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JÓZEF HELLER

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JÓZEF HELLER

Atque inter silvas Academi quaerere verum
Horatius

This issue of Acta Biochimica Polonica and the first number of 1967 are dedicated to Professor Józef Heller on the occasion of his seventieth anniversary, in appreciation of his meritorious contributions to biochemistry and his devoted service to Polish science. All those who are privileged to call Professor Heller their friend, teacher or protector celebrate his jubilee of forty five years of research work and twenty years of unrelenting and successful efforts to rebuild and promote Polish biochemistry.

The path upon which Józef Heller engaged in his early years, and which he still follows, was difficult and unrewarding, yet a path which men with creative intellects chose for themselves. Through adversities, through the turmoils of European wars Józef Heller pursued his youthful dream: to study the mechanisms of life phenomena. He entered medical school while a soldier, and joined the laboratory of J. K. Parnas while an undergraduate. Soon publications bearing Heller's name appeared and in 1924 his first independent work is published on the metabolism of albuminoids in an insect. Insects become the principal object of his investigations, insects treated not as a population, but as individuals, the involved life cycle of which offers fascinating scope for Heller's inquisitive mind. The greater part of his more than one hundred experimental papers deal with insects, principally with hawk moth. Heller applies to his studies contemporary methods of biochemistry, physiology and at times methods borrowed even from medicine as e.g. rentgenology; he designs his own methods and specific apparatus. Soon a wealth of data is published on nitrogen and phosphate metabolism, on metamorphosis, on hereditary and other factors that control metabolism and development. Long before the oxidative phosphorylation had been discovered, Heller draws attention to interdependence between the intensity of metabolism and the level of unesterified phosphate. His is the first discovery of accumulation of inorganic pyrophosphate in an animal, followed by elucidation of its metabolism in the hawk moth.

In quest of general biological formulations, in pursuit of the basic principles common to all living things, Józef Heller tests the results obtained in insects on other biological systems and vice versa. Phosphate metabolism, pentose cycle, respiration and the role of ubiquinone in particular, are among the main lines of investigation by Heller and his school. They are among the first to realize the

fascinating opportunities of studying the genetic code offered by the spinning glands of the silk moth.

Without relaxing his scientific work, Professor Józef Heller devotes himself wholeheartedly to difficult task of rebuilding Polish biochemistry after the liberation from German occupation. A period of intense teaching of under- and postgraduates ensued while many a young and keen future biochemist was offered Professor's competent, exacting yet beneficient tuition. Numerous new laboratories are being created in Poland, new departments of biochemistry are founded, research work gains momentum. The Committee of Biochemistry and Biophysics is formed by the Polish Academy of Science; Professor Józef Heller is called to preside over it. He spares no effort to steer the course of Polish biochemistry along the paths which are most useful to Polish People's Republic and which made it possible for the Polish biochemistry to regain its traditional position in world biochemistry. In 1957, he is nominated the Director of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, the institute which he himself helped to create, to staff and equip.

Serene and helpful, Professor Józef Heller moves among his friends and pupils, for — in the words of Cicero — "*iucundi acti labores*".

Polish Biochemical Society

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BEATRICE KASSELL and M. LASKOWSKI, Sr.*

THE BASIC TRYPSIN INHIBITOR OF BOVINE PANCREAS

VI. SEQUENCE STUDIES AND DISULPHIDE LINKAGES

The Biochemical Laboratory for Cancer Research, Marquette University School of Medicine, Milwaukee, Wisconsin, U.S.A.

1. The primary structure of the basic trypsin inhibitor has been described in a series of short notes. 2. Since other workers have published a somewhat different linear structure, the points of difference have been reexamined and details of the methods are presented. 3. The structure originally proposed has been confirmed.

The pancreatic trypsin inhibitor of Kunitz & Northrop [25] has been the subject of structural studies in three laboratories. The first exact amino acid composition appeared in 1963 [21] and publication of three slightly different linear sequences was almost simultaneous [6, 18, 20, 10]. Our structure [20] is shown in Fig. 1, with the peptides from which it was derived. Recently two substances isolated as kallikrein inactivators from bovine parotid gland and lung [3, 1, 2] were shown to be identical to each other and to the sequence of Fig. 1. We have since reported [19] the location of the three disulphide bridges, which are 5-55, 14-38 and 30-51[1]. The present paper gives the details of the experiments previously summarized in short communications, and some additional data in confirmation of the sequence shown.

EXPERIMENTAL PROCEDURE

Materials. The inhibitor was the same preparation used for amino acid analysis [21].

The reduced, carboxymethyl (RCM[2]) derivative was prepared according to Crestfield *et al.* [8], except that the 50% acetic acid solution obtained in the final desalting [9] was dialysed before freeze-drying (cf. [21], step 9). The derivative was

* American Cancer Society Research Professor. Present address: Roswell Park Memorial Institute, Buffalo, New York.

[1] After this paper had been submitted for publication, the positions of the disulphide bridges were confirmed: Anderer F. A. & Hörnle S., *J. Biol. Chem.* **241**, 1568, 1966.

[2] Abbrevations used are — RCM: reduced, carboxymethyl-; CM-: carboxymethyl-; DNP-: 3,4-dinitrophenyl-; PTH-: phenylthiohydantoin of.

prepared in 500 mg. batches, and each batch analysed. Only traces of cystine and cysteic acid were found. The carboxymethyl cysteine content was close to 6 residues per molecule and all of the other amino acids had the same values as those previously reported [21]. The RCM-derivative had no inhibiting activity.

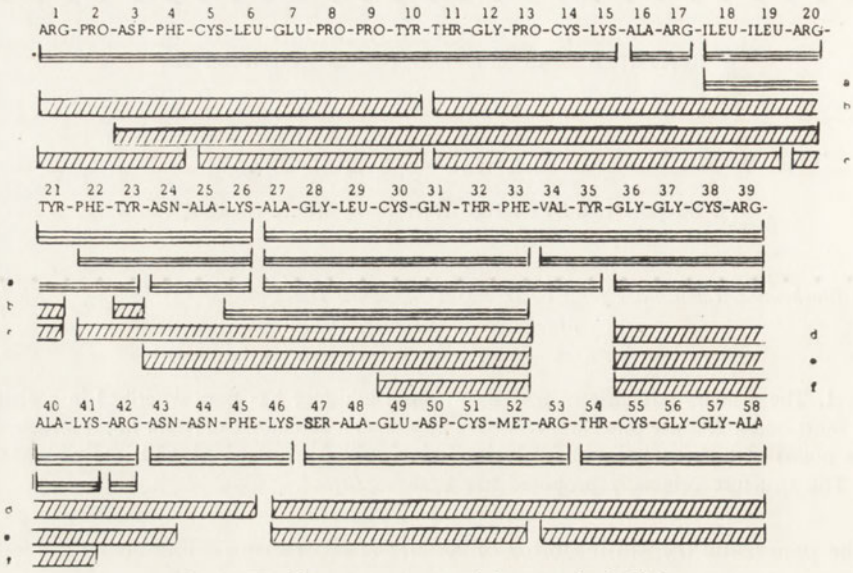

Fig. 1. The linear sequence of the trypsin inhibitor.

The succinylated inhibitor was prepared by the method of Habeeb *et al.* [15]: 574 mg. (88.3 μmoles) of native inhibitor was dissolved in 0.1 M-phosphate buffer, pH 8.0, readjusted to pH 8.0 and cooled to 0°. While the solution was stirred magnetically in an ice bath, 528 mg. (a 60:1 molar ratio) of finely powdered succinic anhydride was gradually added over a period of one-half hour, keeping the pH at 8.0 by intermittent addition of 1 N-NaOH. The product was dialysed against 1 liter of the phosphate buffer overnight, then against 2 × 1 liter of water and freeze-dried; yield 612 mg. The succinylated inhibitor had no activity against trypsin. When tested with ninhydrin [29], it showed only a trace of colour, compared to 7.7 leucine equivalents per mole for the native inhibitor.

The trypsin (EC 3.4.4.4) was the preparation described earlier [21] made from crystalline inhibitor-trypsin complex by trichloroacetic acid precipitation of the trypsin, followed by purification on CM-cellulose [26].

The chymotrypsin B (EC 3.4.4.6) was the sample of highest activity previously used to study the activation of chymotrypsinogen B ([24] Fig. 4). It still contained 0.034% of trypsin which was not removed after the activation.

Acetylated tubing [7] was used for dialysis throughout. Pyridine, *N*-ethylmorpholine, fluorodinitrobenzene, phenylisothiocyanate and all organic solvents were redistilled. Other chemicals were reagent grade.

Digestion with trypsin. A water suspension of 2.09 g. (290 μmoles) of RCM-inhibitor was adjusted to pH 8.5 with 1 N-NH_4OH (thymol blue as indicator).

The volume was brought to 196 ml. and 2 ml. of 1 M-$CaCl_2$ was added. The suspension was stirred mechanically in a 38° bath and a stream of water-saturated nitrogen passed over it during the digestion. Trypsin (30 mg. in 2 ml. of solution) was added. The pH was maintained by frequent addition of 1 N-NH_4OH from a microburette. A second portion of 15 mg. of trypsin was added at 2.5 hr. The digestion was stopped at 5 hr., when the consumption of base had almost ceased. A total of 2.90 ml. of NH_4OH was used, equivalent to about 11 peptide bonds split per mole [32]. The digest was evaporated at 40° in a rotary evaporator to about 30 ml., adjusted to pH 2.2 with 1 N-HCl, and centrifuged. The supernatant fluid was chromatographed at once (Fig. 2). The washed and dried residue amounted to 125 mg.

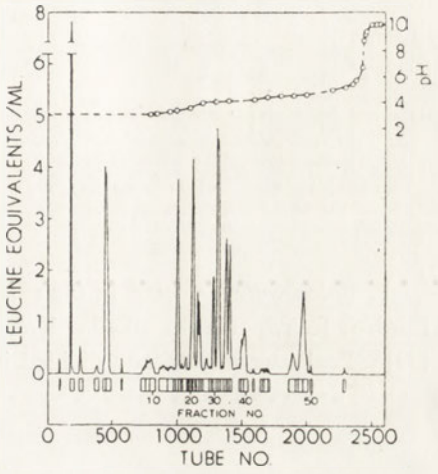

Fig. 2. Elution pattern of peptides obtained by digesting the RCM-inhibitor with trypsin. Digest: 290 μmoles. Column: Dowex 50-X2, 3.8 × 150 cm. Rate: 130 ml. per hour. Fractions: 20 ml. Temp. 38°. Starting buffer: pyridine acetate, pH 3.1, 0.2 M with respect to pyridine. First gradient from tube 807: 14.2 liters starting buffer and 14.2 liters of 2 M-pyridine acetate, pH 5.0, in equal open containers. Second gradient from tube 2145: 2 liters of 2 M-pyridine acetate, pH 5.0 and 2 liters of 2.5 M-pyridine acetate, pH 6.0, as above. Final elution from tube 2305: 1 M-ammonium carbonate solution, pH 10.0. The blocks below the chart indicate the division into fractions. The numbers correspond to the fraction numbers in the second line of Table 1.

Digestion with chymotrypsin B. The general procedure was similar. A water suspension of 506 mg. of RCM-inhibitor (73.7 μmoles) was adjusted to pH 7.8 (cresol red). The volume was brought to 98 ml.; 0.5 ml. of 1 M-$CaCl_2$ and 10 mg. of chymotrypsin B in 1.5 ml. of solution were added. The bath temperature was 40°. A second portion of 5 mg. of chymotrypsin B was added at 5 hr., and the digestion was stopped at 6.5 hr. The base consumption was 0.48 ml. of 1 N-NH_4OH, corresponding to about 7 peptide bonds split per mole [32]. The digest was evaporated to about 12 ml. and 0.2 ml. of thiodiglycol was added [27]; the solution was adjusted to pH 2.2 and centrifuged. The precipitate was negligible. Chromatography was started at once (Fig. 5).

Chromatography. The tryptic and chymotryptic digests of the RCM-inhibitor were separated on columns of Dowex 50-X2 (Beckman/Spinco special peptide resin). A small scale separation of the products of tryptic digestion, under the conditions developed by Margoliash & Smith [28] for the peptides from cytochrome *c*, showed that this method could be used with only minor modifications. Details are given in the legends of Figs. 2 and 5.

Dowex 1-X2 (200 - 400 mesh) was used as described by Schroeder *et al.* [35] for further purification of two of the tryptic peaks; details are given in the legends of Figs. 3 and 4.

Phosphocellulose (Schleicher nda Schuell) was used for the separation of S-S peptides with the ammonium acetate gradient buffers described by Canfield & Anfinsen ([5], cf. [19]).

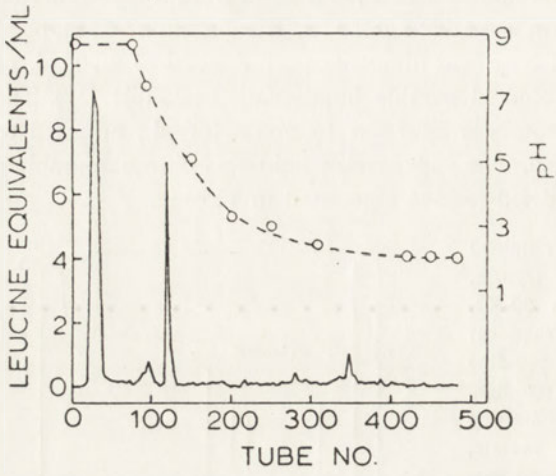

Fig. 3. Rechromatography of fractions T (23 to 26) of Fig. 2. Column: Dowex 1-X2, 0.9 × 92 cm. Rate 6 - 15 ml. per hour. Fractions: 3 ml. Temp. 38°. Starting buffer in a mixing chamber of 135 ml. constant volume and in the reservoir: pyridine 30 parts, *N*-ethylmorpholine 50 parts, glacial acetic acid added to pH 8.7. At fractions 36, 117, 194 and 268, the liquid in the reservoir was changed to 0.1, 0.5, 1.0 and 2.0 N-acetic acid, respectively.

Peptides were detected in the effluent by subjecting 0.2 ml. aliquots from every third or fourth tube to alkaline hydrolysis [17] followed by the ninhydrin reaction [29][3]. Peaks were generally divided into ascending and descending portions. The

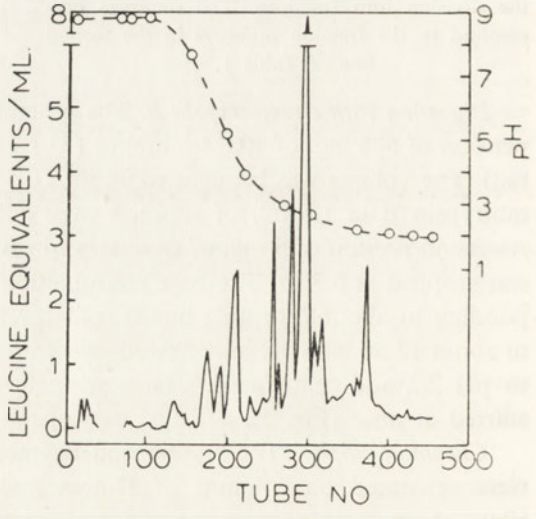

Fig. 4. Rechromatography of peak 32 of Fig. 2. Same conditions as for Fig. 3.

fractions were pooled as indicated in the figures, evaporated to a few ml. on a rotary evaporator, then freeze-dried in weighed tubes. The freeze-dried chymotryptic peaks were extracted with acetone to remove thiodiglycol before weighing.

[3] For the chromatography of the tryptic peptides (Fig. 2), the peaks were also determined without alkaline hydrolysis, but no significant extra peaks were detected.

Amino acid analysis. The method was the same as previously described [21] except that during the course of this work, the Spinco amino acid analyser was converted for accelerated analysis. All chromatographic fractions of 5 mg. or more were analysed.

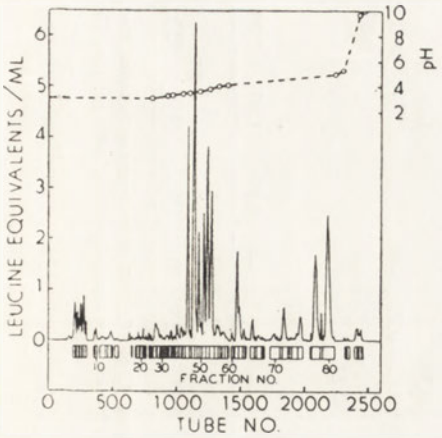

Fig. 5. Elution pattern of peptides obtained by digesting the RCM-inhibitor with chymotrypsin B. Digest: 74 μmoles. Column: 1.9 × 150 cm. Other conditions as in Fig. 2, but with one-fourth of the volumes and rate, and thiodiglycol (2 ml./liter) was added to the buffers. The blocks below the chart indicate the division into fractions. The numbers correspond to the fraction numbers in the second line of Table 3.

Amide groups. The NH_3 derived from the individual peptides on hydrolysis, and determined with the amino acid analyser, was used to locate the four amide groups [21]. Because of the high ammonia content of the laboratory air, the pH 5.28 buffer used for the short column analysis was freed of NH_3 by passing through a Dowex 50 column [31]. The treated buffer was stored on top of the amino acid analyser in a large bottle connected by a siphon and one-hole stopper to the buffer storage bottle inside the compartment. As buffer was used, air entering the top bottle first passed through a sulphuric acid wash bottle. When the short column was not running, it was stoppered and the buffer connector was sealed off to prevent leakage by gravity. Blanks for NH_3 were run occasionally and amounted to about 0.05 μmoles.

In spite of these precautions, all the peptides contained at least small amounts of NH_3, resulting from exposure during collection of chromatographic effluent solutions, transfers, etc. Fractional moles of NH_3 were therefore not considered to be amide groups.

Sequence analysis. The Sanger DNP method was used in some cases for amino terminus determination as described by Fraenkel-Conrat *et al.* [13], but sequences were determined mainly by the subtractive Edman method as described by Konigsberg & Hill [22]. The results were usually confirmed by acid hydrolysis [13] of the PTH-derivatives to free amino acids, which were then determined with the amino acid analyser. Absence of a free amino acid after hydrolysis of the PTH-compound indicated the presence of a hydroxy amino acid [13] or of CM-cysteine. When more than four or five steps of the Edman degradation were required, the later steps were confirmed independently, using an overlapping peptide (see Table 5). Carboxyl-terminal amino acids were determined by hydrazinolysis [30].

Table 1

Amino acid composition[a] of the tryptic peptides of Figs. 2, 3 and 4

Sequence position[b]	1-15	16-17	18-20	18-23	21-26	22-26	24-26	26-33	27-39	27-33	27-35	34-39	36-39	40-41	42	40-42	43-46	47-53	54-58	
Source fraction no.	Fig. 2 31-32	Fig. 2 36	Fig. 2 39-40	Fig. 2 38	Fig. 2 44,48-49	Fig. 2 41-43	Fig. 2 27	Fig. 2 17-18	Fig. 2 21-22	Fig. 2 3	Fig. 2 4	Fig. 2 34B-35B[c]	Fig. 3 23-3	Fig. 2 29-30	Fig. 3 23-1	Fig. 2 46-47	Fig. 2 34A-35A[c]	Fig. 2 8-15	Fig. 2 2	
Ala		**1.00**		0.12	**1.00**	**0.88**	**1.00**	**1.61**	**1.00**	**1.00**	**1.00**		0.34	**1.00**		**1.00**		**1.00**	**1.00**	Ala
Arg	**1.00**	**1.04**	**1.00**	**1.00**		0.21	0.17	0.36	**1.05**			**1.02**	**1.07**		**1.00**	**1.01**		**1.00**		Arg
Asp	**0.99**			0.16	**1.00**	**0.93**	**1.11**	0.36	0.05		0.09		0.29				**2.04**	**1.01**		Asp
CM-Cys	**1.79**					0.11	0.17	**1.31**	**2.04**	**0.90**	**0.91**	**1.00**	**0.77**					**1.04**	**1.04**	CM-Cys
Glu	**0.92**			0.10		0.17	0.17	**1.17**	**1.06**	**1.05**	**1.13**		0.26					**1.04**		Glu
Gly	**1.00**			0.20		0.38	0.23	**1.12**	**3.00**	**1.04**	**1.11**	**2.00**	**2.00**					0.06	**2.02**	Gly
Ile			**1.85**[d]	**0.81**[e]		0.13							0.26							Ile
Leu	**0.96**			0.06		0.13	0.15	**0.95**	**1.00**	**0.97**	**1.02**	0.07	0.26							Leu
Lys	**0.95**			0.13	**1.00**	**1.00**	**1.05**	**1.00**					0.19	**1.00**		**1.00**	**1.00**			Lys
Met								0.20										**0.95**		Met
Phe	**0.97**			**1.28**	**0.99**	**0.79**	0.19	**0.98**	**1.05**	**0.97**	**1.03**		0.38				**1.00**			Phe
Pro	**3.87**					0.31	0.46	0.68	0.06			0.14	0.15	0.06				0.07		Pro
Ser				0.08		0.15		0.20										**0.87**		Ser
Thr	**0.94**			0.07		0.16	0.14	**0.93**	**0.96**	**0.94**	**0.98**		0.27						**0.96**	Thr
Tyr	**0.93**			**2.05**	**1.89**	**1.12**		0.23	**0.94**	0.08	**0.91**	**0.91**								Tyr
Val						0.17			**0.96**		**0.98**	**0.98**								Val
NH_3	0.16	0.18	0.03	0.61	**0.98**	**1.16**	**1.11**	**1.30**	**0.99**	**1.32**	—	—	—		—	0.19	**1.87**	0.23	0.46	NH_3
Residues	15	2	3	6	6	5	3	8	13	7	9	6	4	2	1	3	4	7	5	Residues
Yield	51%	81%	88%	2%	80%	2%	10%	5%	42%	8%	3%	20%	5%	62%	40%	19%	53%	85%	100%	Yield

a Calculated as molar ratios of amino acids without corrections for destruction during hydrolysis. Hydrolysis: 5.7 N-HCl, 110°, 20 hr. except as indicated. Residues present as 0.05 moles or less are omitted. When the same peptide appeared in more than one fraction, the analysis given is for the fraction least contaminated with other peptides. The yield is the sum of the corresponding component of all the fractions which could be clearly identified.

b From Fig. 1.

c See Table 2.

d 72 hr. hydrolysis. After 20 hr. hydrolysis: arginine 1.00, isoleucine 0.91.

e Hydrolysis 20 hr.; assumed to be 2 residues (see footnote *d*).

RESULTS

Isolation and composition of the peptides. The tryptic and chymotryptic peptides in Tables 1 and 3, respectively, are arranged according to their sequence positions in the protein. They may be correlated directly with the fractions, indicated by blocks below Figs. 2 and 5 from the second line of both tables, which gives their positions in the chromatographic patterns.

The majority of the tryptic peptides of Table 1 were pure enough to use directly for sequence analysis. The peptides of positions 34-39 and 43-46 were contained in fractions T (34-35) (a single chromatographic peak). The separate analyses in Table 2 of the ascending and descending portions showed clearly that the two peptides were present in different proportions and had no common amino acids. They were therefore not separated, and one series of Edman degradations yielded both sequences.

Table 2

Analysis of a peak of Fig. 2 which contained two peptides

Amino acid compositions as molar ratios in the ascending (T 34) and descending (T 35) portions of the peak.

Peptide	Fraction			
	T 34		T 35	
	A	B	A	B
Arg		0.44		0.21
Asp	1.99		2.04	
CM-Cys		0.43		0.18
Gly		0.86		0.38
Lys	1.00		1.00	
Phe	0.99		1.00	
Tyr		0.39		0.16
Val		0.42		0.17
NH_3	1.91		1.87	

The compositions of fractions T (23 to 26) of Fig. 2 indicated a small amount of a peptide contaminated with a large amount of free arginine. These fractions were combined and rechromatographed (Fig. 3). The first peak designated T (23-1) was free arginine (Table 1, position 42). The second peak contained only 4.5 mg. of a mixture. The third peak, T (23-3), was the peptide in position 26-39.

The ascending and descending portions of many of the peaks differed in purity, and the separation proved worth-while. Fractions T (31 and 32), forming one peak of Fig. 2 (positions 1-15), were remarkable in this respect. The ascending portion, fraction T 31, contained only 0.5% of impurity, while the descending portion, fraction T 32, was so impure it could not be identified at all. This part was rechromatographed and yielded the complex pattern of Fig. 4. The largest peak starting at fraction 292 and the peak just before it contained peptide T 31 in fairly pure form. The peak starting at fraction 200 contained a mixture of the same two peptides

Table 3

Amino acid composition[a] of the chymotryptic peptides of Fig. 5

Sequence position	1-10	1-4	5-10	3-20	11-19	11-21	20-21	22-23	23	24-33	30-33	34-35	36-45	36-43	36-41	46-58	46-52	53-58	
Fraction no.	53	62	1	65	61	78	72A[b]	48	7	47	4-5	lost	75,80	72B[b],76	63	49-51	45	52	
Ala	0.60	0.09		**0.94**	**1.00**	**1.07**	—	**2.03**		**2.05**			**1.00**	**1.07**	**1.18**	**2.00**	**1.00**	**1.12**	Ala
Arg	**0.92**	**1.00**		**1.63**	**1.05**	**2.05**	**0.94**						**2.24**	**2.26**	**1.28**	**0.87**		**1.00**	Arg
Asp	**1.00**	**1.15**		**1.10**	0.21	0.13		**1.10**		**1.01**			**2.06**	**1.14**		**1.05**	**1.00**	0.08	Asp
CM-Cys	**0.83**		**0.84**	**1.17**[c]	**0.77**	**0.89**		**0.85**		**1.26**	**0.74**		**0.78**	**0.87**	**0.98**	**1.40**[c]	**0.87**	**0.91**	CM-Cys
Glu	**1.00**		**1.03**	**0.78**				**1.09**		**0.94**	**0.93**					**1.06**	**0.94**	0.08	Glu
Gly	0.68	0.13	0.08	**1.00**	**0.98**	**1.12**		**1.00**		**1.00**			**1.78**	**1.72**	**2.00**	**1.87**		**2.09**	Gly
Ile	0.09			**1.46**[c]	**1.42**[c]	**1.29**[c]													Ile
Leu	**0.90**		**1.00**	**0.77**				**0.92**		**1.00**								0.06	Leu
Lys	0.38	0.07		**0.83**	**0.88**	**1.00**		**1.12**		**0.98**			**1.12**	**1.00**	**1.00**	**1.06**	**0.99**	0.10	Lys
Met																**0.69**	**0.75**		Met
Phe	**1.27**	**0.97**		**1.15**	0.20	0.09		**1.97**		**0.99**	**1.00**		**1.02**			0.07			Phe
Pro	**2.58**	**1.26**	**2.35**	**3.23**	**1.02**	**1.10**													Pro
Ser	0.17															**0.92**	**0.81**		Ser
Thr	0.42			**0.74**	**0.83**	**0.80**		**0.90**		**0.89**	**0.92**					**0.90**		**1.01**	Thr
Tyr	**0.70**		**0.87**	**0.65**		**1.02**	**1.00**	**1.05**	**1.00**			**(1)**							Tyr
Val	0.07											**(1)**							Val
NH_3	0.51	0.32	0.17	0.55	0.36	0.47	—	**2.01**		**2.04**	**1.02**		**2.08**	**1.29**	0.25	0.63	0.70	0.25	NH_3
Residues	10	4	6	18	9	11	2	12	1	10	4	2[c]	10	8	6	13	7	6	Residues
Yield	30%	18%	11%	8%	25%	52%	28%	14%	33%	53%	7%	lost	50%	17%	4%	38%	63%	71%	Yield

a Hydrolysis 24 hr. Other conditions as in Table 1, footnote *a*.

b Fraction 72 contained 2 peptides; the total analysis was alanine 1.16, arginine 5.73, aspartic acid 1.15, CM-cysteine 0.83, glycine 1.98, lysine 1.00, tyrosine 4.07, NH_3 1.13. Fraction 72B was the same as 76, the analysis given for position 36-43 was the analysis of 76. Fraction 72A was obtained by difference.

c Two residues deduced from the tryptic peptides.

Table 4a

The sequences determined

Arrows indicate the amino acids identified

Peptide used	Sequence
Whole protein	Amino-terminal Arg-Pro-Asp*
T (1-15)	Arg(Pro,Asp,Phe,CMCys,Leu,Glu,Pro,Pro,Tyr,Thr,Gly,Pro,CMCys)Lys 0.03 M-HCl, 105°, 10hr. Arg-Pro (1 and 1a) Phe-CMCys-Leu-Glu-Pro(Pro,Tyr,Thr,Gly,Pro,CMCys)Lys (7)** Asp (5) Pro-Pro-Tyr-Thr-Gly-Pro-CMCys-Lys (3)** Pro,Pro,Tyr,Thr,Gly (4) (Pro,CMCys)Lys (2) Phe,CMCys,Leu,Glu (8) Thr,Gly,Pro (3A)

* Method: DNP and PTH derivatives, the latter identified as the free amino acids after acid hydrolysis.

** Method: Edman subtractive, and PTH derivatives identified as the free amino acids after acid hydrolysis.

Table 4b

The sequences determined

Arrows indicate the amino acids identified

Peptide used	Sequence	Method
T (16-17)	Ala Arg	Trypsin specificity
T (18-20)	Ileu-Ileu-Arg	Trypsin specificity and Sanger DNP
T (21-26)	Tyr-Phe-Tyr-Asn-Ala-Lys	Edman subtractive and PTH-derivatives*
T (24-26)	Asn-Ala-Lys	Sanger DNP
T (27-33)	Ala-Gly-Leu-Cys-Gln-Thr-Phe	Edman subtractive
C (30-33)	Cys-Gln-Thr-Phe	Edman subtractive and PTH-Glu*
T (34-39)	Val-Tyr-Gly-Gly-Cys-Arg	Edman subtractive and PTH-derivatives*
T (40-42)	Ala-Lys-Arg	Edman subtractive
T (43-46)	Asn-Asn-Phe-Lys	Edman subtractive and PTH-derivatives*
T (47-53)	Ser-Ala-Glu-Asp-Cys-Met-Arg	Edman subtractive and PTH-derivatives*
C (46-52)	Lys-Ser-Ala-Glu-Asp-Cys-Met	Hydrazinolysis
T (54-58)	Thr-Cys-Gly-Gly-Ala	Edman subtractive
Whole protein	Carboxyl-terminal-Ala	Hydrazinolysis

* Identified as the free amino acids after acid hydrolysis.

as in Table 2. The other peaks consisted of smaller amounts of material and were not analysed.

For the tryptic peptides the yields were 51-100% counting overlapping peptides. These yields were not corrected for the residue of 125 mg. remaining after tryptic hydrolysis. This was 6.0% by weight and appeared from the amino acid analysis to be unchanged RCM-inhibitor somewhat contaminated with trypsin. The peaks not shown in Table 1 either contained less than 5 mg. and were not analysed, or were mixtures that could not be clearly identified. None of the weights exceeded 7 mg. and they were not counted in the yields. The low yield of 51% for the amino terminal peptide in position 1-15 is in part accounted for by its spreading over many other peaks, as shown by the contamination of other peptides with proline.

For the chymotryptic peptides (Table 3) the yields, counting overlapping peptides, were also of the order of 50 - 100%.

Sequence analysis

The determination of the sequences (refer to Fig. 1) is summarized in Table 4, and given in detail below.

Terminal groups. Hydrazinolysis [30] yielded 0.2 mole of alanine. Peak 2 of Fig. 2 was the only high yield peptide without a basic group and was therefore the carboxyl terminal peptide (positions 54-58).

The amino terminal amino acid determined by the Sanger method [13] was arginine [14, 21]. The subtractive Edman method [22] was not successful with the whole protein. However, the benzene extracts prepared according to Konigs-

Table 5

Amino acid composition of the peptides derived from T (1-15) by mild acid hydrolysis (see Table 4)

Peptide no. [a]	Molar ratios of amino acids								
	1	1a	2	3	3A[b]	4	5	7	8
Arg	**1.00**	**1.00**		0.28				0.14	
Asp				0.21		0.11	**1.00**	0.12	0.23
CM-Cys			**0.99**	**0.90**	0.24	0.14		**1.40**	**0.76**
Glu								**0.91**	**0.64**
Gly			0.10	**0.93**	**1.09**	**0.89**	0.05	**1.13**	0.30
Leu								**1.14**	**1.19**
Lys		0.19	**1.00**	**1.00**	0.37			**1.00**	0.18
Phe								**1.19**	**1.00**
Pro	**1.06**	**1.02**	**1.17**	**2.60**	**1.00**	**2.00**		**2.69**	0.88[d]
Thr			0.05	**0.85**	**0.94**	**1.13**		(**1.0**)[c]	0.23
Tyr				**0.55**	0.18	**1.14**		**0.97**	

a The peptides were separated on Dowex 1-X2 ([18], Fig. 2). Peptide 6 (omitted) was impure starting peptide.
b Peptide 3A was derived from 3 by further acid hydrolysis and was separated by paper electrophoresis [18].
c This peak was lost on the amino acid analyser.
d This proline is assumed to be part of the impurity, but could be the next residue (position 8).

berg & Hill [22] yielded PTH derivatives of the second and third amino acids. These were determined as free proline and aspartic acid after acid hydrolysis [13] in yields of 24 and 26%, respectively.

Peptide T (1-15). Since all of the proline of the inhibitor was present in this peptide, it was placed in position 1-15. Arginine was identified as the amino terminal amino acid (Sanger method) and lysine as the carboxyl-terminus (hydrazinolysis). This peptide resisted digestion by pepsin and chymotrypsin and was degraded with 0.03 M-HCl under the conditions of Schultz [36] as described previously [18]. The amino acid composition of the peptides obtained is given in Table 5. A protocol of the sequence determination is included in Table 4. This is taken from reference [18], Fig. 3. For details of the separation and Edman degradation of these peptides, see [18]. Confirmation is provided by the composition of the chymotryptic peptides of Fig. 1.

Peptide T (16-17). The order Ala-Arg was assumed from the specificity of trypsin [20]. This was proved by Anderer [1] by sequence determination.

Peptide T (18-20). The order Ileu-Ileu-Arg was originally assumed from the specificity of trypsin [20]. Because of differences with other investigators [6, 10] the DNP-peptide was prepared. After 72 hr. hydrolysis at 110°, 0.43 mole of DNP-isoleucine was recovered by ether extraction. The aqueous layer contained arginine and isoleucine in a ratio of 1.0 to 1.14. These data, combined with the original ratio of arginine 1.0 to isoleucine 1.85 (Table 1), definitely established the presence of two isoleucine residues and a single arginine in this peptide. Anderer [1] also obtained the sequence Ileu-Ileu-Arg from two steps of the Edman degradation.

Peptide T (21-26) and T (24-26). The Edman method gave the following results with T (21-26), using both the subtractive method and identification of the PTH--derivatives. The identification of DNP-aspartic acid from T (24-26) confirmed the order of asparagine and alanine in T (21-26) (Table 6).

Table 6

Step	Molar ratio of amino acids remaining					Free amino acid from PTH-derivative
	Tyr	Phe	Asn	Ala	Lys	
0	1.89	0.99	1.00	1.00	1.00	-----
1	Hydrolysate was lost					...**Tyr** 0.32 mole
2	1.04	**0.32**	0.86	1.00	0.47	
3	**0.14**		0.90	1.00	0.66	
4			**0.55**	1.00	0.81	**Asp**

Peptides T 27-33 and C (30-33)[4]. Three steps of the Edman degradation were carried out with each of these peptides (Table 7).

[4] Part of the analysis was presented previously [19].

Table 7

Step	Molar ratio of amino acids remaining							Free amino acid from PTH-derivatives
	Ala	Gly	Leu	CM-Cys	Gln	Thr	Phe	
T (27-33)								
0	1.00	1.04	0.97	0.90	1.05	0.94	0.97	
1	**0.10**	1.05	0.98	0.86	1.02	0.98	1.00	
2		**0.07**	1.00	0.81	1.02	0.96	1.00	
3			**0.08**	0.75	1.02	0.93	1.00	
C (30-33)								
0				0.74	0.93	0.92	1.00	
1				**0.05**	0.97	0.92	1.00	
2					**0.13**	0.92	1.00	**Glu** 0.48 mole
3						**0.16**	1.00	

Peptide T (34-39). This is peptide 34B of Table 2. Five steps of the Edman degradation established the sequence (Table 8).

Table 8

Step	Molar ratio of amino acids remaining					Free amino acid from PTH-derivative
	Val	Tyr	Gly	CM-Cys	Arg	
0	0.98	0.91	2.00	1.00	1.02	- - - -
1	**0.18**	0.86	1.84	0.69	1.00	**Val**
2		**0.06**	2.02	0.71	1.00	**Tyr**
3			**1.12**	0.41 *	1,00	**Gly**
4			**0.41**	0.48 *	1.00	**Gly**
5				**None**	1.00	

* Part of the CM-cysteine was converted to cysteic acid during prolonged handling of the peptide.

Peptide T (40-42). The Edman degradation was given previously by the subtractive method [18] (Table 9).

Table 9

Step	Molar ratio of amino acids remaining		
	Ala	Lys	Arg
0	1.00	1.00	1.01
1	**0.19**	1.00	1.11
2		**0.07**	1.00

Peptide T (43-46). This is peptide 34A of Table 2. Three steps of the Edman degradation established the sequence (Table 10).

Table 10

Step	Molar ratio of amino acids remaining			Free amino acid from PTH-derivative
	Asn	Phe	Lys	
0	2.04	1.00	1.00	----
1	**1.24**	1.11	1.00	**Asp**
2	**0.71**	1.40	1.00	**Asp**
3		**trace**	1.00	**Phe**

Peptide T (47-53) and C (46-52). The data below give six steps of the Edman degradation for the tryptic peptide. Since the identification of the last two steps left some doubt, the carboxylterminal amino acid of the overlapping chymotrypic peptide was identified as methionine (Table 11).

Table 11

Step	Molar ratio of amino acids remaining							Free amino acid from PTH-derivative
	Ser	Ala	Glu	Asp	CM-Cys	Met	Arg	
0	0.87	1.00	1.04	1.01	1.04	0.95	1.00	----
1	**0.06**	0.91	0.88	0.90	0.73	0.71	1.00	None
2		**0.07**	0.99	0.98	0.82	0.93	1.00	**Ala**
3			**0.17**	0.99	0.84	0.95	1.00	**Glu**
4				**0.33**	0.73	0.88	1.00	**Asp**
5					**0.58**	0.91	1.00	
6						**0.66**	1.00	

Peptide T (54-58). The sequence was established by four steps of the Edman degradation (Table 12).

Table 12

Step	Molar ratio of amino acids remaining				Free amino acid from PTH-derivative
	Thr	CM-CyS	Gly	Ala	
0	0.96	1.04	2.02	1.00	----
1	**0**	0.89	2.26	1.00	None
2		**trace**	2.01	1.00	None
3			**1.03**	1.00	
4			**0.25**	1.00	

The tryptic peptides were put together by using the chymotryptic peptides as overlaps (Fig. 1). This was possible from the amino acid composition of the chymotryptic peptides without further sequence work. In one instance, in which C (34-35) was lost during chromatography, there were fortunately two pairs of overlapping tryptic peptides to establish the sequence.

Disulphide linkages. The derivation of the disulphide linkages was described in detail previously [19] and is summarized here. We were unable to find conditions

to digest the native inhibitor enzymically without exposing it to the possibility of disulphide interchange [33, 34, 37]. Therefore two methods were used to obtain S-S peptides: (1), digestion of the succinylated inhibitor (cf. Materials, above) with pepsin followed by trypsin and (2), dilute HCl hydrolysis of the native inhibitor in the presence of thioglycolic acid [33]. The peptides of both digests were separated on phosphocellulose [5] and the individual peaks were oxidized [16] and separated by paper electrophoresis or subjected to the diagonal paper electrophoresis and oxidation method of Brown & Hartley [4]. Peptides were eluted from the paper by the method of Vanderheiden [38], hydrolysed, and analysed on the amino acid analyser.

The succinylated inhibitor yielded two peptides, both containing the same linkage:

30
CYS-GLN-THR
|
LYS-SER-ALA-GLU-ASP-CYS-MET
51

30
LYS-ALA-GLY-LEU-CYS-GLN-THR-PHE
|
ASP-CYS-MET
51

From the acid hydrolysate, only one peptide could be clearly identified. This was

14
GLY-PRO-CYS-LYS-ALA-ARG-ILEU-ILEU-ARG-TYR-PHE-TYR
|
GLY-CYS-ARG-ALA-LYS-ARG
38

The remaining S-S linkage is therefore 5-55 and the complete structure of the inhibitor is shown in Fig. 6.

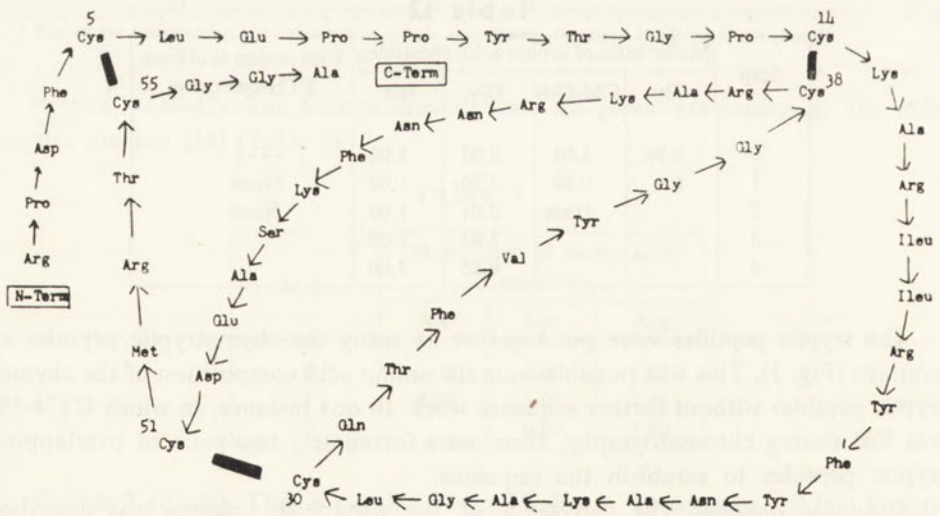

Fig. 6. Complete structure of the basic trypsin inhibitor

DISCUSSION

A comparison of the peptides isolated by four groups of investigators is given in Table 13. Certain differences should be noted. Chauvet *et al.* [6] and Dlouhá *et al.* [10] used performic acid oxidized inhibitor for digestion, while Anderer's [1] and our work was done with reduced carboxymethylated inhibitor. It appears that more linkages are susceptible to digestion in the RCM-derivative than in the oxidized inhibitor. We used chymotrypsin B; the other groups used α-chymotrypsin. In addition, our tryptic and chymotryptic digests were made in the presence of

Table 13

Comparison of the tryptic and chymotryptic peptides found by different workers

Sequence positions are from Fig. 1.

Tryptic peptides				Chymotryptic peptides			
This paper	Chauvet *et al.* [6]	Dlouhá *et al.* [10]	Anderer [1]	This paper	Chauvet *et al.* [6]	Dlouhá *et al.* [10]	Anderer [1]
1-15	1-15	1-15	1-15			1-21[b]	
16-17	16-17	16-17	16-17				1-19
18-20	18-20[a]		18-20	1-10	1-10		1-10
		18-21[b]		1-4			
18-23				5-10			
21-26	21-26		21-26	3-20			
22-26				11-19	11-19		
24-26				11-21			11-21
26-33				20-21	20-21		
							20-22
27-39	27-39	27-39	27-39				20-23
27-33					22-23	22-23	22-23
27-35				22-23			
34-39							23-33
36-39				23			
40-41	40-41		40-41	24-33	24-33	24-33	24-33
42			42	30-33			
40-42		40-42	40-42	(34-35)	34-35	34-35	34-35
			40-46	36-45	36-45		36-45
43-46	43-46	43-46	43-46	36-43			
			47-58	36-41		36-41	
47-53	47-53	47-53	47-53			42-43	
54-58	54-58		54-58			44-45	44-45
				46-58	46-58	46-58	46-58
				46-52			
				53-58			
Total 19	9	7	13	18	8	8	12

a Arginine 42 was erroneously placed in position 21 by Chauvet *et al.* [6]; the numbering between 21 and 42 has been corrected to correspond to the other columns.

b Numbering of this and subsequent peptides is corrected to include 2 isoleucine residues, in order to correspond to the other columns.

