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LIGHT-SENSITIVE DERIVATIVES OF THE CORRINOID-PROTEIN COMPLEXES FROM *PROPIONIBACTERIUM SHERMANII* CELLS*

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To elucidate whether the native corrinoids are bound to specific proteins, or the protein-corrinoid binding is an artifact formed during the isolation procedure, different preparations from *Propionibacterium shermanii* cells have been compared [17]. The isolated protein-bound corrinoids when subjected to different purification procedures showed some variations in the amino acid contents and electrophoretic mobility. The ratio of the protein to the corrinoid was as 1:1 and the absorption spectra of the complexes differed markedly from that of the crystalline α-5,6-dimethylbenzimidazolylcobamide (vitamin B_{12}). Whereas these studies were aimed at isolation of the corrinoid-protein complexes (B_{12}-M)[1], without taking any precautions to avoid light-induced molecular changes, the present work is concerned primarily with the photosensitive form of the corrinoid-protein complex (B_{12}-M-S). By studying the protein-binding capacity of the light-sensitive corrinoids from *P. shermanii* it was hoped to obtain some data concerning the role of specific proteins in the biochemical functions of some vitamin B_{12} coenzymes [22, 6].

MATERIAL AND METHODS

All operations and procedures throughout this work (except the amino acid determination and chromatographic separation after exposure to daylight) were performed in dispersed red light or in a completely dark room.

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[1] The following abbreviations are used throughout this text: B_{12}, crystalline α-5,6-dimethylbenzimidazolylcobamide; B_{12}-M, α-5,6-dimethylbenzimidazolylcobamide-protein complex [17]; B_{12}-M-S, light-sensitive corrinoid-protein preparation; B_{12}-M-S_L, photolysed B_{12}-M-S.

Growth and harvesting of bacteria. As the source of the light-sensitive corrinoid-protein complexes (B_{12}-M-S), *Propionibacterium shermanii*-1 cells were used. The stock culture was maintained on a yeast-glucose medium supplemented with casein hydrolysate [23]. Cultures were incubated in 5 litres of liquid medium composed of acid and enzymic hydrolysates of casein, added with mineral salts, vitamins and glucose [23]. The medium adjusted to pH 6.5 was sterilized for 30 min. at 120°, and to each 4-litre portion 1000 ml. of inoculum was added. The inoculum was previously subjected to threefold transfer at two-day intervals. Then the bacteria were allowed to grow for 8 days at 30°. During the first 24 hr. the dropping pH was adjusted every hour to 6.8-7.0 with concentrated ammonia. At the end of this period, 20 ml. of 60% glucose solution was added per 1 litre of the medium, and after the first 48 hr. of incubation, 16 mg. of 5,6-dimethylbenzimidazole per 1 litre was added. The cells were harvested by centrifugation, washed with distilled water and centrifuged again. One litre of the culture yielded approximately 56 g. of cell wet weight. Before further treatment the material was stored at -20°.

Isolation of B_{12}-M-S. The procedure of Mulli & Schmidt [20, 17] was followed except that the daylight was excluded. The combined phenol extracts were carefully washed with distilled water and treated with 9 volumes of acetone. After several hours, the brick-orange precipitate was separated by centrifugation, washed with ethanol and dried *in vacuo* over concentrated H_2SO_4.

Absorption spectrum. This was measured in 0.01 M-phosphate buffer, pH 0.8, in a Unicam SP-700 automatic recording double-beam spectrophotometer.

Amino acid composition. The preparation was hydrolysed in 6 N-HCl (A. R.) for 22 hr. at 110° in a tube sealed under nitrogen. A 500:1 weight ratio of the acid to the material was applied. After hydrolysis the acid was evaporated under reduced pressure, the residue was added with distilled water (corresponding to 1/4 volume of the acid used) and the solution was evaporated again. The residue was then added with 0.1 M-citrate buffer, pH 2.2, to obtain a concentration corresponding to 1 mg. protein per 1 ml. and the insoluble material was removed by filtration; in the clear solution, amino acids were analysed according to Spackman, Moore & Stein [26] using an automatic amino acid analyser (Phoenix Precision Inst. Co., Model K-5000-A). The solution corresponding to 1 - 4 mg. of the starting material, was loaded on an Amberlite IR-120 (150 × 0.9 cm.) column. A rapid procedure was also applied in which a smaller column (55 × 0.9 cm.) packed with finer particles of Amberlite IR-120 [27] was used. This modification allowed to achieve a complete resolution of the acidic and neutral amino acids within 6 hr. instead of 17 hr.

Paper chromatography. The homogeneity and chromatographic mobilities of the preparations studied, were tested on Whatman no. 1 paper using the descending technique in three solvent systems [8]: (*A*), butan-2-ol - ammonia (23%) - water (100:1:50, by vol.); (*B*), butan-2-ol - ammonia (23%) - water - 5% solution of potassium cyanide (100:1:50:0.25, by vol.); and (*C*), butan-2-ol - acetic acid - water - 5% solution of KCN (100:1:50:0.25, by vol.). In each case the time of development was 20 hr. In preliminary experiments several solvent systems were examined, and the butan-2-ol containing solvents were found to give the best resolution.

Paper electrophoresis. This was performed according to Holdsworth [15] on Whatman no. 3 paper in five solvent systems: (I), 1 N-acetic acid, pH 2.7 [15]; (II), 1 N-acetic acid with 0.015% KCN, pH 2.7; (III), 0.1 M-KCN solution, pH 11 [1]; (IV), phosphate buffer, pH 6.5; and (V), phosphate buffer, pH 6.5, with 0.015% KCN; the voltage gradient being 9 v/cm. and time 10 hr.

Chromatography on CM-Sephadex G-25. The capacity of CM-Sephadex G-25 (Pharmacia, Uppsala, Sweden) to adsorb haemoglobin when equilibrated with sodium phosphate buffer (ionic strength 0.05, pH 6.5) was 0.7 g./g. Before use the exchanger was treated according to Flodin [10], then packed into a tube 2 cm. in diameter to a height of 24 cm. and equilibrated with 0.01 M-potassium-phosphate buffer, pH 6.8. The elution was carried out with 110 - 120 ml. of 0.01 M-phosphate buffer, pH 6.8, and subsequently with 0.05 M-phosphate buffer of the same pH. The flow rate was 60 ml. per hour and fractions of 2 - 4 ml. were collected. The elution was completed within about 5 hr. In all fractions the extinction at 260, 280 and 530 mμ was measured in a Unicam SP-500 spectrophotometer.

Growth factor activity. This was assayed on *Escherichia coli* 113-3 both by plate [12, 16] and tube [9] procedures, the latter being applied in the modification of Hedbom [13]. The results were expressed as percentages of the activity of crystalline vitamin B_{12}.

Photolysis. The light-sensitive complexes in 0.01 M-phosphate solution, pH 6.8, were exposed to direct sunlight for 8 hr., unless otherwise indicated.

RESULTS AND DISCUSSION

The yield of the B_{12}-M-S preparation was 30 - 40 mg. per 1 kg. of cell wet weight. The bright-orange colour of this material differed markedly from that of the B_{12}-M preparation [17] and was typical for the B_{12}-coenzymes [2, 22]. Paper chromatography run in dark chambers showed the heterogeneity of the isolated preparations. In alkaline *sec.*-butanol solvent (Fig. 1, A) the B_{12}-M-S complex separated into four

fractions whereas B_{12}-M into five. None of the spots obtained either from B_{12}-M-S or from B_{12}-M, was located in the position corresponding to that of crystalline vitamin B_{12}. It seems worth noting that the brick-orange colour, characteristic for the light-sensitive forms of corrinoids, remained unaltered even after a prolonged period of separation and exposure to light of the eluate. The main part of the B_{12}-M-S preparation was located on the chromatogram in a position different from that of B_{12}-M. The spots moving somewhat faster than the main fraction of B_{12}-M-S corresponded to the conversion product of B_{12}-M-S into B_{12}-M. In the presence of CN^- ion (Fig. 1, B) the B_{12}-M preparation moved into a position which roughly corresponds to that of vitamin B_{12}, whereas only a small part of B_{12}-M-S was converted into cyanocobalamin, the main part not being cleaved. It seems that the alkaline pH of the solvent, as well as the presence of the protein moiety, protected the B_{12}-M-S against degradation by cyanide. This view appears to be supported by the results obtained with the solvent system *C*. The acidic medium accelerated greatly the cyanide-induced conversion of B_{12}-M-S into the B_{12}-M complex (Fig. 1, C).

By paper electrophoresis, a smaller number of fractions from B_{12}-M-S was obtained than by paper chromatography; the former, however, allowed to obtain larger amounts of fractions thus facilitating further analysis. Electrophoresis of B_{12}-M-S- in 1 N-acetic acid at pH 2.7 in the absence of cyanide (Fig. 2, I) gave one small electroneutral fraction (*1*) and two electropositive fractions (*2* and *3*). The weight ratio of fractions *2* and *3* was 1:3. Fraction *2* was located in the position of B_{12}-M. Crystalline vitamin B_{12} under the same conditions retained its electroneutral character. When the B_{12}-M-S preparation was exposed to daylight for a total of 48 hr. (B_{12}-M-S_L) and then subjected to paper electrophoresis, a spot in the position of B_{12}-M was obtained.

Addition of cyanide to the 1 N-acetic acid solution (Fig. 2, II) resulted in the conversion of the main part of the B_{12}-M complex into cyanocobalamin. B_{12}-M-S, however, was only partially cleaved; the cyanocobalamin spot was smaller than the main fraction, which retained the characteristic orange colour even after prolonged exposure to daylight. The photolysed B_{12}-M-S preparation, during electrophoresis in the presence of KCN was almost completely converted into cyanocobalamin.

The electrophoresis of B_{12}-M-S, B_{12}-M-S_L and B_{12}-M in KCN solution, pH 11 (Fig. 2, III) resulted in total degradation of these complexes into cyanocobalamin. However, the electrophoresis in phosphate buffer, pH 6.5 (without cyanide, Fig. 2, IV), resulted in the conversion of only a part of B_{12}-M-S into vitamin B_{12}, whereas B_{12}-M was almost completely cleaved. The main fraction of B_{12}-M-S retained the characteristic orange colour. When cyanide was added to the phosphate buffer (Fig. 2, V),

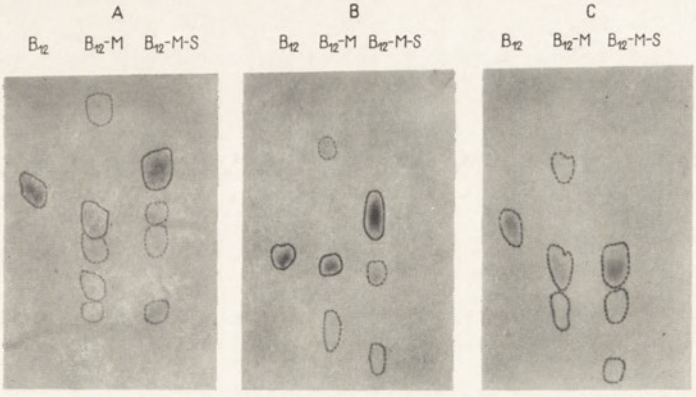

Fig. 1. Paper chromatography of B_{12}-M-S and vitamin B_{12} in (A) butan-2-ol - 23% ammonia - water (100:1:50, by vol.); (B), butan-2-ol - 23% ammonia - water - 5% KCN solution (100:1:50:0.25, by vol.); (C), butan-2-ol - acetic acid - water - 5% KCN solution (100:1:50:0.25, by vol.).

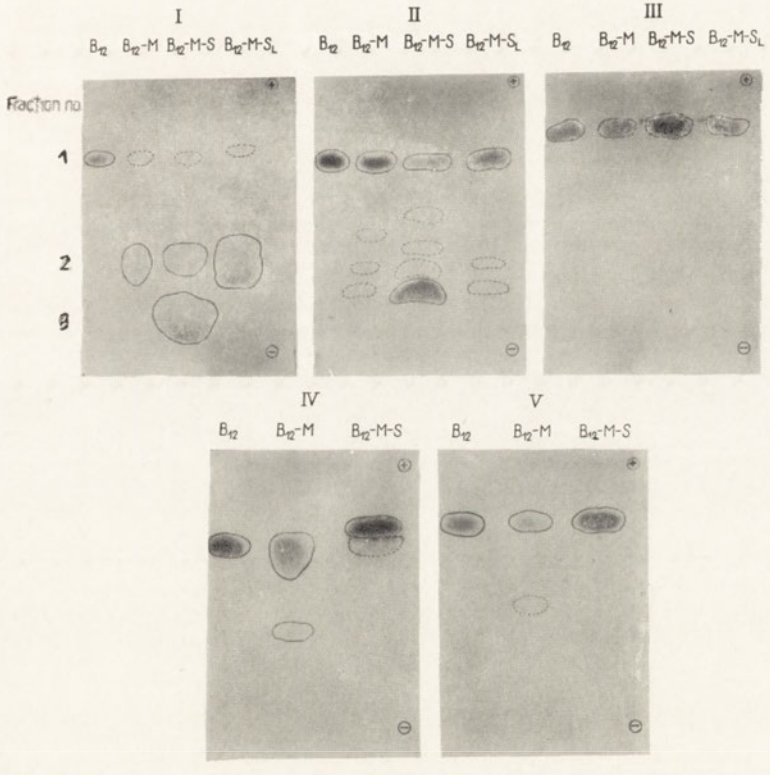

Fig. 2. Electrophoretic patterns of vitamin B_{12}, B_{12}-M, B_{12}-M-S and B_{12}-M-S_L. Electrophoresis was performed in: (I), 1 N-acetic acid, pH 2.7; (II), 1 N-acetic acid - 0.015% KCN solution; (III), 0.1 M-KCN solution, pH 11; (IV), phosphate buffer, pH 6.5; (V), phosphate buffer, pH 6.5, with 0.015% KCN solution.

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a complete conversion of B_{12}-M-S and B_{12}-M into cyanocobalamin took place.

From these studies it appears that the acidic medium had a protecting effect against cyanide-induced conversion of the corrinoid-protein complexes.

The absorption spectra of the crude B_{12}-M-S preparation and its electrophoretic fractions *1* and *3* (cf. Fig. 2, I) are illustrated in Fig. 3. The spectrum of the small fraction *1* was found to be typical for vitamin B_{12}. Presence of this compound in the crude B_{12}-M-S preparation was probably due to the insufficient protection of the preparation against daylight during its isolation and purification. The absorption spectrum of fraction *3* was found to be similar to that of 5,6-dimethylbenzimidazolylcobamide coenzyme [4, 5], indicating the presence of this compound in the B_{12}-M-S complex. The crude B_{12}-M-S preparation containing fraction *1*, had the absorption spectrum slightly different from

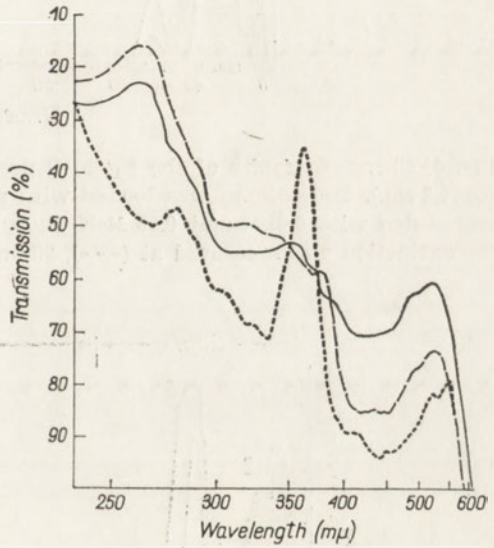

Fig. 3. Absorption spectra: (———), of the crude B_{12}-M-S preparation, and of the electrophoretically separated fractions (- - -), B_{12}-M-S *1* and (— — —), B_{21}-M-S *3*.

that of the vitamin B_{12} coenzyme. This was most apparent in the region of 350 mμ. It is worth to remember that all purified cobamide coenzymes are characterized by a prominent peak at 260 mμ and by absence of an absorption maximum at 350 - 360 mμ, the absorbancy peaks of the coenzymes in the visible region being at lower wavelengths than those of the corresponding vitamins.

For separating the free from bound forms of corrinoids, dialysis [7, 24] and ultrafiltration [11] with the use of radioactive vitamin B_{12} are the most commonly applied methods. These techniques, however, have some disadvantages such as dissociation of the complex [25] and partial denaturation of the protein during the procedure, as well as variability

of results depending on the technique used. Recently, gel filtration and chromatography on Sephadex columns have been introduced [10]. The molecular weight of proteins bound to corrinoids has consistently been found to be above 5 000 [29], therefore Sephadex G-25, which excludes material whose molecular weight exceeds 3 500 - 4 000, is especially

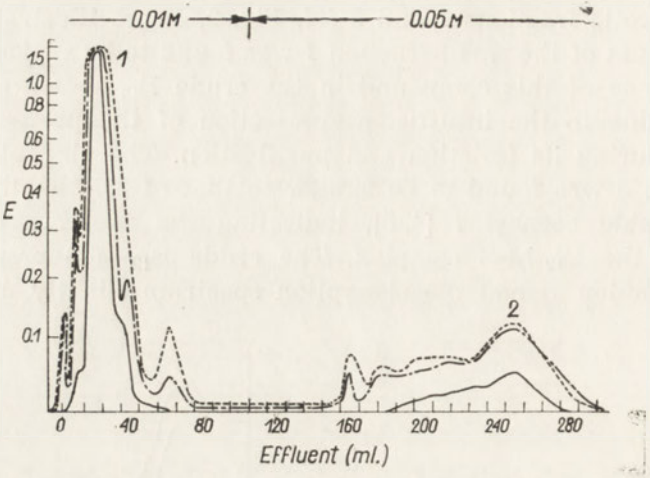

Fig. 4. Chromatography of the B_{12}-M-S preparation on CM-Sephadex G-25 column (24 × 2 cm.). The column was loaded with a solution containing 9.8 mg. of B_{12}-M-S and eluted with 0.01 M and 0.05 M-K-phosphate buffer, pH 6.8. In the effluent the extinction was measured at (- - -), 260 mμ; (— • —) 280 mμ and (———), 530 mμ.

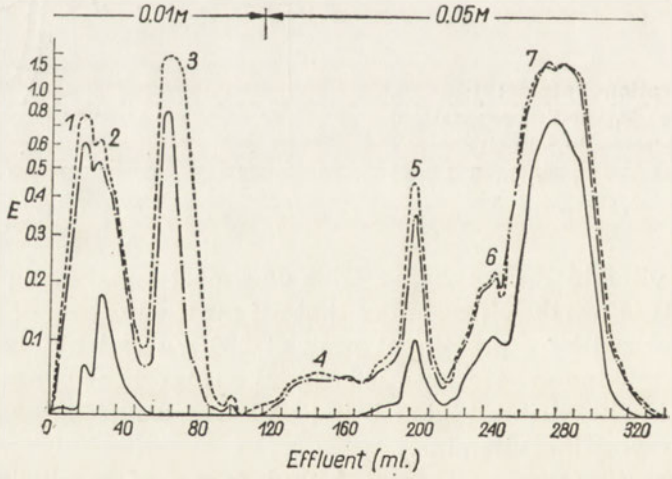

Fig. 5. Chromatography of the photolysed B_{12}-M-S preparation on CM-Sephadex G-25 column (24 × 2 cm.). The column was loaded with a solution containing 9.2 mg. of B_{12}-M-S_L and eluted with 0.01 M and 0.05 M-K-phosphate buffer, pH 6.8. In the effluent the extinction was measured at (- - -), 260 mμ (— • —), 280 mμ; and (———), 530 mμ.

suitable for separating free from bound corrinoids [19]. To obtain additional confirmation of the heterogeneity of the B_{12}-M-S complex, the preparation was chromatographed on a CM-Sephadex G-25 column. In the absence of daylight, B_{12}-M-S separated into two heterogeneous fractions (Fig. 4) and after photolysis into four coloured fractions (Fig. 5, peaks *1, 2, 4, 5, 6* and 7) and one colourless peak. The photolysis changed also the chromatographic distribution of the coloured material. Whereas prior to photolysis the main fraction was eluted with 0.01 M-phosphate buffer, after exposure to light the main coloured fraction was eluted with 0.05 M-phosphate buffer (Fig. 5, peaks *6* and 7). This seems to be due to a shift of the electric charge in the corrinoid moiety, induced by photolysis. The extinction determinations at 280 and 530 mμ in the effluents of the photolysed B_{12}-M-S complex seem to indicate a rather strong binding of the protein to the corrinoid. The lack of a typical protein fraction with E_{max} at 280 mμ in the effluents suggests that light-induced degradation of B_{12}-M-S does not cause the liberation of the native protein.

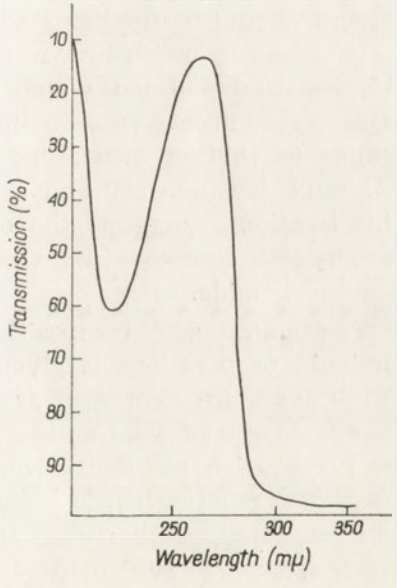

Fig. 6. UV absorption spectrum of the column chromatographic fraction *3* of the photolysed B_{12}-M-S preparation (cf. Fig. 5).

To obtain further data concerning the fractions of B_{12}-M-S_L, the UV absorption spectra of peaks *3, 5* and *7* were determined. The spectrum of the material from peak *3* was typical for adenine with a maximum at 263 mμ at neutral pH (Fig. 6). This indicates that the mechanism of photolysis involves the cleavage of the carbon-cobalt bond within the molecule of the B_{12}-M-S complex, followed by an electron shift resulting in the liberation of an adenosine-like compound. It seems that the 5-deoxyadenosyl moiety in the cobamide coenzymes affects the

co-ordinate bond between the nucleotide and the cobalt atom. One of the characteristic properties of the cobamide coenzymes is their instability in light [28]. Exposure of an aqueous solution of coenzyme B_{12} to light causes a loss of coenzyme activity and a change in the absorption spectrum. Photolytic cleavage of coenzyme B_{12} in the presence of air results in the formation of aquocobalamin and two colourless compounds that have absorption spectra similar to adenosine [3].

Cyanide converts the cobalamin coenzyme into cyanocobalamin by splitting off adenine and *erythro*-3,4-dihydroxy-1-penten-5-al [18]. This reaction is not affected by atmospheric oxygen [5]. Various corrinoid coenzymes are cleaved by light and cyanide in the same manner as the cobalamin coenzyme but at different rates [21]. The isolated protein-containing light-sensitive corrinoid preparation seems to have similar properties. Peak *3* of the photolysis product, which had an absorption maxium at 263 mμ, may be identical with the adenine-*β*-D-*ribo*-pentofuranosyldialdose (adenosine-5′-aldehyde) demonstrated as a product of photolytic cleavage of coenzyme B_{12} [12]. This assumption, however, requires further studies.

Peak *5*, yellowish-brown in colour, showed absorption maxima at 215, 338 and 476 mμ which differed markedly from those of the naturally occurring vitamin B_{12} analogues. The spectrum of peak 7 was similar to that of crystalline vitamin B_{12}, except in having a broad maximum between 260 and 280 mμ, indicating the presence of protein. This fraction 7 suggests the occurrence of light-induced conversion of the B_{12}-M-S complex into a stable protein-bound 5,6-dimethylbenzimidazolylcobamide.

The amino acid composition of protein-containing light-sensitive corrinoid preparations is given in Table 1. The amounts of individual amino acids are expressed as g. per 100 g. of the whole complex and as percentages of total amino acids found. Both the crude B_{12}-M-S and the preparation purified according to Mulli & Schmidt [20] were analysed. The purified B_{12}-M-S during paper electrophoresis in 0.1 N-acetic acid separated, similarly as the crude preparation, into two fractions which were also subjected to amino acid analysis. The electrophoretic fraction *2*, containing a relatively high amount of glutamic acid, was called the glutamate fraction, and fraction *3* rich in glycine, the glycine fraction. In all preparations analysed, 16 amino acids were present but no cysteine and/or cystine was found. Proline and glutamic acid were present in rather high amounts in all the preparations studied. Among the basic amino acids, histidine was the predominant one. These results are similar to those found earlier [17] for the B_{12}-M complex isolated from *P. shermanii* cells under conditions in which light-induced degradation of the B_{12} coenzyme-like compounds could take place.

Table 1

Amino acid composition of the B_{12}-M-S complex and its electrophoretic fractions

From the purified B_{12}-M-S preparations, fractions *2* (glutamate) and *3* (glycine) were separated by electrophoresis (Fig. 2, I). The amount of the respective amino acid is given A, in g. per 100 g. of the complex and B, as percentage of the total amino acids; this latter value, as well as the weight ratio, were calculated without taking into account the NH_3 content.

Amino acid	B_{12}-M-S crude		B_{12}-M-S purified		Glutamate fraction		Glycine fraction	
	A	B	A	B	A	B	A	B
Lysine	0.86	4.32	0.39	3.71	0.20	1.71	0.18	2.96
Histidine	1.10	5.53	0.67	6.38	0.81	6.94	0.28	4.61
NH_3	4.04	—	3.71	—	4.08	—	6.23	—
Arginine	0.34	1.72	0.17	1.62	0.12	1.03	0.06	0.98
Aspartic acid	0.48	2.41	0.32	3.05	0.36	3.09	0.20	3.29
Threonine	0.77	3.87	0.58	5.52	0.94	8.06	0.22	3.62
Serine	0.37	1.86	0.11	1.05	0.40	3.43	0.22	3.62
Glutamic acid	3.43	17.23	1.67	15.50	2.68	22.98	0.90	14.80
Proline	5.45	27.38	3.11	29.62	3.41	29.24	1.20	19.74
Glycine	1.07	5.38	1.10	10.47	0.48	4.12	1.49	24.51
Alanine	0.26	1.31	0.15	1.43	0.23	1.97	0.12	1.99
Valine	1.28	6.43	0.38	3.62	0.28	2.40	0.22	3.62
Methionine +Met-SO_2	0.37	1.86	0.19	1.81	0.20	1.71	0.17	2.80
Isoleucine	0.61	3.07	0.35	3.33	0.18	1.54	0.21	3.45
Leucine	1.59	8.00	0.85	8.09	0.98	8.40	0.30	4.93
Tyrosine	0.31	1.56	0.04	0.38	0.10	0.86	0.06	0.98
Phenylalanine	1.62	8.14	0.51	4.86	0.30	2.51	0.25	4.11
Total minus NH_3	19.91	100.1	10.51	100.44	11.66	100.05	6.08	100.01
Protein to corrinoid wt. ratio	1:4.0		1:8.5		1:7.6		1:15.4	

The B_{12}-M-S complex differs greatly, however, from the B_{12}-M as regards the ratio of the protein to the corrinoid. This value was found to be 1:1 for B_{12}-M whereas for the crude B_{12}-M-S it was 1:4 and for the glycine fraction even 1:15. The lower amount of protein in the B_{12}-M-S complex seems to indicate that only part of the protein present in B_{12}-M combines with the light-sensitive form of corrinoids.

The amount of glycine in the protein of the crude B_{12}-M-S preparation was found to be at least twice as great as that in the protein of the B_{12}-M preparation [17], and in the light-sensitive preparations studied the content of glycine was greater when the content of protein was smaller. This seems to suggest that glycine, or its polypeptide, has a specific function in the structure and biological activity of the B_{12}-M-S

complexes. The amounts of other amino acids present in B_{12}-M-S and B_{12}-M compounds were fairly similar which may indicate that a highly specific protein is required for the formation of these complexes.

The purification of the crude B_{12}-M-S preparation resulted in the loss of half of the protein content, probably due to the removal of loosely bound protein; simultaneously an increase was observed of glycine, threonine, aspartic acid and histidine contents whereas the amounts of tyrosine, phenylalanine, valine, serine and lysine became lower.

The two fractions *2* and *3* obtained by electrophoresis from the purified B_{12}-M-S complex differed markedly in their protein contents. The glutamate fraction contained 12% of protein whereas the glycine one, only 6%. In comparison with the purified B_{12}-M-S complex, the glutamate fraction had significantly higher amounts of serine, tyrosine, threonine, alanine and glutamic acid, the same amounts of histidine, aspartic acid, proline, leucine and methionine, and smaller amounts of the remaining amino acids. On the other hand, the glycine fraction contained more serine, tyrosine, glycine, methionine and alanine, and less leucine, arginine, threonine, proline and histidine, than the B_{12}-M-S complex. The amino acid composition of the glutamate and glycine fractions was similar except for arginine, aspartic acid, serine, alanine and tyrosine. The obtained results seem to indicate that the B_{12}-M-S preparation contained at least two complexes differing in their protein content and amino acid composition.

Table 2

Growth factor activity for Escherichia coli *of the isolated corrinoid--protein complexes*

Fractions *2* and *3* separated by electrophoresis of B_{12}-M-S in 1 N-acetic acid pH 2.7 (Fig. 2, I), were used. Results are mean values from three preparations.

Substance	Activity in relation to vitamin B_{12} (%)	
	Plate assay	Tube assay
B_{12}, crystalline	100	100
Crude B_{12}-M-S	53	24
Electrophoretic fraction *2*	50	28
Electrophoretic fraction *3*	101	62

The crude B_{12}-M-S preparation and its two fractions separated by electrophoresis (Fig. 2, I) were tested for growth factor activity on *E. coli*. The preparations containing less protein were found to have higher activity whereas the preparations with greater protein content were less active (Table 2); it seems therefore that the growth factor activity is a property of the corrinoid moiety.

So far, no naturally occurring light-sensitive protein-containing corrinoids have been isolated from *Propionibacterium shermanii* and characterized. It seems possible that the B_{12}-M-S complexes may act as enzymes in the known reactions depending on B_{12}.

The data presented in this paper may be considered as an attempt to elucidate the problem of the occurrence in nature and biological functions of various light-sensitive protein-containing corrinoids. It is now recognized that all the naturally occurring corrinoids apparently exist in the coenzyme form. This form presumably arises soon after the biosynthesis of the corrin ring and the incorporation of the cobalt atom, perhaps at the stage of pentacarboxylic acid which, like other related polycarboxylic acids, can be converted enzymically into its coenzyme form [6]. The stage at which protein is bound to the corrin ring during the formation of the B_{12}-M-S-type compounds, remains still unknown.

