CLVI. STUDIES ON AMINO-ACID DEHYDROGENASE.

I. PROLINE DEHYDROGENASE.

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It has been shown by many workers, especially by Knoop [1925] and Krebs [1933], that amino-acids are oxidised by tissues *in vitro*. It has later been reported by Bernheim and Bernheim [1934; 1935] and Krebs [1935] that a cell-free extract of tissues can also oxidise aerobically many amino-acids, and the former authors claim to have obtained an enzyme from tissue extracts which can decolorise methylene blue more rapidly in presence of proline, phenylalanine, valine, leucine, serine *etc.*

The present work is a study of the mechanism of oxidative deamination of amino-acids by cell-free dehydrogenases. It has been shown that dehydrogenases such as alcohol dehydrogenase, glucose dehydrogenase, hexosemonophosphate dehydrogenase *etc.* require the presence of a low-molecular co-enzyme; that is to say the active enzyme (holo-dehydrogenase) consists of a high-molecular group (apo-dehydrogenase) and a low-molecular prosthetic group (co-dehydrogenase), the components of the holo-dehydrogenase being in dissociation equilibrium. Up to the present time two co-enzymes (co-dehydrogenases) are known, namely co-enzyme (co-dehydrogenase I) and the co-ferment of Warburg and Christian [1935] (co-dehydrogenase II).² These co-enzymes act by taking up activated hydrogen from the substrate, thereby forming the reduced co-enzymes which are not autoxidisable but are reoxidised by flavin-enzyme. The leucoflavin-enzyme itself can act directly with molecular oxygen or with a reducible dye like methylene blue. It seemed to be of interest to investigate whether amino-acid dehydrogenase has anything to do with co-enzyme-flavin-enzyme catalysis. The fact that amino-acid oxidation by cell-free extracts was not inhibited by KCN made it possible that it might be such a metal-free system in which co-enzyme-flavin-enzyme catalyses the reaction.

In the present work the problem has been studied with reference to proline. None of the known activators like co-enzyme (Euler), co-dehydrogenase II [Warburg and Christian, 1935; Euler and Adler, 1935, 3; 1936], flavin-enzyme [Warburg and Christian, 1932, 1, 2], glutathione, ascorbic acid, adenylypyrophosphate can activate proline dehydrogenase except glutathione in higher concentrations. (This may be due to some inhibiting metal which cannot be bound by lower concentrations of glutathione.) The facts that the dehydrogenase is not catalysed by flavin-enzyme and that anaerobic reduction of dyes is relatively much slower than the oxidation by molecular oxygen seem to indicate that flavin-enzyme is not a necessary component of the system.

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² For the general nomenclature of the components of dehydrogenases see Euler and Adler [1936].

It has been found that the original enzyme solution heated at 80 to 90° for 5 min. at p_H 8.0 can activate proline dehydrogenase. This activator is destroyed when heated at p_H 3.0 and p_H 12.0 for 5 min. at 90° and is also inactivated by ultraviolet irradiation. The chemical nature and the specificity of this activator have not yet been sufficiently studied but further investigations are in progress.

EXPERIMENTAL.

Extraction of the enzyme.

The enzyme was extracted according to Krebs [1935] from acetone-dried pig kidney, 1 g. being extracted with 40 ml. distilled water for 10 min. and centrifuged. The centrifugate could oxidise proline, alanine, phenylalanine and leucine. 2 ml. of this extract were used for every experiment.

Method of experiment.

The oxidation experiments were carried out in Warburg-Barcroft apparatus at 30°. Phosphate buffer (p_H 7.8, $M/20$) was always used with the enzyme solution. Substrate was always added from the side-limb after equilibration of the temperature of the vessels and 0.4 ml. of 8% NaOH was used for absorbing the CO_2 formed.

Purification of the enzyme.

