20 to 2 mM in the presence of 10 mM-Mg^{2+} resulted in an increase of K_m for ATP from 0.55 to 3 mM. The latter value is comparable with the content of ATP in liver (Söling, Kattermann, Schmidt & Kneer, 1966; Start & Newsholme, 1968). Moreover, ADP and P_i, used at concentrations corresponding to physiological values, had a synergistic action and decreased citrate lyase activity five- to several-fold (Tables 2 and 3). Therefore it may be supposed that *in vivo*, when the [ATP] : [ADP] + [P_i] ratio in liver cell cytoplasm is low, the activity of citrate lyase would be considerably inhibited. Thus citrate lyase could be the regulatory step in the formation of acetyl-CoA available for fatty acid synthesis. However, *in vivo* the concentrations of the components of the adenylate system are known to change only over a rather narrow range (Start & Newsholme, 1968), and this cannot explain the observed rather large changes in the rate of fatty acid synthesis occurring in starvation and on refeeding (Foster & Srere, 1968; Goodridge, 1968a,b). Nevertheless, the effect of the adenylate system and P_i on the activity of citrate lyase *in vitro* is in agreement with the general principles of regulation of energy metabolism postulated by Atkinson (1965).

The role of citrate concentration in the regulation of citrate lyase activity *in vivo* should also be envisaged, as it has been demonstrated by Angielski & Szutowicz (1967a), Zakim, Pardini, Herman & Sauberlich (1967) and Start & Newsholme (1968) that citrate concentration in liver differs rather widely in various physiological conditions.

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REGULACJA AKTYWNOŚCI LIAZY ATP CYTRYNIANOWEJ *IN VITRO*

Streszczenie

1. Badano kinetykę częściowo oczyszczonej liazy ATP cytrynianowej (EC 4.1.3.8) z wątroby szczura.

2. Przebieg wykresów Lineweavera-Burka zależności szybkości reakcji od stężenia cytrynianu jest dwufazowy. Wartość K_{m1} (dla niskich stężeń cytrynianu) jest 0.13 mM i nie zależy od stężenia Mg^{2+} i ATP. Natomiast K_{m2} (dla wysokich stężeń cytrynianu) zwiększa się przy wzroście stężenia Mg^{2+} lub przy obniżeniu stężenia ATP, co może być spowodowane inhibicyjnym działaniem jonów Mg^{2+}.

3. DL-Izocytrynian zwiększa K_{m1} dla cytrynianu, a obniża K_{m2}. W zależności od stężenia cytrynianu może on działać jako częściowo kompetytywny inhibitor lub też jako aktywator — przez usuwanie nadmiaru jonów Mg^{2+}.

4. Wartości K_m i V_{max} dla ATP zwiększają się ze wzrostem stałego stężenia Mg. Również wzrasta K_m i V_{max} dla Mg, gdy zmienia się stałe stężenie ATP. Sugeruje to, że aktywnym substratem jest kompleks ATP-Mg^{2-}.

5. ADP i P_i są kompetytywnymi w stosunku do ATP inhibitorami liazy cytrynianowej. Obniżenie stężenia Mg^{2+}, dodanie izocytrynianu lub EDTA zmniejsza inhibicję liazy przez ADP i P_i, co sugeruje, że aktywnymi formami tych inhibitorów mogą być ich kompleksy z Mg^{2+}.

6. Przedyskutowano możliwość regulacji aktywności liazy cytrynianowej *in vivo*.

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THE ESTIMATION OF OXIDIZED AND REDUCED NADP BY AN ENZYMIC RECYCLING SYSTEM, WITH OXIDIZED GLUTATHIONE AS HYDROGEN ACCEPTOR

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1. $NADP^+$ and NADPH extracted from mouse liver and spleen were estimated by an enzymic recycling system consisting of glucose-6-phosphate, G-6-P dehydrogenase, oxidized glutathione and glutathione reductase, in the presence of β-hydroxyethyl-2,4-dinitrophenyldisulphide (HEDD). The reduced glutathione (GSH) formed reacts with HEDD and the liberated 2,4-dinitrothiophenol is estimated at 408 nm.
2. The amounts of $NADP^+$ and NADPH present in tissue extracts are calculated from the differences in the amount of GSH formed in the presence and absence of standard $NADP^+$. The method has proved suitable for determination of 0.01 - 0.2 μg. of NADP.

The coupling of enzymic reduction of $NADP^+$ with simultaneous oxidation of the formed NADPH, has found wide application for determination of minute quantities of the two forms of the coenzyme. In such systems $NADP^+$ is reduced by G-6-P dehydrogenase, and the formed NADPH is immediately utilized for the enzymic or chemical reduction of an appropriate substrate. In this way, the dinucleotide is several times in turn reduced and oxidized, and the amounts of the final product are hundreds or even thousands times as high as the amount of the dinucleotide. By applying, moreover, fluorimetric (Lowry, Passonneau, Schulz & Rock, 1961) or radiometric (Serif, Schnotzer & Butcher, 1966) methods it is possible to estimate quantities of NADP as small as 0.01 - 1.0 mμg.

For oxidation of NADPH, "the old yellow enzyme" (Warburg, Christian & Griese, 1935), cytochrome *c* reductase (Glock & McLean, 1955a), glutamate dehydrogenase in the presence of 2-oxoglutarate (Lowry *et al.*, 1961; Brown & Clarke, 1963; Serif *et al.*, 1966), diaphorase with dichlorophenolindophenol (Villee, 1962) and phenazine methosulphate (Slater & Sawyer, 1962; Slater, Sawyer & Sträuli, 1964; Greenbaum, Clark & McLean, 1965) have been applied.

Conn & Vennesland (1951) observed that GSSG[1] could be enzymically reduced

[1] Abbreviations used: GSSG and GSH, oxidized and reduced glutathione, respectively; HEDD, β-hydroxyethyl-2,4-dinitrophenyldisulphide; 2-PDS, 2,2′-dipyridyl disulphide.

by catalytic amounts of $NADP^+$ when this coenzyme was continuously reduced by a G-6-P - G-6-P dehydrogenase system. On the basis of this observation Grassetti & Murray (1967) elaborated a recycling technique for determination of NADP using a system consisting of G-6-P dehydrogenase, G-6-P glutathione reductase, GSSG and 2,2′-dipyridyl disulphide (2-PDS):

$$\text{G-6-P} + NADP^+ \xrightarrow{\text{G-6-P dehydrogenase}} \text{6-P-gluconate} + NADPH$$

$$GSSG + NADPH \xrightarrow{\text{GSSG reductase}} 2\,GSH + NADP^+$$

$$2\,GSH + \text{2-PDS} \longrightarrow GSSG + 2 \text{ thiopyridine}$$

However, Grassetti & Murray applied only standard $NADP^+$ and did not check the usefulness of this technique for determination of $NADP^+$ and NADPH in biological material.

The aim of the present work was to test the applicability of the above recycling system for estimation of $NADP^+$ and NADPH in animal tissues. Moreover, the possibility of replacement of 2-PDS by HEDD was investigated:

$$GSH + \text{HEDD} \longrightarrow \text{G-S-S-}CH_2CH_2OH + HS\text{-}C_6H_4(NO_2)_2$$

The liberated 2,4-dinitrothiophenol was estimated colorimetrically. $NADP^+$ and NADPH were assayed in mouse liver and spleen. Liver was chosen as the tissue rich in the estimated dinucleotide, and spleen as that containing but small amounts.

MATERIALS AND METHODS

Reagents. G-6-P dehydrogenase (D-glucose-6-phosphate : $NADP^+$ oxidoreductase, EC 1.1.1.49) from yeast was a product of Sigma Chem. Co. (St. Louis, Mo., U.S.A.; series 78-B-737, 2 mg./ml., 250 units/mg.); for experiments, it was appropriately diluted with 0.05 M-glycylglycine buffer, pH 7.6.

GSSG reductase (NAD(P)H : oxidized glutathione oxidoreductase, EC 1.6.4.1) from yeast, type III, suspension in $(NH_4)_2SO_4$, was a product of Sigma Chem. Co. (series 107 B-741-L, 4 mg./ml., 140 units/mg.); it was diluted before use with 200 volumes of 1% bovine serum albumin (Cohn fraction V, Koch-Light, Colnbrook, Bucks., England) solution in 0.05 M-glycylglycine buffer, pH 7.6.

$NADP^+$ sodium salt (C. F. Boehringer, Mannheim, West Germany) was dissolved in water just before use. NADPH was obtained by reducing, in a volume of 1 ml., 1 mg. of $NADP^+$ with 0.1 unit of yeast G-6-P dehydrogenase in the presence of 2 μmoles of G-6-P. The remaining $NADP^+$ was then decomposed by adding NaOH to 0.1 N concentration and heating for 2 min. on a boiling water bath. The solution was neutralized with 0.1 N-HCl, and the concentration of NADPH determined spectrophotometrically at 340 nm.

GSSG was obtained by aeration of GSH (T. Schuchardt, München, West Germany) solution at pH 8 in the presence of catalytic amounts of $FeSO_4$, until negative reaction with nitroprusside. Then the solution was treated with charcoal, adjusted to pH 6, concentrated under reduced pressure, and GSSG was precipitated

with ethanol. The obtained preparation was free of GSH as checked by HEDD. Commercial GSSG obtained from Schuchardt (GL 076) was also used. The GSSG preparations were dissolved in water, neutralized with 0.1 N-NaOH and diluted to 2% concentration with 0.05 M-glycylglycine buffer, pH 7.6.

HEDD was synthesized as described by Bitny-Szlachto (1960). Since HEDD is poorly soluble in water, it was added to the assay mixture as a 25 mM ethanolic solution (6.9 mg./ml.). When the assay sample did not contain HEDD, for estimation of the GSH formed, 0.5 mM-HEDD solution was added after the incubation. This solution was prepared in the following way: 1 ml. of 25 mM ethanolic solution of HEDD was carefully poured into about 40 ml. of bidistilled water, then 5 ml. of 0.5 M-tris-acetate buffer, pH 6.9, and water up to 50 ml. were added.

NAD^+ and NADH were commercial preparations of C. F. Boehringer (Mannheim, West Germany), and Sigma Chem. Co. (St. Louis, Mo., U.S.A.), respectively. G-6-P disodium salt was from Koch-Light Lab. (Colnbrook, Bucks, England) and glycylglycine from Reanal (Budapest, Hungary).

Animals. Male BALB/c mice, 12 - 14 weeks old, fed *at libitum* standard breeding diet, were used. The animals were killed by decapitation, 100 - 200 mg. samples of liver and spleen were removed and immediately homogenized in an all-glass Potter-Elvehjem apparatus at 500 - 1000 rev./min. in 0.1 N-HCl or 0.1 N-NaOH. Prior to the addition of tissue samples the homogenizer, containing 2 ml. of HCl or NaOH solution, was heated for 2 min. on a boiling water bath.

$NADP^+$ and NADPH extraction. The procedure of Glock & McLean (1955a) with some modifications was used. For the extraction of $NADP^+$ with simultaneous destruction of NADPH and NADH, the tissue sample was immersed in hot 0.1 N--HCl in the homogenizer, then heated on the boiling water bath for 30 sec. and homogenized for 1.5 min. The homogenate was cooled on ice, centrifuged for 30 min. at 20 000 *g* at 0°, and to the supernatant (1 - 1.5 ml.) 0.4 ml. of 0.5 M-glycylglycine buffer, pH 7.6, was added. The mixture was adjusted to pH 7.0 - 7.2 (indicator paper) with simultaneous vigorous electromagnetic stirring and cooling on ice. The extract was then diluted with bidistilled water to correspond to 10 mg. of liver or 20 mg. of spleen per 1 ml. The diluted extract was centrifuged for 30 min. at 20 000 *g* at 0°, and the clear supernatant was used for $NADP^+$ determination.

For the extraction of NADPH with simultaneous destruction of $NADP^+$ and NAD^+, the tissue sample was immersed in hot 0.1 N-NaOH solution in the homogenizer, heated on the boiling water bath for 1 min., and homogenized for 1 min. Then 0.4 ml. of 0.5 M-glycylglycine buffer, pH 7.6, was added, then the mixture was neutralized as described above and diluted to correspond to 2 mg. of liver or 10 mg. of spleen per 1 ml. After centrifuging, the clear supernatant was used for estimation of NADPH.

Determination of $NADP^+$ and NADPH in tissue extracts. For each determination, three incubation samples were prepared: *1*, containing the tissue extract; *2*, containing the tissue extract and standard $NADP^+$; *3*, blank, containing neither tissue extract nor added $NADP^+$. The incubation mixture, 1 ml., was composed of 0.1 ml. of G-6-P solution (2 μmoles), 0.1 ml. of G-6-P dehydrogenase (about 0.1 unit),

0.1 ml. of 2% GSSG solution (3 μmoles), 0.1 ml. of glutathione reductase (2 μg., 0.28 unit), 0.1 - 0.5 ml. of tissue extract (sample *1*), 0.05 M-glycylglycine buffer of pH 7.6 up to a volume of 0.97 ml., and 0.03 ml. of 25 mM-HEDD ethanolic solution (0.75 μmole). Incubation sample *2* contained additionally standard $NADP^+$, 0.02 μg. for $NADP^+$ determination, and 0.05 μg. for NADPH determination. After 2 hr. incubation at 30°, the samples were filtered through cotton-wool and the extinction at 408 nm of the 2,4-dinitrothiophenol liberated was measured on a VSU-1 (Zeiss, Jena) spectrophotometer at 0.2 cm. light path. For calculation of the amount of GSH formed, the millimolar extinction coefficient for 2,4-dinitrothiophenol, $m\varepsilon = 13.06$ (Bitny-Szlachto, Kosiński & Niedzielska (1963), was used.

When the incubation mixture did not contain HEDD, 0.05 M-glycylglycine buffer was added to a volume of 1.0 ml., and after incubation, 3.0 ml. of 0.5 mM-HEDD solution was added. After 10 min. the extinction was read at 408 nm. If the samples became turbid, the extinction at 600 nm was subtracted.

Calculation of the content of $NADP^+$ and NADPH in tissue. The content of the dinucleotide was calculated by comparing the amount of GSH formed in the presence and absence of added $NADP^+$:

$$c = \frac{a \cdot E_x}{b\,(E_{x+a} - E_x)}$$

where c is the concentration of the dinucleotide in the tissue (mμg./mg.); a, the amount (mμg.) of standard $NADP^+$ added to the assay sample; b, the amount (mg.) of tissue to which corresponds the amount of the extract used; E_x, extinction of the sample containing tissue extract; E_{x+a}, extinction of the sample containing tissue extract and standard $NADP^+$.

RESULTS

Determination of standard $NADP^+$ and NADPH

The enzymic reduction of GSSG in the presence of the G-6-P - G-6-P dehydrogenase system was linear with the amount of $NADP^+$ added, both in the presence and absence of HEDD in the assay mixture (Fig. 1).

In the experiment presented in Fig. 1A, in which 1 ml. of the incubation mixture contained 0.1 unit of G-6-P dehydrogenase and 2 μg. of glutathione reductase, after 2 hr. incubation with 0.05 μg. (0.06 mμmole) of $NADP^+$ in the presence of HEDD, the increase in GSH formed was 95 mμmoles. Hence, the molar ratio of GSH formed to $NADP^+$ was about 1500. When the amount of G-6-P dehydrogenase was five times higher, 315 mμmoles of GSH was obtained in the presence of 0.05 μg. of $NADP^+$ (Fig. 1B), and the GSH : $NADP^+$ ratio was 5000. This indicates that under these conditions each molecule of $NADP^+$ was on the average 2500 times in turn reduced and oxidized.

The effect of concentration of the enzymes on GSSG reduction is presented in Fig. 2. It is evident that the amount of GSH formed and, consequently, the sensitivity of the system, could be greatly increased by applying larger amounts of G-6-P

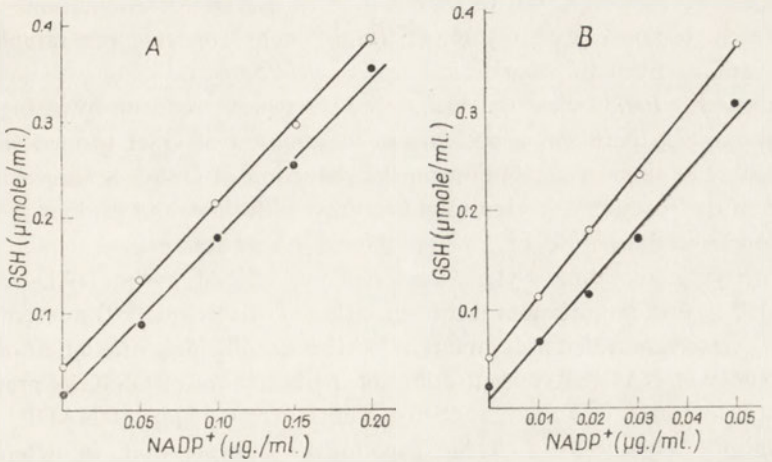

Fig. 1. The effect of $NADP^+$ concentration on GSSG reduction in the presence of *A*, 0.1 unit of G-6-P dehydrogenase; *B*, 0.5 unit of G-6-P dehydrogenase. One milliliter of the incubation mixture contained: 2 μmoles of G-6-P, 3 μmoles of GSSG, 2 μg. (0.28 unit) of glutathione reductase, the indicated amount of $NADP^+$, G-6-P dehydrogenase and glycylglycine buffer, pH 7.6. (●), In the absence of HEDD, and (○), in the presence of 0.75 μmole of HEDD. Incubation 2 hr. at 30°. Mean values from duplicate experiments are given.

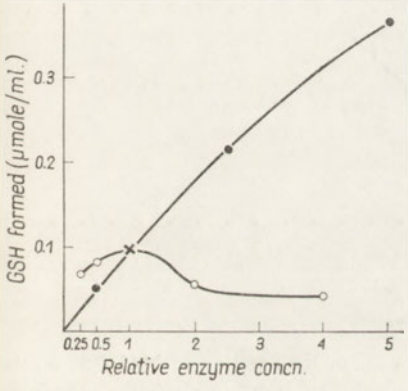

Fig. 2

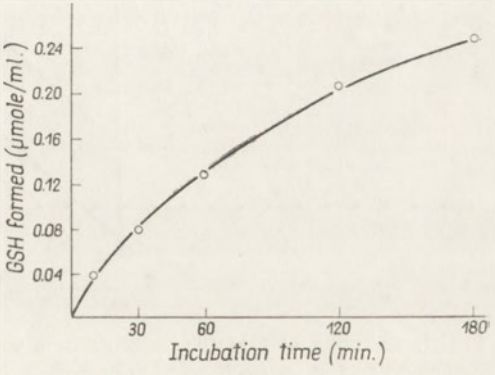

Fig. 3

Fig. 2. The effect of the amount of G-6-P dehydrogenase and glutathione reductase on GSH formation. One milliliter of the incubation mixture contained: 2 μmoles of G-6-P, 0.05 μg. of $NADP^+$, 3 μmoles of GSSG, 0.75 μmole of HEDD, glycylglycine buffer, pH 7.6, and the following amounts of the enzymes: (●), 2 μg. (0.28 unit) of glutathione reductase and 0.05 - 0.5 unit of G-6-P dehydrogenase; (○), 0.1 unit of G-6-P dehydrogenase and 0.5 - 8 μg. (0.07 - 1.12 units) of glutathione reductase. The standard sample (point ×) contained 0.1 unit of G-6-P dehydrogenase and 2 μg. (0.28 unit) of glutathione reductase. Incubation 2 hr., at 30°. The amounts of enzymes are expressed as relative values, that in the standard sample (×) being taken as 1. Mean values from triplicate experiments are given.

Fig. 3. Time-course of the enzymic reduction of GSSG. One milliliter of the incubation mixture contained: 2 μmoles of G-6-P, 0.1 unit of G-6-P dehydrogenase, 3 μmoles of GSSG, 2 μg. (0.28 unit) of glutathione reductase, 0.1 μg. of $NADP^+$, glycylglycine buffer, pH 7.6, and 0.75 μmole of HEDD. Incubation at 30°. Mean values from duplicate experiments are given.

dehydrogenase. However, satisfactory results of NADP determinations in tissue extracts were obtained with 0.1 unit of G-6-P dehydrogenase per sample, which is of advantage from the economic point of view.

On the other hand, when the amount of glutathione reductase was higher than 2 µg. per sample, there was a decrease in the amount of GSH formed (Fig. 2).

The effect of time of incubation on the reduction of GSSG is shown in Fig. 3. The rate of the reaction has been found to drop with time, and prolongation of the incubation over the period of 2 - 3 hr. offered no advantage.

The enzymic recycling system used has proved not to be NAD-dependent (Table 1). This is of importance for the utilization of this technique for determinations on tissue extracts, in which the content of NAD is usually higher than that of NADP.

The effect of NADPH concentration on the reduction of GSSG is presented in Fig. 4. The increase was linear, and the addition of 0.05 µg. of $NADP^+$ resulted in its uniform enhancement. These experiments indicate that, in determination of NADPH in tissue extracts, $NADP^+$ can be used instead of NADPH as an inner standard.

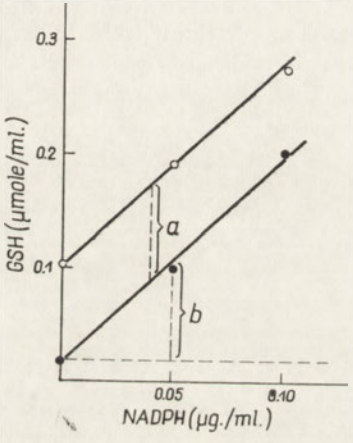

Fig. 4. The effect of NADPH concentration, with and without the addition of $NADP^+$, on the enzymic reduction of GSSG. The samples were incubated for 2 hr. at 30° in a mixture containing in a final volume of 1.0 ml.: 2 µmoles of G-6-P, 0.1 unit of G-6-P dehydrogenase, 3 µmoles of GSSG, 2 µg. (0.28 unit) of glutathione reductase, 0.75 µmole of HEDD, the indicated amounts of NADPH, and glycylglycine buffer, pH 7.6. (●), Without, and (○), with 0.05 µg. of $NADP^+$.

To compare the efficiency of HEDD with that of 2-PDS used by Grassetti & Murray (1967) for trapping the GSH formed, parallel experiments were performed, the results of which are presented in Table 2.

Within the first hour, the reduction of GSSG was the same in the presence of either disulphide, the samples incubated with HEDD having a considerably greater extinction than those with 2-PDS. However, after 3.5 hr. of incubation, the formation of GSH stopped in the assays with HEDD, in contrast to those carried out in the presence of 2-PDS.

Table 1

The effect of oxidized and reduced NAD on the enzymic reduction of GSSG in the presence of NADP+

The samples were incubated for 2 hr. at 30° in a mixture containing in a final volume of 1 ml.: 2 µmoles of G-6-P, 0.1 unit of G-6-P dehydrogenase, 0.1 µg. of NADP+, 3 µmoles of GSSG, 2 µg. of glutathione reductase, glycylglycine buffer, pH 7.6, the indicated amount of NAD+ or NADH, and where indicated 0.75 µmole of HEDD. The values are means from triplicate determinations and are expressed as percentages of the control values.

Amount of NAD+ or NADH added (µg.)	Without HEDD		In the presence of HEDD	
	NAD+	NADH	NAD+	NADH
None (control)	100	100	100	100
0.1	99	96	104	100
0.5	98	95	102	96
1.0	99	95	105	95

Table 2

Comparison of the results obtained with β-hydroxyethyl-2,4-dinitrophenyldisulphide (HEDD) and 2,2'-dipyridyl disulphide (2-PDS)

The incubation mixture contained in 1 ml.: 2 µmoles of G-6-P, 0.05 µg. of NADP+, 0.1 unit of G-6-P dehydrogenase, 3 µmoles of GSSG, 2 µg. of glutathione reductase, 0.75 µmole (0.2 mg.) of HEDD or 0.1 mg. of 2-PDS, and glycylglycine buffer, pH 7.6; temperature 30°. The results are means from triplicate determinations. For calculation of GSH formed, the millimolar extinction coefficients of 2,4-dinitrothiophenol, $m\varepsilon = 13.06$, and of 2-thiopyridine, $m\varepsilon = 7.06$, were used.

Incubation time (hr.)	HEDD (0.7 mM)		2-PDS (0.5 mM)	
	$E^{0.2\text{ cm}}_{408\text{ nm}}$	GSH (µmole/ml.)	$E^{0.2\text{ cm}}_{408\text{ nm}}$	GSH (µmole/ml.)
1	0.198	0.075	0.108	0.075
2	0.258	0.099	0.168	0.120
3.5	0.288	0.111	0.226	0.162
4.5	0.282	0.108	0.246	0.175

Table 3

The content of NADP+ and NADPH in mouse liver and spleen

The conditions were as described in Fig. 5. For NADP+ determination, 0.25 - 0.5 ml. of the acidic extract, corresponding to 5 mg. of tissue was used, and for NADPH determination, 0.25 ml. of the alkaline extract, corresponding to 0.5 mg. liver and 5 mg. of spleen. The results are means from 7 determinations ± S.D., and are expressed as µg./g. of wet tissue weight.

