CXLI. GLUTAMIC ACID DEHYDROGENASE FROM GERMINATING SEEDS¹

By MANAYATH DAMODARAN AND
KESAVAPILLAY RAMAKRISHNAN NAIR

From the Biochemical Laboratory, University of Madras, Madras

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LITTLE definite knowledge exists as to the mechanism of oxidative deamination in plants. That such deamination does occur is to be inferred both from the values of the respiratory quotients of certain germinating seeds [Malhotra, 1933] which indicate protein catabolism, as well as from the well-known accumulation of asparagine and glutamine during germination which has been shown to take place only in the presence of oxygen [Palladin, 1889]. As the amount of asparagine found is usually far in excess of what can be accounted for on the basis of the aspartic acid content of the seed proteins and, further, as the increase in asparagine is concomitant with the disappearance of other amino-acids [Schulze, 1898; 1901] there can be no doubt that its formation takes place at the expense of the latter. It is generally assumed that the ammonia formed by oxidative deamination condenses with $\text{COOH.CO.CH}_2\text{COOH}$ arising either from carbohydrates or from deaminized amino-acid residues to give first aspartic acid and then its amide. In the case of glutamine the position is rather different on account of the high content of the seed proteins in glutamic acid. As Greenhill & Chibnall [1934] have pointed out, with the evidence at present available it is equally valid to assume either that the glutamine present in the seed is the direct product of the hydrolysis of protein [Damodaran *et al.* 1932] or that it arises from the combination of ammonia with a non-nitrogenous residue.

One of the most interesting results of recent work on the oxidation of amino-acids in animal tissues is the hypothesis due to Krebs [1935, 1, 2] that in kidney tissue glutamine synthesis takes place through the intervention of a "deaminase". The disappearance of ammonia during the oxidation of glutamic and aspartic acids by muscle was noted by Needham [1930]. Krebs observed a similar effect with glutamic acid in the presence of kidney slices; he was able to show that the disappearance of ammonia was accompanied by an increase of amide-N determined by the method of Sachsse [1873] and further that the disappearance of added ammonia could be brought about by kidney slices in the presence of α -ketoglutaric acid. These observations taken in conjunction with the existence of a glutaminase in kidney have led Krebs to the inference that in the intact organ the deaminase system studied by him is really concerned, not with the oxidation of glutamic acid, but with the reverse synthetic process, viz. the formation of glutamine from ammonia and ketoglutaric acid, the latter probably arising from carbohydrate oxidation via pyruvic acid and $\alpha\alpha$ -diketoadipic acid [Toenniesen & Brinkmann, 1930].

It is of obvious interest to ascertain if this very attractive hypothesis is applicable to the vegetable organism also, especially in view of the fact that the experimental evidence for the formation of glutamine adduced by Krebs rests

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solely upon the determination of amide-N by acid hydrolysis, a method which can hardly be called specific [cf. Chibnall & Westall, 1932], whereas the formation of glutamine in the plant has been proved beyond doubt by actual isolation by a number of workers.

The present paper describes in the first place experiments carried out to ascertain the existence of deaminases in germinating seeds and secondly the properties of the only amino-acid dehydrogenase which was actually found in the seedlings examined, viz. one specific for glutamic acid.