SUMMARY

A light-sensitive corrinoid-protein complex (B_{12}-M-S) was isolated from *P. shermanii*-1 cells. The colour and absorption spectrum of B_{12}-M-S were both different from those of the preparation isolated on light (B_{12}-M) and indicated the presence of coenzyme B_{12}. B_{12}-M-S on paper chromatography, electrophoresis and CM-Sephadex G-25 column chromatography appeared to be heterogeneous but was not degraded into vitamin B_{12}. On photolysis it was converted to B_{12}-M, and with KCN gave cyanocobalamin.

The amino acid composition of B_{12}-M-S differed from that of B_{12}-M. The ratio of protein to corrinoid in B_{12}-M was as 1:1 and in B_{12}-M-S varied from 1:4 to 1:8, depending upon the grade of purity. The growth factor activity of the B_{12}-M-S preparations for *E. coli* was inversely proportional to the protein contents.

REFERENCES

[1] Armitage J. B., Cannon J. R., Johnson A. W., Parker L. F. J., Smith E. L., Stafford W. H. & Todd A. R. - *J. Chem. Soc.* 3849, 1953.
[2] Barker H. A. - *Fed. Proc.* **20**, 956, 1961.
[3] Barker H. A., Smyth R. D., Weissbach H., Toohey J. I., Ladd J. N. & Volcani B. E. - *J. Biol. Chem.* **235**, 480, 1960.
[4] Barker H. A., Weissbach H. & Smyth R. D. - *Proc. Nat. Acad. Sci. U. S.* **44**, 1093, 1958.
[5] Bernhauer K. & Müller O. - *Biochem. Z.* **334**, 199, 1961.
[6] Bernhauer K., Müller O. & Wagner F. - Angew. Chem. **3**, 200, 1964.
[7] Bird O. D. & Hoevet B. - *J. Biol. Chem.* **190**, 181, 1951.
[8] Brown F. B., Cain J. C., Gant D. E. & Parker L. F. J. - *Biochem. J.* **59**, 82, 1955.

[9] Burkholder P. R. - *Science* **114**, 459, 1951.
[10] Flodin P. - *J. Chromat.* **5**, 103, 1961.
[11] Gregory M. E. & Holdsworth E. S. - *Biochem. J.* **66**, 456, 1957.
[12] Harrison G. F., Lees K. A. & Wood F. - *Analyst* **76**, 696, 1951.
[13] Hedbom A. - *Biochem. J.* **74**, 307, 1960.
[14] Hogenkamp H. P. C., Ladd J. N. & Barker H. A. - *J. Biol. Chem.* **237**, 1950, 1962.
[15] Holdsworth E. S. - *Nature* **171**, 148, 1953.
[16] Iwanowska J., Bogucka J. & Kąkol H. - *Przem. Chem.* **9**, 14, 1953.
[17] Janicki J., Pędziwilk F., Nowakowska K., Kowalczyk J. & Skupin J. - *Bull. Acad. Polon. Sci. Ser. Biol.* **11**, 573, 1963.
[18] Johnson A. W. & Shaw N. - *Proc. Chem. Soc.* (London) 447, 1961.
[19] Kakei M. & Glass G. B. J. - *Proc. Soc. Exp. Biol. Med.* **111**, 270, 1962.
[20] Mulli K. & Schmidt O. J. - *Vit. Horm. Ferm.* **8**, 15, 1957.
[21] Müller O. & Müller G. - *Biochem. Z.* **336**; 299, 1962.
[22] Perlman D. - *Ann. New York Acad. Sci.* **112**, 644, 1964.
[23] Pędziwilk F. - *Prace Komisji Nauk Roln. i Leśn.*, T. P. N. Poznań **11**, 141, 1962.
[24] Rosenblum D., Woodbury D. F. & Reisner E. H., *Proc. II Radioisotope Conf., Oxford, July 19-23, 1954*, p. 287. Butterworths Scien. Publ., London 1954.
[25] Schwartz G. H., *Thesis*, Princenton Univ., 1958.
[26] Spackman D., Moore S. & Stein W. H. - *Analyt. Chem.* **30**, 1190, 1958.
[27] Stark G. R. & Smyth D. G. - *J. Biol. Chem.* **238**, 214, 1963.
[28] Weissbach H., Ladd J. N., Volcani B. E., Smyth R. D. & Barker H. A. - *J. Biol. Chem.* **235**, 1462, 1960.
[29] Wijmenga H. G., in *Vitamin B_{12} und Intrinsic Factor* (H. C. Heinrich, ed.) p. 156. F. Enke, Stuttgart 1957.

ŚWIATŁOCZUŁE POCHODNE KOMPLEKSÓW KORYNOIDOWO-BIAŁKOWYCH Z KOMÓREK *PROPIONIBACTERIUM SHERMANII*

Streszczenie

Z komórek *P. shermanii*-1 wyizolowano światłoczuły kompleks korynoidowo-białkowy (B_{12}-M-S). Barwa i widmo B_{12}-M-S wskazują na obecność koenzymu B_{12} i różnią się od kompleksu izolowanego przy dostępie światła (B_{12}-M). Elektroforeza, chromatografia bibułowa i rozdział na kolumnie CM-Sephadex G-25 wykazały niejednorodność kompleksu B_{12}-M-S; nie stwierdzono jednak rozpadu do witaminy B_{12}. Zidentyfikowano dwie frakcje, z których jedna zawierała dużo kwasu glutaminowego, druga zaś glicyny. Fotoliza B_{12}-M-S powodowała powstanie B_{12}-M, a w obecności cyjanków powstawanie cyjanokobalaminy.

Skład aminokwasowy B_{12}-M-S różnił się od preparatu B_{12}-M. Stosunek białka do korynoidu był w B_{12}-M 1:1, zaś w B_{12}-M-S wynosił od 1:4 do 1:8 w zależności od stopnia oczyszczenia. Aktywność wzrostowa preparatów B_{12}-M-S wobec *Escherichia coli* była odwrotnie proporcjonalna do zawartości białka w kompleksach.

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ENZYMIC CONVERSION OF GLYCEROL INTO β-HYDROXYPROPIONALDEHYDE IN A CELL-FREE EXTRACT FROM *AEROBACTER AEROGENES*

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A number of micro-organisms are known to produce propan-1,3-diol during glycerol fermentation. Already half a century ago Voisenet [14] postulated that β-hydroxypropionaldehyde may be an intermediate in this reaction. However, it was only in 1960 that Abeles *et al.* [1] demonstrated the formation of this compound in a culture of *Aerobacter aerogenes* (ATCC 8724) using semicarbazide as the aldehyde-trapping agent. Nevertheless, Abeles & Lee [2] were unable to demonstrate the formation of β-hydroxypropionaldehyde by the cell-free extract obtained from this strain, although this preparation was able to catalyse similar reactions converting ethylene glycol into acetaldehyde and propan-1,2-diol into propionaldehyde. Smiley & Sobolov [12] were the first to demonstrate *in vitro* the enzymic synthesis of hydroxypropionaldehyde from glycerol, using an extract of acetone-dried *Lactobacillus* 208-A cells.

In 1962 it has been observed in this laboratory that *A. aerogenes* cultured in anaerobic conditions on glycerol-containing medium, is a rich source of enzymes converting ethylene glycol into acetaldehyde [16], propan-1,2-diol into propionaldehyde [9], and glycerol into β-hydroxypropionaldehyde. All these reactions are coenzyme B_{12}-dependent and the conversion of glycerol has been used in this laboratory as an assay system for testing the activity of coenzyme B_{12} analogues [15, 18, 19, 20].

The present work describes the general properties of the enzyme system isolated from *Aerobacter aerogenes* (PZH, strain no. 572) which catalyses the conversion of glycerol into β-hydroxypropionaldehyde.

MATERIALS AND METHODS

Coenzymes. Coenzyme B_{12} and the coenzymic form of cobinamide were isolated from a culture of *Propionibacterium shermanii* [17] and additionally purified by paper electrophoresis and chromatography as

described by Pawełkiewicz *et al.* [8]. Coenzyme B_{12} obtained by chemical synthesis [18] was also used. Co-ethylcobalamin was obtained from cyanocobalamin by the method described for the synthesis of Co-methylcobinamide [15]. Co-5′-deoxyuridylcobalamin was synthesized according to Zagalak & Pawełkiewicz [18].

Reagents. Streptomycin sulphate was a product of Tarchomińskie Zakłady Farmaceutyczne - Polfa (Warszawa-Tarchomin). DL-Tryptophan and DL-serine were from Nutritional Biochemicals Co. (Cleveland, Ohio, U. S. A.); *N*-ethylmaleimide and mercaptoethanol from T. Schuchardt (Munich, Germany); *p*-chloromercuribenzoic acid from L. Light Co. (Colnbrook, England). Other reagents were from Fabryka Odczynników Chemicznych (Gliwice, Poland).

The enzymic hydrolysate of casein was prepared according to Pawełkiewicz & Zodrow [10]. Yeast autolysate was prepared in the following way: 5 kg. of pressed baker's yeast was suspended in 5 litres of tap water, added with 50 ml. of toluene and thoroughly mixed. The mixture was left for 4 days at 37°, the pH being adjusted twice daily with concentrated ammonia to 7 - 8. Then the mixture was steam-heated in a Koch apparatus for 30 min., cooled and centrifuged. From the clear supernatant, the remaining toluene was removed at 80° under reduced pressure, then the autolysate was sterilized for 30 min. at 120°.

Growth of the organism. Aerobacter aerogenes (strain no. 572) was obtained from the collection of the State Institute of Hygiene, Warszawa. Stock cultures were kept on agar slopes and subcultured every 7 - 10 days.

For experiments, the bacteria were cultured at 29° in a medium consisting of: 400 ml. of baker's yeast autolysate, 150 ml. of trypsin hydrolysate of casein, 12 g. of KH_2PO_4, 28 g. of K_2HPO_4, 0.8 g. of $MgSO_4 \cdot 7H_2O$, 10 mg. of $CoSO_4 \cdot 7H_2O$, 195 g. of 85% glycerol, and tap water to a final volume of 8000 ml. The medium was adjusted to pH 7.0 with 0.1 N-NaOH. Then two conical flasks, 25- and 100-ml., were filled with the medium and the remainder was poured into a 10-litre flat-bottomed flask provided with a glass tube reaching to the bottom of the vessel for flushing nitrogen. The media were sterilized for 30 min. at 1 atmosphere pressure. The inoculum was prepared by transferring the bacteria with a platinum loop from the agar to the 25-ml. flask; after 18 hr. the culture was poured into the 100 ml. flask and after further 18 hr., to the 10-litre flask. There the bacteria were allowed to grow for 18 hr., a stream of nitrogen from a high-pressure tank being flushed at a rate of 100 - 150 ml. per minute. The nitrogen was freed of oxygen by passing through a solution of pyrogallol in potassium hydroxide, and from bacteria by passing through a bacterial filter.

Preparation of cell extracts. About 50 ml. of bacteria, harvested by centrifugation for 40 min. at 6000 g., were added with 70 ml. of water,

cooled in an ice bath to 2 - 4°, and the pH adjusted to 8.5 with 1 N-KOH. The cells were disintegrated for 20 min. in the Ultrasonic MSE "Mulard" apparatus (60 W, 20 kc), with simultaneous cooling on the ice bath. Then the suspension was added with 400 mg. of active charcoal (Carbopol H2, pH 7) and after 20 min. on the ice bath, centrifuged for 15 min. at 60 000 *g*. The clear supernatant was added with 5 ml. of 20% streptomycin sulphate (calculated for free base) and after further 20 min. added again with 200 mg. of charcoal, left for 15 min. on the ice bath and centrifuged as above. The clear supernatant was dialysed against distilled water at about 1° for 24 hr. The precipitated globulins were centrifuged off (25 min. at 75 000 *g*) and the slightly opalescent extract obtained, containing 10 - 15 mg. of protein par 1 ml. and less than 0.01% of nucleic acids, was stored at -10°. If a preparation still contained traces of coenzyme B_{12} giving a distinct reaction in the blank test, it was added again with 200 mg. of charcoal and treated as described above.

Analytical assays. Corrin coenzymes were determined after KCN treatment by measuring the extinction at 580 mμ. For all corrinoid dicyanides tested, the molar extinction coefficient 10.1×10^3 [3] was adopted.

Protein was estimated spectrophotometrically at 260 and 280 mμ [6].

Glycerol was determined photocolorimetrically [5] after its oxidation with periodide to formaldehyde (two moles of formaldehyde being formed per one mole of glycerol) and assaying formaldehyde with chromotropic acid. The standard of formaldehyde was prepared by oxidizing DL-serine under the same conditions.

β-Hydroxypropionaldehyde was determined according to Smiley & Sobolov [12] as described previously [15], the extinction being measured at 555 mμ in 1 cm. wide cuvette.

For extinction determinations, a Hilger H 700 spectrophotometer and a Bausch-Lomb Spectronic 20 photocolorimeter were used.

Determination of enzymic activity. The incubation mixture contained in 1 ml.: enzymic extract containing 0.5 - 1.5 mg. of protein, 50 μmoles of glycerol, 40 μmoles of K-phosphate buffer, pH 8, and 0.05 mμmoles of coenzyme B_{12}. The addition of coenzyme B_{12} started the reaction. In the blank sample coenzyme B_{12} was omitted. The incubation was carried out for 10 min. at 37°. The activity was expressed in extinction units of β-hydroxypropionaldehyde formed per sample. The addition of coenzyme B_{12} as well as the incubation were carried out in a darkened room. For serial determinations, a modification was introduced consisting in cooling the samples on an ice bath to about 5° before the addition of coenzyme B_{12}, and thereafter starting the reaction by transferring the samples to a water bath of a temperature of 37°; after incubation the reaction was stopped by cooling on an ice bath.

RESULTS AND DISCUSSION

Incubation of cell-free extract from *Aerobacter aerogenes* with potassium phosphate buffer and glycerol in the presence of coenzyme B_{12} resulted in formation of β-hydroxypropionaldehyde. This was

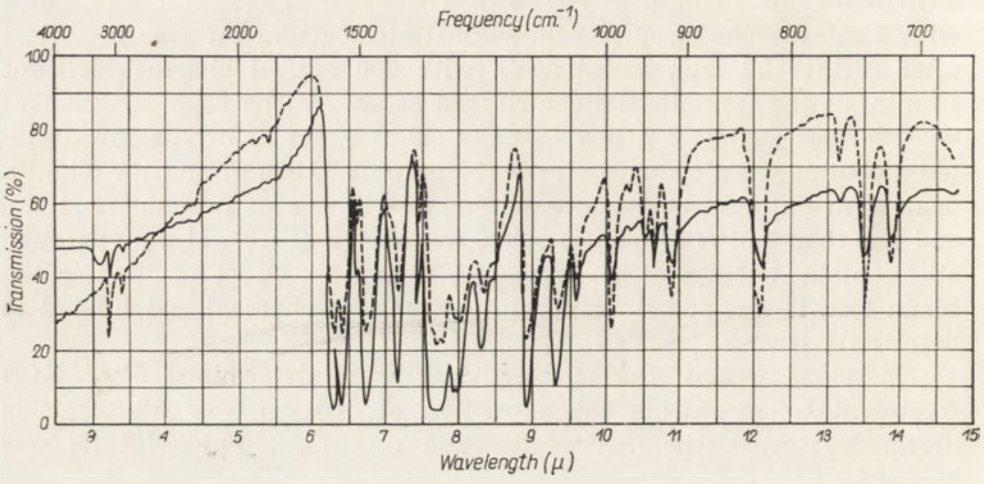


Fig. 1. Infrared absorption spectrum in KBr of 2,4-dinitrophenylhydrazone of acroleine formed from β-hydroxypropionaldehyde during incubation of glycerol with the cell-free preparation from *A. aerogenes*. The incubation mixture was heated with a solution of dinitrophenylhydrazine in 2-N-HCl to a temp. of 60° and the hydrazone formed was extracted from chloroform and crystallized. Acroleine dinitrophenylhydrazone: (- - -), isolated from the incubation mixture; (———), authentic sample.

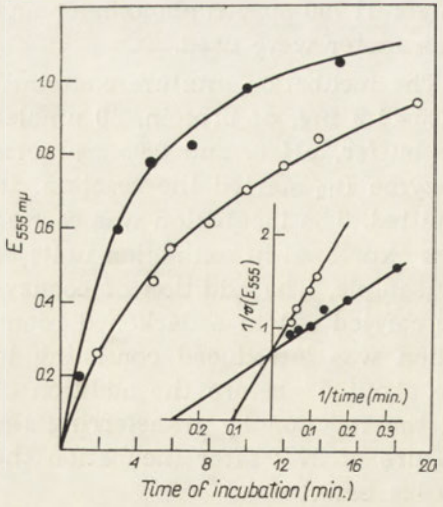


Fig. 2. The effect of time of incubation on the formation of β-hydroxypropionaldehyde from glycerol by the cell-free preparation from *A. aerogenes*. For conditions see Methods. Incubation: (○), at 27° or (●), at 37°. The plot of $1/t$ against $1/v$ is also shown.

demonstrated by transforming β-hydroxypropionaldehyde to acroleine by heating with 2 N-HCl; the acroleine formed was converted to 2,4-dinitrophenylhydrazone which was identified by melting point determination (m. p. 165°) and infrared absorption spectrum [11] (Fig. 1).

The amount of hydroxypropionaldehyde formed increased with the time of incubation but not at a linear rate (Fig. 2). The curve obtained in experiments performed at 27° had the same character suggesting that there was no thermal inactivation of the enzyme during incubation. It should be noted, however, that the plot of 1/E against $1/t$ gave a straight line. This point will be discussed later in connection with the kinetics of glycerol conversion.

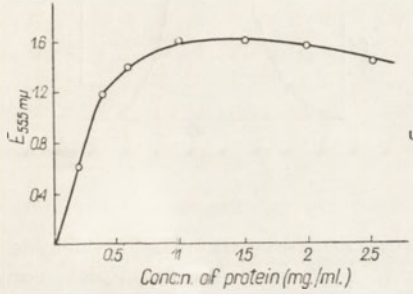


Fig. 3

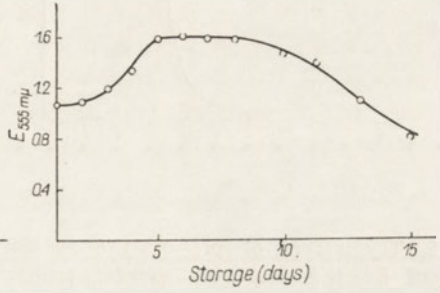


Fig. 4

Fig. 3. The effect of protein concentration on the rate of β-hydroxypropionaldehyde formation from glycerol by the cell-free preparation from *A. aerogenes*. For conditions see Methods.

Fig. 4. Changes in the enzymic activity of the cell-free preparation from *A. aerogenes* during storage at -10°. After thawing, samples containing 1.5 mg. protein were taken and the activity was determined as described under Methods.

With constant coenzyme B_{12} amount, the reaction was proportional to the concentration of protein reaching its maximum when the coenzyme became saturated with the apoenzyme. Further addition of protein did not enhance the reaction and an excess had even an inhibitory effect (Fig. 3). Therefore the amount of the protein per sample was chosen so as to obtain the optimum reaction rate in the presence of 0.05 mμmole of coenzyme B_{12}, this being 0.5 - 1.5 mg. depending on the extract preparation. The activity of preparations stored at -10° increased at first, reaching a maximum between the 5th and 8th day, and then slowly decreased (Fig. 4).

A pH optimum was found at pH 8, the activity at pH 6 being only 10% of the maximum (Fig. 5). These data differ from those obtained by

Smiley & Sobolov [12] for the enzyme extracted from *Lactobacillus* cells which had the pH optimum at pH 5.9 and was inactive at pH values exceeding 7.5.

The effect of temperature on the conversion of glycerol is shown in Fig. 6. For 10 min. incubation, the optimum was found to be at 37° and the reaction rate decreased rapidly both above and below this value.

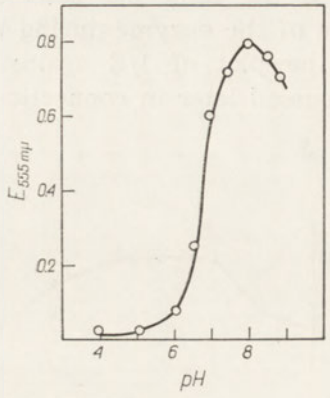


Fig. 5

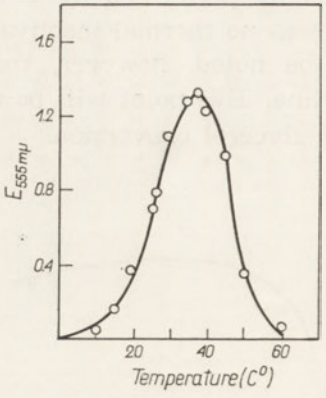


Fig. 6

Fig. 5. The effect of pH on the formation of β-hydroxypropionaldehyde from glycerol by the cell-free extract from *A. aerogenes*. The samples contained 40 M-potassium phosphate buffer of the indicated pH values. For details see Methods.

Fig. 6. The effect of temperature on the formation of β-hydroxypropionaldehyde from glycerol by the cell-free extract from *A. aerogenes*. For conditions see Methods.

The amount of the buffer affects the reaction rate, the optimum being found at a concentration of potassium phosphate exceeding 30 mM (Fig. 7). In tris buffer without the addition of K^+ ion the reaction did not occur. Addition of K^+ ion activated the enzyme which, however, even at a concentration of 100 mM-KCl, did not attain an activity equal

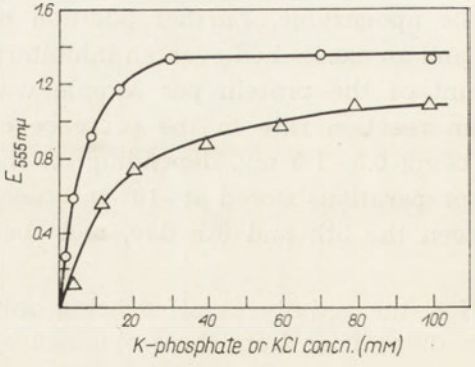


Fig. 7. The effect of concentration of (○), potassium phosphate buffer and (△), KCl concentration in tris buffer, on the formation of β-hydroxypropionaldehyde from glycerol by the cell-free extract from *A. aerogenes*. pH 8.0, for details see Methods.

Table 1

The effect of univalent cations on the enzymic conversion of glycerol into β-hydroxypropionaldehyde

Standard incubation mixture, in which 40 μmoles of K-phosphate buffer were replaced by 10 μmoles of tris buffer, pH 8.0, was incubated with the addition of 20 μmoles of chloride of the indicated cation. For details see text. Activity is expressed in relation to the activity with K^+, taken as 100.

Cation	Activity (%)
K^+	100
Li^+	184
Na^+	0
Rb^+	95
NH_4^+	151

to that in potassium phosphate. K^+ ion could be replaced by Li^+, Rb^+ and NH_4^+; the Li^+ and NH_4^+ ions being even more effective whereas Na^+ had no effect (Table 1). The effect of K^+ and other univalent cations on the enzymic system from *Lactobacillus* has been reported by Smiley & Sobolov [12]. It seems possible that in the conversion of glycerol the univalent cations activate one of the constituent enzymes, similarly as it has been found for the conversion of ethylene glycol into acetaldehyde [16]; in this reaction acetaldol, the intermediate metabolite formed from glycol, is cleaved to acetaldehyde by an aldolase which requires K^+ ion and is inhibited by Na^+.

Table 2

The effect of SH-group reagents on the enzymic conversion of glycerol into β-hydroxypropionaldehyde

The SH-group reagent indicated was added to the standard incubation mixture before the addition of coenzyme B_{12}. For details see text.

Inhibitor	Concentration (mM)	Inhibition (%)
N-Ethylmaleimide	0.01	22
	0.05	35
	1.0	99
	5.0	100
p-Chloromercuribenzoate	0.01	20
	1.0	100

The reaction was inhibited by SH-group reagents, such as *N*-ethylmaleimide and *p*-chloromercuribenzoate (Table 2) which at 1 mM concentration caused a total inhibition. In these experiments, the SH-group reagents were added to the incubation mixture after the

addition of coenzyme B_{12}. If this order was reversed, the inhibition was much less marked; this seems to indicate that the SH groups of the apoenzyme are directly or indirectly involved in the coenzyme binding. The same conclusion has been reached by Smiley & Sobolov [12,13].

The conversion of glycerol is dependent on coenzyme B_{12}, which could be replaced by other corrinoid coenzymes. It was found previously [18] that the naturally occurring derivative of pseudovitamin B_{12} (Co-5′--deoxyadenosyladenylcobamide) was active, whereas no activity was observed with derivatives of corrinoids not possessing the nucleotide group (so-called incomplete analogues), such as the derivatives of cobinamide and cobyrinic acid. This seems to indicate a certain specificity of the enzyme(s) involved. A similar specificity, shown moreover towards the cyanide forms, has been observed in the microbiological assays with *Euglena gracilis* [7].

The effect of coenzyme B_{12} concentration on the reaction velocity can be represented by the Lineweaver-Burk plotting method as a straight line when $1/v$ is plotted against $1/[S]^2$ (Fig. 8), v being the velocity of the reaction in extinction units and S concentration of the coenzyme. This relationship may be deduced from the equation:

$$E + 2S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES_2 \xrightarrow{k_{+2}} E + P$$

and thus, assuming a steady-state

$$\frac{1}{v} = \frac{1}{V_m} + \frac{K_m}{V_m} \cdot \frac{1}{[S]^2} \tag{1}$$

where

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}}, \qquad V_m = k_{+2} E_0,$$

and E_0 total concentration of the enzyme. It has been demonstrated that equation (1) holds too for the data obtained during a 4 min. incubation period of the samples. From Fig. 8 it was found graphically that K_m is 1.3×10^{-15} M^2. These data suggest that one molecule of the apoenzyme binds with two molecules of coenzyme B_{12} which means that the enzyme has two activity centres.

Inhibition of glycerol conversion by some analogues of coenzyme B_{12} seems to confirm the bimolecular reaction between the coenzyme and apoenzyme. As it has been shown previously [15, 18], this reaction is strongly inhibited by a number of Co-substituted analogues of coenzyme B_{12} in which the 5′-deoxyadenosyl group is replaced by another deoxynucleoside or an alkyl group. The reaction inhibited by Co-5′-deoxyuridylcobalamin is shown in Fig. 9 plotted according to Dixon [4]. In this case, also a straight line was obtained provided that the concentra-

tion of the inhibitor was expressed as $[I]^2$. From Fig. 9 it appears that the uridyl analogue of coenzyme B_{12} is a competitive inhibitor and from the intercept on the abscissa the inhibition constant for this analogue was found to be K_i 7.3×10^{-14} M^2. Other analogues of coenzyme B_{12} also inhibited the reaction, Co-ethylcobalamin being one of the most potent inhibitors (K_i 3.5×10^{-16} M^2).

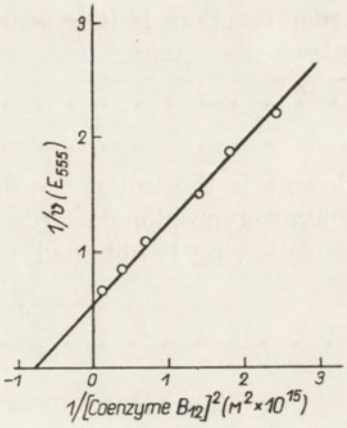


Fig. 8. The effect of coenzyme B_{12} concentration on the formation of β-hydroxypropionaldehyde from glycerol by the cell-free preparation from *A. aerogenes*. For conditions see Methods.

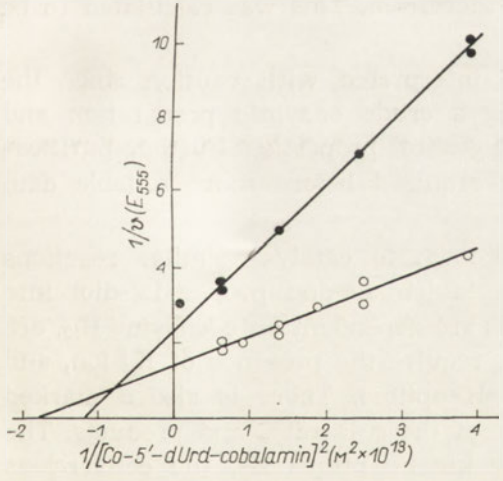


Fig. 9

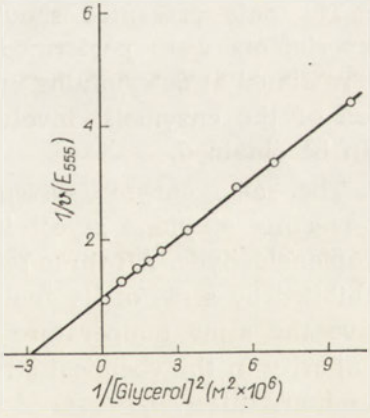


Fig. 10

Fig. 9. Competitive inhibition of conversion of glycerol into β-hydroxypropionaldehyde by Co-5′-deoxyuridylcobalamin, plotted according to Dixon [4]. Concn. of coenzyme B_{12} per 1 ml. of the incubation mixture: (○), 0.0125 mμmoles; (●), 0.0250 mμmoles. For details see Methods.

Fig. 10. The effect of glycerol concentration on β-hydroxypropionaldehyde formation by the cell-free preparation from *A. aerogenes*. For details see Methods.

The presence of two activity centres in the enzyme seems to be supported by the experiments on the effect of glycerol concentration on the rate of hydroxypropionaldehyde formation (Fig. 10). Also in this case the Lineweaver-Burk plot of $1/v$ against $1/[S]^2$ gave a straight line and the value of K_m 3.6×10^{-6} M^2 was found graphically. The data concerning the effect of the time of reaction on the amount of hydroxypropionaldehyde formed (cf. Fig. 2) also seem to confirm the second-order reaction. If it is assumed that the velocity of glycerol conversion follows the equation

$$\frac{dx}{dt} = k\,(a - x)^2 \tag{2}$$

where a is the initial concentration of glycerol and x the concentration of hydroxypropionaldehyde formed during time t, then after integration the following is obtained

$$kt = \frac{a}{a\,(a - x)} \quad \text{that is} \quad \frac{1}{x} = \frac{1}{ka^2} \cdot \frac{1}{t} + \frac{1}{a} \tag{3}$$

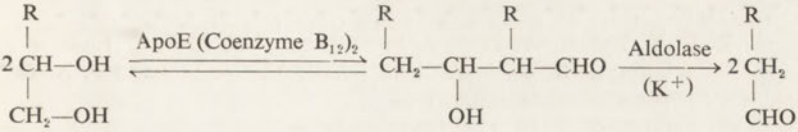
From equation (3) it appears that the plot of $1/x$ against $1/t$ should give a straight line, which is in agreement with the experimental data (Fig. 2). From this plot it is possible to determine the velocity constants k for the reactions taking place at two different temperatures, and the activation energy of the reactions therefrom. This was calculated to be 12.5 Kcal/mole.

