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ISOLATION OF THE SOLUBLE TYROSINASE FROM *CELERIO EUPHORBIAE*

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Highly purified preparations of tyrosinase (*o*-diphenol:O_2 oxidoreductase, EC 1.10.3.1) have been obtained from mushrooms [4, 9, 5, 11, 1] and higher plants [7]. In animals however, especially in insects, during homogenization of the tissues tyrosinase forms insoluble aggregates which interfere with the purification of the enzyme [3].

In insects, chinones produced by oxidation of endogenous phenolic compounds participate in the process of aggregation of tyrosinase *in vitro*. Karlson & Liebau [3] applied L-ascorbic acid as reducing agent to prevent the formation of chinones and from *Calliphora erythrocephala* larvae they obtained a highly purified soluble *o*-diphenol oxidase. Their preparation had, however, no activity towards monophenols which is characteristic for tyrosinase *in vivo*.

In the present work, soluble tyrosinase from *Celerio euphorbiae* pupae was isolated and some properties of the enzyme were studied. As the presence of a large amount of polyphenols in *C. euphorbiae* moth has been demonstrated by Heller & Mochnacka [2], L-ascorbic acid, according to Karlson & Liebau [3], was used as reducing agent.

EXPERIMENTAL

Material. C. euphorbiae diapauzing pupae were used for experiments. The caterpillars which underwent pupation between the 15th of August and the end of September were transferred to a cold room and kept at 4°.

Preparation of the adsorbent. Tyrosinase was chromatographed on calcium phosphate gel. As insect tyrosinase is a very labile enzyme, it seemed advisable to use brushite gel ($CaHPO_4 \cdot H_2O$) which has larger grain than hydroxylapatite applied by Tiselius *et al.* [12] for protein separation. This permitted to limit the time of elution during chromatographic separation of the enzyme. Brushite gel was prepared according to Tiselius *et al.* [12] by mixing equimolar aqueous solutions of $CaCl_2$ and Na_2HPO_4. The $CaHPO_4$ sediment formed was washed 4 times with

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water by decantation. The obtained gel was kept at room temperature in a glass container with a polished stop-cork and was covered with an amount of water of the same height. These conditions ensured more satisfactory stability of the grain than when brushite was held in dry form; reproducible results of chromatographic separation were obtained when the adsorbent was used within 3 days since its preparation. For every experiment, the gel was standardized by estimating the time of sedimentation of a 5 cm. high gel column and determining the flow-rate of 0.005 M-phosphate buffer.

Determination of enzymic activity. The colorimetric method applied to obtain orientative data, consisted in determination of E^{1cm}_{426} increment during the first 2 min. of the enzymic oxidation of substrate per 1 mg. protein. To 1 ml. of the enzymic solution was added 0.5 ml. of 0.06 M-substrate solution. Readings were taken in a VSU-1 Zeiss spectrophotometer at 20°.

The manometric method of Keilin & Mann [4] was used, with the modification of Kertesz & Zito [6] consisting in L-ascorbic acid being added to the incubation mixture. According to Miller *et al.* [10], if phenolic substrates are used without the addition of L-ascorbic acid the manometric method is charged with an error due to non-linearity of the oxygen consumption curve. When, however, L-ascorbic acid was added to prevent aggregation and inactivation of the enzyme, a linear relationship was obtained (cf. Fig. 4).

The determinations were made in a Warburg apparatus at 25°; to the main vessel were added 2 ml. of enzyme solution in 6% sucrose in 0.1 M-phosphate solution ($KH_2PO_4 + Na_2HPO_4$) of pH 7.2, and 0.3 ml. of 0.3% L-ascorbic acid solution. To the side arm was added 0.5 ml. of 0.06 M-solution of the substrate. When tyrosine or *p*-cresol were used as substrate, traces of diphenols were added. The results are expressed as µl. O_2/1 hr./1 mg. protein, the estimations being made during the first 10 min. of oxygen consumption.

Protein determination. In samples with high protein concentration dry weight was determined after precipitation with trichloroacetic acid and drying at 105°. In samples with low protein concentration the Folin & Ciocalteu method of Lowry *et al.* [8] was applied, the protein tested by the gravimetric or Kjeldahl method being used as standard.

Partial purification of the enzyme

Thirty diapauzing pupae, kept for several weeks at 4°, were homogenized in a Waring Blendor for 5 min. in 600 ml. of 0.25 M-sucrose solution added with 0.7% L-ascorbic acid. The pH was adjusted to 7.2 with phosphate buffer. The homogenate was centrifuged in the cold at 30 000 *g* for 10 min., then the supernatant fluid was passed through a Seitz filter. To the obtained opalescent fluid, ammonium sulphate

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in substantia was added to 0.9 saturation, with slow stirring. Foaming was avoided since it has been observed that too energetic stirring of the solution might result in a marked decrease of enzyme activity. After 30 min. the precipitate was centrifuged off in the cold at 25 000 *g* for 7 min. and suspended in 0.005 M-phosphate buffer containing 0.03% of L-ascorbic acid. The suspension was dialysed for 24 hr., with constant stirring, against the same buffer added with L-ascorbic acid. Then a slight precipitate of the denatured protein was centrifuged off and the green opalescent fluid obtained was lyophilized. All these steps of enzyme preparation were performed at about -2°.

The cream-coloured dry powder obtained after lyophilization was kept in tightly covered vessels at -20°. If low temperature was maintained and even traces of humidity were avoided, no decrease of enzyme activity was observed over a two-months period. The activity of the crude extract and purified preparations is shown in Table 1.

Chromatographic separation

In preliminary experiments, the effect of concentration and pH of the eluent were tested. The pH range 7 - 8 was chosen taking into consideration the stability of both the adsorbent and enzyme. Tiselius *et al.* [12] demonstrated that the adsorptive properties of brushite are modified in an acidic medium, therefore pH values lower than 7 could not be applied. The optimum for tyrosinase activity is known to be at pH about 7.2, and the enzyme is easily inactivated at other pH values.

Figure 1 shows the elution pattern of the enzyme and protein by 0.005, 0.15 and 0.25 M-phosphate buffers, of pH 7 (Fig. 1A) and pH 8 (Fig. 1B). The column (3 × 25 cm.) was fitted with a sintered-glass disk G1 and provided with a cooling jacket. To the column, 150 ml. of a suspension containing 15 g. of brushite gel was introduced and allowed to settle. The gel formed a column 9 cm. high; this was sufficient for absorption of 100 mg. of the lyophilized enzyme preparation. The column was equilibrated with 0.005 M-phosphate buffer and cooled to 0°, then 100 mg. of the preparation dissolved in 5 ml. of 0.005 M-phosphate buffer was introduced. The elution lasted for 3 hr.; phosphate buffers of pH 8 were applied in the following concentrations: 0.005, 0.05, 0.1, 0.15, 0.2 and 0.25 M, 70 ml. of each buffer being applied, that is the volume equal to the liquid displacement volume of the column. The temperature maintained during the elution was 0° for buffers of 0.005 - 0.1 M concentration, and 15° for concentrations of 0.15 - 0.25 M, to prevent crystallization of phosphates. Fractions of 10 ml. were collected with a fraction collector and kept at 0°. The protein content and activity of successive fractions were determined semiquantitatively by the colorimetric method using catechol as substrate (Fig. 2).

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With the 0.005 M-phosphate buffer only a part of the non-active protein was eluted and the eluate had a bluish colour. The enzyme began to appear at 0.05 M concentration of the buffer; the eluate of this fraction was yellow. The next four fractions, possessing also the enzymic activity, were colourless.

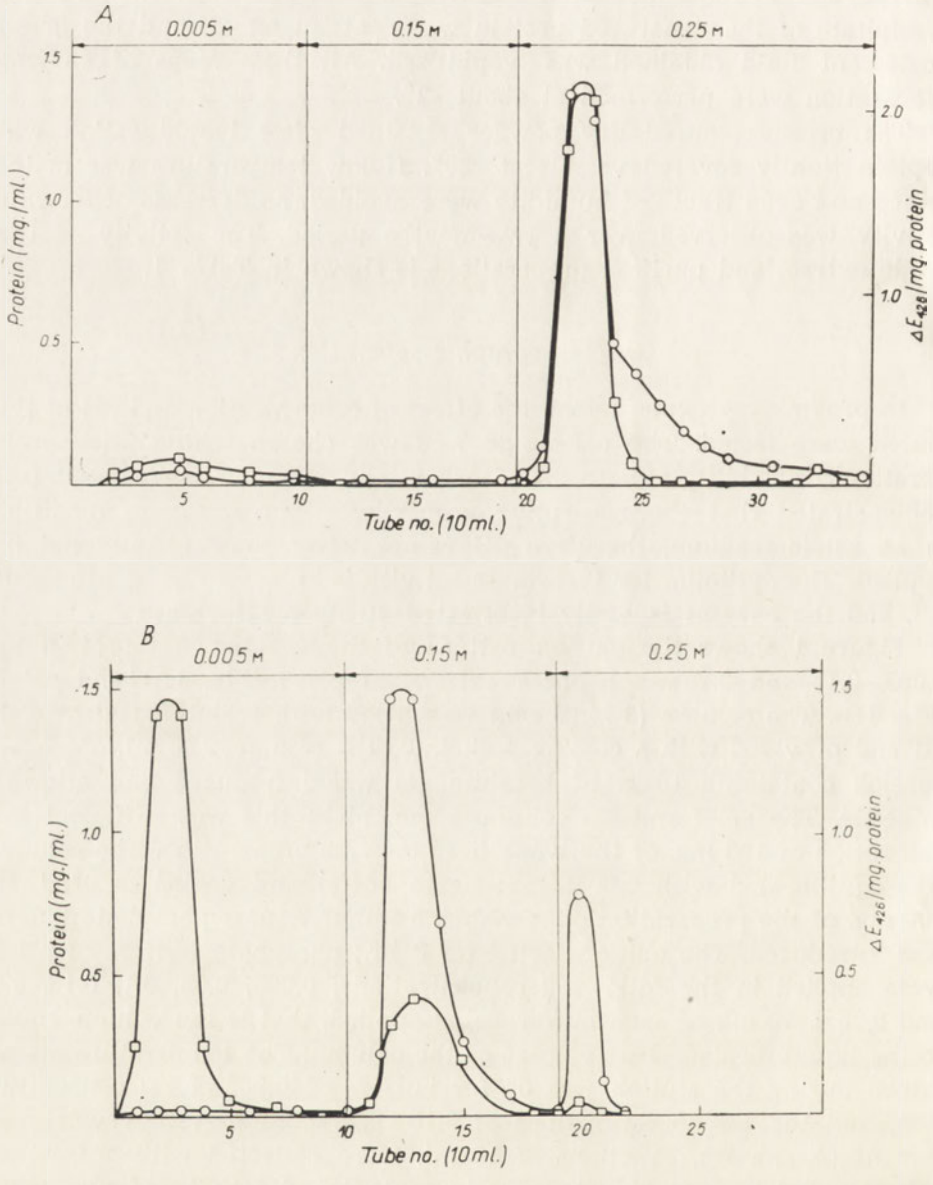

Fig. 1. Elution of tyrosinase from brushite column with 0.005, 0.15 and 0.25 M-phosphate buffers. (*A*), pH 7; (*B*), pH 8. (○), Activity towards catechol; (□), protein content (method of Lowry *et al.*).

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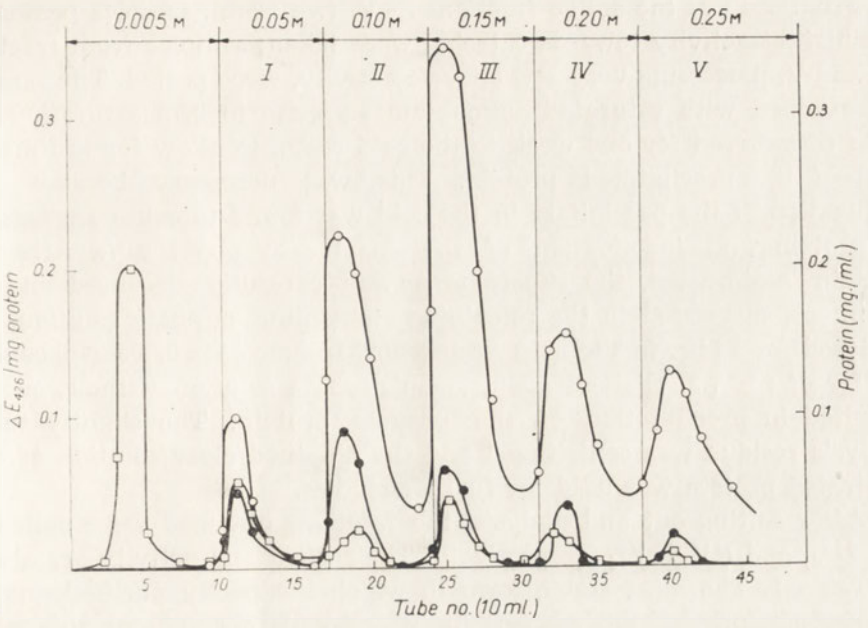

Fig. 2. Elution of tyrosinase from brushite column with six concentrations of phosphate buffer of pH 8. (○), Activity towards catechol; (●), activity towards *p*-cresol; (□), protein content (method of Lowry *et al.*).

Table 1

Activity of tyrosinase after the successive steps of purification

The homogenate prepared from 30 pupae was centrifuged and passed through Seitz filter, then its activity was determined. The preparation was treated with saturated $(NH_4)_2SO_4$, dialysed and then lyophilized. The lyophilizate was chromatographed as described under Experimental and 5 fractions were obtained; the concentration of the eluent is given in parentheses.

Preparation	Total volume (ml.)	Total protein (g).	Activity towards					
			catechol (μl. O_2/hr./mg. protein)	*p*-cresol (μl. O_2/hr./mg. protein)	catechol (Total activity)	*p*-cresol (Total activity)	catechol yield (%)	*p*-cresol yield (%)
Homogenate	600	4.8	115	112	552	537	100	100
Lyophilizate	—	2.3	171	168	393	386.5	71.5	72
Fraction I_d (0.05 M)	1610	0.735	60.5	44	44.5	32	8	6
Fraction II_d (0.1 M)	1610	0.245	94	50	23	12	4	2
Fraction III_d (0.15 M)	1610	0.63	326	196	205	123.5	35	23
Fraction $(IV + V)_d$ (0.2 and 0.25 M)	3220	0.04	1106	820	44	17	8	3

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From each of the active fractions *I* - *V*, two 10 ml. samples possessing the highest activity towards catechol were taken; samples from fractions *IV* and *V*, possessing very low enzyme activity, were pooled. The samples were added with saturated ammonium sulphate to 0.75 saturation and then transferred for one week to the cold room, to allow for sedimentation of the precipitated protein. This was necessary because centrifugation of the precipitate in the cold was found to cause aggregation of protein and inactivation of the enzyme by about 30%. After the protein sedimented, the supernatant was carefully decanted and the sediment, suspended in the remaining ammonium sulphate solution, was dialysed for 24 hr. in the cold against 0.25 M-sucrose in 0.005 M-phosphate buffer of pH 7.2. Dialysis against a phosphate solution without sucrose resulted in precipitation of the enzyme protein. The slightly turbid dialysis residue was centrifuged and the obtained clear solution of concentrated protein was used for further studies.

After salting out and dialysis, the fractions obtained were called I_d, II_d, III_d, and $(IV + V)_d$, respectively. Their yield and activity are shown in Table 1. The most stable form in which the partly purified enzyme could be kept was the suspension in ammonium sulphate; this could be stored at -10° for six weeks without loss of activity. The solutions of the enzyme in 0.25 M-sucrose kept at 4° became inactivated after a few days, and on freezing and thawing lost about 50% of activity.

Characterization of the dialysed active fractions

Determination of the optimum pH. The activity towards catechol and *p*-cresol was determined by the colorimetric method in the pH range of 6 - 9. Solutions of separated fractions in sucrose were added with a double volume of 0.1 M-phosphate buffer of appropriate pH. Two control systems were applied: (1), buffer and enzyme without substrate, to estimate the changes in absorption of the enzyme solution at alkaline pH, and (2), buffer, 0.25 M-sucrose solution in 0.005 M-phosphate buffer, and substrate. The effect of pH on the activity of the enzyme is shown in Fig. 3. The optimum for activity towards *p*-cresol in all the fractions was found to be at pH 8. For the activity towards catechol no pH optimum was observed at the pH values tested.

Substrate specificity. The consumption of oxygen by the enzymic fractions I_d - V_d during incubation with catechol, dioxyphenylalanine, *p*-cresol, and tyrosine was estimated (Table 2). The rate of oxygen consumption by the lyophilizate and fraction $(IV + V)_d$ are shown in Fig. 4; other fractions gave similar results.

Rechromatography. Fractions I_d, II_d, and III_d in sucrose solution, each containing about 50 mg. protein in 10 ml., were rechromatographed on brushite column under the same conditions as described above. The enzymic activity determined colorimetrically in the eluates, is shown in

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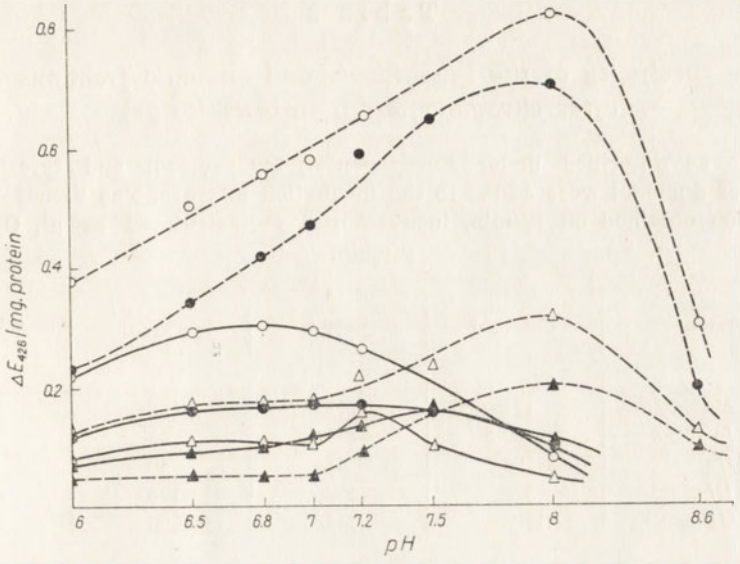

Fig. 3. pH optima for the catalytic activity of fractions obtained by chromatography (see Fig. 2 for elution pattern). (———), Activity towards catechol; (— — —), activity towards p-cresol. Fractions: (▲), I_d; (△), II_d; (●), III_d; (○), fraction $(IV + V)_d$.

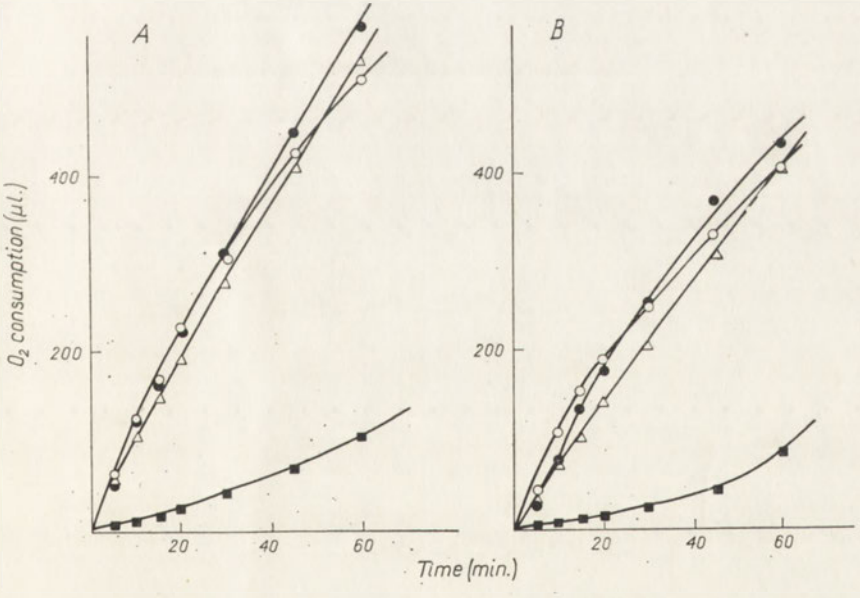

Fig. 4. Oxygen consumption during incubation of (*A*), 4 mg. of lyophilizate; (*B*), 0.6 mg. of the fraction $(IV + V)_d$ with: (○), catechol; (△), DOPA; (●), p-cresol; (■), tyrosine.

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Table 2

Substrate specificity of the lyophilizate and enzymic fractions obtained after chromatography on brushite gel

Conditions as described under Experimental; for *p*-cresol and tyrosine, trace amounts of diphenol were added to the incubation mixture. The values of oxygen consumption obtained on 10-min. incubation were calculated as µl. O_2/1 hr./mg. protein.

Preparation	Catechol	DOPA	*p*-Cresol	Tyrosine
Lyophilizate	171	153	168	18
Fraction I_d	60.5	109.5	44	0
Fraction II_d	94	65	50	0
Fraction III_d	326	209	196	44.5
Fraction $(IV + V)_d$	1106	744	820	72

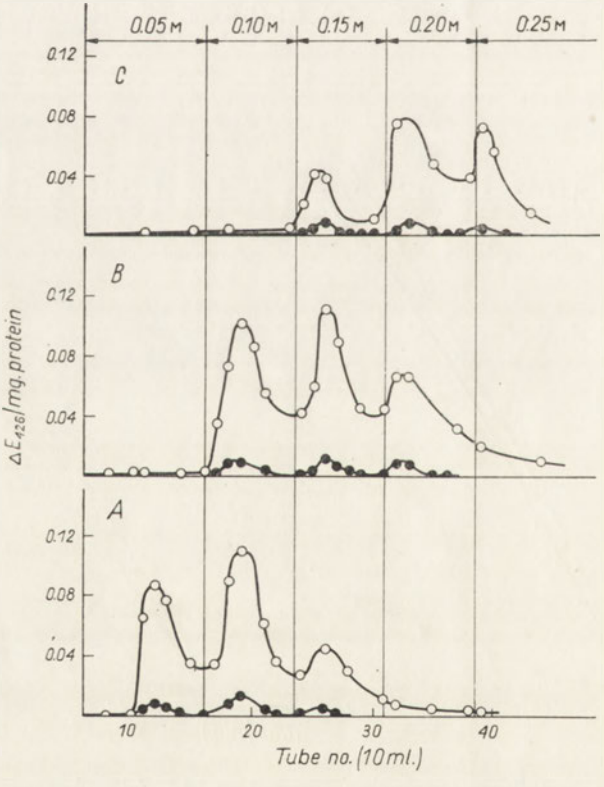

Fig. 5. Rechromatography on brushite gel of the chromatographic fractions: (*A*), I_d; (B), II_d; (C), III_d. Activity towards: (○), catechol; (●), p-cresol.

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Fig. 5. From each fraction, on rechromatography three active peaks were obtained; the first of them corresponded to such a concentration of the buffer at which this fraction had been eluted on the first chromatography. The following two peaks appeared when the higher buffer concentrations were applied. On rechromatography of the eluate obtained with the 0.1 M buffer (i.e. sum of fractions I_d and II_d) and with the 0.15 M buffer (i.e. sum of fractions I_d, II_d and III_d) the elution curves were identical with those for fractions II_d and III_d rechromatographed separately. Rechromatography of the fraction $(IV + V)_d$ could not be performed because its quantity was too small.

DISCUSSION

The preparation of soluble *o*-diphenol oxidase obtained by Karlson & Liebau [3] from *Calliphora erythrocephala* larvae was active only towards diphenols and was inactive towards monophenols. In the present work, from *C. euphorbiae* pupae a preparation was obtained of water-soluble tyrosinase which is active towards catechol and tyrosine as well as towards *p*-cresol. Crude enzyme preparation (yield 70%) was further purified by chromatography on calcium phosphate gel, and the obtained results seem to suggest the presence of multiple form of tyrosinase. This is in agreement with the results reported by other workers. Bouchilloux, McMahill & Mason [1], by chromatography on hydroxylapatite, separated mushroom tyrosinase into 4 fractions and found differences in the ratio of activity towards *p*-cresol and catechol between the individual fractions. Smith & Krueger [11] separated chromatographically plant tyrosinase into 5 fractions with different ratios of activity towards *p*-cresol and towards catechol. On rechromatography, they observed that some overlapping of the fractions occurred during the first chromatography. It is not to be excluded, however, that this effect, observed also in our experiments, is due to multiside absorption. Nevertheless, Smith & Krueger [11] interpret their results as indicating that the plant tyrosinase preparation is "multicomponent in nature". The presented results seem to suggest that the same may be true for the tyrosinase isolated from *Celerio euphorbiae* pupae.

SUMMARY

From pupae of *Celerio euphorbiae*, a soluble and relatively stable (1.5 month at -10°) preparation of tyrosinase was obtained. The procedure consisted of homogenization in 0.25 M-sucrose solution containing L-ascorbic acid, salting out with ammonium phosphate, lyophilization and column chromatography on brushite gel with stepwise elution by

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phosphate buffers of 0.005 - 0.25 M-concentration. In the eluates 5 fractions active both towards catechol and *p*-cresol, were found. Rechromatography resulted in further separation of the obtained fractions.

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IZOLOWANIE ROZPUSZCZALNEJ TYROZYNAZY Z POCZWAREK *CELERIO EUPHORBIAE*

Streszczenie

Z poczwarek motyla *Celerio euphorbiae* otrzymano rozpuszczalny i względnie trwały (przez 1,5 miesiąca w temp. -10°) preparat tyrozynazy. Stadia preparacji obejmowały: homogenizację w 0,25 M roztworze sacharozy z dodatkiem kwasu L-askorbinowego, wysalanie siarczanem amonu, liofilizację oraz chromatografię kolumnową na żelu bruszytowym przy skokowej zmianie stężenia eluenta w zakresie od 0,005 M do 0,25 M buforu fosforanowego. W eluatach znaleziono 5 frakcji tyrozynazy, wykazujących zarówno aktywność katecholazową jak i *p*-krezolazową. W wyniku rechromatografii otrzymano dalszy rozdział poszczególnych frakcji tyrozynazy.

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FURTHER OBSERVATIONS ON THE FRACTIONATION OF DNA BY CHLOROFORM TREATMENT

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It has previously been shown that an aqueous mixture of native and heat-denatured DNA may be fractionated by treatment with chloroform, as a result of which an appreciable fraction of denatured DNA is transferred to the water-chloroform interphase [18]. The procedure is relatively specific with respect to DNA heat-denatured under conditions such that depurination and chain scission are avoided. These results were obtained mainly with transforming DNA (T-DNA) isolated from group H *Streptococcus Challis*, and chloroform treatment of a fully heat-denatured preparation of this DNA left a residual activity in the aqueous phase the specific activity of which was increased 7 - 10 fold.

In the present paper we have extended the above fractionation procedure to a closer examination of the nature of the residual activity of heat-denatured T-DNA and have investigated the influence of various factors on the efficiency of fractionation, e.g. modifications of the aqueous phase, effect of variations of molecular weight of the DNA submitted to fractionation, the degree of fractionation achievable with DNA samples of varying base composition, conditions of denaturation, etc.

It was previously unequivocally established [16, 19, 5, 8, 9, 7, 22] that T-DNA, heat-denatured under conditions where depurination and chain scission are avoided, exhibits a small residual activity. In common with other observers, three possibilities may be envisaged to interpret this phenomenon; viz. the residual activity is a property of (a) some of the twin strands which have not fully unfolded, (b) a small fraction of the molecules which have undergone renaturation even during rapid cooling, (c) single-stranded DNA.

For the *Bacillus subtilis* transforming system it was shown by Rownd *et al.* [21] by means of density gradient centrifugation that single-stranded T-DNA is inactive and that the residual activity is a property of partially unfolded twin strands. On the other hand, the residual

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activity of *Haemophilus influenzae* T-DNA appears to be due to single strands [22], most likely those which possess a non-ordered secondary structure resembling in some respects that of twin-stranded DNA [1]. A somewhat similar situation prevails for *Diplococcus pneumoniae* T-DNA [7], for which the decrease in activity resulting from heat-denaturation parallels the decrease in DNA uptake by the bacterial cells [11].

In the absence of facilities for density gradient centrifugation, we have examined the residual activity of the streptococcal transforming system by other methods, with conclusions similar to those for the *H. influenzae* and *D. pneumoniae* systems.

MATERIALS AND METHODS

Bacterial DNA samples were isolated by the procedure of Marmur [14] and checked by means of their melting profiles and their UV absorption at short wavelengths. Base compositions were estimated from the T_m of the melting profiles [15]. The degree of protein contamination was estimated by means of the Lowry *et al.* [13] reaction and was found to vary between 2 and 4%. RNA contamination was estimated on the basis of the difference between the extinction at 260 mμ and the determination of the DNA content by means of the diphenylamine reaction.

Transformation of *Streptococcus Challis* was carried out as described elsewhere [17].

Heat denaturation was carried out on solutions containing 20 μg./ml. DNA. For samples containing up to 46% guanine-cytosine (G-C) base pairs the salt concentration was 0.15 M-NaCl - 0.015 M-sodium citrate (SSC). For base compositions above 46% G-C, the salt concentration employed was one-tenth of the foregoing (DSC). Samples of 0.5 ml. were heated in sealed tubes at 100° for 3 min. and then cooled rapidly in an ice bath. The importance of a 3-min. heating period, as compared to the 10 min. previously employed by us, and others, will be pointed out below.

Chloroform fractionation was as previously described [18].

DNA preparations of decreased molecular weight were obtained by sonication of solutions of DNA, 20 μg./ml. in SSC, using an MSE ultrasonic disintegrator the output of which was 60 watts at 18 - 20 Kc/sec.

RESULTS

Fractionation of DNA of varying base compositions. Five bacterial strains were employed for isolation of DNA with base compositions varying from 27 to 75% G-C, i.e. the entire range of base compositions

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encountered in bacterial DNA. The results of chloroform treatment of SSC solutions of native and heat-denatured preparations, are presented in Table 1. It will be seen that the behaviour of the native preparations is similar, little or no DNA being removed to the interphase. Following heat denaturation a rough correlation does in fact exist between the G-C content and the amount of DNA left in the aqueous phase following chloroform treatment, i.e. the higher the G-C content, the smaller the amount of denatured DNA transferred to the interphase.