0.01 M and 0.005 M-Ca^{2+}, respectively. Calcium ions are known to increase the activity of these enzymes [cf. 39].

For the tryptic peptides, there is fairly close correspondence between our peptides and Anderer's. With the chymotryptic peptides, only five were the same. This probably indicates a real difference between the specificities of α-chymotrypsin and chymotrypsin B. Chymotrypsin B also hydrolysed more linkages in glucagon than α-chymotrypsin [11]. Further studies of the comparative specificity of these two enzymes would be of value.

Much effort has been made to have the trypsin used in sequence studies as free of chymotryptic activity as possible. In these experiments, in spite of using trypsin which had been purified through the specific inhibitor-trypsin complex, we obtained small quantities of several "chymotryptic peptides" in the tryptic digest. As it turned out, these proved to be a great advantage. Their yields were low, and they in no way interfered with the identification of the main "tryptic peptides". Many were just the right size for sequence studies, e.g., T (27-33), Table 4. Without one of these peptides, T (18-23) (see Fig. 1), it would not have been possible to place the two tryptic peptides Ala-Arg, T (16-17), and Ileu-Ileu-Arg, T (18-20), unequivocally in order. Indeed, Anderer [1] used a third digest with pepsin to do this. While this is not a recommendation to use a "crude" trypsin, the use of specific chymotrypsin inhibitors [23, 12] is probably not desirable for sequence determination in small proteins.

Many of the structural features of the inhibitor have been discussed previously [18, 20, 19] and will not be considered here.

We wish to thank Mrs. Milla Radicevic for excellent work in the operation of the amino acid analyser as well as for other assistance and Mr. Michael Ansfield for his careful chromatography of the peptides of Figs. 2 - 4. This work was supported by Public Health Service Research Grant AM 00535 from the National Institutes of Health, Grant G-7581 from the National Science Foundation, and the institutional grant 5 SO1FR-5434.

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ZASADOWY INHIBITOR TRYPSYNY Z TRZUSTKI WOŁU

VI. BADANIE SEKWENCJI AMINOKWASÓW ORAZ WIĄZANIA DWUSIARCZKOWE

Streszczenie

1. W poprzednich krótkich komunikatach przedstawiono pierwszorzędową strukturę zasadowego inhibitora trypsyny.

2. Ponieważ inni autorzy podali nieco odmienną strukturę, punkty sporne zostały ponownie zbadane; podano również szczegóły zastosowanych metod.

3. Uzyskano potwierdzenie pierwotnie sugerowanej struktury.

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V. J. MARMARAS, C. E. SEKERIS and P. KARLSON

ACTIVITY OF 5-HYDROXYTRYPTOPHAN DECARBOXYLASE DURING DEVELOPMENT OF THE BLOW-FLY *CALLIPHORA ERYTHROCEPHALA*, IN RELATION TO THE ECDYSONE TITER

Physiologisch-Chemisches Institut der Philipps-Universität, Marburg/Lahn, Germany

Dedicated to Prof. J. Heller on the occasion of his 70th birthday

1. The activity of 5-hydroxytryptophan decarboxylase is high during the pupal stage with maxima at the beginning and end of the pupal period. 2. A reverse correlation exists between the enzyme activity and ecdysone titer. 3. Ligation of the larvae leads to enhancement of the 5-hydroxytryptophan decarboxylase activity while injection of ecdysone into the ligated larvae leads to a drop of the enzyme activity.

In insects, the metabolism of aromatic amino acids is often related to special developmental events. Tryptophan is the precursor of eye pigments (Butenandt, 1960); tyrosine metabolites are responsible for sclerotization (Hackmann, 1958; Karlson, Sekeris & Sekeri, 1962; Karlson & Herrlich, 1965; Sekeris & Herrlich, 1966). The activity of some enzymes of tyrosine metabolism during development has been measured (Sekeris, 1964; Shaaya & Sekeris, 1965) and found to be correlated to the ecdysone titer. This is especially true for DOPA decarboxylase (EC 4.1.1.26) which can be induced by ecdysone (Karlson & Sekeris, 1962; Sekeris & Karlson, 1964; Sekeris & Karlson, 1964; Sekeris & Lang, 1964). DOPA decarboxylase has been regarded as being responsible for the decarboxylation of 5-hydroxytryptophan (5-HTP) and 5,6-dihydroxytryptophan (5,6-DiHTP) as well (Sekeris, 1963); this assumption was based on the fact that crude extracts as well as a fivefold purified enzyme preparation could decarboxylate DOPA, 5-HTP and 5,6-DiHTP but not other aromatic amino acids. However, the finding that extracts of *Calliphora* pupae devoid of DOPA decarboxylase activity could transform 5-HTP to serotonin (Marmaras & Sekeris, 1966) led to the conclusion that a special 5-hydroxytryptophan decarboxylase (EC 4.1.1.28) must exist in *Calliphora*.

To further substantiate this conclusion, we followed the 5-HTP decarboxylase activity during development and compared it with the known activity of DOPA decarboxylase and with the ecdysone titer.

MATERIALS AND METHODS

DL-[^{14}C]5-HTP (sp. act. 25 mc/m-mole) was obtained from Amersham Radiochemical Corporation. The ecdysone used was a crystalline preparation. *Calliphora erythrocephala* Meig was reared at 24° and relative humidity of 45%. The eggs hatch within 24 hr. after being laid. The larval stage lasts about 8 days, the pupal 10 - 11 days.

Preparation of enzyme extracts. Ten animals were homogenized in 10 ml. phosphate buffer, 0.066 M, pH 7.0, in an Ultra-Turrax for 1 min. at 0°. The homogenate was centrifuged at 1230 *g* in a Serval centrifuge. The supernatant was used as enzyme preparation.

Determination of 5-HTP decarboxylase activity. A radiochemical test, similar to that used by us previously for the measurement of the DOPA decarboxylase (Sekeris & Karlson, 1962; Sekeris, 1963) was used. The determination is based on the conversion of [^{14}C]5-HTP to [^{14}C]serotonin, separation of the radioactive metabolites by paper chromatography (chromatographic system: butanol-pyridine-water, 1:1:1, by vol.; Schleicher & Schull 2043b paper; R_F: 5-HTP, 0.50, serotonin, 0.70) and measurement of the radioactivity of the metabolites directly on the paper by liquid scintillation counting. Enzyme activity is expressed as percentage transformation of substrate to serotonin. The incubation mixture consisted of 0.02 μc [^{14}C]5-HTP, 5 μmoles of non-labelled 5-HTP, 1 ml. enzyme extract brought to an end volume of 3 ml. with 0.066 M-phosphate buffer, pH 7.0. Incubation time 90 min.

RESULTS

The activity of 5-HTP decarboxylase during development of the blowfly, *Calliphora erythrocephala*, measured as percentage transformation of 5-HTP to serotonin, is shown in Fig. 1a. The activity is high during the pupal stage, with one maximum at the beginning and another one towards the end of the pupal period. In Fig. 1b,

Table 1

Effect of ecdysone on 5-HTP decarboxylase activity in vitro

Experimental conditions as in Methods. The enzyme extract was prepared from 4-day-old pupae. C.U., *Calliphora* units.

	Transformation of 5-HTP to serotonin (%)
Enzyme extract in absence of ecdysone	4
Enzyme extract with 50 C.U. ecdysone	4
Enzyme extract with 150 C.U. ecdysone	4

the activity of DOPA decarboxylase is given (the data are from Shaaya & Sekeris, 1965). It can be seen that the maxima of 5-HTP decarboxylase activity correspond to periods of absence of DOPA decarboxylase activity, at least in the late larvae

and in the pupae. Comparison with the ecdysone titer which is given in Fig. 1c (according to Shaaya & Karlson, 1965) shows that there is a negative correlation between 5-HTP decarboxylase activity and ecdysone concentration.

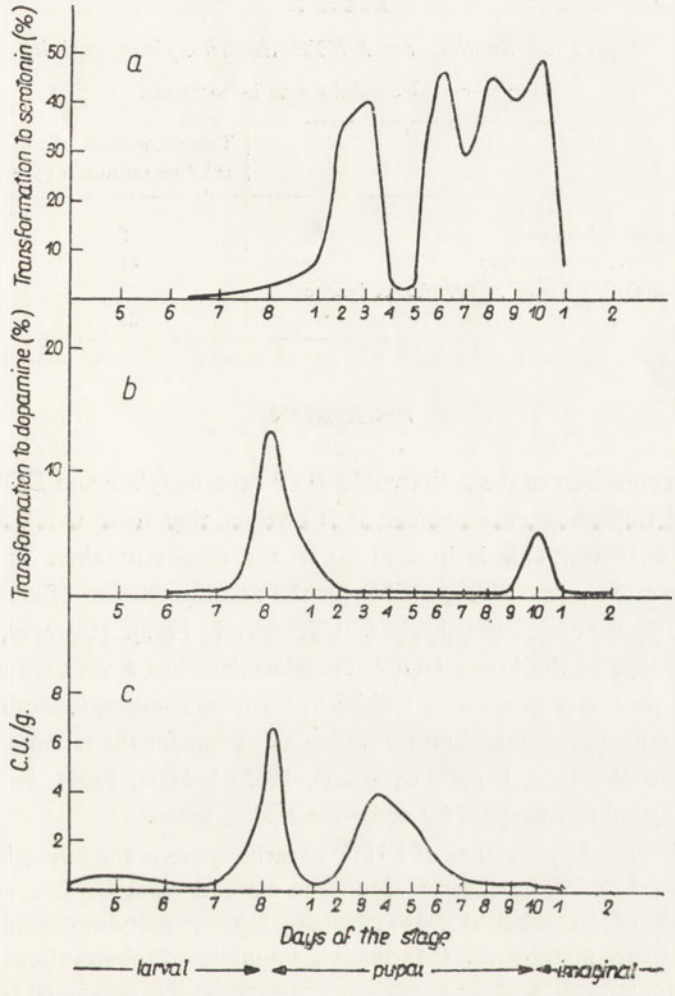

Fig. 1. Activity of: (*a*), 5-HTP decarboxylase; (*b*), DOPA decarboxylase; and, for comparison, (*c*), ecdysone titer, during development of *Calliphora erythrocephala*. Data for (*b*) and (*c*) are derived from Shaaya & Sekeris (1965) and Shaaya & Karlson (1965), respectively. C. U., *Calliphora* units.

Ecdysone added *in vitro* to a preparation of 5-HTP decarboxylase has no influence on the activity of this enzyme (see Table 1). We therefore assumed that the decrease in enzyme activity at the time of ecdysone secretion might be due to enzyme repression, i.e. a reduced enzyme synthesis. This hypothesis was tested further in experiments with larvae deprived of their hormone-producing gland by ligation. One day after ligation, the 5-HTP decarboxylase activity was high, while the non-ligated controls (which had, by that time, already pupated) showed low

activity (Table 2). Moreover, injection of ecdysone into the ligated abdomens resulted in a significant drop of decarboxylase activity within 24 hr.

Table 2

Effect of ligation on 5-HTP decarboxylase activity

Experimental conditions as in Methods.

	Transformation of 5-HTP to serotonin (%)
Control animals	5
24 hr. after ligation	41
Ligated animals 24 hr. after ecdysone injection	25

DISCUSSION

From a comparison of the activity of 5-HTP decarboxylase and DOPA decarboxylase during *Calliphora* development, it is obvious that these two decarboxylases are different enzymes. This is in contrast to the results obtained in mammalian tissues, where one enzyme is responsible for the decarboxylation of all the aromatic amino acids (Lovenberg, Weissbach & Udenfriend, 1962). However, it must be kept in mind that in the insect DOPA decarboxylase has a very special physiological role: it generates dopamine, which, in form of *N*-acetyldopamine, serves as sclerotizing agent during puparium formation as well as for the tanning of the newly emerged imago (Karlson, Sekeris & Sekeri, 1962; Sekeris, 1964). In this respect, the inducibility of this enzyme by ecdysone makes sense.

The physiological significance of 5-HTP decarboxylase in the pupae is not known. The product, serotonin, has been identified in extracts from *Calliphora* pupae after administration of ^{14}C-labelled 5-HTP, along with *N*-acetylserotonin and other, not yet identified metabolites (Marmaras, unpublished observations). Serotonin has been demonstrated to occur in various arthropods; it is presumed to be a neurohormone, but it may have some other, still unknown functions in insect development. In this respect, the inverse relationship to ecdysone is of special interest. Our working hypothesis is that the serotonin-producing enzyme, 5-HTP decarboxylase, is repressed by ecdysone, that is, the genes bearing the information for this protein are kept inactive by the hormone. However, we do not claim to have conclusive evidence for this hypothesis; other interpretations of the data given above are still possible. The phenomenon, however, warrants further study.

V. J. Marmaras wishes to thank the World Health Organization for financial aid.

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ZALEŻNOŚĆ MIĘDZY ILOŚCIĄ EKDYZONU A AKTYWNOŚCIĄ DEKARBOKSYLAZY 5-HYDROKSYTRYPTOFANU W TRAKCIE ROZWOJU MUCHY PLUJKI *CALLIPHORA ERYTHROCEPHALA*

Streszczenie

1. U poczwarki aktywność dekarboksylazy 5-hydroksytryptofanu jest wysoka, z maksimum na początku i końcu okresu przepoczwarzania.

2. Istnieje odwrotna zależność między aktywnością enzymu a ilością ekdyzonu.

3. U larw założenie ligatury prowadzi do wzrostu aktywności dekarboksylazy 5-hydroksytryptofanu, a wstrzyknięcie ekdyzonu powoduje spadek aktywności.

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ZENON SCHNEIDER and JERZY PAWEŁKIEWICZ

THE PROPERTIES OF GLYCEROL DEHYDRATASE ISOLATED FROM *AEROBACTER AEROGENES*, AND THE PROPERTIES OF THE APOENZYME SUBUNITS

Department of Biochemistry, College of Agriculture, Poznań

1. The apoenzyme of the isolated glycerol dehydratase consists of two subunits, A and B, having molecular weights of 22 000 and 240 000, respectively, and which combine in a steicheiometric ratio. 2. The holoenzyme formed from apoenzyme and coenzyme B_{12} undergoes rapid, spontaneous and irreversible inactivation both in the presence and in the absence of substrate. The inactivation occurs also at 1°, when the enzymic reaction does not occur. The inactivated enzyme contains hydroxycobalamin firmly bound to protein. 3. The presence of univalent cations and slightly alkaline pH, which favour the association of proteins A and B into active apoenzyme AB, promote the inactivation of the holoenzyme. The inactivation proceeds in the presence of Na^+ ion, although such a system does not catalyse the enzymic reaction. 4. Protein A is thermostable, exhibiting an interesting minimum of stability at 70°. In solutions of pH lower than 7 and low ionic strength, protein A dissociates into subunits having a molecular weight of 12 000. Protein A does not contain active thiol groups. 5. Protein B is thermostable up to a temperature of 70°. It contains active thiol groups. 6. An efficient method for isolation and stabilization of the enzyme was elaborated.

Aerobacter aerogenes (PZH, strain no. 572) grown on a glycerol-containing medium, produces glycerol dehydratase [7], an enzyme which catalyses the conversion of glycerol to β-hydroxypropionaldehyde. The isolated apoenzyme requires for its activity the presence of coenzyme B_{12}. Recently it has been demonstrated in this laboratory [9] that the apoenzyme consists of two subunits, A and B, having molecular weights of 22 000 and 240 000, respectively. Either of these proteins, when tested in the presence of coenzyme B_{12}, was separately inactive, but together they formed an active complex, AB; its molecular weight, determined by gel filtration, amounted to 240 000 which suggests a 1:1 molar proportion. The binding of proteins A and B was promoted by alkaline pH values (8 - 9), by the presence of potassium or ammonium ions, and also by the presence of glycerol. In neutral or weakly acidic solutions as well as in the absence of K^+ or NH_4^+ ions, dissociation of the apoenzyme into subunits was observed [9].

In the present work, experiments concerning spontaneous inactivation of the enzyme as well as the properties of subunits A and B are described. A more efficient method for preparation and stabilization of purified enzymic extracts is also presented.

MATERIALS AND METHODS

Special reagents. Coenzyme B_{12} was isolated from *Propionibacterium shermanii* cultures or synthesized as described previously [12]. [^{60}Co]Coenzyme B_{12} was isolated from *P. shermanii* cultures grown on a medium containing $^{60}CoCl_2$ (100 μc/l.). One mμmole of the radioactive coenzyme gave, under the conditions applied, 3100 counts/min. Streptomycin sulphate was a product of Tarchomińskie Zakłady Farmaceutyczne Polfa (Warszawa-Tarchomin, Poland). DL-Tryptophan was from Nutritional Biochemicals Co. (Cleveland, Ohio, U.S.A.); *N*-ethylmaleinimide and mercaptoethanol from T. Schuchardt (Munich, Germany); crystalline bovine serum albumin from E. Gurr (London, England); cytochrome *c*, ox-heart preparation, from the Laboratory of Sera and Vaccines (Kraków, Poland); Sephadex G-25, G-100 and G-200, and dextran-blue from Pharmacia (Uppsala, Sweden); DEAE-cellulose DE 50 from Whatman (Balston, England) or prepared after Peterson & Sober [8]. Acrolein was synthesized according to [5]. Other reagents were products of Fabryka Odczynników Chemicznych (Gliwice, Poland).

Analytical determinations. Concentration of coenzyme B_{12} was determined spectrophotometrically by measuring the extinction of solutions at 260 or 520 mμ, using the millimolar extinction coefficients of 34.7 and 8.0, respectively [2], or by treating a sample with potassium cyanate and measuring at 580 mμ the extinction of the dicyanide form of the cobamide formed, using the millimolar extinction coefficient of 10.1 [2].

Protein was determined by the tannin method of Mejbaum-Katzenellenbogen [4]; crystalline bovine serum albumin was used as standard.

β-Hydroxypropionaldehyde was determined by the method of Smiley & Sobolov [11]. The amount of the aldehyde converted to acrolein was calculated from a standard curve of freshly distilled acrolein. The extinction was proportional over the range 0.5 - 3.0 μmoles acrolein per sample.

Extinctions were determined in a Bausch-Lomb Spectronic 20 spectrocolorimeter and Hilger H 700 spectrophotometer. Radioactivity was measured in a scintillation counter with a NaI crystal, type LRS (produced by Akademia Górniczo-Hutnicza, Kraków, Poland).

Determinations of enzymic activity. The incubation mixture contained in 1 ml.: 100 μmoles of glycerol; 100 μmoles of potassium-phosphate buffer, pH 8.6; apoenzyme preparation containing 1 - 3 units, and 0.2 mμmole of coenzyme B_{12}. The coenzyme B_{12} was added in a volume of 0.2 ml. to a mixture which contained all the other components and had been heated to 35°. The sample was incubated for 20 min. at 35°. The activity was expressed as μmoles of the β-hydroxypropionaldehyde formed.

Determination of enzymic activity of protein A and protein B. This was performed in the same way as described for the apoenzyme activity except that to 1 - 3 units of protein A an excess (5 - 10 units) of protein B was added, and for measurements of protein B activity, an excess (5 - 10 units) of protein A was used.

Chromatography on Sephadex G-100. Dry Sephadex G-100 was suspended in 0.1 M-phosphate buffer, pH 8.6, and left to swell for a period of at least one month. Then, to avoid trapping of air bubbles in the column, the suspension was de-aerated in vacuum. A glass tube fitted with a sintered glass disk was half-filled with the buffer, then portions of the swelled and de-aerated suspension of the Sephadex were poured in. After a thick layer a few centimetres high had formed, a tap at the bottom of the tube was opened and, as the fluid flowed out, new portions of the Sephadex suspension were added. In this way gel columns 6 × 50 cm. were prepared. The upper surface was covered with a filter paper disk. Before use, the column was washed for several hours with 0.1 M-phosphate buffer containing usually 2 - 4% of glycerol. Then protein solution, not more than 50 ml., was applied.

Determination of molecular weight. Gel-filtration on Sephadex G-100 column (1.2 × 50 cm.) was used. The column was calibrated as described previously [9] using solutions of proteins of known molecular weight.

Preparation of cell-free extract. Aerobacter aerogenes (PZH, strain no. 572) was grown as described previously [7]. The stock culture was kept at 4° on agar slants prepared as for *E. coli* 113-3 [3], and subcultured at 2-week intervals. The cells were harvested by centrifugation at 6000 *g* for 30 min., then washed twice with 0.05 M-potassium-phosphate buffer, pH 7.1, and centrifuged for 10 min. at 20 000 *g* at 2°. The washed sediment was suspended in 400 ml. of 0.02 M-potassium-phosphate buffer, 10 ml. of glycerol was added and then 1 N-KOH to pH 10 - 11; then 50 ml. portions were sonicated for 3 min. in the Ultrasonic MSE "Mulard" (60 watt, 20 kc) instrument, with simultaneous cooling on an ice-bath, care being taken to maintain the pH at a value not lower than pH 8. The collected solution was treated with 200 mg. of charcoal (Carbopol H2, pH 7) homogenized in a small amount of water. After 10 min. the mixture was centrifuged for 15 - 20 min. at 30 000 *g*. The yellow supernatant was decanted, the sediment suspended in about 10 ml. of glycerol, about 400 ml. of water was added and the pH adjusted to 10 with 1 N-KOH. Then the sample was sonicated as above, centrifuged, and the two supernatants were pooled. To the clear solution was added 10 - 15 ml. of 20% solution of streptomycin sulphate to precipitate nucleic acids and after 10 - 20 min. the sediment formed was centrifuged at 30 000 *g* for 15 - 20 min. The clear supernatant was applied immediately to Sephadex G-25 to remove the excess streptomycin which inactivates protein A. The column of Sephadex G-25 (3.5 × 40 cm.) was equilibrated with 0.05 M-potassium-phosphate buffer, pH 8.6, and the same buffer was used for protein elution. To the eluate, glycerol was added to a final concentration of 4%. The obtained crude enzyme preparation could be stored at -10° for several months without loss of activity. The preparations contained 0.8 - 2.0 mg. of protein per 1 ml. and their specific activity amounted to 10 - 30 units per 1 mg. Those preparations which contained a greater amount of protein, had usually lower specific activity.

Partial purification of the preparation. The crude preparation was freed from glycerol by Sephadex G-25 gel filtration or by dialysis, then the solution was adjusted to pH about 9 with ammonia and saturated with solid ammonium sulphate, care

being taken to maintain an alkaline pH value. The precipitated protein was centrifuged, dissolved in a small amount of water or 0.01 M-mercaptoethanol, and dialysed against water or potassium-phosphate buffer, pH 8.6.

The concentrated crude extract was partly purified by Sephadex G-100 gel-filtration in 0.1 M-potassium-phosphate buffer containing 4% of glycerol. Gel-filtration reduced the amount of protein to 60%, the losses of total activity being but slight. Usually only the most active fractions were collected.

Resolution of the apoenzyme into subunits A and B. The resolution was carried out on a DEAE-cellulose column according to a method described previously [9], using the partly purified preparation. The preparation was fourfold diluted to reduce the concentration of the buffer. Usually 250 mg. of protein was applied to a column (2 × 15 cm.), the column was washed with 0.025 M-potassium phosphate buffer, pH 8.6, then protein A was eluted with about 200 ml. of 0.12 M-buffer, pH 8.0, and subsequently protein B with 300 ml. of 0.20 M buffer, pH 8.0. At this stage the proteins A and B were 1.5 - 2 fold purified in relation to the crude preparation. Before further purification, glycerol was added to 4% concentration (w/v) to the solution of protein A.

Purification of protein A and B. Protein A was purified on DEAE-cellulose and Sephadex G-100 columns according to a method described previously [9]. The activity of the most active preparations amounted to 1100 units/mg. protein. The activity decreased on storage, even in the deep-freeze. Losses of activity were especially marked at pH values below 8.0.

Protein B was purified according to the following procedure: to 100 ml. of fraction B (150 mg. of protein) obtained from the DEAE-cellulose column, was added 0.2 ml. of mercaptoethanol and 1 ml. of glycerol, then the solution was heated for 15 min. on a water bath at 65 - 70°, cooled rapidly, and the denatured protein was centrifuged off for 15 min. at 15 000 *g*. The clear supernatant containing about 60 mg. of protein was saturated with ammonium sulphate at pH 8 - 9. The sedimented protein was centrifuged, dissolved in a small amount of water and the solution dialysed for 2 hr. against water at 4°. To the protein solution (15 ml.) were added 5 ml. of 0.1 M-potassium-phosphate buffer, pH 8.6, and 0.1 ml. of mercaptoethanol, and after 30 min. at 0° the mixture was applied to a Sephadex G-100 column, equilibrated with 0.04 M-potassium-phosphate buffer; 8 ml. fractions were collected. About 25 mg. of protein B was obtained. The activity of the most active fractions amounted to 130 units/mg.

Recombination of the apoenzyme from proteins A and B. After mixing the most active fractions of the purified proteins A and B in steicheiometric amounts, determined according to a method described further in the text, the apoenzyme was obtained. Unless otherwise indicated, this preparation was used to determine the properties of the apoenzyme as well as those of the holoenzyme.

RESULTS

The optimum values of pH, *temperature and potassium-phosphate buffer concentration for the enzymic reaction*

The optimum pH for the purified glycerol dehydratase preparation differed from that determined for a crude preparation and, under the conditions of activity determination used, it was 8.6. The activity decreased rapidly at pH values above 9.5, and less sharply and almost linearly at pH values below the optimum (Fig. 1.).

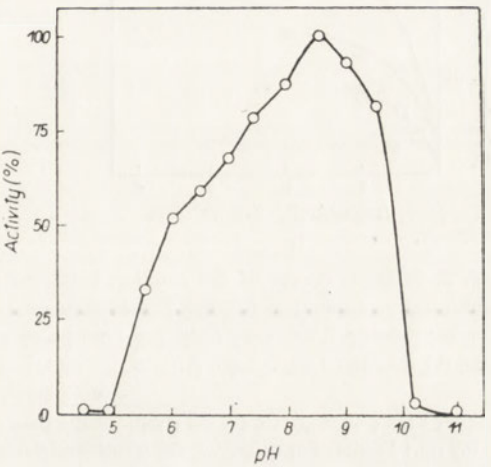

Fig. 1. Effect of pH on the activity of purified glycerol dehydratase. The incubation mixture contained in 1 ml.: 3 units of apoenzyme, 100 μmoles of glycerol, 100 μmoles of potassium-phosphate buffer, and 0.2 mμmole of coenzyme B_{12}. Incubation for 20 min. at 35°. The indicated pH values were determined potentiometrically.

The optimum temperature was 35° for 20 min. incubation. When the time of incubation was prolonged to 30 min., the optimum temperature was 30°. The optimum concentration of potassium-phosphate buffer was within the range 0.08 - 0.15 M.

It was also observed that an excess of glycerol (more than 400 μmoles per 1 ml. sample) had a distinctly inhibitory effect. Moreover, it was found that the crude enzyme preparation contained enzyme(s) which transformed the β-hydroxypropionaldehyde formed into compounds which did not react with the tryptophan reagent.

Spontaneous inactivation of the holoenzyme

It was previously demonstrated [7] that the velocity of conversion of glycerol into β-hydroxypropionaldehyde is not constant, but decreases with the time of reaction, following an equation of second order. Since this decrease of the reaction rate was observed for different initial concentrations of glycerol, it could not be due, as it had been thought, to exhaustion of the substrate. Using the simple test of Selwyn [10] it was found that the decrease was due to inactivation of the enzyme (Fig. 2). For different concentrations of the enzyme (E), the amounts of hydroxypropionaldehyde (P) formed during time (t), did not fall on one curve when plotted

in the system [P]/[E] × t, which is a condition for enzymic reactions in which no inactivation does occur. Since the apoenzyme alone did not undergo inactivation under the conditions of the experiment, it follows that the inactivation could occur

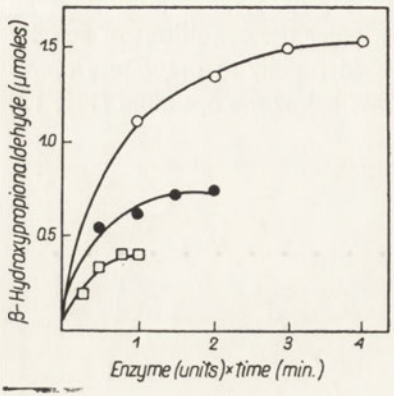

Fig. 2

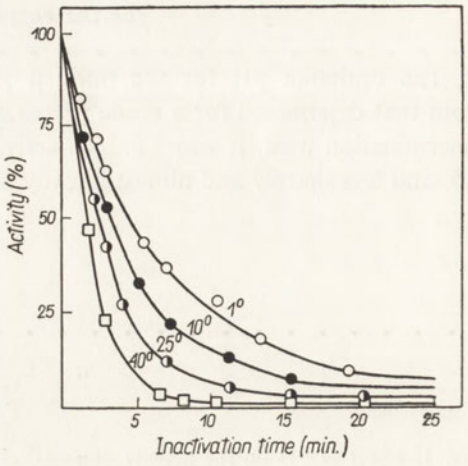

Fig. 3

Fig. 2. Progress curves of the reaction catalysed by glycerol dehydratase, plotted against time multiplied by amount of enzyme. Standard incubation conditions, final volume 1 ml. Enzyme solution (containing 7.5 activity units per 1 ml.) was added in the following amounts: (□), 0.05 ml.; (●), 0.1 ml., and (○), 0.2 ml. After 5 - 10 min. incubation, the β-hydroxypropionaldehyde formed was determined.

Fig. 3. Effect of temperature on spontaneous inactivation of holoenzyme. The samples containing in 0.9 ml.: 3 units of apoenzyme, 100 μmoles of potassium-phosphate buffer, pH 8.6, and 0.2 mμmole of coenzyme B_{12}, were incubated at different temperatures. After indicated time intervals, 100 μmoles of glycerol (0.1 ml.) was added, and after 20 min. at 35° the enzymic activity was determined.

only in the presence of coenzyme B_{12}. In the presence of an excess of coenzyme B_{12} the enzyme became inactivated very rapidly, which is at variance with the observations concerning other enzymes. The inactivation was observed also in the absence of glycerol, that is when the enzymic reaction did not proceed.

The time-course of inactivation at different temperatures is shown in Fig. 3. At 40° the half-inactivation time was about 2 min., and even at 1° it was 4.5 min. The spontaneous inactivation of the enzyme was influenced by potassium ion, in the presence of which most of the enzyme assays in this work were carried out. Over the concentration range 0.1 - 0.2 M, potassium ions accelerated the inactivation to the same degree; at lower concentrations, the effect was smaller (Fig. 4). In the presence of 0.1 M-ammonium ion the inactivation occurred faster than in the presence of the potassium ion at the same concentration (Fig. 5). The half-inactivation time at 20° was, respectively, 1 and 3 min., and for sodium ion it was 6 min. Glycerol in a medium unbuffered and deprived of ion required for the reaction, did not increase the rate of inactivation.

The pH value of the solution also affected the rate of inactivation. Apoenzyme (2 units) incubated with 0.2 mμmole of coenzyme B_{12} in 0.04 M-potassium-phosphate buffer, pH 6.5, at 30° retained after 20 min. about 35% of its initial activity, whereas

in the control sample incubated under the same conditions but at pH 8.6, it was almost completely inactivated.

The process of inactivation is connected with a stable binding of the coenzyme B_{12} to the apoenzyme. After inactivation of the enzyme in the presence of [^{60}Co]coenzyme B_{12}, the labelled corrin compound was not separated by Sephadex G-100 gel filtration from the protein, although free, unbound coenzyme was eluted in the

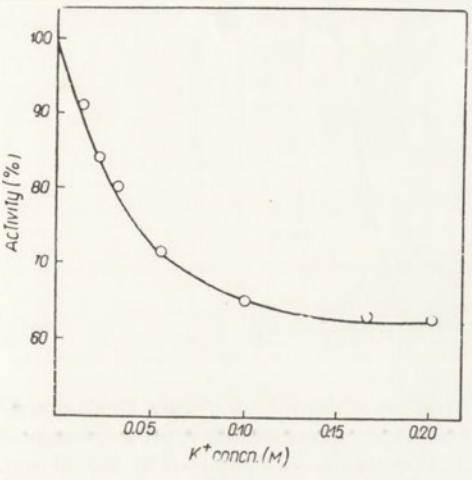

Fig. 4

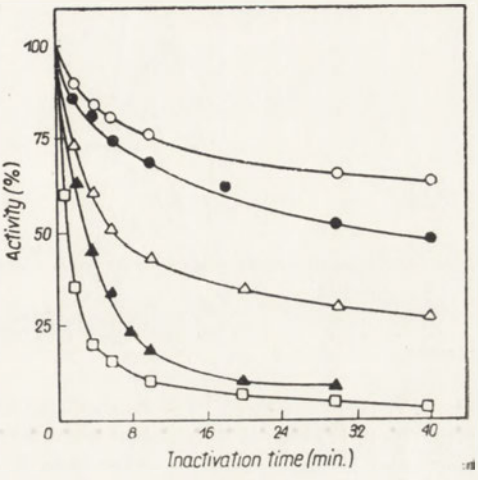

Fig. 5

Fig. 4. Effect of potassium ion concentration on spontaneous inactivation of holoenzyme. The samples contained in 1 ml.: 3 units of apoenzyme, 40 μmoles of tris-HCl buffer, pH 8.6, 0.4 mμmole of coenzyme B_{12} and indicated amounts of KCl. After 10 min. at 20°, the concentration of K^+ ion was brought to 200 μmoles per sample, 100 μmoles of glycerol was added and activity determined. The activity of a sample preincubated without K^+ ion was taken as 100.

Fig. 5. Effect of Na^+, K^+ and NH_4^+ ions and of glycerol on spontaneous inactivation of glycerol dehydratase at 20°. The samples contained in 0.9 ml.: 3 units of apoenzyme, 0.4 mμmole of coenzyme B_{12}, and one of the following components: (△), 40 μmoles of Na-phosphate, pH 8.6; (▲), 100 μmoles of K-phosphate, pH 8.6; (□), 100 μmoles of NH_4-phosphate, pH 8.6; or (○), 100 μmoles of glycerol. The control sample (●) contained only the apoenzyme and coenzyme. At determined time intervals, the lacking components were added so as to restore standard conditions (final volume 1 ml.) and the activity was determined.

fraction of low-molecular compounds separated from proteins. The results of an experiment with gel filtration are shown in Fig. 6. The crude enzyme preparation was incubated for 20 min. with [^{60}Co]coenzyme B_{12} in 0.1 M-potassium or sodium phosphate buffer. In agreement with the previous results, in the presence of potassium ions the inactivation was more pronounced and the amount of coenzyme bound to the protein fraction was higher than when the inactivation was carried out in the presence of Na^+ ion. The peaks of radioactivity were symmetrical and coincided with the symmetrical peak of enzymic activity of the non-inactivated preparation. This suggests the specificity of binding of the coenzyme to the enzyme protein. The coenzyme B_{12} bound during inactivation was not exchangeable with the excess of coenzyme B_{12}. Incubation for 20 min. of the unlabelled inactive complex in 0.1 M-potassium-phosphate buffer with [^{60}Co]coenzyme B_{12} did not lead to incorpo-

ration of the radioactivity into the protein fraction. It was also demonstrated by gel filtration that the subunits A and B of the apoenzyme separately did not associate with [^{60}Co]coenzyme B_{12}, and did not undergo inactivation in its presence.

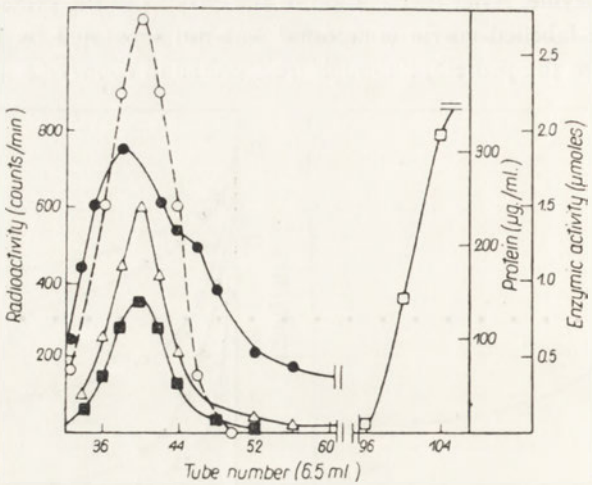

Fig. 6. The elution diagram of Sephadex G-100 gel filtrations of glycerol dehydratase. Crude enzyme preparation, 112 mg., in 40 ml. of 0.1 M-potassium or sodium phosphate buffer, pH 8.6, was incubated with 26 mμmoles of [^{60}Co]coenzyme B_{12} (3100 counts/mμmole/min.) at 20° for 20 min. then filtered through the gel column (50 × 6 cm.) equilibrated with the appropriate 0.1 M buffer. Radioactivity after incubation of the corrin compound bound to protein: (△), in K-phosphate buffer, and (▲), in Na-phosphate buffer; (□), radioactivity of the free, non-bound [^{60}Co]coenzyme B_{12}. (●), Protein concentration. (- - ○ - -), Enzymic activity (the same volume of enzyme preparation not incubated with coenzyme B_{12} was submitted to gel filtration in 0.1 M-K-phosphate buffer).

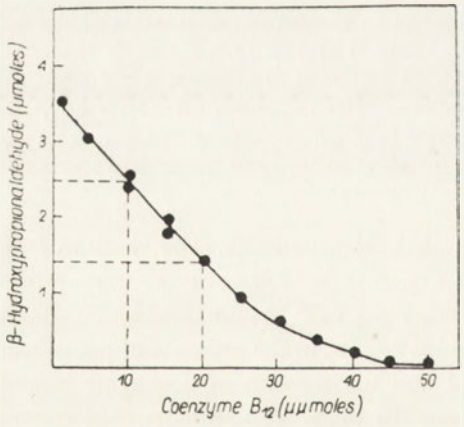

Fig. 7. Effect of coenzyme B_{12} concentration on spontaneous inactivation of the apoenzyme. The sample containing in a volume of 0.9 ml.: 3.5 units of the apoenzyme, 100 μmoles of ammonium phosphate, pH 8.6, and coenzyme B_{12} in amounts indicated, was incubated for 10 hr. at 20°; then 100 μmoles of glycerol and 0.2 mμmole of coenzyme B_{12} were added and the enzymic activity was determined.

The effect of pH on the binding ability of coenzyme with the enzyme protein was assayed by incubating the apoenzyme, prior to gel filtration, for 20 min. with [^{60}Co]coenzyme B_{12} in 0.1 M-potassium-phosphate buffer, either at pH 8.6 or 6.5. In the sample incubated at pH 8.6 the radioactivity of protein was twice that in the sample incubated at pH 6.5.

The process of inactivation takes place at a definite steicheiometric ratio (Fig. 7). When to a certain amount of the apoenzyme, coenzyme B_{12} was added in increasing amounts and the activity was determined after a time long enough for inactivation of the apoenzyme, the enzymic activity was found to be inversely proportional to the amount of coenzyme B_{12}. From the plot it was calculated that 1 μmole of coenzyme B_{12} associated with an amount of the apoenzyme corresponding to 100 000 activity units. Thus, if we assume that one molecule of the enzyme contains one molecule of coenzyme B_{12}, it would follow that one molecule of the holoenzyme is able to convert only 100 000 molecules of glycerol into β-hydroxypropionaldehyde. The above result was obtained for samples incubated in ammonium phosphate buffer. From analogous experiments performed using potassium phosphate buffer, it would follow, according to the same assumption, that one μmole of coenzyme B_{12} is able to convert about 70 000 μmoles of glycerol. The observed difference could be due to differences in the rates of inactivation and the enzymic reaction in the presence of K^+ and NH_4^+ ions. The above steicheiometric relations, expressed graphically in Fig. 7, were utilized for quantitative determination of coenzyme B_{12} and other corrinoids in amounts of 10 μμmoles (in preparation).

To study the fate of coenzyme B_{12} in the process of spontaneous inactivation, the apoenzyme was incubated for 4 hr. with [^{60}Co]coenzyme B_{12} in 0.1 M-potassium-phosphate buffer and then the excess coenzyme was removed from the solution by Sephadex G-100 gel filtration. The protein fraction containing the bound [^{60}Co]-corrinoid was treated on an ice-bath with an equal volume of 10% trichloroacetic acid. The protein sediment was centrifuged off; to the clear supernatant, which contained practically all the radioactivity, were added as non-radioactive carriers: coenzyme B_{12} and hydroxycobalamin (about 2 μmoles of each), then corrinoids were isolated by phenol extraction and separated by electrophoresis in 0.5 M-acetic acid on Whatman paper no. 3 [6]. From the electrophoretograms, the parts corresponding to the fractions of coenzyme B_{12} and hydroxycobalamin were cut out and submitted to radioactivity determinations. In the coenzyme B_{12} fraction only traces of radioactivity were found (290 counts/min.) whereas the fraction of hydroxycobalamin contained practically all the radioactivity (13 600 counts/min.). To avoid photochemical degradation of coenzyme B_{12}, the experiment was performed in a darkened room and all the analytical procedures were carried out in disperse red light.

Thermal inactivation of the apoenzyme

It has been demonstrated [9] that the reversible dissociation of the apoenzyme into subunits A and B was influenced by the presence of potassium ion and glycerol. The effect of those factors on the thermal stability of the apoenzyme was studied to find the optimum conditions for isolation and storage of the apoenzyme preparation. From Fig. 8a, representing the inactivation of the apoenzyme at 50° it appeared that glycerol had no stabilizing effect. A slight stabilizing influence was shown by potassium ion; the half-inactivation times for the samples incubated with

glycerol or potassium-phosphate buffer were, respectively, 1 and 2 min. The stabilizing effect was distinct only in samples incubated in the presence of both glycerol and potassium-phosphate buffer. Under those conditions the half-inactivation time was 8.5 min.

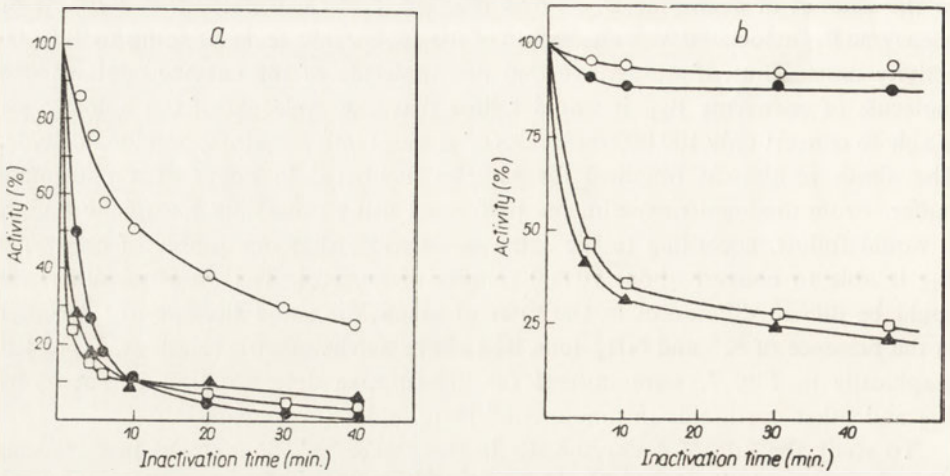

Fig. 8. Effect of K^+ ion and glycerol on inactivation of the apoenzyme at (*a*), 50°, and (*b*), 40°. The sample contained in a volume of 0.5 ml.: 3 units of the apoenzyme and: (●), 100 μmoles of K-phosphate buffer, pH 8.6; (▲), 100 μmoles of glycerol; (○), 100 μmoles of K-phosphate buffer and 100 μmoles of glycerol; (□), a solution of apoenzyme alone. After determined time intervals, the enzymic activity was determined under standard conditions.

On the other hand, the stabilizing effect of the potassium-phosphate buffer alone was apparent at lower temperatures (Fig. 8b). The enzyme activity was only slightly affected after 40-min. incubation at 40° in potassium-phosphate buffer. Glycerol alone, similarly as at 50°, had no effect but it enhanced the protecting effect of the potassium-phosphate buffer. That glycerol alone had no stabilizing effect can be in part explained by the fact that the incubation mixture was unbuffered and therefore had a lower pH value.