Maceration extracts of the seedlings of about a dozen species (mostly of the leguminous family) were tested, by the methylene blue and the manometric methods, for their actions on the following amino-acids: glycine, alanine, leucine, tyrosine, histidine, aspartic acid, glutamic acid and *dl*-proline. Glutamic acid was the only amino-acid that was acted upon and even this action was found to be limited to three out of the twelve species examined, viz. to *Phaseolus mungo*, *P. radiatus* and *Pisum sativum*. In being obtained in cell-free extracts capable of oxidizing the natural *l*(+)-glutamic acid the enzyme found in germinating seeds differs from the deaminases studied by Bernheim and coworkers [1934, 1, 2; 1935, 1, 2] by Krebs [1933, 1, 2; 1934; 1935, 1, 2, 3] and by Weil-Malherbe [1935; 1936] whose preparations in extracts free from cellular material acted only on the optically non-natural amino-acids. Purification of the dehydrogenase by adsorption on kaolin, kieselguhr and aluminium hydroxide proved unsuccessful. Some concentration could be effected by precipitation of the maceration extract by saturation with ammonium sulphate. But the precipitate thus obtained could not be freed from ammonium sulphate as dehydrogenase activity was completely lost during dialysis. For all experiments, therefore, the ammonium sulphate precipitate redissolved in phosphate buffer at pH 7.8 and without dialysis was used. Seedlings of *Phaseolus mungo* two days old were found to be the best starting material. The initial oxygen uptakes of such preparations were quite low being generally about 50 $\mu\text{l./hr./ml.}$ The enzyme was always contaminated with peroxidase. It could, however, be readily demonstrated by means of differential inactivation that peroxidase activity was in no way responsible for the oxidation of glutamic acid. Thus treatment with acetone had very little influence on peroxidase (as determined quantitatively by the oxidation of Nadi reagent) while glutamic acid dehydrogenase was completely destroyed by the process.

Being capable of utilizing either oxygen or methylene blue as hydrogen acceptor the enzyme belongs to the class of oxytropic dehydrogenases. The action in the presence of oxygen is not inhibited by KCN up to a concentration of 0.005 *M* or by the other inhibitors tried, viz. arsenite and fluoride. The following substances were tried on the enzyme system to determine if they could function as hydrogen transporters in the reaction: methylene blue, ascorbic acid, glutathione and dihydroxyphenylalanine. None of these were found to have any accelerating effect on the oxygen consumption.

Inactivation by alcohol precluded the use of the method based on the coupled oxidation of alcohol by which Keilin & Hartree [1936] had demonstrated the formation of hydrogen peroxide in the case of kidney deaminase. The "Abfangsverfahren" of Wieland & Rosenfeld [1930] using ceric sulphate also gave negative results. But taking advantage of the presence of peroxidase in the enzyme preparation used, production of hydrogen peroxide could be demonstrated by the coupled oxidation of nitrite [Thurlow, 1925].

The oxygen uptake corresponds to one atom of oxygen to each molecule of glutamic acid. The end product of the reaction was shown to be α -ketoglutaric

acid which was isolated and identified as the dinitrophenylhydrazone. The reaction taking place is therefore as in the case of kidney deaminase:

The production of ammonia could not, however, be shown as exact ammonia determinations were rendered impossible by the presence of ammonium sulphate in the enzyme preparation used.

EXPERIMENTAL

Methods

Qualitative data such as the distribution of the dehydrogenase in seedlings were obtained by Thunberg's methylene blue technique; tubes of the Keilin type were used. In the use of Barcroft's manometer for the measurement of oxygen uptake the instructions given by Dixon [1934] were followed in detail. The manometers were calibrated both by calculation and by Munzer & Neumann's method; the values of the manometric constant obtained by both methods were in close agreement. 0.4 ml. of a 40% solution of sodium hydroxide and a small roll of No. 42 Whatman filter paper were placed in the centre compartment of the manometric cup. The rate of shaking was kept at 120–180 oscillations per minute according to the rate of oxygen uptake. The substrate was placed in small Keilin cups and added to the reaction mixture after equilibration.

For the estimation of peroxidase a method due to Guthrie [1931] based upon the colorimetric measurement of the amount of indophenol formed in a definite time from a mixture of *p*-phenylenediamine and α -naphthol in the presence of hydrogen peroxide was used. The reagent was made up by adding to 200 ml. of citrate buffer at pH 4.5 200 ml. of water, 1 g. of *p*-phenylenediamine hydrochloride and 20 ml. of a 4% solution of naphthol in 50% alcohol and filtering. To 25 ml. of this in a centrifuge tube were added 0.5 ml. of the solution to be tested and 5 ml. of a 0.1% solution of KCN. The mixture was shaken up with toluene, centrifuged and the solution of the indophenol in toluene compared in the colorimeter with an artificial standard consisting of 1 g. of iodine in 500 ml. of chloroform.