(a) *Adsorption with aluminium oxide $C\gamma$* (Willstätter). 30 ml. of the enzyme solution prepared from acetone-dried pig kidney were stirred with $C\gamma$ suspensions for 15 min. at p_H 6.0 and centrifuged. The centrifugate still contained most of the proline dehydrogenase.

(b) *Adsorption with kaolin*. 40 ml. of the enzyme solution were agitated with 2 g. kaolin for 15 min. at p_H 6.0 and centrifuged. The centrifugate had lost a considerable portion of the proline dehydrogenase.

The enzyme was eluted from the kaolin by phosphate buffer (p_H 7.8, $M/20$). To the kaolin adsorbate 9 ml. of phosphate buffer and 1 ml. of 1% Na_2CO_3 were added, stirred for 30 min. and centrifuged. The centrifugate was found to oxidise proline. The enzyme solution eluted from kaolin in this way was used throughout this work, 1 ml. being employed for all experiments described below.

Nature of the enzyme.

The light brown clear enzyme solution could hardly oxidise *d*-alanine, whilst with *dl*-proline oxidation was extensive. The velocity of reaction was very great at the beginning and then relatively slow. The enzyme was unstable at p_H 8.0 at room temperature but more stable in the cold.

Test for activators.

In order to see whether co-zymase could act as an activator in the oxidation of proline by the enzyme 1 ml. of the enzyme solution freshly prepared from pig kidney was tested in yeast fermentation according to the well-known method of Myrbäck and Euler [1929], but no appreciable evolution of CO_2 was found even after 1 hour.

It has been shown that in fresh tissue extracts an inhibiting substance is present which prevents the reaction with co-zymase and that this inhibitor can be destroyed by heating [Meyerhof, 1918; Euler, Adler and Dahlgren, 1935]. The enzyme solution was therefore heated for 5 min. at 80° and tested as before on fermentation. In this case also however no appreciable amount of CO_2 was formed. Various kidney preparations were tested in this way and only in one preparation was there very slight evolution of CO_2 (0.75 CO_2 /ml.).

1. *Co-zymase*. Co-zymase has been found by many workers, especially by Andersson [1934], to activate many dehydrogenases. This wide range of co-

zymase as an activator suggested its possible rôle in the oxidation of amino-acids. Co-zymase, prepared from yeast according to Euler, Albers and Schlenk [1935], was therefore added to the enzyme but no increased oxygen uptake was found with proline, suggesting that co-zymase plays no part in the activation of proline dehydrogenase. Various preparations of co-zymase have been tried.

Table I.

No.	Enzyme solution ml.	Co-zymase ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	37.0	92.4	147.8	195.4
2	1.0	0.3	1.0	0.1	40.1	92.8	137.4	175.5
3	1.0	0.3	1.0	0.1	41.2	89.7	131.0	172.2
4	1.0	—	1.0	0.1	50.2	105.6	145.2	172.2
5	1.0	0.3	1.0	0.1	55.8	111.5	150.3	179.4
6	1.0	—	1.0	—	—	—	—	—

2. *Co-dehydrogenase II*. (a) Warburg's co-ferment from horse red blood corpuscles, prepared according to Warburg and Christian [1933] acts as co-enzyme in the oxidation of hexosemonophosphate. It has been shown [Das, 1936] that it can also act as co-enzyme to glucose dehydrogenase. It fails however to activate the oxidation of proline (Table II).

(b) *Co-dehydrogenase II from yeast*. It was found by Euler and Adler [1935, 3; 1936] that impure co-zymase from yeast contained an activator for the oxidation of hexosemonophosphate which was named co-dehydrogenase II and was identical with Warburg and Christian's co-ferment from blood-cells. This also could activate glycolic dehydrogenase [Das, 1936] but failed to activate the oxidation of proline (Table II).

Table II.

