LXVI. EXPERIMENTS RELATING TO THE CONSTITUTION OF ALLOXAZINE- ADENINE-DINUCLEOTIDE

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It was shown by Das [1936; 1938] that the *d*-amino-acid oxidase of Krebs [1933] was associated with a coenzyme. The latter substance was found by Straub [1938] to contain flavin and later isolated in a pure condition by Warburg & Christian [1938, 1] and reported to be an alloxazine-adenine-dinucleotide.

This coenzyme is becoming increasingly important. Warburg & Christian stated that in addition to its combination with the apoenzyme of *d*-amino-acid oxidase it formed, with other specific proteins, xanthine oxidase and enzymes capable of transferring hydrogen from dihydropyridine nucleotide to oxygen and methylene blue respectively. It is also the prosthetic group of a flavo-protein, isolated from heart muscle by Straub [1939], which was later reported by Straub *et al.* [1939] to be identical with diaphorase (coenzyme factor).

The constitution given to the substance by Warburg & Christian was based on its elementary composition and the liberation of one molecule of adenine by acid hydrolysis and one molecule of lumiflavin by alkaline photolysis. The suggestion was later made that the "old" yellow enzyme was not a natural product but resulted in the course of preparation by the fission of adenylic acid from the dinucleotide [Warburg & Christian, 1938, 2].

The present work was concerned with splitting the substance by mild acid and alkaline hydrolysis and investigating the products formed by enzymic reactions. In connexion with the adenine-containing part of the molecule use was made of the fact that the adenosine-5-phosphoric acids, which are coenzymes of the phosphorylase described by Euler *et al.* [1936, 1, 2], act as activators in the enzymic dephosphorylation of phosphoglyceric acid. The appearance of co-phosphorylase on hydrolysis showed the presence in the molecule of linkages between adenine, carbohydrate and phosphoric acid which are those of muscle adenylic acid. The flavin part of the molecule was investigated, after hydrolysis, in the presence of the protein component of the "old" yellow enzyme, using Warburg's system for the oxidation of Robison ester. In this system a considerable proportion of the original activity of the dinucleotide remained after treatment which completely destroyed its ability to oxidase *d*-alanine. This indicated the production, on hydrolysis, of lactoflavin-5-phosphoric acid and the presence of this residue in the dinucleotide.

EXPERIMENTAL

Alloxazine-adenine-dinucleotide

The coenzyme was prepared as its Ba salt (24 mg. from 10 kg. yeast) by the method of Warburg & Christian [1938, 1], with whose product it appeared to be identical. The phosphorus content, determined colorimetrically, was 6.5 %

($C_{27}H_{31}O_{15}N_9P_2Ba$ requires P 6.74 %). Spectrophotometric measurements were kindly carried out by Mr G. Günther. At $\lambda = 3650$ a $2.8 \times 10^{-5} M$ solution by weight was found to contain 2.7×10^{-5} mols per litre, on the basis of the curve given by Warburg & Christian.

Phosphorylase system

The reaction measured was the dephosphorylation of phosphoglyceric acid. The enzyme preparation was obtained by acetone precipitation from rat muscle extract. Minced rat muscle was extracted twice with twice its weight of water, the extract dialysed 18 hr. against 0.9 % KCl, precipitated with 10 vol. acetone at -8° , and the precipitate washed with absolute alcohol and dried. For experiment 0.25 g. of the precipitate was extracted with a mixture of 1 ml. 0.04 M $MgSO_4$, 4 ml. of water and 6 ml. of veronal buffer pH 7.4, and the insoluble material separated by centrifuging.

In each determination were used 0.8 ml. of enzyme solution, 0.5 ml. of phosphoglyceric acid (15 mg. of Ba salt per ml. decomposed with $M/10$ Na_2SO_4) and 1.2 ml. of water containing the activator to be investigated. After incubation for 2 hr. at 30° 3 ml. of 10 % trichloroacetic acid was added and the protein removed by centrifuging. Free phosphoric acid was determined in 1 ml. of the resulting solution by the method of Teorell [1931].