Table 1

Fractionation of DNA of varying base compositions

DNA source	G-C (%)	DNA in aqueous phase following chloroform treatment (%)	
		Native DNA	Heat-denatured DNA
Clostridium perfringens type B, Moscow 216	27	93.5	13.4
Streptococcus haemolyticus Challis group H	41	90	3
Bacillus subtilis	46	95.5	14.5
Azotobacter vinelandii	70	96	65
Micrococcus lysodeicticus	75	100	74

However, as noted previously [18], the salt concentration influences to a considerable extent the degree of fractionation. Several experiments were therefore conducted with a view to finding conditions which would permit of the fractionation of denatured DNA with high G-C content.

Influence of salt concentration. The amount of native or denatured DNA removed from the aqueous phase by chloroform treatment, as a function of the salt concentration in the aqueous phase, is shown in Fig. 1. From the various curves in this figure it will be observed that the greater the salt concentration in the aqueous phase, the larger the amount of DNA removed to the interphase, the slopes of the curves being less marked for the native, as compared to the denatured, preparations. By profiting from this effect of salt concentration it is possible in the case of *M. lysodeicticus* DNA, which is only partially fractionated in SSC solution, to achieve a reasonably good partition of native from denatured DNA with a salt concentration of 17.5%. Under these conditions about 13% of the DNA is removed from a native preparation and 85% from a denatured sample.

A comparison of the curves for the salting out of *native* DNA preparations with varying G-C contents shows that, the lower the

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percentage of G-C, the greater the slope of the curve, and the more pronounced is the dependence of the fractionation on the salt content. For example in the case of *Cl. perfringens* DNA, with a G-C content of 27%, a salt concentration of 9.5% suffices to transfer almost all the DNA to the interphase as a result of chloroform treatment; whereas in the case of *Streptococcus Challis* DNA with 41% G-C the same result

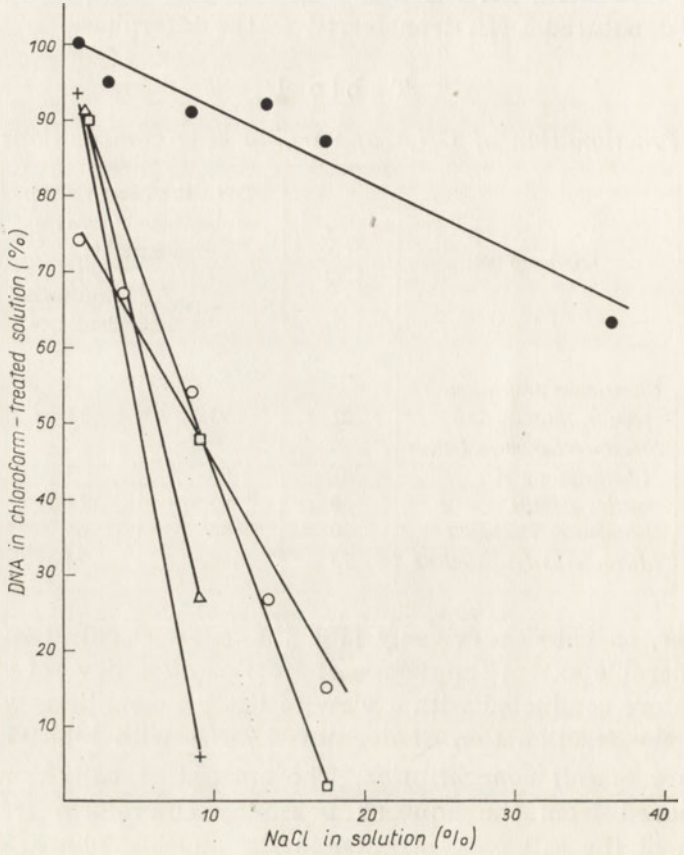

Fig. 1. Influence of salt concentration on the amount of DNA remaining in the aqueous phase following treatment with chloroform as described in text: (●), native DNA *M. lysodeicticus;* (○), denatured DNA *M. lysodeicticus;* (□), native DNA *B. subtilis*; (△), native DNA *Streptococcus Challis*; (+), native DNA *Clostridium perfringens.*

requires a salt concentration of 12.5%; while for *B. subtilis* DNA with 46% G-C about 18% NaCl is required to remove the entire DNA to the interphase. For *M. lysodeicticus* DNA with 75% G-C, even a saturated salt solution is insufficient.

The possibility of readily salting out to the interphase native DNA with low G-C contents suggested that the deproteinization procedures normally applied to cellular lysates during the isolation of DNA may

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conceivably result in some fractionation of the crude DNA preparations, involving the removal of components with a low G-C content. With a view to testing this, a chloroform - isoamyl alcohol mixture (24 : 1, v/v), such as is normally applied for deproteinization according to Sevag *et al.* [25], was tried. With *B. subtilis* DNA in an 18% salt solution, under which conditions chloroform treatment salts out to the interphase all the DNA, the chloroform - isoamyl alcohol mixture removed 30% of the DNA. The influence of salt concentration on the removal of DNA from the aqueous phase is readily interpretable in terms of the properties of the denatured DNA isolated from the water-chloroform interphase: it dissolves with difficulty even in dilute salt solution (DSC), but is readily dissolved in SSC on heating to about 80° or in formamide at room temperature. Both heating and formamide treatment rupture hydrogen bonds, and the observed behaviour of DNA isolated from the interphase clearly demonstrates that it must have undergone aggregation during transfer to the interphase; in addition, it is clear that the stability of DNA aggregates increases with an increase in salt concentration of the solution.

Fractionation of DNA during deproteinization may also be reduced by the mutual interaction of the DNA molecules. This follows from the results of experiments in which chloroform treatment was applied to a mixture of *M. lysodeicticus* and *Cl. perfringens* DNA in 0.15 M-NaCl, i.e. under conditions favourable for the removal to the interphase of *Clostridium* DNA. It was found that the quantity of *Clostridium* DNA was reduced from 50 to only 32%, this result being based on a comparison of the temperature profiles and T_m values of the mixture prior to, and following, chloroform treatment.

Influence of chain scission, or reduction in molecular weight was examined in the case of *Streptococcus Challis* DNA. The decrease in molecular weight of a given preparation as a result of chain scission was estimated roughly from a curve published by Litt *et al.* [12] for *D. pneumoniae* DNA, and relating molecular weight (from sedimentation measurements) to transforming activity. The transforming activity of our preparation was measured using a dihydrostreptomycin resistance marker.

Litt *et al.* [12] established the minimum molecular weight for biologically active T-DNA as 10^6 (no activity was observed below this molecular weight). Since complete inactivation of streptococcal T-DNA occurred after an 8-fold decrease in molecular weight, it follows that the molecular weight of our native preparation was about 8 million, i.e. about that normally encountered with bacterial DNA preparations obtained by the method of Marmur [14].

Control and sonicated DNA samples were heat-denatured and then treated with chloroform. Fig. 2 illustrates the amount of DNA removed

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from the aqueous phase as a function of the decrease in molecular weight. It will be seen that the amount of DNA removed by chloroform treatment decreases with a decrease in molecular weight. A 50% decrease in molecular weight reduces the amount of DNA removed by chloroform treatment from 97 to about 35%. An 8-fold decrease in molecular weight reduces to zero the amount of DNA removed from an SSC solution.

The foregoing results largely explain another of our observations, viz. the dependence of the amount of DNA removed by chloroform treatment on the time of heat denaturation. If the time of heating streptococcal DNA at 100° is reduced from 10 min. (that normally applied by most observers) to 3 min. (applied in this investigation), the amount of DNA remaining in the aqueous phase as a result of chloroform treatment is reduced from 20 to 3%. As will be seen from Fig. 2, 20% of a denatured streptococcal DNA remains in the aqueous

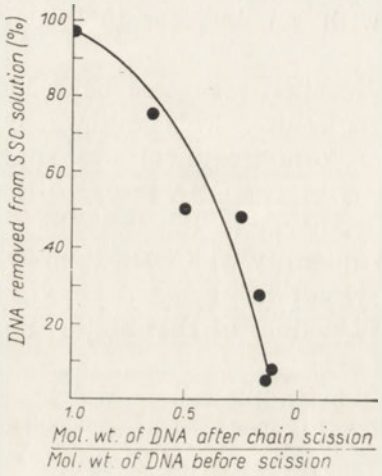

Fig. 2. Influence of chain scission on removal from the aqueous phase by chloroform treatment of heat-denatured streptococcal DNA.

phase following chloroform treatment of a preparation with a 30% lower molecular weight. Heating of DNA at 100° in SSC is known to provoke both depurination and chain scission [6, 3] and a heating period of 10 min. as compared to 3 min. would consequently be expected to result in a measurable decrease in molecular weight, which would explain the increased amount of DNA in the aqueous phase following denaturation and chloroform treatment. It also accounts for the fact that the residual transforming activity is only 1% as compared to 2% after 3 min. heating [18].

Residual activity of T-DNA. Chloroform fractionation of T-DNA denatured for 3 min. at 100° leaves in the aqueous phase 3 - 10% of the original DNA with a 7 to 10-fold increase in specific (residual) activity. The temperature profile of this residual DNA, while typical

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of that for heat-denatured DNA, exhibits only 12% hyperchromicity as compared to 20% for the non-fractionated denatured DNA [18]. The low hyperchromicity of this residual fraction, taken in conjunction with its increased specific activity, effectively excludes renaturation as the source of this activity.

An additional characteristic property of this residual fraction with increased specific activity is its failure to exhibit either renaturation or reactivation. Trials were carried out under optimal conditions for renaturation [16], i.e. at a DNA concentration of 20 μg./ml. in 0.3 - 1.5 M salt. There was, however, only a few percent increase in hyperchromicity with concurrent reactivation of the dihydrostreptomycin resistance marker which was either negligible or completely absent. By contrast the addition of wild-type DNA to this fraction resulted in a 3-fold increase in activity on "reactivation". The failure of this residual fraction with increased specific activity to exhibit reactivation implies that its activity is not due to partially separated twin strands since such strands would be expected to renature and exhibit reactivation at least as efficiently, if not more so, than the unfractionated denatured DNA. This finding is in accord with the suggestion advanced on the basis of the behaviour of the residual activity following inactivation at 100° in 7.2 M-$NaClO_4$ [18], i.e. under conditions where strand separation should be complete even for molecules composed exclusively of guanine--cytosine base pairs.

Consequently, of the three possible interpretations of the source of the residual activity, there remains only that according to which this activity is a property of single strands of denatured DNA. The increase in residual specific activity by chloroform fractionation, as well as the ability of this fraction to undergo reactivation only on addition of wild--type DNA, likewise suggested that the increase in specific activity following fractionation is due to some type of selection at the molecular level either according to the nature of the secondary structure of individual molecules, or the average base composition or sequence, or a combination of both these factors.

The foregoing suggestion was tested qualitatively by examining the absorption spectrum of the residual fraction as a function of pH and comparing with that for the unfractionated denatured DNA. At acid pH, where ionized cytosine residues are placed in evidence, no significant differences could be observed. However at alkaline pH, where thymine residues exhibit marked differences in absorption, the spectral modifications could be interpreted to indicate a lower thymine content for the residual fraction.

An additional criterion for a difference in base composition between the residual and unfractionated denatured DNA was based on the reaction of both fractions with formaldehyde, using 1% formaldehyde

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at pH 7 - 8 at room temperature as described by Stollar & Grossman [26]. The increase in absorption of the residual fraction on reaction with formaldehyde was 12.5% and of the unfractionated DNA 18.5%. When both samples were heated in the presence of formaldehyde to 85° and then cooled, each showed about 20% hyperchromicity (this equality is, of course, purely fortuitous). In the interpretation of this result, it is necessary to bear in mind that the hyperchromicity recorded after heating to 85° in the presence of formaldehyde represents the sum of two effects: (a) that due to complete separation of twin strands, and (b) that resulting from reaction of amino groups with formaldehyde. Since we know from the temperature profiles that the residual fraction possesses only half the hyperchromicity of the unfractionated, denatured DNA, the equal hyperchromicity of both samples following reaction with formaldehyde must be due to the presence of a larger number of amino groups in the residual fraction.

The foregoing, admittedly crude, tests therefore suggest that the residual fraction with increased specific activity differs from the unfractionated sample in that it has a lower thymine content and a higher content of adenine or/and guanine. The increased specific activity of the residual fraction is consequently due to selective fractionation of molecules according to base content and sequence, thus accounting for the difficulty in reactivating this sample and the reactivation observed following addition of wild-type DNA, i.e. following addition of the missing complementary strands.

DISCUSSION

From the results for the salting-out of DNA to the water-chloroform interphase, it is clear that both for native and heat-denatured DNA these are dependent on several factors. The amount of DNA transferred to the chloroform-water interphase increases with an increase (a) in salt concentration, (b) in the adenine-thymine (A-T) content of the DNA sample, and (c) in molecular weight of the DNA. Apart from the decisive influence of secondary structure, the foregoing factors are of some significance in relation to the behaviour of aqueous DNA solutions to chloroform treatment. It must once more be emphasized that the influence of salt concentration on the transfer of DNA to the interphase is much more specific for denatured, as compared to native, DNA; as a result of this it is possible to select the appropriate conditions for fractionation of native or heat-denatured DNA even for samples with a high G-C content.

The observations of Cavalieri *et al.* [2] on the behaviour of chloroform-treated solutions of DNA are in agreement with our findings. They noted that, during deproteinization of DNA solutions according

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to the Sevag method with a mixture of chloroform and octanol, high ionic strength favoured the transfer to the interphase of those molecules with higher molecular weight.

The results presented here are also of interest in relation to reported observations of DNA satellite bands in a density gradient [4, 10, 27, 24, 20]. Of the two observed satellite bands, one possesses a higher, and the second a lower, density than the main band. It is assumed that the higher density band represents a DNA fraction with a less ordered structure (presumably the replicating form of DNA), and the lower density band a DNA-protein complex [20]. Satellite bands are absent in DNA isolated according to the method of Marmur [14, 20], based on deproteinization by means of chloroform and isoamyl alcohol. An additional pertinent finding is the demonstration by Saito & Miura [23] that deproteinization by the phenol method gives DNA preparations with considerably higher molecular weight than those obtained by use of the Sevag method. These observations, and our own results, indicate that the Sevag method leads to the selective removal from a DNA preparation of both DNA complexed with protein and those DNA molecules with less ordered structure.

The findings on the residual activity of streptococcal transforming DNA point to this activity being a property of single strands of denatured DNA. Since chloroform fractionation leads to the selection of a residual fraction with higher specific activity, it is likely that this activity is a property of those molecules the structure of which differs from the others so that they react more readily with the acceptor cells.

Summing up, it may be concluded from the results presented in this, and a preceding paper [18], that chloroform fractionation of DNA is applicable to both native and denatured DNA isolated from various sources, due account being taken of the approximate mean base composition of the sample under test. The possibility of obtaining a 10-fold increase in specific activity of residual activity is a particularly useful property of this procedure, in view of its simplicity. It must be admitted, however, that there is a need for a more precise evaluation of its scope and sphere of application by more refined techniques such as density gradient centrifugation.

SUMMARY

Native DNA, or a mixture of native and heat-denatured DNA, may be fractionated by shaking an aqueous, saline solution with chloroform, as a result of which a portion of the DNA is transferred to the water--chloroform interphase. A study has been made of the influence of

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various factors on the degree of fractionation, taking into account the salt concentration in the aqueous phase, and the molecular weight and base-pair composition of the DNA. The procedure has been extended to establish conditions for fractionation of bacterial DNA preparations covering the entire known range of base-pair compositions.

Chloroform fractionation was utilized for the characterization of the nature of the streptococcal transforming DNA fraction which contains the residual activity resulting from heat denaturation. It has been established that this activity is a property of single-stranded DNA as in the case of *Diplococcus pneumoniae* and *Haemophilus influenzae* DNA.

Indirect evidence is presented suggesting that the observed increase in specific activity of the residual activity resulting from heat denaturation is due to the fractionation of molecules with a base-pair content differing from that of the entire, unfractionated, sample.

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DALSZE BADANIA NAD FRAKCJONOWANIEM DNA ZA POMOCĄ CHLOROFORMU

Streszczenie

Rodzimy DNA lub mieszaninę rodzimego i cieplnie zdenaturowanego DNA frakcjonowano przez wytrząsanie ich wodnych roztworów z chloroformem. W wyniku takiego postępowania część DNA przechodzi do interfazy wodno-chloroformowej. Przebadano wpływ różnych czynników na frakcjonowanie, przy czym wzięto pod uwagę stężenie soli w fazie wodnej, ciężar cząsteczkowy oraz skład zasad DNA.

Zastosowano frakcjonowanie chloroformem preparatów DNA o składzie zasad odpowiadającym zakresowi stwierdzonemu u bakterii.

Wykorzystano frakcjonowanie chloroformem dla scharakteryzowania frakcji DNA posiadającej resztkową aktywność transformującą po denaturacji cieplnej. Ustalono, że aktywność ta w układzie transformującym paciorkowców należy do DNA o pojedynczym łańcuchu, podobnie jak w układach transformujących *D. pneumoniae* i *H. influenzae*. Są wskazówki przemawiające za tym, że obserwowane zwiększenie specyficznej aktywności resztkowej jest połączone z wydzielaniem łańcuchów DNA różniących się od reszty preparatu pod względem składu zasad.

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LIPOLYTIC ACTIVITY OF THE FAT BODY OF THE WAXMOTH LARVAE

I. FATTY ACID COMPOSITION OF THE FAT BODY AND OF THE HAEMOLYMPH LIPIDS AND RELEASE OF FREE FATTY ACIDS FROM THE FAT BODY DURING INCUBATION

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While much work has been done on the metabolism of the adipose tissue of the vertebrates, very little is known about the metabolic aspects of the insect fat body, which combines some of the functions of adipose tissue and of the liver of vertebrates [14]. Evidence has been presented in recent years supporting the view that the insect fat body may be very actively involved in lipid metabolism. The presence of an enzyme system which catalyses incorporation of acetate into long-chain fatty acids has been shown by Zebe & McShan [25] in *Prodenia eridania* and by Tietz [20] in a cell-free preparation of the locust fat body. George & Eapen [6] found lipase activity in the fat body of the desert locust and stated that the concentration of enzyme was more than double the amount of the same enzyme in the pigeon adipose tissue. Uptake of glycerides by the fat body *in vitro* and release of glycerides into the haemolymph was shown by Tietz [21] to occur in the locust, and diglyceride release from the fat body of the *Hyalophora cecropia* silkmoth was observed by Chino [3].

As the waxmoth larvae feed on beecomb and consume large amounts of wax [16] the questions concerning the digestion of this lipid material and the transport of the products absorbed are of major importance in the physiology of this insect. However, very little is known about the mechanism of the transport of lipids in the waxmoth and about the role of the fat body in this process.

The purpose of the present study was to investigate the lipolytic activity of the waxmoth fat body *in vitro* and to examine the free fatty acids (FFA) produced in the tissue and released into the medium during incubation of the fat body. The composition of the fatty acids in the lipids of the fat body was also investigated and compared with

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the fatty acid pattern of the haemolymph lipids. The correlation between the lipolytic activity of the fat body *in vitro* and the concentration of free fatty acids in the haemolymph was also studied.

MATERIAL AND METHODS

Fully grown waxmoth larvae (*Galleria mellonella* L.) weighing about 150 - 180 mg. each were used in the experiments. The larvae were bred on beecomb and kept at 30°. The insects to be starved were ligated just behind the head in order to prevent metamorphosis [17]. Groups of 80 - 100 larvae were used in each experiment.

Haemolymph was aspirated in capillary pipettes after cutting off one of the prolegs of the larva and was collected in weighed small vessels chilled in ice.

The fat body was carefully removed free from most of the accompanying tracheoles. It was rinsed in Krebs-Ringer phosphate buffer, blotted on filter paper, and preserved at 0° up to the time of incubation.

Portions of the fat body were transferred into small vessels containing the incubating medium (2 ml. per 200 mg. of the tissue) and agitated gently in air at 37°. The period of incubation was 3 hr. except where otherwise noted. The incubation was carried out in a medium prepared by dissolving 5% by weight of bovine serum albumin (Sigma, USA, fraction V) in Krebs-Ringer phosphate buffer and adjusting the pH to 7.4. Other additions were as indicated in the legends. At the end of the incubation period the fat body tissue was separated from the medium by filtration and the amounts of FFA were determined in both the tissue and the medium. In the controls the amounts of FFA present in the tissue and in the medium were measured at zero time. The results of FFA analyses were calculated in terms of μmoles of fatty acids produced per gram wet weight of tissue in 3 hours.

Lipids present in the medium were extracted with 25 vol. of ethanol - diethyl ether (3:1, v/v). The mixture was brought to boil, cooled and filtered into a separatory funnel. After addition of water, the ether layer was separated and evaporated under reduced pressure. The residue was re-extracted with petroleum ether. The amount of FFA was determined by titration with 0.1 N-NaOH in a two-phase system using Nile blue as indicator, according to Dole & Meinertz [5].

Lipids from the fat body and from the haemolymph were extracted in the same way, except that the free fatty acids were separated from the total lipid extract before titration. This was attained by washing the petroleum ether extract with 0.1 N-NaOH in 50% ethanol, according to Borgström [1], and extracting the FFA with petroleum ether from the aqueous ethanol after acidification.

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Phospholipids were separated from the neutral lipids by column chromatography on silicic acid (Mallincrodt, 100 mesh). The adsorbent was activated by heating at 110° for 18 hr. and washed successively with methanol, acetone, diethyl ether and petroleum ether before use. Neutral lipids, after separation of the FFA, were eluted from the column with chloroform, and phospholipids with methanol. The nonesterified fatty acids were converted to their methyl esters by heating with water-free methanolic 0.2 N-HCl for 12 hr. The fractions containing total lipids, neutral lipids or phospholipids, respectively, yielded fatty acid methyl esters directly by methanolysis with water-free methanolic 6 N-HCl for 18 hr. at 72°. The composition of fatty acids in individual lipid fractions was determined by gas-liquid chromatography of the corresponding methyl esters, using a Pye Argon Chromatograph (Cambridge, Great Britain). Columns were packed with Celite 545 BDH (100 - 120 mesh) impregnated with polymerized ethylene glycol succinate, in the proportions 10:1 by weight of solid support to liquid phase. The Celite was pretreated with concentrated HCl in order to remove iron, washed free of acid, oven dried, and treated with alcoholic alkali. The operating temperature was 168° and the argon flow was 50 ml. per minute. Samples were dissolved in petroleum ether for injection.

Calculation of the proportions in each mixture was made directly from the relative peak areas on the chromatograms determined by the "triangulation" procedure. The fatty acids were identified on the basis of the retention times of standards run on the same column under the same conditions prior to analysis of a sample.

RESULTS

The splitting of fatty acids from the lipids and their release from the fat body in vitro

Approximately 10.5 μmoles of FFA were found to be present in 1 g. fat body prior to incubation. When incubated in a buffered medium, the tissue produced and released FFA and the final amount of FFA in the tissue and in the medium, taken together, highly exceeded the amount initially present in the fat body. Figure 1 shows the results obtained and the effect of albumin on the release of FFA from the tissue. The production of FFA in the presence of albumin in the medium was much higher than in its absence. It should be noted that no accumulation of FFA within the tissue was observed, and in the presence as well as in the absence of albumin the FFA concentration in the fat body markedly decreased during incubation, while the concentration of FFA in the medium increased.

The incubation was usually performed at pH 7.4, as this pH was mostly applied by other authors for rat adipose tissue [8, 18]. Changing

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the pH in the medium resulted in a pronounced change in FFA production; at a lower pH value (6.5) the amount of FFA released was approximately one-half of that released at pH 7.4, while the amount of FFA liberated at pH 8.5 was about 40% higher. Lowering the temperature of incubation below 37° brought about a marked decrease in the amount of FFA found in the medium after incubation.

The course of the release of FFA from the fat body is shown in Fig. 2. The FFA concentration rose sharply during the first 60 min. of incubation and continued to rise slightly during the following 2 hours. From the total amount of about 20 μmoles FFA released per 1 g. fat body in 3 hr. more than 12 μmoles were found in the first 30 min. of incubation (60%) and 4 μmoles in the following 30 min. Later on, the rate of FFA production was markedly slowed down.

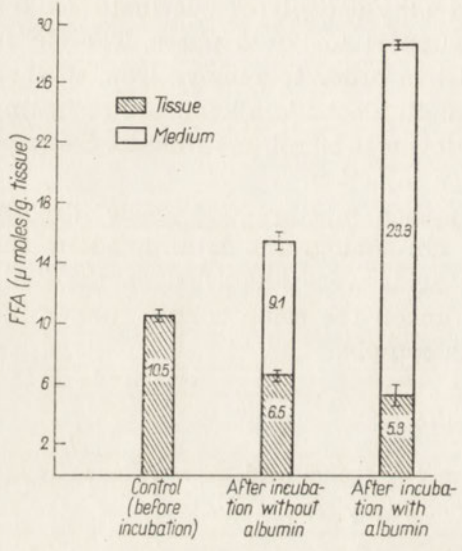

Fig. 1

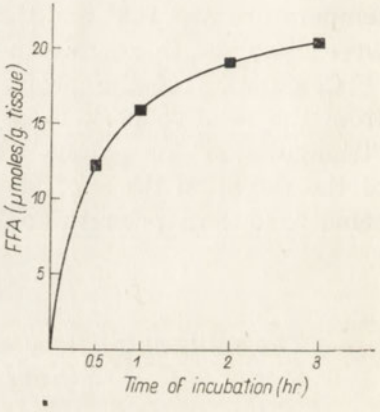

Fig. 2

Fig. 1. Production of free fatty acids by the fat body of the waxmoth larvae *in vitro*. Effect of albumin in the medium. Portions of about 200 mg. of the fat body were incubated at 37° for 3 hr. in 2 ml. of Krebs-Ringer phosphate buffer, pH 7.4, with or without bovine serum albumin (5%). At the end of the incubation period FFA were extracted and determined as described in Methods. Correction was made for the small amount of fatty acids present in the albumin. Mean values of at least 4 experiments.

Fig. 2. The time-course of free fatty acids production by the fat body of the waxmoth larvae *in vitro*. Each point on the curve represents the mean of 3 determinations, the results of which were nearly identical. Portions of the fat body (200 mg.) were incubated at 37° in 2 ml. of Krebs-Ringer phosphate buffer, pH 7.4, containing 5% bovine serum albumin. The increase in the FFA content in the medium and tissue was measured at the times given. For other details see text.

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In order to get more information of the nature of the lipolytic enzymes which are present in the fat body and catalyse the splitting of fatty acids from the lipids, the effect of several substances known to influence the activity of various lipases was studied. The results are summarized in Table 1. As heparin is considered to activate the lipoprotein lipase [12, 2, 10] this substance was included in the incubation mixture in varying amounts. However, in the concentrations used, heparin had no effect on the lipolytic activity of the tissue. Protamine, known to inhibit the lipoprotein lipase, brought about a marked decrease in the amount of FFA produced. The inhibitory effect of 1 M-NaCl was even more pronounced. Both these compounds affected the lipolytic activity of the

Table 1

Effect of various additions on the lipolytic activity of the waxmoth fat body

Portions of about 200 mg. fat body of waxmoth larvae were incubated at 37° for 3 hr. in 2 ml. of Krebs-Ringer phosphate buffer, pH 7.4, containing 5% bovine serum albumin and the substance tested. For other details see text. Mean values of 4 - 6 experiments and the range are given.

Additions (final concn.)		Lipolytic activity as percentage of control	
		Waxmoth fat body	Rat adipose tissue (Cherkes & Gordon, cit. after [14])
None (control)		100	100
Protamine sulphate	20 mg./ml.	43 (22 - 64)	43
	10 mg./ml.	42 (38 - 46)	
NaCl	1 M	20 (15 - 28)	17
NaF	0.2 M		93
KF	0.2 M	69 (55 - 85)	
Heparin	10 µg./ml.	102 (101 - 103)	
	30 µg./ml.	100 (92 - 104)	
	50 µg./ml.	96 (84 - 103)	
	150 µg./ml.	99 (93 - 103)	
Sodium tripolyphosphate*	0.17 mg./ml.	62 (49 - 72)	
	0.33 mg./ml.	62 (59 - 68)	
Glucose	1 mg./ml.	52 (42 - 61)	
Trehalose	1 mg./ml.	52 (44 - 63)	

* $Na_5P_3O_{10} \cdot 6H_2O$.

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fat body in a way very similar to that reported by Cherkes & Gordon [2] for the lipases from rat adipose tissue. On the other hand, fluoride, which in the experiments of Cherkes & Gordon [2] had practically no effect on the lipases of rat adipose tissue, markedly inhibited the lipolytic activity of the waxmoth fat body. Fluorides are known to be inhibitors of the pancreatic lipase but not to influence the lipoprotein lipase.

It was further found (Table 1) that the lipolytic activity of the fat body was also appreciably inhibited by the presence in the medium of glucose, trehalose, and sodium tripolyphosphate.

Effect of fasting on the lipolytic activity of the fat body and on the FFA content in the haemolymph

Figure 3 presents the results of experiments performed on fat bodies removed from the larvae which had previously been starved for 1, 3 or 7 days. As before, the fat bodies were incubated in buffer solution containing albumin. The amount of FFA produced per 1 g. of the tissue from the one day starved insects was consistently lower than in the controls (fed larvae); in three days starved larvae it was somewhat higher, but still lower than in the controls, but after 7 days of fasting the FFA production was almost the same as in the fat bodies of the fed insects.