The data presented should be interpreted with caution since the experiments were performed using a crude enzymic preparation and were aimed at determining only its general properties. Further purification of the enzyme(s) involved is required before more reliable data can be obtained.

The same enzyme system is known to catalyse similar reactions converting ethylene glycol into acetaldehyde and propan-1,2-diol into propionaldehyde. All three reactions are dependent on coenzyme B_{12}, are inhibited by some of its analogues, require the presence of K^+ ion, and have the same temperature and pH optima. There is also a marked similarity in the chemical structure of the substrates and products. The reactions differ, however, in their kinetic properties, the conversions of diols being reactions of first order [18] whereas the conversion of glycerol is a reaction of second order.

The similarities in the enzymic conversion of diols and glycerol permit to assume that their mechanisms as well may be very similar or identical. It has been previously demonstrated [16] that the conversion of ethylene glycol is a complex reaction catalysed by two enzymes, with acetaldol being formed as the intermediate product. It seems probable

that the conversion of glycerol may follow the same pattern; thus the enzymic system studied would catalyse the following reactions



where R, is CH_2OH, H, or CH_3. In the first reaction two molecules of the substrate would be converted into one molecule of a corresponding aldol. In the second reaction the aldol would be cleaved by aldolase into two molecules of aldehyde (hydroxypropionaldehyde, acetaldehyde or propionaldehyde). The order of the overall reaction is determined by the slower reaction. In the conversion of glycerol, this may be the second-order reaction of glycerol conversion into the aldol of hydroxypropionaldehyde, whereas in the conversion of diols, the reaction catalysed by aldolase. In agreement with the experimental data it seems also possible to advance a suggestion concerning the structure of the coenzyme B_{12}-dependent enzyme. It seems possible that this enzyme possesses two active centres with two coenzyme B_{12} molecules and that these centres are in close vicinity. At each of them the substrate could be converted into the corresponding aldehyde and the two adjacent molecules would immediately undergo aldolization, forming the intermediate product of the reaction. This suggestion requires confirmation, and further experiments are in progress.

The authors wish to thank Prof. Dr. M. Wiewiórowski from the Department of Organic Chemistry of the University in Poznań, for performing the infrared spectral analyses.

SUMMARY

A cell-free extract catalysing the coenzyme B_{12}-dependent conversion of glycerol into β-hydroxypropionaldehyde was obtained from *Aerobacter aerogenes* cells grown on a glycerol-containing medium. The optimum pH for the reaction was 8.0, the optimum temperature 37°. The reaction required K^+ ion which could be replaced by Li^+, Rb^+ or NH^+_4 ions but not with Na^+. The reaction was inhibited by SH-group reagents and inhibited competitively by some synthetic analogues of coenzyme B_{12}. Kinetic studies indicated that the conversion of glycerol is a reaction of second order, the K_m value being 3.6×10^{-6} M^2. It has been demonstrated that one molecule of the apoenzyme binds two molecules of coenzyme B_{12}. The K_m value for coenzyme B_{12} was found to be 1.3×10^{-15} M^2. It seems that the enzyme has two active centres and that the conversion of glycerol may take place in two steps catalysed by two enzymes.

REFERENCES

[1] Abeles R. H., Brownstein A. M. & Randles C. H. - *Biochim. Biophys. Acta* **41**, 530, 1960.
[2] Abeles R. H. & Lee H. A. Jr. - *J. Biol. Chem.* **236**, 2347, 1961.
[3] Barker H. A., Smyth R. D., Weissbach H., Toohey J. I., Ladd J. N. & Volcani B. E. - *J. Biol. Chem.* **235**, 480, 1960.
[4] Dixon M. - *Biochem. J.* **55**, 170, 1953.
[5] Frisell W. R., Meech L. A. & Mackenzie C. G. - *J. Biol. Chem.* **207**, 709, 1954.
[6] Kalckar H. M. - *J. Biol. Chem.* **167**, 461, 1947.
[7] Kon S. K. & Pawełkiewicz J., *Proc. Fourth Intern. Congress Biochem. Vienna, 1958*, Vol. 11, p. 115. Pergamon Press, London 1960.
[8] Pawełkiewicz J., Bartosiński B. & Walerych W. - *Acta Biochim. Polon.* **8**, 131, 1961.
[9] Pawełkiewicz J. & Zagalak B. - *Ann. New York Acad. Sci.* **112**, 703, 1964.
[10] Pawełkiewicz J. & Zodrow K. - *Acta Microbiol. Polon.* **6**, 219, 1957.
[11] Ross J. H. - *Anal. Chem.* **25**, 1288, 1953.
[12] Smiley K. L. & Sobolov M. - *Arch. Biochem. Biophys.* **97**, 538, 1962.
[13] Smiley K. L. & Sobolov M. - *Ann. New York Acad. Sci.* **112**, 706, 1964.
[14] Voisenet E. - *Ann. Inst. Pasteur* **28**, 807, 1914.
[15] Zagalak B. - *Acta Biochim. Polon.* **10**, 387, 1963.
[16] Zagalak B. & Pawełkiewicz J. - *Life Sciences* 395, 1962.
[17] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **9**, 315, 1962.
[18] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **11**, 49, 1964.
[19] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **12**, 103, 1965.
[20] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **12**, 219, 1965.

ENZYMATYCZNE PRZEKSZTAŁCENIE GLICEROLU W ALDEHYD β-HYDROKSYPROPIONOWY W BEZKOMÓRKOWYM UKŁADZIE Z *AEROBACTER AEROGENES*

Streszczenie

Z komórek *A. aerogenes* hodowanych na pożywce z glicerolem izolowano i opisano układ enzymatyczny katalizujący zależną od koenzymu B_{12} reakcję przekształcenia glicerolu w aldehyd β-hydroksypropionowy. Optymalne pH reakcji wynosi 8.0, temperatury 37°. Reakcja wymaga obecności jonu potasowego, który można zastąpić jonem Li^+, Rb^+ lub NH^+_4, lecz nie jonem sodowym. Reakcja jest hamowana przez odczynniki reagujące z grupami sulfhydrylowymi, jak również kompetycyjnie przez niektóre syntetyczne analogi koenzymu B_{12}. Badania kinetyczne wykazały, że przemiana glicerolu jest reakcją drugiego stopnia. Wartość K_m dla glicerolu wynosi 3.6×10^{-6} M². Cząsteczka apoenzymu łączy się z dwiema cząsteczkami koenzymu B_{12} i K_m wynosi 1.3×10^{-15} M². Postawiono hipotezę, że enzym zawiera dwa miejsca aktywne i że reakcja przemiany glicerolu przebiega w dwu etapach, katalizowanych przez dwa różne enzymy.

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Note added in proof. Recent observations have shown that the enzyme system transforming glycerol into β-hydroxypropionaldehyde consists of one enzyme only which itself is composed of two different proteins. These proteins appeared to be inactive separately.

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SYNTHESIS AND PROPERTIES OF Co-ADENINE NUCLEOSIDE ANALOGUES OF COENZYME B_{12}*

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The Co-substituted analogues of coenzyme B_{12} may permit to study the relation between the chemical structure and activity of coenzyme B_{12}. As the analogues which instead of the 5′-deoxyadenosyl group of coenzyme B_{12} possess the alkyl, acyl or other 5′-deoxynucleoside groups, have been found to be inactive in the enzyme systems tested and even to inhibit the coenzyme B_{12}-dependent reaction (see ref. [11]), it was concluded that the intact structure of the adenine nucleoside is essential for the coenzymic activity. However, it seemed possible that by some modifications in the adenosyl group active analogues may be obtained permitting to study more closely which structural elements of the deoxyadenosyl group are essential for the coenzymic activity.

Previously [12] the 1-methyladenosine and N^6-methyladenosine analogues were tested. In the present work, the 2′-deoxyadenosine, 3′-deoxyadenosine and isoadenosine (3-*N*-β-D-ribofuranosyladenine)[1] analogues were synthesized and their properties examined.

MATERIALS AND METHODS

Coenzyme B_{12} was synthesized from cyanocobalamin as previously described [11] or by a procedure described in this paper. Cyanocobalamin was isolated from *Propionibacterium shermanii* cultures and crystallized from aqueous acetone [10]. 2′-Deoxyadenosine was a Schuchardt (Munich, Germany) product. 3′-Deoxyadenosine was a gift from Dr. H. Kle-

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[1] Abbreviations used: Co-5′-d(2′-dAdo)-cobalamin, 2′-deoxyadenosine analogue of coenzyme B_{12}: Co-5′-deoxy(2′-deoxyadenosyl)-α-(5,6-dimethylbenzimidazolyl)-cobamide; Co-5′-d(3′-dAdo)-cobalamin, 3′-deoxyadenosine analogue of coenzyme B_{12}; Co-5′-d(iAdo)-cobalamin, isoadenosine analogue of coenzyme B_{12}.

now (The Fibiger Laboratory, Kobenhaven, Denmark), and isoadenosine from Dr. N. J. Leonard (University of Illinois, Illinois, Urbana, U.S.A.). P-Cellulose was prepared according to Peterson & Sober [8]. Other reagents were from Fabryka Odczynników Chemicznych, Gliwice, Poland. For purification and identification of the synthesized corrinoids, paper chromatography and paper electrophoresis in a darkened room were applied. The details are given in Table 1.

The amount of the corrin coenzymes was assayed in the cobalamin dicyanide form by spectrophotometry at 580 mμ. The molar extinction coefficient 10.1×10^3 according to Barker [1] was adopted for calculations. The absorption spectra and extinction determinations were made in a Hilger H 700 spectrophotometer in 10 mm. wide cuvettes. After chromatography or electrophoresis, the corrinoids to be submitted to densitometric measurements were exposed on paper to direct sunlight, and after photolytic degradation of the coenzymic form kept under a glass cover containing hydrogen cyanide, to convert hydroxycobalamin into cyanocobalamin. Densitometric analyses were performed in the Locardt Co. (London, England) apparatus, and the approximate percentage content of the individual corrinoids was determined planimetrically.

The enzymic extract from *Aerobacter aerogenes* (PZH, Warszawa, strain no. 572) was prepared according to the method recently described [7]. The enzyme assays were performed in a darkened room. In the conversion of glycerol, β-hydroxypropionaldehyde was assayed according to Smiley & Sobolov [9] and in the conversion of propan-1,2-diol propionaldehyde was assayed by the method of Böhme & Winkler [2].

RESULTS AND DISCUSSION

Synthesis and properties of the analogues

The synthesis of the nucleoside analogues of coenzyme B_{12} is based on the alkylation of reduced cyanocobalamin with 5′-tosyl derivative of the nucleoside. To obtain the 5′-tosylnucleoside, the hydroxyl groups on C'_2 and C'_3 are blocked. After binding of the nucleoside to the cobalt atom of the corrin, the blocking groups are removed. Johnson *et al.* [5] reported, however, that the adenosine, uridine and inosine analogues may be synthesized without prior blocking of the 2′,3′-hydroxyl groups but the details of the procedure were not given and the yield was rather low. Nevertheless in this way they were able to obtain the 2′-deoxyadenosyl analogue and they described its chromatographic and electrophoretic properties. In our experiments it was found that hydrolytic removal of the blocking propylidene group from the isoadenosyl analogue of coenzyme B_{12} resulted in the decomposition of this compound.

Therefore, taking into account the experiments of Johnson *et al.*, a method was elaborated which permitted to obtain from cyanocobalamin with good yield the coenzyme B_{12} and its analogues.

Several experiments were performed to synthesize coenzyme B_{12} by tosylating directly adenosine in a pyridine solution and treating the obtained product with reduced cyanocobalamin. Satisfactory results were obtained by the following procedure, which was then applied for the synthesis of analogues of coenzyme B_{12}.

Finely ground adenosine was dried for 10 hr. at 105° at 0.1 mm. Hg over P_2O_5 and 40 mg. (0.15 m-mole) was dissolved in 1 ml. of anhydrous pyridine (distilled over KOH). The mixture was gently warmed to accelerate the dissolution of adenosine, then cooled in a salt-ice bath to about -10° and 28 mg. (0.15 m-mole) of *p*-toluenesulphonyl chloride was added. After 10 min. in the cold, the vessel was put into a desiccator over KOH to avoid moisture and left for 16 hr. at room temperature. Then the solution was filtered, pyridine was evaporated at room temperature under reduced pressure and the sirupy residue was dissolved in 2 ml. of methanol. Simultaneously 10 mg. (7 μmoles) of cyanocobalamin was dissolved in 0.5 ml. of 10% ammonium chloride solution and reduced with Zn and Mg powder as described by Zagalak & Pawełkiewicz [11]. The cobalamin hydride obtained was added twice at a 10 min. interval with 0.2 ml. (30 μmoles) of the methanolic solution of 5′-tosyladenosine. Five minutes after the second addition, the mixture was added with water, filtered, and the solution adjusted to pH 5. The corrinoids were extracted with a mixture of phenol and trichloroethylene (1:1, v/v), then re-extracted to water by adding 10 volumes of an ethyl ether - acetone mixture (2:5, v/v) to the phenolic solution. The organic solvents were removed from the water solution under reduced pressure, the solution evaporated to a small volume and the corrinoids were separated by electrophoresis and paper chromatography [11]. From densitometric measurements it was found that 40 - 50% of the present corrinoids represent a compound with physico-chemical and enzymic properties of the natural coenzyme B_{12}.

The same procedure has been applied for the synthesis of coenzyme B_{12} analogues. 2′-Deoxyadenosine was dried for 14 hr. at 80° over P_2O_5 at 0.1 mm. Hg, cobalamin hydride prepared from 50 mg. of cyanocobalamin being used for the synthesis. In relation to cyanocobalamin, the yield of Co-5′-d(2′-dAdo)-cobalamin was 30%. Co-5′-d(3′-dAdo)-cobalamin was obtained in a similar way and with the same yield. For the synthesis of the isoadenosyl analogue, isoadenosine dried for 16 hr. at 65° over P_2O_5 at 0.1 mm. Hg was used; the yield was only 15%.

The presented method for the synthesis of coenzyme B_{12} analogues gave smaller yields than the methods using tosylnucleosides with

blocked hydroxyl groups, but the simplicity of this procedure permits to obtain relatively quickly various analogues of coenzyme B_{12} and may be very useful for investigating corrin coenzymes. It should be mentioned that recently Hogenkamp & Oikawa [3] reported the synthesis of Co-5′-d(2′-dAdo)-cobalamin with a 65% yield but using a rather tedious and laborious procedure.

The chromatographic and electrophoretic properties of the synthesized analogues of coenzyme B_{12} are presented in Table 1. As it could be anticipated, the 2′- and 3′-deoxyadenosine analogues were more hydrofobic than coenzyme B_{12} and in the neutral solvent *B* migrated somewhat faster. In the acidic solvent *A* the isoadenosine analogue migrated most slowly indicating that this compound has a more basic character than coenzyme B_{12} and the two other adenosine analogues. The electrophoretic results point to the same conclusion, as Co-5′-d(iAdo)-cobalamin migrated somewhat faster than the other compounds tested.

Table 1

Paper chromatography and paper electrophoresis of coenzyme B_{12} and its analogues

The chromatograms were developed on Whatman no. 3 paper by the descending technique, using two solvent systems: (*A*), *n*-butanol - propan-2-ol - water - acetic acid (100:70:99:1, by vol.), and (*B*), *n*-butanol - propan-2-ol - water (10:7:10, by vol.). The electrophoresis was carried out on Whatman no. 3 paper in 1 M-acetic acid at 6 - 8 V/cm. R_B is the rate of movement of the compound in relation to that of cobinamide. The electroneutral cyanocobalamin defined the starting point.

Compound	Paper chromatography		Paper electrophoresis
	Solvent *A*	Solvent *B*	
	$R_{coenzyme\ B_{12}}$		R_B
Cyanocobalamin			0.00
Aquocobinamide cyanide			1.00
Coenzyme B_{12} (Co-5′-dAdo-cobalamin)	1.00	1.00	1.18
Co-5′-d(2′-dAdo)-cobalamin	1.13	1.22	1.18
Co-5′-d(3′-dAdo)-cobalamin	0.92	1.11	1.18
Co-5′-d(iAdo)-cobalamin	0.38	0.88	1.22

The absorption spectra of the analogues (Fig. 1) did not differ markedly from the spectrum of coenzyme B_{12}, only the main absorption maximum in the region of 260 mμ was shifted towards 266.5 mμ in the spectrum of Co-5′-d(iAdo)-cobalamin. It should be mentioned that free isoadenosine in aqueous solution has the maximum at 277 mμ [6].

The analogues studied, similarly as coenzyme B_{12}, are light-sensitive and undergo degradation when exposed to light. The colourless degradation products of 2′- and 3′-deoxyadenosyl analogues were isolated on P-cellulose columns as described previously [12]. From the products of each analogue, three colourless fractions were eluted with 0.1 N-HCl, one of the fractions being always predominant. The spectra of these

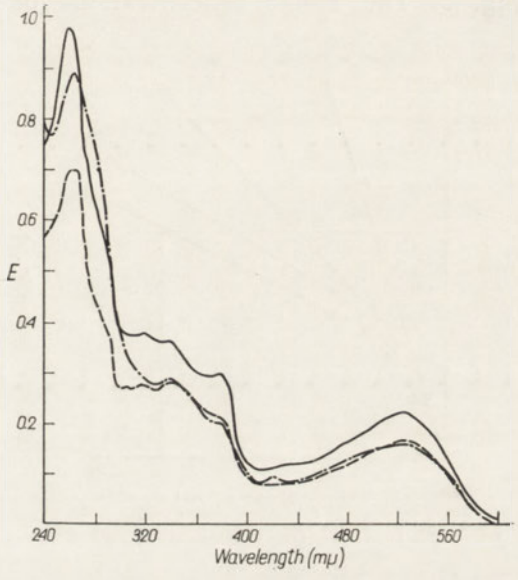

Fig. 1. Absorption spectra of coenzyme B_{12} analogues in aqueous solution. (——), Co-5′-d(2′-dAdo)-cobalamin, concn. 2.67×10^{-5} M; (- - -), Co-5′-d(3′-dAdo)-cobalamin, concn. 2.30×10^{-5} M; (— • —), Co-5′-d(iAdo)--cobalamin, concn. 1.95×10^{-5} M.

two main fractions were similar to those of N_9-substituted adenine derivatives, with absorption maxima at 258 - 260 mμ in 0.1 N-HCl, and at 262 mμ in 0.1 N-NaOH. This indicates that the photolytic degradation of the analogues occurred in the same way as for coenzyme B_{12}.

The obtained three analogues were degraded also by KCN; in the degradation products of each of them a compound was found whose spectrum was identical with that of free adenine, which was isolated as described previously [12]. From these experiments it follows that also the reaction with potassium cyanide occurs in the same way as for coenzyme B_{12}.

The amount of the material was insufficient for further determinations to be made. Nevertheless, the two degradation reactions of the coenzyme B_{12} analogues, together with the method of their synthesis, demonstrate indirectly their chemical structure.

The coenzyme B_{12} activity of the analogues

The enzymic conversion of glycerol into hydroxypropionaldehyde and of propandiol into propionaldehyde by *A. aerogenes* cell-free extracts is coenzyme B_{12}-dependent. Both these reactions have been used in our

laboratory for studying corrin coenzymes [11, 12]. Unlike the synthetic analogues obtained previously, e.g. containing uridyl, cytidyl or alkyl groups, the analogues studied in the present work were active in both these reactions.

The effect of concentration of coenzyme B_{12} and of the three analogues studied, on the conversion rate of propan-1,2-diol is shown in Fig. 2. The molar concentrations of corrinoid coenzymes at which,

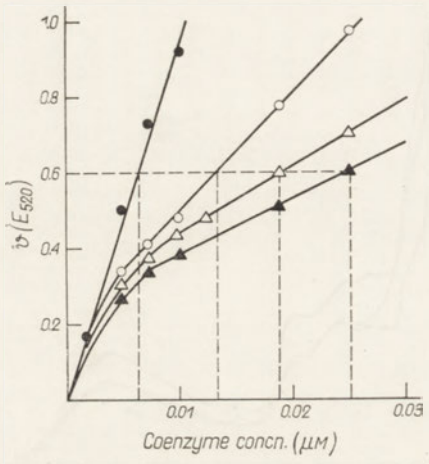

Fig. 2

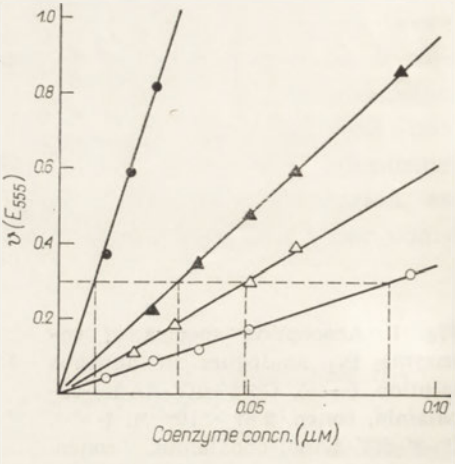

Fig. 3

Fig. 2. The effect of concentration of coenzyme B_{12} and its analogues on the rate of conversion of propan-1,2-diol into propionaldehyde. Conditions: 50 μmoles of propandiol, 40 μmoles of potassium-phosphate buffer, pH 8.0, 1.5 mg. of enzymic protein and coenzyme B_{12} or its analogue in various amounts, were incubated in a final volume of 1 ml. for 5 min. at 37°. Then the reaction mixture was cooled in an ice bath and added with 1 ml. of methanolic solution of 2,4-dinitrophenylhydrazine (50 mg. of dinitrophenylhydrazine in 50 ml. of methanol and 0.4 ml. of conc. HCl). After 30 min., were successively added: 5 ml. of a mixture of pyridine and water (4:1, v/v) and 1 ml. of methanolic solution of KOH (10 g. KOH was dissolved in 20 ml. of water and added with methanol to 100 ml.). The sample was mixed, left for 15 min., then added with water to 10 ml. and immediately the extinction at 520 mμ was determined. (●), Coenzyme B_{12}; (▲), Co-5′-d(3′-dAdo)-cobalamin; (△), Co-5′-d(2′-dAdo)-cobalamin; (○), Co-5′-d(iAdo)-cobalamin.

Fig. 3. The effect of concentration of coenzyme B_{12} and its analogues on the rate of conversion of glycerol into β-hydroxypropionaldehyde. Conditions: 50 μmoles of glycerol, 40 μmoles of potassium-phosphate buffer, pH 8.0, 1.5 mg. of enzymic protein and coenzyme B_{12} or its analogue at the concentration indicated, were incubated in a final volume of 1 ml., for 10 min. at 37°. Then 0.5 ml. of tryptophan solution (300 mg. DL-tryptophan in 100 ml. of 0.1 N-HCl) and 3 ml. of conc. HCl were added and the sample was heated on the water bath at 60° for 5 min. After cooling, the extinction was determined at 555 mμ. (●), Coenzyme B_{12}; (▲), Co-5′-d(3′-dAdo)-cobalamin; (△), Co-5′-d(2′-dAdo)-cobalamin; (○), Co-5′-d(iAdo)-cobalamin.

under the same experimental conditions, the same amounts of propionaldehyde were formed, were graphically determined. The same amount of product (E, 0.6) was formed when the concentrations of coenzyme B_{12} and of the isoadenosyl, 2′-deoxyadenosyl and 3′-deoxyadenosyl analogues were, respectively, 6.3, 13.2, 18.7, and 25.0 mμM. These data served to calculate the molar activity of the analogues in relation to coenzyme B_{12} (Table 2). The effect of concentration of coenzyme B_{12} analogues on the conversion of glycerol into hydroxypropionaldehyde, is shown in Fig. 3, and the molar activity in relation to coenzyme B_{12}, in Table 2. The analogues were found to differ in their effect on the two reactions studied. In the conversion of propandiol, Co-5′-d(iAdo)-cobalamin was the most active, and Co-5′-d(3′-dAdo)-cobalamin the least active compound, while in glycerol conversion the reverse was true. Although the mechanism of the two reactions is similar, they differ in their affinity toward corrin coenzymes. This is in agreement with the previous observations concerning the natural coenzymes [11] as well as the coenzyme B_{12} analogues methylated in the adenosyl group [12].

Table 2

Kinetic properties of coenzyme B_{12} and its analogues in the conversion of propan-1,2-diol into propionaldehyde and glycerol into β-hydroxypropionaldehyde

Values of V_m are expressed in extinction units per reaction time, 15 min. for propandiol and 10 min. for glycerol.

Coenzyme	Conversion of					
	propandiol			glycerol		
	Relative molar activity (%)	K_m (M × 10^7)	V_m	Relative molar activity (%)	K_m (M^2 × 10^{15})	V_m
Coenzyme B_{12} (Co-5′-dAdo-cobalamin)	100	1.4	14.3	100	1.3	15.4
Co-5′-d(2′-dAdo)-cobalamin	34	1.1	2.5	18	1.7	0.44
Co-5′-d(3′-dAdo)-cobalamin	25	1.1	1.7	29	1.9	0.71
Co-5′-d(iAdo)-cobalamin	48	0.6	3.3	10	2.8	0.33

The relationship between the concentration of corrin coenzymes and the reaction rate plotted in the double-reciprocal manner (Lineweaver-Burk) is presented in Figs. 4 - 5. For the formation of propionaldehyde, a straight line is obtained when $1/v$ is plotted against $1/[S]$ whereas for the formation of β-hydroxypropionaldehyde the line is straight when $1/v$ is plotted against $1/[S]^2$, in agreement with the suggestion that this is a second-order reaction. The K_m and V_m values determined graphically

are presented in Table 2. In both reactions, the K_m values were practically identical for all the corrin coenzymes studied. On the other hand, the maximum velocities (V_m) differed markedly. This seems to indicate that the analogues studied are able to form complexes with the apoenzyme as efficiently as the natural coenzyme B_{12}, whereas the catalytic activity of their complexes is much lower. The analogues of coenzyme B_{12} methylated in the adenosyl group were found to have similar properties [12].

The differences in the coenzymic activity of the analogues containing the 2′- or 3′-deoxyadenosine residues, in relation to the activity of coenzyme B_{12} may have two explanations: (1), 2′- and 3′-hydroxyl groups of adenosine may be directly involved; (2), the substitution of

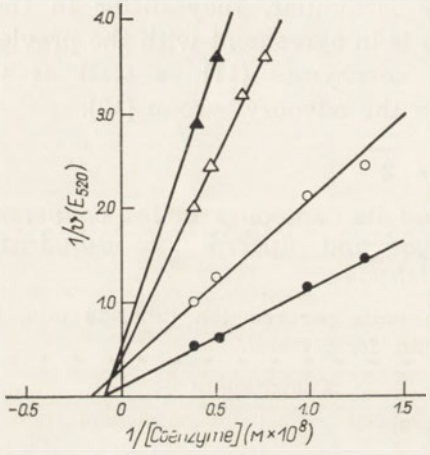

Fig. 4

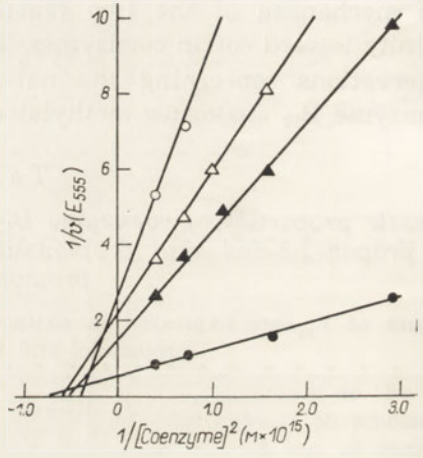

Fig. 5

Fig. 4. The Lineweaver-Burk plot for the conversion of propandiol as a function of coenzyme concentration. Conditions as for Fig. 2.

Fig. 5. The Lineweaver-Burk plot for the conversion of glycerol as a function of coenzyme concentration. Conditions as for Fig. 3.

the 2′- and 3′-hydroxyl groups by hydrogen atoms may induce a change in the conformation of the ribofuranose ring which may affect indirectly the polarization of the Co—C bond, probably essential for the coenzyme function. The latter suggestion seems to be the more probable one, all the more that the data of Jardetzky [4] concerning the conformation of riboside and deoxyriboside indicate the possibility of such changes occurring in coenzyme B_{12}. If this assumption is correct then it could be anticipated that other changes in the molecule of coenzyme B_{12} which indirectly affect the Co—C bond, would affect the coenzymic activity. In corrinoids, trans-effects have been observed in two co-ordinate sites perpendicular to the plane of the corrin ring. For instance,

the exchange of substituents in the benzimidazole could change in this way the activity of coenzyme B_{12}. Preliminary, unpublished results from our laboratory seem to support this suggestion.

The observation that the isoadenosyl analogue, in which the imino form of nitrogen N^6 prevails over the amino form, possessed the coenzymic activity is in agreement with the previous experiments [12] which indicated that the amino group of adenosine may be substituted by a related group without impairing the coenzyme B_{12} activity. Thus the amino group of adenine could be substituted by the imino group. On the other hand, the same imino form seems to prevail in the coenzyme B_{12} analogue methylated in the position N_1 of the purine ring (Co-5′-deoxy(1-methyladenosyl)-cobalamin) and this analogue was found to be inactive as coenzyme [12]. This may suggest that nitrogen N_1 plays an essential role in the catalytic function of this compound. This conclusion, advanced already in previous studies [12], is supported by the results presented in this work.

The authors wish to thank Dr. H. Klenow for a gift of the sample of 3′-deoxyadenosine, and Dr. N. J. Leonard for the samples of isoadenosine and its isopropylidene derivative.