No.	Enzyme solution ml.	Co-dehydrogenase II (ml.)	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	37.0	92.4	147.8	195.4
2	1.0	0.3*	1.0	0.1	32.1	81.6	128.2	171.9
3	1.0	0.3†	1.0	0.1	34.0	86.0	136.0	180.0

* Euler and Adler [1935, 3; 1936].

† Warburg and Christian [1933].

3. *Flavin-enzyme*. Warburg and Christian [1932, 1, 2] isolated a yellow pigment from bottom yeast which is a protein combined with a yellow prosthetic group and is capable of reversible oxidation and reduction. It has been shown that the enzymic oxidation of alcohol and glucose either by molecular oxygen, methylene blue or lactoflavin is catalysed by flavin-enzyme [Euler and Adler, 1934; 1935, 1]. It was also shown by Euler, Adler and Hellström [1935] that reduced co-enzyme was reoxidised by flavin-enzyme, but the latter failed to activate proline dehydrogenase (Table III).

Table III.

No.	Enzyme solution ml.	Flavin-enzyme ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	13.2	42.2	84.5	121.4
2	1.0	0.5	1.0	0.1	24.2	50.9	89.7	126.0
3	1.0	—	1.0	0.1	50.2	105.6	145.2	174.2
4	1.0	0.5	1.0	0.1	59.0	108.9	146.3	174.3

4. *Adenylpyrophosphate (A.T.P.)*. Adenylpyrophosphate is involved in the activation of several reactions such as muscle glycolysis [Lohmann, 1931] and as a

phosphate donator to carbohydrates [Euler and Adler, 1935, 2; Parnas *et al.*, 1935; Lutwak-Mann and Mann, 1935]. A preparation of adenylypyrophosphate from rabbit muscle was incapable of activating the oxidation of proline by the dehydrogenase (Table IV).

Table IV.

No.	Enzyme solution ml.	A.T.P. ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	29.0	79.2	116.2	147.8
2	1.0	0.3	1.0	0.1	33.9	65.5	89.7	109.1

5. *Ascorbic acid*. The reversible oxidation-reduction of ascorbic acid suggests the possibility of its important rôle in oxidation-reduction systems of the body. It has been shown to be capable of activating intracellular proteases of the type of cathepsin, which by washing with acetone and alcohol have been freed from their natural activators [Karrer and Zehender, 1933]. Ascorbic acid however is also unable to activate the oxidation of proline by the dehydrogenase (Table V).

Table V.

No.	Enzyme solution ml.	Ascorbic acid ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					10	20	40	60
1	1.0	—	1.0	0.1	11.7	29.14	72.9	102.0
2	1.0	0.5 (1 mg.)	1.0	0.1	19.4	38.8	65.5	82.4
3	1.0	—	1.0	0.1	32.1	51.2	107.8	145.7
4	1.0	0.5 (2 mg.)	1.0	0.1	30.3	49.5	82.5	107.3

6. *Glutathione*. Since the discovery of glutathione by Hopkins [1929] many speculations have been made as to its rôle in metabolism. It was shown by Mann [1932] that glutathione is reduced by glucose dehydrogenase and later by Meldrum and Tarr [1935] that it is also reduced by the Warburg and Christian hexosemonophosphate oxidising system. It has also been studied as an activator in various reactions, and it therefore seemed to be of importance to study its rôle in the oxidation of amino-acids by the dehydrogenase. Various concentrations were used and it was found that a concentration of $M/300$ activated the system whilst a smaller concentration ($M/1500$) had no effect (Table VI).

Table VI.

No.	Enzyme solution ml.	Glutathione ml.	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	45	60
1	1.0	—	1.0	0.1	21.1	50.2	81.8	108.2
2	1.0	0.1	1.0	0.1	52.6	97.8	137.9	168.0
3	1.0	$M/300$	1.0	0.1	28.0	49.8	77.8	99.6
		$M/1500$						

Action of dyestuffs on the enzyme.