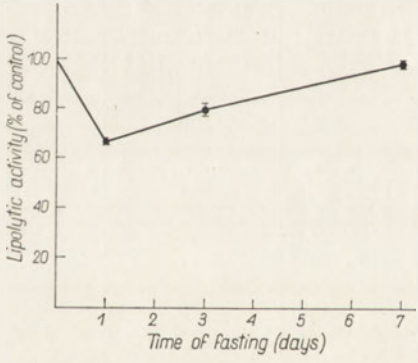

Fig. 3

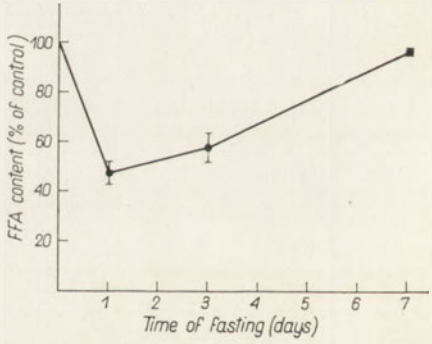

Fig. 4

Fig. 3. The effect of fasting on the production of free fatty acids by the fat body of the waxmoth larvae. Portions of about 200 mg. fat body of the fasted ligated larvae were incubated for 3 hr. at 37° in 2 ml. of Krebs-Ringer phosphate buffer, pH 7.4, containing 5% bovine serum albumin. The production of FFA was measured as described in text. Production of FFA by the fat body of fed larvae was taken as 100%. The vertical lines indicate the range of values for 4 experiments.

Fig. 4. Content of the free fatty acids in the haemolymph of the waxmoth larvae during starvation. The FFA content in the haemolymph of fed larvae was taken as 100%. The vertical lines indicate the range of values for 4 experiments.

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It seemed to be of interest to check whether the changes in lipolytic activity of the fat body during fasting would be reflected by the FFA content in the haemolymph. It appeared that the FFA concentration, which was fairly stable in the fed larvae (7.2 μmoles/g., ± 0.14 S.E.), was changed during starvation in parallel to the changes of the lipolytic activity of the fat body (Fig. 4).

The effect of starvation was studied on larvae which had a ligature applied just behind the head. The experiments were, therefore, difficult to interpret, because the results might be influenced by two additional factors. First, ligation makes the larvae motionless and secondly, it prevents the insect hormones produced in the neurosecretory glands from penetrating the body.

Another series of experiments was, therefore, performed on larvae deprived of food but not ligated. In this case fasting could not exceed 24 hr., because otherwise pupation started. It is seen in Table 2 that, contrary to the results obtained with the ligated larvae, the FFA release from the fat body of the fasted and of the fed insects was very similar; simultaneously the content of free fatty acids in the haemolymph was found to be practically identical with that in the fed larvae (7.0 μmoles/g.).

Table 2

The production of free fatty acids by the fat body of the larvae fasted for 24 hr. and not ligated

Experimental conditions as in Table 1. The results are expressed as μmoles/g./3 hr.

Expt. no.	Fed larvae	Fasted larvae
1	18.7	18.8
2	19.1	18.1
3	18.2	18.5

Fatty acid composition in the lipids of fat body and of haemolymph

Since a correlation was found to exist between the lipolytic activity of the fat body *in vitro* and the concentration of FFA in the haemolymph, the question arose whether the composition of fatty acids in the fat body would be reflected by the composition of the fatty acids released from the tissue and by the FFA in the haemolymph. The following fat body lipids were analysed: glycerides, FFA present in the tissue, and FFA released into the medium during incubation. The lipids of the haemolymph were separated into neutral lipids, phospholipids, and free fatty acids and each fraction was analysed separately.

The results of gas-liquid chromatography of the corresponding fatty acid methyl esters are presented in Tables 3 and 4. The predominant

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fatty acids in the glycerides of the fat body were found to be palmitic and oleic acids which were present in approximately equal amounts and comprised together more than 80% of the total. Stearic acid presented only a very small part of the total fatty acids. The total unsaturated fatty acids comprised about 56% and consisted chiefly of monounsaturated acids, di- and trienoic acids being present in very small amounts. The proportion of acids with a carbon chain of more than C_{18} was about 5%.

Table 3

Fatty acid composition of the lipids of the fat body and of the free fatty acids released into the medium

Mean values of 6 determinations, ± S.E.M., are given.

Fatty acid	Composition (%)		
	Fat body		FFA released into the medium during incubation
	Glycerides	FFA	
$C_{14:0}$	0.3 ± 0.04	0.2 ± 0.01	0.4 ± 0.06
$C_{15:0}$	0.3 ± 0.04	0.2 ± 0.15	0.5 ± 0.07
$C_{16:0}$	40.0 ± 0.71	35.0 ± 1.20	37.7 ± 1.07
$C_{16:1}$	3.4 ± 0.03	3.7 ± 0.78	3.1 ± 0.70
$C_{17:0}$	0.3 ± 0.04	0.5 ± 0.09	0.5 ± 0.19
$C_{17:1}$	0.5 ± 0.17	0.7 ± 0.01	0.6 ± 0.15
$C_{18:0}$	1.4 ± 0.05	2.5 ± 0.60	1.8 ± 0.31
$C_{18:1}$	45.3 ± 1.10	45.8 ± 1.32	42.0 ± 1.17
$C_{18:2}$	1.8 ± 0.22	3.2 ± 0.38	2.2 ± 0.22
$C_{18:3}$	2.1 ± 0.20	4.5 ± 0.74	4.2 ± 0.77
$C_{20:1}$*	3.2 ± 0.45	2.5 ± 0.46	3.9 ± 0.90
$C_{20:2}$*	0.2 ± 0.1	—	—
$C_{20:4}$*	—	<0.2	4.2 ± 1.04
$C_{22.0}$	1.4 ± 0.37	<0.2	—
Saturated	43.7	38.4	40.9
Unsaturated	56.3	60.6	60.2

* Tentative identification.

The composition of free fatty acids present in the fat body before incubation differed somewhat from that of the glycerides. The proportion of palmitic acid was smaller, the unsaturated acids comprised approximately 60% and the percentage contribution of C_{18}-polyunsaturated acids was higher.

The free fatty acids released from the tissue into the medium during 3 hr. incubation were found to be similar in composition to the FFA fraction of the fat body, except that the proportion of $C_{20:4}$ was significantly higher.

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Table 4

Fatty acid composition of total lipids and of some lipid fractions of the haemolymph

Mean values of 6 determinations, ± S.E.M., are given.

Fatty acid	Composition (%)			
	Total lipids	FFA	Neutral lipids	Phospholipids
$C_{14:0}$	0.4 ± 0.09	0.3 ± 0.04	0.4 ± 0.12	0.5 ± 0.21
$C_{15:0}$	0.5 ± 0.07	0.7 ± 0.28	0.6 ± 0.10	0.9 ± 0.20
$C_{16:0}$	35.5 ± 1.7	31.3 ± 2.20	40.4 ± 1.20	31.0 ± 4.02
$C_{16:1}$	3.6 ± 0.3	2.0 ± 0.18	2.8 ± 0.31	2.8 ± 0.50
$C_{17:0}$	1.2 ± 0.2	1.9 ± 0.10	0.8 ± 0.13	2.3 ± 0.70
$C_{17:1}$	1.8 ± 0.2	2.1 ± 0.25	1.3 ± 0.15	2.7 ± 0.62
$C_{18:0}$	2.5 ± 0.1	4.7 ± 0.37	2.4 ± 0.40	3.5 ± 0.53
$C_{18:1}$	38.9 ± 1.3	29.9 ± 1.20	36.6 ± 3.22	32.6 ± 3.00
$C_{18:2}$	3.6 ± 0.5	6.6 ± 0.46	16.1 ± 1.70	8.1 ± 0.91
$C_{18:3}$	5.2 ± 1.1	6.3 ± 0.81	4.9 ± 0.80	9.8 ± 1.72
$C_{20:2}$*	4.5 ± 1.5	2.3 ± 1.10	3.0 ± 0.94	2.8 ± 1.30
$C_{22:0}$	1.5 ± 0.2	1.7 ± 0.35	0.9 ± 0.21	2.5 ± 1.10
Saturated	41.6	40.4	45.5	40.7
Unsaturated	57.6	59.2	54.7	58.8

* Tentative identification.

The fatty acid pattern of the total lipids of the haemolymph resembled that of the fat body glycerides, but the relative contents of linoleic and linolenic acids were higher and the fatty acid tentatively identified as $C_{20:2}$ comprised about 5%. However, the FFA of the haemolymph were found to differ considerably from the fatty acids bound in esters. They contained relatively less palmitic and oleic acids and significantly more stearic acid and C_{18}-polyunsaturated acids, especially linolenic acid. Phospholipids of the haemolymph differed from the neutral lipids by a lower relative content of palmitic acid and by a higher content of linoleic and linolenic acids.

DISCUSSION

The fat body of insects differs in many respects from the adipose tissue of vertebrates and the mechanisms of lipid mobilization in insects and other animals may not be identical. The adipose tissue has been shown to be a rich source of free fatty acids which are released into the blood and, bound to albumin, are possibly the major form in which lipids are transported to other tissues for utilization. Few data only are available concerning the role of insect fat body in fat transport, but they point to a somewhat different mechanism operating in, at least,

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some insects as compared to higher animals. Locust fat body has not been found by Tietz [21] to release any significant amounts of free fatty acids, either into a buffered medium (with or without albumin) or into the haemolymph. When the tissue was incubated in haemolymph, glycerides were released into the medium. According to Tietz, glycerides and not the free fatty acids are mobilized from the fat body and incorporated into lipoproteins of the haemolymph and these compounds may therefore represent the transport form of lipids in the locust. Experiments performed by Chino [3] on *Hyalophora cecropia* and *Melanoplus differentialis* strongly suggest that diglycerides are released from the fat body of these insects, and diglycerides are thought to be the chief form in which lipids are transported by the haemolymph.

In the present experiments, the fat body of the waxmoth larvae was incubated in a buffered medium, not in haemolymph, and appreciable amounts of FFA were found to be liberated from the tissue, especially in the presence of albumin as a fatty acid acceptor. Hence, the waxmoth fat body seems to differ in this respect from that of the locust. It should be noted that the amount of free fatty acids released by 1 g. of the waxmoth fat body was much higher than the amount released by 1 g. of rat adipose tissue incubated in similar conditions [8]. In our experiments, the presence of albumin in the medium strongly enhanced the hydrolysis, since not only the release into the medium but also the production of free fatty acids by the tissue was much smaller when albumin was absent from the medium. These findings are at variance with the results of Hollenberg & Angel [11] who found that in the albumin-free medium the release of FFA from rat adipose tissue was inhibited whereas the accumulation of FFA within the tissue was not prevented. Our results seem to indicate that free fatty acids, not removed from the reaction mixture by binding to albumin, inhibit the enzyme activity and interfere with lipolysis in the waxmoth fat body.

The time-course of lipolysis in the fat body (Fig. 2) shows the reaction to proceed much faster during the first hour of incubation than during the following 2 hours. The amount of FFA released from the tissue at any time may be the dynamic resultant of two opposite reactions, namely the liberation of fatty acids from the lipids and their esterification within the cell. Although the conditions of experiment do not favour the esterification, it is not to be excluded that some fatty acids initially released into the medium may be taken up again by the tissue.

The effects of various substances added to the incubation mixture may give some information about the nature of lipolytic activity in the waxmoth fat body. Sodium chloride solution (1 M) and protamine sulphate, which are known to be inhibitors of lipoprotein lipase [12, 2], were effective in inhibiting the activity of the enzymes present in the fat body (Table 1). Sodium tripolyphosphate inhibited the lipolytic

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activity of the fat body in a similar way as pyrophosphate inhibited the lipoprotein lipase from rat heart and chicken adipose tissue [12, 13]. It seems to be of interest that polyphosphates have been found to occur normally in the waxmoth larvae [15] and their influence on the mobilization of lipids *in vivo* is not to be excluded. The inhibitory effect of glucose on the liberation of FFA from both the rat adipose tissue [19] and the waxmoth fat body may be attributed to its giving rise to glycerophosphate which serves to esterify the fatty acids. Inhibition produced by trehalose, the characteristic carbohydrate of the haemolymph of many insects, may be due to a similar mechanism.

Activation by heparin of the release of fatty acids from the waxmoth fat body has never been observed in our experiments. However, this does not seem surprising, since the nonuniformity of the heparin response between species and tissues has been frequently observed. For example, lipoprotein lipases from chicken adipose tissue [3], rat aorta [12] or mice epididymal fat [9] are reported not to be activated by heparin.

The effect of inhibitors on the production of FFA by the fat body of the waxmoth larvae suggests that an enzyme similar to lipoprotein lipase may be involved in this process. On the other hand, potassium fluoride known to inhibit the pancreatic lipase but not to affect the lipoprotein lipase, was also found to cause a decline in the mobilization of FFA from the fat body. This fact as well as the observation that the inhibition of the lipolytic activity by NaCl and protamine sulphate was not complete seem to indicate that another lipase, distinct from lipoprotein lipase and possessing different characteristics, is also present in the waxmoth fat body. This problem was the subject of a further study, the results of which are presented in another paper [24].

A close correlation seems to exist between the ability of the fat body to release fatty acids and the FFA content in the haemolymph. When, as in the fed larvae, high lipolytic activity was found in the tissue, simultaneously a high content of the free fatty acids was observed in the haemolymph. After one day fasting, the rate of FFA release was markedly diminished and at the same time the FFA concentration in the haemolymph was about one half of that in the fed insects. When, however, the lipolytic activity in the fat body was restored, the FFA content in the blood was the same as in the controls. As reported by Tietz [21], the locust fat body liberates only insignificant amounts of fatty acids, and the haemolymph contains only 0.1 μmole FFA per one ml. A similar relationship between the lipolytic activity of the adipose tissue of the vertebrates and the concentration of FFA in blood has been frequently observed [4, 7]. Gordon [7] was the first who correlated the concentration of FFA in serum with changes in the FFA production and interpreted these data as evidence for the view that fat is released

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from adipose tissue and transported in the blood in form of free fatty acids bound to albumin.

In view of the pronounced lipolytic activity of the fat body and of the fairly high concentration of free fatty acids in the haemolymph one is inclined to suppose that in the waxmoth larvae, like in the vertebrates, free fatty acids represent the form in which lipids are transported from the sites of storage to the sites of utilization. Whether they represent the only transport form and whether glycerides are also involved in the lipid transport cannot be decided so far.

The significance of the marked decline in FFA release from the fat body of the one day fasted larvae is poorly understood. It has been generally accepted that the adipose tissue from fasted animals releases much more fatty acid into the medium than does the tissue from fed animals [8, 18]. The fall in enzyme activity observed in our experiments during the first days of fasting could be due to ligation, because such a decline was not observed in insects fasted and not ligated. Unfortunately, fasting without ligation cannot be continued for more than one day as it results in spinning and pupation. Therefore, it is not known whether further fasting would have activated the fatty acid production or not.

As the release of fatty acids from the adipose tissue is controlled by the concentration of nutrients in blood and by the secretion of hormones [22], one may speculate that the fall in enzyme activity of ligated larvae is due to some lack of hormones caused by ligation and that the increased rate of FFA production in the later period of starvation may be induced by some changes in the concentration of some nutrients in the haemolymph. However, the role of other factors is not to be excluded.

Analyses of the fatty acid composition of the lipids in the fat body and in the haemolymph revealed the presence of more than 50% unsaturated acids. The major component was oleic acid but small amounts of polyunsaturated acids were also present. Since the lipids extracted from the beecomb, which constituted the only food of the waxmoth larvae, were found to contain almost exclusively saturated fatty acids [23], it is reasonable to suppose that desaturation of fatty acids takes place on a large scale in the insect body. The pathway leading to the formation of polyunsaturated fatty acids in this insect is still obscure, the more so as most animals seem to be unable to synthesize these compounds.

Some differences were found to exist between the composition of the fatty acids of the fat body glycerides and the composition of the FFA fraction in the tissue. The relative content of palmitic acid was lower in the free fatty acid fraction, and the unsaturated acids (especially $C_{18:2}$ and $C_{18:3}$) comprised a greater part of the total than in

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the glycerides. These differences may be explained by some selectivity of the enzymes in liberating the polyunsaturated acids, or by differences in solubility of these acids in the cytoplasm of the cells, or perhaps by a desaturation of the liberated fatty acids which is likely to occur in the fat body.

On the other hand, practically no differences were found between the free fatty acids produced in the tissue and those released into the medium during incubation. This finding is at variance with that of Hollenberg & Angel [11] who found significant differences between the free fatty acids accumulated in rat adipose tissue during incubation, and the FFA released into the medium. Two factors may be responsible for these discrepancies. First, it should be taken into account that the morphological structure of the waxmoth fat body is very different from that of the adipose tissue. Rat adipose tissue, when incubated, is exposed to the medium by a rather limited surface and the presence of several compartments which differ in the extent to which the fatty acids enter the medium seems to be likely. On the contrary, the waxmoth fat body consists of a loose meshwork of very small lobes and a close contact is likely to exist between the cells of the tissue and the medium, and this may allow an easy outflow of the FFA from the tissue. Next, the free fatty acids in rat adipose tissue may be more firmly associated with some cellular binding sites and may differ in their availability for transfer out of the cell, while the FFA produced in the fat body may have a stronger affinity to albumin present in the medium than to the cellular binding sites. The failure to observe any accumulation of FFA within the waxmoth fat body during incubation and the marked accumulation of free fatty acids in rat adipose tissue in the experiments of Hollenberg & Angel [11] seem to support both suppositions.

Some differences were found to exist between the composition of FFA of the haemolymph and the composition of free fatty acids produced by the fat body. Thus, the relative content of oleic acid was smaller and those of linoleic and linolenic acids were higher in the haemolymph FFA than in the free fatty acid fraction of the fat body. Several factors may be responsible for these differences: (1), haemolymph circulates in the insect body in a direct contact with all tissues and its composition reflects the biochemical transformations which take place in various tissues. Apart from the fat body, other tissues, and first of all the intestine, may contribute to the FFA pool of the haemolymph. (2), Some metabolic changes may be brought about during the transport of the fatty acid and during its contact with the tissues. (3), Finally, the possibility exists that the affinity of the haemolymph proteins for an individual fatty acid may be higher than the affinity of the cellular binding sites for the same acid. This would result in the easier transfer of this acid into the haemolymph draining the fat body.

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SUMMARY

1. The composition of free and esterified fatty acids in the fat body and haemolymph of the waxmoth larvae was determined by gas-liquid chromatography.

2. The fat body of the larvae was found to release appreciable amounts of free fatty acids when incubated in a buffered medium, especially in the presence of added albumin.

3. The effects of various enzyme inhibitors seem to indicate that at least two different lipases are present in the fat body.

4. Factors which affect the lipolytic activity of the fat body induce some changes in the free fatty acid concentration in the haemolymph of the larvae.

5. The free fatty acid fraction seems to be the major form in which lipids are transported in the haemolymph from the sites of storage to the sites of utilization.

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AKTYWNOŚĆ LIPOLITYCZNA CIAŁA TŁUSZCZOWEGO GĄSIENIC MOLA WOSKOWEGO

I. SKŁAD KWASÓW TŁUSZCZOWYCH W LIPIDACH CIAŁA TŁUSZCZOWEGO I HEMOLIMFY ORAZ POWSTAWANIE WOLNYCH KWASÓW TŁUSZCZOWYCH PODCZAS INKUBACJI CIAŁA TŁUSZCZOWEGO

Streszczenie

1. Przy pomocy chromatografii gazowej zbadano skład wolnych i zestryfikowanych kwasów tłuszczowych w lipidach ciała tłuszczowego i hemolimfy.

2. Stwierdzono, że podczas inkubacji ciała tłuszczowego gąsienic w buforze fosforanowym następuje uwalnianie się kwasów tłuszczowych znacznie intensywniejsze w obecności albuminy surowiczej w środowisku.

3. Wpływ rozmaitych inhibitorów na aktywność lipolityczną ciała tłuszczowego wskazuje na obecność co najmniej dwóch różnych lipaz.

4. Czynniki powodujące zmiany aktywności lipolitycznej ciała tłuszczowego wpływają na zmianę stężenia niezestryfikowanych kwasów tłuszczowych w hemolimfie gąsienic.

5. Niezestryfikowane kwasy tłuszczowe są prawdopodobnie formą transportową lipidów w hemolimfie gąsienic mola woskowego.

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LIPOLYTIC ACTIVITY OF THE FAT BODY OF THE WAXMOTH LARVAE

II. CHARACTERISTICS OF THE TWO DIFFERENT LIPASES IN THE WAXMOTH FAT BODY

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Previous studies [11] on the lipolytic activity of the fat body of the waxmoth larvae suggested that at least two enzymes with different characteristics might be involved in the liberation of fatty acids from the tissue. The partial inhibition of the fatty acid production by potassium fluoride pointed to the presence of an enzyme similar to the pancreatic lipase (glycerol ester hydrolase, EC 3.1.1.3) of higher animals, while the inhibition by protamine sulphate and 1 M-NaCl suggested that another enzyme similar to lipoprotein lipase might also be active in the fat body.

The present experiments were designed in order to characterize in more detail the two types of lipase activity in the fat body of the waxmoth larvae.

METHODS

Fully grown waxmoth larvae (*Galleria mellonella* L.) bred on beecomb and kept at 30° were used in the experiments.

The fat body was removed from the larvae as described earlier [11]. Samples of about 500 mg. of the tissue were incubated for 1 hr. at 37° in 5 ml. of Krebs-Ringer phosphate buffer, pH 7.4, containing 30 μg. heparin per 1 ml. of the solution. After incubation lipase activity was examined both in the fat body and in the incubation medium.

The fat body was separated from the medium by filtration under slight suction, rinsed with Krebs-Ringer buffer solution and homogenized in another 10 ml. of the buffer solution. The homogenate was centrifuged in the cold for 10 min. at 1000 *g* and the water phase between the sediment and the fat layer was collected (this will be referred to later as homogenate). Samples of the incubation medium and of the

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homogenate, respectively, were incubated with equal amounts of a mixture containing 7 parts of 6% serum albumin (Sigma, St. Louis, USA, fraction V) in Krebs-Ringer phosphate buffer, 0.5 part of 1 M-$(NH_4)_2SO_4$, and 2 parts of substrate. The substrate was either a commercial coconut oil emulsion (Ediol [1], Riker Laboratories, Inc. (Schen. Labs.) Northridge, California) or an activated coconut oil emulsion prepared from it by incubation with rat serum for 30 min. at 37°. Both emulsions were diluted to 4% with the phosphate buffer. The pH of the incubation mixtures was adjusted to 8.5. The mixture was essentially the same as that used by Björntrop & Furman [1] and by Cherkes & Gordon [3].

The free fatty acid (FFA) concentration was measured after 1 hr. incubation and at zero time in the controls. The increase in FFA concentration over that found in the control was a measure of lipase activity. A series of control experiments in which the activated coconut oil emulsion was incubated in phosphate buffer without the source of lipase showed no increase in the FFA content. For the FFA determination either the procedure described earlier [11] or the method of Dole & Meinertz [4] was used.

The fat layer which accumulated on the top of solution during centrifuging of the homogenized fat body was also assayed for lipolytic activity. For this purpose the fat was collected and emulsified by grinding in a mortar with gum arabic and phosphate buffer. Portions of this emulsion were incubated with equal amounts of either activated substrate or a mixture containing only albumin, $(NH_4)_2SO_4$ and buffer in the proportions given above. The increase in FFA concentration after 1 hr. incubation was measured.

RESULTS

It is seen in Table 1 that both the waxmoth fat body and the medium in which the tissue had been incubated contain lipolytic activity. However, some differences seem to exist between the enzyme activity which appears in the incubation medium and that which remains in the tissue. The lipase present in the medium strongly hydrolysed the activated coconut oil emulsion but had almost no effect on the non-activated emulsion. On the other hand, in the tissue homogenate the lipolytic activity could be observed, directed toward both the activated and the non-activated substrate.

It has been shown by several authors [7, 3, 1] that oil emulsion activated by preincubation with normal serum is an adequate substrate for the action of lipoprotein lipase and is indistinguishable from chylo-

[1] Ediol is an aqueous fat emulsion containing 50% coconut oil, 12.5% sucrose, 1.5% glyceryl monostearate, and 2% polyoxyethylene sorbitan monostearate.

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Table 1

Lipolytic activity of the waxmoth fat body

Portions of the fat body (about 500 mg.) were incubated in 5 ml. Krebs-Ringer phosphate buffer at 37°. After 1 hr. incubation the tissue was removed from the medium and homogenized in another 10 ml. of the buffer. The lipolytic activity in the homogenate and in the medium in which the tissue had been preincubated was assayed as described in Methods.

The lipolytic activity is expressed as μmoles of FFA released per 1 g. tissue in 1 hr. The values represent the mean and range for 3 - 5 experiments.

Substrate	Lipolytic activity	
	in the medium	in the tissue
Coconut oil emulsion	0.4 (0.0 — 0.6)	4.5 (4.2 — 5.0)
Activated coconut oil emulsion	8.4 (6.5 — 11.0)	4.1 (3.8 — 4.5)

microns or low-density lipoproteins with respect to this enzyme. It seemed, therefore, that the lipolytic activity of the medium might be due almost exclusively to an enzyme similar to lipoprotein lipase. On the other hand, in the tissue homogenate, beside this enzyme, a different one, similar to a "true" lipase, might be also present. For further characterization of the various lipase activities, some lipase inhibitors were added to the incubation mixtures and their effects were investigated (Table 2). It is seen that lipase activity of the medium was completely inhibited by 1 M-NaCl and by protamine sulphate, known inhibitors of the lipoprotein lipase, but was not affected by KF which inhibits the pancreatic lipase. When samples of tissue homogenate were incubated with activated oil emulsion, a similar inhibition by protamine

Table 2

Inhibition of the lipase activity of the medium and of the tissue homogenate

The values represent the percentage of inhibition. Mean values and range for 3 - 4 experiments are given.

Addition (final concn.)	Lipase in the medium	Lipase in the tissue	
	Activated substrate	Activated substrate	Non-activated substrate
Protamine sulphate (475 μg./ml.)	93 (90 — 98)	92 (88 — 95)	8 ([-16]* — 20)
NaCl (1 M)	100	96 (92 — 100)	44 (34 — 44)
KF (0.2 M)	7 (6 — 10)	8 (7 — 9)	99 (98 — 100)

* This value indicates activation.

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sulphate and by 1 M-NaCl and no effect of KF could be observed. On the contrary, in the tissue homogenate incubated with non-activated substrate, lipolysis was almost completely inhibited by fluoride, but not by protamine sulphate. 1 M-NaCl inhibited this activity to approximately half the initial value.

These results seem to indicate that of the two lipases which may be present in the waxmoth fat body only the enzyme similar to a lipoprotein lipase was released into the medium during incubation of the tissue, while the "true" lipase remained within the tissue.

During centrifugation of the homogenized fat body a thick layer of fat accumulated on the top of the homogenate. This fat was collected and tested for its ability to hydrolyse the activated coconut oil emulsion. Table 3 shows that the enzyme(s) present in the fat brought about a pronounced hydrolysis of the oil emulsion. Since the activated oil emulsion was found to be more strongly hydrolysed than the fat layer itself, there are good reasons to suppose that the lipolysis might be, partly at least, attributed to lipoprotein lipase.

Table 3

Lipolytic activity of the fat layer

The fat layer which accumulated on the top during centrifugation of the homogenized fat body (about 500 mg.) was emulsified in 10 ml. of phosphate buffer as described in Methods and incubated for 1 hr. at 37°, pH 8.5. One ml. of the emulsified fat was incubated with 1 ml. of a mixture containing 7 parts of 6% bovine serum albumin, 0.5 part of 1 M-$(NH_4)_2SO_4$ and 2 parts of 4% activated coconut oil emulsion.

The values represent the contents of FFA in the samples expressed in μg.

Incubation mixture	Before incubation	After 1 hr. incubation	Difference
Whole system	390	650	+260
Activated coconut oil omitted	405	509	+104

It has been frequently shown that the release of lipoprotein lipase from various tissues of higher animals is highly stimulated by heparin added to the incubation mixture [5, 3, 1]. The waxmoth fat body and the rat epididymal fat pads were therefore incubated with or without heparin and the results are shown in Table 4. Heparin strongly stimulated the release of lipoprotein lipase from the rat adipose tissue, but not from the waxmoth fat body; the slight increase in the lipolytic activity observed in the medium containing heparin does not seem to be significant.

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Table 4

Effect of heparin on the release of the lipolytic activity into the medium

The waxmoth fat body or portions of rat epididymal fat pad were incubated for 1 hr. at 37° in phosphate buffer pH 7.4 (100 mg./1 ml.) with and without heparin (30 μg./ml.). One ml. of each medium was afterwards incubated for 1 hr. with a mixture containing in 1 ml.: 0.7 ml. 6% bovine serum albumin, 0.2 ml. 4% activated coconut oil emulsion and 0.05 ml. 1 M-$(NH_4)_2SO_4$. The lipolytic activity is expressed as increase in the content of FFA during incubation, in μmoles/g./hr. The results of 2 experiments are given.

Tissue	Lipolytic activity in the medium after incubation of tissue	
	with heparin (30 μg./ml.)	without heparin
Epididymal rat pad	10.0	6.5
	14.0	6.5
Waxmoth fat body	6.6	6.1
	8.1	6.3

Table 5

Lipolytic activity in the haemolymph of the waxmoth larvae

0.2 ml. of haemolymph was incubated with 0.8 ml. of a mixture containing 7 parts of 6% bovine serum albumin in phosphate buffer, 0.5 part of 1 M-$(NH_4)_2SO_4$ and 2 parts of either activated or non-activated 4% coconut oil emulsion. The amounts of released FFA were calculated per 1 ml. of haemolymph.

Substrate	FFA content	
	before incubation (μmoles/ml.)	after 1 hr. incubation (μmoles/ml.)
Activated coconut oil emulsion	10.0	24.0
Non-activated coconut oil emulsion	10.0	25.5

The observed prompt liberation of lipoprotein lipase from the tissue during incubation as well as the finding that no heparin was needed for this liberation, led us to suppose that this enzyme might be normally present in the haemolymph of the waxmoth larvae. In order to check this supposition, samples of haemolymph were incubated with the activated and non-activated oil emulsion and the increase in FFA concentration after incubation was measured. The results shown in Table 5 strongly suggest that the blood of the waxmoth larvae, unlike that of higher animals, normally contains lipoprotein lipase activity together with the "true" lipase activity.

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DISCUSSION

Our previous studies [11] have suggested the presence of at least two lipases in the waxmoth fat body. In the present experiments it has been shown that a lipolytic enzyme is released into the medium during incubation of the fat body in the buffer solution. On the basis of the action of this enzyme on the oil emulsion activated by blood serum, but not on the plain coconut oil emulsion, and on the basis of the inhibitory effect of protamine sulphate and of 1 M-NaCl, there are good reasons to suppose that the enzyme may be a lipoprotein lipase, similar to that found by several authors in the adipose tissue, heart muscle and some other tissues of the vertebrates.