Tissue	NADP+	NADPH	NADPH/NADP+ ratio
Liver	9.4 ± 2.2	141 ± 27	15.0 ± 4.7
Spleen	5.7 ± 2.3	41.5 ± 11.7	8.0 ± 2.7

Determination of NADP⁺ and NADPH in tissue extracts

The elaborated method was applied to alkaline and acidic tissue extracts from mouse liver and spleen (Fig. 5). The reduction of GSSG was linear with the amounts of tissue extract; and the addition of standard NADP⁺ gave a uniform increase,

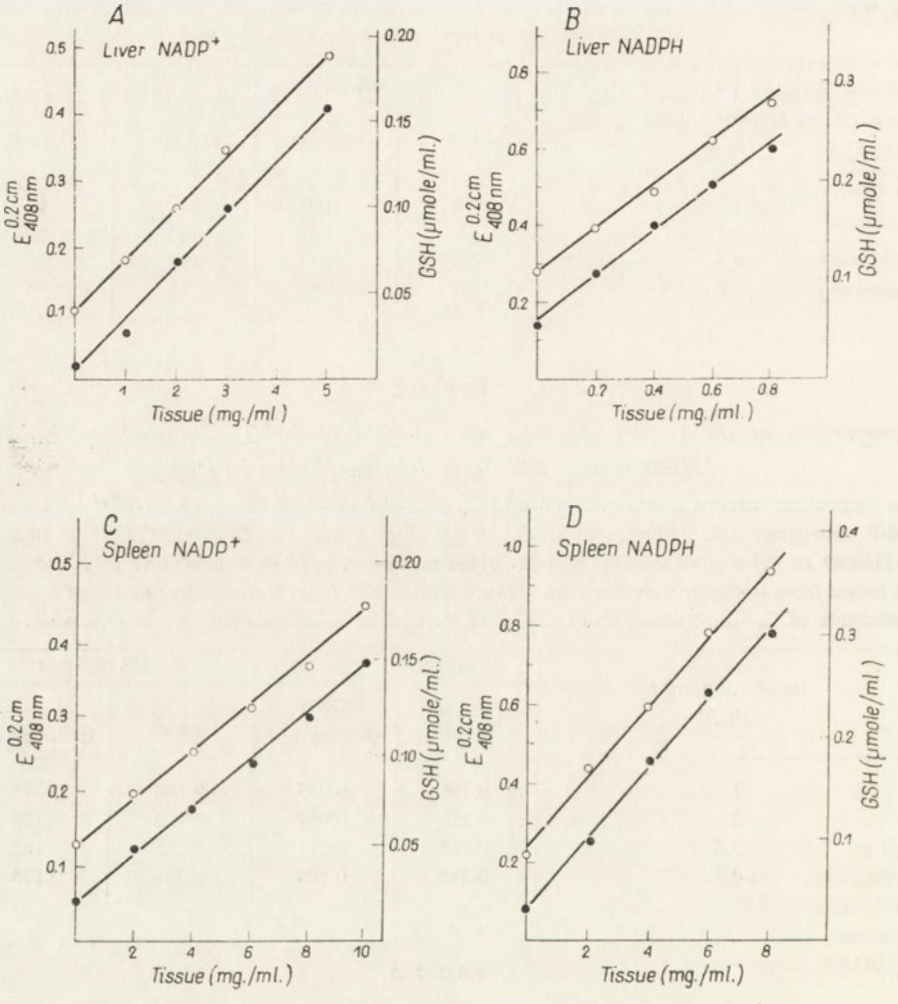

Fig. 5. The determination of NADP⁺ and NADPH in extracts of mouse liver and spleen. The samples were incubated for 2 hr. at 30° in a mixture containing in a final volume of 1 ml.: 2 µmoles of G-6-P, 0.1 unit of G-6-P dehydrogenase, 3 µmoles of GSSG, 2 µg. (0.28 unit) of glutathione reductase, 0.75 µmole of HEDD, tissue extract corresponding to the indicated amount of tissue, 0.05 M-glycylglycine buffer, pH 7.6, and where indicated standard NADP⁺.

A, NADP⁺ extract from liver (10 mg./ml.), (●), without, and (○), with 0.02 µg. of NADP⁺;
B, NADPH extract from liver (2 mg./ml.), (●), without, and (○), with 0.05 µg. of NADP⁺;
C, NADP⁺ extract from spleen (20 mg./ml.), (●), without, and (○), with 0.02 µg. of NADP⁺;
D, NADPH extract from spleen (10 mg./ml.), (●), without, and (○), with 0.05 µg. of NADP⁺.

which indicates that no compound inhibiting the recycling of $NADP^+$ was present in tissue extracts. The amounts of $NADP^+$ and NADPH found are presented in Table 3.

DISCUSSION

In the present work, it has been demonstrated that the enzymic recycling system proposed by Grassetti & Murray (1967) is suitable for determination of $NADP^+$ and NADPH in tissue extracts. The amount of GSH formed was linear with the amount of tissue extract. The addition of a standard sample of $NADP^+$ gave a parallel straight line; thus it proved possible to calculate the content of the dinucleotide in a tissue extract from the difference in the amount of GSH formed in the presence and absence of the added $NADP^+$.

The replacement by HEDD of 2-PDS used by Grassetti & Murray (1967) appeared to be advantageous because 2,4-dinitrothiophenol, liberated in the reaction of GSH with HEDD, can be determined at 408 nm, and not, like 2-thiopyridine, at 343 nm. This makes possible to take the readings on a common spectrophotometer or colorimeter, whereas with 2-PDS a UV-spectrophotometer must be used. Moreover, the molar extinction coefficient for 2,4-dinitrothiophenol is nearly twice as high as that for 2-thiopyridine, which permits to raise the sensitivity of the system.

The contents of $NADP^+$ and NADPH determined in the present work in mouse liver, 9.4 ± 2.2 and 141 ± 27 µg./g. wet weight, respectively, are in reasonable agreement with the data reported in the literature for rat liver, determined in acid and alkaline tissue extracts. For instance, Glock & McLean (1955b) obtained, respectively, 6 and 205 µg./g., and Slater *et al.* (1964), 27 and 190 µg./g.

The data for spleen are very scarce. Using different recycling systems, Glock & McLean (1955b) found in rat spleen less than 2 µg. of $NADP^+$ and 12 µg. of NADPH per one gram of tissue, and Serif *et al.* (1966) 0.9 µg. of $NADP^+$. The values obtained in the present work for mouse spleen are much higher and amount to 5.7 ± 2.3 and 41.4 ± 11.7 µg., respectively. It should be noted that the contents of the two forms of the dinucleotide in spleen of the black-hooded rat (unpublished results) were also found to be of the same order.

This discrepancy might be ascribed to a relatively greater effect of the dinucleotides extracted from the spleen, than of standard $NADP^+$, on the recycling system consisting of G-6-P dehydrogenase and glutathione reductase.

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OZNACZANIE UTLENIONEGO I ZREDUKOWANEGO NADP NA PODSTAWIE AKTYWACJI ENZYMATYCZNEGO UKŁADU ZAWIERAJĄCEGO UTLENIONY GLUTATION JAKO AKCEPTOR WODORU

Streszczenie

1. $NADP^+$ i NADPH wyekstrahowane z wątroby i śledziony myszy oznaczono na podstawie aktywacji enzymatycznego układu zawierającego G-6-P, dehydrogenazę G-6-P, utleniony glutation i reduktazę glutationową w obecności dwusiarczku β-hydroksyetylo-2,4-dwunitrofenylowego (HEDD). Powstający zredukowany glutation (GSH) reagował z HEDD, a wytworzony 2,4-dwunitrotiofenol oznaczono spektrofotometrycznie przy 408 nm.

2. Zawartość $NADP^+$ i NADPH w ekstraktach z tkanek obliczono na podstawie różnicy w ilości wytworzonego GSH w obecności i nieobecności standardowego $NADP^+$.

3. Za pomocą opisanej metody można oznaczać 0,01 - 0,2 μg. NADP.

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IMMUNOELECTROPHORETICALLY HOMOGENEOUS PREPARATION OF ARYLSULPHATASE A FROM HUMAN PLACENTA

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1. A method for the purification of human placental arylsulphatase A (arylsulphate sulphohydrolase, EC 3.1.6.1) is presented. 2. The enzyme preparation was homogeneous on immunoelectrophoresis. Specific activity expressed as mμmoles of substrate hydrolysed per min. per mg. of protein was 124 000. The molecular weight estimated by Sephadex G-200 gel filtration was 109 000. 3. The purified enzyme was highly unstable; its inactivation was accompanied by considerable changes in the immunoelectrophoretic pattern.

So far only arylsulphatase A from ox liver has been obtained in a form homogeneous with respect to sedimentation coefficient and free boundary electrophoresis (Nichol & Roy, 1964). Arylsulphatase A of high specific activity has been also obtained from ox brain (Błeszyński, 1967).

In this work we present the method for preparation of highly purified, immunoelectrophoretically pure arylsulphatase A from human placenta.

MATERIALS AND METHODS

Determination of arylsulphatase A. The substrate, dipotassium salt of 2-hydroxy-5-nitrophenyl sulphuric acid (NCS) was prepared according to Roy (1958) and further recrystallized several times from glass-distilled water.

The activity of arylsulphatase A was determined according to the spectrophotometric method of Robinson, Smith & Williams (1951). To 0.36 ml. of 20 mM-NCS solution and 0.12 ml. of 2 M-sodium acetate-acetic acid buffer, pH 5.0, 0.12 ml. of enzyme solution was added. After incubation at 37° for 1 min., the reaction was stopped with 3 ml. of 10% NaOH. The specific activity is expressed as mμmoles of hydrolysed substrate/min./mg. protein.

Purification of arylsulphatase A. The course of the purification is presented in Table 1. The first steps (1, 2 and 3) of the procedure resemble those previously described (Gniot-Szulżycka & Działoszyński, 1967). Stage 1. Fresh human placenta

(8500 g.) was homogenized in an equal volume of 0.2 M-ammonium acetate, pH 7.0, containing heparin. Then acetone, toluene and ethyl acetate were added to the final concentration of, respectively 10%, 5% and 5%. The mixture was autolysed at 0° - 4° for 72 hr. and the clear filtrate was adjusted to pH 5.0 with acetic acid; then acetone was added at -5° up to 60%. The precipitate containing arylsulphatase A and B was dissolved in water and dialysed against water. Some insoluble proteins were discarded.

Table 1

Purification of arylsulphatase A from human placenta

Stage of the purification procedure	Total protein	Total activity (μmoles/min.)	Specific activity (mμmoles/ /min./mg. of protein)	Yield (%)	Purification factor
Homogenate	927.0 g.	27 800	30	—	—
1 Acetone preparation	99.5 g.	18 360	184	100	—
2 Ppt. at 1.6 M-ammonium sulphate sat.	4.7 g.	6 770	1 440	37	6
3 Dialysis against water	2.5 g.	5 700	2 240	31	12
4 DEAE-cellulose chromatography, pH 7.0	583.0 mg.	6 900	11 850	37.5	64
5 Sephadex G-200 gel filtration, pH 7.0	113.0 mg.	4 300	38 050	23	207
6 Dialysis against acetate buffer, pH 5.0	90.7 mg.	1 980	21 000	11	115
7 DEAE-cellulose chromatography, pH 8.6	43.2 mg.	1 770	41 000	9	223
8 Agar-gel electrophoresis, pH 8.6	7.8 mg.	970	124 000	6	673

Stage 2. The dialysed solution (2200 ml.) was adjusted to pH 7.0 and solid ammonium sulphate was added up to 1.6 M concentration at the temp. of -5° to -9°. After 20 hr. the precipitate was collected, made into a slurry with water and dialysed until free from SO_4^{2-} (final volume 200 ml.).

Stage 3. The dialysed solution was concentrated to a volume of 35 ml. Some proteins appearing as precipitate were discarded. The clear supernatant was diluted fivefold with water and the precipitate of γ-globulins was discarded.

Stage 4. The resulting solution was concentrated to the volume of 35 ml., dialysed against 0.01 M-tris-HCl buffer, pH 7.0, and chromatographed at 10° on DEAE-cellulose column (30×3.6 cm.) equilibrated with 0.01 M-tris-HCl buffer, pH 7.0. Elution was made with stepwise increased gradient of NaCl in the above buffer; 9 ml. fractions were collected. Enzymically active fractions appearing at 0.14 - 0.3 M-NaCl were collected, combined (180 ml.), concentrated to 20 ml., and dialysed against 0.1 M-tris-HCl buffer, pH 7.0.

Stage 5. The dialysed solution was applied to a Sephadex G-200 column (95× ×2.5 cm.) which was equilibrated and eluted with 0.1 M-tris-HCl buffer, pH 7.0, at 10 - 14°. Fractions of 7.5 ml. were collected and those containing the enzyme were combined and concentrated to 5 ml.

Stage 6. This solution was dialysed against 0.2 M-sodium acetate buffer, pH 5.0. It was expected that on dialysis the enzymically active proteins would separate from precipitating non-enzymic impurities (cf. Nichol & Roy, 1964). Unfortunately, both the precipitate and the supernatant were found to be active, and moreover, this step resulted in a considerable inactivation of the enzyme. Therefore the application of dialysis at this stage of enzyme purification is not recommended.

Stage 7. The pH 5.0 soluble and insoluble proteins from stage 6 were extensively dialysed against 0.1 M-tris-HCl buffer, pH 8.6. The clear supernatant after discarding the precipitate, was chromatographed on DEAE-cellulose column (30×3.6 cm.) at 10° to 14°, the column being equilibrated with 0.1 M-tris-HCl buffer, pH 8.6. Elution was performed with linear gradient of NaCl and fractions of 3.6 ml. were collected. Enzymically active fractions appearing at 0.14 - 0.3 M-NaCl were collected (40 ml.), concentrated to 1.4 ml. and dialysed against 0.05 M-veronal buffer, pH 8.6.

As demonstrated by immunoelectrophoresis, the dialysed solution contained two protein fractions, which could be separated by electrophoresis on agar gel.

Stage 8. Electrophoresis was carried in 1.5% agar gel in 0.05 M-veronal buffer, pH 8.6 (19.5×19.5×1.0 cm. agar block was used); the electrophoresis lasted 2 hr., the voltage gradient was 15 V/cm., the current gradient 3.5 mA/cm., the electrophoretic chamber being cooled with ice. After the run, sections about 0.25 cm. wide were cut out from the agar bed. Each section was eluted with a 2 ml. portion of 0.25 M--NaCl in 0.01 M-tris-acetate buffer, pH 7.0. The elution procedure was repeated twice. Enzymically active fractions (Fig. 1, fractions -1 and -2) were collected and concentrated to 3.8 ml.

Immunoelectrophoresis. Difco Bactoagar, 1.5 g., was dissolved in 65 ml. of hot water, then 35 ml. of 0.1 M-veronal-HCl buffer, pH 8.2, was added and 2.5 ml. of this solution was poured onto each microscopic slide; 0.02 ml. samples of proteins from stage 1, 4, 7 and 8 were separated electrophoretically in veronal-HCl buffer, pH 8.6, at 4°. The electrophoretic run lasted 90 min., the current gradient was 8 mA per slide. Immunodiffusion occurred against 0.06 ml. of polyvalent horse anti-human plasma (Biomed, Warszawa, Poland) or rabbit anti-human placenta proteins (rabbits were immunized by two subcutaneous and one intraperitoneal injections of 160 mg. of placental acetone powder proteins each at one week intervals).

The diffusion was allowed to proceed at room temperature until the precipitation bands were fully developed; usually this required 1 to 3 days. Immunoelectrophoretograms were stained with Amido Black.

Estimation of the molecular weight. The method of Andrews (1964) was used. Sephadex G-200 column (1.5×85 cm.) was equilibrated and eluted with 0.05 M--tris-HCl buffer, pH 7.5, in 0.1 M-KCl at 10° to 14°; 3 ml. fractions were collected.

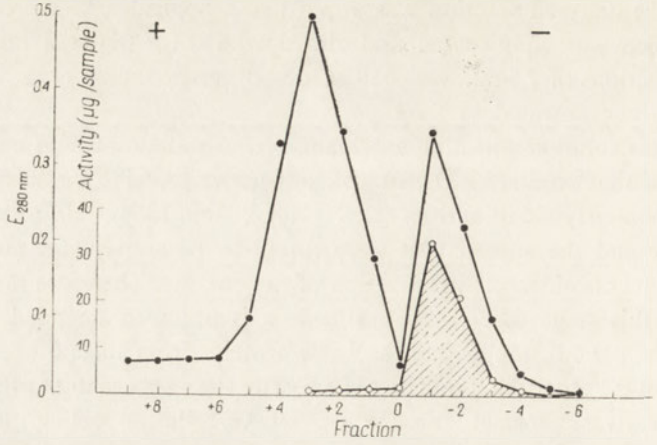

Fig. 1. Separation of arylsulphatase A from the enzymically inactive protein fraction by agar-gel electrophoresis. (●), $E_{280\,nm}$; (○), enzymic activity (µg. of 4-nitrocatechol liberated/sample).

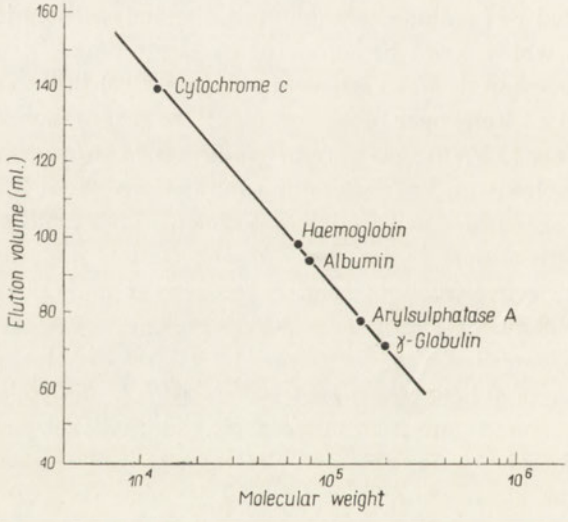

Fig. 2. Estimation of molecular weight of arylsulphatase A from human placenta by gel filtration on Sephadex G-200. Column 85 × 1.5 cm., V_0 55 ml. Separation was carried out at 10 - 14° in 0.05 M--tris-HCl buffer, pH 7.5, containing 0.1 M-KCl; the flow rate was 0.5 ml./min., and fractions of 3 ml. were collected.

For calibration of the column, Blue Dextran 2000 (Pharmacia, Uppsala, Sweden), cytochrome *c* (Biomed, Warszawa, Poland), human haemoglobin (Serva, Heidelberg, Germany), crystalline albumin from ox serum (Sigma, St. Louis, Mo., U.S.A.) and human plasma γ-globulins F II (Calbiochem., Los Angeles, Calif., U.S.A.) were used.

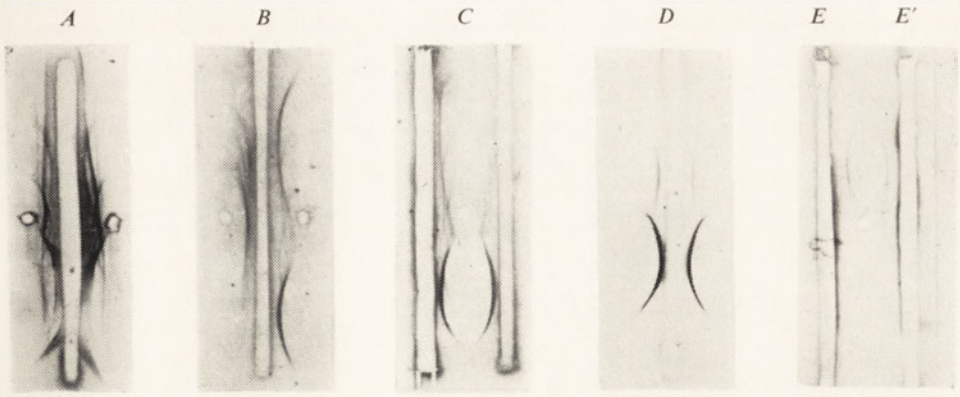

Fig. 3. Immunoelectrophoretic patterns of protein fractions from various stages of the purification of arylsulphatase A from human placenta. Antisera used for immunodiffusion: *A*, *B*, *C*, *D* and *E*, polyvalent horse anti-human plasma proteins serum; E′, rabbit anti-human placenta proteins serum.

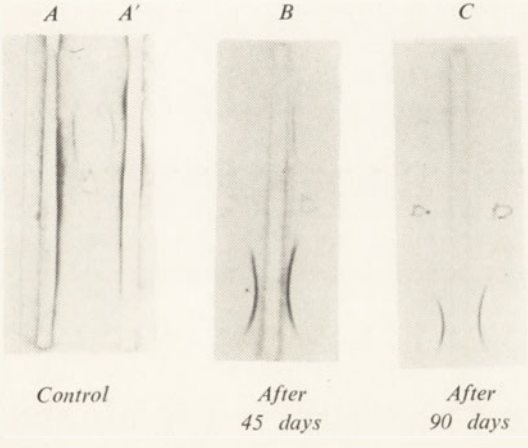

Fig. 4. Time-dependent changes in immunoelectrophoretic pattern of arylsulphatase A stored in frozen state (−16°). The purified preparation (stage 8, sp. act. 96 000) was submitted to immunoelectrophoresis just after isolation, and after storage at −16° for 45 days (sp. act. 60 000) and 90 days. Antisera used for immunodiffusion: *A*, *B* and *C*, polyvalent horse anti-human plasma proteins serum: *A′*, rabbit anti-human placenta proteins serum.

Concentration procedure. Protein solutions were concentrated in dialysing bag in ventilated chamber cooled at the top. A tenfold concentration of 100 ml. of protein solution occurred in 5 hr. at 4°.

Protein determinations were carried out by the spectrophotometric method of Warburg & Christian (1941), protein content being calculated from the Kalckar equation: $1.45\,E_{280} - 0.74\,E_{260} = c$. In some experiments the method of Lowry, Rosebrough, Farr & Randall (1951) was used.

RESULTS AND DISCUSSION

The final preparation of arylsulphatase A from human placenta exhibited a specific activity of 124 000 if proteins were determined by the spectrophotometric method, and 96 000 if proteins were determined by the method of Lowry *et al.* (1951). It should be noted that the specific activity of the most highly purified arylsulphatase A preparation from ox liver was 140 000 when the method of Lowry *et al.* (1951) for protein determination was used (Nichol & Roy, 1964).

The final yield expressed as percentage of the enzymic activity of the acetone-dried preparation, was 6% only. The yield increased twice if stage 6 of the purification procedure was omitted.

The molecular weight 109 000 calculated from the elution volume after Sephadex G-200 gel filtration (Fig. 2) agreed well with the values for arylsulphatase A from ox liver, 107 000 (Nichol & Roy, 1964) and ox brain, 104 000 (Lewosz & Działoszyński, 1967).

Immunoelectrophoretic patterns of samples from successive steps of the purification procedure are presented in Fig. 3. Immunoelectrophoresis was performed as soon as possible after each stage of purification. At stage 7 only two precipitation lines were present and after electrophoresis on agar gel which separated the enzymically active fractions from the inactive one, the final preparation was immunoelectrophoretically homogeneous (Fig. 3E).

The highly purified placental arylsulphatase A with a specific activity of 96 000 lost 30% of the activity after 3 days at 0°. On the other hand, the enzyme preparation with a specific activity of about 30 000 was relatively stable; when stored at -16° it was not completely inactivated even after one year.

Immunoelectrophoretically it has been shown that, as the enzyme inactivation proceeds, the immunoelectrophoretic pattern of the previously homogeneous enzyme changes. New precipitation lines appear which are finally replaced by only one precipitation line of quite different mobility (Fig. 4).

This could be due to denaturation of the protein molecule, or to existence of more than one form of the enzyme. Kinetical abnormalities of arylsulphatase A (unpublished results), high instability at 0° of the purified enzyme of specific activity of 124 000 and relative stability at -16° of the enzyme preparation of specific activity of 30 000, support the second possibility.

The changes in electrophoretic mobility which occur during storage of the enzyme preparation at -16° may represent structural changes of the protein molecule,

namely a transition from tetramer through dimer to monomer or *vice versa*. This view is supported by the results of Nichol & Roy (1964) who demonstrated by the ultracentrifugation techniques that arylsulphatase A from ox liver at pH 7.0 is a monomer and at pH 5.0 a tetramer.

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IMMUNOELEKTROFORETYCZNIE JEDNORODNY PREPARAT ARYLOSULFATAZY A Z ŁOŻYSKA LUDZKIEGO

Streszczenie

1. Przedstawiono metodę oczyszczania arylosulfatazy A z łożyska ludzkiego (sulfohydrolaza arylosiarczanów, EC 3.1.6.1).

2. Uzyskano immunoelektroforetycznie jednorodny preparat enzymu. Specyficzna aktywność enzymu wyrażona w mμmolach substratu zhydrolizowanego w przeciągu minuty przez 1 mg białka wynosi 124 000. Ciężar cząsteczkowy oznaczony sączeniem na żelu Sefadex G-200 wynosi 109 000.

3. Wysokooczyszczony enzym jest nietrwały. Inaktywacji enzymu towarzyszy zanik linii precypitacyjnej odpowiadającej ruchliwości białka enzymatycznego oraz pojawienie się białka o odmiennej ruchliwości elektroforetycznej.

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THE FAST-RENATURING FRACTION OF CALF THYMUS DNA

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1. Calf thymus DNA submitted to thermal denaturation and rapid cooling showed, when chromatographed on hydroxyapatite, a two-peak elution pattern. The second peak appeared in the region where native molecules are eluted, and is referred to as the "native-like" fraction. This fraction constituted about 10% of DNA. 2. The "native-like" fraction possesses some features of native DNA as might be judged by the melting profiles and the electron microscopic observations. Its G+C content amounted to 55.4 mole%. The molecular weight determined from sedimentation velocity in alkaline solution was 10^5, and calculated from the contour length for the bulk of molecules was 2.88×10^6. 3. From kinetic studies on renaturation of calf thymus DNA it is supposed that the "native-like" fraction represents the population of fast-renaturing molecules, resembling satellite DNA.

It is known that some small fractions in natural DNA's can return to a native-like conformation following complete thermal denaturation and rapid cooling (for review see Szybalski, 1967). This fraction, amounting to 1 - 10% of the preparation, seems to contain interstrand cross-links, being in this respect closely related to the fraction responsible for residual transforming activity in denatured bacterial DNA (Alberts & Doty, 1968; Chevallier & Bernardi, 1968).

