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FORMYLTETRAHYDROFOLATE SYNTHETASE FROM AN URICOTELIC INSECT, *GALLERIA MELLONELLA* L. (LEPIDOPTERA)

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This paper is dedicated to Professor Włodzimierz Niemierko in honour of his seventhieth birthday

Properties of an insect formyltetrahydrofolate synthetase were studied. The enzyme appeared to be unstable and thermosensitive with optimum of activity at 36.5 - 37° and pH 8.0 - 8.1, and to be activated synergistically by Mg^{2+} and NH_4^+. The K_m values of the three substrates and of the two cations were calculated. Since K_m values of ATP and Mg^{2+} were of the same order (0.13 and 0.18 mM), and since an excess of ATP or Mg^{2+} inhibited the enzyme, the possibility was discussed that ATP-Mg complex rather than free ATP may be its real substrate. Similarly, the correspondence of K_m values of formate and NH_4^+ (17 and 14 mM) and nearly 50-fold increase in K_m value of formate in the absence of NH_4^+ allowed to consider the role of NH_4^+ in binding formate to the enzyme molecule. The probable mode of enzyme inhibition by aminopterin was also discussed.

Formyltetrahydrofolate synthetase (formate : tetrahydrofolate ligase (ADP), EC 6.3.4.3.) catalyses the reaction

$$\text{formate} + \text{ATP} + \text{tetrahydrofolate} \rightleftharpoons N^{10}\text{-formyltetrahydrofolate} + \text{ADP} + P_i.$$

The mechanism of this reaction was studied in detail by Himes and coworkers who investigated the kinetics of the cation activation (Himes & Wilder, 1965), the equilibrium reaction rates and binding of the substrates with the enzyme molecule using the enzyme crystallized from *Clostridium cylindrosporum* (Joyce & Himes, 1966a, b). Their results indicate that the enzyme binds the ammonium cation before tetrahydrofolate and formate, and all the substrates must be bound before any products are released.

One of the products of the reaction, the N^{10}-formyltetrahydrofolate ($f^{10}FH_4$), serves as a donor of one-carbon unit in the biosynthesis of the purine ring either directly in enzymic formylation of 5-amino-4-imidazolecarboxamide ribotide or after interconversion into N^{5-10}-methenyltetrahydrofolate ($f^{5-10}FH_4$) in formylation of glycinamide ribotide. It is obvious therefore that the activity of the $f^{10}FH_4$ syn-

thetase is very high in organisms or tissues with high rate of nucleotide metabolism. This is also the case with insect tissues (Grzelakowska-Sztabert & Zielińska, 1967). The present study was undertaken to determine the properties of formyltetrahydrofolate synthetase from insect tissues. It was expected that this would provide some evidence as to the action mechanism of the enzyme.

MATERIALS AND METHODS

Source of enzyme. Last instar larvae of the waxmoth *Galleria melonella* L. (Lepidoptera) bred in this Laboratory were taken for experiments. After homogenization of the larvae in Waring blendor in ice-cold tris-HCl buffer (pH 8.0) the extract was centrifuged at 500 *g* in MSE centrifuge for 5 - 10 min. After removal of lipid the sample was centrifuged once more at 10 000 *g* for 20 min. and the supernatant obtained in this way was used for the enzyme assays. If necessary, to remove free multivalent cations the enzyme extracts were treated with resin Dowex 50 W-4 (200 - 400 mesh) or occasionally with Chelex 100. For routine experiments an acetone-dried and ethyl ether-treated preparation was prepared and stored in cold but no longer than for two or three weeks because of inactivation of the enzyme. When such a lipid-free dry material was a source of the enzyme, it was suspended in the buffer solution and centrifuged at 10 000 *g*.

Chemicals. The following chemicals were commercial products: ATP and ADP sodium salt (C.F. Boehringer and Soehne GmbH, Mannheim, Germany), folic acid (British Drug Houses, London, England), tris, DL-cysteine hydrochloride monohydrate (Fluka A.G., Buchs S.G., Switzerland), β-mercaptoethanol and aminopterin (Koch-Light Lab. Ltd., Colnbrook, England). All the other chemicals were of analytic grade.

Tetrahydrofolate (FH_4) prepared after Hatefi, Talbert, Osborn & Huennekens (1960) by catalytic reduction of folate using the modification of the method proposed by O'Dell, Vandenbelt, Bloom & Pfifner (1947) was either kindly given by Dr. K. Slavík from the Laboratory of Protein Metabolism, Charles University, Prague, or produced in the Department of Organic Technology II of Technical Institute, Warsaw. Tetrahydrofolate was stable when stored at 0° *in vacuo* as a powder. Immediately prior to use, a solution of tetrahydrofolate was prepared by dissolving 4.4 mg. of the substance in 1 ml. of equal parts of 0.1% $NaHCO_3$ and 0.6 mM-cysteine solution at pH 7.0.

Analytical procedures. Protein content in the samples was determined either by the nephelometric method described by Bücher (1947) or calculated from the total nitrogen estimated by the Kjeldahl method. All the orthophosphate determinations were performed according to Fiske & Subbarow (1925). In some experiments after acid hydrolysis of the samples the content of the phosphate groups of ATP was calculated.

Enzyme assays. The standard assay mixture (based on the mixture used by Šhejbal, Slavik & Souček, 1962) contained: 0.66 μmole of DL-FH_4, 40 μmoles of

sodium formate, 0.66 μmole of ATP Na salt, 4 μmoles of cysteine, 2.6 μmoles of $MgCl_2$, 33 μmoles of NH_4Cl, 100 μmoles of tris-HCl buffer at pH 8.0, and the enzyme extract (100 - 200 μg. of protein) in a total volume of 1 ml. Immediately after addition of the enzyme, a part of the sample was acidified with 8% perchloric acid and left standing for 10 - 15 min., then any denaturated protein was removed by centrifugation and the solution used as a blank. The rest of the sample was incubated at 36.5° for 15 min., deproteinized and treated similarly as the blank. Under

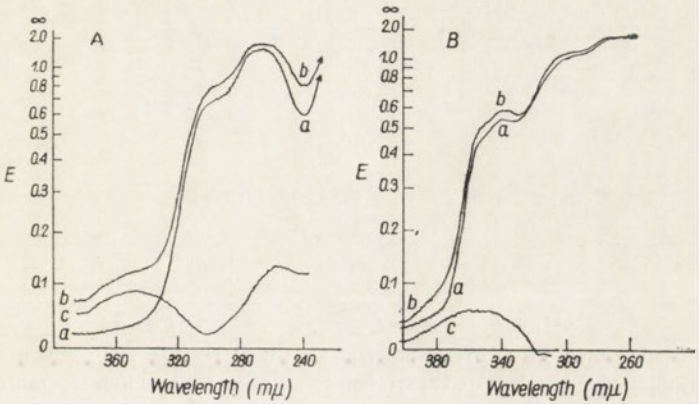

Fig. 1. Absorption spectra (in $HClO_4$), *A*, of the standard reaction mixture (diluted fivefold) used for the assay of the activity of $f^{10}FH_4$ synthetase, and *B*, of the standard mixture with addition of aminopterin (diluted tenfold). Curve *a*, before incubation, and curve *b*, after incubation, both measured against 8% $HClO_4$ used as a blank; curve *c*, the spectrum of the incubated sample measured against the control treated with $HClO_4$ prior to the incubation. The standard reaction mixture in a total volume of 1 ml. contained: 40 μmoles of formate, 0.66 μmole of DL-FH_4, 0.66 μmole of ATP, 4 μmoles of cysteine, 2.6 μmoles of $MgCl_2$, 33 μmoles of NH_4Cl, 100 μmoles of tris-HCl buffer at pH 8.0, and enzyme extract, about 150 μg. of protein.

these conditions the $f^{10}FH_4$ produced in the enzymic reaction undergoes a conversion into $f^{5-10}FH_4$ (Rabinowitz & Pricer; 1956) and the latter compound can be estimated by measuring the light absorption at 355 mμ. Molar extinction coefficient $\varepsilon = 22 \times 10^6$ cm^2. (May *et al.*, 1951) was taken for the calculation and the results were reported as mμmoles of $f^{10}FH_4$ synthesized per mg. of protein in 15 min. Because of high light absorption of aminopterin, the applicability of the method in the presence of this compound was proved (Figs. 1 A and B).

RESULTS

Effect of temperature and pH. Under the conditions of the assays the optimum temperature for the activity of the $f^{10}FH_4$ synthetase was found to be 36.5 - 37° (Fig. 2). At 42° the efficiency of the reaction appeared to be much lower even than that at 25° (Fig. 3). Since this might be due to thermal inactivation of the enzyme,

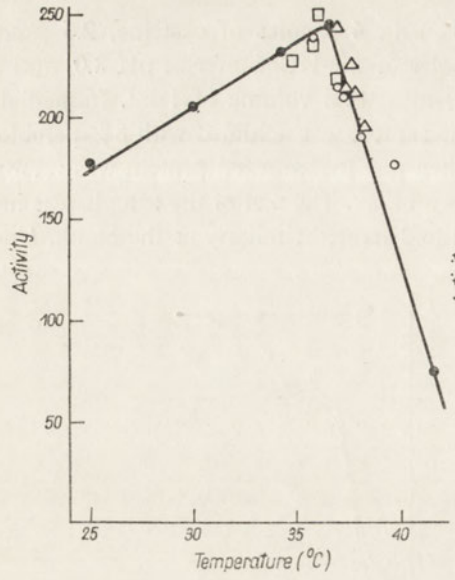

Fig. 2

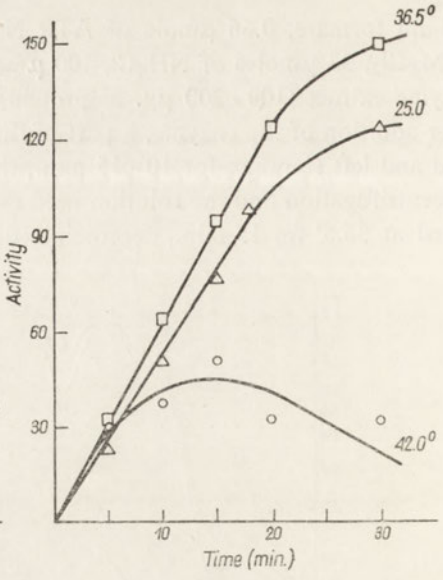

Fig. 3

Fig. 2. Effect of temperature on $f^{10}FH_4$ synthetase activity at pH 8.0. The reaction mixture was the same as that indicated in Fig. 1. Different symbols express the results of four separate experiments. Each point is the mean value from 5 - 10 samples. The S.E.M. values calculated for experiments performed at 25 - 37° did not exceed 1 - 2% of the total amount of $f^{10}FH_4$; in samples incubated at 39 - 42° the S.E.M. values were higher (up to 7% of the formed $f^{10}FH_4$). The activity is expressed as mμmoles of $f^{10}FH_4$ formed/mg. protein/15 min.

Fig. 3. Effect of temperature and time of incubation on the enzymic formylation of tetrahydrofolate. The reaction mixture was the same as that indicated in Fig. 1. The activity is expressed as mμmoles of $f^{10}FH_4$ formed/mg. protein.

Table 1

Efficiency of the formylation of tetrahydrofolate catalysed by the enzyme extracts preincubated with different substrates

The enzyme extract was preincubated at 42° without or with one of the substrates or Mg^{2+} and then incubated for 15 min. at 36.5° with the standard assay mixture containing in a volume of 1 ml.: 0.66 μmole of DL-FH_4, 40 μmoles of sodium formate, 0.66 μmole of ATP (Na salt), 4 μmoles of cysteine, 2.6 μmoles of $MgCl_2$, 33 μmoles of NH_4Cl, 100 μmoles of tris-HCl buffer at pH 8.0. The results are averages of three parallel estimations in one series of experiments. The relative activity is expressed as the percentage of activity in the non-incubated sample.

Preincubation of enzyme extract		Formylation efficiency	
min.	with addition of	$f^{10}FH_4$ formed (mμmoles/mg. protein)	Relative activity (%)
0	None, control	147	100
15	None	109	74
15	Formate	81	55
15	FH_4	87	59
15	ATP	2	0.13
15	Mg^{2+}	15	1.0
15	ATP+Mg^{2+}	25	17.0

in a further set of experiments samples of the enzyme were preincubated at 36.5° and at two higher temperatures before the enzymic reaction was carried out (Fig. 4). The results show that the preincubation at the optimum temperature did not cause any change in the activity of the enzyme, whereas that at 42° and 52° brought about its partial inactivation already in a few minutes (Fig. 4). Since the amounts of the $f^{10}FH_4$ found in the samples incubated at 42° were much smaller (Fig. 3) than it could be expected from the experiments with preincubation of the enzyme, some decomposition of this compound in these conditions could not be excluded.

The formyltetrahydrofolate synthetase from *Micrococcus aerogenes* could be protected against the thermal inactivation at 52° by the product of the reaction, $f^{10}FH_4$, by one of the substrates, ATP, and by the essential divalent cation, Mg^{2+} (Whiteley, Osborn, Talbert & Huennekens, 1959). On the contrary, none of the substrates did protect the *Galleria mellonella* enzyme, and the preincubation even at 42° was always followed by formation of smaller amounts of $f^{10}FH_4$ than in samples assayed without preincubation (Table 1). Moreover, the results suggested that Mg^{2+} and ATP made the enzyme even more thermosensitive.

The apparent activation energy (μ coefficient) for the enzyme was calculated, and this value appeared to be equal to 5100 cal./mole.

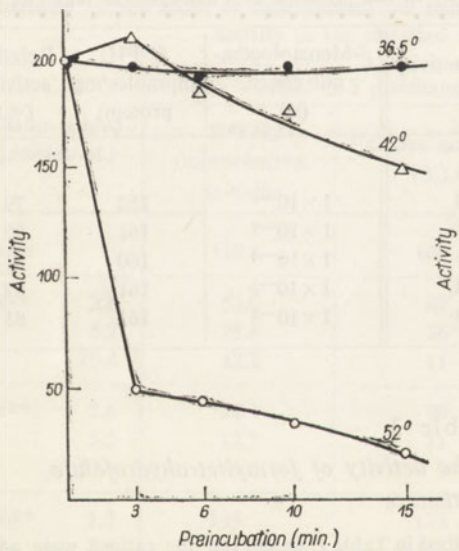

Fig. 4

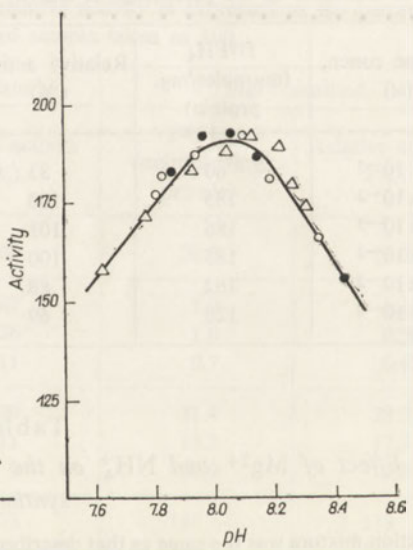

Fig. 5

Fig. 4. Effect of preincubation of enzyme extracts: (●), at 36.5°; (△), at 42° and (○), at 52° on the efficiency of the subsequent formylation of tetrahydrofolate at 36.5°. The reaction mixture was the same as that indicated in Fig. 1. The activity is expressed as mμmoles of $f^{10}FH_4$ formed/mg. protein/15 min. at 36.5°.

Fig. 5. Effect of pH on $f^{10}FH_4$ synthetase activity at 36.5°. The reaction mixture was the same as that given in Fig. 1, except for pH of the buffer solutions. Different symbols express the results of three separate experiments. Each point is the mean value from at least five samples, and S.E.M. did not exceed 2.5% of $f^{10}FH_4$ synthesized. The activity is expressed as mμmoles of $f^{10}FH_4$ formed/mg. protein/15 min. at 36.5°.

Effect of the incubation at different pH values on the activity of the formyltetrahydrofolate synthetase was checked in the range from 7.6 to 8.5 and the optimum was found at pH 8.0 - 8.1 (Fig. 5).

Reducing agents. To prevent the decomposition of tetrahydrofolate its solutions used for the assays contained 0.3 mM-cysteine. It was impossible to substitute cysteine by β-mercaptoethanol or ascorbic acid when dissolving FH_4 because of splitting of this compound.

For studying the properties of the $f^{10}FH_4$ synthetase, more cysteine (4 mM) was added to the samples, because without this supply the efficiency of the formylation of tetrahydrofolate was fairly low, and even in the presence of β-mercaptoethanol reached only 80% of that in the standard conditions with cysteine (Table 2).

Table 2

Effect of cysteine and β-mercaptoethanol on the activity of formyltetrahydrofolate synthetase

The reaction mixture was the same as that described in Table 1. Incubation was for 15 min. at 36.5°. The values are averages of three parallel estimations in one series of experiments. The relative activity is expressed as the percentage of activity in the presence of 4 mM-cysteine taken as 100.

Cysteine concn. (M)	$f^{10}FH_4$ (mμmoles/mg. protein)	Relative activity (%)	β-Mercaptoethanol concn. (M)	$f^{10}FH_4$ (mμmoles/mg. protein)	Relative activity (%)
2×10^{-5}	60	33			
4×10^{-3}	185	100	1×10^{-6}	152	79
8×10^{-3}	186	101	1×10^{-5}	161	82
2×10^{-2}	185	100	1×10^{-4}	160	81
4×10^{-2}	162	88	1×10^{-3}	161	82
8×10^{-2}	128	69	1×10^{-2}	162	83

Table 3

Effect of Mg^{2+} *and* NH_4^+ *on the activity of formyltetrahydrofolate synthetase*

The reaction mixture was the same as that described in Table 1, except that the cations were added in the amounts as indicated. Incubation was for 15 min. at 36.5°. The values are averages from 5 parallel estimations ±S.E.M., in one series of experiments.

Treatment of enzyme	Without cations	Mg^{2+}, 33 μmoles	NH_4^+, 33 μmoles	Mg^{2+}, 33 μmoles NH_4^+, 33 μmoles
	$f^{10}FH_4$ formed (mμmoles/mg. protein)			
None, control	2.5±0.4	23.0±2.9	111.0±1.2	211.0±5.1
Dowex 50	1.6±0.4	9.5±0.6	53.0±3.4	161.0±10.5
Chelex-100	0.5	0.8	0.5	0.6

Cations specificity. The cation requirement of the formyltetrahydrofolate synthetase was checked in experiments with the enzyme extracts treated with Dowex 50 or occasionally with Chelex 100. In the presence of Na^+, the activity of the enzyme was strongly stimulated by NH_4^+ which acted in a synergistic manner, but the complete removal of cations by Chelex 100 inactivated irreversibly the enzyme (Table 3). When using extracts untreated with the resin or treated with Dowex 50, Mn^{2+} cation replaced that of Mg^{2+} whereas Ca^{2+} or Cu^{2+} decreased the efficiency of formylation (Table 4) and Fe^{2+} and Co^{2+} completely inactivated the enzyme. None of the other monovalent cations could be used instead of NH_4^+ (Table 5), while spermine could substitute this cation to some extent (25%).

Table 4

Effect of divalent cations on the activity of formyltetrahydrofolate synthetase

The enzyme extract was treated with Dowex 50. The composition of the standard reaction mixture was the same as that given in Table 1, except the presence or absence of Mg^{2+} and addition of another divalent cation; incubation was for 15 min. at 36.5°. The values are averages from 3 parallel estimations in one series of experiments; the relative activity is expressed as the percentage of activity in the standard sample taken as 100.

Cation added (μmoles/ml.)		Mg^{2+} added (2.6 μmoles/ml.)		Mg^{2+} omitted	
		$f^{10}FH_4$ (mμmoles/mg. protein)	Relative activity (%)	$f^{10}FH_4$ (mμmoles/mg. protein)	Relative activity (%)
None		110.8	100	20.5	18.5
Ca^{2+}	2.6	52.0	47	1.3	1.18
	5.2	28.8	26	1.0	0.90
	10.4	12.2	11	0.7	0.63
Cu^{2+}	2.6	21.9	20	31.4	28.2
	5.2	12.3	11	19.2	17.3
	10.4	9.6	9	10.9	9.8
Mn^{2+}	1.3	148	133	148	133
	2.6	127	115	136	123
	5.2	93	84	123	110
	7.8	89	80	119	108
	10.4	37	33	93	84

Reaction kinetics. The apparent K_m values of all the substrates and activating cations were determined from double-reciprocal plots (Figs. 6 A-E, Table 6) according to the method of Lineweaver & Burk (1934). Since in the presence of Mg^{2+} the ammonium cation activated so strongly the enzyme, the affinity for the particular substrates in the presence and in absence of the latter were calculated (Table 6).

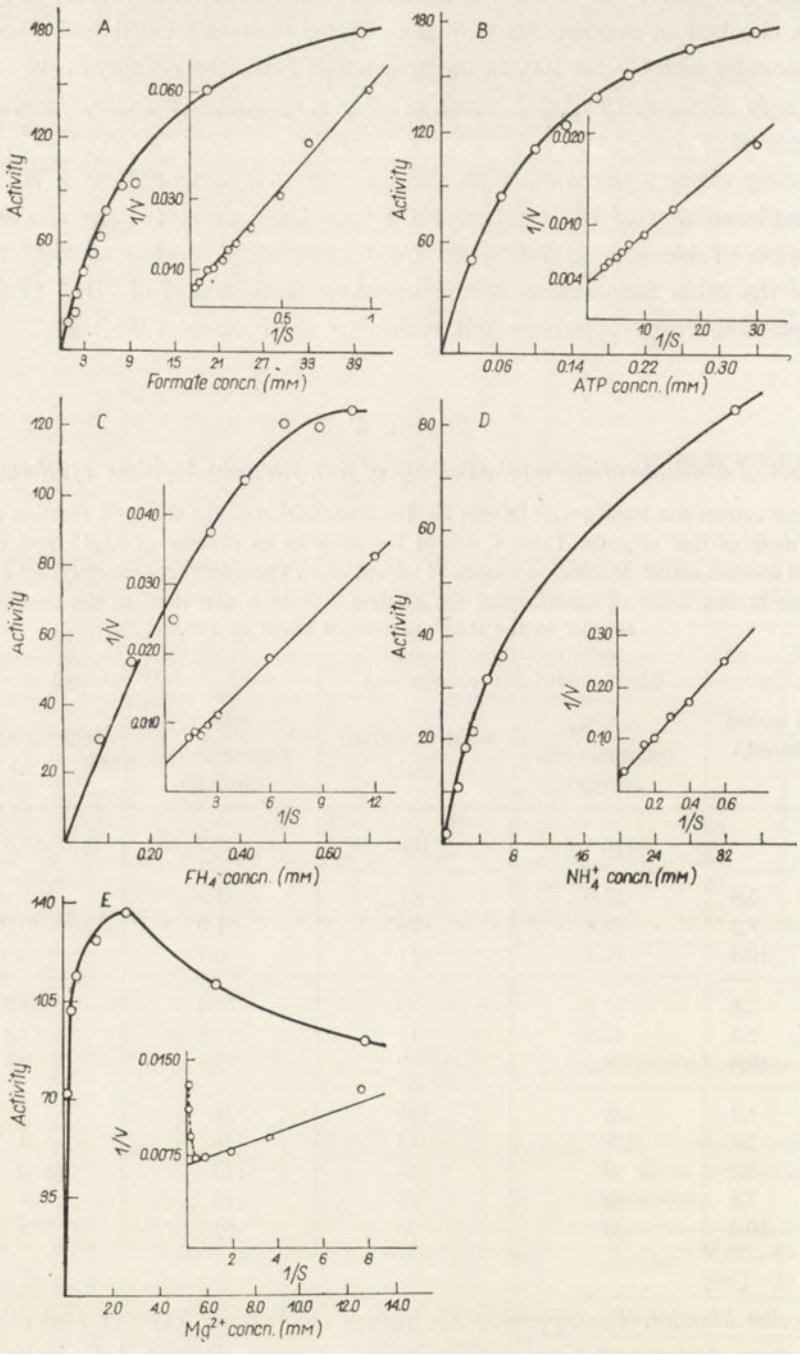

Fig. 6. Dependence of the rate of the reaction catalysed by $f^{10}FH_4$ synthetase upon the concentrations of (*A*), formate; (*B*), ATP; (*C*), FH_4; (*D*), NH_4^+; (*E*), Mg^{2+}. Other constituents of the sample were those indicated in Fig. 1. The Lineweaver-Burk plots are also presented. The activity is expressed as mμmoles of $f^{10}FH_4$ formed/mg. protein/15 min. at 36.5°.

Table 5

Effect of monovalent cations on the activity of formyltetrahydrofolate synthetase

The enzyme extracts were treated with Dowex 50. The composition of the standard sample was the same as that given in Table 1, except the presence or absence of NH_4^+ and addition of another monovalent cation; incubation was for 15 min. at 36.5°. The values are averages from 5 parallel estimations ± S.E.M., or from 3 parallel estimations in one series of experiments; the relative activity is expressed as the percentage of activity in the standard sample taken as 100.

Cation added (μmoles/ml.)		NH_4^+ added (33 μmoles/ml.)		NH_4^+ omitted	
		$f^{10}FH_4$ (mμmoles/mg. protein)	Relative activity (%)	$f^{10}FH_4$ (mμmoles/mg. protein)	Relative activity (%)
None		222±3.9	100	—	—
K^+	33.0	192±1.5	86	16.3±4.7	7.4
None		134	100	15.0	11.2
Li^+	16.5	131	98	6.6	4.9
	33.0	103	77	3.3	2.5
	66.0	89	66	2.2	1.0
Rb^+	33.0	136	101	5.2	2.3

Table 6

K_m values of substrates of formyltetrahydrofolate synthetase as determined in different incubation mixtures

The standard reaction mixture was the same as that given in Table 1 and the incubation was for 15 min. at 36.5°. Because of low enzyme activity in the absence of NH_4^+ the time of incubation was prolonged to 1.5 hr.

Substrate	K_m in the standard reaction mixture (mM)	K_m in the absence of NH_4^+ (mM)
FH_4	0.57	0.98
ATP	0.13	0.28
Formate	17.0	800

The 50-fold increase of K_m values of formate in the absence of NH_4^+ was the most remarkable result. By contrast, when suboptimum amounts of formate were present in the samples, only negligible changes in the K_m values of NH_4^+ could be demonstrated.

Inhibitors. Excess of ADP and P_i in the reaction mixture inhibited the $f^{10}FH_4$ synthetase. Inhibition by 50% was stated when ADP and P_i were used at concentra-

tions of 3 mM and 40 mM, respectively (Fig. 7). In the presence of ATP at concentrations higher than 0.7 mM the amounts of $f^{10}FH_4$ formed in the reaction were somewhat smaller than those in the standard samples. It seems that this effect may be considered as a result of inhibition by the excess of the substrate, because the

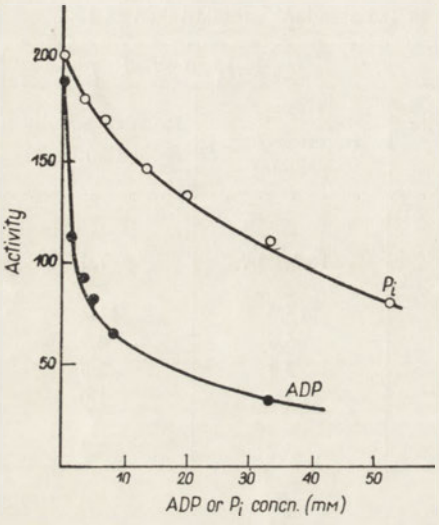

Fig. 7

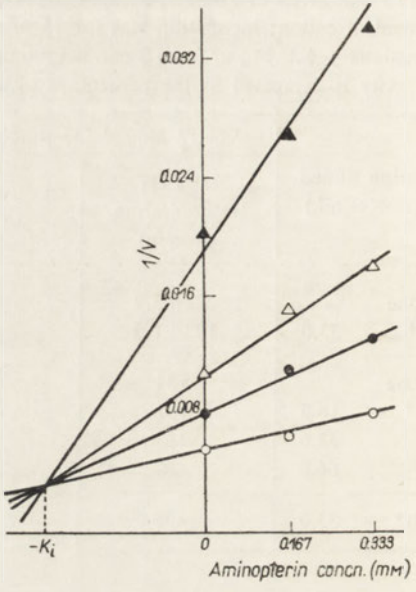

Fig. 8

Fig. 7. Effect of (●), ADP and (○), P_i concentration on the activity of $f^{10}FH_4$ synthetase. The composition of the samples was the same as that given in Fig. 1, except that different amounts of ADP or P_i were added. The activity is expressed as mμmoles of $f^{10}FH_4$ formed/mg. protein/15 min. at 36.5°.

Fig. 8. Competitive inhibition of $f^{10}FH_4$ synthetase by aminopterin in the presence of tetrahydrofolate at concentrations of: (○), 0.416; (●), 0.250; (△), 0.167, and (▲), 0.083 μmole/ml.

level of ATP and P_i after incubation of the samples without FH_4 was found to be constant (0.576±0.021 and 0.019±0.003 μmole/ml., respectively). Similar inhibition by an excess of Mg^{2+} was also stated (Fig. 6E). An aminoanalogue of folate, aminopterin, was found to be a competitive inhibitor of the synthetase from *G. mellonella* (Fig. 8) and its K_i was 0.33 mM.

DISCUSSION

The formyltetrahydrofolate synthetase from *G. mellonella*, an uricotelic insect, appeared to be very unstable, and to lose irreversibly almost all its activity when fractionated by ammonium sulphate or treated with Chelex 100. Therefore in this study the postmitochondrial supernatant or the crude enzyme preparations treated with Dowex 50 were used.

The enzyme from *G. mellonella* shows its maximum activity at 36.5 - 37° and, similarly as formyltetrahydrofolate synthetases of other animals, is a thermolabile

one (Brode & Jaenicke, 1961; Bertino, Simmons & Donohue, 1962). It undergoes a partial inactivation in a few minutes at 42°, that is at optimum temperature for this enzyme from a micro-organism, *Clostridium cylindrosporum* (Rabinowitz & Pricer, 1962). Moreover, ATP and Mg^{2+} could not protect the enzyme of *G. mellonella* against thermal inactivation in contrast to their effect on the enzyme of *Micrococcus aerogenes* (Whiteley & Huennekens, 1962). The rather low value for the apparent energy activation seems to indicate a rather high turnover of the reaction catalysed by the enzyme from *G. mellonella.*

The pH optimum of formyltetrahydrofolate synthetase from different tissues of some invertebrates and from mammalian liver has been found to be between 6.5 and 7.2 (Brode & Jaenicke, 1961; Whiteley, 1960) whereas higher pH optima between 7.5 and 8.1 have been reported for the enzymes from human erythrocytes (Bertino *et al.*, 1962) and uricotelic animals, such as insects (Grzelakowska-Sztabert & Zielińska, 1967) and birds (Jaenicke & Brode, 1961), and from those species of micro-organisms which ferment purines (Himes & Rabinowitz, 1962; Nurmikko, Soini, Taiminen & Kyyhkynen, 1965; Whiteley & Huennekens, 1962).

The evident requirement for reducing agents to protect the activity of the formyltetrahydrofolate synthetase from *G. mellonella* indicates indirectly that some of thiol groups in the enzyme molecule are involved in its activity.

The insect $f^{10}FH_4$ synthe ase requires NH_4^+ and Mg^{2+} ions which act as essential, synergistic activators and cannot be replaced by other mono- or divalent cations, except Mn^{2+}. The synthetases from different animals and micro-organisms differ from one another as to the requirement of the divalent cations other than Mg^{2+} (Bertino *et al.*, 1962; Himes & Rabinowitz, 1962; Whiteley, 1960; Whiteley & Huennekens, 1962). It is worth noting that the $f^{10}FH_4$ synthetase from *G. mellonella* which requires Mg^{2+} and ATP for catalysing the formylation of tetrahydrofolate exhibits no ATPase activity, in contrast to the enzyme from *M. aerogenes* (Whiteley *et al.*, 1959).

The K_m values of the substrates and the essential cations and K_i of aminopterin, could be paired with regard to their values as follows: ATP and Mg^{2+} (0.13 and 0.18 mM), formate and NH_4^+ (17.0 and 14.0 mM), tetrahydrofolate and aminopterin (0.57 and 0.33 mM). Since K_m of ATP and that of Mg^{2+} appeared to be nearly equal, it is probable that the Mg-ATP complex (formed non-enzymically), rather than free ATP is the real substrate for $f^{10}FH_4$ synthetase (Grzelakowska & Zielińska, 1966) as it has been postulated for the crystalline $f^{10}FH_4$ synthetase from *Cl. cylindrosporum* (Himes & Wilder, 1965). This view seems to be supported by the fact of inhibition of $f^{10}FH_4$ synthetase in the presence of an excess of ATP or Mg^{2+} in the incubation mixtures.

Furthermore, K_m values of NH_4^+ and formate are of the same order and in the absence of ammonium cation the K_m value of formate is approximately 50-fold that which has been estimated in the standard conditions. On the other hand, the omission of NH_4^+ has but small influence on K_m values of tetrahydrofolate and ATP. Therefore it is probable that NH_4^+ may be involved, in some way, only in binding formate with the enzyme molecule whereas the binding of tetrahydrofolate

seems to be independent of the presence of this cation. This is not in agreement with the mechanism suggested for the action of the crystalline enzyme from *Cl. cylindrosporum* by Himes & Wilder (1965).

Finally, aminopterin which in substrate concentrations has no influence on the activity of the formyltetrahydrofolate synthetase from other organisms (Slavikova & Slavík, 1961; Whiteley *et al.*, 1959) acts as a competitive inhibitor of this enzyme of *G. mellonella*. Since its K_i value corresponds to that of K_m of tetrahydrofolate it may be assumed that this analogue of folate competes with tetrahydrofolate for the same binding sites in the enzyme molecule.

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SYNTETAZA FORMYLOTETRAHYDROFOLANOWA Z URYKOTELICZNEGO OWADA *G. MELLONELLA* L.

Streszczenie

Badany enzym ulega inaktywacji podczas prób oczyszczenia przy zastosowaniu typowych metod, wykazuje też znaczną termolabilność. Optimum aktywności przypada w 36,5 - 37°, przy pH 8,0 - 8,1 w obecności Mg^{2+} i NH_4^+ działających jako synergistyczne aktywatory. Jednakowe wartości K_m dla ATP i Mg^{2+} sugerują, że nie wolny ATP lecz kompleks ATP-Mg^{2+} stanowi właściwy substrat reakcji. Odpowiednio, wartości K_m dla mrówczanu i NH_4^+, jak również 50-krotny wzrost wartości K_m dla mrówczanu w nieobecności jonu amonowego pozwoliły na przedyskutowanie roli amonu w wiązaniu mrówczanu z cząsteczką enzymu. Podobnie, zbieżność wartości K_i dla aminopteryny i K_m dla FH_4 nasuwają przypuszczenie, że aminopteryna współzawodniczy właśnie z tetrahydrofolanem o to samo miejsce wiążące w cząsteczce enzymu.

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OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA OF THE WAX MOTH AND SOME OTHER INSECTS

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Dedicated to Professor W. Niemierko on the occasion of his seventieth birthday

1. Mitochondria isolated from wax moth (*Galleria mellonella*) larvae contain high amounts of free fatty acids and their phosphorylation ability (with succinate as the respiratory substrate) shows an almost absolute requirement for serum albumin. Isolated mitochondria of muscles of adult insects: wax moth, blowfly (*Calliphora erythrocephala*) and cockroaches (*Periplaneta americana* and *Blaberus giganteus*) contain less free fatty acids and are able to phosphorylate in the absence of serum albumin, but the addition of albumin increases the P:O ratio. 2. The high content of free fatty acids in insect mitochondria is caused in part by lipolytic enzymes present in insect tissues and in part by free fatty acids already present in tissues of living insects and adsorbed by mitochondria during the isolation procedure. 3. Mitochondria of wax moth larvae undergo a rapid spontaneous swelling in 0.125 M-KCl. This swelling can be diminished by serum albumin.

Pioneering studies of Lewis & Slater (1954), Sacktor (1954) and Rees (1954) demonstrated that mitochondria isolated from various insects are able to carry out oxidative phosphorylation, similarly as isolated mitochondria of mammalian tissues. It became, however, apparent that the efficiency of this phosphorylation, as determined by P:O ratio, was lower than in mammalian mitochondria but could be much improved by the addition of serum albumin (Lewis & Slater, 1954; Sacktor, 1954) or of some other proteins (Rees, 1954). The role of serum albumin in maintaining high efficiency of oxidative phosphorylation in insect mitochondria became clear when it was demonstrated (Wojtczak & Wojtczak, 1959, 1960; Lewis & Fowler, 1960) that isolated insect mitochondria contain uncoupling quantities of free fatty acids and that these acids can be bound by serum albumin. Uncoupling properties of fatty acids were first studied in liver mitochondria by Pressman & Lardy (1956) and the binding of fatty acids by serum albumin was described by Goodman (1958).

The present paper describes in more detail the effect of serum albumin on the respiration, phosphorylation and related processes in mitochondria of the wax moth (*Galleria mellonella*). Investigations extended over other insect species show

a correlation between the requirement for albumin and the content of free fatty acids in mitochondria. Furthermore, a negative correlation is demonstrated between the efficiency of oxidative phosphorylation and the activity of lipolytic enzymes in insect tissues. This paper also describes the ability of wax moth mitochondria to undergo swelling and contraction and enables the comparison with analogous properties of mammalian mitochondria (Lehninger, 1959, 1962).

A part of this investigation has already been published in a short communication (Wojtczak, 1963).

MATERIALS AND METHODS

Whole last instar larvae of the wax moth, *Galleria mellonella*, were homogenized in 0.25 M-sucrose and the homogenate was fractionated by centrifugation essentially as described for mammalian liver mitochondria (Hogeboom, 1955). The mitochondria were washed twice and suspended in 0.25 M-sucrose. Some preparations of wax moth mitochondria were isolated in 0.25 M-sucrose containing 5 mM-ATP as described previously (Wojtczak & Wojtczak, 1960), but no essential advantage of this procedure has been noted. Thoracic muscle mitochondria of adult insects (wax moth *Galleria mellonella*, blowfly *Calliphora erythrocephala*, cockroaches *Periplaneta americana* and *Blaberus giganteus*) were isolated in 0.25 M-sucrose according to the procedure of Lewis & Slater (1954). Rat liver mitochondria were isolated in 0.25 M-sucrose according to Hogeboom (1955).

Oxidative activity of mitochondria was determined manometrically. Oxidative phosphorylation, ATP-P_i exchange reaction and ATPase activity were measured by procedures and in media described previously (Wojtczak & Wojtczak, 1960). Swelling and contraction of mitochondria was followed photometrically, as described by Lehninger, Ray & Schneider (1959), in 0.125 M-KCl - 0.02 M-tris-Cl, pH 7.4; total volume was 7.5 ml., temperature 20°.

Free fatty acids in mitochondria were determined as follows. A dense mitochondrial suspension (20 - 40 mg. protein/ml.) was extracted first with a fivefold volume of ethanol, and then the residue was extracted several times with a mixture of ethanol and ethyl ether (3 : 1, v/v). The extracts were combined and the lipids were dissolved in petroleum ether. Free fatty acids were separated by shaking the petroleum ether solution with alkaline ethanol, and their amount was determined by microtitration according to Dole & Meinertz (1960).

Gas-liquid chromatography of fatty acids was performed at 172° on succinic polyester of ethylene glycol on Celite using a Pye Argon Chromatograph. Fatty acids were methylated by heating with methanol-HCl for 18 hr. at 72°.

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

Reagents. Bovine serum albumin fraction V, ATP and hexokinase type III or II were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); ADP from Light Ltd. (Colnbrook, England), and phosphocholine from California Biochemical Corporation (Los Angeles, Calif., U.S.A.). Other chemicals were of analytical grade.

RESULTS

Oxidative phosphorylation and related reactions

Table 1 summarizes the results of studies on respiration, phosphorylation and related reactions of mitochondria of wax moth larvae. It is evident that in the absence of serum albumin these mitochondria are not able to carry out any substantial oxidative phosphorylation with succinate as oxidizable substrate or to catalyse

Table 1

Respiration, oxidative phosphorylation and related reactions in mitochondria of wax moth larvae. Effect of serum albumin

Incubation medium for measurements of the respiratory activity (Q_{O_2}) and the respiratory control contained: 20 mM-KCl, 20 mM-tris-Cl, 20 mM-K-Na-phosphate, 6 mM-$MgCl_2$, 1 mM-EDTA, 50 mM-sucrose, 10 mM the respiratory substrate indicated, 2 - 4 mg. mitochondrial protein, and 10 mg. bovine serum albumin where indicated; 10 mM-glucose, 0.5 mM-ATP (final concentrations) and 200 K.M. units of hexokinase were tipped in from the side arm.Total volume was 1.0 ml, pH 7.2, temperature 25°. Q_{O_2} represents the rate of oxygen uptake in the active state of mitochondria (with ATP, glucose and hexokinase added), while the respiratory control ratio is a ratio of the respiratory rate in the active state to the respiratory rate in the controlled state (before the addition of ATP, glucose and hexokinase). The figures represent the range, with number of experiments in parentheses.

	Without serum albumin		With serum albumin
Q_{O_2} (μatoms/mg. protein/hr.)			
substrates: succinate	0.8 - 1.9	(4)	0.8 - 2.8
α-glycerophosphate	0.7 - 0.8	(2)	1.4 - 1.5
glutamate	0.6 - 1.6	(3)	1.1 - 2.0
pyruvate + malate	0.9 - 1.2	(2)	1.1 - 1.6
Respiratory control ratio			
substrates: succinate	0.8 - 1.0	(5)	1.1 - 1.8
α-glycerophosphate	0.8 - 1.1	(4)	1.1 - 1.9
glutamate	1.7 - 2.3	(4)	1.9 - 2.1
pyruvate + malate	1.1 - 1.5	(4)	1.2 - 2.1
P:O ratio			
substrates: succinate*	0.2		1.3
glutamate**	1.1		1.8
ATP-P_i exchange reaction* (μmoles/mg. protein/hr.)	0.1		2.2
Adenosinetriphosphatase* (μmoles/mg. protein/hr.)	3		

* Data from Wojtczak & Wojtczak (1960).
** Data from Wojtczak & Wojtczak (1959).

Table 2

Oxidative phosphorylation and the content of free fatty acids in mitochondria of various insects and the rat

Oxidative phosphorylation was measured with succinate as the respiratory substrate in a medium described in Table 1 for measurements of the respiration and the respiratory control. The figures represent mean values for 3 to 8 experiments ±S.D. (except for the content of fatty acids in rat liver mitochondria where the range is indicated).

Animal	Tissue	Free fatty acids (μmoles/g. protein)	P:O	
			without albumin	with 10 mg. serum albumin
Galleria mellonella, larva	Whole body	110±50	<0.2	1.2±0.1
Galleria mellonella, imago	Thoracic muscles	70±15	1.0±0.1	1.7±0.1
Calliphora erythrocephala, imago	Thoracic muscles	60±10	0.6±0.1	1.2±0.1
Blaberus giganteus, imago	Thoracic and leg muscles	60±10	1.7±0.1	1.9±0.2
Rat	Heart	50±10	1.5±0.1	1.9±0.2
Rat	Liver	10—20	1.9±0.2	1.9±0.2

the ATP-P_i exchange. Oxidative phosphorylation with glutamate is low (P:O=1.1). A measurable respiratory control can be obtained only with NAD-linked substrates (glutamate, or pyruvate + malate). With succinate and α-glycerophosphate a reverse respiratory control[1] is sometimes observed. Addition of serum albumin to the incubation medium results in a substantial increase of the degree of coupling. The mitochondria become able to catalyse the ATP-P_i exchange and to synthesize ATP during succinate oxidation; the efficiency of oxidative phosphorylation with glutamate is also increased and a positive respiratory control with all substrates tested appears. The addition of albumin also results in an increase of the respiratory activity (Q_{O_2}). The effect of serum albumin on ATPase activity was variable and therefore is not shown in Table 1. In some cases a decrease in the activity was observed after addition of albumin while in other cases there was no change (Wojtczak & Wojtczak, 1960). Similar effects as with bovine albumin have also been obtained with human serum albumin and with bovine β-lactoglobulin (Wojtczak & Wojtczak, 1960). No other proteins have been found active.

As shown previously (Wojtczak & Wojtczak, 1960), the low efficiency of oxidative phosphorylation in mitochondria of wax moth larvae is caused, at least in part, by the presence of free (i.e. non-esterified) fatty acids in these mitochondria,

[1] "Reverse respiratory acceptor-control" designates the situation when ADP decreases, instead of increasing, the rate of respiration (Lehninger & Gregg, 1963). The respiratory control ratio is then lower than unity.

and the beneficial effect of serum albumin is due to its binding properties towards fatty acids. Table 2 shows that serum albumin also increases oxidative phosphorylation in mitochondria of adult wax moth, the blowfly and the South American cockroach, *Blaberus giganteus*. An increase can also be observed with rat heart sarcosomes but not with freshly isolated rat liver mitochondria. It can be noted that P:O ratios and the requirement for serum albumin are correlated with the presence in mitochondria of free fatty acids. In general, the higher is the content of free fatty acids, the lower is the P:O ratio and the greater is the effect of serum albumin. An exception is, however, the blowfly, *Calliphora erythrocephala*, the sarcosomes of which contain approximately the same amount of free fatty acids as sarcosomes of *Blaberus giganteus* and of adult *Galleria mellonella*, but exhibit a low phosphorylation which is not much improved by serum albumin. Table 2 also shows that mitochondria of *Galleria mellonella* larvae contain the highest amount of free fatty acids; and parallel with this is an almost absolute requirement for serum albumin to obtain a synthesis of ATP coupled to the oxidation of succinate in these mitochondria.

By means of paper chromatography, palmitic, stearic, oleic, linoleic and linolenic acids have been previously identified in the fatty acid fraction from wax moth mitochondria (Wojtczak & Wojtczak, 1960). Now, a more precise examination by gas-liquid chromatography revealed the composition of this fraction as shown in Table 3.

