CLXXXIII. THE *l*(+)GLUTAMIC DEHYDROGENASE OF ANIMAL TISSUES

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THUNBERG [1920] observed that natural glutamic acid was oxidized in the presence of frog muscle. Weil-Malherbe [1936] was the first to prepare an enzyme extract which catalysed this oxidation but he did not resolve the system into its catalytic components.

While this present analysis of the *l*(+)glutamic dehydrogenase system was in progress Adler *et al.* [1937] described the properties of the same enzyme in liver. They found that coenzyme I was essential to the system and that the products of the reaction were α-ketoglutaric acid and ammonia. They also demonstrated the reversibility of the reaction.

I. *Preparation of the components*

The *l*(+)glutamic dehydrogenase was prepared from liver or kidney of pig as follows. About 300 g. liver or kidney are minced in a Latapie mincer. To the mince are added 4 vol. cold acetone and the mixture is stirred for about 3 min., filtered on a Büchner funnel and washed in turn with acetone and ether. The material on the filter paper is again mixed with 4 vol. acetone, filtered, washed with acetone and ether and spread out on paper to dry in a current of air. 10 g. of the dry powder are rubbed up with 100 ml. distilled water and the mixture centrifuged. The supernatant fluid after filtration is acidified with 10% acetic acid to *p*H 4·6, the centrifuged precipitate washed with distilled water and finally suspended in 25 ml. *M*/10 phosphate buffer, *p*H 7·3. The enzyme keeps its activity in solution at 0° for 4–5 days. The acetone powder can be kept *in vacuo* for several weeks without loss of activity.

Coenzyme I was prepared by the method outlined by Green *et al.* [1937].

II. *The reaction with molecular oxygen*

A mixture of the dehydrogenase, coenzyme I and *l*(+)glutamic acid in the presence of a carrier such as pyocyanine takes up O_2 readily. The rate of O_2 uptake is dependent on the concentrations of the various reactants, i.e. dehydrogenase, coenzyme, substrate and carrier (cf. Figs. 1–4). As in other coenzyme systems the proportionality between the rate of oxidation and the concentrations of the different components holds only at low concentrations. Above some limiting concentration the system becomes saturated with the component in question.

The Michaelis constant, i.e. the substrate concentration at which half the maximum velocity is reached is approximately *M*/133 (Fig. 3).

Carriers which have been found to be active in the *l*(+)glutamic acid system include pyocyanine, methylene blue, cytochromes *a* and *b*, flavinphosphate and flavoprotein from yeast (Table I). Cytochrome *c* in large concentrations (1·2 mg./ml.) has a small effect on the O_2 uptake. The most efficient carrier

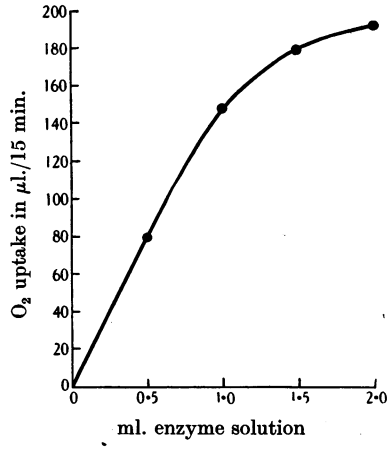

Fig. 1.

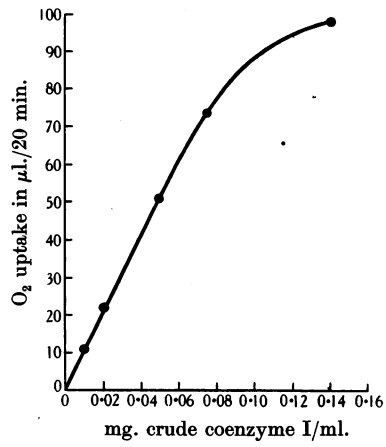

Fig. 2.

Fig. 1. The effect of the concentration of dehydrogenase. Each manometer contained 1·0 ml. 0·3% coenzyme I, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid.

Fig. 2. The effect of the concentration of coenzyme I. Each manometer contained 1·5 ml. enzyme, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid.

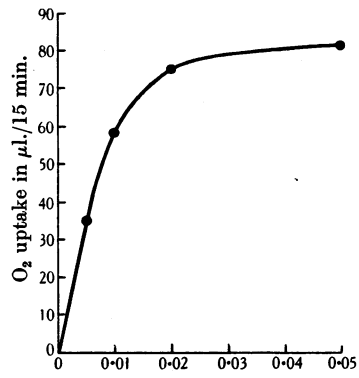

Fig. 3.