In the course of previous studies on the denaturation of calf thymus DNA and fractionation by "thermal chromatography" on hydroxyapatite (Szala & Chorąży, 1969) the existence of a "native-like" fraction, surviving denaturation at 100° in 0.02 M-NaCl and rapid cooling, was observed. The unusual behaviour of this fraction justifies the author's interest in its further characterization.

MATERIALS AND METHODS

DNA preparations. The calf thymus DNA used for the experiments, was purchased from Worthington Biochemical Co. (Freehold, N.J., U.S.A). In some experiments DNA isolated according to Savitsky & Stand (1966) from nuclei of mouse leukaemia L1210 cells and nuclei of liver of CDBA/2 inbred mice, were used. These DNA pre-

parations were additionally digested with RNase (5 × crystallized, Sigma Chemical Co., St. Louis, Mo., U.S.A) at a concentration of 50 μg./ml. in 0.15 M-NaCl for 30 min. at a temp. of 25°, and then by pronase B (Calbiochem., Los Angeles, Calif., U.S.A.) at a concentration of 500 μg./ml. for 5 hr. at 25°. The RNase solution was previously heated for 10 min. at 90° to destroy DNase activity. The solution of pronase before use was incubated overnight at room temperature. After enzymic treatment, the DNA solution was deproteinized by repeated extractions with chloroform - isoamyl alcohol (10 : 1, v/v) followed by ethanol precipitation. Such DNA did not show positive reaction for RNA (orcinol test; Schneider, 1957) or protein (method of Lowry, Rosebrough, Farr & Randall, 1951).

It was assumed that the native DNA has an $E_{260}^{1\%} = 200$ and the denatured DNA $E_{260}^{1\%} = 230$ in 1 cm. light-path.

Denaturation of DNA. Samples of DNA were diluted to a concentration of 50 - - 100 μg./ml. 0.02 M-NaCl, heated in glass ampoules at 100° for 10 min. and then rapidly cooled in an ice bath. Alkaline denaturation was performed as follows: to 9 parts of DNA solution in 0.02 M-NaCl at room temperature, 1 part of cold 1.0 N-NaOH was added. After 15 min., the solution was neutralized by 2 parts of 1 M-KH_2PO_4 and dialysed against several changes of 0.02 M-NaCl.

Renaturation rate of DNA. The kinetics of DNA renaturation was studied according to the method of Britten & Kohne (1968). Samples of DNA in 0.125 M-phosphate buffer, pH 6.8, were degraded by rapid stirring in Sorvall homogenizer at a constant speed 8800 rev./min. for 60 min., as described by Müller & Crothers (1968). The average molecular weight of DNA was lowered to about 1 million daltons, as determined by Zimm & Crothers (1962) viscosimetric method. The degraded DNA samples were denatured at 100° for 10 min. and incubated at 60° for various lengths of time, then submitted to chromatography on hydroxyapatite. The elution was carried out with stepwise increasing molarity of phosphate buffer, as described below, then the amount of renatured DNA was determined.

Chromatography on hydroxyapatite (HA). The HA was prepared and chromatography carried out according to Miyazawa & Thomas (1965). The HA column (3 × 3 cm.) was enclosed in a water-jacket which was heated and kept at a constant temperature (65.0°) with water circulated from an ultrathermostat. The DNA was eluted from the column with sodium phosphate buffer, pH 6.8, with a linear concentration gradient from 0.01 to 0.25 M. The recovery of the material from the column amounted to 95%.

In some experiments, denatured DNA was eluted stepwise with 0.10, 0.125, 0.15 and 0.18 M concentrations of sodium phosphate buffer. The fraction eluted with 0.18 M buffer corresponded, as it might be judged by its melting profile, to the second peak resolved by continuous concentration gradient chromatography.

Thermal chromatography was performed in the following way. The DNA sample was applied to an HA column equilibrated with 0.10 M-sodium phosphate buffer, pH 6.8. The column was perfused with the same buffer at increasing temperature in the range from 60° to 100°. After raising the temperature to the given value (every 2° or 5°), the column was kept for 10 min. at this temperature and then

eluted with 15 ml. of 0.10 M-sodium phosphate buffer, pH 6.8, five 3 ml. fractions being collected (Fig. 5a).

Determinations of melting profiles of DNA. These were obtained in quartz, teflon-stoppered cuvettes in a Beckman DU spectrophotometer equipped with thermospacers. Samples of DNA ($E_{260\ nm} = 0.2 - 0.5$) were heated at a rate of 0.5 - 1.0°/min., and the temperature measured with the accuracy of 0.1° by a copper-constantan thermistor inserted into the cell.

Cross-linking of DNA. DNA was cross-linked according to Becker, Zimmerman & Geiduschek (1964) by treatment with HNO_2 at pH 4.0 for 30 min. Samples were then denatured at 100° for 10 min., rapidly cooled, and fractionated on the HA column. Two peaks were obtained: the first emerged at 0.10 M-sodium phosphate concentration and the second at 0.17 M, i.e. in the region where native DNA is eluted. The fraction from the second peak was considered as artificially cross-linked DNA.

Electron microscopy. For electron microscopic examination DNA was prepared by the method of Kleinschmidt & Zahn (1959) modified as follows. DNA at a concentration of 5 μg./ml. in 1.0 M-ammonium acetate was mixed with the same volume of 0.02% cytochrome *c* (Sigma Chemical Co.) in 1.0 M-ammonium acetate. This solution was layered on the surface of 1.0 M-ammonium acetate. The surface was previously cleaned by talcum atomized onto it. The DNA-cytochrome film was picked up by grids and fixed in ethanol. The grids were then transferred to a JEE 3B type vacuum evaporator and the DNA cytochrome surface was shadowed at an angle of 6° with uranium while being rotated at a speed of 60 rev./min. The specimens were examined in a JEM-5Y (Japan Electron Optics Laboratory Co., Tokyo, Japan) electron microscope. Random-picked fields were photographed, at a direct magnification of 6900 or 15 000. The length of DNA molecules was measured after magnification of the photographic plates 10 times with an optical projector.

Determination of base composition. Acid hydrolysate of DNA (70% $HClO_4$, 100°, 1 hr.) was chromatographed by descending technique on Whatman no. 1 paper using the following freshly prepared solvent: isopropanol - conc. HCl - H_2O (65 : 16.7 : 13.3, by vol.). The spots were located in UV light and the amount of bases after elution with 0.1 N-HCl was determined using the molar coefficients given by Bendich (1957).

Sedimentation velocity. Sedimentation analysis of heat-denatured DNA was carried out in alkaline solution (0.9 M-NaCl - 0.1 N-NaOH) according to Studier (1965) in a Spinco model E analytical ultracentrifuge.

RESULTS

Chromatography on HA column. Figure 1 shows a typical two-peak elution pattern from an HA column of calf thymus DNA denatured at 100° for 10 min. and rapidly cooled. The main fraction was eluted at about 0.10 M-sodium phosphate buffer, pH 6.8, and a small one appeared at 0.17 M, i.e. at the concentration when usually native DNA is eluted.

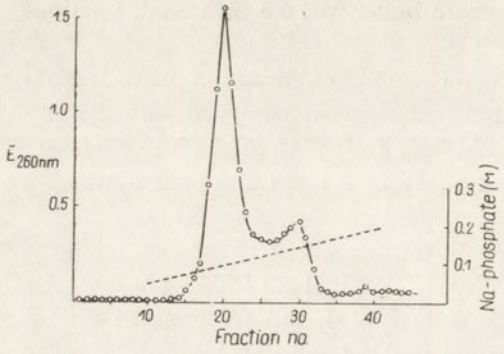

Fig. 1

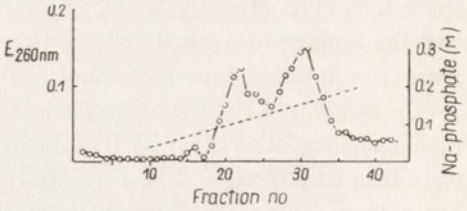

Fig. 2

Fig. 1. Chromatography of denatured DNA from calf thymus on a hydroxyapatite column. DNA in 0.02 M-NaCl at a concentration of 100 μg./ml., was heat-denatured at 100° for 10 min., then cooled rapidly. The sample was loaded on the column and the elution was carried out with a linear molarity gradient (0.01 - 0.25 M) of 150 ml. of sodium phosphate buffer, pH 6.8. The temperature of the column was 65°. Fractions of 3.2 ml. were collected.

Fig. 2. Rechromatography on hydroxyapatite column of the "native-like" fraction (second peak from Fig. 1). To the column, 20 ml. of DNA solution ($E_{260\ nm} = 0.275$) was applied. Other details of the procedure as for Fig. 1.

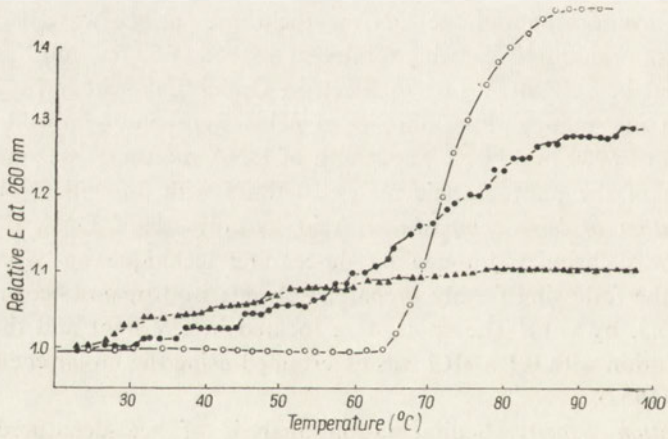

Fig. 3. Melting profiles of (○), native, (△), denatured, and (●), rechromatographed "native-like" DNA from calf thymus. All samples were dissolved in 0.02 M-NaCl. Relative absorbance = $E_{260,t}/E_{260,20°}$.

Rechromatography of the second peak again showed a two-peak pattern (Fig. 2). The first peak contained denatured DNA and was not analysed further. The second one, again eluted at 0.17 M-sodium phosphate, will hereafter be called the "native-like" fraction. This fraction when submitted to second rechromatography gave once more a two-peak distribution pattern with the second peak prevailing.

A two-peak chromatographic pattern was also found in denatured DNA isolated from nuclei of L1210 leukaemia cells and nuclei of CDBA/2 mouse liver. However, further characterization of the fractions from these sources was not carried out.

From the stepwise elution pattern it was calculated that the "native-like" fraction constituted about 10% of the original calf thymus DNA sample. Its magnitude was independent of the DNA concentration used for denaturation as well as of temperature and denaturation time (Table 1). After alkaline denaturation, the amount of the "native-like" fraction obtained was the same. A DNA sample denatured at 100° for 10 min. at a concentration of 0.02 M-NaCl containing 1% of neutralized formaldehyde and subsequently fractionated on an HA column, showed only a single peak consisting of denatured molecules.

Melting profiles of the "native-like" fraction. The melting profile of the rechromatographed "native-like" fraction along with those of untreated native DNA and denatured DNA from the main peak after continuous gradient HA chromatography (see Fig. 1) is shown in Fig. 3. The melting curve of the "native-like" fraction was distinctly different from the curve for completely denatured DNA. The hyperchromic effect steadily increased in the temperature range from 25° to 55°. Above 55° the slope of the curve became sharper, resembling to a degree the transition of native DNA. However, the transition range for the "native-like" fraction was wider (55 - 95°) than for the native sample (65 - 85°) and the hyperchromic effect reached only 30% as compared with 46% for native DNA. From melting profiles it might be supposed that the "native-like" fraction possessed a partially preserved secondary structure.

If the preservation of double-stranded regions was due to cross-links, we should expect the melting profiles of "native-like" DNA and DNA containing artificially induced interstrand links to be similar. Consequently, native DNA was treated with HNO_2 according to Becker *et al.* (1964), then denatured at 100°, rapidly cooled, and chromatographed on an HA column. The fraction from the second peak eluted at 0.17 M-sodium phosphate was dialysed against Geiduschek's buffer: 51% CH_3OH - - 1 mM-NaCl - 1 mM-tris-HCl, pH 7.0 (Geiduschek, 1962). The "native-like" fraction and native DNA were parallelly dialysed against the same buffer.

It is known that in Geiduschek's buffer only those DNA molecules which possess cross-links are able to regain their secondary structure. The comparison of melting profiles of native, artificially cross-linked, and "native-like" DNA reveals distinct differences (Fig. 4). The DNA sample possessing HNO_2-induced cross-links seems to regain a part of its secondary structure as might be judged by the rapid decrease of hyperchromicity during cooling (Fig. 4, curve *b*). This is not observed for native and "native-like" DNA (Fig. 4, curves *a* and *c*). It may be thus inferred that in the "native-like" DNA the strands are kept in register without the participation of natural cross-links. We may therefore suppose that the "native-like" fraction represents the population of fast-renaturing DNA molecules with repeated sequences described recently by Britten & Kohne (1968).

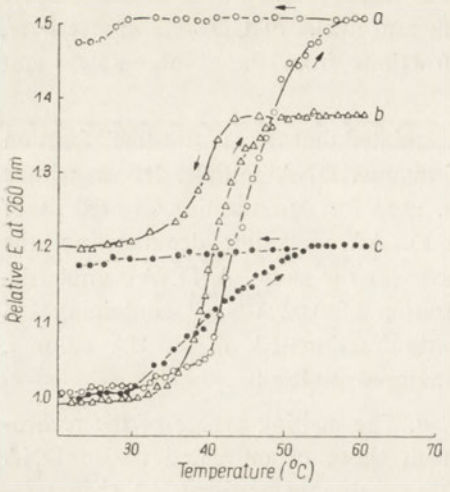

Fig. 4. Melting profiles of (○), native, (●), "native-like" and (△), the artificially cross-linked DNA. DNA samples in 51% methanol - 1 mM-NaCl - 1 mM-tris-HCl buffer, pH 7.0, were used.

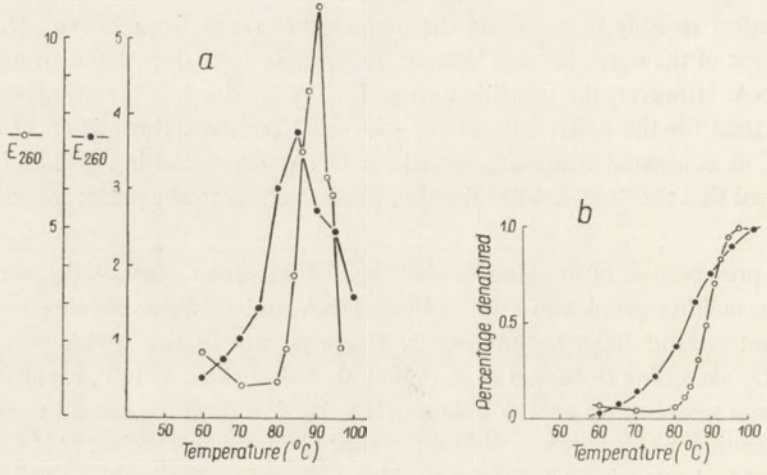

Fig. 5. *a*, Thermal chromatography on a hydroxyapatite column of (○), native DNA, and (●), the "native-like" DNA fraction. Samples of 30 ml. ($E_{260\ nm} = 2.0$) were applied to the column. For details see Methods. *b*, The same results presented in the integrated form.

Thermal chromatography of the "native-like" fraction. In Fig. 5a is shown the profile of the "native-like" fraction and native DNA submitted to thermal chromatography on an HA column. The eluted fractions of both samples have a Gaussian-like distribution curve with a maximum for the "native-like" DNA sample at 85° and for the native one at 90°. These differences are more evident when elution profiles are represented as integration curves (melting curves) as shown in Fig. 5b. From the work of Miyazawa & Thomas (1965) it is known that thermal fractionation on an HA column separates short double-stranded segments of DNA with

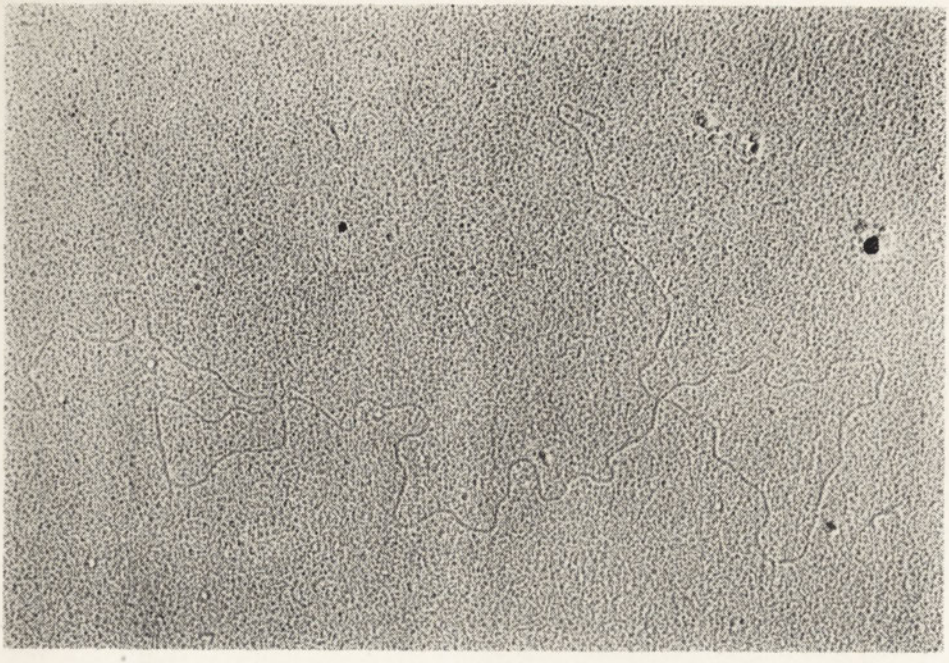

Fig. 6. Electron micrograph of native DNA molecule from calf thymus. ×55500.

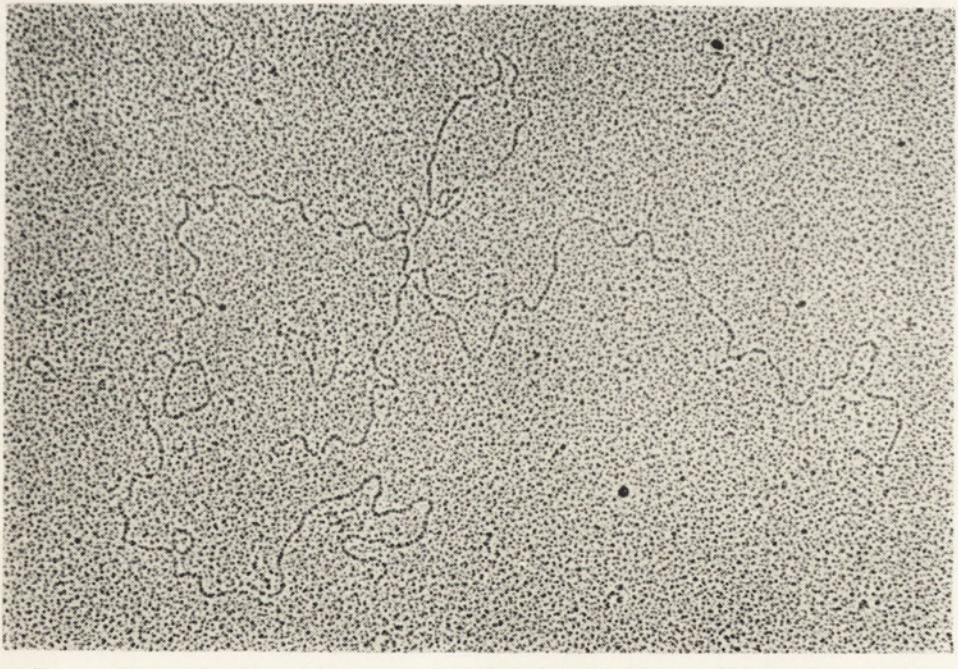

Fig. 7. Electron micrograph of DNA molecules from the "native-like" fraction. ×34500.

Fig. 8. Electron micrograph of the "native-like" fraction, magnification ×28300. *N*, Native molecules; *D*, denatured molecules.

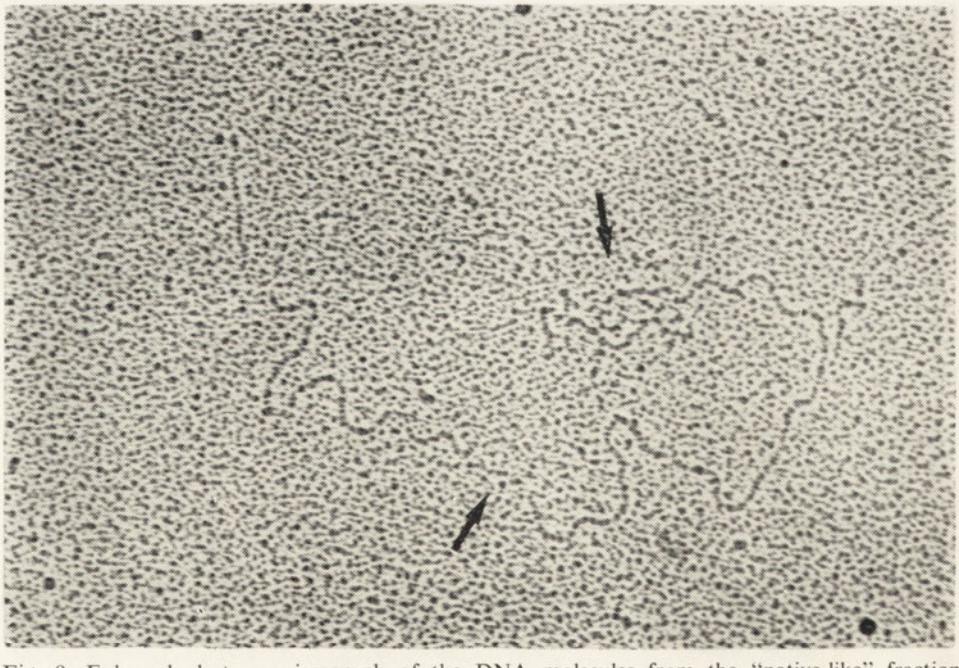

Fig. 9. Enlarged electron micrograph of the DNA molecules from the "native-like" fraction ×69700. Note denatured areas in the middle and at the end of the molecule (arrows).

respect to their nucleotide composition: molecules richer in G+C are released from the column at higher temperatures than those with lower G+C content. Thus our results from thermal chromatography might indicate that the "native-like" fraction has a lower G+C content than the original native sample. However, this is not the case, as the G+C content from three separate analyses of the "native-like" fraction was found to be 55.4±1.9 mole%, as compared with 41.9±0.7 mole% for native DNA. Alternatively, we may conclude that the base-pairing of the "native-like" fraction is less complete than in native DNA. A similar conclusion may be drawn from the analysis of the melting profiles of the "native-like" fraction and native DNA (see Fig. 3).

Electron microscopy of the "native-like" fraction. The electron micrograph of native calf thymus DNA showed single, long, well-dispersed filaments with distinct outlines. A fragment of one typical molecule is shown in Fig. 6. The molecules of the "native-like" fraction are very similar on electron micrographs to native ones (Fig. 7). They also appear as long, well defined threads. The inspection of the larger areas reveals, besides long filaments with native appearance, also short fragments and completely denatured molecules (Fig. 8). In some regions the continuity of the filament seems to be disrupted and a "bushed" area appears. This is particularly noticeable at a greater magnification (Fig. 9). The "native-like" fraction exhibits considerable length polydispersity. The average length of 132 molecules is 2.2±1.6 (S.D.) μ, the length range being from 0.4 μ to 10.9 μ. The main class of molecules has a length of 1.5 μ. Assuming 192 daltons/Å for B configuration (Thomas & MacHattie, 1967), the molecular weight of these molecules is 2.88×10^6. In the length distribution pattern (Fig. 10) one notes an accumulation of length

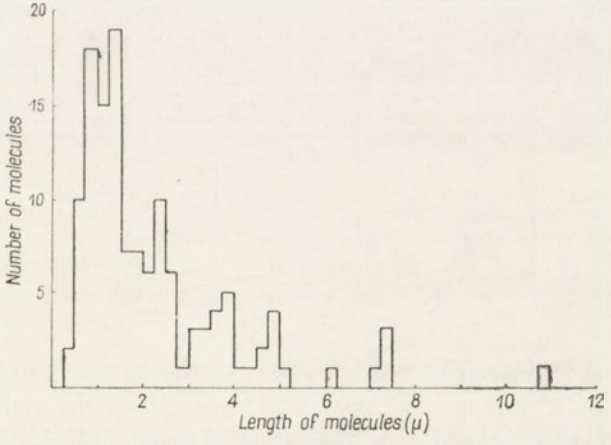

Fig. 10. Length distribution of 132 native DNA molecules from the "native-like" fraction.

around values which are multiples of 0.8 μ, e.g. 1.5, 2.5, 3.5 μ. The extreme values of molecular weight calculated for the shortest and longest molecules from their contour length were 0.77×10^6 and 20.9×10^6, respectively.

Table 1

Effect of DNA concentration, temperature and time of denaturation, on the quantity of "native-like" DNA

Calf thymus DNA was denatured in 0.02 M-NaCl.

DNA concentration (μg./ml.)	Temperature of denaturation (°C)	Time of denaturation (min.)	% of "native-like" DNA
0.76	100	10	10
3.8	100	10	15
7.6	100	10	11.6
34.0	100	10	10
50.0	100	10	12
100.0	100	10	12
50.0	90	10	10
50.0	100	10	11.6
50.0	110	10	11.6
50.0	120	10	9.5
50.0	100	10	10
50.0	100	30	12
50.0	100	60	9
100.0	Alkaline denaturation, pH 13.0	10	10

Table 2

Some parameters characterizing the kinetics of renaturation of calf thymus DNA

$C_0t_{1/2}$ determines the state when 50% of molecules are renatured; k_2, the second order renaturation rate constant is $1/C_0t_{1/2}$.

