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Studies on Bacterial Amino-acid Decarboxylases

4. *l*(-)-HISTIDINE DECARBOXYLASE FROM *CL. WELCHII* TYPE A

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Gale (1941) has shown that strains of *Cl. welchii*, when grown under acid conditions or in the presence of fermentable carbohydrate, decarboxylate *l*(-)-histidine and *l*(+)-glutamic acid to histamine and γ -aminobutyric acid respectively. In this paper the extraction, purification and properties of *l*(-)-histidine decarboxylase from a strain of this organism are described.

ORGANISM AND METHODS

Organism and substrates used. The organism used as a source of *l*(-)-histidine decarboxylase was *Cl. welchii* (Type A) BW 21, National Collection of Type Cultures (N.C.T.C.) no. 6784. Histidine solutions were prepared from *l*(-)-histidine monochloride monohydrate (The British Drug Houses Ltd.), except for the quantitative experiments, in which *l*- and *dl*-compounds as supplied by Roche Products Ltd. were used.

Methods of estimating and expressing enzyme activity. As in the earlier papers of this series (Gale & Epps, 1944a; Epps, 1944) the activity of enzyme preparations was estimated in Warburg manometers. Unless otherwise stated, the reaction temperature was 30°, the gas phase air, the buffer 2 ml. *m*/5 acetate pH 4.5 and the substrate (in side-bulb) 0.5 ml. *m*/30 *l*(-)-histidine solution at pH 4.5.

At 30° the reaction curve is not linear (see below), so the manometers were read 2 min. after the substrate had been tipped into the main reaction vessel and then at 5 min. intervals, the rate of CO₂ evolution being calculated from the first of these 5 min. periods (under the conditions adopted the temperature of the manometer flask had regained 30° within 2 min. of mixing).

Enzyme units. The unit quantity of enzyme liberates 100 μ l. CO₂/5 min.; rates of enzymic activity are expressed as $Q_{CO_2}^0 = \mu$ l. CO₂ evolved/mg. C/hr. C was estimated by the wet combustion micro-method of Van Slyke & Folch (1940).

1. PRELIMINARY WORK ON THE PURIFICATION OF ENZYME PREPARATIONS

Growth of cultures and preparation of acetone powders. For this preliminary work cultures were grown for 17 hr. at 37° in 2% glucose tryptic-casein broth to which a small amount of heart muscle had been added. Anaerobic conditions were ensured by addition after autoclaving of 1 drop thiolacetic acid/l. broth, thiolacetic acid in this concentration having no harmful effect on growth or activity. After growth the bacteria were harvested, washed once, made into a thick suspension with distilled water and stirred into 5 vol. ice-cold acetone. The precipitate was left to flocculate, then filtered off on a small Buchner funnel and washed successively with acetone, a mixture of acetone and ether in equal parts and, finally, with ether. The dry powder was active towards *l*(-)-histidine and also, to a lesser extent, towards *l*(+)-glutamic acid; by altering the conditions of growth and acetone treatment it was possible to reduce this residual glutamic acid decarboxylase activity to zero.

Destruction of *l*(+)-glutamic acid decarboxylase activity during preparation of the acetone powder. The test conditions for activity towards glutamate were the same as for histidine, except for the substrate used.

(1) **Variation in growth time.** Glutamic acid decarboxylase shows no variation with growth times from 12 to 27 hr. at 37°; histidine decarboxylase is maximal when the culture is harvested between 15 and 21 hr.

(2) **Variation in growth temperature.** Powders prepared from cultures grown for 17 hr. at 27 and 37° respectively have the same activity towards glutamic acid; the activity towards histidine is five times greater in the powder from the culture grown at the higher temperature.

(3) **Temperature of acetone treatment.** The histidine decarboxylase activity of the powder is the same whether the suspension is treated with acetone at room temperature or at 0°; the glutamic acid decarboxylase activity is halved by the use of acetone at room temperature.

(4) *Conditions of acetone treatment.* The histidine decarboxylase activity is unaffected by variation in the volume of acetone used or in the time of contact with acetone; the activity towards glutamic acid can be halved by the use of less than 5 vol. acetone for precipitation and again by leaving the suspension in contact with the acetone for periods longer than 15 min.

