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# D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM HUMAN MUSCLE

## SH GROUPS AND INHIBITORS

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1. D-Glyceraldehyde-3-phosphate dehydrogenase from human muscle was found to contain 12 SH groups, of which 4 - 5 reacted rapidly, within 15 min., with *p*-chloromercuribenzoate (PCMB) while the remaining groups reacted very slowly. 2. The enzyme was inactivated by 3 equivalents of PCMB, iodoacetate, tetrathionate or $Ag^+$, by 4 equiv. of $Cu^{2+}$ or by 100 equiv. of $Zn^{2+}$. 3. In the presence of up to 10 equiv. of PCMB, after a longer time of incubation, the enzyme underwent partial spontaneous reactivation. 4. The enzyme from human muscle was found to differ from that of rabbit muscle in its electrophoretic mobility and rate of the reaction of SH groups with inhibitors.

Since the isolation of D-glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate : NAD oxidoreductase (phosphorylating), EC 1.2.1.12) in crystalline form from rabbit muscle (Cori, Slein & Cori, 1948) the enzymic activity has been known to depend on the condition of SH groups. GAPD[1] obtained by earlier methods required activation by incubation with cysteine or mercaptoethanol (see Velick, 1955). By isolating the enzyme in the presence of EDTA, Krimsky & Racker (1952) obtained preparations active without incubation with cysteine. These preparations were used for determination of the number of SH groups and their relation to the enzymic activity. Depending on the method of determination, 9 - 14 SH groups per mole have been found (Segal & Boyer, 1953; Murdock & Koeppe, 1964; Allison & Kaplan, 1964; Cecil & Ferdinand, 1965); the greater part of SH groups reacted with PCMB rapidly, within 15 min., whereas 2 - 3 groups reacted slowly and the reaction was complete within 1 hr. Urea had no effect on the number of SH groups determined. It was also found that at least 3 SH groups were involved in the enzymic reaction, as 3 equiv. of a thiol reagent inactivated the enzyme (Krimsky & Racker, 1952; Velick, 1953; Pihl & Lange, 1962).

[1] Abbreviations used: GAPD, D-glyceraldehyde-3-phosphate dehydrogenase; G3P, D-glyceraldehyde-3-phosphate; PCMB, *p*-chloromercuribenzoate.

In the previous work, GAPD has been isolated from human muscle (Baranowski & Wolny, 1963), and some of its properties determined. In the present work, molecular weight of the enzyme, the isoelectric point, number of SH groups, and the effect of thiol reagents and heavy-metal ions on the enzymic activity, are reported.

## MATERIALS AND METHODS

*Materials.* D-Glyceraldehyde-3-phosphate dehydrogenase from human muscle (12-72 hr. after death) was obtained as described previously (Baranowski & Wolny, 1963), and from rabbit muscle according to Beisenherz *et al.* (1953). Both enzymes were crystalline and homogeneous on free-boundary electrophoresis. Their activity, determined according to Beisenherz *et al.* (1953) but without adding cysteine, was about 60 μmoles of substrate/mg./min.

D-Glyceraldehyde-3-phosphate (G3P) was prepared according to Szewczuk, Wolny, Wolny & Baranowski (1961); the purity of the preparations determined enzymically and by phosphorus assay, was on the average 80%, and the content of inorganic phosphate did not exceed 0.15%. To obtain such a low contamination by inorganic phosphate it was necessary to use for the synthesis a preparation of fructose-1,6-diphosphate free of inorganic phosphate. The calcium salt of G3P stored at -20° was quite stable; after storage for 5 years the content of G3P decreased from 80% to 66% and simultaneously the content of inorganic phosphate rose from 0.05 to 0.42 μmole/mg.

NAD was a commercial preparation of Boehringer & Söhne (Mannheim, Germany). Iodoacetic acid was recrystallized three times and the colourless preparation was used. *p*-Chloromercuribenzoate (Sigma Chem. Co., St. Louis, Mo., U.S.A.) was purified and assayed according to Boyer (1954). $Na_2S_4O_6$ was obtained and determined according to Chinard & Hellerman (1954). Urea was purified in the following way: 1 g. of Dowex 50 ($H^+$) was added to 100 ml. of a urea solution saturated at 50°, the mixture stirred at 50° for 30 min., the resin filtered off, and the solution cooled. The crystallized urea was dried, recrystallized from hot bidistilled methanol, and dried for 72 hr. at 37°. Triethanolamine was distilled under vacuum (5 mm. Hg) at 195° (Beisenherz *et al.*, 1953); for determination of inhibition by heavy-metal ions it was purified additionally after Milstein (1961) in the following modification: Dowex 50 ($H^+$ form) in a column of 10×80 mm., was converted into the triethanolamine form by passing 50 ml. of 0.2 M-triethanolamine buffer, pH 8.75. Then 0.05 M-buffer, pH 8.70, was passed; the first 20 ml. was discarded, and the further portions contained no traces of heavy metals. $Na_2HAsO_4$ (R.A.) was recrystallized from 2 mM-EDTA solution. All solutions to be used for activity determinations were checked for the absence of heavy metals by shaking the aqueous solutions at pH 7.7 - 7.8 with 5 μM-dithizone in chloroform.

*Analytical proceaure.* The activity of the enzyme was determined as described by Baranowski & Wolny (1963) without adding cysteine, unless otherwise indicated in the text.

Protein was determined spectrophotometrically at 280 mμ. The extinction coefficient was calculated from the extinction of a protein solution dialysed against water or buffer, which was then evaporated and dried to constant weight at 105°. $E_{280}$ was found to be 1.02 $cm.^{-1} mg.^{-1}$.

SH groups were determined according to Boyer (1954). The millimolar extinction coefficient for the enzyme-PCMB complex at 255 mμ was found to be 7.0 $cm.^{-1}$. It was obtained by measuring in 0.33 M-acetate buffer, pH 4.6, the maximum change in extinction at 255 mμ resulting from the addition of increasing amounts of enzyme to a constant amount of PCMB. The plot of $E_{255}$ against enzyme concentration was linear up to the point where there were equivalent concentrations of the thiol groups of the enzyme and PCMB.

Free-boundary electrophoresis and determination of the diffusion coefficients were carried out at 4° in a Fokal B (Strubin & Co, Switzerland) instrument.

The isoionic point was determined according to Eastoe & Courts (1953) by measuring the pH of a protein solution deionized with a mixture of Amberlite IR 120 ($H^+$) and IRA 400 ($OH^-$). The protein, about 10 mg./ml., was submitted previously to exhaustive dialysis against water.

All spectrophotometric measurements were carried out in a Jobin-Yvon (France) instrument.

## RESULTS

*Physico-chemical data.* The diffusion coefficient ($D_{20}$) of GAPD from human muscle was found to be $5.45 \times 10^{-7}$ $cm.^2 sec.^{-1}$. The effect of protein concentration on $D_{20}$ is shown in Fig. 1. Since both the diffusion coefficient and the previously determined (Baranowski & Wolny, 1963) sedimentation coefficient $S_{20,w}$ $7.7 \times 10^{-13}$ $sec.^{-1}$, were in good agreement with the values reported for GAPD from rabbit muscle (Fox & Dandliker, 1956; Elias, Garbe & Lamprecht, 1960), the partial specific volume of the human muscle enzyme was accepted after Fox & Dandliker (1956) to be 0.725 $cm.^3/g$. The molecular weight calculated according to the equation of Svedberg, $M_{S,D}$, was 125 000.

Determinations of mobility in free-boundary electrophoresis demonstrated that the isoelectric point varied markedly with the kind of buffer used. In citrate buffer of ionic strength 0.1 the isoelectric point was 4.85 whereas in phosphate buffer, $I$ 0.1, it was 5.80 (Fig. 2). The isoelectric point of GAPD from rabbit muscle in phosphate buffer, $I$ 0.1, determined as a control, was 6.55, in agreement with the value obtained by Velick & Hayes (1953). When a mixture of crystalline preparations of GAPD from muscles of man and rabbit was subjected to electrophoresis in phosphate buffer, $I$ 0.1, pH 6.25, two fractions were obtained with a mobility, respectively, of $-0.75 \times 10^{-5}$ $cm.^2/v.sec.$ and $+0.58 \times 10^{-5}$ $cm.^2/v.sec.$ (Fig. 3). Additionally, determinations of the isoionic point for the enzymes were carried out. Concentrated solutions of GAPD from muscles of man and rabbit, after deionization with mixed ion exchangers, showed identical pH values of 8.30.

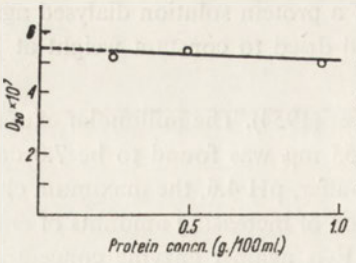


Fig. 1

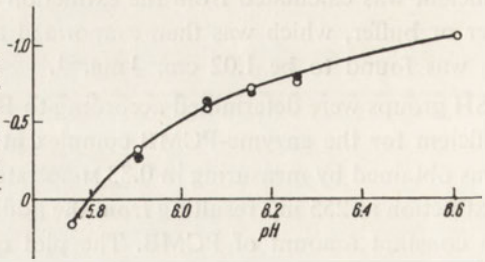


Fig. 2

Fig. 1. The effect of protein concentration on the diffusion coefficient of D-glyceraldehyde-3-phosphate dehydrogenase.

Fig. 2. Electrophoretic mobility of D-glyceraldehyde-3-phosphate dehydrogenase from human muscle as a function of pH, in phosphate buffer, $I$ 0.1. (○), Descending menisci; (●), ascending menisci.

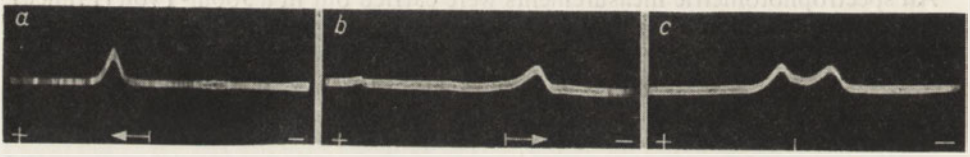


Fig. 3. Electrophoretic pattern of crystalline preparations of D-glyceraldehyde-3-phosphate dehydrogenase, in phosphate buffer, pH 6.25, $I$ 0.1, time of electrophoresis 120 min. (*a*), Enzyme from human muscle; voltage gradient 5.8 v/cm., protein concn. 0.8%; (*b*), enzyme from rabbit muscle; 8.4 v/cm., protein concn. 0.8%; (*c*), mixture of equal amounts of GAPD from human and rabbit muscles; 8.0 v/cm., total protein concn. 1.2%.

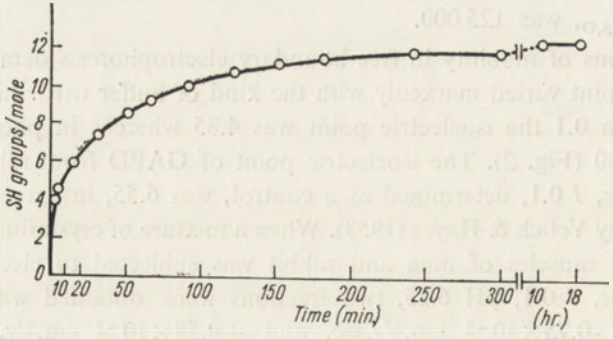


Fig. 4. Time-course of the reaction of D-glyceraldehyde-3-phosphate dehydrogenase from human muscle with PCMB in 0.33 M-acetate buffer, pH 4.6; content of protein 0.011 μmole, PCMB 0.165 μmole, in a final volume of 3.0 ml. Extinction of the enzyme-PCMB complex was measured at 255 mμ.

*Determination of SH groups.* GAPD from rabbit muscle reacted rapidly with PCMB in 0.33 M-acetate buffer, pH 4.6, in agreement with the data of other workers (Koeppe, Boyer & Stuhlberg, 1956; Allison & Kaplan, 1964), and within 5 min. 11 - 12 SH groups per 120 000 g. were titrated. On the other hand, the enzyme from human muscle reacted very slowly, and it was found impossible to accelerate the reaction by changing the pH value or the buffer used. In 0.33 M-acetate buffer, pH 4.6, in which the reaction was relatively fast, 4 - 5 SH groups reacted within 5 min. whereas the completion of the reaction was observed only after 10 hr. (Fig. 4). On the average, 12 SH groups per 125 000 g. were found. Often after a few hours a turbidity appeared which interfered with further measurements, similarly as it had been found by Allison & Kaplan (1964) for GAPD from human heart muscle and muscles of some animals. Urea at a concentration of 8 M accelerated markedly the reaction which was complete within 15 min., the final result being the same.

The enzyme-substrate complex obtained by incubation with an excess of G3P (100 equiv.) and dialysed to remove non-bound G3P, while retaining full enzymic activity, appeared to have 1.8 - 2.0 fewer SH groups than the control sample.

*Inhibition by PCMB.* Inhibition by PCMB of activity of human muscle GAPD (Fig. 5) corresponded to the values for the rabbit muscle enzyme (Velick, 1953; Pihl & Lange, 1962), and was complete with about 3 equiv. of PCMB. Inactivation at pH 7.4 was rapid also at protein concentrations up to 1 mg./ml., and after 5 min. incubation at room temperature it was stable for some time. On prolonged incubation,

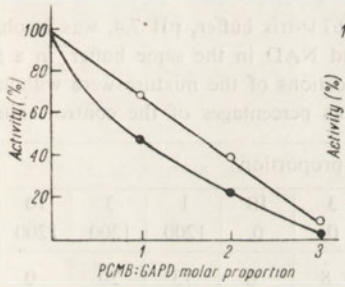


Fig. 5

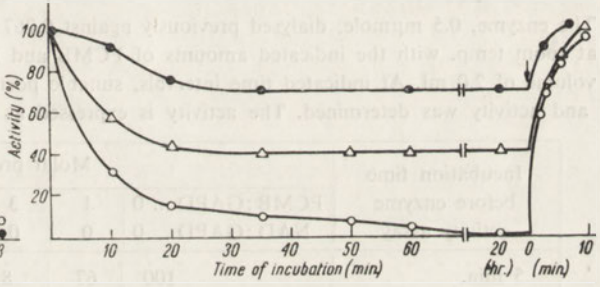


Fig. 6

Fig. 5. The effect of the amount of PCMB on the activity of D-glyceraldehyde-3-phosphate dehydrogenase from human muscle. (○). Enzyme (0.02 mμmole in a final volume of 2.0 ml.) after dialysis against 0.067 M-tris buffer, pH 7.4; (●), GAPD-G3P complex (0.02 mμmole in a final volume of 2.0 ml.) obtained by incubation of the enzyme for 10 min. at 0° with 100 equiv. of G3P in 0.067 M-tris buffer, pH 7.4, then dialysed for 20 hr. against the same buffer. To enzyme preparations, indicated amounts of PCMB were added and after 10 min. at 23° the enzymic activity was determined. The content of SH groups in the enzyme and the GAPD-G3P complex was, respectively, 11.3 and 9.0 per mole of protein.

Fig. 6. Time-course of the inactivation of D-glyceraldehyde-3-phosphate dehydrogenase from human muscle with various amounts of $Na_2S_4O_6$, and the effect of cysteine. The enzyme, 0.5 mμmole, was incubated in 0.067 M-phosphate buffer, pH 7.4, in a final volume of 2.0 ml. with (●), 1; (△), 2; or (○), 3 equiv. of $S_4O_6^{2-}$. At indicated time intervals, portions of the mixture were taken for activity determinations. The arrow indicates the addition of 1 μmole of cysteine.

a slow reactivation occurred, the slope of the inhibition curve decreased, and after 48 hr. with 3 PCMB the inhibition was only 66%, but the relationship between the activity and the amount of the inhibitor continued to be linear. Reactivation of the enzyme did not appear in the presence of 10 or more equiv. of PCMB. The inhibition was completely reversible by cysteine; when a sample of the enzyme was incubated for 48 hr. at room temperature with 3 equiv. of PCMB and then for 10 min. with cysteine in a final concentration of 4 mM, the activity was even by 20% higher than that of the control sample. This was probably due to the protective effect of PCMB against oxidation of those SH groups that are required for the activity. The substrate had no influence on the inhibition and reactivation. As it appears from he data presented in Table 1, NAD had no marked effect on the initial course of inhibition; the enzyme-G3P complex was even slightly more inhibited. On the other hand, NAD but not G3P enhanced the reactivation; after 48 hr. the enzyme inhibited by 3 equiv. of PCMB exhibited in the presence of 1200 equiv. of NAD 75%, and after preincubation with cysteine even 150%, of the activity of the control sample. However, it should be taken into account that the control sample after 48 hr. at room temperature showed only 50 - 60% of the initial activity, and preincubation with cysteine had but a slight enhancing effect.

Table 1

*The effect of PCMB on the activity of* D-*glyceraldehyde-3-phosphate dehydrogenase from human muscle*

The enzyme, 0.5 mμmole, dialysed previously against 0.067 M-tris buffer, pH 7.4, was incubated at room temp. with the indicated amounts of PCMB and NAD in the same buffer, in a final volume of 2.0 ml. At indicated time intervals, suitable portions of the mixture were withdrawn and activity was determined. The activity is expressed as percentages of the control values.

| Incubation time before enzyme activity assay | Molar proportion | | | | | | |
|---|---|---|---|---|---|---|---|
| PCMB : GAPD ... 0 | 1 | 3 | 10 | 1 | 3 | 10 |
| NAD : GAPD ... 0 | 0 | 0 | 0 | 1200 | 1200 | 1200 |
| 5 min. | 100 | 67 | 8 | 0 | 73 | 20 | 0 |
| 200 min. | 100 | 62 | 14.5 | 0 | 85 | 36 | 0 |
| 24 hr. | 100 | 82 | 23 | 0 | 96 | 50 | 0 |
| 48 hr. | 100 | 85 | 35 | 0 | 100 | 75 | 0 |
| 48 hr., then 10 min. with cysteine | 100 | 105 | 120 | 96 | 130 | 155 | 120 |

*Inhibition by iodoacetate.* Iodoacetate is a strong inhibitor also of GAPD from human muscle (Table 2) but the reaction proceeded much slower than with the enzyme from rabbit muscle. In 0.067 M-tris buffer, pH 7.4, after 20 min. even 100 equiv. of iodoacetate did not cause complete inactivation, and with 3 equiv. of iodoacetate a complete inhibition was observed after 24 hr. The presence of substrate protected to some extent from inactivation, but only in the initial period of incubation. NAD even at a great excess had no effect.

Table 2

*The effect of iodoacetate on the activity of* D-*glyceraldehyde-3-phosphate dehydrogenase from human muscle*

The enzyme, 0.5 mμmole, dialysed previously against 0.067 M-tris buffer, pH 7.4, was incubated at room temp. with the indicated amounts of iodoacetate and G3P in the same buffer, in a final volume of 2.0 ml. At indicated time intervals, suitable portions of the mixture were withdrawn and activity was determined. The activity is expressed as percentages of the control values.

| Incubation time before enzyme activity assay | Molar proportion | | | | |
|---|---|---|---|---|---|
| Iodoacetate : GAPD... | 3 | 10 | 100 | 100 | 100 |
| G3P : GAPD... | 0 | 0 | 0 | 400 | 1600 |
| 20 min. | 85 | 55 | 5 | 31 | 47 |
| 150 min. | 50 | 10 | 0 | 0 | 5 |
| 24 hr. | 0 | 0 | 0 | 0 | 0 |

*Inhibition by tetrathionate.* The inhibitory effect of $Na_2S_4O_6$ has been known for a long time (Keilin & Hartree, 1940), but the mechanism of the reaction of tetrathionate with SH groups was studied only in 1962 by Pihl & Lange (1962). They have demonstrated that the reaction with the SH groups of GAPD of rabbit muscle proceeds with formation of sulphenyl thiosulphate groups according to the equation:

$$\text{protein}-SH+S_4O_6^{2-} \rightarrow \text{protein}-S-S_2O_3^{-}+S_2O_3^{2-}+H^+$$

The effect of $Na_2S_4O_6$ on GAPD from human muscle (Fig. 6) was similar to that with the rabbit muscle enzyme (Pihl & Lange, 1962). However, the rate of the inactivation was slightly lower and the maximum for 1 and 2 equiv. was achieved after 35 min. and for 3 equiv. or more, after a longer time. The degree of inhibition did not change during 20 hr. of incubation, and cysteine fully restored the enzymic activity. The relationship between the degree of inhibition and the number of $S_4O_6^{2-}$ equivalents was similar to that for the inhibition by PCMB, and complete inhibition was achieved in the presence of about 3 equiv. of the inhibitor per mole of the enzyme.

*Inhibition by heavy-metal ions.* The inhibition by silver, copper and zinc ions appeared to be dependent on the enzyme-metal ion molar proportion and was almost as high as the inhibition by the thiol reagents studied.

Inhibition by $Cu^{2+}$ and $Ag^+$ was markedly dependent on the time of incubation; after 6 hr. it was complete in the presence of about 4 equiv. of $Cu^{2+}$ and after 2 hr. incubation with 3 $Ag^+$ (Fig. 7). The inhibition, which did not change on further incubation (up to 24 hr.), was fully reversible by cysteine.

Inhibition by $Zn^{2+}$ was not dependent on the time of incubation; the plot of inactivation against $Zn^{2+}$ concentration was the same after 1 min. (Fig. 8) as after a longer time of incubation. About 120 equiv. of $Zn^{2+}$ caused complete inhibition of enzymic activity. Inhibition even by a large excess of $Zn^{2+}$ (about $10^5$ equiv., 100 min.) was completely reversed by incubation with cysteine.

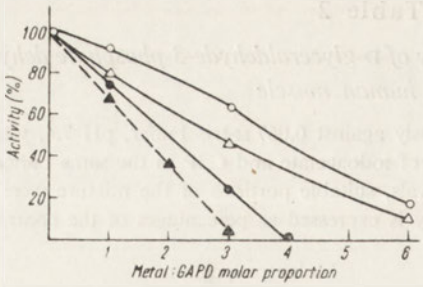


Fig. 7

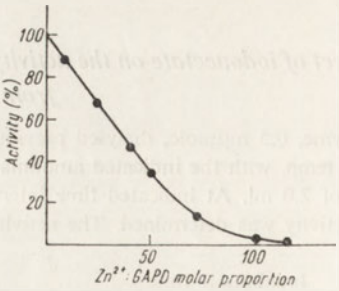


Fig. 8

Fig. 7. The effect of $Ag^+$ and $Cu^{2+}$ on the activity of D-glyceraldehyde-3-phosphate dehydrogenase from human muscle. The enzyme, 0.4 mμmole, was incubated at room temp. in 0.05 M-triethanolamine buffer, pH 7.4, with $Cu^{2+}$ (full lines) or $Ag^+$ (dashed line). After incubation for (○), 15 min., (△), 75 min., (●), 6 hr. and (▲), 2 hr., portions of the mixture were taken for activity determinations.

Fig. 8. The effect of $Zn^{2+}$ ion on the activity of D-glyceraldehyde-3-phosphate dehydrogenase from human muscle. The enzyme, 0.029 mμmole, was incubated at room temp. in a final volume of 2.0 ml. in a mixture containing 0 - 4.5 mμmole of $Zn^{2+}$ and all the compounds used for activity determination except the substrate. After 60 sec. the enzymic reaction was initiated by adding G3P.

The cadmium ion had the smallest effect; full inhibition was achieved at a concentration of 0.1 mM (over the range of 200 - 10 000 ion equiv.); it did not depend on the time of incubation and was reversible by cysteine.

## DISCUSSION

The molecular weight of GAPD from human muscle, 125 000, determined on the basis of sedimentation and diffusion coefficients, is in good agreement with the values reported for the rabbit muscle enzyme, 120 000 (Elias *et al.*, 1960) and 140 000 (Fox & Dandliker, 1956). Gulyi, Dwornikowa, Fedorczenko & Poczenowa (1962) demonstrated by the light scattering method that the enzyme freshly obtained from rabbit muscle is a monomer of a molecular weight about 45 000, which on storage forms a trimer of mol.wt. 136 000 - 144 000. As the sedimentation coefficient for GAPD from human muscle did not change between the tenth day since the beginning of its isolation and the sixth month of storage, it seems that this effect did not appear in the human muscle enzyme.

The isoelectric point of GAPD from human muscle in phosphate buffer, $I$ 0.1, differed by 0.75 pH units from the corresponding value for the rabbit muscle enzyme and the mixture of the two enzymes at pH 6.25 separated into two distinct fractions. The isoionic point for the two enzymes was the same, but this is insufficient to conclude whether the content of basic and acidic amino acids was the same, or whether the differences in electrophoretic mobility were due to different amounts of bound anions. According to Allison & Kaplan (1964) GAPD from human heart muscle possesses fewer basic amino acid residues than does the rabbit muscle

enzyme, and a higher amount of aspartic acid residues. On the other hand, these authors did not observe any differences in mobility of the two enzymes on paper electrophoresis.

The number of SH groups did not differ from 9 - 14 reported for GAPD from rabbit muscle (Velick, 1953; Murdock & Koeppe, 1964; Allison & Kaplan, 1964; Cecil & Ferdinand, 1965). However, a very distinct difference was observed in the reactivity of SH groups with PCMB. The human muscle enzyme reacted with PCMB very slowly; within 5 min. 4 - 5 groups were bound whereas for the remaining ones about 10 hr. were required. Allison & Kaplan (1964) also observed a very slow reaction of GAPD from human heart muscle with PCMB. In 0.1 M-phosphate buffer, pH 7, within 2 min. 6 - 7 SH groups reacted, then the reaction proceeded very slowly, and after 40 min. a sediment appeared which made impossible further determinations. As 3 equiv. of PCMB inactivate rapidly the enzymes both from human and rabbit muscles, it seems possible to suppose that just those three rapidly reacting, easily accessible SH groups are required for the enzymic reaction. The peptide containing the active centre, isolated by Harris, Meriwether & Park (1963) from pig GAPD, contained two cysteine residues one of which reacted rapidly with iodoacetate and the other only in the presence of urea. Blocking of the first one resulted in inactivation of the enzyme.

Spontaneous reactivation of GAPD from human muscle after inactivation with 3 - 10 equiv. of PCMB, is probably caused by movement of the inhibitor molecules on to further SH groups, not involved in the enzymic reaction. They become accessible to the inhibitor only after a longer time of incubation, when a slight alteration in steric structure has occurred due to the action of PCMB on the reactive SH groups. Szabolsci, Biszku & Sajgó (1960) who studied the inhibition by PCMB of GAPD from pig and ox muscles at pH 8.3, observed complete inhibition only in the presence of 7 PCMB. The inactivated enzyme also underwent spontaneous reactivation when the amount of PCMB did not exceed 7 equiv., and the authors explained this effect by displacement of PCMB from one molecule to another. Thus, after a certain time, some molecules would be completely free of the inhibitor and active again whereas other molecules would have a higher content of the inhibitor. The completely blocked protein would not be reactivated by cysteine; according to Elödi (1960) its configuration is changed which leads to irreversible denaturation. Gruber, Warzecha, Pfleiderer & Wieland (1961) observed a similar spontaneous reactivation of lactate dehydrogenase from pig heart muscle. They supposed that this might result from displacement of PCMB within a single enzyme molecule, from the SH groups that are more reactive and involved in enzymic activity to other, non-active groups. GAPD from human muscle is more resistant to the action of PCMB than the enzyme from pig or ox muscles; in almost neutral medium even at 37° no precipitation of protein was observed, and a short incubation with cysteine restored completely the activity even after prolonged action of the inhibitor. These properties seem to be compatible with the mechanism of reactivation proposed by Gruber *et al.* (1961).

Inhibition of GAPD by heavy-metal ions appeared to be much stronger than

it had been hitherto supposed. Weitzel & Schaeg (1959) observed complete inhibition of the enzyme from rabbit muscle by 0.4 μM-PCMB, 5 μM-$Cu^{2+}$ or about 50 μM-$Zn^{2+}$ or $Cd^{2+}$ after 60 sec. of incubation with the protein. The activity was determined using aldolase and fructose-1,6-diphosphate as substrate. Aldolase, which was applied in excess, also possesses SH groups which react with PCMB (Swenson & Boyer, 1957) and thus it could bind a part of heavy-metal ions from the solution, lowering their effect on GAPD. In the present work it was found that on prolonged incubation the extent of inhibition by $Ag^{+}$ and $Cu^{2+}$ reached the values obtained with strong thiol reagents. Undoubtedly the same SH groups were bound that react rapidly with PCMB and are required for the activity. Also Boross (1965) reported that 3 - 4 equiv. of silver ions inactivated completely in a short time GAPD from pig muscle.

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## DEHYDROGENAZA D-GLICERALDEHYDO-3-FOSFORANU Z MIĘŚNI CZŁOWIEKA

### GRUPY SH I INHIBITORY

### Streszczenie

1. Stwierdzono, że dehydrogenaza D-gliceraldehydo-3-fosforanu z mięśni człowieka posiada 12 grup SH, z których 4 - 5 reagują szybko, w ciągu 15 minut, z *p*-chlororteciobenzoesanem (PCMB), natomiast pozostałe grupy reagują bardzo wolno.

2. Enzym jest inaktywowany przez 3 równoważniki PCMB, jodooctanu, czterotionianu i $Ag^+$, 4 równoważniki $Cu^{2+}$ oraz 100 równoważników $Zn^{2+}$.

3. W obecności do 10 równoważników PCMB po dłuższym czasie inkubacji następuje częściowa samoistna reaktywacja enzymu.

4. Stwierdzone dotychczas różnice we właściwościach enzymu z mięśni człowieka i królika odnoszą się do ruchliwości elektroforetycznej i szybkości reakcji z inhibitorami grup SH.

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# THE VARIATIONS OF FREE AND BOUND STEROLS IN *CALENDULA OFFICINALIS* DURING VEGETATION

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1. The quantities of free sterols, sterol esters and sterol glycosides were determined in *Calendula officinalis* plants during vegetation. 2. The period of germination was found to be characterized by hydrolysis of esters to free sterols, the period of flowering by the accumulation of sterols of all three fractions in all plant organs, particularly in flowers, and the period of senescence by hydrolysis of sterol esters in the shoot and by esterification of sterols in the root and seeds.

It has been shown in the previous studies of Kasprzyk & Pyrek (1965) on the composition of triterpenoids of the ether extracts from the flowers of five plant species belonging to the Compositae family that sterols occur in these plants in the form of free and ester bound compounds. The detailed study of the sterol components by Kasprzyk & Pyrek (1967) showed that the majority of them contain besides sterols with double bond at position 5 also those with double bond at position 7. Quantities of the latter in the flowers of arnica and dandelion represent 50% of the sterol fraction. The main sterol of calendula was shown to be stigmasterol, $\Delta^7$-sterols being absent.

In plants, in addition to free and esterified sterols, also sterol glycosides are present. Nicholas (1963) has demonstrated that free and esterified sterols occur in all parts of cabbage and lettuce plants, but sterol glycosides are present only in leaves and root and are absent from the stem. It has been shown by Eichenberger & Menke (1966) that the leaves of *Anthirrinum majus*, *Spinacia oleracea* and *Allium porrum* contain from 0.05 to 0.18% of sterols (on dry weight basis), and that 1/4 of this amount occurs in chloroplasts. It has also been found by these authors that free sterols represent 54 - 78% of the total amount of sterols, sterol esters 10 - 22% in leaves and 22 - 41% in chloroplasts, and sterol glycosides 25% in leaves, being present in chloroplasts in trace amounts. The acid component of the sterol esters has been shown to be palmitic acid, and the sugar components of glycosides, glucose and mannose.

The studies carried out by Kasprzyk & Fonberg-Broczek (1967) on the metabolism of triterpenoids in *Calendula officinalis* during vegetation have shown that the amount

of all sterols obtained by acid and alkaline hydrolysis of the plant material increases with the growth of the young plant up to the appearance of inflorescence buds. Afterwards it is stabilized at 3000 μg. per 1 g. of dry weight in young leaves, being in old leaves and in the root 3 times less. It has also been observed that sterols accumulate in great quantities in flowers.

The aim of the present work was to determine the free, esterified and glycosidically bound sterols and $\Delta^7$-sterols in *Calendula officinalis* during vegetation.

## MATERIAL AND METHODS

*Cultivation of plants.* The material analysed comprised seeds and plants of *Calendula officinalis* var. Rodio. The seeds were obtained from the Plant Selection Center of the Warsaw Horticultural Co-operative and cultivated in the greenhouse at 18 - 20° from January 10 to June 15, 1966. Forty seeds were sown in one pot filled with 10 kg. of sand to which was added a mixture (elaborated by the Department of Plant Physiology of the Warsaw University) containing 0.6 g. of $Ca(H_2PO_4)_2 \cdot H_2O$, 0.8 g. of $NH_4NO_3$, 0.6 g. of $CaCl_2 \cdot H_2O$, 0.4 g. of KCl, 0.6 g. of $MgSO_4 \cdot 7H_2O$, 0.02 g. of ferric citrate and 10 ml. of A-Z Hoagland nutrient solution. Every month the plants were fed with a half of this amount of the mixture. Till the end of March the plants were illuminated with fluorescence lamps to yield a 12-hr. daylight required by calendula plants for normal development. The plants were harvested gradually, and during flowering only 4 plants were left in the pot.

*Collection of samples and extraction.* The samples were always collected in the morning. All plants collected were divided into parts and weighed; for dry weight determination small portions were dried for 20 hr. at 40° and then at 105° to constant weight (about 4 hr.). The remaining plant material was mixed with a fivefold volume of boiling ethanol and homogenized in the Unipan homogenizer at 10 000 rev./min. for 5 min. The homogenate was filtered and the residue was heated for 10 min. with one volume of anhydrous ethanol (with respect to the initial volume of the plant material) and filtered again. Then the residue was twice extracted with double volumes of benzene in a similar way. Then from the two extracts the solvents were removed by distillation and the residues combined.