Protein component of the "old" yellow enzyme

Crude yellow enzyme from yeast was purified by adsorption on alumina $C\gamma$ using the method of Weygand & Stocker [1937]. The prosthetic group was then separated by the procedure of Warburg & Christian [1938, 2]. 4 ml. of a solution containing 0.2×10^{-6} mol. of flavin enzyme were mixed with 4 ml. of saturated ammonium sulphate and cooled to 0° . 0.1 N HCl was then added slowly with shaking until the pH of the solution was approximately 2.8. The precipitated protein was washed at 0° at the centrifuge with 7 ml. of half-saturated ammonium sulphate, dissolved at 0° in 1 ml. of $M/2$ phosphate buffer, pH 7.4 and diluted to 10 ml. The flavin solution was neutralized with $M/2$ Na_2HPO_4 and also diluted to 10 ml. (denoted by F.M. in Table III).

Protein component of d-amino-acid oxidase

From an extract of acetone-dried sheep's kidney [Krebs, 1935] a solid preparation containing the required protein was obtained by the method of Warburg & Christian [1938, 1], separation of the prosthetic group being effected in 35 % saturated ammonium sulphate solution at pH 2.8. 100 ml. of kidney extract yielded 150 mg. of material.

Oxidation of Robison ester and d-alanine

After hydrolysis by $N/20$ HCl and NaOH the activity of the coenzyme, in combination with the specific proteins, was tested in two different systems, the oxidation of *d*-alanine and of Robison ester.

The experiments were carried out in Warburg vessels at 30° . In each case the gas used was pure O_2 and the centre cups contained 0.2 ml. of 5 % KOH. In the Robison ester system there was used in each vessel 5.0×10^{-9} mol. of the co-enzyme (denoted by F. A. D.) and protein corresponding to that obtained by

splitting the same amount of "old" yellow ferment. For the oxidation of alanine the vessels contained, in the case of hydrolysis with HCl, 10 μ g. of the coenzyme and 3 mg. of the protein preparation; the coenzyme was in large excess, parallel experiments showing that the rate of O₂ uptake using 2 μ g. was approximately only 5% less. In the case of hydrolysis with NaOH there was used 0.5 μ g. of the co-enzyme with 4 mg. of protein.

RESULTS

Production of cophosphorylase from the dinucleotide

Cophosphorylase experiments were carried out, in the system described, on the intact coenzyme and after hydrolysis of the latter under mild alkaline and acid conditions. Table I summarizes the results obtained.

Table I

Activator	μ g.-atom P per ml.	% dephosphory- lation
0.0125 mg. adenylic acid	1.66	20.2
0.025 " "	2.70	32.7
0.050 " "	3.29	39.9
0.10 " "	3.95	47.9
0.20 " "	4.44	53.8
0.025 mg. adenosine triphosphoric acid	2.00	24.2
0.10 " "	3.13	37.9
0.40 " "	4.45	53.8
0.636 mg. coenzyme in water	1.84	22.3
0.636 mg. coenzyme after treatment with N/20 NaOH 15 min. at 18° and neutralization with N/20 HCl	3.55	43.1
0.636 mg. coenzyme treated with N/20 NaOH 10 min. at 18° and 5 min. at 95°. Neutralized with N/20 HCl	3.88	47.1
0.636 mg. coenzyme treated with N/20 HCl 10 min. at 95° and neutralized with N/20 NaOH	3.22	39.0
No activator	1.16	14.1

By comparison of the amounts of dephosphorylation when adenylic acid and the coenzyme were used as activators an estimate can be made of the amount of splitting of the latter, in terms of the possible production of adenosine-5-phosphoric acid, under the conditions employed (Table II).