The conditions finally adopted made use of the relative instability of glutamic acid decarboxylase and were as follows: the culture was grown for 17 hr. at 37°; the bacteria were harvested, made into a thick suspension with water and stirred into 3 vol. acetone at room temperature. After 30 min. the precipitate was filtered off and washed as before. Acetone powders thus prepared decarboxylate l(-)-histidine alone of the 16 naturally occurring amino-acids tested by Gale (1940) and when kept in a desiccator maintain their activity for several weeks. The amount of l(-)-histidine decarboxylase present in the powder appears to be greater than that in the original washed suspension (Table 1); a similar apparent increase was observed in the l(-)-tyrosine decarboxylase content of acetone powders from *Strep. faecalis* (Eppe, 1944).

Preparation of cell-free extract. Acetone powders were incubated with buffers over the range pH 4.0-10.0, the suspension was centrifuged and the supernatant tested for l(-)-histidine decarboxylase activity. The enzyme is soluble over the range pH 5.0-10.0; optimum pH for extraction 8.5. Extraction is more rapid at 37° than at 30°, and the percentage extraction is independent of the strength of the suspension between 10 and 50 mg./ml. The maximum activity of the supernatant fluid is reached after 4 hr. at 37° and pH 8.5; further incubation, up to 24 hr., causes no significant loss.

Adsorption and elution. Previous work has shown that l(+)-lysine decarboxylase from coliform organisms can be purified by acid-alcohol precipitation followed by adsorption on to alumina C_γ (Gale & Eppe, 1944a), and l(-)-tyrosine decarboxylase from *Strep. faecalis* by adsorption on to Ca₃(PO₄)₂ (Eppe, 1944). When the extract from *Cl. welchii* BW 21 is adjusted to pH 4.0-4.5, a precipitate is formed which contains all the l(-)-histidine decarboxylase activity, but washing the precipitate with mildly alkaline buffers does not redissolve the active fraction. Addition of ethanol followed by adjustment of the pH to 5.0 results in total loss of activity. The enzyme can be adsorbed from the crude extract on to alumina C_γ or on to Ca₃(PO₄)₂ over a pH range of 5.0-10.0; the pH-adsorption curve for alumina shows optima at pH 5.0 and 9.5, while the enzyme is adsorbed maximally on to Ca₃(PO₄)₂ at pH 5.0. m/5 phosphate pH 7.0 elutes the enzyme from alumina, and 50% saturated (NH₄)₂SO₄ solution from either adsorbent. The greatest degree of purification was obtained when the extract was adjusted to pH 5.0 and the enzyme adsorbed on to alumina, followed by elution with 50% saturated (NH₄)₂SO₄ solution. The enzyme is not adsorbed on to kaolin, Fuller's earth or charcoal from mildly acid, neutral or mildly alkaline solutions.

(NH₄)₂SO₄ precipitation and fractionation. l(-)-Histidine decarboxylase is precipitated from solution when the (NH₄)₂SO₄ concentration of the eluate described above is raised to 75% saturation, and the enzyme may be further purified by fractional precipitation with this salt (details are given in the next section). Fractionation further than that described does not lead to a significant increase in activity.

2. FINAL CONDITIONS FOR PURIFICATION

Table 1 gives the volumes, enzyme content and activity at each stage during the following enzyme preparation.

13 l. of 2% glucose casein-digest broth containing heart muscle and thiolacetic acid (1 drop/l.) were inoculated from a 24 hr. meat-broth culture of *Cl. welchii* BW 21 and incubated at 37° for 17 hr. The culture was strained through glass-wool and passed through a Sharples super-centrifuge. The bacteria were washed, made into a thick suspension with distilled water (Stage 1, Table 1) and then stirred into 3 vol. acetone at room temperature. After 30 min. the precipitate was filtered off on a small Buchner funnel and washed successively with acetone, a mixture of equal parts of acetone and ether and, finally, with ether. The powder was spread out to dry.