The amino-acids used were analytically pure samples prepared in the laboratory with the exception of *dl*-proline which was obtained from Messrs Hoffmann La-Roche. The dibasic amino-acids were neutralized with NaOH before use.

Presence of amino-acid dehydrogenase in seedlings

The seedlings were washed and weighed after removal of the testa. They were ground up for 15 min. with half the weight of 0.87% K_2HPO_4 and a little acid-washed sand, pressed through muslin and centrifuged. The supernatant was tested for amino-acid dehydrogenases using in every case 0.5 ml. of the extract to be tested, 0.2 ml. of an *M*/10 solution of the amino-acid, 0.3 ml. of 1/5000 methylene blue and 2 ml. of *M*/15 phosphate buffer at pH 7.8. Each tube had two controls, one with the boiled extract and the other with no amino-acid added.

Table I gives the results with different seedlings using various amino-acids. Glutamic acid is seen to be the only one of the amino-acids which accelerates the reduction of methylene blue. Even in this the action is confined to three of the seedlings examined, namely *Phaseolus mungo*, *P. radiatus* and *Pisum sativum*.

Where glutamic dehydrogenase is present a feeble action on alanine also is noticeable; but this seems to be too small to be of significance; the Thunberg factor [Thunberg, 1920] is below 2.

Table I. *Time for decoloration of methylene blue in Thunberg tubes*

(Figures in brackets refer to substrate-free controls. In boiled controls the time was the same in all cases.)

	Time in minutes							
	Glycine	Alanine	Tyrosine	Histidine	Aspartic acid	Leucine	Glutamic acid	dl-Proline
Seedling tried								
<i>Phaseolus mungo</i>	23 (23)	17 (21)	28 (28)	28 (28)	19 (19)	23 (24)	6 (18)	22 (22)
<i>Phaseolus radiatus</i>	12 (12)	10 (12)	12 (12)	16 (16)	13 (12)	9 (10)	5 (11)	13 (12)
<i>Dolichos lab lab</i>	22 (22)	25 (25)	25 (25)	30 (29)	28 (28)	23 (23)	23 (23)	23 (23)
<i>Dolichos biflorus</i>	No decoloration in 2 hr.							
<i>Cicer arietinum</i>	6 (6)	6 (6)	8 (8)	8 (8)	9 (8)	8 (8)	5 (6)	6 (6)
<i>Canavalia ensiformis</i>	35 (35)	27 (27)	27 (27)	32 (33)	33 (33)	30 (31)	24 (25)	26 (25)
<i>Canavalia obtusifolia</i>	31 (30)	33 (33)	41 (41)	41 (41)	37 (36)	36 (36)	32 (33)	38 (36)
<i>Pisum sativum</i>	22 (22)	18 (19)	23 (22)	22 (22)	19 (19)	19 (19)	8 (19)	21 (22)
<i>Citrillus colocynthis</i>	82 (85)	78 (76)	82 (85)	76 (76)	78 (76)	81 (82)	80 (82)	69 (69)
Coconut	No decoloration in 2 hr.							
Wheat	No decoloration in 2 hr.							
<i>Citrillus vulgaris</i>	No decoloration in 2 hr.							

Purification of the enzyme

Precipitation with dilute acetic acid, sodium sulphate and acetone and electro dialysis were tried as methods of purification, but all the methods yielded inactive preparations. The enzyme was not adsorbed on kaolin, kieselguhr or aluminium hydroxide. Precipitation by saturation with ammonium sulphate and taking up in phosphate buffer at pH 7.8 yielded an active preparation with a low initial oxygen uptake.

Seedlings between 2 and 3 days' old yielded the most active preparation. 20 ml. of the extract (pH 6.1) were cooled in ice and brought to pH 5.2 by the addition of very dilute acetic acid. After saturation with ammonium sulphate and centrifuging for 10 min. at 2000 r.p.m. the clear supernatant liquid was discarded. The residue was washed with phosphate buffer at pH 5.2 and re-dissolved in phosphate buffer at pH 7.8. The preparation in the absence of glutamic acid did not appreciably reduce methylene blue and had an oxygen uptake of about 50 μ l./hr./ml.