Table 3

*Composition of free fatty acids found in isolated mitochondria of wax moth larvae**

Fatty acid		% composition (range for 7 determinations)
Shorthand designation**	Name	
12:0	Lauric acid	0.0 - 0.4
14:0	Myristic acid	0.2 - 1.0
14:1***	Myristic acid	0.1 - 1.4
15:0***	Myristic acid	0.0 - 0.7
15:1***	Myristic acid	0.0 - 1.1
16:0	Palmitic acid	21.7 - 31.4
16:1	Palmitoleic acid	2.0 - 6.0
17:0***	Margaric acid	0.0 - 1.0
17:1***	Margaric acid	0.1 - 3.4
18:0	Stearic acid	1.3 - 5.0
18:1	Oleic acid	22.3 - 38.9
18:2	Linoleic acid	3.2 - 21.4
18:3	Linolenic acid	2.4 - 17.2
20:0	Arachidic acid	0.0 - 7.6
20:1***	Arachidic acid	0.0 - 4.6
22:0***	Behenic acid	0.0 - 1.6

* Data obtained in collaboration with Dr. J. Zborowski.

** The first number indicates chain length and the second the number of double bonds.

*** Tentative identification.

Lipolytic activity

When mitochondria of wax moth larvae are incubated in 0.25 M-sucrose for 2 hr. at 37° the amount of free fatty acids is approximately doubled. This indicates that the mitochondrial fraction contains lipolytic enzymes acting on mitochondrial own lipids. It seems, however, not likely that these enzymes could be responsible for the accumulation of high amounts of free fatty acids found in freshly isolated mitochondria. Therefore attention was directed to extramitochondrial lipolytic enzymes. Table 4 shows lipolytic activity of the supernatant fractions (containing soluble cytoplasm and microsomes) of various insects. It is evident that these fractions from various insects exhibit a very high lipolytic activity as compared with rat heart and liver. During the isolation procedure mitochondria are in contact for a certain period of time with the whole homogenate and thus are exposed to extramitochondrial lipolytic enzymes. For this reason, the high activity of these enzymes in insect tissues could account, at least in part, for the high content of free fatty acids in isolated mitochondria. Due to a high ability of mitochondria to bind fatty acids, the free fatty acids formed in the homogenate are probably adsorbed by the mitochondria.

Table 4

Lipolytic activity of supernatant fractions from various insects and rat tissues

Supernatants, obtained after removing mitochondria, were incubated at about 20° for 1 hr. at pH 5 with heat-inactivated rat liver mitochondria (added as substrate for the lipolytic enzymes). The numbers (range for 2 to 5 experiments) represent the amount of fatty acids liberated per 100 mg. supernatant protein.

Animal and tissue	Lipolytic activity (μmoles fatty acids/100 mg. protein/hr.)
Galleria mellonella, whole larvae	83 - 92
Galleria mellonella, imago, muscles	70 - 80
Calliphora erythrocephala, imago, muscles	75 - 90
Blaberus giganteus, imago, muscles	64
Rat, heart	traces
Rat, liver	0

It is to be noted, however, that there are no great differences in lipolytic activity between various insects examined (Table 4) although there are differences in the content of free fatty acids in mitochondria (Table 2), those of wax moth larvae being particularly rich in fatty acids. Therefore some other factors must contribute to the high content of free fatty acids in these mitochondria (see Discussion).

In an attempt to characterize the lipolytic enzymes of insect tissues the effect of some substances was studied. It was found that production of free fatty acids by the supernatant fraction of wax moth larvae was inhibited by 0.5 M-NaCl, 0.1 M--pyrophosphate and protamine sulphate (20 mg./ml.); it was strongly enhanced by heparin (30 μg./ml.) and was not affected by 0.2 M-potassium fluoride. These

properties were especially evident when no lipids were added as substrates, i.e. when the enzyme(s) acted on endogenous lipids present in the supernatant fraction. Optimum activity was observed at pH 5.5. The inhibition by protamine sulphate, pyrophosphate and high concentrations of NaCl and the activation by heparin suggest that the enzyme(s) involved may be similar to the lipoprotein lipase described by Korn (1955).

Binding of fatty acids by mitochondria

As mitochondria of wax moth larvae already contain large quantities of free fatty acids, the ability to bind fatty acids was measured with rat liver mitochondria. Various quantities of oleate were added to suspensions of mitochondria in 0.25 M-sucrose, the mixtures were centrifuged, supernatants were carefully decanted and the content of free fatty acids bound to the mitochondrial pellet was determined. The results illustrated in Fig. 1 show that, if 30 μmoles or less oleate is added per

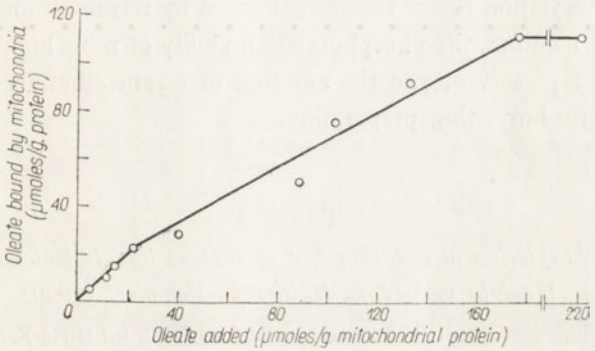

Fig. 1. Binding of oleate by rat liver mitochondria.

gram of mitochondrial protein, all oleate is bound by mitochondria. When the amount of oleate is increased, a part of it remains unbound. However, even with 100 μmoles of oleate added per gram of mitochondrial protein, some 70% is bound. The saturation is attained when 175 μmoles oleate/g. protein is added, 110 μmoles/g. protein being bound by the mitochondria. This is the maximum amount of oleate which can be bound by rat liver mitochondria. However, it should be noted that by this procedure only that portion of oleate can be determined which is bound to whole mitochondria and to sedimentable mitochondrial fragments. Oleate bound to soluble mitochondrial proteins has been regarded as unbound; and it is known (Wojtczak & Załuska, 1967; Drahota & Honová, 1968) that some soluble mitochondrial proteins leak out to the medium at elevated concentrations of oleate. It is therefore

likely that the true amount of oleate bound to mitochondrial proteins is somewhat higher than 110 μmoles/g. protein; it probably amounts to 175 μmoles/g. protein, a value at which the "saturation" is observed.

Effect of isolation media

It has been shown by Gregg, Heisler & Remmert (1960) that, using a complex isolation medium containing sucrose, phosphate and intermediates of the tricarboxylic acid cycle, it is possible to obtain housefly thoracic muscle sarcosomes carrying out oxidative phosphorylation without the addition of serum albumin. When isolated in plain 0.25 M-sucrose, these sarcosomes were completely uncoupled (Sacktor, 1954). It seemed therefore interesting to examine whether replacing 0.25 M-sucrose by the medium described by Gregg and coworkers it would be possible to obtain phosphorylating mitochondria of wax moth larvae. Table 5 shows that using the complex isolation medium improves phosphorylation efficiency of thoracic muscle mitochondria of adult blowfly and wax moth (compare with data in Table 2), and the phosphorylation is not further enhanced by serum albumin. However, on isolation in this medium, the phosphorylation ability of mitochondria of wax moth larvae cannot be preserved, and the addition of serum albumin is still necessary to obtain a phosphorylating preparation.

Table 5

Oxidative phosphorylation in mitochondria of various insects isolated in the complex medium of Gregg, Heisler & Remmert (1960)

The isolation medium contained: 0.25 M-sucrose, 0.05 M-tris-Cl, 0.1 M-Na-K-phosphate, pH 7.4, 3 mM-EDTA, and citrate, pyruvate, α-ketoglutarate and succinate, 6 mM each. Other experimental conditions as in Tables 1 and 2.

Source of mitochondria	Substrate	P:O	
		without albumin	with albumin
Calliphora erythrocephala, imago, thoracic muscles	Succinate	2.1	2.3
	Succinate	2.4	2.3
	α-Glycerophosphate	1.9	1.8
	Pyruvate	2.7	2.6
Galleria mellonella, imago, thoracic muscles	Succinate	1.6	1.8
	Succinate	1.6	1.8
	α-Glycerophosphate	1.5	1.7
Galleria mellonella, whole larvae	Succinate	0	1.3
	α-Glycerophosphate	0	1.5
	α-Glycerophosphate	0	1.5

Swelling and contraction of wax moth mitochondria

As shown in Fig. 2, mitochondria of wax moth larvae undergo a rapid swelling when suspended in buffered 0.125 M-KCl (the so-called spontaneous swelling; Lehninger, Ray & Schneider, 1959). This swelling is much larger than the spontaneous swelling of rat-liver mitochondria (Lehninger *et al.*, 1959) and is not, or is only slightly, increased by agents like phosphate, thyroxine, fatty acids and Ca^{2+} which are known to induce swelling of mammalian mitochondria (Lehninger,

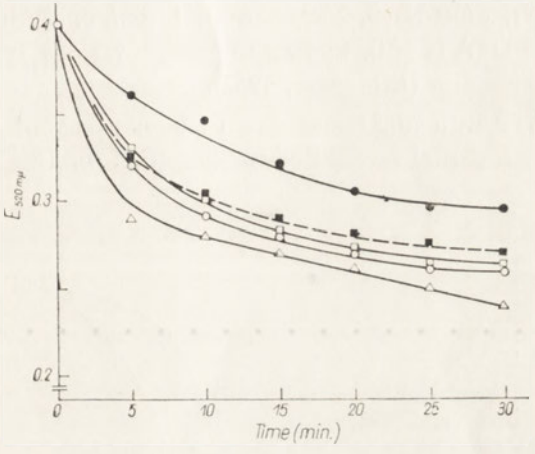

Fig. 2. Swelling of mitochondria of wax moth larvae. Effect of phosphate, oleate and albumin. Swelling medium: 0.125 M-KCl - 0.02 M-tris-Cl, pH 7.4. (○), Spontaneous swelling; (□), 2 mM-phosphate; (△), 10^{-5} M-oleate; (●), spontaneous swelling in the presence of bovine serum albumin, 1 mg./ml.; (■), 2 mM-phosphate - bovine serum albumin, 1 mg./ml.

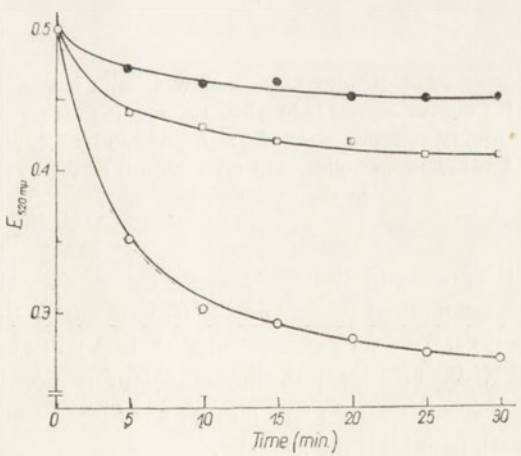

Fig. 3. Swelling of mitochondria of wax moth larvae. Effect of sucrose. (○), Spontaneous swelling in 0.125 M-KCl - 0.02 M-tris-Cl; (●), spontaneous swelling in 0.25 M-sucrose - 0.02 M-tris-Cl, pH 7.4; (□), swelling in 0.25 M-sucrose - 0.02 M-tris-Cl - 8 mM-phosphate.

1962). In analogy to what has been observed with fatty acid-induced swelling of rat-liver mitochondria (Wojtczak & Lehninger, 1961), the swelling of wax moth mitochondria can be diminished by serum albumin (Fig. 2) and only in this case it can be increased by phosphate. The spontaneous swelling of wax moth mitochondria can be largely diminished if 0.125 M-KCl is replaced by 0.25 M-sucrose (Fig. 3) which is known to diminish swelling of liver mitochondria (Lehninger *et al.*, 1959). However, in this case neither oleate, $CaCl_2$ or thyroxine produce any swelling effect. Only phosphate at a relatively high concentration (8 mM) induces a slight swelling (Fig. 3). Swelling in KCl solution is not inhibited by respiratory inhibitors (1 mM--KCN, 2 mM-NaN_3, 2 mM-Na_2S, 2 mM-amytal, 1 μg./ml. antimycin), 2,4-dinitrophenol (1 mM) or EDTA (1 mM), known to inhibit various types of swelling of mammalian mitochondria (Lehninger, 1962).

Addition of ATP with Mg^{2+} and serum albumin to swollen wax moth mitochondria produces a partial reversal of swelling (Fig. 4). This contraction can be

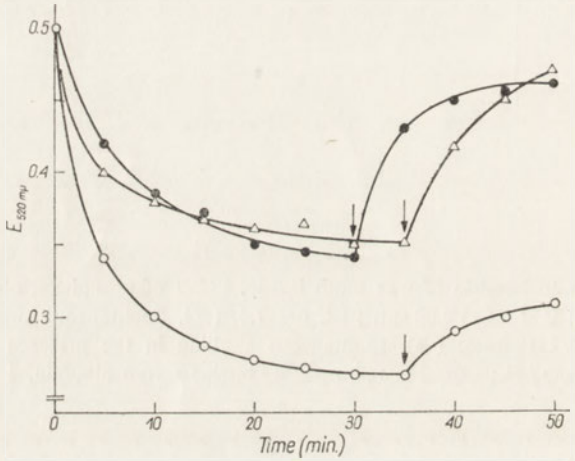

Fig. 4. Swelling and contraction of mitochondria of wax moth larvae. Medium: 0.125 M-KCl - - 0.02 M-tris-Cl, pH 7.4. Contraction was induced by the addition (at arrows) of ATP, $MgCl_2$ and bovine serum albumin to final concentrations of 5 mM, 3 mM and 0.2%, respectively. (○), Spontaneous swelling; (●), 8 mM-phosphocholine, and (△), 0.3 ml. of the supernatant fraction added to the swelling medium.

increased if a small amount of the supernatant fraction is added to the swelling medium. The active component present in the supernatant is thermostable and can be removed by dialysis. The ATP-induced contraction can also be potentiated by phosphocholine (Fig. 4). Neither phosphoethanolamine nor phosphoserine have any effect. The supernatant fraction and phosphocholine not only increase the contraction but also partially inhibit the swelling.

Thoracic muscle sarcosomes of the cockroaches *Periplaneta americana* and *Blaberus giganteus* behave essentially as mitochondria of wax moth larvae in respect of swelling and contraction.

DISCUSSION

It seems now well established that a low efficiency of oxidative phosphorylation generally observed in mitochondria obtained from insect tissues is caused by the presence of non-esterified fatty acids bound to these mitochondria. This is indicated by both the beneficial effect of serum albumin on P:O ratio (Sacktor, 1954; Sacktor, O'Neill & Cochran, 1958; Wojtczak & Wojtczak, 1959) and direct measurements of fatty acids (Wojtczak & Wojtczak, 1960; Lewis & Fowler, 1960; Chefurka, 1963). The present investigation provides further evidence that insect mitochondria are, in general, richer in fatty acids than mitochondria from mammalian tissues (liver, heart) and shows a correlation between P:O ratio and the content of free fatty acids. The content of free (non-esterified) fatty acids is particularly high in mitochondria of wax moth larvae, an insect of exceptionally intense lipid metabolism. Free fatty acids are not only responsible for the lowering of P:O ratio but also for other patterns by which these mitochondria differ from those of rat liver or heart, i. e. the high Mg^{2+}-activated ATPase which is not further stimulated by 2,4-dinitrophenol, the inability to catalyse the ATP-P_i exchange reaction in the absence of serum albumin, and a high rate of swelling in isotonic saline media. Uncoupling effect of fatty acids on oxidative phosphorylation has been suggested as early as in 1950 by Lehninger (1951). The stimulation by fatty acids of mitochondrial ATPase has been studied by Pressman & Lardy (1956), Borst, Loos, Christ & Slater (1962) and Bos & Emmelot (1962); the inhibition of the ATP-P_i exchange reaction was observed by Hülsmann, Elliott & Slater (1960), and Ahmed & Scholefield (1960); and the lowering of P:O ratio was found by Scholefield (1956b), Lehninger & Remmert (1959), and Borst *et al.* (1962). Swelling effect of fatty acids is known from studies by Lehninger & Remmert (1959), Avi-Dor (1960), and Zborowski & Wojtczak (1963). An increase in substrate oxidation by wax moth mitochondria observed after addition of serum albumin can also be ascribed to the removal of free fatty acids. The inhibition by fatty acids of mitochondrial respiration has already been observed (Scholefield, 1956a; Hülsmann *et al.*, 1960; Wojtczak & Załuska, unpublished). The reverse respiratory (acceptor) control, i.e. the lowering, instead of the increasing, of the respiration by ADP, as observed in wax moth mitochondria with some substrates in the absence of albumin, is also presumably an effect of fatty acids. A similar effect produced by fatty acids in rat-liver mitochondria has been observed by Vázquez-Colón, Ziegler & Elliott (1966).

The present investigation provides an explanation of the high content of free fatty acids in insect mitochondria. First, mitochondrial fractions contain enzymes hydrolysing mitochondrial lipids and liberating free fatty acids. This was demonstrated in mitochondria of wax moth larvae. A similar lipolytic activity has also been observed in flight-muscle sarcosomes of the housefly (Chefurka, 1963) and in mammalian liver mitochondria (Hülsmann, 1958; Wojtczak & Lehninger, 1961; Chefurka, 1963). As shown by Chefurka (1963), this activity is several times higher in housefly sarcosomes than in liver mitochondria. It is, however, difficult to evaluate

whether lipolytic enzymes found in the mitochondrial fraction are present in mitochondria themselves or in contaminations, e.g. in lysosomes.

Secondly, in insect tissues there are highly active extramitochondrial lipases and/or phospholipases. They can be demonstrated in homogenates and in supernatants after removal of mitochondria. The activity of these enzymes in insect material is strikingly higher than in mammalian tissues (Table 4). A high activity of lipases in insects has also been observed by George & Eapen (1959) and Włodawer & Barańska (1965a). During the homogenization and the first steps of centrifugation these lipases can hydrolyse mitochondrial lipids and other lipid material present in the homogenate, and the fatty acids produced in this way are bound by mitochondria. This is the second factor responsible for the high content of fatty acids in insect mitochondria.

However, no substantial differences could be observed between lipolytic activities of supernatants from various insect species and developmental stages (Table 4), while there was a great difference in the content of free fatty acids in mitochondria, those of wax moth larvae being particularly rich in fatty acids (Table 2). This fact suggests that the high content of free fatty acids in these mitochondria must be due not only to lipolysis. A factor which contributes to this high content of fatty acids is most probably the presence of free fatty acids in the living insect (Włodawer & Barańska, 1965a; Włodawer, Łągwińska & Barańska, 1966; Włodawer & Łągwińska, 1967). It is well known (Wojtczak, 1961; Reshef & Shapiro, 1962, 1965; and the present paper) that mitochondria exhibit a high binding ability towards long-chain fatty acids and it is to be expected that free acids present in the tissues are bound by mitochondria when the cells are disrupted during homogenization. It seems highly probable that this is the main source of free fatty acids found in isolated mitochondria of wax moth larvae. Similarly, it has been shown by Lewis & Fowler (1960) that free fatty acids found in isolated sarcosomes of the blowfly originate from free fatty acids already present in the insect body.

As shown in Table 3, free fatty acids extracted from mitochondria of wax moth larvae contain a large percentage of unsaturated acids. As it is known that unsaturated fatty acids are, in general, more potent uncouplers and swelling-producing agents than saturated acids (Pressman & Lardy, 1956; Borst *et al.*, 1962; Zborowski & Wojtczak, 1963), this fact may be an additional factor contributing to poor phosphorylation efficiency in isolated insect mitochondria.

The amount of free fatty acids in mitochondria isolated from wax moth larvae is the same as the maximum amount of oleate which can be bound by rat liver mitochondria (Fig. 1). It can be thus supposed that isolated mitochondria of wax moth larvae are "saturated" with fatty acids.

An attempt to characterize lipolytic enzymes of insect tissues revealed in wax moth larvae the presence of enzyme(s) exhibiting properties similar to those of a lipoprotein lipase described by Korn (1955) in mammalian tissues, i.e. inhibition by pyrophosphate, protamine sulphate and high concentrations of NaCl, and activation by heparin. The presence of this enzyme in the wax moth, first shown

by one of us (A.B. Wojtczak, 1961, 1963), has been more recently investigated by Włodawer & Barańska (1965b).

These multiple sources of fatty acids allow to postulate that the high content of fatty acids in isolated insect mitochondria, and consequently the low efficiency, or even the lack, of oxidative phosphorylation, are artifacts and that insect mitochondria *in situ* are as well coupled as are mitochondria of mammalian tissues. It is also evident that 0.25 M-sucrose is not the best isolation medium for insect mitochondria. A medium containing phosphate and respiratory substrates gives much better results (Gregg *et al.* 1960; and the present paper). Although no direct measurements of free fatty acids in mitochondria isolated in this medium have been made, it is reasonable to suppose that in the complex medium conditions exist for an efficient oxidation of fatty acids and therefore no accumulation of these acids can occur. However, this oxidation either is not sufficient or is inhibited in the case of wax moth larvae where high amounts of free fatty acids are present, and in this case only removal of fatty acids by serum albumin is able to re-couple oxidative phosphorylation.

On the basis of experiments with housefly muscle sarcosomes Chance & Sacktor (1958) have postulated that the respiration of flying insects is controlled by the availability of respiratory substrates rather than by the requirement for ATP. Chefurka (1963) has postulated that free fatty acids may be a factor controlling the degree of coupling in insect mitochondria *in situ*. It has been, however, demonstrated that mitochondria isolated from several insect species have a substantial respiratory acceptor-control and are able to carry out oxidative phosphorylation in the absence of serum albumin (Gonda, Taub & Avi-Dor, 1957; Klingenberg & Bücher, 1959; Gregg *et al.*, 1960; Newburgh, Potter & Cheldelin, 1960; Van den Bergh & Slater, 1960, 1962; Cochran, 1963; Michejda, 1962; Michejda & Błotna, 1964). These results together with those of the present investigation allow to postulate that insect mitochondria do not differ substantially from mammalian mitochondria in respect to their *in vivo* phosphorylating ability.

The high rate of swelling of mitochondria of wax moth larvae in 0.125 M-KCl is another effect of the high content of free fatty acids. This swelling can be thus compared with fatty acid-induced swelling of mammalian mitochondria (Lehninger & Remmert, 1959; Zborowski & Wojtczak, 1963). Contrary to other types of swelling, fatty acid-induced swelling of rat liver mitochondria is not inhibited by inhibitors of the respiratory chain (Neubert, Foster & Lehninger, 1962), a property also observed with wax moth mitochondria.

The ATP-induced contraction of wax moth mitochondria is lower as compared with contraction of rat liver mitochondria (Lehninger, 1959) and cannot be increased by serum albumin. This may be due to some irreversible changes caused by fatty acids. The extent of contraction is, however, increased by phosphocholine and by an unidentified dialysable factor present in the supernatant fraction. Stimulation of oxidative phosphorylation by phosphocholine and its protective effect against uncoupling by ageing of mitochondria have been observed by Rossi, Rossi, Sartorelli,

Siliprandi & Siliprandi (1962). These authors explained the effect of phosphocholine by its inhibitory action on phospholipases. It is not clear whether the same explanation may be valid in the case of stimulation by phosphocholine of the ATP-induced contraction of insect mitochondria.

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OKSYDACYJNA FOSFORYLACJA W MITOCHONDRIACH MOLA WOSKOWEGO I NIEKTÓRYCH INNYCH OWADÓW

Streszczenie

1. Mitochondria izolowane z gąsienic mola woskowego (*Galeria mellonella*) zawierają znaczną ilość wolnych kwasów tłuszczowych i są zdolne prowadzić oksydacyjną fosforylację (z bursztynianem jako substratem oddechowym) tylko w obecności albuminy surowiczej. Izolowane mitochondria mięśni owadów dorosłych: mola woskowego, muchy plujki (*Calliphora erythrocephala*) i karaluchów (*Periplaneta americana* i *Blaberus giganteus*) zawierają mniej wolnych kwasów tłuszczowych i mogą fosforylować w nieobecności albuminy surowiczej, jednak dodatek albuminy zwiększa stosunek P:O.

2. Wysoka zawartość wolnych kwasów tłuszczowych w mitochondriach owadów spowodowana jest częściowo działalnością enzymów lipolitycznych, częściowo zaś obecnością wolnych kwasów tłuszczowych w tkankach żywych owadów, które to kwasy są adsorbowane przez mitochondria w czasie procedury izolacji.

3. Mitochondria gąsienic mola woskowego wykazują szybkie pęcznienie spontaniczne w roztworze 0.125 M-KCl. Pęcznienie to można zmniejszyć dodatkiem albuminy surowiczej.

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INFLUENCE OF TEMPERATURE ON ACCUMULATION OF ACETYLCHOLINESTERASE ACTIVITY AT THE ENDS OF TRANSECTED NERVES OF THE FROG

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This paper is dedicated to Professor Włodzimierz Niemierko

1. The acetylcholinesterase (AChE) activity in normal sciatic nerves of the frog increases when the temperature at which the frog is kept, is raised from 7° to 10° and from 18° to 25°, whereas for 18° it is the same as for 10°. 2. After transection of the nerve, AChE activity increases at both ends of the severed nerve, the degree of the increase being dependent on the temperature at which the frog is kept. 3. The effect of temperature on accumulation of AChE at the ends of the transected nerve is discussed in connection with the regeneration of frog nerves, which also is a temperature-dependent process.

It was shown earlier (Lubińska, Niemierko, Oderfeld, Szwarc & Zelenà, 1963b) that in severed peripheral nerves of mammals an increase of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) activity occurs near the place of transection at its both sides. A similar phenomenon was observed by Lubińska, Niemierko, Oderfeld-Nowak & Szwarc (1964) when a segment of the nerve, cut off from cell bodies and from the nerve endings, was left *in situ* for several hours. However, no differences of the total activity of AChE of the whole isolated fragment were found as compared with the activity of the nerve before transection. This result indicated that in the isolated segment neither synthesis nor activation of AChE took place, while the distribution of the enzyme along the segment did change. The increase in AChE activity at the ends of the nerve segment was balanced by a simultaneous drop of activity in the middle part. These findings, as well as many other biochemical, histochemical, and cytological data were interpreted (for review see Lubińska, 1964) as an indication of bidirectional movement of axoplasm and of settling of particles of subcellular membranes containing AChE at the ends of the severed nerve.

It seems that the amount of AChE accumulated at the ends of the nerve fibres might reflect the rate of axoplasmic flow. It could be supposed therefore that

factors affecting the flow, as e.g. temperature of environment (Kamiya, 1959), should also influence the process of accumulation of AChE. In the present experiments, the effect of temperature on the changes in AChE activity at the transected ends of axons of the frog was investigated.

MATERIALS AND METHODS

The experiments were performed on frogs, *Rana esculenta*, caught in October and kept in tanks at 5 - 10°. Before each experiment, the frogs were put separately into jars containing some sand and water, and kept for about 15 days in darkened thermostats at 7°, 10°, 18°, or 25°. The adaptation to 25° was made in two steps. First the animals were transferred to room temperature (about 20°) for 10 days, and thereafter to 25°. This procedure diminished the mortality of the animals at 25° which otherwise was fairly high.

The transection of the sciatic nerves was done under ether anaesthesia. From both legs, segments of nerves 10 mm. long were removed at a distance of about 3 mm. above the branching of sciatic into peroneal and tibial nerves; the AChE activity of these fragments was the initial (control) activity. After the excision of nerve segment and suture of the skin, the frogs were transferred again to the jars and kept at the starting temperature. At time intervals from 3 to 24 hr., the frogs were killed by decapitation and about 2 mm. long segments of proximal and distal nerve stumps were removed and analysed. As AChE activity was related to 1 mm. of nerve length, it was essential that in the two examined portions the number of nerve fibres should be the same as in the control segment. All portions of nerves to be analysed, were thoroughly cleaned under microscope from the connective tissue and blood clots, rinsed in Ringer solution, and the length of the segments was measured with calipers to the nearest 0.1 mm. The details of the procedure were the same as described previously (Lubińska, Niemierko, Oderfeld & Szwarc, 1963a), except that the nerves were analysed in epineurium. The AChE activity was determined in nerve homogenates at 37° by microiodometric method of Niemierko, Gruda & Oderfeld (1960) with acetylthiocholine (Light, Colnbrook, Bucks, England) as substrate. For each AChE activity determination, similar nerve pieces from 1 - 5 frogs were pooled. The activity of the enzyme was expressed in mμmoles of acetylthiocholine split in 2 hr. per 1 mm. of the nerve length.

RESULTS

In the first series of experiments, the AChE activity was estimated in nerves of frogs which had been kept for 15 days at 7°, 10°, 18° or 25°. The estimations were made at a temperature of 37° which was found to be optimal for AChE from all nerves irrespective of the temperature at which the frogs were kept. Table 1 shows that environmental temperature influenced significantly the AChE activity in frog nerves. In frogs kept at 7° the activity was the lowest, but already at 10° it increased

Table 1

Acetylcholinesterase activity in normal nerves of the frogs kept at various temperatures

The activity was measured at 37° and expressed in mμmoles of acetylthiocholine split per 1 mm. of nerve length during 2 hr. Mean values ±S.E.M. are presented. The differences between the successive values were significant at $P < 0.001$, except the difference between 10° and 18° (insignificant). Each analysis was performed on 2 - 8 frog nerves in duplicate or triplicate.

Temperature of environment	AChE activity	Number of experiments
7°	12.3±0.5	33
10°	18.0±0.7	21
18°	17.0±0.6	16
25°	26.9±1.2	27

Table 2

The ratio of acetylcholinesterase activity in 2 mm. long segments of the proximal to those in the distal stump of transected nerve of frogs kept at various temperatures, measured at various time intervals after the transection

Mean values ± S.E.M. are given; in parentheses, the number of experiments.

Temperature of environment	Time after transection			
	3 hr.	6 hr.	12 hr.	24 hr.
7°	1.15±0.06 (3)	1.50±0.09 (5)	1.54±0.07 (6)	1.48±0.09 (6)
10°	—	1.32±0.09 (4)	1.83±0.19 (3)	1.44±0.15 (3)
18°	—	1.31±0.06 (6)	1.45±0.10 (4)	1.53±0.15 (4)
25°	1.47±0.17 (3)	1.58±0.06 (6)	1.40±0.11 (4)	1.26±0.07 (6)

by about 50%. There was no further increase up to 18° whereas at 25° the activity of the enzyme increased again and was twice as high as in frogs kept at 7°. The changes in AChE activity were found to be dependent on environmental temperature in a way similar to that observed by Lubińska & Olekiewicz (1950) for the rate of regeneration of frog nerves. The regeneration began when the temperature was not lower than 8°; its rate did not change markedly over the range from 9° to 16° whereas it was highly increased between 17° and 25°.

In the next series of experiments, AChE activity in terminal 2 mm. long nerve stumps of frogs kept at various temperatures was examined at different time intervals after the transection (Fig. 1), and expressed as percentages of the control values. During the period of 3 - 24 hr. after the transection, AChE accumulated at the ends of both nerve stumps, its amount being always higher in the proximal than in the distal one. The degree of accumulation, similarly as it has been found

for dog nerves (Lubińska *et al.*, 1964), was dependent on the time interval since the transection and on environmental temperature of the frogs. For 7° the AChE activity increased only during the first 6 hr. after the transection to about 250% of the control value proximally, and to about 170% distally to the lesion, and no further increase in enzyme activity with time was observed. For 10° and 18°, AChE activity continued to rise up to 24 hr., the rate of accumulation of the enzyme being the highest during the first 6 hr. For 25° a steady rise of AChE accumulation was observed during 24 hr.

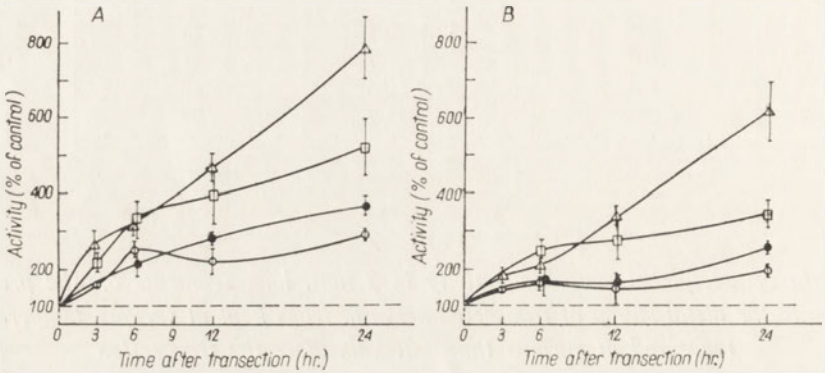

Fig. 1. The effect of environmental temperature on accumulation of acetylcholinesterase activity at (*A*), proximal, and (*B*), distal end (2 mm. long) of severed frog nerve at various time intervals after the transection. Environmental temperature: (○), 7°; (●) 10°; (□), 18°; (△), 25°.

The relative increase of AChE activity after 3 and 6 hr. was nearly the same for 7° and 10°, as well as for 18° and 25°. Later on, however, 12 hr. after the transection, a distinct difference between the degree of accumulation of AChE in nerves of frog kept at 18° and 25° was observed; during the subsequent 12 hr. this difference became still greater.

The activity of AChE at the transected end of the proximal stump reach ed about 500% of the control value at 18° and about 800% at 25°. The difference between the effect of the lower temperatures examined, 7° and 10°, was much smaller and after 24 hr. the respective values amounted to 280% and 350% of the control.

Rather unexpected results were obtained 12 hr. after the transection for frogs kept at 7° and 10°, as AChE activity was found to be lower at this time than 6 hr. after the transection. As the 3- and 6-hr. experiments were carried out in the daytime, and in the 12-hr. experiments the nerves were transected in the evening and the fragments of both stumps removed in the morning, it was thought possible that the accumulation of AChE activity might by influenced by the time of the day. Therefore in two additional 12-hr. experiments with frogs kept at 7°, the nerves were transected in the morning and the stump segments removed at night. In these experiments the AChE activity accumulated during the day at the end of the proximal stump amounted to 267% of the control value, and of the distal stump to

197% (mean from 2 experiments) whereas for the night-time the corresponding values were 200% ± 12 and 128%±18 (n=4). This seems to indicate that in frog nerve during the day a higher accumulation of AChE takes place than during the night. It is to be noted that no effect of day- or night-time on accumulation of AChE activity was observed in experiments on dogs (Niemierko & Lubińska, 1967).

As it may be seen in Fig. 1A and B, the AChE activity was consistently higher in the terminal part of the proximal than of the distal nerve stumps, but for all the temperatures studied the corresponding curves were practically parallel. The ratio of the activity at the ends of the two stumps varied but little (Table 2) and amounted to 1.45±0.03 (n = 61). A similar pattern of AChE accumulation was observed in nerves of some mammals (Lubińska *et al.*, 1963b).

DISCUSSION

The experiments presented in this paper show that raising of the environmental temperature results not only in an increase in AChE activity in normal frog nerves, but also in its enhanced accumulation in severed nerves at both sides of the transection. Accumulation of AChE at the ends of transected nerves was assumed (Lubińska *et al.*, 1963b) to be a consequence of two different processes: bidirectional movement of axoplasm and settling of membraneous particles containing AChE. Although the present experiments do not permit to distinguish the effect of temperature on either of these processes separately, the obtained results might perhaps give some indications concerning their respective roles.

Viscosity of cytoplasm is known to be smaller at higher than at lower temperature (Heilbrunn, 1952). It seems possible that the flow of axoplasm is accelerated when its viscosity becomes lower, and the settling of some subcellular structures might be also dependent in some way on viscosity of axoplasm.

The Q_{10} values can only roughly indicate the changes in the rate of accumulation of AChE in transected nerves at different temperatures. In our experiments, Q_{10} calculated for the temperature of 7° to 10° amounted to 2.2, whereas for higher temperatures, 10 - 18° and 18 - 25° it was 1.6 and 1.8, respectively. The obtained data for Q_{10} as well as the decrease of Q_{10} with raising of temperature, are similar to those found for other biological processes.

As the regeneration of nerves is considered to be connected with movement of axoplasm, it seemed interesting to compare the influence of various temperatures on the regeneration of nerves with their effect on the accumulation of AChE. The process of regeneration of frog nerves has not been observed at temperatures lower than 8° (Lubińska & Olekiewicz, 1950) whereas the accumulation of AChE at the ends of transected nerves was observed also at 7°. This difference additionally indicates that one should not, as it is often done, calculate the rate of axoplasmic flow from data concerning the regeneration. The relationship between these two processes was discussed by Lubińska (1964).

As for the regeneration of the nerves the requirement of axoplasmic material synthesized in cell bodies is increased, it seems possible to assume that for these enhanced synthetic processes a higher temperature is needed than for the axoplasmic movement alone.

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WPŁYW TEMPERATURY NA WZROST AKTYWNOŚCI ESTERAZY ACETYLOCHOLINOWEJ W NERWACH ŻABY W POBLIŻU MIEJSCA PRZECIĘCIA

Streszczenie

1. Aktywność esterazy acetylocholinowej (AChE) w normalnych nerwach kulszowych żaby zwiększa się, gdy temperaturę hodowli podwyższa się z 7° do 10° i z 18° do 25°, natomiast w temperaturze 18° aktywność AChE jest taka sama jak w 10°.

2. Po przecięciu nerwu aktywność AChE wzrasta w obu końcach uszkodzonego nerwu, przy czym stopień wzrostu zależy od temperatury, w której przetrzymywane są żaby.

3. Wpływ temperatury na gromadzenie się AChE w pobliżu miejsca przecięcia nerwu przedyskutowano w powiązaniu z procesem regeneracji nerwów obwodowych żaby, również zależnym od temperatury.

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DETERMINATION OF NUCLEIC ACIDS IN PERIPHERAL NERVES

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This paper is dedicated with esteem and affection to Prof. Dr. W. Niemierko on the occasion of his seventieth birthday

1. A modification of the two-wavelength method of Tsanev & Markov (1960) was applied for estimation of RNA and DNA in peripheral nerves. 2. The method allows to determine small amounts of nucleic acids (corresponding to 0.3 μg. P_{nucl}) in about 10 mg. of nerve tissue.

Estimation of nucleic acids in peripheral nerves is difficult due to their very small concentration and the presence of interfering substances (cf. Hutchinson & Munro, 1962). Logan, Mannel & Rossiter (1952) reported that the method of Schneider (1946) is unsuitable because of the presence of non-nucleotide chromogenic substances which prevent the estimation both of ribose in RNA by orcinol method and of deoxyribose in DNA by diphenylamine method. Besides, the same authors stressed that some degradation products of lipoproteins, containing inositol phosphate, considerably increase the amount of phosphorus, especially in the RNA fraction obtained according to Schmidt & Thannhauser (1945).

As it is generally known, contamination of tissue nucleic acid fractions with protein degradation products formed during isolation of nucleic acids, interferes with the ultraviolet spectrophotometric estimation of RNA and DNA. Elimination of interfering substances, e.g. by adsorption on charcoal or Dowex, is very laborious and quite unsuitable for small amounts of tissue. The interfering effect of protein degradation products is eliminated in the spectrophotometric two-wavelength method elaborated by Tsanev & Markov (1960) for estimation of both RNA and DNA isolated from various animal tissues after mild alkaline hydrolysis. As in the tissues studied (liver, epidermis, muscle and malignant tumours) the extinction of protein degradation products at 260 mμ was found to be equal to that at 286 mμ, and at 268 mμ to 284 mμ, they proposed equations which enabled to determine RNA at 260 and 286 mμ, and DNA at 268 and 284 mμ.

In our attempts to apply the two-wavelength method for determination of nucleic acids in the nerves, we encountered some products of protein breakdown with UV

spectra highly different from those reported by Tsanev and Markov. It seemed therefore necessary to examine more carefully the procedure of isolation of the protein degradation products from the nerve tissue, to characterize their ultraviolet spectra and to modify correspondingly the two-wavelength method for determination of the nucleic acids present in the peripheral nerves.

EXPERIMENTAL

Experiments were performed on the sciatic nerves of adult cats and rats. The animals were anaesthesized with nembutal; the nerves were removed, cleaned from fat tissue and epineurium as described by Lubińska, Niemierko, Oderfeld & Szwarc (1963), and stored at 4° for several hours. The tissue was homogenized at 0° in bidistilled water. The homogenate was extracted twice in the cold with 0.6 N-perchloric acid to remove the acid-soluble compounds, and the residue was dried and delipidated with ethanol and ether according to Bloor (1929).

Characteristics of protein degradation products present in RNA and DNA fractions of the nerve

The protein degradation products were separated from the dry delipidated nerve as described by Tsanev & Markov (1960). For comparison, a corresponding preparation from rat liver was separated in the same way. The ultraviolet absorption

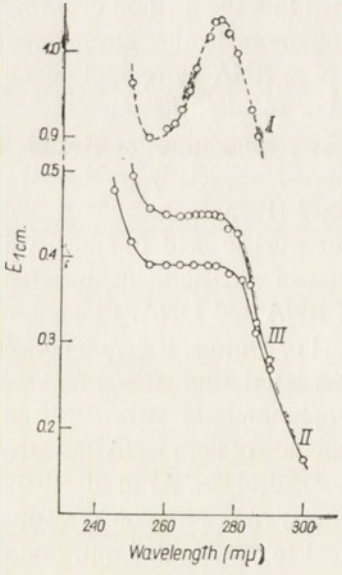

Fig. 1. Ultraviolet absorption spectra of protein degradation products isolated from *I*, rat liver; *II*, rat sciatic nerve; *III*, cat sciatic nerve, according to the procedure of Tsanev & Markov (1960). Acid-insoluble fat-free material obtained from 50 mg. of wet tissue was extracted with 1 N-perchloric acid at 80° six times, for 15 min. each. The residue was washed with water and treated with 1 N-KOH for 1 hr. at 100°. The hydrolysate was cooled and 60% perchloric acid was added to 1 N concentration, and centrifuged. The supernatant was diluted to 5 ml. with 1 N-perchloric acid and the extinction measured.

spectrum of protein degradation products (Fig. 1) obtained for the liver was similar to that reported by Tsanev and Markov, whereas the peak at 275 mμ which corresponds to aromatic amino acids, was not found in the nerve preparation. As the

aromatic amino acids are regularly present in animal proteins, it could be supposed that the preliminary sixfold perchloric acid extraction removed from the nerves not only the nucleic acids but also some proteins.

Therefore the spectra of successive perchloric acid extracts were examined (Fig. 2). The first two extractions removed both from the liver and nerve the whole amount of substances with E_{max} near 260 mμ, i.e. the whole amount of nucleic acids. In the subsequent extracts the maxima were shifted to 275 - 280 mμ, indicating the extraction of proteins or their degradation products. The sixth extract showed only a very small extinction, and no distinct peak of absorption was visible.

To gain some insight into the protein breakdown products present in the DNA and RNA fractions of the nerves, the following procedure was used. Instead of sixfold extraction by hot perchloric acid, the material was extracted only twice. In this way practically all the nucleic acids were removed while the bulk of proteins

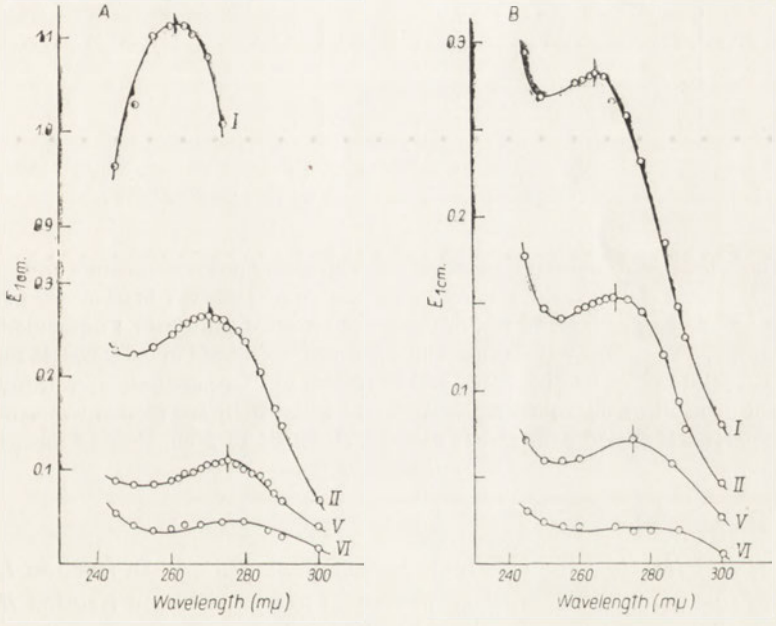

Fig. 2. Ultraviolet absorption spectra of the successive hot perchloric acid extracts of the acid-insoluble fat-free residues from 50 mg. of *A*, rat liver, and *B*, rat sciatic nerve. The first, second, fifth and sixth extracts are shown. The extracts were diluted to 5 ml. with 1 N-perchloric acid and the extinction measured. The short vertical lines indicate the maxima.

still remained in the residue. Then the residue was submitted to the procedure of Schmidt & Thannhauser (1945), which was further used for isolation of nucleic acids, similarly as it was done by Santen & Agranoff (1963) who studied protein degradation products in the brain. According to this procedure, the residue after

alkaline hydrolysis was treated with cold perchloric acid, the soluble fraction was separated by centrifugation and the insoluble material was extracted with hot perchloric acid. Ultraviolet absorption was examined both in the cold and hot perchloric acid-soluble fractions (Fig. 3). Their spectra, unlike those presented in Fig. 1

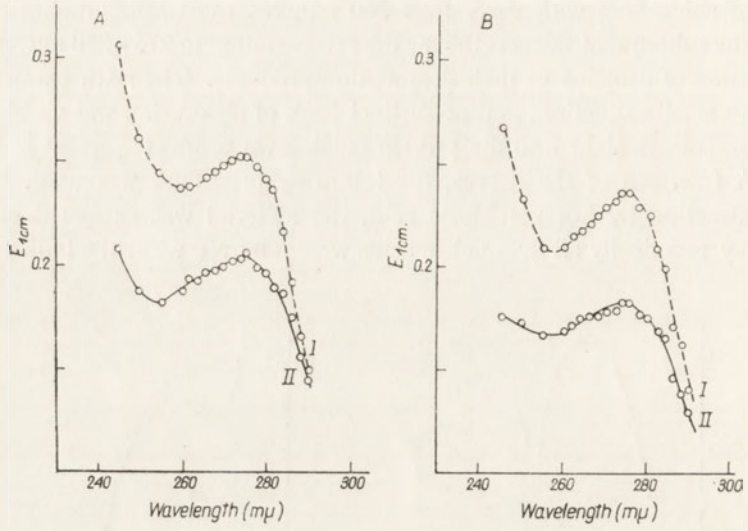

Fig. 3. Ultraviolet absorption spectra of protein degradation products obtained from the sciatic nerve of *A*, rat and *B*, cat by the presented modification of Tsanev & Markov procedure. Acid-insoluble fat-free residue from 50 mg. of tissue was extracted twice with 1 N-perchloric acid for 15 min. at 80°. The residue was washed with water and hydrolysed in 0.2 N-NaOH for 16 hr. at 37°. To the cooled hydrolysate, perchloric acid was added to 1 N concentration, centrifuged and the supernatant diluted to 5 ml. with 1 N-perchloric acid (fraction *I*). The residue was extracted with 1.5 N-perchloric acid for 30 min. at 70°, cooled and diluted to 5 ml. with 1.5 N-perchloric acid (fraction *II*).