So far as we know, the presence of lipoprotein lipase in insects has not been reported and it is uncertain whether this enzyme is common to the whole group of these animals or is restricted to *Galleria mellonella*. As the waxmoth larvae consume large amounts of lipid material (wax) with the beecomb which is their normal food, the physiological role of lipoprotein lipase in assimilation and utilization of lipids by this animal seems to be important.

It seems to be well documented that the lipoprotein lipase of the vertebrates participates in the uptake of chylomicron and lipoprotein glycerides from the circulation [10]. Hydrolysis of the triglycerides of the blood lipoproteins yields free fatty acids and perhaps partial glycerides which may enter the cells of the adipose tissue [10]. It has been suggested that the lipoprotein lipase system is located very near the circulating blood, perhaps in the capillary endothelium [9] and this would explain the prompt appearance of lipase activity in the blood following the injection of heparin.

The ability of adipose tissue of vertebrates to release lipoprotein lipase not only into the blood but also into the incubation medium has been frequently reported [3, 5, 1]. The promptness of liberation of this enzyme led to the hypothesis that it is normally held to the cell surface by electrostatic forces and that "the binding of the enzyme to cell surface is an equilibrium that allows slight dissociation" [3]. The same may be true in the case of the lipoprotein lipase of the waxmoth fat body. The enzyme is rapidly eluted into the incubation medium and its activity in the preincubated tissue falls strongly, leaving another type of activity.

This other type of activity found in the homogenates of the waxmoth fat body differs from lipoprotein lipase; it does not require blood serum proteins other than albumin for its activity, as it acts on non-activated oil emulsion; it is not inhibited by protamine sulphate, only partially by 1 M-NaCl, and completely inhibited by KF; it cannot be eluted into

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the medium during incubation *in vitro*. Thus, this enzyme has several properties in common with a "true" lipase like the pancreatic lipase of the vertebrates.

One may suppose, therefore, that the two lipases present in the fat body of the waxmoth larvae differ in their mode of action. The "lipoprotein lipase" may act extracellularly, presumably by splitting the triglycerides bound to the lipoproteins of the haemolymph. On the other hand, the "true" lipase, which is strongly bound within the cell and is liberated only after disruption of the cell structure, may act intracellularly by hydrolysing the glycerides stored in the fat depot of the insect. In this respect, the fat body of the waxmoth larvae shows a striking similarity to the adipose tissue of the vertebrates where the same two types of lipase activity have been shown [1, 6]. The main difference between the activity of the lipoprotein lipase of the waxmoth larvae and the lipoprotein lipase of the rat epididymal fat pads is the fact that the enzyme of *Galleria mellonella* is not activated by heparin, nor is its release from the tissue increased by the presence of heparin in the medium, as is always the case with the adipose tissue of the rat. It was therefore anticipated that the haemolymph of the waxmoth larvae might normally contain some lipoprotein lipase originating probably from the fat body. This in fact proved to be the case. One may suppose that the enzyme present in the haemolymph may act in transporting the non-esterified fatty acids on the lipoproteins and in binding them into the glycerides. It should be mentioned that Borgström & Carlson [2] found that lipoprotein lipase present in human postheparin plasma is involved in esterification of the free fatty acids to the chylomicron glycerides. As the haemolymph of the waxmoth larvae is very rich in lipids [12], contains a rather high proportion of FFA [11] and contains lipoprotein lipase, the possibility of the transfer of the FFA on the lipoproteins is very likely to exist. This problem is now under study.

Another feature common to the lipoprotein lipase of the waxmoth larvae and to that of higher vertebrates also seems to be of interest. It has been shown by Korn & Quigley [8] that this enzyme when incubated with a triglyceride emulsion is adsorbed onto the oil emulsion. According to the authors, this finding may be explained by assumption that the reaction catalysed by lipoprotein lipase may proceed through an enzyme-substrate complex. In the present experiments, the fat layer which accumulated on the top during centrifugation of the homogenized fat body contained pronounced lipase activity. This may also be due to the adsorption of the enzyme onto the fat layer which may result in formation of an enzyme-substrate complex, similar to that described by Korn & Quigley.

The results of our experiments seem to indicate, therefore, that despite of the pronounced differences between the insect fat body and

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the adipose tissue of the vertebrates some of the enzyme systems involved in lipid metabolism in these tissues have several characteristics in common.

SUMMARY

The fat body of the waxmoth larvae was incubated in Krebs-Ringer phosphate buffer solution, with or without heparin, and the lipase activity present both in the incubation medium and in the tissue after homogenization was measured and characterized.

The lipase found in the incubation medium required serum proteins for its activity and was inhibited by protamine sulphate and by 1 M-NaCl, but not by KF; it was therefore considered to be a lipoprotein lipase.

This enzyme was present also in the preincubated tissue homogenate. In addition, another lipase differing from the former was found in the homogenate. It acted on non-activated oil emulsion and was completely inhibited by KF; it seemed therefore to be a "true" lipase.

The lipoprotein lipase of the waxmoth larvae was insensitive to heparin and it was found to occur normally in the haemolymph. The possible role of the two lipases in lipid metabolism of the waxmoth larvae is discussed.

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AKTYWNOŚĆ LIPOLITYCZNA CIAŁA TŁUSZCZOWEGO GĄSIENIC MOLA WOSKOWEGO

II. WŁASNOŚCI DWÓCH RÓŻNYCH LIPAZ CIAŁA TŁUSZCZOWEGO

Streszczenie

Podczas inkubacji ciała tłuszczowego w buforze fosforanowym do środowiska przechodzi enzym, mający właściwości lipazy lipoproteidowej zwierząt wyższych. Enzym ten wymaga do swego działania białek surowiczych i jest hamowany przez

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protaminę oraz 1 M-NaCl, ale nie jest hamowany przez fluorki. W homogenacie uprzednio inkubowanej tkanki obecny jest ponadto drugi enzym o cechach lipazy właściwej. Hydrolizuje on emulsję tłuszczową w nieobecności białek surowiczych i jest całkowicie hamowany przez fluorki. Enzym ten, normalnie silnie związany w komórce i uwalniający się dopiero po rozbiciu struktury, działa prawdopodobnie wewnątrzkomórkowo, podczas gdy słabo związana lipaza lipoproteidowa może działać pozakomórkowo. Lipaza lipoproteidowa mola woskowego jest niewrażliwa na heparynę i jest normalnie obecna w hemolimfie.

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CITRATE AND ELECTROLYTE EXCRETION IN RATS TREATED WITH 17β-OESTRADIOL

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The results of experiments performed so far indicate that citrate excretion is controlled by sexual hormones. Shorr *et al.* [11] have shown that in women citrate excretion increased during the menstrual flux when the oestrogen level in the organism was higher. Similarly we have observed an increased citrate excretion before parturition i.e. at the time when the oestrogen level is elevated [16]. Also the women who had been treated with therapeutical doses of oestradiol benzoate excreted more citrate in the urine [9]. Among the intact rats subjected to prolonged acidosis, females excreted more citrate than males [17]. However, after administration of large doses of oestradiol benzoate to female rats, a decrease of citrate excretion was observed [15].

As in previous experiments [14] it has been shown that chloride content decreased in the urine at the period of decreased citrate excretion, it seemed reasonable to undertake studies on mineral metabolism in rats treated with oestrogens. Besides, the participation of reproductive and adrenal glands in the process of citrate and minerals excretion was investigated.

METHODS

Urine collection and the estimation of citrate, α-ketoacids, chlorides and total nitrogen in the urine were carried out as described previously [14]. Plasma chlorides were estimated by the method of Van Slyke [4], plasma and urine sodium and potassium with flame photometer Zeiss III, and urine ammonia as described by Connerty *et al.* [2].

The experiments were performed on albino rats kept on a standard diet [17].

Three-month-old rats were submitted to removal of the testes together with epididymides or to ovariectomy; they were used for experiments 2 months later. In female rats aged 4 months, bilateral adrenalectomy was performed by removing adrenal glands together with capsules. For

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six weeks after this treatment, i.e. until the experiment started, and all the time throughout the experiment, the animals were given 1.5% NaCl solution instead of drinking water. The effectiveness of the adrenalectomy was checked both by autopsy after the completion of the experiment and by giving to a group of 6 adrenalectomized rats not NaCl solution but water to drink. No animals of this control group survived longer than 15 - 19 days.

The intact animals used for experiments were 4 - 6 months old.

The steroids for the injections were dissolved in arachis oil and administered intramuscularly every other day in a dose of 26.6 μmoles per kg. body weight.

Statistical evaluation of the results was carried out by comparing the values obtained after the hormone treatment with those obtained before the treatment in the same animals; the coupled Student's t test was used for P calculation as described previously [15].

Reagents: 17β-oestradiol, oestron and progesterone were a gift from Hoechst A. G., Frankfurt am Main. Arachis oil was from Warszawskie Zakłady Farmaceutyczne. 2,4-Dinitrophenylhydrazine was L. Light & Co. (Colnbrook, England) product. Other reagents were purchased from Fabryka Odczynników Chemicznych, Gliwice.

RESULTS

In the first series of experiments the influence of 17β-oestradiol, oestron, stilbestrol, progesterone and cholesterol on citrate and α-ketoacids excretion has been studied, each compound tested being administered to 5 litter-mate female rats. The results presented in Table 1 indicate that 17β-oestradiol, oestron and stilbestrol caused a decrease of both citrate and α-ketoacids excretion whereas the administration of progesterone and cholesterol was without effect.

The changes of cation excretion under the influence of 17β-oestradiol and oestron were followed on two groups of litter-mate female rats of 5 animals each. Five litter-mate rats receiving injections of arachis oil only, served as control. The excretion of sodium and potassium decreased after the rats had been treated with 17β-oestradiol or oestron. However, in relation to total urine nitrogen, only the excretion of sodium decreased while the ratio of potassium to total N was unchanged (Table 2). Since after oestradiol treatment the nitrogen excretion decreased, a possibility existed that the changes observed were caused by the decreased food intake. Therefore in all further experiments the values of the excreted compounds were expressed in relation to that of excreted nitrogen.

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Table 1

The excretion of citrate and α-ketoacids in the urine of female rats after treatment with steroids

Intramuscular injections of 26.6 μmoles of steroids per kg. body wt. were given on the days marked with an asterisk. The excretion of citrate and α-ketoacids is presented as m-moles per kg. body wt. per day. The daily amount of urine is expressed as ml. per kg. body wt. Mean values obtained from 5 animals, ± S.E., are given, with *P* values in parentheses.

Treatment	Excretion	Day of experiment										
		1	2	3*	4	5*	6	7	8	9	10	11
17β-Oestradiol	Citric acid	0.27±0.02	0.31±0.05	0.23±0.05	0.17±0.03 (0.02)	0.21±0.05 (0.2)	0.06±0.01 (0.01)	0.07±0.01 (0.05)	0.18±0.05 (0.05)	0.32±0.08 (0.4)		
	α-Ketoacids	0.20±0.02	0.20±0.04	0.17±0.02	0.11±0.01 (0.02)	0.16±0.04 (0.4)	0.08±0.01 (0.01)	0.06±0.01 (0.001)	0.12±0.03 (0.05)	0.24±0.07 (0.4)		
	Urine	61±8	67±12	51±5	93±18 (0.3)	50±7 (0.2)	55±8 (0.8)	55±5 (0.7)	53±6 (0.4)	62±10 (0.8)		
Oestron	Citric acid	0.46±0.05	0.32±0.03	0.34±0.02	0.19±0.02 (0.001)	0.27±0.05 (0.05)	0.10±0.01 (0.001)	0.15±0.02 (0.001)	0.22±0.02 (0.02)	0.29±0.03 (0.1)		
	α-Ketoacids	0.29±0.03	0.21±0.02	0.21±0.01	0.14±0.01 (0.001)	0.20±0.04 (0.3)	0.09±0.01 (0.001)	0.12±0.04 (0.001)	0.16±0.02 (0.3)	0.18±0.05 (0.5)		
	Urine	86±3	84±4	86±10	97±14 (0.6)	93±11 (0.4)	85±10 (0.6)	96±10 (0.3)	78±3 (0.2)	92±4 (0.2)		

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Treatment	Excretion	Day of experiment										
		1	2	3*	4	5*	6	7	8	9	10	11
Stilbestrol	Citric acid	0.29±0.02	0.22±0.02	0.21±0.05	0.14±0.02	0.13±0.02	0.08±0.01	0.09±0.02	0.10±0.01	0.07±0.01	0.13±0.03	0.17±0.04
					(0.001)	(0.001)	(0.001)	(0.001)	(0.01)	(0.01)	(0.1)	(0.3)
	α-Ketoacids	0.20±0.01	0.16±0.01	0.16±0.03	0.10±0.01	0.10±0.02	0.10±0.02	0.11±0.02	0.10±0.01	0.07±0.00	0.10±0.01	0.12±0.02
					(0.001)	(0.001)	(0.01)	(0.05)	(0.01)	(0.01)	(0.02)	(0.2)
	Urine	91±10	88±8	78±8	62±4	63±10	70±13	76±16	87±14	73±13	87±11	79±10
					(0.02)	(0.01)	(0.3)	(0.5)	(0.8)	(0.5)	(0.2)	(0.2)
Progesteron	Citric acid	0.28±0.04	0.31±0.07	0.30±0.04	0.21±0.03	0.27±0.03	0.24±0.03	0.27±0.04	0.29±0.03	0.25±0.04	0.25±0.04	
					(0.1)	(0.5)	(0.4)	(0.3)	(0.9)	(0.5)	(0.1)	
	α-Ketoacids	0.25±0.04	0.24±0.03	0.18±0.02	0.16±0.02	0.15±0.02	0.11±0.01	0.12±0.01	0.14±0.02	0.14±0.02	0.10±0.01	
					(0.001)	(0.01)	(0.01)	(0.01)	(0.001)	(0.01)	(0.01)	
	Urine	73±3	57±11	71±12	62±7	62±2	60±8	55±9	62±5	55±2	60±9	
					(0.2)	(0.7)	(0.6)	(0.3)	(0.4)	(0.2)	(0.1)	
Cholesterol	Citric acid	0.21±0.04	0.20±0.03	0.17±0.02	0.18±0.01	0.20±0.02	0.19±0.01	0.25±0.03	0.22±0.04	0.19±0.04		
					(0.6)	(0.4)	(0.9)	(0.9)	(0.9)	(0.7)		
	α-Ketoacids	0.14±0.02	0.12±0.02	0.10±0.01	0.12±0.01	0.16±0.02	0.13±0.01	0.16±0.03	0.13±0.02	0.14±0.02		
					(0.7)	(0.1)	(0.9)	(0.1)	(0.8)	(0.2)		
	Urine	53±8	70±13	63±7	68±12	59±10	63±10	77±9	75±15	71±12		
					(0.4)	(0.7)	(0.8)	(0.05)	(0.5)	(0.2)		

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The rats used in the experiments presented in Table 2 were killed on the fourth day after the first injection, i.e. at the time of the maximum decrease of urine citrate excretion. The blood was withdrawn, and no changes in plasma sodium, potassium and chloride and blood citrate and α-ketoacids were observed in the treated animals (Table 3).

Table 2

The influence of 17β-oestradiol and oestron on the excretion of cations in the urine of female rats

Intramuscular injections of 26.6 μmoles 17β-oestradiol or oestron per kg. body wt. were given on the days marked with an asterisk. The values represent m-moles per kg. body wt. per day. The daily amount of urine is expressed as ml. per kg. body wt. Mean values obtained from 5 animals, ± S.E., are given, with *P* values in parentheses.

Treatment	Excretion	Day of experiment				
		1	2*	3	4*	5
Arachis oil (control)	Sodium	13.6±0.9	15.2±0.2	12.3±1.8 (0.5)	11.5±1.3 (0.2)	12.6±1.6 (0.9)
	Potassium	6.4±0.3	7.1±0.3	6.3±0.6 (0.4)	5.6±0.6 (0.2)	5.8±0.7 (0.2)
	Ammonia	3.2±0.3	3.5±0.3	3.7±0.2 (0.8)	2.8±0.3 (0.1)	3.8±0.4 (0.8)
	Total N	114±10	135±6	107±2 (0.1)	100±1 (0.05)	105±12 (0.3)
	Urine	78±5	75±5	67±14 (0.2)	59±9 (0.1)	77±13 (0.5)
17β-Oestradiol	Sodium	15.6±1.0	13.9±1.0	7.8±1.9 (0.01)	7.9±0.7 (0.001)	4.9±0.7 (0.001)
	Potassium	6.9±0.6	7.2±0.6	5.6±0.6 (0.05)	4.1±0.3 (0.01)	3.6±0.3 (0.01)
	Ammonia	3.5±0.1	3.5±0.2	3.1±0.3 (0.3)	2.5±0.3 (0.1)	2.5±0.2 (0.02)
	Total N	131±9	133±12	97±19 (0.1)	79±7 (0.01)	77±11 (0.01)
	Urine	80±15	73±12	79±17 (0.8)	53±9 (0.02)	61±23 (0.1)
Oestron	Sodium	11.4±1.0	12±1.4	6.9±1.0 (0.001)	8.2±1.9 (0.2)	6.5±1.0 (0.01)
	Potassium	5.2±0.5	5.5±0.5	4.5±0.5 (0.02)	4.5±0.6 (0.3)	3.3±0.6 (0.01)
	Ammonia	2.8±0.2	2.6±0.1	2.7±0.3 (0.9)	2.9±0.2 (0.2)	2.8±0.4 (0.7)
	Total N	96±7	111±4	90±5 (0.9)	82±11 (0.2)	73±4 (0.7)
	Urine	51±7	61±4	71±17 (0.4)	56±11 (0.9)	53±12 (0.8)

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To exclude the possibility that the changes in excretion occurring after 17β-oestradiol injection are a secondary effect caused by the changes in hormonal activity of ovaries, ovariectomized rats were submitted to the same treatment. Also in these animals a pronounced decrease of citrate and a less pronounced decrease of α-ketoacids excretion occurred after 17β-oestradiol administration. These changes were accompanied by a decrease of sodium chloride and an increase of ammonia excretion. Nevertheless, potassium excretion did not change (Table 4).

Table 3

Concentration of electrolytes and citrate and α-ketoacids in the blood of female rats treated with 17β-oestradiol or oestron

The animals were treated as described in Table 2. The concentration of all compounds is expressed as m-moles/litre. Mean values obtained from 5 animals, ± S.D., are given.

Excretion	Treatment		
	Arachis oil (control)	17β-oestradiol	Oestron
Blood plasma			
Sodium	140±6.5	140±6.5	143±5.5
Potassium	4.7±0.6	4.9±0.9	5.0±0.4
Chloride	103±3.4	105±5.6	104±3.4
Whole blood			
Citric acid	0.125±0.026	0.120±0.032	0.145±0.031
α-Ketoacids	0.374±0.032	0.350±0.053	0.350±0.03

Further experiments were carried out on castrated and normal male rats. Also in normal animals (two litter-mate groups, numbering 10 males) a decrease of citrate, sodium and chloride excretion could be observed on the third day after the first oestradiol injection. However, the excretion of α-ketoacids, potassium and ammonia did not change (Table 5). Seven castrated litter-mate rats also showed after oestrogen administration a decrease of citrate excretion, which was accompanied by a decrease of sodium and chloride excretion (Table 6). The excretion of α-ketoacids, potassium and ammonia was not changed.

The possibility that the adrenal glands are the factor responsible for the decrease of citrate and sodium chloride excretion after oestradiol treatment was studied by using adrenalectomized female rats (Table 7). No decrease was observed in the ratio of the excreted citrate, α-ketoacids and sodium chloride to total urine nitrogen. Nevertheless, the amount of total nitrogen excreted and urine volume were diminished significantly on the third day after the first oestradiol injection.

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Table 4

The influence of 17β-oestradiol on citrate, α-ketoacids, sodium, potassium, chloride, ammonia and total nitrogen excretion in the urine of ovariectomized rats

Intramuscular injections of 26.6 μmoles of 17β-oestradiol per kg. body wt. were given on the days marked with an asterisk. The nitrogen excreted is expressed as m-moles per kg. body wt. per day. Other values represent m-moles of excreted compound per g. of total N excreted. The daily amount of urine is expressed as ml. per kg. body wt. Mean values obtained from 5 animals, ± ± S.E., are given, with *P* values in parentheses.

Excretion	Day of experiment							
	1	2*	3	4*	5	6	7	8
Citric acid	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.02 (0.5)	0.06 ± 0.01 (0.01)	0.06 ± 0.01 (0.05)	0.05 ± 0.01 (0.001)	0.08 ± 0.01 (0.05)	0.11 ± 0.00 (0.6)
α-Ketoacids	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.00 (0.4)	0.07 ± 0.01 (0.01)	0.07 ± 0.01 (0.05)	0.08 ± 0.00 (0.2)	0.11 ± 0.01 (0.2)	0.13 ± 0.01 (0.05)
Sodium	8.2 ± 0.4	7.2 ± 0.5	7.2 ± 0.6 (0.5)	6.6 ± 0.5 (0.1)	5.2 ± 0.4 (0.01)	6.6 ± 0.4 (0.05)	7.5 ± 0.5 (0.8)	7.1 ± 0.3 (0.09)
Potassium	3.8 ± 0.2	4.3 ± 0.2	4.5 ± 0.4 (0.6)	4.2 ± 0.4 (0.6)	3.7 ± 0.2 (0.5)	4.1 ± 0.2 (0.3)	3.8 ± 0.0 (0.7)	3.9 ± 0.0 (0.9)
Chloride	8.2 ± 0.4	8.1 ± 0.4	5.9 ± 0.6 (0.05)	6.8 ± 0.5 (0.02)	4.5 ± 0.7 (0.01)	6.6 ± 0.5 (0.05)	8.0 ± 0.4 (0.9)	7.3 ± 0.3 (0.5)
Ammonia	1.5 ± 0.1	1.7 ± 0.1	1.4 ± 0.1 (0.4)	2.5 ± 0.2 (0.05)	2.2 ± 0.2 (0.05)	2.1 ± 0.2 (0.05)	1.9 ± 0.2 (0.2)	1.5 ± 0.1 (0.6)
Total N	146 ± 7	140 ± 7	108 ± 7 (0.02)	107 ± 5 (0.02)	79 ± 4 (0.01)	75 ± 5 (0.01)	104 ± 8 (0.05)	124 ± 6 (0.2)
Urine	67 ± 6	67 ± 5	61 ± 7 (0.6)	51 ± 5 (0.1)	48 ± 9 (0.1)	45 ± 6 (0.2)	55 ± 6 (0.1)	61 ± 6 (0.3)

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Table 5

The influence of 17β-oestradiol on citrate, α-ketoacids, sodium, potassium, chloride, ammonia and total nitrogen excretion in the urine of male rats

Intramuscular injections of 26.6 μmoles of 17β-oestradiol per kg. body wt. were given on the days marked with an asterisk. The nitrogen excreted is expressed as m-moles per kg. body wt. per day. Other values represent m-moles of excreted compound per g. of total N excreted. The daily amount of urine is expressed as ml. per kg. body wt. Mean values obtained from 10 animals, ± S.E., are given, with *P* values in parentheses.

Excretion	Day of experiment							
	1	2	3*	4	5*	6	7	8
Citric acid	0.048 ± 0.006	0.048 ± 0.006	0.039 ± 0.005	0.047 ± 0.006 (0.7)	0.047 ± 0.006 (0.6)	0.027 ± 0.002 (0.01)	0.040 ± 0.004 (0.4)	0.067 ± 0.007 (0.01)
α-Ketoacids	0.135 ± 0.010	0.142 ± 0.011	0.115 ± 0.002	0.130 ± 0.003 (0.6)	0.198 ± 0.032 (0.01)	0.152 ± 0.015 (0.01)	0.180 ± 0.018 (0.02)	0.251 ± 0.061 (0.001)
Sodium	9.2 ± 0.5	9.4 ± 0.4	8.9 ± 0.3	8.8 ± 0.3 (0.9)	9.4 ± 0.4 (0.1)	6.5 ± 0.4 (0.001)	7.5 ± 0.3 (0.02)	9.4 ± 0.6 (0.6)
Potassium	3.8 ± 0.5	3.8 ± 0.1	3.6 ± 0.2	3.8 ± 0.1 (0.7)	4.0 ± 0.3 (0.9)	3.7 ± 0.2 (0.7)	3.3 ± 0.3 (0.2)	3.8 ± 0.5 (0.7)
Chloride	9.6 ± 0.5	9.3 ± 0.3	9.1 ± 0.4	9.0 ± 0.4 (0.4)	9.4 ± 0.3 (0.7)	6.9 ± 0.4 (0.01)	6.8 ± 0.4 (0.05)	9.1 ± 0.1 (0.2)
Ammonia	2.2 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.1 ± 0.1 (0.8)	2.0 ± 0.1 (0.9)	2.2 ± 0.1 (0.4)	2.1 ± 0.2 (0.8)	2.2 ± 0.1 (0.4)
Total N	99 ± 5	116 ± 10	120 ± 13	117 ± 9 (0.2)	121 ± 11 (0.5)	121 ± 12 (0.4)	128 ± 17 (0.3)	117 ± 14 (0.6)
Urine	34 ± 3	40 ± 4	45 ± 4	40 ± 3 (0.7)	41 ± 4 (0.6)	40 ± 7 (0.8)	44 ± 8 (0.4)	46 ± 8 (0.4)

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Table 6

The influence of 17β-oestradiol on citrate, α-ketoacids, sodium, potassium, chloride, ammonia and total nitrogen excretion in the urine of castrated male rats

Intramuscular injections of 26.6 μmoles of 17β-oestradiol per kg. body wt. were given on the days marked with an asterisk. The nitrogen excreted is expressed as m-moles per kg. body wt. per day. Other values represent m-moles of excreted compound per g. of total N excreted. The daily amount of urine is expressed as ml. per kg. body wt. Mean values obtained from 7 animals, ± S.E., are given, with *P* values in parentheses.

Excretion	Day of experiment							
	1	2	3*	4	5*	6	7	8
Citric acid	0.029 ± 0.008	0.030 ± 0.005	0.030 ± 0.004	0.052 ± 0.007 (0.1)	0.026 ± 0.001 (0.5)	0.016 ± 0.004 (0.05)	0.022 ± 0.004 (0.2)	0.026 ± 0.004 (0.3)
α-Ketoacids	0.118 ± 0.005	0.163 ± 0.033	0.132 ± 0.023	0.220 ± 0.070 (0.05)	0.167 ± 0.006 (0.1)	0.146 ± 0.008 (0.7)	0.137 ± 0.012 (0.7)	0.154 ± 0.010 (0.3)
Sodium	6.4 ± 0.2	7.3 ± 0.2	6.5 ± 0.3	5.8 ± 0.3 (0.05)	6.8 ± 0.4 (0.5)	5.3 ± 0.3 (0.01)	6.3 ± 0.4 (0.3)	6.8 ± 0.2 (0.8)
Potassium	4.0 ± 0.5	4.1 ± 0.4	3.8 ± 0.1	4.3 ± 0.2 (0.3)	3.8 ± 0.1 (0.1)	3.5 ± 0.3 (0.1)	3.8 ± 0.4 (0.5)	4.0 ± 0.8 (0.8)
Chloride	8.3 ± 0.4	7.4 ± 0.3	7.0 ± 0.1	4.9 ± 0.5 (0.02)	6.5 ± 0.3 (0.7)	6.7 ± 0.4 (0.05)	6.9 ± 0.3 (0.1)	7.0 ± 0.2 (0.2)
Ammonia	2.5 ± 0.2	2.0 ± 0.1	2.2 ± 0.1	1.9 ± 0.1 (0.1)	2.6 ± 0.2 (0.3)	2.6 ± 0.1 (0.2)	2.3 ± 0.2 (0.9)	2.5 ± 0.1 (0.3)
Total N	118 ± 4	126 ± 6	118 ± 4	90 ± 6 (0.01)	100 ± 7 (0.1)	99 ± 7 (0.05)	109 ± 7 (0.3)	123 ± 6 (0.7)
Urine	53 ± 6	53 ± 2	55 ± 5	39 ± 4 (0.05)	42 ± 4 (0.3)	41 ± 5 (0.05)	42 ± 4 (0.2)	51 ± 7 (0.5)

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Table 7

The influence of 17β-oestradiol on citrate, α-ketoacids, sodium, potassium, chloride, ammonia and total nitrogen excretion in the urine of adrenalectomized female rats

Intramuscular injections of 26.6 μmoles of 17β-oestradiol per kg. body wt. were given on the days marked with an asterisk. The nitrogen excreted is expressed as m-moles per kg. body wt. per day. Other values represent m-moles of excreted compound per g. of total N excreted. The daily amount of urine is expressed as ml. per kg. body wt. Mean values obtained from 8 animals, ± S.E., are given, with *P* values in parentheses.