The anaerobic reduction of methylene blue by the enzyme was very slow in absence of proline and was only slightly more rapid in presence of the substrate [Bernheim and Bernheim, 1934; 1935]. The effect of the dye on the aerobic oxidation of proline was then studied and was found to be slightly inhibitory in a concentration of $M/60,000$. A dye with more positive redox potential, 2:6-dichlorophenolindophenol, was also studied but was found to have no effect in a concentration of $M/60,000$ (Table VII).

Table VII.

No.	Enzyme solution ml.	Dyestuffs ml. <i>M</i> /60,000	Phosphate buffer ml.	<i>M</i> proline ml.	μ l. O ₂ uptake after min.			
					10	20	40	60
1	1.0	—	1.0	0.1	57.6	118.0	222.7	288.2
2	1.0	0.3*	1.0	0.1	47.6	102.8	205.6	268.2
3	1.0	0.3†	1.0	0.1	36.4	80.0	167.3	228.0

* 2:6-Dichlorophenolindophenol.

† Methylene blue.

Action of inhibitors on the enzyme.

1. *KCN*. *KCN* in the concentration of *M*/100 had a slight activating effect on the dehydrogenase. This might be due to some metal which was present in the original enzyme solution. The original enzyme solution was therefore treated with *KCN* and kept at room temperature (20°) for 1 hour, but the same slight activating effect was found as when *KCN* was added along with the substrate after equilibration of temperature of the vessels (Fig. 1).

Fig. 1.

Fig. 2.

Fig. 1. Oxygen uptake in μ l. (A) Enzyme and proline; (B) enzyme, proline and *M*/100 *KCN*.

Fig. 2. Oxygen uptake in μ l. (A) Enzyme and proline; (B) enzyme, proline and CuSO_4 (*M*/75); (C) enzyme, proline, CuSO_4 (*M*/75) + *KCN* (*M*/100).

2. *CuSO₄* and *CuSO₄* + *KCN*. Both CuSO_4 and CuSO_4 + *KCN* when added to the enzyme had activating effects for a short time, followed by complete inactivation of the enzyme. The initial activation might be due to the formation of a copper compound of the substrate which had a more labile hydrogen, whilst copper itself acted as a poison to the enzyme. The action of CuSO_4 + *KCN* was the same as that of CuSO_4 itself. The slight increase of initial oxygen uptake with the "Cu-complex" might be due to the slight activating action of *KCN* (Fig. 2).

Action of H₂O₂ on the enzyme.

It was shown by Keilin and Hartree [1936] that the H₂O₂ formed during the oxidation of xanthine by xanthine oxidase destroys the enzyme. It has also been shown that H₂O₂ is formed during oxidation of amino-acids by amino-acid dehydrogenase. To see whether the decrease in velocity of the oxidation of proline after a short time is due to the destruction of the enzyme by H₂O₂ formed during the course of reaction, a solution of H₂O₂ was added with the substrate after equilibration of temperature to make 1% concentration; no inhibition was observed however even after 90 min. (Table VIII).

Table VIII.

No.	Enzyme solution ml.	H ₂ O ₂ 1%	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
					15	30	60	90
1	1.0	—	1.0	0.1	10.6	34.3	76.6	110.9
2	1.0	0.1	1.0	0.1	10.0	31.4	68.1	104.8

Heated enzyme solution as activator.

The possibility was next considered that the activator might have been bound up with some protein carrier which could be split off by heating. The enzyme solution was therefore heated at 80° for 5 min. at p_H 8.0. The heated enzyme solution, when added to the original enzyme, produced a great increase in the oxidation of proline which was not due to the presence of residual enzyme or any substrate.

The ash of an enzyme preparation had no effect on the enzyme, showing that the activation by the heated solution was not due to the presence of any metal (Table IX).

Table IX.