The dry powder was suspended in m/20 borate pH 8.5 (40 mg. powder/ml.) and incubated overnight at 37°. The suspension (Stage 2) was centrifuged at 3000 r.p.m. for 20 min. and the clear, yellow supernatant liquid collected (Stage 3). The pH was adjusted by addition of 2M-acetate pH 5.0 (1 ml. buffer to 5 ml. extract) and neutral alumina C_γ suspension was added (8 mg. alumina for each ml. at Stage 3). After 10 min. the alumina was centrifuged and washed once with distilled water. The enzyme was twice eluted with 50% saturated (NH₄)₂SO₄ solution, each time with 1 ml. eluent/48 mg. alumina, the suspension being kept for 10 min. at room temperature before centrifuging. The eluates were combined (Stage 4).

1.5 vol. saturated (NH₄)₂SO₄ solution was added to the preparation; after half an hour the suspension was centrifuged for 30 min. at 3000 r.p.m. The precipitate was dissolved in distilled water (Stage 5) and sufficient saturated (NH₄)₂SO₄ solution added to bring the final salt concentration successively to 45, 50, 67 and 75% saturation; each precipitate was centrifuged in turn and dissolved in distilled water (Stages 6 a, b, c and d).

Table 1. *Purification of l(-)-histidine decarboxylase*

Stage	Vol. (ml.)	Activity (μl. CO ₂ /ml./5 min.)	Enzyme units	C content (mg./ml.)	Q _{CO₂} ^c
1	114	255*	290	42.7	71
2	300	198	595	16.4	145
3	233	197	459	9.30	254
4	75	434	325	8.90	577
5	50	555	277	9.30	715
6 (a)	0-45%	20	420	84	20.8
(b)	45-50%	10	320	32	4.16
(c)	50-67%	10	1270	127	4.16
(d)	67-75%	5	205	10	0.740
					3330

* Activity measured at pH 2.5, the optimum for the intact organism.

Maximum purification of the enzyme was obtained at Stages 6 *c* and *d*, which were slightly turbid and yellowish in colour; the degree of purification was *c.* 50 times.

3. PROPERTIES OF *l*(-)-HISTIDINE DECARBOXYLASE

The source of enzyme for these studies was from Stages 6 *c* and *d* of various preparations.

Variation of activity with pH. Fig. 1 shows the effect of pH on the activity of enzyme preparations. The activity is the same in acetate or phthalate buffer systems, the optimum being at pH 4.5. At values acid to *c.* pH 4.8 precipitation occurs and

Fig. 1. Variation in activity with pH of *l*(-)-histidine 5.5 decarboxylase. ○—○ Phthalate buffer. ●—● Acetate buffer.

the enzyme is associated with the acid-precipitated protein. Optimum *l*(-)-histidine decarboxylase activity is shown by intact cells in buffer at pH 2.5 (Gale, 1941); a similar apparent change in pH optimum on extraction of an enzyme from the bacterial cell has been recorded by Davies (1943) for acetoacetic acid decarboxylase from *Cl. acetobutylicum* and by Gale & Epps (1944*a*) for *l*(+)-lysine decarboxylase from *E. coli*.

Effect of substrate concentration. Variation of activity with substrate concentration is shown in Fig. 2; the Michaelis constant for the reaction is 0.0075*M*.

Effect of temperature. Fig. 3 shows variation of activity with temperature. By following the CO₂ output it appears that the reaction is not linear at temperatures above 22°; Table 2 shows the percentage decrease in activity at each temperature during the second 5 min. period of the experiment. As in the case of *l*(-)-tyrosine decarboxylase from *Strep. faecalis* (see Epps, 1944), temperature-sensitivity is conditioned by the presence of the substrate; incubation of the acetone powder at 37° for 24 hr.

does not result in loss of activity nor does the presence of added histamine increase the extent of the inhibition. The rate of the reaction is not maintained by addition of codecarboxylase or a boiled aqueous suspension of *Cl. welchii* BW 21.

Fig. 2. Variation in activity of *l*(-)-histidine decarboxylase with substrate concentration.

Fig. 3. Variation of activity of *l*(-)-histidine decarboxylase with temperature.