*Fractionation of the extract.* The combined residues of ethanol and benzene extracts were fractionated in the system water - ethanol - light petroleum, b.p. 40 - 60° (1:1:2, by vol.). The ethanol - water phase contained sterol glycosides, and the upper layer of light petroleum - ethanol contained free and esterified sterols. The latter were separated by countercurrent distribution in 6 sections of the extraction apparatus using hexane - ethanol - water (14:10:1.3, by vol.). The upper layers contained sterol esters and the lower phases, free sterols. The separation of all fractions was checked by thin-layer chromatography on silica gel as described later.

*Isolation of sterols in the form of digitonides.* From the three sterol fractions the solvents were removed by distillation. The fraction containing sterol glycosides was hydrolysed with 10% methanolic HCl solution for 3 hr. at 100°, and the fractions containing free and esterified sterols were hydrolysed with 10% KOH under the

same conditions. Then the hydrolysates were threefold diluted with water, adjusted to pH 8, and the sterols were extracted with light petroleum. The solvents were removed by distillation and the three fractions obtained were dissolved in 95% ethanol. Samples containing 5 mg. of sterols were quantitatively transferred to weighed vessels fitted with a sintered disc (Schott G-4), added with 5 ml. of hot solution of digitonine in 90% ethanol, and left for 20 hr. Then the supernatant was filtered off and the precipitate remaining in the vessel was washed three times with small portions of ethyl ether, and after drying in a vacuum desiccator over $H_2SO_4$ to constant weight, the quantity of sterol was calculated by dividing the weight of digitonide by four.

*Determination of $\Delta^7$-sterols.* Samples containing up to 100 μg. of sterols in 0.5 ml. of carbon tetrachloride were mixed at 0° with 1 ml. of the Liebermann & Burchard reagent composed of acetic anhydride and concentrated sulphuric acid (19:1, v/v), freshly prepared before use. After 10 min. the extinction at 620 mμ was measured in a Zeiss spectrophotometer using a microcell of 1 ml. volume with a light-path of 3 cm. Standard curve was prepared with $\Delta^7$-cholestenol.

*Thin-layer chromatography.* This was performed on plates covered with silica gel (Serva, Heidelberg, Germany) (thickness 0.3 mm.) and on plates with silica gel impregnated with silver nitrate (20% with respect to gel). The plates were activated at 120° for 20 min. The plates with gel alone were developed in chloroform - methanol (99:1, v/v) and those impregnated with silver nitrate in chloroform - ethyl ether - acetic acid (19.4:0.6:0.1, by vol.). The sterol spots were detected with 50% sulphuric acid.

## RESULTS

Seeds and plants separated into parts at the different periods of vegetation as described in Table 1, were analysed. The results of determinations are presented in Figs. 1 - 6 and in Table 2. During germination and the first 18 days of development of young seedlings an insignificant loss of dry weight and of sterols was observed (Fig. 1). The fresh weight of the plant increased, however, during this period due to water uptake. An increase both in fresh and dry weight was observed from the 18th day until the end of the period of observation. An increase in the amount of sterols proceeded up to the stage just before formation of the inflorescence buds (between the 71st and 120th day of vegetation), parallelly to the increase in weight. At the same time the synthesis of sterols became more intensive; their amount increased fivefold whereas the weight of the plant only threefold. During flowering the content of sterols was very high and changed only slightly during the next stage of seed formation. The course of accumulation of $\Delta^7$-sterols had the same character up to the stage of formation of the inflorescence buds. On the other hand, during transformation of buds into flowers the quantity of $\Delta^7$-sterols practically did not change, but it increased considerably during development of the seeds.

The percentage of dry weight of the plant (Fig. 2) increased from 6% at the stage of seedlings to about 15% at the stage of formation of flower buds, and then

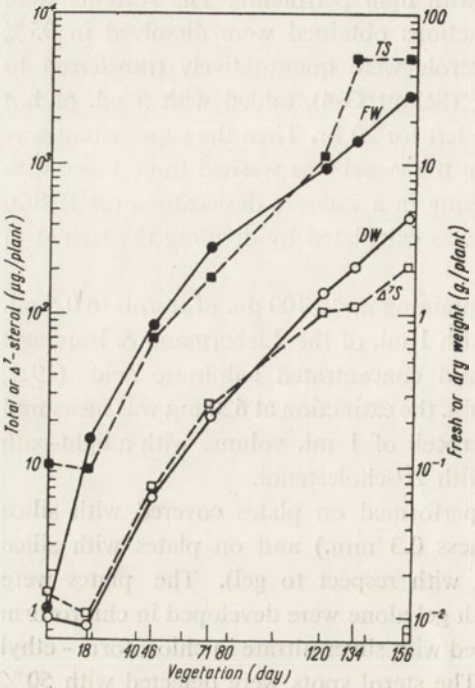



Fig. 1. Changes in weight and content of sterols in *Calendula officinalis* during vegetation. (●), Fresh weight; (○), dry weight; (■), total sterols; (□), $\Delta^7$-sterols.

## Table 1

*Description of the plant material analysed*

| Date of collection | Age (day) | Stage of development | Part analysed | Quantity pooled for analysis |
|---|---|---|---|---|
| 10.I | 0 | Seed | Whole | 500 |
| 28.I | 18 | Seedling without leaves | Whole | 220 |
| 25.II | 46 | Plant with two floors of leaves | Shoot | 141 |
| | | | Root | 141 |
| 22.III | 71 | Plant with four floors of leaves | Shoot | 53 |
| | | | Root | 53 |
| 10.V | 120 | Plant developing flower buds | Inflorescence with flower buds | 180 |
| | | | Shoot | 11 |
| | | | Root | 11 |
| 25.V | 134 | Flowering plant | Inflorescence with flowers | 13 |
| | | | Shoot | 5 |
| | | | Root | 5 |
| 15.VI | 156 | Plant forming seeds | Inflorescence with seeds | 16 |
| | | | Shoot | 3 |
| | | | Root | 3 |

it remained practically unaltered. The total sterols calculated per gram of dry weight increased insignificantly till the 46th day, then between the 80th and 120th day their amount was somewhat lower and attained a maximum during flowering. On the other hand, the amount of $\Delta^7$-sterols steadily decreased during vegetation from 140 to 50 μg.

Figure 3 illustrates changes in free sterols (*F*), sterol esters (*E*) and sterol glycosides (*G*) in the whole plant, in the shoot, root and inflorescence. During germination sterols *E* were transformed into sterols *F*, but the quantity of sterols *G* practically did not change. During further growth of the plant the amount of sterol

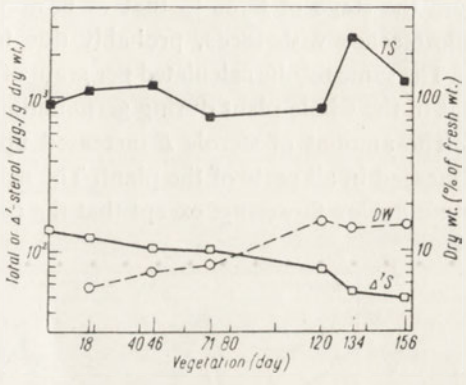



Fig. 2. Changes in the content of sterols per 1 g. of dry weight in *Calendula officinalis* during vegetation. (■), Total sterols; (□), $\Delta^7$-sterols; (○), dry weight.

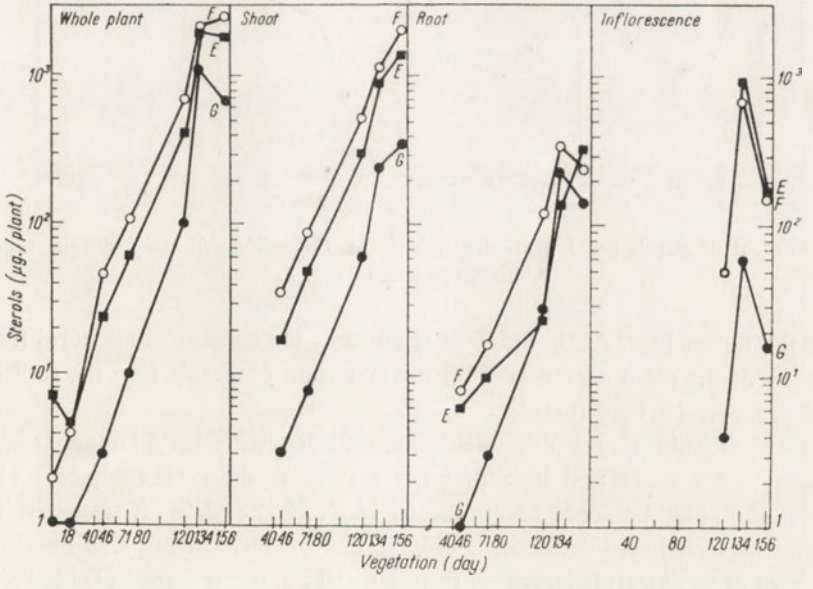



Fig. 3. Content of sterols per one plant (or its part) in *Calendula officinalis* during vegetation. *F*, free sterols; *E*, sterol esters; *G*, sterol glycosides.

of all fractions increased reaching a maximum during flowering. In the old plant, sterols *F* further increased but the quantity of sterols *E* and *G* decreased.

In the shoot the metabolism of the three fractions proceeded from the 46th day up to the stage of flowering similarly as in the whole plant, but during flowering a slower increase of sterols *G* was observed. During senescence the amount of sterols present in all fractions increased at a lower rate.

In the root, up to the formation of flower buds, the greatest increase was observed for sterols *G*. During flowering the amount of sterols *G* exceeded that of sterols *E*, but in the old plant both sterols *G* and *F* decreased whereas sterols *E* increased.

In the inflorescence the amounts of sterols of all fractions increased rapidly from the stage of buds to that of flowering, then a decrease was observed in the inflorescence with seeds, probably due to the loss of the flower coronas.

The same results calculated per gram of dry weight (Fig. 4) support the conclusion that in the whole plant during germination sterols *E* were hydrolysed to give sterols *F*. The amount of sterols *F* increased up to the 46th day of vegetation and then decreased in all parts of the plant. The maximum amount of sterols *E*, *F* and *G* was found during flowering, except that the content of sterols *F* in the flower was lower

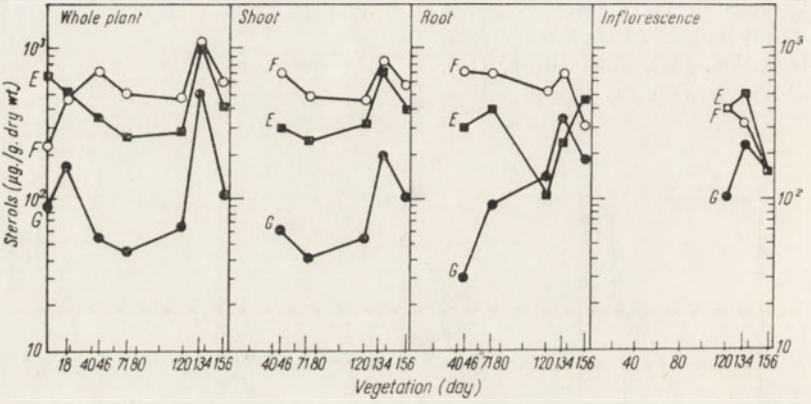



Fig. 4. Content of sterols per 1 g. of dry weight in *Calendula officinalis* during vegetation. Abbreviations as in Fig. 3.

than in the flower bud. At the stage of senescence the content of all sterol fractions in all parts of the plant decreased with the exception of sterols *E* in the root and in the seeds, in which they increased.

The 156-day-old plants developed inflorescence with very young seeds. Ten days later, older seeds and in September mature seeds were analysed. The dry weight of the analysed seeds amounted to 14%, 25% and 94%, respectively. The sterols in the seeds were determined only as digitonides. During seed development the amount of total sterols increased from 3 to 10 μg. per one seed (Table 2). In the initial period mainly sterols *F* and *G* were increasing, the increment of sterols *E* being insignificant. At the final stage of ripening the amount of sterols *F* and *G*

decreased but that of sterols *E* rose considerably. When calculated per dry weight, a steady drop of sterols *F* and *G* was observed, and an increase of sterols *E* at the final stage.

$\Delta^7$-Sterols constituted 15% of all sterols in the seed and in the young plant. During the subsequent growth of the plant the increase of their quantity was much slower than that of other sterols, and in the old plant they constituted only 2% of all sterols. From the results presented in Fig. 5 it appears that in the whole plant at earlier stages of development the course of changes in $\Delta^7$-sterols was similar to that of all sterols. On the other hand, in inflorescence at the stage of flowering there was more of $\Delta^7$-sterols *E* than *F*; the same was observed for the whole plant also during development of flower buds.

Table 2

*The content of sterols in developing seeds*

| Age of seeds | Dry weight (%) | Number of inflorescences | Quantity of seeds pooled for analysis | Sterol fractions | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *F* | *E* | *G* | *F* | *E* | *G* | Total |
| | | | | μg./g. of dry weight | | | μg./one seed | | | |
| Very young | 14 | 11 | 430 | 900 | 1420 | 400 | 1.0 | 1.6 | 0.4 | 3.0 |
| Young | 25 | 15 | 350 | 400 | 320 | 320 | 2.5 | 2.0 | 2.0 | 6.5 |
| Mature | 94 | 13 | 500 | 190 | 680 | 100 | 2.0 | 7.0 | 1.0 | 10.0 |

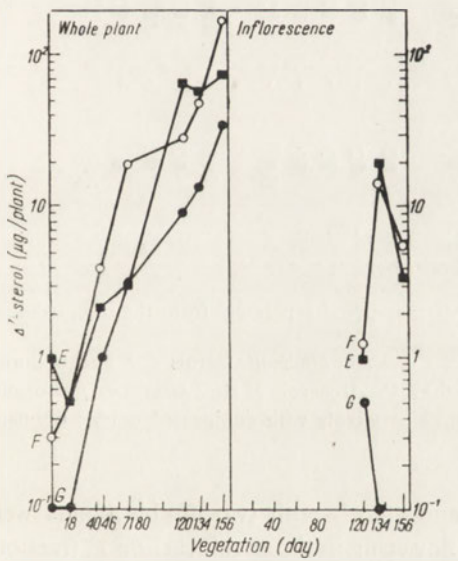



Fig. 5

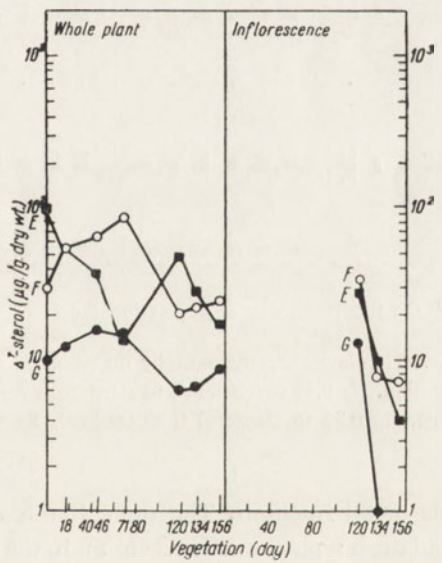


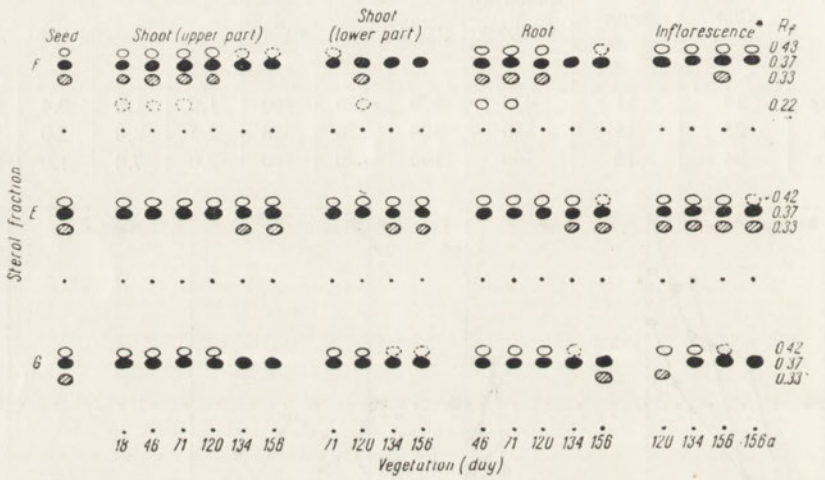

Fig. 6

Fig. 5. Content of $\Delta^7$-sterols per one plant and inflorescence in *Calendula officinalis* during vegetation. Abbreviations as in Fig. 3.

Fig. 6. Content of $\Delta^7$-sterols per 1 g. of dry weight in *Calendula officinalis* during vegetation. Abbreviations as in Fig. 3.

When calculated per dry weight (Fig. 6), the amount of $\Delta^7$-sterols *F* and *G* increased up to the 71st day, then decreased and from the 120th day slightly increased again. The content of sterols *E* dropped down till the 71st day, increased up to the 120th day and then decreased steadily. Changes in the content of $\Delta^7$-sterols *F* and *E* in flowers proceeded similarly to those of total sterols in the appropriate fractions. $\Delta^7$-Sterols *G* occurring in the inflorescence buds disappeared nearly completely after transformation into flowers and then reappeared in mature seeds. The content of $\Delta^7$-sterols *F* and *E* in the inflorescence decreased with its development.

Thin-layer chromatograms on silica gel impregnated with silver nitrate, of sterols of the isolated fractions are presented in Fig. 7. In the system used, the $R_F$ value for $\Delta^7$-sterols was 0.42, for $\Delta^5$-sterols of the stigmasterol or $\beta$-sitosterol type 0.37, for the unidentified $\Delta^5$-sterol 0.33, and for sterols with conjugated double bonds, 0.22. The main sterols of the $\Delta^5$-type ($R_F$ 0.37) occurred during vegetation in all fractions and in all parts of the plant except for the glycosidic fraction in inflorescence buds. $\Delta^7$-Sterols and both types of $\Delta^5$-sterols occurred in mature




* On the 156th day of vegetation the seeds were analysed separately from the receptaculum.

Fig. 7. Thin-layer chromatography on silica gel of *Calendula officinalis* sterols *F*, *E* and *G* during vegetation. $R_F$ 0.42 corresponds to $\Delta^7$-sterols, $R_F$ 0.37 to $\Delta^5$-sterols of stigmasterol or $\beta$-sitosterol type, $R_F$ 0.33 to $\Delta^5$-sterol (unidentified), $R_F$ 0.22 to sterols with conjugated double bonds.

seeds in all fractions. The same sterols and sterols with two double bonds were found during plant development up to the flowering in the sterol fraction *F*, fractions *E* and *G* containing only $\Delta^7$-sterols and main $\Delta^5$-sterols. Characteristic changes of $\Delta^5$-sterols with $R_F$ 0.33 were observed at the stage of development of the inflorescence buds. These compounds disappeared from fraction *F* in all parts of the plant and reappeared in fraction *E*. They were present also in sterols *G* in the root of old plants and in young seeds in the fraction of free sterols.

## DISCUSSION

In the present work, the period of vegetation of calendula plants was longer, particularly before flowering, as compared with the plants cultivated last spring by Kasprzyk & Fonberg-Broczek (1967), mainly because of insufficient illumination in the greenhouse during the winter months. Nevertheless, the total amounts of sterols found at successive stages of development were similar to the corresponding values obtained previously. The results of determinations are presented in the graphs both as amounts of sterols per one plant or its part, and as values calculated per gram of dry weight, because each form of presentation reflects a different phenomenon. The first shows whether during development of the plant there occurs biosynthesis or catabolism of the compound studied, and the second shows the rate of the biosynthetic process with respect to the rate of growth of the whole plant.

It follows from our previous results (Kasprzyk & Fonberg-Broczek, 1967) that the amount of total sterols during vegetation is not constant and changes depending on the organ and stage of development; environmental conditions have little effect on the content of these compounds in the plant.

The main stages of vegetation characterized by quantitative and qualitative changes of free, ester and glycoside sterols, are as follows: 1, germination and growth of the seedlings up to the formation of first leaves (18 days); 2, growth of the young plant up to the appearance of inflorescence buds (from 18th to 120th day); 3, flowering (from 120th to 134th day); 4, seed formation (from 134th to 156th day of vegetation).

The first stage is characterized by an insignificant decrease in the amount of total as well as $\Delta^7$-sterols in the plant as compared with the seed; calculation per dry weight shows, however, an increase which is caused mainly by removal of the seed coats containing a certain amount of sterols. At this stage there is, presumably, no synthesis of sterols, and there occurs an intensive hydrolysis of sterol esters to free sterols (total as well as $\Delta^7$). In the group of $\Delta^5$-sterols this hydrolysis concerns mainly the sterol with $R_F$ 0.33, which occurs in the seeds in all three fractions but which is present in young seedlings only in fraction *F*.

During the second stage of development, a rapid increase of the fresh and dry weight of the plant is observed accompanied by a high increase of sterols (from about 10 μg. to about 1100 μg./plant). The ratio of individual sterol fractions in the whole plant during this stage is $F:E:G = 1:0.5:0.1$. Fraction *F* in the shoot as well as in the root is composed of $\Delta^7$-sterols, both types of $\Delta^5$-sterols and of sterols with two conjugated double bonds. Fractions *E* and *G* contain $\Delta^7$-sterols and main $\Delta^5$-sterols. This stage is characterized by a stabilization of the content, calculated per dry weight, of fractions *F*, *E* and *G* in the shoot and of fraction *F* in the root, this indicating a parallelism of synthesis of sterols of these fractions and growth of the plant. Fraction *E* in the root decreases and fraction *G* increases considerably which suggests the possibility of transformation of a part of sterol esters into sterol glycosides.

The stage of flowering, from formation of flower buds to formation of young seeds, is characterized by quantitative and qualitative changes of all types of sterols. A considerable increase of sterols of all fractions is observed in the shoot and root as well as in the inflorescence, showing maxima during flowering when calculated per dry weight. The amount of sterols of fractions *E* and *G* per one plant increases and the ratio of sterols *F*:*E*:*G* is 1:1:0.5. In the inflorescence the content of fraction *E* exceeds even that of fraction *F*. It is possible that $\Delta^5$-sterol with $R_F$ 0.33 is esterified in this organ as well as in others, as this compound disappears from fraction *F* and appears in fraction *E* in the shoot, root and in the inflorescence buds. In the inflorescence buds it occurs also in fraction *G* but is absent from the flowers. The main $\Delta^5$-sterols are present in fraction *G* in inflorescence at the stage of flowering but are absent from inflorescence buds, what may suggest that they are transported from the shoot to the developing flower. The metabolism of $\Delta^7$-sterols at the second and third stage of development differs slightly from the scheme of metabolism of the sum of sterols, but it should be noted that the determination of these compounds was not precise because of low quantities of the material available. Traces of $\Delta^7$-sterols undeterminable as digitonides could be detected on thin-layer chromatograms in fraction *G* in inflorescence on the 134th and 156th day.

The fourth stage, when the plant is getting old and the seeds are maturing, is characterized by a decrease of sterol synthesis. Calculation per dry weight shows a decrease of all fractions in the shoot, in the inflorescence and in fraction *F* and *G* in the root, only the content of sterols *E* is growing in the root and in the seeds. The results of determinations indicate that during formation of seed the increase in their weight is accompanied by a rise of sterols probably due to their transfer from the green part of the plant, followed by esterification. A similar process is observed at the same time in the root. On the other hand, the amount of sterols *F* in the shoot is increasing at a higher rate than that of other sterol fractions, what is shown by the slope of curves *F*, *E* and *G* on Fig. 3 between the 134th and 156th day. It may indicate hydrolysis of sterols *E* and *G* to sterols *F* in the green part of the plant. In young seeds (dry weight, 14%) the sterols are composed of main $\Delta^5$-sterols and $\Delta^7$-sterols. $\Delta^5$-Sterols with $R_F$ 0.33 occur in them only in fraction *E* and are detected in the two other fractions only in mature seeds.

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## PRZEMIANY STEROLI WOLNYCH I ZWIĄZANYCH W *CALENDULA OFFICINALIS* W CZASIE WEGETACJI

Streszczenie

1. Oznaczono zawartość steroli wolnych, estrów steroli i glikozydów steroli w *Calendula officinalis* w czasie wegetacji.

2. Wykazano, że w okresie kiełkowania zachodzi hydroliza estrów do wolnych steroli; w następnym okresie, do kwitnienia, wzrasta ilość wszystkich frakcji sterolowych, a w okresie wytwarzania nasion zachodzi hydroliza estrów steroli w pędzie, natomiast w korzeniu i dojrzewających nasionach obserwuje się estryfikację tych związków.

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# ENZYMIC REDUCTION OF THIOSULPHATE IN PREPARATIONS FROM BEEF LIVER

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1. Crystalline beef liver rhodanese was observed to catalyse reduction of thiosulphate to sulphite at pH 8.8 in the presence of cyanide, borohydride, dihydrolipoate, cysteine or glutathione, with the relative activity of the substrates decreasing in the order given. 2. Glutathione-dependent reduction of thiosulphate in fresh homogenate of beef liver was found to be carried out by two separate enzymes: rhodanese and thiosulphate reductase. 3. Thiosulphate reductase exhibited a maximum activity at pH 7.4 and was inactive with cyanide, borohydride and cysteine. 4. The estimation of thiosulphate reductase activity in tissue preparations and the separation of both enzymes is described.

Oxidation of thiosulphate to sulphate in animal tissues is initiated by the enzymic reductive breakdown of thiosulphate in the presence of a thiol with subsequent oxidation of sulphite to sulphate (Sörbo, 1964; Koj & Frendo, 1967), while bivalent sulphur is oxidized to provide another molecule of thiosulphate (Koj, Frendo & Janik, 1967). It is known that rhodanese[1] (thiosulphate : cyanide sulphur transferase, EC 2.8.1.1) catalyses the reduction of thiosulphate to sulphide and sulphite by dihydrolipoate (Villarejo & Westley, 1963), but is completely inactive with glutathione or cysteine as substrates (Sörbo, 1962; Villarejo & Westley, 1963). We found recently (Koj & Frendo, 1967) that mitochondria isolated from livers of different animal species incubated with thiosulphate produce appreciable amount of sulphate in the presence of glutathione, and at the same time no strict correlation could be seen between the amount of thiosulphate decomposed and rhodanese content in the tissue preparation. These data supported the idea that in animal tissues exists a specific enzyme different from rhodanese — a glutathione-dependent thiosulphate reductase (Sörbo, 1964).

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[1] The name „rhodanese" is consistently used in the text, because it seems that the approved name, thiosulphate : cyanide sulphur transferase will need revision — the enzyme accepts sulphur from different substrates and transfers it to many acceptors.

Our early attempts to purify this enzyme were unsuccessful because active preparations always contained rhodanese; therefore the acceptor specificity of rhodanese was re-investigated. The results presented here indicate that rhodanese is a polyvalent sulphur transferase able to use different sulphur acceptors including glutathione in suitable reaction conditions. However, the reduction of thiosulphate by glutathione in preparations from animal liver is only partly brought about by rhodanese and evidence will be given for the existence of a separate enzyme and some of its properties will be described.

## MATERIALS AND METHODS

Fresh or frozen beef liver was used for obtaining the enzymic preparations. Crystalline rhodanese was isolated by two independent procedures as described by Sörbo (1955) and Green & Westley (1959). The preparations were indistinguishable in their enzymic properties and contained rhodanese of specific activity 200 - 230 Sörbo units/mg. protein. Just prior to use the enzyme was suitably diluted with 0.05% solution of bovine serum albumin containing 0.005 M-sodium thiosulphate. Rhodanese activity was measured according to Sörbo (1955); 1 unit of enzyme catalyses the formation of 10 μmoles of thiocyanate in standard conditions.

Thiosulphate reduction in the presence of different substrates was estimated by the measurement of sulphite formed during the incubation employing a sensitive colorimetric reaction with rosaniline (Sörbo, 1958). In the preliminary experiments anaerobic conditions were maintained to avoid oxidation of sulphite, but it was found that non-enzymic oxidation of sulphite in the presence of inert proteins, thiosulphate and glutathione was slow, and in tissue preparations containing sulphite oxidase the enzyme was strongly inhibited by the high concentrations of thiosulphate which were used. In the standard procedure 0.5 ml. enzymic preparation was mixed with 0.5 ml. 0.05 M-sodium thiosulphate and the test tube placed in the water bath at 37°. Two minutes later 1 ml. of a freshly prepared solution of the appropriate sulphur acceptor (25 μmoles) was added and incubation allowed to proceed for 15 min. The reaction was stopped by adding 2 ml. 0.2 M-$HgCl_2$, the resulting precipitate was centrifuged off and 0.5 ml. of supernatant used for estimation of sulphite (Sörbo, 1958). Control samples contained all the reagents but in studies with crystalline rhodanese enzyme was replaced by 0.05% albumin solution used for dilutions. When crude tissue preparations were used control samples contained enzymes inactivated by 3 min. heating on the boiling water bath.

All the substrates were dissolved in 0.05 M mixed buffers consisting of ammonium phosphate, tris-HCl or sodium borate, and the final pH was carefully controlled. Enzyme activities were expressed in μmoles of sulphite formed in the sample during 15 min. incubation in the conditions described. Among the substrates tested a good linear relationship between sulphite formation and enzyme concentration was found for cyanide, borohydride and dihydrolipoate but not for cysteine and glutathione even at a concentration of 0.1 M. This may be explained by reversibility of the enzymic decomposition of thiosulphate, inhibition by sulphite formed or

by side reactions between sulphite and persulphides of cysteine and glutathione. It has been observed, however, that enzymic sulphite formation from thiosulphate with glutathione or cysteine as substrates was approximately proportional to the enzyme concentration at a low reaction rate. Therefore in all cases enzymes were suitably diluted prior to incubation to produce not more than 0.3 μmoles of sulphite per sample.

The protein contents of the preparations were measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Dihydrolipoate was prepared freshly before the experiment from DL-lipoic acid (Koch-Light, Bucks, England) by reduction with borohydride in alkaline medium as described by Volini & Westley (1966). After removal of excess borohydride by acidification the pH was suitably adjusted and dihydrolipoate was diluted with an appropriate buffer to a concentration of 25 μmoles/ml. Reduced glutathione and cysteine were supplied by B.D.H. (Poole, England), and other reagents used were of analytical purity.

## RESULTS

Data contained in Table 1 enable a comparison to be made of substrate specificity of beef liver rhodanese incubated with thiosulphate and various sulphur acceptors at pH 8.8.

### Table 1

*Rhodanese-catalysed sulphite formation from thiosulphate in the presence of different sulphur acceptors*

Crystalline beef liver rhodanese prepared by the method of Green & Westley (1959) was incubated for 15 min. in 37° at pH 8.8 with 25 μmoles of thiosulphate and the appropriate sulphur acceptor as described in Methods. Activity of enzyme is expressed in μmoles of sulphite formed by 1 unit of rhodanese (R.U.).

| Sulphur acceptor | Sulphite formed | |
|---|---|---|
| | μmoles/R.U. | Relative activity |
| KCN | 8.02 | 100.00 |
| $NaBH_4$ | 0.556 | 6.93 |
| $Lip(SH)_2$ | 0.456 | 5.68 |
| Cysteine | 0.283 | 3.52 |
| Glutathione | 0.164 | 2.04 |

In these conditions all the substrates tested were active sulphur acceptors although great differences between cyanide and thiols were evident. It appears also that glutathione was less active than cysteine and this is in striking discrepancy with the highest activity of GSH observed in experiments on the metabolism of thiosulphate in mitochondrial preparations (Koj & Frendo, 1967). Therefore the reaction catalysed by rhodanese in the presence of glutathione was studied in detail and its dependence on pH established: an optimum was found in a strongly alkaline

medium (Fig. 1). When fresh homogenate of beef liver was examined at pH 6.9 - 9.3 and the results referred to the rhodanese content of this preparation, an apparently different curve was obtained. The discrepancy between the two curves is clearly visible at lower pH values and can hardly be explained by the presence of unknown activator(s) of rhodanese in tissue preparations. When the observed differences in sulphite formation between the full homogenate and crystalline rhodanese were plotted on the same graph (Fig. 1, dashed line) a characteristic curve was obtained with an apparent optimum near pH 7.4. The most plausible explanation for this is

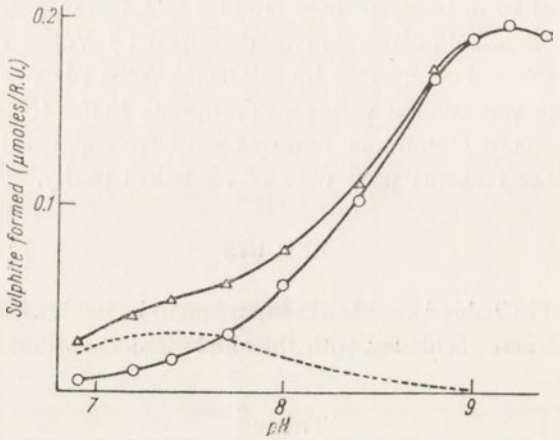


Fig. 1. Reduction of thiosulphate in the presence of glutathione, catalysed by (○), crystalline rhodanese and (△), by fresh homogenate of beef liver. The dashed line denotes the difference between the reduction catalysed by crystalline rhodanese and by the homogenate. The activity is expressed in μmoles of sulphite formed by 1 unit of rhodanese (R.U.).

the existence of a separate enzyme, glutathione-dependent thiosulphate reductase, as was already suggested to explain the oxidation of thiosulphate in animal liver (Sörbo, 1964; Koj & Frendo, 1967).