(curves *II* and *III*), had the maximum extinction at 275 mμ. In fraction *I*, i.e. that soluble in cold perchloric acid, E_{260} was equal to E_{282}, while in fraction *II* (the hot perchloric acid extract) E_{268} was equal to E_{280}. This is at variance with the data of Tsanev & Markov (1960) who, as it has been mentioned above, reported that for protein degradation products of tissues examined by them E_{260} is equal to E_{286} and E_{268} is equal to E_{284}. According to the presented data, the corresponding values for nerve proteins are $E_{286}/E_{260} = 0.81$ and $E_{284}/E_{268} = 0.95$. These values should be taken into account in determinations of nucleic acids in nerves by the method of Tsanev & Markov, similarly as the values of Santen & Agranoff (1963) should be applied in the case of brain. However, in the present analyses the estimations of absorption of RNA and DNA were done at these pairs of wavelengths at which the extinctions of nerve protein degradation products were equal, that is 260 and 282 mμ for RNA and 268 and 280 mμ for DNA.

The modified estimation of RNA and DNA in nerves

A sample of the nerve tissue (about 10 mg. wet weight) deprived of acid-soluble compounds, delipidated and dried as described above, was hydrolysed according to Schmidt & Thannhauser (1945) in 2 ml. of 0.2 N-NaOH at 37° for 16 hr. The hydrolysate was cooled to 0° and after addition of 60% perchloric acid to 1 N concentration the precipitated DNA was separated by centrifugation. The extinction of the supernatant containing RNA was measured at 260 and 282 mμ. The precipitated DNA was extracted with 2 ml. of 1.5 N-perchloric acid at 70° for 30 min. according to Borkowski & Sikorska (1964), and the extinction read at 268 and 280 mμ. The extinction was measured in the Unicam spectrophotometer SP 500. Calf-thymus DNA and *E. coli* RNA were used as the standards (DNA was prepared and kindly offered by Dr. B. Skoczylas of the Nencki Institute of Experimental Biology, and RNA by Mgr. L. Nowak of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw). The DNA preparation contained 7.7% of phosphorus, the ε(P) being 8551 at 268 mμ after heating with 1.5 N-perchloric acid for 30 min. at 70°; the RNA preparation contained 8.5% of phosphorus, the ε(P) being 10 680 at 260 mμ after 16-hr. hydrolysis in 0.2 N-NaOH at 37°.

To calculate the amount of RNA and DNA in nerves, the following modification of Tsanev & Markov's (1960) equations was used:

$$\text{RNA (mg. P/100 g. of tissue)} = K_{\text{RNA}} \frac{(E_{260} - E_{282})v}{w \times l} \tag{1}$$

$$\text{DNA (mg. P/100 g. of tissue)} = K_{\text{DNA}} \frac{(E_{268} - E_{280})v}{w \times l} \tag{2}$$

where v is the volume of the extract (ml.); w, the weight of the analysed nerve tissue (mg.); l, the length of the light path (cm.).

The coefficients K for RNA and DNA respectively were calculated according to the equation of Tsanev & Markov (1960):

$$K = \left(\frac{M}{\varepsilon(\text{P})} \times \frac{r}{r-1} \right) \times 100,$$

where M is g.-atom of phosphorus; ε(P), the molar extinction coefficient of the respective nucleic acid used as standard per g.-atom of phosphorus; r, the ratio of extinctions which for the RNA at 260/282 mμ was 2.10 and for DNA at 268/280 mμ was 1.40. K_{RNA} and K_{DNA} were found to be 554 and 1270, respectively.

Table 1 summarizes the results of determination of RNA and DNA in the sciatic nerve of the cat and rat, obtained by the presented modification of the method of Tsanev and Markov. These results are similar to those we have obtained by other methods. After purification of the alkaline hydrolysate of cat sciatic nerve on charcoal as described by Borkowski (1962), the RNA values from the estimations of

Table 1

Amount of nucleic acids in peripheral nerves estimated by the presented modification of the method of Tsanev & Markov

The values are expressed as mg. P per 100 g. fresh nerve tissue and given as the mean ±S.E.M. Number of animals in parentheses. The amounts of nucleic acids were estimated in the samples of about 10 mg. of nerve tissue, in 4 cm. path length quartz cuvettes. The analyses were done in duplicate or triplicate.

Sciatic nerve of	DNA	RNA
Cat	3.4±0.7 (7)	4.7±0.5 (4)
Rat	3.3±0.2 (7)	4.5±0.2 (6)

E_{260}, ribose by the orcinol method (Mejbaum, 1939) and P according to Macheboeuf & Delsal (1943) were, respectively, 4.4 , 4.8 and 4.3 mg. P per 100 g. of nerve tissue.

The experiments which showed that the absorption spectra of nerve protein breakdown products and of nucleic acids were additive, also proved the suitability

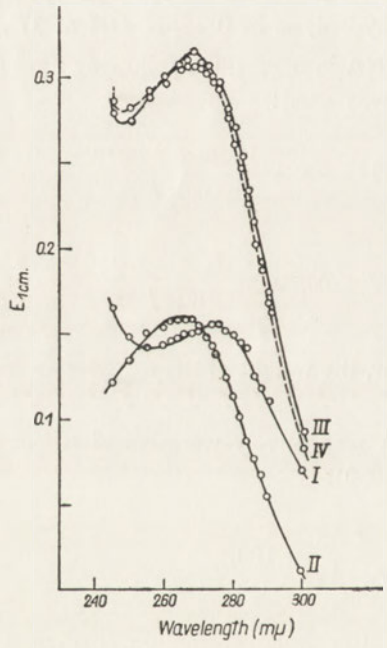

Fig. 4

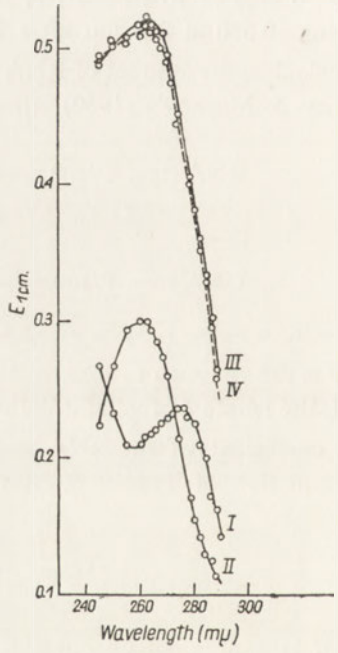

Fig. 5

Fig. 4. Ultraviolet absorption spectra in 1.5 N-perchloric acid of *I*, protein degradation products of the hot perchloric acid-soluble fraction of cat nerve; *II*, calf-thymus DNA hydrolysate (1.5 N-$HClO_4$, 30 min., 70°); *III*, mixture of the protein degradation products and calf-thymus DNA; *IV*, calculated sum of the extinction of the two components of the mixture.

Fig. 5. Ultraviolet absorption spectra in 1.5 N-perchloric acid of *I*, protein degradation products of the cold perchloric acid-soluble fraction of cat nerve; *II*, *E. coli* RNA hydrolysate (0.2 N-NaOH, 16 hr., 37°); *III*, mixture of the protein degradation products and *E. coli* RNA; *IV*, calculated sum of the extinction of the two components of the mixture.

of the applied method (Figs. 4 and 5). Furthermore, when the equations (1) and (2) were applied respectively for RNA and DNA, the recovery of nucleic acids from their mixture was quantitative. The corresponding values (in μg. P) were: DNA taken 3.0, found 3.1; RNA taken 4.5, found 4.4. Although in these experiments *E. coli* RNA and calf-thymus DNA were used instead of purified nucleic acids from the nerve, this seemed to be justified because of the similarity of absorbancy of the respective nucleic acids from different sources.

DISCUSSION

The reported results indicate that severalfold extraction of tissue with hot perchloric acid may remove not only the nucleic acids but also protein degradation products. In the case of the liver a great part of proteins is resistant to this treatment, as after the subsequent alkaline hydrolysis of the residue the spectrum has a marked maximum at 275 mμ, but from the nerve the protein degradation products with E_{max} 275 mμ are completely removed (Fig. 1). This explains why it appeared to be unfeasible to determine RNA and DNA in the nerve by the original method of Tsanev and Markov. In order to apply this very convenient two-wavelength method to nerve, the manner of extraction of the nucleic acids should be chosen very carefully and the ultraviolet spectrum of the protein degradation products present in RNA and DNA fractions should be also examined.

The Tsanev and Markov procedure for isolation of the protein degradation products has already been criticized by Fleck & Munro (1962) with respect to liver and by Santen & Agranoff (1963) with respect to brain. They assumed that the obtained products were not representative of those normally present in nucleic acid fractions. The procedure which we have used for isolation of protein breakdown products seems to be much more suitable, because these products were obtained after removing the nucleic acids from the tissue by the same treatment which was applied for the determination of nucleic acids. Although, even in this case, the isolated protein degradation products are not identical with those occurring in nucleic acid fractions, nevertheless they closely resemble them. This is evident from the fact that the values for RNA, estimated by the present method, are very close to those obtained by other methods, after purification of the RNA fraction on charcoal.

It seems that the presented modification of the two-wavelength method has some advantages in comparison with other methods used for the determination of nucleic acids in the nerve tissue (for example the method of Logan, Mannel & Rossiter, 1952) and the mentioned procedures of purifying the nucleic acid fractions from the interfering substances. It is simpler and allows the determination of small amounts of nucleic acids in the investigated material.

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OZNACZANIE ZAWARTOŚCI KWASÓW NUKLEINOWYCH W NERWACH OBWODOWYCH

Streszczenie

1. Opisano modyfikację spektrofotometrycznej metody Tsaneva i Markova (1960) dla ilościowej analizy RNA i DNA w nerwach obwodowych.

2. Metoda pozwala na oznaczenie małych ilości kwasów nukleinowych, odpowiadających 0.3 μg P_{nukl}, w około 10 mg. tkanki nerwowej.

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W. DRABIKOWSKI and URSZULA RAFAŁOWSKA

ATPase ACTIVITY OF MEMBRANE GHOSTS OBTAINED FROM MITOCHONDRIA

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Dedicated to Prof. W. Niemierko on the occasion of his 70th birthday

1. Membrane ghosts obtained by exhaustive extraction of mitochondria with 0.6 M-KCl contain about 55% of protein and about 35% of lipids calculated for the dry weight. About a half of lipids consist of phospholipids. 2. Membrane ghosts reveal an ATPase activity which is activated by Mg^{2+} (pH optimum about 7.0) and by Ca^{2+} (pH optimum about 8.0). This ATPase is not influenced by dinitrophenol or by mercurials. Azide and Cu^{2+} inhibit its activity. 3. From the respiratory chain in membrane ghosts only cytochrome *c* and cytochrome oxidase seem to be preserved.

In the course of our study on the actomyosin-like protein in mitochondria it has been observed that after complete removal of this protein by exhaustive extraction of mitochondria with 0.6 M-KCl the remaining mitochondrial membrane ghosts still exhibit an ATPase (ATP-phosphohydrolase, EC 3.6.1.3) activity. Since this kind of mitochondria-derived preparations has hitherto not been studied, it seemed worthwhile to examine in detail the properties of the ATPase activity of these preparations.

A preliminary report of some of the results presented below has appeared earlier (Drabikowski & Rafałowska, 1966).

MATERIAL AND METHODS

Mitochondria were prepared according to Hogeboom (1955). Calf liver obtained in the slaughter-house, immediately after killing of the animal, was rapidly transported in cold 0.25 M-sucrose solution to the laboratory. After washing several times with cold sucrose solution, the liver was cut to small pieces, freed of blood vessels, ground in a meatgrinder and finally homogenized in a Potter-Elvehjem homogenizer with a teflon pestle in 0.25 M-sucrose. The fraction sedimenting between 500 and

8000 *g* for 10 min. was collected by centrifugation. Pellets were suspended in the sucrose solution and centrifuged again. This procedure was repeated 3 - 4 times. After each centrifugation the fat layer which accumulated on the top was carefully removed. After the last centrifugation the pellets were suspended in 0.6 M-KCl solution buffered to pH 7.0, or in the Weber-Edsall solution (0.6 M-KCl, 10 mM-Na_2CO_3, 40 mM-$NaHCO_3$) and stirred for 24 hr. at 2°. The insoluble material was removed by centrifugation at 28 000 *g* for 1 hr. The extraction was usually repeated three times. The mitochondrial membrane pellets obtained after the final extraction were washed with water to remove KCl, suspended in water and kept in the frozen state.

ATPase activity was determined at 25° in a medium containing 20 mM-histidine-HCl or tris-HCl of the required pH, and 1 mM-$MgCl_2$ or $CaCl_2$. The reaction was initiated by the addition of ATP to 1 mM final concentration. When the effect of inhibitors was investigated the incubation mixture was preincubated with the inhibitor for 10 min. before addition of ATP. The samples were taken off usually after 5, 10 and 15 min. of incubation and the reaction was stopped by the addition of cold trichloroacetic acid and rapid cooling. After removal of the precipitated protein by centrifugation, in the supernatants the amount of liberated orthophosphate was measured according to Fiske & Subbarow (1925). Oxygen uptake was measured with the Clark oxygen electrode.

For electron microscopy the membrane pellets were fixed with 1% osmium tetroxide buffered to pH 7.4 with veronal buffer, dehydrated in alcohol and finally embedded in methacrylate. Sections were cut with an LKB Ultratome and stained with lead citrate. The specimens were examined with a JEM 6c microscope.

Protein concentration was measured by the biuret method in the modification of Cleland & Slater (1953). Lipids were extracted with a mixture of hot chloroform-methanol (2 : 1, v/v), according to Folch, Less & Sloane Stanley (1957). Purification of lipid extracts, determination of lipid phosphorus and thin-layer chromatography of lipids were performed as described in the previous paper (Drabikowski, Dominas & Dąbrowska, 1966).

All the reagents used were of analytical reagent grade. Sucrose was freed of contaminating bivalent cation by treatment with Dowex 50. ATP disodium salt was purchased from Boehringer and Soehne GmbH, Mannheim, Germany.

RESULTS

It has been found that three 24-hr. extractions of mitochondria with 0.6 M-KCl were sufficient to remove all proteins soluble in these conditions, accounting for about 50% of total mitochondrial proteins. The insoluble residue consisted of mitochondrial membrane ghosts. The electron micrographs (Fig. 1) of this preparation showed two kinds of vesicles: large vesicles, which were most probably unfolded and swollen inner membranes, and small ones formed from the fragments of outer membranes.

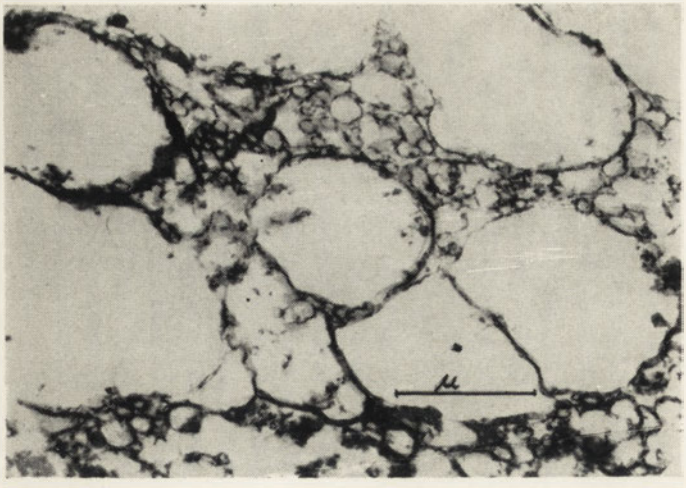

Fig. 1. Electron micrograph of mitochondrial membrane preparation. Mitochondria were extracted with 0.6 M-KCl three times, each for 24 hr. at 0°. The centrifuged pellet after washing out of KCl was prepared for examination by electron microscopy as described in Methods.

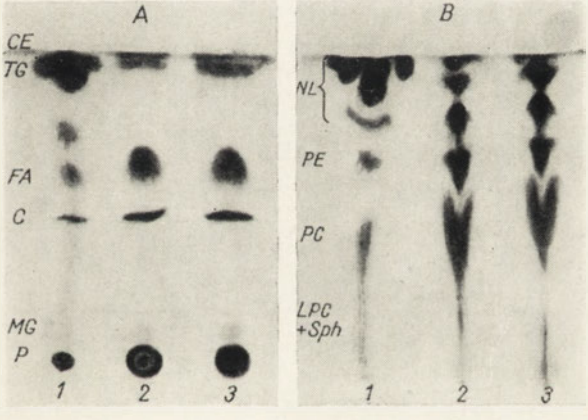

Fig. 2. Thin-layer chromatography of mitochondrial total lipids (*A*) and phospholipids (*B*). Sample *1*, lipid material collecting on the top during centrifugation of the suspension of mitochondria in 0.6 M-KCl, after 24 hr. extraction. Sample *2*, lipids bound to the protein extracted by 0.6 M-KCl and precipitated after dilution. Sample *3*, lipids bound to the membrane ghosts. Glass plate (8.5 × 8.5 cm.) covered with silica gel G. Each sample contained 50 μg. of lipid material. Solvent system in (*A*): petroleum ether - diethyl ether - glacial acetic acid (6.0:4.0:0.2, by vol.), in (*B*): chloroform-methanol - H_2O (6.5:2.5:0.4, by vol.). *CE*, cholesterol esters; *TG*, triglycerides; *FA*, fatty acids; *C*, cholesterol; *MG*, monoglycerides; *P*, phospholipids; *NL*, neutral lipids; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *LPC*, lysophosphatidylcholine; *Sph*, sphingomyelin.

During centrifugation of mitochondria extracted with 0.6 M-KCl some lipids in amount of 1.2 - 1.8% of mitochondrial dry weight, corresponding to about 5% of all lipids, accumulated on the top. They consisted almost entirely of neutral lipids and contained only about 3% of phospholipids (see also Fig. 2). This fraction of lipids seemed to be released from the mitochondria under the influence of high salt concentration, since during the preparation of mitochondria the whole amount of structurally unbound fat was carefully removed. From the remaining 95% of the mitochondrial lipids about one half was bound to the proteins passing into solution during KCl extraction and the second half remained bound with the membrane ghosts.These ghosts were found to contain about 55% of protein and about 35% of lipids per dry weight. About a half of lipids consisted of phospholipids (Table 1). The thin-layer chromatography of total lipids and phospholipids bound both to mitochondrial membranes and to proteins soluble in 0.6 M-KCl revealed a similar pattern of lipids (Fig. 2). In comparison with intact mitochondria the membranes showed much higher content of free fatty acids which were probably formed during the extraction of mitochondria with KCl.

Table 1

Lipid and phospholipid content of mitochondrial membranes

Protein	Total lipids	Phospholipids	Phospholipids
	% of dry weight		% of total lipids
55.5	37.8	21.3	56.4
54.5	33.8	18.6	55.0
55.5	34.4	17.4	50.5
av. 55.1	35.3	19.1	54.0

ATPase activity of the membranes was usually measured at two ionic strength values: at low *I*, in the presence of about 0.003 M-KCl, and at high *I*, in 0.6 M-KCl. At low ionic strength (Fig. 3A), the pH optimum in the presence of 1 mM-Mg^{2+} was about 7.0 and in the presence of 1 mM-Ca^{2+} about 8.0 - 8.5. In the medium containing 0.6 M-KCl the pH optimum was between 7.0 and 7.5 in the presence of either one bivalent cation (Fig. 3B). The comparison of the effect of $MgCl_2$ and $CaCl_2$ at pH 7.0 shows that both cations activated the ATPase activity but $MgCl_2$ at low ionic strength caused slightly higher activation than $CaCl_2$.

The influence of the concentration of Mg^{2+} and Ca^{2+} on the ATPase activity is presented in Fig. 4. One can see that the activity increased in the case of both cations with the increase of their concentrations up to about 5 mM.

The presence of small amounts of bivalent cations was necessary for the ATPase activity of mitochondrial ghosts, since 1 mM-ethylenediaminetetraacetate (EDTA), both at low and high ionic strength, completely inhibited the activity. On the other hand, calcium ions seemed not to be necessary because the addition of 1 mM-1,2-bis-

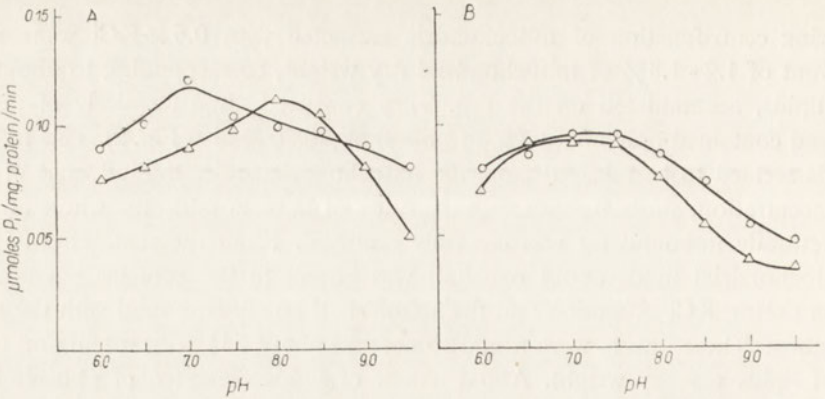

Fig. 3. The effect of pH on the membrane ATPase activity measured (*A*) in 0.003 M-KCl and (*B*) in 0.6 M-KCl. The samples contained: 1 mM-ATP, 20 mM-histidine-HCl, 0.14 mg. of mitochondrial membrane protein per ml., and 1 mM-$MgCl_2$ (○) or 1 mM-$CaCl_2$ (△). Points on the figure are the averages from three parallel determinations.

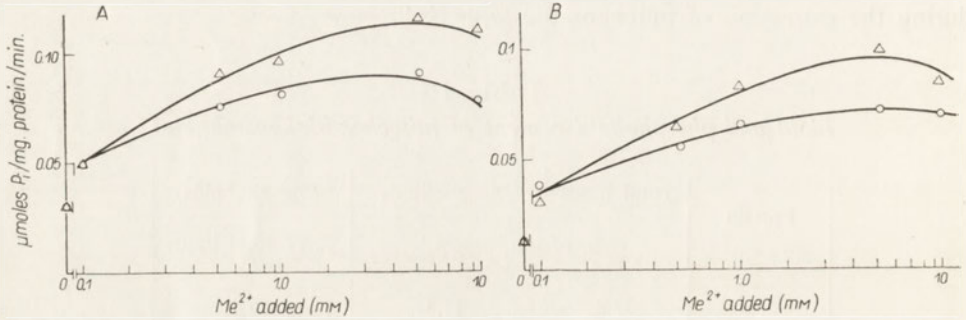

Fig. 4. The effect of the concentration of Mg^{2+} and Ca^{2+} on the ATPase activity of mitochondrial membranes. Incubation mixture contained: 20 mM-tris-HCl, pH 7.0, 1 mM-ATP, 0.7 mg. of mitochondrial membrane protein per ml., $MgCl_2$ (△) or $CaCl_2$ (○) in the concentration indicated on the abscissa, and KCl: 0.003 M in samples (*A*) and 0.6 M in samples (*B*).

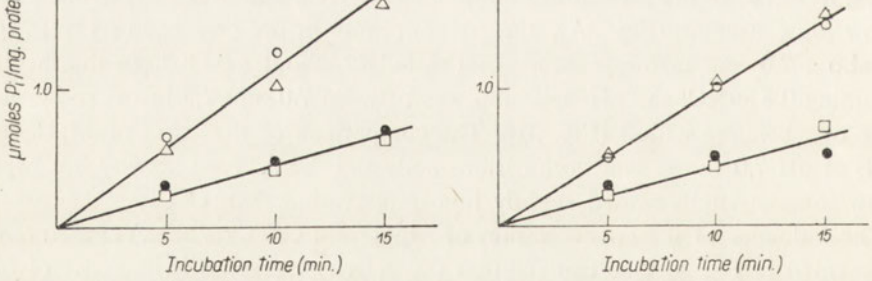

Fig. 5. The effect of dinitrophenol (DNP) on the ATPase activity of mitochondrial membranes measured (*A*) in 0.003 M-KCl and (*B*) in 0.6 M-KCl. Samples containing 20 mM-tris-HCl, pH 7.0, and 0.17 mg. of membrane protein per ml. were preincubated for 10 min. at 25° with 0.1 mM-DNP in the absence (●) or in the presence (△) of 1 mM-$MgCl_2$ in samples (*A*) and 1 mM-$CaCl_2$ in samples (*B*). Control samples without bivalent cations (□), with Ca or Mg (○). The reaction was initiated by the addition of 1 mM-ATP.

(2-dicarboxymethylaminoethoxy)ethane (EGTA), a specific chelator of Ca^{2+}, had no influence on the ATPase activity measured in the presence of 1 mM-Mg^{2+}.

In view of the known effect of 2,4-dinitrophenol, azide and oligomycin on intact mitochondria the influence of these substances on the ATPase activity of mitochondrial ghosts was examined. It was found that dinitrophenol at 0.1 mM concentration had no influence on the ATPase activity at both ionic strengths studied, independently whether or not Mg^{2+} was present (Fig. 5). Sodium azide caused some inhi-

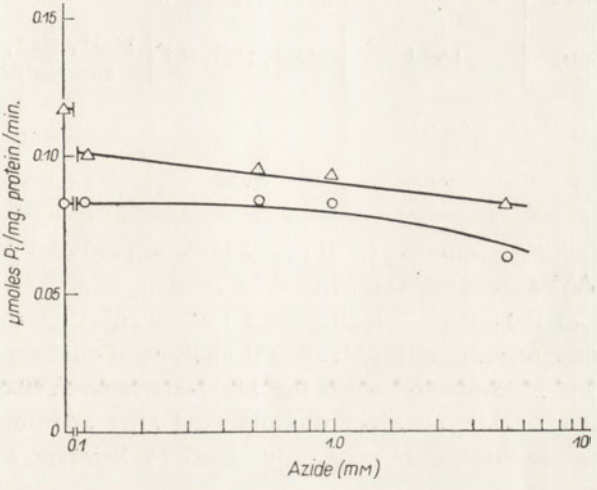

Fig. 6. The effect of the concentration of azide on the ATPase activity. Samples containing 20 mM-tris-HCl, pH 7.0, 0.003 M-KCl, 0.69 mg. of mitochondrial membrane protein per ml., and 1 mM-$MgCl_2$ (△) or 1 mM-$CaCl_2$ (○) were preincubated for 10 min. at 25° with sodium azide and the reaction was initiated by the addition of 1 mM-ATP.

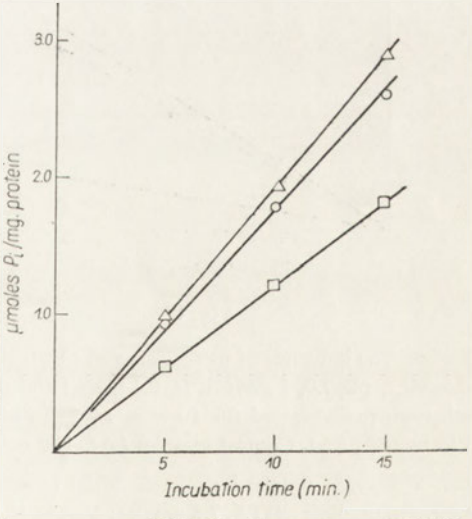

Fig. 7. The effect of oligomycin and azide on the ATPase activity. Samples containing 0.003 M-KCl, 1 mM-$MgCl_2$, 20 mM-tris-HCl, pH 7.0, and 0.23 mg. of mitochondrial membrane protein per ml. were preincubated for 10 min. at 25° with 5 mM-sodium azide (□) or 10 μg. oligomycin/g. protein (○); control samples (△). The reaction was initiated by the addition of 1 mM-ATP.

bition of the ATPase activity (Fig. 6). In the presence of Mg^{2+}, the extent of inhibition increased continuously with the increase of azide concentration, whereas in the presence of $CaCl_2$, only at higher concentration some inhibition was observed.

Table 2

The influence of NaCl + KCl *and ouabain on the* Mg^{2+}*-activated ATPase of mitochondrial membranes*

Incubation mixture contained: 20 mM-tris-HCl, pH 7.0, 1 mM-ATP, 1 mM-$MgCl_2$, 0.33 mg. of mitochondrial membrane protein per ml., and 100 mM-NaCl+20 mM-KCl and 1 mM-ouabain when present.

Azide (mM)	ATPase activity (μmoles P /mg. protein/min.) in the presence of		
	Mg^{2+}	Mg^{2+}, Na^+, K^+	Mg^{2+}, Na^+, K^+ and ouabain
0	0.207	0.206	0.206
5	0.172	0.176	0.165

Oligomycin, added in the amount of 10 μg. of protein, produced only a very small decrease of the ATPase activity (Fig. 7).

The addition of 100 mM-Na^+ + 20 mM-K^+ had no effect on the ATPase activity measured in the presence of Mg^{2+}, and the addition of ouabain did not decrease the activity (Table 2). Similarly, when the Mg^{2+}-activated ATPase was partially inhibited by sodium azide, no effect was observed after addition of Na^+ + K^+ or ouabain. This procedure was previously used by Schwarz & Laseter (1963)

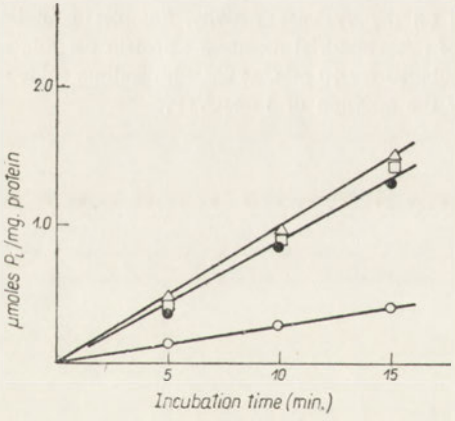

Fig. 8

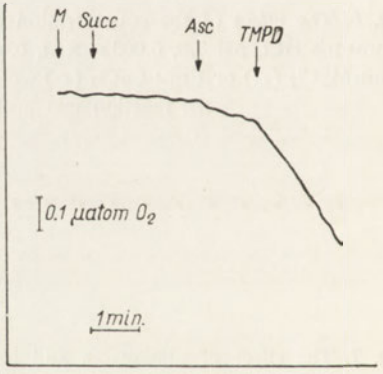

Fig. 9

Fig. 8. The influence of mercurials and $CuCl_2$ on the ATPase activity. Samples containing 20 mM-tris-HCl, pH 7.0, 1 mM-$CaCl_2$, 0.6 M-KCl and 0.16 mg. of mitochondrial membrane protein per ml. were preincubated for 10 min. at 25° with 1 mM-PCMB (●), or 1 mM-salyrgan (□), or 1 mM-$CuCl_2$ (○). Control samples (△). The reaction was initiated by the addition of 1 mM-ATP.

Fig. 9. Oxygen uptake by mitochondrial membranes (polarographic traces). Incubation mixture: KCl, 210 μmoles; EDTA, 7.5 μmoles; $MgCl_2$, 18 μmoles; inorganic phosphate, 30 μmoles; tris-HCl, pH 7.4, 24 μmoles. Final volume 3 ml. Temp. 30°. Additions were made when indicated. Abbreviations: *M*, membranes, 23 mg.; *Succ*, succinate, 20 μmoles; *Asc*, ascorbate, 20 μmoles; *TMPD*, tetramethyl-*p*-phenylenediamine, 0.9 μmole.

and Samaha & Gergely (1965) in order to reveal the $Na^+ + K^+$-dependent ATPase in heart or skeletal muscle microsomes.

ATPase activity of mitochondrial ghosts did not change during storage for a few months at -10° or after repeated freezing and thawing; nor were the stored samples influenced by Na^+ and K^+ ions.

At low ionic strength neither *p*-chloromercuribenzoate, nor salyrgan, nor $CuCl_2$ in 1 mM concentration affected the ATPase activity. *p*-Chloromercuribenzoate and salyrgan in the presence of 0.6 M-KCl were also practically without effect, whereas $CuCl_2$, in this case, decreased the activity to about one third (Fig. 8).

ADP was also hydrolysed by mitochondrial membrane ghosts, and liberation of inorganic phosphate reached an extent of about half of the ATPase activity when measured in the presence of either $MgCl_2$ or $CaCl_2$.

The oxygen uptake by mitochondrial ghosts is presented on Fig. 9. The lack of oxidation of succinate (as well as malate *plus* pyruvate, not shown in the figure) and the oxidation of ascorbate *plus* tetramethyl-*p*-phenylenediamine indicates that only cytochrome *c* and cytochrome oxidase are rather well preserved in the membranes.

DISCUSSION

Several kinds of preparations of mitochondrial fragments are known to exhibit an ATPase activity different from that found in the intact mitochondria. These preparations were obtained by sonic disintegration of mitochondria (Bronk & Kielley, 1957; Lardy, Johnson & McMurray, 1958), fragmentation in Waring-Blendor (Kielley & Kielley, 1953), freezing and thawing (Myers & Slater, 1957), treatment of mitochondria with deoxycholate (Watson & Siekevitz, 1956; Siekevitz, Low, Ernster & Linberg, 1958; Ulrich 1963, 1964, 1965), and by lipid peroxidation induced by ferrous ions (McKnight & Hunter, 1966). All such preparations consist essentially of fragments of mitochondrial membranes deprived of matrix. In contrast to the intact mitochondria all these preparations are characterized by lack of, or very small activation by dinitrophenol and a strong activation by Mg^{2+} ions. On the other hand, Ca^{2+} ions either do not activate ATPase or activate it to a much smaller degree than Mg^{2+} ions (Ulrich, 1965). Apart from several similarities, these mitochondrial fragment preparations differ from each other in some respects. For instance, the effect of ADP is not uniform. Thus, ATPase of mitochondrial fragments, obtained by sonication or by disruption in Waring-Blendor, is inhibited by ADP. Fragments obtained by deoxycholate treatment reveal no ability to hydrolyse ADP. On the other hand, fragments obtained by freezing and thawing or by lipid peroxidation are able to split ADP, which may indicate the presence of myokinase.

Membrane preparations obtained and examined in the present work, were not influenced by dinitrophenol either in the absence or in the presence of $MgCl_2$. They were strongly activated by Mg^{2+} ions, but, in comparison with other mentioned preparations, Ca^{2+} ions also activated the ATPase, essentially to the same degree.

In the presence of Mg^{2+} the ATPase activity of our preparations was of the same order as that of other kind of mitochondrial membranes (cf. Kielley & Kielley, 1953; Ulrich, 1964; McKnight & Hunter, 1966).

Similarly to the fragments obtained by lipid peroxidation, the ATPase of KCl-extracted mitochondria is completely inhibited by EDTA, which indicates an absolute requirement for bivalent cations. On the other hand, Ca^{2+} ions seem not to be necessary, because their removal by EGTA does not change the activity found in the presence of Mg^{2+}.

Mitochondrial membranes obtained after extraction with 0.6 M-KCl exhibit the ability to split ADP. Preliminary experiments with further fractionation of these preparations with 2 M-NaI (Drabikowski & Rafałowska, unpublished) seem to suggest that myokinase is present in small vesicles formed from fragmented outer membranes.

In agreement with the results of Ulrich (1963), who studied mitochondrial membrane preparations obtained by deoxycholate treatment, higher concentrations of NaCl or KCl decreased the ATPase activity, although this effect was in the present work much less pronounced.

It is rather interesting that the ATPase of mitochondrial membranes obtained by KCl-extraction is not affected by mercurials; only $CuCl_2$ in the presence of high concentration of KCl causes an inhibition. The enzyme resembles in this respect the mitochondrial ATPase preparation from beef heart (Pullman, Panefsky, Datta & Racker, 1960) and the "basic" ATPase of sarcoplasmic membranes (Hasselbach & Seraydarian, 1966). On the other hand, the ATPases of mitochondrial fragments obtained either with deoxycholate or by sonication are inhibited by *p*-chloromercuribenzoate. A procedure similar to that used in the present work, i.e. extraction of mitochondria with 0.6 M-KCl, was recently used by Tellez de Inon & Burgos (1966) who obtained preparations containing an ATPase which differed in some properties, e.g. some stimulation by dinitrophenol, from those of our preparation. Since these authors applied much shorter time of extraction, their preparations could contain some mitochondria only partially extracted.

The procedure used in the present work enabled the removal of all mitochondrial proteins soluble both in water and in salt solutions. The insoluble residue, i.e. mitochondrial membrane ghosts, seems to consist chiefly of structural protein which forms the "backbone" of the mitochondria and, according to Green, accounts for about 50% of total mitochondrial protein (see review by Green, 1966). The remainder corresponds to coupling enzymes and active electron carriers (cf. Lehninger, 1964).

The content of proteins and lipids in the KCl-extracted mitochondrial membranes is similar to that found in isolated membrane system in Green's laboratory (cf. Green, 1966), and is somewhat higher than that found by McKnight & Hunter (1966) in their membrane preparation.

The amount of protein passing into solution during extraction of mitochondria with 0.6 M-KCl is similar to that removed by sonication, about 55% (cf. Lehninger, 1964) or solubilized after lipid peroxidation, about 65% (McKnight & Hunter, 1966).

Preliminary experiments (Drabikowski & Rafałowska, 1966) have shown that proteins extracted with 0.6 M-KCl consist of two main fractions: proteins remaining soluble at low ionic strength, and proteins precipitating after dilution. The former fraction, deriving probably mainly from matrix, contains an ATPase, most probably identical with the "soluble-ATPase" of Pullman *et al.* (1960). The latter fraction, corresponding to the so-called actomyosin-like protein, also exhibits an ATPase activity of different properties, partially similar to the ATPase of skeletal muscle actomyosin. The results of these investigations will be published elsewhere. However, it seems worthwhile to mention that these two ATPase systems differ in properties from the ATPase of mitochondrial membrane ghosts studied in this work.

Attempts to demonstrate in mitochondrial membranes an Na+K-dependent and ouabain-inhibited ATPase system, which might be related to the ATPase of active transport, were unsuccessful. Recent work in this laboratory (Rafałowska & Drabikowski, 1967) shows, however, that mitochondrial membranes after treatment with 90% acetone according to Lester & Fleischer (1961), reveal some activation by Na^+ and K^+ ions, which is inhibited by ouabain.

There is a general opinion that there is only one ATPase system in mitochondria manifested by reversal of the reactions involved in the formation of ATP. The procedure used in this work enabled the fractionation of mitochondrial proteins into three fractions, each possessing an ATPase activity of different properties.

During the extraction of mitochondria with 0.6 M-KCl the liberation of about 5% of total mitochondrial lipids, almost entirely neutral lipids, takes place. This part of lipids may correspond to a small part of mitochondrial lipids found to be released from beef-heart mitochondria during their preparation (Jensen & Kofod, 1966). High KCl concentration should cause dissociation of all electrostatic bonds between lipids and proteins. Hence, one can suppose that the majority of mitochondrial lipids combines hydrophobically with proteins. This assumption is in full agreement with the recent view on the kind of interaction of mitochondrial lipids with proteins (cf. Green, 1966). On the other hand, KCl-extracted membranes contain only about one half of all mitochondrial lipids, whereas other authors reported (cf. Lehninger, 1964) that nearly all mitochondrial lipids were present in the membrane fraction.

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AKTYWNOŚĆ ATPazy BŁON MITOCHONDRIALNYCH

Streszczenie

1. Błony mitochondriów otrzymane przez wyczerpującą ekstrakcję mitochondriów 0.6 M-chlorkiem potasu, zawierają około 55% białek i około 35% lipidów na suchą masę. Fosfolipidy stanowią około 50% lipidów.

2. Błony mitochondrialne wykazują obecność ATPazy, aktywowanej jonami Mg^{2+} (o optimum pH około 7,0) i jonami Ca^{2+} (o optimum pH około 8,0). Na aktywność ATPazy nie mają wpływu ani DNP ani organiczne pochodne rtęci. Azydek i jony Cu^{2+} hamują aktywność ATPazy.

3. W błonach mitochondrialnych, po ich wyczerpującej ekstrakcji, z całego łańcucha oddechowego daje się stwierdzić jedynie obecność cytochromu *c* i oksydazy cytochromowej.

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PURIFICATION, PROPERTIES AND INHIBITION OF PLANT ARGINASE

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1. Arginase isolated from plants of bitter lupin has been purified 75-fold. 2. Some physical and chemical properties of the enzyme were described and the Michaelis & Menten constants in the presence of Mn^{2+}, Co^{2+} and Ni^{2+} reported. 3. The natural inhibitor of arginase obtained from sunflower seeds, inhibits in a different degree the activities of the enzyme isolated from plant or animal material. 4. Dissociation constants for the arginase inhibitor from sunflower seeds have been determined at various pH values in the presence of Mn^{2+}, Co^{2+} and Ni^{2+}. 5. Plots according to the equations of Lineweaver & Burk, Hunter & Downs, and Dixon have proved that this inhibition is of the non-competitive character.

Many studies have been published concerning the biology, methods of isolation and purification, and physico-chemical properties of arginase (L-arginine amidinohydrolase, EC 3.5.3.1) from animal tissues. Mammalian liver arginase was particularly extensively investigated since it is a very rich source of the enzyme (Greenberg, 1951). However, very little is known about arginase of plant origin, and no references on its purification and physico-chemical properties could be found in the literature. In this paper we report on partial purification and some properties of the arginase isolated from bitter blue lupin (*Lupinus angustifolius*), and we present some data on the kinetics of arginase inhibition by the inhibitor from sunflower seeds, the occurrence of which has been reported on in previous papers (Reifer & Morawska, 1963; Morawska-Muszyńska & Reifer, 1965).

MATERIAL AND METHODS

Purification of arginase. Seven-day-old plants of bitter blue lupin, variety Bielak, were acetone-dried as described previously (Morawska, Kleczkowski & Reifer, 1963). To 15 g. of the obtained powder 300 ml. of 0.02 M-potassium phosphate buffer, pH 7.2, and 3 ml. of 0.1 M-$MnCl_2$ were added, and the suspension was stirred mechanically with a glass rod for 30 min. Then the insoluble material was centrifuged off and 60 ml. of acetone cooled to -15° was added per 100 ml. of the clear supernatant. The resulting precipitate containing the enzyme was immediately centrifuged

at 4000 rev./min. for 10 min. at -12°. The supernatant was discarded, the sediment drained for 15 min., suspended in 60 ml. of 5 mM-maleate - 2.5 mM-$MnCl_2$ buffer, pH 7.0, and dialysed against the same buffer for 18 hr. The dialysis residue was centrifuged and the sediment discarded. To the clear supernatant, 10 μmoles of cysteine per 1 ml. was added, and then solid ammonium sulphate to 0.3 saturation. The precipitate was centrifuged off at 12 000 rev./min. for 10 min., and to the supernatant solid ammonium sulphate added to 0.5 saturation. The resulting precipitate, which contained most of the arginase activity, was dissolved in about 1 ml. of 0.2 M-potassium phosphate buffer, pH 7.2, applied to a column (30×1.2 cm.) of Sephadex G-100 and eluted with the same buffer at a rate of 1 ml. per 15 min. The fraction eluted between 10 and 15 ml. contained all the arginase, the amount of protein being 8 mg. per 1 ml. The active fraction was divided into 0.2 ml. portions and kept at -10°. The whole procedure was carried out in the cold room at 3 - 5°, and several independent purifications yielded similar results.

Determination of arginase activity. Standard assay conditions: 15 μg. of arginase preparation in 100 μl. of potassium phosphate buffer, pH 7.2, was preincubated with 100 μl. of 10 mM-$MnCl_2$ for 60 min. at 38°. Then 100 μl. of 0.5 M-L(+)arginine solution (brought to pH 10.0 by HCl) was added to the preincubated mixture. After 30 min. incubation at 38°, the sample was cooled rapidly to 2 - 5° in an ice-water bath, and neutralized with 200 μl. of 0.2 N-HCl to pH 7.0.

The amount of urea formed was calculated from the amount of ammonia liberated after addition of 300 μl. of 0.25% urease solution in 0.066 M-phosphate buffer, pH 6.6, addition of water to 1.2 ml., and incubation for 30 min. at 38°. One milliliter of the mixture was withdrawn and distilled statically according to the Conway method (Conway, 1947). All estimations were carried out in triplicate. Arginase activity is expressed as μmoles of urea/mg. protein/30 min. Protein was determined colorimetrically according to Lowry, Rosebrough, Farr & Randall (1951).

K_m and K_i were determined in the presence of Mn^{2+}, Co^{2+} and Ni^{2+} at various pH values. Solution of arginase (15 μg.) was preincubated with 100 μl. of a solution containing 1 μmole of $MnCl_2$, 1 μmole of $CoCl_2$ or 2 μmoles of $NiCl_2$. Then were added 100 μl. of appropriate 0.05 M buffer and 10 to 300 μl. of 0.5 M-L(+)arginine solution (adjusted previously to the required pH by 1.0 or 0.2 N-HCl) and water up to 600 μl. After 30 min. of incubation at 38°, the samples were treated as described above. For incubation at pH 7.0 and 7.85, 0.05 M-potassium phosphate buffer, and at higher pH values, 0.05 M-Na-bicarbonate buffer (Gomori, 1955), were used.

Arginase inhibitor. This was prepared from seeds of the sunflower (*Helianthus annuus*) according to the method described previously (Morawska-Muszyńska & Reifer, 1965). Inhibition of arginase was determined by adding 2.5 μg. of the inhibitor dissolved in 5 μl. of water, to the preincubation mixture.