SUMMARY

An improved method for the synthesis of coenzyme B_{12} and its analogues is described. It consists in direct tosylation of adenosine nucleoside and coupling with reduced cyanocobalamin. The yield of coenzyme B_{12} was 50%, of 2′- and 3′-deoxyadenosine analogues of coenzyme B_{12} 30%, and of isoadenosine analogue 15%. All the analogues were active as coenzymes in a system isolated from *Aerobacter aerogenes* converting propan-1,2-diol into propionaldehyde and glycerol into β-hydroxypropionaldehyde. In both reactions, the analogues differed in their activity but were consistently less active than coenzyme B_{12}. The relation between chemical structure and coenzymic function of coenzyme B_{12} is discussed.

REFERENCES

[1] Barker H. A., Smyth R. D., Weissbach H., Toohey J. I., Ladd J. N. & Volcani B. E. - *J. Biol. Chem.* **235**, 480, 1960.
[2] Böhme H. & Winkler O. - *Z. anal. Chem.* **142**, 1, 1954.
[3] Hogenkamp H. P. C. & Oikawa T. G. - *J. Biol. Chem.* **239**, 1911, 1964.
[4] Jardetzky C. D. - *J. Amer. Chem. Soc.* **83**, 2919, 1961.
[5] Johnson A. W., Mervyn L., Shaw N. & Smith E. L. - *J. Chem. Soc.* 4146, 1963.
[6] Leonard N. J. & Laursen R. A. - *J. Amer. Chem. Soc.* **85**, 2026, 1963.

[7] Pawełkiewicz J. & Zagalak B. - *Acta Biochim. Polon.* **12**, 207, 1965.
[8] Peterson E. A. & Sober H. A. - *J. Amer. Chem. Soc.* **78**, 751, 1956.
[9] Smiley K. L. & Sobolov M. - *Arch. Biochem. Biophys.* **97**, 538, 1962.
[10] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **9**, 315, 1962.
[11] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **11**, 49, 1964.
[12] Zagalak B. & Pawełkiewicz J. - *Acta Biochim. Polon.* **12**, 103, 1965.

SYNTEZA I WŁASNOŚCI Co-ADENINO-NUKLEOZYDOWYCH ANALOGÓW KOENZYMU B_{12}

Streszczenie

W pracy opisano uproszczoną metodę chemicznej syntezy koenzymu B_{12} i jego analogów. Metoda polega na bezpośredniej tozylacji nukleozydów adeniny lub pochodnej adeniny i sprzęganiu produktu reakcji ze zredukowaną cyjanokobalaminą. Koenzym B_{12} otrzymano z wydajnością 50%. Analogi zawierające reszty 2′- i 3′-dezoksyadenozyny otrzymano z wydajnością 30%, a resztę izoadenozyny z wydajnością 15%. Wszystkie analogi okazały się czynne jako koenzymy w układzie izolowanym z *Aerobacter aerogenes* przekształcającym propan-1,2-diol w aldehyd propionowy i glicerol w aldehyd β-hydroksypropionowy. Aktywności analogów są niższe od aktywności koenzymu B_{12} i zróżnicowane względem siebie, w obu przemianach. W pracy przedyskutowano zależność między aktywnością koenzymu B_{12} a jego strukturą chemiczną.

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ISOLATION AND PROPERTIES OF D-LACTATE DEHYDROGENASE FROM YEAST GROWN ANAEROBICALLY

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Three flavin-containing lactate dehydrogenases have been isolated from yeast: two from cells grown aerobically, L(+)lactate dehydrogenase [6, 4] and D(-)lactate dehydrogenase [27, 17], and one from cells grown anaerobically, D(-)lactate dehydrogenase [23, 33, 21]. It has been suggested [33, 26] that during oxygen adaptation of anaerobic yeast, D(-)lactate dehydrogenase is converted into dehydrogenases present in yeast grown aerobically. Unlike animal L-lactate dehydrogenase [30, 34], the yeast lactate dehydrogenases are not NAD-dependent and aerobic L-lactate dehydrogenase is hem-dependent.

The comparison of the three yeast dehydrogenases requires the use of pure enzymes. However, only L-lactate dehydrogenase has been obtained in crystalline form [3, 31] whereas the two D-lactate dehydrogenases have not yet been sufficiently purified [7, 28].

In this paper, a method of isolation of D-lactate dehydrogenase from yeast grown anaerobically is described and some kinetic data of the obtained preparation are presented.

MATERIALS AND METHODS

Organism. Yeast Foam (strain no. 251 - 253 from our collection) was the same as used previously [33]. The cells were cultured on a sterile medium composed of: glucose 5%, peptone 2%, KH_2PO_4 0.1%, $(NH_4)_2SO_4$ 0.12%, yeast extract 0.1%, calcium pantothenate 0.2 mg%, nicotinic acid 0.05 mg%, biotin 0.0025 mg%, Tween 80, and ergosterol (25 mg. of ergosterol and 2.5 ml. of Tween 80 were added with 96% ethanol to a final volume of 10 ml., and added to 10 litres of the medium). To obtain anaerobic conditions, CO_2 from a tank was flushed through the culture, the flask being closed with a U-shaped tube containing mercury. The yeast was grown for 48 hr. at 28°, then centrifuged in the cold. The harvested cells were washed three times with cold water and air-dried at room temperature.

Reagents. FAD, FMN, and BAL were from Fluka (Switzerland); calcium D(-)lactate was from Mann Research Lab. (New York, U.S.A.), the contents of D- and L- forms in the preparation have not been assayed; NAD was from Boerhinger (Mannheim, Germany); $ZnSO_4$, spectrally pure, from the Polytechnic School in Gliwice. Other reagents were of analytical grade and were obtained from Fabryka Odczynników Chemicznych in Gliwice, Poland. Calcium lactates were converted to sodium salts using trisodium phosphate. The organic acids used were adjusted to pH 7 with sodium hydroxide. Cytochrome *c* in a concentration of 54 μmoles per 1 ml., was isolated from horse heart muscle [29]. The preparation of calcium phosphate gel [9] contained 54 mg. dry weight per 1 ml. Phosphate, tris and borate buffers were prepared by standard methods [15], pH of the solutions being checked on an LBS I pH-meter (Eureka, Poland). Ionic strength was adjusted by adding calculated amounts of NaCl *in substantia*. Quartz sand was purified by treatment with concentrated hydrochloric acid, washed with water, dried at 100°, and the fraction passing through a 0.5 mm. mesh sieve was collected.

Protein determination. This was done by the biuret [22] or turbidimetric [22] method.

Enzymic activity determination. The reaction mixture (3 ml.) contained: 66 mM-phosphate buffer, pH 7.9; 16.6 mM-sodium DL-lactate or 8.3 mM-sodium D-lactate, an appropriate electron acceptor and varying amounts of enzyme. In most of the experiments, 0.66 mM-potassium ferricyanide was used as electron acceptor, and its reduction was determined by measuring the change of extinction at 420 mμ. The reduction of other acceptors was measured, respectively: of cytochrome *c* at 550 mμ, of NAD at 340 mμ, of 2,6-dichlorophenol indophenol at 620 mμ. The extinction was read in an SF-5 spectrophotometer (U.S.S.R.). As a unit of enzyme activity was taken that amount of enzyme which caused oxidation of 1 μmole of lactate per 1 min. per 1 mg. protein.

Michaelis constants (K_m) and maximum velocities (V) were calculated by the method of least squares from the double reciprocal form after Lineweaver & Burk [24]

$$\frac{1}{V} = \frac{K_m}{V} \cdot \frac{1}{S} + \frac{1}{V}$$

Inhibitor constants (K_i) were calculated according to Dixon [12] for competitive inhibitors by the formula:

$$K_i = \frac{[I]}{(K_p/K_m) - 1}$$

and for non-competitive inhibitors by

$$K_i = \frac{[I]}{(V/V_p) - 1}$$

K_p and V_p, the apparent Michaelis constant and maximum velocity in the presence of inhibitor, were calculated by the method of least squares.

The negative logarithmic form of ionization constants pK_1 and pK_2 were obtained from the plot of pK_m (i.e. $-\log K_m$) and $\log V$ against pH [11].

RESULTS

Purification of the enzyme

During all procedures involved in the isolation of the enzyme, the preparation was kept on ice. Sediments were obtained by centrifugation at 17 500 *g* in a Servall SS-1 centrifuge placed in a refrigerator.

Extracts of air-dried yeast cells were prepared with 66 mM-phosphate buffer, pH 7.4, by one of the four following procedures. (1), Autolysis: the suspension of yeast, containing 3.5 ml. of buffer per 1 g. of cells, was incubated for 2.5 hr. at 37° on a water bath with continuous stirring. (2), Freezing and thawing: a suspension containing 7 ml. of the buffer per 1 g. of cells, was frozen with solid CO_2 and thawed with tap water. (3), Extraction of acetone-dried cells: air-dried yeast was added with a double volume of acetone at -6°, the precipitate dried in a stream of cold air, and 7 ml. of phosphate buffer was added per 1 g. of the powder. (4), Disruption of cells by grinding with quartz sand: yeast was ground with sand, 7.0 ml. of phosphate buffer being added per 1 g. of cells. The activity and the yield of the extracts are presented in Table 1. The greatest yields were obtained by freezing and thawing and by extraction of acetone-dried cells; the latter extracts, however, were unstable and during purification became inactivated. Therefore in further experiments only the freezing and thawing procedure was used.

Table 1

Extraction of D-*lactate dehydrogenase from anaerobic yeast with phosphate buffer,* pH 7.4

Details of the procedure as described in the section Purification of the enzyme.

Method of extraction	Protein (mg./ml.)	Activity (μmole lactate oxid./min./mg. protein)	Yield (μmole lactate oxid./min./g. dry yeast)
Autolysis	33	0.17	15
Freezing and thawing	45	0.22	54
Extraction from acetone-dried yeast	22	0.40	53
Grinding with quartz sand	21	0.08	7

The yeast extract was added with trypsin to 1% final concentration and incubated for 30 min. at 37°. The digestion of protein and the increase in specific activity during incubation with and without trypsin are shown in Fig. 1. After trypsin digestion, calcium phosphate gel was added in the proportion of 0.5 mg. of dry weight per 1 mg. of the original amount of protein in the extract, and left for 15 min. at room temperature. After centrifuging, the supernatant was treated with solid ammonium sulphate. The fraction precipitated at 0.35 - 0.75 saturation was centrifuged and the sediment dissolved in 66 mM-phosphate buffer, pH 7.4. The solution was added with sodium DL-lactate and $ZnSO_4$ to a final concentration of 50 mM and 0.01 mM, respectively, heated on a water bath for 3 min. at 53°, cooled rapidly and centrifuged. The

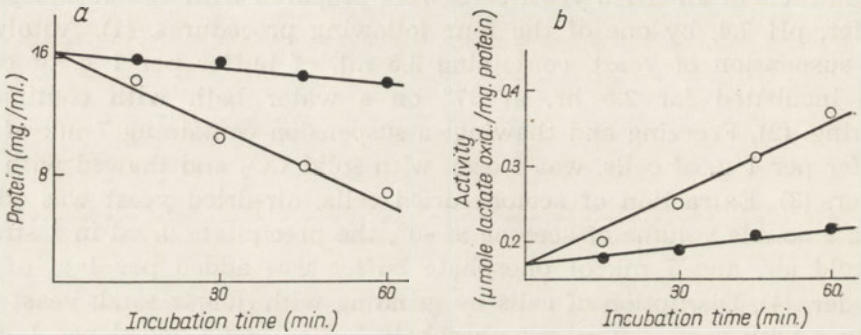

Fig. 1. The effect of trypsin (*a*), on the amount of protein in yeast autolysate and (*b*), on D-lactate dehydrogenase activity. (●), With no trypsin added (control); (○), after addition of trypsin to 1% final concentration.

supernatant was frozen at -18°, then, after thawing, trypsin was added again to a final concentration of 0.2% and the mixture incubated successively for 15 min. at 37°, for 5 min. at 42° and for 5 min. at 47°. After cooling, calcium phosphate gel was added as above, the mixture centrifuged, and the supernatant fractionated with solid ammonium sulphate. Usually the fraction precipitating at 0.50 - 0.55 saturation contained the bulk of the enzyme and was used for further purification. Since, however, slight deviations were sometimes observed, the enzymic activity was determined also in the neighbouring fractions. The active fraction was collected by centrifugation, dissolved in phosphate buffer as described above, and after addition of sodium DL-lactate to a final concentration of 50 mM, was heated for 3 min. at 53°, cooled and centrifuged. The supernatant was divided into small samples which were frozen and stored at -18° until used for experiments. The course of isolation of the enzyme is shown in Table 2.

Table 2

Purification of D-lactate dehydrogenase

Ten grams of air-dried yeast cells were extracted and treated as described in the section Purification of the enzyme.

Stage	Volume (ml.)	Total activity	Protein (mg./ml.)	Activity (μmoles lactate oxid./min./mg. protein)	Purification	Yield (%)
Extraction with phosphate buffer	50	300	30	0.2	1	100
Digestion with trypsin and gel adsorption	55	275	10	0.5	2	91
Ppt. at 0.35 - 0.75 $(NH_4)_2SO_4$ sat.	15	315	21	1.0	5	105
Heating at 53°, 3 min.	12	240	4	5.0	25	80
Second digestion with trypsin and gel adsorption	14	224	2	8.0	40	74
Ppt. at 0.50 - 0.55 $(NH_4)_2SO_4$ sat.	3	140	3	15.5	77	46
Second heating at 53° 3 min.	3	93	1	31.0	155	31

Properties of the purified enzyme

Substrate specificity. The purified dehydrogenase preparation in the presence of potassium ferricyanide as electron acceptor oxidized D-lactate, the K_m value being 0.9 mM. When DL-lactate was used as substrate, the calculated K_m for D-lactate was also 0.9 mM. Among the other substrates tested, D-malate (K_m 0.7 mM) and DL-hydroxybutyrate (K_m 3.5 mM) were oxidized whereas *meso*-tartrate, 3-phosphoglycerate, ethanol and acetaldehyde were not oxidized. In the oxidation of D-lactate, ferricyanide could be replaced by dichlorophenol indophenol but not by cytochrome c or NAD. Moreover, $NADH_2$ was not oxidized in the presence of pyruvate.

The K_m values at 8.3 mM-D-lactate and 16.6 mM-DL-lactate concentrations, were found to be 5 μM for ferricyanide and 2 μM for dichlorophenol indophenol.

The enzymic oxidation of D-lactate in the presence of 0.66 mM-ferricyanide is a zero-order reaction (Fig. 2) as the K_m value of ferricyanide is much lower than the concentration used. The oxidation of D-lactate by the dehydrogenase under the conditions of the experiments corresponded to the reaction:

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P$$

that is to the classical one-substrate kinetics of Briggs-Haldane [8] and not to the kinetics of a two-substrate reaction [1] commonly found for oxidoreductases.

The effect of pH. Using D-lactate as substrate, it was found that the optimum for the enzyme activity was at pH 8.6 - 8.9 (Fig. 3) and that the enzyme was more stable in alkaline medium. The effect of lactate concentration was tested at different pH values, and the Michaelis constants and maximum velocities were calculated. From the plot of log V

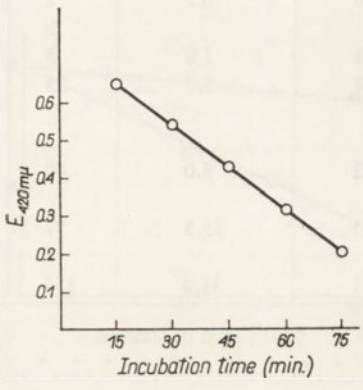

Fig. 2

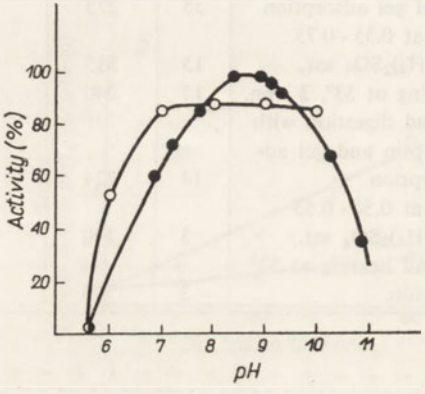

Fig. 3

Fig. 2. Enzymic oxidation of D-lactate in the presence of ferricyanide as electron acceptor. In a sample (3 ml.), 10 μl. of enzymic preparation was present. Details of the procedure as described in the section Materials and Methods.

Fig. 3 The effect of pH on the activity and stability of D-lactate dehydrogenase from anaerobic yeast. (●), Activity depending on the pH value of the medium; (○), activity of the enzyme at pH 7.9 after 30 min. incubation at the indicated pH.

and pK_m against pH (Fig. 4) the pK values of the enzyme were calculated [11] to be pK_1 7, and pK_2 10.6. These values were confirmed by the values obtained when the reciprocal of Michaelis pH function was plotted against pH [13] as shown in Fig. 5.

When the phosphate buffer was replaced by borate buffer at the same concentration, the activity of the enzyme was about 8% lower and in tris buffer 68%. The effect of ionic strength is shown in Fig. 6, the optimum being at I 0.165.

The effect of temperature. To determine the amino acids that correspond to the pK values found, the effect of temperature on the

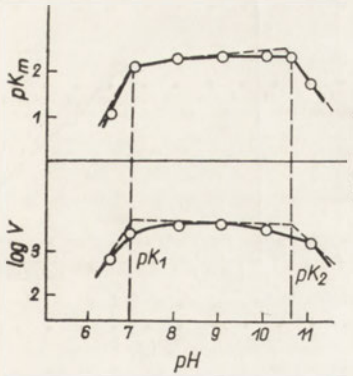

Fig. 4

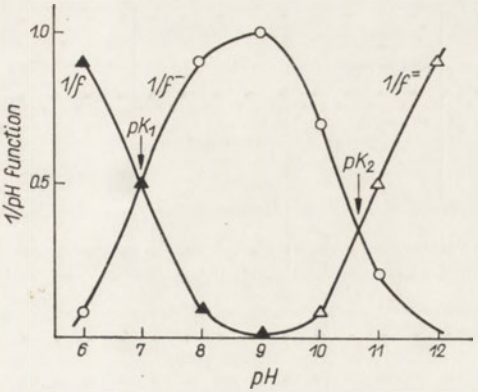

Fig. 5

Fig. 4. The effect of pH on pK_m and log V of D-lactate dehydrogenase from anaerobic yeast.

Fig. 5. Variation of the reciprocals of the Michaelis pH functions with pH. D-Lactate dehydrogenase (▲), in non-ionized form; (○), with one ionizing group dissociated; (△), with two ionizing groups dissociated.

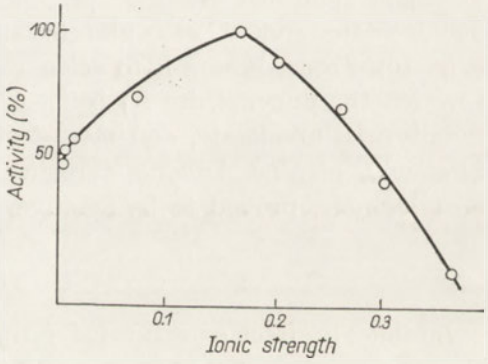

Fig. 6. The effect of ionic strength on the activity of D-lactate dehydrogenase from anaerobic yeast.

maximum velocity and Michaelis constant was studied. The ionization energy (Fig. 7) corresponding to the amino acid situated at the active site of the enzyme was calculated by the formula [16]

$$\Delta H = 2.3\,R\,\frac{dpK}{d(1/T)}\,.$$

For pK_1, ΔH was about 6000 cal. and for pK_2 about 5600 cal.

The activation energy of the enzyme studied, calculated from the Arrhenius plot shown in Fig. 8, was 21 700 cal. per mole.

Inhibitors. One-carboxylic acids and oxalic acid appeared to be competitive inhibitors (Fig. 9) and among them oxalic acid was found to

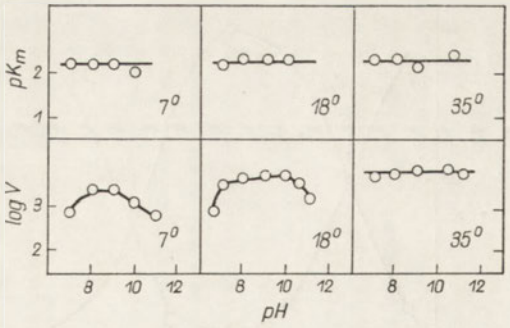

Fig. 7

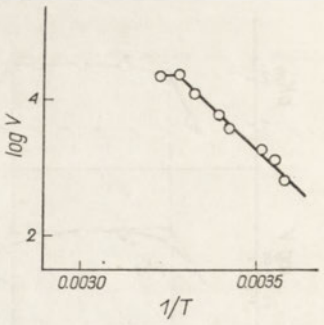

Fig. 8

Fig. 7. The effect of temperature on pK_m, and log V, at different pH values. The temperature of the reaction mixture was obtained by placing the spectrophotometric cells in a double-walled container connected with a Hoepler thermostat.

Fig. 8. The effect of temperature on the activity of D-lactate dehydrogenase from anaerobic yeast. (○), Arrhenius plot, log V of D-lactate conversion as a function of reciprocal of temperature (Kelvin scale).

have the lowest K_i value (Table 3). The inhibitory activity of the acids was not abolished by the presence of an α-keto group; pyruvate, oxaloacetate and α-ketoglutarate also acted as competitive inhibitors (Fig. 10). From these α-keto acids, oxaloacetate had the greatest affinity towards the enzyme, its K_i being 0.7 mM. The hydroxyacids, DL-hydroxybutyrate, DL-malate, and *meso*-tartrate had no effect on the reaction. Similarly, alanine, leucine, tyrosine, glycerol and ethanol, as well as acetaldehyde, proved to be inactive.

Table 3

Inhibitory effect of saturated fatty acids and ketoacids on D-*lactate dehydrogenase from anaerobic yeast*

K_m of D-lactate was 0.9 mM. The acids were added as sodium salts.

Addition	Concn. of compound added (mM)	K_p (mM)	K_i (mM)	r
Acetate	12	10	1	1.2
Butyrate	3	4	0.8	1.0
Octanoate	0.15	3.5	0.16	0.9
Tetradecylate	0.1	2.1	0.1	1.2
Oxalate	3.5	50	0.056	1.1
Pyruvate	30	21	19	1.0
α-Ketoglutarate	30	6	4.6	0.9
Oxaloacetate	2	3.4	0.7	1.1

The number r of molecules of the inhibitor which are bound with the enzyme ($E + rI \rightleftharpoons EIr$) may be calculated from the equation

$$r = \frac{\log\left(\frac{v_0}{v_i} - 1\right) - C}{\log I}, \quad \text{where } C = \log \frac{K_m}{(K_m + S)K_i}$$

[5,14]. The calculated value of r varied from 0.9 to 1.2 indicating a 1:1 molar proportion (Table 3). The chelating compounds studied, EDTA, o-phenanthroline and haematoporphyrin, inhibited the activity by 50% at concentrations of 2.5, 3.4, and 0.11 mM, respectively. The effect of EDTA had a competitive character, K_i being 18 μM, and r 1; PCMB had a similar competitive effect, the calculated K_i being 7 μM, and r 1. Under the conditions used, the inhibitory activity of EDTA and PCMB did not increase with the incubation time during the activity determinations.

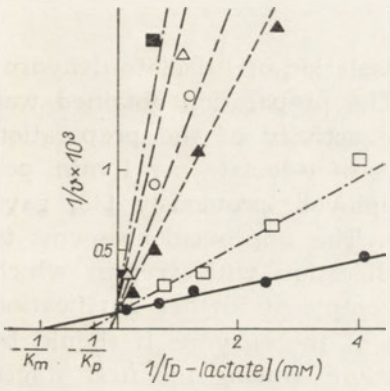

Fig. 9

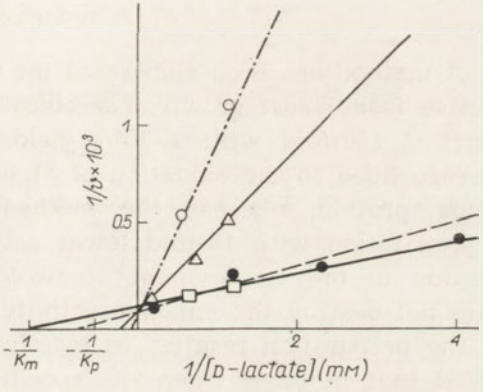

Fig. 10

Fig. 9. The effect of saturated fatty acids on the activity of D-lactate dehydrogenase from anaerobic yeast. (●), Control, no inhibitor added; with the addition of sodium salts of: (□), 0.1 mM-tetradecylate; (▲), 0.15 mM-octanoate; (○), 3 mM-butyrate; (△), 12 mM-acetate; or (■), 2 mM-oxalate.

Fig. 10. The effect of α-ketoacids on the activity of D-lactate dehydrogenase from anaerobic yeast. (●), Control, no inhibitor added; with the addition of sodium salts of: (□), 2 mM-oxaloacetate; (△), 30 mM-α-ketoglutarate or (○), 30 mM-pyruvate.

Activators. The following compounds were tested: 0.3 mM-FAD, 0.3 mM-FMN, 3 mM-NAD; GSH, GSSG, BAL, cysteine, and lipoic acid at 65 mM concentration; and $ZnSO_4$, $FeCl_2$ and $MgSO_4$ at 0.17 mM concentration. No effect of these compounds on the activity was observed

when an active enzyme preparation was used. On the other hand, when the preparation was partially inactivated, some of these compounds had an activating effect.

Dialysis of the purified enzyme preparation for 36 hr. against water resulted in an 80% decrease of activity, and the addition of FMN or FAD caused 38 and 34% reactivation, respectively. A preparation obtained from the fraction precipitated at 0 - 0.7 ammonium sulphate saturation which was 80% inactivated by warming on a water bath at 55° for 10 min., was reactivated by FAD and FMN to 48 and 32% of initial activity, respectively.

DISCUSSION

A method has been elaborated for the isolation of D-lactate dehydrogenase from yeast grown anaerobically. The preparation obtained was purified 150-fold with a 30% yield. The activity of the preparation corresponded to the oxidation of 31 μmoles of D-lactate per 1 min. per 1 mg. protein, whereas the method employed previously [33] gave a preparation with 10-fold lower activity. The improvement seems to be due to the employment of twofold digestion with trypsin which does not destroy the enzymic activity. Attempts at further purification of the preparation resulted in inactivation of the enzyme. It should be noted that in cases when the specific activity during the first stages of purification was high, in further steps a lower degree of purification was achieved.

The obtained preparation of D-lactate dehydrogenase had a specificity similar to that of the preparation of Boeri, Cremona & Singer [7]. The high activation energy found (21 700 cal. per 1 mole) and high Michaelis constant (0.9 mM) may indicate that lactate is not a natural substrate for this enzyme.

Assuming as the maximum specific activity of the enzyme the value of 100 μmoles calculated by Iwatsubo [18], it follows that in our preparation the enzyme is purified by 30%. The molecular weight of the preparation as determined kindly by Dr. Iwatsubo by the Sephadex method of Iwatsubo & Curdel [19] was found to be 100 000, and the preparation was not hydrolysed by trypsin into smaller subunits.

From the data obtained, using the Briggs-Haldane equation [8]:

$$K_m = \frac{k_{-1} + k_{+2}}{k_{+1}}$$

it is possible to determine the velocity constants of the enzymic reaction using the calculation method of Slater [32]. The three velocity constants were: $k_{-1} \cong 0$; $k_{+2} \cong 1.6 \times 10^3$; $k_{+1} \cong 1.7 \times 10^6$. These values are similar to those obtained for other oxidoreductases [32].

Saturated fatty acids (Fig. 9) and ketoacids (Fig. 10) proved to be competitive inhibitors. When the carboxyl group was replaced by a hydroxyl or aldehyde group, the inhibitory activity of the compound was abolished. These data suggest the participation of the carboxyl group of the substrate in the reaction with the enzyme. At the active site of the enzyme, the imidazole group of histidine may participate in this reaction, as indicated by the value of pK_1 7 (Fig. 4, 5) and the ionization energy of about 6000 cal. Similarly as the carboxylic acids, PCMB appeared to be a competitive inhibitor; this may indicate the participation of thiol group in the substrate-enzyme reaction. On the assumption that ferricyanide reacts with the SH groups of the

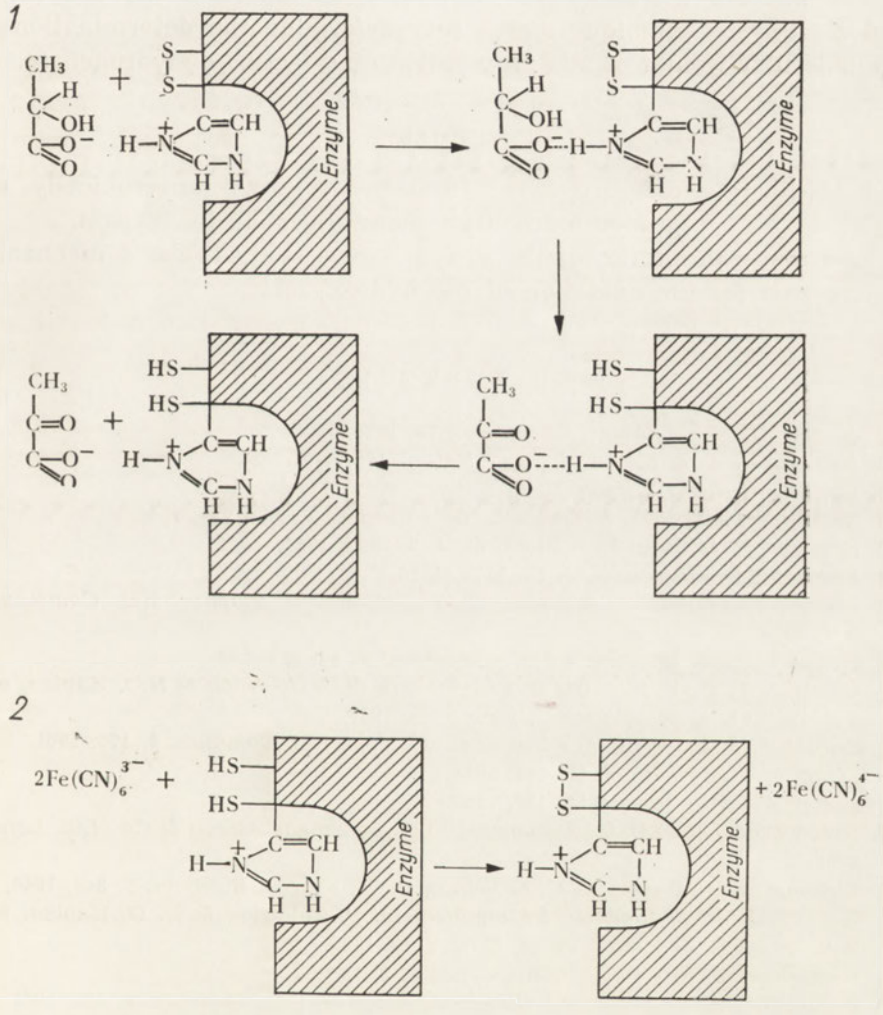

Fig. 11. Proposed mechanism of D-lactate oxidation by lactate dehydrogenase from yeast grown anaerobically, in the presence of ferricyanide as electron acceptor.

respiratory enzymes [20] it seems possible to suggest the oxidation of SH groups to S—S during the enzymic reaction. Oxidized S—S groups may undergo reversible reduction by hydrogens of α-carbon of D-lactate. In experiments *in vitro*, the mechanism of enzyme activity consists in a reversible reduction of S—S group to SH, with ferricyanide serving as electron acceptor. The proposed scheme of the reaction is shown in Fig. 11. The reduction of ferricyanide by SH groups may be inhibited by EDTA in a non-chelating manner [20]. So far, however, neither the role of anaerobic yeast D-lactate dehydrogenase nor the electron acceptors participating in the reaction, are known.