Excretion	Day of experiment								
	1	2	3*	4	5*	6	7	8	9
Citric acid	0.14 ± 0.02	0.13 ± 0.01	0.15 ± 0.02	0.14 ± 0.02 (0.8)	0.15 ± 0.02 (0.3)	0.16 ± 0.02 (0.3)	0.13 ± 0.02 (0.5)	0.14 ± 0.02 (0.8)	0.12 ± 0.02 (0.4)
α-Ketoacids	0.071 ± 0.006	0.081 ± 0.006	0.078 ± 0.006	0.080 ± 0.006 (0.9)	0.091 ± 0.006 (0.001)	0.097 ± 0.011 (0.02)	0.088 ± 0.005 (0.001)	0.099 ± 0.010 (0.02)	0.078 ± 0.010 (0.5)
Sodium	36 ± 3.3	36 ± 3.8	35 ± 4.0	33 ± 4.0 (0.2)	36 ± 3.2 (0.8)	29 ± 3.3 (0.2)	36 ± 3.7 (0.9)	38 ± 7.7 (0.3)	31 ± 1.9 (0.3)
Potassium	4.2 ± 0.1	4.1 ± 0.2	4.4 ± 0.2	4.1 ± 0.1 (0.4)	3.6 ± 0.1 (0.1)	4.1 ± 0.4 (0.2)	3.8 ± 0.2 (0.1)	4.0 ± 0.4 (0.2)	2.8 ± 0.5 (0.05)
Chloride	37 ± 3.1	37 ± 4.1	32 ± 3.3	31 ± 4.0 (0.2)	33 ± 2.9 (0.6)	29 ± 3.1 (0.2)	37 ± 3.7 (0.8)	38 ± 4.4 (0.5)	33 ± 2.2 (0.5)
Ammonia	3.1 ± 0.2	2.5 ± 0.2	2.7 ± 0.2	2.8 ± 0.2 (0.9)	2.7 ± 0.2 (0.6)	3.4 ± 0.3 (0.2)	2.8 ± 0.2 (0.9)	4.1 ± 0.6 (0.4)	2.2 ± 0.1 (0.02)
Total N	120 ± 4	141 ± 6	145 ± 6	122 ± 7 (0.1)	126 ± 6 (0.2)	95 ± 10 (0.01)	115 ± 12 (0.1)	152 ± 9 (0.05)	197 ± 19 (0.02)
Urine	185 ± 25	195 ± 25	185 ± 27	154 ± 28 (0.1)	185 ± 23 (0.8)	123 ± 19 (0.01)	197 ± 31 (0.6)	270 ± 36 (0.05)	270 ± 33 (0.05)

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DISCUSSION

The results obtained indicate that the decreased citrate excretion occurring as a result of oestrogen injection, is connected with an increased sodium and chloride reabsorption. The experiments with ovariectomized females and with both normal and castrated male rats have shown that the effect of 17β-oestradiol is not dependent on the presence of ovaries. The experiments performed on adrenalectomized female animals indicate that the adrenal glands participate in the process leading to the decreased citrate excretion after the administration of oestrogen. The data concerning the effect of oestrogen on the excretion of adrenocorticoids are numerous but controversial [10, 7, 12, 5]; this is due probably to the application of different compounds and to differences in the methods used. After oestrogen treatment, Zondek & Burnstein [13] observed an increase in corticoids excretion and in our previous [15] and present experiments a decrease of citrate excretion was found. Therefore it seems permissible to assume that a parallelism exists between the increased excretion of corticoids and the decreased urine citrate excretion. Mills *et al.* [8] observed an increased 17-hydroxycorticosteroids level in the serum of castrated women after they had been treated with oestrogens, while no increase was found in adrenalectomized subjects or in persons with adrenal insufficiency.

From the experiments dealing with the effect of corticosteroids on the citrate serum level and on the excretion of this compound in the urine it is evident that the increased amount of corticosteroids does influence the excretion of citrate.

In human subjects with Addison disease an increased serum citrate level and increased urine citrate excretion were observed [1, 6]. The assumption that corticosteroids control in some way the excretion and/or metabolism of citrate, is supported by the experiments carried out by Harrison & Harrison [3] on rats. The animals treated with cortisol showed a decrease of serum citrate level and also decreased urine citrate excretion whereas the concentration of citrate in the kidney tissue did not change.

SUMMARY

1. A decrease of urine citrate, sodium and chloride excretion was observed in normal and ovariectomized female rats, as well as in normal and castrated males after the animals had been treated with oestrogens.

2. No decrease of citrate excretion after 17β-oestradiol administration occurred in adrenalectomized female rats.

3. At the time when the decrease of citrate excretion caused by oestrogens reached its maximum, no changes of the concentration of citrate and α-ketoacids in whole blood, and of sodium, potassium and chloride in plasma were observed.

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WYDALANIE KWASU CYTRYNOWEGO I ELEKTROLITÓW U SZCZURÓW PO PODANIU ESTROGENÓW

Streszczenie

1. Po podaniu estrogenów szczurom normalnym i kastrowanym obu płci stwierdzono spadek wydalania kwasu cytrynowego oraz sodu i chlorków.

2. U szczurów płci żeńskiej po usunięciu nadnerczy nie obserwowano zmniejszonego wydalania cytrynianu po podaniu 17β-estradiolu.

3. W dniu maksymalnego spadku wydalania cytrynianu stężenie sodu, potasu i chlorków w osoczu oraz cytrynianu i α-ketokwasów w pełnej krwi pozostało nie zmienione.

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STUDIES ON SULPHYDRYL GROUPS OF TROPOMYOSIN

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Contrary to the numerous results concerning the number and reactivity of sulphydryl groups in myosin and actin, few data exist on the SH groups in tropomyosin. Thus Kominz *et al.* [10] and Szent-Györgyi *et al.* [12] have found that tropomyosin molecule contains two sulphydryl groups and one disulphide bridge. As concerns the role of the SH groups, Chang & Tsao [4] have suggested that SH groups take part in polymerization of tropomyosin.

In the present paper the SH groups in tropomyosin have been investigated by blocking them with β-hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD). During the reaction of SH groups with this compound stoichiometrical amounts of dinitrothiophenol are liberated which can be easily quantitatively determined. This method was previously adapted in this Laboratory to investigate the number and the role of SH groups in actin [5] and myosin [8].

MATERIAL AND METHODS

Tropomyosin was prepared from rabbit muscles according to Bailey [2]. The whole purification cycle was performed twice but in some experiments the determination of SH groups was also performed on once purified tropomyosin, dialysed after purification against water. After the second purification cycle tropomyosin was precipitated with alcohol in the presence of 0.1 M-KCl, dried-down in alcohol and ether, and stored in this form at 2°. Before use it was dissolved in water and clarified by centrifugation for 20 min. at 28 000 g. Preliminary experiments showed, in agreement with the observation of Kominz *et al.* [10], that when commercial $(NH_4)_2SO_4$, usually containing traces of heavy metals, was used for fractionation of tropomyosin the subsequently obtained preparations contained but minute amounts of SH groups. Therefore $(NH_4)_2SO_4$ was purified by passing through a column of Dowex 50, ammonium cycle [10].

The reaction of HEDD with SH groups of tropomyosin took place in conditions previously described for actin [5]. The solutions of tropomyosin

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were incubated with HEDD and the dinitrothiophenol liberated was measured in a Unicam Spectrophotometer S.P. 600 at 408 mμ. Sometimes the solutions became a little cloudy; in these cases the extinction at 600 mμ was also measured and this value was subtracted from that found at 408 mμ.

Protein was determined with a biuret reagent [6]. Viscosity measurements were carried out in Ostwald viscometers with outflow time of approximately 50 sec. at 21°.

RESULTS

The determination of SH groups content in tropomyosin was performed in various conditions: in the absence and in the presence of 0.6 M-KCl; and in the presence of 6 M-urea. The time-course of the reaction of SH groups of tropomyosin with HEDD is presented in Fig. 1. In the presence of urea, when all free SH groups were expected to react with HEDD, the reaction was very fast and it was practically finished within 10 min. On the other hand, in the absence of urea not all free SH groups

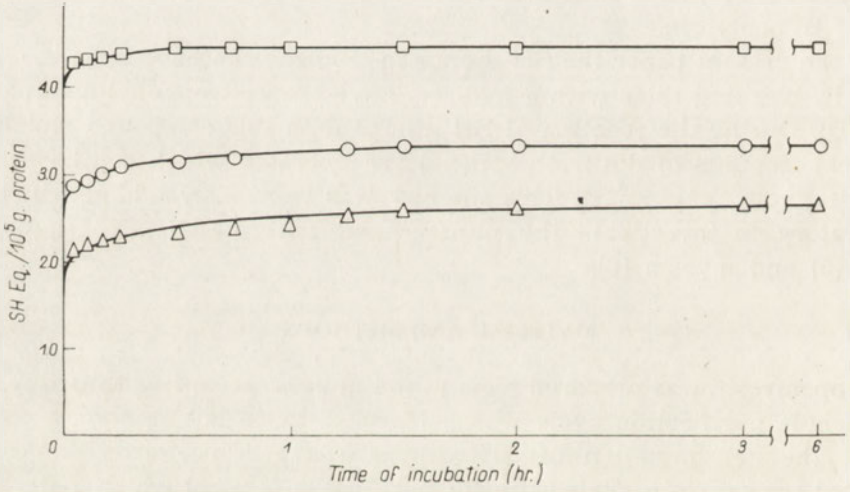

Fig. 1. The time course of the reaction of HEDD with SH groups of tropomyosin. To 2 ml. samples of twice purified tropomyosin dissolved: (△), in water, (○), in 0.6 M-KCl, (□), in 6 M-urea, 4 ml. of 5×10^{-4} M-HEDD in 2 mM-tris buffer, pH 7.0, was added at zero time. Measurements of extinction at 408 mμ were taken at room temperature at the times indicated in the Figure. Final concentration of tropomyosin: 0.36 mg./ml.

were accessible to HEDD. In this case the number of the reacting SH groups depended on the presence of KCl. Thus, in 0.6 M-KCl the final number of SH groups reacting with HEDD was greater than that found without addition of salt, although the rate of the reaction seemed not to depend substantially on the presence of KCl. By far the greater part

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of the accessible SH groups, both in water and in 0.6 M-KCl, reacted very fast with HEDD; afterwards the reaction proceeded slowly so that the endpoint was reached only after about 2 hr. The comparison of several tropomyosin preparations showed that the rates of the reaction of the accessible SH groups seemed not to depend on the fact whether tropomyosin was purified once or twice, or stored in dried form.

On the other hand, several factors decreased the number of reacting SH groups. One of them was the time of storage of alcohol-ether muscle powder itself. Table 1 shows that when tropomyosin was extracted from an alcohol-ether dried muscle powder stored for about a year the values of SH groups found both in the presence and in the absence of urea were markedly decreased. The decrease in SH groups content was similarly observed between preparations purified once and twice. Moreover, the number of SH groups available to HEDD have also continuously decreased when tropomyosin was stored in dehydrated form (Fig. 2).

In all cases, however, independently of the number of SH groups found in a given tropomyosin preparation, the same characteristic feature

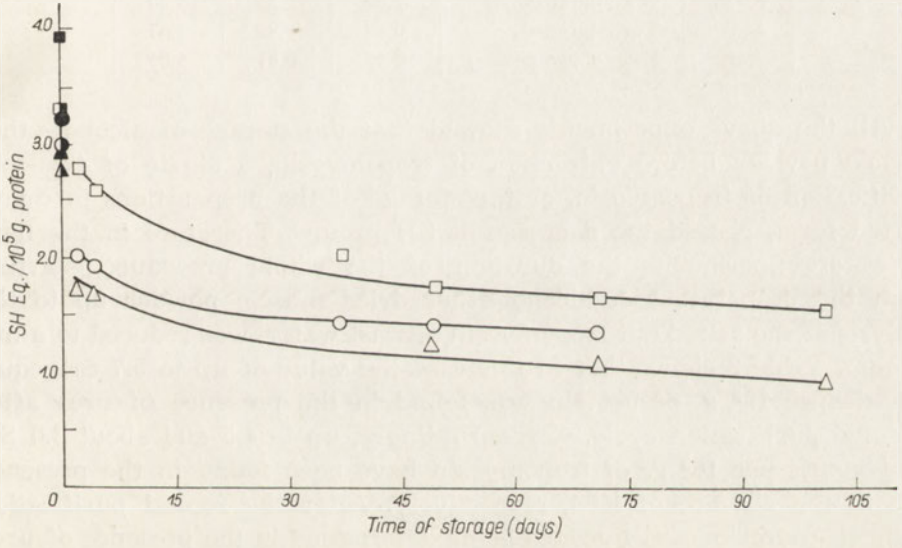

Fig. 2. The influence of the storage of dehydrated tropomyosin on the content of SH groups. Outlined symbols correspond to the tropomyosin preparation which after the second purification cycle was dried-down and stored at 2°. On the days indicated in the Figure samples of this preparation were dissolved and the SH content was measured. The determinations of SH groups were performed as described in the legend to Fig. 1. Final concentration of tropomyosin 0.2 - 0.5 mg./ml. Solvent: (△), water; (○), 0.6 M-KCl; (□); 6 M-urea. For comparison the contents of SH groups in the same tropomyosin preparation after the first purification cycle (filled-in symbols) and after the second purification cycle (half-filled-in symbols) are also presented.

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was observed. The lowest number of SH groups reacted with HEDD in the absence of salt; the number was always higher in 0.6 M-KCl and was the highest in urea (Fig. 1, Table 1).

Table 1

The amount of SH groups in various preparations of tropomyosin

The determination of SH groups was performed as described in the legend to Fig. 1. Final concentration of tropomyosin 0.20 - 0.50 mg./ml. Number of SH equivalents expressed per 10^5 g. protein, is given.

Time of storage of alcohol-ether muscle powder	Preparation	Solvent		
		H_2O	0.6 M-KCl	6 M-urea
Two weeks	Once purified	3.00	3.27	3.60
	Twice purified	2.87	3.00	3.40
	Dried-down powder	1.85	2.03	2.80
About one year	Once purified	2.00	2.25	2.80
	Twice purified	0.88	0.98	1.47
	Dried-down powder	0.59	0.81	1.05

All the above experiments showed that the storage of alcohol-ether muscle powder before extraction of tropomyosin, a single or two-fold purification of tropomyosin, or the storage of the preparations in dehydrated form, caused the decrease of SH groups. Therefore in the next series of experiments the duration of the whole procedure, starting from the preparation of alcohol-ether dried muscle powder up to the dialysis of the salted out tropomyosin against water, was reduced to a minimum. Table 2 shows that in this case the value of up to 5.7 SH equivalents per 10^5 g. of protein was found in the presence of urea after a single purification cycle. Correspondingly, up to 4.5 and about 3.0 SH equivalents per 10^5 g. of tropomyosin have been found in the presence and in the absence of KCl, respectively. After the second purification cycle the number of SH groups being determined in the presence of urea dropped to 3.5 - 4.4 SH equivalents per 10^5 g. of protein.

Heat denaturation of tropomyosin led to some decrease of the number of SH groups reacting with HEDD, especially in the presence of urea (Table 3). The decrease was relatively smaller in tropomyosin redissolved from alcohol-ether powder; in this case the value found after 20 min. of incubation at 100° was similar to that of twice purified tropomyosin.

Regardless of the number of free SH groups found in particular tropomyosin preparations the viscosity was always nearly the same. Table 4 and Fig. 3 show that the dehydration procedure or duration

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Table 2

The content of SH groups in tropomyosin from freshly prepared alcohol--ether muscle powder

The determination of SH groups was performed as described in the legend to Fig. 1. The time between the killing of the rabbit and the determination of SH groups after a single purification cycle was two and a half days. It included 2 hr. of evaporation of alcohol-ether from the muscle powder, two-fold 2 hr. extraction of this powder with 1 M-KCl and, after precipitation at isoelectric point and fractionation with $(NH_4)_2SO_4$, extensive dialysis for 20 hr.

Number of SH equivalents expressed per 10^5 g. protein, is given.

No.	Preparation	Solvent		
		H_2O	0.6 M-KCl	6 M-urea
4	Once purified	3.1	3.5	5.6
5		3.1	4.4	5.7
7		3.2	4.5	5.6
8		3.0	4.3	5.2
9		3,1	4.1	5.3
4	Twice purified	1.3	2.0	3.1
5		2.4	3.1	3.7
7		1.6	2.8	4.3
8		2.4	3.1	4.3
9		2.6	3.3	4.4

Table 3

The influence of heat denaturation on content of SH groups in tropomyosin

The solutions of tropomyosin in water were heated in a boiling water bath. After rapid cooling, SH groups content was determined as described in the legend to Fig. 1.

Number of SH equivalents expressed per 10^5 g. of protein, is given.

No.	Preparation	Solvent					
		H_2O			6 M-urea		
		Control, unheated	Time of heating (min.)		Control, unheated	Time of heating (min.)	
			5	20		5	20
7	Twice purified	1.6	1.2	1.2	4.3	3.6	3.0
8		2.4	1.7	1.4	4.3	3.5	3.2
9		2.6	2.6	2.2	4.4	3.4	3.1
	Dried-down						
8	powder	1.9	1.7	1.4	3.3	3.3	3.0
9		2.0	2.0	2.0	3.1	2.8	2.7

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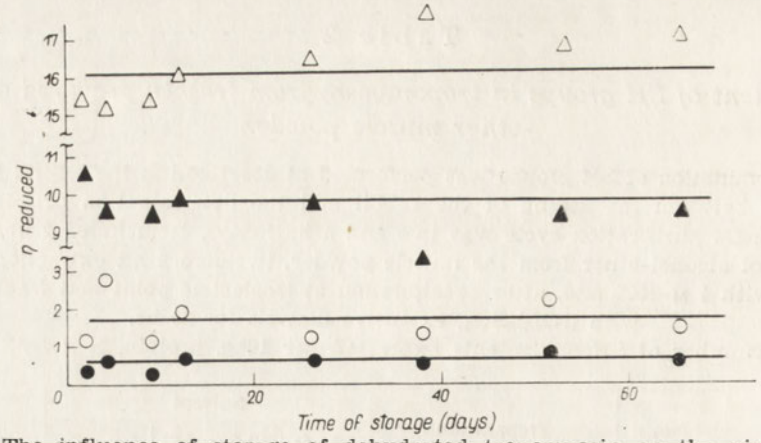

Fig. 3. The influence of storage of dehydrated tropomyosin on the viscosity of its solutions. Dehydrated tropomyosin preparation (the same as that used in the experiments presented in Fig. 2) was stored at 2°. On the days indicated in the Figure samples of this preparation were dissolved in water and the viscosity was measured in 3 ml. aliquots as described in the legend to Table 4. Tropomyosin dissolved in water: (△), in the presence and (▲), in the absence of HEDD. Tropomyosin dissolved in 0.6 M KCl: (○), in the presence and (●), in the absence of HEDD. Final concentration of tropomyosin: 0.2 - 0.5 mg./ml.

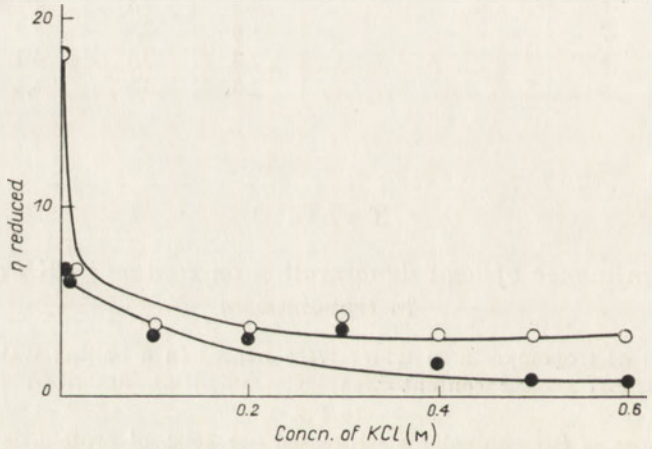

Fig. 4. The influence of HEDD on the viscosity of tropomyosin at various concentrations of KCl. Viscosity was measured in 3 ml. samples which consisted of tropomyosin at the final concentration of 0.35 mg./ml., 1.3 mM-tris buffer, pH 7.0, KCl at concentrations shown in the Figure, with or without 3.3×10^{-4} M-HEDD. In the solutions of tropomyosin containing HEDD a partial precipitation of the protein was observed at certain concentrations of KCl. The precipitation started to occur at 0.01 M-KCl, it reached its maximum at 0.1 M-KCl (at this concentration up to 20% of protein precipitated), and decreased with a further increase of KCl; eventually at 0.4 M-KCl concentration no precipitation took place at all. In cases when in the presence of HEDD a partial precipitation of protein took place the precipitates were centrifuged off and the viscosity was measured in supernatants. For the calculations of reduced viscosity a correction was made for the amount of precipitated protein. Viscosity of tropomyosin: (○), in the presence and (●), in the absence of HEDD.

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of storage of tropomyosin powder have no influence on the viscosity either in the absence or in the presence of 0.6 M-KCl. On the other hand, in the presence of HEDD the viscosity appeared to be increased; this increase of viscosity occurred immediately after addition of HEDD. In the absence of HEDD the viscosity of tropomyosin diminished continuously when the concentration of KCl was increased from 0 to 0.6 M (Fig. 4); in contrast, in the presence of HEDD a considerable decrease of viscosity was observed only after addition of 0.01 M-KCl and the subsequent increase of the concentration of salt had no effect on the viscosity.

Table 4

The comparison between the viscosity of tropomyosin and the number of SH groups reacting with HEDD

The determination of SH groups was performed as described in the legend to Fig. 1. Viscosity was measured in 3 ml. samples consisting of 1 ml. of tropomyosin solution in water or in 0.6 M-KCl, and 2 ml. of 2 mM-tris buffer, pH 7.0, with or without 5×10^{-4} M-HEDD.

No.	Preparation	Number of SH Eq. per 10^5 g. protein		Reduced viscosity			
		Solvent		Solvent			
		H_2O	0.6 M-KCl	H_2O	H_2O + HEDD	0.6 M-KCl	0.6 M-KCl + HEDD
4	Twice purified	1.3	1.9	6.6	13.2	0.5	1.4
5	Twice purified	2.4	3.1	7.0	13.9	0.2	0.8
5	Dried-down powder	1.6	2.8	7.6	15.2	0.3	0.5
7	Dried-down powder	0.5	0.9	7.6	13.4	0.3	0.6
8	Twice purified	2.4	3.1	7.2	13.4	0.8	2.0
9	Twice purified	2.6	3.3	6.8	18.1	0.7	3.1

Table 5

The effect of various reagents on the viscosity of tropomyosin

To 1 ml. of tropomyosin solution in water or in 0.6 M-KCl, 2 ml. of 2 mM-tris buffer, pH 7.0 (alone or with indicated reagents at the concentration of 5×10^{-4} M) was added and the viscosity was measured. Final concentration of tropomyosin 0.20 - 0.50 mg./ml.

Reduced viscosity							
Control		Methylmercuric hydroxide		*N*-Ethylmaleimide		Salyrgan	
H_2O	0.6M-KCl	H_2O	0.6 M-KCl	H_2O	0.6 M-KCl	H_2O	0.6 M-KCl
8.8	0.6	11.2	0.2	11.3	0.2	8.8	0.2
6.2	0.5	10.5	0.3	12.5	0.3	10.7	0.2
6.6	0.5	11.7	0.4	12.6	0.2	8.8	0.3

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The increase of viscosity of tropomyosin solutions in water took place not only in the presence of HEDD but also, although to a smaller degree, after addition of methylmercuric hydroxide or *N*-ethylmaleimide and to some degree in the presence of salyrgan at the same molar concentrations. In the presence of 0.6 M-KCl, however, these three reagents caused a decrease in viscosity (Table 5).

DISCUSSION

Tropomyosin isolated from rabbit skeletal muscle has been found to contain, according to amino acid analysis [2, 9], about 6.5 half-cystine equivalents per 10^5 g. Previous studies of Kominz *et al.* [9] failed to detect any free SH group in tropomyosin but the subsequent investigations of these authors [10], performed in modified conditions, showed the presence of 3 titratable SH equivalents per 10^5 g. protein. Later on Szent-Györgyi *et al.* [12] found a similar value using amperometric titration both in the absence and in the presence of 8 M-urea; on this basis they have postulated that two SH groups per one tropomyosin molecule are free and the two other form a disulphide bridge.

The value of 5.7 SH equivalents per 10^5 g. of protein obtained in the present study in once purified tropomyosin is much higher than the number of SH groups previously found by Kominz [10] and Szent-Györgyi [12]. This value is close to the number of half-cystine residues determined by amino acid analysis [9] which corresponds to about 4 half-cystine residues per one tropomyosin molecule. It does not seem likely that certain impurities, which might still be present in tropomyosin preparations after the first purification cycle, are responsible for such a high content of SH groups. Hence, it seems rather probable that the native tropomyosin molecule contains four free SH groups and no disulphide bridges [1]. This view is in agreement with the earlier results of Locker [11] who, after oxidation of tropomyosin with performic acid, was not able to find any changes in physico-chemical properties of tropomyosin molecule which should be observed if the disulphide bridge were present. Chang & Tsao [4] also found that the helical content of tropomyosin molecule decreased only slightly after oxidation.

From all the SH groups present in the tropomyosin molecule two seem to be extremely susceptible to auto-oxidation, possibly due to favorable steric conditions. This is probably the reason why in the tropomyosin

[1] After this paper had been finished, a reprint of the paper of P. H. Pei, C. I. Sun & N. C. Lin, published in *Acta Biochim. Sinica* **1**, 287, 1958 was obtained by us. As the paper is written in Chinese we do not know any details of the procedure; it follows, however, from the English abstract that in the presence of EDTA four SH groups in tropomyosin molecule have been found to react with PCMB.

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preparations only about two SH equivalents per mole have been usually detected. Two other SH groups seem also to be, partly at least, sensitive to auto-oxidation. Hence a considerable decrease of the groups content has been observed in the present study during prolonged storage of alcohol-ether dried muscle powder or of dried tropomyosin preparations. A similar phenomenon was previously observed during storage of oxyhaemoglobin [7].

The results of Kominz *et al.* [10], confirmed by the observations in the present study, showed that the oxidation of SH groups of tropomyosin was much more pronounced in the presence of traces of heavy metals which might be introduced with commercial ammonium sulphate used for fractionation. The strongly catalysing effect of traces of iron or copper on the oxidation of protein SH groups by molecular oxygen is well known in protein chemistry [cf. 3].

Incubation of tropomyosin solution at 100° does not result in the rendering of all SH groups accessible to HEDD, as it was found for instance with actin [5]; on the contrary, heating of tropomyosin causes a decrease of the number of reacting SH groups. It seems, in agreement with what was mentioned above, that also in this case two SH groups of tropomyosin molecule become rapidly inaccessible to HEDD. During heat denaturation either oxidation of these two SH groups takes place, a phenomenon similar to that observed with carboxyhaemoglobin [1], or the random coil structure formed after denaturation makes some of SH groups inaccessible to HEDD. The appearance of such new unfavourable steric conditions is plausible because the number of SH groups reacting with HEDD after heat denaturation is higher in the presence than in the absence of urea (Table 3).

The oxidation of SH groups in tropomyosin molecule seems to be at least partially reversible. Preliminary experiments have shown that when a tropomyosin solution prepared from a dried-down powder kept for a long period of time was treated with 1 mM-glutathione and next precipitated at the isoelectric point, in order to remove the excess of glutathione, the number of SH groups found subsequently was higher than in the control untreated solution.

In concentrated urea solution, when according to Chang & Tsao [4] the tropomyosin molecule becomes unfolded, all SH groups react with HEDD, similarly as in the case of actin [5]. In the absence of urea not all SH groups react with HEDD. It seems reasonable to assume that the steric hindrance is a primary reason of the inaccessibility of a part of SH groups. Thus, only about 3 SH equivalents per molecule react in 0.6 M-KCl, in conditions when tropomyosin remains practically in the form of monomers [2]. In the absence of KCl, when tropomyosin is in a polymerized form, an additional SH group in the molecule becomes inaccessible to HEDD. This effect may be due to the changes in steric conditions after

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aggregation of tropomyosin molecules. The possibility, however, cannot be excluded that this one SH group might be in some way involved in polymerization process.

The view that SH groups of tropomyosin may be involved in the polymerization of this protein was recently postulated by Chang & Tsao [4]. Their assumption was based on the observations that blocking of SH groups by metal ions or by organic mercurials, or oxidation of SH groups with performic acid markedly retarded the polymerization process. The results of the present study indicate that the partial auto-oxidation of SH groups of tropomyosin seems to have no influence on the viscosity of this protein. On the other hand it was observed, somewhat unexpectedly, that HEDD, *N*-ethylmaleimide, as well as some organic mercurials, led to the increase of viscosity of tropomyosin in the absence of salts. Much more work is, however, needed before these observations, as well as those of Chang & Tsao [4], would be properly explained.

The authors wish to thank Prof. Dr. W. Niemierko for his interest and criticism in the course of this work and Dr. S. Bitny-Szlachto for preparation of HEDD.

SUMMARY

The number of SH groups in tropomyosin has been determined using β-hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD). The sulphydryl groups of tropomyosin seem to be easily auto-oxidizable. In conditions in which auto-oxidation of the SH groups was reduced four SH groups per molecule of tropomyosin were found to react with HEDD in the presence of 6 M-urea. In the absence of urea the number of SH groups accessible to HEDD amounted to three and to two, respectively, when tropomyosin was dissolved in 0.6 M-KCl or in water.

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BADANIA NAD GRUPAMI SULFHYDRYLOWYMI TROPOMIOZYNY

Streszczenie

Oznaczano ilość grup SH w tropomiozynie przy użyciu dwusiarczku β-hydroksyetylo-2,4-dwunitrofenylowego. Grupy SH tropomiozyny wydają się łatwo podlegać autooksydacji. W warunkach, w których autooksydacja grup SH była zredukowana do minimum, cztery grupy SH na cząsteczkę tropomiozyny reagowały z HEDD w obecności 6 M-mocznika. W nieobecności mocznika trzy grupy SH były dostępne dla HEDD, gdy tropomiozyna była rozpuszczona w 0.6 M-KCl, a dwie, gdy białko było w roztworze wodnym.

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DETERMINATION OF ACTIVITY OF NUCLEOTIDE PYROPHOSPHATASE

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The activity of widely occurring nucleotide pyrophosphatase is usually measured by spectrophotometric [7] or manometric [1] determination of the decomposed nicotinamide-adenine dinucleotide. Other nucleotide coenzymes also have been used as substrates, i.e. flavin-adenine dinucleotide [7], coenzyme A [13], uridine diphosphate glucose [14] and adenosine diphosphate ribose [6].

Nucleotide coenzymes are decomposed not only by pyrophosphatase but also by other enzymes; therefore, especially when working with crude extracts, it is necessary to supplement the determinations by an analysis of the products formed.

Great advances in chemical synthesis of asymmetric esters of pyrophosphoric acid have been made during the last ten years as a result of the works of Todd, Cramer and Khorana, and it became possible to use synthetic substrates for the determination of pyrophosphatase activity.

In the present work, the synthesis of P^1-adenosine-P^2-phenylpyrophosphoric acid (ADPPhen) is described. This compound is hydrolysed by nucleotide pyrophosphatase to adenosine-5′-monophosphate (AMP) and monophenylphosphate (PPhen). PPhen formed can be hydrolysed by the addition of a phosphatase and the amount of phenol determined; in this way the activity of pyrophosphatase can be calculated from the amount of phenol released.