Special reagents. L(+)Cysteine (Schuchardt, GMBH & Co., Munich, Germany), urease and beef liver arginase (Nutr. Biochem. Corp., Cleveland, Ohio, U.S.A.), L(+)arginine (Fluka AG, Buchs GG, Switzerland), Sephadex G-100, new special form (Pharmacia, Uppsala, Sweden). Chlorogenic acid was a kind gift from Lund University, Sweden.

RESULTS AND DISCUSSION

Characteristics of lupin arginase

The purified arginase preparation was 75 times more active than the acetone powder from which it was isolated. Table 1 shows the increase of arginase activity with purification. The enzyme was determined in absence and in presence of 16.5 mM-cysteine to estimate the "apparent" and "true" arginase activity (Reifer & Morawska, 1963), because the addition of cysteine increased the activity at each step of purification of the enzyme.

No satisfactory results were obtained on attempts at purification of the enzyme by the classical methods employed for purification of arginase from animal tissues (Greenberg, 1951; Bach & Killip, 1958; Grassman, Hörmann & Janowsky, 1958). The heating step and freeze-drying caused even a partial inactivation of the enzyme at a concentration of 8 mg. protein/ml. buffer.

Solutions of the enzyme preparation kept frozen at -5°, pH 7.0, were rather unstable at a concentration of 0.7 mg. protein/ml. buffer, as 30% of initial activity was lost after 14 days. However, under the same conditions, but at a concentration of 8 mg. protein/ml. buffer, the enzyme was much more stable and after 3 weeks no changes in activity could be observed.

The influence of various buffers and Mn^{2+} ion during preincubation and incubation on the activity of arginase in the acetone-powder extract, is presented in

Table 1

Purification of lupin arginase

Conditions of assay: indicated amount of protein was preincubated for 1 hr. with 3.3 mM-$MnCl_2$ in potassium phosphate buffer, pH 7.2, then incubated for 30 min. with 166 mM-L-arginine at pH 10.0.

Step of purification	Protein (mg./sample)	Activity (μmoles urea/mg. protein)		Recovery (%)	Ratio of "apparent" to "true" activity
		"apparent", without cysteine	"true", with 16.5 mM-cysteine		
Acetone powder extract	2.0	4.8	6.8	100	0.77
Ppt. by 37.5% acetone (after dialysis)	0.73	17.7	22.6	53	0.78
Ppt. at 0.5 $(NH_4)_2SO_4$ sat.	0.125	149	189	32	0.79
Eluate from Sephadex G-100	0.028	352	536	23	0.66

Table 2. Preincubation in the presence of Mn^{2+} was essential to obtain maximum arginase activity. It appeared that phosphate was the most suitable buffer and acetate caused marked inhibition.

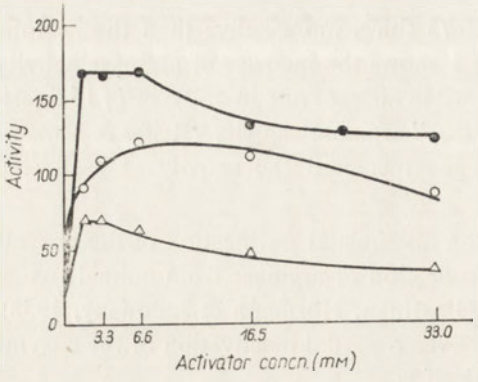

Fig. 1. Influence of various concentrations of (●), Mn^{2+}; (△), Co^{2+}; and (○), Ni^{2+}, on lupin arginase activity at pH 9.95. Standard assay conditions; arginine concentration 165 mM. The activity is expressed as μmoles urea/mg. protein/30 min.

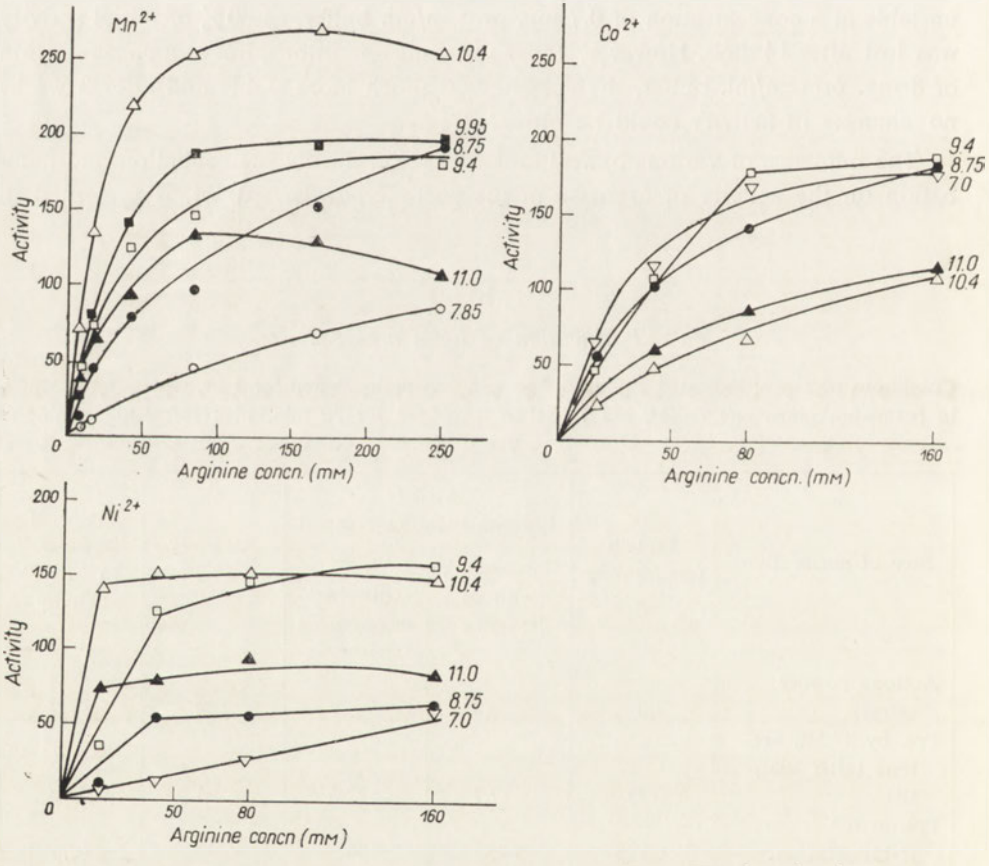

Fig. 2. Michaelis & Menten plots for lupin arginase at various pH values in the presence of 1.6 mM-$MnCl_2$, 1.6 mM-$CoCl_2$, and 3.2 mM-$NiCl_2$. Conditions as described in Methods. The activity is expressed as μmoles urea/mg. protein/30 min.

Figure 1 shows the influence of Mn^{2+}, Co^{2+} and Ni^{2+} on arginase activity at pH 9.95. Low enzyme activity observed in the absence of bivalent cation increased considerably in the presence of 1.65 mM activator. Maximum enzyme activity, especially in the presence of Mn^{2+}, was observed at 1.65 - 6.66 mM concentration, but higher amounts caused a decline of activity. Similar results were obtained by Roche, Thoai & Verrier (1953) with beef liver arginase.

Table 2

Influence of buffer, preincubation, and Mn^{2+} *ion on arginase activity*

Conditions of assay: 100 µl. of 0.05 M appropriate buffer, pH 7, extract from 1.1 mg. of acetone powder and 1.25 µmoles of Mn^{2+} were preincubated for 60 min. After preincubation the samples were alkalized with NaOH to pH 10 and 50 µmoles of arginine, pH 10, was added. Incubation time 2 hr. at 38°, final volume 0.3 ml. The activity is expressed in relation to the activity in maleate buffer without preincubation and in the absence of Mn^{2+}, taken as 100.

Buffer	Without preincubation		After 1 hr. preincubation	
	Mn^{2+} omitted	Mn^{2+} added	Mn^{2+} omitted	Mn^{2+} added
Na-maleate	100	233	125	434
Na-carbonate-bicarbonate	115	197	152	440
K-phosphate	175	333	185	457
Na-acetate	25	184	35	312

Plots according to the equation of Michaelis & Menten in the presence of Mn^{2+} were drawn for reactions carried out over a range of pH values from 7.85 to 11.0, as at pH 7.0 only very slight arginase activity was detected. In the presence of Co^{2+} or Ni^{2+} the plots represent activities over a pH range from 7.0 to 11.0 (Fig. 2). With Mn^{2+}, maximum activity was obtained at pH 10.4, which is in good agreement with the results of Roholt & Greenberg (1956) for liver arginase, and of Ramaley & Bernlohr (1966) for arginase from *Bacillus licheniformis*. With Co^{2+}, pH 7.0 - 9.4, and with Ni^{2+}, pH 9.4 and 10.4 were the most effective values, which also corroborates the results reported by Junowicz-Kocholaty & Kocholaty (1941).

In the presence of Mn^{2+} at pH 9.95 and at higher pH values the V_{max} was reached with 83 mM-arginine, whereas at pH 8.75 and below this value the V_{max} was not obtained even with 240 mM-arginine. At pH 11.0 rising concentrations of the substrate caused even a noticeable decline of enzyme activity. Similar results were obtained by Kleczkowski (1965) in kinetic studies on ornithine carbamoyltransferase.

Table 3 shows the Michaelis & Menten constants plotted according to Lineweaver & Burk (1934) at various pH values in the presence of Mn^{2+}, Co^{2+} or Ni^{2+}. The comparison of K_m at pH 8.75 indicated a decreasing affinity of the enzyme to the substrate in the presence of $Mn^{2+} > Co^{2+} > Ni^{2+}$. K_m in the presence of Mn^{2+}, and even more so in the presence of Co^{2+}, increased with the rise of pH. Similar observations were reported by Bach & Killip (1961) in the case of three

Table 3

K_m *values for lupin arginase in presence of* Mn^{2+}, Co^{2+} *or* Ni^{2+} *at different* pH

Conditions of assay: arginase preparation, 15 μg. protein in 100 μl. of 0.05 M-Na-carbonate-bicarbonate buffer was preincubated for 60 min. with the indicated ion in carbonate-bicarbonate buffer. Then different amounts of arginine were added, and the mixture incubated for 30 min. at 38°. The samples with Mn^{2+} ion contained 8.3 - 250 mM-L-arginine, samples with Co^{2+} or Ni^{2+} contained 16.6 - 166 mM-L-arginine. Volume of the sample 0.6 ml. The values are taken from Figs. 4 and 5.

pH	K_m (mM) in the presence of		
	1.6 mM-Mn^{2+}	1.6 mM-Co^{2+}	3.2 mM-Ni^{2+}
8.75	41	59	151
9.40	32	71	—
9.95	36	—	—
10.40	40	83	—

crystalline mammalian liver arginases, where the K_m, which was high below pH 8.5, reached the lowest value at pH 8.5 - 9.6, rising again in more alkaline media.

The K_m values for lupin arginase in the presence of Mn^{2+} are very close to corresponding K_m for several liver arginases (Mora, Martuscelli, Ortiz-Pineda & Soberón, 1965a; Bascur, Cabello, Véliz & Gonzalez, 1966). However, there are considerable differences reported for K_m of arginases from various animals and some bacteria, oscillating from 2 mM to 200 mM (Mora *et al.*, 1965a; Ramaley & Bernlohr, 1966). This cannot be due to different degrees of purification, as it has been shown that K_m for arginase does not change after purification (Mora, Tarrab, Martuscelli & Soberón, 1965b).

Arginases of different origin show immunological and electrophoretic differences (Cabello, Prajoux & Plaza, 1965), various rates of inhibition by certain amino acids (Mora *et al.*, 1965a) and varying capacity for hydrolysis of L-arginine derivatives (Mora *et al.*, 1965b). Furthermore, the molecular weight of liver arginase from ureotelic animals was reported to be 138 000 (Schimke, 1962) against 278 000 for

Table 4

Inhibitor specificity towards arginase from various sources

Conditions of assay: 15 μg. of arginase preparation, 3.3 mM-$MnCl_2$, 166 mM-arginine, pH 10. Inhibitor concentration 10 μg./ml. Final volume 0.3 ml. Preincubation 1 hr., incubation 30 min. at 38°

Arginase from	Activity (μmoles urea/mg. protein)		Inhibition (%)
	Control	With inhibitor	
Lupin	343	86	75
Vetch	243	175	28
Beef liver	1357	1107	18

arginase from *Neurospora crassa* and 276 000 from chicken liver (Mora, Tarrab & Bojalil, 1966), indicating that arginase may be active in the form either of a monomer or a dimer.

Further differences among arginases of various origin were observed when the effect of the arginase inhibitor from sunflower seeds on this enzyme was studied (Table 4). The inhibitor has shown a high degree of activity towards purified arginases from bitter lupin, lower affinity towards vetch arginase preparation, and beef liver arginase. Other studied arginases: from spinach, beans, wheat, and pumpkin, have shown high affinity towards the inhibitor. It appears that natural inhibitor(s) may contribute towards the recognition of existing differences of structural nature among various isoenzymes.

Arginase inhibitor

The effect on arginase activity of various concentrations (0.8 - 8 μg./ml.) of the inhibitor from sunflower seeds, was studied at two arginine concentrations, i.e. 33 and 66 mM (Fig. 3). In both cases a proportional increase of inhibition up to 60% was observed with inhibitor concentrations from 0.8 to 4.8 μg./ml. Above 60% inhibition, this increase was no longer proportional to the concentration of the inhibitor, which suggested partial stoicheiometric relations for the complex enzyme - inhibitor with very limited capacity for dissociation.

The dissociation constants K_i were plotted according to Lineweaver & Burk at various pH values and in the presence of arginase activators, Mn^{2+}, Co^{2+} and Ni^{2+} (Figs. 4 and 5). K_i according to Hunter & Downs (1945) and Dixon (1953) were determined only in the presence of Mn^{2+} (Figs. 6 and 7).

The dissociation constants according to Hunter & Downs and Dixon were read directly from the plots, and K_i according to Lineweaver & Burk was calculated from the equation:

$$x = \frac{1}{V}\left[1 + \frac{[I]}{K_i}\right]$$

where [I] was the concentration of the inhibitor (4.1 μg./ml.) and x the point of interception of inhibited reaction with the ordinate

$$y = \frac{1}{V}$$

which determines the interception of the non-inhibited reaction with the ordinate.

$$K_i = \frac{[I]\,y}{x - y}$$

As can be seen from the plots, regardless of the pH employed or the activators used, there was no relationship between the degree of inhibition and the concentration of substrate, indicating the non-competitive type of inhibition.

In Table 5 are summarized the results of K_i determinations. The molecular weight of the inhibitor is not yet known, therefore the dissociation constants cannot be expressed in molecular concentrations, and are all reported in μg./ml. Assuming

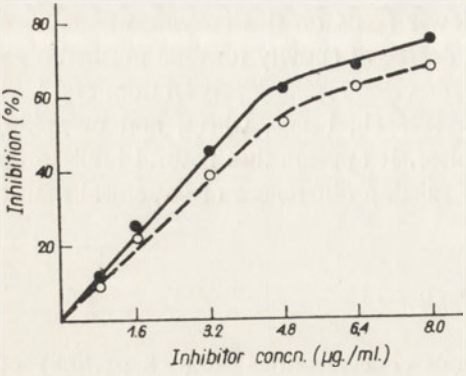

Fig. 3. Influence of concentration of sunflower seed inhibitor on lupin arginase activity. Standard assay conditions. Arginine concentration: (●), 33 mM; (○), 66 mM.

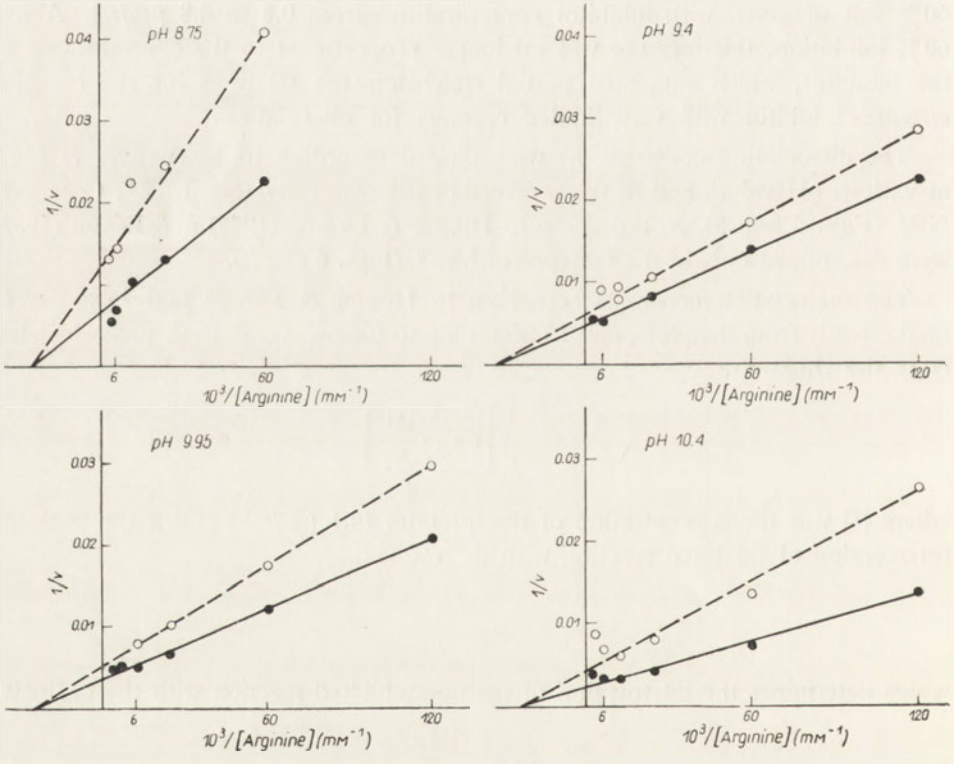

Fig. 4. Graphical determination of K_i by the method of Lineweaver & Burk, in the presence of 1.6 mM-$MnCl_2$, at indicated pH values. (●), Without inhibitor; (○), with 4.1 μg./ml. inhibitor added. The substrate concentrations were in the range of 8 - 240 mM; v is expressed as μmoles urea/mg. protein/30 min.

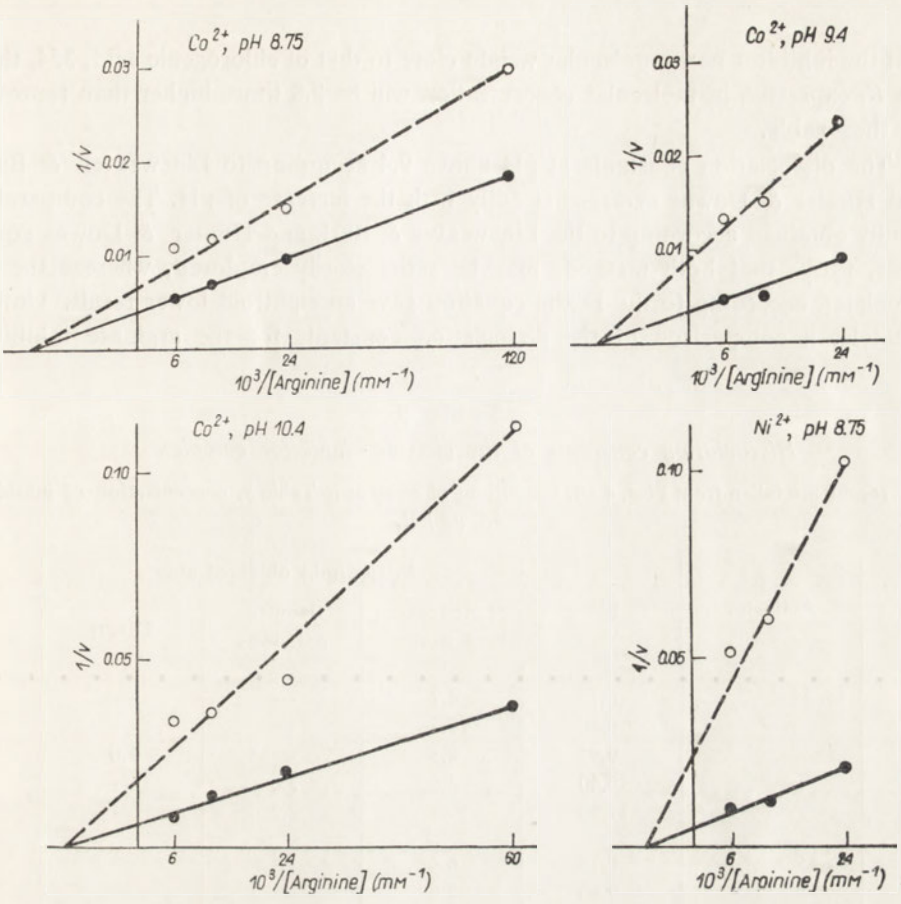

Fig. 5. Graphical determination of K_i by the method of Lineweaver & Burk in the presence of 1.6 mM-$CoCl_2$ or 3.2 mM-$NiCl_2$, at indicated pH values. (●), Without inhibitor; (○), with 4.1 μg./ml. inhibitor added. The substrate concentrations were in the range of 16 - 160 mM; v is expressed as μmoles urea/mg. protein/30 min.

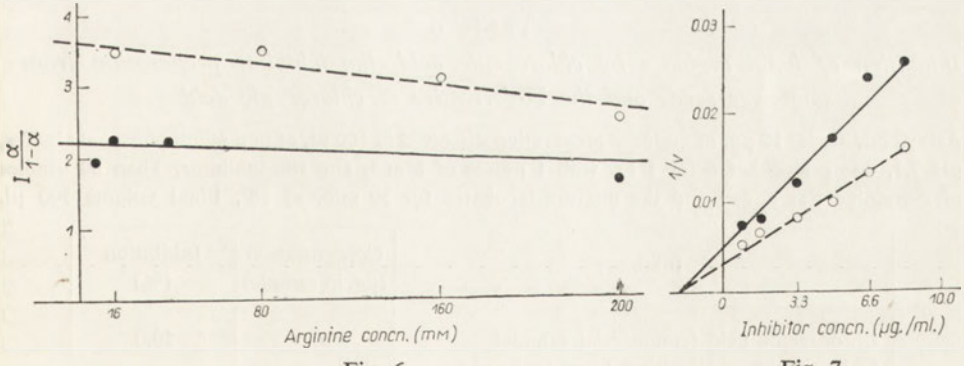

Fig. 6 Fig. 7

Fig. 6. Graphical determination of K_i by the method of Hunter & Downs, at (○), pH 10.4, and (●), pH 11.0. α, Fractional activity.

Fig. 7. Graphical determination of K_i by the method of Dixon at two arginine concentrations (●), 33 mM, and (○), 66 mM; v is expressed as μmoles urea/mg. protein/30 min.

that the inhibitor has a molecular weight close to that of chlorogenic acid, 354, then the K_i expressed in molecular concentration will be 2.8 times higher than reported in the Table.

The dissociation constants at pH above 9.4 according to Lineweaver & Burk and Hunter & Downs decrease steadily with the increase of pH. The comparable results obtained according to the Lineweaver & Burk and Hunter & Downs equations, prove that both methods may be satisfactorily employed, whereas the K_i calculated according to the Dixon equation gave an eightfold lower result. Unfortunately, a comparison of the dissociation constants for the arginase inhibitor

Table 5

Dissociation constants of the enzyme - inhibitor complex

The results are taken from Figs. 4 - 7. Conditions of assay as in Table 3, concentration of inhibitor 4.1 μg./ml.

Activator	pH	K_i (μg./ml.) obtained after		
		Lineweaver & Burk	Hunter & Downs	Dixon
Mn^{2+}	8.75	4.7	—	—
	9.40	13.7	—	—
	9.95	8.3	—	1.0
	10.40	4.6	3.6	—
	11.00	—	2.2	—
Co^{2+}	8.75	6.4	—	—
	9.40	2.6	—	—
	10.40	2.4	—	—
Ni^{2+}	8.75	11.5	—	—

Table 6

Inhibition of lupin arginase by chlorogenic acid, the inhibitor preparation from sunflower seeds and the N*-derivative of chlorogenic acid*

Assay conditions: 15 μg. of arginase preparation dissolved in 100 μl. of potassium phosphate buffer, pH 7.1, was preincubated for 1 hr. with 1 μmole of $MnCl_2$ and the inhibitor. Then 50 μmoles of L-arginine was added and the mixture incubated for 30 min. at 38°. Final volume 300 μl.

Inhibitor	Concentration (μg. in sample)	Inhibition (%)
Chlorogenic acid (commercial sample)	10.0	50.0
Chlorogenic acid isolated from sunflower seed inhibitor	10.0	43.0
Inhibitor preparation from sunflower seeds	2.5	45.0
N-Derivative of chlorogenic acid isolated from sunflower seed inhibitor	0.66	54.0

obtained by the three equations with those for inhibitors of other enzymes is not possible because results reported in literature are generally limited to K_i calculated from one equation only.

At pH 8.75 the lowest K_i value was obtained in the presence of Mn^{2+}, increasing markedly in the presence of Co^{2+} and Ni^{2+}. These values point towards decreasing affinities of the enzyme to the inhibitor in the order $Mn^{2+} > Co^{2+} > Ni^{2+}$. In addition it should be pointed out that the inhibition of arginase is considerably lower in the presence of Co^{2+} and Ni^{2+} than in the presence of Mn^{2+}, which may suggest that Mn^{2+} causes a conformation of arginase molecules most favourable for the affinity of the enzyme to the inhibitor.

The previously reported UV absorption spectra for the arginase inhibitor isolated from sunflower seeds (Morawska-Muszyńska & Reifer, 1965), were strikingly similar to the spectrum of chlorogenic acid (CGA) (Ruckenbrod, 1955). For this reason arginase inhibition was tested against a commercial sample of CGA. Additionally CGA and an *N*-derivative of CGA isolated by Augustyniak & Reifer (unpublished work) from the inhibitor preparation employed in this study, were also tested (Table 6). No marked differences of inhibition were obtained with the commercial CGA and the CGA isolated from the inhibitor preparation. The inhibitor preparation itself was 4 times more active and the *N*-derivative 15 times more active than the free acid, which is quite understandable as the preparation was a mixture of the two substances.

Schwimmer (1958) tested various phenol derivatives for phosphorylase inhibition and found that chlorogenic acid was by far the most potent, inhibiting the enzyme by 50% at a molar concentration of 1 mM. Comparing these results with the inhibition of arginase it is evident that chlorogenic acid is a much stronger inhibitor for arginase than for phosphorylase, as 50% inhibition was obtained at a concentration of 0.1 mM.

As mentioned previously, the *N*-derivative of chlorogenic acid is a much stronger inhibitor of arginase than the free acid. Therefore it is obvious that this compound must be obtained in pure state, the kinetics of its inhibition compared against CGA and the mechanisms of both inhibitions determined.

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OCZYSZCZENIE, WŁASNOŚCI I INHIBICJA ARGINAZY ROŚLINNEJ

Streszczenie

1. Arginazę wyizolowaną z siewek łubinu oczyszczono 75-krotnie.

2. Przebadano niektóre właściwości fizyko-chemiczne arginazy z łubinu i wyznaczono stałe Michaelisa i Menten w obecności jonów Mn^{2+}, Co^{2+} i Ni^{2+} przy różnych wartościach pH.

3. Naturalny inhibitor arginazy z nasion słonecznika w różnym stopniu hamuje działanie izoenzymów roślinnych i zwierzęcych.

4. Wyznaczono stałe dysocjacji kompleksu enzym-inhibitor w różnym pH, w obecności aktywatorów: Mn^{2+}, Co^{2+} i Ni^{2+}.

5. Wykresy otrzymane wg równań Lineweavera i Burka, Dixona oraz Huntera i Downsa dowodzą, że badana inhibicja ma charakter niekompetycyjny.

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PROTEOLYSIS OF LOW-DENSITY LIPOPROTEIN FRACTION OF HEN'S EGG YOLK AND PREPARATION OF GLYCOPEPTIDES FROM ITS PROTEIN MOIETY

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1. From low-density lipoprotein fraction (LDF) of hen's egg yolk, about 60% of protein is released as low-molecular peptides after pronase digestion. 2. The remaining part of the protein, more tightly bound to lipid, is also susceptible to pronase cleavage, a large part of this material (30%) consisting of low-molecular, water-soluble peptides. 3. Glycopeptides are released from native lipoprotein more rapidly than other peptides and practically all hexoses of LDF were recovered after proteolysis as two glycopeptide fractions with a molecular weight of about 2000. 4. A model of LDF structure is proposed.

Along with the development of studies on low-density lipoproteins, different conceptions concerning their structure have been advanced (Oncley, Gurd & Melin, 1950; Zollner, 1958; Vandenheuvel, 1962; Cook & Martin, 1962a, b; Margolis & Langdon, 1966c). At present it seems certain that they form a microemulsion composed of particles possessing a lipid core covered with a layer of protein and phospholipid (Cook & Martin, 1962a, b). Studies of the last few years supplied further data on the properties, composition, function and biosynthesis of lipoproteins (for review see Wehr, 1966). However, much less is known about the structure of the protein component and its organization on the surface of the lipid core.

The low-density lipoprotein fraction (LDF) of hen's egg yolk is a typical representative of that class of lipoproteins. Its general properties and composition have been reported (Turner & Cook, 1958; Martin, Turner & Cook, 1959) and therefore this readily prepared material is particularly suitable for the study of structure of low-density lipoprotein. The aim of the present work was to gain more information on the arrangement of protein on the surface of the lipid core by determining the susceptibility of LDF to the action of proteolytic enzymes, and by studying the glycopeptides released from the native lipoprotein and from the delipidated protein moiety. According to Abraham, Hillyard & Chaikoff (1960), the protein component of LDF, vitellenin, contains 1.3% of hexoses, 0.67% of hexosamines and 0.38% of sialic acid. The distribution of these saccharides along the polypeptide chains may influence the organization of protein in LDF.

Although LDF consists of a system of two mutually overlapping polydisperse fractions (LDF_1 and LDF_2) which differ in average molecular weight, density, and content of lipid and phospholipids (Sugano & Watanabe, 1963; Saari, Powrie & Fennema, 1964; Martin, Augustyniak & Cook, 1964), the same protein is present in both fractions (Augustyniak, Martin & Cook, 1964) and therefore in the present work the unfractionated LDF was used.

EXPERIMENTAL

Reagents. Pepsin, 3 times crystallized, pronase (*Streptomyces griseus*) and cytochrome *c* were Calbiochem. (U.S.A.) products. Trypsin was from Choay (France). Conglutin β was prepared from seeds of *Lupinus angustifolius* by the method of Joubert (1955) in the modification of Wiewiórowski & Augustyniak (1961). Insulin A chain was obtained by oxidation according to Sanger (1949). Sephadex G-50 (fine) and CM-Sephadex C-25 (fine) were from Pharmacia (Uppsala, Sweden). The remaining reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland).

Analytical methods. Hexoses were determined by the orcinol method (Winzler, 1955) or by the anthrone method (Dische, 1955), a mixture of galactose and mannose (2:1) being used as standard. Sialic acid was determined by the method of Svennerholm (1957) except that the transfer of the coloured product to amyl alcohol was omitted, the extinction at 570 mμ being read directly in the aqueous solutions. Protein and peptides were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). In some experiments, the relative concentration of protein in effluents from the column was determined by reading the extinction of the solutions at 280 mμ. For all estimations, Zeiss VSU-1 spectrophotometer was used.

Preparation of LDF and vitellenin

LDF was prepared from hen's egg yolk by ultracentrifugation as described by Martin *et al.* (1964). Vitellenin was obtained by delipidating LDF, using the procedure of Bligh & Dyer (1959). To 0.8 vol. of a 10% solution of LDF in 0.2 M-NaCl, 3 vol. of a methanol - chloroform (2 : 1, v/v) mixture were added with vigorous stirring. The formed suspension was left for 2 hr., then 1 vol. of water and 1 vol. of chloroform were added. The separated chloroform phase, which contained lipids, was carefully removed, the main part of the methanol-water phase was decanted, and vitellenin was collected from the interphase by centrifugation, washed four times with a mixture of methanol - chloroform - water (2 : 1 : 0.8, by vol.), three times with chloroform - methanol (1 : 1, v/v), three times with ethyl ether, and finally dried in vacuum over KOH and P_2O_5.

Vitellenin was also obtained from LDF that was prepared in the following way: 1 vol. of egg yolk was mixed with 1 vol. of water and centrifuged for 2 hr. at 15 000 *g* at 4° to remove granules. The collected supernatant was added with solid ammonium sulphate up to 26% (w/v) concentration and centrifuged again at

15 000 *g* for 5 hr. The top layer containing LDF was collected and mixed with a new portion of 26% (w/v) ammonium sulphate and centrifuged for 5 hr. as above. This procedure was repeated twice more, then LDF was dispersed in water and dialysed against water until free of ammonium sulphate. Then the dialysis residue was centrifuged for 5 hr. at 15 000 *g* to remove insoluble substances which floated in 26% ammonium sulphate solution but sedimented in water. The preparation obtained did not differ in lipid content from LDF obtained by ultracentrifugation.

Pronase digestion of LDF and estimation of extent of proteolysis

A 10% solution of LDF in 0.9% natrium chloride - 0.015 M-calcium acetate, adjusted to pH 6.0 with phosphate, was incubated with pronase at 40° under nitrogen, 1 mg. of the enzyme preparation being added per 100 mg. of LDF protein.

The extent of pronase digestion was estimated as follows:

a. The content of protein in the starting mixture was compared with the amount of peptides soluble in 2.5% trichloroacetic acid after incubation. After a determined time of incubation (0 - 27 hr.), 0.5 ml. samples were withdrawn and added with 1 ml. of water and 0.5 ml. of 10% trichloroacetic acid. The successive filtrates were kept in solid CO_2 until the last sample was collected. For determination of peptides by the Lowry method (1951), 0.5 ml. of the filtrate was added with 3.5 ml. of the carbonate-copper reagent and after 30 min. with 0.2 ml. of the Folin reagent. Then the sample was fourfold diluted with 2% Na_2CO_3 in 1 N-NaOH and after 1 hr. the extinction was read in 1 cm. light-path cuvettes at 600 mμ. For the determination of protein in the starting material 0.5 ml. of the LDF solution was delipidated with the methanol - chloroform mixture, the precipitated protein was dissolved in 3 N-NaOH, the solution after neutralization was adjusted to a volume of 2 ml., and in a 0.5 ml. sample protein was determined as described above.

The extent of proteolysis was expressed as the ratio of extinction of the trichloroacetic acid filtrate (corrected by a value for zero-time incubation) to the extinction of the starting amount of protein.

The time-course of pronase digestion is shown in Fig. 1. After 27 hr. of incubation, the digestion of LDF was practically arrested and 56% of the LDF protein was hydrolysed to peptides soluble in trichloroacetic acid.

b. Four ml. of the mixture which had been incubated for 27 hr., was applied to the Sephadex G-50 column (3.7×98 cm.) equilibrated with 0.9% NaCl, and eluted with the same solution. Fractions of 5 ml. were collected, in which the content of hexoses was determined by the orcinol or anthrone method, and peptides according to Lowry *et al.* (1951). The void volume of the column was 222 ml. The hydrolysate separated into a high-molecular fraction eluted between 195 and 255 ml., and low-molecular fractions (Fig. 2B). The high-molecular peak was eluted at a volume close to that of native LDF (Fig. 2A) and corresponded to 40% of the amount of the LDF protein submitted to pronase digestion. From the remaining 60%,

45% was found in the low-molecular fractions. The ratio of the elution volume of these fractions to the void volume of the column pointed to their molecular weights below 1000.

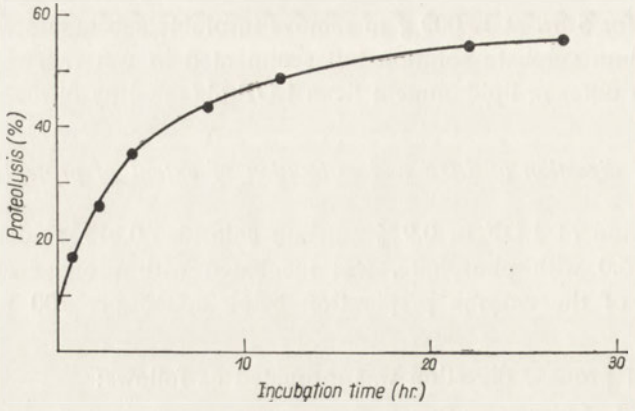

Fig. 1. Time-course of LDF digestion by pronase.

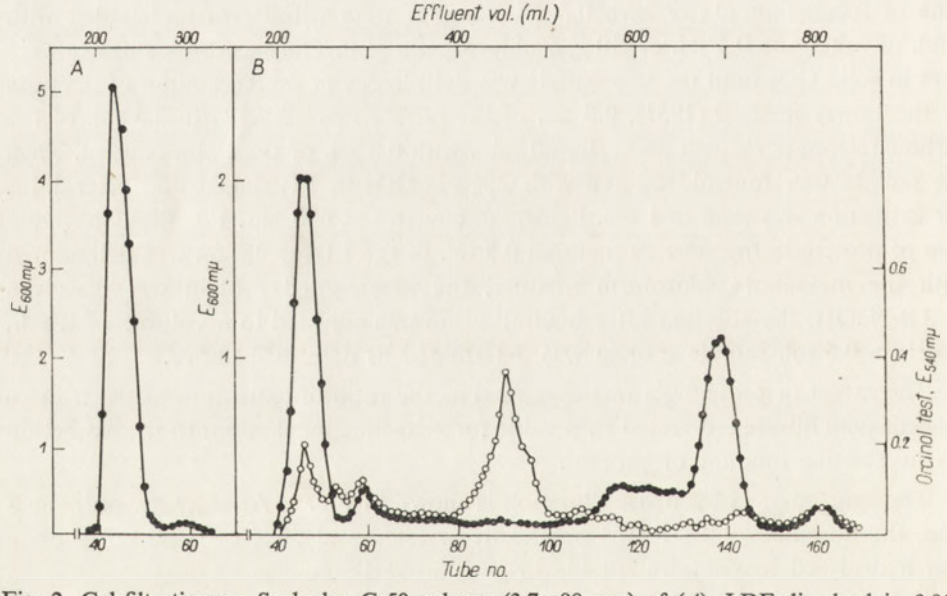

Fig. 2. Gel filtration on Sephadex G-50 column (3.7×98 cm.) of (*A*), LDF dissolved in 0.9% NaCl, and (*B*), LDF pronase digestion products. Protein (●) was determined by the Folin-Lowry test, hexoses (○) by the orcinol test.

In the high-molecular fraction, about 13% of the total amount of hexoses applied to the column was present, and 87% was recovered in other fractions, mainly those eluted at 400 - 500 ml.

c. To the mixture which had been incubated for 27 hr., ammonium sulphate was added to 27% (w/v) concentration and centrifuged for 2 hr. at 15 000 *g*. The

lipid layer collected at the meniscus was purified by twofold redispersion in 27% ammonium sulphate and centrifugation, then dialysed against water until free of salt. The dialysis residue was freeze-dried, and then in the methanol - chloroform (1:1) extract the content of lipid, and in the residue after removal of lipid, total protein and water-soluble peptides were determined by weight.

From 40 g. of native LDF (containing 19 g. of protein per 100 g. of lipid), after pronase digestion about 24.8 g. of a material floating in 27% ammonium sulphate was obtained. This material consisted in 93.4% of lipid and in 6.6% of peptides (7.1 g. of protein per 100 g. of lipid). This indicated that about 63% of the initial amount of protein (19.0 — 7.1 = 11.9 g.) underwent digestion. Although from the initial amount of 33.6 g. of lipid present in 40 g. of native LDF, only 23.2 g. was recovered in the floating fraction of the hydrolysate, these losses did not result from dissolution during digestion of LDF, as no lipids were detected in the chloroform-ethyl ether extract from the subnatant obtained after separation of the floating fraction.

After removal of lipid from the floating fraction of the hydrolysate, about 31% of the protein material present in this fraction was soluble in water. Gel filtration on Sephadex G-50 column showed that the main part of the water-soluble material had a molecular weight of 1000 or less.

Preparation of glycopeptides from vitellenin

Method 1. Ten grams of delipidated vitellenin was mixed with 750 ml. of water, adjusted to pH 2.0 with HCl, then 40 mg. of pepsin was added and the mixture incubated under nitrogen with constant stirring at 30° for 72 hr.; during incubation the pH of the medium was maintained at 2.0 - 2.2. Then the mixture was neutralized and concentrated in a rotary vacuum evaporator to about 60 ml. (a scanty precipitate formed was removed by centrifugation). The digest was then applied to a column (1.9×90 cm.) of CM-Sephadex C-25 equilibrated with 0.1 M-pyridine - acetic acid buffer, pH 5.8, and eluted by a concentration gradient of the above buffer from 0.1 to 0.5 M. In the eluates, peptides were estimated by measuring the extinction at 280 mμ and hexoses by the orcinol test. A typical elution pattern is shown in Fig. 3. Of the hexoses applied to the column, 90 - 94% was recovered in one peak, the appropriate fraction of the effluent being collected, concentrated, dialysed against water and freeze-dried. From 10 g. of vitellenin, 0.63 g. of a material containing about 20% of hexoses was obtained. It was dissolved in 100 ml. of water and, after adjusting the pH to 7.5 with 1 N-NaOH, incubated with 10 mg. of trypsin at 37° under nitrogen for 24 hr., the pH being maintained at 7.4 - 7.6. The hydrolysate was concentrated to about 4 ml. and fractionated on a Sephadex G-50 column (3.7×98 cm.), using water as the eluent (Fig. 4). Glycopeptides, as revealed by estimation of hexoses, were eluted at 400 - 500 ml., at the same elution volume as the glycopeptides obtained by pronase digestion of native LDF (see Fig. 2; for both runs the same column was used). Paper electrophoresis proved that both "400 - 500 ml." fractions contained the same glycopeptides.

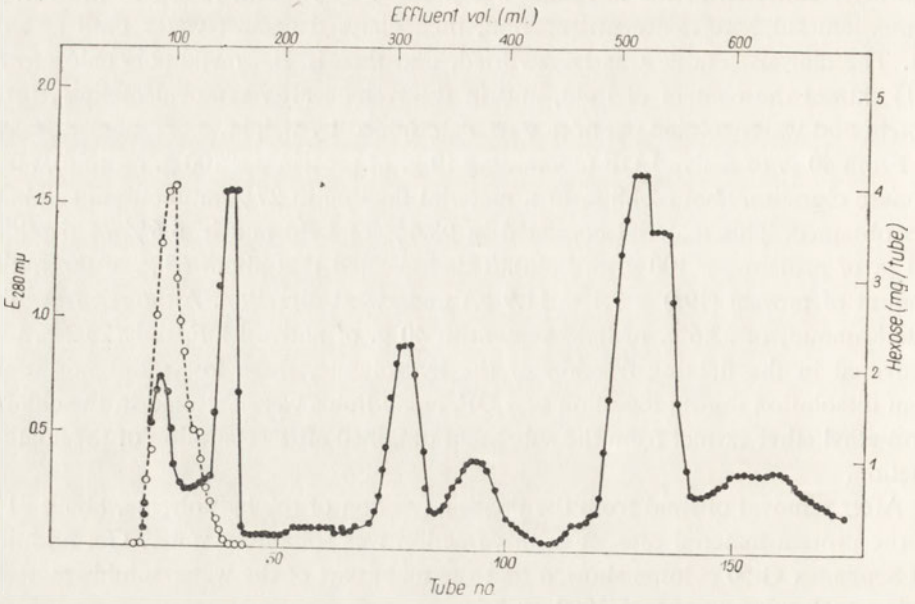

Fig. 3. Separation of the products obtained by digestion of vitellenin (2 g.) with pepsin on CM--Sephadex C-25 column (1.9×90 cm.). Gradient (0.1 to 0.5 M) elution with pyridine - acetic acid buffer, pH 5.8. (●), Protein, $E_{280\,m\mu}$, (○), hexoses.

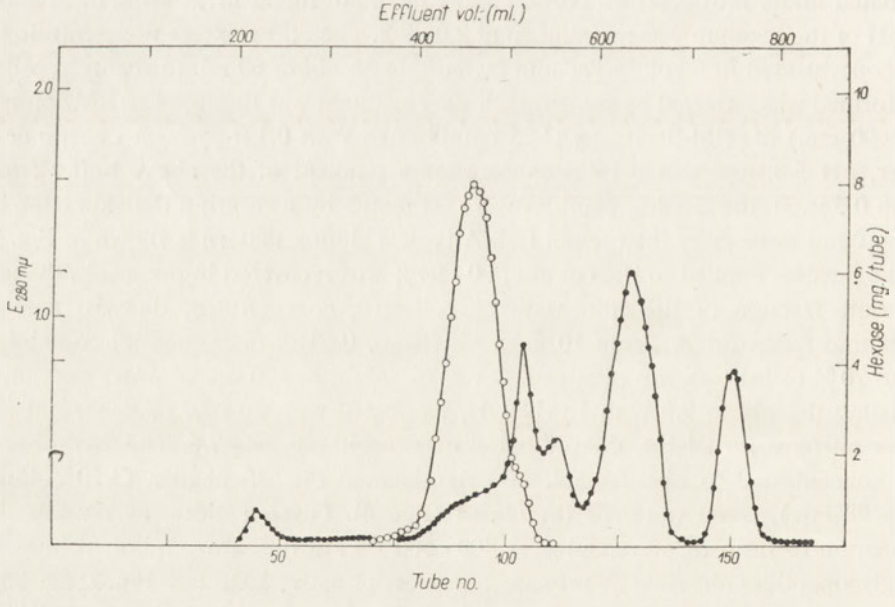

Fig. 4. Gel filtration on Sephadex G-50 column (3.7×98 cm.) of the glycopeptide fraction (see Fig. 3) after digestion with trypsin. (●), Protein, $E_{280\,m\mu}$; (○), hexoses.