The authors wish to thank Dr. M. Iwatsubo from the Institut de Biologie Physico-chimique, Paris, for performing the determinations of the molecular weight of our preparation of D-lactate dehydrogenase.

SUMMARY

1. D(-)lactate dehydrogenase from yeast grown anaerobically was purified by an improved method involving digestion by trypsin.

2. Kinetic properties of the enzyme were studied, and a mechanism is suggested for the oxidation of D-α-hydroxyacids.

REFERENCES

[1] Alberty R. A. - *J. Amer. Chem. Soc.* **75**, 1928, 1953.
[2] Appleby C. A. & Morton R. K. - *Nature* **173**, 749, 1954.
[3] Appleby C. A. & Morton R. K. - *Biochem. J.* **71**, 492, 1959.
[4] Bach S. J., Dixon M. & Zerfas L. G. - *Biochem. J.* **40**, 229, 1946.
[5] Bergman F. & Segal R. - *Biochem. J.* **58**, 692, 1954.
[6] Bernheim F. - *Biochem. J.* **22**, 1179, 1928.
[7] Boeri E., Cremona T. & Singer T. P. - *Biochem. Biophys. Res. Commun*, **2**, 298, 1960.
[8] Briggs G. E. & Haldane J.B.S. - *Biochem. J.* **19**, 338, 1925.
[9] Colowick S. P., in *Methods in Enzymology* (S.P. Colowick & N.O. Kaplan, eds.) vol. 1, p. 91. Academic Press, New York 1955.
[10] Curdel A. & Labeyrie F. - *Biochem. Biophys. Res. Commun.* **4**, 175, 1961.
[11] Dixon M. - *Biochem. J.* **55**, 161, 1953.
[12] Dixon M. - *Biochem. J.* **55**, 170, 1953.
[13] Dixon M. & Webb E. C., *Enzymes*, p. 123. Longmans, Green & Co., Ltd, London 1958.
[14] Ebersole E. R., Guttentag C. & Wilson P. W. - *Arch. Biochem.* **3**, 349, 1944.
[15] Gomori G., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 1, p. 138, Academic Press, New York 1955.
[16] Grabowski Z. R. - *Postępy Biochemii* **8**, 3, 1962.
[17] Gregolin C. & Singer T. P. - *Biochem. Biophys. Res. Commun.* **4**, 189, 1961.
[18] Iwatsubo M. & Labeyrie F. - *Biochim. Biophys. Acta* **59**, 614, 1962.
[19] Iwatsubo M. & Curdel A. - *C. R. Acad. Sc.* **256**, 5224, 1963.

[20] Konecka A. & Tysarowski W. - *Acta Biochim. Polon.* **12**, 251, 1965.
[21] Labeyrie F., Slonimski P. P. & Naslin L. - *Biochim. Biophys. Acta* **34**, 262, 1959.
[22] Layne E., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 3, p. 447. Academic Press, New York 1955.
[23] Lindenmayer A. & Smith L. - *Fed. Proc.* **16**, 212, 1957.
[24] Lineweaver H. & Burk D. - *J. Amer. Chem. Soc.* **56**, 658, 1934.
[25] Michaelis L. & Davidsohn H. - *Biochem. Z.* **35**, 386, 1911.
[26] Nygaard A. P. - *Arch. Biochem. Biophys.* **86**, 317, 1960.
[27] Nygaard A. P. - *Biochim. Biophys. Acta* **40**, 85, 1960.
[28] Nygaard A. P. - *J. Biol. Chem.* **236**, 920, 1961.
[29] Paul K. G., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. 2, p. 749. Academic Press, New York 1955.
[30] Pfleiderer G., Jeckel D. & Wieland T. - *Biochem. Z.* **328**, 187, 1956.
[31] Rippa M. - *Arch. Biochem. Biophys.* **94**, 333, 1961.
[32] Slater E. C. - *Discuss. Faraday Soc.* **20**, 231, 308, 1955.
[33] Slonimski P. P. & Tysarowski W. - *C. R. Acad. Sc.* **246**, 1111, 1958.
[34] Takenaka Y. & Schwert G. W. - *J. Biol. Chem.* **223**, 157, 1958.

IZOLOWANIE I WŁASNOŚCI DEHYDROGENAZY D(-)MLECZANOWEJ DROŻDŻY HODOWANYCH BEZTLENOWO

Streszczenie

1. Opracowano nową metodę oczyszczania dehydrogenazy D(-)mleczanowej drożdży hodowanych beztlenowo z użyciem trypsyny.

2. Zbadano kinetyczne własności tego enzymu, na podstawie których zaproponowano schemat utleniania D-α-hydroksykwasów przez dehydrogenazę.

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L-GLUTAMINE AS DONOR OF CARBAMOYL GROUP NITROGEN FOR THE ENZYMIC SYNTHESIS OF CITRULLINE IN GREEN PEA SEEDLINGS

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Three different pathways are known for the enzymic synthesis of carbamoyl phosphate (CP). In the first one, found in micro-organisms in 1955 by Jones *et al.* [2], CP formation from carbamate and 1 mole of ATP in the presence of Mg^{2+} is catalysed by carbamate kinase (ATP: carbamate phosphotransferase, EC 2.7.2.2). The second one, found by Metzenberg *et al.* [6] in 1958 in ureotelic animals, is catalysed by carbamoyl phosphate synthase (ATP : carbamate phosphotransferase (dephosphorylating) EC 2.7.2.5) in the presence of ammonium bicarbonate, 2 moles of ATP, *N*-acetylglutamate and Mg^{2+}. Finally the third one, found in 1962 by Levenberg [4] in the common mushroom, *Agaricus bisporus*, is also a carbamoyl phosphate synthase [3] but is mediated by L-glutamine in the presence of bicarbonate, ATP and Mg^{2+}.

Preliminary experiments for the present work, done in 1962 in the laboratory of Prof. Dr. P. P. Cohen, University of Wisconsin, Madison, U.S.A., have indicated that in green pea seedlings L-glutamine could serve as a nitrogen donor for the synthesis of carbamoyl group of citrulline. Other plants were also tested, such as seedlings of the lupin, wheat, barley, cucumber and bean, but have shown only negligible activity.

In this paper an L-glutamine-mediated synthesis of citrulline by pea seedlings, has been described.

MATERIALS AND METHODS

Reagents. ATP-Na_2 (Reanal, Hungary and Schuchardt, Germany); L-ornithine-HCl and 3-phosphoglycerate Ba salt (Calbiochem., U.S.A.); for experiments the Ba salt was transferred to K salt; *N*-acetyl-DL-glu-

tamic acid and L-glutamic acid (Sigma Chem. Corp., St. Louis, U.S.A.); tris (Carl Roth, Karlsruhe, Germany). All other reagents were of Polish origin, distributed by Biuro Obrotu Odczynnikami, Gliwice.

Enzyme extracts. Green pea seeds, variety Perła Szlachetna, were soaked overnight in water and germinated on small trays covered with moist cotton wool. Plants were grown at room temperature for 10 - 14 days and the tops (4 - 5 cm. above the roots) were used for the acetone-dried powder preparation. The harvested plant tops were frozen in solid CO_2 and homogenized three times for 15 sec. at 16 000 rev./min. in a cooled Servall Omnimixer, with 7.5 volumes of acetone (cooled in solid CO_2) containing 10 mM-2-mercaptoethanol. The homogenate was quickly filtered through a Schott G-3 sintered glass and again homogenized as above with an excess of acetone (15 - 20 volumes of the initial weight of seedlings). After filtration, the sediment was washed with cold acetone and the light green material spread thinly on filter paper to allow quick evaporation of acetone. The dry preparation kept in an evacuated desiccator over P_2O_5 at 4 - 8° retained its initial enzyme activity for at least two months.

The enzyme solution was prepared just before the experiments by extraction of 100 mg. of the acetone-dried powder in ice with 10 ml. of 0.2 M-tris-HCl buffer, pH 7.6, containing 10 mM-2-mercaptoethanol, by continuous stirring for 5 min. and filtering through cotton wool.

The ATP regenerating enzyme preparation was obtained from rabbit muscle according to Racker [7] using the modification described by Ratner [8].

Incubation mixture: 0.25 ml. of the acetone-dried-powder extract, 0.25 ml. of a solution containing 50 μmoles of tris-HCl buffer, pH 7.6, and 2.5 μmoles of 2-mercaptoethanol; 0.1 ml. of a mixture containing 5 μmoles ATP, 10 μmoles L-ornithine-HCl and 15 μmoles $MgSO_4$, adjusted to pH 7.4 with 0.5 N-KOH; 0.1 ml. of a solution containing 20 μmoles of L-glutamine, 20 μmoles of $NaHCO_3$ and 10 μmoles of 3-P-glycerate; and 0.1 ml. of a solution containing 1.2 mg. of the freeze-dried ATP regenerating enzyme preparation. The mixture was made up with water to 1.1 ml. and incubated for 30 min. at 37°. The reaction was stopped by addition of 0.1 ml. of 60% $HClO_4$, then water was added to 2.6 ml., the precipitated protein was centrifuged off and 2 ml. of the supernatant were used for the determination of citrulline [1]. Control samples were prepared as above, except that the enzyme solution has been inactivated by heating at 100° in a water bath for 5 min. prior to the addition of above substrates.

Protein was determined according to Lowry *et al.* [5], 2.5 - 3.0 mg. of protein being present in 1 ml. of the acetone-dried powder extracts. Enzyme activity was expressed as mμmoles of synthesized citrulline per 1 mg. of protein in experimental conditions.

RESULTS

The synthesis of carbamoyl group was assumed to have occurred when in the incubation mixture citrulline was formed from L-ornithine in the presence of bicarbonate, glutamine and ATP, ornithine transcarbamylase being present in a sufficient amount in the acetone-dried powder extracts [9].

To ascertain the optimum conditions for the reaction studied, three buffer solutions were tried. Glycylglycine was more effective by about 20%, and phosphate buffer less effective by 20%, than tris-HCl buffer. Since supplies of glycylglycine were inadequate, all reported experiments were done in tris buffer.

The pH optimum for the assayed reaction (Fig. 1) lies between 7.4 and 7.6. Linear relation between the quantity of enzyme and citrulline formation was observed only at a narrow range up to 0.25 ml. of the extract used (Fig. 2). The enzymic activity increased proportionally to

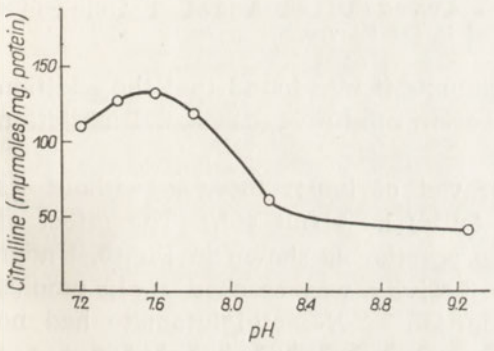

Fig. 1

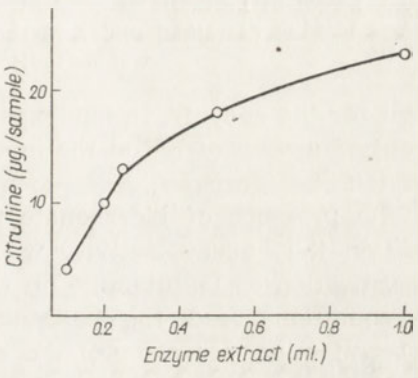

Fig. 2

Fig. 1. Effect of pH on citrulline synthesis. The incubation system was as described under Methods.

Fig. 2. Effect of concentration of acetone-dried powder extract on citrulline synthesis. The incubation system was as described under Methods except that the amount of acetone-dried powder extract was changed as indicated.

the time of incubation for up to one hour (Fig. 3) and no further increase was observed on extending the time of incubation. About 15 μmoles of Mg^{2+} per sample (1.1 ml.) were the optimum amount for the activity. Higher concentrations caused a small decline in the formation of citrulline (Fig. 4).

The effect of metal ions is shown in Table 1. The Mg^{2+} ions proved to be the most efficient activator, followed by Mn^{2+} and Co^{2+}. Nickel ions were practically inactive. The addition of Co^{2+} to Mg^{2+} ions caused a 50% drop in activity. Levenberg [4] used fluoride ion as stabilizing

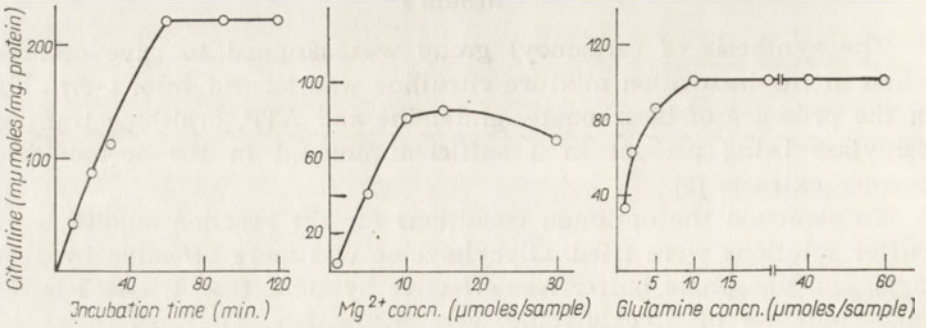

Fig. 3 Fig. 4 Fig. 5

Fig. 3. Effect of time of incubation on citrulline synthesis. The incubation system was as described under Methods, and time of incubation as indicated in the Figure.
Fig. 4. Effect of Mg^{2+} concentration on citrulline synthesis. The incubation system was as described under Methods, except that the amount of $MgSO_4$ was as indicated in the Figure.
Fig. 5. Effect of L-glutamine concentration on citrulline synthesis. The incubation system was as described under Methods except that the amount of L-glutamine was as indicated in the Figure.

agent for the enzyme. In our experiments it was found that the addition of only 3 μmoles of KF to the incubation mixture caused total inhibition of citrulline formation.

The presence of bicarbonate proved of importance, as without its addition the enzyme activity was lower by about 40%. The effect of concentration of L-glutamine on the reaction is shown in Fig. 5. Under the conditions used, the maximum activity was reached at 10 μmoles of L-glutamine per sample; the addition of *N*-acetylglutamate had no effect on citrulline formation.

Table 1

The effect of metal ions on citrulline synthesis

The incubation system contained: acetone-dried powder extract, 0.25 ml.; tris-HCl buffer, pH 7.6, 50 μmoles and 2-mercaptoethanol, 2.5 μmoles; ATP, 5 μmoles; L-ornithine, 5 μmoles; metal ions in amount as shown in Table; L-glutamine, 20 μmoles; $NaHCO_3$, 20 μmoles; 3-P-glycerate, 10 μmoles; ATP regenerating enzyme preparation, 1.2 mg.; water to give a final volume of 1.1 ml. Time of incubation 30 min. at 37°.

Addition	Concentration (μmoles/sample)	Citrulline synthesis (mμmoles/mg. protein)
None		0
Mg^{2+}	15	80
Mn^{2+}	15	20
Co^{2+}	15	10
Ni^{2+}	15	traces
$Mg^{2+}+Co^{2+}$	15+15	40

Table 2

Effect of L-*glutamine,* L-*asparagine,* NH_4^+ *and other nitrogen compounds on citrulline synthesis*

The incubation mixture was as described under Methods except that where indicated L-glutamine was replaced with 20 μmoles per sample of the indicated compound.

Compound	Citrulline synthesis (mμmoles/mg. protein)
L-Glutamine	100
L-Asparagine	30
Formamide	0
Succinate amide	0
Benzoate amide	0
Succinate imide	0
L-Glutamic acid	0
Ammonium carbamate	12
NH_4HCO_3	9
$(NH_4)_2SO_4$	12

Amides other than L-glutamine were tested as nitrogen donors (Table 2). L-Asparagine showed considerable activity but formamide, succinate amide, benzoate amide and succinate imide had practically no effect. Some activity was observed in the presence of inorganic ammonium salts. No citrulline formation was observed when L-glutamine was replaced by L-glutamic acid.

Table 3

Effect of SH-compounds on citrulline synthesis

The incubation mixture was as described under Methods except that the acetone powder was prepared with tris buffer without 2-mercaptoethanol.

Addition (5 μmoles/sample)	Citrulline synthesis (mμmoles/mg. protein)
None	70
2-Mercaptoethanol	99
SH-glutathione	97
Cysteine	60
Control, extraction with 2-mercaptoethanol	126

For the experiments on the effect of SH-containing compounds tris extracts without 2-mercaptoethanol were used, and to the incubation mixture 5 μmoles of 2-mercaptoethanol, reduced glutathione or cysteine was added and compared with the sample extracted in the presence of 2-mercaptoethanol (Table 3). 2-Mercaptoethanol and reduced glutathione

added to the incubation mixture, had an activating effect on the enzyme. It is remarkable that no such effect was observed on addition of cysteine, the reducing activity of which was tested with urease and arginase prior to this experiment.

DISCUSSION

For the synthesis of carbamoyl group of citrulline in green pea seedlings L-glutamine appears to be the favoured donor of nitrogen. As glutamate was found to be inactive, it would seem that the amide group of L-glutamine contributes the necessary nitrogen.

The results reported in this paper confirm largely the findings of Levenberg [4] on the mushroom *Agaricus bisporus*. The role of L-glutamine and Mg^{2+} as well as the optimum pH range appear to be similar both for the mushroom and green pea seedlings. However, several differences have been observed. With the mushroom enzyme Co^{2+} was twice as active as Mg^{2+} whereas with the pea seedling enzyme Co^{2+} had only 10% of the activity of Mg^{2+}. Fluoride had no stabilizing effect on the pea seedling enzyme, on the contrary the addition of 3 μmoles of KF to the incubation mixture caused total inhibition of citrulline formation. In the mushroom, only L-glutamine was effective as nitrogen donor whereas in pea seedlings L-asparagine was also utilized but the reaction was 30% of that with L-glutamine. It would therefore seem that the reaction in pea seedlings is less specific than in *A. bisporus*. Yet the results presented in this paper will have to be confirmed on plant enzyme preparation that had been further purified.

No satisfactory results were obtained with incubation systems commonly used for carbamate kinase or carbamoyl phosphate synthase (unpublished results).

I wish to express my very best thanks to Prof. Dr. P. P. Cohen who facilitated my first experiments in his laboratory in 1962 and to Dr. M. Marshall also from the University of Wisconsin, Madison, U.S.A. for the gift of 3-phosphoglycerate. I would like to express my best thanks to Prof. Dr. I. Reifer for valuable advice and discussion. The skilfull technical assistance of Miss Jadwiga Konaszewska is gratefully acknowledged.

SUMMARY

It was shown that in green pea seedlings L-glutamine is the best nitrogen donor for the formation of carbamoyl group of citrulline. L-Asparagine had 30%, and inorganic ammonium ions about 10%, of L-glutamine activity while *N*-acetylglutamate had no effect. The presence of Mg^{2+} proved to be essential. The optimum pH for synthesis was 7.4 - 7.6.

REFERENCES

[1] Archibald R. M. - *J. Biol. Chem.* **156**, 121, 1944.
[2] Jones M. E., Spector L. & Lipman F. - *J. Amer. Chem. Soc.* **77**, 819, 1955.
[3] Jones M. E. - *Science* **140**, 1373, 1963.
[4] Levenberg B. - *J. Biol. Chem.* **237**, 2590, 1962.
[5] Lowry O. H., Rosebrough N. J., Farr A. L. & Randall R. J. - *J. Biol. Chem.* **193**, 265, 1951.
[6] Metzenberg R. L., Marshall M. & Cohen P. P. - *J. Biol. Chem.* **233**, 1560, 1958.
[7] Racker E. - *J. Biol. Chem.* **167**, 843, 1947.
[8] Ratner S., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. II, p. 358. Academic Press, New York 1955.
[9] Wilkoszewska I., Kleczkowski K. & Reifer I. - *Bull. Acad. Polon. Sci., Ser. Biol.* 9, 1, 1961.

L-GLUTAMINA JAKO DONOR AZOTU W SYNTEZIE KARBAMOILOFOSFORANU W KIEŁKACH GROCHU

Streszczenie

Wykazano, że w kiełkach grochu L-glutamina jest najlepszym donorem azotu dla syntezy grupy karbamoilowej cytruliny. L-Asparagina wykazuje 30%, a nieorganiczne jony amonowe 10% aktywności L-glutaminy, podczas gdy *N*-acetyloglutaminian jest nieczynny. Aktywność układu uwarunkowana jest obecnością jonów Mg^{2+}, a optimum pH wynosi 7,4 - 7,6.

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EDTA AS INHIBITOR OF FERRICYANIDE REDUCTION BY GLUTATHIONE AND CYSTEINE

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Potassium ferricyanide is an artificial electron acceptor widely used for studying respiratory enzymes [14, 10, 12], which, being reduced by SH groups, is also used for the estimation of SH groups of proteins [4].

Three lactate dehydrogenases from yeast studied in our laboratory are known to differ, among others, in the ability to reduce ferricyanide, the lower reduction being accompanied by a lower sensitivity to *p*-chloromercuribenzoate [1] and EDTA [8, 12]. Taking these data into account, it was thought that experiments on the effect of EDTA on the reduction of artificial electron acceptors by SH compounds, may help to elucidate the mechanism of ferricyanide reduction by enzyme systems.

EXPERIMENTAL

Chemicals: Glutathione (GSH) and cysteine (CySH) were obtained from T. Schuchardt (München, West Germany), 2,3-dimercaptopropanol (BAL) from Fluka (Switzerland), β-hydroxyethyl-2,4-dinitrophenyl disulphide (HEDD) was synthesized and kindly given by Doc. Dr. S. Bitny-Szlachto. Chelax 100 was from Calbiochem. (Los Angeles, U.S.A.). Other reagents were of analytical grade, obtained from Fabryka Odczynników Chemicznych, Gliwice, Poland. The complexes of EDTA with Mg^{2+} and Cu^{2+} were prepared according to Pfeiffer & Offerman [9] and the Fe^{3+} complex according to Brintzinger *et al.* [3].

Determination of the reduction rate. The final volume of the reaction mixture was 3 ml., and a sample contained: 100 μmoles of phosphate buffer, pH 7.5; one of three electron acceptors: ferricyanide, 2.0 μmoles, Fe^{3+}-EDTA complex 390 μmoles or 2,6-dichlorophenol indophenol 0.2

[1] Abbreviations used: PCMB, *p*-chloromercuribenzoate; BAL, 2,3-dimercaptopropanol; FeCy, ferricyanide; FeY^{-1}, complex of Fe^{3+} with EDTA; DCPI, 2,6-dichlorophenol indophenol; HEDD, β-hydroxyethyl-2,4-dinitrophenyl disulphide.

μmoles; and one of six electron donors (in μmoles): glutathione 1.5, cysteine 1.0, thioglycollate 1.5, BAL 1.0, hydroquinone 1.5 (for DCPI 2.0), and vitamin C: 0.75 for FeCy, 1.0 for FeY^{-1} and 0.5 for DCPI. EDTA, when used, was added, unless otherwise indicated, in the same molar concentration as the reducing agent.

The rate of the reduction of the electron acceptor was estimated during 1 min. after the addition of the electron donor. The reduction of ferricyanide was determined by measuring the change of extinction at 420 mμ, that of indophenol at 620 mμ and the reduction of the Fe^{3+}-EDTA complex, by measuring the change of extinction at 510 mμ of the coloured compound formed by Fe^{2+} with *o*-phenanthroline [15]; for this purpose, 2.0 μmoles of *o*-phenanthroline were added after 1 min. incubation of the acceptor with electron donor and extinction was read during 1 min. The extinctions were read in an SF-5 spectrophotometer (U.S.S.R.). The inhibitory effect was expressed as percentage according to the equation: $\left(\frac{v_i}{v} - 1\right) \times 100$, and the activating effect as $\left(\frac{v}{v_a} - 1\right) \times 100$; where v_i is the reaction rate with inhibitor, v, reaction rate without inhibitor or activator, and v_a, reaction rate with activator.

Free SH groups were determined by the nitroprusside method [6], with HEDD [2], and by measuring directly the extinction at 230 or 240 mμ [11, 5].

RESULTS

The reduction of ferricyanide by GSH or CySH was inhibited by 90% in the presence of EDTA (Fig. 1), the effect of EDTA being practically instantaneous. The same degree of inhibition was observed with thioglycollate used as the reducing agent. The effect of EDTA concentration on the reduction of FeCy by GSH is shown in Fig. 2; at about 7.5 μM concentration of EDTA the inhibition was 50%.

Table 1

The effect of EDTA on the reduction of different electron acceptors

Conditions as described in the section: Determination of the reduction rate. All reactions were started by the addition of the reducing agent. The results indicate % of inhibition.

Electron acceptor		One-electron donor			Two-electron donor		
		GSH	CySH	Thioglycollate	BAL	Vitamin C	Hydroquinone
Univalent	FeCy	88	89	89	2	0	5
	FeY^{-1}	96	29	71	1	5	—
Bivalent	DCPI	0	2	11	0	0	0

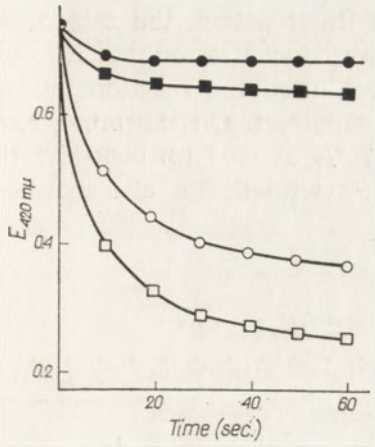

Fig. 1

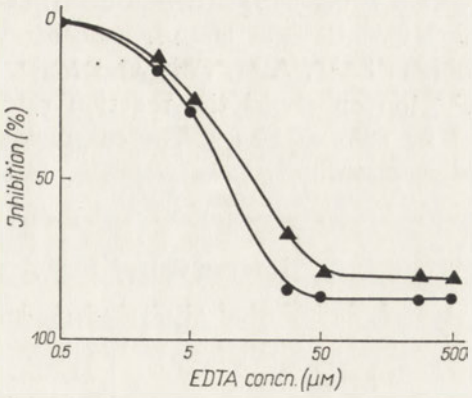

Fig. 2

Fig. 1. The effect of EDTA on the rate of reduction of ferricyanide by glutathione and cysteine. The conditions of the experiment as described in the section: Determination of reduction rate. The reaction was started by the addition of GSH or CySH. (○), GSH; (●), GSH + EDTA; (□), CySH; (■) CySH + EDTA.

Fig. 2. The effect of EDTA concentration on the rate of reduction of ferricyanide by glutathione. The reaction mixture (3 ml.) contained: 2.0 μmoles of FeCy; 100 μmoles of phosphate buffer, pH 7.5; 1.5 μmoles of GSH, and EDTA at the indicated concentration. The reaction was started by adding GSH. (▲), Results obtained when Cu^{2+} had been removed by treatment with dithizone solution in carbotetrachloride; (●), without removing Cu^{2+}.

The inhibitory effect of EDTA on the redox systems studied is summarized in Table 1. In a system composed of FeCy and one-electron donor the inhibition by EDTA was found to be about 90%. In a system containing FeY^{-1} as acceptor, different values of inhibition were obtained with particular one-electron donors: 96% for GSH, 71% for thioglycollate and 29% for CySH. If the bivalent acceptor DCPI was used, EDTA had no effect on the reduction either with uni- or bivalent electron donors. Similarly, EDTA did not affect the reactions of FeCy and FeY^{-1} with a bivalent reducing agent: BAL, vitamin C or hydroquinone.

The lack of inhibition of FeCy and FeY^{-1} reduction by the bivalent electron donors indicated that EDTA did not bind with these two oxidants. Therefore the possibility that the inhibitory effect of EDTA consists in its binding to thiol groups, was examined. However, no decrease in the number of free SH groups of GSH or CySH was observed after addition of EDTA (Table 2).

Since the effect of EDTA could not be explained by the binding with ferricyanide or SH group, it was thought that a metal may participate in the reaction and the effect of various cations was examined (Table 3).

Hg^{2+} ion at 50 μM concentration inhibited the reaction, the rate of the reaction being 56% of the control; at 500 μM concentration, the rate was only 18%. Co^{3+} at 500 μM concentration inhibited the reaction by 60% whereas Zn^{2+}, Al^{3+}, Fe^{3+} and Mg^{2+} had no effect. On the other hand, Cu^{2+} ion enhanced the reaction rate by 20% at 0.05 μM concentration and by 60% at 50 μM. The complex of Cu^{2+} with EDTA also increased the reaction.

Table 2

Interaction of EDTA with the SH groups

Methods as described in text. In the Table, the extinction values are given.

Sample (3 ml.)	Method of determination			
	Nitroprusside	HEDD	$E_{230m\mu}$	$E_{240m\mu}$
Cysteine, 54 μmoles	0.460	—	0.215	0.060
Cysteine, 54 μmoles + EDTA, 54 μmoles	0.490	—	0.235	0.060
Glutathione, 0.9 μmole	—	0.350	—	—
Glutathione, 0.9 μmole + EDTA, 0.9 μmole	—	0.385	—	—

Table 3

The effect of metal ions on the reduction rate

The reaction mixture (3 ml.) contained: 100 μmoles of phosphate buffer, pH 7.5; 2.0 μmoles FeCy; 1.5 μmoles GSH, and the indicated metal ion. The reaction was started by the addition of GSH. The reduction rate with no ions added (control) was 0.320 ΔE^{1cm}_{420}/min.. The results are expressed as % of inhibition (—) or activation (+).