EXPERIMENTAL

Chemicals and substrates

AMP used for synthesis was a Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) product. Other chemicals were purchased from Fabryka Odczynników Chemicznych (Gliwice, Poland).

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N,N'-Dicyclohexylthiourea (m.p. 182°) was prepared according to Lowenstein [8].

N,N'-Dicyclohexylcarbodiimide (DCC) was prepared according to Schmidt & Hitzler [17] by collecting the fraction boiling at 154-156°/11 mm. Hg.

N-Cyclohexylamide of AMP (CAMP) was prepared by the method described by Moffat & Khorana [10] for the synthesis of nucleoside-5'-monophosphoromorpholidate: 0.365 g. of AMP·H_2O (1 m-mole) was dissolved in a mixture consisting of 10 ml. of *tert.*-butanol, 1 ml. of water, and 4.8 ml. (0.3967 g., 4 m-moles) of cyclohexylamine (d, 0.8191) and heated on a water bath under reflux with constant mixing, using an electromagnetic stirrer. To this mixture 0.8253 g. (4 m-moles) of DCC dissolved in 15 ml. of *tert.*-butanol was added dropwise. Vigorous stirring of the reaction mixture permitted to obtain a 100% yield and to limit the duration of this step to 1 hr. Then the mixture was heated for a further 15 min. and the alcohol was distilled off under normal and then under reduced pressure. The almost dry, gummy residue was transferred to a flask, added with 20 ml. of water and stirred to dissolve CAMP; then the insoluble dicyclohexylurea was filtered off and the flask was washed twice with 2.5 ml. of water. The aqueous solution of CAMP was extracted three times with 50 ml. of ethyl ether, the first portion being reextracted with 5 ml. of water. The water phase was evaporated to dryness on a water bath under reduced pressure. The white crystalline mass obtained was dissolved in methanol, transferred to a 50 ml. centrifuge tube, and methanol was evaporated to about 3 ml.; then 40 ml. of dry ethyl ether was added. The precipitate was centrifuged, washed twice with 30 ml. of ether and dried *in vacuo* over P_2O_5 at room temperature. The CAMP was obtained as *N,N',N''*-tricyclohexylguanidinium monohydrate salt, yield 99.2%. (Found: H_2O, 2.32; N, 16.78; P, 3.92%; calculated for $C_{35}H_{60}O_6N_9P \cdot H_2O$: H_2O, 2.39; N, 16.77; P, 4.12%). Paper chromatography revealed the presence of two spots: CAMP (R_F 0.52) and guanidinium base (R_F 0.90) released during chromatography [10].

Monophenyldichlorophosphoric acid was prepared according to Jirousek [5] by collecting the fraction boiling at 116-118°/16 mm. Hg.

Monophenylphosphoric acid (PPhen) was prepared by hydrolysis of 100 g. of monophenyldichlorophosphoric acid by the addition of 20 g. of ice. The mixture, which became warm during hydrolysis, was cooled and the white mass obtained was filtered and crystallized from chloroform as plates, m.p. 99.5°, R_F value 0.27.

Synthesis of P^1-adenosine-P^2-phenylpyrophosphoric acid (ADPPhen) was performed by a modification of the method of Moffat & Khorana [10] for obtaining ADPPhen from *N*-substituted AMP-phosphoramidates. One m-mole (0.734 g.) of tricyclohexylguanidinium salt of CAMP, dried

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at 100 - 105°, was twice dissolved in 5 ml. of pyridine (dried with BaO) and evaporated under reduced pressure. Then 4 m-moles (0.694 g.) of PPhen (dried *in vacuo* over P_2O_5) was added and traces of water were removed by three-fold evaporation from 5 ml. of pyridine under reduced pressure. The gummy residue was adjusted with 0.2 N-HCl to pH about 6.5 and the sediment of guanidinium hydrochloride was filtered off and washed twice with small portions of water. The filtrate was added with acetone, the precipitate was centrifuged and dissolved in a small amount of dry methanol, and again added with acetone; the precipitate formed was washed twice with acetone and dried *in vacuo* over P_2O_5 at room temp. The dihydrate of ADPPhen was obtained (found: H_2O, 6.20; N, 12.66; P, 11.09%; calculated for $C_{16}H_{19}O_{10}N_5P_2 \cdot 2\,H_2O$: H_2O, 6.68; N, 12.98; P, 11.48%). On paper chromatogram one spot with R_F 0.32, characteristic for ADPPhen [10] was found.

Even the crude product contained no AMP; this seems to be due to high reactivity of CAMP in the formation of the pyrophosphate bond with PPhen, and to the large excess of PPhen. Under these conditions it was possible to limit the purification of ADPPhen to the removal of the guanidinium base and of PPhen by acetone extraction. The reaction of CAMP occurred immediately after the addition of PPhen showing that the reactivity of CAMP was greater than that of the *N*-substituted AMP-phosphoramidates described by Moffat & Khorana [10].

Cobinamide phosphate and guanosine pyrophosphate cobinamide were prepared in our Laboratory by J. Pawełkiewicz from cultures of *P. shermanii* [15].

Enzymes and activity determinations

Alkaline phosphatase from calf intestine was a Sigma (St. Louis, Miss., U.S.A.) product.

Acid phosphatase was prepared from potato by ammonium sulphate fractionation according to Naganna *et al.* [12]; the fraction used contained 40 enzymic units per 1 ml.

Potato nucleotide pyrophosphatase was prepared from "Dary" potato according to Kornberg & Pricer [7]. The fraction after the first adsorption on phosphate gel (prepared after [2]) was used. For tobacco nucleotide pyrophosphatase determination, the mitochondrial fraction from the roots of *Nicotiana tabacum* L. Virginia Joyner, at the stage before flowering of the plants, was used. The homogenate and mitochondrial fraction were prepared by the method of Clayton & Hanselman [1]. Lupin pyrophosphatase was prepared from roots of two to four-week-old plants of bitter yellow lupin, according to Hasse & Schleyer [4]. The fraction precipitated by 0.35 $(NH_4)_2SO_4$ saturation was used.

The activity of acid phosphatase was determined in 0.1 M-acetate buffer, pH 5.0, and of alkaline phosphatase in 0.05 M-carbonate buffer,

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pH 9.5, in either case 0.02 M-solution of PPhen, adjusted with NaOH to pH 7, being used as substrate. The composition of the samples was: 2 ml. of buffer solution, 0.25 ml. of PPhen, and 0.25 ml. of phosphatase solution. The samples were incubated for 30 min. at 38°, then added with 0.5 ml. of 10% trichloroacetic acid (TCA). To the control sample, phosphatase was added after incubation and TCA addition. The precipitated protein was centrifuged off and phenol was assayed in the clear supernatant by the Folin method of Lowry *et al.* [9]. The unit of activity for alkaline and acid phosphatase was defined as that amount of enzyme which caused the release from PPhen of 1 μmole of phenol per 1 hr. at 38°.

To test the activity of the nucleotide pyrophosphatase preparations studied, a natural substrate, guanosine pyrophosphate cobinamide was used. One ml. (about 20 μg.) of the solution of this compound was incubated for 4 hr. at 38° with a mixture of 1.0 ml. of 0.1 M-Na-phosphate buffer, pH 7.5, and 1.0 ml. of pyrophosphatase solution. To the control sample, the enzyme inactivated by boiling was added. After incubation, the substrate and hydrolysis products were isolated and assayed according to Pawełkiewicz *et al.* [16]: guanosine pyrophosphate cobinamide, phosphate cobinamide and cobinamide were separated by descending chromatography on Whatman no. 2 paper with the solvent system of butan-1-ol - propan-2-ol - 5% HCN solution - water - 1 M-acetic acid (100:70:3:97:1, by vol.), and by electrophoresis on Whatman no. 3 paper with 0.01% NaCN in 1 M-acetic acid. The results indicate that after 4 hr. incubation with nucleotide pyrophosphatase preparations, guanosine pyrophosphate cobinamide was completely hydrolysed to cobinamide phosphate and guanosine monophosphate, in agreement with the observations of Pawełkiewicz [16] on snake venom pyrophosphatase.

The phosphatase preparations used in the present work hydrolysed cobinamide phosphate to cobinamide and inorganic phosphate.

PPhen, AMP, CAMP, ADPPhen and guanidinium bases were determined by descending chromatography on Whatman no. 1 paper using the solvent system of propan-2-ol - ammonium hydroxide (d, 0.9) - - water (7:1:2, by vol.). The chromatograms were dried, sprayed with 0.5% ninhydrin solution in butan-1-ol and dried again at 90° for 20 min.; the appearing spots were of a blue-violet colour and showed high absorbancy in UV-light.

Phosphorus was assayed according to Fogg & Wilkinson [3]. Nitrogen and protein concentrations above 0.1 mg./ml. were determined by the Kjeldahl method. Phenol and protein at concn. below 0.1 mg./ml. were determined by the Folin method of Lowry *et al.* [9]. A Pulfrich photometer provided with an Elpho equipment and K66 filter was used.

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RESULTS AND DISCUSSION

Determination of nucleotide pyrophosphatase activity using ADPPhen and phosphatase

The following final procedure was established: To 1 ml. of 0.1 M-Na--phosphate buffer, pH 7.5, was added 0.25 ml. of 0.02 M-ADPPhen (adjusted with NH_4OH to pH 7.0) and 0.25 ml. of pyrophosphatase solution (about 5 units). In the control sample, pyrophosphatase was omitted. Samples were incubated for 30 min. at 38°. If alkaline phosphatase was to be used, the samples were added with 2 ml. of 0.05 M-carbonate buffer, pH 10.5 (resulting in an increase of pH to 9.5), and then 0.25 ml. (10 units) of alkaline phosphatase solution to hydrolyse the PPhen formed. After incubation for 30 min. at 38°, 0.5 ml. of 10% TCA was added, and then to the control sample also 0.25 ml. of pyrophosphatase solution. The precipitate formed was centrifuged off and phenol was determined in the clear supernatant by the Folin reagent, the colorimeter being adjusted to zero for the control sample. When acid phosphatase was to be used for PPhen hydrolysis, the incubated samples were boiled, cooled under tap water, and added with 2.0 ml. of 0.1 M-acetate buffer of pH 4.0 (the pH of the sample then decreasing to 5.0), and 0.25 ml. (10 units) of acid phosphatase solution. Then it was incubated for 30 min. at 38°, deproteinized with TCA, and the amount of phenol determined by the Folin reagent.

When small amounts of protein were present in the sample, the phosphatase activity could be stopped by addition of HCl or NaOH, but if protein was present in greater amount it interfered with phenol estimations and had to be removed by TCA. Since many cations, except Na^+ and NH_4^+, in concentrations exceeding 1 mM form sediments with the Folin reagent, it was necessary to remove the interfering cations (e.g. with Na_2CO_3) prior to phenol determination.

In all experiments the amount of phosphatase added was sufficient to hydrolyse such an amount of PPhen as could be theoretically released from ADPPhen present.

Non-enzymic hydrolysis of ADPPhen

The effect of time, temperature and pH was assayed in samples containing 5 μmoles of ADPPhen, the amount of PPhen liberated being determined after hydrolysis with phosphatase (Table 1). When the sample of ADPPhen was kept for a week at 0 - 2° at pH 7.0, it was hydrolysed by 13% (0.66 μmole); when it was kept at 38° at pH 7.5, the amount hydrolysed within 24 hr. was 8% (0.4 μmole). The half-time of ADPPhen decomposition (Table 2) at 38° at pH values of 5.0 and 9.5 was, respectively, about 23 and 10 hr.

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Table 1

Non-enzymic hydrolysis of ADPPhen in water solution

Expt. I: 0.02 M-aqueous solution of ADPPhen, neutralized with NH_4OH to pH 7.0, was kept at 0 - 2°. Expt. II: 0.25 ml. of 0.02 M-ADPPhen neutralized solution was incubated with 1.0 ml. of 0.1 M-Na-phosphate buffer, pH 7.5. At determined time intervals PPhen was assayed by alkaline phosphatase.

The results are expressed as μmoles of phenol released from 5 μmoles of ADPPhen.

Time of incubation	Expt. I, pH 7.0, 0-2°	Expt. II, pH 7.5, 38°
0	0.09	0.09
30 min.	—	0.12
1 hr.	0.09	0.13
6 hr.	0.10	0.20
12 hr.	0.12	0.34
24 hr.	0.21	0.40
2 days	0.21	—
4 days	0.24	—
6 days	0.54	—
7 days	0.66	

Table 2

Half-time of non-enzymic hydrolysis of ADPPhen at 38°

To 1.0 ml. samples of 0.1 M-acetate buffer, pH 5.0, or 0.05 M-carbonate buffer, pH 9.5, was added 0.25 ml. of 0.02 M-ADPPhen solution, pH 7.0. The samples were incubated at 38° and at determined time intervals the PPhen formed was estimated by acid or alkaline phosphatase, resp.

The results are expressed as μmoles of phenol released from 5 μmoles of ADPPhen.

Time of incubation	pH 9.5	pH 5.0
0	0.10	0.08
10 min.	0.46	0.12
20 min.	0.66	0.13
30 min.	0.88	0.18
45 min.	1.14	0.20
1 hr.	1.36	0.26
2 hr.	1.62	0.40
3 hr.	1.74	0.67
6 hr.	1.94	0.96
10 hr.	2.36	1.32
12 hr.	—	1.47
23 hr.	—	2.32

The assays of non-enzymic hydrolysis indicate that the solution of ADPPhen of pH 7 kept at 0 - 2° is relatively stable. During incubation for 30 min. at 38° at pH 7.5 and 5.0 the amount of ADPPhen hydrolysed was but slight, while at pH 9.5 ADPPhen was hydrolysed by about 16%.

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Enzymic hydrolysis of ADPPhen

The effect of nucleotide pyrophosphatase on ADPPhen. ADPPhen (5 μmoles) was incubated with nucleotide pyrophosphatase preparations from potato, tobacco and lupin in Na-phosphate buffer, pH 7.5, for 30 min. at 38°. In the incubation mixture ADPPhen, AMP, and PPhen were found by paper chromatography. Analysis of the incubation mixture containing boiled enzyme preparation showed a much smaller amount of AMP and PPhen, their presence being due only to the non-enzymic hydrolysis. In no case the presence of free phenol was observed indicating that at pH 7.5 the preparations showed no phosphatase activity.

The effect of phosphatases on ADPPhen. Ten units of alkaline or acid phosphatase were incubated with 0.25 ml. of 0.02 M-ADPPhen solution under the conditions described in Methods. In the control samples, containing phosphatases inactivated by boiling, ADPPhen, AMP, and PPhen were found by paper chromatography. In the samples containing active phosphatases, no PPhen was present and the amounts of phenol were the same as those released during 30 min. of non-enzymic hydrolysis of ADPPhen at pH 9.5 and 5.0, resp. These results indicate

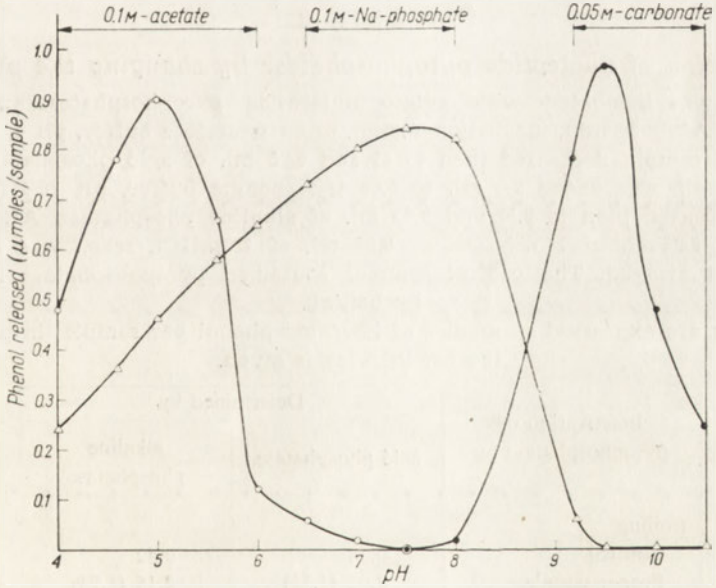

Fig. 1. pH optima for potato nucleotide pyrophosphatase and for alkaline and acid phosphatases. Activity of acid phosphatase was assayed at pH 4.0 - 7.5, of alkaline phosphatase at pH 7.0 - 10.4, and of pyrophosphatase at pH 4.0 - 10.4. (○), Acid phosphatase, with PPhen as substrate; (●), alkaline phosphatase, with PPhen as substrate; (△), nucleotide pyrophosphatase from potato, with ADPPhen as substrate.

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that neither of the phosphatase preparations tested was active towards ADPPhen.

pH *optima*. The aim of the experiments was to determine such pH values at which the activity of phosphatases used for nucleotide pyrophosphatase activity determinations, is high and the pyrophosphatase is inactive. The optimum pH values (Fig. 1) in agreement with the results of other authors, were for acid phosphatase pH 5.0 [12], for alkaline phosphatase pH 9.5 [11] and for potato nucleotide pyrophosphatase the optimum pH was 7.5 [7]. At pH 9.5 pyrophosphatase was inactive, while at pH 5.0 its activity amounted to 50% of the maximum. At pH 7.5 both alkaline and acid phosphatases were inactive. The results for pyrophosphatases prepared from tobacco and lupin roots were similar to those for potato pyrophosphatase.

Inactivation of nucleotide pyrophosphatase. Since nucleotide pyrophosphatase was inactive at pH 9.5, no other inactivation procedure except the change of pH was necessary before further incubation with alkaline phosphatase (Table 3). However, at pH 5 nucleotide pyrophosphatase showed considerable activity and therefore it was necessary to inactivate the enzyme by boiling before incubation with acid phosphatase (Table 4).

Table 3

Inactivation of nucleotide pyrophosphatase by changing the pH *value*

ADPPhen was incubated with potato nucleotide pyrophosphatase in standard conditions. After 30 min. incubation, 2.0 ml. of 0.1 M-acetate buffer, pH 4, was added (pH of the sample decreased then to 5) and 0.25 ml. of acid phosphatase. To the second sample was added 2.0 ml. of 0.05 M-carbonate buffer, pH 10.4 (pH of the sample increased then to 9.5) and 0.25 ml. of alkaline phosphatase. After 30 min. incubation, 0.25 ml. of 1 N-NaOH or 0.25 ml. of 1 N-HCl, resp., was added and phenol was assayed. The control samples contained pyrophosphatase inactivated by boiling.

The results are expressed as μmoles of liberated phenol per sample; in parentheses the net increase is given.

Inactivation of pyrophosphatase by	Determined by	
	acid phosphatase	alkaline phosphatase
Boiling		
Control	0.28	0.42
Proper sample	2.02 (1.74)	2.15 (1.73)
Changing the pH to 5		
Control	0.11	
Proper sample	2.42 (2.31)	
Changing the pH to 9.5		
Control		0.08
Proper sample		1.82 (1.74)

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Table 4

Inactivation of nucleotide pyrophosphatase by boiling

ADPPhen was incubated with potato nucleotide pyrophosphatase in standard conditions for 30 min. and boiled. Then in one sample the amount of released PPhen was assayed immediately with alkaline phosphatase, and in the second sample after 30 min. In the control samples, pyrophosphatase inactivated by boiling was used.

Sample	Phenol liberated (μmoles per sample)		
	Control	Proper sample	Difference
Incubated for 30 min.	0.12	2.15	2.03
Incubated for 30 min., boiled, incubated for further 30 min.	0.34	2.38	2.04

The effect of boiling on pyrophosphatase activity and on non-enzymic hydrolysis of ADPPhen was assayed by alkaline phosphatase just after boiling and 30 min. later, and the same activity of pyrophosphatase was found in both samples. The amount of ADPPhen hydrolysed non-enzymically during boiling at pH 7.5 was small, being only about 10% of the value for enzymic hydrolysis.

The results presented indicate that when acid phosphatase was used for the determination of nucleotide pyrophosphatase activity, it was necessary to inactivate pyrophosphatase by boiling; when using alkaline phosphatase, it was sufficient to change the pH from 7.5 to 9.5.

Crude extracts of nucleotide pyrophosphatase contain usually a considerable admixture of acid phosphatase. This permits to demonstrate rapidly the presence of pyrophosphatase by incubating the crude extract first with ADPPhen at pH 7.5 and then at pH 5.0.

Table 5

The effect of ions on potato nucleotide pyrophosphatase activity

Activity of a sample containing no added ions was taken as 100%.

Addition (0.5 mM)	Activity (%)
Mg^{2+}	100
Ca^{2+}	100
Mn^{2+}	90
Fe^{2+}	81
Co^{2+}	80
CN^{-}	100
F^{-}	65

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The effect of ions on the activity of potato nucleotide pyrophosphatase. Salt solutions were added to the incubation medium to final concentration of 0.5 mM. Cations interfering with the determinations of phenol by the Folin reagent were precipitated with 10% Na_2CO_3 solution. Since the ions added could influence both the activity of pyrophosphatase and phosphatase, the control sample after incubation at pH 7.5 was added with the respective ions to assure the same conditions for PPhen hydrolysis as in the proper sample. The results presented in Table 5 are in agreement with those of Kornberg & Pricer [7].

SUMMARY

1. Adenosine-5′-monophosphate *N*-cyclohexylamide was prepared with 100% yield and used for the synthesis of P^1-adenosine-P^2-phenylpyrophosphoric acid (ADPPhen).

2. Nucleotide pyrophosphatase preparations from potato, tobacco and lupin hydrolysed ADPPhen to AMP and monophenylphosphate (PPhen) and were also active towards a natural substrate, guanosinepyrophosphate cobinamide.

3. A method for the determination of nucleotide pyrophosphatase activity is described. The amount of PPhen liberated is determined from the amount of phenol released by acid or alkaline phosphatase.

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OZNACZANIE AKTYWNOŚCI PIROFOSFATAZY NUKLEOTYDOWEJ

Streszczenie

1. Otrzymano ze 100% wydajnością *N*-cykloheksyloamid kwasu 5′-adenozyno-monofosforowego i użyto go do syntezy kwasu P^1-adenozyno-P^2-fenylo-pirofosforowego (ADPPhen).

2. Stwierdzono, że ADPPhen jest rozkładany na AMP i monofenylofosforan (PPhen) przez preparaty pirofosfatazy nukleotydowej z ziemniaków, korzeni tytoniu i łubinu; preparaty te są aktywne również wobec substratu pochodzenia naturalnego, guanozyno-pirofosforanu kobinamidu.

3. Opracowano metodę oznaczania aktywności pirofosfataz nukleotydowych. Metoda polega na oznaczeniu ilości uwolnionego enzymatycznie PPhen, z którego, po hydrolizie fosfatazą alkaliczną lub kwaśną, uwolniony fenol oznacza się odczynnikiem Folina.

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THE EFFECT OF IONIZING RADIATION ON THE ACTIVITY OF ADENOSINE TRIPHOSPHATASE IN MITOCHONDRIA OF RAT LIVER, SPLEEN AND HEART

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The inhibition of ATP synthesis induced by ionizing radiation may be regarded as one of the primary radiation effects, evoking further disturbances of many metabolic reactions. The existing evidence [22] indicates that the generation of ATP in the process of oxidative phosphorylation in mitochondria is coupled to three distinct sites of the electron transport chain. Although the general mechanism of ATP synthesis:

$$A \sim X + ADP + P_i \overset{\text{synthetase}}{\rightleftharpoons} A + X + ATP$$

is common for all three sites, it has been shown recently [26] that separate and site-specific coupling factors possessing ATP synthetase activity, are involved. ATP may be generated in the cell also by other reactions and the question arises whether all of these reactions are equally impaired by radiation; the effect of radiation on reactions utilizing and/or degrading ATP should also be taken into account. The difference between synthesis of ATP and its utilization or hydrolysis in mitochondria is usually termed ATPase activity. In heart mitochondria this activity was found by Myers & Slater [13] to have three pH optima which was interpreted [22, 25] as reflecting the presence of three different enzyme systems of ATP synthesis.

MATERIALS AND METHODS

Rats were killed by decapitation, organs removed and liver and spleen mitochondria were prepared according to Hogeboom [10]. Heart sarcosomes were prepared according to Cleland & Slater [6] in a medium composed of KCl, 0.115 M; Na-phosphate buffer, 0.02 M; and sodium versenate, 0.01 M; phosphate being removed by two-fold washing with

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0.25 M-sucrose solution. The final suspension of mitochondria in 0.25 M-sucrose solution used for experiments, corresponded to 100 mg. of tissue per 1 ml. of the suspension.

ATP disodium salt was purchased from L. Light & Co. Ltd. (Great Britain); in some experiments Myotriphos, the ATP preparation of Tarchomińskie Zakłady Farmaceutyczne (Poland) was used. The chromatographic analysis of Myotriphos has shown that beside ATP it contained also ADP, AMP and traces of inorganic orthophosphate; however, it was used without further purification since no difference was found between the two preparations in ATPase activity.

All other chemicals used were of analytical grade. Twice glass-distilled water was used throughout.

Suspensions of freshly isolated mitochondria placed in an ice-bath were irradiated *in vitro* with γ-rays delivered from a ^{60}Co source. The dose-rate in the irradiation chamber was approximately 1840 rads/min. The control, non-irradiated samples were kept in similar bath conditions.

Whole rats were irradiated *in vivo* with Stabilipan X-ray apparatus operated at 160 kV and 15 mA. The radiation filtered through a 0.5 Cu filter gave at the distance of 60 cm. a dose-rate of approximately 20 rtg./min. in air, as measured with the Siemens Universal Dosimeter. Rats to be irradiated were placed in an appropriate wooden box and a dose of 750 rtg. was applied. The rats were killed by decapitation 15 - 30 min., 24 or 48 hr. after exposure and mitochondria from studied organs were isolated immediately.

The activity of ATPase was assayed in irradiated and non-irradiated material in duplicate samples. The activity of the enzyme was expressed in μmoles of orthophosphate liberated per mg. of protein nitrogen or per 0.1 g. of wet tissue, during 1 hr. at 25°. The incubation mixture, according to Myers & Slater [13], consisted of: mitochondria equivalent to about 2 mg. of protein nitrogen; KCl, 75 mM; tris buffer, 50 mM, pH 5.0 to 10.0; ATP disodium salt, 2 m_M; $MgCl_2$, 1 mM and sodium versenate, 0.7 mM; final volume 1.5 ml. Samples were incubated for 15 min. at 25° and the reaction was stopped by adding 1.5 ml. of 10% TCA solution. The precipitate was centrifuged off and the inorganic phosphate (P_i) was determined according to Fiske & Subbarow [8] in 1.5 ml. of the supernatant. Protein concentration in mitochondria was determined by the indirect biuret method [9] and occasionally checked by the determination of protein nitrogen by the Kjeldahl method.

RESULTS

Irradiation in vitro

The activity of ATPase in irradiated mitochondria was assayed either immediately after irradiation or 24 hr. later, the mitochondrial suspensions being kept during this time at 2 to 4°. The irradiation of

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the liver, spleen and heart mitochondria (Tables 1, 3 and 4, resp.) with 10 000, 30 000 and 40 000 rads had no effect on ATPase activity assayed at different pH values. The ageing of irradiated liver mitochondria for 24 hr. did not result in marked changes in ATPase activity (Table 2). The observed differences of activity were not statistically significant.

The stimulating effect of 2,4-dinitrophenol (2,4-DNP) on ATPase activity was also tested on irradiated and non-irradiated liver and heart mitochondria (Table 5) and no differences in the stimulation were observed.

Irradiation in vivo

In liver mitochondria (Fig. 1) isolated from rats killed within 30 min. after irradiation, the activity of ATPase was lower than in the controls, the decrease being dependent on the pH of the incubation medium and most marked at pH 6.5 and 8.5. In animals killed 24 hr. after exposure, the activity of ATPase returned to normal values except for the determination made at pH 8.5 which showed a statistically significant increase. After 48 hr., the activity was at an approximately normal level at all pH values studied.