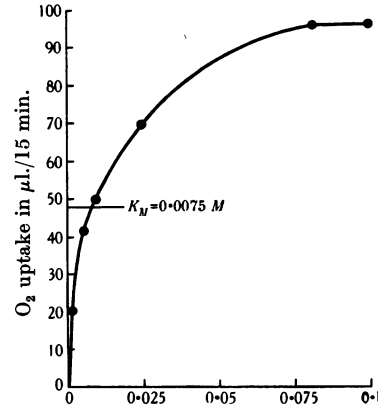

Fig. 4.

Fig. 3. The effect of the concentration of pyocyanine. Each manometer contained 1·5 ml. enzyme, 1·0 ml. coenzyme I and 0·5 ml. *M*/3 *l*(+)glutamic acid.

Fig. 4. The effect of the concentration of substrate. Each manometer contained 1·0 ml. enzyme, 1·0 ml. 0·3% coenzyme I and 0·2 ml. 0·1% pyocyanine. The total volume was 3·3 ml.

Table I. *The effect of carriers on the reaction with O_2*

The system contained 1·5 ml. enzyme; 1·0 ml. 0·3% coenzyme and 0·5 ml. *M*/3 *l*(+)glutamic acid. The total volume was 3·3 ml.

	μl. O_2 in 15 min.
l(+)glutamic system without carrier	0
System + 1·0 mg. methylene blue	56
System + 0·2 mg. pyocyanine	84
System + 0·5 mg. flavinphosphate	24
System + 2·5 mg. pure flavoprotein (yeast)	56
System + 0·3 ml. cytochromes *a* and *b* preparation	125

system was the cytochrome *a* and *b* system of Keilin and Hartree (cf. Dewan & Green [1938] for method of preparation). It has already been shown in other coenzyme systems that the coenzyme factor rapidly catalyses the oxidation of reduced coenzyme by cytochromes *a* and *b* [Dewan & Green, 1938].

M/100 cyanide inhibited the *l*(+)glutamic system when the cytochromes were used as carriers (Table II). The inhibition was practically complete during the first 5 min. but became progressively less as the reaction proceeded owing to the accumulation of α-ketoglutaric acid and subsequent cyanohydrin formation. *M*/500 cyanide reduced the rate of O_2 uptake by about 66% in the first 5 min. Here again the extent of inhibition decreased as the reaction progressed. Cyanide even in *M*/10 concentration had no inhibitory effect when pyocyanine was used as carrier.

Table II. *The effect of cyanide on the* l(+)*glutamic system with cytochromes* a *and* b *as carriers*

The complete system contained 1·5 ml. enzyme, 1·0 ml. 0·3% coenzyme I, 0·5 ml. preparation of cytochromes *a* and *b* and 0·3 ml. *M*/3 *l*(+)glutamic acid.

	μl. O_2 in 5 min.	μl. O_2 in 10 min.
Complete *l*(+)glutamic system	30	53
With *M*/500 HCN	11	25
With *M*/100 HCN	0	5

Ketone fixatives such as hydrazine, semicarbazide and cyanide were tested for their effect on the velocity of oxidation of *l*(+)glutamic acid. Hydrazine had no appreciable effect. Semicarbazide and cyanide did not influence the

Table III. *The effect of fixatives*

The system contained 1·5 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1% pyocyanine, 0·4 ml. *M*/3 *l*(+)glutamic acid. The fixative solutions were neutralized.

	μl. O_2		
	10 min.	20 min.	30 min.
l(+)Glutamic system	142	200	226
+0·2 ml. *M*/2 semicarbazide	157	222	260
+0·2 ml. 2*M* HCN	162	246	300

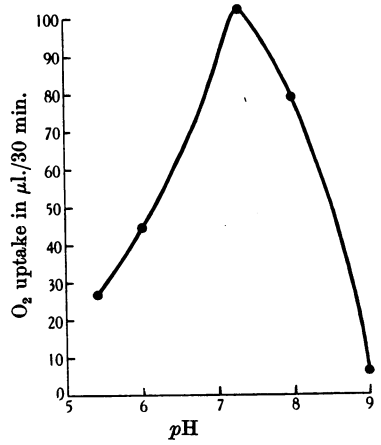

Fig. 5. The effect of *p*H. Each manometer contained 1·0 ml. enzyme, 0·8 ml. coenzyme, 0·2 ml. 0·1% pyocyanine and 0·3 ml. *M*/3 *l*(+)glutamic acid.

initial velocity but had a slight effect in preventing falling off in velocity as the reaction proceeded (cf. Table III). α-Ketoglutaric acid inhibits the oxidation of *l*(+)glutamic only when present in high concentration. This indicates that the glutamic system is much more negative in potential than the coenzyme system.