Table II

Treatment	% of theoretical splitting
Coenzyme in water	<5
Coenzyme in N/20 NaOH 15 min. at 18°	28
Coenzyme in N/20 NaOH 10 min. at 18° and 5 min. at 95°	40
Coenzyme in N/20 HCl 10 min. at 95°	20

Effect of hydrolysis of the coenzyme on its activity in combination with the protein components of d-amino-acid oxidase and the "old" yellow enzyme

(1) *Hydrolysis with acid.* The results are shown in Table III. In exps. (a) and (b) different samples of Robison ester and "Zwischenferment" were used in the one system and a fresh protein preparation in the other.

In the oxidation of Robison ester the activity of the coenzyme, after treatment with $N/20$ HCl, was very similar whether the time of hydrolysis was 30 or 120 min., the rate of O_2 uptake, after subtraction of the blank values, being about 70 % of that with the unchanged substance. Under the same conditions the coenzyme activity with respect to the oxidation of *d*-alanine was completely destroyed.

Table III

(1) Oxidation of Robison ester					
Protein solution	0.25 ml.				
$M/2$ phosphate pH 7.4	0.2 ml.				
Zwischenferment	0.5 ml.	→	→	→	→
Robison ester (0.1 m.)	0.4 ml.				
$N/20$ KCN	0.2 ml.				
H_2O	0.45 ml.	/	/	/	0.2 ml.
Coenzyme	/	0.45 ml. F.A.D.	0.45 ml. F.A.D. (30 min. in $N/20$ HCl at 95°)	0.45 ml. F.A.D. (120 min. in $N/20$ HCl at 95°)	0.25 ml. "split" F.M.
Co_{II} (in side cup)	0.2 ml. (0.05 mg.)	→	→	→	→
Time (min.)	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$
(a) 10	8.8	41.9	32.3	32.8	37.6
20	14.0	67.6	51.0	54.1	61.6
30	21.1	91.8	70.5	75.4	83.8
40	25.5	114.3	88.4	94.3	102.6
(b) 10	7.5	45.9	34.0	30.6	—
(2) Oxidation of <i>dl</i> -alanine					
Protein solution	3 mg. in 1 ml. $N/10$ pyrophos- phate pH 8.3	→	→	→	
H_2O	/	/	/	/	1.0 ml.
Coenzyme	1.0 ml. F.A.D.	1.0 ml. F.A.D. (30 min. in $N/20$ HCl at 95°)	1.0 ml. F.A.D. (120 min. in $N/20$ HCl at 95°)	/	/
4.5 % alanine (in side cup)	0.2 ml.	→	→	→	
Time (min.)	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$	$\mu l. O_2$
(a) 10	11.3	0	0	0	0
20	24.1	0	0	0	0
30	35.4	0	0	0	0
40	46.7	0	0	0	0
(b) 10	14.2	0	0	0	0

(2) *Hydrolysis with alkali.* Treatment of the coenzyme with $N/20$ NaOH was carried out at room temperature, as heating at 95° brought about rapid disappearance of the flavine colour. But even under these conditions it did not appear possible to destroy selectively, as in the case of acid hydrolysis, the activity with respect to the oxidation of *d*-alanine. Table IV gives the values for the O_2 uptake. Under the conditions employed, hydrolysis for 15 and 60 min. reduced the rate of O_2 uptake in the Robison ester system to about 75 % and 45 % respectively of that obtained using the untreated coenzyme. In the oxidation of alanine the corresponding values were about 93 and 58 %.