Properties of proteins A and B

Susceptibility to thiol reagent. *N*-Ethylmaleinimide was used as the SH group-binding reagent. Fraction A, 9 ml. (5 mg. of protein) obtained by resolution of the apoenzyme on the DEAE-cellulose column, was diluted to 0.02 M-potassium-phosphate buffer concentration, pH 8.6; then 0.2 g. of glycerol was added and, at 2°, 1 ml. of 0.1 M-solution of *N*-ethylmaleinimide. After 3 hr. of incubation at 2° the mixture was applied to a short column (1 × 1.5 cm.) of DEAE-cellulose equilibrated with 0.02 M-potassium-phosphate buffer, pH 8.6. The column was carefully washed, then protein A was eluted with 0.12 M buffer, pH 8.6, and the enzymic activity (after addition of an excess of protein B) was determined. It was found that protein A, after treatment with *N*-ethylmaleinimide retained about 25% of the

activity of the control sample, treated in the same way but without *N*-ethylmaleinimide.

In an analogous experiment, protein B was found to be completely inactivated by *N*-ethylmaleinimide. The thiol nature of protein B was supported by the reactivation by mercaptoethanol during its preparation. In those samples the excess reagent was removed by Sephadex G-25 gel filtration prior to activity determinations because mercaptoethanol interferes with the formation of a colour reaction used for determination of hydroxypropionaldehyde.

Thermal inactivation. Figure 9 presents the dependence of activity of protein A on the time of heating in potassium-phosphate buffer at 50° and 100°. Despite marked dilution (15 μg./ml.) protein A was fairly stable; the half-inactivation time was 4.5 min. at 100°, and at 50° it was 10 min. An analogous experiment performed at 70° gave rather unexpected results. Already after a few minutes of heating a complete inactivation was observed. To elucidate this phenomenon, the effect of one-minute heating of the solution of protein A at different temperatures, was studied (Fig. 10).

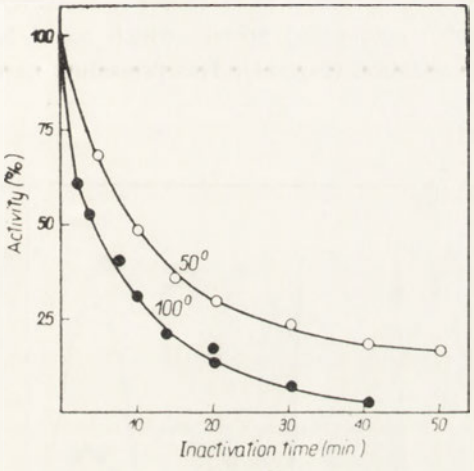

Fig. 9

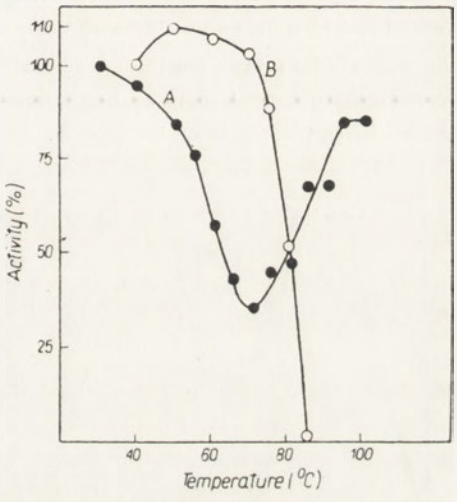

Fig. 10

Fig. 9. The rate of inactivation of protein A at 50° and 100°. The sample contained in a volume of 0.5 ml.: 3 units of the apoenzyme, 100 μmoles of K-phosphate buffer, pH 8.6, and 100 μmoles of glycerol. After incubation at the indicated temperature and for given time intervals, 5 units of protein B and 0.2 mμmole of coenzyme B_{12} were added and the activity of protein A was determined under standard conditions.

Fig. 10. Effect of temperature on the activity of proteins A and B. (●), Protein A, 3.5 units, or (○), protein B, 2.5 units, were incubated for 1 min. at the indicated temperature. A sample contained in 0.5 ml.: 100 μmoles of K-phosphate buffer and 100 μmoles of glycerol, and the activity was determined under standard conditions.

It appeared that at 70° protein A was most unstable, the inactivation being the greatest. By heating at 100° the sample inactivated at 70°, or by storing it at low temperature, e.g. at -10°, the activity could be partly restored (Table 1).

Table 1

Inactivation at 70° and reactivation of protein A

Treatment before enzyme determination	Activity	
	units	%
None	3.3	100
Incubation at 70°, 2 min.	0.85	25.8
Incubation at 70°, 2 min. then at 100°, 1 min.	1.65	50
Incubation at 70°, 2 min. then storage at -10°, 7 days	1.25	38

Protein B, when incubated for 1 min. at 50°, underwent even a slight activation (Fig. 10). A rapid inactivation occurred above 75°. The marked thermal stability of protein B was utilized in this work for its purification from the preparation partly purified on the DEAE-cellulose column (see Methods). The protein B fraction was heated for 15 min. at 65 - 70° and the precipitated proteins were removed. However, the supernatant still contained some soluble aggregated protein which could be separated from protein B by Sephadex gel filtration (Fig. 11). This procedure gave a 13-fold purification of protein B.

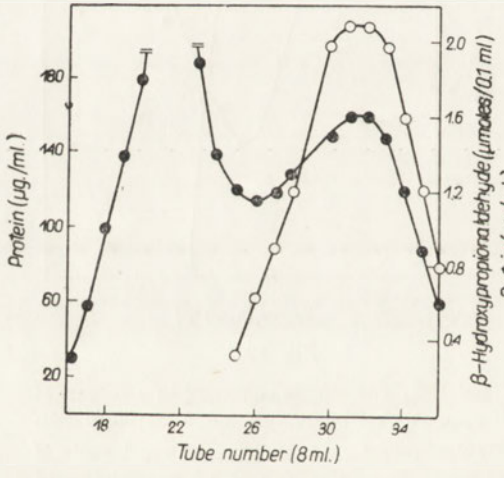

Fig. 11

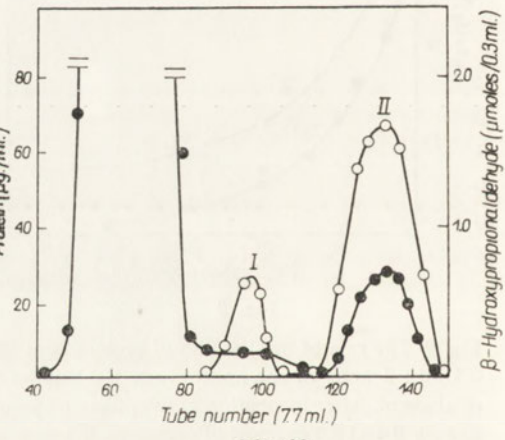

Fig. 12

Fig. 11. Gel filtration of protein B preparation after heating at 65 - 70° for 15 min. (for details see Methods). To a Sephadex G-100 column equilibrated with 0.1 M-K-phosphate buffer, pH 8.6, a solution of protein B (25 mg.) in the above buffer was applied. In the collected fractions (●), protein concentration was determined, and (○), in a volume of 0.1 ml., the enzymic activity of protein B was assayed.

Fig. 12. Sephadex G-100 gel filtration of a purified preparation of protein A, in 0.02 M-potassium-phosphate buffer, pH 6.7. About 30 mg. of protein A separated by DEAE-cellulose column chromatography, was applied to the gel column (50 × 6 cm.). In the fractions (●), protein concentration was determined, and (○), in 0.3 ml. enzymic activity was assayed. Peak *I* corresponds to a protein having the molecular weight of 22 000, peak *II* to a protein having the molecular weight of 12 000.

Dissociation of protein A into subunits. During purification of protein A by gel filtration in 0.1 M-potassium-phosphate buffer, pH 8.6, traces of protein A activity were always found to be present in the fraction of a molecular weight lower than that of protein A. By modifying the conditions of gel filtration, it was found that the appearance of the low-molecular fraction with protein A activity depends on the pH and low ionic strength. In 0.02 M-potassium-phosphate buffer, pH 6.7, a greater part of protein A dissociated to subunits of molecular weight 12 000 (Fig. 12). As the molecular weight of protein A was found to be 22 000 [9], the weight of the subunits was practically half that of protein A.

The steicheiometric ratio of binding of proteins A and B. To determine the weight ratio of proteins A and B binding to form the apoenzyme, two methods were applied. One of them consisted in determining the protein A to B ratio at which the highest activity of the apoenzyme is obtained. The second method was based on the inactivation of the apoenzyme by coenzyme B_{12}; the protein subunit which was given in excess remained unbound, and its amount was determined enzymically. For these experiments, the most purified preparations of protein fractions A and B were used. The details are given in the legends to Fig. 13 and 14. Both methods gave

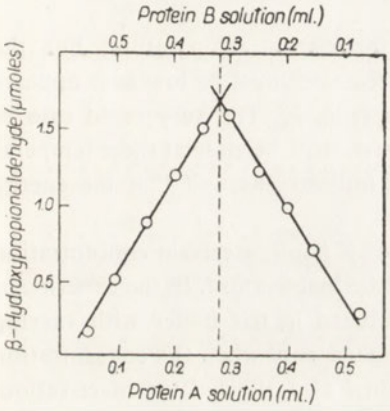

Fig. 13

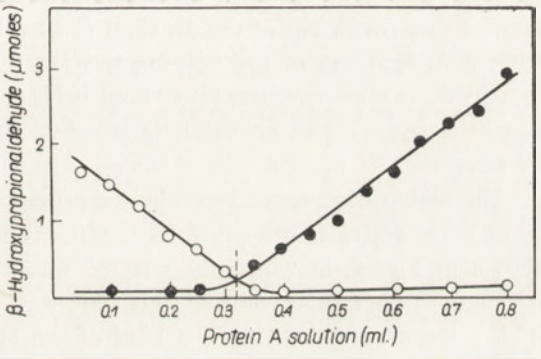

Fig. 14

Fig. 13. Effect of the ratio of protein A and B on the apoenzyme activity. To a solution of protein A (5.6 μg./ml., sp. act. 1100 units/mg.) different amounts of protein B solution (10 μg./ml., sp. act. 130 units/mg.) were added, the total volume being 0.6 ml. Then were added 100 μmoles of K-phosphate buffer, pH 8.6, 100 μmoles of glycerol and 0.2 mμmole of coenzyme B_{12}, and the enzymic activity was determined.

Fig. 14. Spontaneous inactivation of the holoenzyme at varying protein A and B ratios. To 0.4 ml. of protein B solution (40 μg./ml., sp. act. 130 units/mg.) increasing amounts, from 0 to 0.8 ml., of protein A solution (5.6 μg./ml., sp. act. 1060 units/mg.) were added. After addition of 200 μmoles of ammonium phosphate buffer, pH 8.6, and 0.4 mμmole of coenzyme B_{12}, the sample was incubated for 1 hr. at 20°; then 200 μmoles of glycerol was added and the volume adjusted to 2 ml. The incubation was carried out in duplicate, then the enzymic activity of (●), protein A was determined in one sample, and that of protein B (○), in another.

practically the same results and demonstrated that the subunits combined in a definite weight ratio. In the described experiments, 1 part of protein A having an activity of 1100 units/mg. combined, depending on the method used, with 8.1 or 8.9

parts of protein B having an activity of 130 units/mg. This ratio roughly corresponds to the molar ratio of subunits A and B. Assuming the molar ratio of the two proteins to be 1:1, this suggests the same degree of purification of the two components in the obtained preparations.

DISCUSSION

One of the characteristic features of glycerol dehydratase isolated from *Aerobacter aerogenes*, is its rapid spontaneous inactivation (Fig. 2). Incubation of holoenzyme at 35° for 20 min. in potassium-phosphate buffer, pH 8.6, leads to its practically complete inactivation. The apoenzyme alone is stable under these conditions. The rate of inactivation of the enzyme is influenced by a number of factors, such as temperature, univalent ions, and pH of the medium.

Because of this very rapid inactivation of the enzyme, the unit of glycerol dehydratase has been defined in a way which is not generally accepted. In the present work as a unit of activity was taken the amount of enzyme which, under standard conditions, till its inactivation catalyses the transformation of 1 μmole of glycerol into β-hydroxypropionaldehyde.

The rate of inactivation increased with an increase in temperature, but the temperature coefficient of the constant k for this reaction must be low as it appears from a comparison of the half-inactivation time (Fig. 3). The very rapid rate of inactivation at temperatures lower than 10° is characteristic because at these temperatures the enzyme had no catalytic activity. This indicates as well that the energy of activation of this process is low.

The inactivation is enhanced by the presence of K^+ ion, a certain concentration being necessary to assure optimum conditions for the inactivation. In the experiment shown in Fig. 4, in which the enzyme was incubated in tris buffer with varying amounts of potassium chloride, this state was reached only when the concentration of K^+ ion exceeded 0.1 M. The kind of ion applied also affected the inactivation. Ammonium ion accelerated the inactivation even more than did potassium ion. There was a marked similarity between the rate of inactivation and the rate of the reaction catalysed by glycerol dehydratase. It has been found previously [7] that NH_4^+ ion activated the reaction 1.5 times as strongly as did the K^+ ion. On the other hand, the Na^+ ion did not prevent inactivation although in its presence the enzymic reaction did not occur. Nevertheless, the inactivation in the presence of sodium phosphate proceeded much slower than in potassium phosphate.

As it has been shown previously [9], K^+ or NH_4^+ ions are required to maintain the association of subunits A and B, and this explains their effect on the spontaneous inactivation of the enzyme. In the presence of Na^+ ion the apoenzyme dissociated into subunits A and B, which could be separated, e.g. on a gel column. The inactivation of the enzyme in the presence of sodium salts indicates, however, the existence of an equilibrium between the associated and dissociated subunits $A+B \rightleftarrows AB$. A part of the apoenzyme can bind with coenzyme B_{12} and undergo inactivation. On the other hand, the lack of catalytic activity of the enzymic system containing

Na^+ but no K^+ or NH_4^+ ions, suggests that K^+ or NH_4^+ ions might bring about some further changes of the enzyme structure, in addition to the association of subunits A and B; therefore these ions seem to be indispensable for the enzymic reaction, although they are not indispensable for the reaction of inactivation. It has been demonstrated previously [9] that subunits A and B associate in the presence of Na^+ ion and glycerol but the complex formed under these conditions is enzymically inactive.

The effect of pH on inactivation of the holoenzyme can be also explained by the existence of a dissociation - association equilibrium, which is also dependent on hydrogen ion concentration. At pH 6.5 the apoenzyme undergoes partial dissociation to A and B components which can be separated by the molecular sieve technique [9]. At this pH, however, the holoenzyme also undergoes inactivation, but when the medium contains K^+ ion, the non-dissociated part of the apoenzyme combines with the coenzyme giving the active holoenzyme (Fig. 1). This supports the suggestion of an additional role of K^+ ion in the formation of an enzymically active structure in the already formed complex of apoenzyme and coenzyme.

The equilibrium $A + B \rightleftharpoons AB$ seems to be influenced also by temperature, the increase of which can increase the dissociation of the apoenzyme. This suggestion arises from the observation of the protective effect of potassium-phosphate buffer, alone or with the addition of glycerol, against thermal inactivation of apoenzyme (Fig. 8a, b). These factors, as it has been shown previously [9], promote the association of subunits A and B.

So far, the nature of the spontaneous inactivation has not been elucidated; in the present work, however, hydroxycobalamin was identified as one of the products formed during inactivation. Recently Abeles & Lee [1] obtained similar results studying a diol dehydratase isolated from a different strain of *A. aerogenes* which, however, was inactive towards glycerol. Formation of hydroxycobalamin from coenzyme B_{12} involves breaking of the bond between the atom of cobalt of the corrin ring, and the $C'_{(5)}$ of the 5'-deoxyadenosyl group. The observation that a number of factors (presence of univalent ions, pH, temperature) influence in a similar manner both the enzymic reaction catalysed by glycerol dehydratase and the inactivation of this enzyme, seems to indicate that the bond Co-C may participate directly in the enzymic process, in the same way as it takes part in the process of inactivation.

Inactivation of the holoenzyme is connected with the formation of a stable binding between the protein molecule and the arising hydroxycobalamin. The inactive complex can be easily separated from the free coenzyme B_{12} by gel filtration (Fig. 6).

On the basis of the presented data, the reaction of inactivation can be expressed by equation (I) in which $[AB\text{-}CoB_{12}]'$ and $[AB\text{-}CoB_{12}]''$ are, respectively, an enzymically inactive complex of apoenzyme with coenzyme B_{12}, and a complex activated by K^+ ions; $AB\text{-}B_{12b}$ is the inactive complex, composed of apoenzyme and hydroxycobalamin (vitamin B_{12b}). Formation of this last compound appeared to be irreversible. However, it is difficult to assume

that such a process could occur in a living cell, without any possibility of reactivation of apoenzyme and coenzyme, the more so that in *A. aerogenes* growing on a glycerol-containing medium glycerol dehydratase is an important enzyme.

$$\begin{array}{l} A + B \rightleftharpoons AB \\ AB + CoB_{12} \rightleftharpoons \left[AB-CoB_{12}\right]' \longrightarrow \\ \qquad\qquad +K^+ \downarrow \uparrow -K^+ \qquad\qquad AB-B_{12b} \\ \qquad\qquad \left[AB-CoB_{12}\right]'' \longrightarrow \end{array} \qquad (I)$$

Therefore it should be assumed that either a reactivation takes place in the cells of *A. aerogenes*, or the inactivation does not occur in a living cell. Elucidation of this point requires further study.

The subunits A and B of glycerol dehydratase differ markedly in their molecular weight (resp. 22 000 and 240 000). This property, along with dissociation of the dehydratase and association of the subunits into active apoenzyme, permits to isolate the component A in a very pure state, probably even as a homogeneous protein [9], and to determine some of its properties. When diluted to a greater extent, protein A appeared to be rather unstable. In 0.1 M-potassium-phosphate buffer, pH 8.6, protein A at a concentration of 5 μg./ml. at 0° lost daily about 10 - 25% of its initial activity. In more diluted buffers and at lower pH values, the activity decreased more rapidly. This loss of activity can be explained, at least in part, by decomposition of protein A into two further subunits which appear in solution of low ionic strength and at pH values lower than 7, and have a molecular weight of 12 000 (Fig. 12). These new subunits seem to be even more labile than protein A. It seems possible to suppose that protein A is a dimer composed of two identical subunits.

The preparations of protein A obtained in this work had a specific activity of not more than 1100 units/mg. As this activity could be expected to be about 3200 units/mg $\left(\frac{70\,000}{22}\right)$, it seems that protein A became inactivated already during the isolation procedure. The above calculation was based on the assumption that 1 μmole of coenzyme B_{12}, when it combines with 1 μmole of apoenzyme (composed of proteins A and B in a proportion of 1:1), converts about 70 000 μmoles of glycerol into hydroxypropionaldehyde. Thus the specific activity of most active protein A preparation obtained amounted to about 34% of the theoretical value.

Protein A in potassium-phosphate buffer was relatively resistant to short exposure to higher temperature (Figs. 9 and 10) exhibiting a characteristic minimum of stability at 70°. The differences in the stability at various temperatures can be explained by the presence of an equilibrium of three different forms of protein A which are stable, respectively, at temperatures below 70° (A_I), at 70° (A_{70}), and above 70° (A_{II}):

$$A_I \rightleftharpoons A_{70} \rightleftharpoons A_{II}$$

Only the forms A_I and A_{II} are able to associate with protein B to give the apoenzyme. The form A_{70} seems to be inactive. The conversions of these forms are reversible

as indicated by the reactivation of protein A after its inactivation at 70°, by heating at 100° or by prolonged storage at low temperature (Table 1). From the observations made in the present work it cannot be concluded whether the forms A_I and A_{II} are identical or different, or in which way the subunits of protein A participate in their formation. On the basis of the observed marked lability of subunits of protein A it can be only suggested that the form A_{70} may be to a large extent dissociated into subunits which subsequently at a higher temperature, after internal regrouping, can combine again to give the active form A_{II}.

The stability of protein A at 100° can be utilized for its isolation. Rapid heating of the apoenzyme to 100° results in complete inactivation of protein B, the activity of protein A being retained. On the other hand, heating of the apoenzyme for 10 min. at 70° causes complete inactivation of protein A without altering the activity of protein B. From these results it follows that at higher temperature the apoenzyme undergoes dissociation into its components, which behave further as independent units.

Inactivation of component B, with unaffected activity of protein A, occurs also during 30-min. incubation of the apoenzyme at 0° in 6 M-urea solution. After incubation the active component could be separated by Sephadex G-100 gel filtration.

These experiments demonstrated a rather marked stability of the secondary and tertiary structure of protein A. The effect of temperature on protein B was quite different. At 70°, when protein A underwent inactivation, protein B was even slightly activated. This behaviour was utilized for elaborating a simple method for purification of component B. The specific activity of the most purified fractions amounted to 130 units/mg., which corresponds to 43% of the theoretical value calculated on the basis of the assumptions described above. In contrast to protein A, the presence of free SH groups is essential for the activity of protein B.

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WŁASNOŚCI DEHYDRATAZY GLICEROLOWEJ IZOLOWANEJ Z *AEROBACTER AEROGENES* I WŁASNOŚCI PODJEDNOSTEK APOENZYMU

Streszczenie

1. Apoenzym wyizolowanej dehydratazy glicerolowej składa się z dwóch podjednostek A i B, o ciężarach cząsteczkowych 22 000 i 240 000, łączących się w stechiometrycznym stosunku.

2. Holoenzym utworzony z apoenzymu i koenzymu B_{12} ulega szybkiej, samorzutnej i nieodwracalnej inaktywacji, zarówno w obecności jak i pod nieobecność substratu. Inaktywacja zachodzi nawet w temp. 1°, w której nie zachodzi reakcja enzymatyczna. Zinaktywowany enzym zawiera hydroksykobalaminę trwale związaną z białkiem.

3. Jednowartościowe jony i słabo alkaliczne pH środowiska, które sprzyjają asocjacji podednostek w aktywny apoenzym, przyśpieszają inaktywację holoenzymu. Inaktywacja enzymu biegnie w obecności jonów Na^+, mimo że układ taki nie katalizuje reakcji enzymatycznej.

4. Podjednostka A jest białkiem termostabilnym wykazującym ciekawe minimum stabilności w temp. 70°; w roztworach o pH niższym od 7 i niskiej sile jonowej rozpada się na dalsze podjednostki o ciężarze cząsteczkowym równym 12 000. Białko A nie ma czynnych grup SH.

5. Podjednostka B jest białkiem odpornym na działanie temperatur do 70°. Zawiera ono czynne grupy SH.

6. Opracowano wydajną metodę izolowania i stabilizacji enzymu.

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V. JANDA Jr., F. SEHNAL and V. ŠIMEK

CHANGES IN SOME CHEMICAL CONSTITUENTS OF *GALLERIA MELLONELLA* LARVAE IN RELATION TO GROWTH AND MORPHOGENESIS

Department of Comparative Physiology and General Zoology, J. E. Purkyně University, Brno, and Department of Physiology, Institute of Entomology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

1. The growth of the body and the changes in water, dry matter, protein and residual nitrogen, fat, glycogen, and uric acid content were studied in larvae of *Galleria mellonella* L. 2. These changes were different in the last larval instar from those in the penultimate one, in connection with the onset of metamorphosis in the last instar. 3. The relationship between the chemical body composition and the processes of morphogenesis and histolysis is discussed.

More or less pronounced changes in the body composition may be found throughout the ontogenetic development of insects, depending especially on the food intake and food utilization. Therefore, the content of structural and reserve substances changes considerably during each feeding period of larval development. A great difference occurs, however, between the last larval instar, when metamorphosis begins, and the other, typically larval instars [19]; this difference does not seem to be related only to the rate of food ingestion. Such change in the chemical composition of the body seems to depend on specific physiological and biochemical conditions related to metamorphosis, and its causes are more complicated.

In this paper we have tried to study the relation of the chemical body composition to such phenomena as ecdysis, excretion, spinning, etc. Special attention was paid to morphogenetic processes, because their intensity and character determine the difference between the last and the other larval instars. Since this difference is most pronounced in Endopterygota insects, the larvae of *Galleria mellonella* were chosen for the experiments.

MATERIAL AND METHODS

Larvae of *Galleria mellonella* L. were kept at 30° on an artificial diet according to Balázs [2]. In optimal conditions, development of the larvae proceeds in seven, well defined instars [17]. Freshly moulted larvae of the VIth and VIIth instars were

taken daily from the stock and then kept in Petri dishes in smaller groups. In this way the regular development of the larvae and the homogeneity of the experimental material was secured.

The duration of the VIth instar was less than four days, that of the last (VIIth) instar was 8 days. The last-instar larvae empty their intestines after five days of development and begin to spin the cocoon on the sixth day. The cocoon is finished between the seventh and eighth day. On the eighth day there are already immobile prepupae in the cocoons, which moult into pupae within 24 hr.

The growth was investigated on the basis of regular weighing of a group of 35 larvae during their development in penultimate and ultimate instars. The same values were, however, obtained by weighing all the experimental specimens in the relevant phases of development. The results of the determinations were then related directly to the average weight. Twenty to fourty specimens were used in each determination and a total of about 2000 specimens was investigated.

Fresh material was used for the determination of protein and residual nitrogen and lipids; glycogen and uric acid were determined in dry matter. For nitrogen determinations the larvae were homogenized in 10% trichloroacetic acid. The homogenate was centrifuged and the residual nitrogen was determined in the supernatant by colorimetry with Nessler reagent. The sediment was washed and digested with 1 N-NaOH; the protein nitrogen was determined in the solution by the same method. Lipids were extracted with the methanol-chloroform mixture, refluxed with ethyl ether and determined gravimetrically. Glycogen was determined colorimetrically by means of the anthrone reagent following hydrolysis by 30% KOH and precipitation by 96% ethanol. Uric acid was analysed by extraction with borate buffer solution (pH 9.0) using colorimetry with arsenophosphotungstene reagent. The procedures have been described in more detail by Janda & Sláma [13].

RESULTS

The body weight increases intensively during the first two-thirds of the penultimate instar, whereas during the last third the gain in body weight slows down or stops completely. A further increase of the body weight sets in after moult, i.e., with the beginning of the last instar. The caterpillars attain their maximal weight during the second half of the last instar before spinning; then the body weight decreases till the pupal ecdysis. The dry matter undergoes similar changes (Fig. 1).

The relative water content decreases slowly during the first half of the VIth instar, then it increases slowly until moulting. Much greater changes occur in the VIIth instar. The water content decreases intensively until the onset of spinning, whereas during spinning it begins to rise again. The changes in the relative dry matter content show an opposite course (Fig. 2).

The amount of both protein and residual nitrogen per specimen increases gradually from the beginning of the VIth instar up to the onset of spinning (with the exception of a short period at the end of the VIth instar). The protein nitrogen decreases continuously from the beginning of spinning to pupation. On the other

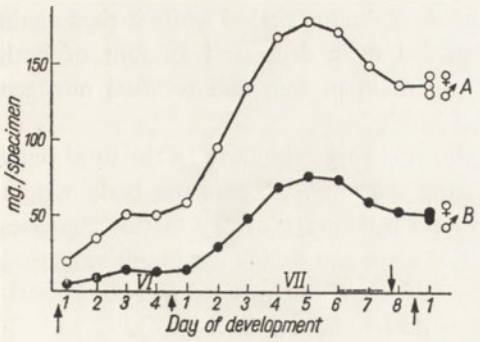

Fig. 1

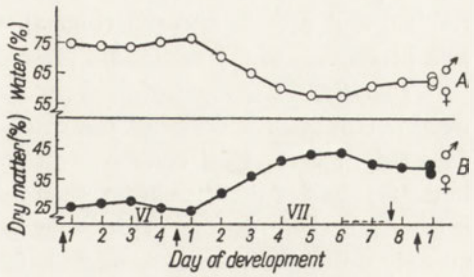

Fig. 2

Fig. 1. Changes in (*A*), body weight and (*B*), dry matter per specimen during the penultimate and the last larval instars of *Galleria mellonella*. In this and subsequent Figures arrows indicate ecdyses (↑) and prepupal stage (↓). The period of spinning is indicated by a broken line (- - - -).

Fig. 2. Changes in (*A*), relative water and (*B*), dry matter content in larvae of the penultimate and the last instars.

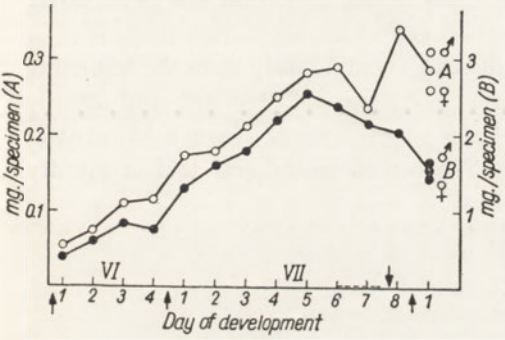

Fig. 3

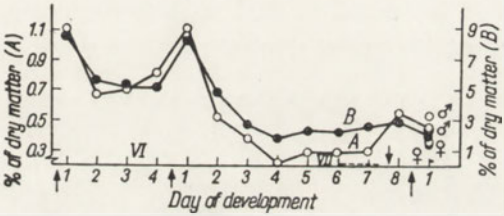

Fig. 4

Fig. 3. The amount of (*A*), residual and (*B*), protein nitrogen in larvae of the penultimate and the last instars.

Fig. 4. The (*A*), residual and (*B*), protein nitrogen expressed as percentage of the dry matter in larvae of the penultimate and the last instars.

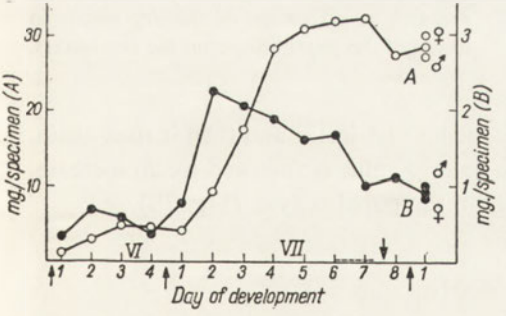

Fig. 5

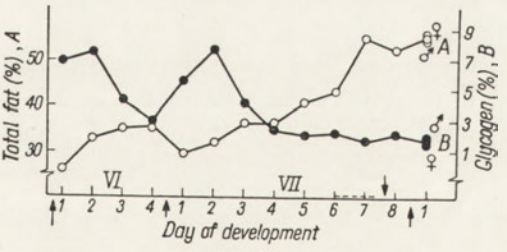

Fig. 6

Fig. 5. Changes in (*A*), total fat and (*B*), glycogen content per specimen during the penultimate and the last larval instars.

Fig. 6. The content of (*A*), total fat and (*B*), glycogen percentage of the dry matter in larvae of the penultimate and the last instars.

hand, the residual nitrogen decreases during the spinning period while it rises again before pupation. Pupal ecdysis is accompanied by a decreased amount of both residual and protein nitrogen (Fig. 3). Changes in protein and residual nitrogen as a percentage of dry matter are presented in Fig. 4.

Generally, the amount of lipid per specimen changes similarly to the total body weight. However, it does not decrease during spinning, whereas the body weight does (Fig. 5). The lipid content expressed as a percentage of dry matter increases in the first half of the VIth instar, then the rise stops and during the moulting period it decreases again. In the VIIth instar the relative amount of lipid increases continuously up to the cocoon formation, falling down afterwards (Fig. 6).

The amount of glycogen per specimen increases quickly at the beginning of each instar and decreases towards the next ecdysis. A more profound decrease occurs during spinning (Fig. 5). The relatively high glycogen content in the dry matter, found at the beginning of the VIth instar, decreases approximately to one half in the course of this instar. During the first half of the last instar it rises temporarily to the original maximum, while it decreases again during the second half of the instar and then remains at a low level until pupation (Fig. 6).

The amount of uric acid per specimen increases continuously from the beginning of the VIth instar up to the middle of the last one. It decreases afterwards and actually disappears from the body before spinning. Later on, the content of uric acid increases again, especially after pupation (Fig. 7A). The percentage of uric acid in the dry

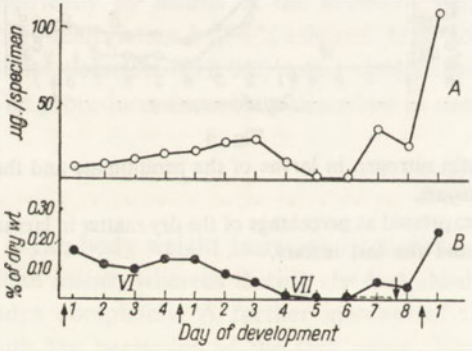

Fig. 7. Uric acid content (*A*), per specimen and (*B*), as percentage of the dry matter in larvae of the penultimate and the last instars.

matter decreases during the first two-thirds of the VIth instar and then it rises again. In the VIIth instar a steady decrease down to zero value is followed by an increase, which proceeds from the onset of spinning till the pupal ecdysis (Fig. 7B).

DISCUSSION

The body weight of *Galleria mellonella* larvae increases in a way typical for insects [11, 12, 19] (Fig. 1). There is a transitory decrease of the growth rate during the moulting period, which is connected with the reduced food intake. The permanent decrease of body weight at the end of the last larval instar depends also on other

factors. These are, for instance, emptying of the intestine, cocoon formation, water loss, etc. In *Galleria* spinning of the cocoon seems to be most important, because the percentage of water does not decrease, or it may even increase (Fig. 2). In this respect larvae of *Galleria* differ from caterpillars of other species of butterflies, like *Deilephila euphorbiae* [9], *Bombyx mori* [15], *Hyphantria cunea* [7].

The relative increase in the protein nitrogen content (Figs. 4, 3) during the larval ecdysis is due not only to protein synthesis; it seems rather to reflect the significant decrease in the amount of non-protein substances (lipid, glycogen), which are utilized by the organism to a greater extent during this period (Figs. 5, 6). On the other hand, the decrease in body weight during spinning is due mainly to a decreased amount of protein, and, to a smaller extent, to the loss of other substances. These results indicate different changes in metabolism accompanying larval and pupal moults. Contrary to the protein nitrogen, which decreases at the end of the VIIth instar, the residual nitrogen shows more complicated changes. The intensive decrease in residual nitrogen during the spinning period is clearly connected with the synthesis in the silk glands. The subsequent increment of the residual nitrogen content is initiated by histolytic processes in larval tissues [5], which start in this period in *Galleria* (F. Sehnal, to be published). A similar relationship between the haemolymph amino acid content, spinning, and histolytic processes has also been pointed out by Gilbert & Schneiderman [8]. The final decrease of the residual nitrogen in the last instar seems to be connected with the process of pupal moult and the formation of a new cuticle.

In caterpillars of various butterflies the fat content decreases already at the beginning of spinning: *Bombyx mori* [15, 10], *Hyalophora cecropia* [1], *Pieris napi* and *Pieris rapae* [18], whereas in *Galleria* the fat content continues to rise or does not change even during spinning. The relative fat content decreases temporarily during larval moult, similarly as in *Bombyx mori* [15] or in larvae of caddisflies [16]. During this period the fat is probably utilized as a source of energy. Since the larvae were fed on a predominantly carbohydrate-containing diet, the reserve fat had to be formed from carbohydrate sources [3, 6]. From Fig. 6 the connection between the changes of the fat and glycogen content is quite obvious. In periods when the glycogen decreases, fat is formed. A similar connection was found also by Ditman [4] in prepupae of *Heliothis obsoleta*. It is probable that in *Galleria* glycogen also represents the source of carbohydrates for lipid synthesis.

The almost constant content of uric acid per specimen in the penultimate and ultimate larval instars proves the continuous excretion of uric acid. Even during the moulting period there are no significant changes in the uric acid content, though the gut remains full all the time [14], and the amount of excrements decreases (F. Sehnal, unpublished observations). We may, therefore, accept that the uric acid content would increase during this period, similarly as has been found in larvae of *Neodiprion sertifer* [12]. The decrease of uric acid or its total disappearance is probably connected with the complete emptying of the intestine and the Malpighian tubes. As there is no formation of excrements during metamorphosis since

the beginning of the spinning period, no more uric acid is excreted from the body, so that its amount greatly increases.

The results of investigations of all body components studied during the larval development of *Galleria mellonella* have proven again that there is a significant difference between the penultimate and the last larval instar, as has been found by Teissier [19]. This difference is connected with the onset of metamorphosis during the last instar. The chemical composition of the body is much influenced by the processes accompanying metamorphosis (e.g. emptying of the gut and silk glands), but also by morphogenetic and histolytic processes themselves (e.g. relations between the formation of the pupal cuticle by differentiated epidermal cells and the residual nitrogen, or relation between the uric acid content and the histolysis of the Malpighian tubes and of the gut).

More pronounced structural changes occur in the second half of the last instar (F. Sehnal, to be published). The chemical composition of larvae of the last and the penultimate instars is, however, different already from the beginning of the instar (cf. the content of fat or water). It would be, therefore, possible that the metabolism of nutrients changes in the cells under the influence of hormones the effect of which at that time is not yet manifested morphologically. This problem is dealt with in another paper (F. Sehnal, V. Janda Jr. & V. Šašínková, to be published).

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ZMIANY W PEWNYCH SKŁADNIKACH CHEMICZNYCH U LARWY *GALLERIA MELLONELLA* W ZALEŻNOŚCI OD WZROSTU I MORFOGENEZY

Streszczenie

1. U larwy *Galleria mellonella* L. zbadano przyrost wagi ciała, zmiany ilości wody, suchej masy, białka i azotu resztkowego, oraz zawartości tłuszczu, glikogenu i kwasu moczowego.

2. Zmiany te w ostatnim stadium laiwalnym różnią się, w związku z zachodzącą w tym stadium metamorfozą, od odpowiednich zmian w stadium przedostatnim.

3. Omówiono zależność między chemicznym składem ciała a procesami morfogenezy i histolizy.

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A C T A B I O C H I M I C A P O L O N I C A

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P. REMY, G. DIRHEIMER and J. P. EBEL

ANALOGUES OF NUCLEOSIDE POLYPHOSPHATES

SYNTHESIS OF AN ANALOGUE OF ATP CONTAINING A HYPOPHOSPHORIC BOND

Laboratoires de Chimie Biologique, Faculté des Sciences et Faculté de Pharmacie, Strasbourg, France

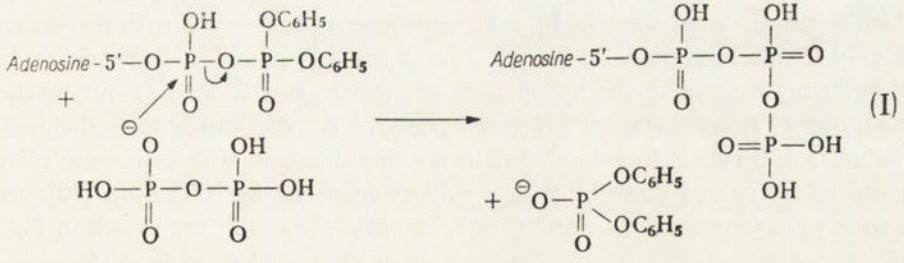
1. From P_1-5′-adenosyl,P_2-diphenyl pyrophosphate by an anionic exchange reaction a compound was obtained that, on the basis of chemical analysis, was tentatively identified as adenosine-5′-phosphohypophosphate.

Synthesis of analogues of adenosine-5′-triphosphate may be interesting in many respects. Myers, Nakamura & Danielzadeh [11] and Simon, Myers & Mednieks [12] have described the synthesis of 5′-adenylylmethylenediphosphonate and studied the behaviour of this compound as a substrate of the enzymic synthesis of polyribonucleotides.

We thought that it would be interesting to synthetize an analogue of ATP in which the tripolyphosphate chain would be replaced by a chain containing phosphorus atoms of lower oxidation state.

We have tried to synthetize an analogue of ATP in which a P-O-P bond is replaced by a P-P bond.

We used the anionic exchange reaction described by Michelson [9]. Letters & Michelson [7] synthetized adenosine polyphosphates by displacing diphenylphosphate group of P_1-5′-adenosyl,P_2-diphenyl pyrophosphate by polyphosphates of various chain-lengths, according to reaction (I):



(I)

We performed a similar reaction, replacing pyrophosphate by hypophosphate.

METHODS

Hypophosphoric acid was prepared from disodium hypophosphate by cationic exchange. The latter was prepared by oxidation of elemental red phosphorus with sodium chlorite, according to Blaser & Worms [2].

P_1-5′-Adenosyl,P_2-diphenyl pyrophosphate was obtained by the method of Michelson [10].

The anionic exchange reaction was performed under the following conditions: 3 m-moles of hypophosphoric acid and 12 m-moles of tri-*n*-butylamine dissolved in 4.5 ml. of pyridine and 2 ml. of dimethylformamide were added to 2 m-moles of P_1-5′-adenosyl,P_2-diphenyl pyrophosphate dissolved in 2 ml. of dimethylformamide and 3 ml. of dioxan. All the reagents used were anhydrous.

The mixture was allowed to react for one hour at room temperature and the nucleotidic materials were extracted from the reaction mixture by evaporation of solvents under vacuum.

The liquid residue, containing essentially tri-*n*-butylamine in excess and phosphorus compounds, was triturated with anhydrous diethylether. Phosphorus compounds gave a gummy precipitate which was washed once more with diethylether.

CHARACTERIZATION OF ADENOSYL NUCLEOTIDES

Chromatographic analysis of the crude reaction product

Ascending one-dimensional chromatography. The ascending chromatography on Schleicher & Schull 2043b paper was performed with isobutyric solvent [6]. Three spots absorbing ultraviolet light were found. Two of them had, respectively, the R_F values of adenosine and AMP. The third one had a R_F close to that of ATP (Fig. 1). The spots were cut out and eluted. The extinction of each eluate, measured at 260 mμ, allowed us to determine the percentage of each compound. The results expressed as adenine, are: 7% at the level of adenosine, 36% at the level of AMP, 14% at the level of ADP, 44% at a level close to ATP. Spraying of the chromatogram with Lucena-Conde & Prat's reagent [8], which gives a blue colour with the hypophosphoric ion (Yoza & Ohashi, [13]), revealed immediately two spots. Their R_F values were close, respectively, to those of ATP and of hypophosphoric acid (which migrates slower than ATP). ATP does give a positive test with this reagent, but only after a long time. So it seems that a new compound, different from ATP and probably containing the hypophosphoric group, was formed in our reaction.

Ascending two-dimensional chromatography. A chromatogram (Schleicher & Schull 2043b paper) was developed in the first direction with isobutyric solvent [6] and in the second direction with a solvent described by Michelson [10]: *tert.*-pentyl alcohol - formic acid - water (3:2:1, by vol). The results are shown in Fig. 2.

The spot migrating in the isobutyric solvent to a level close to ATP, gives in the second solvent a single elongated spot at the level of AMP. We think that this is due to a hydrolysis of the compound which first migrated to the level of ATP,

because of the strong acidic pH of Michelson's solvent. In fact, we noticed that this solvent also hydrolyses ATP and ADP with formation of AMP.

The spot which migrated to the level of AMP in the first solvent, was separated into three spots in the second solvent. They probably are AMP (theoretical R_F 0.35; found 0.30) and diadenosinepyrophosphate (theoretical R_F 0.13; found 0.12). The third compound remained unidentified (R_F 0.06).

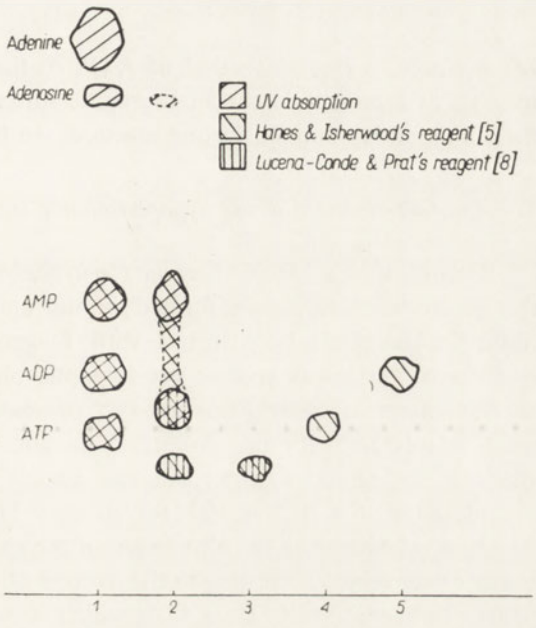


Fig. 1. Ascending one-dimensional chromatography on Schleicher and Schull 2043b paper in the isobutyric acid - water - ammonium hydroxide (66:33:1, by vol.) solvent [6]. (*1*), Adenine, adenosine, AMP, ADP and ATP standard mixture; (*2*), reaction mixture; (*3*), hypophosphate; (*4*), phosphate; (*5*), phosphite.