Differentiation from peroxidase activity

Peroxidase was invariably found in the seedlings and persisted in the preparation obtained as described above. Previous attempts at purification had shown that drying with acetone destroyed dehydrogenase activity. A comparison was therefore made of the effects of acetone treatment on the peroxidase activity and on the oxidation of glutamic acid.

20 g. of the seedlings were extracted with 20 ml. 0.87% K_2HPO_4 . The extract was poured slowly with stirring into five times the volume of acetone cooled in ice. The precipitate was washed on the centrifuge with 5 ml. of acetone, spread out on a Petri dish and the acetone driven off by aeration. The yellowish white powder so obtained was suspended in 20 ml. of phosphate buffer at pH 7.8

and the activities of the dehydrogenase and peroxidase determined by the manometric method and by Guthrie's method respectively. For comparison the same determinations were done on the fresh extract of the seedlings. The results of three different experiments are given in Table II.

Table II. *Effects of acetone treatment on dehydrogenase and peroxidase*

Peroxidase			Dehydrogenase		
Length of column in colorimetric tube (standard at 20 mm.)			Oxygen uptake with glutamic acid ($\mu\text{l./hr.}$)		
Before	After	% peroxidase	Before	After	% dehydrogenase
18.2	26.1	76.6	238.2	0	0
29.5	38.9	76.0	250.0	0	0
10.2	13.1	77.7	242.0	0	0

The results clearly indicate that the oxidation of glutamic acid is unconnected with peroxidase. Though more than 75% of the peroxidase remained after acetone-drying the manometric oxygen uptake was zero.

Variation of activity with pH

The variation of the oxygen uptake with pH is illustrated in Fig. 1. Below pH 5.9 the enzyme is totally inactive. The optimum pH is 7.8–8.0. The reduction of methylene blue in Thunberg tubes does not appear to be so susceptible to changes in pH as the oxygen uptake (Table III); at pH 5.9, however, the reduction of methylene blue also ceases.

Fig. 1.

Table III. *pH and time of decoloration of methylene blue*

No.	pH of medium	Time of decoloration
1	5.9	∞
2	6.5	23 min.
3	6.8	23 min.
4	7.2	24 min.
5	7.8	23 min.
6	8.7	24 min.

Optimum substrate concentration

Fig. 2 shows the effect on the rate of oxygen uptake and Table IV on the time for reduction of methylene blue of variations in glutamic acid concentration. 0.045 *M* is the optimal substrate concentration as determined from the graph.

Fig. 2.

Table IV. *Decoloration of methylene blue and substrate concentration*

Tubes	...	1	2	3	4	5	6
Enzyme		0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.	0.5 ml.
Methylene blue 1/5000		0.3 ml.	0.3 ml.	0.3 ml.	0.3 ml.	0.3 ml.	0.3 ml.
Glutamic acid <i>M</i> /10		2.0 ml.	—	—	—	—	—
Glutamic acid <i>M</i> /20		—	1.0 ml.	0.5 ml.	—	—	—
Glutamic acid <i>M</i> /100		—	—	—	1.0 ml.	0.5 ml.	—
Buffer pH 7.8		1.2 ml.	2.2 ml.	2.7 ml.	2.2 ml.	2.7 ml.	3.2 ml.
Time for decoloration		19 min.	24 min.	31 min.	39 min.	42 min.	127 min.

Inactivation by heat

10 ml. of the enzyme solution were placed in each of several test tubes kept immersed for different periods in baths at different temperatures. The solutions were cooled and the dehydrogenase activity in each tested manometrically. Slight inactivation was evident even at 37°. At 50° and above the destruction of the enzyme was very rapid (Table V).