Addition	0.05 μM	0.5 μM	5 μM	50 μM	500 μM
Zn^{2+}	+3	+1	—6	—4	0
Fe^{3+}	—7	+1	—7	—7	+9
Mg^{2+}	+4	—13	—2	—11	0
Hg^{2+}	—10	—13	—4	—44	—82
Al^{3+}	—9	—3	—5	—10	—10
Cu^{2+}	+21	+31	+46	+62	—
Co^{3+}	+10	+1	+9	+1	—60
Cu^{2+}-EDTA complex	—	—	—	—	+40

As Cu^{2+} ion was found to accelerate the reduction of FeCy, experiments were performed in which special procedures were applied to remove copper from the water and reagents used (Table 4). No change

in the reaction rate was observed when the water had been bidistilled from glass, and when the reagents had been treated with Chelax 100 [16]. But if the water had been distilled in the presence of EDTA the reaction rate was lower by 34%; the treatment of the reagents with a solution of dithizone in carbotetrachloride [13] reduced the rate by 66% and the treatment of the reagent solutions with Mg^{2+}-EDTA complex for 24 hr. resulted in a decrease by 39%. Nevertheless in all the experiments in which Cu^{2+} had been removed, the addition of 1.5 μmoles EDTA to the reaction mixture inhibited the reduction of FeCy by 70 - 90%.

Table 4

The effect of removal of Cu^{2+} and of adding EDTA on the reduction of ferricyanide by glutathione

The reaction mixture (3 ml.) contained: 100 μmoles of phosphate buffer, pH 7.5; 2.0 μmoles of FeCy and 1.5 μmoles GSH. The amount of EDTA added was 1.5 μmoles. The reaction was started by adding GSH.

Treatment of the reagents	Reduction rate		Inhibition (%)
	no EDTA ($\Delta E^{1\ cm}_{420}$/min.)	with EDTA ($\Delta E^{1\ cm}_{420}$/min.)	
Dissolved in distilled water	0.350	0.040	88
Dissolved in bidistilled water	0.345	0.035	90
Dissolved in distilled water in the presence of EDTA	0.230	0.040	83
The reagent solution treated with Chelax 100	0.330	0.030	91
The reagent solution treated with dithizone in carbotetrachloride	0.120	0.030	71
The reagent solution added with Mg^{2+}-EDTA complex (500 μM) and left for 24 hr.	0.215	0.040	81

When Cu^{2+} had been removed by dithizone, EDTA in about 10 μM concentration inhibited by 50% the reduction of FeCy by GSH (Fig. 2); without removing the Cu^{2+} ion the same degree of inhibition was obtained with 7.5 μM-EDTA. The concentration of copper in bidistilled water, determined spectrophotometrically, was 0.088 μM, being thus 1/100 of that concentration of EDTA which inhibited the reaction by 50%.

The inhibition of ferricyanide reduction by EDTA was fully reversible by the addition of an equimolar amount of $CuSO_4$ (Fig. 3). On the assumption that the ratio of EDTA to copper in the complex formed is as 1 : 1, it was possible to calculate the theoretical concentration of free EDTA inhibiting the reaction. For the inhibition by 50%, this value was calculated to be 10 μM, which is in agreement with the experimental results.

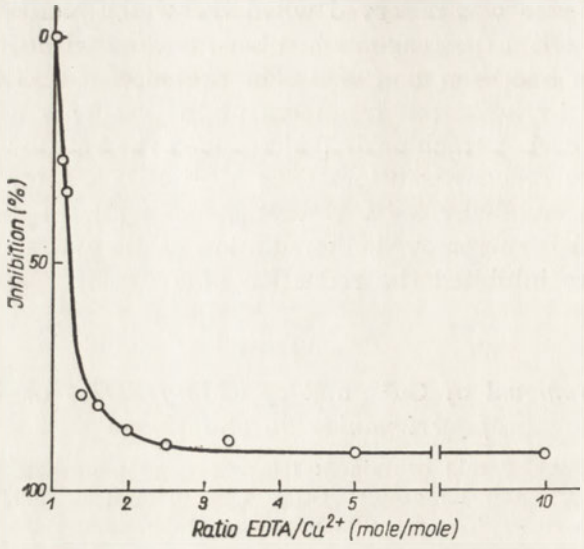

Fig. 3. The effect of Cu^{2+} on the EDTA-induced inhibition of ferricyanide reduction. The reaction mixture (3 ml.) contained: 2.0 μmoles of FeCy; 100 μmoles of phosphate buffer, pH 7.5; 1.5 μmoles GSH; 0.15 μmoles EDTA; and $CuSO_4$ ranging from 0.015 to 0.15 μmoles. The reaction was started by adding GSH.

DISCUSSION

The presented experiments indicate that the inhibition by EDTA of ferricyanide reduction by SH compounds is not due to the interaction of EDTA with the electron donor or acceptor. Two other mechanisms of EDTA activity were therefore considered: chelation of traces of metal ion catalysing the reduction, or the interaction of EDTA with an intermediate formed during the reaction.

Among the metal ions tested, only Cu^{2+} accelerated the reduction of ferricyanide and seemed to participate in the oxidation of SH groups by ferricyanide. It should be noted that Barron [1] and Michaelis [7] reported a catalytic effect of copper on the autoxidation of cysteine. In our experiments, the rate of ferricyanide reduction decreased after removal of copper from the reaction mixture. It was observed, however, that also in this case the reaction was inhibited by EDTA, therefore the chelating property of EDTA cannot account for the whole of the observed inhibition, and it seems justified to postulate an interaction of EDTA with an intermediate product:

$$GSH \rightarrow GS^- + H^+ \quad (1)$$

$$GS^- + FeCy \rightarrow (GS^-FeCy) \quad (2)$$

$$(GS^-FeCy) + EDTA \rightarrow (GS^-FeCy\text{-}EDTA) \quad (3)$$

The intermediate formed in the reaction (2) would be bound by EDTA. Since this intermediate is formed in a small amount and EDTA at a con-

centration corresponding to 1/60 of the used concentrations of GSH and FeCy has been found to inhibit the reaction by 50%, it seems possible to suggest a stoicheiometric reaction of EDTA with the intermediate. In this case EDTA would have a non-chelating effect.

From the presented experiments it follows that in the reaction between GSH and FeCy, the inhibition by EDTA seems to be due to two independent mechanisms: the chelating effect (removal of copper), and the non-chelating blocking of further conversion of the intermediate.

EDTA did not inhibit the reduction of the bivalent electron acceptor DCPI by compounds possessing one SH group. This may suggest another pattern of the reduction mechanism. The reduction of ferricyanide by GSH would proceed as follows:

$$2\,GS^- + 2\,Fe^{3+}Cy \rightarrow 2\,(GS^-Fe^{3+}Cy) \rightarrow 2\,(GS^0Fe^{2+}Cy) \rightarrow \rightarrow GSSG + 2\,Fe^{2+}Cy$$

whereas the reduction of 2,6-dichlorophenol indophenol seems to proceed through the following steps:

$$2\,GS^- + DCPI \longrightarrow \begin{pmatrix} GS^- \\ GS^- \end{pmatrix} DCPI \longrightarrow \begin{pmatrix} GS^0 \\ GS^0 \end{pmatrix} \text{reduced DCPI} \longrightarrow$$

$$\longrightarrow GSSG + \text{reduced DCPI}$$

The proposed mechanisms may explain the effect of EDTA. With a bivalent electron acceptor, the intermediate formed is of the type $\begin{pmatrix} GS^- \\ GS^- \end{pmatrix}$ indophenol$\Big)$. The two SH groups binding with one molecule of the acceptor, become situated in close proximity and EDTA cannot prevent their oxidation, and so EDTA could not affect the reduction of the bivalent electron acceptor. The lack of an inhibitory effect of EDTA on the reduction of univalent electron acceptors by BAL which possesses two SH groups per molecule, may be explained in a similar way. On the other hand, in the reaction between one-electron donor and one--electron acceptor the oxidized product is formed from two molecules of the intermediate (GS^-FeCy). EDTA interacts with GS^-FeCy and thus prevents the formation of the oxidized product.

The presented experiments suggest that in biological studies on the inhibitory effect of EDTA, the possibility of its non-chelating activity should be taken into account.

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SUMMARY

The effect of EDTA on the reduction of univalent and bivalent electron acceptors by thiol compounds was studied. EDTA inhibited the reaction of a univalent electron acceptor with a reducing agent containing one SH group; removal of copper did not overcome the inhibition. With 2,6-dichlorophenol indophenol as a bivalent electron acceptor, EDTA had no effect.

REFERENCES

[1] Barron E. S. G., in *Enzymes* (P. D. Boyer, H. Lardy & K. Myrback, eds.) vol. I, p. 525, Academic Press, New York 1959.
[2] Bitny-Szlachto S., Kosiński J. & Niedzielska M. - *Acta Polon. Pharm.* **20**, 365, 1963.
[3] Brintzinger H., Thiele H. & Muller U. - *Z. Anorg. Allgem. Chemie* **251**, 285, 1943.
[4] Chinard F. P. & Hellerman L., in *Methods of Biochemical Analysis* (D. Glick, ed.) vol. I, p. 12. Interscience Publ. Inc., New York 1954.
[5] de Deken R. H., Broekhuysen J., Bechet J. & Mortier A. - *Biochim. Biophys. Acta* **19**, 45, 1956.
[6] Grunert R. P. & Philips P. H. - *J. Biol. Chem.* **181**, 820, 1949.
[7] Michaelis L., in *Enzymes* (P. D. Boyer, H. Lardy & K. Myrback, eds.) vol. I, p. 525. Academic Press, New York 1959.
[8] Nygaard A. P., in *Haematin Enzymes* (J. E. Falk, R. Lemberg & R. K. Morton, eds.) part 2, p. 541. Pergamon Press, New York, London 1961.
[9] Pfeiffer P. & Offerman W. - *Ber. D. Chem. Gesellschaft* **75**, 1, 1942.
[10] Quastel J. H., in *Methods in Enzymology* (S. P. Colowick & N. O. Kaplan, eds.) vol. IV, p. 331. Academic Press, New York 1957.
[11] Racker E. - *J. Biol. Chem.* **190**, 685, 1951.
[12] Rytka J. & Tysarowski W. - *Acta Biochim. Polon.* **12**, 229, 1965.
[13] Sandell E. B., *Colorimetric Determination of Traces of Metals* (Third edition). Interscience Publ. Inc., New York 1959.
[14] Slater E. C. & Benner W. D. - *Biochem. J.* **52**, 185, 1952.
[15] Tysarowski W. & Kwiek S. - *Acta Biochim. Polon.* **3**, 55, 1956.
[16] Weber A. & Winicur S. - *J. Biol. Chem.* **236**, 3198, 1961.

EDTA JAKO INHIBITOR REDUKCJI ŻELAZICYJANKU PRZEZ GLUTATION I CYSTEINĘ

Streszczenie

Badano wpływ EDTA na szybkość reakcji redukcji jednoelektronowych i dwuelektronowych akceptorów przez związki sulfhydrylowe. EDTA wywołuje efekt hamowania w układach: jednoelektronowy akceptor - reduktor zawierający jedną grupę SH. Efekt hamowania po usunięciu miedzi różnymi sposobami jest taki sam jak bez jej usunięcia. EDTA nie hamuje redukcji dwuelektronowych akceptorów elektronów.

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HYDROLYSIS OF FATTY ACYL ESTERS OF *p*-NITROPHENOL BY HOMOGENATES OF INTESTINAL MUCOSA OF DIFFERENT ANIMAL SPECIES

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Carboxylic ester hydrolases (EC 3.1.1.1) are widely distributed in nature. Animal tissues contain two types of these enzymes: type A, resistant to organophosphate inhibitors, and type B, sensitive to these compounds [1]. Esterases of low specificity, acting on both aliphatic and aromatic substrates, belong to both these types. The distribution of these enzymes in the rat was studied by Aldridge [2]. This author found that rat intestinal mucosa contains chiefly organophosphate-sensitive esterase hydrolysing phenyl butyrate more rapidly than phenyl acetate. More detailed studies concerning substrate specificity were carried out by Hofstee [6, 7] on esterases from liver and pancreas. He found that the rate of hydrolysis of *m*-hydroxybenzoic acid esters increases with the elongation of the fatty acyl chain, reaching a maximum between C_5 and C_9, or even at higher numbers of carbon atoms in the case of the liver enzyme.

The present paper describes the hydrolysis of a series of fatty acyl esters of *p*-nitrophenol differing in chain length, by crude extracts of small-intestine mucosa of six animal species, i.e. chicken, rat, rabbit, pig, ox, and man.

MATERIALS AND METHODS

Preparation of mucosal extracts. Sections of the small intestine, mainly from the part proximal to the duodenum, were used for experiments. Chickens, rabbits and rats deprived of food for 12 hr. were killed in the laboratory, and the intestines were removed immediately, rinsed with ice-cold 0.9% NaCl solution, and opened longitudinally on a glass plate. The mucosa was then scrapped off with a glass slide, weighed, and homogenized in a glass homogenizer with an appropriate volume of 0.1 M-acetate buffer, pH 5.6, containing 1 mM-EDTA. The

homogenate was centrifuged for 15 min. at 10 000 rev./min. in a MSE refrigerated centrifuge. Ox and pig intestines were obtained from a slaughterhouse, transported on ice, and then treated in the same way. Human intestine (a section 5 cm. long) was taken in the course of a surgery.

Substrates. *p*-Nitrophenyl esters of acetic, propionic, butyric, valeric, hexanoic, heptanoic, octanoic, decanoic and dodecanoic acids were synthesized according to the method of Huggins & Lapides [9] designed for *p*-nitrophenyl acetate synthesis. According to this method, a solution of *p*-nitrophenol in benzene was refluxed with the appropriate acyl chloride in the presence of magnesium turnings. The mixture was taken to ethyl ether and washed repeatedly with diluted sodium bicarbonate solution and water; then the solvents were removed by distillation under reduced pressure. The solid esters (acetate, propionate, decanoate and dodecanoate) were recrystallized from methanol until free from unesterified phenol. The remaining, liquid esters were purified by repeated distillation *in vacuo*.

Other reagents. Diisopropylfluorophosphate (DFP) was a product of L. Light & Co. (Colnbrook, Bucks, England). The reagents used for preparation of buffer solutions were of analytical grade.

Determination of esterase activity. A mixture of 2.5 ml. of suitably diluted mucosal extract and 1.5 ml. of 0.066 M-phosphate buffer, pH 7.0, was equilibrated for 5 min. at 25°, then 1.0 ml. of 0.5 mM-substrate solution was added. After 10 to 20 min. incubation, the extinction of the yellow colour of the liberated *p*-nitrophenol was read at 430 mμ in a Coleman Junior Spectrophotometer. The incubation samples were kept at 25° up to the moment of extinction reading, and the exact time of incubation was then noted. Six parallel tests were run simultaneously, with two controls for non-enzymic hydrolysis. All substrates were tested with the same dilution of the mucosal extract within one series of determinations. The activity was expressed as μmoles of the substrate decomposed per 1 min. per 1 mg. protein.

Protein determination. This was carried out with the Folin & Ciocalteu phenol reagent [4] according to the procedure previously described [8].

RESULTS

The activity patterns of mucosal extracts related to the length of the fatty acyl chain of *p*-nitrophenyl esters, are presented in Fig. 1. The general appearance of the patterns is similar for all animals studied. The activity increases at first with the increase of the chain length, attains a maximum for esters between C_5 and C_7, and falls with the further increase in the number of carbon atoms. There are, however,

distinct differences between the species studied with respect to the character of the activity patterns, localization of the maxima and the intensity of enzymic activity.

The maximum of activity was located at C_5 ester for man and ox, at C_6 for rat and rabbit, and at C_7 for chicken and pig. The activity decreased almost symmetrically at chain lengths below and above those corresponding to the maximum activity. For the ox, the activity pattern was flattened and the maximum was less pronounced than in other species.

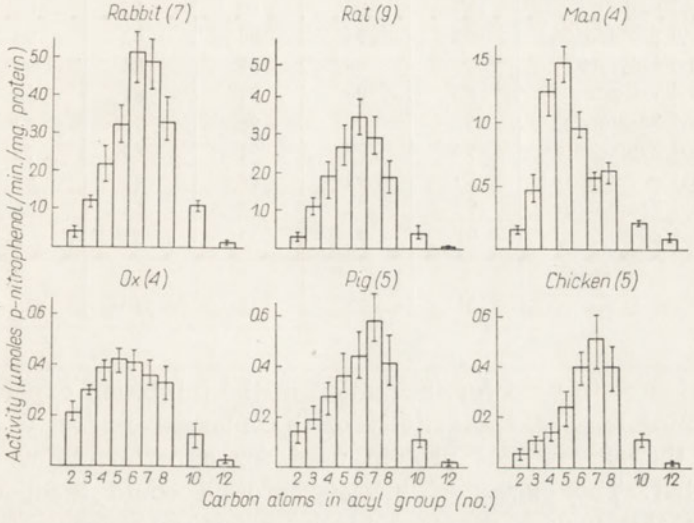

Fig. 1. Activity of crude mucosal extracts of the small intestine towards a homologous series of *n*-fatty acyl esters of *p*-nitrophenol. The mean and limit values are given, the number of determinations being indicated in parentheses. The conditions of assay as described in the text.

The activity per milligram protein was the highest in the rabbit and rat, lower by two thirds in man, and still lower, of the order of one tenth of that of the rabbit, in the chicken, pig and ox.

The hydrolysis of *p*-nitrophenyl esters was inhibited by low concentrations of DFP in all species studied (Table 1). The inhibition was always the greatest for the esters grouped around the activity maximum but definitely lower for esters less susceptible to enzymic hydrolysis. Also in this respect there were noticeable differences between the animals studied. Thus the inhibition of acetate hydrolysis was smaller for the pig and chicken than for other animals, whereas for dodecanoate the inhibition was the least pronounced in the case of ox intestine.

Higher inhibition observed in the rabbit and rat may be related to the higher activity characteristic for these species and thus smaller

Table 1

Effect of diisopropylfluorophosphate on the esterolytic activity of crude extracts of intestinal mucosa

The samples of mucosal extracts were preincubated with 0.066 M-phosphate buffer, pH 7.0, and DFP for 20 min. at 25°. The concentration of DFP in the incubation mixture was 6 μM. All values are the means of two series of experiments differing by 1 - 4%. The conditions of assay as described in the text.

Substrate	Rabbit	Rat	Man	Pig	Ox	Chicken
	Inhibition (%)					
p-Nitrophenyl acetate	89	93	80	55	85	42
p-Nitrophenyl propionate	94	98	94	62	90	70
p-Nitrophenyl butyrate	95	99	98	84	90	89
p-Nitrophenyl valerate	96	98	97	81	90	91
p-Nitrophenyl hexanoate	98	99	98	78	87	89
p-Nitrophenyl heptanoate	96	99	91	83	79	88
p-Nitrophenyl octanoate	97	99	88	81	85	86
p-Nitrophenyl decanoate	96	93	62	55	66	69
p-Nitrophenyl dodecanoate	91	72	33	25	9	29
Protein in mucosal extract (μg./ml.)	21	23	42	350	136	132

amounts of protein used for incubation in the inhibition experiments. In the remaining species, the activity of the mucosal extract was low and therefore the amounts of protein used for experiments were several times higher. The excess of inactive protein could react with DFP causing in this way a decrease in the effective concentration of the inhibitor.

DISCUSSION

The observed differences in the activity patterns and the degree of inhibition by DFP, seem to indicate that more than one enzyme is involved in the esterolytic activity of intestinal mucosa. If one enzyme would catalyse the hydrolysis of several substrates then the inhibitor should affect the hydrolysis of all these substrates to the same degree.

As the activity towards the most actively hydrolysed substrates was almost completely inhibited, it seems that in all species studied the organophosphate-sensitive esterase of B-type is predominating. If we assume that only one esterase of B-type is present then it would appear that this enzyme exhibits species-dependent differences with respect to the activity centre, as shown by variations in the activity pattern. These differences, however, may be interpreted also as the presence, in varying amounts, of two or three DFP-sensitive esterases differing with respect to the most actively hydrolysed substrate.

It has also been observed that hydrolysis of some substrates, i.e. acetate, dodecanoate and decanoate, was inhibited by DFP to a much smaller degree. This may suggest the presence of small amounts of organophosphate-resistant esterases, responsible for the part of total hydrolysis of these esters not inhibited by DFP. Probably distinct enzymes hydrolyse esters of long and short chain acids. The enzyme resembling serum A-esterase [1] may be responsible for DFP-resistant hydrolysis of acetate observed in pig and chicken. The presence of a lipase-like enzyme may account for the resistant part of decanoate and dodecanoate hydrolysis. This enzyme seems to be present in most of the species studied, with the exception of rabbit. The occurrence of lipase in intestinal mucosa has been reported by DiNella *et al.* [3] but the activity of this enzyme toward *p*-nitrophenyl esters used in the present experiments, has not been tested. It is known that pancreatic lipase does not hydrolyse aromatic esters [5], on the other hand gastric lipase showed considerable activity toward *p*-nitrophenyl esters [10].

On the basis of the obtained results it seems possible to suggest the complex character of esterolytic activity in intestinal mucosa, but more detailed studies are necessary to elucidate this problem.

SUMMARY

Extracts of small-intestine mucosa of six animal species were tested for the ability to hydrolyse *p*-nitrophenyl esters containing 2 to 12 carbon atoms in fatty acyl chain. The activity per mg. protein was the highest in the rabbit and rat, lower in man, and the lowest in the pig, chicken and ox. The maximum of activity was observed for the C_5, C_6 or C_7 ester. It is suggested that the differences observed in the inhibitory effect of diisopropylfluorophosphate may be interpreted as indicating the occurrence of several esterases in the intestinal mucosa.

REFERENCES

[1] Aldridge W. N. - *Biochem. J.* **53**, 110, 1953.
[2] Aldridge W. N. - *Biochem. J.* **57**, 692, 1954.
[3] DiNella R. R., Meng H. C. & Park C. R. - *J. Biol. Chem.* **235**, 3076, 1960.
[4] Folin O. & Ciocalteu V. - *J. Biol. Chem.* **73**, 627, 1927.
[5] Hofstee B. H. J. - *J. Biol. Chem.* **199**, 357, 1952.
[6] Hofstee B. H. J. - *J. Biol. Chem.* **199**, 365, 1952.
[7] Hofstee B. H. J. - *J. Biol. Chem.* **207**, 219, 1954.
[8] Homańska-Szafranowa H., Oleksy J. & Szafran Z. - *Acta Physiol. Polon*, **9**, 233, 1958.
[9] Huggins C. & Lapides J. - *J. Biol. Chem.* **170**, 467, 1947.
[10] Popiela T., Szafran H. & Szafran Z. - *Clin. Chim. Acta* **11**, 283, 1965.

HYDROLIZA ESTRÓW *p*-NITROFENOLU Z KWASAMI TŁUSZCZOWYMI PRZEZ HOMOGENATY BŁONY ŚLUZOWEJ JELITA CIENKIEGO RÓŻNYCH GATUNKÓW ZWIERZĄT

Streszczenie

Badano aktywność ekstraktów błony śluzowej jelita cienkiego sześciu gatunków zwierząt w stosunku do estrów *p*-nitrofenolu z kwasami tłuszczowymi zawierającymi od 2 do 12 atomów węgla w łańcuchu. Najwyższą aktywność na mg. białka stwierdzono u królika i szczura, niższą u człowieka, najniższą u świni, kurczęcia i wołu. Maksimum aktywności obserwowano dla estrów C_5, C_6 i C_7. Na podstawie tych wyników oraz zauważonych różnic w działaniu hamującym dwuizopropylofluorofosforanu przedyskutowano możliwość występowania kilku esteraz w błonie śluzowej jelita.

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LOW-MOLECULAR WEIGHT PEPTIDES SYNTHESIZED IN RIBOSOMAL PREPARATIONS AS POSSIBLE PRECURSORS OF PROTEINS*

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A generally accepted theory of protein biosynthesis postulates the stepwise growing of the polypeptide chain beginning from one of its ends [4, 2, 1]. Therefore it is logical to suppose that at any given time the ribosomal template contains peptides of different length which are the precursors of proteins synthesized in the system.

It is well known that bound amino acids, presumably peptide or nucleotide-peptide in character, are present in micro-organisms [5] and in mammalian tissues [10]. Our earlier observations [9] showed that [^{14}C]amino acids are rapidly incorporated into these compounds in a cell-free system derived from guinea pig liver. In order to establish the relation of these compounds to the newly synthesized proteins, in the present study the specific activities of labelled amino acids were compared in low-molecular weight peptides, total trichloroacetic acid (TCA) insoluble protein, and in the pool of free amino acids isolated from incubation mixtures after incorporation experiments.

A preliminary account of this work has been presented at the VIth International Congress of Biochemistry (New York, 1964, I-7).

EXPERIMENTAL

DL-[1-^{14}C]Glutamic acid (5 mc per m-mole) was purchased from Amersham Radiochemical Centre; L-[^{14}C]alanine uniformly labelled (123 mc per m-mole) was a product of Phylips-Duphar, Holland.

Ribosomes were prepared from guinea pig livers by the method of Lingrel & Webster [7]. Microsomes were obtained by differential centri-

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fugation of 10% homogenates of liver in Lingrel & Webster's Medium A between 10 000 and 95 000 *g*.

Sephadex-treated supernatant was prepared by passing the 95 000 *g* supernatant through a 20 × 2 cm. column packed with Sephadex G-25 and equilibrated with 0.05 M-tris buffer, pH 7.8; the protein fraction was collected and used as soon as possible.

Hydrolysis of proteins and peptides was carried out in 6 N-HCl at 105° in sealed glass tubes for 18 hr. Alanine was isolated as its 2,4-dinitrophenyl (DNP) derivative by column chromatography. Partition chromatography on Hyflo Super-Cel with the upper phase of a mixture of ethyl acetate and tris-NaCl buffer at pH 8.4 was employed as described by Matheson [8]. For the isolation of DNP-glutamic acid a minor modification of this method was necessary: a column half the size of Matheson's "standard column" was used, and after the alanine band had emerged the eluent was changed; it consisted of the "pH 8.4 top phase" equilibrated with the "bottom phase" adjusted previously to pH 4.9 with strong acetic acid. The amount of DNP-amino acids was estimated spectrophotometrically and prepared for counting essentially as described by Matheson except that the final dry preparation was dissolved in toluene and counted in a liquid scintillation counter instead of plating and counting in end-window counter.

Paper electrophoresis was run on Whatman 3MM paper at 40 V/cm. in 1.3% formic acid - 3.9% acetic acid at pH 1.9 [3] for 180 min. and in 0.05 M-ammonium acetate, pH 4.7, for 300 min. in an apparatus cooled with tap water.

Incubation conditions. The incubation mixture contained: 0.4 M-KCl, 0.018 M-$MgCl_2$, a mixture of 20 amino acids (in this 1.0 μc per ml. of either [1-^{14}C]Glu or [^{14}C]Ala) 0.1 mM each, 9 mM-ATP, 0.4 mM-GTP, 0.05 M-phosphoenolpyruvate, phosphoenolpyruvate kinase 0.3 mg. per ml., 0.01 M-GSH, 0.05 M-tris-HCl buffer, pH 7.8, ribosomes or microsomes 4.0 mg. of protein per ml., and Sephadex-treated supernatant 3.0 mg. of protein per ml. The incubation was carried out for 15 min. at 37°.

Isolation and analysis of peptides and proteins. At the end of the incubation trichloroacetic acid was added to the final concentration of 5%; the precipitate was washed four times with TCA, once with acetone, once with ethanol-chloroform (1:3, v/v) and with ether; it was called protein fraction.

TCA extracts were pooled, extracted four times with ether, and the aqueous phase concentrated by freeze-drying. It was then subjected to paper electrophoresis at pH 1.9. On radiography three radioactive fractions appeared beside the free amino acid. They were termed E_I, E_{II}, and E_{III}. The results of this fractionation are shown in Fig. 1.

Labelled fractions that migrated behind the free amino acids were

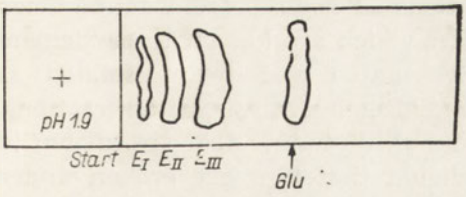

Fig. 1. Electrophoretic pattern at pH 1.9 of labelled fractions isolated from the incubation mixture of liver microsomal systems with [^{14}C]glutamic acid. Autoradiography.

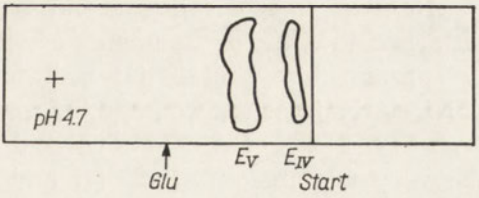

Fig. 2. Electrophoretic behaviour at pH 4.7 of fraction E_I eluted from the electrophoretogram presented in Fig. 1. Autoradiography. Glu, the position of free glutamic acid run in the same conditions.

ninhydrin-positive, absorbed ultraviolet light and on hydrolysis gave several amino acids. Fraction E_I could be further separated if subjected to electrophoresis at pH 4.7 giving two fractions (Fig. 2) one of which, fraction E_V, did not contain any ultraviolet-absorbing material but contained bound amino acids probably in the form of peptides.

Radioactive spots were eluted from the paper, subjected to complete hydrolysis and the specific activity of the amino acid incorporated into them determined as described under Methods. The spot containing free radioactive amino acid was also eluted and its specific activity determined. The washed TCA-insoluble residue was hydrolysed and the specific activity of the labelled amino acid determined in the hydrolysate. The results of typical experiments are presented in Tables 1 and 2. It is evident from these data that the specific activity of amino acids incorporated into fractions of low-molecular weight peptides is of the same order as the specific activity in the pool of free amino acids and is about a thousand times greater than in protein. This indicates that compounds of this type are not break-down products of finished polypeptides but probably the precursors of some bigger molecules.