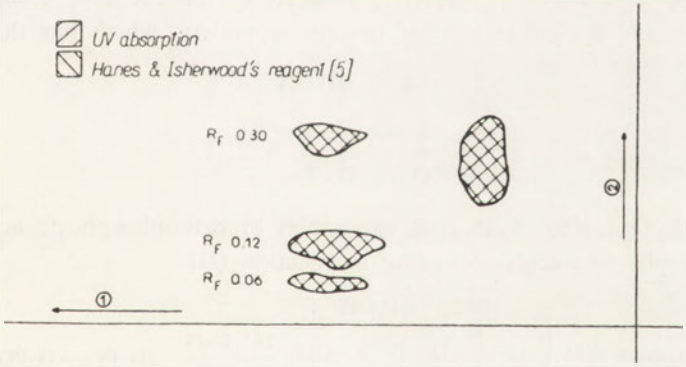
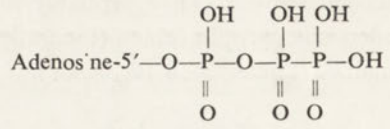


Fig. 2. Ascending two-dimensional chromatography on Schleicher and Schull 2043b paper with the solvents: 1st direction: isobutyric acid - water - ammonium hydroxide (66:33:1, by vol.) [6]; 2nd direction: solvent described by Michelson [10]: *tert.*-pentyl alcohol - formic acid - water (3:2:1, by vol.).

These results show that the action of the hypophosphoric ion on P_1-5′-adenosyl,-P_2-diphenyl pyrophosphate leads to the transformation of about 44% of the AMP (which is the starting material to prepare P_1-adenosyl,P_2-diphenyl pyrophosphate)

into a new compound, somewhat similar to ATP, but containing the hypophosphoric group.

The compound we were trying to synthetize:



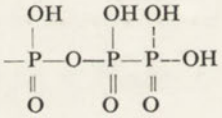
has a structure similar to that of ATP, so that it should migrate to a level close to ATP in some acidic chromatographic solvents. As this was the case for one of the compounds obtained in our reaction, we tried to identify this new compound.

Characterization of the hypophosphoric structure of the adenosyl compound

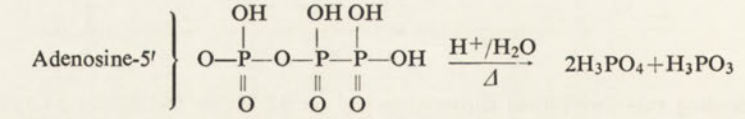
Reaction with a specific reagent for hypophosphate anion. As already mentioned, the nucleotidic compound formed in the anionic displacement reaction gave at room temperature a positive test with Lucena-Conde & Prat's reagent [8], which in these conditions is specific for hypophosphate anion.

Determination of the phosphorus to adenine ratio. After chromatography of the reaction mixture with the isobutyric solvent, the spot corresponding to the new adenosyl compound was cut out and eluted. After concentration, the eluate was hydrolysed by 6 N-HCl at 100° for 30 min. The concentration of adenine was determined by measuring the absorption of the solution at 260 mμ and the phosphorus content determined according to the method of Ames & Dubin [1]. We found a phosphorus to adenine ratio close to 3, which is consistent with the assumed structure.

Studies on the nature of hydrolysis products of the adenosyl compound. The phosphorus chain, on position 5′ of the ribose moiety, which has the structure:



should give by complete hydrolysis two moles of orthophosphoric acid and one mole of phosphorous acid, according to reaction (II).



(II)

The hydrolysis was performed on a chromatographic eluate, under the following conditions: 6 N-HCl, 100°, 2 hr., under nitrogen. The hydrolysate was analysed by paper chromatography (Schleicher & Schull, 2040a) with the alkaline solvent described by Ebel [4]. Two spots were revealed by the method of Hanes & Isherwood [5], which respectively corresponded exactly to phosphorous and phosphoric acids.

Determination of the P_{total} *to* $P_{orthophosphoric}$ *ratio.* An eluate from the isobutyric chromatogram was hydrolysed under the above mentioned conditions. Total phosphorus was determined by the method of Ames & Dubin [1], which involves an oxidation step with magnesium nitrate, and acidic hydrolysis of the polyphosphates formed during the ashing procedure.

The same titration performed without the oxidation step, allowed to determine the orthophosphoric phosphorus.

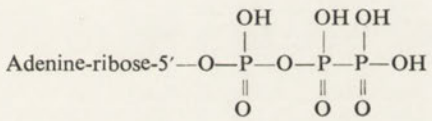
The P_{total} to $P_{orthophosphoric}$ ratio was found to be 1.5, and this again is consistent with the assumed structure.

Characterization of two free hydroxyl groups in 2′ *and* 3′. As the synthesis reaction was performed on AMP and as the *cis*-hydroxyls of its ribose moiety were not protected, it was necessary to check whether these hydroxyl groups were or were not substituted during the synthesis reaction. The isobutyric chromatogram was sprayed with benzidine-periodate reagent [3]. The reaction was immediately positive, which demonstrates that the *cis*-hydroxyls in 2′ and 3′ were not modified.

DISCUSSION

The presented results show that the action of the hypophosphoric anion on P_1-5′-adenosyl,P_2-diphenyl pyrophosphate leads to a new compound, somewhat similar to ATP. However, this compound seems to be different from ATP because it gives an immediate positive test with the hypophosphate reagent and because it leads, after complete hydrolysis, to one mole of phosphorous acid and two moles of phosphoric acid.

These results allow us to infer the formation of adenosine-5′-phosphohypophosphate, which should have the following structure:



The synthesis also leads to formation of diadenosine pyrophosphate.

We are now preparing this compound in larger amounts, in order to study its properties and its behaviour in enzymic reactions usually involving ATP.

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ANALOGI POLIFOSFORANOW NUKLEOZYDOWYCH

SYNTEZA ANALOGU ATP ZAWIERAJĄCEGO HYPOFOSFORAN

Streszczenie

1. Z P_1-5′-adenozylo-P_2-dwufenylopyrofosforanu reakcją wymiany anionów otrzymano związek, który na podstawie analizy chemicznej zidentyfikowano wstępnie jako adenozyno-5′-fosfohypofosforan.

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W. OSTROWSKI, MIROSŁAWA WEBER and JANINA RYBARSKA

IMMUNOCHEMICAL PROPERTIES OF ACID PHOSPHOMONOESTERASE FROM HUMAN PROSTATE GLAND

Department of Physiological Chemistry, Medical School, Kraków

1. A preparation of the enzyme, homogeneous on physico-chemical examination, showed properties of a single antigen and gave with the specific antibody of polyvalent rabbit anti-serum a specific precipitate which retained full enzymic activity toward *p*-nitrophenylphosphate as substrate. 2. The enzyme treated with 6 - 8 M-urea or heated at 55° at pH 7.0, lost irreversibly the enzymic activity as well as the ability to react with antibody. 3. Heat denaturation at pH 5.0, treatment with iodine or 0.2 M-2-mercaptoethanol inactivated partially or completely the enzymic activity without affecting the immunochemical reactivity.

The immunochemical method is one of the techniques used for studying homogeneity and structure of proteins. Little is known so far about the participation of individual chemical groupings in the antigenic specificity of proteins, nevertheless on the basis of the obtained data it seems reasonable to suppose that this activity is due not only to the primary structure but also to the internal structure and conformation of the antigen molecule [1, 22, 7, 13, 18]. In this paper are presented general antigenic properties of acid phosphomonoesterase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2.) obtained in this laboratory from human prostate in a highly purified state [16, 15]. The enzyme reacted with the specific antibody of rabbit serum to give an insoluble complex which was active toward *p*-nitrophenylphosphate. The effect of urea treatment, heat denaturation at different pH values and iodine monochloride or 2-mercaptoethanol treatment on immunochemical reactivity of the enzyme are also presented.

MATERIALS AND METHODS

Enzyme preparation. A highly purified preparation of acid phosphomonoesterase obtained from human prostate as described previously [16, 15] was used.

Determination of enzymic activity. This was done as described previously [16] with *p*-nitrophenylphosphate as substrate (Sigma 104, Biochem. Corp., St. Louis, U.S.A.). The liberated *p*-nitrophenol was assayed in a Uvispec spectrophotometer

(Hilger & Watts, London) at 400 mμ and determined with the reference to a standard curve prepared with analytical grade *p*-nitrophenol. The activity was expressed as μmoles of the substrate split off during 1 min. at 25° by 1 ml. of the solution.

Rabbit antiserum. For immunization cross-bred rabbits, females, about 18 months old and weighing about 2 kg., were used. In preliminary experiments it was found that the serum obtained after immunization with a purified preparation of acid phosphomonoesterase from human prostate, showed very low precipitin titers, as tested by the capillary method, and had no effect on enzymic activity. Therefore a partly purified enzyme preparation, designated as fraction F-II in a previous paper [16], was used as antigen. The enzyme preparation contained 4 - 5 mg. of protein in 1 ml. of physiological saline and had an activity of 0.1 m-mole per 1 mg. per min. at 25°. One ml. of this solution was mixed with 1 ml. of Freund's adjuvant [2] and injected intramuscularly twice a week for three consecutive weeks. Simultaneously with the first injection, a single dose of 10 μg. of *E. coli* endotoxin [20] (a purified lipopolysaccharide obtained from the Laboratory of Sera and Vaccines, Kraków, Poland) was applied intravenously. After a 10-day rest period the second series of injections was applied: 10 mg. of the antigen with 1 ml. of Freund adjuvant once a week for three weeks, and again 10 μg. of the endotoxin of *E. coli* simultaneously with the first injection. A week after the last injection, blood was withdrawn by heart puncture and the precipitin reaction was carried out by the ring test of Uhlenhut [8] or by the double-diffusion technique in agar gel according to Ouchterlony [17].

Immunoelectrophoresis. This was carried out on glass plates (76 × 25 mm.) covered with a layer 1 - 1.5 mm. thick of 1% agar (Bacto agar, Difco, Detroit, U.S.A.) or agarose gel (Agarose, I.B.F., Gennevillieres, France) in 0.025 M-veronal buffer, pH 8.2. The electrode vessels were filled with 0.05 M-veronal buffer, pH 8.2. Into a well made in the gel near the cathodic end, 2 - 5 μl. of 0.3% antigen solution was introduced and the separation was carried out for 75 - 90 min. in agar gel, and 60 min. in agarose; a voltage gradient of 6 v/cm. and a current of 60 - 65 mA were applied.

When the electrophoretic separation was completed, undiluted antiserum was introduced into a groove cut in the gel and the plates were left in a moist chamber at room temp. for 24 - 48 hr. After the appearance of the precipitation arcs, the plates were soaked in 0.9% NaCl solution for two days, then washed with distilled water, air-dried, stained with Amido Black 10B solution [21] and, after washing out the excess of the dye with 2% acetic acid, air-dried and then photographed.

Starch-gel electrophoresis. This was performed according to Smithies [19] as described previously [16].

RESULTS

The homogeneity of the purified acid phosphomonoesterase was judged by chromatography, by free electrophoresis at pH 5 and 7, by starch-gel electrophoresis at pH 4.5 and 8.5, by ultracentrifugation in sucrose density gradient and by immuno-

electrophoresis. A comparison of migration of the enzyme and human blood serum albumin on starch gel is shown in Fig. 1. Acid phosphomonoesterase migrated as a homogeneous band with the mobility corresponding to that of α_2 or β_1-globulins

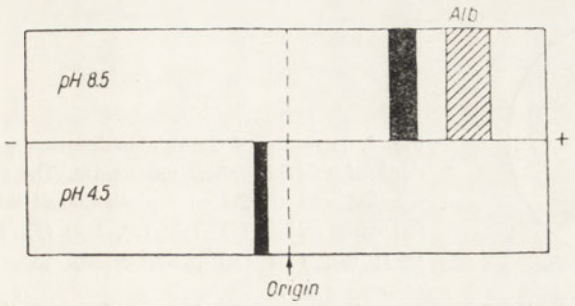

Fig. 1. Electrophoresis of acid phosphomonoesterase on starch gel in 0.03 M-phosphate buffer, pH 8.5, and in 0.05 M-acetate buffer, pH 4.5. *Alb*, human blood serum albumin (for comparison). Conditions: 10 v/cm., 6 mA, 6 hr., room temp.

of human serum. The result of immunoelectrophoresis is shown in Fig. 2. The precipitation arcs were regular and symmetric, which indicated a single antigen-antibody complex. The same results were obtained both when the serum was twofold diluted and when the time of electrophoresis was markedly prolonged. From Fig. 2 it can also be seen that the enzyme migrated more rapidly in the agarose than in the agar gel, the result of immunoelectrophoresis being in either case the same.

To check further the homogeneity of the enzyme preparation and to see whether it reacts only with one specific antibody of a polyvalent antiserum, the double-diffusion test on a Petri dish according to Ouchterlony [17] was applied. Six dilutions of the enzyme solution were used and one precipitation arc, independently of dilution and time of diffusion, was always obtained (Fig. 3).

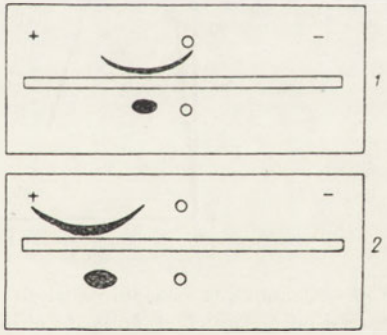

Fig. 2. Immunoelectrophoresis and electrophoresis of acid phosphomonoesterase: (*1*), on agar, and (*2*), on agarose, under the same conditions: 6 v/cm., 65 mA, 75 min.

Quantitative precipitin analysis. This was done according to a commonly used procedure [7]. To 0.2 ml. of antiserum were added increasing amounts of acid phosphomonoesterase in 0.1 M-phosphate buffer, pH 7, and 0.9% NaCl solution to a final volume of 1 ml., and left for 1 hr. at 37° and for further 72 hr. at 2°. After centrifuging, the precipitate was washed three times with 0.5 ml. portions of 0.9% NaCl solution, dissolved in 0.2 ml. of 0.01 N-NaOH and the amount of protein

was determined by the method of Lowry *et al.* [9]. A precipitin curve was obtained over the range from excess antibody to excess antigen. From Fig. 4 it can be seen that the point of equivalence of the reaction between acid phosphomonoesterase

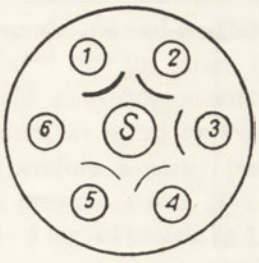

Fig. 3. Diagram of the double-diffusion test at different dilutions of acid phosphomonoesterase. The initial 0.3% enzyme solution was diluted with physiological saline solution in the following ratios: (*1*), 1:2; (*2*), 1:4; (*3*), 1:8; (*4*), 1:16; (*5*), 1:32; (*6*), 1:64 (no precipitation). (*S*), Antiserum.

and the specific antibody present in the polyvalent antiserum, i.e. when in the solution neither antigen nor antibody could be found, corresponded to antigen concentration of 30 μg./ml. At a concentration of 150 μg./ml. the precipitation was inhibited by about 70%. The antibody/antigen ratio in relationship to antigen

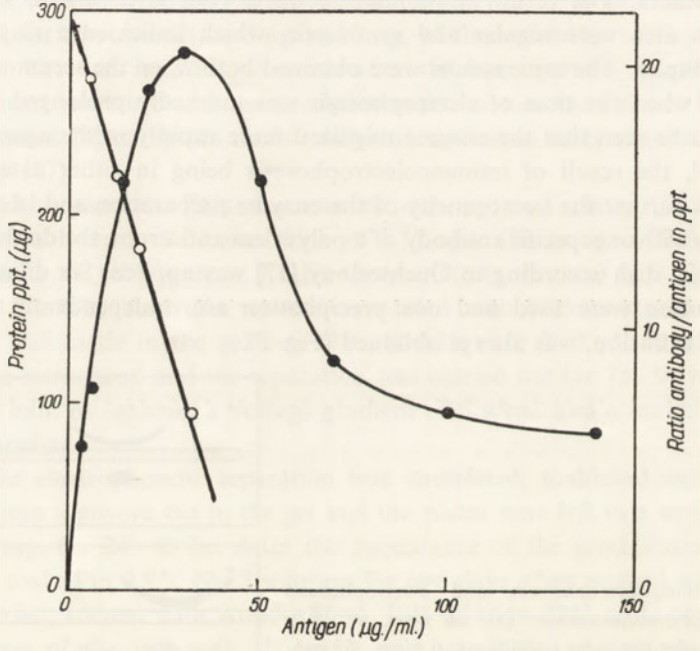

Fig. 4. Quantitative precipitin analysis of acid phosphomonoesterase with rabbit antiserum. (●), Protein precipitate; (○), antibody nitrogen to antigen nitrogen ratio in the specific precipitate.

concentration gave a straight line which, when extrapolated to the equivalence point, gave the value of R [5] equal to 8. Almost hundred percent inhibition of enzyme activity in the supernatant was observed at the equivalence point. This problem is discussed in detail in the next section.

The effect of antibody on enzyme activity. To a constant volume of the enzyme (10 μl. containing 15 μg. of protein) were added increasing amounts of antiserum;

the samples were made up to 0.5 ml. with 0.9% NaCl in 0.1 M-phosphate buffer, pH 7, and incubated for 2 hr. at 18° and then for 2.5 hr. at 2°. Control samples contained normal, non-immunized rabbit serum. The precipitate was centrifuged off in the cold and the enzyme activity was determined in the supernatant. The results presented in Fig. 5 showed that a sharp drop of activity appeared at a certain

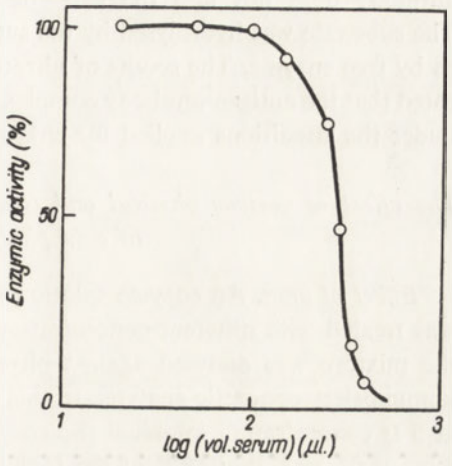

Fig. 5. The effect of the amount of antiserum on the activity of acid phosphomonoesterase in the supernatant.

concentration of the antibody, and the activity in the supernatant was completely lost when, at the equivalence point, the ratio of antibody to antigen, R, was equal to 8.

In order to check the enzymic activity of the specific precipitate, 30 µg. of the enzyme was incubated with 200 µl. of antiserum in 0.1 M-phosphate buffer, pH 7.0, containing 0.9% NaCl, made up to a final volume of 0.58 ml. The incubation was carried out at 37° for 60 min., then for 60 hr. at 2°. In the control sample a non-immunized rabbit serum was present. In the proper sample, the precipitated sediment was centrifuged in the cold, washed four times with physiological saline, pH 7.0, and suspended in 0.1 M-citrate buffer, pH 5.0. The enzyme activity was determined in the supernatant, in the pooled washings and in the sediment. The incubation was carried out at 37° for 2 min. in the presence of 0.02 M-*p*-nitrophenylphosphate as substrate. The activity of the enzyme was recovered in the sediment and in the washings (about 11%), the sum of the two values being slightly greater than that of the activity found after incubation of the enzyme with normal rabbit serum. When the sediment was washed with 0.1 M-citrate buffer, pH 5.0, less than 2% of the activity passed to the solution, the remaining activity being present in the precipitate.

To test whether the active antigen-antibody complex becomes dissolved in the presence of an excess of antigen or during incubation in 0.1 M-citrate buffer, pH 5.0, appropriate solutions were submitted to ultracentrifugation in a sucrose gradient according to Martin & Ames [10] and to gel filtration on a Sephadex G-100 column. In the sample with antigen excess, only free enzyme, having a molecular weight of about 100 000 [15] was present, and no compound of greater molecular weight

corresponding to the enzyme-antibody complex was found. During the extraction of the specific precipitate at pH 5.0, only very small amounts of the enzyme, consisting probably of molecules more strongly adsorbed on the specific precipitate, passed to the solution.

From these data it follows that acid phosphomonoesterase bound to the specific antibody does not lose the catalytic activity towards *p*-nitrophenylphosphate. The substrate was hydrolysed by the suspension of the insoluble complex as rapidly as by free enzyme. The results of ultracentrifugation and dextran gel filtration indicated that the antigen-antibody complex does not undergo dissociation or dissolution under the conditions applied for enzyme assay.

The effect of various physical and chemical factors on immunochemical reactivity of acid phosphomonoesterase

Effect of urea. An enzyme solution containing about 1.5 mg. protein per 1 ml., was treated with different concentrations of urea at pH 7 and 20°, for 1 hr. Then the mixture was dialysed against physiological saline solution and submitted to immunoelectrophoretic analysis. In this study it has been observed that urea already at 5 M concentration inhibited the enzyme activity by about 90% within about 15 min. at 0°, and only 15% of the inhibited activity was recovered after removal of

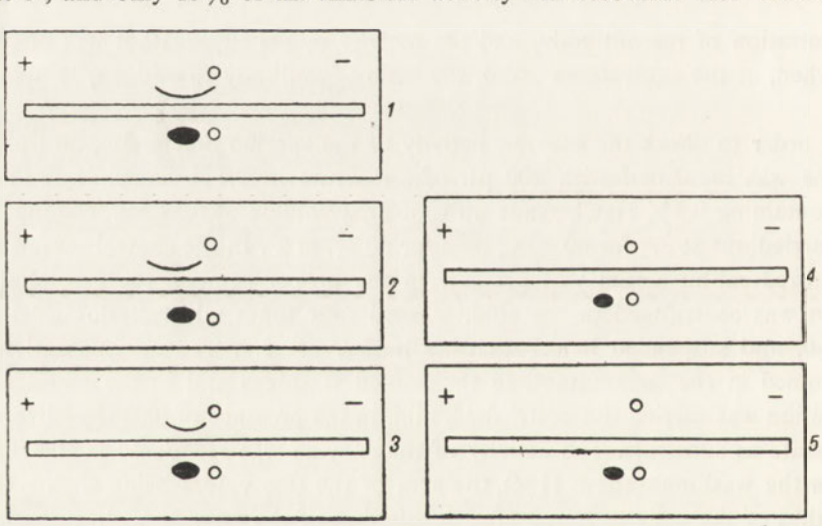

Fig. 6. Immunoelectrophoresis and electrophoresis on agar gel of acid phosphomonoesterase after denaturation with urea at different concentrations. (*1*), Native enzyme. After treatment with: (*2*), 2 M-urea; (*3*), 4 M-urea; (*4*), 6 M-urea; (*5*), 8 M-urea.

the urea by dialysis. At higher concentrations of urea the inactivation at 0° was complete within 15 min., and dialysis had no effect on the restoration of enzymic activity. Also, the immunoreactivity was affected by urea at concentrations exceeding 2 M (Fig. 6). In the presence of 4 M-urea the reaction was somewhat weaker, the precipitation arc being indistinct, whereas after treatment with 6 M or 8 M-urea the enzyme did not react at all with the antibody.

Effect of heat denaturation. Acid phosphomonoesterase lost irreversibly the enzymic activity within about 1 min. at 55° and pH 7.0, whereas heating at 55° but at lower pH values had little effect on biological activity of the enzyme. An enzyme solution containing 1.5 mg. protein/ml. was heated at 55° in 0.05 M-sodium phosphate buffer, pH 7.0, or in 0.05 M-acetate buffer, pH 5.0. At different time intervals, samples of the enzyme were taken and immediately cooled to 0°; then the enzyme activity and the reactivity towards the specific antibody were determined. From the results presented in Fig. 7 it appeared that the enzyme in anionic form

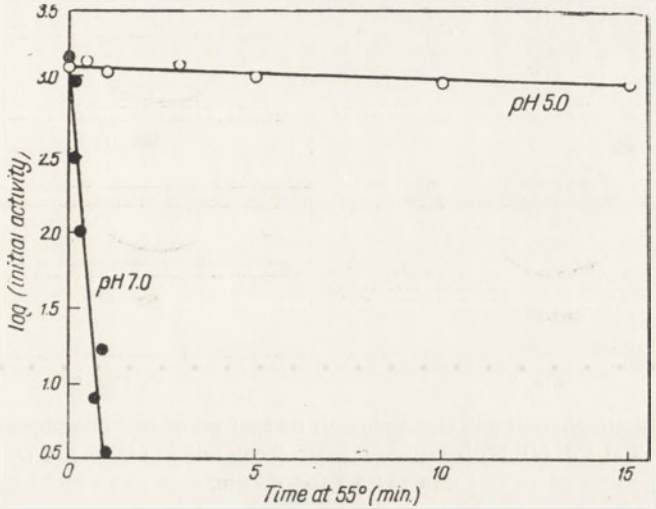

Fig. 7. Changes in the enzymic activity of acid phosphomonoesterase after denaturation by heating at 55° at: (○), pH 5.0, and (●), pH 7.0.

underwent denaturation at 55° already within a few seconds, whereas at a pH value close to the isoelectric point [14] heating for 1 hr. caused only a 10% decrease in activity. This observation was confirmed also by immunoelectrophoresis (Fig. 8); the enzyme heated at pH 7 for 1 min. was unable to react with the antibody and migrated much more rapidly toward the anode, whereas heating for 15 min. at pH 5.0 did not affect the ability to form the precipitation arc and did not alter the electrophoretic mobility of the protein.

The effect of iodine monochloride. In our studies (K. Bobrzecka, W. Ostrowski & J. Rybarska, in preparation) on the role of aromatic amino acids on the enzymic activity of acid phosphomonoesterase it has been found that treatment with iodine monochloride over the pH range 4 - 8 caused complete inactivation of the enzyme at 8×10^{-5} M-ICl concentration. The inactivation was completed within 2 - 3 min. and appeared to be irreversible as no recovery of enzyme activity was observed after removal of excess ICl by dialysis or filtration on Sephadex G-25. On the other hand, the iodination of the enzyme had no effect on the electrophoretic mobility and on the ability to precipitate with antibody (Fig. 9).

Effect of 2-*mercaptoethanol.* On the basis of sulphitolysis of acid phosphomonoesterase denatured by urea, and amperometric titration of liberated thiol groups,

it was demonstrated [3] that one enzyme molecule contains 10 disulphide bridges. Reduction of the enzyme with 2-mercaptoethanol and alkylation with iodoacetate resulted only in partial inactivation, a 50% decrease in activity being obtained by

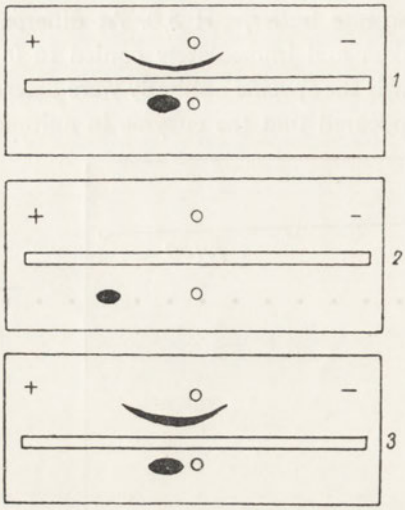

Fig. 8

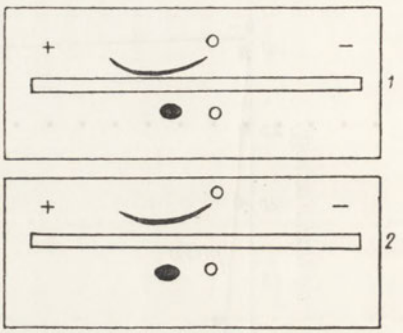

Fig. 9

Fig. 8. Immunoelectrophoresis and electrophoresis on agar gel of acid phosphomonoesterase after heat denaturation at 55°. (*1*), Native enzyme. After denaturation: (*2*), at pH 7.0 for 60 sec.; (*3*), at pH 5.0 for 15 min.

Fig. 9. Immunoelectrophoresis and electrophoresis on agar gel of acid phosphomonoesterase inhibited by 8×10^{-5} M-ICl. (*1*), Native enzyme; (*2*), ICl-treated enzyme.

treatment with 0.2 M-mercaptoethanol. To test the effect of reduction on immunochemical properties of the enzyme, a solution of acid phosphomonoesterase containing 1.5 mg. protein/ml. in tris-HCl buffer, pH 8.0, was treated with 0.02, 0.04 and 0.2 M-mercaptoethanol. After 60 min. at 37°, the mixture was dialysed overnight in a cold-room against physiological saline solution containing 0.01 M-iodoacetate

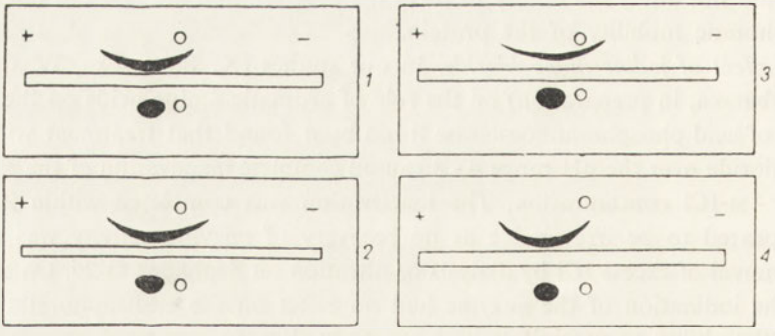

Fig. 10. Immunoelectrophoresis and electrophoresis on agar gel of acid phosphomonoesterase treated with 2-mercaptoethanol. (*1*), Native enzyme. After treatment with: (*2*), 0.02 M; (*3*), 0.04 M; and (*4*), 0.2 M-mercaptoethanol.

and submitted to immunoelectrophoresis. From Fig. 10 it can be seen that treatment with different concentrations of 2-mercaptoethanol did not alter the antigenic properties of the enzyme, nor did it affect its electrophoretic mobility.

DISCUSSION

To obtain a high titer of specific antiserum, it was necessary to immunize the rabbit with a partially purified preparation of acid phosphomonoesterase from human prostate. The immunization with a highly purified enzyme was much less effective. According to the method of Johnson *et al.* [6] and McKenna & Stevens [11], simultaneously with the first antigen injection, *E. coli* endotoxin was administered and a polyvalent serum was obtained. It has been established that the endotoxins from gram-negative bacteria increase markedly the content of serum antibodies. The endotoxins stimulate the antibody-producing cells [20] but their role has not been elucidated.

For the experiments *in vitro*, the highly purified homogeneous enzyme preparation was used. The course of quantitative precipitation of the enzyme with the antibody and determination of the enzymic activity in systems with excess antibody, excess antigen and at equivalence, indicated that the reaction occurred between acid phosphomonoesterase and specific antibody. The relationship of the antibody/antigen ratio to antigen concentration was linear [5] and the antibody/antigen ratio at the equivalence point amounted to 8, which indicates that more than one molecule of antibody reacted with one molecule of antigen [4].

The enzymic activity of the insoluble antigen-antibody complex at pH 7 and pH 5 indicates that the substrate-binding site of the enzyme is different from the antibody-binding site. This assumption is supported by the results of iodine and 2-mercaptoethanol treatment, urea denaturation and heat denaturation. Iodine and mercaptoethanol, although they inhibited the enzymic activity, did not affect the ability to react with antibody. On the other hand, general disorganization of the molecule by urea treatment or heating at pH 7 inhibited both the enzymic activity and the immunoreactivity. It seems interesting that treatment of the enzyme with mercaptoethanol did not abolish the reaction with antibody, and in this respect acid phosphomonoesterase behaved in a way similar to that of egg albumin, in which splitting of disulphide bridges does not alter the immunological properties of the protein [12].

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IMMUNOCHEMICZNE WŁASNOŚCI KWAŚNEJ FOSFOMONOESTERAZY STERCZU LUDZKIEGO

Streszczenie

1. Otrzymany preparat enzymu, jednorodny pod względem fizyko-chemicznym, wykazuje własności pojedynczego antygenu i daje ze swoistym przeciwciałem poliwalentnej antysurowicy królika specyficzny osad, który w dalszym ciągu zachowuje pełną aktywność enzymatyczną przy użyciu *p*-nitrofenylofosforanu jako substratu.

2. Enzym poddany działaniu 6 - 8 M-mocznika oraz ogrzewaniu w temperaturze 55° przy pH 7.0 traci nieodwracalnie aktywność enzymatyczną, jak również zdolność reagowania z przeciwciałem.

3. Denaturacja cieplna przy pH 5.0, jodowanie oraz działanie 0.2 M-2-merkaptoetanolem powodują częściową lub całkowitą inaktywację enzymu, nie wpływając na jego immunochemiczną reaktywność.

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J. ILAN* and F. LIPMANN

A CELL-FREE PROTEIN SYNTHESIS SYSTEM FROM PUPAE OF *TENEBRIO MOLITOR*

The Rockefeller University, New York, N. Y. 10021, U.S.A.

1. A cell-free system for the study of amino acid incorporation was prepared from *Tenebrio molitor* pupae. Inhibition of blackening due to phenol oxidase using phenylthiourea was found essential to obtain a viable system. From microsomes, a ribosomal fraction was prepared by washing with deoxycholate buffer and high salt. The ribosomal preparation was dependent on addition of supernatant to polymerize amino acids from amino acyl sRNA; insect supernatant and rat liver supernatant were equally active. Crude *E. coli* supernatant stimulated somewhat, but lost activity on purification. 2. The tyrosine content of cuticular protein is unusually high; this protein appears at the end of metamorphosis. Therefore, relative rates for tyrosine incorporation were determined with cell-free preparations from various stages of metamorphosis. The tyrosine:leucine ratio of incorporation rises steeply to 10-fold during the last two days; this might be attributed to completion at that time of templates for the synthesis of cuticular protein.

Systems of insect tissues which are active in protein synthesis have been isolated from *Bombyx* larvae (Suzuka, Tanaka & Shimura, 1962), and *Drosophila* adults (Takayama, Ilo & Miura, 1958). A rather extensive study was published recently by Fox, Kan, Kang & Wallis (1965) using preparations from *Drosophila melanogaster* adults.

The present study was undertaken to explore the possibility of following metamorphosis by testing cell-free protein synthesis. During metamorphosis in the course of pupation the activities of many enzymes change. Some enzyme activities connected with the respiratory chain and with glycolysis drop to low levels and reappear before adult emergence (Gilmour, 1961). Shappirio & Williams (1957) found that cytochrome *b* and *c* disappear in *Cecropia* pupae, to reappear on initiation of adult development. Pronounced changes are observed in partial reactions taking part in cuticle formation. Adult cuticular protein, which appears at the end of pupation, differs in its amino acid spectrum from that of larvae and pupae. In the metamorphosis of *Prodenia eridania* (Jaworski, Wang & Marco, 1963), chitin synthetase was found to reach peak levels in the final larval stage.

* Present address: Department of Biology, Temple University Philadelphia, Pa. 19122.

With *Tenebrio molitor*, Ilan, Ilan & Quastel (1966) observed that actinomycin D injected into pupae prevents chitin formation in the abdominal region only, indicating a regional difference in predevelopmental messenger RNA maturation. *Tenebrio molitor* larvae are easily available in large quantities in this country. The following study deals with the characteristics of cell-free protein synthesis in preparations of this useful organism.

We are happy to dedicate this contribution with our best wishes to Dr. Joseph Heller on his anniversary. His pioneering work on insect metabolism was instrumental in opening up this fascinating field.

METHODS AND MATERIALS

Preparations of insect microsomes and 105 000 g *extract. Tenebrio* larvae were obtained from a local biological supply house and handled as previously described (Ilan *et al.*, 1966). Pupae were incubated at 28°, at which temperature pupation lasts 7 days. For homogenization, 100 g. of pupae were suspended in 250 ml. of a solution (buffer *A*) containing 0.01 M-$MgCl_2$, 0.025 M-KCl, 0.035 M-tris pH 7.6, 0.25 M-sucrose, 0.004 M-GSH, and 0.005 M-phenylthiourea to inhibit the interfering phenol oxidase. The suspension was kept for 40 sec. at 4° in a Sorvall Omni-Mixer at speed control 5. The homogenate was filtered through four layers of cheesecloth, and centrifuged for 5 min. at 3000 *g*. A lipid layer was removed by suction, and the supernatant fraction collected and centrifuged for 20 min. at 20 000 *g*; the upper two thirds of the supernatant fraction was centrifuged at 105 000 *g* for 2 hr., yielding a microsomal pellet. The supernatant fluid was passed through a column of Sephadex G-25; it represents the 105 000 *g* fraction used. Rabbit liver supernatant was prepared analogously.

The microsomal pellet was resuspended in 10 ml. of buffer *A* by gentle manual homogenization; it was then centrifuged for 2 hr. at 105 000 *g*, and the washing with buffer *A* was repeated four times. Microsomes were kept as a pellet at -20° for at least 3 months without loss of activity, and were resuspended in 1 ml. of buffer *A* before use.

Ribosomes were prepared by resuspension of the microsomes in buffer *A* containing 0.5 M-KCl. After centrifugation at 105 000 *g* for 2 hr., the supernatant fraction was removed and dialysed against 0.01 M-tris, pH 7.6, containing 5 mM-phenylthiourea and 4 mM-β-mercaptoethanol. This fraction was used as supernatant complement for polymerization. The microsomal pellet was resuspended in buffer *A* containing 1% deoxycholate, and was layered on 5 ml. of 1 M-sucrose containing 0.01 M-tris, pH 7.6, 0.5 M-KCl, 0.01 M-$MgCl_2$, 0.004 M-β-mercaptoethanol, and 0.005 M-phenylthiourea (buffer *B*). After centrifugation for 2 hr. at 105 000 *g*, the pellet was resuspended in buffer *B*, less sucrose, and centrifuged again for 2 hr. The pellet was then resuspended in a small volume of buffer *B* and quickly frozen. When phenylthiourea is excluded from the preparation, an otherwise appreciable endogenous incorporation is destroyed, but these ribosomes are stimulated by the addition of poly U + phenylalanine.

[^{14}C]Leucyl sRNA, charged with 19 [^{12}C]amino acids, and [^{14}C]phenylalanyl sRNA were prepared from *E. coli* sRNA according to Conway (1964).

Amino acid incorporation. The incubation mixture for microsomes contained: 2 mg. microsomal RNA/ml. of insect microsomes; 5×10^{-4} M-GTP; 8×10^{-3} M-GSH; 2×10^{-3} M-ATP; 8×10^{-3} M-phosphoenolpyruvate; 40 μg./ml. pyruvate kinase; 0.04 M-KCl; 0.035 M-tris, pH 7.6; 0.006 M-$MgCl_2$; 6×10^{-5} M 19 [^{12}C]amino acids; 0.2 μc of [^{14}C]leucine (222 μc/μmole) or 0.2 μc of [^{14}C]tyrosine (375 μc/μmole); and 1×10^{-3} M-phenylthiourea. The total volume was 0.25 ml. and the temperature 30°; for ribosomes, the KCl concentration was changed to the optimal value of 0.12 M. The concentrations of amino acyl sRNA are indicated in the legends of Tables 2 and 3. The incorporation was nearly proportional to the amount of microsomal RNA up to 0.5 mg./0.25 ml. Although in the experiments described the actual concentration of ribosomes per 0.25 ml. was varied as indicated in the legends, to equalize the data they were adjusted to incorporation for 1 mg. of ribosomal RNA.

Determination of amino acid incorporation into protein. The reaction was stopped by the addition of 5 ml. of 5% trichloroacetic acid and the suspension heated for 20 min. at 90°. Samples were passed through Millipore filters with an average pore size of 0.45 μ, which were then glued onto planchets, dried, and counted in a thin-window, gas-flow Nuclear-Chicago counter (22% efficiency).

L-[^{14}C]Leucine, L-[^{14}C]tyrosine, and L-[^{14}C]phenylalanine were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. RNase, 3 × crystallized, was a product of Worthington Biochemical Corp., Freehold, N.J., U.S.A.; Sephadex G-25 was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J., U.S.A. Poly U was obtained from Miles Chemical Co., Elkhart, Ind., U.S.A., and *E. coli* B sRNA from General Biochemicals, Chagrin Falls, Ohio, U.S.A.

RESULTS

The Tenebrio *microsomal system.* When the complete reaction mixture described under Methods was incubated at 30° (Fig. 1), incorporation of [^{14}C]leucine was relatively rapid and linear for 10 min., continuing then at a steadily decreasing rate. The usual dependence on Mg^{2+}, ATP, and GTP is shown in Table 1. Addition of the amino acid mixture did not increase incorporation. Insect supernatant does not stimulate, but *E. coli* or rabbit liver supernatant fractions do stimulate the system about 5-fold; as will appear from experiments with amino acyl sRNAs, this effect appears to be due mainly to the supply of sRNA and activating enzymes. RNase abolishes incorporation. When phenylthiourea was omitted from the medium during the preparation of microsomes, the blackish extract thus obtained proved to be inactive (Table 1). Previously, phenylthiourea had been used extensively in many biological dissections of insects to prevent blackening by inhibition of phenol oxidases, and lately it has been used by Brookes & Williams (1965) in the extraction of thymidylate kinase from insect material.

When activated amino acids, i.e. amino acyl sRNAs, with [^{14}C]leucine as the marker, were used with the microsomal system, the stimulation by *E. coli* or liver supernatant disappeared, presumably because the microsomes were saturated with

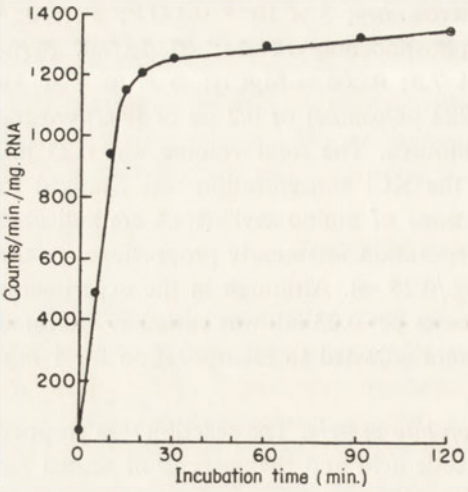

Fig. 1. Time curve of [^{14}C]leucine incorporation into insect microsomes. Experimental conditions are described in Methods.

Table 1

Incorporation of [^{14}C]*leucine into polypeptide on insect microsomes*

Incubations were carried out for 30 min. at 30° in a final vol. of 0.25 ml. Experimental conditions were as described in Methods. The concentration of supernatant, when added, was 1.6 mg. protein/ml. The complete system contained 0.5 mg. microsomal RNA/0.25 ml. of incubation medium.

Conditions	Incorporation (counts/min./mg. RNA)
Complete system	1420
GTP omitted	50
amino acids omitted	1400
insect supernatant added	1450
rabbit liver supernatant added	5120
rabbit liver supernatant added, phenylthiourea omitted	150

supernatant factors for polymerization from activated amino acids (Table 2). Characteristically, puromycin completely inhibited incorporation.

The ribosomal system. With insect ribosomes obtained by treating microsomes as described under Methods, the polymerization became fully dependent on addition of supernatant (Table 3). In these experiments the polymerization from phenylalanyl sRNA on poly U-charged ribosomes was assayed, and they responded very well. Insect supernatant as well as liver supernatant activated in this system. A small, reproducible activation was also observed using crude *E. coli* supernatant, but the purified transfer fractions T and G (Nishizuka & Lipmann, 1966), kindly supplied by Dr. J. Gordon in this laboratory, failed to stimulate.

Table 2

Polymerization of leucine from [^{14}C]*leucyl sRNA on insect microsomes*

Incubation was carried out for 30 min. at 30° in a final vol. of 0.25 ml. Each tube contained 0.5 mg. of microsomal RNA and 0.1 mg. of *E. coli* sRNA charged with 19 [^{12}C]amino acids and [^{14}C] leucine (1700 counts/min./tube).