Table 1

Effect of γ-irradiation on ATPase activity in rat liver mitochondria

ATPase activity was determined at indicated pH values. The components of the incubation medium as described under Methods. Results are expressed as μmoles of inorganic phosphate liberated from ATP, per mg. of protein per 1 hr., ± S. D. Number of determinations in each group is indicated in parentheses.

pH	Non-irradiated, control (8)	Irradiation dose (rad)		
		10 000 (4)	30 000 (4)	40 000 (2)
5.0	3.50 ± 0.59	3.09 ± 0.65	—	3.00 ± 0.71
5.5	4.12 ± 0.13	3.69 ± 0.52	—	5.35 ± 0.65
6.0	4.18 ± 0.56	2.94 ± 1.02	2.97 ± 1.10	5.29 ± 0.88
6.5	5.60 ± 1.16	5.77 ± 0.88	4.73 ± 1.12	6.52 ± 0.84
7.0	4.41 ± 1.03	4.49 ± 0.65	3.76 ± 0.98	4.76 ± 0.84
7.5	4.65 ± 1.03	5.60 ± 0.40	4.42 ± 1.02	4.78 ± 0.90
8.0	6.76 ± 1.70	6.36 ± 1.00	—	6.00 ± 0.76
8.5	6.85 ± 1.51	6.42 ± 0.96	5.55 ± 1.15	5.90 ± 1.01
9.0	6.46 ± 1.49	5.85 ± 1.12	—	6.19 ± 1.35
9.5	5.36 ± 1.17	4.79 ± 1.07	4.54 ± 1.10	5.98 ± 1.01
10.0	4.36 ± 1.14	3.46 ± 0.73	—	5.03 ± 0.89

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Table 2

Effect of ageing on ATPase activity in irradiated liver mitochondria

Assays were performed 24 hr. after exposure; other conditions as in Table 1. Results are expressed as μmoles of inorganic phosphate liberated from ATP, per mg. of protein per 1 hr., ± S. D. Number of determinations in each group is indicated in parentheses.

pH	Non-irradiated, control (4)	Irradiation dose (rad)	
		10 000 (4)	40 000 (4)
5.0	4.60 ± 0.88	4.12 ± 0.72	4.43 ± 0.82
5.5	4.75 ± 0.57	4.39 ± 0.68	5.26 ± 1.11
6.0	5.54 ± 1.36	4.96 ± 1.01	5.44 ± 1.16
6.5	6.72 ± 0.90	6.60 ± 0.73	7.50 ± 1.20
7.0	6.03 ± 0.43	6.32 ± 0.88	6.46 ± 0.82
7.5	7.28 ± 1.20	6.72 ± 1.17	7.58 ± 0.98
8.0	9.88 ± 1.12	8.58 ± 1.36	9.75 ± 1.05
8.5	10.98 ± 1.40	9.83 ± 1.50	10.46 ± 1.19
9.0	9.38 ± 1.12	7.76 ± 1.21	8.63 ± 0.85
9.5	6.47 ± 0.84	6.22 ± 0.96	6.38 ± 0.84
10.0	4.92 ± 0.45	5.27 ± 0.82	5.40 ± 0.77

Table 3

Effect of γ-irradiation in vitro *on ATPase activity in rat heart sarcosomes*

ATPase activity was determined at indicated pH values. Components of the incubation medium as described under Methods. Results are expressed as μmoles of inorganic phosphate liberated from ATP, per mg. of protein per 1 hr., ± S. D. Number of determinations in each group is indicated in parentheses.

pH	Non-irradiated, control (8)	Irradiation dose (rad)		
		10 000 (4)	20 000 (4)	40 000 (4)
5.0	7.23 ± 0.98	7.13 ± 1.03	9.32 ± 1.30	7.78 ± 1.12
5.5	7.36 ± 1.25	9.50 ± 1.35	7.81 ± 0.98	6.23 ± 1.15
6.0	5.70 ± 1.21	5.06 ± 0.89	6.81 ± 1.10	6.23 ± 0.93
6.5	6.14 ± 0.98	6.35 ± 1.01	6.47 ± 0.88	7.50 ± 1.20
7.0	5.00 ± 1.08	6.00 ± 1.12	5.68 ± 1.01	7.26 ± 1.40
7.5	5.38 ± 0.81	5.26 ± 0.98	4.92 ± 1.11	7.23 ± 1.38
8.0	6.06 ± 0.78	6.81 ± 1.05	6.21 ± 0.87	6.99 ± 1.02
8.5	6.22 ± 0.81	5.55 ± 1.13	7.80 ± 1.21	7.08 ± 0.89
9.0	5.24 ± 0.82	4.92 ± 1.03	6.96 ± 0.94	4.78 ± 0.68
9.5	4.44 ± 0.71	3.75 ± 0.98	4.49 ± 0.73	5.45 ± 0.88
10.0	4.23 ± 0.95	4.33 ± 0.85	4.86 ± 1.01	4.84 ± 1.05

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The activation of ATPase by 2,4-DNP in mitochondria isolated from rat liver 24 hr. after irradiation did not differ from the values for non-irradiated animals (Table 5).

In spleen mitochondria within 30 min. after exposure a slight decrease of ATPase activity was observed but the difference was not statistically significant. In the mitochondria isolated 24 hr. after exposure, a statistically significant increase was observed at pH 7.5 and 5.5. An

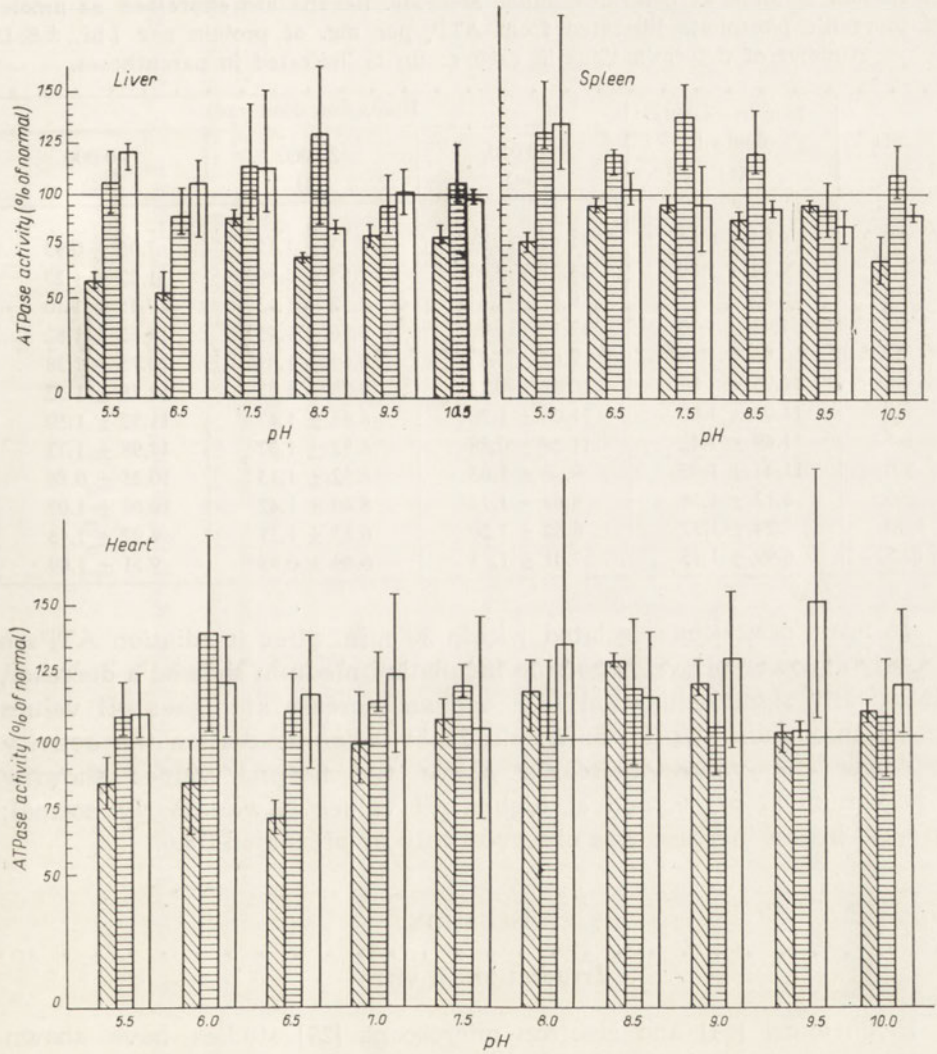

Fig. 1. Activity of ATPase in liver, spleen and heart mitochondria isolated from rats irradiated with 750 rtg. of X-rays. Mean values from 3 duplicate determinations and the range are given. The activity, expressed as percentage of the normal values taken as 100, was estimated at indicated pH values at time intervals of: 30 min. (diagonally hatched), 24 hr. (horizontally hatched) and 48 hr. after irradiation (outlined column).

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increase of activity was also found at pH 5.5 in mitochondria isolated 48 hr. after irradiation when at other pH values ATPase activity was normal.

Table 4

Effect of γ-irradiation on ATPase activity in rat spleen mitochondria

ATPase activity was determined at indicated pH values. Components of the incubation medium as described under Methods. Results are expressed as μmoles of inorganic phosphate liberated from ATP, per mg. of protein per 1 hr., ± S. D. Number of determinations in each group is indicated in parentheses.

pH	Non-irradiated, control (8)	Irradiation dose (rad)		
		10 000 (4)	20 000 (4)	40 000 (4)
5.0	8.95 ± 0.80	11.64 ± 1.68	8.73 ± 1.12	7.05 ± 0.93
5.5	9.14 ± 1.59	13.21 ± 2.07	7.85 ± 0.90	11.22 ± 1.27
6.0	11.16 ± 1.47	9.17 ± 1.61	8.52 ± 1.13	10.55 ± 1.10
6.5	11.42 ± 1.37	12.16 ± 1.08	8.96 ± 1.45	14.62 ± 1.82
7.0	10.86 ± 1.79	9.63 ± 1.27	6.69 ± 1.61	10.75 ± 1.38
7.5	11.75 ± 1.87	10.43 ± 1.11	9.25 ± 1.73	12.78 ± 1.52
8.0	11.08 ± 1.80	11.00 ± 1.20	8.83 ± 1.47	11.52 ± 1.50
8.5	11.69 ± 1.42	11.29 ± 0.88	8.83 ± 1.37	14.98 ± 1.73
9.0	11.44 ± 1.05	9.98 ± 1.03	8.52 ± 1.13	10.25 ± 0.86
9.5	8.12 ± 1.78	8.69 ± 1.14	8.40 ± 1.42	10.08 ± 1.07
10.0	7.24 ± 1.37	6.52 ± 1.20	6.33 ± 1.31	9.97 ± 1.13
10.5	6.99 ± 1.15	7.01 ± 1.13	6.69 ± 0.93	9.11 ± 1.09

In heart sarcosomes isolated within 30 min. after irradiation ATPase activity at lower pH values of the incubation medium showed a decrease, statistically significant at pH 6.5, and an increase at higher pH values with a maximum at pH 8.5. Within 24 hr. after irradiation the activity at lower pH values increased above the normal values showing a maximum at pH 6, and at higher pH values it was nearly normal; after 48 hr. an increase was observed only at pH 9.5 and 8.0.

DISCUSSION

Irradiation in vitro

Biochemical [14] and electron microscope [27] studies have shown that some structural changes appear in irradiated mitochondria. Bacq & Alexander [2] interpreted the observed increase of activity of some enzymes as indicating the release of the enzyme. This suggestion was not supported by Scaife & Alexander [20] who studied the effect of ionizing radiation on permeability of mitochondria, and by our present experiments on ATPase activity in irradiated mitochondria.

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Table 5

Effect of 2,4-dinitrophenol on ATPase activity in mitochondria irradiated in vitro *and* in vivo

ATPase activity was assayed with and without addition of 2,4-DNP to the incubation medium. Other conditions as described under Methods. Results are expressed as µmoles of inorganic phosphate liberated from ATP, per ml. of mitochondrial suspension (equivalent to 0.1 g. of wet tissue) per hour.

Mitochondrial preparation	2,4-DNP (mM)	Radiation dose (rad)	pH of the incubation system										
			5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
Liver fresh	0.05	None	5.89	7.13	16.55	14.89	11.82	9.47	13.44	14.36	14.16	7.69	8.10
	0.05	40 000	4.54	8.62	14.41	14.29	9.78	10.78	17.60	12.94	9.09	8.66	7.98
aged	0.05	None	4.56	12.69	10.70	7.83	8.08	11.04	15.09	12.78	13.96	8.17	8.61
	0.05	40 000	6.70	9.46	8.70	8.45	8.88	10.70	19.59	16.71	10.71	8.28	7.27
Heart	0.6	None	5.99	7.68	8.23	9.38	11.04	9.57	11.10	8.41	6.44	5.37	4.81
	0.6	10 000		7.23		10.59		8.73		7.71		5.27	
	0.6	20 000		9.23		10.01		9.37		9.05		5.85	
	0.6	40 000		9.14		8.46		7.16		9.14		5.70	
Liver 20 hr. after irradiation *in vivo*	None	None				5.53		9.35		12.40		8.88	
	1	None				10.29		14.60		18.44		14.03	
	1	750				9.47		12.89		16.07		11.89	

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The irradiation *in vitro* of liver, spleen and heart mitochondria had no effect on ATPase activity tested just after irradiation and 24 hr. later at different pH values of the medium. This is in agreement with the observation of other workers that irradiation of isolated organs [4, 17], homogenates [15] or mitochondria [12, 15] is not followed by any changes in ATPase activity.

The isolated from mitochondria, purified soluble ATPase differs from the enzyme present in intact mitochondria. The ATPase bound to some component of mitochondria is stimulated by 2,4-DNP while the soluble ATPase is unaffected [21]. In our experiments, irradiation of mitochondria did not influence the stimulation of ATPase activity by 2,4-DNP. This suggests that no solubilization of mitochondrial ATPase occurs during irradiation.

Irradiation in vivo

Several workers observed an increased activity of ATPase in tissue homogenates or mitochondria isolated from irradiated animals. Most of the results showed activation of ATPase in the spleen [1, 7, 3, 5] and no change in the liver [4, 5]. According to Ord & Stocken [15], 90 min. after irradiation of rats with 1000 rtg. of X-rays the ATPase activity in spleen mitochondria was increased by 30%. Other authors found similar activation 3 and 4 hr. after irradiation [1, 18] or even later [23]. This activation lasted usually for 24 hr. [7] or longer [23]. Our results confirm the activation of ATPase in the spleen 24 hr. after the exposure. Moreover, we have also observed activation of ATPase in liver and heart mitochondria.

The mechanism of ATPase activation by radiation is not known. Certainly, the effects observed 24 hr. after the exposure only remotely reflect the changes induced by irradiation in numerous metabolic processes of the living cell. Many authors studied the relation between radiation effects on ATPase and on oxidative phosphorylation but found no correlation between the two processes [cf. 16]. Since the experimentally determined ATPase activity is in reality the difference between the synthesis and hydrolysis of ATP, it seems permissible to assume, in spite of the lack of correlation of the two processes, that the effect of ionizing radiation on oxidative phosphorylation in mitochondria participates in the observed effect of radiation on ATPase activity.

An attempt was made to study more direct effect of radiation on ATPase activity, or at least the effect appearing immediately after irradiation, by carrying out the assays on mitochondria isolated from animals killed within 30 min. after exposure. In liver mitochondria a decrease of activity determined at pH 6.5 and 8.5, and in sarcosomes at pH 6.5, were found In spleen mitochondria no changes were observed, while in sar-

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cosomes the activity at pH 8.5 was not decreased but increased. It should be recalled that according to Myers & Slater [13] the pH optima for the ATPase activity induced by 2,4-DNP in mitochondria are at 6.5, 7.5 and 8.5. In the present work, the greatest effect of irradiation on ATPase activity was observed at the pH optima reported by Myers & Slater. This fact can be taken as an additional indication for the existence of different ATPases and/or different phosphorylation processes within the mitochondrion. The reason why in liver mitochondria 30 min. after the exposure the activity at pH 8.5 decreased while in heart mitochondria it increased, cannot be explained on the basis of the available data. It should be noted that Thomson *et al.* [24] did not find significant changes in ATPase activity of the thymus after irradiation; Rabassini *et al.* [19] observed in the brain a decrease of the activity dependent on the radiation dose delivered, while Mandell & Schmitt [11] found an increase after local irradiation of eye lenses.

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SUMMARY

ATPase activity of rat liver, spleen and heart mitochondria irradiated *in vitro* with the dose of 10 000 to 40 000 rads did not differ from control values. ATPase activity in organs of rats irradiated *in vivo* with 750 rtg. of X-rays, tested 30 min., 24 hr. and 48 hr. after exposure, showed differences dependent on the pH of the incubation system.

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WPŁYW PROMIENIOWANIA JONIZUJĄCEGO NA AKTYWNOŚĆ ADENOZYNOTRÓJFOSFATAZY W MITOCHONDRIACH WĄTROBY, ŚLEDZIONY I SERCA SZCZURA

Streszczenie

Badano w różnych pH aktywność ATPazy mitochondriów napromienionych *in vitro* dawkami 10 000 do 40 000 radów oraz izolowanych w 1/2, 24 lub 48 godzin po napromienieniu zwierząt dawką 750 rtg. promieniowania X. Aktywność mitochondriów napromienionych *in vitro* nie różniła się od wartości kontrolnych. Wyniki uzyskane po napromienieniu *in vivo* wskazują, że zmiany w aktywności ATPazy w badanych narządach zależą od pH układu inkubacyjnego.

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ALKALINE PHOSPHATASE ACTIVITY IN SUBCELLULAR FRACTIONS OF THE INTESTINE OF THE FROG DURING STARVATION AND FEEDING

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It has been shown by several authors, by means of chemical or histochemical methods, that the activity of alkaline phosphatase (EC 3.1.3.1) in the intestinal tissue of various groups of animals appears to be highly increased after feeding with fat. Such results have been obtained with mammals [10, 25, 1, 17, 8], amphibia [6, 8] and with insects [18]. In a recent histochemical study from this laboratory [8] it has been found that in the intestine of starved frogs the alkaline phosphatase is seen only in the striated border; whereas after feeding with fats, but not with other food, it becomes clearly visible also in the Golgi region of the epithelial cells.

In relation to these findings it seemed to be interesting to study more closely the distribution of alkaline phosphatase in subcellular fractions of the intestinal epithelium. Special attention has been paid to the much discussed question of the presence of alkaline phosphatase in the nuclei [cf. 20].

MATERIAL AND METHODS

The frogs (*Rana esculenta*) used for experiments were caught in autumn and kept during winter at 4 - 10° without feeding. Before the experiments the animals were transferred for two weeks to room temperature.

Three types of experiments were performed: (1), on starved animals; (2), on animals which were given orally by means of a pipette 0.2 ml. of olive oil; and (3), on animals which were given orally a piece of about 50 mg. of boiled egg white. Two days after feeding, most probably at the height of food absorption [7], the frogs were killed by decapitation, the small intestine was dissected and its content was removed by pressing out and rinsing in 0.25 M-saccharose at 0°.

Preparation of subcellular fractions. The small intestines collected from five to seven frogs were cut into small pieces and homogenized

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at 0° in 0.25 M-saccharose in a Potter-Elvehjem type teflon homogenizer. The homogenate, after filtration through gauze, was centrifuged for 10 min. at 600 *g* at 0°. The sediment contained the nuclear fraction contaminated with cell debris. The mitochondrial fraction was separated on centrifugation for 10 min. at 7000 *g* and the supernatant was separated by centrifugation for 1 hr. at 92 000 *g* in a preparative Spinco centrifuge (model L) into the microsomal fraction and the final supernatant.

In some experiments not the whole intestine was used but only the mucosa which was scrapped off with a plastic spatula and weighed. The mucosal scrappings were homogenized in 0.25 M-saccharose containing various amounts of $CaCl_2$ or EDTA. The nuclear fraction obtained from the mucosa was purified using the method of Hogeboom & Schneider [11]. In some experiments, the nuclei present in the partly purified nuclear fraction were destroyed mechanically by a more intense homogenization in a glass homogenizer or by treatment with 0.26% sodium deoxycholate [22].

Determination of alkaline phosphatase. The activity in each fraction was expressed as the amount of inorganic phosphate (P_i) liberated from sodium β-glycerophosphate during 1 hr. incubation at 37° par 1 mg. protein nitrogen. The incubation mixture consisted of: 0.1 to 1.0 ml. suspension of the examined fraction; 1.0 ml. of 0.03 M-sodium β-glycerophosphate; 3 ml. of 0.2 M-carbonate buffer, pH 9.6; 0.5 ml of 0.1 M-$MgCl_2$, and water to the final volume of 6 ml. The amount of particular fraction added to the incubation mixture was chosen to split not more than 10% of the substrate during the incubation period [21]. In control samples only the substrate was omitted. After 1 hr. incubation the reaction was stopped by the addition of 1 ml. of 50% TCA. The precipitated protein after centrifugation was rinsed with 5% TCA and the mixture was again centrifuged. In the combined TCA extracts, after neutralization, P_i was measured according to Fiske & Subbarow [9].

In the protein precipitates, after removal of lipids with a hot mixture of methanol and chloroform (3:1, v/v) the nitrogen content was determined by the micro-Kjeldahl method.

Morphological and cytochemical observations. Smears of the nuclear fraction were stained with methyl green with or without pyronine. For determination of alkaline phosphatase activity both in smears and in tissue slices the method of azo-coupling or the Gomori method were used [15].

RESULTS

The results of determinations of alkaline phosphatase activity in various subcellular fractions of the frog intestine are presented in Table 1. The figures show that nearly all activity of the enzyme, both in the starved and in the fed animals, was found in the microsomal fraction;

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the activity of the supernatant obtained at 92 000 *g* was especially low. It can also be seen that after feeding with olive oil but not with protein, a marked increase (by about 50%) of the activity of the enzyme was found in each of the subcellular fractions of the intestine, as compared with starved animals.

Table 1

Activity of alkaline phosphatase in subcellular fractions in the intestine of starved and fed frogs

The incubation mixture contained: either samples of the whole homogenate of the intestinal tissue, 5 to 25 mg., or suspensions of particular subcellular fractions, 0.1 to 1.0 ml., obtained from 2.50 g. tissue in 50 ml. of 0.25 M-saccharose (for details see Methods); sodium β-glycerophosphate, 30 µmoles; carbonate buffer, 0.6 m-mole, pH 9.6; $MgCl_2$, 50 µmoles; final volume, 6.0 ml. Time of incubation 1 hr. at 37°. The mean values, ± S. E. M., from 5 experiments are given, expressed as mg. P_i liberated per mg. nitrogen during 1 hr. at 37°.

Fraction	Starved	Fed with protein	Fed with olive oil
Whole homogenate	0.235 ± 0.009	0.251 ± 0.001	0.365 ± 0.006
Nuclei	0.253 ± 0.011	0.200 ± 0.007	0.295 ± 0.005
Mitochondria	0.312 ± 0.012	0.330 ± 0.005	0.509 ± 0.007
Microsomes	3.225 ± 0.210	3.700 ± 0.190	5.033 ± 0.234
Supernatant at 92 000 *g*	0.041 ± 0.001	0.039 ± 0.001	0.057 ± 0.002

The results of experiments performed on frogs which were starved during one to twelve months showed that the duration of fasting had no influence on the level of alkaline phosphatase activity in the intestine: the variations did not exceed 7%. The rate and the degree of the increase of the enzymic activity after feeding with fat were also not influenced by the duration of the preceding fasting; and two days after administration of olive oil, in all experiments a similar increase of the activity up to about 50% was observed.

Table 2 shows that although about 75% of the total alkaline phosphatase activity of the tissue was found in the microsomal fraction the amount of protein in this fraction was small. On the other hand, most of the protein of the cells was present in the supernatant fluid obtained after centrifugation at 92 000 *g* while the alkaline phosphatase activity of this fraction seemed to be very low.

Phosphatase activity in the nuclear fraction

An attempt was made to find out whether the activity of the enzyme found in the nuclear fraction was really present in the nuclei or whether it was due to a contamination with other fractions. Purification of the nuclei by the method of Hogeboom & Schneider [11] or by a modification

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Table 2

Distribution of alkaline phosphatase activity and of the amount of nitrogen in subcellular fractions of the intestine of starved and fed frogs

The results are expressed as percentages, in relation to the activity and nitrogen content of the whole homogenate taken as 100%.

Fraction	Starved		Fed with protein		Fed with olive oil	
	Activity (%)	Nitrogen (%)	Activity (%)	Nitrogen (%)	Activity (%)	Nitrogen (%)
Whole homogenate	100.0	100.0	100.0	100.0	100.0	100.0
Nuclei (600 *g*)	14.0	12.8	13.0	14.0	17.8	13.4
Mitochondria (7000 *g*)	4.0	5.2	6.0	5.0	5.4	4.5
Microsomes	73.5	2.9	73.0	3.8	75.0	4.0
Supernatant fluid (at 92 000 *g*)	5.7	79.0	6.0	74.0	6.0	76.0

of this method (lower concentration of $CaCl_2$ and EDTA) gave no satisfactory results: either fragments of the cytoplasm appeared to adhere still to the nuclei, or the nuclei themselves were damaged.

It could be supposed that if the nuclei contained the enzyme then, after rupturing of the nuclear structure, a higher activity of alkaline phosphatase should be found as a result of an easier contact of the enzyme with the substrate present in the medium.

The experiments showed that after destruction of the nuclei by intense homogenization or after treatment with 0.26% deoxycholate the activity of alkaline phosphatase did not change. These results seem to suggest that the enzyme is absent from the nuclei. Such view was also supported by a tentative cytochemical examination which showed that a positive reaction for the phosphatase activity was visible only in the cytoplasm adhering to the surface of the nuclei.

DISCUSSION

Distribution of alkaline phosphatase in subcellular fractions of the intestinal tissue

From the findings presented above it is evident that the activity of alkaline phosphatase in the intestine is mainly associated with the microsomal fraction. As it is well known, however, this fraction is very heterogeneous; it contains endoplasmic reticulum and membranes of brush border and of Golgi apparatus. Thus, the enzymic activity of the total microsomal fraction might be connected with some or with all the subcellular structures mentioned above.

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The results demonstrating that alkaline phosphatase is present mainly in the microsomal fraction are in accordance with our own histochemical findings as well as with those of some other authors. Thus, alkaline phosphatase was observed by electron and light microscopy on brush border of the intestine of mice [3], in the zone of Golgi apparatus [1, 8] and on lateral membranes of the epithelial cells [12]. The slight phosphatase activity found in the 92 000 *g* supernatant fluid depends most probably on the presence of some small fragments of membranes of endoplasmic reticulum, brush border or Golgi apparatus, which can never be completely separated by centrifugation.

A contamination of mitochondria with the microsomal fraction also seems to be probable: this view is supported by the electron micrographs of mitochondria in which the histochemical tests for phosphatases were negative [3, 12].

The most controversial point is the presence of alkaline phosphatase in the nuclei. As it was mentioned above, a microscopic observation showed that the nuclear fraction was never pure; it was contaminated by cytoplasmic fragments and by mucus. It is possible that the phosphatase activity found in the nuclear fraction was in reality only present in these contaminations. Isolation of pure nuclei from the intestine is much more difficult than from other tissues. This may be connected with the peculiar morphology of the intestinal epithelium in which the poles of nuclei in cylindrical epithelial cells are very strongly bound to the cytoplasm. The association is so close that even after a thorough homogenization of the intestine it was not possible to obtain pure nuclei without fragments of cytoplasm. Experiments in which some chemical reagents (e.g. deoxycholate) were used to purify the nuclei gave no positive results; although these agents were able to split the adhering cytoplasm they always destroyed the structure of the nuclei to a considerable extent.

Although no success has been achieved in obtaining and examining the pure nuclei it seems likely that they possess no alkaline phosphatase. This was proved indirectly by experiments in which the structure of the nuclei was destroyed and no increase of the enzymic activity was found. The negative results of histochemical tests performed on smears of the nuclear fraction also support this view.

The data in the literature concerning this problem are divergent. According to Roodyn [20] the activity of alkaline phosphatase in nuclei of the hepatic cells amounts to 10 - 40% of that found in the homogenate of the whole tissue. Baumann *et al.* [2] observed in the nuclear fraction from rat liver a phosphatase different from that of microsomal fraction: the pH optima and the activation by magnesium ions were different for the two enzymes. On the other hand, Stern *et al.* [23] applying a special technique for isolation of dehydrated nuclei, were able to show that in

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the nuclear fraction the activity of alkaline phosphatase is only 4% of the total. According to these authors this slight activity is due to contamination. The histochemical tests performed on tissue slices by means of azo-coupling were in the nuclei always negative [18].

A distribution of alkaline phosphatase similar to that described in this paper was observed in mucosa of the calf [13] and in the rat intestine [24, 19]. As the characteristics of the enzyme (Michaelis constant, pH optimum and activation by Mg^{2+} ions) were similar in all the cellular fractions, Triantaphyllopoulos & Tuba [24] also suggested that alkaline phosphatase is present in the microsomal fraction only and that the activity found in other fractions was due to contaminations. On the other hand, Clark & Porteous [4] indicate that alkaline phosphatase "is located in the microvilli and/or in the nuclei rather than in the endoplasmic reticulum of intestine epithelium cells" whereas according to Doell & Kretchmer [5] even most of the enzymic activity is present in the nuclear fraction. One may suppose that the conflicting conclusions discussed above depend most probably on the kind of the applied technique and hence on a different degree of purity of the fractions obtained.

Increase of the activity of alkaline phosphatase in the intestinal tissue after fat administration

The increase of the alkaline phosphatase activity after administration of lipid was observed by several authors in various animals. Motzok & Branion [15] suppose that in the intestine of the chicken under influence of food, which normally contains lipids, a synthesis of alkaline phosphatase takes place. This enzyme seems to be different from that present in the intestine just after hatching.

Tuba & Robinson [25] who have performed experiments on rats also suggest that two different alkaline phosphatases are present in the intestine; one of them is active permanently whereas the second one, the adaptive phosphatase, appears only after administration of fat.

The results of the present investigation are not incompatible with this view. Accordingly, the non-adaptive phosphatase would be the enzyme whose activity did not change even during a prolonged starvation of the frog. On the other hand, the extra activity of the enzyme found in the intestine after fat administration would correspond to the adaptive enzyme.

There is no sufficient experimental evidence so far to elucidate the mechanism of the considerable increase in the activity of the alkaline phosphatase discussed above. It seems to be premature to decide whether such large quantities of the enzyme could be synthesized in the intestine in a day or two after fat administration. Perhaps "the idea of changes in protein conformation causing changes in enzymic activity", as sug-

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gested by Popják [16] in relation to some enzymes, might be the more probable. Such changes could, perhaps, take place under the influence of some activators which would appear in the intestinal tissue in the course of fat absorption.

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SUMMARY

After separation of the subcellular fractions of the intestinal tissue of the frog nearly all activity of the alkaline phosphatase was found in the microsomal fraction. The slight activity observed in nuclear and mitochondrial fractions, and traces of the activity present in the amorphous supernatant fluid, might be considered as due to contamination derived from the microsomal fraction.

In about two days after feeding the frog with fat, the activity of alkaline phosphatase in the intestine was increased by approximately 50%, but no increase was observed after administration of protein.

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AKTYWNOŚĆ FOSFATAZY ALKALICZNEJ WE FRAKCJACH SUBKOMÓRKOWYCH JELITA ŻABY W CZASIE GŁODU I ŻYWIENIA

Streszczenie

Badano rozmieszczenie i aktywność fosfatazy alkalicznej we frakcjach komórkowych jelita żab głodzonych i karmionych. Stwierdzono, że aktywność tego enzymu związana jest głównie, a być może nawet wyłącznie, z frakcją mikrosomalną. Aktywność enzymatyczna znaleziona w innych frakcjach komórkowych pochodzi przypuszczalnie z zanieczyszczeń fragmentami mikrosomalnymi. Stwierdzono także, że po dwóch dniach od podania zwierzętom pokarmu lipidowego następuje wzrost aktywności fosfatazy alkalicznej o około 50%, natomiast podanie pokarmu białkowego nie wywoływało tego zjawiska. Otrzymane wyniki nasuwają przypuszczenie, że być może w jelicie znajduje się stała i adaptacyjna fosfataza alkaliczna.

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RECENZJE KSIĄŻEK

THE POPULATION CRISIS AND THE USE OF WORLD RESOURCES (Stuart Muud, ed.). Dr W. Junk Publishers - The Hague, 1964; str. 362+XIX.