No.	Enzyme solution ml.	Heated enzyme solution ml.	Ash of the enzyme solution	Buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
						15	30	45	60
1	1.0	—	—	1.0	0.1	29.0	79.2	116.2	147.8
2	1.0	0.5	—	1.0	0.1	78.7	163.2	224.4	268.1
3	1.0	—	0.5 (5 mg.)	1.0	0.1	37.6	82.7	127.9	162.0
4	—	0.5	—	1.0	0.1	—	—	—	—
5	1.0	0.5	—	1.0	—	—	—	—	—

That the activation was not due to co-zymase or co-dehydrogenase II was also proved by testing the heated enzyme preparation on yeast fermentation as well as on the hexosemonophosphate oxidising system; furthermore, KCN did not inhibit the activation, showing that neither catalase nor peroxidase is responsible (Table X).

Table X.

No.	Enzyme solution ml.	Heated enzyme solution ml.	KCN M/110	Phosphate buffer ml.	M proline ml.	μ l. O ₂ uptake after min.			
						15	30	45	60
1	1.0	—	—	1.0	0.1	10.6	34.3	76.6	110.9
2	1.0	—	0.3	1.0	0.1	32.1	67.0	107.8	145.7
3	1.0	0.5	—	1.0	0.1	50.1	100.1	170.5	228.1
4	1.0	0.5	0.3	1.0	0.1	65.4	126.0	184.2	242.4

Negative nitroprusside tests or tests of an intensity lower than that corresponding with effective concentrations of glutathione were given by the heated solution; it is clear therefore that the natural activator cannot be glutathione.

Nature of the activator.

1. *Stability of the activator.* In order to study the stability of the activator the original enzyme preparation was heated at 90° for 5 min. at p_H 3, 5, 6, 7, 8, 9, 10, 11, 12. It was found that the activity was partially destroyed at p_H 11.0 and completely destroyed at p_H 3.0 and 12.0, whilst at other p_H values the activity was fairly constant.

When the enzyme was heated at $p_H < 8.0$ a heavy precipitate was formed; the clear colourless filtrate contained the activator.

2. *Destruction by ultraviolet rays.* 3 ml. of the heated enzyme solution were exposed to ultraviolet rays in an open small crystallising dish for 15 min. The activator was found to be completely destroyed.

3. *Dialysis.* The activator was found to be very slowly dialysable. After 4 hours' dialysis in a cellophane tube against running water about 10 % and after 20 hours' dialysis about 80 % of the activity was found to be lost.

Anaerobic reduction of dyestuffs.

Though both methylene blue and 2:6-dichlorophenolindophenol were reduced very slowly in presence of the enzyme and proline, the reduction was comparatively rapid in presence of the heated enzyme, showing that the heated enzyme could also act as an activator in the anaerobic reduction of dyes (Table XI).

Table XI.

Enzyme solution heated at 90° for 5 min. at p_H 8.0 was used as the activator solution. Substrate: *M* proline. *M*/20 phosphate buffer at p_H 7.8 and 2:6-dichlorophenolindophenol 1 : 5000. Total volume of the fluid 3 ml. Thunberg technique was followed.

No.	Enzyme solution ml.	Activator solution ml.	Phosphate buffer ml.	<i>M</i> proline ml.	Dye 1/5000 ml.	Time of decoloration in min.
1	1.0	0.25	0.4	0.1	0.5	58
2	1.0	—	0.4	0.1	0.5	32
3	1.0	0.25	0.4	—	0.5	>180

SUMMARY.

1. A purified enzyme has been studied which can oxidise *DL*-proline more than *D*-alanine, *DL*-phenylalanine and leucine.

2. Several known activators such as co-enzyme (co-dehydrogenase I), Warburg co-ferment (co-dehydrogenase II), flavin-enzyme, glutathione, adenylypyrophosphate and ascorbic acid failed to activate the proline dehydrogenase.

3. When the original enzyme solution was heated at 80° at p_H 8.0 for 5 min. and added to the original enzyme an activation of the enzymic oxidation of proline was found.

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