Additions	Counts/min./mg. microsomal RNA
None	536
Insect supernatant	558
Liver supernatant	526
Puromycin, 2×10^{-4} M	58

Table 3

Effect of various supernatant fractions on polymerization of [^{14}C]*phenylalanyl sRNA on insect and liver ribosomes + poly U*

Incubations were carried out for 10 min. at 30° in a final vol. of 0.25 ml. containing 32.5 μg. of poly U, 0.1 mg. of *E. coli* [^{14}C]phenylalanyl sRNA (2000 counts/min.), and ribosomes at a concentration of 0.2 mg. RNA/0.25 ml. Insect ribosomes were prepared without phenylthiourea in order to destroy endogenous template activity. Insect supernatant was used at a concentration of 1 mg. protein/ml. *E. coli* supernatant and liver supernatant were used at a concentration of 1.6 mg. protein/ml.

Supernatant fraction added	Counts/min./mg. RNA	
	Insect	Liver
None	175	215
Insect	1580	2055
Liver	1550	1240
E. coli	435	100

Table 4

Ratio of [^{14}C]*tyrosine*:[^{14}C]*leucine incorporated into* Tenebrio *microsomes during different days of pupation*

Incubations were carried out for 8 min. at 30° in a final vol. of 0.25 ml. Experimental conditions are described in Methods. Microsomes were used at a concentration of 0.5 mg. RNA/0.25 ml. Rabbit liver supernatant was added at a concentration of 1.6 mg. protein/ml. of incubation medium.

Day of pupation	Exp. no.	Microsomes (counts/min./mg. RNA)					
		No supernatant added			Liver supernatant added		
		Leu	Tyr	Tyr:Leu	Leu	Tyr	Tyr:Leu
1	1	1282	252	0.19	6788	1240	0.18
	2	1382	238	0.17	7250	1190	0.16
3	1	908	126	0.14	4064	620	0.15
	2	770	114	0.16	3700	608	0.16
5	1	1040	492	0.47	4832	2500	0.52
	2	860	476	0.55	3602	2462	0.68
7	1	254	660	2.6	990	2250	2.3
	2	358	780	2.2	860	2426	2.7

Change of ratio of [^{14}C]*tyrosine to* [^{14}C]*leucine incorporation during pupation.* Adult cuticular protein formed in the last days of pupation is known to contain an unusually high amount of tyrosine (Hackman & Goldberg, 1958). As an approximation to testing for a specific change in protein pattern during metamorphosis, it was decided to determine during this period the relative rates of incorporation of tyrosine and an evenly distributed amino acid such as leucine. Microsomes from different days of pupation were isolated and incubated with [^{14}C]leucine or [^{14}C]-tyrosine, and the ratio of tyrosine:leucine was measured (Table 4). As can be seen from the Table, the rate of [^{14}C]leucine incorporation is nearly unchanged during the first five of seven days of pupation, but diminishes during the last two days. On the other hand, the rate of tyrosine incorporation into protein is low at the beginning of pupation and increases towards the end. Therefore, the ratio of tyrosine: leucine increases 10-fold during pupation. Addition of rabbit liver supernatant, which stimulates the incorporation 5-fold throughout, does not significantly affect the ratio.

DISCUSSION

The properties of the *Tenebrio* microsomal cell-free system described here do not show unusual features, except for an inhibition due to the presence in insect tissues of a very potent phenol oxidase which oxidizes endogenous phenols and causes blackening. Only by addition of phenylthiourea, an apparently innocuous inhibitor of phenol oxidase, is it possible to obtain a viable system.

Rabbit liver or *E. coli*, but not insect, supernatant fractions stimulate a crude homogenate 5-fold. This stimulation is largely caused by an increased supply of amino acyl sRNA because, when amino acyl sRNA is used with the microsomes, the stimulation disappears, therefore the microsomes seem to be saturated with transfer factors. However, if deoxycholate-washed microsomes, i.e. ribosomes, are used, the polymerization factors are essential. Here, the liver and insect systems showed interchangeability; rat liver supernatant supplements insect ribosomes and insect supernatant supplements liver ribosomes.

Microsomes from silk glands of *Bombyx* (Suzuka *et al.*, 1962) have been reported to incorporate [^{14}C]amino acids in ratios resembling the amino acid composition of silk protein, and phenol-extracted RNA from silk gland tested on *E. coli* ribosomes (Szafrański, Lutowicz & Pużyńska, 1963) showed that the amino acids incorporated reflect the amino acid composition of the silk protein, sericin. In the experiments reported here, an increased rate of tyrosine incorporation was found with microsomes of pupae towards the end of metamorphosis. Such tests were undertaken because the cuticular protein that is formed at that stage contains much tyrosine. The rise in tyrosine incorporation at the period of cuticle formation may thus reflect the emergence of a messenger RNA expected to appear at this time in metamorphosis.

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SYNTEZA BIAŁKA W BEZKOMÓRKOWYM UKŁADZIE OTRZYMANYM Z POCZWARKI *TENEBRIO MOLITOR*

Streszczenie

1. Z poczwarki *Tenebrio molitor* przygotowano bezkomórkowy układ do badań nad włączaniem aminokwasów. Stwierdzono, że dla otrzymania aktywnego układu niezbędne jest dodanie fenylotiomocznika w celu zahamowania oksydazy fenolowej katalizującej powstawanie ciemnego zabarwienia. Frakcję rybosomalną otrzymano z mikrosomów przez przemycie buforem dezoksycholanowym i roztworem soli o dużej sile jonowej. Polimeryzacja aminokwasów z aminoacylo-sRNA przez preparat rybosomalny zależna była od dodania supernatantu; supernatant z owadów i supernatant z wątroby szczura posiadały jednakową aktywność. Surowy supernatant z *E. coli* stymulował tylko w niewielkim stopniu włączanie aminokwasów, ale tracił aktywność w czasie oczyszczania.

2. Zawartość tyrozyny w białku kutikularnym jest niezwykle wysoka i białko to pojawia się przy końcu metamorfozy. Z tego względu badano włączanie tyrozyny przez bezkomórkowe układy otrzymane z poczwarek znajdujących się w różnych stadiach metamorfozy. Stosunek włączonej tyrozyny do leucyny wzrasta gwałtownie do 10-krotnej wartości podczas dwóch ostatnich dni, co można przypisać skompletowaniu w tym czasie matryc dla syntezy kutikularnego białka.

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M. A. SMITH*, MARGARITA SALAS**, W. M. STANLEY, Jr., A. J. WAHBA, and S. OCHOA

EFFECT OF POLYADENYLIC ACID CHAIN LENGTH ON THE SIZE DISTRIBUTION OF LYSINE PEPTIDES

Department of Biochemistry, New York University School of Medicine, New York, New York 10016, *U.S.A.*

1. Purified *E. coli* ribosomes and *L. arabinosus* supernatant were used in an amino acid incorporating system to study the effect of polyadenylic acid chain length on the size distributions of the lysine peptides formed. 2. Oligoadenylic acids of increasing chain length were prepared by partial alkaline hydrolysis of polyadenylic acid, followed by treatment with acid and phosphomonoesterase, and chromatography in 8 M-urea on DEAE-cellulose. 3. The size distribution of lysine peptides was determined by chromatography on carboxymethyl cellulose. 4. The most abundant peptide formed with each oligoadenylic acid of chain length between 12 and 20 indicated a triplet code.

Recently it was shown that polyadenylic acid of relatively low molecular weight promotes the synthesis of lysine peptides in a cell-free *E. coli* system (Jones, Townsend, Sober & Heppel, 1964; Smith, 1964). Since the size distributions of polyribonucleotides and lysine peptides are easily determined by ion exchange chromatography (Tomlinson & Tenner, 1962; Stewart & Stahmann, 1962) an attempt was made to determine the coding ratio by comparing the length of the polylysines synthesized, with short polyadenylic acids of known chain length used as messengers. Such estimations of the coding ratio are only valid provided the *in vitro* system is devoid of nucleases and peptidases. The amino acid incorporating system employed throughout this work was shown to be lacking any such activities (Salas, Smith, Stanley, Wahba & Ochoa, 1965). This system consisted of chromatographically purified *E. coli* ribosomes and a supernatant fraction derived from *Lactobacillus arabinosus*. The size distribution of lysine peptides synthesized in the presence of individual oligoadenylic acids of chain lengths between 12 and 20 indicated a coding ratio of 3. This is in agreement with genetic evidence (Crick, Barnett, Brenner & Watts-

* Postdoctoral fellow of the National Institutes of Health, U.S. Public Health Service.

** International postdoctoral fellow of the National Institutes of Health, U.S. Public Health Service. Permanent address: Instituto Maranon, Centro de Investigaciones Biologicas, C.S.I.C., Madrid, Spain.

Tobin, 1961) and with *in vitro* studies in the presence of synthetic polyribonucleotides containing repeating base sequences (Khorana, 1965). A preliminary account of these results has been presented (Smith, Salas, Stanley & Wahba, 1965).

MATERIALS

Polyadenylic acid was purchased from Miles Chemical Company, Elkhart, Indiana. ^{14}C-Labelled lysine (specific radioactivity, 200 μc per μmole) was purchased from New England Nuclear Corporation, Boston, Mass. Poly-L-lysine hydrobromide was from Pilot Chemicals, Watertown, Mass. We are greatly indebted to Professor Gerhard Schmidt, Tufts University, for generous gifts of phosphomonoesterase [highly purified yeast acid phosphatase (Schmidt, Bartsch, Laumont, Herman & Liss, 1963)]. Carboxymethyl cellulose and DEAE-cellulose were obtained from Bio-Rad, Richmond, California.

METHODS

Hydrolysis of polyadenylic acid. Oligoadenylic acids of different chain lengths were obtained from partial alkaline hydrolysates of polyadenylic acid. A solution of high molecular weight polyadenylic acid (100 mg. in 5 ml. of 0.1 M-ammonium bicarbonate adjusted to pH 10 with concentrated NH_4OH) was sealed in a pyrex test tube and heated in boiling water for 15 min. After removal of the ammonium bicarbonate by rotary evaporation, the oligonucleotides were dissolved in 15 ml. of water, titrated to pH 1.5 with HCl (12 M), and incubated for 30 min. at 25° to hydrolyse terminal 2′,3′-cyclic phosphates. This was followed by treatment with yeast acid phosphomonoesterase (Schmidt, Bartsch, Laumont, Herman & Liss, 1963) to remove terminal 2′(3′)-phosphate residues.

Chromatography of oligoadenylic acids. Prior to chromatography at room temperature, the oligonucleotide solution was diluted to 100 ml. in 8 M-urea, 0.01 M-tris-HCl, and adjusted to pH 7.8. This solution was applied to a DEAE-cellulose column (1.4 × 70 cm., 0.9 meq./g.). Elution according to chain length was achieved by using an exponential gradient (8 M-urea, 0.01 M-tris-HCl, pH 7.8, in a 1 liter constant volume mixer; 8 M-urea, 0.7 M-NaCl, 0.01 M-tris-HCl, pH 7.8, in the reservoir; at a flow rate of 30 ml./hr.). Column eluates were continuously monitored at 260 mμ, and fractions to be subsequently tested were dialysed extensively against water and concentrated by rotary evaporation.

Chain length determination. The chain length of individual oligonucleotide fractions were assigned on the basis of cochromatography on DEAE-cellulose with oligonucleotides of similar composition and of known degree of polymerization. The characterization of these reference compounds has been reported previously (Salas, Smith, Stanley, Wahba & Ochoa, 1965).

Analysis of the products of the in vitro *system.* Oligonucleotide fractions were used as messengers to stimulate the incorporation of lysine in an *in vitro* system

consisting of chromatographically purified *E. coli* ribosomes and a supernatant fraction derived from *Lactobacillus arabinosus* (Salas, Smith, Stanley, Wahba & Ochoa, 1965). Following incubation (40 min., 37°), samples (0.25 ml.) were made 0.3 M in KOH, left at 37° for 1 hr. and neutralized with $HClO_4$. The precipitates were discarded, the supernatants diluted 20-fold, 10 mg. of a partial acid hydrolysis of polylysine added to each and the solutions were applied to columns (0.9 × 40 cm.) of carboxymethyl cellulose (0.58 meq./g.). Lysine peptides were eluted at 25° according to chain length with an exponential gradient of NaCl [water in a one liter constant volume mixer; 0.8 M-NaCl in the reservoir; at a flow rate of 1 ml./min. (Stewart & Stahmann, 1962)]. Elution positions of carrier oligolysines were determined by monitoring the column eluate at 220 mμ. The distribution of the [^{14}C]peptides was determined by adding 1 ml. of each 10 ml. fraction to 10 ml. of Bray's scintillation mixture (Bray, 1960) and counting in a scintillation spectrometer.

RESULTS AND DISCUSSION

The distributions of lysine peptides as a function of the chain length of the oligoadenylic acid messengers are shown in Table 1. Within each distribution, the degree of polymerization of the most abundant peptide was found to be related to the chain length of the messenger. Recent work from this laboratory utilizing polyribonucleotides of specified base sequences has indicated that although attachment of ribosomes occurs at the 5′-end of the messenger, the initiation of translation does not occur until the second triplet (Smith, Salas, Stanley, Wahba & Ochoa, 1966). In Table 1 are listed the physical lengths of each oligoadenylic acid used; it should be remembered, however, that the number of residues capable of being translated will be three residues less.

Although each oligoadenylic acid gave rise to a family of oligolysines, it may be seen in Table 1 that with the short messengers, e.g. poly $(A)_{\overline{12}}$, poly$(A)_{\overline{14}}$, poly$(A)_{\overline{19}}$ and poly$(A)_{\overline{20}}$, the peptides formed, in relation to the number of adenylic acid residues translated, had an abundance of tri-, tetra-, penta-, and hexalysine, respectively, in agreement with a triplet code. With poly A of 28 residues and higher, heptalysine was the most abundant polypeptide. A possible explanation for the results with longer peptides is that beyond a certain length the growth of the polylysine chain may be inhibited through electrostatic interaction between the positively charged polypeptide and the negatively charged polynucleotide template and/or ribosome. It may also be seen that, with regard to the total incorporation of lysine, the efficiency of polyadenylic acid greatly increases with increasing chain length. The formation of oligolysines of chain length shorter than that which would be predicted for a triplet code may be due to the fact that oligoadenylic acids, possibly for lack of specific signals for polypeptide chain start and termination, are not always read throughout their whole length. When the reaction is stopped, unfinished peptide chains may also contribute small oligolysines to the population of peptides. The formation of peptides of chain length higher than that which corresponds to a triplet code is more difficult to explain. This phenomenon was previously observed

Table 1

Synthesis of polylysine with polyadenylic acids of increasing chain length

Values expressed in μμmoles of [^{14}C]lysine incorporated/ml. reaction mixture. With each polymer [^{14}C]lysine was maximally incorporated into oligolysine of a given chain length. These values are shown in bold face type.

No. of residues* per molecule of polylysine	Polymer							
	None	$A_{\overline{12}}$	$A_{\overline{14}}$	$A_{\overline{19}}$	$A_{\overline{20}}$	$A_{\overline{28}}$	$A_{\overline{37}}$	Poly A
3	12	**200**	240	248	236	180	304	732
4	3	112	**252**	264	240	156	324	1244
5	1	40	180	**280**	276	164	404	1484
6		18	92	272	**340**	224	576	3864
7		8	44	168	288	**256**	**612**	**6480**
8			18	128	128	156	396	3372
9			8	19	60	108	184	2076
10				8	21	72	96	1072
11					16	44	68	736
12						37		700
13						28		592
14						25		295
15								118
16								40
Total incorporation	16	378	834	1387	1605	1450	2964	22 805
Coding ratio		3.0	2.8	3.2	2.8	3.6	4.9	

* Values for dilysine are not reported since in incubations without polymer, large amounts of material were synthesized which eluted from carboxymethyl cellulose in the region of dilysine.

by Smith (1964), and it may be due to reiteration during translation. This is not unlike the synthesis of long polydeoxyribonucleotides by DNA nucleotidyl transferase with oligodeoxyribonucleotides as templates (Khorana, 1965).

We wish to thank Mr. H. Lozina for help in the preparation of tRNA and ribosomal and supernatant fractions. This work was aided by grants AM-01845, AM-08953, and FR-05399 from the National Institutes of Health, U.S. Public Health Service, and E. I. du Pont de Nemours and Company, Inc.

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WPŁYW DŁUGOŚCI ŁAŃCUCHA KWASU POLIADENYLOWEGO NA WIELKOŚĆ PEPTYDÓW LIZYNY

Streszczenie

1. Wpływ długości łańcucha kwasu poliadenylowego na wielkość utworzonych peptydów lizyny w układzie włączającym aminokwasy badano przy użyciu oczyszczonych rybosomów z *E. coli* oraz supernatantu z *L. arabinosus*.

2. Kwasy oligoadenylowe o wzrastającej długości łańcucha otrzymano w następujący sposób: kwas poliadenylowy poddawano częściowej hydrolizie alkalicznej, następnie działano kwasem i fosfomonoesterazą, po czym rozdzielano na kolumnie z DEAE-celulozy w 8 M-moczniku.

3. Ilości peptydów lizyny o poszczególnych wielkościach oznaczano chromatografią na kolumnie z karboksymetylocelulozy.

4. Długość peptydu powstającego w największej ilości w obecności kwasu oligoadenylowego o długości łańcucha od 12 do 20 wskazuje na kod tripletowy.

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H. BREMER* and G. S. STENT

BINDING OF *E. COLI* TRANSFER RNA TO *E. COLI* RNA POLYMERASE

Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720, U.S.A.

1. *E. coli* transfer RNA (tRNA), labelled either uniformly with ^{32}P *in vivo* or specifically with [^{3}H]leucine by enzymic aminoacylation *in vitro*, was allowed to react with purified *E. coli* RNA polymerase. The reactants and the reaction product were then resolved by passage through Sephadex G-200 columns or by zone sedimentation in sucrose gradients. 2. Both [^{32}P]tRNA and [^{3}H]leucyl-tRNA were found to form a stable complex with the polymerase, the approximate binding capacity of 1 μg. polymerase protein ranging from 0.027 to 0.06 μg. tRNA, suggesting that one polymerase molecule can bind of the order of one tRNA molecule. 3. Though the polymerase-tRNA complexes do not dissociate spontaneously at an appreciable rate, labelled tRNA bound in such complexes can be readily exchanged for subsequently added non-labelled tRNA. This suggests that a co-operative, multi-site reaction intervenes in the formation of the complex. 4. A fraction of the polymerase-bound tRNA is not exchangeable, however, and this fraction rises with ratio of tRNA/polymerase in the reaction mixture. This suggests that the tRNA population is heterogeneous in regard to its polymerase binding, a small fraction (about 5%) of the tRNA entering into a non-exchangeable complex if exposed to an excess of enzyme protein.

It has been proposed that the degree of aminoacylation of transfer RNA (tRNA) determines the rate of bacterial RNA synthesis (Stent & Brenner, 1961; Kurland & Maaløe, 1962) and that this regulation is effected through the inhibition by tRNA of the bacterial RNA polymerase (Tissieres, Bourgeois & Gros, 1963). In support of this notion it was found by *in vitro* experiments that exposure of *Escherichia coli* RNA polymerase to *E. coli* tRNA prior to addition of its DNA template effectively inhibits later RNA synthesis in the complete reaction mixture, and that the inhibitory power of aminoacyl-tRNA is somewhat less than that of uncharged tRNA (Tissieres, Bourgeois & Gros, 1963; Bremer, Yegian & Konrad, 1966). However, the magnitude of the observed difference in relative *in vitro* inhibition between these two states of tRNA, though significant, does not appear to be great enough to account for the observed changes in the rate of *in vivo* RNA synthesis induced by changing nutritional conditions. Nevertheless, since it is very difficult to obtain a tRNA preparation in which all RNA molecules are aminoacylated it is possible that the high residual inhibitory power of the aminoacyl-tRNA used in

* Present address: Graduate Research Center of the Southwest, Dallas, Texas (U.S.A.).

those experiments actually derived from the presence of contaminating uncharged RNA molecules, and that aminoacyl-tRNA molecules are, in fact, non-inhibitory. To explore this possibility, experiments to be reported here were carried out, in which the binding of uncharged tRNA and leucyl-tRNA to *E. coli* RNA polymerase was studied directly. These binding studies revealed two different types of stable binding of both uncharged and leucyl-tRNA to polymerase, one exchangeable with free tRNA and the other not. These results are in general agreement with those of Fox, Gumport & Weiss (1965), who described formation of a stoicheiometric complex between *Micrococcus lysodeikticus* RNA polymerase and tRNA and with those of Richardson (1966) who found that both uncharged as well as aminoacyl--tRNA are bound by *E. coli* RNA polymerase.

MATERIALS AND METHODS

Purified *E. coli* RNA polymerase and T4 phage template DNA were prepared as described previously (Chamberlin & Berg, 1962; Bremer & Konrad, 1964). The specific activity of the enzyme preparation was 2500 units of enzyme activity (Chamberlin & Berg, 1962) per unit of extinction at 280 mμ. It is assumed that 1 E_{280} unit corresponds to about 1 mg. protein. (It should be noted, however, that recent unpublished measurements of J. Milligan on highly purified *E. coli* RNA polymerase indicate that 1 E_{280} unit corresponds to 1.9 mg. of enzyme protein).

Unlabelled tRNA was isolated from *E. coli* according to the method previously described (Martin, Yegian & Stent, 1963). Aqueous solutions of the tRNA were made immediately before use from the freeze-dried preparation.

[^{32}P]tRNA was isolated from *E. coli* grown in a medium containing $^{32}PO_4$ at 10 μc/μmole by the method previously described (Martin, Yegian & Stent, 1963) and further purified by two successive elutions from a Sephadex G-200 column (8 cm. height, 10 ml. volume) in which only the material eluting after a delay was retained. The purified tRNA preparation was precipitated with 70% ethanol, redissolved at a concentration of 100 μg./ml. in 0.02 M-sodium cacodylate buffer, pH 6.9, and kept frozen at -10°. Over 95% of the ^{32}P-activity of this preparation was rendered soluble in cold acid by alkaline hydrolysis. "[^{3}H]leucyl-tRNA" was prepared by enzymic aminoacylation of the uncharged, unlabelled tRNA with ^{3}H-labelled leucine. The charging mixture contained per ml.: 100 μmoles sodium cacodylate buffer, pH 6.9, 5 μmoles $MgCl_2$, 1 μmole ATP, 25 μmoles [^{3}H]leucine (about 1 mc/μmole L-leucine), 1 mg. tRNA and 150 μg. charging enzymes, prepared by C. D. Yegian, as previously described (Martin, Yegian & Stent, 1963). The aminoacylation reaction was carried out for 60 min. at 37° and the extent of reaction was determined by measurement of the incorporation of radioactivity into cold acid-insoluble material. About 6% of the tRNA molecules accepted leucine under these conditions and 9% at higher concentration of leucine. The RNA of the aminoacylation mixture was then precipitated with 70% ethanol, redissolved to a concentration of 1 mg./ml. in 0.02 M-sodium acetate buffer, pH 4.7, and kept frozen at -10°.

EXPERIMENTAL

Demonstration of a polymerase-tRNA complex by passage through a Sephadex G-200 *column.* Fig. 1a presents the result of an experiment in which the passage of purified *E. coli* RNA polymerase through a Sephadex G-200 column was followed by measurement of the enzymic activity of samples of the effluent. It can be seen that the large polymerase molecule is not significantly retarded by this column and appears in the front of the effluent. Figs. 1b and 1c show the elution kinetics of *E. coli* tRNA from the same column, as determined by measurement of either the UV extinction (in the case of unlabelled tRNA), or the radioactivity (in the case of a mixture of [^{32}P]tRNA and [^{3}H]leucyl-tRNA) of the effluent. The tRNA molecules are clearly retarded by the column and appear in the effluent later than the polymerase molecules. Fig. 2a shows the result of an experiment in which the passage of a mixture of polymerase and [^{32}P]tRNA and [^{3}H]leucyl-tRNA is followed. It can be seen that here more than 90% of ^{32}P and ^{3}H-label are eluted with the polymerase, indicating that both tRNA and aminoacyl-tRNA have formed a complex with the enzyme protein. Figs. 2b and 2c show the result of similar experiments, in which greater amounts of tRNA were added to a fixed amount of polymerase. Here it can be seen that the tRNA elution kinetics are diphasic, one portion of the tRNA being eluted early with the polymerase and another portion being eluted later at the time characteristic of free tRNA, indicating that the tRNA binding capacity of polymerase has been exceeded. From the amount of ^{32}P-label bound to polymerase in the experiment of Fig. 2c it may be estimated that this capacity is 0.03 μg. tRNA per μg. polymerase protein. Since the molecular weight of the polymerase appears to be an order of magnitude greater than that of the tRNA (Fuchs, Zillig, Hofschneider & Preuss, 1964), this capacity suggests that each polymerase molecule can bind of the order of one tRNA molecule, in agreement with Richardson's (1966) estimate.

Studies on the rate of formation of the polymerase-tRNA complex were carried out, in which polymerase and tRNA were allowed to react for various times before being passed through the Sephadex column. These studies showed that in the concentration range of 20 - 200 μg./ml. polymerase and 10 - 100 μg./ml. tRNA formation of the complex is complete within 3 min., in agreement with the previously determined rate of functional inactivation of polymerase by tRNA (Bremer, Yegian & Konrad, 1966).

An experiment was carried out to test whether the binding of tRNA to polymerase is reversible. For this purpose, polymerase and [^{32}P]tRNA and [^{3}H]leucyl-tRNA were allowed to react under conditions similar to those of the experiment of Fig. 2a, and the mixture was then divided into two portions. An excess of non-labelled tRNA was added to one sample and both samples were passed through the Sephadex column. Figs. 3a and 3c showing the elution kinetics of ^{32}P and ^{3}H in the sample without additional unlabelled tRNA reveal joint elution of polymerase and tRNA as in Fig. 2a. Figs. 3b and 3d show that secondary addition of unlabelled tRNA

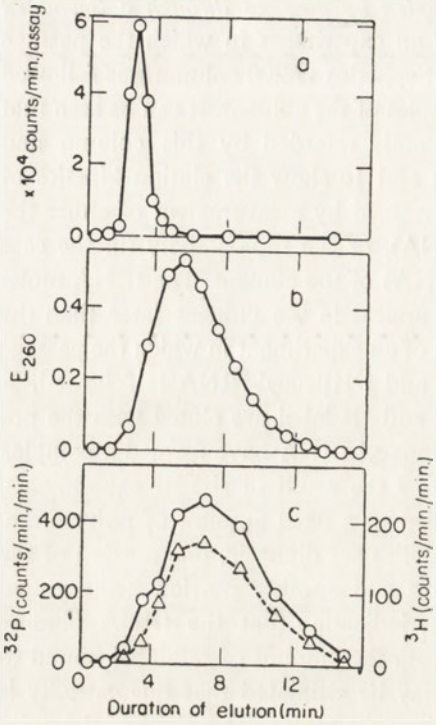

Fig. 1

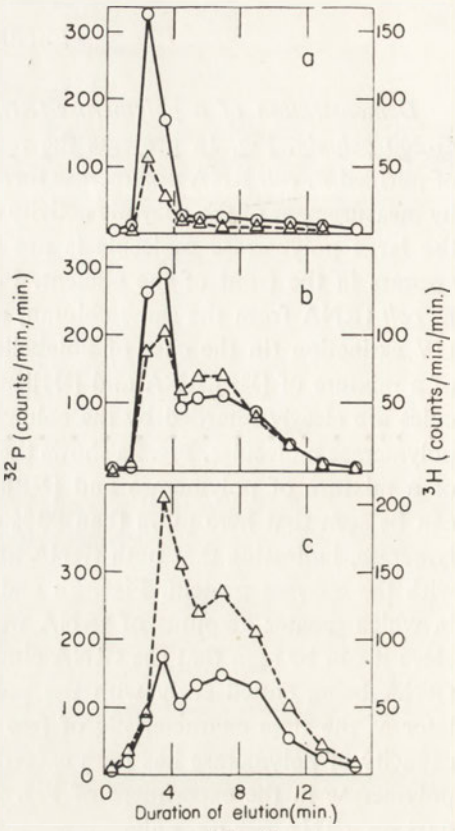

Fig. 2

Fig. 1. Sephadex column passage of (a), RNA polymerase (12 μg.); (b), uncharged, unlabelled tRNA (100 μg.); and (c), a mixture of uncharged [^{32}P]tRNA (0.1 μg. labelled with 3.5×10^4 counts/min./μg., ○) and [^{3}H]leucyl-tRNA (2 μg. labelled with 575 counts/min./μg. RNA, △). The material was applied to a Sephadex G-200 column (3 cm. long, 4.5 ml. volume) in 50 μl. of elution buffer (0.01 M-Na-cacodylate buffer, pH 6.9, 0.01 M-$MgCl_2$), and eluted at a flow rate of 0.75 ml./min. at 25°. Fractions were collected at various times. For analysis of the polymerase activity in the elution profile of part (a) 0.25 ml. of each fraction was added to 0.1 ml. of an assay mixture for RNA synthesis (containing per ml. 240 mμmoles of each GTP, UTP and CTP, 4 mμmoles [^{3}H]ATP [6×10^5 counts/min./mμmole]; 20 μg. T4 DNA, 1 μmole $MnCl_2$, 4 μmoles $MgCl_2$, 50 μmoles tris-HCl buffer, pH 7.9) and incubated for 25 min. at 30°. After addition of 100 μg. carrier DNA and 4 ml. 0.5 M-trichloroacetic acid (TCA) at 0°, the precipitates formed were collected on Millipore filters and the ^{3}H-activity retained on the filters was counted in a liquid scintillation spectrometer. About 75% of the polymerase activity applied to the column was recovered in the eluate. The distribution of unlabelled tRNA in part (b) was ascertained by measuring the E_{260} of each fraction. The distribution of the labelled tRNA in part (c) was ascertained by precipitating the nucleic acids of each fraction with TCA after addition of carrier DNA, collecting the precipitates on filters and counting ^{3}H and ^{32}P activities, as described under (a) above.

Fig. 2. The binding capacity of polymerase for uncharged [^{32}P]tRNA (○) and [^{3}H]leucyl-tRNA (△) as analysed by Sephadex G-200 column passage. Samples of 15 μg. polymerase in 50 μl. elution buffer were incubated for 3 min. at 30° with mixtures of (a), 0.25 μg. [^{3}H]leucyl-tRNA and 0.025 μg. [^{32}P]tRNA; (b), 1.25 μg. leucyl-tRNA and 0.05 μg. [^{32}P]tRNA; and (c), 2 μg. [^{3}H]leucyl-tRNA and 0.05 μg. [^{32}P]tRNA. Specific activities and analytical procedures as described for Fig. 1c.

has produced change in the elution pattern, and that a major portion of the labelled tRNA has been displaced from its polymerase complex and is now eluted at the slower rate of free tRNA. The tRNA in the complex is, therefore, exchangeable with free tRNA.

Study of the polymerase-tRNA complex by zone sedimentation. Although the existence of a tRNA-polymerase complex can readily be demonstrated by Sephadex column passage, a much better separation of polymerase and free tRNA, and hence a better quantitative study of the complex, can be attained by sucrose density gradient zone sedimentation. Fig. 4a shows the distribution of polymerase activity

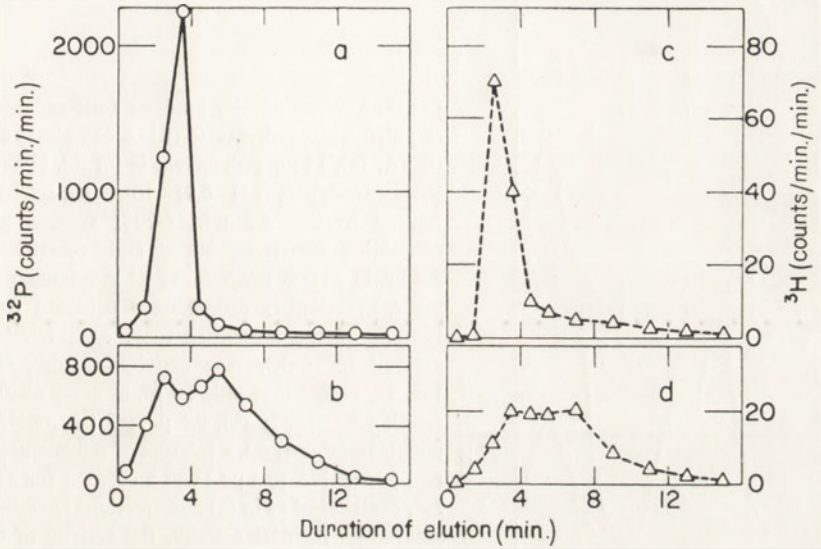

Fig. 3. Exchange of tRNA from the polymerase-tRNA complex in presence of excess tRNA, as analysed by Sephadex G-200 column passage. (a), Incubation of 0.25 μg. uncharged [^{32}P]tRNA for 3 min. at 30° with 15μg. polymerase in 50 ml. elution buffer; (b), incubation of 0.25 μg. uncharged [^{32}P]tRNA and 15 μg. polymerase for 3 min. as in (a) followed by addition of 100 μg. uncharged, unlabelled tRNA and further incubation for 3 min.; (c) as (a), except that 0.25 μg. [^{3}H]leucyl-tRNA was used instead of [^{32}P]tRNA. (d) as (b), except that 0.25 μg. [^{3}H]leucyl-tRNA was used. Specific activities and analytical procedures as described for Fig. 1c.

in a sucrose density gradient after sedimentation of an enzyme solution initially layered on the top of the sucrose gradient. This distribution has a major band corresponding to a sedimentation velocity of about 15 *S* (as determined by its position relative to a reference peak of *E. coli* ribosomal RNA not shown here) and two minor bands corresponding to sedimentation velocities of about 10 *S* and 20 *S*. This finding agrees with the heterogeneity in sedimentation velocity of *E. coli* RNA polymerase preparations previously reported by Fuchs *et al.* (1964), who attributed this behaviour to the aggregation of active enzyme molecules into stable aggregates containing up to six units. Fig. 4b shows the distribution of [^{32}P]tRNA in a similar sucrose gradient; evidently the tRNA has not moved very far from the top of the gradient, in accord with its sedimentation velocity of 4 *S*. Fig. 4c shows the distri-

bution of [^{32}P]tRNA in a similar sucrose gradient after 0.5 μg. of [^{32}P]tRNA had been allowed to react with 6 μg. polymerase. This distribution clearly contains two main components: one in the domain of the rapidly moving polymerase and one

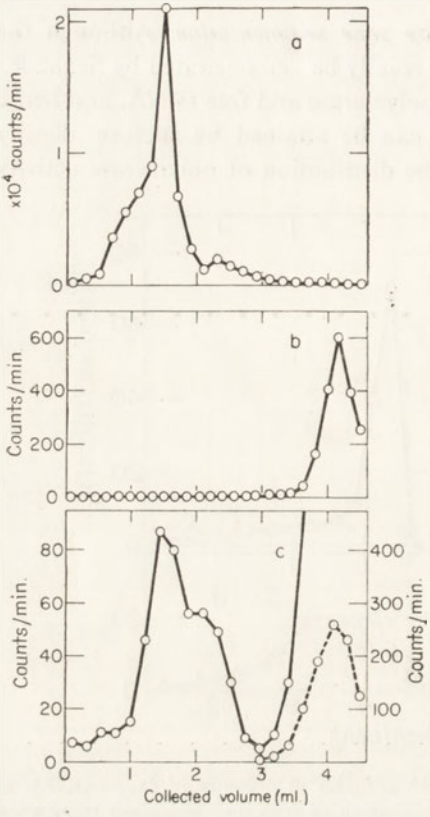

Fig. 4. Sucrose density gradient zone sedimentation of polymerase, polymerase-tRNA complexes and free tRNA. (a), 12 μg. polymerase in 0.1 ml. buffer (10^{-2} M-Na-cacodylate, pH 6.9; 10^{-2} M-$MgCl_2$) were layered over a 4.5 ml., 4-20% sucrose gradient containing the same buffer and centrifuged for 5.5 hr. at 39 000 rev./min. at 4° in a Spinco SW 39 rotor. Fractions (3 drops) were collected from the bottom of the centrifuge tube, each fraction was assayed for polymerase activity as described for Fig. 1a, except that only 50 μl. of assay mixture per fraction was used; (b), 0.5 μg. of [^{32}P]tRNA (3600 counts/min./μg.); (c), a mixture of polymerase (6 μg.) and [^{32}P]tRNA (0.5 μg.) was incubated for 3 min. at 30°, centrifuged as in (a) and fractions (3 drops) were assayed for their ^{32}P activity, the activity of the last fractions having been plotted at a reduced scale (broken line, right ordinate).

in the domain of the slowly moving tRNA. Thus the existence of the polymerase-tRNA complex can be demonstrated also by this method. Comparison of the amounts of [^{32}P]tRNA and RNA synthetic activity at various positions in the polymerase domain reveals, moreover, that the minor 10 *S* polymerase band of Fig. 4a binds several times more tRNA per unit of enzyme activity than the major 15 *S* polymerase band. This finding could mean either that the specific enzyme activity per unit of protein capable of binding tRNA is lower in the 10 *S* than in the 15 *S* band, or that reaction with tRNA dissociates the 15 *S* polymerase aggregate into 10 *S* subunits. In the experiment of Fig. 4c a total of 0.16 μg. of [^{32}P]tTRNA was found to be complexed to the 6 μg. of polymerase protein added to the reaction mixture, leading to a binding capacity of 0.027 μg. tRNA per μg. polymerase protein, in good agreement with the estimate based on Sephadex column analysis.

Three further experiments similar to that of Fig. 4c were carried out in which 0.1 μg., 0.2 μg. and 0.5 μg. of [^{32}P]tRNA were added to 3 μg. polymerase prior to zone sedimentation. The result of these experiments was that 45%, 38% and 18% of the respective [^{32}P]tRNA input was found in the polymerase complex.

The nature of this dependence of fraction of tRNA bound on relative tRNA input suggests that about half the tRNA molecules cannot form a complex with the polymerase population sufficiently stable to be detected by zone sedimentation.

Exchange of tRNA in the polymerase complex. Since the zone sedimentation proceeds during several hours, the detection of the polymerase-tRNA complex in the final density gradient means that this complex is quite stable and does not rapidly dissociate, though, as had been seen in the experiment of Fig. 3, its tRNA is exchangeable. This, on first sight paradoxical finding resembles the non-enzymic binding of tRNA to *E. coli* ribosomes, which is similarly stable yet readily exchangeable (Cannon, Krug & Gilbert, 1963). To investigate this unusual property of the polymerase-tRNA complex further, an experiment similar to that of Fig. 3 but employing zone sedimentation analysis was carried out. The results are shown in Fig. 5. It can be seen that in agreement with the result of Fig. 3, later addition of an excees of non-labelled tRNA to a polymerase [^{32}P]tRNA mixture greatly reduces the amount of [^{32}P]tRNA found in the polymerase band of the sucrose gradient sedimentation distribution. This experiment included an additional reaction mixture in which an excess of non-labelled tRNA was first presented to the polymerase and [^{32}P]tRNA added only later. As can be seen in Fig. 5, practically no [^{32}P]tRNA becomes bound to polymerase under this program, showing that the fraction of [^{32}P]tRNA which is retained in the polymerase complex under the first program is irreversibly bound.

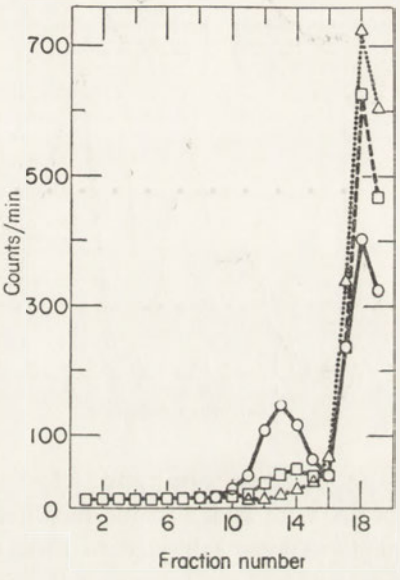

Fig. 5. Exchange of tRNA from the polymerase-tRNA complex in presence of excess tRNA, as analysed by zone sedimentation. To three solutions of 6 μg. polymerase in 100 μl. cacodylate buffer tRNA was added according to three different incubation programs at 30° before the mixtures were layered on sucrose gradients, centrifuged and analysed as described for Fig. 4c, except that here centrifugation was carried out for only 2 hr. Mixture 1 (○) received 0.5 μg. [^{32}P]tRNA (3400 counts/min./μg.) and was incubated for 10 min.; mixture 2 (□) received 0.5 μg. [^{32}P]tRNA, was incubated for 4 min., then received 100 μg. non-labelled tRNA and was incubated for another 6 min.; mixture 3 (△) received 100 μg. non-labelled tRNA, was incubated for 4 min., then received 0.5 μg. [^{32}P]tRNA and was incubated for another 6 min.

Further experiments were carried out, not to be presented here in detail, in which it was found that most of the [^{32}P]tRNA can be displaced from the polymerase complex also by unlabelled *E. coli* ribosomal RNA and by T4 phage DNA. The sedimentation profiles of [^{32}P]tRNA obtained in these experiments were more complicated, however, and thus have not been analysed. They could be interpreted

to mean that some polymerase molecules can enter a complex with two nucleic acid molecules.

The non-exchangeably bound tRNA fraction was studied in more detail in the experiment presented in Fig. 6. Here 1, 2 and 5 μg. of [^{32}P]tRNA were added to

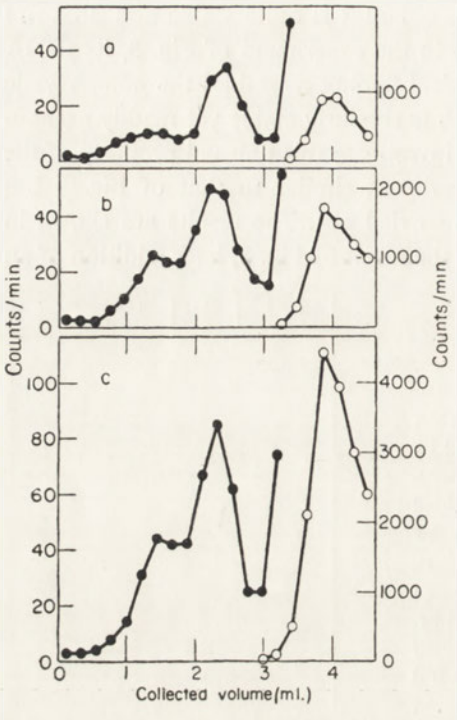

Fig. 6. Dependence of the formation of non-exchangeable polymerase-tRNA complexes on the tRNA/polymerase input ratio. Mixtures of 3 μg. polymerase and (a), 1 μg.; (b), 2 μg.; and (c), 5 μg. of [^{32}P]tRNA (3200 counts/min./μg) were incubated for 3 min. at 30° in 0.1 ml. cacodylate buffer, followed by addition of 100 μg. unlabelled tRNA, further incubation for 3 min. and zone sedimentation analysis, as described for Fig. 4c.

3 μg. of polymerase; after allowing the primary reaction, 100 μg. of non-labelled tRNA were added to the mixture, and after allowing the secondary reaction, the mixtures were subjected to zone sedimentation. It can be seen that 4.9%, 4.4% and 2.8% of the respective [^{32}P]tRNA inputs remained non-exchangeably bound to the polymerase. These results appear to indicate that about 5% of the tRNA molecules bind irreversibly to polymerase. Furthermore, the proportion of total polymerase binding capacity occupied by such non-exchangeable molecules rises with the tRNA/polymerase input ratio, approaching 100% as that ratio exceeds twenty times the binding capacity.

It is to be noted, however, that the fraction of 5% of input tRNA bound irreversibly to an excess of polymerase could very well be a reflection of the as yet poorly characterized heterogeneity of polymerase molecules in regard to their capacity to bind different species of tRNA molecules (Bremer, Yegian & Konrad, 1966). It is possible, therefore, that a fraction much greater than 5% of tRNA molecules can form an irreversible complex with polymerase, provided that each tRNA molecule is given an opportunity to react with that specific polymerase molecule to which it can be irreversibly bound.