Renatured DNA fractions	Amount (%)	$C_0t_{1/2}$	k_2	Number of repeated sequences
A	10 - 15	10^{-3}	10^3	5×10^6
B	15 - 20	5×10^{-2}	20	10^5
C	70	5×10^3	2×10^{-4}	1

Sedimentation analysis. The sedimentation velocity of the "native-like" fraction in 0.9 M-NaCl - 0.1N-NaOH solution was used to determine the molecular weight of single strands of "native-like" DNA. The estimated $S_{20,w}$ at pH 13, for "native-like" DNA was 5.4.

The molecular weight of the "native-like" DNA was calculated from the sedimentation velocity according to the equation $S_{20,w} = 0.0528 \times M^{0.400}$ given by Studier (1965). From this equation a molecular weight of about 10^5 daltons was obtained. This low molecular weight of the "native-like" DNA is probably due

to chain degradation during thermal denaturation, especially when using DNA dispersed in 0.02 M-NaCl solutions. This possibility is strongly stressed by Bagchi, Mistra, Basu & Das Gupta (1969) in their electron microscopic studies on the conformation of denatured and renatured *E. coli* DNA.

Kinetics of renaturation. The kinetics of renaturation of calf thymus DNA was followed as described by Britten & Kohne (1968). The percentage of renatured DNA was plotted *versus* C_0t (Fig. 11) where: C_0 is DNA concentration (mole/l.) used for renaturation, and t, renaturation time (sec.). The curve illustrating the rate

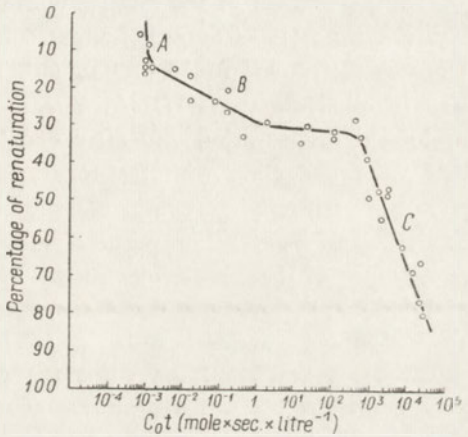

Fig. 11. The kinetics of renaturation of calf thymus DNA. Samples of DNA at a concentration of 20 - 8300 μg./ml. of 0.125 M-sodium phosphate buffer, pH 6.8, were denatured at 100° for 10 min., then renatured at 60° during different periods of time.

of renaturation of calf thymus DNA is composed of three distinct parts: *A*, *B* and *C*. Part *A* represents renaturation rate of the fast-renaturing DNA fraction constituting 10 - 15% of total DNA and having $C_0t_{1/2} = 10^{-3}$ mole×sec.×litre^{-1}, and second-order renaturation constant $k_2 = 1000$ litre×mole^{-1}×sec.$^{-1}$. Component *B* amounting to 15 - 20% of total DNA has $C_0t_{1/2} = 5\times10^{-2}$ mole×sec.×litre^{-1} and $k_2 = 20$ litre×mole^{-1}×sec.$^{-1}$. The largest DNA fraction (70% of total DNA) represented by part *C* of the curve, has the slowest renaturation rate ($C_0t_{1/2} = 5\times10^3$ mole×sec.×litre^{-1} and $k_2 = 2\times10^{-4}$ litre×mole^{-1}×sec.$^{-1}$).

The values of $C_0t_{1/2}$, k_2 and the approximate amounts of each component of three DNA's are listed in Table 2. From comparison of $C_0t_{1/2}$ of fractions *A*, *B* and *C* (Britten & Kohne, 1968) it is calculated that renaturation rate of fraction *A* is about 5×10^6 times faster, and fraction *B* is renaturing 10^5 times faster than fraction *C*.

If one assumes the correctness of reasoning of Britten & Kohne (1968) that the slow-renaturing fraction *C* consists of molecules with non-repeating sequences and that the renaturation rate is proportional to the number of base pairs in the genome, then sequences of fraction *A* repeat 5×10^6 times, and these of fraction *B* — 10^5 times as compared to *C* component.

DISCUSSION

The results presented in this communication indicate that DNA isolated from calf thymus contains a class of molecules which after heat denaturation and cooling reveal a native-like secondary structure. This "native-like" class constitutes about 10% of the total DNA and can be partly separated from the bulk of denatured molecules by chromatography on hydroxyapatite. A "native-like" fraction was also found to be present in the nuclear DNA of mouse lymphatic leukaemia L1210 cells and liver cells of CDBA/2 inbred mice.

The percentage of the "native-like" fraction was not dependent on the denaturation time (10 - 60 min.), temperature (90 - 120°), or on the concentration of DNA in the range of 0.76 - 100 μg./ml. The presence of this fraction was observed also in alkali-denatured DNA. The presence of the "native-like" fraction after prolonged denaturation and after denaturation at elevated temperature indicates that we are dealing with fast-renaturing molecules rather than with molecules possessing natural interstrand cross-links. If the "native-like" molecules occurred as a result of renaturation proceeding according to a typical second-order reaction, the amount of these molecules should be expected to depend on the concentration of DNA. This, however, was not the case.

A "stable" fraction, similar to the "native-like" fraction, has been observed by Walker & McLaren (1965) in DNA from mouse liver and mouse L cells; they concluded that the stability of this fraction was due to cross-links. The "stable" fraction was later on identified with the rapidly renaturing mouse satellite DNA (Waring & Britten, 1966; Bond, Flamm, Burr & Bond, 1967; Schildkraut & Maio, 1968; Flamm, Walker & McCallum, 1969). On the other hand, Alberts & Doty (1968) showed that some of the DNA molecules of calf thymus are naturally cross-linked. If our "native-like" fraction represented native cross-linked molecules then it should have renaturation features similar to artificially cross-linked (HNO_2 treated) DNA molecules. However, the melting profile of the HNO_2-treated DNA reveals distinct differences when compared with that of the "native-like" fraction (Fig. 4). Similarly, the differences seen in the cooling curves strongly suggest that the cross-links are not involved in the maintenance of the secondary structure of the "native-like" fraction.

The G+C content of the "native-like" fraction of calf thymus DNA was found to be 55.4 mole%. Polli, Corneo, Ginelli & Bianchi (1965) found that calf thymus satellite DNA has a density of 1.712 g./cm^3, which corresponds to 52 - 54 mole% of G+C. The G+C content of this DNA estimated from T_m was found by these authors to be in the range 50 - 58 mole%. According to Polli *et al.* (1965), calf thymus satellite DNA has the ability for fast renaturation. It may be supposed that our "native-like" fraction corresponds to, or is identical with, those calf thymus satellite DNA molecules which were able to recover their secondary structure after denaturation.

The restitution of double-strands, as might be judged from the melting profile and thermal chromatography on an HA column of the "native-like" fraction, was

not complete. However, electron micrographs clearly indicate that the majority of molecules in the "native-like" fraction are long, linear filaments with all the features of typical native molecules, only some of them possessing denatured regions (Fig. 9). The diffuse melting curve (Fig. 3) of the "native-like" fraction could be explained by the presence in this fraction of denatured DNA molecules visible in the electron photographs (Fig. 8).

The length distribution (Fig. 10) shows that the main class of molecules consists of those 1.5 μ in length. Molecular weight calculated for this class is about 2.88×10^6, and determined from the sedimentation rate in alkaline solution was 10^5 daltons. This discrepancy could be explained as follows. In alkaline solution the molecules of the "native-like" fraction exist as separate segments about 300 nucleotides long. Probably, due to repeated sequences, these molecules during renaturation can form elongated filaments in a process similar to "concatenation". End-to-end elongation of mouse satellite DNA molecules was suggested recently by Salomon, Kaye & Herzberg (1969).

If sequences in "native-like" fraction constitute about 10% of total DNA and contain about 300 nucleotides each (10^5 daltons), then in calf thymus genome containing 5×109 base pairs, these sequences will include 5×108 base pairs, and will occur about $5 \times 108/300 = 1.6 \times 10^6$ times per genome.

The "native-like" fraction could be further identified with fast-renaturing ($k_2 = 10^3$ litre $\times$ mole$^{-1} \times$ sec.$^{-1}$) component *A* (see Fig. 11). The C_0t value roughly estimated for the "native-like" fraction to be 10^{-3} mole $\times$ sec. $\times$ litre^{-1} fits also in the range of C_0t found for component *A* of both the "native-like" fraction and the total preparation. From the kinetic studies it was estimated that the sequences of component *A* are repeated in calf thymus genome about 5×10^6 times (Table 2), this value being close to that calculated for the "native-like" fraction. The sequences of the much slower renaturing fraction *B* exist in calf thymus genome in some 10^5 copies, whereas only single copies of sequences of component *C* may be assumed. These considerations provide further support for the identity of the "native-like" fraction with satellite DNA and component *A*.

We wish to express our sincere thanks to Prof. Dr. K. Zakrzewski (Serum and Vaccine Research Laboratory, Warszawa) for performing the sedimentation analyses of DNA samples. The skilful technical assistance of Miss E. Gara is acknowledged.

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SZYBKO RENATURUJĄCA FRAKCJA DNA GRASICY CIELĘCEJ

Streszczenie

1. DNA grasicy cielęcej, poddany denaturacji termicznej i szybkiemu oziębieniu, w trakcie chromatografii na hydroksyapatycie rozdzielał się na dwie frakcje. Drugi szczyt pojawiał się w tym rejonie, gdzie eluowane są natywne cząsteczki DNA; nazwano go frakcją „podobną do natywnej". Frakcja ta stanowi około 10% DNA.

2. Frakcja „podobna do natywnej" posiada niektóre cechy natywnego DNA, jak to można sądzić po badaniach krzywych topnienia i badaniach w mikroskopie elektronowym. Zawartość G+C tej frakcji wynosi 55.4 mol%. Ciężar cząsteczkowy oznaczony z szybkości sedymentacji w alkalicznym roztworze wynosi 10^5, a wyliczony z długości konturów dla przeważającej liczby cząsteczek wynosi $2{,}88 \times 10^6$.

3. Na podstawie badań kinetyki renaturacji DNA grasicy cielęcej można przypuszczać, że frakcja „podobna do natywnej" jest populacją cząsteczek szybko renaturujących, przypominających satelitarny DNA.

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STUDIES ON THE ORIGIN OF UREA IN SUBMAXILLARY AND SUBLINGUAL GLANDS OF THE RAT

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1. The submaxillary gland differed from the sublingual one in the amount of extractable protein (63 and 18 mg./g. of tissue, respectively), and in arginase activity (10 and 1.5 μmoles/min./g. tissue, resp.). 2. Arginase was located in nuclei and mitochondria; it was activated by Mn^{2+} ions and had two pH optima, at 7.5 and 9.5. 3. Some activity of guanidinobutyrate ureohydrolase was found. 4. Neither ornithine carbamoyltransferase nor the enzymic system catalysing the formation of arginine from citrulline and aspartate, have been found in either gland, indicating the absence of ureogenesis.

The occurrence of arginase (EC 3.5.3.1) in all three salivary glands of the rat has been demonstrated by Kochakian, Endahl & Hall (1955) and confirmed histologically by Malmgren & Syloen (1959). Urea can arise not only from arginine but also from γ-guanidinobutyrate by the action of a specific enzyme, guanidinobutyrate ureohydrolase. The activity of this enzyme was first demonstrated by Thomas (1913) and later was investigated by many authors (for review see Porembska Gąsiorowska & Mochnacka, 1968). The presence of this enzyme in tissues of numerous invertebrates and vertebrates has been demonstrated by Baret, Mourgue & Broc (1967). However, the occurrence of guanidinobutyrate ureohydrolase in salivary glands has not so far been reported.

In the present work, submaxillary and sublingual glands of the rat were examined for the presence of enzymes of the ornithine cycle. The content of arginase and its subcellular distribution in the glands were determined and some properties of this enzyme were studied. Moreover, the presence of γ-guanidinobutyrate ureohydrolase in salivary glands has been demonstrated.

MATERIALS AND METHODS

Reagents. Carbamoylphosphate dilithium salt, L-arginine hydrochloride, L-ornithine hydrochloride and L-citrulline were from Sigma Chem. Co. (St. Louis, Mo., U.S.A.); γ-aminobutyric acid from Fluka A. G. (Buchs S. G., Switzerland); L-aspartic

acid from Reanal (Budapest, Hungary); γ-guanidinobutyric acid and ATP from Calbiochem. (Los Angeles, Calif., U.S.A.); cetyl trimethylammonium bromide (CTB) from British Drug Houses (Poole, Dorset, England); xanthone from Merck GmbH (München, West Germany). A partially purified arginase preparation from rat liver was obtained according to Ratner (1955); 1 ml. of the solution contained 6 mg. of protein.

Solutions of the reagents to be used as components of the incubation mixture, were adjusted to pH 7.

Animals and tissue extracts. Wistar rats, 8 - 10 weeks old, weighing 150 - 200 g., were used for experiments. The rats were fed *ad libitum* standard L.S.M. mixture (Bacutil, Warszawa, Poland) supplemented with carrot. The animals were stunned, the spine at the neck cut through and, after bleeding, the capsulae containing the submaxillary and sublingual glands were isolated. The capsula was removed, the glands were separated and rinsed with 0.9% NaCl solution at 0° and blotted in filter paper. The glands were homogenized on ice in a glass-teflon Potter homogenizer at 1900 rev./min., twice for 1 min., in 9 volumes of 0.1% solution of CTB (pH 6.5). The homogenate was left for 1 hr. on ice with gentle stirring, centrifuged for 20 min. at 7000 rev./min. in a cold room, and the supernatant was directly used for determinations of enzymic activity.

Subcellular fractions were prepared by the method of Hogeboom (1955).

Enzyme assays. The activity of arginase (EC 3.5.3.1) was measured in a mixture containing in 1 ml.: 50 μmoles of glycine buffer, pH 9.5, 25 μmoles of arginine, 5 μmoles of $MnCl_2$ and 0.1 - 0.3 ml. of tissue extract, containing 0.05 - 0.2 mg. of protein. After 30 min. incubation at 37°, the sample was placed in a boiling water bath for 7 min., cooled, and the precipitated protein was centrifuged. In the clear supernatant, the increase of ornithine was determined according to Chinard (1952). The control samples contained no arginine or no tissue extract. The activity of arginase was expressed as μmoles of ornithine formed/mg. protein/min. Hydrolysis of arginine was linear with time for at least 60 min. and with protein concentration from 0.02 to at least 0.7 mg.

The activity of ornithine carbamoyltransferase (EC 2.1.3.3) was measured in a mixture containing in 2 ml.: 50 μmoles of glycine buffer, pH 8.5, 20 μmoles of ornithine, 20 μmoles of carbamoylphosphate and tissue extract (2 - 4 mg. of protein). After 30 min. incubation at 37°, the reaction was stopped by adding 2 ml. of 1 M-$HClO_4$ and citrulline was determined in the supernatant according to Lazarev (1950).

Synthesis of arginine from aspartate and citrulline (adenylosuccinate synthetase, EC 6.3.4.4, and argininosuccinate lyase, EC 4.3.2.1) was assayed in a mixture containing in 1.2 ml.: 50 μmoles of tris-HCl buffer, pH 7.2, 5 μmoles of citrulline, 5 μmoles of aspartate, 5 μmoles of ATP, 5 μmoles of $MgSO_4$, 5 μg. of arginase (partly purified preparation from rat liver), and tissue extract (2 - 4 mg. of protein). After 1 hr. incubation at 25°, 1.5 ml. of 83% acetic acid was added, the mixture placed for 7 min. in a boiling water bath, cooled, centrifuged, and in the supernatant urea was determined by the xanthydryl method (Engel & Engel, 1947) and ornithine by the method of Chinard (1952).

The activity of guanidinobutyrate ureohydrolase was assayed in a mixture containing in a volume of 2 ml.: 50 µmoles of glycine buffer, pH 9.5, 10 µmoles of γ-guanidinobutyrate, 5 µmoles of $MnCl_2$ and tissue extract (2 - 4 mg. of protein). After 1 hr. incubation at 37°, 2 ml. of 83% acetic acid was added, the precipitated protein was centrifuged off and the urea formed was determined by the xanthydryl method.

Protein was determined according to Lowry *et al.* (1951).

Paper chromatography. The tissue, 550 mg. of the submaxillary gland or 160 mg. of the sublingual gland, was homogenized in 60% ethanol at a ratio of 50 mg./1 ml. The extract obtained after centrifuging was concentrated in vacuum at room temperature to a volume of 1 ml., and 20 - 50 µl. was applied to Whatman no. 1 paper; amino acid standards (0.5% solution) were applied in amounts of 5 µl. The chromatograms were developed with butanol - acetic acid - water (2 : 2 : 1, by vol.) for 24 hr. γ-Aminobutyric acid was located with 0.2% ninhydrin solution in acetone at 100° and γ-guanidinobutyrate with a mixture composed of 16 ml. of water, 2 ml. of 40% NaOH, 2 ml. of 1% α-naphthol solution and 1 drop of diacetyl (Roche, Thoai, Felix & Robin, 1951).

RESULTS AND DISCUSSION

Protein. The two salivary glands differed in the amount of protein extracted with 0.1% CTB, pH 6.5. The extract obtained from 1 g. of the submaxillary gland contained on the average 63 mg. of protein, whereas that from the sublingual gland only 18 mg. (Table 1). These differences are probably related to different protein composition of the two glands.

Table 1

The content of protein and the activity of arginase in extracts from salivary glands

The glands were homogenized with 0.1% CTB and extracted for 1 hr. The content of protein and arginase activity were determined as described in Methods. The results are mean values ± S.E. from 12 individual glands.

Gland	Protein extracted (mg./g. of tissue)	Arginase activity (µmole/min.)	
		per mg. protein	per g. of tissue
Submaxillary	63.1 ± 1.0	0.17 ± 0.0076	10.73 ± 0.3616
Sublingual	18.0 ± 0.99	0.096 ± 0.0063	1.52 ± 0.0293

Enzymes of the ornithine cycle. Arginase was the only enzyme of the urea biosynthesis cycle found in the submaxillary and sublingual glands, either in tissue extracts or in subcellular fractions. Neither the enzymes catalysing the formation of citrulline from ornithine and carbamoylphosphate, nor those involved in the formation of arginine from citrulline and aspartate could be found. Thus the two salivary glands of the ureotelic rat appear to belong to those tissues which, in contrast to liver, do not synthesize urea from ammonia and carbon dioxide although

they exhibit arginase activity; the arginase present in those tissues serves probably only to hydrolyse arginine derived from the decomposition of protein. Jones, Anderson, Anderson & Hodes (1961) reported some activity of ornithine carbamoyltransferase in rat salivary gland (without indicating in which of the three glands). However, in our experiments neither in extracts nor in subcellular fractions of the submaxillary or sublingual glands, any activity of this enzyme could be detected.

Arginase. The activity of arginase per 1 mg. of protein was about twice as high in the extracts from the submaxillary gland as in those from the sublingual one (Table 1), the mean values being, respectively, 0.17 and 0.092 μmole/g. protein/min. The activity calculated per 1 g. of fresh tissue was about seven times higher in the submaxillary than in the sublingual gland.

The arginases of both glands had two pH optima, at 7.5 and 9.5, similarly as it has been found for arginase of rat brain and kidney (Gąsiorowska, Porembska, Jachimowicz & Mochnacka, 1970), and in contrast to the liver arginase which exhibited a single optimum at pH 9.5. Like the arginases from all other tissues studied, the arginase of both salivary glands was activated by Mn^{2+} ion.

The localization of arginase in subcellular fractions of the two glands was similar. Almost all the activity was found in the nuclear and mitochondrial fractions, and only 12 - 15% of the activity was present in the post-mitochondrial supernatant (Table 2). A similar intracellular distribution of arginase has been found in rat kidney and brain (Gąsiorowska *et al.*, 1970). It should be noted that in rat liver 28 - 37% of the activity was found in microsomes (Mora, Martuscelli, Ortiz-Pineda & Soberón, 1965; Gąsiorowska *et al.*, 1970).

Table 2

Percentage distribution of arginase activity in subcellular fractions of salivary glands

The composition of the incubation mixture for arginase assay was as described in Methods. Three fractionation experiments were performed, and typical results are presented. They are expressed as percentages of the activity found in the whole homogenate.

Fraction	Arginase activity (%)	
	Submaxillary gland	Sublingual gland
Nuclei	47	59
Mitochondria	35	26
Microsomes	8	15
Supernatant	4	

Both the observed two pH optima and intracellular distribution of the arginase from salivary glands are similar to those of the enzyme from the tissues which are incapable of urea biosynthesis (Gąsiorowska *et al.*, 1970).

Guanidinobutyrate ureohydrolase. The activity of this enzyme was found both in sublingual and submaxillary glands, but it was much lower than the activity of arginase. Guanidinobutyrate ureohydrolase is more difficult to extract than arginase, and even in the presence of a detergent, 0.1% CTB, it was necessary to

prolong the extraction time up to 1 hr. This enzyme, in contrast to arginase, is relatively stable at elevated temperature. Heating of the extract at 70° for 15 min. had no effect on its activity, whereas this procedure completely inactivated arginase (Table 3).

In the submaxillary gland, the guanidinobutyrate ureohydrolase activity per 1 g. of tissue amounted on the average to 0.1 μmole/min. (Table 4) which corresponds to about 1% of the arginase activity. In the sublingual gland, the activity was lower, 0.035 μmole/g./min., which in relation to arginase amounts to about 2.3%.

Table 3

The effect of temperature on the activity of arginase and guanidinobutyrate ureohydrolase in extracts of rat salivary glands

The extract was heated at 70° for 15 min. and after cooling the activity was determined as described in Methods. Samples containing unheated extracts served as control. Results of a typical experiment are given.

Gland	Arginase (μmoles/30 min.)		Guanidinobutyrate ureohydrolase (μmoles/30 min.)	
	Control	After heating	Control	After heating
Submaxillary	4.5	0.0	0.05	0.05
Sublingual	2.5	0.0	0.09	0.09

Table 4

The activity of guanidinobutyrate ureohydrolase in extracts of rat salivary glands

The enzyme activity was determined as described in Methods. The results are means from 4 determinations, limit values being given in parentheses.

Gland	Activity	
	μmole/min./mg. protein	μmole/min./g. of tissue
Submaxillary	0.0015 (0.0011 - 0.0016)	0.1 (0.073 - 0.11)
Sublingual	0.0029 (0.002 - 0.0033)	0.035 (0.024 - 0.04)

Chromatographic analysis of ethanolic extracts from the two salivary glands gave no indication of the presence of γ-guanidinobutyrate but demonstrated the presence of γ-aminobutyrate, which, however, may be formed not only from γ--guanidinobutyrate but also through another metabolic pathway. Thus, both the low activity of guanidinobutyrate ureohydrolase, in comparison with that of arginase, and lack of the substrate in salivary glands seem to indicate that guanidinobutyrate ureohydrolase does not participate to any significant extent in formation of urea in these glands.

The presented data indicate that there is no ureogenesis from ammonia and carbon dioxide in either of the salivary glands studied, and that formation of urea is closely related to the activity of arginase and the amount of arginine arising from other catabolic processes.

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BADANIA NAD POCHODZENIEM MOCZNIKA W GRUCZOLE ŚLINOWYM PODŻUCHWOWYM I PODJĘZYKOWYM SZCZURA

Streszczenie

1. Wyciągi z obu ślinianek różniły się ilością białka. Wyciąg z gruczołu podżuchwowego zawierał 63 mg, a z gruczołu podjęzykowego 18 mg białka w przeliczeniu na 1 g tkanki. Aktywność arginazy wynosiła odpowiednio 10 i 1,5 μmoli/min./g tkanki.

2. Arginaza obu ślinianek występuje w jądrach i mitochondriach, aktywuje się jonami Mn^{2+} oraz ma dwa optima przy pH 7,5 i 9,5.

3. Wykazano nieznaczną aktywność ureohydrolazy kwasu γ-guanidynomasłowego w śliniankach.

4. Nie stwierdzono natomiast ani karbamoilotransferazy ornitynowej ani powstawania argininy z cytruliny i asparaginianu; wskazuje to na brak biosyntezy mocznika z amoniaku i CO_2 w śliniankach.

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CHANGES IN PROPERTIES OF CONTRACTILE PROTEINS AND MEMBRANE SYSTEM OF DRIED AND REHYDRATED FROG MUSCLES

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1. The sartorii muscles of the frog were dehydrated using silica gel and rehydrated in Ringer's solution. It was found that the ATPase activity of the actomyosin extracted from fresh muscles and from dehydrated ones was similar; however, actomyosin obtained from the muscles almost completely dried prior to rehydration was EGTA insensitive. 2. The membrane system of the muscle fibres was affected by a loss of about 60% of the initial water content. This was shown by the lack of contraction after electric stimulation, by diffusion of inorganic phosphate and proteins out of the cells, and by an inflow of sucrose into the cells during rehydration. 3. The importance of water bound to the cell structures is discussed.

Specific properties of dried and rehydrated frog muscles were demonstrated in a previous paper (Dydyńska, 1966). The slow spontaneous contraction was the main phenomenon observed during rehydration of the muscles which had lost more than 70% water. This was accompanied by splitting of inorganic phosphate from phosphocreatine and ATP and the outflow of some small molecular weight compounds to the rehydrating Ringer's solution. Rehydration during 20 - 30 min. at room temperature was sufficient for the complete contracture characterized by shortening of the muscle to 60% of its initial length. It was suggested that these phenomena might reflect the damage to the cell organization brought about by the loss of water.