If rhodanese is responsible only for a part of the sulphite formed in the presence of glutathione, characteristic changes in the rate of reaction may be anticipated during the purification of rhodanese from beef liver homogenate. This is illustrated by the data contained in Table 2.

It can be readily observed that the most marked fall in the rate of thiosulphate reduction by the enzymic preparation occurred during ammonium sulphate fractionation in acid solution, and this might be used in attempts to separate the two enzymes. Preliminary experiments, however, showed that thiosulphate reductase was easily inactivated by many of the techniques commonly used for purification of enzymes, especially at pH values below 5.0. Thus attempts to purify thiosulphate reductase were only partially successful and are summarized in Table 3. All the operations were carried out at 4° unless otherwise stated.

*Step 1.* Beef liver was homogenized with 2 volumes of acetate-glycine buffer, both 0.1 M (pH 5.0), and insoluble material was centrifuged off at 12 000 *g* for 20 min.

Table 2

*Changes in rate of sulphite formation from thiosulphate and glutathione observed during purification of beef liver rhodanese*

Enzyme was isolated from the frozen beef liver by the method of Sörbo (1955) and the different fractions correspond to the main steps in the purification procedure. Sulphite formation in the appropriate fraction was measured with thiosulphate and glutathione at pH 7.4 as described in Methods.

| Fraction | Rhodanese activity R.U./mg. protein | μmoles sulphite/R.U. |
|---|---|---|
| Full homogenate | 0.65 | 0.062 |
| $(NH_4)_2SO_4$, pH 3.8 | 14.9 | 0.032 |
| $(NH_4)_2SO_4$, pH 7.9 | 31.4 | 0.024 |
| Acetone fractionation | 79.6 | 0.018 |
| $(NH_4)_2SO_4$, pH 4.5 | 193.2 | 0.017 |
| Crystalline enzyme | 228.0 | 0.017 |

Table 3

*Separation of beef liver thiosulphate reductase from rhodanese*

Activity of enzymes expressed in μmoles of sulphite formed at pH 7.4 in the presence of glutathione as described in Methods. Rhodanese content in the preparations was estimated by the method of Sörbo (1955), and sulphite formed by rhodanese was calculated assuming that 1 R.U. corresponds to 0.017 μmoles $SO_3^{2-}$ (see Table 2). Sulphite formed by thiosulphate reductase represents the difference between the total amount of $SO_3^{2-}$ produced in the sample and the amount of sulphite deriving from the action of rhodanese.

| Preparation | Sulphite formed by 1 ml. of preparation | | | | | Thiosulphate reductase | |
|---|---|---|---|---|---|---|---|
| | Total (μmoles) | formed by rhodanese | | formed by thiosulphate reductase | | μmoles sulphite/mg. protein | Recovery (%) |
| | | μmoles | % of total | μmoles | % of total | | |
| Homogenate | 1.81 | 0.65 | 36.0 | 1.16 | 64.0 | 0.026 | 100 |
| Acetate-glycine extraction | 1.60 | 0.59 | 37.0 | 1.01 | 63.0 | 0.045 | 79 |
| $(NH_4)_2SO_4$, pH 5.0 | 2.95 | 0.67 | 22.5 | 2.28 | 77.5 | 0.069 | 51 |
| $(NH_4)_2SO_4$, pH 7.9 | 2.82 | 0.38 | 13.5 | 2.44 | 86.5 | 0.092 | 39 |
| Acetone fractionation | 1.46 | 0.10 | 7.0 | 1.36 | 93.0 | 0.053 | 18 |
| Gel filtration (Sephadex) | 0.73 | 0.01 | 1.4 | 0.72 | 98.6 | 0.009 | 3 |

*Step 2.* To the supernatant solid $(NH_4)_2SO_4$ was added (15 g./100 ml.) and the pH adjusted to 5.0 with cold 1 N-HCl. The precipitate obtained was centrifuged off and discarded. The concentration of ammonium sulphate was then increased by adding 20 g. $(NH_4)_2SO_4$ to 100 ml. of supernatant and after 2 hr. the active precipitate

was collected, dissolved in 0.05 M-$(NH_4)_2HPO_4$ containing 0.005 M-thiosulphate and dialysed against this solution.

*Step 3a.* To 1 volume of enzyme preparation from Step 2, 0.5 volume of 3.25 M-ammonium sulphate, pH 7.9, was added. After 1 hr. the precipitate was centrifuged off and discarded, and the ammonium sulphate concentration was increased by adding 0.8 volume of 3.25 M-ammonium sulphate per volume of initial enzyme solution. The active precipitate was collected after 1 hr. and dialysed against 0.05 M-$(NH_4)_2HPO_4$ containing 0.005 M-thiosulphate.

*Step 3b.* The fractionation with an alkaline solution of ammonium sulphate was repeated by mixing 1 volume of enzyme preparation from Step 3a with 1.3 volume of 3.25 M-ammonium sulphate at pH 7.9. After 2 hr. the precipitate was collected and dialysed as above.

*Step 4a.* The enzyme preparation from Step 3b was dialysed for 8 to 10 hr. against 50 volumes of 0.02 M-acetate-glycine buffer, pH 5.0. Any precipitate was centrifuged off and cold acetone (-10°) was added to 25% concentration. The deposit after centrifuging at 10 000 *g* for 10 min. in -10° was suspended in 0.05 M-$(NH_4)_2HPO_4$ containing 0.005 M-thiosulphate and dialysed against this solution. After removing the insoluble material by centrifuging, an opalescent greenish solution was obtained.

*Step 4b.* As an alternative procedure the enzyme preparation after Step 3b was subjected to gel filtration on a column of Sephadex G-150. The solution used for equilibration and elution contained 0.3 M-NaCl, 0.05 M-$(NH_4)_2HPO_4$ and 0.005 M-thiosulphate. 15 ml. of enzyme preparation (equivalent to 0.5 g. protein) was applied to the column (5×60 cm.) and separation carried out in the cold room using a self-recording fraction collector (Uvicord). The effluent was checked for rhodanese activity and pooled into 4 major fractions which were concentrated by pressure dialysis. Rhodanese was confined to the last fraction and its specific activity

Table 4

*Reduction of thiosulphate by beef liver thiosulphate reductase and rhodanese in the presence of different sulphur acceptors at* pH *7.4*

Enzymes used in the experiment: crystalline beef liver rhodanese prepared by the method of Sörbo (1955) and thiosulphate reductase after acetone fractionation with suitable corrections for rhodanese present in the preparation (see Table 3).

| Substrate | Thiosulphate reductase | | Rhodanese | |
|---|---|---|---|---|
| | μmoles sulphite/mg. protein | relative activity | μmoles sulphite/R.U. | relative activity |
| $Lip(SH)_2$ | 0.120 | 1.00 | 0.044 | 1.00 |
| Glutathione | 0.054 | 0.45 | 0.017 | 0.39 |
| Cysteine | 0 | 0 | 0.034 | 0.78 |
| KCN | 0 | 0 | 4.80 | 109.0 |
| $NaBH_4$ | 0 | 0 | 0 | 0 |

was increased about eightfold by this procedure. Thiosulphate reductase virtually free of rhodanese could be detected in the first fraction containing proteins of high molecular weight, but only a small proportion of initial activity was recovered. No better recovery could be achieved by changing the pH and composition of the elution buffer. Also mixing the protein fractions to reconstitute the initial enzyme solution did not reactivate the thiosulphate reductase.

Preparations obtained after acetone fractionation or gel filtration were used for studying substrate specificity of the enzyme. A broad plateau of maximum activity was observed around pH 7.4 as might be anticipated from Fig. 1 (dashed line). Thus all the experiments were carried out at this pH and results are summarized in Table 4 for comparison with crystalline beef liver rhodanese.

## DISCUSSION

Sulphite is a common product in all the reactions leading to the reductive breakdown of thiosulphate regardless of the fate of the bivalent sulphur atom which can be transferred either to cyanide with formation of thiocyanate, or to a thiol compound with formation of persulphide (Sörbo, 1962; Villarejo & Westley, 1963) or even directly reduced to hydrogen sulphide by borohydride. Thus the measurement of sulphite formation enabled a direct comparison of rhodanese specificity toward different sulphur acceptors with thiosulphate as a sulphur donor (Table 1). From the biological point of view the rhodanese-catalysed reaction between thiosulphate and cyanide seems to be of minor importance and the data from present experiments and in the current literature summarized in Table 5 emphasize the real function of rhodanese as a polyvalent sulphur transferase.

Table 5

*Rhodanese as a polyvalent sulphur transferase*

| Sulphur donors | Sulphur acceptors | Products |
|---|---|---|
| Thiosulphate[a] | Cyanide[a] | Thiocyanate |
| Organic thiosulphonate[b,c] | Sulphite[c,e] | Thiosulphate |
| | Sulphinates[c] | Thiosulphonates |
| Bisulphides, polysulphides, | Borohydride[d] | Hydrogen sulphide |
| organic persulphides[d,e,f,g] | Dihydrolipoate[d] | Organic persulphides |
| | Glutathione | generally unstable |
| | Cysteine | yielding organic |
| | Other thiols[c,d] | disulphide and |
| | | hydrogen sulphide |

[a]Lang (1933). [b]Sörbo (1957). [c]Sörbo (1962). [d]Villarejo & Westley (1963). [e]Szczepkowski (1961). [f]Sörbo (1960). [g]Szczepkowski & Wood (1967).

It can be easily observed that some of the products in the rhodanese-catalysed trans-sulphuration are also substrates and in such a case the reaction is apparently reversible with the equilibrium dependent on relative concentrations of particular

substrates. Szczepkowski & Wood (1967) pointed out recently that thiocysteine, thiocystine and corresponding persulphide of glutathione are very efficient sulphur donors for rhodanese. This may explain why cysteine and glutathione were consistently not recognized as sulphur acceptors for rhodanese (Sörbo, 1962; Villarejo & Westley, 1963): decomposition of thiosulphate in the presence of these thiols is slow due to formation of reactive organic persulphides. Nevertheless, a sensitive method based on sulphite estimation indicates that rhodanese present in liver homogenate substantially participates in the glutathione-dependent reduction of thiosulphate (Fig. 1). From the observed ratio of thiosulphate reduction in the complete homogenate and by corresponding amounts of crystalline rhodanese it is possible to calculate that at pH 7.4 nearly 35% of the sulphite formed may be attributed to the action of rhodanese and 65% to thiosulphate reductase (see also Table 3).

The existence in animal liver of a separate enzyme — thiosulphate reductase distinct from rhodanese, was postulated during studies on oxidation of thiosulphate (Sörbo, 1964; Koj & Frendo, 1967) but decisive proof was provided by the recognition of different optimum pH values for both enzymes (Fig. 1). Further evidence is connected with substrate specificity: thiosulphate reductase from beef liver is inactive using cyanide and cysteine as sulphur acceptors (Table 4). Measurement of thiosulphate reductase activity in tissue preparations containing also rhodanese is possible by estimation of the amount of sulphite formed in the presence of glutathione or dihydrolipoate and making an allowance for the rhodanese-catalysed reaction (the rhodanese content must be measured independently, preferably with cyanide as the sulphur acceptor).

Although thiosulphate reductase virtually free of rhodanese was isolated from beef liver (Table 3) the efficiency of the procedure was unsatisfactory. At present it is not known whether the observed inactivation of the enzyme was due to denaturation of protein or loss of some activators. Further studies on the properties of thiosulphate reductase will only be profitable after isolation of highly active enzyme free of rhodanese.

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## ENZYMATYCZNA REDUKCJA TIOSIARCZANU W PREPARATACH Z WĄTROBY WOŁU

Streszczenie

1. Krystaliczna „rhodanese" z wątroby wołu katalizuje redukcję tiosiarczanu do siarczynu w pH 8.8 w obecności cyjanku, borowodorku, dwuhydrolipoinianu, cysteiny lub glutationu, przy względnej aktywności substratów zmniejszającej się w podanej kolejności.

2. Zależna od glutationu redukcja tiosiarczanu obserwowana w świeżym homogenacie z wątroby wołu katalizowana jest przez dwa enzymy: „rhodanese" i reduktazę tiosiarczanu.

3. Reduktaza tiosiarczanu wykazuje maksimum aktywności w pH 7.4 i jest aktywna wobec glutationu i dwuhydrolipoinianu, a nieaktywna wobec cyjanku, borowodorku i cysteiny.

4. Podano metodę oznaczania aktywności reduktazy tiosiarczanu w preparatach tkankowych oraz rozdziału tego enzymu od „rhodanese".

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ZOFIA POREMBSKA, INA GĄSIOROWSKA and IRENA MOCHNACKA

# ISOLATION OF ARGINASE AND GUANIDINOBUTYRATE UREOHYDROLASE FROM HEPATOPANCREAS OF *HELIX POMATIA*

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1. In extract from hepatopancreas of *Helix pomatia*, urea was found to be formed not only from arginine but also from γ-guanidinobutyrate. 2. Arginase and guanidinobutyrate ureohydrolase were isolated and purified 200- and 10-fold, respectively. 3. Both enzymes had the same pH optimum at 9.5 and both were activated by $Mn^{2+}$ ion. 4. Arginase activity was found in the postmicrosomal supernatant (60%) and in mitochondria (30%); guanidinobutyrate ureohydrolase activity was present mainly in mitochondria (80%), the remainder being found in nuclei.

Similarly to arginine, also other guanidino compounds may undergo enzymic hydrolysis to urea in tissues of some animals, in fungi and bacteria. Already Thomas (1913) demonstrated the formation of urea from γ-guanidinobutyrate in preparations from dog and rabbit livers. Sano (1941) demonstrated the degradation of various guanidino compounds in rabbit intestine mucosa, and suggested the presence of an enzyme possessing a wide specificity, which he called heteroarginase. The activity of such an enzyme has been demonstrated by Akamatsu & Kobayashi (1951) in rabbit and hog liver, by Roche, Mourgue & Baret (1953) in chicken kidney and by Pisano, Abraham & Udenfriend (1963) in hog kidney. Baret, Mourgue & Broc (1961, 1962) observed that in extracts from the liver of a fish, *Raia clavata*, after heating at 68° for 15 min, the activity of arginase disappeared while the activity toward γ-guanidinobutyrate persisted. Mora, Tarrab, Martuscelli & Soberón (1965) from livers of two uricotelic species, lizard and chicken, separated from arginase a protein active toward γ-guanidinobutyrate. In *Streptomyces griseus*, which does not possess arginase, Thoai, Thome-Beau & Dang Ba Pho (1962) and Thoai, Thome-Beau & Olomucki (1965) demonstrated the formation of urea exclusively from γ-guanidinobutyrate.

In hepatopancreas of *Helix pomatia*, Baldwin (1935a,b) demonstrated the occurrence of arginase; the enzyme has been found by Porembska & Heller (1962) also in other tissues of this snail. In the present work it has been found that extracts from hepatopancreas of *Helix pomatia* catalyse formation of urea not only

from arginine but also from γ-guanidinobutyrate. Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) and guanidinobutyrate ureohydrolase were isolated and the properties of the purified enzymes were studied.

## MATERIALS AND METHODS

*Reagents.* L-Arginine hydrochloride and L-ornithine hydrochloride (Sigma Chemical Co., St. Louis, Mo., U.S.A.), L-glycine (Biuro Obrotu Odczynnikami Chemicznymi, Gliwice, Poland), cetyl trimethylammonium bromide (British Drug Houses, Ltd., Poole, Dorset, England), xanthone (E. Merck GMBH, Munchen, Germany), tris (Fluka A.G., Buchs SG., Switzerland), ninhydrin (Politechnika Śląska, Gliwice, Poland), crystalline bovine serum albumin (Pentox. Incorp., Kankakee, U.S.A.), Whatman DEAE-cellulose DE 11 (Balston Ltd., England), DEAE-Sephadex A-50 medium (Pharmacia, Uppsala, Sweden). γ-Guanidinobutyric acid was prepared after Schütte (1943).

*Extract from hepatopancreas.* For experiments, hibernating snails *Helix pomatia* were used; they were collected in autumn and kept at 5°. The snails were dissected, the hepatopancreas was isolated and homogenized with 10 volumes of 0.01% solution of cetyl trimethylammonium bromide in a glass Potter-type homogenizer with a teflon pestle. After homogenization at 1900 rev./min. twice for 2 min., the homogenate was left for 1 hr. at 0° with gentle stirring. Then it was centrifuged for 20 min. at 5000 rev./min., the sediment was discarded, and the extract used for experiments.

*Enzyme assays.* Arginase activity was determined in 1 ml. of a mixture containing the enzyme preparation, 50 μmoles of L-arginine, 5 μmoles of $MnCl_2$ and 50 μmoles of glycine buffer, pH 9.5. After 10 min. incubation at 25°, 2 ml. of 83% (v/v) acetic acid was added and after centrifuging, in the supernatant urea was determined by the xanthydrol method according to Engel & Engel (1947), or ornithine was determined by the method of Chinard (1952).

Guanidinobutyrate ureohydrolase activity was determined by incubating the enzyme preparation with 25 μmoles of guanidinobutyrate, 5 μmoles of $MnCl_2$ and 50 μmoles of glycine buffer, pH 9.5. Final volume of the mixture was 1.5 ml., temperature 25°. After 1 hr. the reaction was stopped by adding 2 ml. of 83% acetic acid, and after centrifuging, in the supernatant urea was determined according to Engel & Engel (1947).

Arginase activity was expressed in μmoles of urea or ornithine formed per 1 mg. of protein during 1 min., and activity of guanidinobutyrate ureohydrolase in μmoles of urea formed per 1 mg. of protein per 1 hr.

*Protein determination.* Most of the determinations were carried out according to Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as standard. For the subcellular fractions the same method was used, but the protein was previously precipitated with 5% trichloroacetic acid, washed twice, and dissolved in 0.1 N-NaOH. In the course of purification of the enzymes, the content of protein

was assayed by measuring the extinction at 260 and 280 mμ according to Warburg & Christian (1941).

*Subcellular fractions.* These were prepared according to Hogeboom (1960). The hepatopancreas was homogenized with 10 vol. of 0.25 M-sucrose in a Potter-type glass homogenizer with a teflon pestle. After differential centrifugation, the separated fractions were washed and suspended in 0.05 M-Na,K-phosphate buffer, pH 7.5, gently homogenized and then frozen. On the next day the activity of the two enzymes was assayed.

## RESULTS

### *Activity of the extract from hepatopancreas*

To extract arginase from the hepatopancreas of *Helix pomatia* it sufficed to homogenize the tissue twice for 2 min. in a 0.01% solution of cetyl trimethylammonium bromide. However, to obtain in the extract a maximal activity toward γ-guanidinobutyrate, it was necessary to keep the homogenate for 1 hr. at a temperature of about 0° with gentle stirring. In the extract obtained in this way, the arginase activity exceeded greatly that of guanidinobutyrate ureohydrolase. The extract containing 1 mg. of protein decomposed within 10 min. 20 μmoles of arginine and only 0.06 μmole of γ-guanidinobutyrate. Therefore for estimation of the activity of guanidinobutyrate ureohydrolase, a much greater amount of protein was taken, and the time of incubation was prolonged to 1 hour.

Degradation of arginine was proportional to the amount of protein in the sample over the range of 0.5 - 3 μg., and with 2 μg. of protein it was linear with time for 10 min. of incubation (Fig. 1a,b). Hydrolysis of γ-guanidinobutyrate was proportional with 1 - 8 mg. of protein, and with 6 mg. it was linear with time for 1 hr. of incubation (Fig. 2a,b). The optimum pH values for the two enzymic reactions were the same (Fig. 1c and 2c).

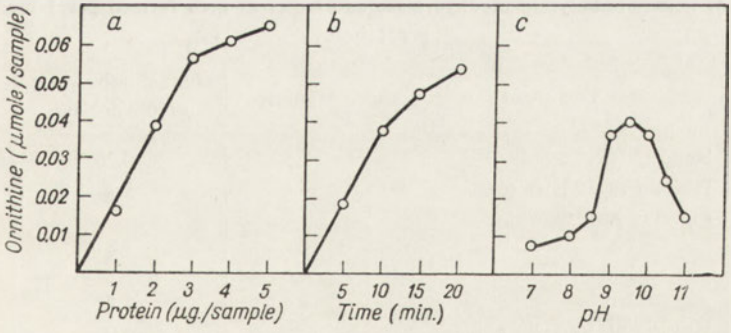


Fig. 1. Effect on arginase activity in hepatopancreas extract of (*a*), protein concentration, (*b*), time of incubation, and (*c*), pH value. The incubation mixture contained in 1 ml.: 50 μmoles of L-arginine, 50 μmoles of glycine buffer, and (*a*), varied amount of protein, pH 9.5, incubation 10 min; (*b*), 2 μg. of protein, pH 9.5; (*c*), 2 μg. of protein, varied pH value, incubation 10 min.

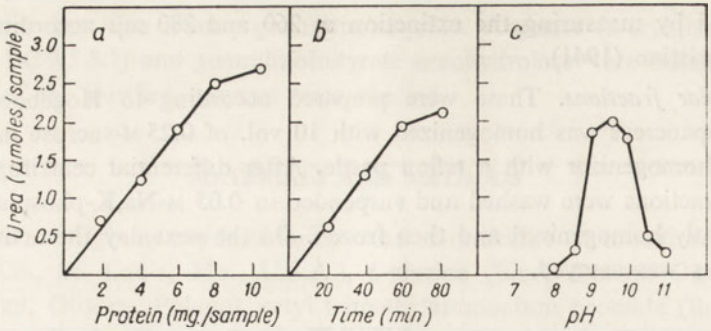


Fig. 2. Effect on guanidinobutyrate ureohydrolase activity in hepatopancreas extract of (*a*), protein concentration, (*b*), time of incubation, and (*c*), pH value. The incubation mixture contained in 1.5 ml.: 25 μmoles of γ-guanidinobutyrate, 50 μmoles of glycine buffer, and (*a*), varied amounts of protein, pH 9.5, incubation 1 hr.; (*b*), 6 mg. of protein, pH 9.5; (*c*), 6 mg. of protein, incubation 1 hour.

The extract kept at 0 - 2° for 2 - 3 days retained full activity toward both substrates. Repeated (6 - 8 times) freezing and thawing of the extract did not affect the activities. The protein after precipitation with ammonium sulphate could be stored for at least 3 months.

Heating of the extract for 90 sec. at 80° or 100° increased the specific activities of both enzymes and had no effect on total activities (Table 1). However, freezing and thawing following heating to 100°, destroyed the arginase and had no effect on guanidinobutyrate ureohydrolase. Its activity was unaltered even after storage in frozen state for several months and repeated freezing and thawing.

Table 1

*The effect of heating on the activities of arginase and guanidinobutyrate ureohydrolase in hepatopancreas extracts*

The extract after heating and after freezing (-10°) and thawing cycle was centrifuged to remove the insoluble material. Activities of the enzymes were determined as described in Methods. The activity of arginase is expressed as μmoles of ornithine formed per 1 mg. of protein per 1 min., the activity of guanidinobutyrate ureohydrolase as μmoles of urea formed per 1 mg. of protein per 1 hour.

| Treatment | Arginase | Guanidinobutyrate ureohydrolase |
|---|---|---|
| None | 2.5 | 0.26 |
| Heating at 80°, 90 sec. | 4.4 | 1.2 |
| Freezing and thawing | | |
| 1st | 4.4 | 1.8 |
| 2nd | 4.4 | 1.8 |
| 3rd | 4.4 | 1.8 |
| Heating at 100°, 90 sec. | 72 | 3.0 |
| Freezing and thawing | | |
| 1st | 20 | 3.5 |
| 2nd | 0 | 3.6 |
| 3rd | 0 | 3.6 |

Almost all the arginase activity of the crude extract was precipitated by ammonium sulphate at 0.25 - 0.45 saturation. On the other hand, guanidinobutyrate ureohydrolase activity was present in the fraction precipitated at 0.45 - 0.75 saturation.

The extract from muscle of *Helix pomatia*, which possessed arginase activity, did not hydrolyse $\gamma$-guanidinobutyrate.

### *Isolation of arginase*

All manipulations were carried out in a cold room at 4°. The course of a typical purification is presented in Table 2.

*Ammonium sulphate precipitation.* To a crude extract from 12 g. of hepatopancreas, ammonium sulphate was added to 0.25 saturation at pH 7. After 1 hr. the inactive sediment was centrifuged off and the supernatant was brought to 0.45 saturation. After 1 hr. the sediment was collected, suspended in 20 ml. of 0.01 M-tris-HCl buffer, pH 7, and dialysed overnight against 5 litres of the same buffer. The preparation contained almost 80% of the arginase activity present in the crude extract, and only 5% of the guanidinobutyrate ureohydrolase activity.

*Heating.* The dialysed solution was heated to 60°, kept for 15 min. at this temperature with gentle stirring, then cooled rapidly to 0 - 2°. The sediment was removed by centrifuging. In the supernatant, total arginase activity was recovered, the specific activity being threefold increased.

Table 2

*Purification of arginase from hepatopancreas of* Helix pomatia

For isolation, 12 g. of hepatopancreas was taken. Details of each step are described in the text.

| Step of purification | Volume (ml.) | Total protein (mg.) | Arginase | | | | Guanidinobutyrate ureohydrolase | |
|---|---|---|---|---|---|---|---|---|
| | | | Total act. | Sp. act. | Purification factor | Recovery (%) | Total act. | Sp. act. |
| 1. Extract | 110 | 1760 | 4576 | 2.6 | — | 100 | 440 | 0.25 |
| 2. Ppt. at 0.25 - 0.45 $(NH_4)_2SO_4$ sat. | 20 | 800 | 3360 | 4.2 | 1.6 | 73 | 24 | 0.03 |
| 3. Heating at 60°, 15 min. | 19 | 361 | 3249 | 9.0 | 3.4 | 71 | 21 | 0.06 |
| 4. Ppt. by acetone | 10 | 183 | 3294 | 18.0 | 6.9 | 71 | 11 | 0.06 |
| 5. DEAE-cellulose fractions *II* and *III* | 120 | 13 | 2860 | 220 | 85 | 62 | 9 | 0.08 |
| 6. Ppt. at 0.75 $(NH_4)_2SO_4$ sat. | 5 | 9 | 2430 | 270 | 104 | 53 | 4.5 | 0.05 |
| 7. DEAE-Sephadex | 50 | 4.2 | 2168 | 516 | 198 | 47 | 0 | 0 |
| 8. Ppt. at 0.75 $(NH_4)_2SO_4$ sat. | 5 | 3.8 | 2128 | 560 | 216 | 46 | 0 | 0 |

*Acetone precipitation.* To the supernatant obtained after heating, 1.5 vol. of acetone was slowly added with stirring at -10°. The sediment was centrifuged at -10° and air-dried at room temperature. Then it was dissolved in 10 ml. of 0.01 M-tris-HCl buffer, pH 8.3, and dialysed for 2 hr. at 0° against 2 litres of the same buffer under constant stirring. Acetone precipitated total arginase activity, resulting in its twofold purification. Simultaneously, contamination by guanidinobutyrate ureohydrolase was reduced.

*Chromatography on DEAE-cellulose.* The dialysed acetone precipitate was applied to a column (1.5×35 cm.) of DEAE-cellulose equilibrated with 0.005 M-tris-HCl buffer, pH 8.3. Elution was with the same buffer alone and then with a gradient of KCl up to 0.3 M. The flow rate was 16 - 18 ml. per hour. Ten-ml. portions were collected, in which protein and the activity of both enzymes were determined (Fig. 3). From 183 mg. of protein applied to the column, 17.5 mg. was eluted, and the whole arginase activity. Four protein peaks were obtained. The first was eluted with the buffer alone; it represented about 26% of the recovered protein and contained 15% of the total arginase activity (fraction *I*). Further protein peaks were eluted with a KCl gradient, and the arginase activity was separated into two fractions (*II* and *III*). Fraction *III* contained some guanidinobutyrate ureohydrolase activity.

*Ammonium sulphate fractionation.* DEAE-cellulose fractions *II* and *III* (protein eluted between 0.06 and 0.175 M-KCl gradient) were pooled. Then ammonium sulphate was added to 0.75 saturation; the precipitated protein was dissolved in 4 ml. of 0.005 M-tris-HCl buffer, pH 7.0, and dialysed overnight against 5 litres of the same buffer. The arginase preparation was purified 100-fold in relation to the crude extract but it still contained guanidinobutyrate ureohydrolase.

*Chromatography on DEAE-Sephadex.* The dialysed solution was applied to the DEAE-Sephadex A-50 column (1×22 cm.). The elution was performed with a gradient of KCl in 0.005 M-tris-HCl buffer, pH 7. One protein peak was obtained (Fig. 4) which contained arginase activity and was free of guanidinobutyrate ureohydrolase. The enzymic preparation was purified about 200-fold in relation to the activity of the crude extract, and its specific activity exceeded 500 μmoles/mg. protein/min., the yield being almost 50%. The purified enzyme precipitated with ammonium sulphate retained the activity for at least 6 weeks at 0°.

### *Isolation of guanidinobutyrate ureohydrolase*

The course of purification of the enzyme is presented in Table 3.

*Ammonium sulphate fractionation.* The crude extract from 12 g. of hepatopancreas was fractionated in the cold with ammonium sulphate and the precipitate at 0.45 - 0.75 saturation was collected, then suspended in 20 ml. of 0.01 M-tris-HCl buffer, pH 7, and dialysed overnight against 5 litres of the same buffer. Almost all guanidinobutyrate ureohydrolase activity was recovered, and about 5% of the arginase activity present in the extract.

*Heating.* The dialysed solution was heated in a boiling water bath for 90 sec., then cooled rapidly to 0°, and the abundant sediment was centrifuged off. The

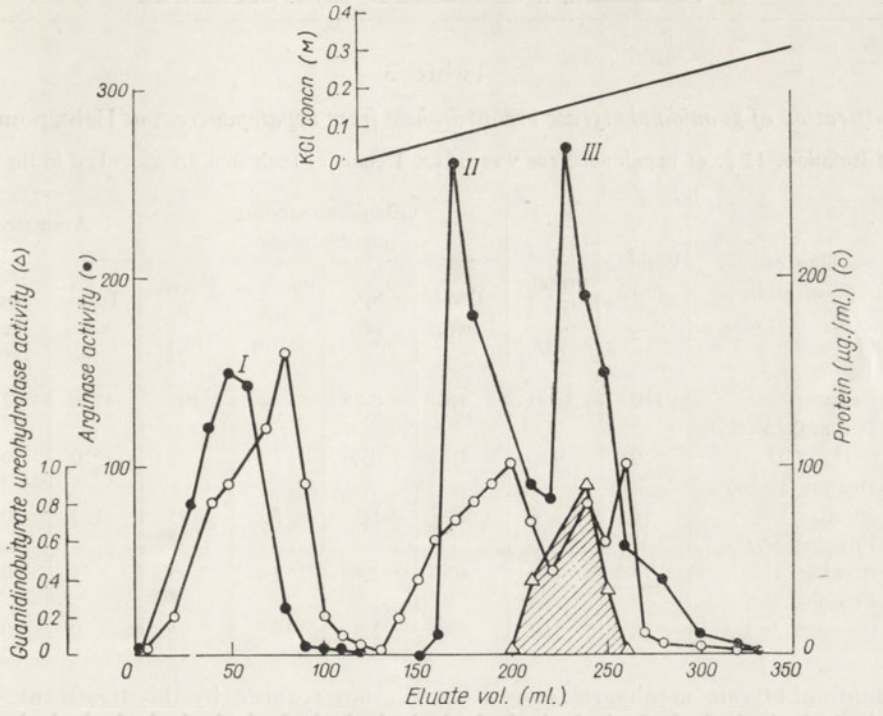


Fig. 3. Chromatography on DEAE-cellulose. The arginase preparation obtained by acetone precipitation, step 4 (183 mg. of protein) in 10 ml. of 0.005 M-tris-HCl buffer, pH 8.3, was applied to the column (1.5×35 cm.) and eluted with the same buffer alone and then with a KCl concentration gradient. (●), Arginase activity, μmoles of ornithine/mg. protein/min.; (△), guanidinobutyrate ureohydrolase activity, μmoles of urea/mg. protein/hr.; (○), protein, μg./ml. of eluate.

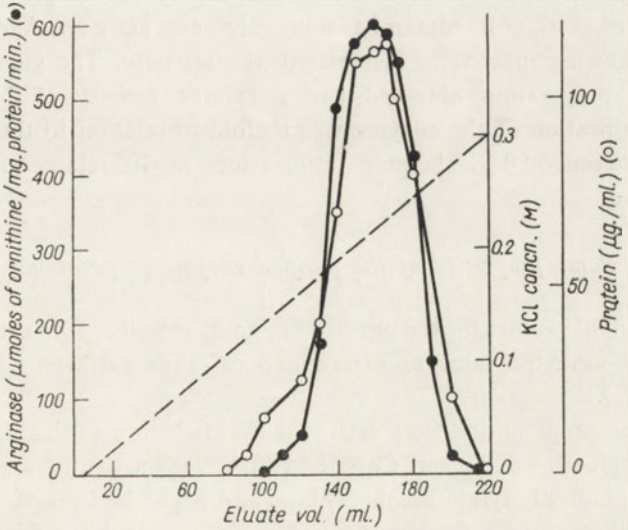


Fig. 4. DEAE-Sephadex chromatography of arginase preparation. The DEAE-cellulose fractions *II* and *III* (Fig. 3) were pooled, the protein precipitated by 0.75 ammonium sulphate sat. was dialysed, then 5 ml. (9 mg. in 0.005 M-tris-HCl buffer, pH 7.0) was applied to the column (1×22 cm.). The elution was carried out with a KCl concentration gradient from 0 to 0.3 M. (●), Arginase activity; (○), protein.