Table 2. Temperature sensitivity of enzyme preparation

Temperature (°)	17	22	27	31	38	44
Decrease in rate during second 5 min. period (%)	0	0	8	12.5	24.5	34

Effect of enzyme concentration. The initial rate of the reaction bears a linear relation to the amount of enzyme preparation used over the range covered by these manometric tests (Fig. 4).

Quantitative nature of the reaction and specificity of enzyme preparations. Table 3 shows the mean total CO₂ output in a number of experiments with 0.5 ml. *M*/30 solution of *l*- and *dl*-histidine as sub-

strates. The figures show that the enzyme is specific for the naturally occurring stereoisomer and the reaction quantitative.

Fig. 4. Variation of activity with l(-)-histidine decarboxylase concentration.

Table 3. Stereospecificity of enzyme and quantitative nature of reaction

Figures refer to total CO₂ output in μl. from 0.5 ml. M/30 solution of appropriate substrate after subtraction of blank; theory ≡ 373 μl. CO₂.

Substrate	No. of observations	Mean	Standard deviation	Range	% of calculated
l(-)-Histidine	6	345	2	341-347	93
dl-Histidine	8	173	4	167-180	46.5

Enzyme preparations are inactive towards the other 15 naturally occurring amino-acids used by Gale (1940); thiol-histidine and carnosine (β-alanyl histidine) are not attacked, nor do they inhibit the reaction with l(-)-histidine. Geiger (1944), using a strain of *E. coli* that decarboxylates l(-)-histidine, has found that the acetyl and benzoyl derivatives are not attacked. It would appear that any possible substrate must contain at least the iminazole ring and free NH₂ group of histidine itself.

Isolation of reaction products. A Krebs vessel containing 20 ml. M/60 l(-)-histidine, 20 ml. M/20 phthalate pH 4.5 and 5 ml. enzyme preparation was incubated at 30°; the course of the reaction was followed in a control manometric experiment. When gas evolution had ceased (c. 60 min.) the contents of the Krebs vessel were boiled, deproteinized with Fe₂(SO₄)₃, and histamine dipicrate was isolated as described by Gale (1941) and recrystallized from water. The product (109 mg.), m.p. 239° (corr.), gave on analysis (Weiler) C, 36.08; H, 2.68; N, 21.3%. C₁₇H₁₅N₉O₁₄ requires C, 35.97; H, 2.64; N, 22.2%.

Effect of inhibitors. Table 4 shows that M/100 sulphanilamide caused 51% inhibition; this effect is specific for sulphanilamide from among this series of drugs, since no inhibition is observed with M/100 sulphaguanidine or with saturated solutions of sulphathiazole, sulphapyridine or sulphasuxidine. The Zn-protein carbonic anhydrase is also inhibited specifically by sulphanilamide but the degree of inhibition is some 1000-fold greater. Keilin & Mann (1940) found that carbonic anhydrase is inhibited by benzene sulphonamide and *p*-acetylamino-benzene sulphonamide, while sulphanic acid and *p*-aminobenzene sulphonacetamide are without effect. None of these compounds in M/100 concentration has any effect on l(-)-histidine decarboxylase activity and it seems probable that the mechanism of inhibition of the two enzymes is different. The sulphanilamide inhibition of l(-)-histidine decarboxylase is not reversed by addition to the enzyme preparation of *p*-aminobenzoic acid or of a boiled aqueous suspension of *Cl. welchii* BW 21.

Table 4. Effect of inhibitors

Inhibitor	pI*	Inhibition (%)
AgNO ₃	4	100
	5	13
HgCl ₂	3	94
	4	58
CuSO ₄	2	92
	3	61
FeCl ₃	1	97
	2	15
Pb(CH ₃ COO) ₂	1	86
Fe(NH ₄) ₂ (SO ₄) ₂	1	25
NH ₂ OH	3	100
	4	54
KMnO ₄	3	94
	4	15
Chloramine T	2	97
	3	15
HCN	2	97
	3	20
Sulphanilamide	2	51
<i>p</i> -Phenylenediamine	2	0
N ₂ H ₄	1	88
Iodoacetate	1	67
Na ₂ S	1	42
NaN ₃	1	38
NaF	1	11
Urea	1	0
Urethane	1	0

* -log₁₀ [I], where [I] is molar concentration of inhibitor.