Table V. *Inactivation by heat*

Time of heating min.	Temperatures					
	37° μl.	40° μl.	50° μl.	60° μl.	70° μl.	80° μl.
5	362.0	352.5	182.5	17.5	0	0
30	341.0	245.0	0	0	0	0
60	328.5	97.5	0	0	0	0

Formation of hydrogen peroxide

Fixation of hydrogen peroxide by oxidation of cerium hydroxide to cerium peroxide [Wieland & Rosenfeld, 1930] and determination of the cerium peroxide by permanganate titration failed to yield any clear-cut evidence of the formation of hydrogen peroxide. The coupled oxidation of alcohol was also inapplicable as the alcohol concentration used by Keilin & Hartree [1936] was found to retard the dehydrogenase action. It was found, however, that the method used by Thurlow [1925] for xanthine oxidase, based on the oxidation of nitrite to nitrate by peroxidase at the expense of the hydrogen peroxide formed by dehydrogenation, could be applied here on account of the presence of peroxidase in the enzyme preparation. The enzyme was allowed to act upon glutamic acid in the presence of added sodium nitrite, the reaction mixture being aerated for a definite period. At the end the solutions were centrifuged and the amount of unoxidized nitrite determined colorimetrically using Griess-Ilosvay's reagent. Table VI gives the composition of the reaction mixture and the colorimetric readings in a typical experiment in which the time of aeration was 45 min.

Table VI. *Coupled oxidation of nitrite*

Flasks	...	...	1	2	3
Enzyme			10 ml.	10 ml.	—
Glutamic acid <i>M</i> /10			5 ml.	—	5 ml.
Sodium nitrite 0.02%			2 ml.	2 ml.	2 ml.
Buffer <i>pH</i> 7.8			—	5 ml.	10 ml.
Unoxidized nitrite (as length of column in colorimetric tube to balance standard set at 5 mm.)			35.5 mm.	6.2 mm.	5.1 mm.

Effect of various hydrogen transporters

Appropriate experiments showed that methylene blue, ascorbic acid, glutathione and dihydroxyphenylalanine were incapable of acting as intermediate carriers in the oxidation of glutamic acid by the dehydrogenase. In no case was a significant acceleration of oxygen uptake observed on the addition of these substances.

Inhibiting agents

KCN up to 0.005 *M* had little effect on the oxidation (Table VII and Fig. 3). With higher concentrations the rate fell appreciably but the oxygen consumption was not completely inhibited even by a cyanide concentration of 0.2 *M*. Arsenite

Fig. 3. a =without (CN); b =0.001 M (CN); c =0.002 M (CN); d =0.005 M (CN); e =0.01 M (CN); f =0.1 M (CN); g =0.2 M (CN).

Fig. 4. a and b =no arsenite; c =0.001 M arsenite; d =0.002 M arsenite; e =0.02 M arsenite.

(Fig. 4) and fluoride in the concentrations generally employed did not lower the oxygen consumption to any extent. According to the accepted ideas this would indicate that oxygen activation is not involved in the action of the enzyme.

Table VII. *Effect of potassium cyanide on the O₂ uptake*

Manometers ...	1	2	3	4	5	6	7	8
Enzyme	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.
Glutamic acid	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.	1.0 ml.
Cyanide <i>M</i>	0.6 ml.	0.3 ml.	—	—	—	—	—	—
Cyanide 0.1 <i>M</i>	—	—	0.6 ml.	0.3 ml.	—	—	—	—
Cyanide 0.05 <i>M</i>	—	—	—	—	0.3 ml.	—	—	—
Cyanide 0.01 <i>M</i>	—	—	—	—	—	0.6 ml.	0.3 ml.	—
Buffer pH 7.8	0.4 ml.	0.7 ml.	0.4 ml.	0.7 ml.	0.7 ml.	0.4 ml.	0.7 ml.	1.0 ml.
O ₂ uptake (μl./hr.)	97.2	115.0	117.0	196.0	251.5	270.0	298.0	296.0.

Amount of oxygen utilized per molecule of glutamic acid

The rate of oxygen uptake of the enzyme during the complete oxidation of 1 ml. of *M*/50 glutamic acid was studied. The completion of the oxidation was shown by the cessation of the oxygen consumption (Table VIII and Fig. 5).