Table 3

*Purification of guanidinobutyrate ureohydrolase from hepatopancreas of* Helix pomatia

For isolation, 12 g. of hepatopancreas was taken. Details of each step are described in the text.

| Step of purification | Volume (ml.) | Total protein (mg.) | Guanidinobutyrate ureohydrolase | | | | Arginase | |
|---|---|---|---|---|---|---|---|---|
| | | | Total act. | Sp. act. | Purification factor | Recovery (%) | Total act. | Sp. act. |
| 1. Extract | 110 | 1760 | 440 | 0.25 | — | 100 | 4576 | 2.6 |
| 2. Ppt. at 0.45 - 0.75 $(NH_4)_2SO_4$ sat. | 20 | 900 | 414 | 0.46 | 1.8 | 94 | 270 | 0.3 |
| 3. Heating at 100°, 90 sec. | 19 | 314 | 408 | 1.3 | 5.2 | 93 | 193 | 0.6 |
| 4. Freezing and thawing, 1st | 18.3 | 170 | 408 | 2.4 | 9.6 | 93 | 51 | 0.3 |
| 5. Freezing and thawing, 2nd | 18 | 160 | 400 | 2.5 | 10 | 91 | 0 | 0 |

guanidinobutyrate ureohydrolase activity was not reduced by this treatment, and the specific activity was increased fivefold in relation to the crude extract.

*Freezing and thawing.* The preparation obtained by heating was frozen and kept overnight at -10°. After thawing, the scanty sediment formed was centrifuged off. Total guanidinobutyrate ureohydrolase activity remained unaltered whereas the specific activity increased almost twofold compared with the previous step. The preparation, however, still contained some arginase activity. Another cycle of freezing and thawing inactivated completely the arginase. The guanidinobutyrate ureohydrolase preparation obtained had a specific activity of 2.5 μmoles/mg. protein/hr. Purification of the enzyme was tenfold in relation to the crude extract and the yield exceeded 90%. The preparation stored at -10° retained full activity for several months.

### *Some properties of the purified enzyme preparations*

The optimum pH values for the purified arginase, as well as for guanidinobutyrate ureohydrolase, were the same as determined in crude extracts, i.e. pH 9.5 (see Figs. 1c and 2c).

Both enzyme preparations were activated by $Mn^{2+}$ ion (Table 4) although the activation of arginase was higher. $Co^{2+}$ and $Cd^{2+}$ ions activated arginase and had no effect on guanidinobutyrate ureohydrolase, and $Mg^{2+}$ had no effect on arginase.

The Michaelis constant for the purified arginase calculated from Lineweaver-Burk plots was 2.6 mM (Fig. 5).

*Subcellular distribution of arginase and guanidinobutyrate ureohydrolase.* The results of determination of the activity of the two enzymes in the subcellular fractions of hepatopancreas, are shown in Table 5. Guanidinobutyrate ureohydrolase activity

## Table 4

*The effect of bivalent cations on arginase and guanidinobutyrate ureohydrolase*

The experiments were performed on the purified arginase preparation (step 5), and the guanidinobutyrate ureohydrolase preparation obtained by step 5. Composition of the incubation mixture as described in Methods.

| Addition (5 mM) | Arginase | | Guanidinobutyrate ureohydrolase | |
|---|---|---|---|---|
| | Specific activity | Activation factor | Specific activity | Activation factor |
| None | 68 | | 1.8 | |
| $Mn^{2+}$ | 231 | 3.4 | 2.5 | 1.4 |
| $Co^{2+}$ | 147 | 2.2 | 1.8 | 0 |
| $Cd^{2+}$ | 120 | 1.8 | 1.8 | 0 |
| $Mg^{2+}$ | 85 | 1.2 | — | — |

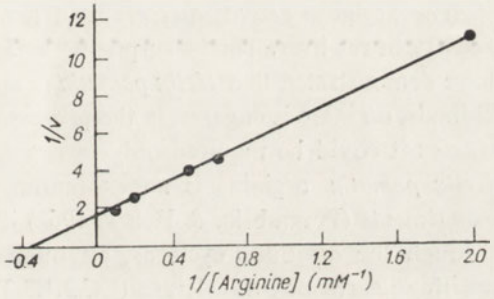


Fig. 5. Lineweaver-Burk plot of the effect of substrate concentration on the activity of arginase. The incubation mixture contained in 1 ml.: 0.8 μg. of protein of the purified arginase preparation, 5 μmoles of $MnCl_2$, 50 μmoles of glycine buffer, pH 9.5, and varying amounts of arginine. After 10 min., the activity of the enzyme was determined by measuring the amount of ornithine formed.

## Table 5

*Subcellular distribution of arginase and guanidinobutyrate ureohydrolase*

Subcellular fractions of hepatopancreas were obtained according to Hogeboom (1960).

| Fraction | Protein (mg.) | Arginase | | | Guanidinobutyrate ureohydrolase | | |
|---|---|---|---|---|---|---|---|
| | | Sp. act. | Total act. | Distribution (%) | Sp. act. | Total act. | Distribution (%) |
| Homogenate | 275 | 4.2 | 1155 | 100 | 1.3 | 357 | 100 |
| Nuclei, 600 *g* | 72 | 1.1 | 77 | 5 | 1.1 | 79 | 22 |
| Mitochondria, 12 000 *g* | 93 | 3.6 | 334 | 30 | 3.1 | 275 | 77 |
| Microsomes, 105 000 *g*, 90 min. | 28.6 | 1.0 | 28 | 2 | 0 | 0 | 0 |
| Supernatant | 71.4 | 9.1 | 646 | 57 | 0 | 0 | 0 |

was present mainly in mitochondria (up to 80% of the activity of the homogenate), the remaining 20% being found in nuclei, whereas of the arginase activity only 30% was present in mitochondria and about 60% in the postmicrosomal supernatant. The arginase activity in nuclei and microsomes was negligible.

## DISCUSSION

The hepatopancreas of *Helix pomatia* was found to possess two separate enzymes catalysing the formation of urea: arginase and guanidinobutyrate ureohydrolase. The two enzymes were separated by ammonium sulphate fractionation, column chromatography, and by taking advantage of the great heat resistance of guanidinobutyrate ureohydrolase.

The role of guanidinobutyrate ureohydrolase remains obscure. Chromatographic analysis of the hepatopancreas extract has demonstrated the presence of $\gamma$-guanidinobutyrate, which gives some indication concerning the physiological role of guanidinobutyrate ureohydrolase. $\gamma$-Guanidinobutyrate may arise from the degradation of arginine by arginine deaminase and decarboxylase, as these enzymes have been found in tissues of many invertebrates (Thoai, Roche & Robin, 1952, 1953).

The arginase activity per gram of hepatopancreas is very high, exceeding the activity in rat liver. This is surprising as Jeżewska, Gorzkowski & Heller (1963a,b) have demonstrated that *Helix pomatia* is uricotelic during its whole life. In ureotelic animals, arginase is engaged in the process of urea biosynthesis whereas in uricotelic species it could be required only to degrade arginine. It is not certain whether in *Helix pomatia* arginine is an exogenous or endogenous amino acid. From our experiments (Porembska & Heller, 1962) it appears that arginine cannot be formed through the ornithine cycle as, although we have demonstrated the presence of ornithine carbamoyltransferase (EC 2.1.3.3), we could not detect argininosuccinate synthetase (EC 6.3.4.5). This may either indicate that arginine is formed through a pathway other than that observed in vertebrates, or point to its exogenous character.

It seems of interest that distribution of arginase in subcellular fractions of the hepatopancreas differs from that found in the ureotelic rat. In contrast to the results of Mora, Martuscelli, Ortiz-Pineda & Soberón (1965) who have demonstrated that in rat liver arginase is present in nuclei, mitochondria and microsomes, and is absent from the supernatant, the hepatopancreas arginase is present in the postmicrosomal supernatant and in mitochondria. This difference is probably related to the different role of the two enzymes.

The Michaelis constants of arginases of ureotelic animals are between 7 and 20 mM (Bach & Killip, 1961; Mora, Martuscelli *et al.*, 1965), and those of arginases of uricotelic species, lizard, chicken, rattlesnake, 100 - 200 mM (Mora, Martuscelli *et al.*, 1965). The $K_m$ of arginase of the uricotelic *Helix pomatia* is of the same order of magnitude as that for "ureotelic" arginase.

The resolution of arginase activity on DEAE-cellulose column (Fig. 3) may indicate that isoenzymes of arginase occur in the hepatopancreas, similarly as it

has been found for arginase from human liver (Bascur, Cabello, Véliz & Gonzáles, 1966; Kalab & Pelican, 1966). It seems possible that the activities found in mitochondria and the supernatant are due to two arginases differing from each other.

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## IZOLOWANIE ARGINAZY I UREOHYDROLAZY GWANIDYNOMAŚLANU Z HEPATOPANCREAS *HELIX POMATIA*


### Streszczenie

1. W wyciągach z hepatopancreas *Helix pomatia* stwierdzono powstawanie mocznika nie tylko z argininy lecz również z $\gamma$-gwanidynomaślanu.

2. Wyizolowano arginazę i ureohydrolazę gwanidynomaślanu i oczyszczono je odpowiednio 200- i 10-krotnie.

3. Oba enzymy posiadają to samo optimum pH 9.5 i oba są aktywowane jonami $Mn^{2+}$.

4. Arginazę stwierdzono w supernatancie pomikrosomalnym (60%) i w mitochondriach (30%), ureohydrolazę gwanidynomaślanu głównie w mitochondriach (80%), a pozostałą część w jądrach komórkowych.


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# ISOLATION OF ISOLEUCINE-SPECIFIC TRANSFER RIBONUCLEIC ACID FROM YELLOW LUPIN SEEDS

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1. By DEAE-cellulose chromatography at 72°, reverse-phase chromatography and countercurrent distribution, an isoleucine-specific tRNA, 80% purity, was isolated from tRNA of *Lupinus luteus* seeds. 2. The isolated $tRNA^{Ile}$ was found to be a mixture of two species which could not be resolved by the isolation procedure but separated partially on hydroxyapatite column chromatography. 3. Some properties and nucleotide composition of $tRNA^{Ile}$ were studied; its similarity to purified tRNA's from other sources is discussed.

In the previous work (Legocki, Szymkowiak, Pech & Pawełkiewicz, 1967a) it has been found that a fraction rich in isoleucine-specific tRNA can be isolated from tRNA of yellow lupin seeds by DEAE-cellulose column chromatography at elevated temperature. In the present work, by combining the above method with the reverse-phase chromatography of Kelmers, Novelli & Stulberg (1965) and with the countercurrent distribution according to Doctor, Apgar & Holley (1961), isoleucine-specific tRNA of 80% purity has been isolated and some properties of the obtained preparation are described. A preliminary account has been presented (Legocki, Szymkowiak, Pech & Pawełkiewicz, 1967b).

## MATERIALS AND METHODS

*Materials.* ATP disodium salt was a product of Reanal (Budapest, Hungary), GTP and GSH were from Sigma Chem. Co. (St. Louis, Mo., U.S.A.), L-amino acids were from Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A.) or Sigma Chem. Co.; tris(hydroxymethyl)aminomethane from British Drug Houses (Poole, Dorset, England), dimethyldilaurylammonium chloride (Aliquat 204, lot no. 5, DW 348) was a product of General Mills (Kankakee, Ill., U.S.A.), Chromosorb W (60 - 80 mesh, acid washed) was obtained from Matheson, Coleman & Ball (Norwood, Cincinnati, Ohio, U.S.A.), 2,5-diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)-benzene from Pilot Chem. Inc. (Watertown, Mass., U.S.A.), DEAE-cellulose from

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Balston (England), Sephadex G-25, and G-200 from Pharmacia (Uppsala, Sweden), cetyltrimethylammonium bromide from Chemapol (Prague, Czechoslovakia), [U-$^{14}$C]isoleucine (36 mc/m-mole) from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia). Yeast RNA was a product of E. Merck (Darmstadt, Germany) and was additionally purified as described by Legocki *et al.* (1967a). Other reagents were obtained from Fabryka Odczynników Chemicznych (Gliwice, Poland).

Transfer RNA (tRNA) from seeds of yellow lupin (*Lupinus luteus*, variety Express) was isolated as described previously (Legocki *et al.*, 1967a) and the fraction purified on Sephadex G-200 column was used for experiments.

Isoleucyl-tRNA synthetase (L-isoleucine : sRNA ligase (AMP), EC 6.1.1.5) was isolated from yellow lupin seeds as described by Legocki & Pawełkiewicz (1967a). For estimation of acceptor activity, a 70-fold purified enzyme preparation was used.

*Analytical methods.* Radioactivity was measured in the liquid scintillator of Bray (1960) with the scintillation counter SE 2 (BUTJ, Warszawa, Poland); the efficiency of counting was 47%. Protein was determined by the turbidimetric method of Mejbaum-Katzenellenbogen (1955). RNA was determined spectrophotometrically at 260 mμ, taking $E^{1\%}_{260}$ to be 192 (Legocki *et al.*, 1967a). Temperature profiles for RNA were determined in a Hilger H 700 spectrophotometer provided with an ultrathermostated water jacket. Temperature was measured with a calibrated thermistor placed in a dummy cuvette.

For determination of the nucleotide composition of tRNA$^{Ile}$, 2 mg. of the preparation was hydrolysed in 0.5 ml. of 0.2 N-NaOH for 45 min. at 80°. The alkaline hydrolysate was immediately submitted to Dowex 1 (formate form) column chromatography according to Holt, Joel & Herbert (1966).

*Determination of acceptor activity of* tRNA$^{Ile}$. This was determined as described previously (Legocki *et al.*, 1967a), 50 - 250 μg. of tRNA depending on the degree of purification, and 0.2 mg. of isoleucyl-tRNA synthetase being taken.

*Isolation of* [$^{14}$C]*isoleucyl*-tRNA$^{Ile}$. This was carried out according to Moldave (1963) as described previously (Legocki *et al.*, 1967a), using 60 $E_{260}$ units of the purified preparation of tRNA$^{Ile}$, 0.6 μmole of [$^{14}$C]isoleucine (6 μc) and 2 mg. of isoleucyl-tRNA synthetase. The isolated [$^{14}$C]isoleucyl-tRNA had a specific activity of about 750 counts/min./$E_{260}$ unit.

*Chromatography of* [$^{14}$C]*isoleucyl*-tRNA$^{Ile}$ *on protamine-coated kieselguhr.* The preparation, 16 $E_{260}$ units (about 12 000 counts/min.), was chromatographed as described previously (Legocki *et al.*, 1967a) on a column (0.65×20 cm.) of protaminated kieselguhr, equilibrated with 0.05 M-potassium phosphate buffer, pH 7.05, containing 0.2 M-NaCl. The elution was done with the same buffer with an NaCl gradient of 0.2 - 1.6 M, fractions of 2.7 ml. being collected.

*Chromatography of* [$^{14}$C]*isoleucyl*-tRNA$^{Ile}$ *on hydroxyapatite column.* The preparation, 18 $E_{260}$ units (about 14 000 counts/min.), was chromatographed as described by Legocki & Pawełkiewicz (1967b) on hydroxyapatite column (1×16 cm.) equilibrated with 0.05 M-sodium phosphate buffer, pH 6.8, and tRNA was eluted by a 0.05 - 0.18 M gradient of the same buffer. Fractions of 3 ml. were collected,

the flow rate being 12 ml./hr. After measuring the extinction at 260 mμ, 0.5 mg. of yeast RNA (in 0.1 ml. of water) was added as carrier to each sample, then RNA was precipitated with 0.6 ml. of 30% trichloroacetic acid. The sediment was centrifuged, washed twice with ethanol, once with ether, and dried at 40°. For radioactivity assay, the RNA was dissolved in 0.3 ml. of water and 6 ml. of the liquid scintillator was added.

*Dissociation of RNA aggregates after countercurrent distribution.* RNA, 20 $E_{260}$ units, was incubated for 15 min. at 70° in 4 M-urea solution containing 0.01 M-potassium phosphate buffer, pH 7.3. The solution was cooled and the nucleic acids were separated from low-molecular compounds by gel filtration on Sephadex G-25 column (1.4×68 cm.), then concentrated until dry under reduced pressure at a temperature not exceeding 40°. The dry residue was dissolved in 1.5 ml. of 1 M-NaCl and chromatographed on a Sephadex G-200 column (1×145 cm.) equilibrated with 1 M-NaCl; fractions of 2.8 ml. were collected. Simultaneously, control samples of RNA which had not been submitted to the action of 4 M-urea, were subjected to gel filtration.

*Purification of isoleucine-specific tRNA*

*Chromatography on DEAE-cellulose column at 72°.* The DEAE-cellulose column (4.2×65 cm.) provided with a water jacket maintained at 72°, was equilibrated with 0.02 M-Na-acetate buffer, pH 6.0, containing 5 mM-$MgCl_2$ and 0.58 M-NaCl. Unfractionated tRNA, 2 g., dissolved in 100 ml. of the above buffer, was applied to the column and eluted with a gradient of NaCl from 0.6 to 1.8 M. Fractions of 17 ml. were collected and in 0.1 ml. portions RNA concentration and acceptor activity were determined. The results of one fractionation are presented in Fig. 1.

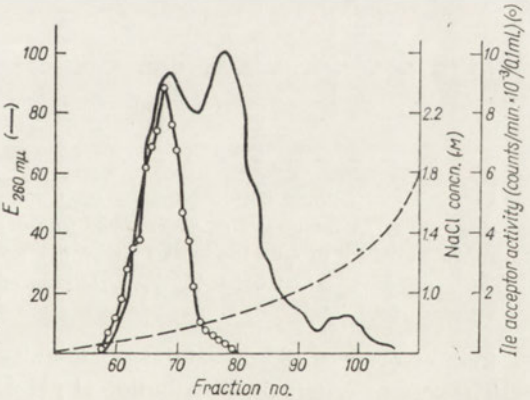


Fig. 1. Fractionation of tRNA from *L. luteus* seeds on DEAE-cellulose column at 72°. tRNA, 2 g., was dissolved in 100 ml. of 5 mM-$MgCl_2$ - 0.58 M-NaCl - 20 mM-sodium acetate buffer, pH 6.0, and applied to a column (4.2×65 cm.) provided with a water jacket and equilibrated with the same buffer. The elution was with 0.6 - 1.8 M-NaCl gradient, fractions of 17 ml. being collected. In the fractions, acceptor activity toward [$^{14}$C]isoleucine was determined.

The $tRNA^{Ile}$-containing fractions eluted between 59 - 73 ml. were pooled and precipitated with 2 vol. of ethanol in the presence of 2% potassium acetate. The sediment was dissolved in a small volume of water and after dialysis freeze-dried. Usually from 2 g. of unfractionated tRNA 450 mg. of the preparation was obtained.

*Reverse-phase chromatography.* The sorbent for reverse-phase chromatography was prepared after Kelmers *et al.* (1965) except that Chromosorb W 60 - 80 mesh

(instead of 120 - 140 mesh) was used, and that the organic phase consisting of 4% (w/v) dimethyldilaurylammonium chloride in isoamyl acetate was washed twice successively with 3 volumes of 1 N-NaOH, 1 N-HCl, 1 M-NaCl and 0.25 M-NaCl. The sorbent was suspended in 0.05 M-tris-HCl buffer, pH 7.25, containing 0.25 M-NaCl and 0.015 M-$MgCl_2$ saturated with isoamyl acetate; it was freed from small particles by repeated decantations, and finally de-aerated under reduced pressure. Then a column (2.1×260 cm.) was prepared and 2 g. of the $tRNA^{Ile}$ preparation, obtained from DEAE-cellulose column chromatography, was applied. The elution was with a gradient of NaCl from 0.25 to 0.75 M in 0.015 M-$MgCl_2$ - 0.05 M-tris-HCl buffer, pH 7.25, saturated with isoamyl acetate; fractions of 17.5 ml. were collected. The distribution diagram is presented in Fig. 2. The $tRNA^{Ile}$-containing fractions eluted between 76 and 105 ml., were pooled and precipitated as described above; 320 mg. of 6.5-fold purified $tRNA^{Ile}$ was recovered.

*Countercurrent distribution.* A fully automatic, thermostated apparatus for 200 transfers, constructed in this laboratory, was used. The volume of phases in the tubes was 6 ml. each. The phases were shaken by applying 30 oscillations of the

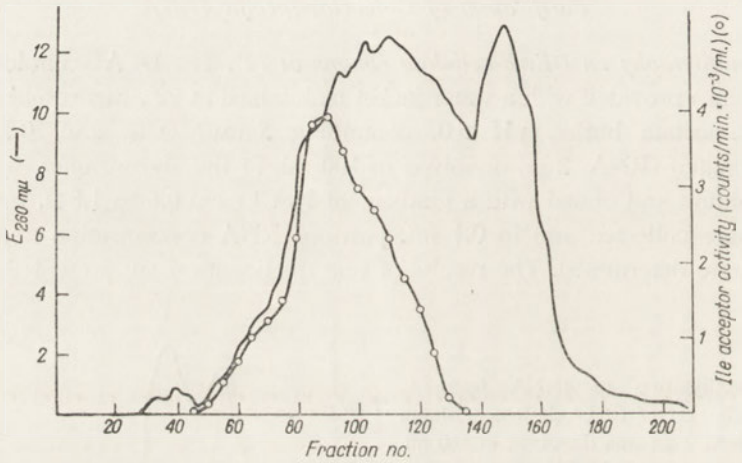


Fig. 2. Reverse-phase chromatography on Chromosorb W of DEAE-cellulose fractions 59 - 73 (Fig. 1). tRNA fraction, 2 g., was applied to a column (2.1×260 cm.) and eluted with 0.25 - 0.75 M-NaCl gradient in 0.05 M-tris-HCl buffer, pH 7.5, saturated with isoamyl acetate and containing 0.015 M-$MgCl_2$. Fractions of 17.5 ml. were collected.

train at a rate of 18 oscillations per minute. The time of phase separation was 8 min. The solvent system for distribution at pH 6.0 was prepared after Doctor *et al.* (1961) in the following way: 555 g. of $K_2HPO_4$ and 870 g. of $NaH_2PO_4 \cdot H_2O$ was dissolved in 5 litres of bidistilled water and $MgCl_2$ was added to a final concentration of 1 mM; then to the solution was added 500 ml. of formamide (freshly distilled under reduced pressure in an atmosphere of nitrogen) and 2200 ml. of isopropanol. The mixture was shaken and left for 10 hr. at 24°. The ratio of the volumes of the upper and lower phases was 2 : 1.

To avoid perturbations in the distribution due to slight differences in the volume

of the individual tubes, the apparatus was filled with the lower phase and 25 transfers of the upper phase were carried out. Then the solvent was removed from the first four tubes and tRNA solution (20 - 45 mg. per tube) was introduced, and 200 transfers were made during 30 hr. at 24°.

The solution of tRNA to be submitted to countercurrent distribution was prepared from the preparation obtained after reverse-phase chromatography, as follows: tRNA preparation, 200 mg., was dissolved in 6 ml. of water, and then were added: 24 ml. of phosphate buffer, pH 6.0 (7 g. of $K_2HPO_4$, 10.9 g. of $NaH_2PO_4 \cdot H_2O$, 0.06 ml. of 1 M-$MgCl_2$, adjusted to 50 ml. with water), 3 ml. of formamide, 13.2 ml. of isopropanol, and 20 ml. of the lower phase of the solvent system and 5 ml. of the upper phase. The mixture was shaken for 12 hr. at 24°, then centrifuged for 10 min. at 6000 *g*, and the insoluble interphase containing about 20% of the applied tRNA, was discarded.

To determine the distribution of tRNA and isoleucine-specific activity, the contents of every fifth tube were shaken with 1 vol. of peroxide-free ethyl ether, then the aqueous phase was desalted on Sephadex G-25 column (3.5×47 cm.) using for elution 0.01 M-potassium acetate, pH 7.0. The ultraviolet-absorbing fractions were flash evaporated under reduced pressure at 35°. The dry residue was dissolved in 2 ml. of bidistilled water, and RNA and acceptor activity were determined in 0.1 ml. samples. The results of countercurrent distribution of $tRNA^{Ile}$ are shown in Fig. 3. Fractions from tubes 40 - 50 were pooled, desalted on Sephadex G-25,

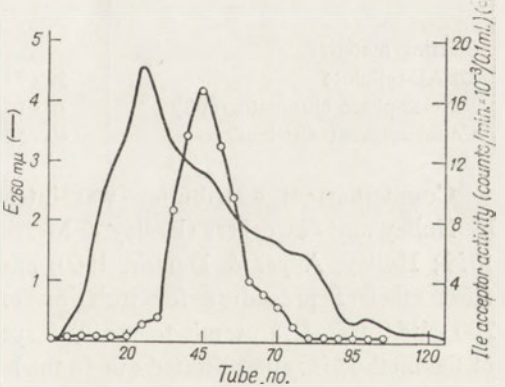


Fig. 3. Countercurrent distribution of reverse-phase chromatography fractions 76 - 105 (Fig. 2). $tRNA^{Ile}$ fraction, 200 mg., was submitted to 200 transfers in phosphate solvent at pH 6.0 and 24°. Details of the distribution are described in the Materials and Methods section.

and $tRNA^{Ile}$ was isolated as described above. In some experiments the procedure described by Zachau, Dütting & Feldmann (1966) was used for recovery of tRNA: the combined upper and lower phases were extracted with ethyl ether, the aqueous phase was diluted with a threefold volume of water in a narrow cylinder and RNA precipitated with cetyltrimethylammonium bromide (4.5 mg./mg. RNA). Then a layer of ethyl ether (0.5 - 1 cm. high) was added, and the mixture was heated for 30 min. on a water bath at 35°. The interphase contained the RNA salts. After siphoning off the aqueous layer, the sediment was collected on a sintered glass filter, washed carefully with a small volume of water to remove inorganic salts, then dissolved in a small volume of ethanol. The solution was made 2% in respect to

sodium acetate and the precipitated RNA after centrifuging and washing with ethanol was dried in vacuum over $P_2O_5$ at room temperature. The recovery was about 90%.

## RESULTS AND DISCUSSION

The increase in specific activity of $tRNA^{Ile}$ at the successive stages of purification is presented in Table 1. The purity of the preparations was calculated taking the molecular weight of $tRNA^{Ile}$ to be 27 000. From 8 g. of unfractionated tRNA, about 25 mg. of the 80% pure $tRNA^{Ile}$ preparation was obtained, which corresponds to 5% of its content in the starting preparation. However, it was not possible to calculate the yield exactly because the isoleucine acceptor activity estimated in the unfractionated tRNA was the sum of activities of all four isoleucine-specific tRNA's, whereas the final product was a mixture of only two. Thus the yield was really somewhat higher. Nevertheless, marked losses occurred during the purification procedure, especially in the reverse-phase chromatography and countercurrent distribution.

Table 1

*Purification of* $tRNA^{Ile}$ *from yellow lupin seeds*

| Purification stage | Fractions or transfer number | Acceptor activity (mμmoles/mg. RNA) | Activity purification factor | Purity (%) |
|---|---|---|---|---|
| Starting material | | 2.4 | 1.0 | 6.5 |
| DEAE-cellulose | 59 - 73 | 6.8 | 2.8 | 18.5 |
| Reverse-phase chromatography | 76 - 105 | 15.8 | 6.5 | 42.7 |
| Countercurrent distribution | 40 - 50 | 28.8 | 12.0 | 77.8 |

Countercurrent distribution was introduced for isolation of specific tRNA's by Holley and coworkers (Holley & Merrill, 1959; Holley, Doctor, Merrill & Saad, 1959; Holley, Apgar & Doctor, 1960) and was found by many authors to be the most efficient procedure for isolation of amino acid-specific tRNA's. Usually 200 - 1000 transfers permit to purify a specific tRNA. On the other hand, the use of the method is rather limited due to the high cost of the apparatus and its restricted capacity. Moreover, the method itself and the isolation of the purified preparation are time-consuming, and the aggregation of tRNA's lowers the yield (Schleich & Goldstein, 1964). That is why in the present work countercurrent distribution was applied only for the final purification of $tRNA^{Ile}$.

In preliminary experiments on countercurrent distribution of unfractionated tRNA, two solvent systems, at pH 6.0 and 8.15, of Doctor *et al.* (1961) were tested. At pH 6.0 (Fig. 4) in contrast to the distribution at pH 8.15 (Fig. 5), the ultraviolet-absorbing material was favourably distributed and three $tRNA^{Ile}$ fractions were obtained. This also confirms the observation of Doctor *et al.* (1961) for tRNA of plant origin that better resolution can be obtained by countercurrent distribution at pH 6.0 than at higher pH values. The obtained three tRNA fractions were

designated by successive numbers (Fig. 4); since the second fraction, as demonstrated in the accompanying paper (Legocki, Szymkowiak, Hierowski & Pawełkiewicz, 1968) was partially resolved on hydroxyapatite column into two subfractions, it was designated $tRNA^{Ile}_{2,3}$.

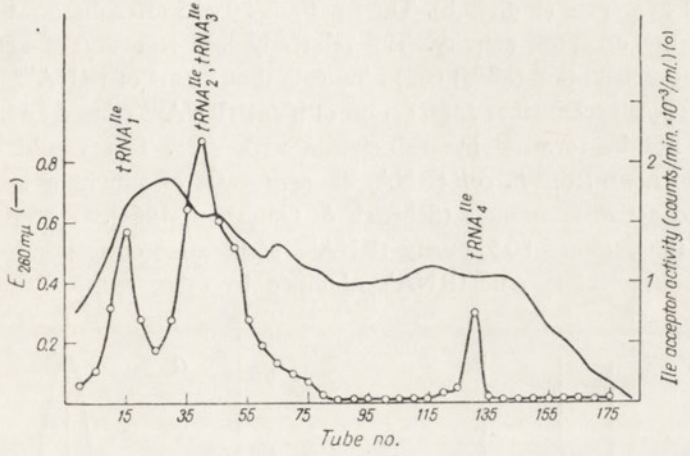


Fig. 4. The profile of 200-transfer countercurrent distribution at pH 6.0 of 330 mg. of unfractionated tRNA from *L. luteus*.

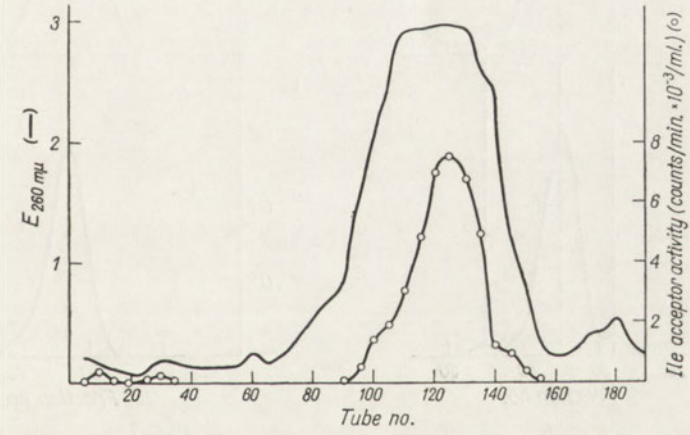


Fig. 5. The profile of 200-transfer countercurrent distribution at pH 8.15 of 100 mg. of unfractionated tRNA from *L. luteus*.

When the preparation partially purified by DEAE-cellulose and reverse-phase chromatography, was submitted to countercurrent distribution, only one $tRNA^{Ile}$ fraction emerged (Fig. 3) in the position corresponding to that of fraction $tRNA^{Ile}_{2,3}$. Thus it appears that the two first purification steps either remove the other $tRNA^{Ile}$ species or modify them in such a way that they cannot be separated from $tRNA^{Ile}_{2,3}$.

Marked aggregation of tRNA was observed in the countercurrent distribution fractions 20 - 35 (Fig. 3), that is the fractions showing the greatest absorption at 260 mμ. After heating at pH 7.3 in 4 M-urea (Schleich & Goldstein, 1964), RNA

underwent dissociation as indicated by the disappearance in the Sephadex G-200 effluent of the peak corresponding to the high-molecular compounds and by increase of the peak corresponding to low-molecular compounds (Fig. 6a,b). An analogous experiment performed with fraction $tRNA^{Ile}_{2,3}$ demonstrated that it contained only traces of aggregate (Fig. 7a,b). The fractions 20 - 35 after dissociation in urea solution lost the acceptor activity. The relatively low recovery of activity after countercurrent distribution (28%) could indicate that a part of $tRNA^{Ile}$ underwent aggregation. Thus it seems that aggregation of plant $tRNA^{Ile}$ caused an inactivation which could not be reversed by dissociation with urea. This would distinguish the $tRNA^{Ile}$ of lupin from *E. coli* tRNA, the aggregates of which in urea solution dissociate into active monomers (Schleich & Goldstein, 1964).