The activity of l(+)-lysine decarboxylase is inhibited by the presence of certain salts (Gale & Epps, 1944a) but (NH₄)₂SO₄ solution, up to 33% saturation, has no effect on l(-)-histidine decarboxylase activity.

Resolution of the enzyme. It has previously been shown that *l*(+)-lysine decarboxylase from coliform organisms and *l*(-)-tyrosine decarboxylase from *Strep. faecalis* contain the same coenzyme, codecarboxylase, whose isolation from yeast has been described (Gale & Epps, 1944*a, b*; Epps, 1944). Dissociation of *l*(+)-lysine decarboxylase can be induced by precipitation of the protein with 66 % saturated $(\text{NH}_4)_2\text{SO}_4$ solution containing 10 % NH_4OH and dissociation of *l*(-)-tyrosine decarboxylase by precipitation with ammoniacal $(\text{NH}_4)_2\text{SO}_4$ solution, by dialysis against distilled water at 0° or by keeping the enzyme preparation in the ice-chest for several days.

l(-)-Histidine decarboxylase preparations were subjected to the following treatments in attempts to dissociate the enzyme: (a) Protein was thrice precipitated by addition of 3 vol. saturated $(\text{NH}_4)_2\text{SO}_4$ solution containing 10 % NH_4OH . (b) Enzyme preparations were dialyzed at 0° for 24 hr. against distilled water and against 45 % saturated $(\text{NH}_4)_2\text{SO}_4$ solution. (c) Enzyme preparations were kept in the ice-chest for periods up to 8 weeks. (d) Protein was precipitated at pH 3.0 with 40 % saturated $(\text{NH}_4)_2\text{SO}_4$ solution and washed with 75 % saturated $(\text{NH}_4)_2\text{SO}_4$ solution. (e) The preparation was made *m* with respect to KCN, protein precipitated by addition of 3 vol. saturated $(\text{NH}_4)_2\text{SO}_4$ solution and the precipitate washed twice with 75 % saturated $(\text{NH}_4)_2\text{SO}_4$. After treatments (a), (b) and (c) there was only small

loss of activity while after treatments (d) and (e) the preparations were almost inactive; in no case was activity increased by addition of codecarboxylase preparation or a boiled aqueous suspension of *Cl. welchii* BW 21. It has also been shown that activity can be restored to *l*(-)-tyrosine decarboxylase apo-enzyme by addition of a boiled preparation of *l*(+)-lysine decarboxylase (Gale & Epps, 1944*b*), whereas addition of boiled *l*(-)-histidine decarboxylase preparation (≈ 13 units enzyme) has no such effect; the presence of codecarboxylase can, however, be shown in a suspension of *Cl. welchii* BW 21. These results suggest that codecarboxylase is not part of *l*(-)-histidine decarboxylase from *Cl. welchii* Type A.

SUMMARY

1. The extraction, purification and properties of *l*(-)-histidine decarboxylase from a strain of *Cl. welchii* Type A are described.
2. Acetone powders and enzyme preparations specifically attack *l*(-)-histidine, which is decarboxylated quantitatively to histamine.
3. It has not been possible to bring about dissociation of the enzyme, which would appear to be devoid of codecarboxylase.

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Studies on Bacterial Amino-acid Decarboxylases

5. THE USE OF SPECIFIC DECARBOXYLASE PREPARATIONS IN THE ESTIMATION OF AMINO-ACIDS AND IN PROTEIN ANALYSIS

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Previous papers of this series have described specific enzyme preparations for the decarboxylation of *l*(+)-lysine (Gale & Epps, 1944), of *l*(-)-tyrosine (Epps, 1944) and of *l*(-)-histidine (Epps, 1945). The present communication shows the application of these and other preparations to the rapid analysis of protein hydrolysates for the amino-acids concerned.

METHODS

Manometric arrangements. The method depends in every case upon the manometric measurement of the CO_2 liberated from the amino-acid by the specific decarboxylase at the optimum pH and 30°. Warburg manometers were used containing measured portions of amino-acid solution or protein