Fig. 5.

Sodium fluoride to a final concentration of 1/300 (0.2 ml. of *M*/20 solution) was added to each cup to ensure freedom from bacterial contamination. With 1 ml. of *M*/50 glutamic acid the oxygen consumed was 230 μl. This corresponds to 11.5 l. of oxygen per mol. or slightly over one atom of oxygen per molecule of glutamic acid.

Table VIII. *Oxygen consumption with 1 ml. of M/50 glutamic acid*

Time in min.	Exp.	μ l. oxygen taken up	
		Control	Difference
10	30.6	10.1	20.5
20	45.7	20.5	25.2
30	98.7	31.7	67.0
40	122.6	38.9	83.7
50	153.1	43.1	110.0
60	193.2	51.0	142.0
70	216.7	61.7	155.0
80	240.7	68.2	172.5
90	260.5	73.5	187.0
100	285.3	80.1	205.2
110	308.2	86.2	222.0
120	320.0	90.0	230.0
130	325.5	95.5	230.0
140	328.7	98.5	230.2

Isolation of α -ketoglutaric acid as the end product of the oxidation

To 250 ml. of the enzyme solution in *M*/15 phosphate buffer at *pH* 7.8, 100 ml. of *M*/10 glutamic acid neutralized with NaOH and 0.079 g. of arsenic trioxide as sodium arsenite were added and the mixture gently shaken for 2 hr. in a stoppered 500 ml. flask. It was found helpful to add 10 ml. of 1/1000 methylene blue to the mixture, the disappearance of the colour indicating the necessity of admitting fresh air and its permanence marking the end point of the reaction. At the end of 2 hr. the proteins were precipitated by adding 50 ml. of 50 % trichloroacetic acid, the liquid filtered at the pump using filter paper impregnated with kieselguhr and the filtrate concentrated to 150 ml. *in vacuo*. 100 ml. of a 2 % solution of 2:4-dinitrophenylhydrazine in 2*N* HCl were now added and the mixture kept in the refrigerator for 12 hr. The crystalline yellow precipitate which formed was filtered and purified by dissolving in 2*N* NaOH and reprecipitating with HCl. The precipitate thus obtained was separated on the centrifuge, washed several times with water and recrystallized from ethyl acetate. Golden yellow needle-shaped crystals of the dinitrophenylhydrazone of α -ketoglutaric acid melting at 223° were obtained. Yield 700 mg.

In a control experiment without the addition of glutamic acid 11 mg. of the same product were obtained. It is assumed that this arose probably from the glutamic acid formed in the breakdown of protein which was not completely removed by the method adopted for the purification of the enzyme.

SUMMARY

A number of germinating seeds have been examined for the presence of amino-acid dehydrogenases. The only such dehydrogenase found is one specific for glutamic acid which occurs in the seedlings of *Phaseolus mungo*, *P. radiatus* and *Pisum sativum*.

The enzyme is readily obtained in aqueous extracts free from cellular material and can be concentrated by precipitation with ammonium sulphate and re-dissolving in phosphate buffer. In this condition it actively oxidizes the naturally occurring *l*(+)-glutamic acid, differing in this respect from deaminases from animal sources hitherto studied, which in cell-free extracts act only on the optically non-natural isomerides of the amino-acids.

The enzyme belongs to the class of aerobic dehydrogenases, being capable of oxidizing glutamic acid either in the presence of methylene blue or of molecular

oxygen. Hydrogen peroxide formation has been demonstrated by the coupled oxidation of nitrite. Cyanide, arsenite and fluoride do not inhibit the enzyme nor is it activated by methylene blue, ascorbic acid, glutathione or dihydroxy-phenylalanine.

The oxygen uptake corresponds to 1 atom of oxygen per molecule of glutamic acid. The end product of the oxidation is α -ketoglutaric acid, which has been isolated and identified as the 2:4-dinitrophenylhydrazone.

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