The collected glycopeptide peak, which accounted for about 92% of the saccharides applied to the column, was concentrated and fractionated on a CM-Sephadex C-25 column equilibrated with 0.1 M-pyridine - acetic acid buffer, pH 5.8, and eluted with the same buffer. Glycopeptides were located in the effluent using the orcinol test for hexoses, and the resorcinol test for sialic acid (Fig. 5). It appeared that the

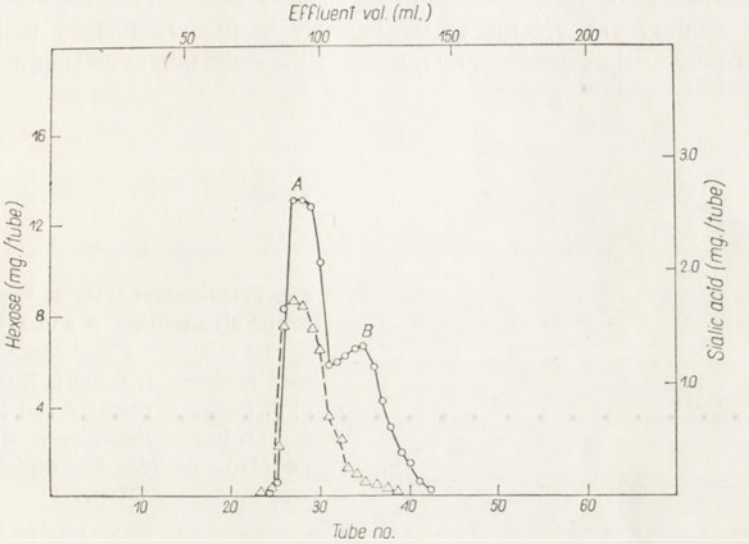

Fig. 5. Chromatography of glycopeptides (see Fig. 4) on CM-Sephadex C-25 column (1.8× 80 cm.). The material was eluted with 0.1 M-pyridine - acetic acid buffer, pH 5.8. (○), Hexoses; (△), sialic acid.

material separated into two fractions, glycopeptide *A* containing sialic acid, and glycopeptide *B* which contained only hexoses. Glycopeptide *A* accounted for 46% and glycopeptide *B* for 41% of the total amount of saccharides applied to the column.

Method 2. Ten grams of vitellenin was suspended in 300 ml. of 0.15 M-calcium acetate, adjusted with phosphate to pH 6.0, and incubated with 100 mg. of pronase preparation at 37° under nitrogen for 48 hr. Then 40 mg. of the enzyme was added and the incubation continued for a further 24 hr. The hydrolysate was concentrated to about 60 ml. and fractionated as described in Method 1 except that trypsin digestion was omitted. Fractionation patterns of the pronase digestion products on CM-Sephadex and Sephadex G-50 columns were the same as those obtained by Method 1.

By Method 2, from 10 g. of vitellenin 115 mg. of glycopeptide *A* and 75 mg. of glycopeptide *B* were obtained, containing respectively 51% and 34% of the total amount of saccharides present in the vitellenin preparation.

Estimation of molecular weight of glycopeptides

The molecular weight of the two glycopeptides was estimated by the method of Whitaker (1963) adapted for Sephadex G-50. To the column (1.5×127 cm.) equilibrated with 0.1 M-acetate buffer, pH 6.0, was applied 10 mg. of the sample dissolved in 1 ml. of the above buffer, and the same buffer was used for elution. Pepsin, cytochrome *c*, insulin A chain, and oxidized and reduced glutathione were used as standards. The volume of the effluents was determined by weight, and the concentration of the substances by measuring the extinction at 280 mμ or 230 mμ.

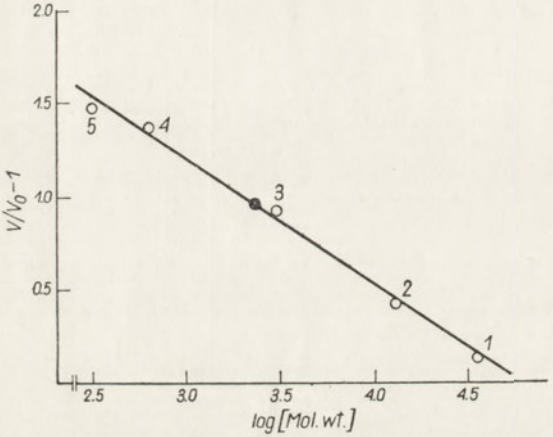

Fig. 6. Estimation of the molecular weight of the glycopeptides *A* and *B* from their elution volumes on Sephadex G-50. Standard proteins: *1*, pepsin; *2*, cytochrome *c*; *3*, insulin A chain; *4* and *5*, oxidized and reduced glutathione, respectively. (●), Glycopeptides. For explanations see text.

The elution volume (v) was determined from the elution pattern assuming that v is equal to the number of milliliters of the buffer required to elute a half of the material that had been applied to the column. The void volume of the column (v_0) was determined using conglutin β. On the basis of the correlation between the ratio $v/v_0 - 1$ and the logarithm of molecular weight for the standard substances, the molecular weight of the two glycopeptides studied was calculated to be about 2300 (Fig. 6).

DISCUSSION

Proteolysis of native lipoproteins permits to study the organization of the protein covering the lipid core of the molecule. Some authors who studied the action o proteolytic enzymes on blood-serum low-density lipoproteins (Bernfeld & Kel ey, 1964; Margolis & Langdon, 1966c) assumed that the amount of the released peptides gives a measure of the degree of digestion of the protein moiety of lipoproteins. This interpretation of the results of proteolysis, together with observations on the reactivity of some amino acid residues, led Margolis & Langdon (1966a, b, c) to the conclusion that a molecule of blood-serum β_1-lipoprotein is built of a number of spherical subunits, each covered by polypeptide chain, and that partial only proteolysis and limited reactivity of amino acid residues could be explained by spatial arrangement of the subunits resulting in inaccessibility of the peptide bonds to the proteolytic enzymes.

Saari *et al.* (1964) studied the effect of papain on LDF and found that after 4.5 hr. of incubation papain released 36% of protein from the fraction LDF_2 and no detectable peptides from fraction LDF_1. Probably, after a longer time of digestion of the two LDF fractions with papain, the amount of released peptides would be closer to the values obtained in the present work; nevertheless, it seems desirable to study more in detail the effect of proteases on separated fractions LDF_1 and LDF_2. According to the conception of Saari *et al.* (1964), protein-phospholipid interactions may be predominant in LDF_1 and render the polypeptides resistant to proteolysis, while some large protein-protein aggregates in LDF_2 would be susceptible to papain digestion.

In the presented experiments it was found that when native LDF was digested by pronase for 27 hr., low-molecular weight compounds soluble in water and in 2.5% trichloroacetic acid, corresponding to 60% of the initial amount of protein, separated from the lipids. From the "protein" which remained bound to the lipid, 30% was soluble in water and had a molecular weight below 1000. This indicated that not all the peptides which were formed by the action of pronase on LDF, separated from the lipid. This, in agreement with the suggestion of Saari *et al.* (1964) concerning different binding of polypeptides with lipid, leads to the conclusion that some fragments of the polypeptide chains might form folds on the surface of the LDF molecule and be relatively weakly bound, whereas other segments would be bound more tightly. On enzymic hydrolysis, the weakly bound fragments would separate more easily than the extended parts, strongly bound to lipids. In such a model of the lipoprotein molecule, resistance of a region of the polypeptide chain to proteolytic enzymes could be explained by overlapping of folds over the extended segments of the polypeptide chain, and by interactions of protein with lipids.

The observations on glycopeptides obtained by enzymic digestion of vitellenin, are in agreement with the proposed model of LDF structure. Digestion of delipidated vitellenin resulted in release of two glycopeptides which contained almost all saccharides of this protein. Similar glycopeptide fractions were obtained, although in a lower yield, when native LDF was digested by pronase. Both glycopeptides had molecular weights of about 2000, whereas the molecular weight of the heteropolysaccharide isolated from the glycopeptides was about 1700 (Augustyniak & Martin, unpublished data); thus practically all the sugars in vitellenin are accumulated in a few large heteropolysaccharide groups. Hydrophilic properties of saccharides seem to exclude the possibility of their direct contact with the lipid core and suggest that the saccharide moieties are bound to the folded fragments of the polypeptide chain. The protruding hydrophilic parts of vitellenin should be more rapidly hydrolysed and more easily separated from the molecule than other peptide segments. Digestion of native LDF by pronase confirms this supposition, as after 27 hr. only 13% of the initial amount of hexoses remained bound in the undigested residues which, however, still contained as much as 40% of the original amount of protein material. A similar trend has been reported by Margolis & Langdon (1966c) for proteolysis of blood serum β_1-lipoproteins.

In the present work it was found that the whole amount of sialic acid was present in one of the two glycopeptide fractions obtained from vitellenin (Fig. 5). This indicates that LDF contains two different heteropolysaccharide groups. Analysis of glycopeptides has demonstrated (Augustyniak & Martin, unpublished experiments) that these groups are bound to identical peptide fragments, but further studies are necessary to elucidate whether these fragments derive from one, or from two different polypeptide chains.

The author, much indebted to Dr. W. H. Cook, Director of the Division of Biosciences, National Research Council of Canada, Ottawa, for making possible some previous work on LDF in his Division and under his direction, would like to dedicate this paper to him on the occasion of his sixty fifth birthday.

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PROTEOLIZA FRAKCJI LIPOPROTEIDOWEJ O NISKIEJ GĘSTOŚCI (LDF) Z ŻÓŁTKA JAJA KURZEGO I PREPARATYKA GLIKOPROTEIDÓW Z JEJ CZĘŚCI BIAŁKOWEJ

Streszczenie

1. Po działaniu pronazą na LDF, około 60% białka uwalnia się w postaci niskocząsteczkowych peptydów.

2. Pozostałe białko, które jest mocniej związane z częścią lipidową, również jest dostępne dla enzymu. Po trawieniu znaczna jego część (30%) przechodzi w niskocząsteczkowe, rozpuszczalne w wodzie peptydy, zasocjowane z tłuszczowym jądrem kompleksu.

3. Z natywnego lipoproteidu najszybciej uwalniają się glikopeptydy, i praktycznie wszystkie heksozy związane z LDF odzyskuje się w postaci dwu glikopeptydów o masach cząsteczkowych około 2000.

4. Na podstawie uzyskanych wyników przedyskutowano model struktury LDF.

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INDUCTION OF MANNITOL DISSIMILATION IN *MYCOBACTERIUM PHLEI*

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1. A soluble NAD-linked dehydrogenase which catalyses the oxidation of D-mannitol to fructose has been found in *Mycobacterium phlei*. Mannitol-grown cells gave several-fold more active extracts than the cells cultivated on glucose. 2. Induction of the enzyme was followed under non-growth conditions with mannitol as inducer. 3. Extract of mannitol-adapted *M. phlei* cells was subjected to fractionation and some properties of mannitol dehydrogenase were studied. The enzyme proved unstable upon storage and lost its activity after 1-min. heating at 60°. The optimum pH was at 8.5 and the apparent K_m value for mannitol in tris-HCl buffer was 15 mM. 4. Besides homologous dehydrogenase, mannitol-adapted cells contained increased activity of specific ATP-fructokinase.

Although growth and metabolic activity of *Mycobacterium* species at the expense of various carbon compounds have frequently been tested (see Edson, 1951; Sweeney & Jann, 1962; Tsukamura, 1966), little attention was paid to enzymes engaged in the metabolism of sugars other than glucose. Nor has adaptation of mycobacteria toward sugars and sugar alcohols been satisfactorily studied. The attempts to adapt *M. tuberculosis* $H_{37}Rv$ to fructose, galactose and arabinose by growing the bacilli in appropriate sugar media were unsuccessful (Sweeney & Jann, 1962).

On the other hand, in a recent study from this laboratory evidence was obtained for the occurrence of inducible fructokinases in *M. phlei* (Szymona & Szumiło, 1966). The present work extends the study to mannitol.

According to the literature (Horecker, 1962), mannitol can either be phosphorylated with subsequent oxidation of mannitol phosphate, or oxidized with subsequent phosphorylation of fructose. It seemed desirable to establish which of the two routes might occur in *Mycobacterium* and whether an adaptation mechanism is involved.

EXPERIMENTAL

Micro-organism and cultivation. A strain of *M. phlei* which had been maintained in this laboratory for several years was used. The medium and growth conditions were as described earlier (Szymona & Szumiło, 1966). Where indicated, fructose

was replaced by mannitol, mannose or glucose. In some cases Sauton's glycerol medium was employed.

Chemicals. The following polyols were used: D-mannitol (B.H.D., Poole, England), D-sorbitol (L. Light Co., Colnbrook, England), dulcitol (Eastman Kodak Co., Rochester, U.S.A.), erythritol (C.A.F. Kahlbaum, Adlershof, Germany). The sugars were: D-glucose from Toxa (Warszawa, Poland), D-fructose from Veb Berlin-Chemie (Adlershof, Germany) and D-mannose from Difco (Detroit, U.S.A.). Tris(hydroxymethyl)aminomethane was purchased from L. Light Co. (Colnbrook, England). NAD, $NADH_2$ and NADP were products of Boehringer (Mannheim, Germany). Deoxyribonuclease (EC 3.1.4.5) was obtained from Mann Research Lab. (New York, U.S.A.). Sephadex G-100 was from Pharmacia (Uppsala, Sweden). Corund no. 3/320 (A) was supplied by Biuro Sprzedaży Artykułów Ściernych (Bielsko-Biała, Poland); it was washed with dilute HCl and water before use. ATP (sodium salt) was obtained from the Sigma Chemical Company (St. Louis, U.S.A.). All other chemicals were commercial preparations of analytical grade. Glass-distilled water was used throughout.

Preparation of cell-free extracts. One gram of packed (wet) cells to which 3 g. of corund had been added, was disintegrated by hand grinding for 2 - 3 min. in a chilled mortar. The resulting paste was suspended in 2 ml. of ice-cold 0.05 M-tris--HCl buffer, pH 7.4 and after 5-min. stirring, centrifuged in a MSE refrigerated centrifuge at 9 000 rev./min. for 30 min. The opalescent yellow supernatant fluid (cell-free extract) was used for enzyme determinations. It contained about 5 mg. protein/ml.

Analytical methods. If not otherwise stated, the mannitol dehydrogenase assay system contained in a total volume of 2.9 ml.: 200 μmoles of tris-HCl buffer (pH 8.5), 1 μmole of NAD, 0.1 ml. of cell-free extract and 50 μmoles of mannitol. Extinction was read at 340 mμ in a Hilger spectrophotometer before and after addition of the substrate (0.1 ml. of 0.5 M-solution). The reaction was followed for 6 min. at room temp., the initial rate being taken for measurement of enzyme activity. A unit of the enzyme was defined as that amount which effects an increase in extinction (ΔE_{340}) of 0.01 per min. under the above conditions. The reverse reaction was checked under the same conditions with $NADH_2$ and fructose.

ATP-fructokinase and inorganic polyphosphate glucokinase activities were assayed in Sephadex G-100 eluates as described previously (Szymona & Szumiło, 1966).

Determination of protein was carried out according to Lowry, Rosebrough, Farr & Randall (1951) using a Chinese photocolorimeter, model no. 581.

Reducing compounds in culture media and in deproteinized cell-free extracts were analysed chromatographically on Whatman no. 1 paper with butanol - pyridine - water (3:2:1.5, by vol.) as solvent. After 18-hr. run, the spots were detected with the alkaline silver nitrate reagent of Trevelyan, Proctor & Harrison (1950) and fructose also with the resorcinol reagent (Forsyth, 1948).

Utilization of mannitol for growth

Preliminary experiments showed that *M. phlei* was able to grow without a lag phase on mannitol as well as on fructose or mannose media (Table 1). Paper chromatography of the culture medium revealed progressive consumption of mannitol. No additional spots were detectable by the $AgNO_3$-NaOH reagent, which indicates that no reducing intermediates accumulated during incubation.

Table 1

Effect of various substrates on growth of M. phlei

The medium had the following composition: substrate 1%, glutamic acid 1%, citric acid 0.2%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, ferric ammonium citrate 0.005%, $ZnSO_4 \cdot 7H_2O$ 0.002%. The pH was adjusted to 7.4 with 2 N-KOH.

Substrate	Cell wet wt. (g./20 ml. of medium)	
	Expt. 1	Expt. 2
Mannitol	1.10	1.26
Mannose	1.15	1.23
Fructose	1.21	1.25

When 100 µl. of a deproteinized cell-free extract from a 4-day-old mannitol-grown culture were chromatographed, two spots appeared one of which, near the starting line, could be identified as hexose-6-phosphate and the other as glucose. The occurrence of either mannitol or unphosphorylated fructose was ruled out on the basis both of negative resorcinol test and different behaviour on paper chromatograms, i.e. the spot in question reduced the $AgNO_3$-NaOH reagent much more readily than that of authentic mannitol run in parallel. Cell-free extracts from older cultures contained little or no reducing substances. The results suggest that mannitol was utilized without being accumulated in the cell plasm.

Mannitol dehydrogenase activity

The attempts to demonstrate the phosphorylation of mannitol by ATP were unsuccessful, therefore direct oxidation to fructose was tested. Fig. 1 shows that cell-free extract from mannitol-grown cells was able to reduce NAD in the presence of 17 mM-mannitol added, the initial rate amounting to ΔE 0.25/min./mg. protein. At the above concentration of substrate and with 0.05 - 0.10 ml. of the extract, the reaction was linear only during the first 3 min. of incubation. In the extract, after addition of 0.35 µmole of $NADH_2$ and 1 µmole of fructose, a reversal of this reaction was observed (Fig. 1).

Dehydrogenation of mannitol was confirmed in a separate experiment, in which the appearance of fructose was followed by paper chromatography and determined

by the Roe method (1934). As shown in Fig. 2, the extract from mannitol-grown cells produced fructose at a rate of 0.06 μmole/min./mg. protein.

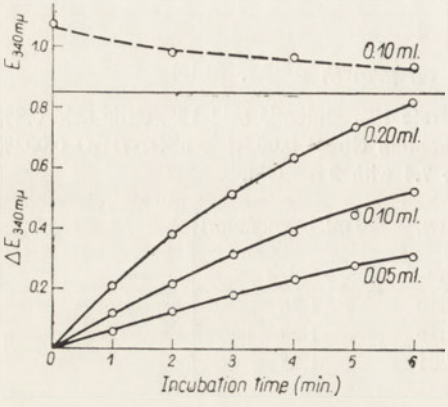


Fig. 1

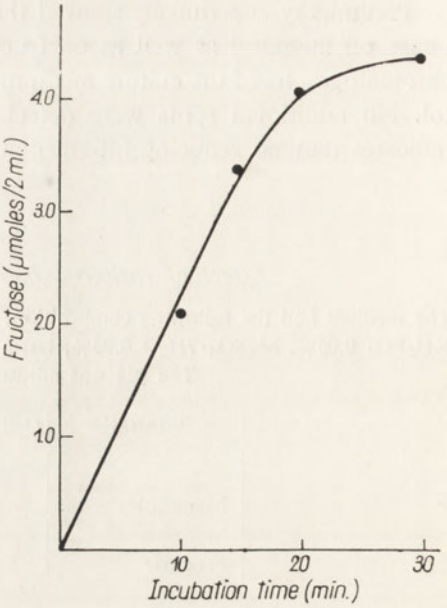


Fig. 2

Fig. 1. NAD reduction as a function of time and enzyme content. Standard assay conditions except for varying amounts of extract from mannitol-grown cells; total volume 2.9 ml. Reaction was started by addition of mannitol (0.1 ml.). Dashed curve shows reverse reaction in the presence of 0.35 μmole of $NADH_2$, 1 μmole of fructose, and 0.1 ml. of extract which started the reaction.

Fig. 2. Fructose formation in the presence of 100 μmoles of tris-HCl buffer, pH 8.5; 5 μmoles of NAD; 40 μmoles of mannitol and 0.8 ml. of cell-free extract (3.8 mg. protein) in a total volume of 2 ml. After deproteinization with trichloroacetic acid, fructose was determined by the method of Roe (1934).

Fractionation of cell-free extract

All operations were conducted in a cold room at about 4°. Mannitol-grown cells were broken up with corund and the homogenate centrifuged for 30 min. in a Servall centrifuge at 18 000 *g*. The supernatant fluid was then made 1 mM with respect to $MgCl_2$, about 0.3 mg. of crystalline deoxyribonuclease was added per 100 ml. and the mixture incubated for 15 min. at room temp. Then solid ammonium sulphate was added, the protein precipitating between 0.35 and 0.75 saturation collected, dissolved in a small volume of water and dialysed for 4 hr. against 0.05 M--tris-HCl buffer (pH 7.4). The resulting preparation was then passed through the Sephadex G-100 column equilibrated with the same buffer. Fractions of 2 ml. were

collected and assayed for enzyme activities. The results showed (Fig. 3) that mannitol dehydrogenase closely followed polyphosphate glucokinase, the molecular weight of which had been found to be about 100 000 (Szymona & Ostrowski, 1964).

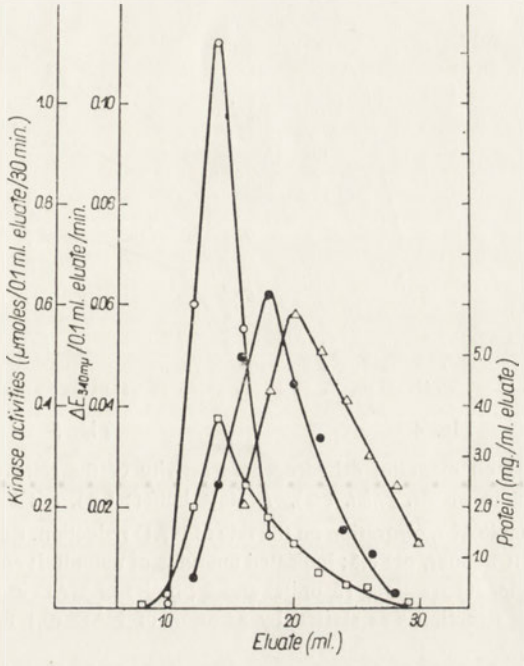


Fig. 3. Fractionation of dialysed 0.35 - 0.75 ammonium sulphate sat. enzyme preparation (2.5 ml., 28.9 mg. protein) on Sephadex G-100 column (19×1.1 cm.). (□), Protein concentration; (●), mannitol dehydrogenase activity; (○), polyphosphate glucokinase activity; (△), ATP-fructokinase activity. Fractions of 2 ml. were collected at a flow rate of 21 ml./hr.

At the same time a threefold increase in specific activity of mannitol dehydrogenase was attained in the peak fraction. The activity of ATP-fructokinase was as high as that of an analogous preparation from fructose-grown cells (Szymona & Szumiło, 1966).

Properties of mannitol dehydrogenase

Storage of washed cells in the frozen state for 2 weeks caused no appreciable loss of activity. The extracts, however, became inactivated within 6 days at 3°, and Sephadex G-100 eluates proved completely inactive when tested after 4 weeks of storage in the frozen state. Heating the eluate (pH 7.4) at 50° for 1 min. caused a decrease in activity by 30%; heating at 60° for 1 min. resulted in a total inactivation. Extracts from acetone-dried cells showed very small activity.

In the cell-free extracts, a sharp pH optimum at 8.5 was observed (Fig. 4) which corresponded to that of mannitol dehydrogenase of *Azotobacter agilis* (Marcus & Marr, 1961) and *Lactobacillus brevis* (Martinez, Barker & Horecker, 1962).

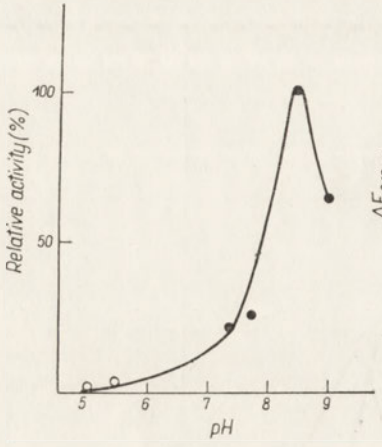


Fig. 4

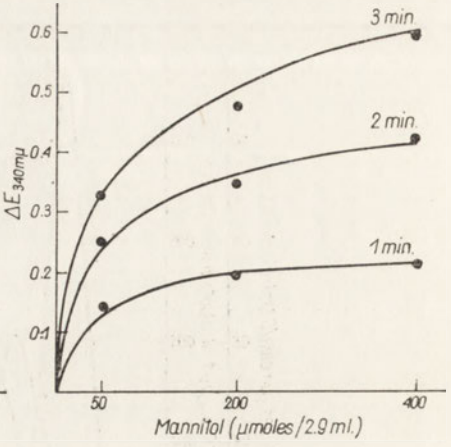


Fig. 5

Fig. 4. Effect of pH on mannitol dehydrogenase activity of the cell-free extract prepared from mannitol-grown *M. phlei.* (○), Acetate buffer; (●), tris buffer.

Fig. 5. Effect of mannitol concentration on the rate of NAD reduction. Reaction mixtures contained 200 μmoles of tris-HCl buffer, pH 8.5; indicated amounts of mannitol; enzyme (dialysed 0.35 - 0.75 sat. ammonium sulphate fraction, 15 units) and 2 μmoles of NAD in a total volume of 2.9 ml. Reaction was started by addition of NAD (0.1 ml.).

Specificity experiments were carried out with cell-free extracts using 0.66 mM-polyalcohols and 0.33 mM-NAD. The enzyme was inactive toward glycerol and erythritol, trace of activity was found with dulcitol, and the activity toward sorbitol was 21% of that toward mannitol. When NADP was substituted for NAD at pH 8.5 with mannitol as substrate, no appreciable reduction was observed.

The effect of varying mannitol concentration on the enzyme activity is shown in Fig. 5. From these data an average K_m for mannitol was found to be 15 mM.

Induction of mannitol dehydrogenase

The results of the above described experiments have shown that mannitol is utilized as effectively as the other substrates tested. Extracts from cells which had been grown on various substrates showed, however, differences with respect to mannitol dehydrogenase activity (Fig. 6). The extract from glycerol-grown cells (Sauton's medium) had little activity while extracts from mannitol- and mannose-grown cells had about sevenfold higher activities. The presence of fructose in the medium proved less efficient than either mannitol or mannose, and glucose-grown cells exhibited still lower values. These results suggest that mannitol dehydrogenase of *M. phlei* is an inducible enzyme.

To study more closely the adaptation to mannitol, mycobacteria which had been grown on glucose were brought in contact with mannitol under non-growth (resting) conditions and enzyme activity was assayed in cell-free extracts. The majority of experiments were performed as follows. Complete glucose medium was distributed into a series of 500-ml. flasks, each fitted with a side tube at the bottom,

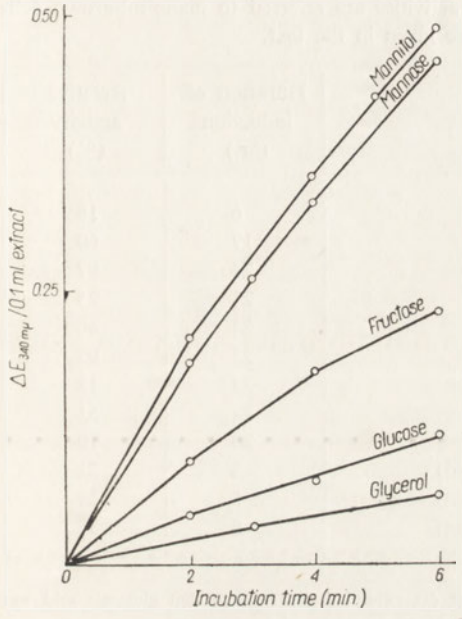


Fig. 6

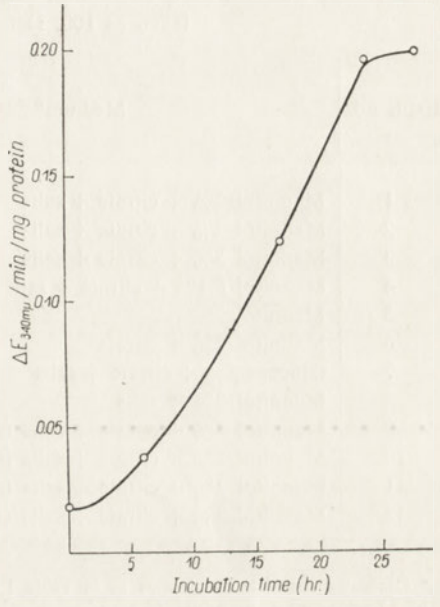


Fig. 7

Fig. 6. Mannitol dehydrogenase activity of cell-free extracts of *M. phlei* grown for 7 days on liquid media with indicated substrates. Standard assay conditions.

Fig. 7. Increase in mannitol dehydrogenase of *M. phlei* during adaptation from glucose media to media containing mannitol. At time zero, cells grown on the surface of glucose media were underlayered aseptically with the medium containing: mannitol 2%, citric acid 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05%, ferric ammonium citrate 0.005% and $ZnSO_4 \cdot 7H_2O$ 0.002%, and incubated at 37° for indicated periods of time.

and after inoculation incubated as usual. After 6 days of static growth, the medium of each flask was siphoned off aseptically by means of a bent glass tube and equal volume of sterilized water (250 ml.) was introduced through the side tube as gently as to leave the pellicle undisturbed. The washing fluid was then removed by siphoning and 250 ml. of glutamate-free mannitol medium added as described above. The underlayered cultures were subsequently incubated for varying periods of time at 37°. The cells were harvested, washed with water and the extracts used for enzyme activity determinations. In some cases, glucose-grown cells were suspended in glutamate-free mannitol medium (1 g. wet weight/100 ml.) and shaken aerobically at 37° for a few hours. The results are summarized in Table 2 and Fig. 7. It is

Table 2

Induction of mannitol dehydrogenase in M. phlei

Cells were grown on glucose medium for 6 days, then washed once with water and underlayered with non-growth medium. In expts. 10 - 12, the glucose-grown cells were suspended in the glutamate-free medium and shaken aerobically. After indicated time of incubation, the cells were collected by centrifugation, washed with water, and enzyme activity was determined in the extract under standard assay conditions. Relative activities are referred to mannitol-grown cells taken as 100. Details are given in the text.

Expt. no.	Medium*	Duration of induction (hr.)	Relative activity (%)
1	Mannitol 2% + citrate + salts	6	18
2	Mannitol 2% + citrate + salts	17	60
3	Mannitol 2% + citrate + salts	24	97
4	Mannitol 0.1% + citrate + salts	21	25
5	Mannitol 2%	24	46
6	Mannitol 2% + citrate	23	92
7	Glucose 2% + citrate + salts	24	12
8	Mannitol 2% + salts	24	51
9	Mannitol 2% + citrate + salts (anaerobic)	20	10
10	Mannitol 2% + citrate + salts (aerated)	3	21
11	Mannitol 2% + citrate + salts (aerated)	5	29
12	Mannitol 2% + citrate + salts (aerated)	6	35

* Concentrations of the components as in Table 1 (if not otherwise indicated) except that glutamic acid was omitted to prevent cells from growing.

clear that the activity of mannitol dehydrogenase depends on the presence of mannitol in medium, citrate being needed for obtaining the maximum effect. The induction process under non-growth conditions took 24 hr. to reach a value about as high as that of mannitol-grown cells.

As one would expect, there was no induction under anaerobic conditions. When glucose-grown cells in a flask were washed with water as above and then suspended in mannitol medium to make them settle on the bottom, no increase in activity could be stated. On the other hand, no change in mannitol dehydrogenase activity resulted from leaving mannitol-grown cells immersed in 1% mannitol medium for 24 hr.

DISCUSSION

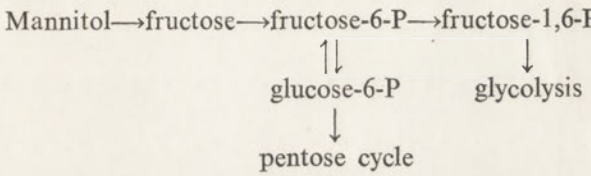
The experiments described above have shown that *M. phlei* is capable of adaptation to mannitol. Mannitol was utilized for growth as efficiently as fructose or mannose. The first step in mannitol catabolism by the strain studied appears to be a NAD-linked dehydrogenation. The amount of enzyme was dependent on the kind of carbon compound present in the growth medium. Of the substrates tested, mannitol and mannose brought about the highest activities; fructose influenced

dehydrogenase activity to a lesser extent and glucose- or glycerol-grown cells gave several-fold lower values.

The effect of mannitol was studied in some detail with glucose-grown cells using a medium deprived of glutamic acid as nitrogen source. In such cases the growth of bacteria was inhibited and thus any marked increase in enzyme activity could be recognized as a result of induction rather than development of active mutants.

Under non-growth conditions the induction process occurred slowly, taking 24 hr. to reach maximum activity. It is of interest to note that relatively high concentrations of mannitol (2%) were required; besides the inducer, citrate was needed for full effect.

The observation that mannitol dehydrogenase was detectable even in the cultures which had not previously been exposed to the inducer, allows to admit a semi-adaptive character of the enzyme studied. Perhaps other enzymes attacking sugars or polyalcohols in mycobacteria behave in a similar way. In this work ATP-fructokinase activity of mannitol-adapted *M. phlei* was found to be increased and comparable with that of fructose-grown cells. This fact is interpreted to mean that mannitol provoked successive formation of both mannitol dehydrogenase and fructokinase. Accordingly, the utilization of mannitol by mycobacteria appears to proceed as follows:



Further research might elucidate the metabolic controls operative in induction and repression of the enzymes involved.

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INDUKCJA ZUŻYTKOWYWANIA MANNITOLU U *MYCOBACTERIUM PHLEI*

Streszczenie

1. W ekstraktach z *M. phlei* stwierdzono występowanie dehydrogenazy katalizującej przemianę mannitolu do fruktozy przy udziale NAD jako koenzymu. Prątki z podłoża mannitolowego wykazywały kilkakrotnie większą aktywność niż z podłoża zawierającego glukozę.

2. W komórkach otrzymanych z hodowli glukozowej badano indukcję dehydrogenazy mannitolu stosując bezazotowe podłoże i mannitol jako induktor.

3. Ekstrakt z *M. phlei* frakcjonowano przy użyciu $(NH_4)_2SO_4$ i Sefadeksu G-100. Otrzymane preparaty enzymatyczne były nietrwałe i ulegały całkowitej inaktywacji podczas 1-minutowego ogrzewania w 60°. Optimum pH wynosiło 8,5; przybliżona wartość K_m 15 mM.

4. Oprócz dehydrogenazy mannitolowej, w komórkach indukowanych mannitolem obserwowano podwyższoną aktywność specyficznej fruktokinazy.

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EFFECT OF SOME METAL IONS ON CITRULLINE-SYNTHESIZING ACTIVITY AND ARSENOLYTIC AND PHOSPHOROLYTIC CITRULLINE DEGRADATION IN HIGHER PLANTS

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1. Arsenolytic and phosphorolytic degradation of citrulline to ornithine in plant homogenates and in partially purified enzyme preparations from young pea plants were studied. The arsenolytic activity is always higher than the phosphorolytic one. Mercaptoethanol has an activating effect on arsenolysis and no effect on phosphorolysis. Cysteine has no effect on either of the above mentioned activities. 2. Inhibitory effect of Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and Zn^{2+} on citrulline synthesis and its arsenolytic degradation to ornithine, ammonia and CO_2 was studied. The inhibition of citrulline synthesis is of the competitive type. The inhibitory effect of the above metal ions can be diminished or completely removed by EDTA. 3. Phosphorolytic citrulline degradation is activated by Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+}. 4. Fe^{2+} and Mn^{2+} in very small concentrations (0.025 to 0.1 mM) have an activating effect on the synthesis of citrulline.

In many reports it has been shown that mammalian livers and micro-organisms contain an activity that catalyses the degradation of citrulline in the presence of phosphate or arsenate into ornithine, ammonia and CO_2 (Knivett, 1952, 1953, 1954a,b,c; Korzenovsky & Werkman, 1953; Krebs, Eggleston & Knivett, 1955; Oginsky & Gehrig 1953; Slade, 1953; Slade, Doughty & Slamp, 1954). Krebs & Eggleston (1955) have shown the above mentioned activity in soaked seeds of *Pisum sativum* and *Phaseolus vulgaris*. Reichard (1957) in his study on citrulline synthesis in rat liver has demonstrated that the purified preparation of ornithine carbamoyltransferase (carbamoyl phosphate : L-ornithine carbamoyltransferase, EC 2.1.3.3) catalyses also the arsenolytic degradation of citrulline.

In the present work, experiments were carried out on arsenolysis and phosphorolysis of citrulline in homogenates from various plants as well as in a partially purified ornithine carbamoyltransferase (OCT) preparation from young pea plants. The effect of some metal ions on citrulline synthesis and degradation was also studied.

MATERIAL AND METHODS

Reagents. L-Citrulline (Reanal, Budapest, Hungary); L-ornithine-HCl (Merck, Darmstadt, Germany); 2-mercaptoethanol (Koch-Light, Colnbrook, England); diacetylmonoxime (Fluka, Buchs, Switzerland); carbamoyl phosphate, ammonium salt prepared according to Metzenberg, Marshall & Cohen (1960). All other reagents were purchased from Biuro Obrotu Odczynnikami Chemicznymi (Gliwice, Poland).

Enzyme extracts. Wheat, soya bean, lupin, pea and cucumber seeds were soaked overnight in water and germinated on small trays covered with moist cotton wool. Plants were grown at room temp. for 12 - 18 days and the young plants were homogenized in a cooled mortar with two volumes of 0.1 M-Na-phosphate buffer, pH 7.1, or 0.1 M-Na-arsenate buffer, pH 6.7. The homogenate was passed through cotton wool and the filtrate used as the enzyme source. In the same way the homogenate from rabbit liver was prepared.

Purification of ornithine carbamoyltransferase (OCT). The partially purified enzyme was prepared from pea plants according to Kleczkowski & Cohen (1964) except that ammonium sulphate fractionation in tris-HCl buffer, pH 8.45 (step III) and in phosphate buffer, pH 7.2 (step IV) were omitted. The procedure was as follows: the centrifuged homogenate was treated with ammonium sulphate and the precipitate between 0.45 - 0.6 saturation was dissolved in 0.1 M-Na-phosphate buffer, pH 7.2, containing 2 mM-L-ornithine. The enzyme solution containing 40 - 50 mg. of protein per 1 ml. was heated to 61° in a water bath at 75° under continuous stirring, held at this temp. 1 min. and cooled rapidly to 4 - 6° in an ice bath. After centrifugation, the precipitate was washed twice with one-third of the original volume of the buffer. The combined supernatants were treated with ammonium sulphate to 0.45 saturation, after 1 - 2 hr. centrifuged, the precipitate dissolved in a small volume of 1 mM-Na-phosphate buffer, and ammonium sulphate was added under stirring until the solution became turbid. Very dilute buffer had to be employed at this stage because various buffers were to be used for estimations of enzymic activity. The preparation suspended in ammonium sulphate was stored at -8 to -10° for several months without loss of activity. All stages of purification were carried out in the cold room at a temperature of 4 - 5°.

Determination of arsenolysis and phosphorolysis. The incubation system contained in 1 ml.: the homogenate (10 - 20 mg. of protein) or the purified enzyme preparation (0.35 - 1 mg. of protein); 56 μmoles of L-citrulline dissolved in 0.1 M-Na-phosphate buffer, pH 7.1, or in 0.1 M-Na-arsenate buffer, pH 6.7; and the appropriate buffer to a final amount of 100 μmoles. In experiments with metal ions, the appropriate salt solution was neutralized with KOH to the required pH prior to addition to the incubation mixture. Two blanks were run for each experiment: without enzyme (as citrulline is usually contaminated with ornithine) and with the enzyme but without citrulline. After 2 hr. incubation at 37° the reaction was stopped either by heating the samples for 3 min. in a boiling water bath (for ornithine determination) or by addition of 0.1 ml. of 60% perchloric acid (for ammonia determination).

Ornithine was determined chromatographically according to Reifer & Buraczewska (1959) with a slight modification introduced to obtain a better separation of ornithine from citrulline. Ascending chromatography on Whatman no. 1 paper with *n*-propanol - water (64:36, v/v), was employed; the R_F for ornithine was 0.06 and for citrulline 0.26. Arsenate did not separate from ornithine causing discoloration of the ornithine test. Therefore equivalent amounts of L-ascorbic acid were added to each sample eluted from the chromatograms prior to addition of HCl.

Ammonia was determined with the Nessler reagent in 0.01% solution of gum arabic, which prevented turbidity of the solution. In the experiments in which ammonia was to be determined, the enzyme preparation suspended in ammonium sulphate was desalted on Sephadex G-25 (medium), just prior to use. The blank samples with enzyme contained about 2 μg. of ammonia, and the proper samples after incubation, about 4 μg. In experiments with homogenates the desalting procedure was omitted; the corresponding values of ammonia were 4 and 7 μg., respectively.

Since ammonia determination is much more troublesome than ornithine determination, ammonia was determined only in each new purified enzyme preparation; the obtained results were similar to those for ornithine estimation.

The arsenolytic and phosphorolytic activities are expressed in micromoles of ornithine formed per mg. of protein per 2 hr. at 37°.

Citrulline-synthesizing activity. This was assayed in diethanolamine - acetic acid buffer according to Kleczkowski & Cohen (1964) using the method of Archibald (1944) for citrulline determination. In some of the experiments, 3% dimethylglioxime in conc. sulphuric acid was applied instead of diacetylmonoxime. Standard curves for citrulline with both reagents were identical. The activity is expressed in micromoles of citrulline synthesized per mg. of protein per 15 min. at 37°.

Protein content was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Five plant species were assayed for arsenolytic cleavage of citrulline (Table 1) and the highest activity was found in the *Papilionaceae* (soya, pea, lupin). As it was difficult, under laboratory conditions, to grow soya plants which possess the highest activity, pea plants were used to obtain the purified OCT preparation.

Comparison of arsenolytic degradation of citrulline in homogenates of pea plants and rabbit liver showed a similar activity tested both by ornithine and ammonia formation (Table 2).

The partially purified OCT preparation was tested also for phosphorolytic activity, which was found to be lower by 30 - 50% than the arsenolytic one (Table 3). 2-Mercaptoethanol in 1 mM concentration activated the arsenolytic activity by about 30%, and had no effect on phosphorolysis (Table 4). Cysteine in concentrations of 5 and 15 mM had no effect on either of the two activities. The influence of SH-gluta-

Table 1

Arsenolysis of citrulline in homogenates of young plants

Assay conditions as described in Methods.

Plant	Activity (μmoles ornithine/mg. protein/2 hr.)
Wheat	0.04
Cucumber	0.05
Soya	0.23
Lupin	0.10
Pea	0.16

Table 2

Arsenolysis of citrulline by liver and plant homogenates

Assay conditions as described in Methods

Source of enzyme	Ornithine	Ammonia
	(μmoles/mg. protein/2 hr.)	
Rabbit liver homogenate	0.13	0.11
Pea plants homogenate	0.16	0.15
Purified preparation from pea plants	4.00	4.40

Table 3

Arsenolytic and phosphorolytic activity in relation to the age of pea plants

Purified enzyme preparations were used. Assay conditions as described in Methods.

Reaction	Age of the plants	
	12 days	18 days
	Ornithine (μmoles/mg. protein/2 hr.)	
Arsenolysis	8.1	4.8
Phosphorolysis	5.6	2.5

thione could not be determined because its presence interfered with the determination both of ornithine and ammonia.

The effect of metal ions was studied using the purified OCT preparations. Specific activity of the preparations was: for citrulline synthesis 60 - 100 μmoles/mg. protein/15 min., for arsenolysis 4 - 8 μmoles/mg. protein/2 hr. and for phosphorolysis 2 - 5.6 μmoles/mg. protein/2 hr. The highest activity was found usually in the preparations from 12-day-old pea plants.

Table 4

Effect of 2-mercaptoethanol on arsenolytic and phosphorolytic citrulline degradation by the purified enzyme preparation

Assay conditions as described in Methods.

Reaction	Ornithine (μmoles/mg. protein/2 hr.)	
	Control	with 1 mM-mercaptoethanol
Arsenolysis	4.0	5.7
Phosphorolysis	2.0	2.0

Table 5

Effect of metal ions on citrulline synthesis and arsenolytic degradation by a purified preparation of ornithine carbamoyltransferase from pea plants

Assay for citrulline synthesis: diethanolamine - acetic acid buffer, pH 8.4, 100 μmoles, about 7 μg. of enzyme protein, 5 μmoles of carbamoyl phosphate, 20 μmoles of L-ornithine and metal ions in amount as indicated. Assay for arsenolytic citrulline degradation: Na-arsenate buffer, pH 6.5, 100 μmoles, 350 - 450 μg. of enzyme protein, 56 μmoles of L-citrulline and metal ions as above. The final volume of incubate 1 ml.

Metal ion	Metal ion concn. (mM)									
	2		5		10		20		40	
	Inhibition (%) of									
	synth.	degrad.	synth.	degrad.	synth.	degrad.	synth.	degrad.	synth.	degrad.
$MgSO_4$	0	0	0	0	0	0	0	0	0	0
$MnSO_4$	32	23	60	30	85	63	86	100	87	100
$CoCl_2$	19	6	26	6	63	6	95	65	95	100
$NiSO_4$	11	0	53	0	100	56	100	90	100	100
$FeCl_2$	0	0	50	30	65	35	100	50	100	76
$ZnSO_4$	30	0	50	0	50	0	75	0	86	0
Li_2SO_4	0	0	0	0	0	0	0	0	0	0

The influence of metal ions on citrulline synthesis and its arsenolytic degradation is presented in Table 5. Mg^{2+} and Li^{1+} ions had no effect, and Mn^{2+} inhibited both activities. Up to a concentration of 20 mM, Mn^{2+} had a higher inhibitory effect on citrulline synthesis than on arsenolysis; higher concentration did not further inhibit the synthesis of citrulline but arsenolysis was completely inhibited.

Co^{2+} at 2 - 20 mM concentration inhibited citrulline synthesis from 19 to 95%. Arsenolysis, however, was inhibited only by 6% even at 10 mM-Co^{2+}; nevertheless a complete inhibition was observed at 40 mM-Co^{2+} concentration. Ni^{2+} was the strongest inhibitor of citrulline synthesis. Complete inhibition was achieved already with 10 mM-Ni^{2+} and 53% with 5 mM. For the inhibition of arsenolysis, higher

Table 6

Effect of metal ions on phosphorolytic citrulline degradation by a purified preparation of ornithine carbamoyltransferase from pea plants

The incubation system contained: 200 μmoles of Na-phosphate, pH 6.75, about 1 mg. of enzyme protein, 50 μmoles of L-citrulline, metal ions as indicated in the Table and water to 1 ml.