In the present experiments with dried and rehydrated muscles the extractability and enzymic activity of proteins as well as properties of cell membranes were investigated.

MATERIAL AND METHODS

Experiments were performed on the frogs *Rana esculenta*. The sartorius muscles after excision were weighed either after draining them up the side of a glass beaker (drained weight) or after blotting with filter paper (blotted weight) (Dydyńska & Wilkie, 1963).

Water content and water partition in the muscles were calculated in the following manner: extracellular water (ECW)[1] was estimated as "sucrose space" according to Dydyńska & Wilkie (1963), total water content was calculated after drying the muscles at 105°, and muscle fibre water (FW) by subtracting the quantity of ECW from the total water content.

Powdered silica gel was used as the dehydrating agent. For partial dehydration the muscles were placed over SiO_2, while for complete desiccation of the tissue the muscles were wrapped in a cellophane sheet and covered with SiO_2. The temperature of dehydration was 4°. The water loss from the muscles at the particular degree of dehydration was estimated by weighing. The examination of the process of dehydration over SiO_2 presented in the previous paper (Dydyńska, 1966) led to the conclusion that there was no gradient of water content inside the muscles during drying. One could assume therefore that the degree of water loss from the total muscle referred also to its fibre water. Figure 1 shows that this was actually the case when the fresh muscles were weighed after blotting, but when some water was left on the muscle surface before dehydration it evaporated initially, leaving water inside the muscle still intact. On the basis of estimations of surface water in the drained muscles (Dydyńska, unpublished experiments) it was possible to calculate the degree of muscle dehydration as the loss of water from the fibres.

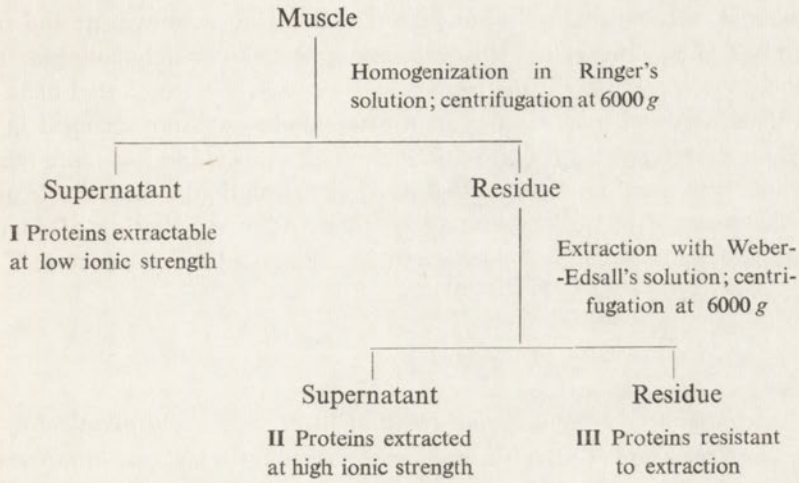
Dried muscles were rehydrated in Ringer's solution (115 mM-NaCl, 2.0 mM-KCl, 1.8 mM-$CaCl_2$ with $NaHCO_3$ added to pH 7) during 20 - 30 min. at room temperature.

For the extraction of proteins, fresh muscles as well as dehydrated and rehydrated ones[2] were ground in glass mortars with 10 volumes of Ringer's solution at 0°. The homogenate was centrifuged at 6000 *g* and the residue was extracted overnight with Weber-Edsall's solution (600 mM-KCl, 40 mM-$KHCO_2$, 10 mM-K_2CO_3). The extract was separated from muscle débris by centrifugation. Actomyosin was precipitated from the supernatant by addition of 10 volumes of water. The proteins soluble in the solutions of high ionic strength were extracted quantitatively from the muscle residue by repeated extractions with Weber-Edsall's solution. After 5 - 8 extractions during a 20 hr. period the supernatant showed only traces of protein. In one series of experiments, the extraction was carried out with 1 M-KI according to Helander (1957). The procedure is illustrated in Scheme 1. All extractions and centrifugations were run at 4°. The content of proteins in each fraction was estimated by the Kjeldahl method or by biuret reaction (Gornall, Bardawill & David, 1949).

The media used for assays of the actomyosin ATPase activity and for superprecipitation of actomyosin by ATP added, are described in legends to the respective figures. Orthophosphate split from ATP was determined by method of Fiske & Subbarow (1925). Outflow of proteins from single rehydrated muscles to the Ringer's solution was estimated by amidoblack method (Niemierko, Gruda & Oderfeld, 1962).

[1] The following abbreviations are used: ECW, extracellular water; FW, fibre water; P_i, inorganic phosphate; EGTA, 1,2-bis(2-dicarboxylaminoethoxy)-ethane.

[2] The muscles which were dried and rehydrated will be referred to later as: rehydrated.



Scheme 1

The excitability of muscles was checked by applying 6 volts A.C. during 0.5 sec. through Ringer's solution in which the muscles were immersed. The tension and shortening of the muscles were recorded by isometric or isotonic myographs.

All reagents used were of analytical reagent grade. ATP and silica gel were imported by B.O.O. (Gliwice, Poland). EGTA was from Geigy Industrial Chemicals (New York, U.S.A.).

RESULTS

Changes in the content and properties of muscle proteins after drying and rehydration. Three protein fractions were obtained by successive extractions of muscle homogenate by Ringer's solution followed by Weber-Edsall's solution according to Scheme 1. They were: fraction I containing the sarcoplasmic proteins as well

Table 1

Protein partition in sartorius muscle of the frog

Fresh muscle and its paired muscle dehydrated to lose more than 80% of fibre water, were examined. Mean values ± S.D. from 8 experiments are given. Total protein content estimated in other experiments (n = 33) is also included.

Protein fractions	Fresh muscle		Dehydrated muscle	
	mg./100 mg. fresh wt.	% of total	mg./100 mg. fresh wt.	% of total
I. Sarcoplasmic proteins	4.9 ± 1.4	27	4.0 ± 1.3	26
II. Contractile proteins	7.2 ± 1.3	47	7.6 ± 1.0	49
III. Residual proteins	3.7 ± 0.8	24	3.9 ± 0.4	25
Total protein (sum)	15.1		15.5	
Total protein	15.2 ± 3.5			

as fragmented sarcoplasmic reticulum; fraction II mainly actomyosin; and fraction III composed of proteins resistant to extraction, probably mainly collagen. Table 1 shows the protein partition in the fresh muscles and in the dehydrated ones. It can be seen that the usual pattern of protein extractability was not changed in the dehydrated muscles. On the other hand, in the muscles which had lost more than 50% of their fibre water and were rehydrated in Ringer's solution, a measurable quantity of protein appeared in the rehydrating medium. After rehydration of completely dried muscles the proteins in Ringer's solution amounted up to about 11% of the total protein of the muscle (Table 2).

Table 2

Outflow of protein during rehydration of dehydrated muscle

Single experiments, except where the number of experiments is indicated in parentheses.

FW loss (% of the initial)	Protein content		
	in the muscle	in Ringer's solution	
	mg./100 mg.	mg./100 mg.	% of the total protein content
54	13.4	0.2	1.5
62	13.1	0.4	3.0
67	13.2	0.4	2.9
75	12.2	0.5	3.9
80	12.2	0.5	3.9
85	17.2	0.9	5.0
90 (2)	11.4	0.8	6.6
100	10.7	1.4	11.6
100	—	1.7 (4)	11.2*

* Calculated as percent of the mean protein content, 15.2 mg.

It was demonstrated in the previous paper (Dydyńska, 1966) that the contraction of dried muscles during rehydration was accompanied by splitting of ATP. In order to determine whether this splitting came from the ATPase activity of the contractile system, the enzymic activity of actomyosin extracted from the dehydrated muscles was estimated. It was found to be almost as high as that of actomyosin extracted from fresh muscles (Fig. 2). On the other hand, the characteristic property of natural actomyosin, its sensitivity to the presence of EGTA, manifested by a decrease in ATPase activity and slowing down of the superprecipitation phenomenon (Ebashi, 1963), was lost when the actomyosin was extracted from muscles which had been almost completely dehydrated and then rehydrated (Figs. 3 and 4). This could be observed only when the extraction of proteins with Weber-Edsall's solution was preceded by removal of the proteins soluble in Ringer's solution. Actomyosin precipitated from the direct extract of the muscles with high ionic strength solution preserved its EGTA sensitivity even when the muscles were completely dehydrated prior to rehydration and extraction.

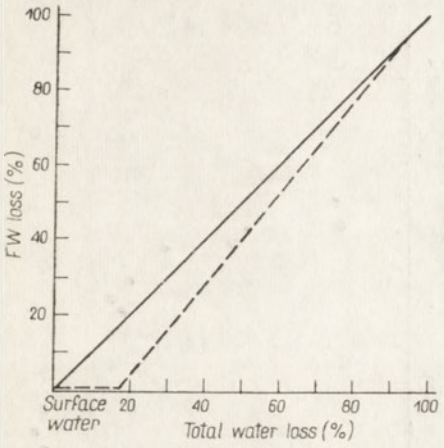


Fig. 1

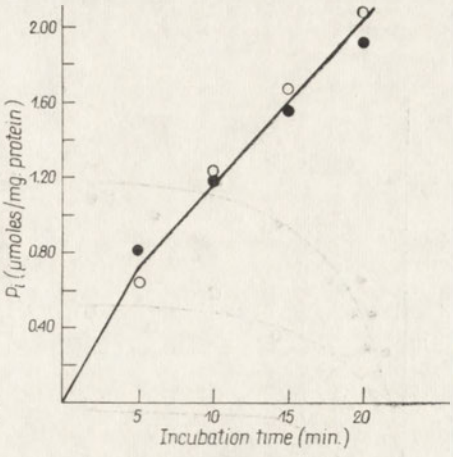


Fig. 2

Fig. 1. Loss of water from muscle fibres in relation to total water loss, in percent of the initial content. (———), Fresh muscles weighed after blotting; (- - -), fresh muscles weighed after draining
Fig. 2. ATPase activity of the actomyosin extracted from: (●), fresh muscles; and (○), muscles completely dehydrated over SiO_2. The incubation mixture was composed of: 0.03 M-KCl, 0.04 M-tris buffer, pH 7.4, 0.005 M-$MgCl_2$ and 0.005 M-ATP; protein concentration 0.1 - 0.3 mg./ml. Temperature 25°.

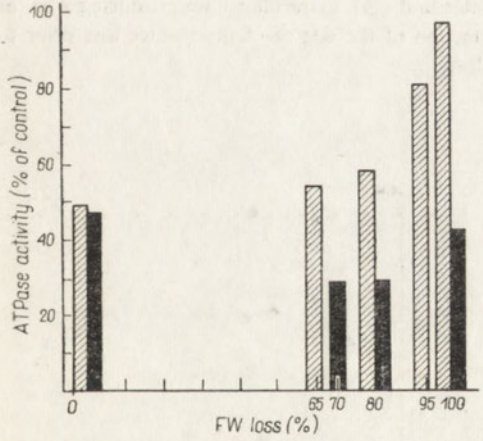


Fig. 3

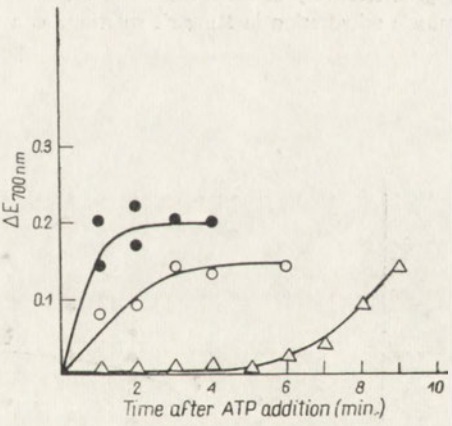


Fig. 4

Fig. 3. EGTA sensitivity of natural actomyosin obtained from fresh and rehydrated muscles. Shadowed bars, actomyosin extracted from the muscle homogenate after previous removal of the proteins soluble in Ringer's solution. Black bars, actomyosin precipitated from the direct extract with Weber-Edsall's solution. The composition of the incubation mixture was as described for Fig. 2, plus 0.001 M-EGTA. Incubation time 5 min. ATPase activity is expressed in % of the activity in the absence of EGTA.

Fig. 4. Superprecipitation of actomyosin obtained from fresh and rehydrated muscles measured by increase of turbidity at 700 mμ on addition of ATP. The incubation mixture was composed of: 0.06 M-KCl, 0.05 M-tris buffer, pH 7.4, 0.001 M-$MgCl_2$, 0.001 M-ATP and 0.001 M-EGTA. Protein concentration was 0.7 mg./ml. (●), actomyosin from fresh and rehydrated muscles in the absence of EGTA; (○), actomyosin from rehydrated muscles in the presence of EGTA; (△), actomyosin from fresh muscles in the presence of EGTA.

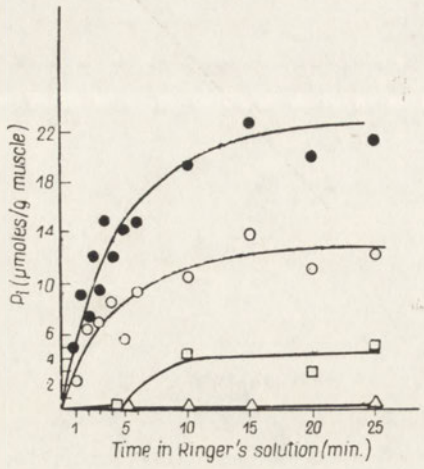


Fig. 5

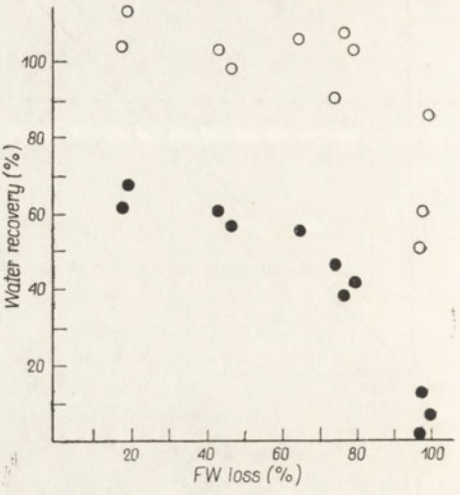


Fig. 6

Fig. 5. Outflow of P_i from muscle fibres during rehydration. Fibre water loss from the dehydrated muscles prior to the immersion in Ringer's solution was: (△), 60%; (□) 75%; (○) 90%; (●) more than 95% of the initial content.

Fig. 6. Recovery of the content of (●), fibre water and (○), extracellular water, during 2 hr. of muscle rehydration in Ringer's solution, as a function of the degree of fibre water loss prior to rehydration.

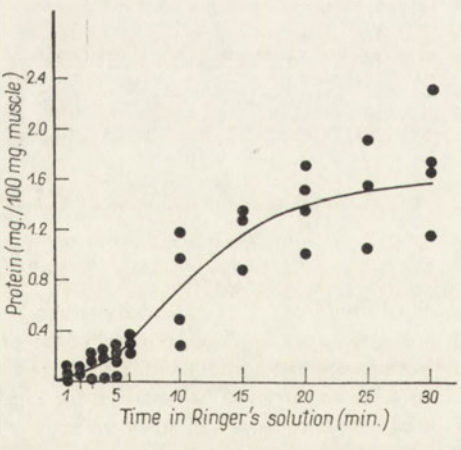


Fig. 7

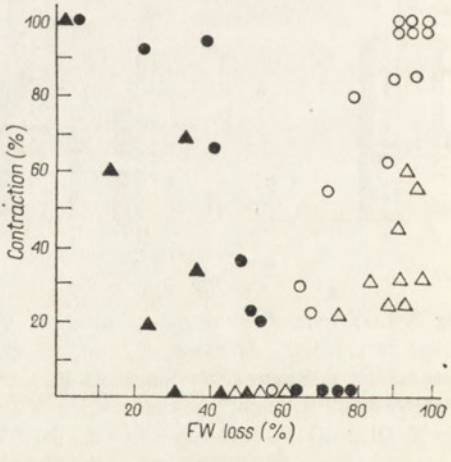


Fig. 8

Fig. 7. Outflow of proteins from completely dried muscles during rehydration in Ringer's solution.

Fig. 8. Contractile activity of rehydrated muscles in relation to the degree of fibre water loss during dehydration. Isotonic contractions: (●), after stimulation; (○), spontaneous during rehydration. Isometric contractions: (▲), after stimulation; (△), spontaneous during rehydration. The contraction is expressed in percent of the maximum contraction of the fresh muscles.

Changes in the properties of membrane system of muscle cell. It was observed that drying and rehydration caused the fibre membranes to become permeable for the substances which otherwise could not flow out of the fibre, e.g. when fresh muscles were immersed in the Ringer's solution (Dydyńska, 1966). This can be seen in Fig. 5 which shows the outflow of inorganic phosphate from the muscle fibres during the rehydration of the muscles of various degrees of FW loss. It can be seen that if the muscle fibres had lost no more than 60% of their water they still maintained the normal membrane impermeability for inorganic phosphate, but further dehydration led to the loss of this property.

In one series of experiments ECW as "sucrose space" and FW were determined in the same muscle before drying and after rehydration. It can be seen in Figure 6 that complete recovery of FW was impossible on rehydration of the muscles in which its content had been reduced by more than 60%. This was apparently due to the membrane damage to large number of fibres followed by the penetration of sucrose and increase of "sucrose space".

The damaged membranes of completely dehydrated muscles enabled the proteins to diffuse out of the fibres during rehydration as it is shown in Fig. 7. The total amount of proteins flowing out of the fibres during 25 min. of immersion in the Ringer's solution amounted to about 11% of the total protein content of the muscles (see Table 2).

Figure 8 shows the changes in the excitability of the muscles which had been dried and rehydrated. It can be seen that the loss of about 60% of FW was the critical degree of dehydration of the muscle fibres. When such muscles were immersed in Ringer's solution, the fibres responded to electrical stimulation neither by developing tension nor by shortening. Spontaneous contraction appeared to be the greatest when the fibres had lost more than 90% of water prior to rehydration.

DISCUSSION

It was found in the present experiments that the contractile proteins and membrane system of the muscle fibres showed different threshold of sensitivity to water loss. The normal properties of the membrane system were changed when more than 60% of water was lost by the fibre; but it was necessary to dehydrate the muscles up to 90% to be able to show some distortion of the properties of contractile proteins.

It should be mentioned that there is only a seeming discrepancy between the present findings and those from an earlier paper (Dydyńska, 1966) because the critical loss of water was calculated previously as 70% from the total muscle (drained weight) which corresponds to 60% of water loss from the fibres of the muscle weighed after blotting (see Fig. 1).

In order to investigate the extractability of proteins from the muscles after drying and rehydration, it was necessary to find out the total protein content and partition in the frog muscle, which in contrast to those of rabbit muscles are not very well known so far.

It seems that the total protein content of the frog sartorii found as 15.2±3.5% of fresh muscle weight could be placed at the lowest level usually found in the rabbit muscle (Huxley & Hanson, 1957; Hanson & Lowy, 1963; Helander, 1957; Iwanow, 1968). From this quantity of proteins, 27% found as fraction I (extracted with solutions of low ionic strength) was about as much as found in rabbit muscles, and 24% as fraction III (resistant to extraction) was significantly more than the 10 - 15% postulated for rabbit muscles, but the whole collagen content of the muscles must be ascribed to this fraction. This rather rough estimation of protein partition showed that about half of the total proteins was extracted by solutions of high ionic strength, the fraction II. This fraction consists of proteins of the contractile system, such as actomyosin, and some other proteins associated with it, e.g. native tropomyosin. There are no detailed studies about myosin content in frog muscles except the indication in a paper by Seraydarian, Abbott & Williams (1961) that one may expect to find 40 - 50 mg. of myosin in 1 g. of the frog sartorii. The actin content of the muscles could be calculated somewhat more precisely from the estimations of firmly bound nucleotide. Earlier studies by Biró & Nagy (1955) showed that 0.38 μmole of inorganic phosphate was hydrolysed during 7 min. in 1 N-H_2SO_4 from a labile compound bound to 1 g. of the muscles. It was found recently by Dydyńska (1968) that 1 g. of frog sartorii extracted exhaustively by 50% glycerol contained 0.43±0.11 μmoles (S.D., n = 30) of a nucleotide which chromatographically was shown to be ADP. This amount was taken as a basis for calculation of the actin content in the frog muscles assuming, as it is generally accepted, that actin binds one nucleotide to its one globular monomer. Taking molecular weight of G-actin as 47 000 (Rees & Young, 1967) one can calculate its quantity in muscle as about 1.5 - 2.0 mg./100 mg. of the tissue. The content of total contractile proteins found in the present experiments was 7.2 mg. in 100 mg. of muscle (see Table 1). This value covers the calculated quantity of actin added to 4 - 5 mg. myosin, and there still remains about 1 mg. for proteins associated to actomyosin. Taking into account that the total protein content of the frog muscle is smaller than that of the rabbit, these data are reasonably close to those reported by Ebashi (1968) for rabbit muscles (mg./100 mg. muscle): 5.5 - 6.0 for myosin, about 2.0 for actin and 1.7 - 2.2 for associated proteins.

As shown in Table 1, protein partition of frog muscles does not change after muscle dehydration. After rehydration of dried muscles (Table 2) the changes are limited to the observed outflow into the Ringer's solution of some part of the proteins, probably those which are soluble at low ionic strength.

The splitting of ATP during contracture of rehydrated muscles demonstrated in the previous paper, seems to indicate that ATPase activity of the contractile system was preserved even in an almost completely dehydrated muscle. This was observed already by Hunt & Matheson (1958) who showed that actomyosin obtained from dried strips of bovine and fish meat exhibited ATPase activity. In the present experiments actomyosin extracted from rehydrated frog muscles showed no decrease of enzymic activity compared with that obtained from fresh muscles (Fig. 2). Analogous studies were undertaken by Biró & Dekany (1964) who investi-

gated ATPase activity of fresh and freeze-dried myosin, actomyosin and myofibrils and found a significant decrease in the activity of myosin only.

The finding that actomyosin was insensitive to EGTA when it was extracted from the muscles dehydrated to lose more than 90% of fibre water and then rehydrated (Figs. 3 and 4), was rather unexpected. Usually the tropomyosin and troponin complex, which plays the role of EGTA sensitizing factor, dissociates from actomyosin only after a long alkaline extraction at low ionic strength. It seems that in the case of completely dried muscles short homogenization in Ringer's solution caused the dissociation of this complex from actomyosin. The observation that actomyosin extracted directly with Weber-Edsall's solution from dried and rehydrated muscles behaved as natural actomyosin does seem to indicate that the loss of water did not cause the denaturation of EGTA sensitizing factor. The changes in the state of natural actomyosin from frog muscles conditioning the dissociation of the EGTA sensitizing factor need further examination.

The membrane system of the muscle cell seemed to be more sensitive to water loss than the contractile system. This is illustrated by the outflow of inorganic phosphate, usually not penetrating through the undamaged cell membranes (Fig. 5), by the drastic changes in sucrose penetration into the fibres (Fig. 6) and by the outflow of proteins from muscle fibres during rehydration of the dried muscles (Fig. 7). The propagation of the impulses, the fibre activity linked with the properties of the whole membrane system of muscle cell, appeared especially sensitive to the dehydration of the fibres. When the muscles had lost about 60% of their water, after rehydration they were no more excitable by electric stimulation. However, a higher degree of water loss from the fibres, prior to rehydration, was needed for maximum spontaneous contracture (Fig. 8). These facts could indicate the changes in the membranes, probably involving the transverse system as well as sarcoplasmic reticulum.

This seemed contradictory to our earlier experiments (Dydyńska & Wilkie, 1963) in which partial recovery of mechanical response was found after water loss from the fibres, induced by hypertonic sucrose-Ringer's solution, even to the degree of 75% of their initial content. This discrepancy may be due to the low temperature at which earlier experiments were run (0°) and the time of recovery being as long as 24 hr.

The studies with use of electron microscopy of the dried and rehydrated muscles (Wroniszewska, Dydyńska & Drabikowski, in press) have shown that damage to the membrane system of muscle cells was observed after rehydration of the muscles whose fibres had lost more than 60% of their water. The fine structure of myofibrils remained apparently unchanged in these conditions.

The content of water bound to the structure of the muscle cell has been widely discussed and A. V. Hill (1930) postulated 4% of the total muscle water as bound, what seemed to be confirmed by Pócsik (1967). It was concluded from physico-chemical experiments in which binding of water by macromolecules was investigated (Bull & Breese, 1968) that 1 g. of protein can bind as much as 0.3 g. of water.

Taking the mean value of fibre water in 100 mg. of muscle as 50 mg. and the protein content as 15 mg., the bound water could amount to 4.5 mg. which makes 9% of the FW content and 5.5% of the total water of the muscle. A considerable part of this bound water was represented by water hydrating the proteins, but as was shown by experiments presented above the loss of this part of water does not bring about drastic changes in protein properties. On the other hand, a certain definite amount of water involved in the structures of the membrane system of muscle cell seemed to be necessary for its normal activity. There are no data about water content of the membrane system of living cell but one finding concerning artificial membranes was recently reported. Lakshminarayanaiah (1967) found that polyethylene-styrene membranes need 59% water content for the optimum conditions of their permeability to cations.

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ZMIANY WŁASNOŚCI BIAŁEK KURCZLIWYCH I UKŁADU MEMBRANOWEGO SUSZONYCH I UWADNIANYCH MIĘŚNI ŻABY

Streszczenie

1. Wykazano, że aktomiozyna ekstrahowana z mięśni żaby suszonych i uwadnianych w płynie Ringera wykazuje aktywność ATP-atyczną na tym samym poziomie co aktomiozyna ze świeżych mięśni. Jednakże aktomiozyna otrzymana z mięśni kompletnie wysuszonych przed uwadnianiem utraciła tzw. „wrażliwość" na EGTA, czynnik chelatujący wapń.