Although the obtained 80% purity $tRNA^{Ile}$ preparation corresponded in purity to the preparations of specific tRNA's obtained by other authors and used for

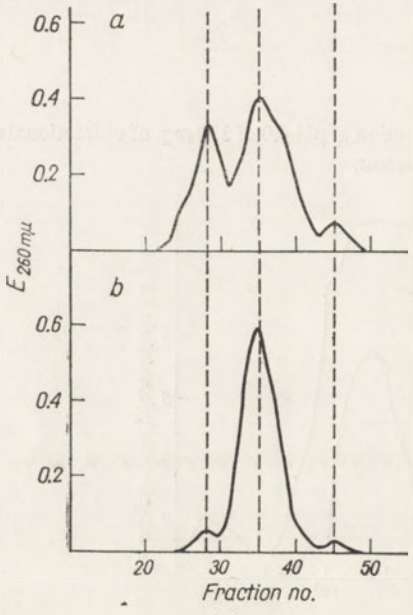


Fig. 6

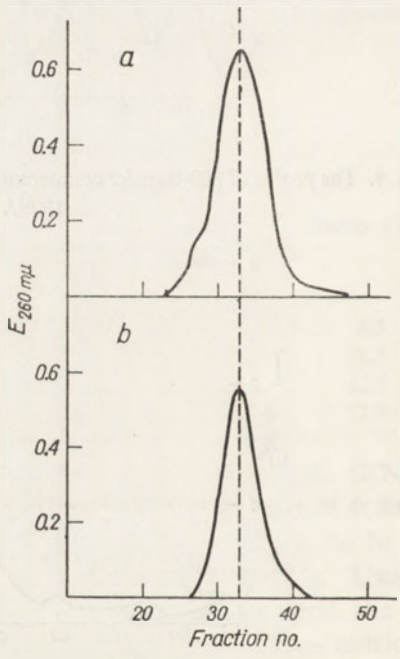


Fig. 7

Fig. 6. Dissociation of aggregated tRNA of countercurrent distribution fractions 20 - 35 (Fig. 4). Twenty $E_{260}$ units were filtered through a Sephadex G-200 column (1.4×68 cm.) in 1 M-NaCl, (*a*), before and (*b*), after treatment with urea. Fractions of 2.8 ml. were collected at a flow rate of 13 ml./hr.

Fig. 7. Gel filtration on Sephadex G-200 of purified $tRNA^{Ile}_{2,3}$ (Fig. 4), (*a*), before and (*b*), after treatment with urea. Conditions as described for Fig. 6.

structural studies, the absorption curve at 260 mμ after countercurrent distribution did not coincide with the acceptor activity curve (Fig. 3). This discrepancy cannot be explained by the presence of aggregated nucleic acids. However, it is not to be excluded that some low-molecular oligonucleotides and mononucleotides were

present as products of hydrolysis of tRNA due to incidentally introduced ribonucleases (for instance, from human fingers, cf. Apgar, Holley & Merrill, 1962).

Chromatography on DEAE-cellulose at 72° was applied as the first step of $tRNA^{Ile}$ purification because in earlier experiments (Legocki *et al.*, 1967a) a broadening of the elution profiles with increase in temperature had been observed. The application of a temperature of 72° appeared to be convenient because in the first RNA fractions a sharp peak of $tRNA^{Ile}$ was obtained. Since at elevated temperatures tRNA undergoes structural changes which may be irreversible, it cannot be excluded that the obtained tRNA differs from the native compound.

Reverse-phase chromatography was first elaborated by Kelmers *et al.* (1965) for fractionation of bacterial tRNA. They have found that the elution profiles of nucleic acids and of acceptor activities are influenced markedly by magnesium ion, and Kelmers (1966) reported the effect of temperature and pH value. In the present work the influence of $Mg^{2+}$ was confirmed also for tRNA of plant origin. Elution profiles of unfractionated tRNA obtained with a 0.25 - 0.75 M-NaCl gradient without $Mg^{2+}$, with 0.015 M-$MgCl_2$, or with a simultaneous gradient of $MgCl_2$ from 0 to 0.024 M, are shown in Fig. 8. In each case three distinct peaks of $tRNA^{Ile}$

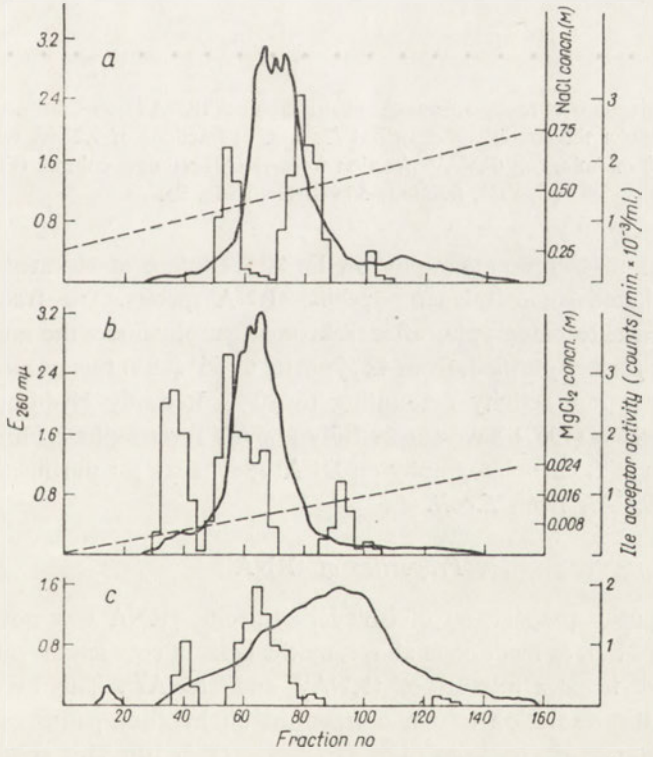


Fig. 8. The effect of $Mg^{2+}$ ion on reverse-phase chromatography. Unfractionated tRNA, 25 mg., was applied to a Chromosorb W column (0.9×100 cm.). The elution was with 0.05 M-tris-HCl buffer saturated with isoamyl acetate, pH 7.25, at a linear NaCl concentration gradient from 0.25 to 0.75 M; (*a*), in the absence of $Mg^{2+}$ ion; (*b*), with a $Mg^{2+}$ gradient (0 - 0.024 M-$MgCl_2$); and (*c*), in the presence of 0.015 M-$MgCl_2$. Fractions of 5 ml. were collected at a flow rate of 0.6 ml./min.

were obtained, the best resolution being observed with the $MgCl_2$ concentration of 0.015 M (Fig. 8c). Therefore this elution system was applied for the isolation of purified $tRNA^{Ile}$. However, the preparation from DEAE-cellulose column was not resolved by reverse-phase chromatography into three $tRNA^{Ile}$ fractions but emerged as a single broad peak (Fig. 2). A similar result was obtained when the fractionation was carried out with much smaller amounts of tRNA (Fig. 9a). This

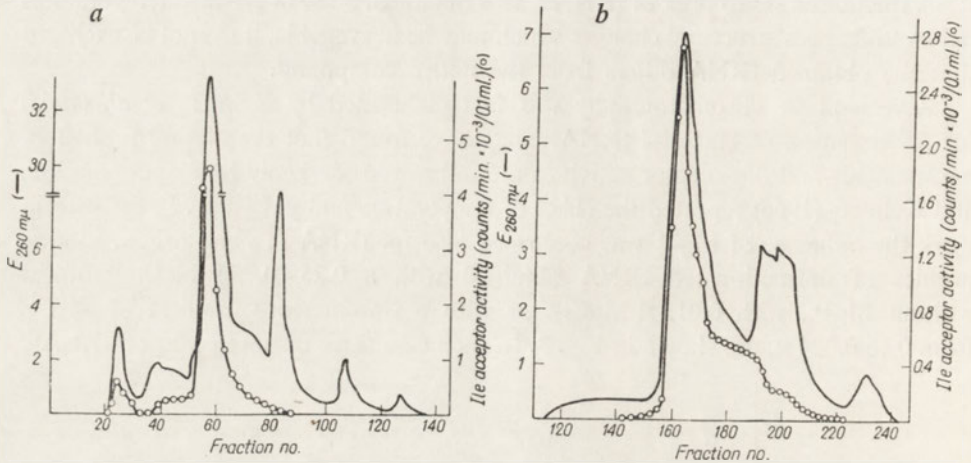


Fig. 9. Elution diagrams of reverse-phase chromatography of $tRNA^{Ile}$ on Chromosorb W column (1.1×200 cm.), under the conditions described for Fig. 2; fractions of 7.2 ml. were collected at a flow rate of 0.8 ml./min. (*a*), $tRNA^{Ile}$ fraction from DEAE-cellulose column (Fig. 1), 250 mg.; (*b*), fractions 56 - 64 from Fig. 9a.

result indicates that chromatography on DEAE-cellulose at elevated temperature removed or altered some isoleucine-specific tRNA species. The fraction purified by reverse-phase chromatography, after rechromatography under the same conditions appeared to be further purified, from 42% purity to 56%, but this procedure resulted in losses of acceptor activity amounting to 80%. Recently Nishimura, Harada, Narushima & Seno (1967) have successfully applied reverse-phase chromatography in combination with chromatography on DEAE-Sephadex for purification of amino acid-specific tRNA's from *E.coli*.

### *Properties of* $tRNA^{Ile}$

The 80% purity preparation of isoleucine-specific tRNA was not resolved by the method used but, as mentioned above, on the basis of countercurrent distribution it was supposed to be a mixture of $tRNA_2^{Ile}$ and $tRNA_3^{Ile}$. This assumption was confirmed by the resolution into two fractions on hydroxyapatite column of the $tRNA^{Ile}$ preparation charged with [$^{14}$C]isoleucine (Fig. 10). This complex was not resolved on protaminated kieselguhr (Fig. 11). The difficulties in separation of the preparation into $tRNA_2^{Ile}$ and $tRNA_3^{Ile}$ indicate that these two fractions differ but slightly from each other, possibly by one nucleotide only, or by some spatial arrangement.

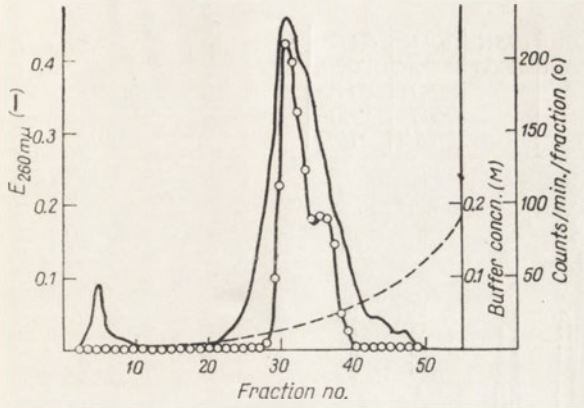


Fig. 10

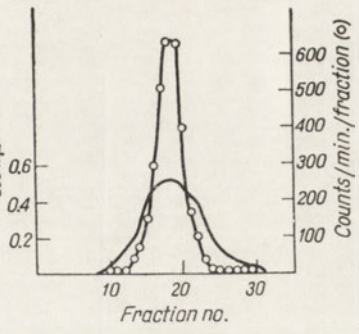


Fig. 11

Fig. 10. Fractionation of [$^{14}$C]isoleucyl-tRNA$^{Ile}_{2,3}$ on a hydroxyapatite column. The preparation, 18 $E_{260}$ units (about 14 000 counts/min.), was chromatographed on a column (1 × 16 cm.) equilibrated with 0.05 M-sodium phosphate buffer, pH 6.8, in a 0.05 - 0.18 M gradient of the same buffer. Fractions of 3 ml. were collected at a flow rate of 12 ml./hr.

Fig. 11. Elution profile of [$^{14}$C]isoleucyl-tRNA$^{Ile}_{2,3}$ on protaminated kieselguhr column. The preparation, 16 $E_{260}$ units (about 12 000 counts/min.), was applied to a column (0.65 × 20 cm.) in 0.05 M-potassium phosphate buffer, pH 7.05, containing 0.2 M-NaCl, and eluted with 0.2 - 1.6 M-NaCl gradient. Fractions of 2.7 ml. were collected at a flow rate of 0.75 ml./min.

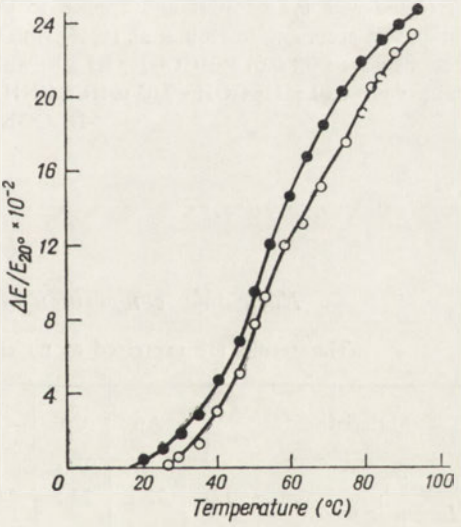


Fig. 12. Thermal denaturation curves of (○), unfractionated tRNA from *L. luteus*, and (●), purified tRNA$^{Ile}$. The measurements were carried out in 0.015 M-potassium phosphate buffer, pH 7.0, containing 0.15 M-NaCl.

The temperature profile of the purified tRNA$^{Ile}$ (Fig. 12) was very similar to that of unfractionated tRNA, which is of interest as marked differences were observed by Nishimura *et al.* (1967) with some amino acid-specific tRNA's isolated from *E. coli*.

The nucleotide composition of the alkaline hydrolysate of tRNA$^{Ile}_{2,3}$ (Fig. 13 and Table 2) did not differ from that of tRNA's originating from bacteria or yeast, reported in the literature.

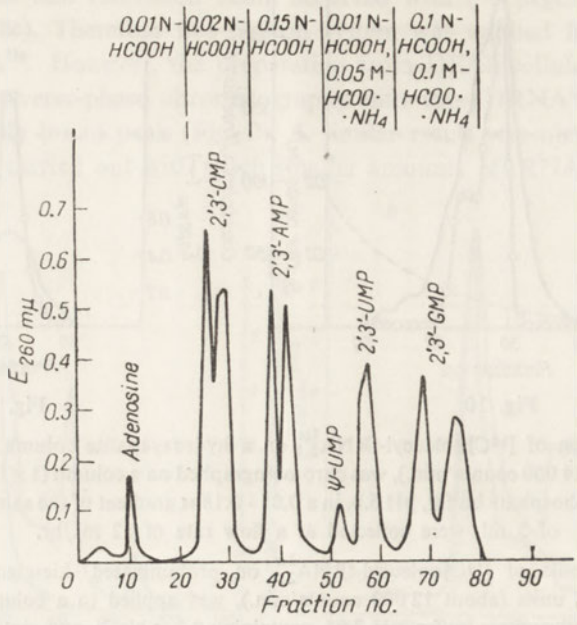


Fig. 13. Chromatography of alkaline hydrolysate of $tRNA^{Ile}_{2,3}$. $tRNA^{Ile}$, 2 mg. (0.5 ml.) was hydrolysed with 0.2 N-NaOH and applied to Dowex 1 (formate form) column (0.3×14 cm.). Elution was according to Holt *et al.* (1966) with the indicated eluents. The volume of the collected fractions was: with 0.01 N-HCOOH, 1.5 ml.; with 0.02 N-HCOOH, 2.5 ml.; with 0.15 N-HCOOH, 6 ml.; with 0.01 N-HCOOH - 0.05 M-$HCOONH_4$, 6 ml.; and with 0.01 N-HCOOH - 0.1 M-$HCOONH_4$, 6 ml.

## Table 2

### *Nucleotide composition of* tRNA *of different origin*

The results are expressed as moles/100 moles of nucleotide recovered

| Material | Adenosine | Ap | Cp | Gp | Up | ψUp | Other nucleotides | Reference |
|---|---|---|---|---|---|---|---|---|
| $tRNA^{Ile}_{2,3}$, *L. luteus* seeds | 1.0 | 22.1 | 24.5 | 28.8 | 18.5 | 4.8 | not determined | this paper |
| $tRNA^{Ser}$, brewer's yeast | 1.1 | 19.4 | 21.3 | 24.2 | 17.8 | 4.0 | 5.6 | Karau & Zachau (1964) |
| tRNA, *E. coli* | | 20.3[a] | 28.9 | 32.1 | 15.0 | 2.1 | 1.6 | Dunn *et al.* (1960) |
| tRNA, rabbit reticulocyte | 1.2 | 17.9 | 27.4 | 31.3 | 18.6 | 3.4 | not determined | Holt *et al.* (1966) |

[a] Value includes terminal adenosine.

The properties of unfractionated lupin tRNA, its behaviour on fractionation, as well as the properties of the purified isoleucine-specific tRNA indicate its close similarity to the amino acid-specific tRNA's isolated from other sources.

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## IZOLOWANIE IZOLEUCYLO-SPECYFICZNEGO PRZENOSZĄCEGO KWASU RYBONUKLEINOWEGO Z NASION ŁUBINU ŻÓŁTEGO

### Streszczenie

1. Z tRNA z nasion *Lupinus luteus*, stosując kolejno chromatografię na DEAE-celulozie w temp. 72°, rozdział w odwróconej fazie chromatograficznej oraz 200-przeniesieniowy rozdział przeciwprądowy, wyodrębniono tRNA specyficzny dla izoleucyny o czystości co najmniej 80%.

2. Oczyszczony $tRNA^{Ile}$ zidentyfikowano jako mieszaninę dwóch rodzajów tRNA, nierozdzielających się w użytej metodzie natomiast częściowo rozdzielanych na kolumnie hydroksyapatytowej.

3. Przebadano niektóre własności oraz skład nukleotydowy $tRNA^{Ile}$, wykazując jego znaczne podobieństwo do innych oczyszczonych tRNA pochodzących z różnych źródeł.

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# HETEROGENEITY OF TRANSFER RIBONUCLEIC ACIDS FROM YELLOW LUPIN SEEDS

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1. The countercurrent distribution technique and chromatography on hydroxyapatite column were applied to demonstrate heterogeneity of single amino acid-specific tRNA from seeds of *Lupinus luteus*. 2. Two tRNA fractions specific for valine, three for leucine and four for isoleucine, were obtained, and only one fraction of tRNA specific for methionine. 3. Transfer properties of two $tRNA^{Ile}$ fractions were studied in *E. coli* cell-free system with synthetic polynucleotides. One of the fractions recognized codon AUU, and the second AUU and AUC.

Chromatography on protamine-coated kieselguhr (Legocki, Szymkowiak, Pech & Pawełkiewicz, 1967) and hydroxyapatite (Legocki & Pawełkiewicz, 1967a) of transfer ribonucleic acid (tRNA) from lupin seeds have demonstrated that similarly as in bacteria and animals, also in plants multiple tRNA's specific for a single amino acid are present.

In the present work, the heterogeneity of lupin tRNA was reinvestigated using the countercurrent distribution technique and chromatography on hydroxyapatite. Recognition of codons by two isoleucine-specific tRNA fractions isolated by countercurrent distribution, was also studied. A preliminary account has been presented (Legocki & Pawełkiewicz, 1967c).

## MATERIAL AND METHODS

Sodium 2-phosphoenolpyruvate and *E. coli* sRNA were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); pyruvate kinase from Boehringer & Soehne (Mannheim, Germany); ADP, UDP and CDP were products of Calbiochem. (Los Angeles, Calif., U.S.A.); deoxyribonuclease (EC 3.1.4.5) was obtained from the Mann Research Lab. (New York, U.S.A.); *Micrococcus lysodeikticus*, dry cell preparation,

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was obtained from Nutr. Biochem. Corp. (Cleveland, Ohio, U.S.A.); casein enzymic hydrolysate was prepared according to Pawełkiewicz & Zodrow (1957); Alumina Alcoa A-303 was from Aluminium Comp. of America (U.S.A.); L-[U-$^{14}$C]isoleucine (36 and 79.3 mc/m-mole) was from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia); L-[U-$^{14}$C]valine (6.9 mc/m-mole) and L-[U-$^{14}$C]leucine (35 mc/m-mole) were from the Radiochemical Centre (Amersham, England); and DL-[1-$^{14}$C]methionine (9.8 mc/m-mole) from Service Molecules Marquées CEA (Gif-sur-Yvette, France). Other reagents were from the sources indicated in the accompanying paper (Legocki, Szymkowiak & Pawełkiewicz, 1968).

tRNA from yellow lupin seeds was isolated as described previously (Legocki *et al.*, 1967), and the preparation purified by Sephadex G-200 gel filtration was used. Aminoacyl-tRNA synthetases were isolated from yellow lupin seeds (Legocki & Pawełkiewicz, 1967b). Synthetic polynucleotides: poly-UAC(6 : 1 : 1), poly-UA(6 : 1), poly-UA(1 : 3) and poly-UA(1 : 6) were prepared according to Singer & Guss (1962).

Radioactivity, RNA and protein concentration were determined as described in the accompanying paper (Legocki *et al.*, 1968).

Determinations of acceptor activity of tRNA toward isoleucine, leucine, methionine and valine were carried out as described by Legocki & Pawełkiewicz (1967b) taking for incubation 0.1 ml. of the tRNA fraction studied (0.1 - 0.6 mg. of tRNA) and 0.1 ml. (0.2 mg. of protein) of the appropriate synthetase.

[$^{14}$C]Aminoacyl-tRNA and [$^{12}$C]aminoacyl-tRNA were prepared as in the accompanying paper (Legocki *et al.*, 1968). The isolated radioactive derivatives had a specific activity of 10 000 - 12 000 counts/min./mg. RNA. [$^{14}$C]Isoleucyl-tRNA$^{Ile}_{I}$ and [$^{14}$C]isoleucyl-tRNA$^{Ile}_{II}$ used in the incorporation experiments, had higher specific activities: 82 000 and 64 000 counts/min./mg. RNA, respectively. [$^{14}$C]Aminoacyl-tRNA was chromatographed on hydroxyapatite column as described in the accompanying paper (Legocki *et al.*, 1968); countercurrent distribution of tRNA was carried out at pH 6.0 according to Doctor, Apgar & Holley (1961) as described by Legocki *et al.* (1968).

*E. coli* strain B was cultured at 30° in a medium containing 1.1% of $K_2HPO_4$, 0.85% of $KH_2PO_4$, 0.1% of yeast extract (Difco), 0.05% of casein acid hydrolysate (Difco), 1% of glucose and 25 ml. of casein enzymic hydrolysate per 1 litre, in a 40-litre aerated vessel. After 2.5 hr. growth, the bacterial pellet was centrifuged and washed with the buffer used for extraction; the yield was 1 g. per 1 litre of the culture.

Fraction S-30 from *E. coli* cells was prepared according to Matthaei & Nirenberg (1961). The bacterial pellet, 40 g., was ground in a cooled mortar with 50 g. of Alumina and 50 ml. of 0.006 M-KCl - 0.014 M-magnesium acetate - 0.01 M-2-mercaptoethanol - 0.01 M-tris-HCl buffer, pH 7.8. After 20 min. extraction, 350 μg. of DNase was added and the mixture centrifuged at 30 000 *g* for 30 min. For incorporation experiments, the supernatant (38 ml.) was preincubated for 40 min. at 37° with the following components (in μmoles): tris-HCl buffer, pH 7.8, 3000; magnesium acetate, 550; KCl, 600; GSH, 600; twenty L-amino acids, 5 each; ATP, 200; 2-phosphoenolpyruvate, 200; GTP, 100; and 1.2 mg. of pyruvate kinase,

100 μg. of DNase (free of RNase) and 12.5 mg. of sRNA from *E. coli* B. After preincubation, the mixture was dialysed for 15 hr. at 1° against 80 volumes of the buffer used for extraction. In the dialysis residue the content of protein was 28 mg./ml.

Incorporation of isoleucine from [$^{14}$C]isoleucyl-tRNA was studied in the system described by Nathans, Natani, Schwartz & Zinder (1962). The incubation mixture contained in the final volume of 0.25 ml. the following components (in μmoles): tris-HCl buffer, pH 7.4, 25; $MgCl_2$, 5; KCl, 12.5; ATP, 0.5; GTP, 0.2; 2-phosphoenolpyruvate, 2.5; 2-mercaptoethanol, 1.5; and 40 μg. of pyruvate kinase, 50 μl. of fraction S-30 (1.4 mg. protein), 0.2 mg. of [$^{12}$C]aminoacyl-tRNA's (except isoleucine), 0.22 mg. of [$^{14}$C]isoleucyl-tRNA$^{Ile}_{I}$ (82 000 counts/min./mg. RNA) or 0.15 mg. of [$^{14}$C]isoleucyl-tRNA$^{Ile}_{II}$ (64 000 counts/min./mg. RNA), and 10 μg. of one of the synthetic polynucleotides. The incubation was carried out at 30°; after 15 min. 2.5 ml. of 10% trichloroacetic acid was added, the sample was heated for 10 min. in a boiling water bath, the precipitate collected on Millipore HA filters (0.45 μ), washed with 10% trichloroacetic acid containing non-radioactive isoleucine, dried at room temperature, and the radioactivity measured.

## RESULTS AND DISCUSSION

The diagram of countercurrent distribution of unfractionated lupin tRNA in the phosphate system at pH 6.0 according to Doctor, Apgar & Holley (1961) after 200 transfers, is presented in Fig. 1. Out of four specific tRNA's studied, only tRNA for methionine was not resolved. The valine-specific and leucine-specific tRNA's separated into two fractions and isoleucine-specific tRNA, into three fractions. First peak of valyl-tRNA (tRNA$^{Val}_{I}$) was much higher than the second peak (tRNA$^{Val}_{II}$). Similarly, the two peaks of tRNA$^{Ile}$ (*I* and *II*) were higher than the third (*III*) peak.

The acceptor activities of tRNA's toward the four amino acids were assayed using homologous aminoacyl-tRNA synthetases isolated from yellow lupin seeds. This seemed to be important because it had been repeatedly demonstrated that heterologous synthetases not always catalysed the transfer of amino acids to a tRNA of different origin (cf. Berg, Bergmann, Ofengand & Dieckmann, 1961; Connelly & Doctor, 1966).

Countercurrent distribution as applied in this work (200 transfers) is usually insufficient for satisfactory resolution of tRNA. Most often, therefore, this procedure is repeated with some modification. In the present work, chromatography on hydroxyapatite column was chosen for further resolution of the obtained fractions, as its suitability for fractionation of plant tRNA had been proved by Legocki & Pawełkiewicz (1967a).

For the resolution on hydroxyapatite column, the specific fractions separated by countercurrent distribution were esterified by appropriate [$^{14}$C]amino acids. The results of fractionation are presented in Fig. 2. On rechromatography on hydroxyapatite, fractions tRNA$^{Val}_{I}$, tRNA$^{Val}_{II}$, tRNA$^{Met}$, tRNA$^{Leu}_{I}$ and tRNA$^{Ile}_{I}$ appeared to be homogeneous whereas fractions tRNA$^{Leu}_{II}$ and tRNA$^{Ile}_{II}$ partially

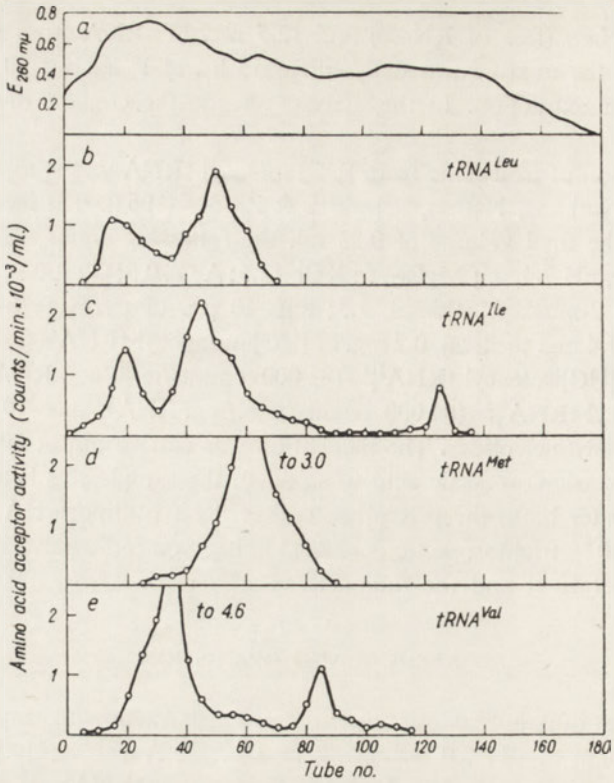


Fig. 1. Countercurrent distribution of unfractionated tRNA from *L. luteus* seeds. tRNA, 300 mg., was separated by 200 transfers in phosphate solvent, pH 6.0, at 24°. (*a*), $E_{260\,m\mu}$. Profiles of acceptor activity for: (*b*), leucine; (*c*), isoleucine; (*d*), methionine and (*e*), valine. For details see Materials and Methods.

separated, each into two subfractions ($tRNA_2^{Leu}$ and $tRNA_3^{Leu}$, and $tRNA_2^{Ile}$ and $tRNA_3^{Ile}$). Because of the small amount of the material available, fraction $tRNA_{II}^{Ile}$ was not submitted to hydroxyapatite chromatography.

By the described technique, only methionine-specific tRNA could not be resolved. However, our earlier experiments proved its heterogeneity on chromatography on protaminated kieselguhr (Legocki *et al.*, 1967). Thus each of the four yellow-lupin-seed tRNA's studied, specific for a single amino acid, represents a mixture of several species. There are at least two kinds of tRNA for methionine and for valine, three for leucine and even four for isoleucine.

These observations are in agreement with the results of other authors who studied heterogeneity of transfer RNA's of bacterial, yeast and animal origin (Weisblum, Gonano, von Ehrenstein & Benzer, 1965; Bennett, Goldstein & Lipmann, 1965).

To study the specificity of fractions $tRNA_I^{Ile}$ and $tRNA_{II}^{Ile}$ separated by countercurrent distribution, for recognition of codons, the incorporation of isoleucine from both [$^{14}$C]isoleucyl-$tRNA^{Ile}$ complexes into polypeptide was carried out in the presence of different synthetic polynucleotides (Table 1). At present, codons

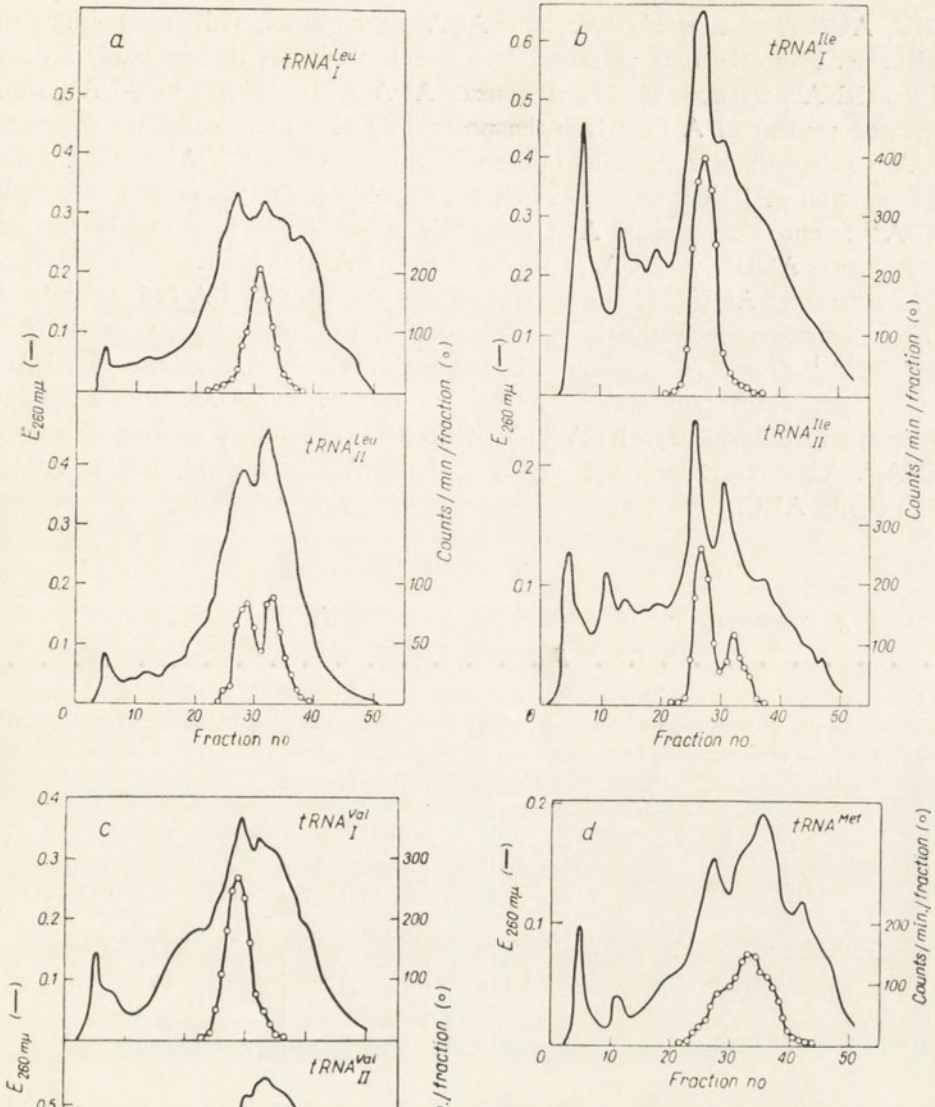


Fig. 2. Elution profiles from hydroxyapatite column of countercurrent distribution fractions, esterified by appropriate [$^{14}$C]amino acid. About 30 $E_{260}$ units of the appropriate fraction charged with [$^{14}$C]amino acid (about 12 000 counts/min./mg. RNA) was applied to a column (1 × 16 cm.) equilibrated with 0.05 M-sodium phosphate buffer, pH 6.8. RNA was eluted by a linear concentration gradient of the same buffer from 0.05 to 0.18 M. Fractions of 3 ml. were collected at a flow rate of 12 ml./hr. (*a*), Leucyl-tRNA; (*b*), isoleucyl--tRNA; (*c*), valyl-tRNA; (*d*), methionyl-tRNA. The respective countercurrent distribution fractions are indicated in the Figure.

AUU, AUC (Trupin *et al.*, 1965) and AUA (Smith *et al.*, 1967) are ascribed to isoleucine. From the data presented in Table 1 it appears that probably neither of the $tRNA^{Ile}$ fractions studied recognizes AUA, since different poly-UA's with increased content of A (in which the probability of occurrence of the sequence AUA is greater than in polynucleotides with a lower content of A) stimulated the incorporation of isoleucine to a much smaller extent. On the other hand, both $tRNA^{Ile}$ fractions recognized AUU, and this in proportion to the probability of occurrence of AUU in poly-UA. For instance, poly-UA(1 : 3), in which the probability of occurrence of AUU is about threefold higher than in poly-UA(1 : 6), stimulated three times more efficiently the incorporation of isoleucine, both with $tRNA_I^{Ile}$ and $tRNA_{II}^{Ile}$. These two fractions showed different behaviour in the poly-UAC (6 : 1 : 1) dependent polypeptide synthesizing system. Poly-UAC stimulated incorporation from isoleucyl-$tRNA_{II}^{Ile}$ about 1.5 times as strongly as from isoleucyl-$tRNA_I^{Ile}$. This result indicated clearly that $tRNA_{II}^{Ile}$ recognized, beside AUU, additionally AUC.