Fig. 5 shows the dependence of the rate of oxidation of *l*(+)glutamic acid on the *p*H of the solution. The maximum velocity is observed at about *p*H 7·3, the rate falling off rapidly above and below this value.

Effect of coenzyme factor

The presence of an enzyme in animal tissues which catalyses the oxidation of reduced coenzyme by carriers was demonstrated by Dewan & Green [1938] and Adler *et al.* [1937]. Coenzyme factor is also necessary in the *l*(+)glutamic system. To obtain a dehydrogenase preparation free from the factor, pig kidney, after mincing in a Latapie mincer, was allowed to stand 30 min. in 2 vol. iced water and pressed through muslin. The filtrate was mixed with 3 vol. cold acetone, the precipitate washed with acetone and ether and rubbed up in a mortar until dry. The acetone powder was then treated in the usual manner of preparing the *l*(+)glutamic dehydrogenase. The enzyme solution before using was filtered through kieselguhr and charcoal to remove last traces of coenzyme factor. The factor was prepared from rabbit skeletal muscle [cf. Dewan & Green, 1938].

Table IV shows that coenzyme factor is necessary for the aerobic oxidation of *l*(+)glutamic acid in presence of dehydrogenase, coenzyme I and pyocyanine.

Table IV. *Effect of coenzyme factor*

The complete system contained 0·5 ml. enzyme, 1·0 ml. coenzyme, 0·05 ml. 0·1% pyocyanine, 1·0 ml. factor and 0·5 ml. *M*/3 *l*(+)glutamic acid.

	μl. O_2 in 30 min.
System	31
Without factor	3
Without enzyme	0
Without glutamic acid	0

III. *The products of oxidation*

l(+)Glutamic acid on oxidation yields α-ketoglutaric acid and NH_3. The ketonic acid was isolated and identified as follows. A mixture containing 100 ml. enzyme, 50 ml. 0·3% coenzyme I, 10 ml. 0·1% pyocyanine and 25 ml. *M*/3 *l*(+)glutamic acid was aerated vigorously at 37° for 90 min., deproteinized with 50% trichloroacetic acid and filtered. The filtrate was concentrated *in vacuo* to 25 ml. 1 g. 2:4-dinitrophenylhydrazine in 100 ml. 2*N* HCl was added to the filtrate when crystals of the 2:4-dinitrophenylhydrazone began forming at once. The mixture was left at 0° for 10 hr. The precipitate was washed with 2*N* HCl and dried. Just sufficient ethyl acetate was added to dissolve the crystals and a small brown residue was filtered off. To the filtrate were added 4 vol. ligroin. The precipitate was centrifuged, washed with ligroin and finally dried *in vacuo*. M.P. 218°; M.P. of synthetic α-ketoglutaric acid 2:4-dinitrophenylhydrazone 218°; mixed M.P. 218°. Found (Weiler): C, 40·81%; H, 3·31%; N, 17·1%. $C_{11}H_{10}O_8N_4$ requires C, 40·44%; H, 3·09%; N, 17·18%.

The ratio of α-ketoglutaric acid formed to O_2 absorbed was determined by estimation of the $NaHSO_3$-binding of the deproteinized system after recording the O_2 uptake. Table V shows that for every atom of oxygen absorbed, approximately 1 mol. α-ketoglutaric acid was formed.

Table V. *Estimation of α-ketoglutaric acid formed by oxidation of l(+)glutamic acid*

The complete system contained 1·5 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid. Control had no substrate.

mg. α-ketoglutaric acid found by measuring $NaHSO_3$-binding power	2·2
μl. O_2 absorbed during the oxidation of the *l*(+)glutamic acid	172
Theoretical amount (in mg.) of α-ketoglutaric acid, assuming 1 mol. α-ketoglutaric acid is produced for each atom O absorbed	2·25

Further confirmation that 1 atom O is absorbed for each mol. *l*(+)glutamic acid oxidized is obtained from the O_2 equivalence of small amounts of *l*(+)-glutamic acid. Table VI shows the close agreement between the O_2 uptake for known amounts of *l*(+)glutamic acid and the theoretical uptake, calculated on this basis. With larger amounts of substrate the O_2 uptake was somewhat lower than the theoretical value, due to the fact that enzymic activity ceases before the whole of the glutamic acid is oxidized.