Table 1

Specific activity of glutamic acid in protein and peptides after the incubation of microsomes

Fraction	Counts/min./mg. Glu $\times 10^{-5}$
Protein	0.0035
Free glutamic acid	8.7
E_I	5.2
E_{II}	3.1
E_{III}	7.2
E_{IV}	3.1
E_V	8.1

In order to ascertain that our fractions do not represent amino acids attached to AMP or fragments of sRNA which are known to participate in protein biosynthesis [6] radioactive amino acids were isolated as DNP-derivatives and counted before and after hydrolysis of the fractions. The acyl bonds of AMP-amino acid and sRNA-amino acid are known to be very unstable in alkali [6] and should therefore get broken under the conditions used for dinitrophenylation. The results of this experiment (Table 3) show that although the fractions contained a small amount of amino acids bound by alkali-labile bonds, complete acid hydrolysis liberated four to eight times more radioactive amino acids suggesting thus that they are bound by peptide bonds.

Table 2

Specific activity of alanine in protein and peptides after the incubation of microsomes and ribosomes

Fraction	Counts/min./mg. Ala $\times 10^{-5}$
Protein, after incubation of ribosomes	0.17
Free alanine, after incubation of microsomes	602
Free alanine, after incubation of ribosomes	578
E_I, after incubation of microsomes	204
E_I, after incubation of ribosomes	356

Table 3

Total activity of DNP-amino acids in peptide fractions before and after complete acid hydrolysis

Fraction	DNP-Glu (counts/min./mg. $\times 10^{-5}$)		DNP-Ala (counts/min./mg. $\times 10^{-5}$)	
	before hydrolysis	after hydrolysis	before hydrolysis	after hydrolysis
E_{IV}, after incubation of microsomes	2.6	16.2	40.5	116.3
E_V, after incubation of microsomes	32.2	124.0	—	—
E_I, after incubation of ribosomes	—	—	13.2	110.0

It is known that reticulocyte ribosomes treated with puromycin [2] or NH_2OH [1] release polypeptides which are considered to be later intermediates in haemoglobin synthesis. However, the approximate determinations of molecular weight performed on the extracts used in the present study by gel-filtration on Sephadex G-25 showed that radioactive peptides were distributed in the fractions the molecular weight of which was in the range of 4 to 6 thousands. This indicates that the

compounds studied in this investigation are probably much earlier intermediates than those isolated by Allen & Zamecnick from reticulocyte ribosomes [2, 1].

SUMMARY

Bound amino acids, probably peptides or nucleotide-peptides, were isolated from the incubation mixtures used for studying the incorporation of amino acids *in vitro*. The specific activity of the radioactive amino acid incorporated under these conditions into low-molecular weight peptides was of the same order as that of the free amino acid; it was about a thousand times greater than in the fraction of total ribosomal or microsomal protein which suggests that these peptides are not break-down products of completed proteins but probably the precursors of some bigger molecules. These fractions do not represent amino acids bound to AMP or sRNA since very little radioactivity is released from them on direct dinitrophenylation.

REFERENCES

[1] Allen B. W. - *Biochim. Biophys. Acta* **68**, 418, 1963.
[2] Allen B. W. & Zamecnick P. C. - *Biochim. Biophys. Acta* **55**, 805, 1962.
[3] Atfield G. N. & Morris C. J. O. R., in *Protides of Biological Fluids, Proc. of 8th Coll. Bruges 1960*, p. 78. Elsevier Publ. Co., Amsterdam 1961.
[4] Bishop J., Leahy J. & Schweet R. - *Proc. Natl. Acad. Sci. U.S.* **44**, 1029, 1958.
[5] Davies J. W. & Harris G. - *Proc. Roy. Soc. B.* **151**, 537, 1960.
[6] Hoagland M. B., Stephenson M., Scott J. F., Hecht L. I. & Zamecnick P. C. - *J. Biol. Chem.* **231**, 241, 1958.
[7] Lingrel J. B. & Webster G. - *Biochim. Biophys. Acta* **61**, 942, 1962.
[8] Matheson N. A. - *Biochem. J.* **88**, 146, 1963.
[9] Szafrański P. & Bagdasarian M. - *Nature* **190**, 719, 1961.
[10] Szafrański P., Sułkowski E., Gołaszewski T. & Heller J. - *Acta Biochim. Polon.* **7**, 151, 1960.

DROBNOCZĄSTECZKOWE PEPTYDY POWSTAJĄCE W PREPARATACH RYBOSOMALNYCH JAKO PRZYPUSZCZALNE PREKURSORY BIAŁEK

Streszczenie

Z mieszanin inkubacyjnych używanych do badania włączania aminokwasów do białek *in vitro* wydzielono związane aminokwasy, prawdopodobnie peptydy lub nukleotydo-peptydy. Aktywność właściwa radioaktywnych aminokwasów wbudowanych do frakcji drobnocząsteczkowych peptydów była tego samego rzędu co aktywność wolnych aminokwasów dodanych do mieszaniny inkubacyjnej; aktywność ta przewyższała właściwą aktywność aminokwasu wbudowanego w białka rybosomów lub mikrosomów około tysiąc razy, co wskazuje, iż badane peptydy nie stanowią produktów degradacji gotowych białek, lecz raczej są prekursorami polipeptydów. Badane frakcje nie są aminokwasami związanymi z AMP lub sRNA, ponieważ bardzo niewiele radioaktywności uwalnia się z nich przy bezpośrednim dwunitrofenylowaniu.

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ZOFIA LASSOTA

URIC ACID IN NORMAL AND γ-IRRADIATED EGGS OF *BOMBYX MORI*

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Wharton & Wharton [16] have reported that in the irradiated adults of *Periplaneta americana* L. the disturbances in nitrogen metabolism occurred and the amount of excreted uric acid increased. It is generally accepted that uric acid is the main endproduct of nitrogen metabolism in insects and its presence in blood and excreta is well known. The informations about uric acid in the eggs of insects are scarce, it was found, however, to be present in the eggs of two species (*Oncopeltus fasciatus* Dallas and *Lucilla sericata* M.G.) and in both it was reported to accumulate during embryogenesis [2, 11].

Our aim was to investigate whether the radiation damage of embryonal metabolism of an insect would result in increase in uric acid content of the egg. Since we are interested in the effects of radiation especially when applied at diapause, we have studied the eggs of a monovoltine race of *Bombyx mori*.

MATERIALS AND METHODS

Material. The diapausing eggs of *Bombyx mori*, "warska" race, were purchased from an industrial culture. The eggs collected during the last days of September were stored for the first 120 days at 15° and then for about 100 days at 4°. The postdiapausal development was promoted by raising gradually the storage temperature to, and maintaining it at, 25°.

The samples to be irradiated were taken out from the whole batch in December. A single dose of 20 000 r of γ-rays was delivered from a ^{60}Co source (about 48 c), the dose rate being about 200 r/min., and thereafter both the normal and the irradiated eggs were treated in the same manner. It has been shown previously [12] that doses higher than 10 000 r when applied at diapause result in damaging the embryogenesis.

Analytical methods. Uric acid was identified spectrophotometrically after the separation on Dowex 1 × 8 column as described for nucleotides

[8] and chromatographically on Whatman no. 3 paper after Leone & Guerritore [13] using *N*-2,6-trichlorbenzoquinoneimine [7] for locating spots. The presence of uric acid riboside was checked as described by Heller & Jeżewska [10].

Quantitatively uric acid was determined by the colorimetric method of Caraway [3]. The specificity of the colour test was checked each time by the decomposition of uric acid with uricase prepared after Leone [14]. No chromogen detectable by this method was left after the incubation of samples with uricase.

The total nitrogen was determined by the Kjeldahl method.

Egg extracts. Usually 100 mg. of whole eggs were homogenized at room temperature with three 2 ml. portions of 0.5 N-$HClO_4$. After centrifugation at about 1000 *g* the supernatant was neutralized with KOH, the resulting precipitate was spun off and the volume of the supernatant was completed to 8 ml. with water. Uric acid was determined in the samples of extracts.

The efficiency of uric acid extraction with $HClO_4$ has been checked by comparison with the results obtained in the extracts prepared with 0.2 M-borate buffer, pH 10, at 100° or with saturated $LiHCO_3$ solution at room temperature. Three 4 ml. portions of either solution were used to extract 100 mg. of eggs. The alkaline extracts were neutralized, completed to 15 ml. with water and deproteinized after Sevag *et al.* [15]. No differences in uric acid content of the eggs, depending upon the method of extraction were stated.

For the separation on Dowex column the portions of $HClO_4$ extracts equivalent to about 1 g. of eggs, neutralized and centrifuged were freeze-dried, dissolved in water and the soluble material was applied on the column.

RESULTS

Identification of uric acid. The presence of uric acid in the eggs of *Bombyx mori* was tested by spectrophotometry and chromatography. When the crude extract, prepared with $HClO_4$, has been adsorbed on Dowex 1 × 8 ($HCOO^-$ form) and the column was eluted with HCOOH gradient (0 - 0.5 N) the first UV-absorbing fraction gave the absorption spectrum shown in Fig. 1. The minimum at 255 mμ and the maximum at 285 mμ as well as the ratio E_{280}/E_{260} amounting to 2.7 indicated the presence of uric acid.

When tested by paper chromatography, the $HClO_4$ extracts from the eggs gave a distinct, UV-absorbing spot, staining orange on spraying, identical with the spot of uric acid assayed simultaneously (R_F 0.40).

The presence of uric acid riboside was checked in the diapausing eggs because this compound has been found in *Sphinx pinastri* and in

Celerio euphorbiae during the pupal diapause [10]. However, neither in the $HClO_4$ extracts nor in the extracts prepared as described by Heller & Jeżewska [10] the characteristic spot of uric acid riboside was found.

Uric acid content of the eggs. The data reported in this paper represent the results of experiments continued during three consecutive years. The eggs were investigated during the period of the arrest of embryogenesis (October - April) as well as during the active postdiapausal development (May). However, also during the period of arrested embryogenesis some changes in the eggs do occur and therefore it appeared necessary to subdivide the whole period of developmental arrest. The period including December and January was named: deep diapause after Chino [4] who stated that glycogen content of the eggs of *Bombyx mori* falls at the beginning and raises towards the end of

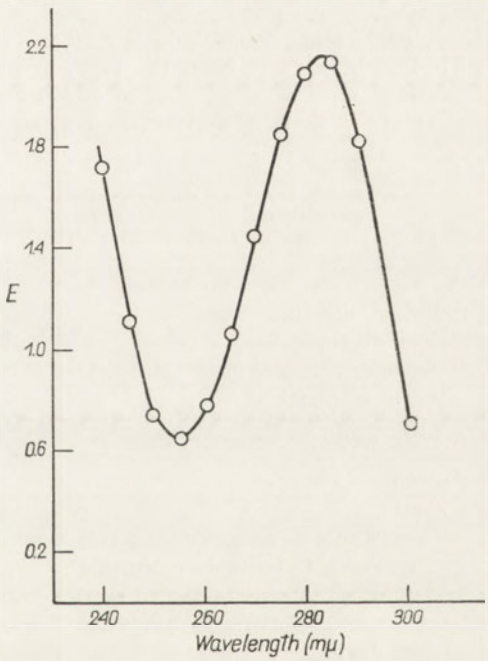

Fig. 1. Spectrophotometric identification of uric acid in the eggs of *Bombyx mori*. The absorption spectrum of the fraction separated by column chromatography on Dowex 1×8 is given.

diapause and is at a minimum between the 60th and the 120th day of storage at room temperature. The whole period from October to March was named after Harvey [9] obligatory diapause. The obligatory diapause included 3 months of storage at 15° and two months of storage at 4° since according to Chino [5] the ability for postdiapausal embryogenesis in the eggs of *Bombyx mori* appears after about 60 days of storage at low temperature. In April the eggs were ready to, but did not, develop because kept below the temperature threshold for embryogenesis.

Table 1 shows the results obtained with the normal as well as with the irradiated eggs. The statistical analysis of data proved that no significant differences existed between the normal and the irradiated eggs till the end of incubation at 25°, resulting in hatching of larvae from normal eggs.

The uric acid content of the normal eggs did not change as long as the obligatory diapause persisted. The increase in uric acid was observed in April, however on incubation at temperatures above the threshold for embryogenesis the uric acid content decreased again. The observed differences were found to be statistically significant.

Table 1

Diapausal and postdiapausal uric acid content of normal and γ-irradiated eggs of Bombyx mori

Uric acid was determined by the colorimetric method of Caraway [3] in 0.5 N-$HClO_4$ extracts from the eggs, prepared as described in the text. Eggs were irradiated with a single dose of 20 000 r at the beginning of deep diapause. Mean values ± S. D. are given, and in parentheses the number of experiments.

Period	Storage temp.	Uric acid (mg./g. of eggs)	
		normal eggs	irradiated eggs
Obligatory diapause			
October - November	15°	3.23 ± 0.406 (8)	
December - January (deep diapause)	15°	3.27 ± 0.545 (9)	3.05 ± 0.187 (5)
February - March	4°	3.69 ± 0.381 (11)	4.20 ± 0.454 (4)
Average value for obligatory diapause		3.42 ± 0.492 (28)	3.52 ± 0.676 (9)
Postdiapausal storage			
April	4°	4.64 ± 0.514 (6)	5.08 ± 0.141 (3)
significance of difference from average value for obligatory diapause		$P = 0.1\%$	$P = 0.1\%$
May (embryogenesis in normal eggs)	25°	3.85 ± 0.293 (14)	3.85 ± 0.313 (12)
significance of difference from value for April		$P = 0.1\%$	$P = 0.1\%$

The assays of uric acid content of the irradiated eggs were extended beyond the incubation period needed for normal embryogenesis. The storage at 25° was prolonged by one week and thereafter the eggs were transferred to, and stored at, 4° for 60 days. The results (Table 2) proved that the uric acid content of the irradiated eggs increased during the prolonged incubation at 25°. This increase continued during the following storage at 4° so that the uric acid content of these eggs doubled,

as compared with the value characteristic for the obligatory diapause. To check whether the increase observed in irradiated eggs was characteristic for the radiation damage, assays were performed on the diapausing eggs which had completely lost their hatchability in consequence of 10 month-long storage at 4°. Uric acid did not accumulate in these "age-dead" eggs as long as they were stored at 4°. When, however, the "age-dead" eggs were incubated at 25° as for normal embryogenesis and then stored again at 4°, the uric acid increased as in the similarly treated irradiated eggs.

Table 2

The increase in uric acid content of irradiated and "age-dead" eggs of Bombyx mori, *after their postdiapausal incubation at 25°*

Uric acid was estimated as described in Table 1. Mean values ± S. D. are given, and in parentheses the number of experiments.

Material	Treatment	Uric acid (mg./g. of eggs)
Irradiated eggs incubated for 10 days at 25°	None	3.85 ± 0.313 (12)
	One week at 25°	4.65 ± 0.160 (6)
	One week at 25° followed by 60 days at 4°	6.42 ± 0.743 (18)
Non-irradiated eggs stored for 10 months at 4° ("age-dead")	None	3.61 ± 0.580 (6)
	Two weeks at 25° followed by 60 days at 4°	6.24 ± 1.71 (4)

Table 3

The total and acid-soluble nitrogen of the eggs of Bombyx mori

The Kjeldahl method was used. Mean values ± S. D. are given, and in parentheses the number of experiments.

Material	Nitrogen (mg./g. of eggs)	
	Whole eggs	0.5 N-$HClO_4$ extracts
Diapausing eggs	40.5 ± 3.44 (5)	5.15 ± 0.613 (10)
Normal eggs during embryogenesis	42.6 ± 3.96 (5)	4.94 ± 0.706 (9)
Irradiated eggs during postdiapausal incubation at 25°	40.0 ± 0.410 (3)	5.05 ± 0.350 (8)

The total N *content of the eggs and of the extracts.* In order to ascertain that in our experiments we are dealing with a closed system as far as nitrogen is concerned, the total N of the whole eggs and of $HClO_4$ extracts was examined. The results (Table 3) indicate that the acid-soluble N and the total N calculated per gram of eggs were reasonably constant during the diapause as well as during the postdiapausal development. The applied method of extraction removed about 12% of total nitrogen and the uric acid N accounted for about 20% of the acid-soluble nitrogen.

The weight of the eggs. Since uric acid was estimated per weight unit of the eggs, the changes in their weight would influence the interpretation of results. The average weight of the eggs was reasonably constant during the diapause and normal storage at 4°. The values of 75.9 ± 0.886 mg. and of 77.3 ± 2.04 mg. were found for 100 normal and irradiated eggs, resp., the difference between the normal and irradiated eggs being statistically not significant. During the postdiapausal embryogenesis the weight of the normal as well as of the irradiated eggs decreased by about 5%.

DISCUSSION

The applied dose of 20 000 r of γ-rays damaged the embryogenesis in *B. mori* so that the hatching of larvae was completely inhibited. However, neither at diapause nor during the subsequent incubation required for normal embryogenesis, was the radiation injury reflected in the uric acid content of the eggs. Moreover, the increase in uric acid found in irradiated eggs on prolonged incubation at 25° and following storage at 4° was shown not to be a characteristic effect of radiation damage since similarly treated "age-dead" eggs showed the same increase. This increase seems to be the result of degradation processes occurring in the dead eggs, irrespective of the cause of death. The formation of uric acid was not inhibited in the irradiated eggs as indicated by the temporary increase observed in April. Since Brown [2] found the activity of uricase in the eggs of *Lucilla sericata* M.G. and Ashbel [1] reported that in the diapausing eggs of *B. mori* as much as 0.46 g./kg./hr. of gaseous ammonia was released, the possibility of concomitant degradation by this way of the eventually arising uric acid had to be considered. However, the observed stability of total N content of the eggs at diapause as well as during the postdiapausal incubation invalidate this supposition. Thus the effect of radiation observed in adult insects by Wharton & Wharton [16] was not revealed at the egg stage.

During the obligatory diapause the uric acid content remained constant and did not parallel the periodicity observed in carbohydrate

metabolism by Chino [4, 5]. The increase amounting to 40% of diapausal value could be observed only when the eggs became ready for further development but did not develop because of storage temperature below the threshold for embryogenesis. This increase can be interpreted as a result of deamination processes involving purines, known to occur in insects [6]. The mechanism of subsequent decrease in uric acid remains unexplained. The accumulation of uric acid during the embryogenesis reported to occur in eggs of *Oncopeltus fasciatus* Dallas [11] and of *Lucilla sericata* M. G. [2] was not stated in the eggs of *Bombyx mori.*

Since the temporary increase in uric acid observed in April was a result of the artificial arrest of embryogenesis it can be stated that the uric acid content of the eggs of *Bombyx mori* remains constant during the normal diapause and development. The role of uric acid as the endproduct of embryonal metabolism seems questionable.

SUMMARY

Uric acid content of *B. mori* eggs, amounting to 3.5 mg./g. of whole eggs remained constant during the diapause as well as during postdiapausal embryogenesis. The irradiation at diapause with 20 kr of γ-rays, which inhibited the hatching of larvae, did not influence the uric acid content. Uric acid accumulated in the dead eggs, regardless of the cause of death, only after 10 days of postdiapausal incubation at 25°.

REFERENCES

[1] Ashbel R. - *Boll. Soc. Ital. Biol. Sper.* **4**, 1, 1929.
[2] Brown A. W. A. - *Biochem. J.* **32**, 895, 1938.
[3] Caraway W. T. - *Amer. J. Clin. Path.* **5**, 840, 1955.
[4] Chino H. - *J. Insect Physiol.* **6**, 231, 1961.
[5] Chino H. - *Arch. Biochem. Biophys.* **102**, 400, 1963.
[6] Florkin M. & Duchateau Gh. - *Arch. Intern. Physiol.* **53**, 267, 1943.
[7] Forrest H. S., Haftield D. & Lagowski J. M. - *J. Amer. Chem. Soc.* **3**, 963, 1961.
[8] Harth S. & Mandel P. - *Bull. Soc. Chim. Biol.* **43**, 969, 1961.
[9] Harvey W. R. - *Ann. Rev. Entomol.* **7**, 57, 1961.
[10] Heller J. & Jeżewska M. M. - *Acta Biochim. Polon.* **7**, 469, 1960.
[11] Hudson B. W., Bortel A. H. & Craig R. - *J. Insect Physiol.* **3**, 63, 1959.
[12] Lassota Z. - *Acta Biochim. Polon.* **10**, 379, 1963.
[13] Leone E. & Guerritore B. - *Boll. Soc. Ital. Biol. Sper.* **26**, 609, 1950.
[14] Leone E. - *Biochem. J.* **54**, 393, 1953.
[15] Sevag M. G., Lackman D. B. & Smolens J. - *J. Biol. Chem.* **124**, 425, 1938.
[16] Wharton D. R. A. & Wharton M. L. - *Radiat. Res.* **14**, 423, 1961.

KWAS MOCZOWY W JAJACH *BOMBYX MORI* NORMALNYCH I NAŚWIETLANYCH PROMIENIAMI GAMMA

Streszczenie

Zawartość kwasu moczowego w jajach *Bombyx mori* wynosi około 3,5 mg/g całych jaj i nie ulega zmianom w czasie diapauzy ani podiapauzalnego okresu embriogenezy. Naświetlanie jaj w okresie diapauzy 20 kr promieni gamma, hamujące wylęg gąsienic, nie wpływa na poziom kwasu moczowego w jajach. Kwas moczowy nagromadza się w martwych jajach, jeśli w okresie podiapauzalnym inkubuje się je w 25°. Nagromadzanie się kwasu moczowego w martwych jajach następuje niezależnie od przyczyny ich śmierci.

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RECENZJE KSIĄŻEK

R. A. Peters, BIOCHEMICAL LESIONS AND LETHAL SYNTHESIS. Pergamon Press, Oxford-London-New York-Paris 1963; str. X + 321; cena 70 s.

Autor jest znanym biochemikiem, wieloletnim uczniem i asystentem F. G. Hopkinsa w Cambridge. W pracowni Hopkinsa Peters zaczął interesować się zagadnieniami żywienia, a w szczególności rolą tiaminy. W roku 1922, w wieku lat 33, powołany został na kierownika Katedry Biochemii w Oksfordzie i przez 31 lat kierował tą placówką.

Recenzowana książka jest zbiorem krótkich esejów i wykładów wygłaszanych przez Autora, zebranych obecnie i uzupełnionych ostatnimi osiągnięciami w omawianych dziedzinach.

We wstępie Autor definiuje termin "biochemical lesion" jako pewne niekorzystne biochemiczne zmiany w komórkach tkanek, zmiany niedostrzegalne w świetle mikroskopu. Termin ten został wprowadzony przez Petersa w roku 1931, w czasie jego prac nad biochemicznymi zaburzeniami w organizmie zwierzęcia wywołanymi niedoborem tiaminy w pożywieniu. W tym czasie w świecie naukowym krystalizował się pogląd, że przyczyn wielu patologicznych zmian w tkankach należy szukać w zaburzeniach biochemicznych. Analiza chemiczna pozwala często na wykrywanie zakłóceń, np. w procesach enzymatycznych, zanim jeszcze uwidocznią się zmiany histologiczne. W powiązanych reakcjach biochemicznych wystarczy czasami uszkodzenie jednego z ogniw łańcucha przemian, aby nagromadzające się produkty reakcji spowodowały uszkodzenie tkanek, a nawet śmierć organizmu. Tak na przykład trujące działanie cyjanku polega na zablokowaniu jednego ogniwa w łańcuchu oddechowym, co prowadzi do zahamowania transportu elektronów.

Biochemiczne podejście do patologii jest, zdaniem Autora, szczególnie istotne w diagnostyce lekarskiej. Pozwala ono niejednokrotnie na ustalenie przyczyny choroby zanim powstałe zmiany staną się nieodwracalne. Oczywiście przyczyną choroby nie koniecznie muszą być zaburzenia w układach enzymatycznych, najczęściej jednak tu należy szukać zakłóceń.

W pierwszej części książki Peters szeroko opisuje swoje dawne badania nad zaburzeniami biochemicznymi wywołanymi niedoborem tiaminy, bądź jej koenzymu, pirofosforanu tiaminy. Stwierdzono wówczas, że zaburzenia polegają na zahamowaniu procesu utleniania kwasu pirogronowego. W mózgu gołębia z typowymi objawami awitaminozy nie stwierdzono pod mikroskopem żadnych zmian histologicznych, co wskazywało, że pierwotnym zaburzeniem są zmiany biochemiczne. Podanie ptakom w odpowiednim czasie witaminy powodowało znikanie objawów chorobowych.

W następnych rozdziałach książki Peters opisuje dalsze badania nad uszkodzeniami biochemicznymi wywołanymi substancjami blokującymi enzymy bądź układy enzymatyczne. Przykładem są jego badania nad trującym działaniem związków arsenowych np. luizytu, który, jak stwierdził, również hamuje przemianę kwasu pirogronowego. Luizyt blokuje tiolowe grupy kwasu liponowego, akceptora acetylowej reszty pirogronianu. Tego rodzaju zablokowania mogą zostać usunięte

działaniem związków bogatych w grupy tiolowe, np. 2,3-dwumerkaptopropanolu (BAL) lub innych podobnych, podanych w odpowiednim czasie.

W drugiej części książki Peters omawia nowy, również przez niego zaproponowany termin "lethal synthesis" (śmiertelna lub szkodliwa synteza), który zyskał już prawo obywatelstwa w anglosaskim piśmiennictwie. Pod tym terminem rozumieć należy nietoksyczne substancje, których szkodliwe działanie uwidocznia się dopiero w trakcie ich dalszego metabolizmu i blokowania lub wiązania enzymu bądź układu enzymatycznego. W przykładzie omawianym przez Petersa figuruje fluorooctan, substancja nietoksyczna, której szkodliwość ujawnia się dopiero po połączeniu się jej z kwasem cytrynowym. Powstały kwas fluorocytrynowy blokuje działanie hydro-liazy cytrynianu, zwanej dawniej akonitazą. Odkrycie tego typu zjawiska rzuciło wiele światła na mechanizm działania różnych naturalnych trucizn oraz otwierało nowe możliwości badania pośrednich metabolitów. Tą drogą stwierdzono np. zaburzenia syntezy skwalenu po podaniu kwasu fluoromewalonowego, zaburzenia syntezy RNA i zaburzenia w tworzeniu się wirusów po podaniu 5-fluorouracylu. Ten ostatni związek hamuje również tworzenie tymidylanu i syntezę DNA. Zamiast kwasu tymidylowego powstaje 5-fluoro-2-dezoksyurydyno-5-monofosforan. Podobnie działają inne analogony, po wbudowaniu których powstają pośrednie związki mogące działać jako antymetabolity. Zdaniem Petersa wbudowywanie analogonów nie zawsze prowadzi do powstawania szkodliwych związków, a wbudowywanie ich do łańcucha białkowego nie zawsze prowadzi do utraty aktywności enzymatycznej.

Końcowe rozdziały, zawierające treść wykładów wygłaszanych przez Petersa w latach 1929—1930, poświęcone są ówczesnym poglądom na rolę witamin grupy B w komórkach. Omawia również Peters zależności pomiędzy strukturą błon komórkowych a aktywnością komórki. Jeden z wykładów, wygłoszony w roku 1962, poświęcony jest roli błon komórkowych, a zwłaszcza półpłynnych błon wewnątrzkomórkowych, w kierowaniu procesami biochemicznymi komórki, głównie poprzez kontrolę układów enzymatycznych. Przyjęcie poglądu o występowaniu tego typu błon prowadzi z kolei do sugestii, że działanie substancji i czynników wywołujących nowotwory lub biochemiczne uszkodzenia polegać może na uszkodzeniu tych błon. To z kolei może prowadzić do długotrwałych zmian w toczących się w komórce procesach biochemicznych.

Bronisław Filipowicz

F. T. G. Prunty, CHEMISTRY AND TREATMENT OF ADRENOCORTICAL DISEASES. Charles C. Thomas Publ., Springfield (Ill.) 1964; str. 380.

Zrozumienie wielu stanów chorobowych wymaga znajomości ich patomechanizmów, w których zaburzenia w przemianach ustrojowych odgrywają niepoślednią rolę. Wymaga to znajomości podstawowych pojęć chemicznych, a w szczególnych zespołach patologicznych znajomości biochemii. Dlatego też w Stanach Zjednoczonych przystąpiono do wydawania serii publikacji znanej pod nazwą American Lectures in Living Chemistry (co można w wolnym tłumaczeniu określić: żywa biochemia), której wydawcą jest znana i zasłużona firma wydawnicza Charles C. Thomas. Wzajemne powiązanie medycyny z biochemią, jak wyjaśnia to redaktor I. N. Kugelmass, jest tak duże, że lekarze coraz częściej zwracają się ku chemii, a chemicy ku medycynie w celu zrozumienia procesów leżących u podstaw życia i choroby. Choroby kory nadnerczy są wymownym tego przykładem, wysuwając wiele zagadnień do rozwiązania wspólnie klinicyście i chemikowi.

Znajomość więc problemów chemicznych może przyczynić się do powodzenia w zabiegach terapeutycznych. Ten punkt widzenia określa układ książki, tj. w pierwszej części zawarto głównie zagadnienia związane z biochemią i fizjologią hormonów kory nadnerczy, w dalszych omówiono patofizjologię oraz metody wykrywania zaburzeń czynności nadnerczy i to zarówno chemiczne, jak i biologiczne, wreszcie przedstawiono klinikę chorób nadnerczy.

Całość materiału zawarto w XIV rozdziałach, z których niektóre należy nieco szerzej omówić. Rozdział I, poświęcony syntezie sterydów nadnerczowych, podkreśla, że punktem wyjściowym syntezy hormonów kory nadnerczy są octan i cholesterol. Najlepiej poznana jest synteza kortyzolu, gdzie główny ciąg syntezy biegnie z pregnenolonu poprzez progesteron, 17-hydroksyprogesteron, 11-dezoksykortyzol do kortyzolu, zachowując kolejność hydroksylacji węgli C_{17}, C_{21} i C_{11}. Znacznie gorzej jest poznana droga syntezy aldosteronu i sterydów 19-węglowych. W związku z procesami syntezy hormonów nadnerczowych omówiono pobudzający wpływ kortykotropiny na korę nadnerczy oraz rolę układów enzymatycznych w procesach syntezy.

W rozdziale II szczegółowo omówiono naturę chemiczną ACTH, znaczenie niektórych czynników humoralnych i nerwowych w jego wyzwalaniu, oraz metody oznaczania. Szczególnie dużo uwagi poświęcono mechanizmowi sekrecji aldosteronu.