Table 1

*Coding characteristics of isoleucine-specific* tRNA *fractions obtained by countercurrent distribution*

The composition of the incubation mixture is given in Materials and Methods. Each sample contained 10 μg. of the indicated polynucleotide. The samples were incubated for 15 min. at 30°. The results have been corrected by subtracting the values obtained with appropriate control samples containing no synthetic polynucleotides.

| Preparation | Synthetic polynucleotide | | | |
|---|---|---|---|---|
| | poly-UAC (6 : 1 : 1) | poly-UA (6 : 1) | poly-UA (1 : 3) | poly-UA (1 : 6) |
| | Incorporation (counts/min.) | | | |
| [$^{14}$C]Isoleucyl-$tRNA_I^{Ile}$ | 185 | 944 | 299 | 108 |
| [$^{14}$C]Isoleucyl-$tRNA_{II}^{Ile}$ | 271 | 618 | 287 | 111 |

Although a non-homologous incorporation system was used, it seems possible to suggest that AUU is the codon for $tRNA_I^{Ile}$, whereas AUU and AUC are codons for $tRNA_{II}^{Ile}$. Recognition of both AUU and AUC is in agreement with the observation that the letters U, C and A in the third position may be recognized as a group (Khorana, 1967). On the other hand, it may suggest that the two species of $tRNA_{II}^{Ile}$, $tRNA_2^{Ile}$ and $tRNA_3^{Ile}$, recognize either AUU or AUC.

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## HETEROGENNOŚĆ PRZENOSZĄCYCH KWASÓW RYBONUKLEINOWYCH Z NASION ŁUBINU ŻÓŁTEGO

### Streszczenie

1. Zastosowano metodę rozdziału przeciwprądowego oraz metodę chromatografii na hydroksyapatycie dla wykazania heterogenności tRNA z nasion *Lupinus luteus*.

2. Uzyskano 2 frakcje tRNA specyficznego dla waliny, 3 dla leucyny i 4 dla izoleucyny. tRNA dla metioniny był w zastosowanych warunkach rozdziału jednorodny.

3. Przebadano własności przenoszące dwu frakcji tRNA specyficznego dla izoleucyny w układzie bezkomórkowym z *E. coli* przy użyciu szeregu syntetycznych polinukleotydów. Wykazano, że jedna z frakcji, $tRNA_I^{Ile}$, odpowiada kodonowi AUU, zaś druga, $tRNA_{II}^{Ile}$, kodonom AUU i AUC.

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# SULPHOSALICYLIC ACID-SOLUBLE PROTEINS AND THEIR DISTRIBUTION IN SUBCELLULAR FRACTIONS OF CALF THYMUS

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1. It has been demonstrated that histones rich in lysine are extracted with sulphosalicylic acid from sucrose homogenate and nuclei of calf thymus. 2. From nuclei, a pure preparation of lysine-rich histone was obtained by the tannin-caffeine procedure.

Animal tissues contain very small amounts of proteins extractable with 0.15 M-sulphosalicylic acid which react with tannin and can be purified and concentrated by the tannin-caffeine procedure of Mejbaum-Katzenellenbogen (1959a,b). These proteins, obtained from kidney (Mejbaum-Katzenellenbogen & Wieczorek, 1966), skeletal muscle (Mejbaum-Katzenellenbogen, Kozar & Maskos, 1967) and anterior lobe of pituitary gland (Kubicz & Mejbaum-Katzenellenbogen, 1964), are characterized by resolution on paper electrophoresis in weakly acidic medium into several distinctly separated fractions moving towards the cathode. Rzeczycki, Grudzińska & Hillar (1962) and Rzeczycki, Grudzińska, Hillar & Wszelaki-Lass (1963) by applying the tannin-caffeine procedure and CM-cellulose column chromatography, from the sulphosalicylic acid (SSA) extract of hog kidney mitochondria isolated a lysine-rich fraction which they called mitochondrial basic protein. This fraction was found to have an inhibitory effect on cytochrome oxidase and succinate oxidase (Rzeczycki, 1963), and to possess RNA depolymerase activity (Bardoń & Rzeczycki, 1966).

Since basic proteins extractable with sulphosalicylic acid have been found to occur in many animal tissues, it might be reasonably supposed that they contain nuclear proteins, and especially the lysine-rich histone. De Nooij & Westenbrink (1962) reported that, in contrast to histone rich in arginine, the lysine-rich histone was readily soluble in 5% trichloroacetic acid. They presented a method for isolating a pure fraction from calf thymus. The obtained protein had the same properties as the lysine-rich histone prepared by the method of Bijvoet (1957), which consists in fractionation with ethanol of the protein obtained by extraction with 0.2 N-HCl.

The method of de Nooij & Westenbrink (1962) was recently modified by Kinkade & Cole (1966) who extracted thymus proteins with 8% trichloroacetic acid, and from the extract precipitated lysine-rich histone with 20% trichloroacetic acid. So far, sulphosalicylic acid has not been applied for extraction of histones from nuclei.

In the present work an attempt was made to isolate the proteins extracted with SSA from the homogenate and subcellular fractions of calf thymus, and to compare them with the lysine-rich histone prepared by the method of Kinkade & Cole (1966). For experiments, calf thymus was chosen because its nuclei are relatively large and therefore lysine-rich histones, if they proved to be soluble in SSA, could be expected to predominate in the extract.

## MATERIALS AND METHODS

Calf thymus was obtained in the slaughterhouse within a few minutes after killing of the animal, and immediately cooled to 0°. All manipulations were carried out at a temperature not exceeding 4°. The gland was freed of lymphatic nodes, fat tissue and major vessels.

*Subcellular fractions.* For their isolation the method of Hogeboom (1955) was used. The thymus was homogenized with a tenfold volume of 0.25 M-sucrose containing 1.8 mM-$CaCl_2$ in Waring blendor type homogenizer for 2 min. The homogenate was filtered through several layers of gauze and layered over 0.34 M-sucrose - 1.8 mM-$CaCl_2$ solution, centrifuged for 10 min. at 800 *g*, the sediment was suspended and layered again over 0.34 M-sucrose - 1.8 mM-$CaCl_2$ and then twice over 0.40 M-sucrose - 1.8 mM-$CaCl_2$.

The obtained nuclei were 5 - 8 times washed by suspending in 0.25 M-sucrose- - 1.8 mM-$CaCl_2$ solution.

The supernatant after sedimentation of nuclei from homogenate was layered over 0.34 M-sucrose - 1.8 mM-$CaCl_2$ and centrifuged at 15 000 *g* for 15 min., and the obtained mitochondrial pellet was washed 3 - 4 times with 0.25 M-sucrose. The post-mitochondrial supernatant was collected and used without further separation.

*Isolation of SSA-soluble proteins by the tannin-caffeine procedure from thymus homogenate and subcellular fractions.* To the sucrose homogenate, post-mitochondrial supernatant, and to the nuclei and mitochondria suspended in water, 0.45 M-SSA was added to a final concentration of 0.15 M. After homogenization in a glass Potter-type homogenizer at 1000 rev./min. for 3 min., the samples were left for 30 min.; then the precipitated proteins were centrifuged off at 2000 *g* and the supernatants were filtered through Whatman no. 1 paper. In the filtrate, protein was determined and then precipitated by a tenfold excess of tannin. On the next day the protein-tannin complex was spun down (20 min., 4000 *g*), washed three times with water, and a twofold excess of caffeine in relation to protein was added (Mejbaum-Katzenellenbogen, 1959a,b). After 3 hr. the insoluble tannin-caffeine complex was centrifuged off at 20 000 *g*. The supernatants contained about 4% of protein, the recovery being about 70%.

*Isolation of SSA-soluble proteins directly from thymus.* The thymus was disintegrated in a meat-grinder and homogenized with 4 volumes of 0.15 M-SSA in a Waring blendor type homogenizer for 3 min. After 30 min. the homogenate was centrifuged for 15 min. at 1000 *g* and the supernatant filtered twice through Whatman no. 1 paper. The obtained slightly opalescent extract contained on the average 0.1% of protein which was then purified and concentrated by the tannin-caffeine procedure as described above, the recovery being about 60%. From 100 g. of thymus 300 mg. of protein was obtained.

*Isolation of lysine-rich histone by the method of Kinkade & Cole.* Thymus, 100 g., was homogenized in 400 ml. of 10% trichloroacetic acid in Waring blendor type homogenizer for 2 min. The homogenate was centrifuged for 10 min. at 1100 *g*; to 380 ml. of the supernatant was added 50 ml. of 100% (w/v) trichloroacetic acid, stirred mechanically for 5 min. and then centrifuged as above. The histone sediment was washed 3 - 4 times with acetone and dried to constant weight in a vacuum desiccator over sulphuric acid at room temperature. Further drying in the vacuum desiccator over $P_2O_5$ at 105° for 24 hr. caused no further loss in weight. From 100 g. of thymus, was obtained 220 mg. of a preparation containing 50% of proteins soluble in water, 0.9% NaCl and in dilute acids.

*Analytical methods.* Protein was determined by the tannin micromethod (Mejbaum-Katzenellenbogen, 1955) using gelatin as standard; the turbidity was read in a Pulfrich apparatus using a $S_{61}$ filter, the extinction of 100 μg. of gelatin per 1 ml. in 1 cm. light-path cuvettes being 1.3.

Paper electrophoresis was carried out in an apparatus manufactured by C. Zeiss (Jena, Germany) equipped with a recording photodensitometer (model ERI 10); 0.15 M-citrate - phosphate buffer, pH 4.4 (McIlvaine, 1921) and strips of Whatman no. 1 paper (4×30 cm.) were used. A voltage of 200 v was applied for 9 hr. The electrophoretograms were air-dried, stained with bromophenol blue in tannin reagent, eluted with 0.1 N-NaOH, and protein was determined (Mejbaum-Katzenellenbogen & Dobryszycka, 1959). The electrophoretograms which were to be submitted to direct photometry were heated in an oven at 100° for 20 min. and stained with Amido black by the method of Grassman as described by Dittmer (1961). The percentage distribution of the individual fractions was calculated from an automatically scanned integral curve.

The electrophoretograms were stained for sugars by the method of Köiv and Grönvall as described by Wunderly & Piller (1954), and for lipids with Sudan black according to Swahn (1952).

Free-boundary electrophoresis was performed in an apparatus of C. Zeiss (Jena), model 35. About 1% (w/v) solution of the protein in 0.15 M-citrate - phosphate buffer, pH 4.4, was used. The protein was dialysed previously for 12 hr. at 5° against the same buffer. The run was performed for 2 hr. at 105 v and 3.5 mA.

*Reagents*: Tannin (U.S.S.R.), gelatin and Amido black 10 B (Grübler, Leipzig, Germany), alkaline fuchsine (British Drug Houses, Poole, Dorset, England), Sudan black 10 B (E. Merck, Darmstadt, Germany). Other reagents were of analytical grade, produced in Poland.

## RESULTS

*Sulphosalicylic acid-soluble proteins.* From 100 g. of calf thymus, 0.484±0.05 g. of protein was extracted with 0.15 M-SSA. The amount of protein that had been concentrated by the tannin-caffeine procedure, did not change on prolonged dialysis, indicating that no easily diffusible material reacting with tannin was present. On free-boundary electrophoresis, the preparation was resolved into three fractions migrating toward the cathode (Fig. 1a). The fastest fraction accounted for 86%, the middle fraction for 3%, and the slowest for 11%, of the protein applied. On paper electrophoresis at the same pH value five fractions moving toward the cathode

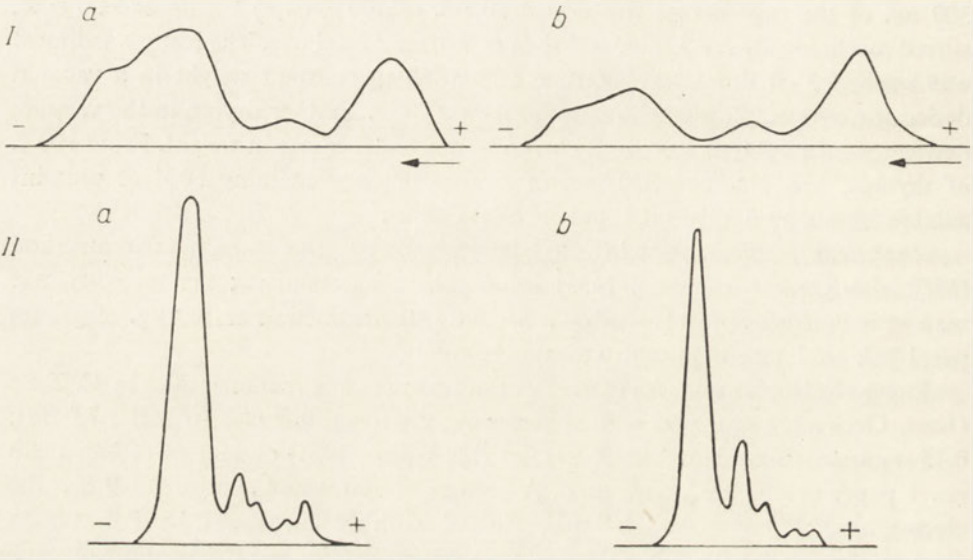


Fig. 1. Electrophoresis of (*a*), SSA-soluble protein from calf thymus; (*b*), lysine-rich histone from calf thymus. (*I*), Free boundary electrophoresis; (*II*), paper electrophoresis (densitometric curves). Conditions of electrophoresis as described in Methods.

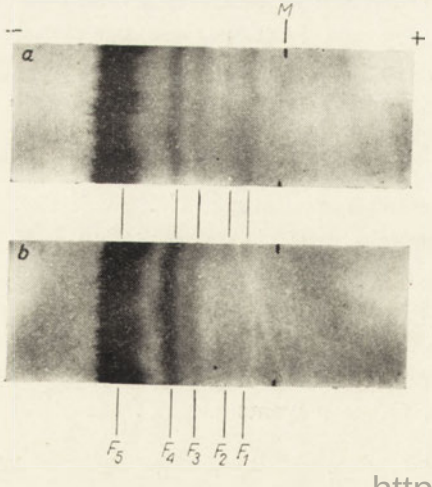


Fig. 2. Paper electrophoresis at pH 4.4 of (*a*), SSA-soluble protein, (*b*), lysine-rich histone from thymus. Conditions as described in Methods; *M*, middle of the strip. Staining with Amido black.

were obtained (Fig. 1c and 2a). They stained well with Amido black or bromophenol blue-tannin reagent. Staining with fuchsine or Sudan black did not reveal the presence of sugar or lipid in any fraction.

From air-dried electrophoretograms, total protein applied was eluted with 0.1 N-NaOH, whereas after drying at 100° for 20 min. only 55% of the starting material was recovered and the percentage distribution of fractions was altered. The fastest-migrating fraction ($F_5$) eluted from the air-dried strips amounted to 90%, whereas after drying at 100°, only to 82%, the latter value being close to those obtained by direct densitometry of the strips dried at 100° and stained with Amido black. The percentage distribution of the fractions, estimated in the eluates by the tannin method, as well as the results obtained by direct photometry after Amido black staining, are summarized in Table 1.

Table 1

*Percentage distribution of paper electrophoretic fractions of SSA-soluble protein and lysine-rich histone of calf thymus at* pH *4.4*

The protein was prepared from the SSA-extract by the tannin-caffeine procedure according to Mejbaum-Katzenellenbogen (1959a). Lysine-rich histone was prepared with trichloroacetic acid by the method of Kinkade & Cole (1966). Electrophoresis was carried out as described in Methods, 0.5 - 1 mg. of protein being used. From the strips stained according to Mejbaum-Katzenellenbogen & Dobryszycka (1959), protein was eluted with 0.1 N-NaOH, and 96% of the protein material applied, was recovered. Direct densitometry was performed on the strips stained with Amido black. The fractions are designated by relative mobility from $F_1$ (the slowest) to $F_5$ (the fastest) in migration toward the cathode. The results are mean values from analyses of 6 preparations, ±S.D.

| Material | Method | Distribution (%) | | | | |
|---|---|---|---|---|---|---|
| | | $F_1$ | $F_2$ | $F_3$ | $F_4$ | $F_5$ |
| SSA-soluble protein | Elution | 1.1±0.4 | 1.1±0.2 | 1.9±0.5 | 5.8±0.3 | 90.1±0.8 |
| | Densitometry | 3.1±0.5 | 2.1±0.4 | 3.8±0.7 | 9.0±0.6 | 82.5±0.6 |
| Lysine-rich histone | Elution | 1.6±0.2 | 1.9±0.4 | 3.0±0.5 | 12.4±0.3 | 81.0±1.1 |
| | Densitometry | 1.0±0.3 | 2.1±0.4 | 4.4±0.4 | 18.0±1.0 | 74.5±0.9 |

From 100 g. of nuclei, on the average 0.6 g. of protein reacting with tannin was extracted with SSA, and from mitochondria only 0.03 g. Although the mitochondrial protein was precipitated by tannin, it was scarcely regenerated from the complex by caffeine. In none of the several preparations was it possible to recover by caffeine more than 10% of the starting material. Figure 3 presents a diagram of the electrophoresis pattern of SSA-soluble protein from the sucrose homogenate and the subcellular fractions. The protein extracted by SSA from the homogenate corresponded in its electrophoretic behaviour to the preparation obtained directly from an SSA-extract from thymus. The nuclei contained only the fraction $F_5$, from which separated a faster-moving fraction, $F_6$. Mitochondria contained two fractions, $F_4$ and a smaller one, $F_3$. The post-mitochondrial supernatant contained fraction $F_1$ and a weakly visible fracton $F_2$.

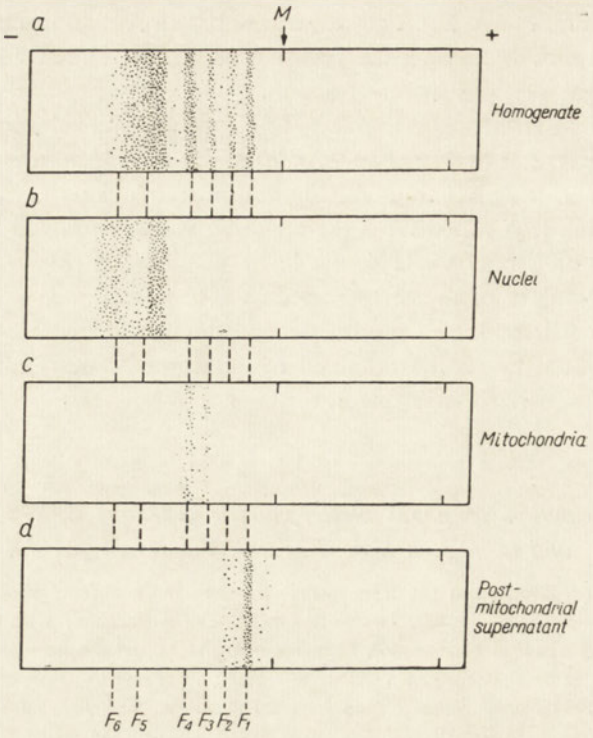


Fig. 3. Diagram of paper electrophoresis of SSA-soluble protein from subcellular fractions of calf thymus. pH 4.4, staining with Amido black; *M*, middle of the strip.

*Lysine-rich histone.* The preparation obtained by the method of Kinkade & Cole (1966) appeared to be heterogeneous; on free-boundary electrophoresis it was resolved into three fractions (Fig. 1b) and on paper electrophoresis into five fractions moving toward the cathode (Figs 1a and 2b). The percentage distribution of paper electrophoretic fractions estimated after elution with NaOH by direct densitometry (Table 1) was similar to the distribution of SSA-fractions, except that fraction $F_4$ was significantly higher, and fraction $F_5$ lower, than in the SSA preparations.

Under the conditions applied for SSA-soluble proteins, the lysine-rich histones prepared after Kinkade & Cole (1966) were precipitated by tannin and regenerated by caffeine. After this procedure, no significant changes were found in their electrophoretic pattern. From 100 g. of thymus, on the average 0.08 g. of protein was extracted with 8% trichloroacetic acid. When this protein was precipitated by tannin and regenerated by caffeine (the recovery being 70%), the preparation obtained did not differ on paper electrophoresis from histone preparations obtained by the method of Kinkade & Cole (1966). From nuclei, one fraction with the electrophoretic mobility of fraction $F_5$, was extracted with 8% trichloroacetic acid, similarly as with SSA.

## DISCUSSION

Preparation of lysine-rich histone obtained from calf thymus by the method of Kinkade & Cole (1966) and preparation obtained by the tannin-caffeine procedure from the sulphosalicylic acid extract, give at pH 4.4 the same patterns both in free-boundary and paper electrophoresis. According to Johns & Butler (1962), lysine-rich histone is extracted from thymus also with 5% perchloric acid. From our experiments it follows that sulphosalicylic acid can also be applied for extraction of lysine-rich histone.

Extraction by acid causes dissociation of nucleoprotein. Sulphosalicylic acid, similarly as trichloroacetic acid and perchloric acid, extracts only lysine-rich histone, whereas nucleic acids, protein other than histone and arginine-rich histone remain in the sediment. Tannin was found to be a suitable reagent for isolating lysine-rich histone from trichloroacetic acid or SSA extracts. Utilization of sulphosalicylic acid for extraction, and then the tannin-caffeine procedure, permitted to obtain histone directly from the thymus, from sucrose homogenates or from nuclei, in a high yield. Histone preparations obtained from thymus homogenates contained about 10% of protein derived from mitochondria and other subcellular structures.

The SSA-soluble thymus protein isolated by the tannin-caffeine procedure gave an electrophoretic pattern closely similar to the pattern obtained with SSA-soluble proteins from kidney (Mejbaum-Katzenellenbogen & Wieczorek, 1966) or from skeletal muscle (Mejbaum-Katzenellenbogen *et al.*, 1967; Mejbaum-Katzenellenbogen, Łomako & Maskos, to be published), except that the fastest-moving fraction amounted to 90% as compared with 30% for kidney and skeletal muscle. This seems to be due to the greater size of nuclei in thymus cells.

From the unpublished experiments of E. Wieczorek it appears that rabbit-kidney nuclei contain a cathodic fraction corresponding to the lysine-rich thymus histone.

According to Rzeczycki *et al.* (1962, 1963), the lysine-rich fraction present in SSA-soluble protein from hog kidney originated from mitochondria. These authors assumed that histones are not extracted with sulphosalicylic acid, as from 5 times washed nuclei only a very small amount of protein was obtained by SSA-extraction. The results obtained in our work indicate that lysine-rich histones are extracted with sulphosalicylic acid and form the fastest-moving fraction of thymus SSA-soluble proteins.

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## BIAŁKA ROZPUSZCZALNE W KWASIE SULFOSALICYLOWYM I ICH LOKALIZACJA W STRUKTURACH PODKOMÓRKOWYCH GRASICY CIELĘCEJ

### Streszczenie

1. Wykazano, że histony bogate w lizynę ekstrahują się kwasem sulfosalicylowym z homogenatów cukrozowych i jąder komórkowych grasicy cielęcej.

2. Stosując metodę taninowo-kofeinową uzyskano z jąder komórkowych z dobrą wydajnością czysty preparat histonów bogatych w lizynę.

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# FURTHER CHARACTERIZATION OF ACID PHOSPHOMONOESTERASE OF HUMAN PROSTATE

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1. Isolation of highly purified preparation of acid phosphomonoesterase from human prostate is presented. 2. The preparation was homogeneous in free-boundary electrophoresis, during sedimentation in ultracentrifuge and during immunoelectrophoresis. 3. The molecular weight of the enzyme determined by sucrose gradient centrifugation is about 96 000.

The isolation of highly purified acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) from human prostate makes possible the use of this enzyme for splitting off terminal phosphate when determining the structure of oligo- and polynucleotides (Markham & Smith, 1952; Privat de Garilhe, Cunningham, Laurila & Laskowski, 1957; Vanecko & Laskowski, 1962; Staehelin, 1964; Novogrodsky & Hurwitz, 1966). Availability of the pure enzyme will also make possible further studies on the physico-chemical and enzymic properties of the phosphatase.

By combining the previously described method of purification (Ostrowski & Tsugita, 1961) with filtration on dextran gel (Ostrowski & Rybarska, 1965), a preparation of phosphomonoesterase has been obtained which is homogeneous when examined in the ultracentrifuge, in free-boundary electrophoresis, disk electrophoresis, and immunoelectrophoresis. The obtained enzyme has a molecular weight of about 96 000 and is free of appreciable contamination by phosphodiesterase (EC 3.1.4.1) and ribonuclease (EC 2.7.7.16).

## MATERIALS AND METHODS

Human hypertrophic prostate glands were obtained from the Urological Clinic of the Medical School in Cracow. Immediately after removal at operation, the tissue was frozen and stored at -25°.

DEAE-cellulose, Whatman powder DE-50, was obtained from W. & R. Balston Ltd. (England). Before use, the adsorbent was treated as previously described (Ostrowski & Tsugita, 1961). DE-52-cellulose, Whatman microgranular, preswollen,

was obtained from the same company; before use it was only washed with the starting buffer solution in which adsorption was carried out. Sephadex G-100, 140 - 400 mesh, was a product of Pharmacia (Uppsala, Sweden). Bovine-liver catalase (EC 1.11.1.6) was purchased from Light & Koch Co. (Colnbrook, England).

Horse $HbO_2$, crystallized three times from water containing 12% ethanol, was prepared by the method described by Heidelberger (1922).

Homogeneity of acid phosphomonoesterase was examined by the use of a Spinco model E ultracentrifuge equipped with a phase plate as optical schlieren diaphragm. In all the experiments, the AnD rotor with double-sector cells, 12 mm. light-pathway, was used. Centrifugation was performed in phosphate buffer of pH 7.4 and ionic strength 0.1 at 50 740 rev./min. at 20°.

Ultracentrifugation in sucrose gradient was carried out on a Martin-Christ (West Germany) Omega type preparative ultracentrifuge using a swinging bucket rotor, no. 001. The linear gradient of sucrose from 5 to 20% (w/v) was prepared in 0.05 M-tris-HCl buffer of pH 6.5 according to Britten & Roberts (1960). A sample of phosphomonoesterase (100 µl.) containing about 0.2 mg. of protein per ml., 1 mg. of $HbO_2$ and 0.5 mg. of catalase was dialysed against the same buffer, layered on the top of the gradient (4.65 ml.) and centrifuged at about 5° for 8 - 16 hr. at 38 000 rev./min. Then the bottom of the tube was punctured with a thin injection needle as described by Szybalski (1960), fractions (7 drops each) were collected, and analysed for phosphomonoesterase activity, for $HbO_2$ by measuring extinction at 417 mµ, and for catalase by measuring the rate of decomposition of $H_2O_2$ at 240 mµ (Martin & Ames, 1961). Analysis of the results and calculation of molecular weight were made by the method described by Martin & Ames (1961).

Moving-boundary electrophoresis was carried out in a C. Zeiss (Jena, East Germany), type 35 apparatus equipped with schlieren optics. Separation was carried out at 4°.

Disk electrophoresis was performed on an apparatus similar to the one manufactured by Canalco Co. (Canal Industrial Corp., Rockville, Ma., USA) in standard 7.5% polyacrylamide gel (Canalco Co.). Separation was done in 0.01 M-tris-glycine buffer of pH 8.4, at 1.5 mA per tube and 190 v. Preparation of the gel, separation and staining of protein were performed by the method described by Ornstein & Davis (1964).

Immunoelectrophoretic analysis was performed, and rabbit antiserum against acid phosphomonoesterase was obtained, as previously described (Ostrowski, Weber & Rybarska, 1966).

The activity of phosphomonoesterase and phosphodiesterase was estimated as described by Ostrowski & Tsugita (1961) using as substrate, respectively, *p*-nitrophenylphosphate and bis-*p*-nitrophenylphosphate (both products of Sigma Biochem. Co., St. Louis, Mo., U.S.A.). The activity of both enzymes was expressed in µmoles or m-moles of *p*-nitrophenol split off by 1 ml. of enzyme solution at 25° within one minute for phosphomonoesterase, and 60 min. for phosphodiesterase.

Protein was assayed by measuring extinction at 280 mµ on an Uvispec (Hilger & Watts, London, England) spectrophotometer.

## RESULTS

### *Purification of acid phosphomonoesterase*

*Extraction of the enzyme.* All the procedures involved in purification of the enzyme were carried out in a cold-room at 3°. The frozen prostate glands were cut into thin (0.5 mm.) slices with a sharp knife and treated with 0.01% (v/v) solution of Tween 80. For each 100 g. of slices, 300 ml. of detergent solution was added, and the mixture was set aside overnight with continuous stirring by means of a slowly rotating stirrer, to avoid foaming of the mixture. Then it was filtered through three layers of gauze, the slices were discarded and the filtrate fractionated with ammonium sulphate.

*Fractionation with ammonium sulphate.* Solid ammonium sulphate was added to the filtrate to 55% saturation, and the mixture was set aside for several hours. Then the sediment was centrifuged off in a cooled centrifuge at 3000 *g* for 20 min. and discarded; solid ammonium sulphate was again added to the solution to 68% saturation. The mixture was set aside overnight, then the sediment was collected by centrifuging at 22 000 *g* for 30 min. and extracted with 10 volumes of 0.1 M-citrate buffer (citric acid - NaOH) of pH 4.0; after centrifugation at 22 000 *g* for 30 min., the sediment was extracted again under the same conditions. The two supernatants were combined and dialysed overnight against water. The sediment precipitated during dialysis was centrifuged off, and the clear solution was dialysed for 12 hr. against 0.05 M-tris-HCl buffer of pH 6.5 containing 0.1 M-KCl. If a precipitate formed during dialysis, it was centrifuged off and discarded. The enzyme solution obtained in this way contained about 3 mg. of protein per ml., and 1 ml. of the solution split off 0.175 m-mole of *p*-nitrophenol within 1 min. at 25°.

*Filtration on Sephadex G-100.* To a column (3.5 × 120 cm.) filled with Sephadex G-100 and equilibrated with 0.1 M-KCl - 0.05 M-tris-HCl buffer, pH 6.5, 10 ml. of the enzyme solution was carefully applied. After the sample had soaked into the gel and after washing the walls of the column with 2 ml. of buffer, the enzyme was eluted with the same buffer, 5-ml. fractions being collected at a rate of 20 ml./hr. Three protein fractions were obtained, of which the middle one with the smallest protein content possessed phosphomonoesterase activity (Fig. 1). Phosphodiesterase was present in the third fraction together with haemoglobin, which was always found in extracts of the prostate gland. The yield of enzymic activity and protein during elution from the column attained nearly 100% of the amount applied.

*Chromatography on DEAE-cellulose.* Sephadex G-100 fractions with the highest phosphomonoesterase activity (shaded area in Fig. 1) were pooled, and dialysed for 24 hr. against 0.0175 M-Na-phosphate buffer of pH 7.0. The dialysed solution (about 100 ml. containing 0.3 - 0.5 mg. protein/ml.) was adsorbed on the DEAE-cellulose column (21 × 1.0 cm.), equilibrated with the same buffer. After washing the column with 150 ml. of the starting buffer, the enzyme was eluted with a convex pH gradient of 0.07 M-Na-phosphate buffer of pH 6.0, the mixing chamber with

constant capacity containing 250 ml. of starting buffer. A typical course of elution of the enzyme is illustrated in Fig. 2. Fractions with constant specific activity (105 - 140) were combined and dialysed overnight against 0.0175 M-phosphate buffer of pH 7.0. The inactive proteins remaining on the column were eluted by passing 0.1 M-phosphate buffer of pH 6.0 containing 0.5 M-NaCl. This fraction

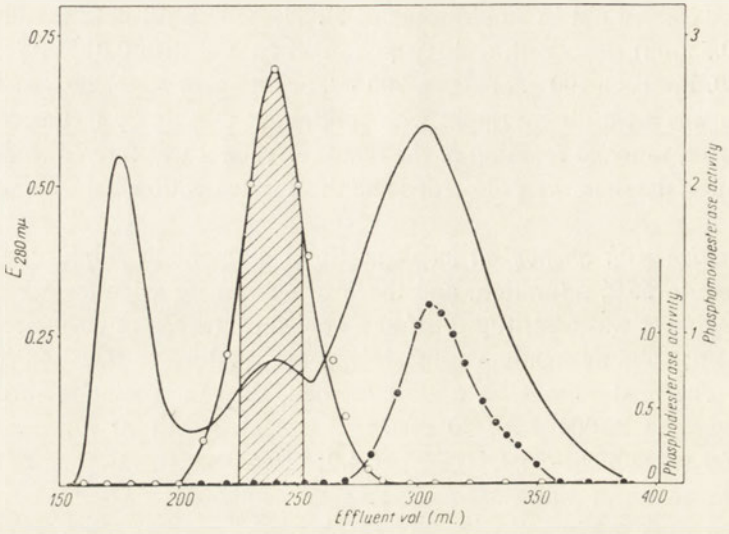


Fig. 1. Gel filtration on Sephadex G-100 column of ammonium sulphate fraction extracted at pH 4. Conditions as described in the text. (—), Protein; (○), activity of phosphomonoesterase, m-moles/ml./min.; (●), activity of phosphodiesterase, m-moles/ml./60 min.