Metal ion	Metal ion concn. (mM)				
	2	5	10	20	40
	(+), Activation or (—), inhibition (%)				
$MgSO_4$	0	+17	+45	+71	+100
$MnSO_4$	0	0	0	+30	—48
$FeCl_2$	0	0	0	0	—56
$ZnSO_4$	+26	+22	+22	+22	+10
C_oCl_2	+67	+80	+120	+55	—13
$NiSO_4$	0	0	0	0	—45

Table 7

K_m *and* K_i *values obtained from the data presented in Fig.* 1

Metal ion concn. (mM)	K_m (mM)	K_i (mM)
Mn^{2+}, 1	1.7	0.2
Zn^{2+}, 1	1.8	0.2
Fe^{2+}, 1	3.0	1.2
Co^{2+}, 1	1.5	1.0
Ni^{2+}, 1.5	2.5	0.5

Table 8

Effect of EDTA on inhibition of citrulline synthesis by metal ions

Experimental conditions as described in Table 5.

Metal ion concn. (mM)	EDTA concn. (mM)							
	0	1	1.5	2	2.5	3	4	5
	Inhibition (%)							
$MnSO_4$, 1	80	70	39	22	12	—	—	—
$FeCl_2$, 4	80	76	—	—	—	53	—	0
$NiSO_4$, 4	50	40	—	30	—	25	—	14
$ZnSO_4$, 4	75	75	—	70	—	47	—	12
$CoCl_2$, 4	30	—	—	13	—	—	13	8

concentrations of Ni^{2+} were required: 5 mM-Ni^{2+} had no effect and 20 mM gave 90% inhibition. Fe^{2+} inhibited more strongly citrulline synthesis than its degradation. The most interesting of the investigated metal ions was Zn^{2+} which inhibited only the synthesis of citrulline and had no effect on arsenolysis.

Unexpectedly Mn^{2+} and Fe^{2+} used in lower concentrations, 0.025 - 0.1 mM, had an activating effect on the citrulline-synthesizing activity (30 - 40%). Such effect was not observed in the case of arsenolytic citrulline degradation.

At variance with their inhibitory effect on arsenolysis and citrulline synthesis, all metal ions studied, except Fe^{2+} and Ni^{2+}, activated the phosphorolytic degradation of citrulline (Table 6). The most effective was Co^{2+} which at 2 mM concentration activated the reaction in 67% and at 10 mM in 120%. However, with increasing concentration of Co^{2+} the activation declined and at 40 mM even an inhibition was observed. Mn^{2+} at 20 mM concentration activated phosphorolysis by 30% and at 40 mM inhibited by 48%. The only metal ion that caused activation with rising

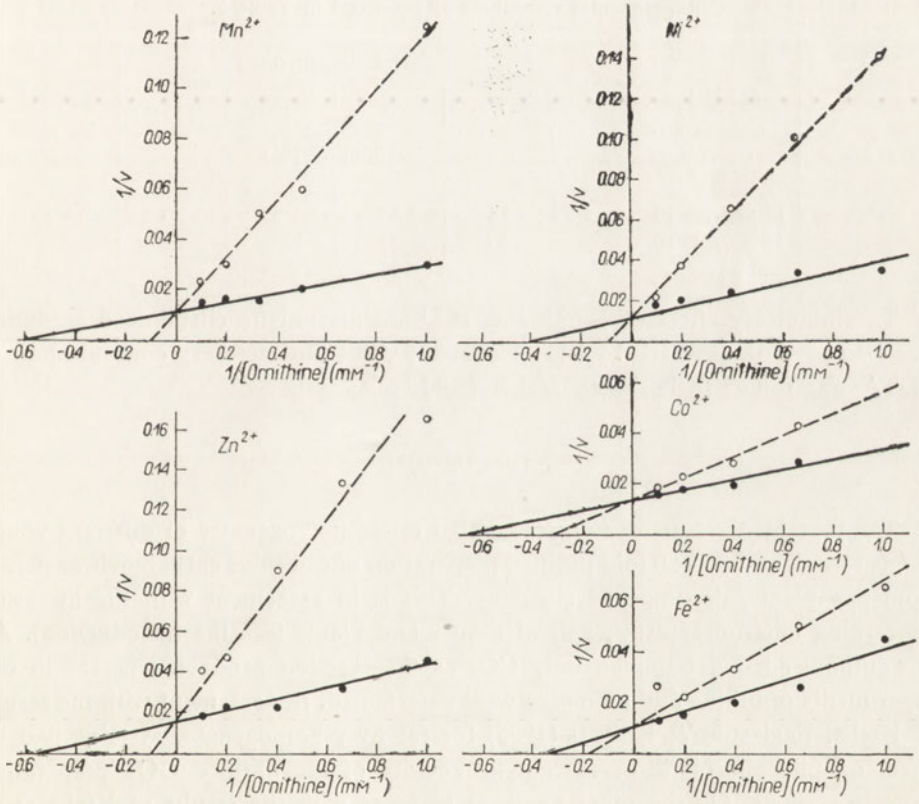

Fig. 1. Lineweaver & Burk plots for 1 mM-$MnSO_4$, 1.5 mM-$NiSO_4$, 1 mM-$ZnSO_4$, 1 mM-$CoCl_2$ and 1 mM-$FeCl_2$ inhibitory effect on citrulline synthesis. The reaction volume was 1 ml., containing: carbamoyl phosphate, 5 μmoles; diethanolamine - acetic acid buffer, pH 8.4, 100 μmoles; enzyme protein about 5 μg.; the appropriate metal ion, and L-ornithine, 1 - 10 μmoles.

concentration was Mg^{2+} which doubled the rate of phosphorolysis at a concentration of 40 mM.

In further experiments some kinetic characteristics of inhibition of the citrulline-synthesizing activity by metal ions were studied. The Lineweaver & Burk (1934) plots demonstrated that in all cases the inhibition was of the competitive type (Fig. 1). The K_i values ranged from 1.2 mM for Fe^{2+} to 0.2 mM for Mn^{2+} and Zn^{2+}, and K_m values were from 1.5 to 3.0 mM (Table 7).

EDTA removed partially or completely the inhibitory effect of the metal ions on citrulline synthesis (Table 8). Mn^{2+} inhibition was most effectively removed by relatively low EDTA concentration. With 1 mM-Mn^{2+}, 1.5 mM-EDTA restored about 50%, and 2.5 mM-EDTA 85% of the inhibited citrulline synthesis. The inhibitory effect of other metals was reversed up to 70 - 100% by 5 mM-EDTA.

Table 9

Effect of EDTA on inhibition of arsenolytic citrulline degradation by metal ions

Experimental conditions as described in Table 5.

Metal ion concn. (mM)	EDTA concn. (mM)			
	0	5	10	15
	Inhibition (%)			
$MnSO_4$, 10	58	35	13	0
$NiSO_4$, 10	60	53	33	22

The inhibitory effect of Mn^{2+} and Ni^{2+} on arsenolytic citrulline degradation was also partially reversed by EDTA (Table 9), but the necessary concentration of EDTA was found to be much higher (5 - 15 mM).

DISCUSSION

The presented results demonstrated that crude homogenates of different young plants as well as a 25 - 50 fold purified preparation of ornithine carbamoyltransferase from pea plants, decomposed citrulline. This is in agreement with the literature concerning micro-organisms, animal tissues and plants (see the introduction). All the authors cited determined only CO_2 as the reaction product, whereas in our experiments ornithine and ammonia were determined. In agreement with the results of Krebs, Eggleston & Knivett (1955) for rat liver homogenate, cysteine had no effect on phosphorolytic or arsenolytic activity of the purified OCT preparation from pea plants. On the other hand, at variance with the results of Krebs *et al.* (1955), 2-mercaptoethanol did not inhibit arsenolysis but promoted it.

In the present work it was found that $MnSO_4$, $CoCl_2$, $NiSO_4$ and $FeCl_2$ inhibited citrulline synthesis and its arsenolytic degradation by the partially purified OCT preparation from pea plants. On the other hand, $ZnSO_4$ inhibited strongly citrulline

synthesis but had no effect on arsenolysis. The metal ions studied, except Fe^{2+} and Ni^{2+}, promoted phosphorolytic citrulline degradation. The inhibitory effect on arsenolysis and the activating effect on phosphorolysis of the same metals may be due to the anion of the buffer used. Our results concerning the activation of phosphorolysis by some metals are in agreement with those of Korzenovsky & Werkman (1953) and Slade (1953). On the other hand, Krebs *et al.* (1955) have reported that in liver homogenates Fe^{2+} and Be^{2+} have no effect on arsenolysis and Ca^{2+}, Mg^{2+}, Fe^{2+} and Be^{2+} on phosphorolysis.

The inhibition by metal ions of citrulline synthesis is of the competitive type. This competition concerns only ornithine because at constant amounts of ornithine and various carbamoyl phosphate concentrations, no change in the inhibitory effect was observed. These results may suggest that ornithine and carbamoyl phosphate have two separate binding sites on the enzyme. Joseph, Baldwin & Watts (1963) have arrived at the same conclusion on the basis of kinetic experiments with various ornithine and carbamoyl phosphate concentrations in studies on inhibitory effect of phosphate on purified OCT preparation from ox liver.

The kinetic data on the inhibition of arsenolytic citrulline degradation are difficult to interpret. The metal ions used formed almost insoluble arsenate or phosphate salts and a cloudy suspension usually appeared in the incubation mixtures. Possibly, the minute amounts of dissociated metal ions are bound to the enzyme or substrate, which promotes further dissolution of the undissolved salt. In this way further amounts of metal ions become accessible for the reaction.

As the citrulline-synthesizing activity and phosphorolytic and arsenolytic activities were found to be present in the partially purified OCT preparation, its seems possible that both synthesis and degradation of citrulline are catalysed by the same enzyme, as suggested by Reichard (1957).

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WPŁYW NIEKTÓRYCH JONÓW METALI NA SYNTEZĘ CYTRULINY ORAZ JEJ ARSENOLITYCZNY I FOSFOROLITYCZNY ROZPAD U ROŚLIN WYŻSZYCH

Streszczenie

1. Badano arsenolityczną i fosforolityczną degradację cytruliny do ornityny w homogenatach siewek szeregu roślin, jak również w częściowo oczyszczonych preparatach z siewek grochu. Aktywność arsenolityczna była zawsze wyższa niż fosforolityczna. 2-Merkaptoetanol aktywował arsenolizę, pozostawał natomiast bez wpływu na fosforolizę. Cysteina nie miała wpływu na oba w/w procesy.

2. Badano również hamujący wpływ Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} i Zn^{2+} na syntezę cytruliny oraz na jej arsenolityczny rozkład do ornityny, amoniaku i CO_2. Hamowanie syntezy cytruliny miało charakter współzawodniczy. EDTA zmniejszał lub usuwał całkowicie hamujący wpływ wyżej wymienionych metali.

3. Fosforolityczny rozpad cytruliny był aktywowany jonami Mg^{2+}, Mn^{2+}, Zn^{2+} i Co^{2+}.

4. Fe^{2+} i Mn^{2+} w bardzo niskich stężeniach (0.025 - 0.1 mM) aktywowały syntezę cytruliny z ornityny.

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PEPTIDIC COMPOUNDS IN DEPROTEINIZED EXTRACTS OF OX LIVER

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1. Two peptidic compounds, one peptide and one UDP-peptide, have been isolated from the deproteinized extract from ox liver. Their molecular weights are about 6000. 2. Incorporation of labelled amino acids indicates that these compounds do not represent the breakdown products of protein or nucleoprotein, but are synthesized directly from free amino acids. 3. The isolated peptidic compounds do not affect L-[^{14}C]phenylalanine incorporation in a cell-free system and it seems that they are not regulators in protein biosynthesis.

Numerous studies on peptides and nucleotide-bound peptides point to a wide distribution of these compounds, which have been found in bacteria (Brown, 1958; Strominger, 1962), plants (Davies & Harris, 1960; Koningsberger, 1961; Brown & Silver, 1966), and mammalian cells (Szafrański & Bagdasarian, 1961; Wilken & Hansen, 1961). Some of these compounds participate in the synthesis and assembly of cell walls, other are regarded as components of peptidyl-tRNA detached from ribosomes (Bagdasarian, 1965). However, the physiological role of most of these compounds which apparently do not fall into either of the foregoing groups, remains obscure.

For any structural or metabolic studies which might throw light on the role of nucleotide-peptides in living cells, suitable methods of isolation of individual components from complex mixtures are required. In the present paper the isolation and partial characterization of two peptidic compounds from deproteinized extracts of ox liver is described, and attempts to elucidate their possible metabolic function are made.

MATERIALS AND METHODS

Special reagents. All the chemicals used were commercial products: carboxypeptidase (crystalline, DFP-treated) and tris(hydroxymethyl)aminomethane of Sigma Chemical Co. (St. Louis, Mo., USA), 2,4-dinitro-1-fluorobenzene of T. Schu-

chardt GMBH & Co. (München, Germany), DEAE-cellulose of Whatman (England), Sephadex G-25 of Pharmacia (Uppsala, Sweden), ethylenediaminetetraacetic acid (EDTA) of Fabryka Odczynników Chemicznych (Gliwice, Poland). Uniformly labelled L-[^{14}C]phenylalanine, 48 mc/m-mole, of the Radiochemical Centre (Amersham, England). Labelled amino acids were prepared by hydrolysis of ^{14}C-labelled protein of *Chlorella pyrenoidosa* (Szafrański & Sułkowski, 1959).

Methods. Ox liver, collected in ice immediately after the slaughter of the animal, was used as starting material for the isolation of peptides. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Amino acids were determined by the ninhydrin method (Jacobs, 1956).

Electrophoresis was carried out on Whatman 3 *MM* paper (20×55 cm.) in 3.9% acetic acid - 1.3% formic acid, at pH 1.9. For paper chromatography the following solvents were used: solvent I: isobutyric acid - water - conc. NH_3 (66:33:1, by vol.); solvent II: butan-1-ol - acetic acid - water (4:1:1, by vol.); solvent III: propan-2-ol - conc. HCl - water (453:113:96, by vol); solvent IV: propan-1-ol - ethyl acetate - water - acetic acid (7:1:2:0.16, by vol.); solvent V: 1 M-ammonium acetate containing 0.1 M-EDTA - 90% ethanol (60:140, v/v).

Preparation of liver cell-free system and incorporation of amino acids into protein were carried out according to Hoagland, Scornik & Pfefferkorn (1964). L-[U-^{14}C]Phenylalanine was used in this experiment. Incorporation was stopped by the addition of trichloroacetic acid (TCA), the precipitate was washed as described by Siekevitz (1952) and counted in a SE-1 liquid scintillation counter. Dinitrophenylation of amino acids was performed according to Matheson (1963) and the isolation of dinitrophenyl glutamic acid (DNP-Glu) was carried out by a modification of the above method (Bagdasarian, 1965) or by a paper chromatographic method (Biserte, Holleman, Holleman de Hove & Santiere, 1960).

Hydrolysis of peptides was carried out in 6 N-HCl at 105° in sealed glass tubes for 18 hr.

The amino acid composition of peptides was determined by the method of Moore & Stein (1954) on a Technicon Amino Acid Analyser.

EXPERIMENTAL

Isolation and analysis of two peptidic components

Liver extract. All operations were performed in the cold room at 4°. Ox liver, 400 g., was homogenized in a Waring blendor with an equal volume of 0.001 M-tris-HCl buffer, pH 7.2, containing 0.001 M-$MgCl_2$, then 80% TCA was added to the homogenate to a final concentration of 5% and left in the cold for 1 hr. The precipitated protein was centrifuged off and TCA was removed from the supernatant by repeated extraction with ether until the pH of the extract rose to about 4. The extract was then freeze-dried and kept at -15° until needed.

Filtration on Sephadex G-25. The dry extract was dissolved in about 10 ml. of water, the insoluble material removed by centrifugation and the clear solution applied on top of a Sephadex G-25 column (4×95 cm.), equilibrated with water. Water was used as the eluent and 10 ml. fractions were collected at a rate of 3 ml. per minute. In some experiments 0.05 M-ammonium acetate buffer, pH 5.0, was used for equilibration and elution of the column, and similar results were obtained. In the fractions, the extinction was measured at 260 mμ, and amino acids were determined by the ninhydrin test (Fig. 1). The main ninhydrin-positive peak contained

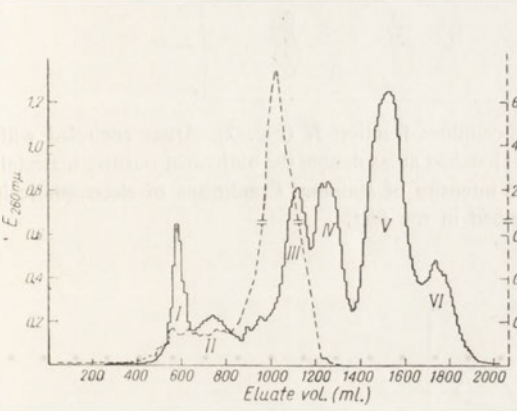

Fig. 1

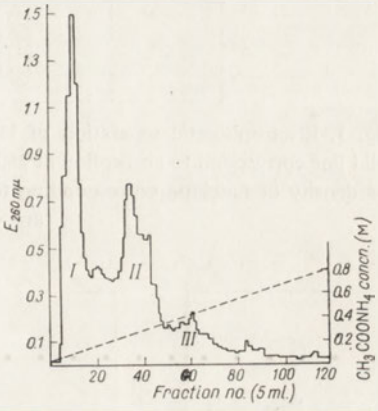

Fig. 2

Fig. 1. Elution diagram of deproteinized extract of ox liver from a column of Sephadex G-25 (4×95 cm.). (———), E_{260}; (- - - -), E_{570} after reaction with ninhydrin. The elution was performed with water, 10 ml. fractions being collected. Void volume established with cytochrome *c* was 560 ml.

Fig. 2. Elution diagram of the Sephadex G-25 fraction *II* from a DEAE-cellulose column, acetate form (2×25 cm.). (———), E_{260}.

free amino acids as revealed by paper chromatography in solvent II. The UV-absorption peaks *I* and *II* emerging in front of the main ninhydrin-positive peak contained peptides as judged by the liberation of free amino acids on acid hydrolysis. Peaks *III* to *VI* contained most of the pigments of the extract and represented a negligible percentage of the dry weight. The material in peak *II* was freeze-dried and submitted to further fractionation.

DEAE-cellulose chromatography. The freeze-dried material from Sephadex G-25 peak *II* (380 mg., about 300 E_{260} units) dissolved in 10 ml. of 0.05 M-ammonium acetate buffer, pH 5.8, was applied to the DEAE-cellulose column (2×25 cm.), acetate form, equilibrated with the same buffer. Elution was performed with a linear concentration gradient of the starting buffer from 0.05 to 0.8 M (Fig. 2). Three UV-absorbing peaks were obtained. Peak *I* contained carbohydrates and other substances that were not adsorbed by the column and was not further analysed. The peak designated *DEAE II* was fractionated by preparative paper electrophoresis.

Electrophoresis and paper chromatography. The freeze-dried material from peak *DEAE II* was applied as a long line on Whatman 3 *MM* paper and subjected to

electrophoresis for 3 hr. at 10 v/cm. and then for 2 hr. at 20 v/cm. This procedure gave better resolution than the application of high voltage from the beginning, because some desalting had probably occurred during the first hours of low voltage electrophoresis. The ultraviolet absorbing spots were visualized under an ultraviolet lamp (260 mμ). Strips were then cut off from both sides of the electrophoretogram and sprayed with ninhydrin. At least seven ninhydrin-positive bands were obtained (Fig. 3). Three of these bands contained the UV-absorbing components, other

Fig. 3. Electrophoretic separation of DEAE-cellulose fraction *II* (Fig. 2). Areas encircled with solid line correspond to absorption at 260 mμ; hatched areas denote the ninhydrin-positive material, the density of hatching corresponding to the intensity of staining. Conditions of electrophoresis are described in the text.

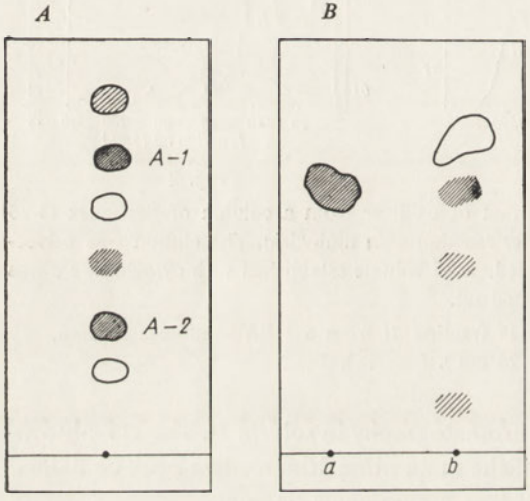

Fig. 4. Paper chromatography in solvent I of (*A*), peptidic fraction *A* separated by paper electrophoresis, and (*B*), rechromatography of fraction *A-1* (*a*), before and (*b*), after alkaline hydrolysis. Areas encircled with solid line correspond to absorption at 260 mμ, hatched areas denote the ninhydrin-positive material.

appeared to correspond to free peptidyl compounds. Individual electrophoretic fractions were eluted from the paper by descending chromatography and freeze-dried. Fractions *A* and *B* were further analysed.

Fraction *A* was found to contain several components when examined by paper chromatography in solvent I (Fig. 4A). One of these spots, *A-1*, was homogeneous on rechromatography in solvent IV and contained both ninhydrin-positive and UV-absorbing material. The separation of the two components was achieved only after hydrolysis in ammonium hydroxide at pH 10 or in 1 N-KOH for 15 hr. at room temperature, followed by chromatography in solvent I (Fig. 4B). This suggests that compound *A-1* might represent a peptide connected to a nucleotide by an alkali-labile bond, such as e.g. carboxyl-anhydride or ester linkage. The UV-absorption spectra of the compound *A-1* at pH 5 and 12 pointed to its being a uridine derivative. On paper chromatography in solvent V it behaved like 5′-UDP.

Table 1

Amino acid composition of compound A-1 *separated on chromatography (Fig. 4) and compound* B *separated on electrophoresis (Fig. 3)*

The amounts of amino acids are expressed as moles per 100 moles of all amino acids found; a correction has been made for losses of amino acids due to hydrolysis in HCl.

Amino acid	*A-1*	*B*
Alanine	2.7	8.3
Aspartic acid (and/or asparagine)	5.9	11.5
Glycine	33.4	19.1
Glutamic acid (and/or glutamine)	25.1	19.6
Isoleucine	0.0	1.7
Leucine	0.0	3.7
Lysine	0.0	3.6
Proline	0.0	8.9
Serine	9.0	7.5
Threonine	2.3	3.3
Tyrosine	7.9	0.0
Valine	0.0	4.4
Unidentified compound	13.7	8.4

The electrophoretic fraction *B* was homogeneous on chromatography in three different solvents (I, II, III). After chromatographic purification it contained no UV-absorbing material, and it was concluded to be a free peptide. The peptide *B* appeared to be susceptible to carboxypeptidase; after incubation with this enzyme at 37° in 0.04 M-tris buffer, pH 7.4, the following free amino acids were detected by paper chromatography in solvent II (in decreasing order): leucine or isoleucine, alanine, and valine. It can be concluded therefore that the *C*-terminal amino acid of the peptide *B* is leucine or isoleucine.

The amino acid composition after total acid hydrolysis of the compound *A-1* and peptide *B* is presented in Table 1.

Incorporation of [^{14}C]*amino acids into peptidic compounds*

To establish whether the peptidic components present in the deproteinized extracts of liver cells are synthesized from the pool of free amino acids or represent the breakdown products of some large molecules an experiment was designed to follow the synthesis of these peptides from [^{14}C]amino acids.

Ox liver, 100 g., was cut into small pieces and squeezed through a fine sieve. Then the cells were suspended in the cold glucose-salt medium (Monier, 1962) and washed by centrifugation. The sediment was suspended in 100 ml. of the glucose-salt medium, 250 μc of a complete acid hydrolysate of *Chlorella* protein was added, and the mixture incubated at 37° for 10 min. Then the labelled cells were extracted and the extract fractionated as described above. Most of the components of the paper electrophoresis fraction *A* separated by paper chromatography in solvent I,

appeared labelled on autoradiography. This indicates that peptidic components were rapidly synthesized under the above conditions.

Nucleotide-peptide *A-1* separated from the mixture of radioactive peptidic compounds was hydrolysed, treated with 2,4-dinitro-1-fluorobenzene and the specific activity of isolated DNP-glutamic acid determined. A value of 2846 counts/min./μmole of DNP-glutamic acid was obtained. When DNP-glutamic acid was isolated from the mixture of liver cells at the beginning of the incubation with radioactive amino acids, its specific activity was 2540 counts/min./μmole. The comparison of the two above values points to a very rapid turnover of the peptidyl moiety of the compound *A-1* and suggests that it did not arise as a breakdown product of proteins or nucleoproteins but was synthesized directly from the pool of free amino acids.

Suggestions have been put forward (Van Dam, Slavenburg & Koningsberger, 1964; Millin & Saltmarsh-Andrew, 1965) that nucleotide-peptides might perform regulatory functions in biosynthesis of inducible enzymes. It was of interest therefore to test the effect of an isolated nucleotide-peptide *A-1* on the incorporation of amino acids in a cell-free system. Compounds *A-1* and *A-2* eluted from paper chromatography (Fig. 4) were added to an amino acid incorporating system isolated from bovine liver according to Hoagland *et al.* (1964). No effect of either of these compounds on the incorporation of [^{14}C]phenylalanine was observed when they were added to the incubation mixtures in concentrations of 75 - 300 μg. of UDP equivalent per 1 ml..

These results do not support the supposition that nucleotide-peptides perform regulatory functions as repressors of protein biosynthesis.

DISCUSSION

The isolation procedure used in the present work allows to separate and purify a number of peptides and nucleotide-peptides from deproteinized tissue extracts. It permits also to obtain these compounds in quantities high enough for performing structural studies. Gel filtration used as the first step of fractionation permits an estimation of the molecular size of the peptidic compounds studied. In the case of peptidic compounds *A-1* and *B*, gel filtration indicates that both have molecular size smaller than that of cytochrome *c* (mol. wt. 13 000). The minimal molecular weight of the *A-1* can be calculated from the content of threonine, and for peptide *B* from isoleucine content (Table 1). Molecular weights of the order of 5650 for the *A-1* and 6850 for *B* are obtained. Theoretically the value for *A-1* might be twice the minimal molecular weight, this however seems unlikely in view of the rather wide separation of these compounds from cytochrome *c*.

The amino acid analysis both of compound *A-1* and *B* revealed an unidentified component, which behaves in the Amino Acid Analyser like hydroxyproline, methionine sulphoxide, cysteic acid or taurine. It is interesting to note that Wilken & Hansen (1961) have found both taurine and cysteic acid in an adenosinediphos-

pho-peptide from bovine liver. This might point to some structural and probably functional similarity of compounds discussed. Taurine and cysteic acid are not normal constituents of proteins and it is difficult to explain at present the significance of this finding.

Experiments with the incorporation of labelled amino acids into compound *A-1* suggest that it is not a breakdown product of proteins. It cannot, however, be considered as a fragment of tRNA-peptide detached from the ribosomes, because in that case the peptide would be expected to be attached to an adenine nucleotide. Since the isolated compounds do not affect incorporation of amino acids in a cell-free system, it seems that they do not represent regulators in protein synthesis, and ther role in the cell remains to be elucidated.

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ZWIĄZKI PEPTYDOWE W ODBIAŁCZONYCH EKSTRAKTACH WĄTROBY WOŁOWEJ

Streszczenie

1. Z odbiałczonych ekstraktów wątroby wołowej wyizolowano dwa związki, z których jeden jest wolnym peptydem a drugi nukleotydo-peptydem zawierającym w części nukleotydowej 5′-UDP. Ich masa cząsteczkowa jest rzędu 6000.

2. Stwierdzono, że związki te nie są produktem degradacji białek lub nukleoproteidów, lecz że syntetyzują się z wolnych aminokwasów.

3. Wyizolowane związki nie mają wpływu na włączanie L-[^{14}C]fenyloalaniny do białek syntetyzowanych *in vitro*, nie można więc przypisać im funkcji regulatorów w biosyntezie białka.

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STUDIES ON POSSIBLE MECHANISMS OF HYDROXYLAMINE MUTAGENESIS

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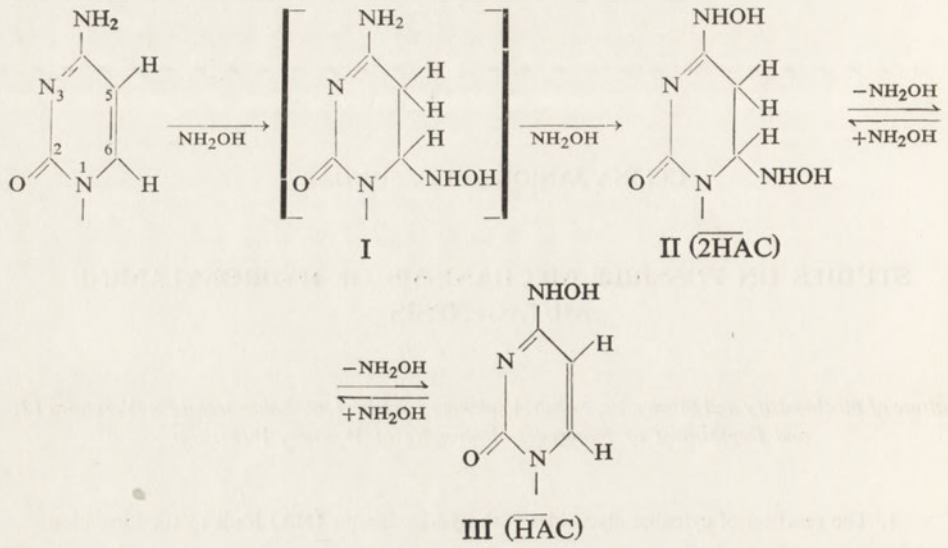
1. The reaction of cytosine glycosides with hydroxylamine (HA) leads to the formation of two products: 4,6-dihydroxylamine-5,6-dihydrocytidine (2HAC) and 4-hydroxylaminocytidine (HAC). The same products are formed from cytidine residues in poly-C. An investigation has been made of the influence of pH and hydroxylamine concentration on the relative percentages of these two products formed during reaction with cytidine-2′(3′)-phosphate and poly-C. Even at approximately neutral pH (6.5) both products, HAC and 2HAC, make their appearance simultaneously, the former in relatively low yield. At acid pH the formation of HAC predominates and, under these conditions, it is possible to adjust the reaction conditions so that this is the major product. These conditions were made use of to obtain HACDP. 2. Poly-2HAC was prepared by reaction of poly-C with hydroxylamine at neutral pH, whereas poly-HAC was isolated following reaction of poly-C with hydroxylamine at acid pH. The latter polymer was also obtained by polymerization of HACDP, which proved to be a substrate for polynucleotide phosphorylase. The structures of these polymers, which were rather low, were examined by optical absorption methods. Both of them were hydrolysed to mononucleotides by pancreatic ribonuclease. Neither of them formed complexes, i.e. base-pairs, with poly-A, poly-U, poly-I or poly-C. 3. Semicarbazide (SC), which is known to react with cytosine glycosides to form 4-semicarbazido cytosine glycosides (SCC), a formal analogue of HAC, was found to react with poly-C at acid pH to give poly-SCC. Some of the properties of this polymer were examined. It was susceptible to ribonuclease and did not complex with poly-A, poly-U, poly-I or poly-C. 4. The significance of the overall results, in relation to the mechanism of hydroxylamine mutagenesis, is discussed.

It has been reasonably well established that, in aqueous medium, hydroxylamine, HA[1], reacts only with cytosine residues in DNA, the mechanism of the reaction

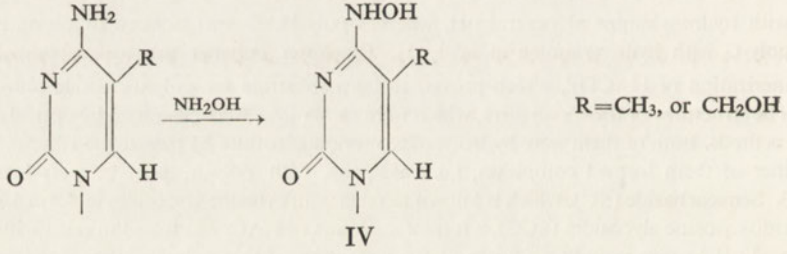
[1] The following terminology is used in this text: HA, hydroxylamine; SC, semicarbazide; C, cytidine; Cp, cytidine-2′(3′)-phosphate; CMP, cytidine-5′-phosphate; CDP, cytidine-5′-diphosphate; poly-U, polyuridylic acid; poly-A, polyadenylic acid; poly-I, polyinosinic acid; poly-C, polycytidylic acid; HAC, 4-hydroxylamino-cytidine; 2HAC, 4,6-dihydroxylamino-5,6-dihydrocytidine; SCC, 4-semicarbazidocytidine.

being as follows (Scheme 1) for cytosine or its glycosides (Verwoerd, Kohlhage & Zillig, 1961; Brown & Schell, 1961; Janion & Shugar, 1965):



Scheme 1.

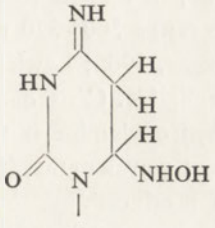
Form I is a hypothetical intermediate which has not been isolated, while, as will be shown, the equilibrium between II and III is dependent on the $\overline{HA}$ concentration and the pH. The transition from II to III is relatively rapid in acid medium. For 5-substituted cytosine residues in aqueous medium, only the following reaction occurs (Janion & Shugar, 1965) (Scheme 2):



Scheme 2.

In view of the known mutagenic activity of hydroxylamine, it becomes of interest to determine whether forms II or III, or both, are those which result in mutations, presumably *via* base-pair transitions. In the case of 5-substituted cytosines it is most likely that IV (which is structurally equivalent to III) is the responsible form, since hydroxylamine is actively mutagenic against the T-even bacteriophages (Freese, Bautz & Bautz-Freese, 1961; Schuster & Vielmetter, 1961; Champe & Benzer, 1962), which contain only 5-substituted (free and/or glucosylated 5-hydroxymethyl)cytosine residues.

It has been proposed that form I is the modified species of cytosine leading to mutations by replication errors (Phillips, Brown, Adman & Grossman, 1965). This proposal, which was based on observations of the template properties of hydroxylamine treated poly-C, was subsequently revised in favour of the imino tautomer of I, i.e. Ia (Phillips, Brown & Grossman, 1966) (Scheme 3) which was presumed to pair readily with adenine. However the failure to isolate I, due to its presumed instability (Brown & Schell, 1961), and the lack of any direct information as to its tautomeric form, raise some doubts as to the validity of this proposal. Form III



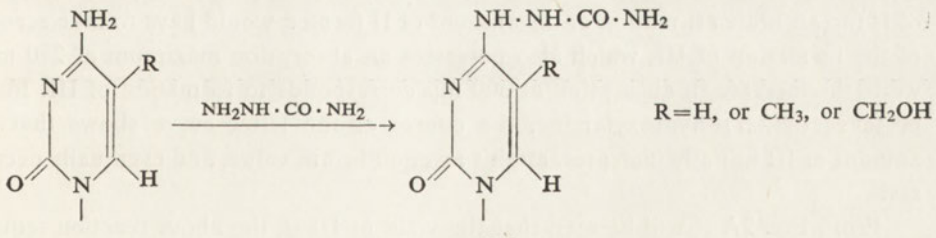
Ia

Scheme 3.

(or IV) has been proposed as the responsible mutagenic reaction product (Janion & Shugar, 1965; Lawley, 1967) on the grounds that this is the only product formed with 5-substituted cytosines (Janion & Shugar, 1965) and is also one of the initial products of reaction of poly-C with hydroxylamine, albeit in lower yield than form II (Lawley, 1967; see also below).

In view of the above, it seemed desirable to prepare synthetic homopolymers containing base residues of forms II and/or III, and to examine the properties of such homopolymers, particularly their base-pairing affinities for other potentially complementary homopolymers. The demonstration of such complexing abilities for either of these two forms might be expected to constitute evidence as to whether one or the other is involved in the mutagenic process.

In relation to the foregoing, some experiments have also been carried out with semicarbazide, which is known to react specifically with cytosine (Hayatsu & Ukita, 1964; Hayatsu, Takeishi & Ukita, 1966) and 5-substituted cytosine (Janion and Shugar, unpublished) residues, both in the free form and in nucleic acids, as follows (Scheme 4).



V (SCC)

Scheme 4.

RESULTS

From the sequence shown in Scheme 1, it is clear that the reaction of cytosine with hydroxylamine may lead to the formation not only of II but, even in the presence of elevated concentrations of hydroxylamine, also of III. Since compound II possesses a saturated 5,6 bond, its UV absorption in the neighbourhood of the absorption maximum of cytosine (270 mμ) will be low or negligible. Compound III, on the other hand, possesses three conjugated double bonds and should absorb selectively at longer wavelengths; in fact its long wavelength absorption band in neutral medium extends to the range 300 - 320 mμ (Fox *et al.*, 1959; Schuster & Vielmetter, 1961; Janion & Shugar, 1965; Lawley, 1967), where the absorption of cytosine and its glycosides is negligible. Consequently, if the course of the reaction of a cytosine derivative with hydroxylamine is followed by measurements of the changes in absorption at different wavelengths, it becomes feasible to evaluate the relative amounts of II and III produced.

The foregoing considerations were applied in this study, as well as by Lawley (1967), and it was demonstrated that the reaction of cytosine analogues with hydroxylamine leads to the appearance of compound III, along with compound II, even in the initial phase of the reaction. This once again points to the difficulty of determining which of the two products is involved in the mutagenic process.

The ratio of the two products formed is dependent on the reaction conditions. At pH 6.5 and 37°, the transformation of 10^{-3} M-Cp in 2.5 M-hydroxylamine is rapid, and is almost complete in about 2 hr. Under these conditions the main product is II, whereas the amount of III formed is relatively low, about 10% in one experiment, but even lower in others. Lawley (1967) reports the formation, under analogous conditions, of about 25% of III, in the reaction of deoxycytidine with 2.1 M-hydroxylamine. The reason for this discrepancy is not immediately obvious, but may be due to the use by the latter author of much higher concentrations of deoxycytidine (25×10^{-3} M) in the reaction mixture.

If the pH of the reaction mixture is reduced to pH 5.2, the percentage of III formed is appreciably increased, as might be expected from the fact that the transformation of II to III is an acid-base catalysed reaction (Brown & Schell, 1961). Fig. 1 exhibits the changes in absorption at two selected wavelengths, 270 mμ and 300 mμ, during the reaction of Cp with 2.5 M-hydroxylamine at 37° and pH 5.2. The decrease in absorption at 270 mμ corresponds approximately to formation of II (an accurate estimation of the amount of II formed would have to take account of the formation of III, which also possesses an absorption maximum at 270 mμ), while the increase in absorption at 300 mμ corresponds to formation of III. In the presence of 2.5 M-hydroxylamine, the course of the latter curve shows that the amount of III initially increases, attains an equilibrium value, and eventually decreases.

From Fig. 2A it will be seen that the yield of III in the above reaction sequence may be augmented at the same pH value by reducing the concentration of hydroxylamine. Note that in the presence of 1 M-hydroxylamine the yield of III conti-

nues to increase during the entire course of the reaction. Fig. 2B exhibits the UV absorption spectrum of the reaction products of Cp with 1 M-hydroxylamine at pH 5.1 following total transformation of Cp (22 hr.). The shape of the curve shows that form III predominates under these conditions. Using a value of $\varepsilon_{270\ m\mu}$ of 9.43×10^3 for Cp at pH 5.1, and $\varepsilon_{270\ m\mu}$ of 6.2×10^3 for $\overline{HA}$Cp (Brown & Phillips, 1965), the percentage of III is found in this case to be about 60%. The foregoing reaction conditions were employed to prepare the 4-hydroxylamino derivative of CDP, i.e. $\overline{HA}$CDP, in even higher yield. Several experiments conducted at a pH of about 4.5 demonstrated formation of a higher percentage of III, attaining values of over 77% after several hours reaction. However, these conditions were not employed

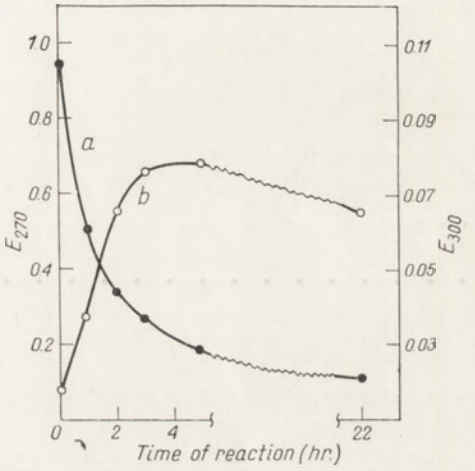


Fig. 1. Course of reaction of cytidine-2′(3′)-phosphate (10^{-3} M) with 2.5 M-hydroxylamine at pH 5.2 and 37°, followed spectrally (●), at 270 mμ (curve *a*, formation of II) and (○), at 300 mμ (curve *b*, formation of III).

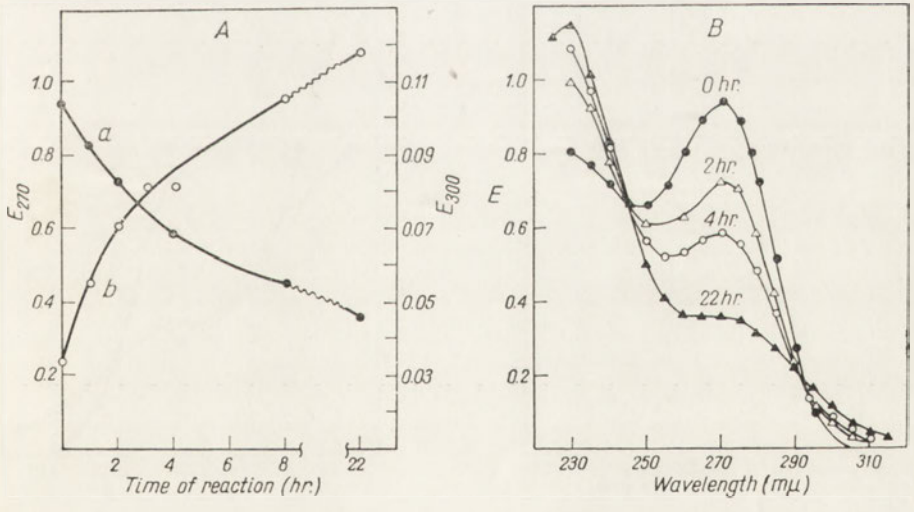


Fig. 2. Course of reaction of cytidine-2′(3′)-phosphate (10^{-3} M) with 1 M-hydroxylamine at pH 5.1 and 37°: (*A*), changes in extinction at 270 mμ (curve *a*, formation of II) and at 300 mμ (curve *b*, formation of III); (*B*), changes in UV absorption spectrum during course of reaction.

for the reaction with CDP because of appreciable hydrolysis of the pyrophosphate to monophosphate.

Similar variations in the relative formation of products II and III, depending on the reaction conditions, are observed in hydroxylamine-treated poly-C. Fig. 3 exhibits the changes in extinction at two wavelengths of poly-C in the presence of 2.5 M-hydroxylamine at pH 6.5 and 37°. The initial lag in the decrease in absorption at 270 mμ, previously observed by Brown & Phillips (1965), is undoubtedly the resultant of two simultaneous effects, the reaction of the cytidine residues with hydroxylamine and the accompanying change in hypochromicity of the treated polymer. As will be shown subsequently, polymers containing residues of II or III exhibit negligible hypochromicity at 270 mμ. Compound III appears in

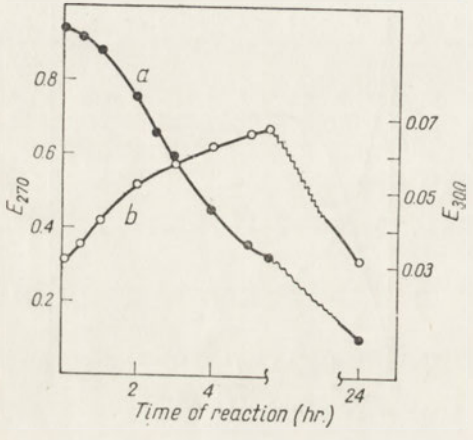


Fig. 3. Course of reaction of poly-C (10^{-3} M) with 2.5 M-hydroxylamine at pH 6.5 and 37°, followed spectrally (●), at 270 mμ (curve *a*, approximate formation of residues of form II) and (○), at 300 mμ (curve *b*, formation of residues with form III).

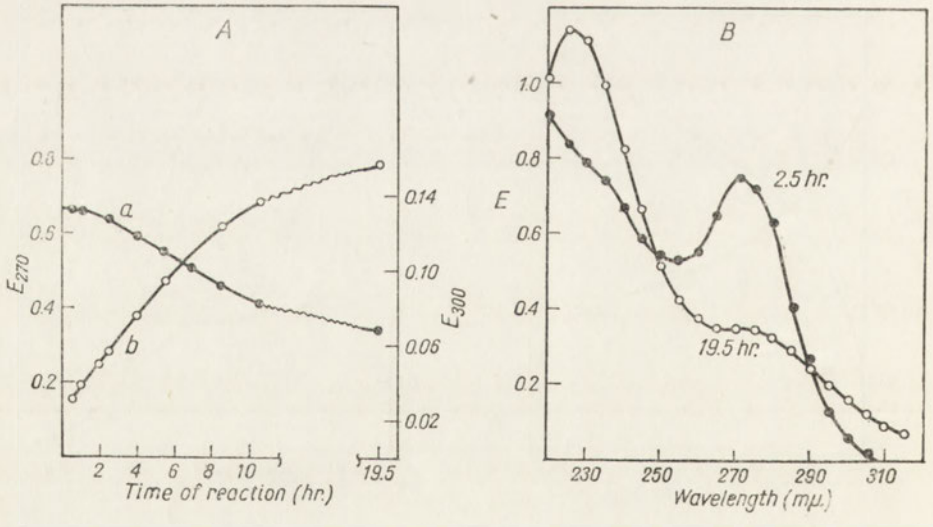


Fig. 4. Course of reaction of poly-C (10^{-3} M) with 1 M-hydroxylamine at pH 5.2 and 37°: (*A*), changes in extinction at 270 mμ (curve *a*, formation of II) and at 300 mμ (curve *b*, formation of III); (*B*) absorption spectra after 2.5 hr. and 19.5 hr. reaction time.

the initial phase of the reaction (increase in absorption at 300 mμ) and, as in the case of Cp, at first increases and subsequently diminishes.