Recenzowana książka jest drugim wydawnictwem Światowej Akademii Sztuki i Nauki (World Academy of Art and Science); pierwszym była książka „Science and the Future of Mankind", omówiona poprzednio w Acta Biochimica Polonica (vol. 10, R1, 1963).

Zagadnienie, któremu jest poświęcone recenzowane dzieło, dotyczy zakłócenia równowagi ekologicznej, wywołanego przez to, że z wydatnym zmniejszeniem śmiertelności, będącym skutkiem zastosowania nowoczesnej medycyny, nie idzie w parze redukcja urodzin; obecny roczny przyrost ludności świata wynosi około 50 milionów, a brak jest podstaw, które pozwalałyby przypuszczać, że w ciągu najbliższych lat dziesięciu przyrost ten ulegnie zmniejszeniu lub nawet tylko utrzyma się na obecnym poziomie. Postęp techniczny nie może nadążyć w dostarczeniu żywności dla stale rosnącej liczby ludzi. W oparciu o stojące dziś do dyspozycji przesłanki należy liczyć się z tym, że liczba głodnych i niedożywionych ludzi będzie rosła w ciągu najbliższych 20 lat, nawet gdyby zrobiono wszystko, co wydaje się dziś możliwe do zrobienia. Stwarza to sytuację szczególnie niebezpieczną, zwłaszcza wobec istnienia nowoczesnych środków zagłady, które mogłyby zachwianą równowagę przywrócić przez szaleństwo wojny, związane z nie dającym się wyrazić ogromem ludzkiego cierpienia. Najwyższy już czas postawić ten problem odważnie i rozpatrzyć go starannie opierając się na ścisłych danych. Omawiana książka takie właśnie dane przedstawia.

Wprowadzeniem w istotę zagadnienia są trzy artykuły wstępne, napisane przez Adlai E. Stevensona, Bertranda Russella i Juliana Huxleya. Następne czterdzieści kilka artykułów jest podzielone na dwie grupy. W pierwszej omówiono kryzys ludnościowy, w drugiej użycie zasobów świata, dostępnych obecnie ludzkości lub takich, które będzie można udostępnić w najbliższych dziesiątkach lat. W części pierwszej omówiono następujące zagadnienia: dane liczbowe dotyczące geograficznego rozmieszczenia ludności ziemi, jej wzrostu, zwłaszcza w ostatnich dziesięcioleciach, charakteru tego wzrostu w poszczególnych krajach; przeprowadzono analizę ekonomiczną, socjalną i polityczną zagadnienia; podano dane dotyczące kryzysu ludnościowego Indii, Pakistanu, Zjednoczonej Republiki Arabskiej, Chin, Japonii, Ameryki Łacińskiej i Stanów Zjednoczonych; przedstawiono zagadnienia biologiczne związane z problemem ludnościowym, wreszcie w siedmiu pracach omówiono program działania w skali międzynarodowej. Część druga książki (dwanaście artykułów) zajmuje się takimi sprawami, jak plan instytutu badań nad lepszym wyzyskaniem zasobów naszego globu; sprawa właściwego wyzyskania gleby i wód oraz ochrony ich przez niszczeniem; naturalne zasoby krajów rozwijających się; praktyczne znaczenie energii jądrowej; zestawienie zasobów świata dostępnych obecnie i w przyszłości.

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Myśli i fakty podane w omawianej książce zdają się dobitnie wskazywać, że dalsze zaniedbywanie problemu ludnościowego grozi katastrofą; znalezienie drogi postępowania, które pozwoliłoby na uniknięcie tej katastrofy, chociaż wcale nie łatwe, nie zdaje się być niemożliwe. Piszącemu tę ocenę wydaje się, że najwłaściwiej istotę tych trudności ujął Adlai E. Stevenson w słowach: „Nauczyliśmy się kierować światem fizycznym prędzej, zanim nauczyliśmy się kontroli nas samych".

Włodzimierz Mozołowski

BRAIN LIPIDS, LIPOPROTEINS AND THE LEUCODYSTROPHIES (J. Folch-Pi & H. Bauer, eds.) Elsevier Publishing Co., Amsterdam, London, New York, 1963.

Omawiana książka stanowi zbiór referatów przedstawionych na Sympozjum Neurochemii VII-go Międzynarodowego Kongresu Neurologii, który odbył się w 1961 r. w Rzymie. Zakres zagadnień omawianych w książce jest bardzo szeroki, począwszy od biochemii lipoproteidów osocza i tkanki nerwowej do szczegółowego przedstawienia obrazów klinicznych leukodystrofii. Leukodystrofie są to wrodzone zmiany chorobowe, których podłoże stanowi brak określonego enzymu. W schorzeniach tych dochodzi do głębokich zmian w składzie lipidowym tkanki nerwowej, głównie substancji białej. Zamierzeniem organizatorów Sympozjum było zilustrowanie dotychczasowych i rozwinięcie dalszych powiązań biochemii z neurologią na przykładzie właśnie leukodystrofii. O ile jednak umieszczenie referatów z różnych dziedzin w ramach jednego sympozjum może być celowe, gdyż pozwala na rozwinięcie szerszej dyskusji wśród uczestników, publikowanie tych samych materiałów jako jednego tomu wydawniczego nie przynosi już tej korzyści, tym bardziej, że w książce nie umieszczono wypowiedzi w dyskusji nad poszczególnymi referatami.

Pierwszą część książki stanowią referaty poświęcone biochemii kompleksów lipidowo-białkowych, związków charakterystycznych dla tkanki nerwowej, badaniom ultrastruktury mieliny oraz badaniom nad właściwościami antygenowymi sfingolipidów. W rozdziałach tych zawarte są niepublikowane gdzie indziej oryginalne dane, np. o rozmieszczeniu proteolipidów w tkance nerwowej i metodzie ich rozdziału chromatograficznego. Autorzy referatów zgodnie uważają, że proteolipidy stanowią ważne zagadnienie nie tylko w biochemii lipidów lecz i w biochemii białek. Prawdopodobnie wszystkie białka tkankowe występują w stanie rodzimym jako lipoproteidy. Zaburzenia biochemiczne w leukodystrofiach są tematem obszernego referatu L. Svennerholma. Podane tam są zmiany składu lipidowego obserwowane w tych schorzeniach ze szczególnym uwzględnieniem rodzaju kwasów tłuszczowych występujących w tłuszczach złożonych. Badania te pozwoliły Autorowi wyróżnić dwa typy syntezy kwasów tłuszczowych w tkance nerwowej: proces cytoplazmatyczny prowadzący do wytworzenia kwasów tłuszczowych C_{18} i proces zachodzący w błonach komórkowych prowadzący do powstania kwasów tłuszczowych C_{24}. Blisko połowę objętości książki stanowią referaty zajmujące się histopatologią i kliniką leukodystrofii.

Każdy z referatów zawiera spis cytowanej literatury. Książka zaopatrzona jest w indeks nazwisk i indeks rzeczowy. Omawiana książka zasługuje na zainteresowanie biochemików i lekarzy neurologów.

Tadeusz Chojnacki

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K. E. Malten and R. L. Zielhuis, INDUSTRIAL TOXICOLOGY AND DERMATOLOGY IN THE PRODUCTION AND PROCESSING OF PLASTICS. Elsevier Publishing Company, Amsterdam, London, New York 1964; stron XIV+258; cena Dfl. 25, 50 s., 28 DM.

Książka K. E. Maltena i R. L. Zielhuisa jest kolejną pozycją z serii znanych i wysoko cenionych monografii, wydawanych przez Elsevier Publ. Comp., dotyczących działania substancji toksycznych na ustrój człowieka. Część ogólna wprowadza w zagadnienia związane z metodami i etapami syntezy omawianych związków wielkocząsteczkowych (polimeryzacja, poliaddycja i polikondensacja), budową chemiczną oraz zależnością działania biologicznego od struktury i własności fizyko-chemicznych. Informuje ona ponadto o ważnych z toksykologicznego punktu widzenia substancjach pomocniczych, stosowanych w technologii mas plastycznych, oraz o charakterze chorób skóry występujących w wyniku kontaktu z tymi związkami. To ostatnie zagadnienie obejmuje problematykę kryteriów diagnostycznych, mechanizmu patogenetycznego ze szczególnym uwzględnieniem uczulenia alergicznego, tolerancji nabytej, metodyki i interpretacji testów skórnych oraz ogólnych zasad leczenia różnych postaci zawodowych uszkodzeń skóry, występujących u pracowników przemysłu mas plastycznych.

Część szczegółowa omawia w zasadzie wszystkie rodzaje tworzyw sztucznych i substancji pomocniczych wytwarzanych przez współczesny przemysł z wyjątkiem syntetycznej gumy. Uwzględnia ona informacje dotyczące własności fizyko-chemicznych i zastosowania omawianych związków, dane z zakresu toksykologii ogólnej, zarówno doświadczalnej, jak i klinicznej, swoistego działania na skórę oraz metod zapobiegania. Poszczególne rozdziały poświęcone są masom fenolowym i aminowym, żywicom alkidowym, nienasyconym poliestrom, izocyjanianom, żywicom etoksylinowym (epoksydowym), polimerom: winylowym, akrylowym i organofluorowym oraz włóknom syntetycznym i szklanym.

W zakresie substancji pomocniczych monografia omawia szczegółowo zagadnienia doświadczalne i kliniczne związane ze stosowaniem, jako wypełniacza mas plastycznych, bezpostaciowego dwutlenku krzemu, starterów polimeryzacji z grupy organicznych nadtlenków, metalicznych stabilizatorów używanych pod postacią mydeł, soli i związków metaloorganicznych, katalizatorów i akceleratorów, takich jak połączenia glinowo-alkilowe i związki boru. Ponadto uwzględnia szereg innych substancji odgrywających rolę w technologii tworzyw sztucznych, a w szczególności związki pieniące, zapobiegające utlenianiu i plastyfikatory, a wśród tych ostatnich przede wszystkim ftalany, niektóre organiczne estry kwasu fosforowego, związki epoksydowe, chlorowane parafiny, oleje antracenowe, glikole i ich pochodne.

Ostatni rozdział poświęcony jest sposobom zapobiegania dermatozom występującym u pracowników przemysłu mas plastycznych.

Duża wartość monografii polega przede wszystkim na szczegółowym opisie właściwości biologicznych związków, których zastosowanie w przemyśle i w życiu codziennym wzrasta niezwykle szybko, a których nie uwzględniają w dostatecznym stopniu nawet obszerne klasyczne podręczniki toksykologii klinicznej. Dzięki temu, jak też i bogatemu piśmiennictwu, podręcznik spełnia zasadniczą intencję Autorów, by z jednej strony służyć pionowi lekarskiemu jako przewodnik w ocenie skutków biologicznych mogących powstać w wyniku kontaktu z masami plastycznymi, z drugiej zaś — udzielić niezbędnych informacji pionowi technicznemu w zakresie wartości parametrów toksykologicznych oraz zabezpieczenia ludzi przed szkodliwym działaniem tych związków.

Kazimierz Spett

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W przeciwieństwie do tomów obrazujących dorobek wcześniejszych sympozjów neurochemicznych, omawiany tom niestety nie zawiera wypowiedzi w dyskusji.

Na podkreślenie zasługuje staranna redakcja i bardzo ładna szata zewnętrzna książki, co jest szczególnie ważne ze względu na zamieszczone zdjęcia z mikroskopu elektronowego.

Stella Niemierko

D. C. Darrow, A GUIDE TO LEARNING FLUID THERAPY. Charles C. Thomas Publ., Springfield (Ill.) 1964; str. 280; cena $ 9.75.

Zagadnienia związane z gospodarką wodno-mineralną weszły głęboko do praktyki klinicznej dnia codziennego, szczególnie w tych stanach, które czy to wskutek nagłych urazów, czy z powodu przewlekłych procesów chorobowych, prowadzą do zasadniczych w tym zakresie zakłóceń. Jednak u podstawy wszelkich ocen, jak i działalności praktycznej, musi stać dobra znajomość praw biochemicznych i ich fizjologicznych dla ustroju skutków. Dlatego Autor w pierwszej, ogólnej części książki przypomina i wyjaśnia podstawowe pojęcia z zakresu fizykochemii roztworów, zasady dyfuzji i osmozy oraz prawa rządzące równowagą kwasowo-zasadową w roztworach wodnych, dając im matematyczne ujęcie. Skład płynów ustrojowych podlega jednak stosunkowo niedużym wahaniom dzięki regulującej czynności nerek oraz krążeniu, które z jednej strony decyduje o funkcji nerek, z drugiej zaś strony zapewnia normalną metaboliczną czynność struktur komórkowych. Dlatego też tym dwom dziedzinom Autor poświęca szczególnie dużo uwagi, omawiając szczegółowo zarówno właściwości krążenia, jak i fizjologiczną czynność nerek z punktu widzenia ich wpływu na gospodarkę wodno-mineralną. Od sprawności krążenia zależy nie tylko dostateczne zaopatrzenie całego ustroju w niezbędny dla jego czynności tlen, ale przede wszystkim wszelkie procesy wymiany dokonujące się na obwodzie dzięki sprawnej czynności całego układu włośniczkowego. Wszelkie w tym zakresie zaburzenia, zarówno ostre, jak i przewlekłe, które Autor szczegółowo omawia, w sposób decydujący wpływają na rozmieszczenie płynów w różnych przestrzeniach pozakomórkowych, a w dalszej konsekwencji i na skład płynów wewnątrzkomórkowych.

Właściwa jednak regulacja składu wewnętrznego ustroju jest najważniejszą czynnością nerek. Dla jej zrozumienia Autor omawia czynność regulującą zarówno kłębków, jak i kanalików nerkowych, zwracając szczególną uwagę na jej znaczenie dla zapewnienia stałości składu środowiska wewnętrznego, zwłaszcza dla utrzymania równowagi kwasowo-zasadowej. Na tę równowagę, jak i wszelkie odmiany jej zwichnięcia, Autor zwraca dużą uwagę, poświęcając jej — niezależnie od bieżącej odcinkowej dyskusji — specjalne rozdziały. Autor, zainteresowany zagadnieniami pediatrycznymi, szczegółowo omawia wpływ ostrych zaburzeń jelitowych na gospodarkę wodno-mineralną. Wobec coraz częstszych uszkodzeń termicznych, jeden rozdział Autor poświęca zagadnieniom oparzeń. Ponadto przedmiotem szczegółowych rozważań są zmiany w równowadze wodno-mineralnej u chorych po zabiegach chirurgicznych w ostrej i przewlekłej niedomodze nerek oraz mechanizmy obrzęków i udział w nich regulacji hormonalnej, szczególnie hormonów sterydowych.

Każdy z tych stanów jest traktowany z punktu widzenia możliwości jego leczenia przy pomocy odpowiednio do potrzeb zestawionych płynów leczniczych, których składowi Autor poświęca jeden rozdział.

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Jak wynika z przeglądu treści książki, D. C. Darrow zwraca dużą uwagę na podstawowe zagadnienia związane z gospodarką wodną i mineralną człowieka w stanie zdrowia i choroby. Właściwe rozplanowanie dzieła z szerokim omówieniem podstaw fizyko-chemicznych pozwala na głębsze wniknięcie w złożony mechanizm dokonywających się przemian i tym samym na oparcie zabiegów terapeutycznych na pewniejszych podstawach. Wydaje się jednak, że pewna niejasność wywodów częściowo osłabia zamierzony efekt. Być może, że jest to wynikiem nadmiernej zwięzłości pierwszej części dzieła, omawiającej właśnie fizyko-chemiczne podstawy regulacji wodnej i mineralnej, a wynikającej z założenia, że czytelnik temat ten ma doskonale opanowany i należy mu przypomnieć tylko zasady, by już bez trudności mógł wniknąć w gąszcz najrozmaitszych regulacji. Doświadczenie jednak życia codziennego pozwala na stwierdzenie, że niejeden lekarz zainteresowany sprawą racjonalnego leczenia zaburzeń wodno-mineralnych właśnie tych wiadomości nie posiada w wystarczającej mierze, gdyż albo już ich nie pamięta albo — jeśli należy do starszego pokolenia — z zagadnieniami tymi mógł się nie zetknąć. Dlatego należałoby Autorowi tej pięknej książki zasugerować, by w następnym wydaniu uwagi te zechciał wziąć pod rozwagę i by tę część swej książki rozszerzył. Czytelnik jednak, który zada sobie trud, by te problemy ogólne sobie przyswoić lub ich znajomość odświeżyć, przeczyta całość z wielką dla siebie korzyścią, znajdując tu wiele wskazówek dla postępowania leczniczego.

Książka D. C. Darrow'a stanowi cenną pozycję, z którą powinni zapoznać się lekarze licznych specjalności, gdyż sprawa leczenia zaburzeń w gospodarce wodnej należy do zagadnień życia codziennego. Badacz natomiast znajdzie cenne zestawienie bibliograficzne, obejmujące nieomal wszystkie poważniejsze publikacje amerykańskie dotyczące zaburzeń w gospodarce wodnej i mineralnej. Niewątpliwym mankamentem jest zupełne pominięcie prac pochodzących z obszaru poza-amerykańskiego.

Szata graficzna dzieła jest bardzo staranna.

Marian Górski

A. Soffer, H. Spencer, M. Rubin, M. Chenoweth, L. Eichhorn and B. Rosoff, CHELATION THERAPY. Charles C. Thomas Publisher; Springfield (Ill.) 1964; str. IX + 163; 37 ryc. i 14 tabel; cena $ 8.00.

W ciągu ostatniego dziesięciolecia obserwuje się wśród klinicystów stale wzrastające zainteresowanie związkami, które mają zdolność tworzenia połączeń chelatowych z różnymi metalami. Zainteresowanie to wynika stąd, że niektóre spośród tych związków znalazły zastosowanie w leczeniu pewnych stanów chorobowych. Zadaniem, jakie podjęli Autorzy omawianej książki, była ocena wartości i przydatności niektórych nowszych środków chelatujących w terapii schorzeń internistycznych. Materiał zawarty w książce podzielony jest na siedem rozdziałów, z których każdy opracowany został przez jednego lub dwóch autorów i każdy stanowi zamkniętą całość. Wprawdzie materiał przedstawiony w poszczególnych rozdziałach oparty jest przede wszystkim na własnym, bogatym doświadczeniu autorów, to jednak w dyskusji poszczególnych zagadnień zarówno wyniki badań, jak i poglądy innych autorów znalazły należne im miejsce.

Pierwszy rozdział poświęcony jest omówieniu związku między tworzeniem połączeń chelatowych a działaniem leków. Bardzo interesujące są sugestie dotyczące zależności działania niektórych antybiotyków i kortykosterydów od tworzenia połączeń chelatowych, jak również te, które odnoszą się do ewentualnej roli związ-

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ków chelatowych w przenoszeniu metali przez błony komórkowe podczas normalnej przemiany związków mineralnych.

W rozdziale drugim omówione zostały te efekty działania pochodnych kwasu etyleno-dwuamino-czterooctowego (EDTA) w organizmie żywym, które są niezależne od wiązania jonów wapnia. Rozdział ten zawiera również opis działania farmakologicznego 2,3-dwumerkapto-propanolu (BAL).

Rozdział trzeci jest napewno najbardziej interesujący dla internisty praktyka, omawia bowiem zastosowanie soli sodowej EDTA w leczeniu chorób układu krążenia. Wpływ ten, związany ze zdolnością wiązania jonów wapnia przez EDTA, wyraża się między innymi polepszeniem przewodnictwa przedsionkowo-komorowego w blokach serca a także usuwaniem ektopicznych, komorowych skurczów dodatkowych oraz częstoskurczu komorowego. Znaczenie praktyczne Na_2EDTA w leczeniu wymienionych stanów chorobowych wydaje się nie podlegać żadnej dyskusji tym bardziej, że związek ten daje tylko bardzo nieznaczne i rzadko występujące objawy uboczne.

Rozdział czwarty podzielony jest na dwie części. W pierwszej z nich omówiony jest wpływ różnych związków tworzących połączenia chelatowe na wydalanie izotopów promieniotwórczych u ludzi. Przedstawione dane wskazują na wyraźny wpływ niektórych spośród tych związków na wzrost wydalania pierwiastków ziem rzadkich oraz cynku i strontu. W drugiej części rozdziału omówiono szczegółowo objawy uboczne, które mogą wystąpić u ludzi podczas podawania środków chelatujących (głównie pochodnych EDTA).

Rozdział następny poświęcony jest roli połączeń chelatowych w przemianie żelaza. To bardzo interesujące zagadnienie przedstawione zostało przede wszystkim z punktu widzenia zastosowania środków chelatujących w tych stanach chorobowych, w których postępowanie terapeutyczne ma na celu zmniejszenie absorpcji żelaza z przewodu pokarmowego lub zwiększenie jego wydalania wraz z moczem. Rozdział szósty jest właściwie uzupełnieniem rozdziału trzeciego, ponieważ dyskutowany w nim jest mechanizm działania EDTA na mięsień sercowy. W odróżnieniu od poglądów przyjmujących, że związek ten wywiera wpływ na akcję serca przez zmianę stosunku ilościowego między stężeniem potasu i wapnia, Autor stoi na stanowisku, że efekt działania EDTA jest spowodowany zmniejszeniem bezwzględnej zawartości wapnia zjonizowanego w płynach pozakomórkowych. Wydaje się, że ten rozdział, w odróżnieniu od pozostałych, potraktowany został zbyt powierzchownie.

W rozdziale ostatnim przedstawione zostały podstawowe dane z chemii połączeń chelatowych.

Całość napisana została w sposób jasny, zwięzły i interesujący. Uwagi krytyczne, które nasuwają się przy czytaniu omawianej książki, są nieliczne i w niczym nie zmniejszają jej dużej wartości. Dotyczą one przede wszystkim powtarzania się (zresztą nie częstego) niektórych wiadomości w różnych rozdziałach podręcznika. Przypuszczalnie jest to wynikiem niezbyt szczegółowego uzgodnienia zakresu przedstawionego materiału między Autorami poszczególnych rozdziałów. Dalsza uwaga dotyczy kolejności rozdziałów w książce. Wydaje się, że bardziej logiczne byłoby umieszczenie dwóch ostatnich rozdziałów (chemia połączeń chelatowych i fizjologiczne działanie EDTA) na początku książki. Wreszcie należy podkreślić, że zarówno po przeczytaniu poszczególnych rozdziałów, jak i całej książki odczuwa się wrażenie niedosytu i uczucie rozczarowania, że pewne zagadnienia poruszane przez Autorów nie zostały potraktowane w sposób bardziej wyczerpujący. Wprawdzie dla zainteresowanych czytelników Autorzy podają dużo odsyłaczy do bogatej literatury, to jednak z prawdziwą przyjemnością przyjęłoby się rozszerzenie przez Autorów całego szeregu poruszonych przez nich tematów dyskusyjnych.

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Omawiana książka przeznaczona jest przede wszystkim dla klinicystów, wśród których znajdzie ona na pewno bardzo licznych i wdzięcznych odbiorców. Wydaje się jednak, że powinna ona spotkać się również z głębokim zainteresowaniem i dużym uznaniem farmakologów, fizjologów i biochemików.

Andrzej Manitius

S. H. Lawrence, THE ZYMOGRAM IN CLINICAL MEDICINE. Charles C. Thomas Publisher, Springfield (Ill.) 1964; str. XV + 100; cena $ 5.75.

Książka S. H. Lawrence'a jest 587-mą publikacją z serii American Lecture Series i jest przeznaczona dla biochemików klinicznych oraz lekarzy stosujących metody enzymologiczne jako narzędzie w diagnostyce lub pracach doświadczalnych.

Autor zebrał i przedstawił w sposób bardzo krótki i zwięzły dostępne dane o izozymach przeszło trzydziestu różnych enzymów dających się wykryć na zonalnych elektroforogramach surowic ludzkich. Główny nacisk położony jest przy tym na wnioski diagnostyczne, jakie wyciągnąć można z otrzymanych tym sposobem „zymogramów".

W pierwszym rozdziale przedstawiono krótki rys historyczy zagadnienia heterogenności enzymów i roli, jaką w jego rozwoju odegrała elektroforeza na żelu skrobiowym oraz zastosowanie barwników histochemicznych dla wywoływania tych elektroforogramów. W drugim rozdziale Autor omawia nomenklaturę izozymów, podając sposoby oznaczania frakcji na zymogramach, stosowane przez różne grupy badaczy. Niestety, jak zwykle w sprawach nazewnictwa, nie ma tu prób uogólnienia i ustalenia jakiegoś określonego układu odniesienia umożliwiającego porównywanie wyników.

Rozdziały od III do VIII są poświęcone omawianiu poszczególnych grup enzymów. Przytoczone są schematy zymogramów i opisy przypadków zilustrowane fotografiami odpowiednich zymogramów oraz podane są własności poszczególnych izozymów, a także obszerne piśmiennictwo.

Rozdział IX, omawiający znaczenie zymogramów w diagnostyce lekarskiej, jest właściwie obszerną tablicą, wskazującą jakie zmiany zymogramów spotykane są w różnych stanach chorobowych. Niestety brak jest krytycznej analizy lub sugestii wyboru odpowiednich metod, tak że czytelnikowi pozostawiona jest właściwie tylko możliwość uczenia się na własnych błędach.

Ostatnie dwa rozdziały, oddzielone od siebie nieco sztucznie, wydają się zbędne. Rozdział X o znaczeniu zymogramu dla genetyki zawiera informacje bardzo fragmentaryczne i niekompletne, połowa zaś rozdziału składa się z rysunku zawierającego wiadomości o DNA, RNA i rybozomach w zakresie popularno-naukowym.

Interesującą i wartościową częścią książki jest zamieszczone na końcu zestawienie zawierające opisy metod elektroforezy zonalnej na żelu skrobiowym oraz immunoelektroforezy, a także tablice dla sporządzania buforów i opisy techniki wywoływania różnych enzymów na elektroforogramach. Żałować należy jedynie, że opisy metod są zbyt lakoniczne.

Na szczęście drobne usterki nie zmieniają faktu, że książka S. H. Lawrence'a jest monografią wartościową dla każdego, kto interesuje się sprawą heterogenności enzymów, zwłaszcza w odniesieniu do patologii ludzkiej.

Michał Bagdasarian

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BONE AND TOOTH. Proceedings of the First European Symposium held at Somerville College, Oxford, April 1963 (H. J. J. Blackwood, ed.). Pergamon Press, Oxford, London, New York, Paris, 1964; str. 425; cena 5 £.

Materiały sympozjalne dotyczące tkanki kostnej i zębów obejmują wiele ciekawych i aktualnie ważnych zagadnień. Podkreślenia godną cechą tego sympozjum jest zarówno jego wysoki poziom naukowy w dziedzinach ściśle teoretycznych, jak i duże zrozumienie dla zagadnień praktycznych, zarówno z dziedziny radiobiologii, jak i kliniki układu tkanek mineralizujących.

L. B. H. Tarlo dyskutuje z punktu widzenia paleozoologa genezę tkanki kostnej, łącząc jej ewolucję z tkanką kostną występującą w skórze wymarłych gatunków najwcześniejszych ssaków morskich.

W szeregu referatów wykazano użyteczność posługiwania się tetracyklinowymi antybiotykami przy badaniu przebudowy tkanki kostnej. Związki te wbudowują się do nowopowstających blaszek kostnych i dają się łatwo ujawniać w mikroskopie fluorescencyjnym. Przykładem prac z tej dziedziny są ilościowe badania R. Amprino i G. Marottiego nad przebudową szkieletu. Praca R. Steendijka poświęcona była badaniu niejasnego nadal mechanizmu wiązania się tetracyklin z nowopowstającą kością. Praca H. A. Sissona i W. R. Lee jest przykładem dyskusji nad zastosowaniem klinicznym tetracyklin w badaniu układu kostnego, a także zawiera ciekawe porównanie stosowania tej metody z metodą badania przemiany układu kostnego przy pomocy wapnia ^{45}Ca.

Wiele prac poświęcono oczywiście sprawom radiobiologicznym, a w szczególności metabolizmowi radioizotopów o wybitnym powinowactwie do kości. Autoradiografia śladowa cząsteczek alfa, zastosowana w pracy M. Williamson i J. Vaugham, pozwoliła na szczegółowe przedyskutowanie miejsca odkładania się takich izotopów jak ^{239}Pu, ^{241}Am i ^{91}Y. Wśród licznych prac na ten temat wybija się nieco ryzykancka praca J. Rundo i współpracowników wykonana na trzech ochotnikach, którym przez miesiąc podawano w diecie dziennie 0,5 μc ^{85}Sr. Mimo znikomych dawek i stosunkowo krótkiego okresu połowicznego rozpadu użytego izotopu, brak w pracy komentarzy na temat ew. szkodliwości takich doświadczeń dla ochotników jest rażący.

Znaczna część prac sympozjalnych dotyczyła regulacji gospodarki fosforanowo-wapniowej. Do najciekawszych należy praca MacIntyre'a i współpracowników omawiająca krytycznie odkrycie kalcytoniny przez Coppa w 1962 r. Kalcytonina jest niewyosobnionym jeszcze hormonem przytarczyc, antagonistycznym co do swego wpływu na poziom wapnia w osoczu w stosunku do parathormonu.

W związku z zagadnieniami regulacji gospodarki fosforanowo-wapniowej wygłoszono na sympozjum szereg podstawowych prac fizykochemicznych dotyczących mechanizmu mineralizacji tkanki kostnej. Rola kollagenu i zasadowej fosfatazy badana była na modelowym doświadczeniu *in vitro*, wykonanym przez H. Fleischa i S. Bisaz. Wykład Honor Fell dotyczył wpływu witaminy A na metabolizm chrząstki. Badania prowadzone od kilku lat w Cambridge nad mechanizmem tych procesów wskazują na wpływ regulacyjny witaminy A na aparat lizosomalny chondrocytów. Autorka podkreśla przydatność metody hodowli narządowej dla badań czynnościowych.

Trzeba wreszcie wspomnieć o szeregu interesujących prac teoretycznych dotyczących struktury i metabolizmu zębów.

Należy zaznaczyć, że w książce zawarta jest również treść dyskusji nad referatami. Niektóre z wypowiedzi, jak np. prof. Gaillarda dyskutującego wykład H. Fell, są nie mniejszej wartości niż teksty oryginalne.

Szata wydawnicza książki jest na najwyższym poziomie.

Kazimierz Ostrowski

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