It seemed conceivable that the non-exchangeably-bound RNA is not tRNA at all, but some ^{32}P-labelled contaminant, for instance fragments of ribosomal RNA, possibly present in the tRNA extract. To test this possibility the polymerase binding of [^{3}H]leucyl-tRNA was compared with that of [^{32}P]tRNA by the zone sedimentation technique, both before (Fig. 7a) and after (Fig. 7b) addition of excess

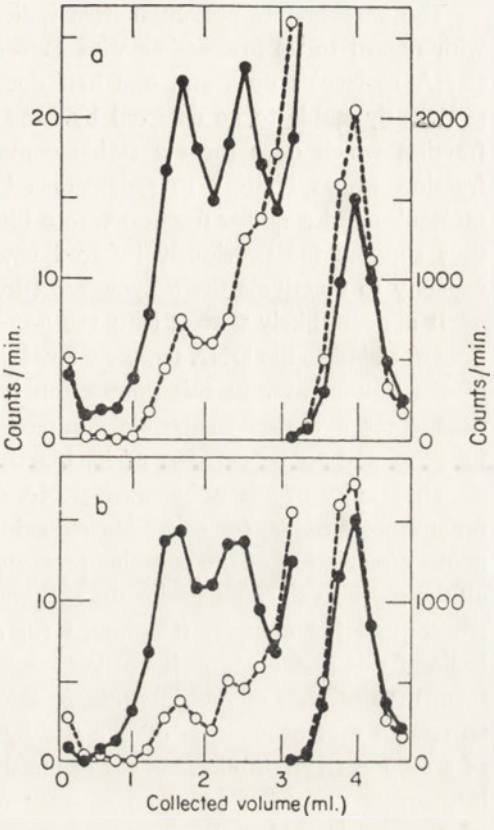

Fig. 7. Binding and exchange of uncharged and leucyl-tRNA to and from polymerase. 6 μg. of polymerase, 1 μg. of [^{32}P]tRNA (4600 counts/min./μg.) and 10 μg. of [^{3}H]leucyl-tRNA (570 counts/min./μg. RNA; containing uncharged tRNA of the other amino acid species) were incubated for 3 min. at 30° in 0.1 ml. cacodylate buffer and subjected to zone sedimentation (a) immediately, (b) after addition of 100 μg. of unlabelled tRNA and further incubation for 3 min. The analysis was carried out as described for Fig. 4c, except that here nucleic acids in fractions were precipitated with cold TCA and counted on Millipore filters in a liquid scintillation spectrometer, as described under Fig. 1. The activity of the last fractions is plotted at a reduced scale (right ordinate). ^{32}P activity: closed symbols; ^{3}H activity: open symbols.

unlabelled tRNA to the reaction mixture initially containing the very high tRNA/polymerase ratio of 11 μg. tRNA per 6 μg. polymerase. As can be seen in Fig. 7a the ^{32}P activity is found in three main bands, corresponding to the 15 *S* and 10 *S* polymerase components and to the free 4 *S* tRNA. The distribution of the ^{3}H activity is qualitatively similar to that of the ^{32}P activity, indicating in confirmation of the Sephadex experiments, that leucyl-tRNA also enters a stable complex with the polymerase. However, it is to be noted that a much smaller fraction of the [^{3}H]leucyl-tRNA input is present in the polymerase bands. As can be seen in Fig. 7b, addition of excess unlabelled tRNA to the reaction mixture reduces the amounts of both [^{32}P]- and [^{3}H]leucyl-tRNA by only 40%, indicating that the major portion of the initially bound tRNA molecules of both types had bound non-exchangeably. Thus it can be concluded that the non-exchangeable fraction represents some species of tRNA molecules and not an extraneous ^{32}P-labelled contaminant. The total amount of tRNA bound to polymerase in the experiment of Fig. 7a appears to be

0.055 μg. tRNA per μg. polymerase protein, which is twice as great as the polymerase capacity inferred from the sedimentation analysis of Fig. 4, involving very much lower tRNA/polymerase input ratios.

DISCUSSION

The experiments presented here indicate that *E. coli* tRNA is heterogeneous with regard to its interaction with *E. coli* RNA polymerase: about one half the tRNA molecules does and one half does not form a complex with polymerase sufficiently stable to be detected by zone sedimentation. Furthermore, that tRNA fraction which does form a stable complex comprises two subclasses: a major fraction whose binding to polymerase is exchangeable with other nucleic acid molecules and a minor fraction whose binding is not exchangeable. These findings thus support the previously inferred heterogeneity of tRNA with regard to its capacity to inactivate the polymerase (Bremer, Yegian & Konrad, 1966).

It appears likely that at least the non-exchangeable tRNA-polymerase complex does not bind to the DNA template any longer and thus inactivates enzymic function of the polymerase. In this connection it is important to note that leucyl-tRNA does enter a non-exchangeable complex with the polymerase, and hence would be presumed to be capable of its inactivation.

The ability of the polymerase-tRNA complex to exchange its tRNA molecule for a second tRNA (or other nucleic acid) molecule in the absence of any spontaneous dissociation of the complex presents an interesting physicochemical problem, all the more so since an analogous exchange reaction exists in the case of the *E. coli* ribosome-tRNA complex (Cannon, Krug & Gilbert, 1963). Although the molecular basis of this phenomenon is not yet known, it seems plausible that it could derive from the existence of multiple nucleic acid binding sites on the polymerase protein so related that occupation of one site by a tRNA molecule weakens the bonding of a second tRNA molecule held at another site. Furthermore, if this multiple site interaction depended in some manner on the purine-pyrimidine base sequence of the nucleic acid molecules to be exchanged, for example when both molecules were trapped irreversibly if part of their base sequences happened to be complementary, some interesting possibilities would exist for the specificity of functional regulation of the polymerase by polynucleotides. Such specificity in the exchange reaction might also explain the previously observed heterogeneity of *E. coli* polymerase molecules with regard to binding to different "starting" sites on their DNA template molecules (Bremer, Konrad & Bruner, 1966) and to different inactivating tRNA molecules (Bremer, Yegian & Konrad, 1966). For it is not unlikely that many of the polymerase molecules carry various fragments of firmly bound polynucleotides through the purification procedure of the enzyme protein.

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TWORZENIE KOMPLEKSU MIĘDZY PRZENOSZĄCYM RNA Z *E. COLI* A POLIMERAZĄ RNA Z *E. COLI*

Streszczenie

1. Przeprowadzono reakcję między oczyszczoną polimerazą z *E. coli* a przenoszącym RNA (tRNA) z *E. coli* znakowanym jednolicie ^{32}P *in vivo* lub znakowanym specyficznie [^{3}H]leucyną na drodze enzymatycznej aminoacylacji *in vitro*. Związki reagujące oddzielano od produktu reakcji na kolumnach z Sephadexu G-200 albo przez wirowanie w gradiencie sacharozy.

2. Stwierdzono, że zarówno [^{32}P]tRNA jak i [^{3}H]leucyl-tRNA tworzą trwały kompleks z polimerazą. Przybliżona zdolność wiązania tRNA przez 1 μg. białka polimerazy wynosi 0,027 - 0,06 μg., co sugeruje, że jedna cząsteczka polimerazy może wiązać około jednej cząsteczki tRNA.

3. Chociaż samorzutna dysocjacja kompleksu polimerazy z tRNA jest nieznaczna, to jednak związany w kompleksie znakowany tRNA daje się łatwo wymienić z nieznakowanym tRNA. Sugeruje to, że przy tworzeniu kompleksu współdziała reakcja wielocentrowa (multi-site).

4. Część tRNA związanego z polimerazą nie ulega wymianie i ilość jej wzrasta równolegle ze wzrostem ilości tRNA w stosunku do polimerazy w mieszaninie reagującej. Wskazuje to, że populacja tRNA jest heterogenna pod względem łączenia się z polimerazą, przy czym mała frakcja, stanowiąca ok. 5% całego tRNA, wchodzi w trwały, niewymienny kompleks w obecności nadmiaru enzymatycznego białka.

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T. SHINOMIYA, A. KIKUCHI, T. UCHIDA and F. EGAMI

ISOLATION OF DNA FROM THE ERYTHROCYTE NUCLEI OF *ANADARA INFLATA*, A MARINE BIVALVE MOLLUSC

Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo, Japan

Dedicated to Professor J. Heller on the occasion of his 70th birthday.

1. DNA was isolated from the erythrocyte nuclei of *Anadara inflata*. 2. Physical and chemical properties of the DNA were investigated.

There have been few reports [12, 14] of the isolation of DNA from invertebrates, and it is expected from the point of view of comparative biochemistry that more extensive studies on invertebrate DNA will be carried out.

This report deals with the isolation and properties of DNA from the erythrocyte nuclei of a marine bivalve mollusc, *Anadara inflata*.

MATERIALS AND METHODS

Organism. Living specimens of *Anadara inflata* were purchased from a fish merchant in Tokyo in October, 1965.

Analytical methods. Protein was determined by the method of Lowry *et al.* [7], using ovalbumin (recrystallized three times in our laboratory) as a standard. RNA was determined by the orcinol reaction, which was modified so as not to be disturbed by DNA [15], and high-molecular-weight yeast RNA (prepared from the pressed baker's yeast in our laboratory) was used as a standard. Polysaccharide was determined by the diphenylamine reaction which was modified by Segovia *et al.* [11] in order not to react with DNA, and glucose was used as a standard hexose.

DNA and RNA analysis of the erythrocytes and erythrocyte nuclei. The erythrocytes and erythrocyte nuclei were fractionated by Schmidt-Thannhauser's method [10]. DNA and RNA contents were estimated by indole reaction [2] and orcinol reaction [1], respectively.

Base analysis of DNA. DNA was hydrolysed by formic acid (99%) at 175 ± 1° for 30 min. [13]. The hydrolysate was dried *in vacuo*, and dissolved in 0.1 ml. of 1 N-HCl. Base composition was determined by ascending paper chromatography

on Toyo paper 51A using methanol - HCl - H_2O (7:2:1, by vol.) [5] or isopropanol - HCl - H_2O [13] as the solvent systems. Each sample was analysed by the two solvent systems and the results were averaged.

Ultraviolet absorption. Molecular absorption coefficient with respect to phosphate, ε(P), was calculated both from UV absorption spectrum and from phosphate content of DNA solution. The value of ε(P) at the absorption maximum (260 mμ), the ratio of one absorbancy to another and a curve of UV absorption spectrum gave the information of the purity of DNA. Absorption spectrum was determined by the Hitachi automatic spectrophotometer (Model EPS-2), and phosphate was determined by the method of Lindberg *et al.* [6], using KH_2PO_4 as a standard.

Melting curve. Ito's spectrophotometer (Beckmann type) with the apparatus to circulate water at a given temperature was used to measure absorbancy. The measurement was carried out in Prof. M. Tsuboi's Laboratory, Faculty of Pharmaceutical Sciences, the University of Tokyo.

Sedimentation constant. The sedimentation velocity constant at low concentration was measured by means of UV absorption with Spinco (Model E), and automatic microdensitometer.

RESULTS

Isolation of erythrocyte nuclei. Haemolymph of *Anadara inflata* was collected by heart puncture after breaking a part of the shell with a small hammer. The red haemolymph was filtered twice through a gauze layer, and centrifuged at low speed (about 1000 rev./min.) for 5 min. The sedimented erythrocytes were washed twice with 0.5 M-NaCl by centrifugation, suspended in the same solution. The collected erythrocytes were disrupted by osmotic shock, suspended in 0.15 M-sodium chloride - 0.002 M-sodium citrate. The pellet obtained by centrifugation at 3000 rev./min. for 20 min. was washed twice with the same saline-citrate, and the suspension was kept still for some time. Then the upper portion (about 90% of the whole volume) of the suspension was centrifuged. The pellet was used as the preparation of erythrocyte nuclei. Plate 1 and 2 are the microscopical photographs of the erythrocytes and the isolated nuclei.

Table 1

Nucleic acid composition of erythrocytes and nuclei

The results are expressed as percentages of the dry weight.

	DNA	RNA
Erythrocytes	0.94	1.1
Nuclei	7.9	1.9

Nucleic acid composition of erythrocytes and nuclei. The composition of nucleic acids of the erythrocytes and the isolated nuclei is shown in Table 1.

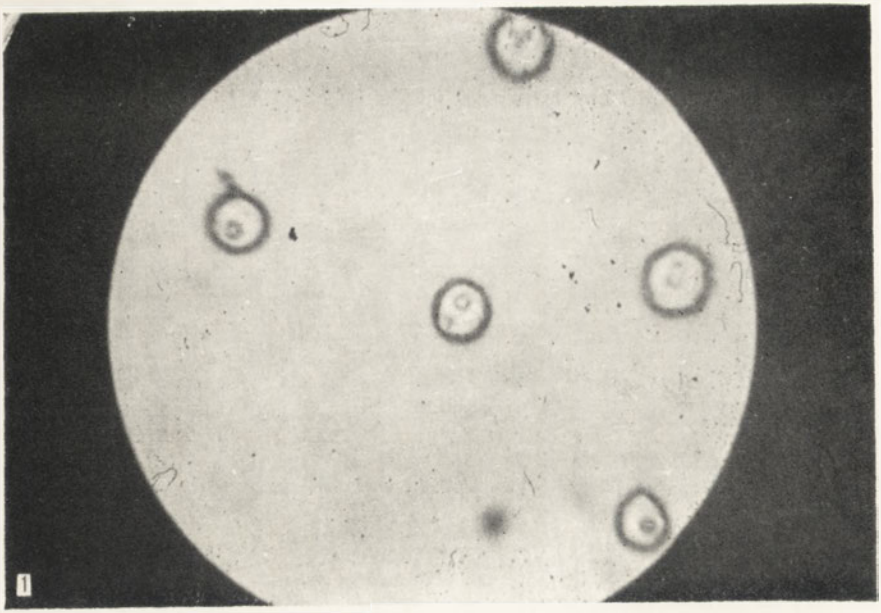

Plate 1. Microscopical photograph of erythrocytes (× ca. 750).

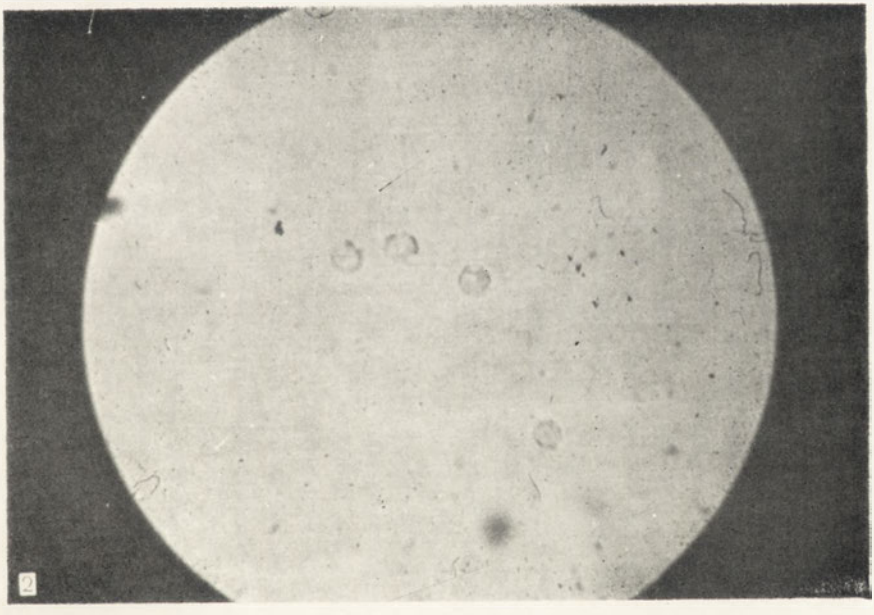

Plate 2. Microscopical photograph of isolated nuclei (× ca. 750).

T. Shinomiya *et al.* (*facing p.* 380).

Isolation of DNA. DNA was prepared from erythrocyte nuclei of *Anadara inflata* by the modified method of Colter *et al.* [3]. Precipitated nuclei were suspended in 1.0 M-sodium chloride - 0.02 M-phosphate buffer, pH 7.3 (about 20 ml. to one hundred specimens of the organism), along with sufficient volume of a 10% aqueous solution of sodium deoxycholate to give a final concentration of 0.5% and a volume of phenol saturated with 0.02 M-phosphate buffer (about 75%) equal to that of the aqueous phase. Being cooled in an ice box, the suspension was homogenized at 2000 rev./min. for 5 min. in Teflon homogenizer (Potter-Elvehjem type). Then the emulsion was centrifuged at 15 000 rev./min. for 10 min., and the upper aqueous layer containing the DNA was carefully removed from the lower phenol phase containing denatured proteins. The lower phase was treated again with equal volume of 0.1 M-sodium chloride - 0.02 M-phosphate buffer, pH 7.3, and the upper phase separated by centrifugation was added to the first obtained upper layer. The DNA solution was subjected to two additional extractions with equal volumes of phenol saturated with phosphate buffer by hand shaking. These were of 5 min. duration and were carried out carefully in the cold. After each extraction the phenol and aqueous phase were separated by centrifugation at 3500 rev./min. for 10 min., and the latter was again centrifuged at 15000 rev./min. for 10 min. DNA was separated from the clear supernatant by adding gradually an equal volume of 95% cold ethanol, cooling in an ice bath. Fibrous precipitates were spooled up with glass rod, washed through 80% ethanol, dissolved in 0.15 M-sodium chloride - 0.015 M-sodium citrate (SSC) and dialysed against the same solution.

Table 2

Base composition of DNA

The results are expressed as percentages of the sum of four bases; each value is an average of two samples.

Treatment by RNase	G	A	C	T	GC	Pu/Py	$\frac{A+C}{G+T}$
None	18.0	34.1	18.2	29.7	36.2	1.09	1.09
Treated	16.1	35.4	15.8	32.7	31.9	1.06	1.05

This DNA solution was incubated at 37° for 45 min. with 20 μl. of RNase I-A solution (purchased from Worthington Biochemical Corporation, 1 mg./ml.) and 20 μl. of RNase T_1 solution (prepared from Taka-Diastase powder in our laboratory, 0.75 mg./ml.). After adding sodium chloride to make a concentration of 1.0 M, the RNase-treated DNA solution was subjected twice to phenol treatment in the same way as described above. DNA was dissolved in SSC to give the final concentration of 1 - 2 mg./ml., and dialysed several times against SSC. This solution was saturated with chloroform and stored at 4°, and all the experiments were carried out with this solution.

Base composition of DNA. Base composition of DNA was chemically determined as described above. The results are given in Table 2.

Chemical and physical properties of erythrocyte DNA. Chemical analysis of DNA is shown in Table 3, and UV absorption spectrum curve is shown in Fig. 1.

Table 3

Chemical and physical properties of DNA

The contents of RNA, protein and hexose are percentages of DNA calculated from UV absorption; $E^{1.0\%}_{260\ m\mu} = 200$.

ε(P) max. = 260 mμ	6800
$\varepsilon_{max.}$: $\varepsilon_{min.}$: ε_{280}	1:0.39:0.53
RNA content	0.5%
Protein content	6.0%
Hexose (glucose) content	10.0%

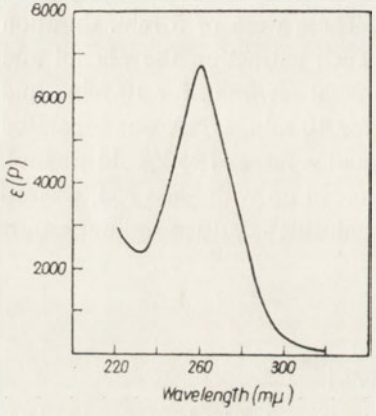

Fig. 1

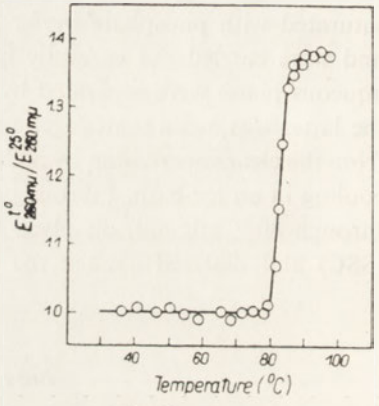

Fig. 2

Fig. 1. UV absorption spectrum of DNA in SSC-solution.
Fig. 2. Melting profile of DNA in SSC-solution. T_m value estimated from the above is 82.5°.

Melting temperature profile is shown in Fig. 2, from which melting temperatuer was deduced to be 82.5°. According to the experimental formula given by Marmur & Doty [8], this value means that GC content of the DNA is 33%. Sedimentation velocity constant depending on concentration is shown in Fig. 3, and coefficient at zero concentration, $S^0_{20,w}$, extrapolated to a value of 28.0 S. Molecular weight of DNA is estimated to be about 1.0×10^7, according to the experimental formula given by Doty [4].

DISCUSSION

A highly viscous DNA preparation was obtained from the erythrocyte nuclei of *Anadara inflata*. It is a typical AT type DNA. The carbohydrate content of the preparation is higher than in usual DNA preparations. Segovia *et al.* [11] have reported, however, that the DNA prepared from mouse liver and HeLa cells by

Colter's method is contaminated by the cytoplasmic polysaccharide. Considering that our procedure for preparation of erythrocyte nuclei is not satisfactory to obtain sufficiently pure nuclei, it is as yet premature to conclude that the carbohydrate is

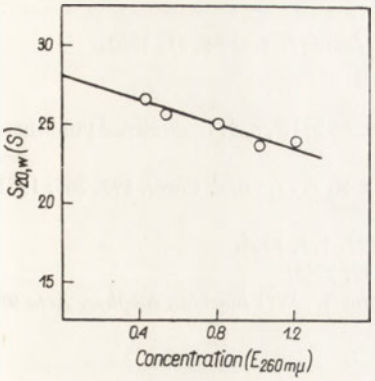

Fig. 3. Variation of sedimentation coefficient with concentration. All runs were performed at 25 980 rev./min. and near 20°. DNA was dissolved in SSC.

not a contaminant but a component of a natural DNA-polysaccharide complex. DNA has to be prepared from erythrocyte nuclei of the organisms collected at various seasons and has to be further purified by different methods.

Sueoka and Chiang kindly carried out a preliminary CsCl density gradient analysis with a sample of the DNA preparation and found that it consists of two components, of which the buoyant density and GC content calculated from the density are shown below:

DNA from erythrocyte nuclei of Anadara inflata

DNA component	Buoyant density in CsCl (assuming *E. coli* DNA = 1.710)	GC (%)	Relative amount (%)
Major band α	1.694	34.4	81
Minor band β	1.710	51.4	19

The existence of a minor component with higher GC content seems to be remarkable and it is expected that these investigations will be further continued. The major component may be regarded as chromatin DNA of the nuclei. We have as yet no idea as to the source of the minor component. It might be also a chromatin DNA or nucleolus DNA, and it cannot be excluded that it might originate from some contaminants in the nuclei preparation. In this connection the existence of DNA in the membrane fraction of mammalian erythrocytes has to be taken into consideration [9].

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IZOLOWANIE DNA Z JĄDER ERYTROCYTÓW SKORUPIAKA MORSKIEGO *ANADARA INFLATA*

Streszczenie

1. Izolowano DNA z jąder erytrocytów *Anadara inflata*.
2. Zbadano fizyczne i chemiczne własności izolowanego DNA.

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F. ŠORM, J. VESELÝ and A. ČIHÁK

BIOCHEMICAL MECHANISMS OF DRUG RESISTANCE

III. DIFFERENCES IN THE METABOLISM OF MOUSE LEUKAEMIC CELLS SENSITIVE AND RESISTANT TO 5-AZACYTIDINE

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

1. AKR leukaemic cells resistant to 5-azacytidine differ remarkably from the wild-type strain by the diminished incorporation *in vivo* of cytidine, thymidine and 5-azacytidine, into nucleic acids. Thymidine incorporation into acid soluble pool was, however, enhanced. 2. The particle-free supernatants from the resistant mutant cells showed a somewhat decreased thymidine kinase activity and the degradation of thymidine and thymidine 5′-diphosphate and 5′-triphosphate was more pronounced than in 5-azacytidine-sensitive leukaemic cells. Thymidine synthetase activity was unaltered. Probable significance of these findings is discussed.

The antileukaemic [12, 13] and bacteriostatic [2] properties of 5-azacytidine [9] have been described previously. In mice, its effects are directed primarily to lymphoid tissue and bone marrow, in which considerable depression of deoxyribonucleic acid and ribonucleic acid synthesis is seen, especially in large lymphocytes and in more mature myeloid cells [15]. Furthermore, it has been shown that 5-azacytidine is converted by mammalian cells to its 5′-phosphates, which are incorporated into nucleic acids [5].

The present report describes certain biochemical changes in the metabolism of the nucleic acid precursors associated with the development of resistance to 5-azacytidine in AKR leukaemic mice.

MATERIALS AND METHODS

Chemicals. 5-Azacytidine was synthesized by Dr. A. Píškala of this Institute. [4-^{14}C]5-Azacytidine (4.6 mc/m-mole) was kindly supplied by Dr. J. Morávek of this Institute. [6-^{14}C]Orotic acid (2.3 mc/m-mole), [G-^{14}C]cytidine (1.37 mc/m-mole), [7-^{14}C]thymidine (24 mc/m-mole) and [2-^{14}C]2′-deoxyuridine (6.5 mc/m-mole) were purchased from the Institute for the Research, Production and Utilization of Radioisotopes, Prague. [^{3}H]Cytidine (1 250 mc/m-mole) and [^{3}H]thymidine (2 420 mc/m-mole) were supplied by The Radiochemical Centre, Amersham, England.

Disodium salt of adenosine 5′-triphosphate was obtained from Calbiochem, Lucerne, Switzerland. 5-Fluoro-2′-deoxyuridine was kindly supplied by F. Hoffmann-La Roche, Basel, Switzerland. Methotrexate sodium was a commercial product of American Cyanamid Co., Wayne, U.S.A. 5,10-Methylenetetrahydrofolic acid sealed in glass ampoules *in vacuo* was obtained through the courtesy of Dr. K. Slavík, Institute of Hematology, Prague.

Animals. Inbred AKR leukaemic mice 6 - 7 days after inoculation with leukaemic cells were used throughout the experiment. Resistance to 5-azacytidine in these animals was obtained as described elsewhere [16].

Autoradiography. The livers of leukaemic mice, which received 200 μc (0.16 μmole) of [^{3}H]cytidine, 9.2 μc (2 μmoles) of [4-^{14}C]5-azacytidine and 80 μc (16 mμmoles) of [^{3}H]thymidine per mouse intraperitoneally 95 - 30 min. before killing, were gently homogenized in 3.5% polyvinylpyrrolidone. The cell suspensions were filtered through double layer gauze, centrifuged in the cold, the supernatant fraction decanted, and brush-smeared preparations of the sediments were made on glass slides. After fixation in methanol the slides were processed with stripping film Kodak, AR.10, and exposed for 12 - 15 days. After developing and fixing bath they were stained with Giemsa or with methyl green pyronin. 500 cells were counted in each experimental group.

The incorporation of 5-azacytidine. [4-^{14}C]5-Azacytidine (1 μc/0.5 μmole/mouse) was administered intraperitoneally to groups of seven mice, 120 min. before killing. The livers were homogenized at 4° in a solution containing equal volumes of 0.02 M-$MgCl_2$ and 0.001 M-EDTA. The homogenate was centrifuged for 30 min. at 10 000 *g* at 5°, and the supernatant was further centrifuged for 90 min. at 105 000 *g* at 5° (MSE 40). Isolation of ribonucleic acid was carried out according to Kirby [6]. Radioactivity of several times purified ribonucleic acids was measured after combustion to carbon dioxide [14].

The incorporation of orotic acid. [6-^{14}C]Orotic acid (1 μc/μmole/mouse) was administered to the group of four mice 2 hr. before killing. The same amount of 5-azacytidine (1 μmole) was injected simultaneously with orotic acid. The livers were homogenized twice in the cold in 3 volumes of 0.2 N-$HClO_4$. After centrifugation (0°, 3000 *g*, 20 min.) 3 volumes of 1 N-KOH were added to the sediment and incubated for 18 hr. at 20°. Further treatment of the hydrolysate and the isolation of uridine and cytidine 3′-phosphates were carried out in the manner described by Škoda *et al.* [11]. The extinction of the eluted nucleotides was measured in 0.01 N-HCl with a Unicam SP 700 spectrophotometer. Radioactivity of the eluted aliquots was assayed with a Frieseke-Hoepfner gas flow counter. The specific radioactivity of the isolated nucleotides was expressed as counts/min./μmole of nucleotide.

The incorporation of thymidine. [^{3}H]Thymidine (80 μc/16 μmoles/mouse) was administered 30 min. before killing. Methotrexate (single dose 0.1 μmole) and 5-fluoro-2′-deoxyuridine (single dose 2.65 μmoles) were injected twice: 2.5 hr. before thymidine and simultaneously with its application. Each experimental group included four animals. The livers were homogenized in physiological saline, the

cell suspensions were centrifuged (all operations at 4°) and washed three times in saline containing an excess of carrier. After the last wash the sediment was extracted for 3 min. with ice-cold 5% trichloroacetic acid and for 20 min. at 90° with 5% trichloroacetic acid. The extracts corresponding to the acid-soluble pool and combined nucleic acid fraction were treated with ether and aliquots were assayed for their radioactivity which was expressed per extinction unit at 260 mμ.

The phosphorylation of thymidine by particle-free supernatants. Supernatant fractions from leukaemic cells were used as a source of enzymic activity. Leukaemic livers were homogenized in the cold with a plastic pestle in 0.01 M-tris-HCl (pH 8.1) containing 0.15 M-KCl. The number of cells was determined by direct counting in a haemocytometer. The cell suspension was subjected to sonication (1 min., 4°, MSE sonic disintegrator 1.2 Kc), then centrifuged at 105 000 *g* (Spinco) for 1 hr. Samples of the supernatant fraction (usually equivalent to 3×10^7 cells) were either used immediately or kept at -70° not longer than 3 days. The medium for incubation at 37° (Dubnoff shaking incubator) contained the following substances at final concentrations indicated: adenosine 5′-triphosphate, 9 mM; $MgCl_2$, 2 mM; tris-HCl (pH 8.1), 50 mM; and [7-^{14}C]thymidine, 0.042 mM. To this medium, 0.3 ml. of the supernatant fraction was added so that the final volume of the reaction mixture was 0.5 ml. The reaction was stopped by boiling for 30 sec. Thymidine and its derivatives were chromatographed in the solvent system composed [8] of isobutyric acid - ammonium hydroxide - water (66:1.5:33, by vol.). The compounds were either eluted with water and counted with gas flow counter or the chromatograms were scanned in a beta 2π scaler. In the case of elution the results were expressed as mμmoles of thymidine phosphorylated by the particle-free supernatant equivalent to 10^9 cells.

The determination of thymidylate synthetase. Supernatant fractions were prepared in a similar fashion as those which were employed for thymidine and thymidylate kinase assay with the exception that the sonication buffer contained 0.003 M-mercaptoethanol. [2-^{14}C]2′-Deoxyuridine-5′-phosphate for the synthetase assay was prepared from [2-^{14}C]2′-deoxyuridine. 0.15 μmole [2-^{14}C]2′-deoxyuridine (2.1 μc) was incubated with particle-free supernatant from leukaemic cells in 0.25 M-tris-HCl buffer, pH 8.1 for 30 min. at 37° in the presence of 5 μmoles adenosine 5′-triphosphate and 1 μmole $MgCl_2$ in final volume of 1 ml. The resulting [2-^{14}C]-2′-deoxyuridine-5′-phosphate was isolated and purified chromatographically (isobutyric acid - ammonium hydroxide - water, and *n*-butanol saturated with water). The medium of the thymidylate synthetase assay (carried out essentially according to Kit *et al.* [7]; 37°, 15 min. of incubation) contained in a volume of 1 ml. the following substances at final concentrations indicated: [2-^{14}C]2′-deoxyuridine-5′-phosphate, 1.54 μM; $MgCl_2$, 2 mM; tris-HCl buffer, pH 8.1, 70 mM; 5,10-methylenetetrahydrofolic acid, 0.45 mM; mercaptoethanol, 64 mM; and formaldehyde, 20 mM. The reaction was stopped by boiling for 30 sec.; after centrifugation samples of supernatants were evaporated *in vacuo* and hydrolysed at 100° for 60 min. in 70% $HClO_4$. Products of hydrolysis were separated chromatographically in a solvent system consisting of isopropylalcohol - hydrochloric acid - water (170:41:39, by vol.).

Uracil and thymine were eluted and counted. The activity of thymidylate synthetase was expressed as mμmoles of 2′-deoxythymidine-5′-phosphate formed by cell equivalent of 10^8 cells after 60 min.

RESULTS

The incorporation of [^{3}H]*cytidine into leukaemic cells of mice* in vivo

The autoradiographic study after [^{3}H]cytidine administration *in vivo* revealed a decreased incorporation of the precursor into individual cells of 5-azacytidine-resistant animals (Table 1); however, the proportion of labelled cells in the population [16] did not change.

Table 1

The incorporation of [^{3}H]*cytidine into leukaemic cells of AKR mice sensitive and resistant to 5-azacytidine, determined by autoradiography*

[^{3}H]Cytidine (0.16 μmole per mouse) was administered to mice 90 min. before killing.

Leukaemic cells	Labelled cells (%)	Number of grains*	Synthetic index**	Depression (%)
Sensitive	100	26.6	2 660	—
Resistant	100	19.5	1 950	36.5

* Number of grains per one average labelled cell.

** Percent of labelled cells × number of grains per one average labelled cell.

The incorporation of [4-^{14}C]5-*azacytidine into ribonucleic acids of leukaemic mice* in vivo

The results of the autoradiographic experiments are shown in Table 2. Radioactivity has been found to be distributed nearly equally among individual 5-azacytidine-sensitive and -resistant leukaemic cells. At the same time, the fraction of labelled cells in 5-azacytidine-resistant animals dropped by 45.6%.

Table 2

The incorporation of [4-^{14}C]5-*azacytidine into leukaemic cells of AKR mice sensitive and resistant to 5-azacytidine, determined by autoradiography*

[4-^{14}C]5-Azacytidine (2 μmoles per mouse) was administered intraperitoneally to four AKR inbred leukaemic mice 90 min. before killing.

Leukaemic cells	Labelled cells (%)	Number of grains*	Synthetic index*	Depression (%)
Sensitive	24.3	5	121.5	—
Resistant	13.2	4.6	60.7	50

* See Table 1.

Direct evidence for the diminished incorporation of 5-azacytidine in 5-azacytidine-resistant leukaemic mice was obtained by isolating liver ribonucleic acids after the administration of the labelled analogue *in vivo*. Table 3 indicates that the incorporation of [4-^{14}C]5-azacytidine into the soluble and ribosomal ribonucleic acids of mutant cells is decreased by 43 and 61%, respectively.

Table 3

The incorporation of [4-^{14}C]5-*azacytidine into ribonucleic acids of AKR leukaemic mice sensitive and resistant to* 5-*azacytidine*

[4-^{14}C]5-Azacytidine (0.5 μmole per mouse) was administered intraperitoneally to groups of seven AKR inbred mice 120 min. before killing.

Leukaemic mice	Incorporated [4-^{14}C]5-azacytidine (mμmoles/ mg. RNA)	
	Soluble RNA	Ribosomal RNA
Sensitive	1.30	1.07
Resistant	0.74	0,41

The incorporation of [6-^{14}C]*orotic acid into liver ribonucleic acid of leukaemic mice* in vivo

[6-^{14}C]Orotic acid was incorporated *in vivo* into ribonucleic acids of mutant leukaemic cells to a slightly lower degree than into their wild-type counterparts. Both of the cell variants reacted to 5-azacytidine that was injected simultaneously with orotic acid by a very pronounced decline of its utilization for ribonucleic acid synthesis (Table 4).

Table 4

Inhibitory effect of 5-*azacytidine on the incorporation of* [6-^{14}C]-*orotic acid* in vivo *into liver ribonucleic acids of AKR leukaemic mice sensitive and resistant to* 5-*azacytidine*

[6-^{14}C]Orotic acid (1 μc per μmole per mouse) was administered to groups of four mice intraperitoneally 2 hr. before killing, and simultaneously 5-azacytidine (1 μmole per mouse) or physiological saline.

Leukaemic mice	Uridine 3′-phosphate (counts/min./μmole)		Cytidine 3′-phosphate (counts/min./μmole)	
	Control	5-Azacytidine	Control	5-Azacytidine
Sensitive	1 318.4	693.1	120.2	68.1
Resistant	966.6	563.5	88.5	51.4

The incorporation of [^{3}H]*thymidine into leukaemic cells of mice* in vivo

Results presented in Fig. 1 show that thymidine was incorporated into deoxyribonucleic acid and into whole cells of 5-azacytidine-resistant leukaemic mice less

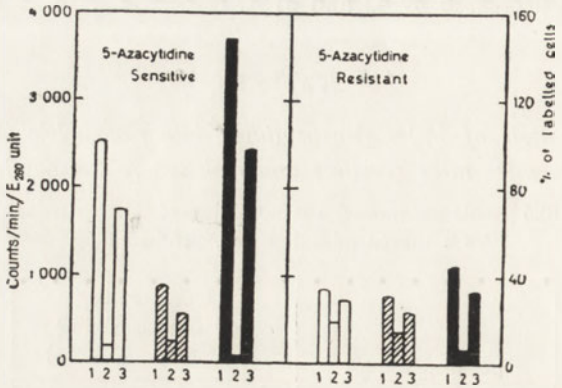

Fig. 1. Incorporation of [^{3}H]thymidine into leukaemic cells of 5-azacytidine-sensitive and -resistant AKR mice *in vivo*. The animals were killed 30 min. after application of [^{3}H]thymidine (16 mμmoles per mouse). Methotrexate (0.1 μmole single dose) and 5-fluoro-2′-deoxyuridine (2.65 μmoles) were injected 2.5 hr. before, and simultaneously with thymidine administration. Radioactivity of: 1, hot trichloroacetic acid extract; 2, cold trichloroacetic acid extract; 3, fraction of labelled cells as obtained by autoradiography. Injection of: (□), [^{3}H]thymidine alone; (▨), with methotrexate; (■), with 5-fluoro-2′-deoxyuridine.

extensively than in 5-azacytidine-sensitive animals. However, the amount of radioactivity which was present in the acid-soluble pool of the mutant cells was greater than that of the parent wild-type population. This holds also for the cells treated with 5-fluoro-2′-deoxyuridine or with methotrexate.

The phosphorylation of [7-^{14}C]*thymidine by particle-free supernatants from leukaemic cells of mice*

Products of thymidine phosphorylation are shown in Fig. 2. During 3 min. thymidine was anabolized to its 5′-monophosphate to a greater extent by the wild-type cell enzyme, whereas kinases from mutant cells were more active in forming 5′-diphosphate and 5′-triphosphate (Fig. 2A). It is also readily seen that the supernatants from 5-azacytidine-resistant leukaemic cells were more efficient in degrading thymidine to thymine. A small peak corresponding by its mobility to thymine riboside was clearly registered.

After 20 min. of incubation (Fig. 2B) more thymidine was degraded to thymine which was simultaneously anabolized to thymine riboside. These reactions were more pronounced in the case of the supernatant from mutant cells where the degradation of thymidine to thymine was enhanced; at the same time thymidine 5′-monophosphate was formed in very considerable amounts but thymidine 5′-diphosphate formation was very low, and no 5′-triphosphate was present. Figures

3 and 4 show an analogous experiment in which the thymidine metabolites have been eluted and counted. The results are in agreement with what has been demonstrated by scanning the chromatograms.

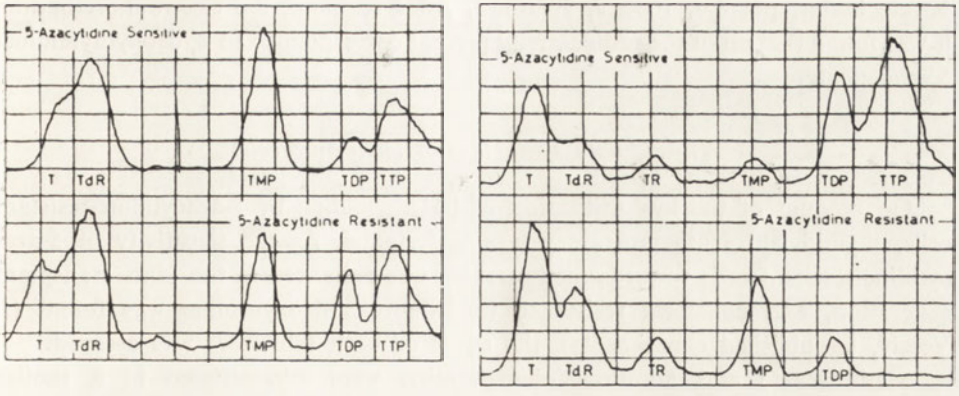

Fig. 2. Phosphorylation of [7-^{14}C]thymidine for: (A), 3 min., and (B), 20 min., by particle-free supernatants isolated from leukaemic cells of AKR mice sensitive and resistant to 5-azacytidine. Composition of the incubation mixture: 9 mM-ATP, 2 mM-$MgCl_2$, 50 mM-tris-HCl (pH 8.1) and 0.042 mM-[7-^{14}C]thymidine (0.2 μc). To 0.2 ml. of this medium, 0.3 ml. of the supernatant was added. Temp. 37°. The compounds were separated chromatographically and scanned in an automatic 2 π scaler.

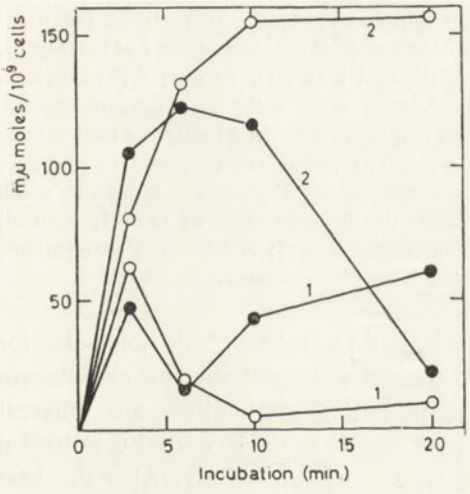

Fig. 3

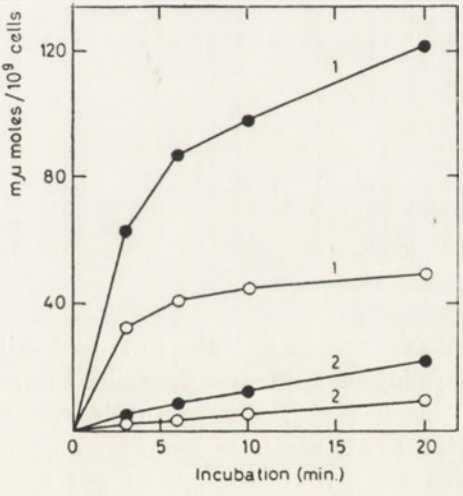

Fig. 4

Fig. 3. Time course of [7-^{14}C]thymidine phosphorylation by particle-free supernatants isolated from leukaemic cells of AKR mice (○), sensitive and (●), resistant to 5-azacytidine. Thymidine 5′-phosphates were eluted and counted after chromatographic separation. Results are expressed as mμmoles of thymidine (0.01 μc, 0.042 mM) phosphorylated by supernatant fraction equivalent to 10^9 cells. 1, Thymidine 5′-phosphate; 2, thymidine 5′-diphosphate and 5′-triphosphate.

Fig. 4. Time course of [7-^{14}C]thymidine splitting and subsequent ribosylation by particle-free supernatants isolated from leukaemic cells of AKR mice (○), sensitive and (●), resistant to 5-azacytidine. Conditions are the same as in Fig. 3. 1, Thymine; 2, thymine riboside.

To exclude the possibility that thymine riboside which was present at 20 min. of incubation was further phosphorylated, spots corresponding to 2′-deoxythymidine-5′-phosphate were eluted, and the eluates subjected to electrophoresis (0.02 M-borate buffer, pH 8.9, 1000 v, 90 min.). After scanning the electrophoretogram, it was found that all of the radioactivity present corresponded to 2′-deoxythymidine-5′-phosphate.

Thymidylate synthetase of leukaemic cells

On account of the low utilization of [^{3}H]thymidine by 5-azacytidine-resistant cells, thymidylate synthetase assay was undertaken. As the sensitivity of 5-azacytidine-resistant cells towards methotrexate was enhanced in the 20th transplant generation, and collateral resistance to 5-fluoro-2′-deoxyuridine was found (J. Veselý, unpublished experiments), the effect of both inhibitors was assayed.