Table VI. *The O_2 equivalence of* l(+)*glutamic acid*

Each manometer contained 2·0 ml. enzyme, 1·0 ml. coenzyme I and 0·2 ml. 0·1% pyocyanine. The substrate was placed in Keilin cups which were introduced into the main body of the fluid after equilibration. The control O_2 uptake without substrate has been subtracted from the experimental values.

Millimol. *l*(+)glutamic acid	0·25	0·5	0·75	1·0
μl. O_2 absorbed	26	58	81	94
Theoretical uptake assuming that 1 atom O reacts with 1 mol. glutamic acid	28	56	84	112

NH_3 estimations. The O_2 uptake of the complete glutamic system was measured manometrically. A control experiment containing the whole system with the exception of substrate was carried out simultaneously. After a run of 90 min. NH_3 was estimated by the Parnas method. Table VII shows that the amount of NH_3 found correlates well with the theoretical value calculated from the O_2 uptake.

Table VII. *NH_3 estimations*

Complete system contained 1·0 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid.

mg. NH_3 found (corrected for control)	Theoretical NH_3 assuming that for 1 atom O absorbed 1 mol. NH_3 is produced	% theory
(1) 0·17	0·18	94
(2) 0·26	0·28	92

IV. *Specificity of substrate*

The glutamic dehydrogenase specifically catalyses the oxidation of *l*(+)-glutamic acid to α-ketoglutaric acid and NH_3. The unnatural isomeride is not oxidized.

dl-β-Hydroxyglutamic acid was attacked at one-tenth the rate of *l*(+)glutamic acid. Alanine, phenylalanine, valine, histidine and leucine of the *l*-series are not oxidized in the presence of the dehydrogenase preparation. Some preparations catalysed the oxidation of *l*-aspartic acid and *l*-cystine but these oxidations were found to be unconnected with the glutamic dehydrogenase. A sample of glutamine showed slight activity, but as it contained small amounts of glutamic acid it was difficult to assess the results.

V. *Specificity of coenzyme*

Coenzyme I cannot be replaced by coenzyme II in the *l*(+)glutamic system (cf. Table VIII). Coenzyme II was prepared from horse red blood cells and its activity checked with the hexosemonophosphate system.

Table VIII. *Specificity of coenzyme*

Experiments were carried out under anaerobic conditions in Thunberg tubes. The *l*(+)glutamic system contained 2 ml. enzyme, 0·2 ml. 0·5% methylene blue and 0·5 ml. of *M*/3 *l*(+)glutamic acid. The Thunberg tubes after evacuation were immersed in a water bath at 38°.

	Reduction time of methylene blue min.
System	∞
+0·15 mg. coenzyme I	5
+0·15 mg. coenzyme II	∞

VI. *Reduction of coenzyme I*

Coenzyme I was reduced by *l*(+)glutamic acid in the presence of the dehydrogenase (cf. Table IX). The reduction was demonstrated spectrophotometrically by the appearance of the characteristic band of reduced coenzyme with a peak at 340 mμ.

Table IX. *Reduction of coenzyme I by* l(+)*glutamic acid in presence of the dehydrogenase*

The system contained 1·0 ml. 0·3% oxidized coenzyme I, 0·3 ml. *l*(+)glutamic acid and 3·1 ml. 0·25% $NaHCO_3$. Total vol. 8 ml. Incubated in Thunberg tubes at 38° for 5 min. Contents then boiled and filtered through kieselguhr. The clear filtrate was used directly for analysis in the Hilger Spekker spectrophotometer [cf. Green & Dewan, 1937]. Two controls were done, one containing the system without *l*(+)glutamic acid, the other without the dehydrogenase.

	log I_0/I at 340 mμ
System	0·7
System without *l*(+)glutamic acid	0·1
System without dehydrogenase	0·1
Oxidized coenzyme	0·1

VII. *Distribution*

Various tissues of pig were investigated for the presence of *l*(+)glutamic dehydrogenase. Acetone powders of the tissues were made in the usual way. 4 g. of the powder in each case were ground up in a mortar with 40 ml. distilled water, centrifuged, the supernatant liquid filtered from fat and precipitated with 10% acetic acid at *p*H 4·6. The sediment was washed with distilled water and resuspended in 10 ml. *M*/10 phosphate buffer, *p*H 7·3. The system contained 2 ml. enzyme, 1·0 ml. 0·3% coenzyme I, 0·2 ml. 0·1% pyocyanine and 0·5 ml. *M*/3 *l*(+)glutamic acid. A control containing the system without *l*(+)glutamic acid was carried out in each case and the O_2 uptake subtracted from the value obtained with the complete system.