Figures 4A and 4B present the changes in extinction at selected wavelengths, as well as changes in the entire absorption spectrum, accompanying the reaction of poly-C with 1 M-hydroxylamine at pH 5.2. Note that at this pH the formation of III is also favoured over II.

Reaction of semicarbazide with poly-C. The action of $\overline{SC}$ is even more specific than that of $\overline{HA}$, in that it reacts only with cytosine residues in both DNA and RNA (Hayatsu & Ukita, 1966), as well as with 5-substituted cytosines (Janion & Shugar, unpublished). The optimum pH for this reaction is about 4.2, leading to replacement of the exo amino group by an $\overline{SC}$ residue as shown in Scheme 4. It will be seen that form V is structurally equivalent to form III or IV. This reaction proceeds readily with the free base or its glycosides (Hayatsu, Takeishi & Ukita, 1966), as well as with tRNA, the latter undergoing a decrease in secondary structure (Hayatsu & Ukita, 1966).

Figure 5 exhibits the change in absorption spectrum of poly-C during the course of reaction with 2.5 M-$\overline{SC}$ at pH 4.2 and 37°. The similarity of the absorption spectrum after 24 hr. reaction to that at zero time suggests that the increase in overall extinction is chiefly due to a reduction in hyperchromicity resulting from a decrease

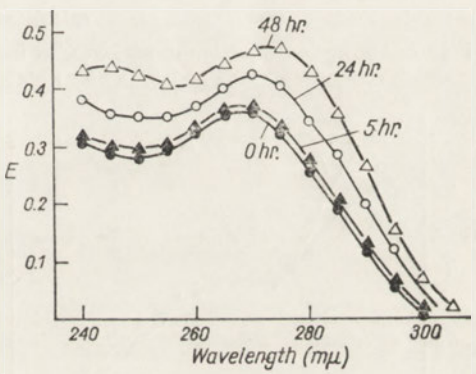


Fig. 5. Course of reaction of poly-C (2×10^{-3} M) with 2.5 M-semicarbazide at pH 4.5 and 37°. At the time intervals shown in the figure, 50 μl. of the reaction mixture was added to 1 ml. of 0.1 M-phosphate buffer, pH 8, in a semi-micro 10-mm. cuvette and the absorption spectrum determined.

in secondary structure of the modified polymer. Extension of the reaction time beyond 48 hr. did not result in any further increase in extinction; however, the reaction was usually prolonged to 92 hr. to ensure that all cytosine residues had reacted.

Properties of modified polymers. The properties of the following three modified homopolymers, prepared as described in Experimental, below, were examined: poly-$\overline{HA}$C (in which the base residues are 4-hydroxylamino-2-ketopyrimidine); poly-2$\overline{HA}$C (in which the base residues are 4,6-dihydroxylamino-5,6-dihydro-2-ketopyrimidine); and poly-$\overline{SC}$C (base residues 4-semicarbazido-2-ketopyrimidine).

In 0.01 M-tris buffer, pH 7, with 0.1 M-NaCl, none of the above homopolymers exhibited any temperature hyperchromicity over the range 15° - 80°, measured at the wavelengths of both the short and long wavelength absorption bands.

The significance of the foregoing observation was made clearer by an examination of the absorption spectra of the polymers at different pH values, for poly-$\overline{\text{HAC}}$ and poly-$\overline{\text{SCC}}$. The results are shown in Figs. 6 and 7. It will be seen that for both polymers the absorption spectra are similar to those of the monomeric nucleoside components. Furthermore for both polymers the neutral and protonated forms intersect at points which are isosbestic and which, together with additional curves

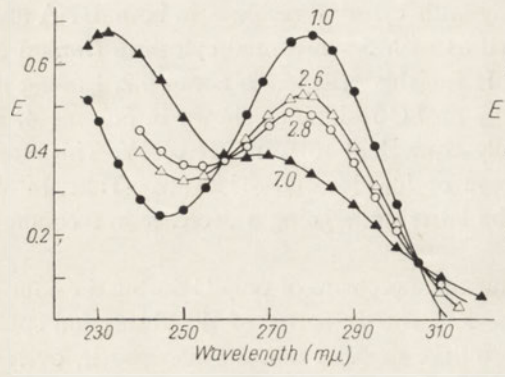


Fig. 6

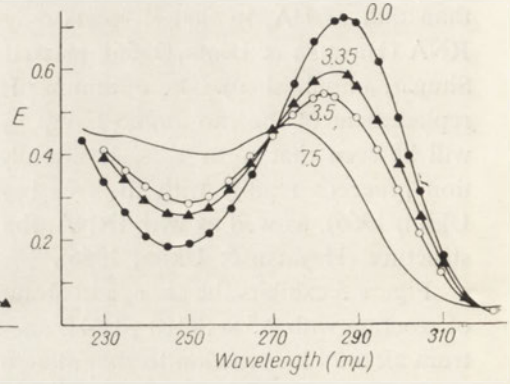


Fig. 7

Fig. 6. Absorption spectrum of poly-$\overline{\text{HAC}}$ at indicated pH values; apparent pK of $\overline{\text{HAC}}$ residues calculated as ~ 2.7.

Fig. 7. Absorption spectrum of poly-$\overline{\text{SCC}}$ at indicated pH values; calculated apparent pK of $\overline{\text{SCC}}$ residues, ~3.5.

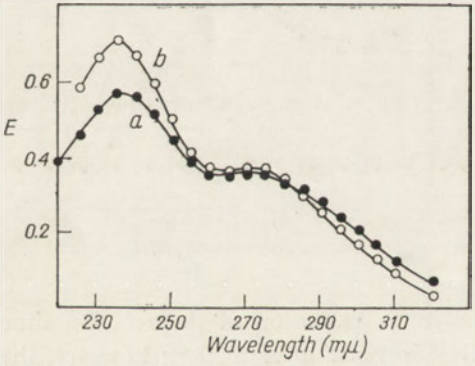


Fig. 8

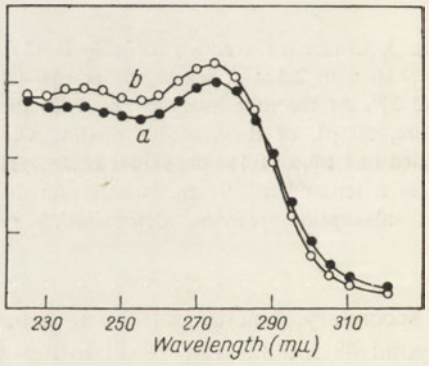


Fig. 9

Fig. 8. Absorption spectrum of poly-$\overline{\text{HAC}}$ in 0.01 M-tris buffer, pH 7.7, (*a*) before and (*b*) after hydrolysis with pancreatic ribonuclease.

Fig. 9. Absorption spectrum of poly-$\overline{\text{SCC}}$ in 0.01 M-tris buffer pH 7.7, (*a*) before and (*b*) after hydrolysis with pancreatic ribonuclease.

at intermediate pH values, lead to pK values for the monomer nucleoside residues of 2.7 and 3.5; these are to be compared with those for the free ribosides of 2.26 and 2.7, respectively. These findings would seem to imply that there is some degree of interaction between the base residues in the polymer chains (see below).

Spectral measurements on the polymers at acid pH proved to be somewhat difficult, due to aggregation. Poly-SCC, for example, rapidly precipitated in 0.1 N-HCl. It did dissolve well on careful addition to 1 N-HCl, and spectral measurements were carried out in this medium as quickly as possible to avoid effects due to hydrolysis of internucleotide linkages. As regards poly-HAC, measurements could be made only to pH 1 or 2; below this pH the polymer precipitated at once.

All three polymers were readily degraded to mononucleotides by pancreatic RNase (EC 2.7.7.16), as shown by paper chromatography. Enzymic hydrolysis was accompanied by some spectral modifications in the case of poly-HAC and poly-SCC, pointing to the presence in these polymers of some structural form, most likely due to base stacking.

Figure 8 presents the spectra of poly-HAC prior to and following enzymic hydrolysis to mononucleotides. The changes in absorption in the region of the long wavelength band at 275 mμ are rather small. By contrast the short wavelength band at 235 mμ undergoes a marked increase in extinction, almost 25%. Furthermore, the ratio of the optical density at 235 mμ to that at 275 mμ for the hydrolysed polymer corresponds quantitatively to that for the free mononucleotide. It must therefore be concluded that poly-HAC does possess some degree of structure, the nature of which remains to be determined, but is probably due to base stacking, as for poly-U at room temperature.

Support for this contention is provided by the following observations. Several preparations of poly-HAC exhibited a lower hypochromicity at 235 mμ, which was enhanced on addition of 2×10^{-3} M-Mg^{2+}, but without affecting the peak at 270 mμ.

By contrast, all preparations of poly-SCC exhibited a uniform value for the residual hyperchromicity on enzymic hydrolysis, about 8.5% at 275 mμ (Fig. 9).

Attempts at complex formation. In the light of the arguments advanced in the introduction to this paper (see above), considerable efforts were devoted to an examination of the potential complexing abilities of the modified polymers. From the results of Troll, Belman & Levine (1963) on the decrease in T_m of hydroxylamine-treated DNA, it may be inferred that the ability of the modified cytosine residues to bond with guanine is appreciably decreased, if not altogether abolished. Analogous conclusions may be drawn from the observations of Hayatsu & Ukita (1966) on the influence of semicarbazide treatment on the T_m of yeast tRNA.

The complexing abilities of the modified homopolymers described above were examined in 0.01 M-tris buffer, pH 7.7, with 0.1 M-NaCl, using as potential complementary homopolymers poly-U, poly-A, poly-I and poly-C. The results showed that the modified polymers had not only completely lost their ability to complex with poly-I, but also failed to form any detectable complexes with poly-A, poly-U or poly-C. Prolongation of the reaction times, decrease of temperature to 0°, or addition of Mg^{2+}, were all without effect.

In view of the above findings, several controls were carried out in order to check whether the lack of complexing abilities of the modified polymers was not due to

side reactions, e.g. decrease in chain length. The effect of dilute acid treatment on poly-C was examined; it was found that treatment of poly-C at pH 4.5 and 37° (as in the reaction with semicarbazide), or at pH 3.0 (as for elimination of hydroxylamine from the 5,6 bond during dialysis) did not affect its complexing ability with poly-I. It was also established that, under the conditions employed by Matsuda & Ogoshi (1966) for deamination of DNA with sodium nitrite, poly-$\overline{HAC}$ was quantitatively transformed to poly-U; such a preparation of poly-U exhibited the expected absorption spectrum and also readily complexed with poly-A under the appropriate conditions.

EXPERIMENTAL

The hydrochloride salts of hydroxylamine and semicarbazide were commercial products which were recrystallized from ethanol. The sodium salt of CDP was a Koch-Light (England) preparation. *Escherichia coli* polynucleotide phosphorylase (Littauer & Kornberg, 1957), with an activity of 110 units of phosphorolysis per 10 μl., was a gift of Dr. U. Z. Littauer; the analogous enzyme from *Micrococcus lysodeicticus* was made available by Dr. J. H. Matthaei. The synthetic homopolymers employed were either Miles (U.S.A.) products, or preparations made available by Dr. J. H. Matthaei and Dr. H. G. Zachau. Bovine pancreatic ribonuclease was obtained from Koch-Light.

All spectral measurements were carried out on a Unicam SP-500 instrument, fitted with a specially constructed heated block compartment through which circulated a glycol-water mixture from a Hoeppler ultrathermostat. A thermistor in a dummy cuvette served for temperature measurements. A Radiometer (Copenhagen) PHM28 meter, with a glass microelectrode, was used for pH control.

Measurements of course of reaction. The course of the reaction of hydroxylamine with Cp or poly-C was followed in 1-mm. quartz spectrophotometer cuvettes in the thermostated compartment of the spectrophotometer at 37°. The reaction was usually initiated by addition of several microlitres of a stock solution of Cp or poly-C to an aqueous solution of hydroxylamine at the appropriate concentration and pH. The control cuvette contained the same solution of hydroxylamine.

Preparation of poly-$\overline{2HAC}$. A stock solution of poly-C was added to a 2.5 M-solution of hydroxylamine at pH 6.5 so that the concentration in poly-C was 400 μg./ml. The reaction was then followed by the decrease in absorption at 270 mμ (in a 1-mm. cuvette) at 37°. The reaction was considered complete after 24 hr., at which time the extinction at 270 mμ did not decrease further (Fig. 3). The reaction mixture was then dialysed in the cold room for 24 - 48 hr. against several changes of 0.1 M-NaCl. Dialysis against salt solution was employed following the observation that under these conditions the elimination of hydroxylamine from the 5,6 bond of the cytosine residues was appreciably inhibited. The resulting solution of poly-$\overline{2HAC}$ exhibited only end absorption in the UV, with a maximum at 225 mμ at neutral pH, in accordance with expectations for a polymer with residues containing a 5,6

saturated bond. Note that in this particular experiment the absorption at 300 mμ after 24 hr. had reverted to its initial value (Fig. 3), indicative of the absence of form III.

It is perhaps worth noting that storage of this polymer solution in the deep-freeze for several months led to the disappearance of the short wavelength maximum at 225 mμ. The reason for this was made clear by paper chromatography with a solvent consisting of a 2:1 mixture of isopropanol and 1% ammonium sulphate. Spraying of the chromatogram with a 1% ethanolic solution of *p*-dimethylaminobenzaldehyde containing 1 ml. conc. HCl per 100 ml. solution gave a characteristic yellow spot with R_F 0.37, corresponding to urea. It follows that both fission of the glycosidic linkages, and opening of the pyrimidine rings, had occurred. Because of this apparent lability, experiments with poly-$\overline{2HA}$C were performed immediately after its preparation.

Preparation of poly-$\overline{HA}$C. This polymer was prepared by two independent methods. One of these was based on the polymerization with polynucleotide phosphorylase of the 4-hydroxylamino derivative of CDP, described in the next section. The second was based on the reaction of poly-C with 1 M-hydroxylamine, at about pH 5.2, under conditions where most of the cytosine residues are transformed to form III (see Fig. 2B). The modified polymer was then dialysed against 10^{-3} N-HCl for 24 hr. at room temperature; these conditions sufficed to eliminate hydroxylamine from the remaining $\overline{2HA}$C residues.

Table 1

Paper chromatography, ascending, of 4-hydroxylamino derivatives of CMP and CDP

Whatman paper no. 1; solvent systems: *A*, 1% ammonium sulphate, isopropanol (1:2, v/v); *B*, isobutyric acid, 28.5 ml.; water, 19.5 ml.; conc. NH_4OH, 2 ml.

Compound	R_F	
	Solvent *A*	Solvent *B*
CMP	0.43	0.47
CDP	0.27	0.32
$\overline{HA}$CMP	0.51	0.32
$\overline{HA}$CDP	0.33	0.24

Preparation of $\overline{HA}$CDP. CDP, 30 mg., was dissolved in 0.45 ml. of a 1 M-solution of hydroxylamine, pH 5.2. This solution was kept at 37° for 24 hr., then cooled and the reaction product precipitated with 3 vol. ethanol. The precipitate was collected by centrifugation, dried, dissolved in 0.1 ml. water and again precipitated with 3 vol. ethanol. The final yield of product was 20 mg. The precipitation with ethanol not only frees the product from hydroxylamine, but also removes traces of the 4-hydroxylamino derivative of CMP formed during the reaction. Appropriate R_F values of these compounds are shown in Table 1. Occasionally the preparations contained traces of $\overline{2HA}$CDP, but this was ignored when it was found in separate

experiments that this compound was only a feeble substrate, if at all, for the polynucleotide phosphorylase.

Polymerization of $\overline{HA}$CDP. A typical incubation mixture was as follows: 2 mg. $\overline{HA}$CDP, 400 µl. 0.15 M-tris buffer, pH 8.2, 25 µl. 0.1 M-$MgCl_2$, 25 µl. 0.01 M-EDTA and 15 µl. *E. coli* polynucleotide phosphorylase. At 37° equilibrium was attained in about 1 hr., the yield of polymer material being in the range 22 - 40%. With longer incubation periods the yield decreased, possibly due to the presence of traces of nucleolytic enzymes. The reaction was therefore followed by paper chromatography with the following solvent system: 48 ml. of saturated ammonium sulphate, 1.2 ml. isopropanol, 10.8 ml. 8.2% ammonium acetate; with this system monomer material separated from polymeric material in 20 - 30 min. Elution and estimation of the amount of monomer gave the yield of polymer by difference.

Prior to isolation of the polymer, 30 µg. pronase was added to the incubation mixture for 1 hr. at 37° to destroy possible traces of nucleases.

The polymer was then isolated by the phenol method, using freshly distilled, neutralized phenol. Traces of phenol were extracted with ether, the latter removed by evaporation, and the solution exhaustively dialysed, first against decreasing concentrations of NaCl and EDTA, and finally against water.

With purified polynucleotide phosphorylase from *Micrococcus lysodeicticus* (Matthaei *et al.*, 1967), the maximum yield of polymer, under the conditions described by the foregoing authors, was about 23% after 18 hr. incubation. No attempt was made to improve on this by variation of the reaction conditions.

Preparation of poly-$\overline{SCC}$. A preparation of poly-C was dissolved in 2.5 M-semicarbazide hydrochloride, previously adjusted to pH 4.2, at a concentration of 400 µg./ml. Occasionally it proved necessary to filter the solution of semicarbazide to remove traces of insoluble matter. After 3 to 4 days at 37°, the reaction mixture was dialysed exhaustively against 0.1 M-NaCl.

Ribonuclease hydrolysis of polymers. To about 60 µg.. of polymer in 300 µl. of 0.01 M-tris buffer, pH 7.7, was added 10 µl. of a 2 mg./ml. solution of pancreatic RNase, and the solution incubated for 2 hr. at 37°. The absorption spectrum, prior to and after addition of RNase, was followed in a 1-mm. cuvette against a suitable control. Extent of hydrolysis was followed by paper chromatography, with the solvent system butanol - ethanol - 5 N-HCl (3:2:2, by vol.), the R_F values for Cp, Up, $\overline{HA}$Cp and $\overline{SCC}$p being 0.43, 0.71, 0.51 and 0.28, respectively. In all instances there was only one product of hydrolysis (see Figs. 8, 9).

DISCUSSION

It is obvious that the reaction of hydroxylamine with cytosine residues in polynucleotides is more complex than hitherto considered, in that it is dependent not only on the concentration of hydroxylamine but also on the ratio of the concentration of cytosine residues to hydroxylamine. The pH is also an important factor. In the

pH range 6.2 - 6.5, which has been reported as optimal for the reaction (Verwoerd *et al.*, 1961), it is the formation of II which appears to be predominant, while the extent of formation of III is variable and probably largely dependent on the hydroxylamine concentration. As the pH is made more acid, the extent of formation of III increases considerably and may, as in several experiments described here, be the major or sole product. There is little doubt but that a more extensive investigation will have to be made of the kinetics of the reaction of hydroxylamine with cytosine glycosides, and model polynucleotides, at a series of pH values from 7.0 to about 4.0, in order to better define the rates of formation of the two products. The potential importance of such detailed kinetic studies is underlined by the observations of Bautz-Freese & Freese (1964) on the increased rate of mutagenesis with decrease in pH to about 4.2.

In fact, the increased rate of mutagenesis at acid pH, together with our observation that the proportion of III appreciably increases at acid pH, might be regarded as evidence for participation of form III in the mutagenic process. However, this cannot be considered as excluding the significance of II, nor even of I (or Ia) if we accept the indirect evidence of Brown & Phillips (1965) for formation of I (or Ia) in hydroxylamine-treated poly-C.

On the basis of a kinetic analysis, Lawley (1967) concluded that at pH 6.5 the formation of III does not proceed *via* II, as in Scheme 1, but directly, as in Scheme 2. While we have no direct evidence for or against this suggestion, the influence of pH seems to us to suggest rather that III is formed from II as in Scheme 1 (cf. Brown & Schell, 1961). It is possible that a comparison of reaction rates of hydroxylamine with cytosine and 5-methyl cytosine (where formation of II does not occur) may assist in resolving this problem.

The failure to find any direct evidence for complex formation between either poly-$\overline{2HA}C$ or poly-$\overline{HA}C$ on the one hand, and poly-A, poly-U, poly-C or poly-I (which is an analogue of poly-G) on the other, implies that neither $\overline{2HA}C$ nor $\overline{HA}C$ residues readily base pair with the residues naturally occurring in nucleic acids. This is, perhaps, understandable in the case of $\overline{2HA}C$ residues (form II), which possess a saturated 5,6 bond so that the pyrimidine ring has lost its aromatic character. But, if this is indeed the case, it is equally difficult to see how form I, which also contains a 5,6 saturated bond, can more readily base pair with the naturally occurring base residues as proposed by Phillips, Brown & Grossman (1966). It is, on the other hand, conceivable that an $\overline{HA}C$ residue formed in a natural nucleic acid with adjacent natural base residues may be constrained to form some other type of base pairing (cf. Crick, 1966). For this reason it might be desirable to examine the complexing ability of a copolymer of $\overline{HA}C$ with either A, U, C or I (or G); in order to avoid ambiguity, such copolymers would have to be prepared with polynucleotide phosphorylase, using the pyrophosphates of the foregoing nucleoside residues as substrates.

Furthermore, in view of the fact that it is possible to prepare the homopolymers of $\overline{HA}C$ and $\overline{2HA}C$, it would be highly desirable to examine the template properties of these polymers in the system described by Phillips *et al.* (1965) and Wilson &

Caicuts (1966). Such experiments would provide additional evidence for or against the involvement of forms II (and perhaps I) and III in the mutagenic process.

Finally, attention should be drawn to the fact that, although poly-$\overline{\text{HAC}}$ and poly-$\overline{\text{SCC}}$ appear to be devoid of secondary structure, this conclusion is based largely on the absence of temperature hyperchromicity of the UV absorption bands. The fact that there is some residual hyperchromicity, revealed by enzymic hydrolysis, shows that there must be some degree of base stacking in these polymers, similar in magnitude to that in poly-U. The appreciable residual hyperchromicity exhibited by poly-$\overline{\text{HAC}}$ at the short wavelength band (Fig. 8) reinforces this conclusion and the utility of a more extensive investigation of these polymers.

We are indebted to Dr. H. Matthaei, Dr. H. G. Zachau and Dr. U. Z. Littauer for gifts of synthetic polynucleotides and polynucleotide phosphorylase; to Mrs. Krystyna Myszkowska for technical assistance; and to the World Health Organization, the Wellcome Trust and the International Atomic Energy Agency for support of this investigation.

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BADANIA NAD MECHANIZMEM MUTAGENEZY HYDROKSYLAMINOWEJ

Streszczenie

1. Reakcja między glikozydami cytozyny a hydroksylaminą (HA) prowadzi do wytworzenia dwóch produktów: 4,6-dwuhydroksylamino-5,6-dwuhydrocytydyny (2HAC) i 4-hydroksylaminocytydyny (HAC). Te same produkty są wytwarzane z reszt cytydynowych w poli-C. Przebadano wpływ pH i stężenia hydroksylaminy na stosunek wzajemny tych dwóch produktów powstających w czasie reakcji hydroksylaminy z kwasem cytydyno-2′(3′)-fosforanowym i z poli-C. Nawet w pH obojętnym (6.5) obydwa te produkty, HAC i 2HAC, pojawiają się jednocześnie. Pierwszy z nich powstaje ze względnie niską wydajnością. W pH kwaśnym dominuje tworzenie HAC i można tak dobrać warunki reakcji, że głównym produktem jest HAC. Te warunki wykorzystano do otrzymania HACDP.

2. Poli-2HAC otrzymano przez reakcję poli-C z hydroksylaminą w pH obojętnym, zaś poli-HAC po reakcji poli-C z hydroksylaminą w pH kwaśnym. Poli-HAC otrzymywano również przez polimeryzację HACDP, który, jak stwierdzono, jest substratem dla fosforylazy polinukleotydowej. Strukturę tych polimerów, która jest raczej mało zorganizowana, badano metodą absorpcyjną. Rybonukleaza trzustkowa hydrolizowała obydwa polimery do mononukleotydów. Żaden z nich nie dawał kompleksów z poli-A, poli-U, poli-I lub poli-C.

3. Semikarbazyd (SC), który po reakcji z glikozydami cytozyny daje glikozydy 4-semikarbazydocytozyny (SCC), związki analogiczne do HAC, w pH kwaśnym reaguje z poli-C dając poli-SCC. Zbadano niektóre właściwości tego polimeru; jest on hydrolizowany przez rybonukleazę i nie ulega kompleksowaniu z poli-A, poli-U, poli-I i poli-C.

4. Znaczenie powyższych wyników przedyskutowano w związku z mechanizmem mutagenezy hydroksylaminy.

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STUDIES ON STREPTOKINASE

PURIFICATION AND SOME MOLECULAR PROPERTIES

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1. Streptokinase was purified by Sephadex G-100 column filtration. Starch-gel electrophoresis, polyacrylamide-gel electrophoresis and ultracentrifugation showed the preparation to be homogeneous. 2. Molecular weight was 49 600. 3. Amino acid analysis showed a total absence of cysteinyl residues.

Streptokinase (SK) is an extracellular protein elaborated by various strains of *Streptococci.* It has the ability to activate plasminogen to the proteolytic enzyme, plasmin. The plasminogen activating properties of SK were subject of numerous and often controversial studies (Ablondi & Hagan, 1960; De Renzo, Barg, Boggino, Englert & Davies, 1963; Marcus & Werkheiser, 1964; Klein, 1966). It is generally assumed that the activation is a non-enzymic process; if this is so then the mechanism of SK action is a process entirely different from other known activations of proteolytic proenzymes.

The studies on the mechanism of plasminogen activation by SK have been seriously hampered by the lack of understanding of the structure of SK. Until very recently all that has been known, was its molecular weight and isoelectric point, 50 000 and pH 4.7 - 5.0, respectively, as estimated by Fletcher & Johnson (1957).

In 1967, De Renzo, Siiteri, Hutchings & Bell (1967) purified SK by repeated chromatography on DEAE-cellulose and by sucrose gradient electrophoresis. The preparation obtained proved to be homogeneous in ultracentrifuge and electrophoresis. Its molecular weight estimated from the sedimentation coefficient and diffusion was 47 000. The authors have also established a complete amino acid composition of their purified SK.

In order to gain insight into the mechanism of SK activity, we have undertaken systematic studies on the purification and properties of SK. Isolation and gross molecular properties are presented in this paper, having been previously briefly communicated (Biliński & Łoch, 1966). In the accompanying paper (Łoch, Biliński & Zakrzewski, 1968) the results of the studies on the conformation of SK are reported.

MATERIALS AND METHODS

Streptokinase. The starting material was a semipurified product used in the manufacture of a commercial preparation of streptokinase for intravenous injections, kindly supplied by Biomed, Warsaw. Most of the contaminating proteins and nucleic acids were removed by the manufacturers. The preparation was further purified by gel filtration as described under Results and Discussion.

Proteins for column calibration. Myoglobin, twice crystallized from horse skeletal muscle was kindly donated by Professor K. Murawski, Institute of Haematology, Warsaw. Human serum albumin and human serum γ-globulin were purchased from Biomed, Warsaw. Chymotrypsin, crystallized, and ovalbumin, crystallized, were the products of Worthington (New Jersey, U.S.A.).

Gel filtration was carried out on columns filled with Sephadex G-100 (Pharmacia, Uppsala, Sweden). Sephadex was equilibrated with pH 7.6 sodium chloride, 0.15 M, containing about 1 mM-sodium hydrogen phosphate. SK was dissolved in the same solvent.

Activity of SK was determined by a fibrinolytic method (Z. S. Latałło, private communication). A standardized mixture of crude human fibrinogen and plasminogen was added, together with 1 National Institute of Health unit of purified bovine thrombin (Biomed), to the SK solution containing between 5 and 25 international units of SK. Under conditions employed a linear relationship between log SK concentration and the time-of-lysis was obtained.

Protein concentration was determined from Kjeldahl nitrogen analysis using a factor of 6.25 to calculate the amount of protein.

Electrophoresis in starch gel was carried out in borate buffer, pH 8.6, with or without urea, in the vertical apparatus as described by Smithies (1959). Electrophoresis in polyacrylamide gel was carried out as described by Davis (1964).

Amino acid analysis was performed using the Technicon Amino-Acid Analyser. For the detection of sulphur-containing amino acids, the ascending paper chromatography was employed using *n*-butanol - methanol - water solvent (75:15:10, by vol.). Sulphur-containing amino acids were detected with the Feigl stain (Chargaff, Levine & Green, 1948).

Spectrophotometric measurements were carried out on the Unicam SP 500 instrument at ambient temperature.

Sedimentation velocity was determined on the Beckmann-Spinco Model E ultracentrifuge.

RESULTS AND DISCUSSION

Purification. Semi-purified SK was filtered through Sephadex G-100 column (5×90 cm.), using phosphate-buffered sodium chloride, pH 7.6. The effluent was collected in 10 ml. fractions at a rate of 2.0 ml./min. A clear-cut separation of contaminating material can be seen in Fig. 1. The residual quantities of streptolysin and of streptococcal deoxyribonuclease emerged from the column just prior and just after the peak containing SK, respectively.

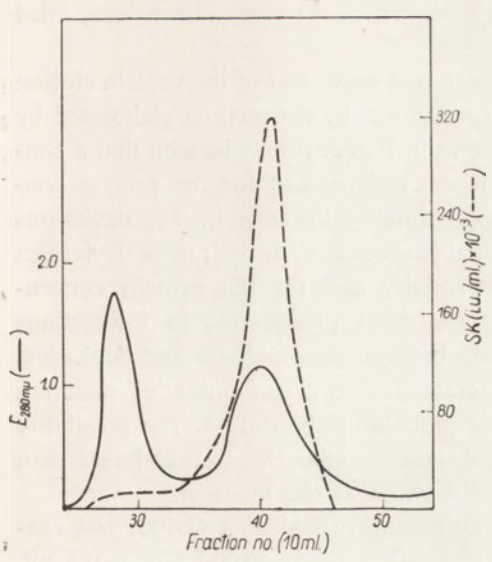

Fig. 1

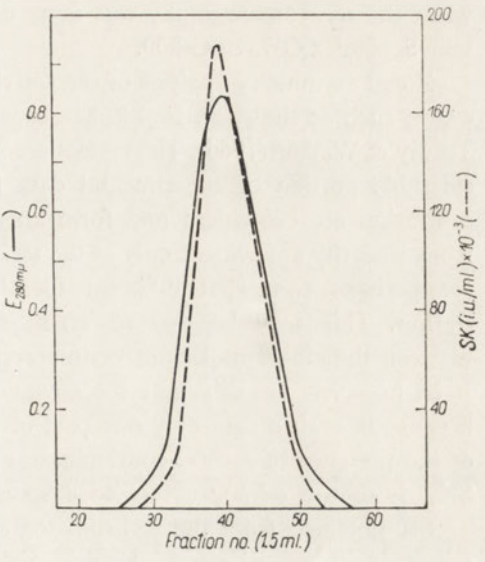

Fig. 2

Fig. 1. Gel filtration of semipurified streptokinase. Sephadex G-100 column (5×90 cm.) was equilibrated and eluted with pH 7.6, 0.15 M-sodium chloride containing 1 mM-sodium hydrogen phosphate; elution rate 2 ml./min. Total content of SK was 15×10^6 i.u.

Fig. 2. Rechromatography of the streptokinase fraction shown in Fig. 1, on Sephadex G-100 column (1.25×90 cm.) equilibrated and eluted with pH 7.6, 0.15 M-sodium chloride containing 1 mM-sodium hydrogen phosphate; elution rate 0.25 ml./min.

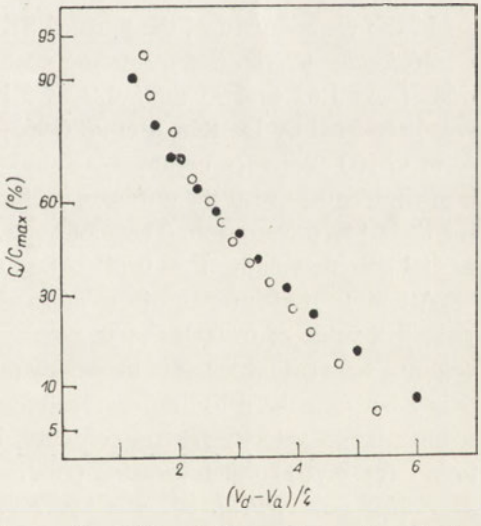

Fig. 3. Zonal asymmetry analysis of gel-elution pattern of purified streptokinase by the method of Tracey & Winzor (1966). ○, Activity; ●, extinction, 280 mμ.

Homogeneity. The peak containing SK was then filtered through another Sephadex column, identical with the former one in all respects but having a diameter of 1.25 cm. The rate of elution for rechromatography was 0.25 ml./min. A single peak emerged (Fig. 2) in which the extinctions appeared to coincide with the activities in the collected fractions. A single and symmetrical peak was also obtained

when the rechromatography was done on columns, of the same dimensions, filled with Sephadex G-75 or G-200.

Zonal asymmetry analysis of the activity elution curve and of the protein elution curve, such as shown in Fig. 2, has been carried out by the method elaborated by Tracey & Winzor (1966). The results are shown in Fig. 3. It may be seen that a considerable portion of experimental data for the activity and for the protein concentration are coincident and form an approximately linear plot. The deviations from linearity are evident only at the terminal parts of both curves; these deviations are perhaps somewhat different for the activity and for the protein concentration. This may be due to errors in analytical procedures, to interactions between the eluted molecules or interactions between streptokinase and Sephadex. It is, however, unlikely that the deviations are due to a contaminating material, because in such a case only one part of the plot should be shifted. The possibility of the presence of several contaminating substances, smaller as well as larger than SK, is of too *ad hoc* nature to deserve discussion at the moment.

SK obtained from the rechromatography columns (Sephadex G-100) was examined by starch-gel electrophoresis. SK, containing about 50 000 i.u. in 0.3 ml. of borate buffer, pH 8.6, was placed in starch-gel, pH 8.6, borate buffer. As it may be seen in Fig. 4, a single fraction was present. A single fraction was also detected on starch-gel electrophoresis in the same buffer but in the presence of 2 M-urea.

A single band was also detected when a sample of purified SK (20 000 i.u. in 0.2 ml.) was analysed by polyacrylamide electrophoresis (Fig. 5). However, just prior to complete removal of the unbound dye from the gel, in some preparations one additional very faint band could be detected.

In the ultracentrifuge, the purified SK sedimented as a single peak (Fig. 6).

Molecular weight. Sedimentation coefficient, S_{20}, determined for SK concentrations from 1.69 to 5.93 mg./ml. was $3.1 \pm 0.2 \times 10^{-13}$, which is identical with the value reported by De Renzo *et al.* (1963). The diffusion coefficient was determined from G-100 Sephadex columns, as shown in Fig. 7. The value found, 5.9×10^{-7}, is again identical with the diffusion coefficient determined by De Renzo *et al.* (1967) for their SK preparation. The molecular weight computed from these data, using partial specific volume $\bar{V} = 0.745$ cm./g. (De Renzo *et al.*, 1967) is 49 600.

Attempts at estimation of the molecular weight using Sephadex G-100 columns yielded a value of 67 000. For the molecular weight determinations, the Sephadex columns were calibrated with the highly purified proteins, listed in Fig. 7. The discrepancy between the gel-filtration results and the molecular weight determined from sedimentation velocity remains obscure, but similar discrepancies have also been found for several other proteins (Andrews, 1965).

Amino acid content. SK was hydrolysed under reflux condenser at the boiling temperature. The results of amino acid analysis are presented in Table 1.

Although the amino acid composition of SK, determined in such a relatively crude way, should be treated as approximate only, the similarity between our results and those reported by De Renzo *et al.* (1967) is quite striking. Neither of SK preparations contained cysteinyl residues. A total absence of thiol groups was confirmed

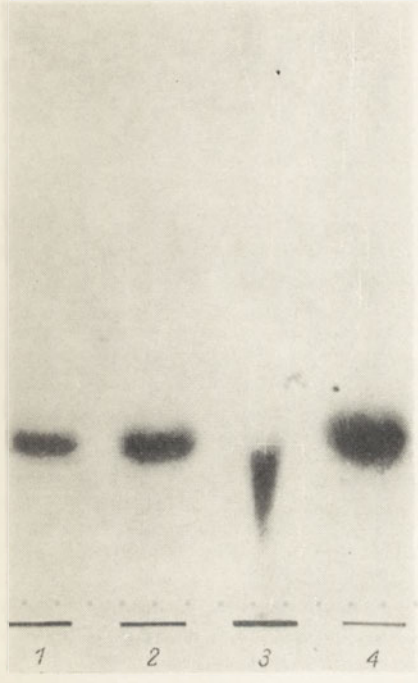

Fig. 4

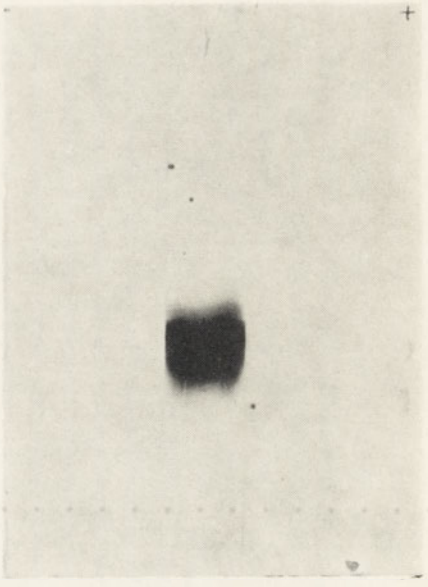

Fig. 5

Fig. 4. Starch-gel electrophoresis of purified streptokinase. Borate buffer, pH 8.6. Migration from bottom to the top. The samples contained: *1*, 25 000 i.u. of streptokinase; *2*, 50 000 i.u. of streptokinase; *3*, 100 000 i.u. of streptococcal deoxyribonuclease; *4*, 100 000 i.u. of streptokinase.

Fig. 5. Polyacrylamide-gel electrophoresis of purified streptokinase. Tris-glycine buffer, pH 8.3; 20 000 i.u. of streptokinase.

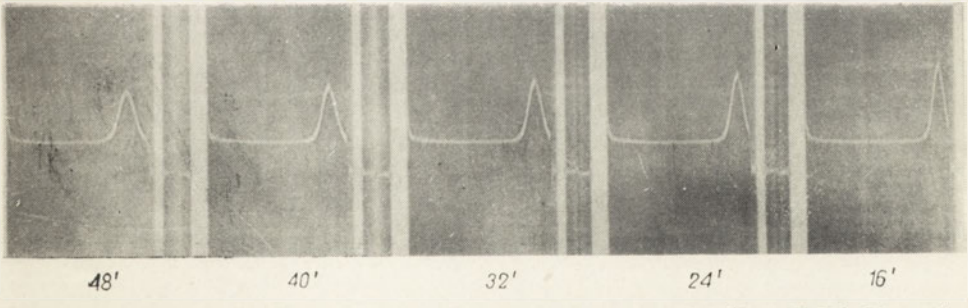

Fig. 6. Sedimentation diagrams of streptokinase in 0.15 M-sodium chloride, pH 7.6. From the right: successive exposures at 8 min. intervals, the first 16 min. from the start. Schlieren optics, 60° angle of slit inclination; temperature 20°.

Table 1

Amino acid composition of the purified streptokinase preparation

Amino acid residue	Residues per molecule	Molecular weight of residues
Alanine	27	1919
Arginine	20	3124
Aspartic acid	62	7135
Cysteine, cystine	0	0
Phenylalanine	15	2208
Glycine	25	1426
Glutamic acid	59	7618
Histidine	9	1234
Isoleucine	23	2603
Leucine	42	4753
Lysine	33	4230
Methionine*	+	
Proline	24	2331
Serine	27	2351
Threonine	31	3134
Tryptophan **	2	372
Tyrosine**	21	3426
Valine	26	2577
		Total: 50441

* See Results and Discussion.

** Tyrosine and tryptophan were determined according to Beaven & Holiday (1957).

by colorimetric titration (Bitny-Szlachto, Kosiński & Niedzielska, 1963). The presence of methionine, which under the conditions of hydrolysis employed for this study was, of course, largely destroyed, has been confirmed by paper chromatography

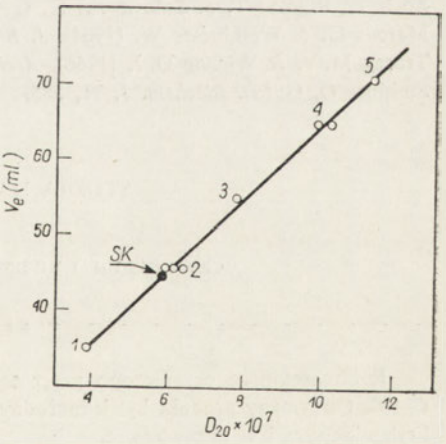

Fig. 7. Determination of the diffusion coefficient D_{20}, of streptokinase by gel filtration. V_e, elution volume. Conditions as in Fig. 2. Proteins for column calibration: *1*, human γ-globulin; *2*, human serum albumin; *3*, ovalbumin, crystallized; *4*, chymotrypsin, crystallized; *5*, horse skeletal muscle myoglobin, crystallized; *SK*, streptokinase.

(cf. Materials and Methods). The content of glutamate appears to be lower, and the content of aspartate somewhat higher than those reported by De Renzo *et al.* (1967).

From the sum of the residues of alanine, valine, proline, leucine, isoleucine, methionine, phenylalanine, tyrosine and tryptophan, the fraction of hydrophobic amino acids in SK was calculated to be 40%.

The authors wish to express their gratitude to Professor Z. Latałło, Institute of Nuclear Research, Warsaw, for his interest in this work and for the help in carrying out fibrinolytic assays. Thanks are also due to Dr. T. B. Grela of the Institute of Plant Protection, Poznań, for his cooperation in performing the sedimentation velocity determination, and to Dr. M. Rakowska and Dr. B. Pliszka of the Institute of Food and Nutrition, Warsaw, for carrying out the amino acid analysis.

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STUDIA NAD STREPTOKINAZĄ

OCZYSZCZENIE I NIEKTÓRE WŁAŚCIWOŚCI MOLEKULARNE

Streszczenie

1. Streptokinazę oczyszczono przez sączenie na kolumnach wypełnionych żelem Sephadex G-100. Otrzymany produkt był jednorodny w elektroforezie na żelu skrobiowym, na żelu polyakrylamidowym i w ultrawirówce.
2. Ciężar molekularny streptokinazy oznaczono na 49 600.
3. Analiza składu aminokwasowego wykazała całkowity brak reszt cysteinylowych.

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TERESA ŁOCH, T. BILIŃSKI and K. ZAKRZEWSKI

STUDIES ON STREPTOKINASE

THE CONFORMATION

Serum and Vaccine Research Laboratory, ul. Chelmska 30, *Warszawa, and the Department of Radiobiology, Institute of Nuclear Research, Warszawa*

1. A highly purified, homogeneous preparation of streptokinase was studied by means of polarimetric and spectrophotometric methods. 2. Tyrosyl residues in streptokinase titrate with pK_{app} 10.6 in aqueous solutions, and with pK_{app} 9.8 in 4 M-guanidine. By solvent perturbation technique only 25% of all tyrosyl residues have been found accessible for glycerol. 3. Optical rotatory dispersion parameters indicated that streptokinase has a predominantly random coil conformation. 4. Between pH 9 and 11.5 a structural transition has been observed in streptokinase, coincident with tyrosyl ionization and irreversible inactivation.

In the previous paper of this series (Biliński, Łoch & Zakrzewski, 1968) it has been shown that streptokinase (SK) purified by Sephadex gel filtration is satisfactorily free of contaminating proteins, and homogeneous in gel electrophoreses and in the ultracentrifuge. In the present paper the results are presented of investigation of the conformation of streptokinase. These studies have been considerably facilitated by the finding that streptokinase is free of cysteinyl residues and has a low tryptophyl content (De Renzo, Siiteri, Hutchings & Bell, 1967; Biliński *et al.*, 1968).

MATERIALS AND METHODS

Streptokinase. SK, described in the accompanying paper (Biliński *et al.*, 1968), has been used in this work. The specific activity of SK was about 90 000 i.u. per mg. of protein. The concentrations of SK were determined from extinction at 280 mμ, assuming the extinction of 7.5 for 1% (w/v) solution of SK in 1 cm. light-path cuvettes. All calculations were based on molecular weight 49 000, and on the content of tyrosyl residues 18 per mole SK.

Optical rotatory power was measured on Rudolph Model 80 spectropolarimeter equipped with a rocking polarizer, which was set at about 1° for the present experi-

ments. The readings were taken at 365, 405, 435, 546 and 578 mμ, using the filters supplied by the manufacturers. All measurements were carried out in 1 dm. polarimeter tubes at ambient temperature. The optical rotatory dispersion (ORD) parameters were computed by the least squares method using the equation of Moffit & Yang (1956). The dispersion of the refractive index has been neglected.

Spectrophotometry. Extinctions were measured on Unicam SP 500, and the difference spectra were recorded on Unicam SP 700 instruments. For the latter, the 100% line was set with empty cuvettes in both beams of the spectrophotometer. Four cuvettes have been used for the difference spectra, with two cuvettes, placed closely together, in each beam. Extinctions of solvents and solutes have been compensated by appropriate arrangement of solutions in the four cuvettes.

pH was measured on the Radiometer TTT1 Titrator using the Radiometer glass electrode B. The sodium error was corrected whenever required using the nonograms supplied by the manufacturers.