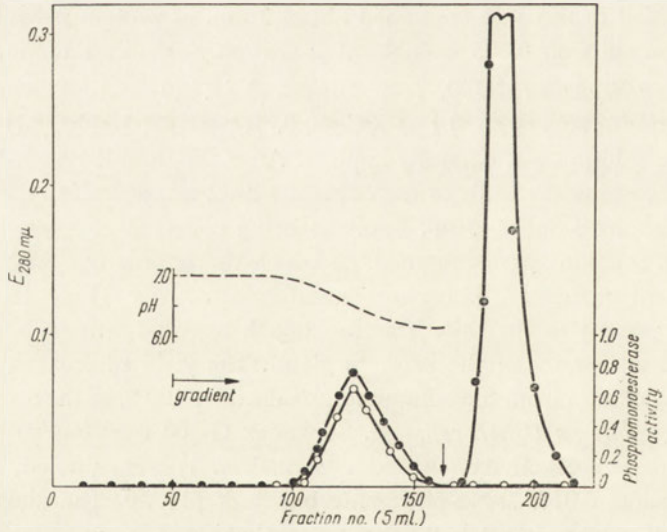


Fig. 2. DEAE-cellulose chromatography of phosphomonoesterase fraction from gel filtration (shaded area from Fig. 1). Conditions as described in the text. (●), Protein; (○), activity of phosphomonoesterase, μmoles/ml./min.; (— — —), pH gradient. Arrow shows the change to 0.1 M-phosphate buffer, pH 6, containing 0.5 M-NaCl.

contained slight phosphomonoesterase activity, not exceeding 10% of the activity applied to the column.

*Concentration of the enzyme solution.* The dialysed solution, obtained as described above, was adsorbed on a small column with DE-52-cellulose (volume of the adsorbent about 1 ml.) equilibrated with 0.0175 M-phosphate buffer of pH 7.0. The enzyme was eluted with 2 ml. of 0.2 M-phosphate buffer of pH 6.0. About 90% of phosphomonoesterase activity was recovered, the protein concentration being about 5 mg./ml.

*Refiltration on Sephadex G-100.* The concentrated enzyme solution was applied to a high column with Sephadex G-100 (150×0.9 cm.) under conditions identical with those used for the first filtration. A typical example of separation, with only small amounts of balast proteins emerging from the column, is shown in Fig. 3. Fractions with constant specific activity were pooled, giving the preparation with which further experiments, described in this paper, were performed.

*Remarks on the purification procedure.* The procedure described above gave about 100-fold purification of the enzyme in respect to the crude extract of tissue, with 15 - 18% yield. Optimal parameters of purification were achieved when about 100 g. of tissue was taken to start with, and columns of the above given dimensions

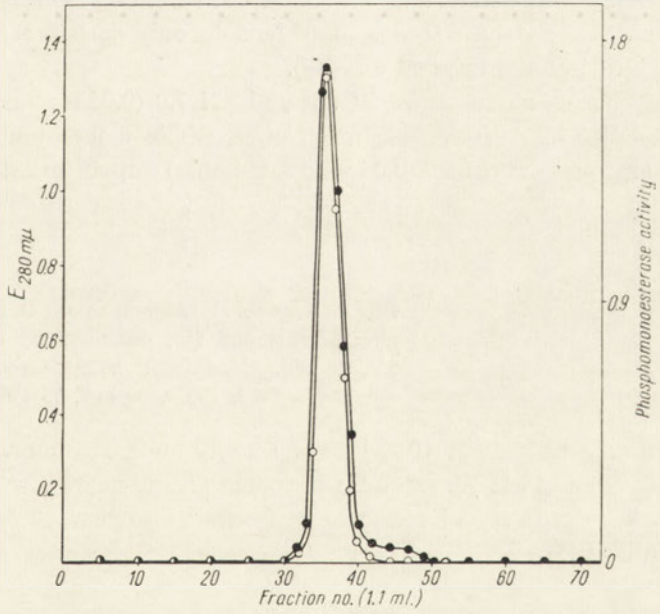


Fig. 3. Refiltration of DEAE-cellulose fraction on Sephadex G-100 column. Active fractions from Fig. 2 were concentrated to about 2 ml. as described in the text. The elution was at the rate of 10 ml./hr. (●), Protein; (○), phosphomonoesterase activity, m-moles/ml./min.

were used. The purified preparation was homogeneous (see below) and its specific activity was 1.65 m-moles of *p*-nitrophenol liberated by 1 mg. of enzyme protein at 25° during 1 min. incubation at optimal ratio of substrate to enzyme.

The highest yield of purification was obtained by filtration on Sephadex G-100,

on which most of phosphodiesterase and Hb were separated from phosphomonoesterase. The excellent separation of phosphomonoesterase from phosphodiesterase was mainly due to the marked difference between the molecular weights of the two enzymes. It has been found from the sedimentation rate in sucrose gradient of the two enzymes that the molecular weight of phosphodiesterase is nearly one-half that of phosphomonoesterase (about 50 000). The greatest loss of phosphomonoesterase, attaining 50%, occurred in the stage of fractionation with ammonium sulphate due to the wide range of solubility of the enzyme in neutral salt solution (London & Hudson, 1953).

*Stability of phosphomonoesterase.* In solutions of pH 6.0 frozen at -25° the enzyme is stable for several years. No activity was lost during repeated freezing and thawing. Freshly isolated and concentrated enzyme, after freezing showed a slight increment, about 10%, of activity on the first few weeks of storage, presumably owing to partial deformation of the tertiary structure of the molecule during purification, and return to the entirely active configuration under the conditions of storage of the enzyme.

In urea solution the enzyme was unstable, and at 3 M concentration at 25° and pH 7.0 (0.05 M-phosphate buffer) after one minute lost more than 90% of its activity. At 0° a similar effect was obtained at 6 M concentration of urea. Dilution or dialysis of the enzyme denatured by 6 M-urea at 0° lead to only partial reactivation (to approximately 15% of the original activity).

Heating of phosphomonoesterase at 55° and pH 7.0 (0.05 M-phosphate buffer) resulted in complete and irreversible inactivation within a few minutes. Heating at the same temperature at pH 5.0 (0.05 M-acetate buffer) caused very slow denatura-

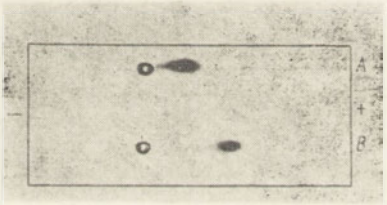


Fig. 4. Agar gel electrophoresis of (*A*), native phosphomonoesterase and (*B*), denatured by heat at pH 7, 55°, 5 min. Conditions: 0.025 M-veronal buffer, pH 8.2, 180 v, 60 mA, 75 min.

tion of the enzyme and about 10% loss of activity after one hour. The enzyme thermally inactivated at pH 7.0 exhibited increased anodic migration in the electric field (Fig. 4). This property of phosphatase is similar to that of β-lactoglobulin (Briggs & Hull, 1945).

### *Physical data on homogeneity of phosphomonoesterase*

*Chromatographic homogeneity of purified enzyme.* One of the criteria of purity of phosphomonoesterase is based on protein content and enzymic activity of fractions eluted from DEAE-cellulose at continuous pH gradient. Figure 5 shows the result of rechromatography of the concentrated enzyme preparation on DE--52-cellulose column. The protein peak and enzymic activity coincide entirely, and specific activity is virtually identical in all the fractions obtained during elution.

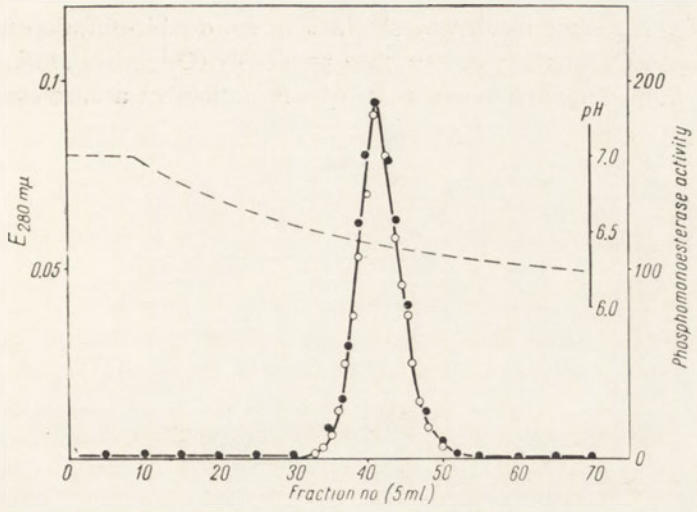


Fig. 5. Rechromatography of 12 mg. sample of phosphomonoesterase on DE-52-cellulose. A 0.9 × 21 cm. column was equilibrated with 0.0175 M-sodium phosphate buffer, pH 7. After a volume of about 100 ml. of starting buffer had passed, a convex gradient was started to 0.1 M-phosphate buffer, pH 6. Five-ml. fractions were collected at the rate of 10 ml./hr. (●), Protein; (○), phosphomonoesterase activity, μmoles/ml./min.; (— — —), pH gradient.

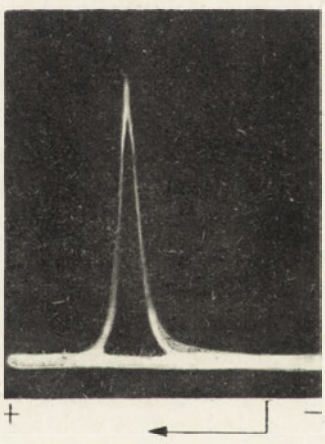


Fig. 6. Free-boundary electrophoresis of purified phosphomonoesterase. Ascending boundaries; 115 min. of migration from right to left. Protein concentration was 1%.

*Free-boundary electrophoresis.* The rechromatographed and concentrated preparation of acid phosphomonoesterase dialysed overnight against one litre of Na-phosphate buffer, pH 7.0, *I* 0.1, and then separated at 100 v and 8.5 mA at 4°, gave a symmetrical single band migrating toward the anode (Fig. 6) with the mobility of $3.98 \times 10^{-5}$ cm.$^2$v$^{-1}$sec.$^{-1}$.

*Disk electrophoresis.* Separation performed in conditions described under Methods indicated homogeneity of the phosphomonoesterase preparation, as can be seen in Fig. 7.

*Immunoelectrophoretic analysis and precipitin reaction.* On immunoelectrophoresis, the phosphomonoesterase preparation gave a single, regular precipitin

arc (Fig. 8). The same result was obtained in the double-diffusion test on agar gel according to Ouchterlony as described previously (Ostrowski *et al.*, 1966). The reaction of phosphomonoesterase with its specific antibody was also examined with

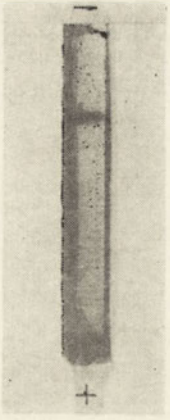


Fig. 7.

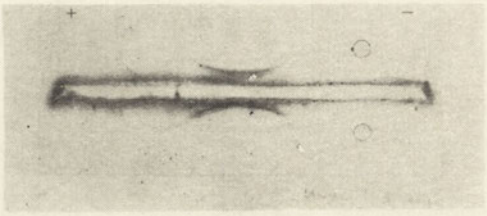


Fig. 8.

Fig. 7. Polyacrylamide gel electrophoresis of phosphomonoesterase. Conditions were as outlined in Methods.

Fig. 8. Immunoelectrophoretic analysis of phosphomonoesterase in agarose gel. Glass plates (76×25 mm.) covered with a layer of 1% agarose gel (Light & Koch, England) in 0.025 M-veronal buffer, pH 8.2, were used (Ostrowski *et al.*, 1966).

reference to precipitation and inactivation of the enzyme by antiserum. Some of the experiments pertinent to these problems have already been published (Ostrowski *et al.*, 1966). It has been demonstrated that the antigen-antibody precipitate formed in agar gel or directly in the test tube after mixing the enzyme with antiserum, retains its full enzymic activity toward *p*-nitrophenylphosphate. Phosphomonoesterase activity remaining in the supernatant after reaction of antiserum with serial dilutions of the pure enzyme, is shown in Fig. 9. On comparing the ratio obtained with control serum (serum of a non-immunized rabbit) it can be seen that the amount of antiserum used precipitated all enzymic activity from the solution up to a concentration of 30 μg./ml. This value, corresponding to the equivalence point, is identical with the value obtained in the quantitative precipitin reaction of antigen with antibody as well as in studies by the double-diffusion method in agar gel (Ostrowski *et al.*, 1966).

*Sedimentation and molecular weight of phosphomonoesterase.* In the analytical ultracentrifuge, the purified and concentrated preparation (5 mg. of protein per 1 ml.) showed a single symmetrical peak with $S_{20,w}$ 5.6, and no detectable contaminations were found during sedimentation (Fig. 10). A detailed study on sedimentation in the ultracentrifuge and dissociation of the enzyme molecule into subunits (M. Derechin, W. Ostrowski & E. A. Barnard) is to be published. Centrifugation in sucrose gradient (Fig. 11) indicates that the phosphatase preparations show density homogeneity as evidenced by the Gaussian character of the activity distribution near its effective density. In the homogeneous preparation a linear relationship

should be obtained by plotting the natural logarithm of phosphatase concentration against the square of the distance of each fraction from the region of maximum concentration of the enzyme (Krentz & Levy, 1963; Spragg & Rankin, 1967). The

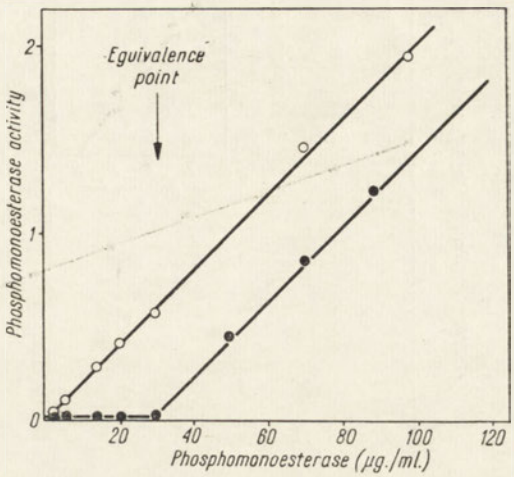


Fig. 9. Titration plot of phosphomonoesterase activity with specific antiserum. The plot shows relationship of enzyme activity remaining in supernatant with respect to antigen concentration after reaction with (○), control serum and (●), antiserum.

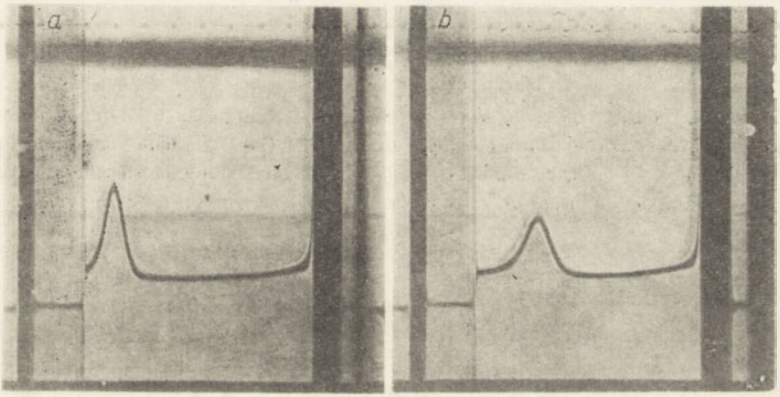


Fig. 10. Sedimentation patterns of phosphomonoesterase, taken (*a*), 25 min., and (*b*), 55 min., after reaching final speed of 50 740 rev./min. Protein concentration was about 0.5%. Bar angle was 70°.

plot presented as inset in the figure indicates the high degree of density homogeneity of the obtained enzyme preparation.

The results of sedimentation of phosphomonoesterase in sucrose gradient with reference to standard proteins are illustrated in Fig. 12. The values of R, sedimentation coefficient $S_{20,w}$, and molecular weights calculated in relation to standard proteins are summarized in Table 1. In relation to catalase, $S_{20,w}$ was 5.684 and molecular weight 89 100, and in relation to $HbO_2$ the respective values were 5.786 and 102 500.

To study the relationship between the distance of displacement of the phosphomonoesterase band in the gradient as a function of time of centrifugation, and

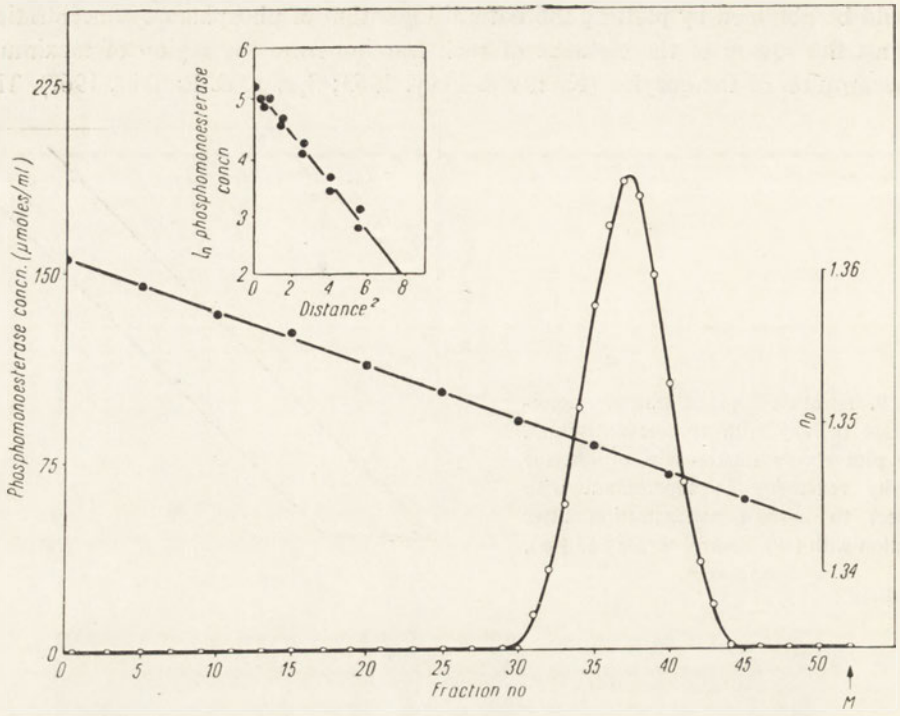


Fig. 11. Sucrose density centrifugation of phosphomonoesterase. (○), Phosphomonoesterase concentration, expressed in activity units, μmoles/ml.; (●), gradient, determined using Zeiss Abbé refractometer. In inset, relationship between ln of phosphomonoesterase concentration and square of distance from maximum concentration. *M*, Meniscus.

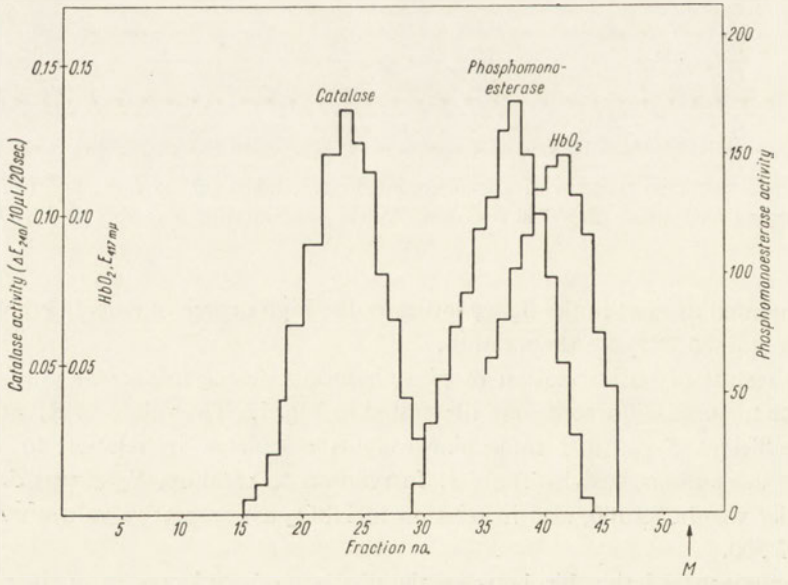


Fig. 12. Molecular weight determination of phosphomonoesterase by sucrose-gradient ultracentrifugation. See Methods for details. *M*, Meniscus.

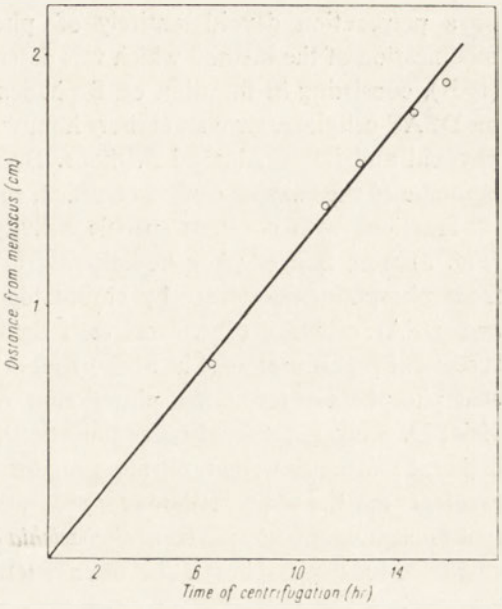


Fig. 13. Sedimentation behaviour of phosphomonoesterase. Each point represents a centrifugation experiment similar to the one shown in Fig. 12.

Table 1

*Sucrose-gradient centrifugation of phosphomonoesterase*

Values for phosphomonoesterase calculated in relation to standard proteins, are given.

| | Standard protein | |
|---|---|---|
| | Catalase | $HbO_2$ |
| R* | 0.496 | 1.315 |
| $S_{20,w}$ | 5.684 | 5.786 |
| Mol. wt. | 89 100 | 102 500 |
| Mean value | 95 800 | |

* R, ratio of displacement of unknown and standard protein during centrifugation.

thereby to check the reproducibility of results of centrifugation, the different samples of the enzyme were centrifuged under identical conditions for different time intervals. As can be seen in Fig. 13, all the points lie on a straight line which passes through the origin of the co-ordinate system. On the basis of the data obtained, it was possible to calculate for phosphomonoesterase the mean value of sedimentation, which at pH 6.5 and 38 000 rev./min. amounted to 0.125 cm./hr.

## DISCUSSION

Of the many methods described for purifying acid phosphomonoesterase from human prostate (Kerr & Chernigoy, 1957; Boman, 1958; Davidson & Fishman, 1959; Andreu, Fernandez Alvarez & Lora-Tamyo, 1960), none gives an entirely homogeneous preparation when tested by different physico-chemical methods,

or a preparation devoid entirely of phosphodiesterase as contaminant. The modification of the method which was described previously by Ostrowski & Tsugita (1961), consisting in filtration on Sephadex G-100 in addition to chromatography on DEAE-cellulose, gave an entirely homogeneous preparation as judged by various physical and immunological methods. By means of the described method, larger amounts of the enzyme can be obtained, sufficient for chemical analysis of protein.

Fractions with constant specific activity after refiltration on Sephadex G-100 (Fig. 3) were free of phosphodiesterase activity. Separation of phosphodiesterase from phosphomonoesterase by chromatography on Dowex 50X2 (Boman, 1958) and DEAE-cellulose (Ostrowski & Tsugita, 1961) is a tedious and not always successful procedure, as it has been found by different authors who used prostatic acid phosphatase for dephosphorylation of oligonucleotides (Petersen & Burton, 1964; D. Shugar, personal communication). Since the two enzymes have markedly different molecular weight, filtration on dextran gel or ultracentrifugation in sucrose gradient are the main methods for separating these two activities. Although at present phosphomonoesterase from *Escherichia coli* (Garen & Levinthal, 1960) is usually employed for dephosphorylation when determining the sequence of oligonucleotides, nevertheless the characteristic properties of prostatic acid phosphatase such as high stability at low temperature and slightly acid pH, high specific activity, and rapid thermal inactivation at pH values above 7, make it a convenient reagent for studies on the structure of nucleic acids and phosphoproteins.

In the present work, determination of the molecular weight of the enzyme was based on centrifugation in sucrose gradient. The main advantage of this technique is that the homogeneity of the studied preparation as well as its molecular weight can be determined in the same, simple experiment. The results obtained by this method are concordant with the data obtained by filtration on Sephadex G-100 which gave a molecular weight of 109 000, and with the results obtained in the analytical ultracentrifuge, which gave a molecular weight of about 100 000 by the sedimentation equilibrium method (Derechin *et al.*, to be published). However, it should be emphasized that the molecular weight determined by sucrose-gradient centrifugation is only approximate, because in the equation of Martin & Ames (1961) shape factors of the protein molecule are not taken into account. Nevertheless, the mean value of the sedimentation coefficient obtained by this method, amounting to 5.7, was very near the value of 5.9 obtained by Boman (1958), and 5.65 recently obtained by Derechin *et al.* (to be published). All these values of the sedimentation coefficient are higher than those reported by Vernon *et al.* (1965) for both active fractions of the enzyme obtained from human semen. This discrepancy requires further studies on a larger amount of pure material.

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This work was aided by a grant from the Committee of Biochemistry and Biophysics of the Polish Academy of Sciences. The author is indebted to Professor E. Michałowski for prostatic tissue supply, to Dr. Mirosława Weber for help in immunoelectrophoretic analysis, and to Mrs. Maria Łabędź for technical assistance.

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## DALSZA CHARAKTERYSTYKA KWAŚNEJ FOSFOMONOESTERAZY STERCZU LUDZKIEGO

### Streszczenie

1. Opisano sposób przygotowania kwaśnej fosfomonoesterazy ze sterczu ludzkiego, wolnej od zanieczyszczeń fosfodwuesterazą.

2. Preparat enzymu jest jednorodny przy badaniu za pomocą wolnej elektroforezy, w ultrawirówce oraz podczas immunoelektroforezy.

3. Ciężar cząsteczkowy enzymu oznaczony na podstawie ultrawirowania w gradiencie sacharozy wynosi ok. 96 000.

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# ON THE FORMATION OF ACETOACETATE BY RAT-LIVER MITOCHONDRIA

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1. Acetoacetate formation from oleate by rat-liver mitochondria was studied in a saline medium containing $Mg^{2+}$ and phosphate but without ATP and „sparker". 2. Two maxima of acetoacetate formation were observed: one at 0.1 - 0.2 μmole oleate per 5 - 10 mg. mitochondrial protein, and the other at ten times that concentration. 3. Oxidative phosphorylation could be found only in the region of the first maximum, while acetoacetate formation corresponding to the second maximum proceeded under conditions of uncoupled oxidative phosphorylation. 4. Acetoacetate formation at low oleate concentrations (first maximum) was inhibited by 2,4-dinitrophenol, oligomycin and azide, and by omission of phosphate in the incubation medium. At higher oleate concentration (second maximum) the formation of acetoacetate was stimulated by oligomycin and azide, and was not affected by 2,4-dinitrophenol and by the omission of phosphate.

Fatty acids can be oxidized by isolated liver mitochondria in simple saline media in the absence of added adenine nucleotides, CoA, nicotinamide nucleotides, cytochrome *c*, and intermediates of the tricarboxylic-acid cycle (Wojtczak, Załuska & Drahota, 1965).

It has been shown previously (Wojtczak *et al.*, 1965; Wojtczak, Drahota, Załuska & Zborowski, 1966) that the incorporation of fatty acids into phospholipids and the oxidation of fatty acids are not inhibited by oligomycin and are only partially depressed by 2,4-dinitrophenol. Hence, it has been suggested that high-energy intermediates of oxidative phosphorylation can serve as a source of energy for fatty acid activation.

Rossi & Gibbson (1964) demonstrated a GTP-dependent thiokinase in mitochondria. Van den Bergh (1965, 1966), who has also found an ATP-independent system that activates fatty acids, claims that this system is identical with the GTP-thiokinase, and utilizes energy produced by substrate-level phosphorylation in the tricarboxylic-acid cycle. Finally, Beattie & Basford (1966a,b) conclude that in brain mitochondria fatty acids can be activated by high-energy intermediates of oxidative phosphorylation, but for incorporation into phospholipids ATP is necessary.

In the presented work acetoacetate formation by isolated liver mitochondria was

taken as an indicator of oleate degradation and of the oxidation of fatty acids under conditions of uncoupled oxidative phosphorylation. Further evidence is presented to show that fatty acid oxidation can occur even in uncoupled mitochondria. This oxidation occurring at higher concentration of oleate is not inhibited by 2,4-dinitrophenol, is activated by oligomycin and azide, and is independent of the presence of inorganic phosphate.

## MATERIAL AND METHODS

Rat-liver mitochondria were isolated as described by Hogeboom (1955). The density of mitochondrial suspension was adjusted on the basis of turbidimetric measurements of a sample in 0.02 M-tris-HCl, pH 7.4, so that the ratio between the amount of mitochondrial protein and added oleate could be maintained constant.

Mitochondria were incubated at 30°, the gas phase being air, in a total volume of 2.0 ml. containing: 50 mM-KCl, 8 mM-tris-HCl (pH 7.4), 6 mM-$MgCl_2$, 14 mM-potassium phosphate (pH 7.4), 25 mM-sucrose and about 8 mg. of mitochondrial protein. The procedure for measuring oxidative phosphorylation was that described by Slater & Holton (1953). Oleate oxidation was measured polarographically with the Clark oxygen electrode. The activity of succinate oxidase was determined manometrically according to Potter & Schneider (1943).

Acetoacetate was determined colorimetrically according to Walker (1954). Sodium acetoacetate used for the calibration curve was prepared according to the method of Ljungren (1924) and was standardized according to Edson (1935).

In isotope experiments [1-$^{14}$C]oleic acid (Radiochemical Centre, Amersham, England) was used. $CO_2$ evolved was absorbed by NaOH solution and was measured by an ultra-thin window Geiger counter. Acetoacetate was decarboxylated according to Edson (1935) and $^{14}CO_2$ absorbed in NaOH. All values were corrected for self-absorption.

Protein was determined according to Lowry, Rosebrough, Farr & Randall (1951).

Oligomycin was a generous gift of Dr. A. L. Lehninger and hexokinase was a gift of Sigma Chem. Co. (St. Louis, Mo., U.S.A.).

## RESULTS

Two peaks of the rate of acetoacetate formation in dependence on the oleate concentration were found, one at 0.1 - 0.2 μmole oleate per 5 - 10 mg. of mitochondrial protein and the other at ten times that concentration (Fig. 1A). Oxidative phosphorylation was found only in the region of the first peak (Fig. 1A, B). The fall in the rate of acetoacetate formation parallels the decline in phosphorylating activity (1st peak) and is the result of inhibition of the initial reactions in the fatty acid oxidase system, since the activity in the respiratory chain determined as succinate oxidase is not affected by the same concentration of oleate (Fig. 1C). The fall in acetoacetate production with higher oleate concentrations (2nd peak)

is accompanied by a decline in succinate oxidase activity (Fig. 1C) and by a release of intramitochondrial soluble proteins (Fig. 1D), i.e. it is due to the destruction of mitochondria.

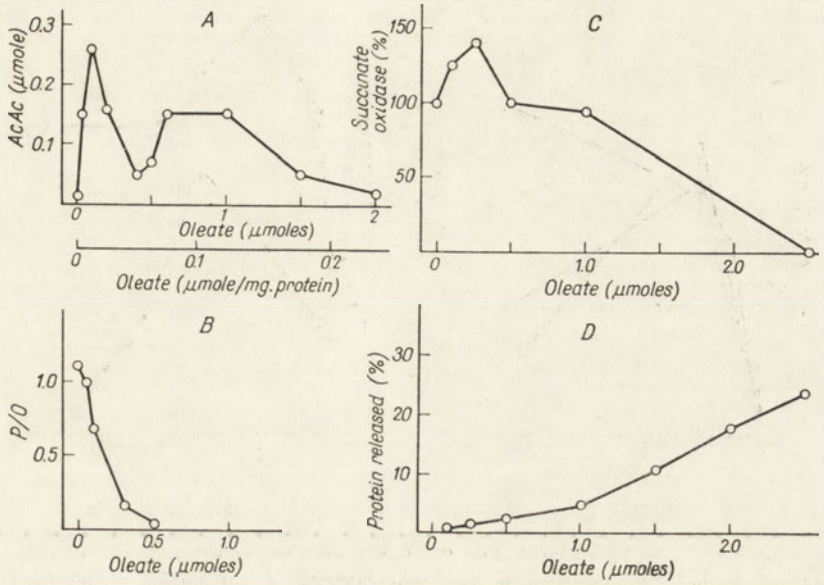


Fig. 1. Acetoacetate (AcAc) formation from oleate (*A*), and the effect of oleate on: (*B*), the P/O ratio; (*C*), succinate oxidase, and (*D*), release of soluble mitochondrial proteins. Succinate was used as a substrate for P/O ratio measurements (*B*); succinate oxidase is expressed in percentages of the activity without oleate, and the release of mitochondrial proteins (*D*) as increase of protein (in percent of total mitochondrial protein) in the supernatant after centrifugation for 5 min. at 20 000 *g* at room temperature. All measurements were made with the same mitochondrial preparation. Samples of 8.4 mg. of mitochondrial protein were incubated for 20 min.

Cyanide completely blocks acetoacetate formation. Hexokinase and glucose are slightly inhibitory in the region of the first peak and are without effect in that of the second peak. Elimination of $Mg^{2+}$ from the medium decreases acetoacetate formation. Acetoacetate production from oleate is linear for the first 20 min. in the first peak and for 60 - 90 min. in the second peak.

Addition of ATP does not prevent the decrease of acetoacetate formation on increasing concentration of oleate. Evidently the affinity of the oleate-induced ATPase to ATP is higher than the affinity of the system activating fatty acids. Only addition of oligomycin with ATP prevents this fall in acetoacetate formation (Fig. 2). Oligomycin itself has an inhibitory effect on the first peak and an activating one when fatty acid concentration is higher. Azide has a similar effect, being inhibitory at low oleate concentrations and stimulatory at higher ones (Fig. 3).