2. Błona sarkoplazmatyczna i elementy układów kanalików wewnątrzkomórkowych ulegały prawdopodobnie uszkodzeniu, gdy komórka mięśniowa utraciła 60% wody. Wskazuje na to brak pobudliwości na bodziec elektryczny, dyfuzja białek i fosforanów nieorganicznych z komórek do płynu Ringera oraz powiększenie tzw. przestrzeni sacharozowej obserwowane podczas uwadniania mięśnia.

3. Wysuwa się przypuszczenie, że najbardziej wrażliwe na odwadnianie są układy błon, które tracą swe własności już gdy z komórki zostanie usunięte około 60% wody.

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THIOL GROUP CONTENT IN TROPOMYOSIN AND TROPONIN

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1. The number of thiol groups in tropomyosin has been determined with β-hydroxyethyl-2,4-dinitrophenyl disulphide under various conditions, leading to depolymerization or to dissociation of tropomyosin and preventing autooxidation. 2. In the absence of reducing agents the number of thiol groups in tropomyosin amounts at low and high ionic strength only to about 1 per 10^5 g. protein and increases only slightly in the presence of urea. 3. After treatment with dithiothreithol, followed by the removal of its excess on Sephadex G-25 columns, about 4 thiol groups per 10^5 g. protein in the presence of 0.6 M-KCl or of urea were found. 4. Thiol groups in troponin were also determined under analogous conditions. Troponin contains about 2.5, 3.5 and 6.0 thiol groups per 10^5 g. protein at low ionic strength, in the presence of 0.6 M-KCl, and urea, respectively. Treatment with dithiothreithol increases the number of thiol groups to about 6.0 per 10^5 g. at low ionic strength and to about 10.0 in the presence of urea.

It has been shown in our previous studies (Drabikowski & Nowak, 1965) that the number of free SH groups in crude tropomyosin preparations is much higher than that usually found in purified tropomyosin. Since the obtained value was close to the number of half-cystine residues determined by amino acid analysis, we came to the conclusion that in tropomyosin all SH groups were free and that only during repeated purification cycles the number of titratable SH groups diminished due to autooxidation. At the time our investigations were performed, nothing was known about troponin, a myofibrillar protein discovered by Ebashi & Kodama (1965), which was subsequently shown by Drabikowski, Dąbrowska & Nowak (1969a) to be present in crude tropomyosin prepared according to a classical procedure of Bailey (1948). Such preparations, being in fact complexes of tropomyosin and troponin, were found to contain a higher number of SH groups per unit weight than tropomyosin (Drabikowski *et al.*, 1969a). This observation might suggest that troponin contained many more SH groups than tropomyosin, and, hence, that the contamination by troponin could be the reason for relatively high SH groups content found previously by us in unpurified tropomyosin preparations. In view of this assumption it seemed necessary to reexamine the number

of SH groups in pure tropomyosin preparations under conditions preventing auto-oxidation, and this was one of the aims of the present study. Since only scarce and divergent data concerning the SH groups content in troponin have been reported (Ebashi, Kodama & Ebashi, 1968; Staprans, Arai & Watanabe, 1968; Yasui, Fuchs & Briggs, 1968) their number and reactivity was also determined.

Similarly to the previous studies (Drabikowski & Nowak, 1965) SH groups were determined with the use of HEDD[1].

MATERIAL AND METHODS

Tropomyosin and troponin were prepared from low ionic strength extract of rabbit muscle residue devoid of myosin (denoted in the text as preparation A). As the starting material, crude actin extracted at 25° (preparation B) or 1 M-KCl extract from ethanol-ether muscle powder (preparation C) were also used. Preparation C corresponded to the first step of preparation of tropomyosin according to the classical procedure of Bailey (1948) and was used in our previous studies (Drabikowski & Nowak, 1965). From all kinds of preparations the fractions salting out between 0.4 and 0.6 ammonium sulphate saturation were collected, dissolved in 1 M-KCl and adjusted to pH 4.6; under these conditions tropomyosin precipitated, whereas troponin remained in the supernatant. (For details of the whole procedure see Drabikowski *et al.*, 1969a). For purification of tropomyosin at least three cycles of repeated fractionation with ammonium sulphate between 0.5 and 0.6 saturation and isoelectric precipitation were employed.

All steps of preparation were carried out in the presence of 1 mM-β-mercaptoethanol.

For the determination of the number of SH groups, samples of protein were incubated with HEDD for two hours at room temperature under the required conditions (see below) and dinitrothiophenol liberated was measured in a Unicam Spectrophotometer S.P. 600 at 408 nm. Protein concentration was determined according to Gornall, Bardawill & David (1949), or according to Lowry, Rosebrough, Farr & Randall (1951).

DTT was purchased from Koch-Light Lab. Ltd (Colnbrook, Bucks, England).

RESULTS

As in our previous studies (Drabikowski & Nowak, 1965), the determination of SH groups in tropomyosin was performed under various conditions: at low ionic strength, in the presence of high concentration of KCl causing depolymerization of the aggregates of this protein, and in the presence of urea leading to dissociation of tropomyosin monomers into subunits (Woods, 1966; 1967). The results presented in Table 1 show that the number of SH groups in tropomyosin reacting with

[1] Abbreviations: HEDD, β-hydroxyethyl-2,4-dinitrophenyl disulphide; DTT, dithiothreithol.

Table 1

The content of SH groups in tropomyosin and troponin

For determination of SH groups, to one ml. (2 - 6 mg.) of protein (preincubated for 1 - 2 hr. at room temperature with either 1.8 M-KCl, 6 M-urea or 10 mM-tris-HCl, pH 7.5, alone), two ml. of 0.5 mM-HEDD in 2 mM-tris-HCl, pH 7.5, were added and after 2 hr. the extinction at 408 nm was measured. Samples preincubated with 0.5 mM-DTT (24 hr., 0°) were subsequently dialysed exhaustively against 10 mM-tris-HCl, pH 7.5, prior to the addition of KCl or urea. Tropomyosin and troponin were prepared as described in Methods.

Preparation	Reactive SH groups (equivalents per 10^5 g. protein)					
	direct measurement			after treatment with DTT		
	Tris-HCl	KCl	Urea	Tris-HCl	KCl	Urea
			Tropomyosin			
A	—	1.10	0.90	—	—	—
	—	0.60	1.03	—	—	—
	—	1.64	1.12	—	—	—
	—	2.11	2.16	—	—	—
	—	1.03	0.93	—	—	—
	0.96	1.46	1.20	—	—	—
B	1.12	—	2.46	1.78	—	3.01
C	0.84	0.97	1.98	1.05	1.71	2.85
	0.75	0.93	2.00	1.18	1.64	2.42
	1.09	1.54	2.78	1.58	—	3.04
	—	1.38	1.20	—	—	—
Average	0.95	1.27	1.61	1.40	1.68	2.83
			Troponin			
A	1.61	2.33	7.06	2.16	3.16	7.27
	3.58	4.55	6.93	3.78	4.74	7.87
	2.02	3.22	6.40	2.05	3.90	7.20
	3.26	4.75	6.54	2.77	5.04	6.28
	3.40	5.32	6.35	—	—	—
*	2.36	—	5.55	3.52	—	8.09
B	2.22	3.16	6.40	2.03	3.10	7.10
*	2.43	2.65	6.20	3.43	5.45	8.35
C	2.02	3.14	6.95	2.52	3.54	—
	1.63	3.93	6.80	1.60	4.00	7.00
	2.63	3.70	5.85	2.89	4.60	6.70
Average	2.46	3.67	6.27	2.67	4.17	7.32

* Preparations additionally purified on Sephadex G-200 column (Drabikowski *et al.*, 1969a).

HEDD at low ionic strength amounted only to about one per 10^5 g. protein. At high ionic strength this value was only slightly higher. Preincubation with 6 M-urea caused some increase of accessibility of SH groups, but not in all preparations. As it was shown previously (Drabikowski & Nowak, 1965) the reaction of tropomyosin

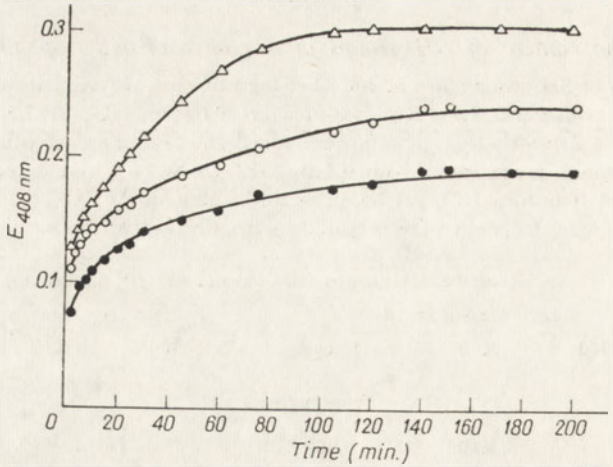

Fig. 1. Time-course of the reaction of HEDD with troponin. One ml. samples containing 4 mg. of protein were preincubated for 2 hr. at room temperature in 10 mM-tris-HCl pH 7.5 (●), alone, and with the addition of (○), 1.8 M-KCl or (△), 6 M-urea. Two ml. of 0.5 mM-HEDD in 2 mM-tris-HCl, pH 7.5, was added at zero time and extinction at 408 nm was measured.

SH groups with HEDD followed first order kinetics and velocity of the reaction was only slightly slower than that with β-mercaptoethanol or DTT.

The determination of SH groups of troponin was performed under the same conditions as in the case of tropomyosin. Fig. 1 shows the time-course of the reaction. The examination of the kinetics of the reaction seems to suggest that troponin contains only one class of thiol groups, which, however, react with HEDD much more slowly than those of low molecular weight thiol compounds (the apparent $t_{1/2}$ for troponin SH groups was 20 to 30 min., depending on the conditions).

Table 1 shows that neither the kind of preparation of troponin, nor the additional purification of this protein on Sephadex G-200 produced significant differences in the number of SH groups. On the other hand, the number of reacting SH groups varied slightly from preparation to preparation. On the average about 2.5 SH residues per 10^5 g. troponin reacted with HEDD at low ionic strength. The presence of 1 mM-ethylenediamine tetraacetate did not increase this number, indicating that the removal of bound Ca (Ebashi *et al.*, 1968; Yasui *et al.*, 1968; Drabikowski, Baryłko, Dąbrowska & Nowak, 1968) from troponin had no influence on the accessibility of SH groups. On the other hand, at high ionic strength the number of reacting SH groups increased to about 3.7 per 10^5 g. protein, whereas after the treatment with urea up to about 6.3 SH residues per 10^5 g. protein were found.

The number of SH groups in troponin and tropomyosin reacting under all conditions tested increased rather slightly following preincubation with 0.5 mM-DTT. This lack of significant effect of DTT could be due to the partial reoxidation of SH groups during prolonged dialysis employed for removal of the excess of DTT, especially in the case of tropomyosin where the tendency to autooxidation is well

known. To ensure that at the time of reaction with HEDD full reduction had taken place, in the next series of experiments another technique was adopted. After incubation of tropomyosin and troponin with much higher — 0.1 M — concentration of DTT, the excess of this reagent was removed from protein on Sephadex G-25 column (Fig. 2) and the SH groups content was immediately determined in the samples collected from the peak containing protein. In this case the reaction with DTT caused a significant increase of the number of SH groups, both in troponin and tropomyosin (Table 2). In the absence of urea about 6 SH equivalents, and in its presence about 10 SH equivalents per 10^5 g. protein was found in troponin. In

Table 2

The content of SH groups in tropomyosin and troponin preparations treated with 0.1 M-DTT

Samples of protein were incubated for 2 hr. at 0° with 0.1 M-DTT; subsequently KCl was added up to a concentration of 1.8 M or urea to 6 M, and the incubation proceeded for another 24 hr. at 0°. Control samples were incubated under the same conditions but without DTT. One to two ml. of solutions were loaded on Sephadex G-25 column equilibrated either with 0.6 M-KCl, 2 M-urea or 10 mM-tris-HCl, pH 7.5, and eluted with the same solvent. In the samples, protein and the content of SH groups were determined. The number of SH groups in the control samples (not shown in the Table) was close to that found by direct measurement (see Table 1).

Preparation	Reactive SH groups (equivalents per 10^5 g. protein)		
	Tris-HCl	KCl	Urea
Tropomyosin			
A	—	3.58	3.94
	—	4.70	4.84
	—	3.27	3.72
	—	3.74	4.17
	—	2.82	4.50
		(1.18)*	4.21*
C		4.05	4.10
Average		3.70	4.20
Troponin			
A	4.98	—	8.20
	—	—	10.30
	5.00	—	11.25
	5.71	—	8.55
	5.40	—	9.75
	8.90	—	11.00
	7.60	—	8.95
	6.25	—	9.57
Average	6.26	—	9.70

* Preparation of tropomyosin kept frozen for 6 months. The value in parenthesis was not used for calculation of the average.

tropomyosin the number of SH equivalents reacting with HEDD both at high ionic strength and after the treatment with urea amounted to about four. In this case no determinations were performed at low ionic strength since gel filtration technique could not be used due to a high viscosity of tropomyosin solutions.

DISCUSSION

The number of accessible SH groups in tropomyosin determined previously by various authors with the use of amperometric titration, or titration with *p*-chloromercuribenzoate, *N*-ethylmaleimide or HEDD was variable, ranging from 0 to 4 (usually about 3) SH residues per 10^5 g. protein (Kominz, Saad, Gladner & Laki,

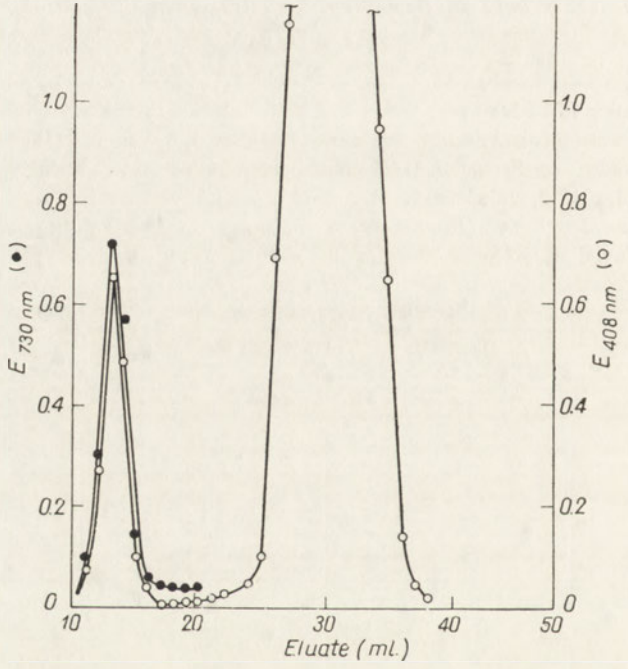

Fig. 2. Sephadex G-25 gel filtration of DTT-treated tropomyosin. One ml. (3 mg.) of tropomyosin was preincubated with 0.1 M-DTT and 6 M-urea for 24 hr. at 0°, then loaded on a Sephadex G-25 column (50×1 cm) equilibrated with 6 M-urea in 10 mM-tris-HCl, pH 7.5, and eluted with the same solvent. One ml. samples were collected in which (●), protein according to Lowry et al. (1951) and (○), SH group content with HEDD, were determined.

1957; Tan, Sun & Lin, 1958; Szent Györgyi & Benesch, 1959; Drabikowski & Nowak, 1965; Tsou, Lu & Tsao, 1966; Carsten, 1968). In full agreement with these observations the number of SH groups in tropomyosin preparations not treated with reducing agents was found in this work to be very small. On the other hand, the highest value obtained under the conditions favouring the reduction of all possible —S—S— bonds present accounted for about 4.5 SH residues per 10^5 g. tropomyosin.

It seems at present evident that much higher values of SH groups obtained previously (Drabikowski & Nowak, 1965) in unpurified tropomyosin and by Mueller (1966) in apparently pure tropomyosin were due to a troponin contamination. Since the latter protein contains much higher amount of SH groups per unit weight than tropomyosin, even its relatively small quantity considerably increases the determined value of the number of SH groups. On the other hand, the value of 10 SH residues per 10^5 g. protein found recently by Yasui *et al.* (1968) in troponin-free tropomyosin prepared in the presence of 0.1 M-DTT must be overestimated since it is even much higher than the reported values of half-cystine residues based on amino acid analysis. The simplest explanation of this result might be the assumption that in spite of precautions used by these authors some traces of remaining DTT were responsible for the obtained values.

The results of this paper show that the number of reactive SH groups in tropomyosin depends only slightly on ionic strength, which suggests that no SH groups become masked during polymerization of tropomyosin. However, some increase of the number of SH groups after the treatment with urea, even in the absence of DTT, may indicate that during autooxidation, besides formation of —S—S— bridges, some SH groups may become buried inside the random aggregates.The latter seem to be more easily deaggregated by urea than by high concentrations of KCl. On the other hand, after reduction of disulphide bridges all SH groups are easily accessible to HEDD even in the absence of urea.

Bailey (1948), Kominz, Hough, Symonds & Laki (1954) and Kominz *et al.* (1957) reported earlier that the number of half-cystine residues in rabbit skeletal muscle tropomyosin was about 6.5 moles per 10^5 g. protein; similar values were recently obtained by McCubbin, Kouba & Kay (1967) and by Carsten (1968) for bovine cardiac and sheep skeletal muscle tropomyosin. On the other hand, Jen, Hsü & Tsao (1965) and Carsten (1968) found only 3.4 and 2.7 half-cystine residues, respectively, per 10^5 g. in rabbit skeletal muscle tropomyosin. Also Woods (1969) found quite recently only 2.8 - 4.7 half-cystine residues per 10^5 g. tropomyosin obtained from muscles of various animals. Taking into account the value of 6.5 half-cystine residues per 10^5 g. one obtains 4.0 to about 4.5 SH groups per tropomyosin molecule, depending on molecular weight of this protein used for calculation. The lower reported values of half-cystine residues correspond, however, only to 2 - 3 SH residues per tropomyosin molecule. The latter value is similar to the amount of titratable SH groups found in this work in DTT-treated tropomyosin. On the basis of the difference between the value of the number of half-cystine residues and the number of titratable SH groups Szent Györgyi & Benesch (1959) postulated that native tropomyosin molecule had one disulphide bridge. Recent studies, however, indicate that two α-helical chains which form tropomyosin molecule by twisting about one another are not covalently linked by a —S—S— bond and that the tropomyosin molecule can be dissociated into subunits without a reducing agent (Holtzer, Clark & Lowey, 1965; Woods, 1966, 1967, 1969). On the other hand, a tendency of tropomyosin to form aggregates by random oxidation of SH groups is at present already well established, and this is the most probable reason for the

rather low values of free SH groups usually found in tropomyosin preparations. The differences in the obtained values for the number of half-cystine residues remain, however, to be elucidated. One cannot exclude the possibility that the higher values of the number of half-cystine in tropomyosin reported mainly in earlier papers were due to some troponin contamination. On the other hand, the odd number of SH groups per one tropomyosin molecule obtained from calculation may indicate the presence of a single SH group only in one of the tropomyosin chains, which, in turn, would indicate a non-identity of the two subunits (cf. Bodwell, 1967).

The data concerning the content of SH groups in troponin are very scarce. Staprans *et al.* (1968) and Ebashi *et al.* (1968) found up to about 5 SH groups per 10^5 g. of this protein reacting with *p*-chloromercuribenzoate. A similar value was found by Yasui *et al.* (1968) in troponin prepared in the absence of DTT. When during preparation 0.5 mM-DTT was present, the authors found about 12 SH groups per 10^5 g., whereas in troponin treated with 0.1 M-DTT as many as 33 groups per 10^5 g. protein were found. The latter value, however, seems also to be overestimated, similarly as in the case of tropomyosin. The values of SH groups in troponin prepared in the absence of DTT found in this work are similar to those obtained by Ebashi *et al.* (1968) and Staprans *et al.* (1968). Urea increases the number of accessible SH groups significantly, both in the absence and in the presence of DTT, up to the value of about 10 SH equivalents per 10^5 g. troponin in the latter case. Thus, the results of the present work clearly show that some of the SH groups in troponin are buried. It is interesting that in the presence of higher KCl concentration more SH groups react with HEDD, indicating that some conformational changes are induced by salt and not only by urea. The results also show that troponin contains some disulphide bridges, perhaps also formed as the result of autooxidation, which can be reduced by DTT. The reduction seems to be complete only in the presence of urea. However, the lack of determination of half-cystine residues in troponin preparations by amino acid analysis precludes any comparisons between the values obtained in this work and the total amount of half-cystine residues.

Practically nothing is known about the molecular properties of troponin, and, moreover, recent studies of Hartshorne, Theiner & Mueller (1969) and Drabikowski, Dąbrowska, Baryłko, Graeser & Gergely (1969b) indicate the heterogeneity of this protein. Although the determinations of the number and reactivity of SH groups in troponin had been started by us before this fact was known, it seemed useful to continue these studies since all fractions of troponin seem to be necessary for its specific function: in the complex with tropomyosin — to sensitize actomyosin to calcium ions. However, there is a discrepancy in the literature concerning the necessity of SH groups for this specific activity (comp. Ebashi *et al.*, 1968; Staprans *et al.*, 1968; Yasui *et al.*, 1968).

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ZAWARTOŚĆ GRUP TIOLOWYCH W TROPOMIOZYNIE I TROPONINIE

Streszczenie

1. Oznaczono ilość grup tiolowych w tropomiozynie przy użyciu dwusiarczku β-hydroksyetylo-2,4-dwunitrofenylowego (HEDD) w różnych warunkach prowadzących do depolimeryzacji lub do dysocjacji, oraz zapobiegających autooksydacji.

2. W nieobecności czynników redukujących, ilość grup tiolowych w tropomiozynie wynosi około jednej na 10^5 g białka zarówno w niskiej jak i w wysokiej sile jonowej i wzrasta jedynie nieznacznie w obecności mocznika.

3. Po działaniu dwutiotreitolu i usunięciu nadmiaru tego związku na kolumnach z Sephadex G-25 znaleziono około czterech grup tiolowych na 10^5 g tropomiozyny w obecności zarówno KCl jak i mocznika.

4. Grupy tiolowe troponiny oznaczono w analogicznych warunkach. Troponina zawiera około 2,5 grup SH na 10^5 g w niskiej sile jonowej, 3.5 w wysokiej sile jonowej, a 6.0 w obecności mocznika. Dwutiotreitol podwyższa ilość grup tiolowych do około 6.0 na 10^5 g w niskiej sile jonowej i do około 10.0 w obecności mocznika.

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FORMATION OF COMPLEXES OF THIOL AND AMINO COMPOUNDS AS STUDIED BY AMPEROMETRIC TITRATION AND INFRARED SPECTROMETRY

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1. The interaction between thiol compounds and some organic amines in ethanol--water solutions was studied. 2. The complex formation between cysteine, reduced glutathione and organic bases was demonstrated using amperometric titration and infrared spectrometry. 3. Possible bonds between the thiol and amino compounds, and their occurrence in some unreactive SH groups in proteins, are discussed.

In the course of studies on the internal structure of proteins, the presence of unreactive, or masked, SH groups has been postulated (Anson & Mirsky, 1931); the unreactivity of these groups has been ascribed to steric hindrance preventing the access of the thiol reagent to the interior of the native protein molecule (Cecil & McPhee, 1959). By the use of amperometric titration, it has been demonstrated that all SH groups of proteins form mercaptides, but at different rates; the terms slow- and fast-reacting SH groups have been then introduced (Benesch, Lardy & Benesch, 1956).

The differences in the reaction velocity of protein SH groups have given rise to many hypotheses. Among others, it has been postulated that a thiazoline ring is formed within the protein molecule in the reaction of the thiol with the carbonyl group of the peptide linkage (Linderstrøm-Lang & Jacobson, 1941), or that thiol esters (Smith, 1958) are formed. These assumptions, however, are not supported by the experimental results; mild factors such as surface-active agents or lowering of pH to about 3.5, liberate unreactive thiol groups, but cannot be held responsible for the disruption of covalent bonds involved in formation of this internal linkage (Cecil & Thomas, 1965). It has also been suggested that SH groups form hydrogen bonds with free amino groups (Cecil, 1950; Benesch, Benesch & Rogers, 1954; De Deken, Brockhuysen, Bechet & Mortier, 1956) but this view has not been substantiated by later studies (Wright, 1956; Edsall, 1963; Cecil & Thomas, 1965).

Cecil & Thomas (1965), who studied the reactivity of SH groups in haemoglobin, found that protein SH groups in the presence of low concentration of alcohol be-

come unmasked without causing any changes in the spectrum and optical rotatory dispersion, whereas chemical modification of SH groups leads to changes in configuration and to dissociation of the protein into subunits. Similar observations were made in a study on the denaturation of haemoglobin with propanol (Konieczny & Domański, 1964). These results seems to imply that SH groups are involved in some type of intramolecular non-covalent bonds, i.e. in hydrophobic interaction with aliphatic non-polar amino acid residues, as suggested by Cecil (1963) and Cecil & Thomas (1965).

To study the character of these bonds, experiments were carried out on the ability of thiol group to react with amino acids and other organic amines (imidazole, benzidine, aniline, ethylenediamine and *p*-aminophenol). Formation of complexes between cysteine, reduced glutathione or cysteamine, and amino compounds was studied by amperometric titration and, in some cases, also by infrared spectrometry in ethanol-water solution.