Methotrexate and 5-fluoro-2′-deoxyuridine were administered in a similar fashion as for thymidine incorporation. Experimental results (Fig. 5) indicated

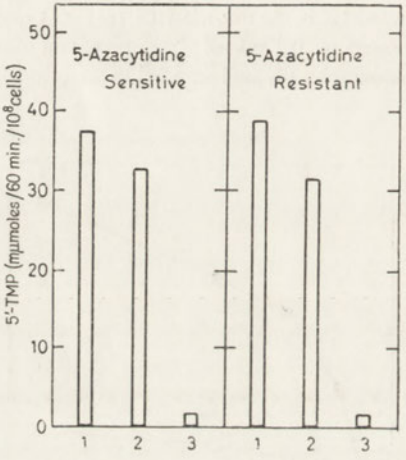

Fig. 5. Thymidylate synthetase from AKR leukaemic cells sensitive and resistant to 5-azacytidine. The reaction mixture (37°, 15 min.) contained the following substances at final concentrations indicated: [2-^{14}C]2′-deoxyuridine-5′-phosphate, 1.54 μM; $MgCl_2$, 2 mM; tris-HCl (pH 8.1), 70 mM; 5,10-methylene-tetrahydrofolic acid, 0.45 mM; mercaptoethanol, 64 mM; and formaldehyde, 20 mM. The final volume was 1 ml. The results are expressed as mμmoles of 2′-deoxythymidine-5′-phosphate formed by a cell equivalent of 10^8 cells after 60 min. 1, Control; 2, methotrexate treated; 3, 5-fluoro-2′-deoxyuridine treated.

that the enzyme activities from both cell variants do not differ. This holds also for thymidylate synthetase determined in mice treated with methotrexate or 5-fluoro-2′-deoxyuridine, although their synthetase inhibiting mechanisms are different [4, 10]. K_m constants plotted according to Lineweaver-Burk were 1.6×10^{-5} M and 1.4×10^{-5} M in mutant and in wild-type cells, respectively. Also the heat inactivation experiments revealed an analogous decline of the enzyme activities in the wild-type as well as in the mutant cells.

DISCUSSION

To obtain an insight into the population of 5-azacytidine-resistant leukaemic cells, autoradiography with [^{3}H]cytidine and with [4-^{14}C]5-azacytidine was undertaken. In the first instance all of the mutant and wild-type cells were labelled, and

the number of grains per average labelled cell was decreased (36.5%) in mutants (Table 1). The fraction of positive cells after [4-^{14}C]5-azacytidine treatment dropped in mutants by 45.6% (Table 2), while the number of grains per average cell decreased but slightly. Any comparison of the incorporation patterns of ^{3}H cytidine and of its ^{14}C-labelled aza-analogue is difficult not only because of their different specific radioactivities and energies but also owing to different amounts of radioactivity which were administered. Nevertheless, autoradiography indicated that 5-azacytidine-resistant leukaemic cells were impaired in their ability to anabolize cytidine as well as 5-azacytidine.

Direct evidence for the diminished incorporation of the analogue (Table 3) into ribonucleic acid confirmed autoradiographic data and suggested that, as in other antimetabolites [1], its decreased uptake was likely to be associated with the phenomenon of resistance in the cells studied.

The low utilization of [^{3}H]thymidine for the synthesis of deoxyribonucleic acid in 5-azacytidine-resistant leukaemic cells which was associated with the increased radioactivity in the acid-soluble pool (Fig. 1), and with the lack of difference in the thymidylate synthesis *de novo* between the two cell variants (Fig. 5) led us to investigate their capacity to phosphorylate exogenous [7-^{14}C]thymidine. The enhanced degradation of thymidine and of its 5′-diphosphate as well as 5′-triphosphate (Figs. 2 - 4) by the supernatants isolated from the mutant cells could explain these findings. However, the comparison of cell-free and whole cell systems is rather dubious and any extrapolation hardly conclusive.

Various biochemical lesions which have been found in 5-azacytidine-resistant leukaemic cells are difficult to reconcile with the supposed one-step mutation [16] underlying the change to the resistance. The mutational genetic basis of this change seems to be well founded because of its stability not only in leukaemic cells (J. Veselý, unpublished) but also in bacteria (V. Fučík, unpublished).

New metabolic traits observed in the mutant cells are probably due to a single genetic deviation which has become dominant in the cell population by the process of clonal selection.

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BIOCHEMICZNE MECHANIZMY OPORNOŚCI NA LEKI

III. RÓŻNICE W METABOLIZMIE LEUKEMICZNYCH KOMÓREK MYSZY WRAŻLIWYCH I OPORNYCH NA 5-AZOCYTYDYNĘ

Streszczenie

1. Komórki leukemiczne AKR oporne na 5-azocytydynę różnią się znacznie od szczepu dzikiego zmniejszonym włączaniem *in vivo* do kwasów nukleinowych następujących związków: cytydyny, tymidyny i 5-azocytydyny. Włączanie tymidyny do frakcji rozpuszczalnej w kwasach było zwiększone.

2. Wolny od cząstek supernatant z komórek opornego mutanta wykazywał zmniejszoną aktywność kinazy tymidyny, zaś rozkład tymidyny, tymidyno-5′-dwufosforanu i -5′-trójfosforanu był wyraźniejszy niż w komórkach leukemicznych szczepu wrażliwego na 5-azocytydynę. Aktywność syntetazy tymidylanowej była niezmieniona. Omówiono również przypuszczalne znaczenie tych wyników.

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BIRGIT HAGLUND and S. LØVTRUP

ANAESTHESIA AND WATER PERMEATION

Department of Zoophysiology, University of Umeå, Umeå, Sweden

1. It has been found that the local anaesthetics procaine and nupercaine in their physiologically active range depress the permeability coefficient for water through the membrane of the amphibian egg. 2. Butyl alcohol also influences the water permeation, but in this case the rate is enhanced. 3. The water permeation was determined by a D_2O-H_2O exchange method, using an automatic electromagnetical diver balance to register the exchange.

The conduction of nerve impulses is known to be blocked by narcotics and anaesthetics of various kinds. The process of impulse conduction has been shown (Hodgkin, 1951) to be associated with a transient increase in the permeability to Na^+ of the axon membrane. These facts have led to the assumption "that the membrane at which the local anaesthetics exert their blocking action is the axon membrane, and that it is the physical changes in the lipid part of the membrane due to the penetration of the drugs that prevents the increase in permeability to Na^+ and thereby block the impulse" (Skou, 1961).

A permeability effect of this kind may either be specific for the passage of ions, an idea seemingly supported by Skou (1961), or it may be of a more general nature, in which case the permeation of uncharged molecules should be affected as well. In order to test this point we have determined the influence of some substances with local-anaesthetic effect upon the passage of water through the cell membrane in the frog's egg.

MATERIAL AND METHODS

The experiments have been made on coelomic eggs of *Rana temporaria*, obtained through pituitary injections.

The isotope exchange method developed by Pigon & Zeuthen (1951) and Løvtrup & Pigon (1951) was used for the determination of the permeability coefficient for water, with the modification that the Cartesian diver balance was substituted by an automatic diver balance (Løvtrup & Larsson, 1965; Larsson & Løvtrup, 1966; cf. Fig. 1).

In preliminary experiments separate eggs were used as controls, but as it was found by experience that the permeability coefficient may vary both between and within batches, a different approach was consequently adopted according to which

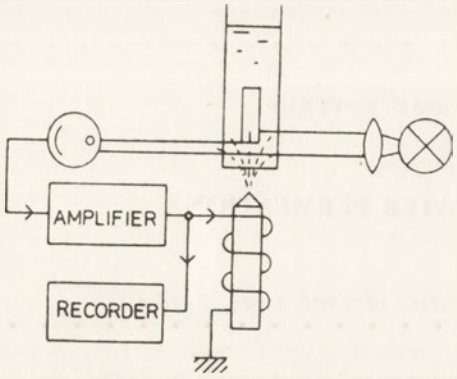

Fig. 1. Diagram of the operating principle of the automatic diver balance.

each egg was used as its own control. An egg was placed in Ringer solution equilibrated in the thermostat for at least half an hour; at zero time it was transferred to a small volume of Ringer solution (pH $\approx$ 7.5) in which the water phase contained 20% D_2O, and from here it was rapidly transferred to the diver, floating in the same medium. When the exchange curve had been recorded (Fig. 2) the egg was transferred

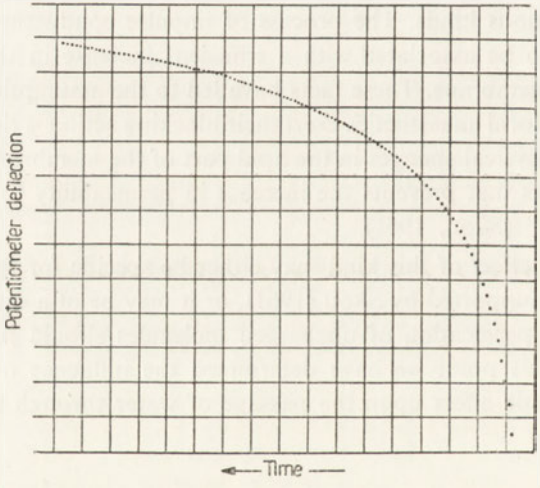

Fig. 2. The H_2O-D_2O exchange in a frog's egg, recorded by means of the automatic diver balance.

to Ringer solution for a time long enough for the D_2O to exchange with H_2O, and subsequently the procedure was repeated, this time with solutions containing the substance the effect of which was to be established. In order to minimize the amount of fluid transferred together with the eggs, the latter were handled with watchmaker's forceps, in which the tips were replaced by spoonlike extensions made of platinum.

The theory of the water permeation determinations has been developed by Løvtrup & Pigon (1951) and Løvtrup (1963).

RESULTS

Skou (1954a) has studied the potency of blocking the impulse conduction in the sciatic nerve of the frog (*Rana esculenta*) for a number of local anaesthetics and the narcotic, butyl alcohol. Among the former, which chemically are tertiary amines, we have chosen to work with the most active, nupercaine, and the least active, procaine, together with butyl alcohol. The results obtained with procaine and nupercaine are shown in Figs. 3a and 3b. The limits of the minimum blocking concentration (MBC) and minimum toxic concentration (MTC) are taken from Skou (1954a; 1954b), corrected to a pH value of 7.5. It is seen that in this range both anaesthetics reduce the rate of water permeation; one of the points in Fig. 3b deviates from the

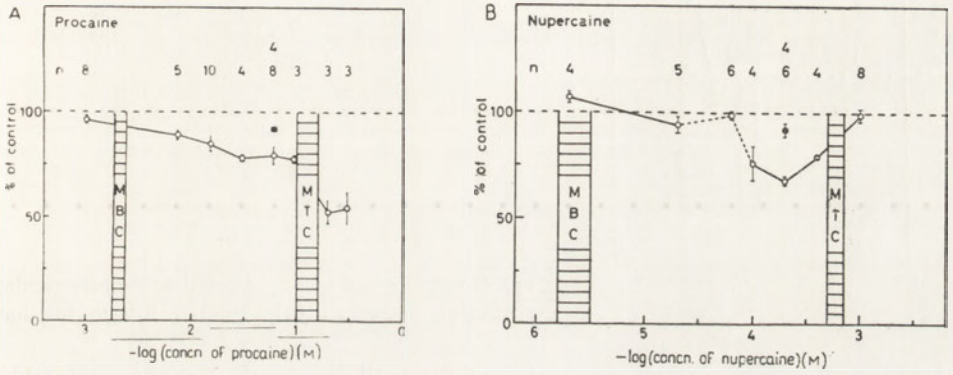

Fig. 3. The influence of A, procaine and B, nupercaine on the permeability coefficient to water. (○), Normal Ringer solution; (●), 7.5% Ringer solution; MBC, minimum blocking concentration; MTC, minimum toxic concentration.

general picture. Below the MBC there is no effect, at or above the MTC the results do not seem to agree. This point needs further investigation. During this part of the work it was found that the eggs could be classed in two groups, some with a low control value for the permeability coefficient E, and others with a high value, the limit lying around $E \approx 2 \times 10^{-4}$ cm. sec.$^{-1}$. The results in the figures are from the latter group; the eggs with low permeability were found to be insensitive to the anaesthetics. This finding seems to be in agreement with the observation that the effect of the substances studied were much slighter in 7.5% Ringer solution (cf. Figs. 3a and 3b), as the permeability to water is reduced in hypotonic solutions (Berntsson, Haglund & Løvtrup, 1964).

In contrast to these results, butyl alcohol (Fig. 4) increased the rate of water permeation and as the minimum toxic concentration was approached, we found $E \to \infty$, suggesting that the diffusion barrier to water was eliminated. This effect corresponds to the one observed with digitonin (Haglund & Løvtrup, 1965).

DISCUSSION

According to the classical theory of narcosis, developed by Höber, Lillie & Winterstein (cf. Heilbrunn, 1952) narcotic substances may act by decreasing the permeability of the plasma membrane. This contention has not stood up to experimental verification; both Jacobs & Parpart (1937) and Bärlund (1938) have found that

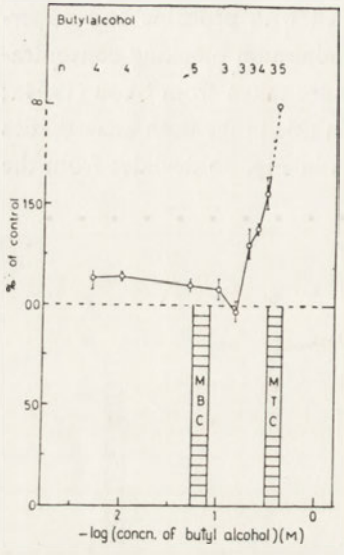

Fig. 4. The influence of butyl alcohol on the permeability coefficient to water. (○), Normal Ringer solution.

the effect of narcotics on the permeability of the cell membranes varies considerably, depending both on the permeating substance and the species investigated. The problem of narcosis or anaesthesia is obviously extremely evasive, as shown also by the great number of theories advanced at the colloquium on "Mécanisme de la narcose" (1951). In several instances these theories establish a correlation between certain chemical or physical properties and the narcotic action of various groups of substances, but this does not seem to bring us closer to a solution of the crucial problem, the basic mechanism.

To take up the study of the problem of the effect upon the water permeation may seem to return to an already abandoned theory. However, as has been shown recently, in many of the classical studies on permeability it has not been possible to distinguish between the effect of the mechanical properties of the cell cortex and the permeability of the cell membrane (Løvtrup, 1963). It may therefore be warranted to approach this question once more, with a method which circumvents this difficulty.

The results obtained with the local anaesthetics show that in the range of their physiological activity the permeability to water is reduced. This possible parallelity between the effects on water and Na^+ suggests that the permeability effect may be a general lowering of the surface area available for diffusion. The circumstance that the anaesthetics interfere with the lipid phase of the membranes (Skou, 1961)

and that the restriction of the water permeation also seems to depend upon the integrity of the lipid phase, does not directly support the contention that proteins or protein covered pores are controlling these permeation processes.

The results with butyl alcohol show that the permeation to water is slightly increased already before any influence on the nerve conduction is observable, and in the active range a substantial increase in the water permeation occurs. Presuming that this substance also inhibits the sodium permeation, it is obvious that the mechanism of action must be different from the one exerted by the anaesthetics. A possible explanation may be that butyl alcohol invades the lipid phase of the membrane, and there presents a vehicle for the diffusion of water, but not for Na^+, thus reducing the area available for the latter process. Further experiments are required to settle whether our observations are representative for the two groups of substances, anaesthetics and narcotics, respectively.

Our results do not disprove the contention that narcosis or anaesthesia is associated with a change in the permeability towards sodium, but they show that the mechanism of action cannot be the same for all substances exerting effects of this kind. Therefore, as the matters stand at the moment, the present observations may add one more complication to the problem of anaesthesia.

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ZNIECZULENIE A PRZEPUSZCZALNOŚĆ DLA WODY

Streszczenie

1. Stwierdzono, że prokaina i nuperkaina, miejscowe środki znieczulające, w stężeniach fizjologicznie aktywnych obniżają współczynnik przepuszczalności dla wody przez błonę jaj płazów.

2. Alkohol butylowy również wpływa na przepuszczalność dla wody, powodując jej zwiększenie.

3. Przepuszczalność dla wody określono metodą wymiany D_2O - H_2O; wymianę rejestrowano przy użyciu automatycznego, elektromagnetycznego pływaka zanurzeniowego.

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H. R. SCHÜTTE, W. MAIER and K. MOTHES

METHYLPUTRESCINE AS POSSIBLE PRECURSOR OF NICOTINE IN *NICOTIANA RUSTICA*

Institute for Biochemistry of Plants at Halle of the German Academy of Science in Berlin

Dedicated to Prof. J. Heller on the occasion of his 70th birthday

[Me-^{14}C,1-^{15}N]*N*-Methylputrescine was fed to *Nicotiana rustica*. The incorporation of ^{14}C and ^{15}N into nicotine was the same. It was found that only the methyl group was labelled. These facts permit the conclusion that the methylputrescine was incorporated intact without degradation.

Our present knowledge on the biosynthesis of nicotine is insufficient to state whether nornicotine represents a biogenetic precursor of nicotine or whether nicotine might be synthesized as well *via* a direct pathway (Mothes & Schütte, 1963; Leete, 1965; Rapoport, 1966). On account of the high-rate and quick incorporation of tritium into nornicotine and on the basis of experiments with [^{15}N]nornicotine, Tso & Jeffrey (1959; 1962) presume that this compound represents the biogenetic precursor of nicotine. The numerous transmethylations of a precursor of nicotine, especially with [Me-^{14}C]methionine (Ladesic & Tso, 1964; Dewey, Byerrum & Ball, 1954) and the demethylation of nicotine (Schröter, 1966), resulting e.g. in the recovery of the ^{14}C-labelled methyl group of nicotine in choline (Leete & Bell, 1959), only prove the lability of the *N*-methyl group and indicate a dynamic balance between nornicotine and nicotine in the plant. In principle, therefore, a transformation of nornicotine to nicotine is possible. The problem, however, remains unsolved whether cyclization requires a precedent methylation or whether it is favoured by it.

H. Rapoport and coworkers (Rapoport, 1966; Alworth & Rapoport, 1965; Alworth, Liebman & Rapoport, 1964; Alworth, Deselms & Rapoport, 1964) performed $^{14}CO_2$-assimilation experiments with *Nicotiana glutinosa* and compared the distribution and the radioactivity of the alkaloids. They reject the concept of an exclusive formation of nicotine by methylation of nornicotine, since nicotine was always found in higher amounts and with a higher radioactivity. Furthermore, the methyl group of nicotine should contain relatively high radioactivity if methylation be the last reaction step of biosynthesis. This, however, was not the case, neither in the work of the cited authors nor in our own experiments. After a 48 hr. assimila-

tion experiment with $^{14}CO_2$, using *Nicotiana rustica*, we found only 4.75% of radioactivity in the methyl group. Also the results of Mizusaki, Kisaki & Tamaki (1965) indicate a synthesis of nicotine without nornicotine as an intermediate.

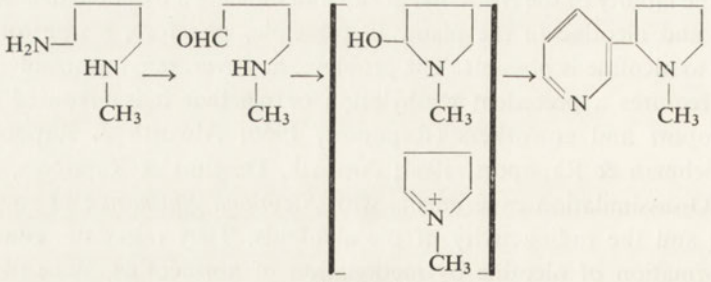
In connection with a rather extensive series of experiments on cyclization in the biosynthesis of alkaloids, which was started in our laboratory, we tried to find out using double-labelled [Me-^{14}C,1-^{15}N]*N*-methylputrescine (Maier, Neumann, Schröter & Schütte, 1966) whether the methyl group of nicotine is incorporated already before cyclization into the amino group of a pyrrolidine precursor.

Very good precursors appeared to be ornithine (Dewey, Byerrum & Ball, 1955; Leete, 1955; Leete, Gros & Gilbertson, 1964) and putrescine (Leete, 1958). After feeding [2-^{14}C]ornithine to *N. rustica* or *N. tabacum*, radioactive nicotine can be isolated in which radioactivity is distributed to the C-atoms 2 and 5 of the pyrrolidine ring. Therefore, a symmetrical intermediate is to be postulated for the synthesis. This appears to be in contradiction to the unsymmetrical incorporation of [2-^{14}C]-lysine into the closely related anabasine, where the total activity is located at C-2 of the piperidine ring (Leete, 1956). Schröter & Neumann (1966) found a considerable incorporation of [Me-^{14}C]α-*N*-methylornithine in nicotine (*N. rustica*), but they could recover only half of the radioactivity in the methyl group.

We fed [Me-^{14}C,1-^{15}N]*N*-methylputrescine to intact plants and to rooted leaf-cuttings of *N. rustica* with incubation periods of 28 and 96 hr. (expt. 1 and 2 in Table 1). The precursor was incorporated with a relatively high rate and the isolated nicotine was labelled only in its methyl group. The good consistency of the ^{15}N and ^{14}C incorporation rates indicates that the methyl group was incorporated into nicotine, without being split off before, and that in this case nornicotine certainly did not represent an intermediate.

If the precursors were, however, applied to the shoot (expt. 3) the ^{15}N and ^{14}C incorporation rates did not show a similar consistency. On the way from the shoot to the root a partial demethylation of methylputrescine might have taken place.

The methylated pyrrolidine ring might be formed *via* γ-methylaminobutyraldehyde and *N*-methyl-2-hydroxypyrrolidine, in which process an exclusive deamination of the δ-amino group must be presumed (Scheme 1).



Scheme 1.

Further experiments are necessary to see how these results correspond with the previous results indicating a symmetrical incorporation of ornithine and putrescine. Possibly there exist different ways of biosynthesis for nicotine.

Experimental procedure

[Me-^{14}C,1-^{15}N]*N*-Methylputrescine was obtained as follows. γ-Chlorobutyronitril was condensed with a solution of [^{14}C,^{15}N]methylamine in alcohol at 85°, and the formed [Me-^{14}C,1-^{15}N]γ-methylaminobutyronitril was reduced with sodium and alcohol to [Me-^{14}C,1-^{15}N]*N*-methylputrescine (Maier, Neumann, Schröter & Schütte, 1966).

Table 1

Incorporation of [Me-^{14}C,1-^{15}N]N-*methylputrescine into nicotine in* Nicotiana rustica

[Me-^{14}C,1-^{15}N]*N*-Methylputrescine hydrochloride, sp. act. 2.3×10^9 counts/min./m-mole; ^{15}N-excess in the CH_3-N group: 49.5%. The labelled compound was applied in the following way: expt. 1, through the roots, to two intact plants five months old, 8 mg. during 28 hr.; expt. 2, to two rooted leaf cuttings, three months old, 6 mg. during 4 days; expt. 3, *via* a cotton thread, to the lower part of the stem, 4 mg.

Expt. no.	Mode of application	Amount (mg.)	Nicotine isolated		^{14}C-Incorporation rate (%)	% of radioactivity in *N*-methyl	^{15}N-excess in nicotine	^{15}N-Incorporation rate*
			(mg.)	(counts/min./m-mole)				
1	To root	8	76	1.5×10^6	0.065	96	0.02	0.08
2	To rooted leaf cutting	6	26	1.43×10^7	0.62	95	0.16	0.64
3	By thread	4	40	3×10^6	0.13	94.5	0.07	0.28

* Related to pyrrolidine ring N.

The feeding experiments were done during the second half of February, as described in Table 1. After feeding, the plant material was dried at 50°, ground, and after alkalization with ammonia extracted for 12 hr. with chloroform. The extract was shaken with 1 N-H_2SO_4, the acid solution alkalized with NaOH and shaken with chloroform. After drying over sodium sulphate and evaporating under reduced pressure, the chloroform extract has been purified on a silica column. The chromatographically pure nicotine (thin-layer chromatography, silica G buffered at pH 8, solvent: chloroform - alcohol, 8:2, by vol.) was precipitated as picrate and crystallized from methanol to a constant specific radioactivity. For measuring the radioactivity a flow-counter of the firm Friesecke & Höpfner, Erlangen-Bruck, was used. In order to determine the radioactivity of the *N*-methyl group we transformed the nicotine picrate with an anion exchange column (Wofatit L 150) to the free base, which was heated with hydroiodic acid in the apparatus of Sirotenko (1955). According to the method of Alworth, Liebman & Rapoport (1964) and Alworth, Deselms & Rapoport (1964) the methyliodide formed was passed with nitrogen gas into an alcoholic solution of triethylamine. Finally, the methyltriethylammoniumiodide formed was twice crystallized from a small amount of isopropanol and its radioactivity determined. The method was checked with [Me-^{14}C]nicotine, which was chemically synthesized.

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METYLOPUTRESCYNA JAKO MOŻLIWY PREKURSOR NIKOTYNY U *NICOTIANA RUSTICA*

Streszczenie

1. Znakowaną [Me-^{14}C,1-^{15}N]*N*-metyloputrescynę podawano roślinom *Nicotiana rustica*.
2. Stwierdzono, że włączanie ^{14}C i ^{15}N było takie samo oraz że jedynie grupa metylowa była znakowana.
3. Fakty te wskazują, że metyloputrescyna została włączona do nikotyny bez uprzedniej degradacji.

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L. LEVENBOOK

ORGANIC ACIDS IN INSECTS

III. CITRATE OXIDATION AND TURNOVER DURING METAMORPHOSIS OF THE SOUTHERN ARMYWORM, *PRODENIA ERIDANIA*

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014, U.S.A.

Dedicated to Professor J. Heller in honour of his 70th birthday

1. The dynamics of citrate metabolism at various stages of the life cycle of the Southern Armyworm *Prodenia eridania* have been studied following the injection of [1,5-^{14}C]citrate. 2. At all stages of development [^{14}C]citrate was oxidized to $^{14}CO_2$ and converted to labelled glutamate. These reactions indicate the functioning of the Krebs cycle, and were employed to assess its activity from larva to adult. 3. Both cycle activity and total radiochemical yield decreased in the order: adult > larva = early pupa > developing (pharate) adult. Oxidation rates and radiochemical yields followed distinctly U-shaped curves during adult development. 4. Kinetic measurements on the larva indicated a miscible citrate pool of 8.1 μmoles, with a half-life of 1.6 hr. and a turnover rate of 3.5 μmoles/hr. 5. The data suggest that the decrease in citrate pool during metamorphosis is due to a decreased rate of citrate synthesis.

In an earlier paper of this series [8] the occurrence of high levels of citrate in the haemolymph of various insects was recorded. In all cases the haemolymph citrate concentration of larval insects was significantly higher than that of the corresponding adult stages of the same species. This finding, together with the fact that during adult development of the blowfly *Phormia regina* and the Southern Armyworm *Prodenia eridania* the citrate content of the entire organism declines markedly, suggested that such a decrease may well be characteristic of insect metamorphosis. The high citrate content of adult Diptera was confirmed by Zahavi & Tahori [17], who showed further that the citrate level increased during aging of the flies.

The purpose of the present work was to answer two questions: first, the extent to which citrate in the Southern Armyworm is in a dynamic state during metamorphosis, and secondly, whether the decrease in citrate level during adult development could be explained by a lowered rate of synthesis, an increased rate of utilization, or disproportionate changes in both. These two questions are clearly related, and can theoretically be answered by employing the same experimental approach, viz. a kinetic treatment of the fate of injected ^{14}C-labelled citrate, at various stages of development.

MATERIAL AND METHODS

Material. Southern Armyworm larvae were reared as previously described [6]. "Pupae"[1] were maintained at 30° in peat-moss at which temperature the time for adult development, from the prepupal stage to adult emergence, occupies about 200 hr. This time span is denoted as 100% in the Figures.

[1,5-^{14}C]Citric acid (Tracerlab, Inc.) was neutralized with NaOH, freeze-dried, and subsequently dissolved in H_2O to give a final concentration of about 0.2 M. The specific activity was about 1 mc/m-mole.

Methods. Lang-Levy type constriction pipettes with needle tips were constructed in the laboratory, calibrated with water gravimetrically, and used for injection. Larvae were first immobilized and relaxed by chilling in crushed ice; a loose silk ligature was tied round the base of a proleg, and [^{14}C]citrate injected into the foot-pad by oral pressure. As the pipette was then gradually withdrawn, the ligature was simultaneously tightened, thereby preventing any bleeding or loss of isotope. Operated larvae, when returned to room temperature, regained normal activity in 5 - 7 min.

A similar procedure was employed for subsequent collections of haemolymph; an adjoining foot-pad was incised, and the droplet of haemolymph which appeared upon gently squeezing the larva was aspirated directly into a 5 µl. Lang-Levy pipette, and the proleg then ligatured. In this manner it was possible to obtain up to five consecutive blood samples from the same larva.

Pupae and developing adults of known chronological age were injected under CO_2 narcosis. The intersegmental membrane between the 5th and 6th abdominal segments was first pierced dorso-laterally with a fine steel needle, and the injection made through the aperture. After injection the wound was sealed with a drop of molten paraffin. Judged by their subsequent ability to make abdominal movements when returned to air, pupae and developing adults also recovered from narcosis in 5 - 7 min. In all experiments to be described "zero time" is therefore taken as 5 min. following removal of the insect from either ice or CO_2.

Adult moths were injected in a manner similar to that for pupae except that the Lang-Levy pipette was inserted directly into the abdomen.

Collection and counting of $^{14}CO_2$. Injected insects were placed individually in a lucite chamber of about 50 ml. capacity containing a disc of moist filter paper, and the chamber inserted into a small constant temperature box maintained at $30 \pm 0.5°$. Air scrubbed free of CO_2 by washing with NaOH was sucked through the chamber at a rate of about 50 ml. per min. through two narrow-bore lucite tubes cemented into the top of the chamber. CO_2 in the effluent air was absorbed in 0.2 N-NaOH (containing carrier carbonate when necessary) by bubbling through a bead tower [1]. The absorbed CO_2 was precipitated with barium, and the $BaCO_3$ filtered onto tared sintered stainless-steel planchets prior to drying, weighing and

[1] The term "pupa" as opposed to pupa is employed loosely to denote a quiescent, non-feeding stage enclosed in its hardened pupal case. It thus includes the true pupal and developing (pharate) adult stages.

counting. All counts were converted to "infinitely thin" equivalents from a previously derived correction curve.

Following a period of CO_2 collection the insects were removed from the chamber; pupae were promptly frozen in methylcellosolve-dry ice prior to storage at -20° pending subsequent chemical analysis, whereas larvae were either treated similarly, or chilled in cracked ice for removal of a haemolymph sample before being replaced in the chamber for further CO_2 measurements.

Determination of larval haemolymph [^{14}C]*citrate specific activity.* The 5 µl. samples of haemolymph were deproteinized with 0.2 ml. of 1% (v/v) perchloric acid, and the extracts, after neutralization with KOH, applied onto a 0.25 cm. × 5 cm. column of Dowex-1 formate (cf. [12]). Twenty bed volumes of distilled H_2O were passed through the column to remove cations and neutral substances, and the acid components were eluted with 1.2 ml. of 6 N-formic acid. The entire formic acid eluate was collected as a single compact spot on a sheet of Whatman no. 1 chromatography paper by careful control of the flow rate and efficient drying of the formic acid on the paper. A number of extracts was spotted onto a sheet of paper and the organic acids separated by ascending chromatography in ether - formic acid - H_2O (13:3:1, by vol.) [12]. At the end of the run the paper was dried for several hours at room temperature and final traces of formate removed by autoclaving for 5 min. The individual acids were located by spraying with bromthymol blue and the yellow citrate "spot" changed to blue by exposure to NH_3 vapour, in order to minimize quenching in the Packard scintillation counter. The citrate "spots" were cut out, and the paper discs placed in scintillation vials for counting [16, 2]. In order to obviate any "positioning error" each sample was counted three times and the values averaged. The maximum variation attributable to a "positioning effect" did not exceed 5%.

After counting, the paper discs were removed from the scintillation fluid and dried. They were then cut into small pieces and extracted overnight in 1.5 ml. of 0.1 N-HCl, and for an additional hour on a shaking machine. After centrifugation, 1.0 ml. samples were analysed for citrate [8].

Determination of pupal and adult moth [^{14}C]*citrate specific activity.* Isolation of [^{14}C]citrate from pupae, developing adults and moths for the determination of its specific activity were performed in a manner similar to that described above, but with the following differences: (1) individual insects were first homogenized in 3.0 ml. of 4% $HClO_4$ and the entire extraction procedure suitably scaled upwards; (2) 1/10th of the final extract (about 0.4 ml.) was applied to the ion-exchange column; (3) the citrate "spots" were eluted immediately after their location on the chromatograms and 0.1 - 0.2 ml. samples were counted on stainless steel planchets at "infinitely thin" in a windowless proportional gas-flow counter. A factor relating these measurements to those obtained by scintillation counting was experimentally determined.

Determination and counting of [^{14}C]*glutamate.* The sum of glutamate plus glutamine, henceforth termed glutamate, was determined manometrically on 1.0 ml. samples of insect extracts according to Levenbook [7]. To measure the radioactivity

of the $^{14}CO_2$ released during the decarboxylation reaction, the manometers were removed from the waterbath *without* opening the stopcocks. The end of the closed manometer limb was connected with Tygon tubing to a specially designed glass U-tube trap containing 5 ml. of 0.2 N-NaOH and carrier carbonate. The manometer stopcock was now opened and the $^{14}CO_2$ swept out of the reaction vessel by a stream of N_2 introduced through the vented side arm. The expelled gas ascended as a stream of bubbles through the alkali trap. After 5 min. of flushing the alkali was collected through a teflon stopcock at the bottom of the trap, and the apparatus rinsed twice with 5 ml. portions of distilled H_2O. $Ba^{14}CO_3$ was then precipitated in the usual manner and counted.

RESULTS

Larvae

Oxidation of [^{14}C]*citrate to* $^{14}CO_2$. The time course of $^{14}CO_2$ production by [^{14}C]citrate injected *P. eridania* larvae indicates that citrate is rapidly and extensively oxidized. Typical progress curves for 3 individual larvae injected with varying amounts of labelled citrate are shown in Fig. 1A. It will be noted that the extent of oxidation is independent of the amount injected, some 60% of the [^{14}C]citrate counts appearing in the expired $^{14}CO_2$ 18 - 22 hr. after injection. By this time less than 10% of the administered counts remained in the larval citrate pool. Analysis

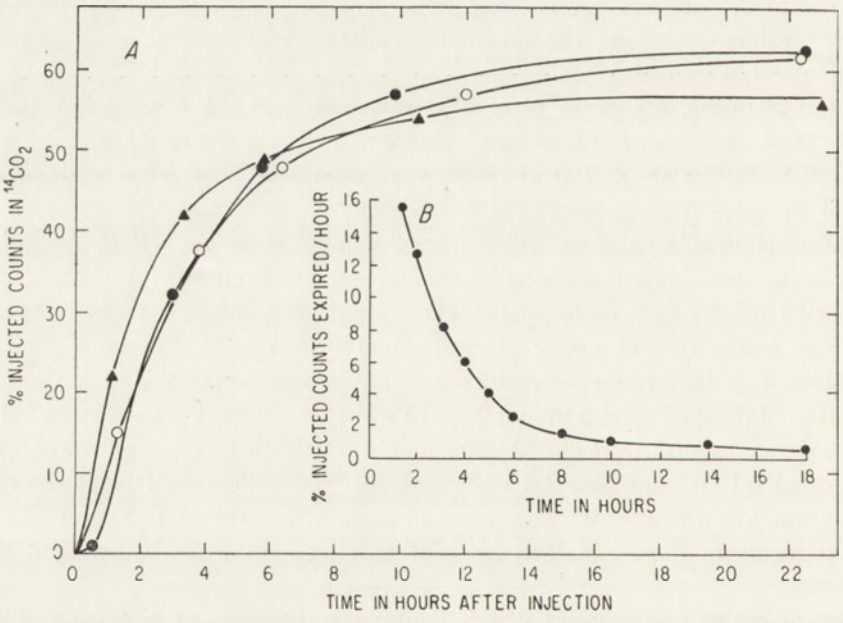

Fig. 1. *A*, Time course for $^{14}CO_2$ formation by *P. eridania* larvae injected with [1,5-^{14}C]citrate. (●), 365 × 10^3 counts/min.; (○), 650 × 10^3 counts/min.; (▲), 1025 × 10^3 counts/min. *B*, Rate of $^{14}CO_2$ formation computed from the mean of the curves depicted in *A*.

of the excrement and filter paper in the chamber at the end of three such experiments revealed that only 1.7 - 5.3% of the injected counts were excreted.

To avoid complications due to incomplete initial mixing of the injected tracer with the miscible citrate pool, the earliest $^{14}CO_2$ measurements were generally made no sooner than 1 hr. after injection. However, as suggested by the early phases of the progress curves, and from a few measurements made during this first hour but not presented here, it appears that the mixing time does not exceed 30 min. and in some larvae, perhaps particularly active individuals, could be as short as 10 - 15 min. Within the limits of these uncertainties, the rate of citrate oxidation is maximal during the first hour after injection and declines asymptotically thereafter (Fig. 1B).

The size of the miscible citrate pool and its rate of turnover. The finding that the rate of [^{14}C]citrate oxidation is proportional to the amount injected suggests that the labelled tracer is mixing with an endogenous pool which is being turned over according to first order kinetics. This conclusion is supported by the observation that the log of decrease in haemolymph specific activity with time approximates a straight line (Fig. 2); extrapolation of such a plot to zero time yields a value for

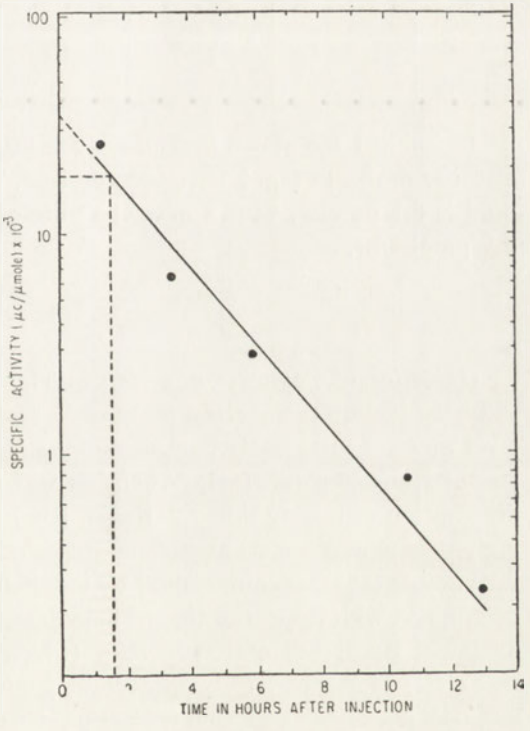

Fig. 2. A typical experiment demonstrating the change in larval haemolymph citrate specific activity with time after injection of 0.94 μmole [1,5-^{14}C]citrate (333 000 counts/min.) into the Southern Armyworm. The experimental curve has been extrapolated to zero time, and the half-life based on the ordinate value thus determined.

the theoretical citrate specific activity (C_0) which would have been observed had the injected citrate mixed instantaneously and completely with the miscible citrate pool.

From a knowledge of C_0, the specific activity (C_i) and the amount (a) of the injected tracer, the miscible pool (P) can be calculated from the formula [15]:

$$P = a\,(C_i/C_0 - 1)$$

Furthermore, the time for C_0 to decrease by a half ($t_{1/2}$) is, by definition, the biological half life of the citrate pool, and $\log_e 2/t_{1/2}$ = the turnover rate K, where K is the fraction of miscible pool turned over per hour. The rate of citrate turnover, in μmoles/hr. is then PK.

Table 1

Citrate pool turnover in the Southern Armyworm larva

Stock citrate injected contained 0.2 μmoles/μl., with a specific activity (counted on paper discs in a scintillation counter) of 374 000 counts/min./μmole. For further details see text.

Larva no.	Vol. injected (μl.)	Intercept C_0 (counts/ min./μmole)	Miscible pool P (μmoles)	$t_{1/2}$ (hr.)	K (fractional pool/hr.)	KP (μmoles/hr.)
1	3.8	26 200	10.1	1.9	0.37	3.7
2	2.4	25 200	6.6	1.6	0.43	2.8
3	4.7	37 100	8.5	1.7	0.41	3.5
4	3.8	40 000	6.4	1.3	0.53	3.4
5	3.8	30 100	8.7	1.5	0.46	4.0
Mean	—	—	8.1	1.6	0.44	3.5

In Table 1 the above parameters are listed for five larvae for which data such as those shown in Fig. 2 have been derived. The mean value for the miscible citrate pool is 8.1 μmoles, with a mean half-life of 1.6 hr. and a mean turnover rate of 3.5 μmoles/hr.

"*Pupae*"

Oxidation of [^{14}C]*citrate to* $^{14}CO_2$. The rate of respiratory metabolism of the virtually immobile *P. eridania* "pupa" during adult development attains a low level which is fortunately unchanged by any "injury metabolism" induced by experimental injection (cf. [11]). Attention was first directed towards determining the distribution or mixing time of injected [^{14}C]citrate throughout the "pupa". Accordingly, pupae and insects at various stages of adult development were injected with 0.29 μmole (443 000 counts/min.) by infinitely thin plating of the tracer, and $^{14}CO_2$ determined with respect to time. These data, presented in Fig. 3, indicate that after an initial lag period of 10 - 30 min. (which is presumed to represent the mixing time), the course of $^{14}CO_2$ production attains a maximal and more or less linear rate, the magnitude of which is clearly related to stage of adult development. There is also some indication that the mixing time may be inversely proportional to rate of citrate oxidation.

A more detailed study of the relationship between rate of citrate oxidation and development is shown in Fig. 4A. In these experiments animals of known age were injected with 0.29 μmole of [^{14}C]citrate and the $^{14}CO_2$ determined for 1 hr. at 30°. The curve is markedly U-shaped during adult development; some 20% of the

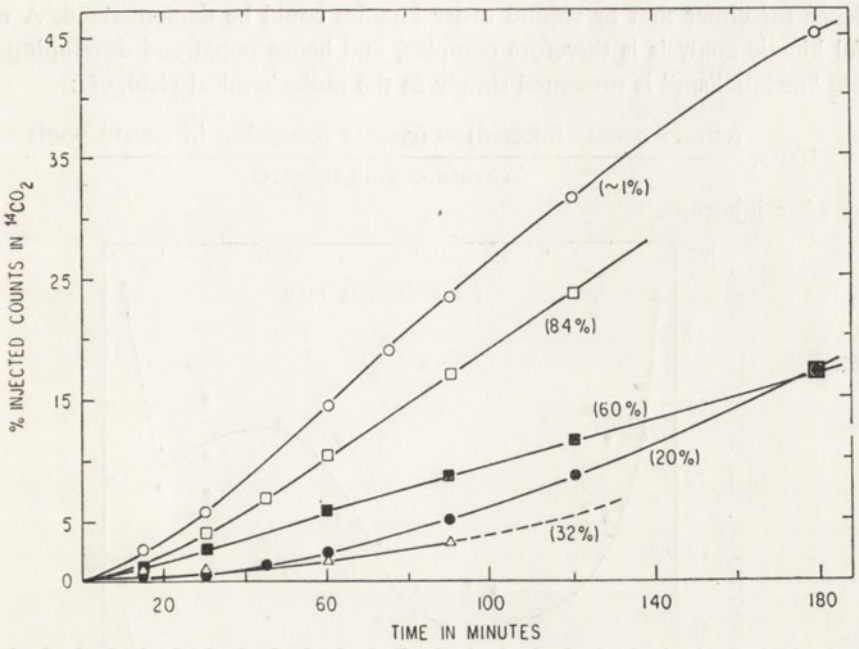

Fig. 3. Time course of $^{14}CO_2$ formation by pupae (~1%) and developing adults of *P. eridania* after injection with [1,5-^{14}C]citrate. Figures in parentheses indicate the percentage of total adult development time commencing from the initiation of pupation

injected counts appear in the expired $^{14}CO_2$ at the onset of metamorphosis, and this value decreases to ca. 2% at about a third of adult development time, gradually increasing thereafter to about 15 - 16% shortly before adult emergence.