Table IV

(1) Oxidation of Robison ester				
Protein solution	0.25 ml.			
<i>M</i> /2 phosphate pH 7.4	0.2 ml.			
Zwischenferment	0.5 ml.	→	→	→
Robison ester (0.1 <i>M</i>)	0.4 ml.			
<i>N</i> /20 KCN	0.2 ml.			
H ₂ O	0.45 ml.	/	/	/
Coenzyme	/	0.45 ml. F.A.D.	0.45 ml. F.A.D. (15 min. in <i>N</i> /20 NaOH at 18°)	0.45 ml. F.A.D. (60 min. in <i>N</i> /20 NaOH at 18°)
Co _{II} (in side cup)	0.2 ml. (0.05 mg.)	→	→	→
Time (min.)	μl. O ₂	μl. O ₂	μl. O ₂	μl. O ₂
10	10.6	45.3	37.8	26.2
20	16.7	71.8	59.6	41.0
30	21.2	94.0	78.9	54.1
40	23.8	112.9	91.8	64.0
(2) Oxidation of <i>dl</i> -alanine				
Protein solution	4 mg. in 1 ml. <i>N</i> /10 pyrophos- phate pH 8.3	→	→	→
H ₂ O	/	/	/	1.0 ml.
Co-enzyme	1.0 ml. F.A.D.	1.0 ml. F.A.D. (15 min. in <i>N</i> /20 NaOH at 18°)	1.0 ml. F.A.D. (60 min. in <i>N</i> /20 NaOH at 18°)	/
4.5% alanine (in side cup)	0.2 ml.	→	→	→
Time (min.)	μl. O ₂	μl. O ₂	μl. O ₂	μl. O ₂
10	11.7	10.5	6.3	0
20	22.5	20.9	12.7	0
30	33.4	32.2	19.9	0
40	44.3	41.9	27.1	0

After treatment of the coenzyme with *N*/20 HCl at 95 for 30 min. or with *N*/20 NaOH at 18° for 60 min. no visible yellow colour was extracted from the resulting solutions, at pH 5, by benzyl alcohol. As this solvent has been shown by Emmerie [1938] to extract flavin, though not flavin phosphoric acid, it appeared that significant amounts of the former substance were not produced under these conditions.

CONCLUSIONS

The experiments on the production of cophosphorylase show that adenosine-5-monophosphoric acid is a constituent of the new dinucleotide and is involved with a linkage which is easily hydrolysed, especially under alkaline conditions. The very small amount of cophosphorylase present after using the untreated substance is possibly due to enzymic splitting in the system employed.

The formation of adenosine-5-phosphoric acid, in conjunction with the results obtained in the Robison ester and alanine systems, indicates that in the first stage of acid hydrolysis a considerable proportion of the coenzyme breaks down into two mononucleotides, the reaction which was suggested by Warburg & Christian [1938, 2] to occur enzymically in Lebedew-Saft. The fact that little difference was found in the activity of the coenzyme, with respect to the oxidation of Robison ester, whether treated with *N*/20 HCl for 30 or 120 min. is explained by the slow rate of hydrolysis of lactoflavin-5'-monophosphoric

acid in acid solution [Kuhn & Rudy, 1936]. The difficulty of destroying selectively, in NaOH solution, the activity of the coenzyme as the prosthetic group of *d*-alanine oxidase, may be assigned to the lability of lactoflavin towards alkali.

SUMMARY

1. The coenzyme of *d*-amino-acid oxidase has been prepared by the method of Warburg & Christian with whose product it was identical in phosphorus and flavin contents.

2. Hydrolysis of the coenzyme with dilute acid and alkali lead to the production of cophosphorylase, showing the presence in the molecule of adenosine-5-monophosphoric acid.

3. By acid hydrolysis it was possible to destroy the activity of the coenzyme with regard to the oxidation of *d*-alanine while maintaining, to a considerable extent, its ability to transfer hydrogen from dihydropyridine nucleotide. This indicated the liberation, by hydrolysis, of lactoflavin-5-phosphoric acid.

4. It has been suggested that the coenzyme can be split enzymically into two mononucleotides, adenylic acid and lactoflavin-5-phosphoric acid [Warburg & Christian, 1938, 2]. It now appears that this reaction can occur on acid hydrolysis.

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