Our data thus give further support to the assumption that there are two independent mechanisms for fatty acid activation. One of them is active when respiratory chain-level oxidative phosphorylation is functioning, and the other is completely independent of energy formation by that phosphorylation. The two

activating systems can be distinguished by using low and high concentrations of oleate. Similarly, Van den Bergh (1966) has distinguished the systems activating palmitate by using inorganic phosphate and 2,4-dinitrophenol. One of these systems

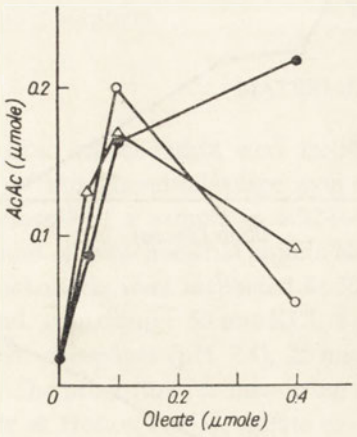


Fig. 2

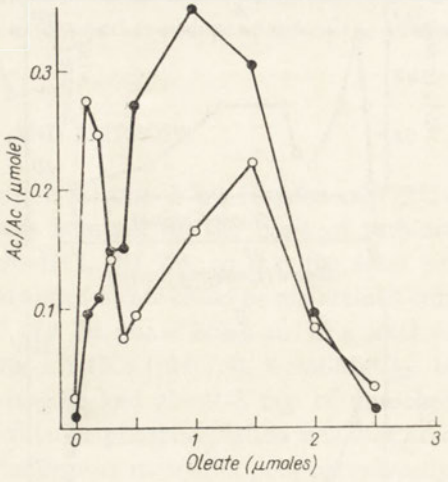


Fig. 3

Fig. 2. The effect of (△), ATP (5 mM), and (●), ATP (5 mM) with oligomycin (1.5 μg./mg. protein) on acetoacetate formation by rat-liver mitochondria. (○), Control, no additions.

Fig. 3. The effect of (●), azide (2 mM) on acetoacetate formation by rat-liver mitochondria. (○), Control.

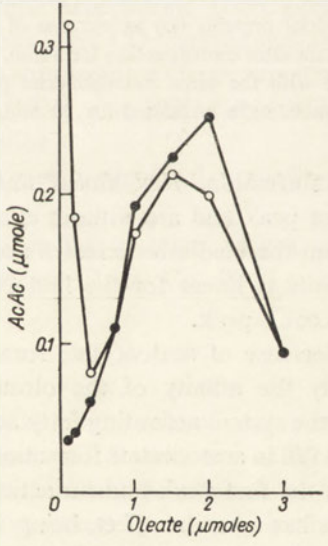


Fig. 4

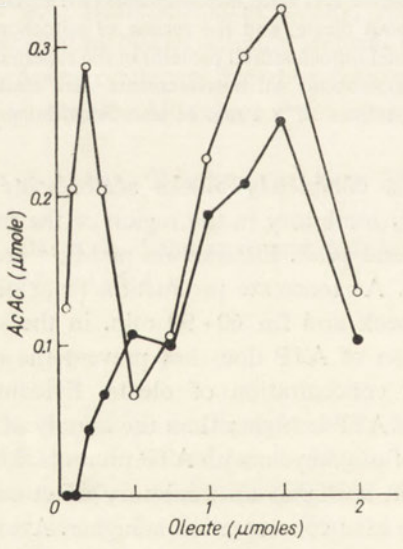


Fig. 5

Fig. 4. The effect of phosphate on acetoacetate formation. (○), 14 mM-Phosphate; (●), no phosphate (concentration of tris-HCl increased to 16 mM).

Fig. 5. The effect of (●), 2,4-dinitrophenol (0.4 mM) on acetoacetate formation by rat-liver mitochondria. (○), Control, no additions.

is characterized by the fact that oxidation of palmitate occurs in the presence of phosphate and is inhibited by 2,4-dinitrophenol, while the other system is dinitrophenol-resistant and occurs in the absence of externally added phosphate only.

Under our conditions both oleate-activating systems can be demonstrated in the presence of inorganic phosphate. The elimination of phosphate considerably decreases acetoacetate formation in the region of the first peak, but has no effect at higher oleate concentrations (Fig. 4). 2,4-Dinitrophenol in the presence of phosphate inhibits acetoacetate formation at lower oleate concentrations and has no effect at higher concentrations (Fig. 5). In the absence of inorganic phosphate, 2,4-dinitrophenol has no effect on the rate of acetoacetate formation.

Evidently, substrate-level phosphorylation serves as an energy source for fatty acid activation. The activity of the tricarboxylic-acid cycle is not diminished at higher oleate concentrations, the percentage of oleate oxidized in the cycle being even increased. This can be concluded from the experiment shown in Fig. 6 where

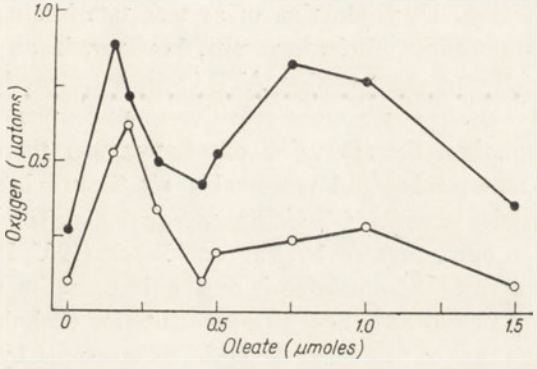


Fig. 6. Oxygen consumption (●), and acetoacetate formation (○) expressed as oxygen necessary for equivalent oleate oxidation.

the difference between total oxygen consumption (full circles) and $O_2$ consumption equivalent to acetoacetate formation (open circles) corresponds to oleate oxidized *via* the tricarboxylic-acid cycle. Using [$^{14}$C]oleate it could be shown that twice as much activity appears in $CO_2$ as in acetoacetate under these conditions.

## DISCUSSION

The formation of acetoacetate from fatty acids under conditions of the non-operation of the tricarboxylic-acid cycle can be a good measure of the oxidation of fatty acids. As it is well known, the oxidation of fatty acids requires their previous activation in the form of acyl-CoA. The oxidation must be therefore coupled to a process providing energy in the form which can be available for the activation. On the other hand, it is known (Pressman & Lardy, 1956; Hülsmann, Elliott &

Slater, 1960; Wojtczak & Wojtczak, 1960; Borst, Loos, Christ & Slater, 1962) that fatty acids uncouple respiratory chain-level oxidative phosphorylation thus cutting off the generation of ATP. In the present investigation oleate was the sole respiratory substrate and the picture was complicated by the fact that oleate is one of the most powerfully uncoupling fatty acids (Borst *et al.*, 1962).

The present study reveals two maxima of acetoacetate formation as plotted against the concentration of oleate, and indicates that at least two mechanisms of the activation of oleate are involved. One of these mechanisms is mainly responsible for the production of acetoacetate at low concentrations of oleate or rather at low values of the oleate/mitochondrial protein ratio. Energy needed for this activation is provided by ATP. This is indicated by the fact that the inhibition of ATP synthesis by either omission of phosphate in the medium or addition of uncouplers or inhibitors of oxidative phosphorylation inhibits the formation of acetoacetate. It should also be noted that at these low concentrations of oleate oxidative phosphorylation is not fully uncoupled.

When the concentration of oleate is increased and oxidative phosphorylation becomes uncoupled (Fig. 1), production of acetoacetate is diminished. A further increase in the concentration of oleate results, however, in an increase of acetoacetate formation. Due to the fact that ATP is no more produced and that the production of acetoacetate is no more sensitive to uncouplers and inhibitors of oxidative phosphorylation, the activation of oleate under these conditions must proceed by a different mechanism, presumably *via* the mechanism described by Rossi & Gibbson (1964) in which GTP is the energy donor. The importance of this mechanism has been demonstrated by Van den Bergh (1965, 1966). GTP needed for this process is generated in mitochondria during the operation of the tricarboxylic acid cycle as a result of the oxidation of α-ketoglutarate (Lehninger, 1945). It can be calculated (Fig. 6) that operation of the cycle, as measured by the uptake of $O_2$, is high enough to account for the activation of oleate due to the formation of GTP.

There is, however, one point in which the results of the present investigation are not in a full agreement with the postulate that at the uncoupling concentrations of fatty acids the activation proceeds exclusively by the GTP-dependent mechanism. It has been shown by Van den Bergh (1966) that the GTP-dependent activation of fatty acids is strongly inhibited by phosphate and only at high concentrations of the fatty acid can this inhibition be overcome. Meanwhile in the present investigation the formation of acetoacetate, which was independent of oxidative phosphorylation, was the same in the presence as in the absence of inorganic phosphate, and no indication of any inhibition by phosphate has ever been noted. The absence of phosphate abolished only that portion of acetoacetate formation which was dependent on the formation of ATP, i.e. at low oleate concentrations.

The isotopic experiments and the comparison of acetoacetate formation and oxygen uptake (Fig. 6) show that a part of oleate is oxidized *via* tricarboxylic-acid cycle in spite that no "sparker" is added. It should be therefore concluded that endogenous intermediates of the tricarboxylic-acid cycle can support oleate oxidation in our conditions. The operation of the cycle is higher at high concentration of

oleate, i.e. under conditions of uncoupling of oxidative phosphorylation. This is in agreement with observations of Shepherd, Yates & Garland (1965) who found that only in the uncoupled state can the synthesis of citrate proceed at full rate.

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## TWORZENIE ACETOOCTANU W MITOCHONDRIACH WĄTROBY SZCZURA

### Streszczenie

1. Badano tworzenie acetooctanu z oleinianu przez mitochondria wątroby szczura w środowisku zawierającym $Mg^{2+}$ i ortofosforan, lecz nie zawierającym ani ATP, ani intermediatów cyklu kwasów trójkarboksylowych.

2. Zaobserwowano dwa maksima tworzenia acetooctanu: jedno przy zawartości oleinianu

wynoszącej 0.1 - 0.2 μmola na 5 - 10 mg białka mitochondrialnego, drugie przy dziesięciokrotnie większej zawartości oleinianu.

3. Oksydacyjna fosforylacja zachodziła tylko w warunkach pierwszego maksimum, natomiast tworzenie acetooctanu odpowiadające drugiemu maksimum przebiegało w warunkach rozprzężonej fosforylacji.

4. Tworzenie acetooctanu przy niskich stężeniach oleinianu (pierwsze maksimum) było hamowane przez 2,4-dwunitrofenol, oligomycynę i azydek, a także przez usunięcie fosforanu ze środowiska inkubacyjnego; natomiast w obecności wyższych stężeń oleinianu (drugie maksimum) oligomycyna i azydek wzmagały tworzenie acetooctanu, a 2,4-dwunitrofenol lub usunięcie fosforanu były bez wpływu.

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## RECENZJE KSIĄŻEK

PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY (editors J. A. V. Butler and H. E. Huxley), Vol. 17, Pergamon Press, London and New York, 1967; 390 pp., price £ 6.

The editors of the latest volume in this series are to be complimented on their assembly of another valuable series of review articles which are of interest to a wide audience. The subject matter of this volume covers a rather broad range of topics, including the contractile mechanism of insect fibrillar muscle (by J. W. S. Pringle); ribonucleic acid and hormonal control of protein synthesis (by A. Korner); formation of radicals in nucleic acids and their constituents by ionizing radiations (by A. Muller); genes and cellular differentiation (by H. Ursprung and R. C. Huang); photochemical and macromolecular aspects of vision (by E. W. Abrahamson and S. E. Ostrov); ultracentrifugal determinations of molecular weights (by J. M. Creeth and R. H. Pain); effects of radiations on DNA biosynthesis (by K. V. Shooter); and the structure and interactions of myosin (by S. V. Perry).

Several of the chapters are presented in a form which is fairly accessible to the non-specialist, for example that of Abrahamson and Ostrov on photochemical and macromolecular aspects of vision. However, the same cannot be said for all the chapters in this book. That on ultracentrifugation methods for determination of molecular weights, although well organized and excellently written by two well-known specialists in the field, may prove rough going for many biologists who routinely use the ultracentrifuge as a tool without delving too deeply into the theoretical aspects of the subject. On the other hand, it may very well stimulate some to examine more critically the data which they accumulate. The value of this chapter is, furthermore considerably enhanced by the extensive and detailed list of contents, as well as a comprehensive and critical discussion on the molecular weights and shape of nucleic acids.

The review by Muller on the formation of radicals by ionizing radiations in nucleic acid, nucleoproteins and their constituents is very comprehensive and up-to-date, and should prove useful to many who work in the field of radiation chemistry or on the effects of radiations on living cells. While such studies have relied up to the present for the most part on the use of electron spin resonance spectroscopy, it should be borne in mind that newer techniques such as pulse radiolysis may be expected in the near future to provide information not readily accessible by means of ESR.

On the whole, this volume should be a useful adjunct to one's personal library, and will certainly not be out of date in a hurry. The inclusion of the titles to original papers in the bibliographies of most of the chapters is a valuable feature; and the publishers are to be complimented on having organized the material in a very pleasing and readable form.

*David Shugar*

A. E. Needham: THE UNIQUENESS OF BIOLOGICAL MATERIALS. Pergamon Press, Oxford, London, 1965; str. 593+XX, cena £ 5.—

Książkę A. E. Needhama można nawiązać do dyskusji na temat pochodzenia życia na Ziemi, która się rozwinęła po Moskiewskiej konferencji w r. 1958. Charakterystyczną cechą tej zorganizowanej przez Oparina konferencji był udział przedstawicieli różnych gałęzi nauki, którzy prze-

nieśli następnie tę tematykę do swych dyscyplin. Tym razem głos zabiera wykładowca zoologii w Uniwersytecie Oxfordzkim, a książka ukazała się jako 25. tom Zoologicznej Sekcji Międzynarodowych Monografii Biologii Czystej i Stosowanej.

Autor dyskutuje zagadnienie, czy unikalność życia wynika z unikalnych własności atomów i cząsteczek wchodzących w skład żywej substancji, czy też jest wynikiem jakiejś unikalnej organizacji, jakiejś specyficznej struktury, której elementarne cegiełki mogły być bez szkody zastąpione przez inne, pokrewne. Autor rozpatruje więc własności fizyko-chemiczne „biogennych" pierwiastków, ich prostych związków oraz ich związków występujących w żywej substancji. Uwypuklając te własności, które mają znaczenie w procesach biologicznych, autor porównuje je z analogicznymi własnościami pierwiastków sąsiadujących z biogennymi w naturalnym układzie. Wynik porównania pozwala autorowi stwierdzić, że żaden z biogennych pierwiastków nie może być zastąpiony, w warunkach życia ziemskiego, przez inny, pokrewny pierwiastek. Stąd już krok tylko do ryzykownego, zdaniem recenzenta, twierdzenia, że życie wynikło jako prosta konsekwencja unikalnych własności podstawowych pierwiastków biogennych i ich związków, i że raczej graniczyłoby z cudem, gdyby życie w tych warunkach nie powstało. Zdaje mi się, że autor nie docenił znaczenia organizacji, której poświęcił zaledwie 14 stron swej książki. Wynika to, być może, stąd, że nasza wiedza o strukturze i jej powiązaniu z funkcją biologiczną jest jeszcze w początkach, o czym dobitnie świadczy wciąż jeszcze silne zróżnicowanie nauk biologicznych na morfologiczne i fizjologiczne.

Książka Needhama składa się z 32 rozdziałów. Po pierwszym, wstępnym, omawia specyficzne własności wody, wodoru i tlenu oraz dyskutuje unikalne własności węgla w porównaniu z innymi pierwiastkami IV grupy. Następnie, w 19 kolejnych rozdziałach omawiane są cukry, tłuszcze, białka, niebiałkowe składniki azotowe oraz kwasy nukleinowe i pterydyny.

Dalsze rozdziały omawiają kolejno siarkę, fosfor, „fizjologiczne jony nieorganiczne" (Na, K, Mg, Ca, Cl), następnie metale katalityczne, halogeny, wreszcie krzem i bor. Rozdział 29. omawia krótko wyższe stopnie organizacji.

Trzy ostatnie rozdziały mają charakter ogólny. Zastanawia się w nich autor nad zasięgiem zasady unikalności, spekuluje na temat możliwości innych form życia w warunkach odbiegających od ziemskich, na koniec zaś włącza się bezpośrednio do dyskusji na temat pochodzenia życia.

Książka Needhama zainteresuje również i tych biochemików, którzy nie śledzą dyskusji o początkach życia na ziemi. Stanowi ona prawdziwą skarbnicę rozproszonych w piśmiennictwie wiadomości o występowaniu różnych związków w świecie zwierzęcym i roślinnym. Daje poza tym przegląd własności fizyko-chemicznych pierwiastków, także niebiogennych, i ich najważniejszych związków, a podstawy biochemii przedstawia w sposób przystępny dla chemika lub biologa. Książka, ujęta raczej literacko, pełna cytatów z literatury pięknej i z Biblii, jest łatwa w czytaniu i pobudza do myślenia.

*Józef Heller*

F. M. Snell, S. Shulman, R. P. Spencer and C. Moos: BIOPHYSICAL PRINCIPLES OF STRUCTURE AND FUNCTION. Addison-Wesley Publishing Comp. Inc., Reading, Mass. Palo Alto, London, 1965; str. 390+10, cena $ 12.50.

Książka ta, oparta na wykładach wygłoszonych w uniwersytecie w Buffalo, przeznaczona jest dla absolwentów fizyki, chemii lub biologii, którzy zamierzają poświęcić się pracy badawczej w dziedzinie biofizyki. Celem autorów było uzupełnienie wiadomości uzyskanych w jednym z wymienionych kierunków — podstawowymi wiadomościami z kierunków pozostałych. Autorzy zajmują się w pierwszej części strukturą — począwszy od atomu a kończąc na komórce, w drugiej zaś funkcjami biologicznymi rozpatrywanymi na zasadzie praw termodynamiki i kinetyki chemicznej.

Rozdział I poświęcono ogólnym wiadomościom o komórce i metodach jej badania, dwa następne budowie atomów i wiązaniom chemicznym. Rozdział IV omawia szczegółowo własności wody, zaś rozdziały V - VIII aminokwasy, białka i kwasy nukleinowe. Rozdział IX opisuje mechanizm przenoszenia informacji, X mówi o wirusach. Dwa następne rozdziały traktują o wielocukrach i tłuszczowcach, zaś rozdział XIII omawia strukturę komórki i błon.

W części drugiej w sześciu kolejnych rozdziałach omówione zostały: podstawy termodynamiki; stany równowagi a funkcje energetyczne; stany równowagi fizycznej i chemicznej; równowaga kwasowo-zasadowa; równowaga poprzez błony; procesy nierównowagi (dyfuzja). W dwóch dalszych rozdziałach omówiono transport, a w dwóch ostatnich kinetykę prostych oraz enzymatycznych reakcji chemicznych.

Jak widać z powyższego, zakres książki pokrywa się prawie zupełnie z kursem biochemii, jednak akcenty są inaczej rozłożone. Strona fizyko-chemiczna, bardzo szeroko uwzględniana w starszych podręcznikach „chemii fizjologicznej", np. u Hamarstena, została stopniowo w miarę penetracji biochemii w głąb i narastania materiału — prawie zupełnie usunięta z podręczników nowszej daty. W omawianej książce natomiast wysuwa się na plan pierwszy. Również nawiązania do biologii, do struktury komórkowej są tu bez porównania ściślejsze, niż w podręcznikach biochemii.

Wydawało mi się celowe szczegółowe zapoznanie Czytelnika z zakresem omawianej książki, ponieważ ciągle jeszcze toczy się u nas dyskusja nad tym, czym jest biofizyka. Autorzy omawianej książki uważają, że głównym celem biofizyki powinno być wyjaśnienie takich procesów biologicznych, które — wskutek złożoności struktury żywych organizmów — *pozornie* stoją w sprzeczności z podstawowymi prawami fizyki i chemii. Można by dyskutować nad słusznością takiego ujęcia, ale nie ulega wątpliwości, że i tak pojęta biofizyka nie da się wyraźnie od biochemii oddzielić.

Omawianą książkę poleciłbym gorąco jako ciekawą i pożyteczną lekturę, uzupełniającą podstawowe studia biochemiczne.

*Józef Heller*

E. R. Hecht: LIPIDS IN BLOOD CLOTTING. C. C. Thomas Publ., Springfield (I11.) 1965; stron 328, cena 11.75 $.

„Godny współczucia jest ten, kto będzie musiał studiować krzepnięcie krwi przez następne czterdzieści lat". Powyższe słowa autora we wstępie książki dobrze obrazują obecny stan wiedzy oraz prognozy w tej obszernej i zawikłanej dziedzinie. Liczba publikacji umieszczanych w różnych periodykach jest olbrzymia, co utrudnia śledzenie całej lub w przybliżeniu całej literatury dotyczącej krzepnięcia. Autor postąpił słusznie, koncentrując uwagę na jednym tylko aspekcie krzepnięcia. Wybór tematu uzasadniony jest wzrostem znaczenia, jakie przypisuje się lipidom w krzepnięciu krwi, oraz obserwacjami i doświadczeniem własnym autora.

Książka podzielona jest na dwa zasadnicze działy, poprzedzone krótkim przeglądem ogólnych wiadomości o krzepnięciu. Aktywatory krzepnięcia omówione są w pierwszej części, podczas gdy druga część poświęcona jest inhibitorom. Ostatni rozdział, luźno związany z poprzednimi, zawiera krytyczne spojrzenie na problemy związane z hemofilią. Cytowane piśmiennictwo (863 pozycje) obejmuje prace do r. 1964.

Czytelnik ogólnie zorientowany w problemach krzepnięcia często utyka w powodzi faktów i opinii różnych autorów. Słabo uwypuklone są opinie autora książki, co przy braku syntetycznych podsumowań omawianych problemów stwarza wrażenie encyklopedycznego nagromadzenia dotychczasowych osiągnięć. Rysunki i tabele nie poprawiają sytuacji, bowiem większość z nich stanowi jedynie ilustracje cytowanych prac oryginalnych. Dla osób zajmujących się krzepnięciem krwi książka może dostarczyć cennych informacji i źródłowego piśmiennictwa.

*Ryszard Niemiro*

A LABORATORY MANUAL OF ANALYTICAL METHODS OF PROTEIN CHEMISTRY, vol. 4 (P. Alexander & H. P. Lundgren, eds.) Pergamon Press, Oxford 1966; str. 233, cena 55 s.

Tom czwarty „Manual" zawiera sześć rozdziałów, opracowanych przez różnych autorów.

Rozdział I (S. J. Leach, *Oznaczanie grup tiolowych i dwusiarczkowych*) jest najobszerniejszy. Autor proponuje uporządkowanie nazewnictwa grup funkcjonalnych zawierających siarkę, jak też

sugeruje sposób wyrażania udziału tych grup w badanym materiale. Następnie dokonuje szczegółowego i systematycznego przeglądu współczesnych metod oznaczania grup -SH i pokrewnych, głównie w białkach, przed i po hydrolizie.

Metody oznaczania zostały podzielone na trzy grupy: kolorymetryczne, wśród których dano pierwszeństwo metodom, w których stosowane są jednofunkcjonalne związki rtęci typu RHgX; spektrofotometryczne, gdzie najbardziej reprezentatywną jest metoda stosująca związki fenilortęciowe; amperometryczne, szczególnie nadające się do barwnych i mętnych roztworów. Podkreślono tu wyższość kroplowej elektrody rtęciowej nad rotacyjną, platynową. Z titransów najbardziej odpowiedni jest jodek metylortęciowy.

W dalszej części rozdziału podano szereg szczegółowych przepisów, łącznie z metodami radiochemicznymi, którym niewiele uwagi poświęcił autor w poprzednich częściach.

Rozdział II (S. Blackburn, *Mikrotechniki analizy aminokwasów i rozdziału peptydów oparte na elektroforezie wysokonapięciowej*) zawiera krytyczne omówienie dotychczasowych technik wysokonapięciowej elektroforezy. Krótko opisano dwa zasadnicze typy aparatów: typ podany przez Michla (1951 r.), gdzie jako systemu chłodzącego używa się cieczy organicznych; oraz typ podany przez Grossa oraz Atfielda i Morrisa (1961 r.) z dwiema płytami chłodzącymi. Artykuł jest w zasadzie poświęcony temu typowi aparatów. Autor podaje bardzo dokładne i szczegółowe przepisy całej procedury oznaczania aminokwasów. Równie szczegółowo opisano rozdział peptydów, przy czym podano oryginalny sposób elucji peptydów z wycinków bibuły. Omówiono również dwuwymiarową elektroforezę peptydów. Pierwszy rozdział dokonywany jest w pH 1,85, drugi w pH 4,5.

Rozdział III (D. A. Darcy, *Oznaczanie swoistych białek w mieszaninach metodą strącania na żelu*) omawia szczegółowo metody precypitacyjne na żelu, m.in. zastosowalność tych metod, sporządzanie surowic zawierających przeciwciała, ich mianowanie, identyfikację swoistego strątu itp. Opisana na końcu rozdziału mikrometoda jest szczególnie cenna. Do jej wykonania potrzebna jest tylko 1/8 ilości poszczególnych odczynników i wykonuje się ją w znacznie krótszym czasie niż makrometodę.

Rozdział IV (S. W. Fox i K. Harada, *Termiczna polikondensacja α-aminokwasów*) jest zbiorem przepisów polimeryzacji aminokwasów na drodze termicznej kondensacji. Podane są tu sposoby otrzymywania homopolimerów: kwasu asparaginowego i argininy, z heteropolimerów opisano kondensacje: kwasu glutaminowego z glicyną, kwasu glutaminowego z alaniną, kwasu asparaginowego z kwasem glutaminowym i kwasu jabłkowego z kwasem glutaminowym. Opisano również sposoby oznaczania składu aminokwasowego opisanych polimerów i oznaczania końcowych grup $-NH_2$. Osobno omówiono termiczną polikondensację lizyny. Dość szeroko opisano proteinoidy, ich oczyszczanie i analizę aminokwasową. Na zakończenie podane są polimeryzacje aminokwasów z kwasem *o*-fosforowym i polifosforowym.

Rozdział V (W. H. Ward, *Skład aminokwasowy wybranych białek i polipeptydów*) jest to tabelaryczny przegląd składu aminokwasowego około 80 białek i polipeptydów. Podano sekwencje aminokwasowe około 30 tych substancji. Wykaz sporządzony jest na podstawie piśmiennictwa do 1964 r.

Rozdział VI (D. Rosen, *Dielektryczne pomiary białek*) poświęcony jest przede wszystkim problemom pomiarów stałych dielektrycznych białek, marginesowo traktuje krytykę uzyskiwanych wyników. Po krótkim wstępie teoretycznym opisane zostają kolejno pomiary roztworów białek w niskich częstotliwościach, następnie w wysokich częstotliwościach, wreszcie pomiary dielektryczne białek suchych. Opisy są bardzo dokładne, przytoczone są schematy ideowe komórek pomiarowych i wymaganego ustawienia mostka pomiarowego. Podano również techniczny rysunek komory pomiarowej. Autor nie podał natomiast ani jednego wyniku oznaczenia jakiegokolwiek białka.

Książka zakończona jest starannymi indeksami: autorów i rzeczowym. Godna podkreślenia jest niezwykle staranna szata graficzna, jak też przejrzystość poszczególnych rozdziałów. Omawiana książka jest niezbędnym podręcznikiem dla każdego badacza, zajmującego się badaniem własności białek.

*Juliusz Popowicz*

PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS (R. T. Holman, ed.) vol. 8. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Paris, Braunschweig 1966; str. 436, cena £ 6.—

Kolejny tom tego seryjnego wydawnictwa jest dedykowany pamięci zmarłego w 1965 r. prof. T. P. Hilditcha, którego ponad 50-letnia praca naukowa w dziedzinie chemii lipidów wywarła duży wpływ na rozwój tej gałęzi nauki. Tom zawiera siedem artykułów o bardzo różnorodnej tematyce. Pierwszym z nich jest artykuł L. L. M. Van Deenena, p.t.: *Phospholipids and Biomembranes*. Jest to chyba najbardziej wyczerpująca monografia omawiająca biosyntezę fosfolipidów, jaka ukazała się w ciągu ostatnich dwu-trzech lat; stanowi ona bardzo cenne zestawienie wiadomości na temat składu lipidowego różnego rodzaju błon biologicznych oraz dane o wpływie zmiany tego składu na charakter błon. Artykuł ten zawiera 115 stron druku i ponad 500 pozycji bibliograficznych, niestety obejmujących pozycje literatury tylko do 1964 r. Artykuł p.t.: *Recent Progress in Carotenoid Chemistry* (S. Liaanen-Jensen & A. Jensen) omawia metody izolacji i identyfikacji karotenoidów oraz zawiera szczegółowe dane o strukturze szeregu nowoodkrytych związków tej grupy. W artykule omówione są również metody chemicznej syntezy karotenoidów oraz drogi ich biosyntezy. Artykuł C. Y. Hopkinsa omawia zastosowanie techniki magnetycznego rezonansu jądrowego w badaniach nad kwasami tłuszczowymi i glicerydami. Badania nad konformacją długołańcuchowych związków węglowych (głównie węglowodorów) są tematem artykułu J. S. Showella. Trzy ostatnie artykuły poświęcone są omówieniu najnowszych osiągnięć w dziedzinie technik chromatograficznych lipidów (D. C. Malins — chromatografia cienkowarstwowa, J. S. Hamilton — chromatografia bibułowa oraz A. Stein i V. Slavson — chromatografia kolumnowa). Tom zawiera indeks autorów i indeks rzeczowy. Z pewnością książka ta stanowić będzie wartościową pozycję dla wszystkich chemików i biochemików zajmujących się lipidami.

*Tadeusz Korzybski*

T. Korzybski, Z. Kowszyk-Gindifer and W. Kuryłowicz: ANTIBIOTICS. ORIGIN, NATURE AND PROPERTIES. Pergamon Press and PWN - Polish Scientific Publishers, Oxford, London, Edinburgh, New York, Toronto, Sydney, Paris, Braunschweig, Warszawa 1967; str. 1651+xxxvii., cena t. I/II £ 14.14, $ 45.

Książka trzech polskich autorów jest rozszerzonym i uzupełnionym III wydaniem w języku angielskim książki T. Korzybskiego i W. Kuryłowicza pt. „Antybiotyki". I wydanie w języku polskim ukazało się nakładem PZWL w r. 1955, II wydanie nakładem PWN w r. 1959 i zostało przetłumaczone na język niemiecki. W opracowaniu znajduje się IV wydanie książki w języku rosyjskim, oparte na danych literaturowych do r. 1967.

Liczba nakładów książki i umowy wydawnicze z firmami zagranicznymi dowodzą wielkiej użyteczności książki dla chemików, biochemików, farmakologów i fizjologów. Ma ona bowiem charakter stale uzupełnianej encyklopedii o ponad 1000 antybiotykach, sklasyfikowanych w 7 grupach na podstawie źródeł ich wytwarzania. Wyodrębniono antybiotyki wytwarzane przez bakterie, Actinomycetales, fungi imperfecti, Basidomycetes i Ascomycetes, glony i mchy, rośliny wyższe oraz zwierzęta. W obrębie tych grup posłużono się tym samym rodzajem podziału, wyjątek stanowią promieniowce, które są głównymi „producentami" związków antybiotycznych. W grupie tej zastosowano wyróżnienie uwzględniające budowę chemiczną (antybiotyki makrolidowe, antybiotyki polipeptydowe, tetra-, penta-, heksa-, i heptapolienowe antybiotyki przeciwgrzybowe) oraz działanie fizjologiczne (działanie przeciw Gram-dodatnim drobnoustrojom, kwasoodpornym pałeczkom, grzybom, pierwotniakom, wirusom, nowotworom). W ten sposób w grupach antybiotyków o podobnym działaniu znalazły się związki o bardzo różnej budowie chemicznej, jak również szereg związków, których budowa jest nieznana. Dlatego niezbędną pomoc w korzystaniu z książki stanowi wyczerpujący i starannie opracowany indeks organizmów wytwarzających antybiotyki, jak również indeks nazw antybiotyków, ich pochodnych, produktów degradacji i produktów pośrednich w syntezie. Książka zawiera także indeks autorów. Zamieszczone odnośniki lite-

raturowe wraz z pełnym tytułem pracy ułatwiają bezpośrednie sięgnięcie do informacji źródłowych. Wydanie obecne obejmuje dane zebrane z 10 periodyków poświęconych antybiotykom, jak również z najpoważniejszych ogólnych czasopism mikrobiologicznych, chemicznych, biochemicznych i literatury patentowej, ogłoszonych do r. 1965. Podane informacje dotyczą charakterystyki szczepu czy organizmu produkującego dany antybiotyk, składu pożywki, metod hodowli, izolacji, oczyszczania, własności fizycznych, chemicznych i biologicznych oraz uwzględniają znaczenie terapeutyczne.

Informując krótko i wszechstronnie o wszystkich wykrytych substancjach antybiotycznych, książka spełnia specjalną rolę. Rozszerza zakres wiadomości o danym antybiotyku każdemu interesującemu się tym związkiem z określonego, wąskiego punktu widzenia, umożliwia szerokie porównanie z innymi związkami o podobnym działaniu lub podobnej strukturze chemicznej. Jest cennym informatorem przy coraz bardziej rozszerzającym się zastosowaniu antybiotyków w badaniach nad metabolizmem komórki oraz w pracach nad mechanizmem ich działania.

*Konstancja Raczyńska-Bojanowska*