RESULTS

Optical rotatory dispersion. The Moffit parameter b_0, calculated from eight experiments, was -45±10 S.D. The a_0 parameter, for the same eight experiments, was -345±10 S.D. The gross conformation of the molecule was determined from these data, using a simplified assumption that only three conformations can exist in a protein molecule, namely α-helix, β-structure, and random coil. The equations employed by Timasheff, Townend & Mescanti (1966) have been applied:

$$b_0 = f_\beta(b_0)_\beta + f_R(b_0)_R + f_\alpha(b_0)_\alpha$$

$$a_0 = f_\beta(a_0)_\beta + f_R(a_0)_R + f_\alpha(a_0)_\alpha$$

$$f_\beta + f_R + f_\alpha = 1$$

in which f denotes the fraction in the given conformation, the subscripts α, β, and R refer to α-helix, β-structure, and random coil, respectively. The following contributions to a_0 and b_0 parameters were assumed: $(a_0)_\alpha = 0$; $(a_0)_R = -650$; $(b_0)_\alpha = -630$; $(b_0)_R = 0$ (Urnes & Doty, 1961); $(a_0)_\beta = +400$; $(b_0)_\beta = 0$ (Ikeda, Maeda & Isemura, 1964).

On that basis, the fraction of SK in β-structure was calculated to be 20%, and that in random coil conformation, 73%. The fraction of SK in the helical conformation appeared to be negligible, about 7%.

A low value of the helical conformation, as assessed from the Moffit b_0 parameter, may be due not only to the lack of helices but also to the compensating amounts of right- and left-handed helices. Such helices might have different stabilities (Huggins, 1952) and their presence could become apparent when ORD measurements were carried out in an unfolding solvent, such as concentrated urea solution. It will be seen in Table 1 that this was not the case for SK. Thus the conclusion that SK is lar-

Table 1

Effect of unfolding agents on optical rotatory dispersion of streptokinase

Solvent	$[\alpha]_{365}$	a_0	b_0
0.05 M-NaCl	—193	—335	—36
3 M-Urea	—228	—415	—1
6 M-Urea	—301	—540	—18
0.05 M-NaCl	—224	—388	—42
4 M-Guanidine	—352	—647	—9

gely free of helical conformation is tentatively supported. However, the apparent lack of helicity will have to be confirmed by more direct methods, such as peptide band hypochromism and short-ultraviolet Cotton effect[1].

Despite the absence of helical conformation, SK exhibits features which indicate that its molecule possesses a considerable degree of internal organization, as it will be shown below. This is not in contradiction to the predominantly random coil nature of the protein, since the random coil conformation in proteins must be considerably restricted for the stereochemical reasons (Schellmann & Schellmann, 1964).

Solvent perturbation. Herskovits & Laskowski (1962) elaborated an elegant method for differentiation between the exposed and buried chromophores in proteins. The method consists essentially in the perturbation of chromophores with neutral solutes. The perturbation of the spectrum is manifested by the long wavelength shift of the tyrosyl absorption band (the so-called red shift), and is best detected by the difference spectral technique. The magnitude of the molar difference extinction coefficient depends on the degree of exposure of the chromophores. Either a totally unfolded protein or an equivalent mixture of amino acids can be used as a reference sample, i.e. as "a 100% exposed" control.

In the present studies glycerol 20% (v/v) has been employed as perturbant. Glycerol when added to a neutral solution of SK generates a typical red shift spectrum, shown in Fig. 1. The perturbation parameter $\Delta\varepsilon_{285\text{-}288}/\varepsilon_{280} = 0.06$ was determined from SK solution in 8 M-urea in the presence of 20% glycerol. SK in 8 M-urea was assumed to be totally unfolded; the reduction with thioglycollate was, of course, unnecessary in view of the absence of cysteinyl residues in SK.

The perturbation spectrum of SK in 0.15 M-sodium chloride at pH 7.6 is represented by the lower curve in Fig. 1. Again, the typical red shift spectrum is apparent. From the difference extinction at 285 - 288 mμ, the accessibility of tyrosyl residues was estimated to be about 30%. More precisely, the exposure of tyrosyl residues in SK may be anywhere between 6 tyrosyls being completely exposed (i.e. 15 tyrosyls completely buried) and all tyrosyls being only partially exposed to the solvent.

[1] *Note added in proof*: Taylor & Botts (*Biochemistry*, 1967, 7, 232) have recently reported that the per cent helix in purified streptokinase was 10 - 12%, as determined from the magnitude of Cotton effect in deep ultraviolet.

Spectrophotometric titration of tyrosyl residues. All titrations reported below have been carried out in sodium chloride solutions adjusted to the desired pH with 1 M-sodium hydroxide. The ionic strength of all solutions was between 0.15 and 0.25 (for more alkaline samples) except for the sample assumed to be at pH 14 which was dissolved in 1 M-sodium hydroxide.

Molar extinction coefficient at 295 mμ of SK in 1 M-sodium hydroxide was 49 000, which yields the molar extinction coefficient for phenolate ion $\varepsilon_{295} = 2350$, assuming

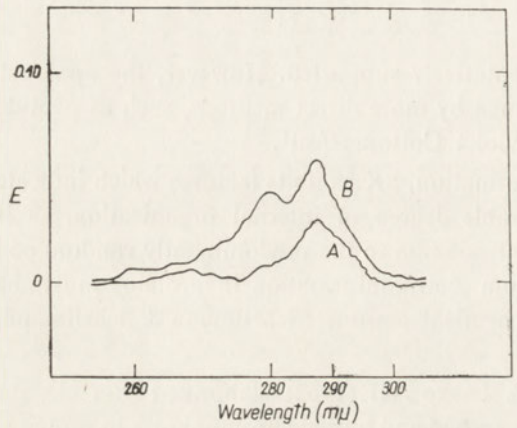

Fig. 1. Glycerol perturbation of streptokinase absorption spectrum. Glycerol 29% (v/v). *A*, streptokinase, 2.13 mg./ml. in 0.15 M-NaCl; *B*, streptokinase, 1.3 mg./ml. in 8 M-urea.

the presence of 21 tyrosyl residues per mole SK. The apparent pK of tyrosyls in SK was estimated from the usual formula

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{app}} + \log \frac{\alpha}{1-\alpha}$$

in which

$$\alpha = \frac{\Delta\varepsilon_{\mathrm{pH}}}{\Delta\varepsilon_{\mathrm{max}}}$$

$\Delta\varepsilon_{\mathrm{max}}$ used in the computation was the molar absorbancy of SK dissolved in 1 M-sodium hydroxide. $\Delta\varepsilon_{\mathrm{pH}}$ values at higher pH were determined graphically by extrapolation from several measurements of the extinction, carried out during 15 min. at ambient temperature. Extinctions were read as difference extinctions using SK in 0.15 M-sodium chloride, pH 7.6, as the reference solution.

All tyrosyls in SK titrate between pH 9 and pH 13. From Fig. 2 it will be seen that the tyrosyl residues titrate with pK_{app} 10.7, which is only slightly above the pK for free tyrosine. However, in 4 M-guanidine a considerable increase of the acidity of phenolic hydroxyl was observed (see lower curve, Fig. 2). In 4 M-guanidine, the tyrosyl residues in SK titrate with pK_{app} 9.8 which is practically equal to the pK_{int} of tyrosyl residues in several proteins (Tanford, 1962), despite the fact that in computing pK for SK-tyrosyls the electrostatic interactions have not been taken into account. This is an interesting feature of SK, perhaps indicating that tyrosyl residues are quite distant from other charged groups.

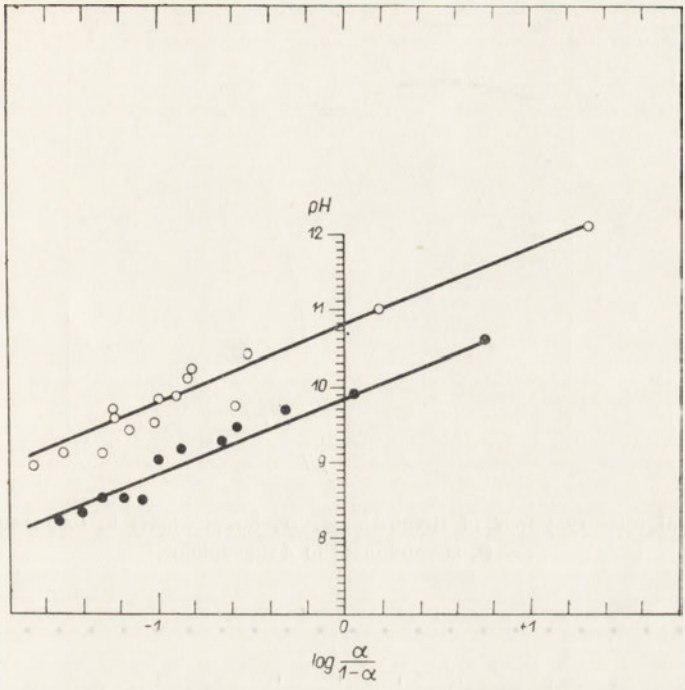

Fig. 2. Phenolic hydroxyl ionization in streptokinase. ○, Streptokinase in 0.05 M-sodium chloride; ●, streptokinase in 4 M-guanidine.

The comparison of the titration behaviour of tyrosyls in SK with and without guanidine shows that the ionization of phenolic hydroxyl is somewhat hindered. One possible explanation of that hindrance is that the tyrosyls may be involved in a hydrogen bonding. An attempt has been made at finding a hydrogen acceptor group for such bonds. Potentiometric titrations, however, did not yield reliable results owing to insolubility of SK in the range of pH 7 to pH 2.

Polarimetric titration. The optical rotatory power of SK in 0.15 M-sodium chloride remains constant between pH 7 and 10. In more alkaline solutions, the levorotation sharply increased, as shown in Fig. 3. The lowest $[\alpha]_\lambda$ for all wavelengths employed (see Materials and Methods) was reached at or slightly above pH 11.5. In still more alkaline solutions, the levorotation somewhat decreased. The optical rotatory power could not be measured between pH 7 and about 2, owing to the insolubility of SK. However, in 0.1 M-hydrochloric acid, SK yielded a perfectly clear solution; its $[\alpha]_{365}$ was -330 ± 18 S.D., i.e. in a strongly acidic solution the levorotation of SK was higher than in a neutral solution, similarly to what has been observed in the alkaline range.

From the profile of the plot of $[\alpha]_{365}$ *versus* pH it is evident that SK undergoes a structural transition between pH 9 and 12. The mid-point of this transition is at about pH 10.7, which is exactly the pK_{app} of tyrosyl residues in SK in the same solvent. The reversibility of the structural transition could not be unequivocally

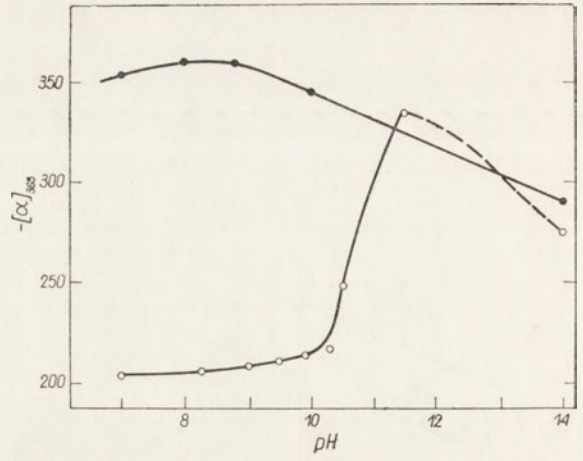

Fig. 3. Optical rotatory power of streptokinase. ○, Streptokinase in 0.05 M-sodium chloride; ●, streptokinase in 4 M-guanidine.

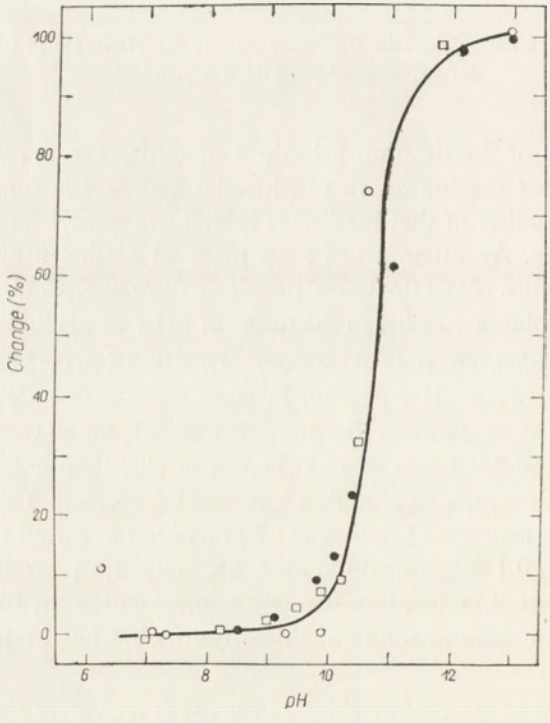

Fig. 4. Effect of pH on the activity, rotatory power, and tyrosyl ionization of streptokinase. ○, Inactivation (after 24 hr. at room temperature); □, optical rotatory power, $[\alpha]_{365}$; ●, tyrosyl dissociation.

evidenced. SK brought to pH 12 and reverted to pH 7 yielded the $[\alpha]_{365}$ as well as a_0 and b_0 parameters which were equal, within the limits of experimental error, to those found for the control sample of SK, i.e. for SK which was not exposed to high pH.

The increase in levorotation is usually interpreted as showing a progressive disorganization of protein molecule. In the case of SK, the change in the rotatory power was found to be reflected only in the Moffit a_0 parameter, the b_0 parameter remaining constant at its very low level. From the reasoning, similar to that of Tanford & Taggart (1960), it may be concluded that during the alkaline structural transition some of the hydrophobic surfaces disappear in the molecule of SK.

Stability of the activity of SK. Solutions of SK in 0.15 M-sodium chloride at various pH values were left overnight at room temperature. The activity of the samples was determined prior and after the incubation by the fibrinolytic method (see Biliński *et al.*, 1968). The activity of the solutions between pH 7 and 10 remained constant, but rapidly decreased at higher pH values. No activity could have been detected in the sample which was incubated at pH 12. Unexpectedly, a similar relationship between the pH and the activity was found for SK dissolved in 6 M-urea. It may be deduced from these observations that the disruption of the hydrogen bonds, in which tyrosyl residues participate, is not sufficient by itself to irreversibly denature SK. That some other mechanism is involved in the irreversible inactivation of SK at elevated pH, is indicated by the finding that SK is quite stable at pH 2, i.e. at a pH in which the tyrosyl hydrogen bonds should be broken owing to the dissociation of the hydrogen acceptor group.

DISCUSSION

The experiments reported in the present paper show that the SK molecule has an internal structure which, however, is not a helical conformation. The bonds which restrict what appears to be a predominantly random coil protein are probably hydrogen bonds formed between tyrosyl residues and unknown hydrogen acceptor group. The disruption of these bonds gives rise to a structural transition in SK.

The profiles of alkaline structural transition, of alkaline inactivation of SK, and of tyrosyl ionization are sufficiently similar to suggest that these three phenomena may be interrelated. In order to investigate this supposition in detail, a normalized plot of the three parameters have been constructed, assuming that: 1st, the optical rotatory power of SK changes from 0% to 100% between pH 9 and 12; 2nd, the fraction of ionized tyrosyl residues changes from 0% to 100% between pH 9 and 13; 3rd, the inactivation of SK is 100% at pH 12, and no inactivation occurs at pH 9. The plot is presented in Fig. 4. It will be seen that a smooth sigmoid curve satisfactorily accounts for all the plotted experimental data. Thus it may be concluded that a common molecular mechanism underlies the structural transition, the tyrosyl ionization, and the inactivation of streptokinase. It seems likely that this mechanism involves the disruption of the tyrosyl hydrogen bonds.

PROGRESS IN BIOPHYSICS AND MOLECULAR BIOLOGY, vol. 16 (J. A. V. Butler and H. E. Huxley, eds). Pergamon Press, 1966; cena 84 s.

Kolejny, 16-ty tom *Progress in Biophysics and Molecular Biology* przynosi 8 artykułów poglądowych o dość zróżnicowanej tematyce: od zagadnień transkrypcji informacji genetycznej do elektroforetycznego zachowania się komórek. Można jednak śmiało powiedzieć, że większość miejsca poświęcono, zgodnie z panującą ostatnio w biologii atmosferą, szeroko pojętym zagadnieniom biosyntezy białek, informacji genetycznej i struktury subkomórkowej.

Krótki artykuł N. Mitchisona poświęcony jest bardzo wąskiemu zagadnieniu rozpoznawania antygenu przez komórki i jest napisany bez wątpienia dla immunologów. Autor ogranicza się do omawiania wyłącznie wyników uzyskanych techniką immunologiczną i stwarza u czytelnika wrażenie, że problem ten nie wkroczył jeszcze w fazę badania go przy pomocy metod bardziej bezpośrednich.

A. Sibatani pisze w obszernym artykule o transkrypcji informacji genetycznej. Zagadnienie to jest potraktowane bardzo szeroko. Na początku artykułu podane są możliwe modele transkrypcji, które następnie są szczegółowo omówione. Dokładnie przedyskutowany jest aktualny problem polarności transkrypcji, sporo uwagi poświęcono enzymatycznej syntezie RNA przez polimerazę, metodom badania powstałego RNA oraz problemom syntezy i regulacji powstawania poszczególnych rodzajów kwasów rybonukleinowych, jak rybosomalnego, przenoszącego i informacyjnego RNA. Artykuł stanowi wyczerpujący przegląd współczesnego stanu zagadnienia, jest napisany jasnym i zwięzłym stylem, łączy w sobie zdobycze zarówno genetyki, jak i biochemii oraz biofizyki. Jest on bez wątpienia cenną pozycją, która zainteresuje dość szeroki krąg czytelników.

Artykuł G. V. Sherbeta o cybernetycznych interakcjach w epigenetyce jest jak gdyby kontynuacją cyklu rozpoczętego przez artykuł Bracheta w poprzednim tomie *Progress in Biophysics and Molecular Biology*. Omówione są w nim wzajemne oddziaływania jądra i cytoplazmy oraz zjawiska indukcji embrionalnej jako mechanizmy regulujące rozwój embrionalny i różnicowanie komórek. Czynniki przyczynowe doprowadzające do różnicowania komórek nie są dokładnie poznane, można jednak na podstawie logicznego rozumowania przypuszczać, że powinny one bezpośrednio lub pośrednio oddziaływać na aparat genetyczny komórki i jego układ syntetyzujący białka. Biorąc to pod uwagę, autor stara się wytłumaczyć obserwacje dotyczące różnicowania, indukcji embrionalnej oraz kompetencji komórek do przyjęcia bodźców wywołujących i kontrolujących różnicowanie, w oparciu o współczesne poglądy na funkcję genomu i regulację syntezy białek.

David Smith pisze o organizacji i funkcji retikulum sarkoplazmatycznego oraz tzw. układu T w komórkach mięśniowych. Szybkość procesów molekularnych doprowadzających do skurczu i rozkurczu mięśni poprzecznie prążkowanych jest zbyt wielka, aby można było przypuszczać, że jest ona spowodowana zwykłą dyfuzją aktywującej substancji do wnętrza komórki mięśniowej o średnicy około 50 μ. Poszukiwania układu, który mogłby doprowadzić bodźce szybko i sprawnie do wnętrza komórek, doprowadziły do dokładnego poznania struktury retikulum oraz układu T (transverse system) t.j. poprzecznych kanalików przechodzących przez cysterny reticulum i łączących się najprawdopodobniej z płynem zewnątrzkomórkowym. W artykule omówiona jest nie tylko morfologia tych struktur, ilustrowana doskonałymi fotografiami preparatów elektrono-mikroskopowych, lecz także wyniki doświadczeń cytofizjologicznych i cytochemicznych, dotyczących funkcji siateczki sarkoplazmatycznej. Osobna sekcja artykułu jest poświęcona omówieniu kontroli skurczu i relaksacji na poziomie molekularnym.

Praca J. B. Fineana o molekularnej organizacji membran komórkowych składa się z dwóch części. Część pierwsza poświęcona jest krótkiemu omówieniu własności fizyko-chemicznych fosfolipidów z punktu widzenia ich zdolności do tworzenia membranowych warstw podwójnych i ich interakcji z wodą i białkami. Druga część omawia różne rodzaje membran, jak warstwy mielinowe, membrany krwinek, mitochondriów i mikrosomów. Koncepcje dotyczące zależności funkcji od struktury poszczególnych komponent oparte są na wynikach badań fizyko-chemicznych oraz fizycznych a w szczególności mikroskopii elektronowej i dyfrakcji promieni rentgena.

Ostatnie osiągnięcia w dziedzinie autoradiografii o wielkiej zdolności rozdzielczej są przedmiotem artykułu Lucien Caro. Jest to artykuł raczej metodyczny omawiający emulsje srebrowe i inne rodzaje detektorów stosowanych w autoradiografii w połączeniu z mikroskopią elektronową a także przygotowywanie preparatów, ich przechowywanie i wywoływanie, czułość emulsji oraz zdolność rozdzielczą. Ostatnia sekcja artykułu poświęcona jest zastosowaniom tego typu autoradiografii, jej możliwościom i ograniczeniom. Artykuł ten niewątpliwie zainteresuje tych wszystkich biologów w szerokim znaczeniu tego słowa, dla których mikroskopia elektronowa jest potężnym narzędziem badawczym.

Simon Silver omawia w swym artykule genetykę bakterii i bakteriofagów. Problem ten jest w chwili obecnej tak obszerny, że może stanowić przedmiot kilkutomowej monografii. Intencją autora jednakże było, jak sam stwierdza we wstępie, przedstawienie wprowadzenia do genetyki molekularnej dla tych, którzy nie zajmują się tym zagadnieniem bezpośrednio lub też rozpoczynają studiowanie tej dziedziny. Należy powiedzieć, że cel ten został w pełni osiągnięty i że rzadko można spotkać artykuł, w którym tak wiele informacji zawarte byłoby w tak niewielkiej przestrzeni z zachowaniem jasności i prostoty stylu, jednakże bez nadmiernych uproszczeń i wulgaryzacji problemu. Szereg dobrze przemyślanych schematów bardzo ułatwia czytanie i zrozumienie tekstu.

Ostatni artykuł tomu dotyczy elektroforezy komórek. E. J. Ambrose przedstawia w nim interesujący problem, który — chociaż sam przez się nie nowy — nie wzbudzał dotychczas wiele zainteresowania. Autor opisuje na początku artykułu szereg różnych aparatów stosowanych do badania ruchliwości elektroforetycznej komórek, a następnie przechodzi do omówienia wyników badań dotyczących komórek rozmaitego pochodzenia. Biologiczna rola błony komórkowej została obecnie należycie doceniona, zaś elektroforeza pozwala badać szereg zjawisk fizyko-chemicznych dotyczących właśnie błony i jej ładunków na nieuszkodzonych komórkach.

W sumie należy przyznać, że wybór artykułów zamieszczonych w 16 tomie *Progress in Biophysics and Molecular Biology* w pełni odpowiada wymogom stawianym przez współczesny stan wiedzy i kierunki rozwoju biologii molekularnej. Artykuły te nie stanowią przeglądu uznanych i bezdyskusyjnych faktów, nadających się do umieszczenia w podręcznikach, lecz raczej stymulują do dalszych poszukiwań w pewnych określonych kierunkach.

Szata graficzna tomu jest tradycyjnie doskonała.

Michał Bagdasarian

RESEARCH IN PROTOZOOLOGY (Tze-Tuan Chen, ed.) vol. 1. Pergamon Press, 1967; str. 428, cena Ł 5, 10 s.

Książka wydana przez Tze-Tuan Chen'a stanowi pierwszy tom dzieła, które ma objąć w sumie cztery tomy. Ostatnie obszerne opracowanie wyników badań protistologicznych *Protozoa in Biological Research* Calkins'a i Summers'a ukazało się w 1941 r., istniała więc pilna potrzeba wydania nowej monografii uwzględniającej współczesny stan wiedzy w tym zakresie. *Research in Protozoology* jest pracą zbiorową; na tom pierwszy składa się pięć rozdziałów napisanych przez sześciu autorów: Organoidy i wtręty cytoplazmatyczne Protozoa (E. Anderson); Zdolności ruchowe Protozoa (T. L. Jahn i E. C. Bovee); Metabolizm oddechowy (W. F. Danforth); Wodniczki kurczliwe, regulacja jonowa i wydalanie (J. A. Kitching) i Odżywianie się i wzrost Protozoa (R. P. Hall). Każdy z wymienionych rozdziałów jest zamkniętą całością, poprzedzoną często wstępem a z reguły spisem treści zawierającym działy szczegółowe i kończącą się obszernym spisem piśmiennictwa uwzględniającym najnowsze pozycje literatury światowej. Najobszerniejszy jest rozdział poświęcony ruchowi pierwotniaków — jego objętość wynosi 160 str. a cytowane piśmiennictwo aż 1005 pozycji, wśród których znajdują się także publikacje polskich badaczy (M. Doroszewski, S. Dryl, A. Grębecki i inni). Rozdział ten dotyczy ogólnego omówienia ruchu jako sposobu zachowania się pierwotniaków, wpływu różnych czynników fizycznych i chemicznych na ruch (promieniowanie, prąd elektryczny, związki chemiczne, czynniki mechaniczne, prądy wody, pole magnetyczne i współdziałanie czynników fizycznych i chemicznych). Bardzo interesująco choć krótko omówiony

jest dział mechanizmu ruchowego — przeprowadzenie analogii ruchu włókienek kurczliwych z pracą mięśni i przedstawienie roli adenozynotrójfosforanu w ruchu pierwotniaków, co ma znaną wartość w rozważaniach ewolucyjnych. Najbardziej wyczerpująco zreferowano w omawianym rozdziale typy ruchowe Protozoa.

Tych kilka uwag o treści książki przedstawiam przykładowo w odniesieniu do jednego, największego rozdziału. Zastanawiając się nad tym, do jakiego odbiorcy adresowana jest omawiana publikacja, należy całkowicie zgodzić się z treścią przedmowy, która wskazuje przede wszystkim na studentów i badaczy biologów ze specjalnością protozoologiczną a także pracowników służby zdrowia, fizjologów i biochemików. Lekarz znajdzie w tej książce niewątpliwie wiele pożytecznych informacji choćby z rozdziału przedstawiającego odżywianie się i wzrost pierwotniaków. Zawarte tam wiadomości dotyczą bowiem nie tylko form wolnożyjących lecz także i pasożytniczych.

Syntetyczne i zwięzłe ujęcie całej książki wydanej na pięknym papierze, staranne przygotowanie graficzne, doskonałe zdjęcia ultramikroskopowe, dużo danych biochemicznych pozwalają wydać bardzo pochlebną opinię o pierwszym tomie *Research in Protozoology*, który zaciekawi z pewnością nie tylko wąskich specjalistów. W dalszych tomach zapowiedziane są opracowania problemów stanowiących obiekt żywych zainteresowań protoparazytologów lekarskich i weterynaryjnych, między innymi metabolizm pasożytniczych pierwotniaków i mechanizm ich działania chorobotwórczego, pasożyty Protozoa i inne.

Bogdan Czapliński

B Cinader: ANTIBODIES TO BIOLOGICALLY ACTIVE MOLECULES. Pergamon Press, London, 1967; str. 424, cena Ł 6.

Książka obejmuje 12 referatów wygłoszonych na II Zjeździe FEBS w Wiedniu w 1965 r. w ramach Sympozjum dotyczącego przedstawionego w tytule tematu. W ostatnich kilkudziesięciu latach rozwój immunologii tak dalece poszedł w kierunku biochemicznym, że dziś rozpatruje się tę dziedzinę wiedzy jako jedną z gałęzi biochemii. Współczesne metody immunochemiczne umożliwiają śledzenie struktury I-rzędowej i wewnętrznej białek, przy czym możliwe staje się również badanie struktury aktywnego ugrupowania enzymów, hormonów, toksyn, określanie determinantów antygenów białkowych, kwasów nukleinowych, wirusów i wreszcie badanie struktury samych przeciwciał.

Poszczególne rozdziały monografii omawiają szereg zagadnień ogólniejszych lub dotyczących bardziej szczegółowych problemów immunochemii. B. Cinader i I. H. Lepow omawiają trójskładnikowy system immunologiczny: enzym - substrat - przeciwciało. Rozważane są zarówno zmiany konformacyjne w cząsteczce enzymu pod wpływem przeciwciała, jak i kompetycja steryczna zachodząca pomiędzy składnikami systemu. Zmiana aktywności enzymu lub wirusa w obecności przeciwciała zależy m.in. od ilości cząsteczek przeciwciała wiążącego się z antygenem i od rodzaju przeciwciała powstającego podczas procesu immunizacji. Szeroko omówiono rolę komplementów reakcji enzym - antyciało.

Serologiczna aktywność kwasów nukleinowych (L. Levine i H. Van Vunakis) jest najnowszym zagadnieniem współczesnej immunologii. Z badań wynika, że DNA wykazuje własności antygenowe (obecność przeciwdział w surowicy krwi chorych na *Lupus erythematosus*), przy czym zdenaturowany termicznie DNA jest silniejszym antygenem niż polinukleotyd natywny. Hydroliza DNA dezoksyrybonukleazą powoduje utratę aktywności immunochemicznej. Inaktywację biologicznej aktywności DNA przez specyficzne przeciwciała wykazano m.in. na systemie polimerazy DNA z grasicy cielęcej: DNA jako *primer* jest nieczynny w obecności antyciała.

Różnice w reaktywności immunologicznej białek włókienkowych i globularnych wydają się być istotne (M. J. Crumpton). Antygenowość pierwszej grupy białek zależy głównie od struktury I-rzędowej, podczas gdy antygenowa aktywność białek kłębuszkowych jest uwarunkowana również konformacją cząsteczki. Z badań nad modyfikacją cząsteczek białka przez alkilację, acetylację, estryfikację lub dezaminację można wnosić, że białka globularne posiadają szereg immunologicznie aktywnych centrów charakteryzujących się różną swoistością serologiczną. Szczególnie dużo informacji w tym zakresie dają studia nad białkami złożonymi, jak mioglobina, flawoproteidy i inne.

Badania B. Cinadera i jego współpracowników nad mechanizmem inaktywacji enzymów przez swoiste przeciwciała dostarczają szczególnie interesujących danych zarówno na temat struktury antygenów, jak i swoistości i charakteru przeciwciał z nimi reagujących. Można rozróżnić trzy typy przeciwciał reagujących z antygenami o własnościach enzymatycznych: antyciała reagujące, ale nie powodujące inaktywacji enzymu; antyciała hamujące aktywność enzymatyczną; i antyciała reagujące z enzymem a nie inaktywujące go, ale uniemożliwiające równocześnie reakcję przeciwciała hamującego aktywność enzymatyczną antygenu. Obecność substratu lub koenzymu w układzie enzym - przeciwciało powoduje zazwyczaj zmniejszenie efektu hamowania lub całkowicie go znosi. Jest to przykład współzawodniczenia substratu lub koenzymu i przeciwciała z aktywnym centrum enzymu.

Antyciała otrzymane przeciwko niektórym enzymom (penicylinazy z *Bacillus licheniformis*) wykazują bądź inaktywację, bądź też aktywację enzymów. Oba rodzaje przeciwciał można od siebie oddzielić przez różnicową adsorbcję przy pomocy swoistych antygenów (M. R. Pollock, J. Fleming i S. Petrie). Innym ciekawym zagadnieniem poruszonym w książce są własności antygenowe enzymów proteolitycznych (R. Arnon). Jak zachowuje się przeciwciało po związaniu z antygenem, które dla niego jest zarazem substratem; wpływ naturalnego substratu na wiązanie przeciwciało - enzym proteolityczny — są to istotne problemy współczesnej biochemii. Dalsze rozdziały monografii poświęcone są serologicznej aktywności toksyn bakteryjnych (M. Raynaud), hormonów białkowych (S. Wilson oraz J. P. Felber i A. Micheli), wirusów (S. E. Svehag), zjawiskom immunologicznym towarzyszącym transplantacji tkanek (G. Möller i E. Möller) i wreszcie perspektywom rozwoju immunochemii (A. E. Bussard).

Książka jest wydana bardzo starannie i doskonale ilustrowana. Ok. 1400 pozycji cytowanego piśmiennictwa umożliwia szybkie i dokładne zapoznanie się z omawianymi problemami immunochemii w odniesieniu do enzymów, kwasów nukleinowych, hormonów, wirusów, etc. Książka dobrze wprowadza w niezmiernie ciekawą i szybko rozwijającą się dziedzinę biochemii — immunologię.

Włodzimierz Ostrowski

KURZGEFASSTES LEHRBUCH DER PHYSIOLOGIE (D. Keidel, ed.) Georg Thieme Verlag, Stuttgart, 1967; str. 502, cena DM 39.80.

Autorzy tego przeznaczonego dla studentów podręcznika postawili sobie za zadanie przedstawienie całości współczesnych zagadnień fizjologii w możliwie najbardziej zwięzły sposób. Jest to zadanie trudne, gdyż granica pomiędzy dydaktycznym uproszczeniem a wulgaryzacją i wynikającymi z niej nieraz merytorycznymi błędami jest bardzo płynna. Niestety autorzy wielu rozdziałów podręcznika nie uniknęli tego niebezpieczeństwa. Trudno tu wymienić wszystkie nadmierne uproszczenia i błędy, wskazane zostaną tu tylko najbardziej jaskrawe.

W rozdziale o fizjologii krwi (H. Bartels) nie podano punktów uchwytu i mechanizmów działania erytropoetyny, witaminy B_{12} oraz kwasu foliowego. Nie podano miejsca wydzielania erytropoetyny, chociaż jest ono już dostatecznie ustalone. Ten sam autor w rozdziale o oddychaniu pisze, że czynność komórek ośrodka oddechowego jest m.in. regulowana przez ciśnienie parcjalne tlenu w dopływającej doń krwi, co nie jest zgodne z prawdą. Błędna jest również informacja, że komórki ośrodka oddechowego są bezpośrednio wrażliwe na ciśnienie parcjalne CO_2. Strefy chemowrażliwe znajdują się na przednio-brzusznej powierzchni rdzenia przedłużonego, a nie pod dnem IV komory.

W rozdziale o sercu (E. Bauereisen) błędnie podano, że przewodzenie w węźle przedsionkowo-komorowym serca może zachodzić tylko w jednym kierunku. Nie napisano za to nic o strefie granicznej oddzielającej węzeł od mięśnia przedsionka, chociaż ma ona ogromne znaczenie w fizjologii i patologii serca. Nie podano nic o mechanizmie okresu refrakcji i jego związku z procesami membranowymi, chociaż zrozumienie tych zjawisk jest dla współczesnego lekarza niezbędne (mechanizmy migotania, resuscitacja serca). Okres refrakcji opisany jest jedynie w synchronizacji ze skurczem mechanicznym a nie z repolaryzacją, co byłoby dobre w początkach naszego wieku.

Dalej błędnie podano, że rozciągnięcie lewego przedsionka i prawej komory wywołuje odruchowe przyspieszenie czynności serca, podczas gdy w rzeczywistości odruchy te wywołują bradykardię. Podano również, że mediatorami pozazwojowych włókien sympatycznych unerwiających serce są zarówno noradrenalina jak i adrenalina, podczas gdy dobrze wiadomo, że zakończenia te wydzielają tylko noradrenalinę. Ze zjawisk wynikających z zależności siły skurczu od rytmu serca opisano jedynie "schody" Bowditch'a, pomijając całkowicie tak ważne dla współczesnej teoretycznej i klinicznej kardiologii zagadnienie jak potencjacja postekstrasystoliczna.

Również nadmiernie uproszczony wydaje się rozdział poświęcony fizjologii ośrodkowego układu nerwowego (45 stron, podczas gdy fizjologia nerwów obwodowych zajmuje 41 stron).

Do najlepszych rozdziałów należy fizjologia nerek oraz przemiana wodno-elektrolitowa (K. J. Ullrich), fizjologia mięśni szkieletowych (K. Brecht) oraz nerwów obwodowych (H. Lullies). Są one bardzo zwięzłe, ale omawiają wszystkie najistotniejsze zagadnienia w prosty i jasny sposób nie dopuszczając do jakiejkolwiek wulgaryzacji.

Pewne zastrzeżenia budzi również układ podręcznika. Na przykład wstępny rozdział o fizjologii komórkowej napisany przez A. Fleckensteina jest zupełnie zbędny, gdyż wszystkie zawarte w nim informacje znacznie dokładniej podane zostały w rozdziałach o fizjologii nerwów i mięśni. Umieszczenie rozdziału dotyczącego fizjologii pracy pomiędzy rozdziałami o mięśniach szkieletowych i nerwach obwodowych oddziela od siebie zagadnienia ściśle ze sobą związane. Wreszcie umieszczenie rozdziału o fizjologii zmysłów przed rozdziałem o fizjologii ośrodkowego układu nerwowego a w szczególności rdzenia kręgowego musi wprowadzić w zakłopotanie niedoświadczonego czytelnika.

W sumie jest to podręcznik bardzo nierówny, z widocznymi brakami w pracy redakcyjnej, którego ze względu na nadmierne uproszczenia, pomijanie istotnych zagadnień oraz merytoryczne błędy zawarte w wielu rozdziałach nie należy polecać naszym studentom.

Bohdan Lewartowski

CALLUS FORMATION. Symposium on the Biology of Fracture Healing (St. Krompecher and E. Kerner, eds.). Symposia Biologica Hungarica, vol. 7. Akadémiai KIADÓ, Budapest; str. VII+ 420.

Sympozjum poświęcone biologii gojenia się złamań odbyło się w Debreczynie w okresie od 5 do 8 lipca 1965 r. i zostało zorganizowane przez Wydział Biologii Węgierskiej Akademii Nauk, z której ramienia działał prof. dr. St. Krompecher. W Sympozjum brało udział około 80 osób z 12 krajów. Najliczniej byli oczywiście reprezentowani gospodarze. Przedstawiono 62 referaty, których część była poświęcona biologii i biochemii chrząstki, kości i gojenia się złamań a część poruszała zagadnienia kliniczne związane z leczeniem złamań kości. Wobec tak dużej liczby referatów nie sposób omówić je wszystkie w krótkiej recenzji.

We wstępnej części Sympozjum V. Zambotti i L. Bolognani przedstawili skład chemiczny chrząstki i kości oraz przebieg niektórych dróg metabolicznych, np. cykl kwasów trójkarboksylowych, biosyntezę mukopolisacharydów i kwasów nukleinowych w tych tkankach. Osobny rozdział był poświęcony procesowi mineralizacji chrząstki i kości. Biosynteza mukopolisacharydów była szerzej omówiona w oddzielnym referacie wygłoszonym przez H. Schmidta z Halle.

Bardzo ogólny i przeglądowy referat, którego temat był głównym motywem całego Sympozjum, przedstawił prof. dr St. Krompecher. Omówił on filogenetyczne i ontogenetyczne aspekty tworzenia się kości oraz czynniki, które mogłyby kierować metabolizm ku syntezie mukopolisacharydów. Tkanki zwierząt o dużej zawartości mukopolisacharydów wykazują niską aktywność oksydazy cytochromowej i odwrotnie. Rozwój kości wymaga oksybiotycznego metabolizmu, związanego z rozwojem naczyń krwionośnych oraz niektórych gruczołów dokrewnych, które kierują metabolizm ku oddychaniu tlenowemu. Tworzenie się chrząstki, zawierającej dużo mukopolisacharydów, nie wymaga obecności naczyń krwionośnych i dlatego ta tkanka wcześniej pojawia się w rozwoju filogenetycznym i ontogenetycznym. Autor powoływał się na wyniki badań doświadczalnych przeprowadzonych w jego Zakładzie, jak i na obserwacje przypadków patologicznych w klinice. Tak

więc dr Cs. Hadhazy, E. H. Olah, M. B. Laszlo i K. S. Kostenszky w jednym zbiorowym referacie opisali biogenezę regenerującej chrząstki w układzie doświadczalnym opracowanym dla stawu kolanowego psa. Autorzy dochodzą do wniosku, że uprzednie zmniejszenie unaczynienia tkanki jest istotne dla możliwości tworzenia się chrząstki w tym miejscu. W takiej tkance cykl glikolityczny zaczyna przeważać nad oddychaniem tlenowym. Dr P. Landanyi i M. B. Laszlo badali zawartość oksydazy cytochromowej w narządach ryb, płazów, gadów i u zwierząt bezkręgowych. E. H. Olah i B. Allemand starali się znaleźć u bezkręgowych zwierząt morskich korelację aktywności cyklu glikolitycznego z zawartością w nich aminowielocukrów. J. Landanyi zajmował się rozwojem naczyń krwionośnych w kostninie podczas gojenia się złamania kości.

Różne aspekty osteogenezy, chondrogenezy oraz gojenia się ran oraz złamanych kości zostały przedstawione przez innych uczestników Sympozium. I tak J. Lindner i współpracownicy zajmowali się przebiegiem tworzenia się elementów międzykomórkowych podczas gojenia się rany. R. Schenk i H. Willenegger stwierdzili, że bardzo dokładne zbliżenie przeciętych albo złamanych końców kości prowadzi do zrośnięcia się odłamków niemal bez powstawania kostniny. Badania te były poparte doskonałymi zdjęciami preparatów histologicznych ilustrujących powyższe stwierdzenia. Łączenie się oddalonych od siebie odcinków złamanej kości, np. w przypadku jej ubytków, omówili V. Petrkov i współpracownicy.

Topograficzne i czasowe zależności procesów w normalnej osteogenezie, obserwowane w mikroskopie elektronowym, omówił K. H. Knese. Opisał on również histochemiczne zmiany w przebiegu tego procesu. Histologią i histochemią kostniny w doświadczalnych pseudoartrozach zajmował się Y. Yashimura, M. Kono i M. Yamagishi. Regenerację kości w złamaniach doświadczalnych u zwierząt można śledzić, używając tetracyklinę jako wskaźnik aktualnie toczącej się osteogenezy. W ten sam sposób daje się również śledzić przebudowę kości, co na zdjęciach z pięknie wykonanych preparatów szlifów kości pokazali W. Eger i H. Kämmerer. E. V. S. Koskinen bardziej ogólnie omówił działanie hormonów na rozwój kostniny w złamaniach doświadczalnych, uwzględniając wpływ hormonu wzrostowego, kortizonu, hormonów płciowych i tyroksyny. Hormon wzrostowy, szczególnie gdy jest wspomagany równoczesnym podawaniem hormonu tyreotropowego, wybitnie zwiększa osteogenezę, co potwierdzono również obserwacjami klinicznymi u pacjentów. Wpływ kortizonu na gojenie się złamań przedstawili J. Lindner i współpracownicy, którzy proces ten śledzili posługując się metodami histochemicznymi, między innymi autoradiografią. Dwie grupy badaczy zajmowały się również dość oryginalnym pomysłem miejscowego wzmożenia osteogenezy przez wypełnianie ubytków kostnych mieszaniną skorupek z jaj i gipsu. Zarówno G. H. Schumacher i H. G. Wischhusen, jak i T. Tomory i E. Tarsoly stwierdzili korzystny wpływ tej mieszaniny na szybkość gojenia się ubytków. K. Ostrowski w zbiorowym referacie przedstawił wyniki badań swoich i współpracowników nad indukcją kości przeszczepami błony śluzowej pęcherza moczowego oraz nad odczynami w obwodowych węzłach chłonnych, które wywoływano przeszczepami kostnymi. To ostatnie zjawisko można wykorzystać dla oceny stopnia antygenowości przeszczepu. Przeszczepami izolowanych chondrocytów i odczynami po tych przeszczepach zajmowali się J. Kawiak i S. Moskalewski.

W przebiegu Sympozjum wygłoszono również szereg doniesień klinicznych łączących się z problemem gojenia się złamań kości, jak np. doniesienie o późnych wynikach leczenia gruźliczych ubytków w kościach kręgosłupa przeszczepami kostnymi, o istnieniu okolic kości mniej korzystnych dla gojenia się złamań, o wydłużaniu kości dla celów rehabilitacji w przypadkach skrócenia kończyny, o gojeniu się złamań rzepki i kości piszczelowej.

Sympozjum zamknął K. H. Knese, który po wysłuchaniu tak wielu referatów wyraził pogląd, że znamy wiele fragmentów omawianego problemu. Należy jednak szukać sposobów łączenia tych wiadomości w całość, w jedną wspólną teorię. Dla dalszych badań nad kostnieniem konieczna jest współpraca i wymiana myśli wśród przedstawicieli wielu dyscyplin, to też wysunięto propozycję ponownego zebrania się w tym celu.

Jerzy Kawiak

A. W. Rogers, TECHNIQUES OF AUTORADIOGRAPHY. Elsevier Publishing Company, Amsterdam 1967; str. XI+335, cena 55 Dfl.

Omawiana książka jest pozycją bardzo cenną z uwagi na ogromny rozwój techniki autoradiografii w ostatnich latach, jak również z powodu braku aktualnego podręcznika w tej dziedzinie. Po *Autoradiography in Biology and Medicine* G. B. Boyd'a, jest to druga książka, w której omówione są teoretyczne podstawy autoradiografii i wszystkie metody stosowane dotychczas w tym zakresie. W książce podane są również wnikliwe uwagi praktyczne, dzięki czemu mogą z niej korzystać nie tylko pracownicy bardzo zaawansowani w tej dziedzinie, lecz również początkujący.

Książkę można podzielić na dwie części. W pierwszej autor podaje teoretyczne podstawy, na których opiera się autoradiografia, i jej powiązania z innymi metodami radioizotopowymi, a następnie opisuje typy emulsji autoradiograficznej i procesy fizyko-chemiczne zachodzące w czasie bombardowania cząstkami jonizującymi. Omawia również zdolność rozdzielczą i wydajność autoradiografii oraz zagadnienia tła i sposoby jego zmniejszenia.

Druga część książki zawiera opisy poszczególnych grup technik autoradiograficznych, poczynając od najprostszych a kończąc na szeroko opisanej autoradiografii pod mikroskopem elektronowym.

A. W. Rogers szczegółowo omawia zarówno zalety, jak i wady poszczególnych metod autoradiograficznych, sugeruje ich wybór i zastosowanie. Metody, które podaje autor, są opisane tak dokładnie, że wydaje się, że może z nich korzystać każdy nawet bez wcześniejszego doświadczenia. Poszczególne rozdziały zaopatrzone są w obszerny spis piśmiennictwa, przy czym szczególnie przyjemny dla polskiego czytelnika jest fakt częstego cytowania polskich badaczy.

Książka jest bardzo starannie wydana i bogato ilustrowana. Można przypuszczać, że omawiana pozycja będzie pożyteczna zarówno dla histologów, biochemików, jak i innych badaczy pragnących zapoznać się z tą ciekawą metodą.

Marek Ombach