	μl. O_2 in 30 min.
System using liver enzyme	274
System using kidney enzyme	270
System using heart enzyme	25

The enzyme was detected in muscle and brain but in concentrations too small for accurate measurements.

VIII. *Reversibility*

The reaction between glutamic acid and coenzyme I may be formulated as follows:

$$l(+)\text{Glutamic acid} + \text{coenzyme I} \rightarrow \alpha\text{-ketoglutaric acid} + NH_3 + \text{reduced coenzyme I.}$$

If this reaction is reversible it should be possible to obtain glutamic acid from a mixture of the reduced coenzyme I, NH_3 and α-ketoglutaric acid. Reduced coenzyme I can be obtained either enzymically or by reduction with hyposulphite. In the following procedure the β-hydroxybutyric system was used to reduce the coenzyme, and the production of acetoacetic acid under anaerobic conditions was used as a measure of the reaction between reduced coenzyme on the one hand, and NH_3 and α-ketoglutaric acid on the other. For the theory of this method, cf. Dewan & Green [1937] on coenzyme-linked reactions. Acetoacetic acid was measured by the aniline citrate method of Ostern [1933]. The enzyme was prepared from pig's heart [cf. Green *et al.* 1937]. This preparation contains dehydrogenases for both *l*-β-hydroxybutyric acid and *l*(+)glutamic acid. Table X shows that the reversed reaction takes place when all components of both systems are present. The small blank in the absence of NH_4Cl is due to the reversibility of the α-hydroxyglutaric dehydrogenase system which has been shown by Weil-Malherbe [1937] to be present in heart.

Table X. *Production of acetoacetic acid in the reaction between β-hydroxybutyric acid, α-ketoglutaric acid and NH_3*

The complete system contained 0·2 ml. *M dl*-β-hydroxybutyric acid, 0·8 ml. *M*/3 NH_4Cl, 0·2 ml. *M*/6 α-ketoglutaric acid, 1·5 ml. heart enzyme preparation and 1 ml. coenzyme I. The substrates were neutralized before using.

	CO_2 production in 60 min.
Complete system	190
Without *dl*-β-hydroxybutyric acid	0
Without NH_4Cl	27
Without α-ketoglutaric acid	0
Without heart enzyme preparation	0
Without coenzyme I	0

Further proof of the reversibility of the *l*(+)glutamic system is derived from the demonstration of the formation of amino-N when all the components of the coenzyme-linked reaction were present. Three mixtures were set up in Thunberg tubes under anaerobic conditions; one contained the complete system, a second was without NH_4Cl and a third without α-ketoglutaric acid. After 1–2 hr. incubation at 38° the contents of each tube were deproteinized and the filtrates made up to the same final volumes. Acetoacetic acid estimations were made on one half of the filtrates and amino-N (Van Slyke method) on the other half.

Table XI. *Production of amino-N in the reaction between β-hydroxybutyric acid, α-ketoglutaric acid and NH_3*

Complete system contained 3·0 ml. heart enzyme preparation, 1·5 ml. 0·3% coenzyme I, 0·2 ml. *dl*-hydroxybutyric acid, 1·0 ml. *M*/3 NH_4Cl and 0·2 ml. *M*/6 α-ketoglutaric acid. Two controls were done, one without NH_4Cl, the other without α-ketoglutaric acid.

	(1)	(2)
Amino-N found	0·04 mg.	0·088 mg.
Theoretical amount of amino-N expected from CO_2 production assuming that 1 atom amino-N is formed for each mol. CO_2 produced.	0·05 mg.	0·081 mg.

The small amino-N values in the controls were averaged and this value was subtracted from the amount obtained in the complete system. Table XI shows that the amino-N found agrees well with the theoretical amount expected from the acetoacetic acid production on the assumption that 1 atom amino-N is formed for each mol. acetoacetic acid produced.

Summary

1. The preparation and properties of *l*(+)glutamic dehydrogenase of animal tissues are described. Coenzyme I is a necessary component of the system.

2. The dehydrogenase catalyses the oxidation of *l*(+)glutamic acid to α-ketoglutaric acid and NH_3. The reversibility of this reaction has been demonstrated.

I should like to express my thanks to Prof. Sir F. G. Hopkins for the interest he has shown in the progress of this work and to Dr D. E. Green for his constant advice, to Prof. C. R. Harington for the gift of a sample of *dl*-β-hydroxyglutamic acid and to Mr S. Williamson for some of the chemical preparations and the amino-N estimations.

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