MATERIALS AND METHODS

Reagents: L(+)Cysteine was a product of R.C.B. (Bruxelles, Belgium); glutathione, reduced, of Schuchardt (München, West Germany); cysteamine·HCl (chem. pur.) and DL-histidine (puriss.) of Fluka AG. (Buchs S. G., Switzerland); DL-arginine, DL-serine, L-glutamine of Reanal (Budapest, Hungary); DL-lysine and tris of Loba Chemie (Wien, Austria); L(+)valine of F. Hoffmann-La Roche (Basle, Switzerland); imidazole of Light (Colnbrook, Bucks., England); $AgNO_3$ of E. Merck (Darmstadt, West Germany); benzidine, aniline and *p*-aminophenol of Fabryka Odczynników Chemicznych (Gliwice, Poland); ethanol 96% "Analar" of Łódzkie Zakłady Spirytusowe (Łódź, Poland). All chemicals used were of analytical grade or as indicated.

The reaction mixture. A saturated solution in ethanol or water of the amino compound studied was added, with vigorous stirring, to 1 ml. of 1 mM solution of cysteine, reduced glutathione or cysteamine in 95% ethanol. The amino compound was added at a 10- to 1000-fold molar excess with respect to the thiol compound. Through all solutions before mixing nitrogen was bubbled carefully to remove oxygen and traces of CO_2, and the samples were kept in N_2 atmosphere throughout the experiment. After 15 min. incubation at room temperature, 0.1 ml. of the mixture was diluted with ethanol to a volume of 10 ml. then 2 ml. of 4 M-tris-HNO_3 buffer solution, pH 7.4, was added, and free SH groups were titrated with 1 mM-$AgNO_3$, using rotating platinum electrode. It should be emphasized that the time of incubation and the order in which the reagents are mixed, are critical for complex formation, e.g. tris-HNO_3 buffer should not be added prior to mixing of the thiol compound with the amino compound.

Amperometric titration of SH groups was carried out according to Benesch *et al.* (1956) using a rotating platinum electrode (the apparatus was constructed in our Department).

Infrared spectrometry. The spectra were taken in UR-10 infrared spectrophotometer (Zeiss, Jena, VEB, German Democratic Republic). In all experiments, standard As_2S_3 cuvettes of light-path 0.05 mm. were used.

RESULTS AND DISCUSSION

Amperometric titration of thiol compounds in the presence of various organic bases. Under the above described conditions, the determination of SH groups in cysteine was not affected by glycine, alanine, valine, leucine, isoleucine, serine, threonine, aspartate, glutamate, asparagine, glutamine, methionine, cystine, phenylalanine, tyrosine, tryptophan, histidine, proline, hydroxyproline, citrulline, aniline and *p*-aminophenol. On the other hand, arginine, lysine (applied in the free form and not as hydrochlorides), imidazole and benzidine completely masked all the titratable SH groups, i.e. those capable of binding the Ag^+ ions complexed with tris (Fig. 1). In the absence of tris, titration of cysteine gave the theoretical value indicating that no oxidation of SH groups occurred during the experiment.

Glutathione was not affected by arginine, lysine, imidazole and benzidine, whereas ethylenediamine blocked the SH groups (Fig. 2), and also in the absence of tris all SH groups were titratable. It should be noted that titration of cysteamine (free or cysteamine hydrochloride) under the same conditions showed only unreactive SH groups, which was possibly due to formation of an inner hydrogen bridge between thiol and $-NH_2$ group.

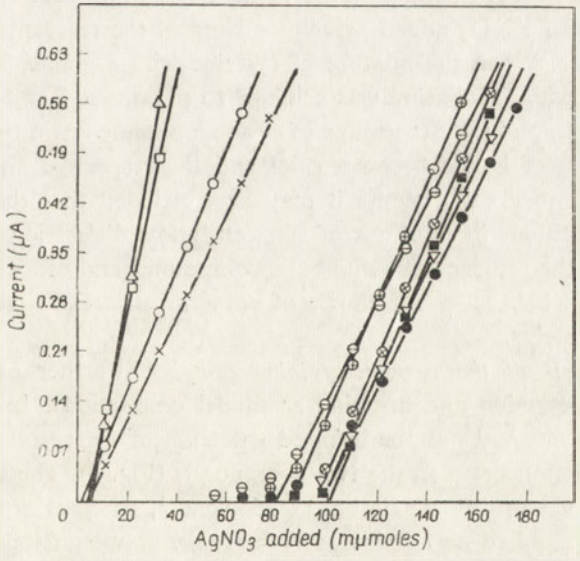

Fig. 1. Amperometric titration of cysteine (1×10^{-7} moles) in the presence of different amino compounds (1×10^{-5} moles): (○), arginine; (□), lysine; (△), imidazole; (×), benzidine; (●), serine; (■), histidine; (▽), valine; (⊗), glutamine, (⊖), aniline; (⊕), *p*-aminophenol. The samples were incubated for 15 min. at room temperature and then, prior to titration with Ag^+ ions, tris-HNO_3 buffer, pH 7.4, and ethanol were added to final concentration 0.6 M and 86.7% vol., respectively.

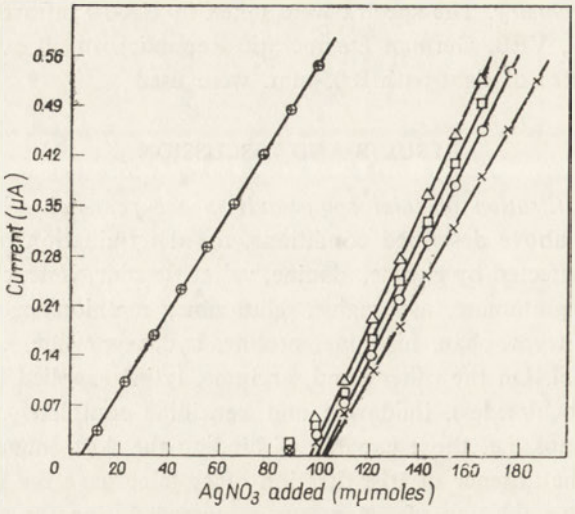

Fig. 2. Titration of glutathione (1×10^{-7} moles) in the presence of: (○), arginine; (□), lysine; (△), imidazole; (×), benzidine; (⊕), ethylenediamine. All bases were in the final concentration of 1×10^{-5} moles. Other conditions as in Fig. 1.

The masking of SH groups of cysteine by the effective amino compounds was not influenced by changes of pH over the range from 6 to 9, when solutions of low ionic strength were used. In the presence of high salt concentration, i.e. saturation of the solution with KNO_3 added prior to mixing of the reactants, all SH groups remained titratable. When the mixture of cysteine with arginine, lysine, imidazole or benzidine following incubation was acidified to pH about 1 and urea was added to 8 M concentration, all the SH groups of cysteine became again titratable (Fig. 3), indicating splitting of bonds between thiol and the respective amino compound.

From the described experiments it may be concluded that the SH groups of cysteine and glutathione in solutions of sufficiently low dielectric constant and low ionic strength, in the presence of some basic compounds and of a complexing agent for Ag^+ ion (tris), behave in the course of amperometric titration like unreactive SH groups of proteins.

Spectral analysis of the cysteine-arginine complex. Further experiments were carried out with cysteine and arginine as model compounds. In the UV region no changes were observed but the infrared spectrum of the reaction product indicated some interaction between the two compounds (Fig. 4). The spectrum of cysteine showed an absorption maximum at 2560 cm^{-1}, whereas arginine under the same conditions exhibited no maximum in the range between 2400 and 2700 cm^{-1}. The ethanolic solution of the mixture of cysteine and arginine incubated for 15 min. had an absorption maximum at about 2500 cm^{-1}. Such a change in the absorption maximum corresponds to formation of a hydrogen bond, as it was observed by Gordy & Stanford (1940) in the reaction of aromatic thiols with amines. When the mixture of cysteine and arginine was analysed after a short time of incubation

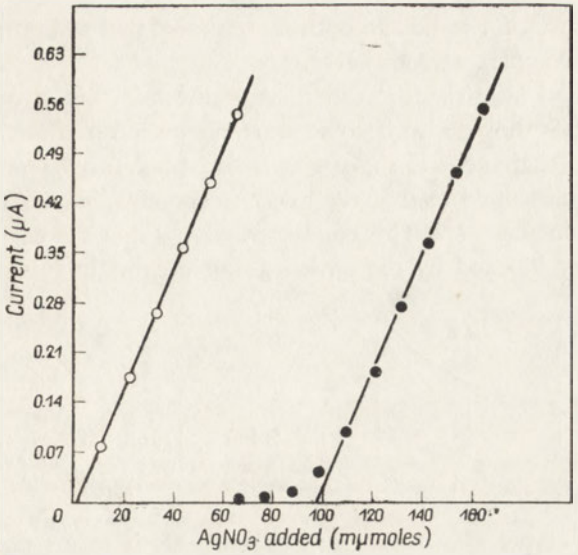

Fig. 3. Titration of cysteine-arginine complex (○), before and (●), after incubation (10 min.) at pH 1.0 in 8 M-urea solution and brought to pH 7.4 with tris. The sample contained 1×10^{-7} moles of cysteine, 1×10^{-5} moles of arginine and 86.7% vol. of ethanol.

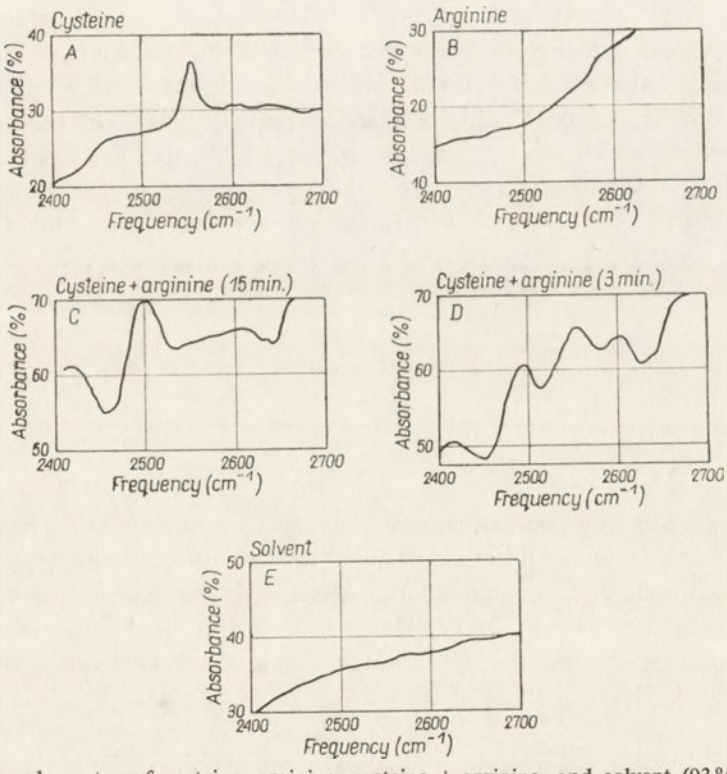

Fig. 4. Infrared spectra of cysteine, arginine, cysteine + arginine, and solvent (93% ethanol in water). Concentrations of the amino acids were: cysteine 4×10^{-3} M, arginine 1×10^{-2} M. Cysteine + arginine were incubated in *C* for 15 min. and in *D* for 3 min. Following incubation, spectra were recorded at 0.02 mm. light-path, except in *E* (0.2 mm.).

(2 - 3 min.), the spectrum contained both maxima, i.e. that of cysteine at 2560 cm^{-1} and that of the complex at 2500 cm^{-1}.

As it was found by titrimetric experiments, the thiol group of cysteamine was unreactive, and this should also become apparent on infrared analysis. Cysteamine alone showed no characteristic sharp absorption peak for SH group (Fig. 5) but a rather wide band shifted toward the lower frequencies, similar to that found for the cysteine-arginine complex. This confirms the view that the thiol group of cysteamine is probably blocked by the amino group present in this compound.

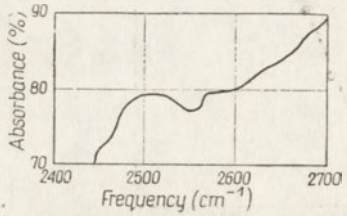

Fig. 5. Infrared spectrum of cysteamine alone. The spectrum was recorded from the KBr pellet.

On the basis of the above described experiments it seems possible to suggest that the low reactivity of at least a part of the unreactive thiol groups in proteins may be due to chemical interaction with ε-amino groups of lysine and guanidine groups of arginine residues in the hydrophobic regions of the polypeptides. This suggestion finds support in the results of other workers, concerning the so-called thiol salts, especially those of aromatic thiols with amines, which have the character of complexes with relatively strong hydrogen bonds (Gordy & Standford, 1940; Grillot & Brooks, 1950; Oswald, Noel & Stephenson, 1961). On the other hand, aliphatic thiols, which are less acid, nevertheless form also hydrogen bonds with alkylamines (Oswald *et al.*, 1961). The shift of hydrogen to the proton-acceptor enhances polarity of the system and formation of a fairly strong hydrogen bond, SH → N:

$$\overset{\delta+}{R_3N} \ldots \overset{\delta-}{H\text{-}SH} \rightleftharpoons \overset{+}{R_3NH} + \overset{-}{SR}$$

The formation of this anion is probably responsible for the ready oxidation of complexes in air giving rise to disulphides and regeneration of amine (Oswald & Wallace, 1966):

$$4\,R_3N \ldots HSR + O_2 \rightarrow 2\,RSSR + 2\,H_2O + 4\,R_3N$$

This may explain why our preliminary attempts to demonstrate the cysteine-arginine complex in solution by other methods (e.g. partition chromatography, high-voltage electrophoresis, extraction with organic solvents) have not been successful.

When considering the above postulated hydrogen bridge binding the SH group with the amino group, the possibility of formation of bonds of salt type should be also envisaged. The hypothesis of the occurrence of hydrogen bonds is supported, in addition to the results of infrared analysis, also by other observations. At low pH values, at which SH groups are unreactive, the cysteine thiol group, as demonstrated by Benesch & Benesch (1955), is not dissociated; consequently, under these conditions the bonding of the salt type cannot be formed. The low dielectric con-

stant of the medium used for experiments does not favour the dissociation of SH group. Moreover, the occurrence of the hydrogen bond between the atoms of nitrogen and sulphur has been recently demonstrated in crystalline phase by the roentgenographic method (Donohue, 1969).

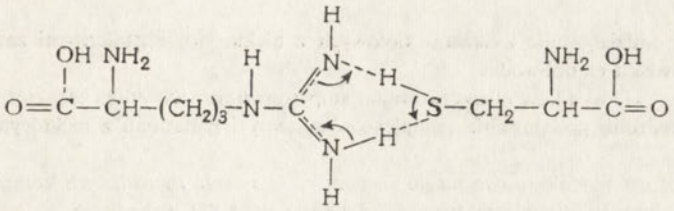

Fig. 6. Proposed mechanism of hydrogen bonding in cysteine-arginine complex in hydrophobic conditions, i.e. in the absence of solvating agent.

Virden & Watts (1966) who studied the role of SH groups in the mechanism of action of arginine kinase, also postulated an interaction of the SH groups essential for enzymic activity with the guanidine groups of arginine. Another possible mechanism of interaction between SH groups of cysteine and guanidine group of arginine is presented in Fig. 6, which shows hydrogen bond formation by both nitrogen atoms of guanidine residues. As it may be seen from the experiments presented above, our results support the possibility of this type of bonds in proteins, and of their formation in reactions involving catalytic activity of some enzymes.

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AMPEROMETRYCZNE I SPEKTROFOTOMETRYCZNE BADANIA NAD TWORZENIEM KOMPLEKSU POMIĘDZY ZWIĄZKAMI TIOLOWYMI I AMINOWYMI

Streszczenie

1. Badano oddziaływanie związków tiolowych z niektórymi organicznymi zasadami aminowymi w środowisku etanol-woda.

2. Na podstawie wyników miareczkowania amperometrycznego oraz charakteru widm w podczerwieni stwierdzono powstawanie kompleksów cysteiny i glutationu z niektórymi spośród badanych amin.

3. W związku z postulowanym mechanizmem powstawania omawianych kompleksów, dyskutowano przyczyny zjawiska niereaktywności pewnych grup SH w białkach.

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EFFECT OF AMYL AZIDE ON RESPIRATION AND OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

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1. Amyl azide strongly inhibits state 3 respiration of rat liver mitochondria with NAD-linked substrates (50% inhibition at about 3×10^{-5} M), is much less inhibitory to respiration with succinate (50% inhibition at 3×10^{-3} M) and at concentrations up to 3×10^{-3} M has virtually no inhibitory effect on the oxidation of ascorbate + + tetramethyl-*p*-phenylenediamine. Amyl azide strongly inhibits the reduction of mitochondrial ubiquinone by NADH and has no effect on the reduction by succinate. 2. Amyl azide increases state 4 oxidation of succinate and ascorbate + tetramethyl--*p*-phenylenediamine. It also partly abolishes the inhibition of state 3 oxidation of succinate by oligomycin. 3. Amyl azide at millimolar concentration decreases P : O ratio with various substrates by a value of 0.5 to 1 and strongly stimulates mitochondrial ATPase. 4. It is concluded that at low concentrations amyl azide preferentially inhibits electron transport between NADH and ubiquinone and uncouples energy conservation, most likely at sites 2 and 3 or at site 3 only.

The mechanism of action of inorganic azides on energy metabolism of mitochondria has been critically re-examined during the last few years by several authors (Bogucka & Wojtczak, 1966; Wilson & Chance, 1966, 1967; Wilson, 1967; Palmieri & Klingenberg, 1967; Vigers & Ziegler, 1968; for references to earlier literature see Bogucka & Wojtczak, 1966). According to our results (Bogucka & Wojtczak, 1966) and those of Wilson & Chance (1966, 1967) azide directly interferes with the energy-coupling system of mitochondria, independently of its inhibitory effect on cytochrome oxidase (EC 1.9.3.1). Contrary to this, Palmieri & Klingenberg (1967) postulated that the apparent effect of sodium azide on the energy-coupling system could be entirely explained on the basis of its effect on cytochrome oxidase and the ability of mitochondria to accumulate azide by an energy-dependent mechanism. Zvyagilskaya, Bogucka & Wojtczak (1969) have been, in fact, able to demonstrate directly the energy-dependent accumulation of azide in rat liver mitochondria, but have shown that some of its effects on mitochondrial metabolism may also indicate a direct action on the energy coupling, viz. the uncoupling effect and the inhibition of energy transport (an oligomycin-like effect), as postulated before (Bogucka & Wojtczak, 1966).

In order to investigate further the action of N_3 moiety on respiration and energy metabolism of mitochondria we studied the effect of organic derivatives of azide. The present investigation concerns the action of amyl azide $CH_3(CH_2)_4N_3$. Toxic effects of this substance are well known. Werle & Fried (1952) have demonstrated its bacteriostatic properties and its action on blood pressure.

In preliminary experiments we have found that amyl azide, in contrast to inorganic azides, does not inhibit cytochrome oxidase. Therefore its effect on energy coupling and on other parts of the respiratory chain should not be obscured by the inhibition of the terminal step of electron transport, as is the case with inorganic azide. The present paper describes the effect of amyl azide on energy metabolism of mitochondria and shows that they differ from those of inorganic azide.

MATERIAL AND METHODS

Rat liver mitochondria were obtained by the procedure described by Hogeboom (1955) and rat heart mitochondria according to Szarkowska & Klingenberg (1963).

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

Esterification of inorganic phosphate was determined isotopically (Nielsen & Lehninger, 1955).

ATPase activity was determined by measuring the formation of inorganic phosphate by the procedure of Fiske & Subbarow (1925).

Reduction of ubiquinone was measured by the procedure described by Pumphrey & Redfearn (1960).

Protein was determined by the biuret method (Gornall, Bardawill & David, 1949) after solubilizing mitochondria with deoxycholate.

Amyl azide was obtained by heating amyl iodide with silver azide as described by Werle & Fried (1952). The product was extracted with ethyl ether and purified by distillation (b.p. 121° - 130°). Amyl azide was added to the test systems as ethanolic solution. Final concentration of ethanol in incubation media did not exceed 1% which was shown to have no effect on the processes under investigation.

Chemicals: Hexokinase (from yeast, types III and IV), β-hydroxybutyrate, oligomycin and ubiquinone-30 were from Sigma (St. Louis, Mo., U.S.A.); ATP, ADP and NADH from Boehringer and Sons (Mannheim, West Germany); ascorbic acid and amyl iodide from Polskie Odczynniki Chemiczne (Gliwice, Poland); and succinate from British Drug Houses (Poole, England). Other chemicals were of analytical grade. Carrier-free [^{32}P]phosphoric acid was purchased from the Institute of Nuclear Research (Świerk, Poland).

RESULTS

Effect of amyl azide on mitochondrial respiration. The effect of amyl azide on the oxidation of various substrates in two different metabolic states of mitochondria is shown in Fig. 1. It is evident that in the active state (state 3 according to Chance & Williams, 1956) amyl azide had practically no effect on the oxidation of ascor-

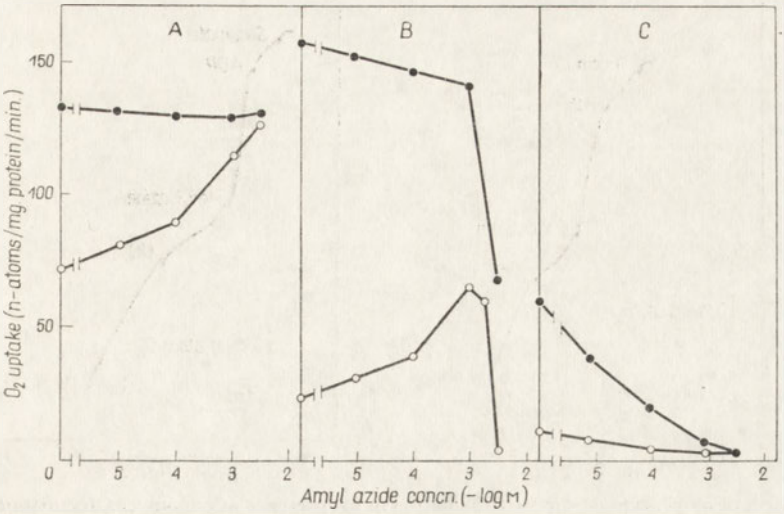

Fig. 1. Effect of amyl azide on the respiration of rat liver mitochondria: (○), in state 4, and (●), in state 3 (+ 1 mM-ADP). Incubation medium: 70 mM-KCl, 5 - 10 mM-sucrose, 6 mM-$MgCl_2$, 2.5 mM-EDTA, 10 mM-Na,K-phosphate, 32 mM-tris-HCl (pH 7.4); other additions: *A*, 6.7 mM-ascorbate + 0.3 mM-TMPD + 1 μM-rotenone; *B*, 6.7 mM-succinate + 1 μM-rotenone; *C*, 6.7 mM-β-hydroxybutyrate; total volume was 3.0 ml.; mitochondria: 4 - 6 mg. protein in *A* and *B*, 6 - 8 mg. protein in *C*. Temperature 30°.

bate + TMPD[1] over the whole concentration range studied, i.e. up to 3 mM (higher concentrations could not be used because of low solubility of amyl azide in water). With succinate as substrate, the oxidation was only negligibly inhibited by concentrations up to 1 mM, but when the concentration was increased to 3 mM the inhibition amounted to 60%. The oxidation of β-hydroxybutyrate and other NAD-linked substrates (not shown in Fig. 1) was partly inhibited already by 10^{-5} M amyl azide and the degree of inhibition strongly increased with increasing concentration, being over 90% at 1 mM amyl azide. A similar inhibition was also obtained in the uncoupled state (not shown).

In the controlled state (state 4 according to Chance & Williams, 1956) amyl azide increased the oxidation of ascorbate + TMPD. In concentrations up to 1 mM it also increased the oxidation of succinate, while was inhibitory at higher concentrations. Contrary to this, the oxidation of β-hydroxybutyrate was inhibited by all concentrations tested (Fig. 1).

Amyl azide at 1 mM concentration partly abolished the inhibition by oligomycin of the state 3 oxidation of succinate (Fig. 2). This effect was similar to that produced by typical uncouplers.

The data shown in Figs. 1 and 2 suggest a dual effect of amyl azide: (1) an uncoupling effect on sites 2 and/or 3 of the energy-coupling system, and (2) an inhibition of NAD-linked oxidation and, at higher concentrations, of succinate oxi-

[1] Abbreviations: TMPD, tetramethyl-*p*-phenylenediamine; DNP, 2,4-dinitrophenol.

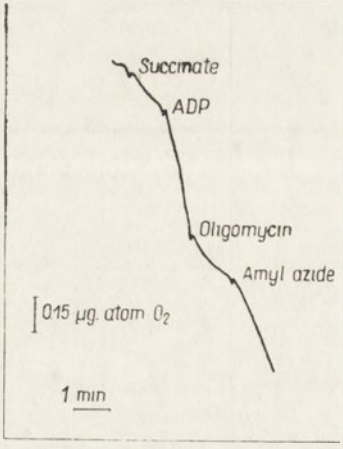

Fig. 2

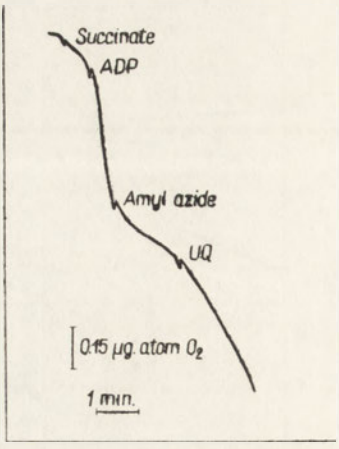

Fig. 3

Fig. 2. Effect of amyl azide on succinate oxidation in the presence of oligomycin. Incubation medium and conditions as in Fig. 1B. Additions: mitochondria, 7.2 mg. protein; succinate, 6.7 mM; ADP, 1 mM; oligomycin, 8 μg.; amyl azide 1 mM.

Fig. 3. Effect of ubiquinone on succinate oxidation inhibited by amyl azide. Incubation medium and conditions as in Fig. 1B. Additions: mitochondria, 6.9 mg. protein; succinate, 6.7 mM; ADP, 1 mM; amyl azide, 3 mM; ubiquinone-30 (UQ), 0.8 μmole/mg. protein.