Due to the mixing time lag in the rate of citrate oxidation, the above figures are obviously lower than the *true* rates obtaining during the hour, i.e. if mixing had been instantaneous. The necessary upward corrections, however, probably do not exceed 10 - 15% for the higher values, and 30 - 40% for the lower ones, and hence would not materially alter the over-all shape of the citrate oxidation curve.

Citrate utilization. Metamorphosis of the Southern Armyworm is accompanied by a marked diminution in body weight — in our stock, from a mature larva weighing about 0.9 g. to a pupa of about 0.3 g. (cf. [13]), and by extensive morphological reconstruction. Thus the small size, absence of a discrete haemolymph phase, and progressive decrease in citrate content during adult development all combine to make it impractical to obtain from a single individual consecutive samples of the internal milieu for citrate determination. Each "pupa", therefore, analysed in its entirety, furnishes but a single sample.

In a series of preliminary experiments a number of insects at different stages of adult development was injected with varying amounts of [^{14}C]citrate and the citrate specific activities determined at 20, 40, 60 and 120 min. thereafter. It should be noted that in these cases the endogenous citrate pool was significantly increased by the amount of labelled citrate injected. Unlike the larval data, no consistent

evidence for either first or second order kinetics could be demonstrated. A meaningful kinetic analysis is therefore complex, and hence pupal and developing adult citrate "metabolism" is presented simply as the radiochemical yield, *viz*:

$$100 \times \frac{\text{(citrate counts injected)} - \text{(counts remaining in citrate pool)}}{\text{citrate counts injected}}$$

1 hr. after injection.

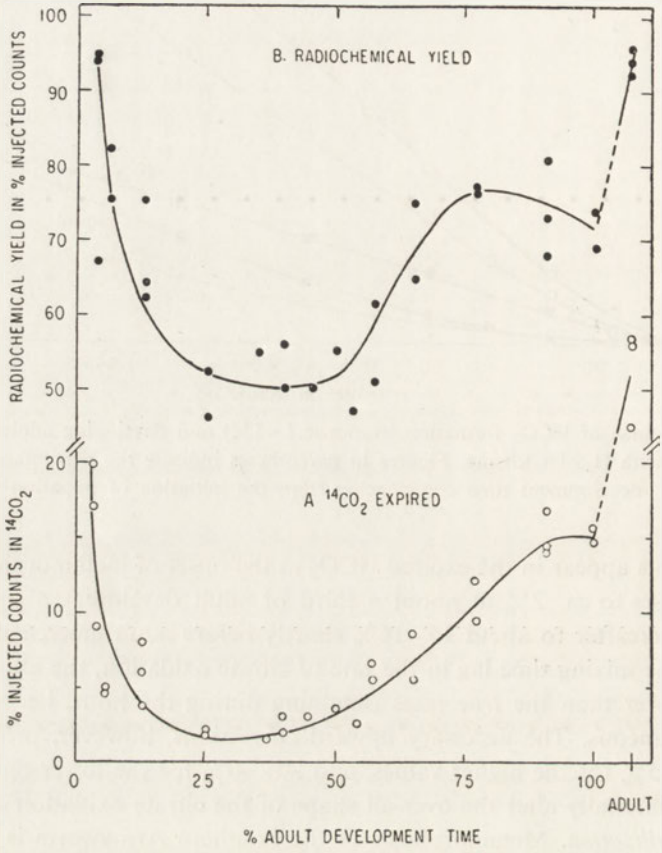

Fig. 4. *A*, $^{14}CO_2$ expired per hour and *B*, radiochemical yield (for definition see text) following injection of 0.29 μmole (443 000 counts/min.) [1,5-^{14}C]citrate into *P. eridania* at various stages of development.

The radiochemical yield during adult development is shown in Fig. 4B. There is considerable scatter in the experimental points, each of which includes any errors in citrate and radioactivity measurements in addition to possible differences in adult development time and normal biological variations. Nevertheless, this curve is also distinctly U-shaped, and indicates that the rate of citrate utilization is maximal at the onset of metamorphosis, decreases to a minimum at between 30 - 40% of adult development time, and then increases during the latter half of the period. There appears to be little change in the rate of citrate utilization during the 1 - 2 days prior to adult emergence.

The rates of citrate oxidation and overall utilization thus follow similar patterns during *P. eridania* adult development, although the magnitude of the relative changes is considerably greater with respect to oxidation than for utilization. During the last third of adult development the continuous increase in rate of citrate oxidation is not reflected in a proportional change in citrate utilization.

Conversion of citrate to glutamate. The Krebs cycle is important not only for the oxidation of citrate but also for its conversion to amino acids (cf. [5, 4]), and hence it would be anticipated that label should appear in glutamate following the administration of tagged citrate to *P. eridania*. Employing [1,5-^{14}C]citrate it would also be expected that C atoms 1 and 5 of glutamate would become the most rapidly labelled; but continued cycling of 4-carbon intermediates around the cycle will result in unequal labelling of the glutamate carboxyl groups [14]. Since neither the degree of cycling nor the extent of unequal labelling is known in the present case, it appears justifiable to employ the easily measured specific activity of the α-C of glutamate as a measure of this conversion.

The specific activity of the α-C of the combined glutamate-plus-glutamine pool 1 hr. after injection of 0.29 μmole (443 000 counts/min.) of [^{14}C]citrate at various stages of adult development is shown in Fig. 5. As might be expected, the

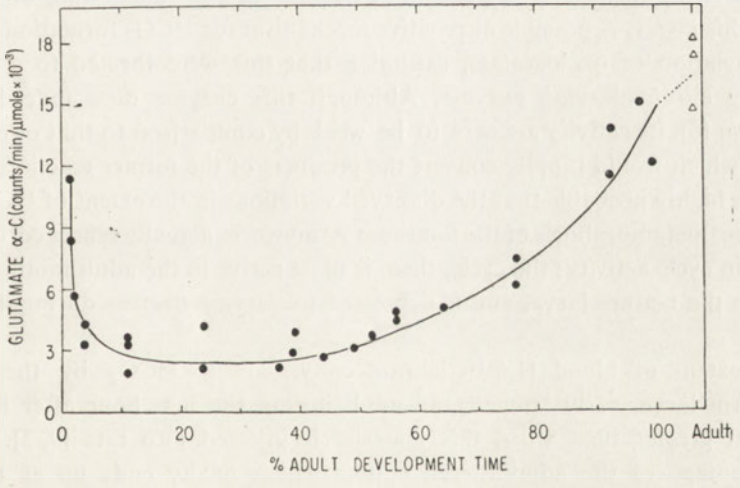

Fig. 5. Conversion of labelled citrate to glutamate one hour after injection of 0.29 μmole (443 000 counts/min.) [1,5-^{14}C]citrate into *P. eridania* at various stages of development

curve is again U-shaped although less so than the curves for citrate oxidation or radiochemical yield. The conversion of citrate to glutamate, undoubtedly *via* the Krebs cycle and subsequent transamination of α-oxoglutarate, is clearly demonstrated. Unfortunately, the data do not permit of any meaningful kinetic determinations, and, in particular, no rates of conversion can be calculated.

Adult moth

[^{14}C]Citrate metabolism has been studied in only three adult moths aged less than 12 hr. after emergence. However, the data for these individuals were in good agreement, and of unexpected interest.

The results for the rates of citrate oxidation and utilization, and the counts in the α-C of glutamate are shown on the right hand parts of the graphs beyond 100% adult development time in Figs. 4 and 5, respectively.

All the imaginal values are markedly higher than those for the virtually fully developed adult still enclosed in its pupal case. Thus, the rate of citrate oxidation is increased over threefold, about half the injected citrate being oxidized to $^{14}CO_2$ in the first hour. The radiochemical yield during this period is over 95%, i.e. only about 5% of the injected counts remain in the citrate pool. The total citrate pool of two of the three moths 1 hr. after injection was 0.19 and 0.28 μmoles, i.e. less than the amount of citrate injected, and in the third individual it was only slightly greater, 0.34 μmole.

DISCUSSION

The formation of labelled CO_2 following injection of [1,5-^{14}C]citrate into the Southern Armyworm affords plausible evidence for the functioning of the Krebs cycle in this insect. A possible alternative mechanism for $^{14}CO_2$ formation is through decarboxylation of oxaloacetate, assuming that this were formed to a significant extent by citrate-cleaving enzyme. Although this enzyme does indeed occur in *P. eridania* [6], its activity appears to be weak by comparison to that of condensing enzyme, which would rapidly convert the products of the former reaction to citrate. Thus it is highly probable that the observed variations in the extent of $^{14}CO_2$ formation during metamorphosis of the Southern Armyworm actually reflect corresponding changes in cycle activity; the cycle, then, is most active in the adult moth, somewhat less so in the mature larva, and is depressed to varying degrees during adult development.

The extent of blood [1,5-^{14}C]citrate conversion to $^{14}CO_2$ by the Southern Armyworm larva, early "pupa" and adult during the first hour after injection is somewhat greater than when this material is injected into rats [9, 3]. However, the percentage of the administered counts which finally ends up as respiratory $^{14}CO_2$ is about the same in rat and insect, *viz.* 50 - 60%.

As already mentioned, the conversion of labelled citrate to glutamate, as measured by the specific activity of the glutamate α-C one hour after injection cannot be subjected to a meaningful kinetic treatment. Similar considerations apply to the kinetics of $^{14}CO_2$ formation. Inspection of the Krebs cycle [14] reveals that of the two molecules of CO_2 released per turn of the cycle, the first comes from C atom 3 of citrate, and the second from C atom 1. In considering the fate of a citrate molecule entering the cycle, it would thus appear that only the second molecule of CO_2 should be radioactive. However, due to randomization of the terminal carboxyl

groups between succinate and fumarate, the resulting molecule of citrate formed by condensation of non-radioactive acetyl-CoA with the oxaloacetate residue will be labelled in positions 1 and 3, with each of these C atoms having a specific activity half that of the original [^{14}C]citrate atoms. Hence in subsequent turns of the cycle, both molecules of CO_2 will be radioactive, but of progressively diminishing specific activities. The radiochemical yield of $^{14}CO_2$ thus depends not only on the activity of the cycle *per se*, but also on the extent of competition between the tracer citrate and *de novo* synthesized and/or endogenous citrate molecules for entry into the cycle.

The present findings leave no doubt that citrate in *P. eridania*, from larva to adult, is an actively turning over metabolite. From radioactivity measurements the size of the miscible citrate pool of the larva is estimated to be about 8.1 μmoles, a value in excellent agreement with that determined by actual chemical analysis [8]. The kinetic data indicate that almost half this pool is replaced per hour which, for a constant pool size, implies that this is the rate of utilization and synthesis. Although comparable data for other stages of *P. eridania* development are lacking, measurements of the expired $^{14}CO_2$ and radiochemical yield throughout metamorphosis suggest a low turnover rate during the period from 20 to 60% of adult development time, and an increasing rate thereafter. Significantly, the citrate pool of the insect decreases markedly during this same period; a decreasing rate of utilization (i.e., $^{14}CO_2$ and radiochemical yield) accompanied by decreasing pool size can be explained only by a disproportionately slow rate of citrate synthesis during the earlier stages of adult development. In view of the higher $^{14}CO_2$ production and radiochemical yield at the later stages of development, and particularly in the adult, it would seem that both utilization and synthesis increase concurrently if the pool remains constant.

The high rate of citrate metabolism by the newly emerged adult as compared with the essentially fully developed adult still enclosed in its pupal case is quite remarkable. It would seem improbable that this difference is attributable to a synthesis of Krebs cycle enzymes, or to an assumed rate-limiting enzyme, in the short period of about 12 hr. separating the two "stages". Rather, it would appear that the act of adult eclosion stimulates some triggering effect on the entire Krebs cycle. An analogous situation is observed in the housefly, where, irrespective of changes in the myosin content, a several-fold increase in myosin ATP-ase activity occurs immediately after adult emergence as compared with the corresponding titre just prior to eclosion [10]. The sluggish behavior of newly emerged adult insects thus belies their covert high metabolism.

It should like to thank Miss Barbara Burkett for her competent technical assistance.

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KWASY ORGANICZNE U OWADÓW

III. UTLENIANIE CYTRYNIANU I JEGO PRZEMIANY PODCZAS METAMORFOZY POCZWARKI *PRODENIA ERIDANIA*

Streszczenie

1. Zbadano dynamikę metabolizmu cytrynianu w różnych stadiach cyklu życiowego *Prodenia eridania* po injekcji znakowanego [1,5-^{14}C]cytrynianu.

2. We wszystkich stadiach rozwoju [^{14}C]cytrynian ulegał utlenieniu do $^{14}CO_2$ i przekształceniu w znakowany glutaminian. Reakcje te, wskazujące na występowanie cyklu Krebsa, zostały wykorzystane do oznaczenia aktywności cyklu w czasie rozwoju owada od stadium larwy do formy dojrzałej.

3. Zarówno aktywność cyklu jak i odzysk całkowitej radioaktywności maleją w następującej kolejności: forma dojrzała > larwa = wczesne stadium poczwarki > rozwijająca się forma dorosła (pharate). Wartości utleniania i odzysk radioaktywności układają się, w czasie rozwoju formy dorosłej, w charakterystyczną krzywą o kształcie litery U.

4. Kinetyczne oznaczenia wykazały u larwy istnienie reaktywnej puli cytrynianu o wartości 8,1 μmola, czasie połowicznego rozkładu 1,6 godz. i szybkości obrotu 3,5 μmole/godz.

5. Powyższe dane sugerują, że zmniejszenie puli cytrynianowej w czasie metamorfozy spowodowane jest zmniejszeniem szybkości syntezy cytrynianu.

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D. E. GREEN and KRYSTYNA KOPACZYK

THE REPEATING UNITS OF MEMBRANES

Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin, U.S.A.

In honour of Professor Josef Heller

The mitochondrial membranes, inner and outer, are built from macromolecular repeating units which are lipoprotein in nature. Both membranes contain multiple species of repeating units and multiple enzymic activities. The electron transfer chain is associated with the basepieces of the repeating units of the inner membrane. The lipid-containing complexes of the chain form vesicular membranes. The lipid-free complexes aggregate randomly, but the capacity for vesicularization is restored by reintroduction of lipid. The specific localization of lipid in the repeating units leads to the formation of a two-dimensional and oriented membrane continuum. The membrane state is required for molecularization of the repeating units, and the molecular state, for demonstration of enzymic activities. Reconstitution requires that the interacting repeating units be in the same vesicular membrane system. This is achieved first by disaggregating membranes in which these complexes are contained, and then by reformation of a membrane in which the several species of repeating units are present. Mitochondrial swelling reflects rearrangements in the membrane modality, from a tube-like to a balloon-like structure. This transition can be induced by changes in the moiety of the repeating units.

A large body of evidence has been accumulated for the thesis that biological membranes are built up by the fusion or nesting of macromolecular repeating units, lipoprotein in nature, and that the fused membrane continuum is one particle thick [10, 11]. According to this thesis, the repeating units are the only structured elements present in the membrane. The properties of membranes are, thus, expressions of the properties of the repeating units. In a given membrane, all the repeating units have the same or at least complementary form and size, but there are usually multiple species of repeating units [11]. These different species account for the multiple enzymic capabilities of a membrane.

The present communication is concerned with some of the consequences of the repeating unit concept for membrane structure in general, and for the mitochondrial membrane, in particular. The questions to be raised are: why is phospholipid required for certain integrated enzymic activities of the mitochondrion?; how does phospholipid influence membrane formation?; what is the nature of the reconstitution of the electron transfer chain?; and what is the connection between mitochondrial swelling and the state of the repeating units of the mitochondrial inner membrane?

Essentiality of phospholipid for electron transfer activity and membrane formation

The integrated enzymic activities of the mitochondrial inner membrane, such as electron transfer and oxidative phosphorylation, have been shown to require the presence of phospholipids [2, 7]. The suggestion has been made [4] that lipid determines the conformation and the structural state of proteins in a membrane; hence, its essentiality for activity. However, there is no evidence that the removal of lipid leads to any intrinsic change in the spectroscopic properties of any of the oxidation-reduction components of the electron transfer chain, or in the electron spin resonance characteristics of copper and non-haeme iron [24]. If lipid makes an important contribution to the conformation of any of the complexes of the electron transfer chain, then the removal of lipid should have a profound influence on the visible, ultraviolet and microwave spectra. The absence of such an influence indicates that the requirement of lipid for activity has to be explained in terms other than conformational.

Two important developments have compelled a reexamination of the problem of lipid essentiality for the activity of the electron transfer chain: (a) the demonstration that the complexes of the electron transfer chain are identical with the basepieces of the repeating units of the inner membrane of the mitochondrion [12]; and (b) the essentiality of lipid for the formation of vesicular membranes by the disaggregated basepieces of the repeating units [19].

The individual repeating units of the mitochondrial inner membrane are made up of three sectors [6]: the basepiece or membrane-forming sector; the headpiece which does not contribute to the membrane continuum, and the stalk which attaches basepiece to headpiece (Fig. 1). Each of the complexes of the electron transfer chain corresponds in size and mass to the basepieces of the repeating units of the inner membrane (Table 1). The complexes, as isolated from the mitochondria, contain

Table 1

Molecular weights of the complexes of the electron transfer chain

Data by D. E. Green & A. Tzagoloff [12].

	Computed molecular weight*	Molecular weight of a sphere, d = 1.25
Complex II	340 000	80 Å 202 000
Complex III	418 000	90 Å 287 000
Complex IV	260 000	100 Å 394 000
	360 000	

* Normalized for 36% lipid from light scattering and composition data.

about 27% of phospholipid [8] and form vesicular membranes when freed of bile salts by dialysis or by Sephadex treatment. The lipid-free complexes, which can be prepared by acetone treatment of the preparations [3] or by refractionation in high concentrations of cholate [24], lack this property. They polymerize in a random

fashion but when lipid is reintroduced, the capacity for vesicularization is fully restored [19]. These findings suggest that the essentiality of lipid for electron transfer is connected with the essentiality of lipid for membrane formation. Furthermore,

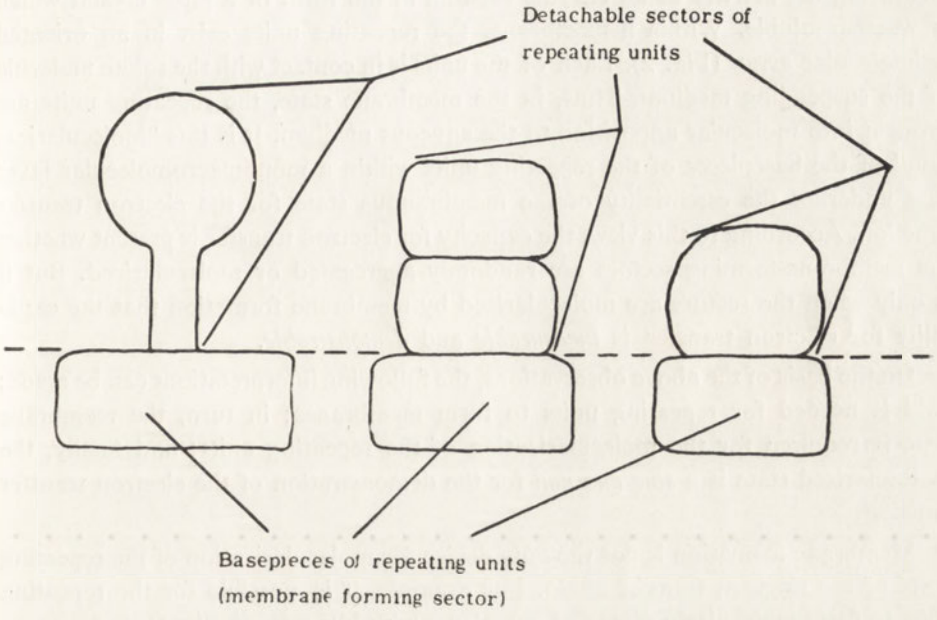

Fig. 1. Diagrammatic representation of different possible forms of repeating units. Taken from D. E. Green & J. Perdue [11].

they indicate that the membrane represents the preferred state for the exercise of the electron transfer function.

The aggregates formed by the lipid-free repeating units are random three-dimensional conglomerates, which may be looked upon as bulk phases (Fig. 2).

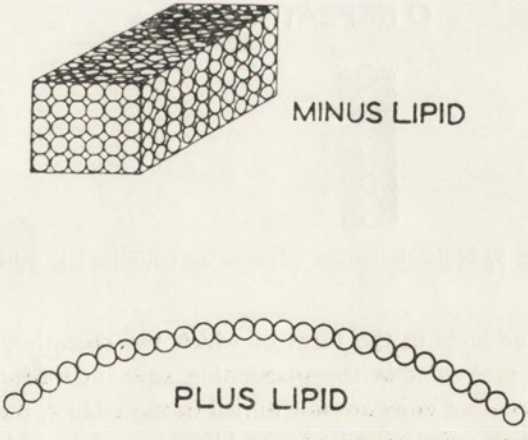

Fig. 2. Repeating units in a random three-dimensional network or bulk phase (MINUS LIPID), and in an oriented membrane or unimolecular film (PLUS LIPID).

Enzymic activities are greatly diminished and often cannot be demonstrated in such bulk phases, because the vast majority of the active sites of the repeating units are buried in the interior of the conglomerates. It would be comparable to measuring the activity of an enzyme existing in the form of a large crystal which is water-insoluble. Within a membrane, the repeating units exist in an oriented unimolecular array (Fig. 2). Each of the units is in contact with the solute molecule of the suspending medium. Thus, in the membrane state, the repeating units are brought into molecular apposition to the aqueous medium. It is this "molecularization" of the basepieces of the repeating units within a monomacromolecular layer that underlies the essentiality of the membranous state for the electron transfer function. According to this view, the capacity for electron transfer is present whether the membrane-forming sectors are randomly aggregated or molecularized. But it is only when the sectors are molecularized by membrane formation that the capability for electron transfer is *measurable* and *demonstrable.*

On the basis of the above observations, the following interpretations can be made: lipid is needed for repeating units to form membranes; in turn, the membrane state is required for the molecularization of the repeating units; and finally, the molecularized state is a *sine qua non* for the demonstration of the electron transfer function.

Membrane formation is not the only device for molecularization of the repeating units of the electron transfer chain. For example, it is possible for the repeating units to form molecularly dispersed complexes with bile salt micelles (Fig. 3). These

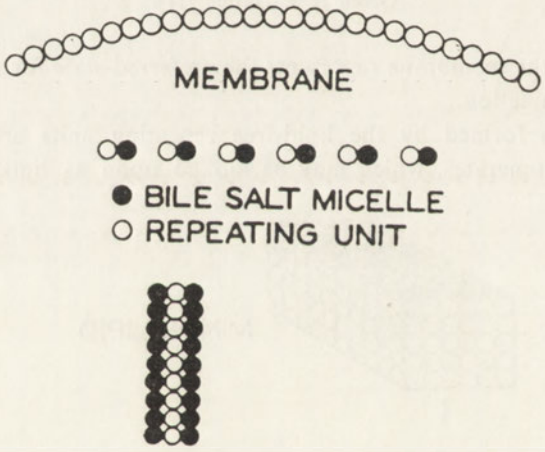

Fig. 3. Molecularization of repeating units by bile salts.

are paucimolecular adducts in the form of which the repeating units can readily interact with solute molecules in the suspending aqueous medium. Providing the active sites of the repeating units are not buried in the adduct, the electron transfer activity of the repeating units micellized with bile salts can be as high as that within the framework of a membrane [25].

Whereas many of the integrated activities of the inner membrane of the mitochondrion have been shown to be lipid-dependent [7], only one of the integrated activities of the outer membrane shows this essentiality [16]. This discrepancy can be explained in terms of solubility characteristics of the enzymes of the outer membrane. The repeating units of the outer membrane can be disrupted into water-soluble complexes without loss of integrated enzymic functions. Thus, pyruvic and α-ketoglutaric dehydrogenase activities are preserved after the particular repeating units of the outer membrane, in which these activities are localized, are fragmented into water-soluble subunits [1]. Water solubility, in this case, achieves for the activities of the disrupted repeating units of the outer membrane what membrane formation accomplishes for the activities of the repeating units of the inner membrane. The only exception among the outer membrane systems is the β-hydroxybutyric dehydrogenase which requires lecithin for activity [16]. The repeating units in which this enzyme is localized cannot be disrupted into water-soluble moieties. Thus, for this activity the membrane is a device (in fact, the physiological device) for molecularization of the enzyme.

It is plausible to generalize further that any enzymic function that can be exercised only at the level of a repeating unit of a membrane will automatically show lipid essentiality. This will be true for any membrane activity. It is only when the activity can be preserved after fragmentation of the repeating unit into water-soluble subunit proteins that lipid ceases to be essential. For example, the pyruvic and α-ketoglutaric dehydrogenase activities of the outer membrane preparations, depleted of lipid by acetone extraction, show a requirement for lipid (E. Bachmann, D. W. Allmann & D. E. Green, unpublished work) whereas the same activities in water soluble preparations are lipid-independent [1].

Role of phospholipid in membrane formation

The lipid-free repeating units form random three-dimensional aggregates, whereas those containing lipid combine with one another into a two-dimensional and oriented membrane continuum. Clearly, the presence of lipid in a repeating unit restricts the number of possibilities for combination, permitting only those to occur which lead to an alignment in one plane only. This restriction of combining possibilities, so effectively achieved by lipid, can be explained in terms of the hypothesis formulated pictorically in Fig. 4. If phospholipid were to combine preferentially with the top and bottom faces of a lipid-free cuboid repeating unit, the combination would be limited to the sites on the faces not occupied by lipid and the formation of a membrane would be automatic. The restriction thus imposed prevents an "above and below" stacking of the repeating units, but it imposes no barrier to a "side to side" nesting that leads to the membrane continuum.

Mitochondrial phospholipid is negatively charged. The localization of negatively charged phospholipid molecules on the top or the bottom face of a repeating unit would, therefore, effectively prevent combination at these sites. The charge would repel a unit which presents a "phospholipid face" for reaction (electrostatic repul-

sion). Moreover, there would be no tendency for a phospholipid-covered face of one repeating unit to unite with the hydrophobic face of a second unit. This representation is predicated on the assumption that the nesting of the repeating units

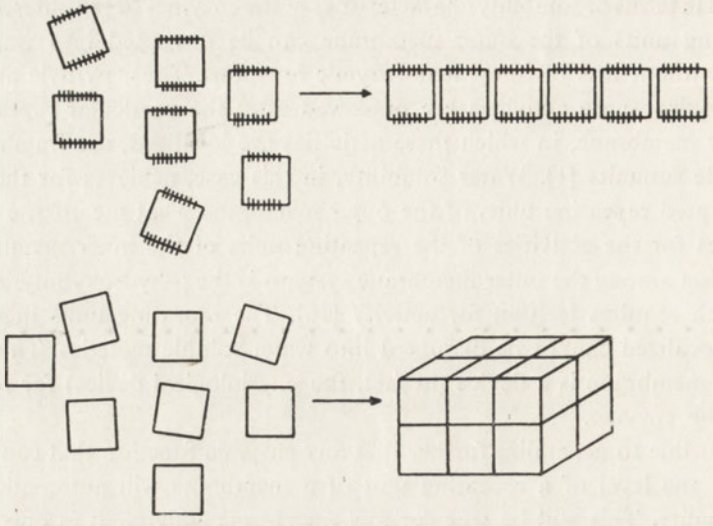

Fig. 4. The role of lipid in restricting modes of interaction between repeating units. The phospholipids are represented by segmented bars at right angles to the face of repeating units.

within a membrane involves predominantly hydrophobic, protein-protein, interactions, and that all of the other sites of the units have a hydrophobic character. The only significant areas of net charge in a repeating unit would then be those that have a coating of hydrophobically bound phospholipid.

The asymmetric localization of lipid within the repeating units of the mitochondrial inner membrane was first inferred from its essentiality for membrane formation, but later this localization was demonstrated both chemically and electron-microscopically. For example, in membranes formed by a preparation of Complex II of the electron transfer chain, the repeating units show structures above and below the plane of the membrane, and these might correspond to phospholipid (Fig. 5). Furthermore, it was possible to combine cytochrome *c* (electrostatically) with phospholipid. Cytochrome *c* added on to the very structures in Complex II that had been equated with phospholipid (J. Perdue, K. Kopaczyk & D. E. Green, unpublished work).

Reconstitution of the electron transfer chain in relation to membrane formation

Hatefi and his colleagues [14] were the first to discover the phenomenon of reconstitution of the electron transfer activities. They had demonstrated that the four primary complexes of the chain could be recombined to give a larger unit in which electron transfer from DPNH and from succinate to oxygen was efficiently reconstituted. Not only the overall system, but any desired portion thereof, could

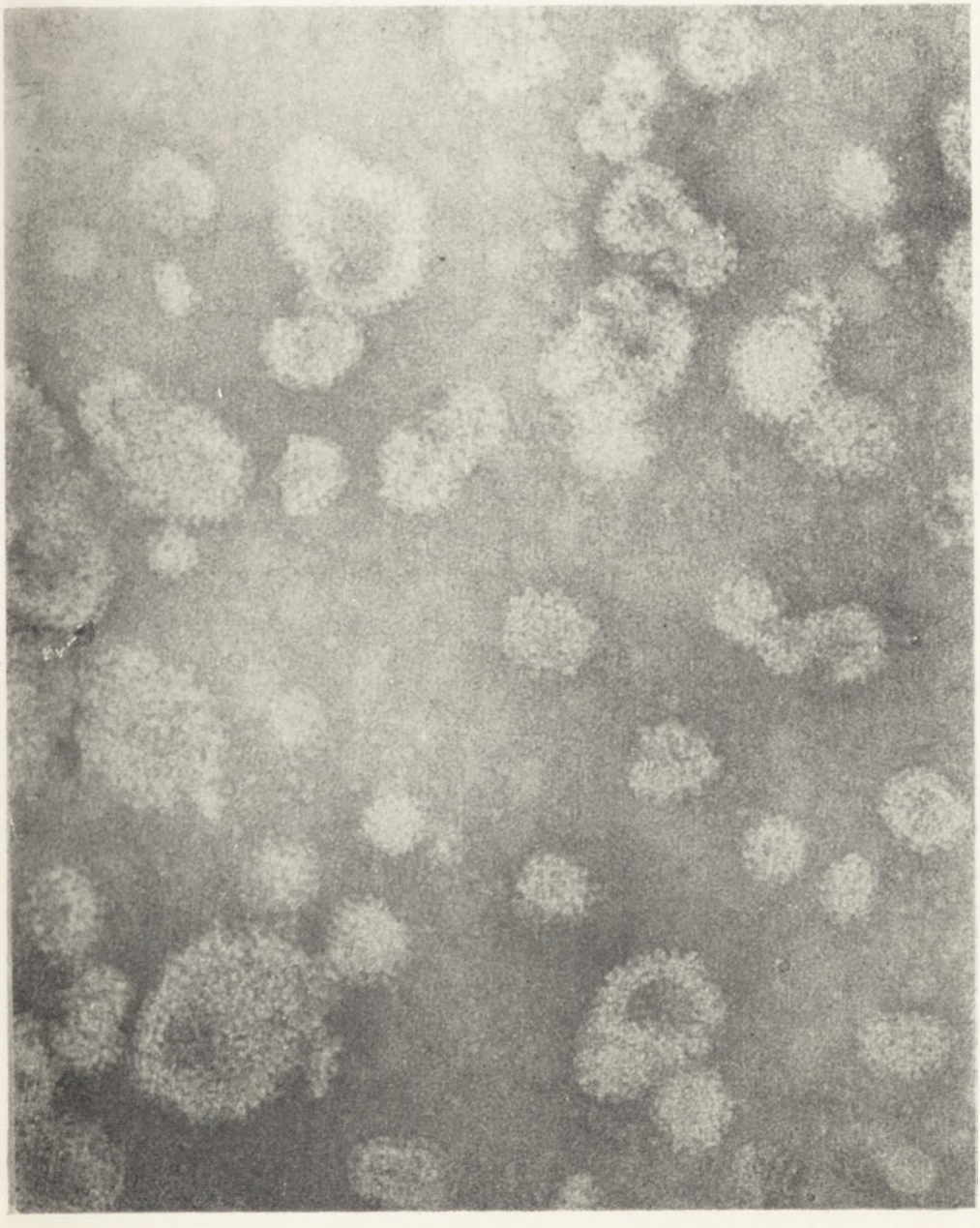

Fig. 5. Electron micrograph of Complex II (succinate-coenzyme Q reductase). Magnification: 258 000 ×. Negatively stained with phosphotungstate. Unpublished work of J. Perdue

be reconstructed. The reconstituted activities resulted from a particular mode of interaction between the participant enzyme complexes. The reconstitution required a very specific set of conditions, namely, mixing of the complexes in concentrated solutions and *then* dilution of the mixture. The mere mixing of the diluted preparations of the individual complexes did not result in reconstitution [9].

It is significant that the reconstitution of the activities of the electron transfer chain takes place under the very conditions which first lead to disaggregation of membranes into the individual repeating units (complexes), and then to reformation of membranes. The rationale of reconstitution may thus be stated as follows. Two complexes (or more) do not interact with one another when each occurs in a separate membrane (Fig. 6), that is, the electrons cannot be transferred from one membrane

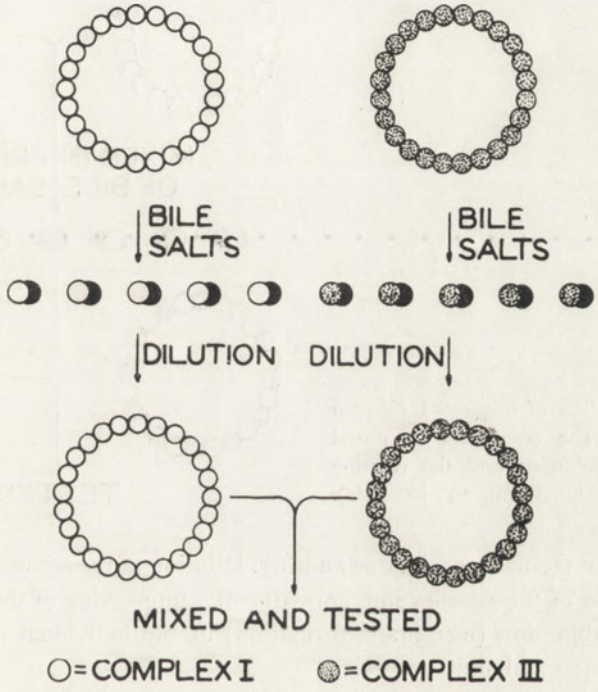

Fig. 6. Absence of reconstitution of integrated electron transfer activity. (The complexes are mixed in dilute solutions and, therefore, each exists in a separate membrane).

to another. The process of a transfer of electrons requires an interaction within the same membrane. The molecules which serve as electronic links between the complexes are coenzyme Q and cytochrome *c*. These components are mobile within *a vesicular membrane*, but do not move between separate membranes. If the complexes, used for the reconstitution of the chain, are first mixed while disaggregated and then are diluted out, the individual vesicular membranes thus formed contain all of the complexes capable of interaction in a sequential manner (Fig. 7). Because the mobile constituents facilitate an interaction between any of the component

repeating units of a given membrane, the sequence itself of these units within that membrane is not critical. This means that, in order for the flow of electrons to take place, Complex I (or II) need not be in the proximity of Complex III, and that the complexes do not have to be aligned in a vectorial fashion. According to the present interpretation, regardless of the sequence of the repeating units, coenzyme Q can shuttle electrons between the Complexes I (or II) and III; and cytochrome *c* does the same in the region of the Complexes III and IV.

The complexes are isolated from the mitochondria as soluble preparations, containing high levels of bile salts. Solubilization is an expression of the micellizing (disaggregation) of lipoproteins by bile salts. While in this micellized form, the

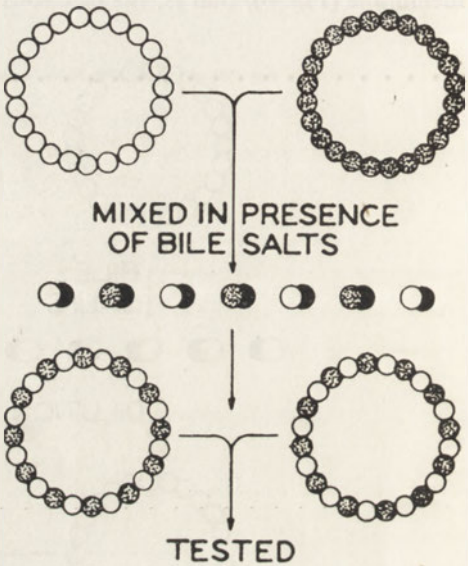

Fig. 7. Reconstitution of integrated electron transfer activity. (The complexes are mixed in concentrated solutions and the reaction mixture is then diluted out 50 - 100 fold).

complexes cannot recombine with one another. Dilution of the reconstitution mixture causes disruption of the micelles and, consequently, unmasking of the binding sites. Hence, a recombination (membrane formation) of the individual segments of the chain into a functional unit occurs.

Conditions leading to reconstitution will also lead to the formation of vesicular membranes, each containing repeating units of all complexes (Fig. 7). Without reconstitution, the complexes remain segregated within separate and separable membranes (Fig. 6). Reconstitution of the electron transfer chain, thus, requires that the interacting repeating units be *in the same* vesicular membrane system. This is achieved by mixing the preparations of the complexes in concentrated solutions and subsequently diluting the reaction mixture.

Originally, reconstitution of the electron transfer chain was interpreted in terms of a stoicheiometric molecular interaction of the individual complexes [14]. Our recent work in this area indicates that stoicheiometry, as a prerequisite for an interaction, is of no special significance. There are actually no restrictions as to the proportions in which the preparations of the complexes can be mixed, provided

precautions are taken to reduce bile salts to low levels during membrane formation. We have observed that the reconstituted membranes will contain the various complexes in the same proportions in which they were chosen for an interaction (A. Tzagoloff & D. H. MacLennan, unpublished work). This means that any combination of the repeating units leads to the formation of an enzymically active membrane. For example, succinate-cytochrome *c* reductase activity can be reconstituted, with an equal effectiveness, whether the ratio (on protein basis) of Complex II to Complex III is 1.0:1.5 or *vice versa*.

Mitochondrial swelling in relation to changes in the shape of repeating units

It has long been known that mitochondria can undergo extensive changes in volume under a variety of experimental conditions [22, 15, 18, 5]. These changes have been interpreted in terms of osmotic [23] or contractile phenomena [21], but the explanations in either of these terms have not been convincing. Extensive swelling can be observed even when no appreciable osmotic gradient is generated [13]. The notion of a contractile system in the mitochondrion has some supporting evidence under a limited number of the experimental conditions [17, 20], but the presence of an actomyosin-like protein in mitochondria is very dubious.

All the phenomena encompassed under the heading of mitochondrial swelling and shrinking are readily rationalized in terms of changes in the form of the inner membrane, consequent on changes in the form of the repeating units (G. A. Blondin & D. E. Green, unpublished work). The inner membrane under normal conditions is a vesicular structure with numerous invaginations into the interior known as cristae (Fig. 8). Each of these invaginations takes the form of a *flattened* tube (tubular arrangement). While it is not possible to specify the arrangement of repeating

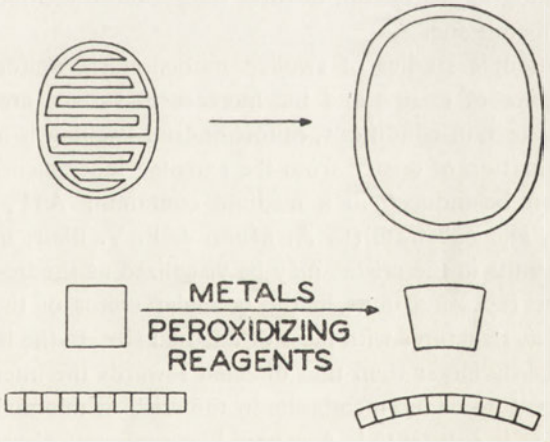

Fig. 8. Swelling as a consequence of changes in form of repeating units.

units in the cristae, the arrangement must be different from that of repeating units in the vesicular periphery of the inner membrane (spherical balloon arrangement). There are thus at least two modalities of arrangement of repeating units — the

spherical balloon modality of a vesicular membrane and the tubular modality of the cristae. It is conceivable that the transition from one modality to another can be brought about by changes in the phospholipid moiety of repeating units (Fig. 8).

Without considering the mechanism by which various reagents modify the structure of the phospholipid associated with repeating units, let us examine the consequences of such modifications. Apparently the structural requirements of the tubular modality of the cristae are exacting, and any changes in the lipid may well lead to a rearrangement of the inner membrane. The disc-like, tubular invaginations will be absorbed into the vesicular sector of the membrane until eventually one much larger vesicular membrane is formed without any invaginations. This extended membrane will contain a much greater interior volume than the original membrane with invaginations. Thus, the water content of the swollen non-invaginated membrane could be 5 - 10 times larger than that of the collapsed membrane with multiple invaginations. Swelling thus reflects rearrangements in the membrane modality, from a tube-like to a balloon-like structure, and this rearrangement can be triggered by a wide variety of reagents.

Calcium ions, which induce mitochondrial swelling under specific conditions, have a profound influence on the structure of phospholipid micelles (E. A. Munn, unpublished work). The structural arrangement of phospholipid molecules chelated to calcium ions is quite different from that of unchelated molecules. This modification in structural arrangement of phospholipids is reflected in a macro change in the form and the gross organization of the entire micellar structure. Much the same applies to the phospholipid of repeating units. When the structure of the phospholipids is altered by metals (monovalent and divalent), lipid peroxidation, or organic solvents, the structure of the repeating unit is correspondingly modified. When repeating units are altered in form, their nesting characteristics change. This change in the nesting modality can, in turn, trigger the transition from the tubular to the vesicular membrane.

Electron-microscopic studies of swollen mitochondria uniformly bear out the virtual disappearance of cristae and the increase in surface area of the vesicular membrane. Under certain conditions, mitochondrial swelling is a reversible phenomenon. The reformation of cristae from the extented inner membrane of a swollen mitochondrion can be induced in a medium containing ATP, magnesium ions, various cofactors, and substrate (E. A. Munn & P. V. Blair, unpublished work).

The repeating units in the cristae may be visualized as square-like, cuboid structures, whereas the repeating units in the vesicular sector of the inner membrane may be portrayed as structures with faces of unequal size. In the latter, the exteriorly directed site is slightly larger than that directed towards the interior (Fig. 8). Consequently, the curvature of the membrane in the crista is minimal, whereas that in the vesicular sector is substantial. A square-like symmetrical repeating unit is required for the formation of cristae; an asymmetric repeating unit is required for the formation of vesicular membranes.

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POWTARZAJĄCE SIĘ JEDNOSTKI TWORZĄCE BŁONY MITOCHONDRIALNE

Streszczenie

Wewnętrzna i zewnętrzna błona mitochondrialna zbudowana jest z makromolekularnych powtarzających się jednostek lipoproteidowych. Obie błony zawierają różne rodzaje powtarzających się jednostek i różnorodne aktywności enzymatyczne. Łańcuch przenoszący elektrony jest związany z podstawowymi częściami składowymi powtarzających się jednostek błony wewnętrznej. Kompleksy łańcucha zawierające lipidy tworzą pęcherzykowate błony. Po usunięciu lipidów kompleksy agregują bezładnie, ale ponowne wprowadzenie lipidów przywraca im zdolność tworzenia pęcherzyków. Specyficzna lokalizacja lipidów w powtarzających się jednostkach prowadzi do utworzenia dwuwymiarowej i zorientowanej ciągłej błony. Istnienie błony jest konieczne do ułożenia się cząsteczek w powtarzające się jednostki, co z kolei jest konieczne dla wykazania aktywności enzymatycznej. Dla odtworzenia łańcucha przenoszącego elektrony konieczne jest, by wszystkie reagujące ze sobą powtarzające się jednostki znajdowały się w tej samej błonie pęcherzykowej. Osiąga się to przez dezagregację błon zawierających te kompleksy, a następnie przez ponowne sformowanie błony, w której są obecne powtarzające się jednostki odpowiednich rodzajów. Pęcznienie mitochondriów jest wyrazem przegrupowania zachodzącego w błonie wewnętrznej, które prowadzi do zmiany struktury rurkowatej na strukturę balonowatą. Przejście to może być indukowane przez zmiany wewnątrz powtarzających się jednostek.

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