Rozdział III przedstawia niezmiernie ważne, a rzadko tak dokładnie omawiane, zagadnienie dalszych losów hormonów sterydowych w ustroju. Przyjęto na ogół, że zasadniczymi miejscami, w których hormony nadnerczowe podlegają dalszym przemianom, są wątroba i nerki, chociaż kortozyl może ulegać przemianie w kortyzon poza tymi narządami. Badania zaś ilościowej konwersji podanych hormonów nadnerczowych w odpowiednie metabolity dały podstawy do wyliczeń, na podstawie których można dokładnie określić ilość ich sterydowych prekursorów. Wszystkie metabolity nadnerczowych sterydów są wydalane do moczu w formie zestryfikowanej jako glukuronidy i siarczany, w nieznacznej zaś ilości w połączeniu z kwasem fosforowym lub aminokwasami.

W rozdziale IV omówiono sprawę działania sterydów nadnerczowych i ich wpływu na metabolizm węglowodanowy, białkowy oraz elektrolitowy ze szczególnym uwzględnieniem roli nerek w jego mechanizmie. Natomiast zasady, na których oparto metody oznaczania ilościowego sterydów i ich metabolitów przedstawiono w rozdziale V, aspekty zaś ilościowe wydzielania, transportu i wydalania zawarto w następnym, VI rozdziale. Pozostałych dziewięć rozdziałów poświęcono zaburzeniom metabolizmu sterydów pozanadnerczowych, zagadnieniu pobudzenia i hamowania wydzielania hormonów nadnerczowych, hypofunkcji nadnerczy pochodzenia przysadkowego, chorobie Addisona, chorobie Cushinga, zespołowi aldosteronizmu, wirylizmowi i wreszcie guzom nadnerczy.

Wszystkie rozdziały napisane są z niezwykłą jasnością i prostotą, dzięki której bez zbędnej symplifikacji zagadnienia można je bez zastrzeżeń zrozumieć. Całość przedstawia współczesny stan wiedzy z zakresu chemii, kliniki i terapii chorób kory nadnerczy, co tym bardziej godne jest podkreślenia, że Autor oparł się na piśmiennictwie do końca 1962 r., a piśmiennictwo jest uwzględnione w bardzo szerokim zakresie, bo zamknięto je pokaźną liczbą 1090 pozycji.

Książka, poza znakomitą treścią, podaną jasno i bezbłędnie, odznacza się doskonałą formą edytorską. Wydana na bardzo dobrym papierze, ma dobrze zróżnicowaną czcionkę drukarską, dzięki czemu łatwo wychwytuje się te zagadnienia, które Autor uważa za ważne. Ponadto korekta jest bezbłędna, co jest wyrazem troskliwości wydawcy. Książkę tę pragnę jak najgoręcej polecić wszystkim zainteresowanym sprawami endokrynologii nadnerczowej.

Marian Górski

E. E. Levitt, H. Persky and J. P. Brady, HYPNOTIC INDUCTION OF ANXIETY: A PSYCHO-ENDOCRINE INVESTIGATION Charles C. Thomas Publ., Springfield (Ill.) 1964; str. 134; cena $ 6.50.

W książce tej, która powstała w wyniku współpracy trzech amerykańskich Autorów, psychologa, endokrynologa i psychiatry, przedstawiono wyniki badań własnych nad lękiem. Oryginalna metoda badań polegała na sugerowaniu badanym osobom w hipnozie stanów lękowych. Badania przeprowadzono na 78 osobach wybranych spośród uczennic szkoły pielęgniarskiej i studentów medycyny. Przed hipnozą, podczas trwania hipnozy, w czasie zasugerowanego w hipnozie stanu lękowego oraz po wyjściu z hipnozy, wykonywano kilka testów psychologicznych zezwalających na określenie stanu lękowego, np. test Rohrschacha, Thematic Aperception Test (TAT), Manifest Anxiety Scale (MAS), skala oceny klinicznej i in., oraz następujące badania endokrynologiczne: oznaczenie hydrokortyzonu w surowicy krwi u wszystkich badanych, oznaczenie ACTH we krwi oraz oznaczenie w surowicy czynnika utrzymującego wagę nadnerczy (plasma adrenal weight-maintenance factor) u niektórych badanych.

We wstępie Autorzy dokładnie uzasadniają stosowaną przez nich technikę doboru materiału, zezwalającą na przyjęcie, że materiał ten nie różnił się od ogółu populacji z badanego środowiska. W rozdziałach następnych przedstawiono szczegółowo różne warianty metody badawczej, m.in. wariant polegający na podaniu hydrokortyzonu przed hipnozą. Po przedstawieniu każdego z wariantów metodycznych, Autorzy szczegółowo analizują wyniki, stosując nie tylko metody statystyczne, ale także opierając się na prawie wyjściowych wartości Wildera (law of initial values). Ostatnie trzy rozdziały poświęcone są omówieniu wyników testów psychologicznych, wyników badań endokrynologicznych i perspektywom badawczym badań nad lękiem.

Najważniejsze wnioski Autorów są następujące:

1. Wyniki badań testowych, zwłaszcza niektórymi testami, jak np. test Rohrschacha, TAT, skala oceny klinicznej, wskazują na istnienie wyraźnego stanu lękowego podczas sugerowania lęku w hipnozie.

2. Wyniki badań endokrynologicznych wskazują przede wszystkim na to, że stan lęku w hipnozie wywołuje wyraźny wzrost poziomu hydrokortyzonu w surowicy krwi. Wzrost ten jest tym wyraźniejszy, im wyjściowy poziom hydrokortyzonu jest niższy przed hipnozą. Wysoki wyjściowy poziom hydrokortyzonu warunkuje długotrwałość stanów lękowych.

3. Istnieją jeszcze inne korelacje endokrynologiczne ze stanem lęku, wymagają one jednak dla ostatecznego sformułowania uzupełnienia obserwacji na większym materiale.

Dalsze perspektywy badań nad lękiem widzą Autorzy w rozszerzeniu badań endokrynologicznych i w prowadzeniu analizy zmian wegetatywnych badanych osób.

Książka, obejmująca 134 strony, posiada załączony bardzo bogaty i dobrze dobrany spis piśmiennictwa (136 pozycji) oraz załączone opisy niektórych stosowanych testów psychologicznych.

Jest to praca będąca bardzo cennym wkładem zarówno psychologicznym, jak endokrynologicznym w badaniach nad zmianami stanów emocjonalnych u ludzi. Zaproponowana przez Autorów metoda badań dała możność badania zmian stanu emocjonalnego i wywołujących go zmian endokrynologicznych w stanie niejako „czystym", „laboratoryjnym", w którym stan lęku nie jest związany z innymi zmianami stanu psychicznego.

Andrzej Jus

NEW PERSPECTIVES IN BIOLOGY (M. Sela, ed.) Elsevier Publishing Co., Amsterdam-London-New York 1964; str. XVIII + 285; cena Dfl. 40.-, sh. 80, DM 44.50.

Niezwykle bujny rozwój biologii molekularnej oraz wspaniałe osiągnięcia tej gałęzi nauki powodują, że każda nowa publikacja z tej dziedziny wzbudza ogromne zainteresowanie nie tylko w wąskim gronie specjalistów lecz wśród większości pracowników nauk biologicznych. Dlatego też z uznaniem należy powitać wydanie referatów wygłoszonych na sympozjum zorganizowanym z okazji inauguracji Ullmann Institute of Life Sciences w dniach 10-17 czerwca 1963 r. w Rehovoth w Izraelu. Publikacja ta stanowi 4 tom serii BBA Library.

W sześciu rozdziałach książki zgrupowano 21 referatów wygłoszonych w czasie sympozjum. Pierwszy rozdział: Nowe perspektywy w badaniach nad białkami, jest podsumowaniem najnowszych osiągnięć z dziedziny struktury cząsteczki białkowej oraz funkcji białek na poziomie molekularnym. Rozdział ten zawiera 6 referatów: J. T. Edsalla o ostatnich osiągnięciach w badaniach nad trzecio- i czwartorzędową budową białek; J. C. Kendrew o budowie mioglobiny oraz hemoglobiny, omawiający również problemy zastosowania tej samej techniki do badania budowy innych białek; H. Neuratha o zależności funkcji enzymów proteolitycznych od budowy, poświęcony głównie zmianom konformacji tych białek przy aktywacji; Ch. B. Anfinsena o możliwości przewidywania trzeciorzędowej budowy białka z jego budowy pierwszorzędowej, rozważający trzeciorzędową budowę rybonukleazy, oraz E. Katchalski'ego omawiający niezwykle interesujące wyniki badań nad syntezą poliaminokwasów i zastosowaniem ich jako modelowych związków w badaniach nad mechanizmem działania enzymów proteolitycznych, jako inhibitorów enzymów, czynników antywirusowych i antybakteryjnych, a także jako modelowych związków w immunochemii. Ostatni referat tej sekcji, wygłoszony przez T. Lipmanna, omawia mechanizmy biosyntezy białek, głównie etap polimeryzacji aminokwasów, zachodzącej w rybosomach.

Drugi rozdział książki poświęcony jest budowie i funkcji kwasów nukleinowych. Znalazły się w nim referaty E. Chargaffa o niektórych aspektach sekwencji nukleotydów w DNA i D. Elsona o badaniach nad enzymami związanymi z rybosomami, omówione jest m.in. zagadnienie obecności RNA-azy w cząsteczkach 30 S. Autor omawia obszernie przypuszczalną rolę poszczególnych komponent rybosomów w syntezie białek oraz znaczenie, jakie posiada obecność RNA-azy w tych cząstkach. Dalsze szczegóły o mechanizmie syntezy białek podane są w następnym artykule zatytułowanym „O funkcji rybosomów", w którym A. Girer omawia kolejność łączenia się aminokwasów oraz rolę polisomów w tej syntezie na przykładzie retikulocytów królika, najlepiej poznanego układu syntezy białek. Ostatnim artykułem tej sekcji jest referat S. Ochoa o kodzie genetycznym, w którym omówiono metodę badania kodu genetycznego przy użyciu syntetycznych homo- lub kopolimerów nukleotydów.

Trzeci rozdział zawiera 4 referaty poświęcone funkcji i organizacji układów enzymatycznych i struktur podkomórkowych. F. Lynen opisuje w nim koordynację ciągów metabolicznych przez kompleksy wieloenzymowe. H. Theorell przedstawia badania nad powstawaniem kompleksów dehydrogenazy alkoholowej z substratem, koenzymem oraz inhibitorem. H. Wieter omawia tzw. aktywny transport jonów przez błony komórkowe oraz rolę ATP w tym procesie, zaś D. Nachmanson wywoływane przez acetylocholinę zmiany przepuszczalności błon dla jonów i znaczenie tego zjawiska w procesie przewodnictwa impulsów. Rozdział IV zawiera tylko jeden artykuł E. Chaina o nowych penicylinach. Rozdział V poświęcony jest immunochemii i zawiera artykuły o konstytucyjnej i immunologicznej swoistości oraz o syntetycznych polipeptydowych antygenach.

Ostatni rozdział: Komórkowe, podkomórkowe i molekularne aspekty różnicowania, zawiera artykuły omawiające genetyczne określenie morfogenezy bakteriofaga, mechanizmy regulacyjne różnicowania komórek oraz genetyczne aspekty powstawania nowotworów. Są to problemy, które wchodzą dopiero w fazę badań na poziomie molekularnym i na ich przykładzie tym jaśniej widoczne są ogromne perspektywy otwierające się przed biologią molekularną w tych dziedzinach.

Niektóre referaty przedstawiono w sposób bardzo zwięzły i skondensowany, jednakże nie tracą one przez to jasności. Żałować należy jedynie, że referaty J. Monod o allosterycznych mechanizmach regulacji przemian oraz P. Doty o powstawaniu hybrydów kwasów nukleinowych, wygłoszone w czasie sympozjum, nie zostały umieszczone w tym wydawnictwie. Brak ich natychmiast wyczuwa się przy czytaniu.

W sumie jednak książka stanowi świetny przegląd najbardziej aktualnych zagadnień biochemii molekularnej. Zawiera ona również przeszło 700 pozycji piśmiennictwa obejmującego ostatnie lata aż do roku 1963 włącznie. Można śmiało powiedzieć, że powinna ona znaleźć się w każdej bibliotece biochemicznej.

Michał Bagdasarian

I. N. Kugelmass. BIOCHEMICAL DISEASES (Chemical Pediatrics) Charles C. Thomas Publ., Springield (Ill.) 1964; str. 1229, cena $ 35.50.

We wstępie Autor podkreśla coraz ściślejsze powiązanie biochemii z medycyną. Biochemik zwraca się do medycyny, a lekarz do biochemii w celu uzyskania lepszego zrozumienia podstawowych procesów zdrowia i choroby. Biochemia przekształca empiryczną praktykę w kliniczną wiedzę przez wprowadzenie faktów dla wyjaśnienia wieloznacznych objawów, pomiarów zamiast szacunkowych ocen i dowodów w miejsce wrażeń. Przez to uzyskuje się racjonalne podstawy leczenia.

Autor wysuwa pogląd, że choroba jest rozkojarzeniem biochemicznych procesów fizjologicznych. Doskonale odpowiadają temu ujęciu choroby dziedziczne, zwłaszcza wszelkie bloki metaboliczne. Ciekawe jest zdanie: „wiemy więcej o chorobach, niż je rozumiemy, a zrozumienie wprowadza, przynajmniej w swoim zakresie, biochemia".

Zdumienie wzbudza ujęcie prawie całości medycyny pediatrycznej z punktu widzenia biochemicznego przez jednego Autora w postaci jednego ogromnego tomu. Od wielu lat panuje słuszna tendencja do zespołowego opracowywania różnorodnych dziedzin. Książka ta stanowi wyłom w tej zasadzie. To z góry narzuca pewne konsekwencje, zarówno dodatnie jak i ujemne: jednolitość ujęcia, ale też niejednolity poziom, gdyż jest rzeczą niemożliwą znać wszystko jednakowo głęboko i szczegółowo. Integracja w tym kierunku polega na prawidłowej selekcji materiału informacyjnego, zatrzymywaniu informacji wiodących, wiążących fenomenologię kliniczną z zasadami biochemicznymi. Autor potrafił osiągnąć ten cel i zachował właściwe proporcje między kliniką a biochemią.

Interesujący jest ogólny podział materiału. Część I obejmuje układy integracyjne, zaburzenia psychiczne, neurologiczne, hormonalne. Część II omawia układy konstytucjonalne, choroby alergiczne, kolagenowe, barwikowe, żywieniowe, elektrolitowe, nieprawidłowości hemoglobiny, krwinek czerwonych, białaczki, skazy krwotocznej. Część III obejmuje zaburzenia efektorów narządowych: wątroby, przewodu pokarmowego, płuc, serca, nerek, mięśni, choroby chirurgiczne i choroby wynikłe z działania zewnętrznych czynników otoczenia.

Książkę rozpoczyna kalendarz odkryć biochemicznych XX wieku począwszy od sformułowania polipeptydowej natury białek przez Hofmeistera i Fischera

w 1902 r., skończywszy na ustaleniu przez Cricka w 1964 r. składu i sekwencji aminokwasów w krystalicznych białkach przy pomocy analizy rentgenowskiej Właściwy tekst poprzedza spis skrótów biochemicznych, metrycznych, klinicznych, obejmujący około 150 pozycji.

Podział materiału dokonany jest z klinicznego punktu widzenia, ujęcie tego materiału natomiast jest biochemiczne, znakomicie zresztą dostosowane do potrzeb kliniki. Wartość książki podnoszą dość liczne zestawienia tabelaryczne zarówno z dziedziny biochemii i fizjologii, jak i samej kliniki: symptomatologia, diagnostyka różnicowa, objawy biochemiczne, próby czynnościowe. Nie ma natomiast w tej książce żadnych przepisów metodycznych, nie ma ani jednego wzoru chemicznego. Każdy rozdział zakończony jest bardzo obszernym piśmiennictwem do 1961 r. włącznie.

Styl książki jest nieledwie telegraficzny, zdania krótkie, orzekające, zawężone do stwierdzeń. Hipotezy omówione zwięźle, główny nacisk położony na to, co się wie w oparciu o dowodowe dane. Mimo to książka jest ogromnym dziełem, zdumiewającym, jako twór jednego autora, rozległością informacji, dokumentacją, ogromem piśmiennictwa.

Książka I. N. Kugelmassa będzie niewątpliwie cennym nabytkiem w bibliotekach szpitalnych i klinicznych, w bibliotekach domowych lekarzy klinicystów biochemicznie zorientowanych oraz na półkach kierowników i pracowników laboratoriów i pracowników teoretycznych dziedzin paraklinicznych.

Leszek Tomaszewski

J. S. Brimacombe and J. M. Webber: MUCOPOLYSACCHARIDES. Chemical Structure, Distribution and Isolation. Elsevier Publishing Co., Amsterdam 1964; str. IX + 181; cena Dfl. 27.50, sh. 55, DM 31.

Recenzowana książka jest szóstym tomem BBA Library. Poprzedni V tom omawiał glikoproteidy, a więc związki bardzo blisko związane z mukopolisacharydami. Nie można ściśle przeprowadzić granicy pomiędzy tymi grupami związków. Nazwą mukopolisacharydy, wprowadzoną przez Meyera na określenie polisacharydów zawierających heksozaminę, z biegiem lat zaczęto nazywać i inne związki polisacharydowe nie zawierające heksozaminy. Dlatego też Autorzy po rozdziale wprowadzającym dokładnie precyzują, jakie związki będą omawiane w dalszych rozdziałach.

Książka głównie omawia strukturę chemiczną, występowanie, metody izolacji oraz sposoby oznaczania mukopolisacharydów. Można jednak tam znaleźć wiadomości dotyczące ich biosyntezy oraz roli fizjologicznej. W oddzielnych rozdziałach zebrano wiadomości dotyczące chityny, kwasu hyaluronowego, kwasów chondroitynosiarkowych, heparyny oraz substancji grupowych krwi. W osobnym rozdziale omówiono rzadziej występujące mukopolisacharydy, takie jak keratosiarczan, siarczan heparyny, kwas mukoitynosiarkowy, kwas tejchurowy oraz różnego rodzaju mukopolisacharydy występujące u bezkręgowców. Jeden z rozdziałów obejmuje substancje grupowe krwi, które zostały omówione szerzej tylko pod względem struktury chemicznej, ponieważ inne aspekty tego zagadnienia przedstawił Gottschalk w poprzednim tomie BBA Library.

Każdy z rozdziałów zaopatrzony jest w spis literatury obejmujący przeciętnie sto kilkadziesiąt, a niekiedy i więcej, pozycji. Przedyskutowanie tylu pozycji literatury czyni książkę bardzo wartościową dla specjalisty, ponieważ pozwala mu łatwo zapoznać się ze wszystkimi ważniejszymi odkryciami w tej dziedzinie. Z dru-

giej jednak strony dla czytelnika, który chce się tylko ogólnie zorientować w zagadnieniu, może to stanowić pewne utrudnienie.

Omawiana książka jest doskonałym źródłem wiadomości dotyczących budowy chemicznej, występowania, sposobów izolacji oraz metod oznaczania różnych mukopolisacharydów w materiale biologicznym.

Wiktor Rzeczycki

S. Abrahamsson, S. Ställberg-Stenhagen and E. Stenhagen. PROGRESS IN THE CHEMISTRY OF FATS AND OTHER LIPIDS. Vol. VII, Part. I, The Higher Saturated Branched Chain Fatty Acids. Pergamon Press, Oxford-London-New York-Paris 1963; str. 164, cena 40 s.

Kwasy tłuszczowe o łańcuchu rozgałęzionym stanowią niewielki jedynie procent w mieszaninie kwasów otrzymanych drogą hydrolizy tłuszczów naturalnych. Na szerszą skalę, w czasie drugiej wojny światowej w Niemczech produkowano tłuszcze syntetyczne z dużą zawartością kwasów rozgałęzionych. Znaczenie kwasów rozgałęzionych i ich wpływ na ustroje zwierzęce jest mało poznany. Do czasu wprowadzenia chromatografii gazowej, w stanie czystym izolowano zaledwie kilka kwasów tłuszczowych rozgałęzionych, głównie z bakterii. Obecnie lista kwasów z różnych źródeł obejmuje dziesiątki pozycji.

Książka zajmuje się głównie chemią i fizyko-chemią wyższych nasyconych rozgałęzionych kwasów tłuszczowych. Obszernie omawia metody syntezy oraz badania nad strukturą kwasów. Autorzy poświęcili dużo uwagi zagadnieniu zależności pomiędzy strukturą a własnościami fizycznymi omawianych kwasów. Dział ten poparty jest wnikliwą analizą danych doświadczalnych, ich interpretacją oraz rozważaniami teoretycznymi. Śledzenie wywodów ułatwiają liczne rysunki i schematy. Bardzo pożyteczne są tabele zestawiające najważniejsze dane fizyczne poszczególnych kwasów rozgałęzionych i ich pochodnych. Tablice te zajmują ponad 30 stron. Dla niektórych kwasów podano także wykresy widma w podczerwieni. W sumie jest to cenne źródło wielu danych fizyko-chemicznych o rozgałęzionych kwasach tłuszczowych. Bardzo skromnie natomiast potraktowano rozdziały dotyczące własności biologicznych kwasów rozgałęzionych i ich występowania. Zebrane wiadomości dają jedynie krótki i niepełny obraz zarysowujących się tu problemów.

Książka zainteresować może chemików, fizyko-chemików i biochemików zajmujących się wyższymi kwasami tłuszczowymi, ich rozdziałem, izolacją i identyfikacją. Piśmiennictwo obejmuje pozycje do roku 1960. Książka uzupełniona jest wykazem blisko 400 prac cytowanych w tekście oraz alfabetycznym skorowidzem rzeczowym.

Ryszard Niemiro

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Ю. ЯНИЦКИ, Ф. ПЕНДЗИВИЛЬК, Я. СКУПИН, Е. КОВАЛЬЧИК,
Кристина НОВАКОВСКА и Кристина ТРОЯНОВСКА

СВЕТОЧУВСТВИТЕЛЬНЫЕ ПРОИЗВОДНЫЕ КОРИНОИДНО-БЕЛКОВЫХ КОМПЛЕКСОВ ИЗ КЛЕТОК PROPIONIBACTERIUM SHERMANII

Резюме

Из клеток P. shermanii-1 выделен светочувствительный кориноидно-белковый комплекс (B_{12}-M-S). Цвет и кривая поглощения света B_{12}-M-S указывает на наличие в нем коэнзима B_{12}. Эти свойства отличаются в комплексе выделенном при свете (B_{12}-M). Электрофорез, хроматография на бумаге и разделение на колонке CM-Sephadex G-25 показали, что комплекс B_{12}-M-S не однороден; не обнаружено, однако, его разложения до витамина B_{12}. Были идентифицированы две фракции, из которых одна содержала значительные количества глютаминовой кислоты, а другая — глицина. Фотолиз B_{12}-M-S вызывал образование B_{12}-M, а в присутствии цианидов — цианокобаламина.

Аминокислотный состав B_{12}-M-S отличается от состава препарата B_{12}-M. Соотношение белок: кориноид в препарате B_{12}-M составляло 1 : 1, а в препарате B_{12}-M-S 1 : 4 до 1 : 8 в зависимости от системы очищения. Активность препаратов B_{12}-M-S в качестве факторов роста для E. coli была обратно пропорциональна содержанию белка в этих препаратах.

Е. ПАВЕЛКЕВИЧ и Б. ЗАГАЛЯК

ФЕРМЕНТАТИВНОЕ ПРЕВРАЩЕНИЕ ГЛИЦЕРОЛА В β-ГИДРОКСИПРОПИОНОВЫЙ АЛЬДЕГИД В БЕЗКЛЕТОЧНОЙ СИСТЕМЕ ИЗ AEROBACTER AEROGENES

Резюме

Из клеток A. aerogenes культивируемых на среде с глицеролом выделена и описана ферментативная система катализирущая зависящю от коэнзима B_{12} реакцию превращения глицерода в β-гидроксипропионовый альдегид. Оптимум pH для этой реакции составляет 8.0, оптимум температуры 37°. Для реакции необходимо наличие ионов калия, которые можно заменить ионами Li^+, Rb^+ или H^+, но нельзя заменить ионами натрия. Реакция тормозится

реактивами реагирующими с сульфгидрильными группами, а также конкурентно тормозится некоторыми синтетическими аналогами коэнзима B_{12}. Кинетические исследования показали, что превращение глицерола является реакцией второго порядка. Величина K_m для лгицерола составляет 3.6×10^{-6}М². Молекула апофермента соединяется с двумя молекулами кофермента B_{12}, а K_m для этой реакции составляет 1.3×10^{-15}М².

Авторы выдвинули гипотезу, что фермент содержит два активных центра и что реакция превращения глицерола протекает в двух этапах, катализируемых двумя различными ферментами.

Б. ЗАГАЛЯК и Е. ПАВЕЛКЕВИЧ

СИНТЕЗ И СВОЙСТВА Co-АДЕНИН-НУКЛЕОЗИДНЫХ АНАЛОГОВ КОФЕРМЕНТА B_{12}

Резюме

В работе описан упрощенный метод химического синтеза кофермента B_{12} и его аналогов. Метод состоит в непосредственном тозилировании нуклеозидов аденина или производных аденина и соединении продуктов реакции с восстановленным цианкобаламином. Кофермент B_{12} был получен с выходом 50%. Аналоги содержащие радикалы 2′- и 3′-дезоксиаденозина получены с выходом 30%, а содержащие радикал изоаденозина с выходом 15%. Все эти аналоги оказались активными в качестве коферментов в системе выделенной из Aerobakter aerogenes превращающей пропан-1,2-диол в пропионовый альдегид и глицерол в β-гидроксипропионовый альдегид. Активности аналогов меньше, чем активность кофермента B_{12} и отличны между собой в обеих реакциях. Обсуждается зависимость между активностью кофермента B_{12} и его химической структурой.

Иоанна РЫТКА и В. ТЫСАРОВСКИ

ВЫДЕЛЕНИЕ И СВОЙСТВА ДЕГИДРОГЕНАЗЫ D(-)МОЛОЧНОЙ КИСЛОТЫ ИЗ ДРОЖЖЕЙ КУЛЬТИВИРУЕМЫХ В АНАЭРОБНЫХ УСЛОВИЯХ

Резюме

1. Разработан новый метод очищения дегидрогеназы D(-)молочной кислоты из дрожжей культивируемых в анаэробных условиях с применением трипсина.

2. Определены кинетические свойства этого фермента, на основании которых предлагается схема окисления D-α-гидроксикислот дегидрогеназой.

К. КЛЕЧКОВСКИ

L-ГЛЮТАМИН КАК ДОНОР АЗОТА ПРИ СИНТЕЗЕ КАРБАМИЛФОСФАТА В ПРОРОСТКАХ ГОРОХА

Резюме

Установлено, что в проростках гороха L-глютамин является наилучшим донором азота для синтеза карбамиловой группы цитрулина. L-Аспарагин обладает 30% а неорганические ионы аммония 10% активностью по отношению к L-глютамину, тогда как *N*-ацетилглютамин вовсе не активен. Активность системы зависит от наличия ионов Mg^{2+}, а оптимум pH составляет 7,4-7,6.

В. ТЫСАРОВСКИ и Анна КОНЕЦКА

EDTA КАК ИНГИБИТОР ВОССТАНОВЛЕНИЯ ФЕРРИЦИАНИДА ГЛЮТАТИОНОМ И ЦИСТЕИНОМ

Резюме

Исследовалось влияние EDTA на скорость реакции восстановления одноэлектронных или двуэлектронных акцепторов сульфгидрильными соединениями. EDTA вызывает торможение в системе: одноэлектронный акцептор — восстановитель содержащий одну группу SH.

Эффекты торможения после удаления меди различными методами и без удаления меди идентичны. EDTA не тормозит восстановления двуэлектронных акцепторов.

З. ШАФРАН

ГИДРОЛИЗ ЭФИРОВ *n*-НИТРОФЕНОЛА И ЖИРНЫХ КИСЛОТ ГОМОГЕНАТАМИ СЛИЗИСТОЙ ОБОЛОЧКИ ТОНКОЙ КИШКИ РАЗНЫХ ВИДОВ ЖИВОТНЫХ

Резюме

Исследовалась активность экстрактов слизистой оболочки тонкой кишки разных животных по отношению к эфирам *n*-нитрофенола и жирных кислот, содержащих от 2 до 12 атомов углерода. Наивысшая удельная активность наблюдалась у кролика и крысы, низшая — у человека и наиболее низкая активность — у свиньи, курицы и быка. Максимальная активность наблюдалась для эфиров C_5, C_6 и C_7.

На основании этих результатов, а также различий тормозящего действия диизопропилфторфосфата обсуждается возможность наличия нескольких эстераз в слизистой оболочке кишки.

М. БАГДАСАРЬЯН

НИЗКОМОЛЕКУЛЯРНЫЕ ПЕПТИДЫ ОБРАЗУЮЩИЕСЯ В РИБОЗОМАЛЬНЫХ ПРЕПАРАТАХ КАК ПРЕДПОЛАГАЕМЫЕ ПРЕКУРСОРЫ БЕЛКОВ

Резюме

Из инкубационной смеси применяемой для исследования включения аминокислот в белки in vitro выделены связанные аминокислоты, повидимому, пептиды или нуклеотидо-пептиды. Удельная активность радиоактивных аминокислот включаемых во фракцию низкомолекулярных пептидов того же порядка, что и удельная активность свободных аминокислот инкубационной смеси; эта активность превысшала удельную активность аминокислот, включенных в белки рибозом и микрозом примерно в тысячу раз. Это указывает на то, что исследуемые пептиды не являются продуктом деградации готовых белков, а скорее прекурсорами полипептидов. Исследуемые фракции не являются аминокислотами, связанными с AMP или s-RNA, так как при непосредственном динитрофенилировании из них освобождается немного радиоактивных аминокислот.

Зофия ЛЯССОТА

МОЧЕВАЯ КИСЛОТА В НОРМАЛЬНЫХ И ОБЛУЧЕННЫХ γ-ЛУЧАМИ ЯЙЦАХ BOMBYX MORI

Резюме

Содержание мочевой кислоты в яйцах Bombyx mori составляет в среднем 3,5 мг/г цельных яиц и не изменяется во время диапаузы и после диапаузы в периоде эмбриогенезиса. Облучение яиц в периоде диапаузы дозой 20 кг γ-излучения, тормозящее образование гусениц не влияет на содержание мочевой кислоты в яйцах. Мочевая кислота накапливается в мертвых яйцах, если в постдиапаузальном периоде они инкубируются при 25°. Накопление мочевой кислоты в яйцах происходит независимо от причины их гибели.