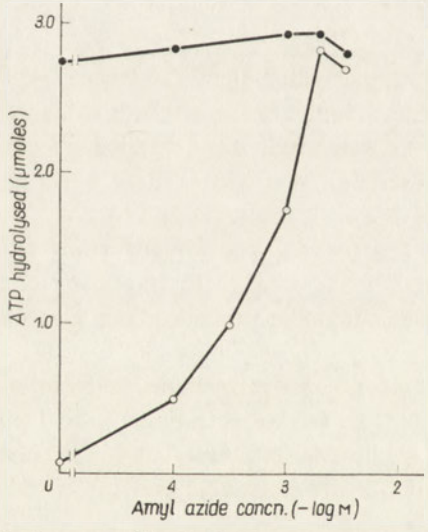

Fig. 4. Effect of amyl azide on mitochondrial ATPase: (○), latent and (●), stimulated by 0.1 mM-DNP. Incubation medium: 70 mM-KCl, 12.5 mM-sucrose, 3 mM-$MgCl_2$, 1 mM-EDTA, 4 mM-ATP, 40 mM-tris-HCl (pH 7.4), and 3.3 mg. mitochondrial protein. Total volume was 1.0 ml., temperature 20°, incubation time 15 min.

dation as well. The inhibition of succinate oxidation could be partly released by high amounts of ubiquinone (Fig. 3).

Effect of amyl azide on oxidative phosphorylation and ATPase. From Table 1 it is evident that 1 mM-amyl azide decreases P : O ratio with both succinate and ascorbate + TMPD as respiratory substrates. Phosphorylation accompanying oxidation of NAD-linked substrates could not be precisely measured because of the strong inhibition of this oxidation by amyl azide. Nevertheless, a decrease of P : O ratio by a value of unity was also noted.

Table 1

Effect of amyl azide on oxidative phosphorylation

Incubation medium as in Fig. 1A and B, respectively, except that it also contained 20 mM-glucose, 200 Kunitz-McDonald (1945 - 1946) units of hexokinase and [^{32}P]phosphate equivalent to 6×10^5 counts/min.; mitochondria 4.8 mg. protein, temperature 30°, incubation time 3 min.

Addition	Substrate	
	Ascorbate + TMPD P : O	Succinate P : O
None, control	0.9	1.8
Amyl azide, 1 mM	0.4	0.9

Table 2

Effect of amyl azide on the reduction of endogenous ubiquinone by NADH and succinate in rat liver mitochondria

Incubation medium: 10 mM-sucrose, 1 mM-KCN and 20 mM-P_i (pH 7.2). Incubation time 6 min.; temperature 20°.

Additions	Oxidized ubiquinone (nmoles/mg. protein)
None	1.86
Amyl azide, 5 mM	1.92
NADH, 5 mM	0.82
NADH, 5 mM; amyl azide, 2 mM	1.76
Succinate, 5 mM	0.56
Succinate, 5 mM; amyl azide, 5 mM	0.52

Table 3

Effect of amyl azide on the reduction of endogenous ubiquinone by NADH and succinate in rat heart mitochondria

Incubation medium: 300 mM-sucrose, 1 mM-EDTA and 20 mM-triethanolamine hydrochloride (pH 7.2). Incubation time 3 min.; temperature 20°.

Additions	Oxidized ubiquinone (nmoles/mg. protein)	
	Expt. 1	Expt. 2
None	3.30	2.35
KCN, 1 mM	2.89	2.30
KCN, 1 mM; NADH, 5 mM	1.10	
KCN, 1 mM; NADH, 5 mM; amyl azide, 2 mM	2.38	
KCN, 1 mM; succinate, 5 mM		0.40
KCN, 1 mM; succinate, 5 mM; amyl azide, 2 mM		0.30
Succinate, 5 mM		1.06
Succinate, 5 mM; amyl azide, 2 mM		2.20

Mitochondrial ATPase was strongly stimulated by amyl azide (Fig. 4). Maximum stimulation, attaining the degree of the stimulation produced by 2,4-dinitrophenol, was obtained at concentration 2 mM, equal to that maximally stimulating state 4 oxidation of succinate. The DNP-stimulated ATPase was not affected by amyl azide at any concentration tested.

Effect of amyl azide on redox state of mitochondrial ubiquinone. This is shown in Tables 2 and 3. It is known (cf. Szarkowska & Klingenberg, 1963) that an appreciable reduction of endogenous mitochondrial ubiquinone by NAD-linked substrates can be obtained only under conditions of terminally inhibited electron transport. Therefore, potassium cyanide was present throughout in the experiment shown in Table 2 and in some experiments of Table 3.

It is evident that 2 mM-amyl azide decreased the reduction of ubiquinone by NADH (i.e. it abolished the decrease in the amount of oxidized ubiquinone produced by NADH) in both rat liver (Table 2) and rat heart mitochondria (Table 3), indicating an inhibition of electron flow between these two coenzymes. Contrary to this, the reduction of ubiquinone by succinate was not affected. In the absence of cyanide when a steady state level of oxidized ubiquinone during succinate oxidation could be measured, amyl azide produced a dramatic increase of the oxidized form of ubiquinone, almost to the values obtained in the absence of succinate (Table 3). As in the presence of cyanide no inhibition of ubiquinone reduction by succinate could be observed, this increase in the oxidized steady state can be interpreted as activation of the reoxidation of ubiquinone. This is compatible with the uncoupling effect of amyl azide. In fact, a similar effect on the redox state of mitochondrial ubiquinone could be obtained with DNP (not shown).

DISCUSSION

Chemical properties of azide are greatly altered by substituting hydrogen atom of the free acid HN_3 by an alkyl radical. Parallel to this, the effects on energy metabolism of mitochondria are also changed. Apart from differences in chemical properties between inorganic azide and its alkyl derivatives, e.g. chelating ability, these compounds also differ in their hydrophilic vs. lipophilic properties. Amyl azide, being strongly lipophilic, probably penetrates more easily into the lipoprotein membrane of the mitochondrion than does inorganic azide whose penetration is facilitated only by an ion pump (Palmieri & Klingenberg, 1967).

On the basis of the present investigation the following effects of amyl azide can be postulated: (1) inhibition of electron transport between NADH and ubiquinone; (2) inhibition, by high concentrations of amyl azide, of electron transport between succinate and cytochrome *c*, this inhibition being partially abolished by added ubiquinone; and (3) uncoupling of energy conservation. The exact site(s) of uncoupling is difficult to determine. On the basis of the decrease of P : O ratio by a value of unity with succinate (Table 1) and β-hydroxybutyrate (not shown) and by 0.5 with ascorbate + TMPD (Table 1) it can be supposed that sites 2 and 3, or possibly

site 3 only, are affected. Thus, the effects of amyl azide strongly differ from those of inorganic azide which uncouples preferentially at site 1 (Bogucka & Wojtczak, 1966) and has no inhibitory effect on the respiratory chain below cytochrome oxidase. Also contrary to inorganic azide, amyl azide does not inhibit cytochrome oxidase and has no "oligomycin-like" effect on the energy-coupling system.

The mechanism of action of amyl azide on mitochondrial oxidation and phosphorylation processes is not clear. One may speculate that the inhibition of electron transport at the level of NADH dehydrogenase might be due to an interaction with non-haem iron, this interaction being facilitated by the lipophilic character of the inhibitor. On the other hand, there is a striking similarity between the effects of amyl azide and those of piericidin A (Kosaka & Ishikawa, 1968; Vallin & Löw, 1968). Also similarly to the inhibition by piericidin A (Hall *et al.*, 1966), the inhibition of succinate oxidation by amyl azide can be partly reversed by added ubiquinone. The mechanism of the action of piericidin A on mitochondrial electron transport and energy conservation is, however, not clear (cf. Vallin & Löw, 1969).

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WPŁYW AZYDKU AMYLU NA PROCESY ODDECHOWE I OKSYDACYJNĄ FOSFORYLACJĘ W MITOCHONDRIACH

Streszczenie

1. Azydek amylu silnie hamuje oddychanie mitochondriów wątroby szczura w stanie 3 z substratami zależnymi of NAD (50% hamowania przy stężeniu 3×10^{-5} M), znacznie słabiej działa na utlenianie bursztynianu (50% hamowania przy 3×10^{-3} M) i nie działa, w stężeniach do 3×10^{-3} M, na utlenianie askorbinianu + czterometylo-*p*-fenylenodwuamina. Azydek amylu silnie hamuje redukcję mitochondrialnego ubichinonu przez NADH, a nie wpływa na redukcję przez bursztynian.

2. Azydek amylu wzmaga utlenianie bursztynianu i askorbinianu + czterometylo-*p*-fenylenodwuamina w stanie 4. Znosi również częściowo hamowanie przez oligomycynę utleniania bursztynianu w stanie 3.

3. Azydek amylu obniża stosunek P : O z różnymi substratami o wartość 0.5 do 1 i silnie stymuluje mitochondrialną ATP-azę.

4. Uzyskane wyniki pozwalają przypuszczać, że azydek amylu wybiórczo hamuje transport elektronów między NADH a ubichinonem i rozprzęga oksydacyjną fosforylację, prawdopodobnie na drugim i trzecim, a być może tylko na trzecim miejscu sprzężenia.

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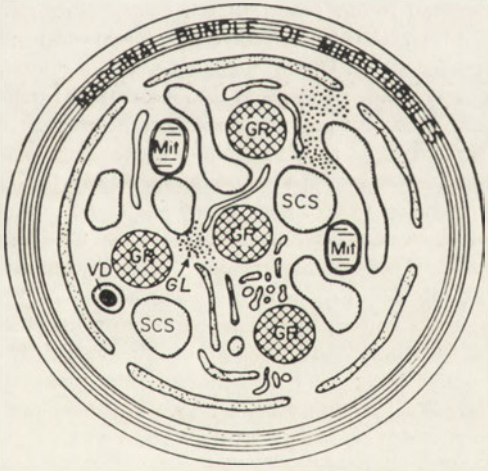
RECENZJE KSIĄŻEK

METABOLISM AND MEMBRANE PERMEABILITY OF ERYTHROCYTES AND THROMBOCYTES (E. Deutsch, E. Gerlach & K. Moser, eds.) G. Thieme Verlag, Stuttgart 1968; str. 479, rys. 407, tablic 101; cena 110,— DM.

W dniach od 17 do 20 czerwca 1968 r. w Wiedniu odbyło się pierwsze międzynarodowe sympozjum poświęcone metabolizmowi oraz zagadnieniom strukturalnym i przepuszczalności membran czerwonych ciałek krwi i trombocytów. Niniejsza książka zawiera właśnie wszystkie wykłady, komunikaty i dyskusje z tego sympozjum.

Rozwój metod biochemicznych i biofizycznych oraz badań klinicznych spowodował znaczne nagromadzenie najrozmaitszych faktów, dotyczących zarówno fizjologicznych jak i patologicznych procesów w elementach morfotycznych krwi. Procesy metaboliczne toczące się wewnątrz komórki są uwarunkowane pasywnym i aktywnym transportem przez membrany, przeto oba te zagadnienia potraktowano łącznie przy układaniu programu sympozjum. Podczas sympozjum wygłoszono blisko 100 referatów dotyczących metabolizmu erytrocytów i trombocytów prawidłowych i patologicznych, struktury membran obu typów komórek oraz przepuszczalności tych membran dla różnych jonów i substratów. Należy podkreślić, że w sympozjum brali również czynny udział biochemicy z Warszawy i Białegostoku.



Schemat ultrastruktury trombocytu krwi ludzkiej. Mit, mitochondria; GR, ziarnistości α; VD, ciałka o dużej gęstości właściwej; GL, ziarna glikogenu; SCS, system połączeń z powierzchnią płytki.

Pierwszy rozdział książki poświęcony jest mechanizmom regulacyjnym przemian energetycznych i innych w erytrocycie. Otwiera go referat S. Rapoporta i G. Jacobascha z Berlina, a następnie szereg autorów omawia procesy glikolizy, cykl fosfotriozowy, system reduktazy methemoglobinowej i glutationu oraz proces syntezy hemu, jak również pomniejsze zagadnienia. W drugim rozdziale

najpierw H. D. Waller z Tybingi klasyfikuje defekty enzymatyczne w erytrocytach, które bardziej szczegółowo przedstawiono w kolejnych doniesieniach. Defekty enzymatyczne w erytrocytach stwierdza się w zakresie glikolizy beztlenowej, w zakresie redukcji i syntezy glutationu oraz w innych procesach, które zostały omówione w kilkunastu referatach. Następny rozdział o przemianach normalnych i patologicznych trombocytów otwiera referat G. W. Löhra (Marburg), w którym i w następnych referatach omówiono strukturę (por. Rysunek), skład chemiczny oraz niektóre przemiany w płytkach krwi. Poruszono m. in. mechanizm uwalniania serotoniny z płytek pod wpływem różnych farmaceutyków, własności i funkcje trombosteniny — kurczliwego białka płytek spełniającego ważną rolę w retrakcji skrzepu i tworzeniu agregatu trombocytu. Kilka doniesień traktuje również o roli trombocytów w procesie krzepnięcia krwi w ogóle. Ostatnie dwa rozdziały obejmujące 27 doniesień poświęcone są strukturze i przepuszczalności membrany erytrocytów. Najbardziej wyczerpująco zagadnienia strukturalne omówili G. Uhlenbruck i G. J. Pardoe (Kolonia, Birmingham). Następnie M. D. Poulik (Detroit) przedstawił metody izolowania i charakterystykę rozpuszczalnych białek membran erytrocytów, a A. Rothstein (Rochester, N.Y.) przedstawił najnowsze badania nad ich przepuszczalnością. Pozostałe referaty dobrze uzupełniają szczegółami powyższe tematy.

W sumie książka bardzo ciekawa, doskonale edytorsko opracowana i wydrukowana, zawiera wiele najnowszych osiągnięć dotyczących metabolizmu i struktury membran tych elementów morfotycznych krwi, które są odpowiedzialne za transport gazów, krzepnięcie i szereg innych funkcji interesujących biochemika, fizjologa i klinicystę.

Włodzimierz Ostrowski

STRUKTURELLE GRUNDLAGEN DER BIOLOGISCHEN FUNKTION DER PROTEINE (T. Dévényi, P. Elödi, T. Keleti & G. Szabolcsi, eds.) Akadémiai Kiadó, Budapest 1969; str. 735.

Książka węgierskich autorów, wydana w języku niemieckim, tylko częściowo odpowiada swoją treścią bardzo ogólnemu tytułowi. Wiadomo wprawdzie, że dopiero kilkanaście lat temu zostały otworzone możliwości badania prawdziwych strukturalnych podstaw biologicznej funkcji białek; stało się to dzięki opracowaniu metod pozwalających na poznanie pierwszorzędowej struktury białek, a potem także konformacji łańcucha polipeptydowego, jego wtórnego pofałdowania i budowy cząsteczek białkowych z podjednostek. Olbrzymi postęp, jaki osiągnięto w ciągu tych kilkunastu lat, pozwolił nie tylko na zapoczątkowanie wglądu w molekularny mechanizm reakcji enzymatycznych, ale także w mechanizm skurczu mięśnia, w biologiczne znaczenie kolagenowych struktur tkanki łącznej, czy — w skromnym na razie zakresie — w chemiczne podstawy zjawisk immunologicznych. Omawiana książka przedstawia strukturalne podstawy biologicznej funkcji białek jedynie na przykładzie enzymów. Jest to więc nowoczesny podręcznik enzymologii ogólnej, nie traktujący wprawdzie ani o systematyce i nomenklaturze enzymów, ani też o układach wieloenzymowych, ale poszerzony o dwa rozdziały poświęcone metodom badania struktury białek.

Książka rozpoczyna się rozdziałem na temat pierwszorzędowej struktury białek globularnych, w którym pobieżnie omówiono najważniejsze i najbardziej owocne metody badania sekwencji aminokwasów oraz przykłady wyników badania tej sekwencji. Przykłady dobrano w taki sposób, że pozwoliły one na zwrócenie uwagi na powszechność kodu genetycznego i na filogenetyczne aspekty badania pierwszorzędowej struktury białka. Tym zagadnieniom poświęcony jest osobny rozdział (czwarty), napisany bardzo ciekawie przez Pál Elödi'ego a zatytułowany: „Wykształcenie gatunkowych własności białek w toku ewolucji". Wydaje się, że ten właśnie rozdział przydaje szczególnej wartości książce; pozwoli on bowiem upowszechnić coraz wyraźniej zarysowującą się dążność współczesnej biologii do oparcia zarówno taksonomii żywych ustrojów, jak i różnych rozważań filogenetycznych i ontogenetycznych, na zdobyczach genetyki molekularnej i chemii białek.

Drugi rozdział książki jest zatytułowany „Przestrzenna struktura białek globularnych". Pál Elödi podaje w nim przykłady konformacji łańcucha polipeptydowego oraz dość obszerny przegląd metod stosowanych współcześnie do badania tej konformacji. Trzeci rozdział jest poświęcony kinetyce enzymów, mechanizmowi ich działania oraz aktywnemu centrum. Bardzo liczne przykłady

ilustrujące zagadnienia omawiane w tym rozdziale dotyczą prawie wyłącznie NAD-zależnych dehydrogenaz. Wspomniany już rozdział czwarty dotyczy biochemii ewolucyjnej białek, ostatni zaś rozdział piąty nosi tytuł: „O strukturalnych podstawach działania enzymów". Omówiono w nim głównie wpływ chemicznych modyfikacji cząsteczki białka na działanie enzymów oraz modyfikujący wpływ ligandów na cząsteczkę enzymu, kończąc omówieniem zjawiska allosterii. W krótki i przystępny sposób przedstawiono w tym rozdziale zarówno teorię Changeux jak i model Koshlanda zjawiska allosterii enzymów.

Każdy z pięciu rozdziałów zakończony jest obszernym wykazem piśmiennictwa, a na końcu dzieła umieszczono alfabetyczny spis nazwisk cytowanych autorów i alfabetyczny skorowidz rzeczowy. Stanowi to dodatkowy walor książki, która dzięki tym obszernym wykazom i skorowidzom może służyć jako podręczne dzieło do wyszukiwania obszerniejszych źródeł szczegółowych informacji.

Pomimo że książka napisana jest przez czterech autorów, nie zauważa się niejednolitości ujęcia. Liczne schematy i tablice ułatwiają czytanie i ilustrują omawiane doświadczenia.

Jak pisze we wstępie profesor Bruno F. Straub, omawiana książka przeznaczona jest „dla tych biologów, chemików i fizyków, którzy z dzisiejszego stanu enzymologii chcą czerpać nie tylko uproszczony materiał do nauki, ale upatrywać w niej chcą bliskie życiu bogactwo wiedzy i bieg rozwoju myśli". Wynika też z książki, że przyszłość enzymologii jest jeszcze bogatsza aniżeli jej stan obecny; słuszne jest więc stwierdzenie, że fizjologia i jej stosowane gałęzie: nauki medyczne i rolnicze, będą coraz obficiej czerpały materiał do swojego praktycznego działania z bogactwa odkryć enzymologicznych.

Mariusz Żydowo

Maria Wollemann, METABOLISME DES MÉDIATEURS CHIMIQES DU SYSTEME NERVEUX. Masson et Cie, Paris; Akadémiai Kiadó, Budapest 1970. Str. 162.

Omawianą książkę wstępem poprzedził prof. H. Laborit, współtwórca sztucznej hibernacji, przedstawiając w ciepłych słowach autorkę, która przez pewien czas pracowała pod jego kierunkiem w Paryżu nad mechanizmem działania fenotiazyn. Temat ten stanowi jeden z lepszych rozdziałów książki.

Monografia w podtytule informuje, iż jest bieżącym przeglądem wiedzy fizjologiczno-patologicznej na temat tzw. neurohormonów lub mediatorów układu nerwowego.

Po wstępie historycznym, informującym o etapach odkryć związków chemicznych i reakcji biochemicznych występujących w tkance nerwowej oraz definiującym pojęcia przekaźnictwa w układzie nerwowym centralnym i obwodowym oraz rolę mediatorów chemicznych, w pierwszej części książki autorka omawia kolejno:

1) acetylocholinę, jej biosyntezę, inne estry choliny, cholinesterazy, ich aktywność i lokalizację, izoenzymy oraz rolę acetylocholiny w układzie nerwowym;
2) katecholaminy, ich syntezę, lokalizację, uwalnianie i hamowanie oraz pokrótce — ich rolę w neuronach i zwojach;
3) serotoninę (zaledwie 4 strony);
4) kwas gamma amino-masłowy (GABA) i jego hydroksypochodne — jako przykład przenośników w układzie hamowania nerwowego;
5) ATP, histaminę i substancję P (kilka stron na temat roli i znaczenia tych związków).

Pierwszą część kończy rozdział poświęcony omówieniu mechanizmu działania fenotiazyn oraz ich wpływu na metabolizm niektórych neurohormonów. Część drugą stanowi omówienie metabolizmu mediatorów w warunkach patologicznych, a więc padaczce, guzach mózgu, chorobie Parkinsona, miasteniach różnego rodzaju oraz niektórych chorobach psychicznych. Jakkolwiek autorka nie wykracza tu poza naukowe truizmy i powołuje się na nienajświeższe piśmiennictwo (734 pozycje), istotną wartość stanowią tablice, zawierające wartości liczbowe poziomów acetylocholiny i jej pochodnych, aktywności cholinesteraz, fosfatazy kwaśnej, dehydrogenazy mlekowej i innych enzymów w różnych przypadkach guzów mózgu.

Rozdział ten ilustrują doskonałe elektroforogramy frakcji białkowych poszczególnych enzymów i izoenzymów na amidonie oraz histogramy szeregu interesujących przypadków nietypowych zmian w centralnym układzie nerwowym. Natomiast zmiany poziomów katecholamin i serotoniny dotyczące guzów układu nerwowego poruszono dosłownie w kilkunastu wierszach.

Niewiele więcej miejsca poświęcono aktywności niektórych enzymów i poziomom neurohormonów występujących w innych chorobach układu nerwowego ze schizofrenią włącznie.

Omówienie i wnioski drugiej części książki nie wykraczają poza granice przeciętnych wiadomości lekarzy lub biochemików zainteresowanych czynnością układu nerwowego. I gdyby nie rozdział dotyczący wpływu fenotiazyn na mechanizmy hamowania niektórych reakcji enzymatycznych w pierwszej części książki oraz szereg interesujących własnych danych dotyczących poziomu i aktywności pochodnych acetylocholiny, przypadków klinicznych w części drugiej — całość stanowiłaby przykład książki bez adresata: na monografię ciekawych przypadków własnych — za mało danych klinicznych, zaś na wstęp biochemiczny dla lekarzy specjalizujących się w histochemii — zbyt wiele ogólników, bez szczegółowego piśmiennictwa dotyczącego metodyk stosowanych w biochemii klinicznej.

Janusz Wysokowski

B.J. Haywood, ELECTROPHORESIS, TECHNICAL APPLICATIONS. Ann Arbor-Humphrey Science Publishers, Inc., Ann Arbor-London 1969; str. 440.

Elektroforeza zwłaszcza w nośnikach stałych znalazła w ostatnich dwu dziesiątkach lat szerokie zastosowanie w badaniach biochemicznych, klinicznych i w dyscyplinach technicznych. Można ją stosować do rozdziału w celach analitycznych i preparatywnych, jonów prostych i substancji wielkocząsteczkowych, oraz różnych elementów morfotycznych z komórkami włącznie. Elektroforeza stosowana jest jako metoda do rozdziału mieszanin na skalę przemysłową, oraz jako ultramikrometoda przy frakcjonowaniu i ilościowym określaniu substancji w granicach 10^{-12} g.

Z tak różnorodnym i powszechnym zastosowaniem wiąże się też ogromna ilość publikacji na temat różnych technik elektroforetycznych, które w ostatnich latach się ukazały, jak również licznych sympozjów i konferencji poświęconych teorii, zagadnieniom metodycznym i zastosowaniu tej powszechnie użytecznej metody fizyko-chemicznej. Jedną z cenniejszych i oryginalnych publikacji w tym zakresie jest książka B. J. Haywooda. Składa się ona z dwóch części: w pierwszej autor przedstawił streszczenia prac o tematyce elektroforetycznej, które ukazały się w latach 1965—1969; w drugiej — wyselekcjonowano streszczenia tych prac, począwszy od roku 1955, które według autora mają znaczenie podstawowe w różnych technikach elektroforetycznych. Abstrakty w obu częściach książki dotyczą prawie wszystkich stosowanych obecnie technik elektroforetycznych (elektroforeza bibułowa, wysokonapięciowa, kolumnowa, elektroforeza w roztworach swobodnych, w gradiencie gęstości, izoelektrycznego zogniskowania, cienkowarstwowa, elektroforeza w żelu agarowym, poliakrylamidowym, skrobiowym, na folii z octanu celulozy, w stałych blokach, immunoelektroforeza i inne) i są tak przygotowane, że eksperyment można powtórzyć względnie dostosować do własnych potrzeb nie koniecznie sięgając do oryginalnych publikacji.

W pierwszej części książki autor referuje ok. 1100 pozycji, w drugiej ok. 600, przy czym cennym jest to, że znaczna część tych pozycji pochodzi z trudno u nas dostępnych, wydawanych na różnych kontynentach, czasopism specjalistycznych z zakresu medycyny, dyscyplin biologicznych i technicznych.

Poza tym, że w tekście spotyka się sporo pomyłek w pisowni nazwisk autorów nie anglosaskich, książka odznacza się bardzo przyjemną szatą graficzną i jest przejrzyście wydrukowana. W sumie, stanowi cenną pozycję w bibliotece „Elektroforeza".

Włodzimierz Ostrowski