logous series, but the two-dimensional chromatogram is especially convenient, in that it shows at a glance information that can be gained otherwise only as the result of numerous experiments. The position of unknown substances on chromatograms developed with suitable pairs of solvents will also provide a clue to the existence of certain groups in the molecules, e.g. hydroxyl, acidic, basic or cyclic.

A few peptides have been shown to give satisfactory bands and also satisfactory colours with ninhydrin. In fact the work reported here was undertaken as a preliminary to the study of the products of the partial hydrolysis of proteins.

SUMMARY

1. A method of separating amino-acids on partition chromatograms by the use of water in cellulose (filter paper) as the stationary phase is described. Ninhydrin is used to reveal the amino-acids.

2. Phenol, collidine and *n*-butanol benzyl alcohol mixture (1:1 v/v) have proved useful as mobile phases. Other solvents have been investigated.

3. The partition coefficients calculated, normal water content of the paper being assumed, are close to those directly measured, showing that the cellulose acts as an inert support.

4. Two-dimensional chromatograms on sheets of filter paper are described; first one solvent is run in one direction, then, after the paper has been dried, another solvent is run in a direction at right angles to the first.

5. The presence of most of the amino-acids in wool, or in an artificial mixture of 22 amino-acids, can be demonstrated in a single experiment; all can be shown by suitable additional experiments. 200–400 μg. of protein are sufficient.

6. Hydroxy-amino-acids move more slowly than the corresponding amino-acids in phenol, but in collidine the rates are similar.

7. Ammonia selectively slows aspartic and glutamic acids and hastens the basic amino-acids. Acid has the reverse effect.

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Studies on Bacterial Amino-acid Decarboxylases

1. *l*(+)-LYSINE DECARBOXYLASE

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Amino-acid decarboxylases are produced by certain bacteria in response to growth in an acid environment (Gale, 1940, 1941), and it has been suggested that the neutralizing effect of the decarboxylation serves to protect the cell against the external acidity (Gale & Epps, 1942). The distribution of the decarboxylases among species and strains would indicate that each activity is produced by an enzyme specific for one amino-acid. The present communication deals with the extraction from the cell of *l*(+)-lysine decarboxylase, its partial purification, the properties of the partially purified enzyme and its resolution into the specific protein and a hitherto undescribed coenzyme. *l*(+)-Lysine decarboxylase is a component of the potential enzyme constitution of many of the coliform organisms, and two such organisms have been used as a source of enzyme in these studies. A preliminary note on the preparation of the specific enzyme and coenzyme has been published (Gale & Epps, 1943).

ORGANISMS USED

1. *Escherichia coli*. National Collection of Type Cultures no. 86.

2. *Bact. cadaveris*. This organism was isolated from pieces of meat left to putrify in phthalate buffer at pH 4. The organism was obtained by plating on to casein-digest-glucose-agar; we are indebted to Dr Tomlinson of the Emergency Public Health Service for the following characterization tests: acid and gas (24 hr.) in glucose, maltose, mannose; (5 days) in sucrose; no fermentation in lactose or salicin; indol negative; non-motile Gram-negative rod: the organism may therefore be classified in the paracolon group. A culture has been deposited with the National Collection under the classification no. 6578.

METHODS

Measurement of activity. The decarboxylation of *l*(+)-lysine was followed manometrically. The main cup of the Warburg manometer contained 2 ml. M/5 phosphate buffer pH 6·0 and 0·5 ml. enzyme preparation, with 0·5 ml. M/15 *l*(+)-lysine dihydrochloride (Roche Products Ltd.), neutralized to pH 6·0, in the side-bulb. The manometers were shaken in a bath at 30°, and after 15 min. equilibration the substrate was tipped in from the side-bulb. The CO_2 evolution was measured for the 15 min. after tipping. One unit of enzyme is defined as the amount which liberates 100 µl. CO_2 from *l*(+)-lysine in 5 min. at pH 6·0 and 30°. The purity of the preparations was followed by estimation of carbon content by the wet combustion method of Van Slyke & Folch (1940) and the activity expressed as $Q^{C}_{CO_2}$ = µl. CO_2 liberated from *l*(+)-lysine at pH 6·0 and 30°/hr./mg. C of enzyme preparation.

Growth conditions for optimum production of enzyme. Previous work on washed suspensions of intact organisms has shown that the decarboxylase activity of coliform organisms is greatest when growth occurs in an acid medium and at a low temperature (Gale, 1940). These findings may be due to variation of enzyme content or alteration of the permeability of the cell membrane with growth conditions. Since the lysine decarboxylase activity of the cells is unaffected by acetone drying, it is possible to investigate this point with acetone powders of the organisms grown under varying conditions. Table 1, A shows the effect of the pH of the growth medium on the lysine decarboxylase activity of washed suspensions and of acetone-dried organisms: in either case the activity increases with decreasing growth pH so that there is an actual increase of enzyme in the cell in response to acid growth conditions. Highest activities are obtained when growth occurs in a casein-digest medium initially adjusted to pH 5·0, but this is near the acid limit of tolerance and yields are small; the presence of 2% glucose in the medium greatly increases the crop and provides, by the acidity of fermentation, organisms of a satisfactory activity level. Table 1, B shows the effect of growth temperature: low temperatures give rise to increased formation of enzyme; the highest activity was given by organisms grown at room temperature (*c.* 20°), but as this is too variable for satisfactory work, other cultures have been grown at 25°. A third factor of importance in growth conditions became apparent when we began to study the extraction of the enzyme from the acetone-dried organisms (as below): at first we found that our extractions varied from 10 to 75% of the total enzyme present from culture to culture. One of the causes of this variation appears to lie in the age of the culture at harvesting and Table 1, C shows that satisfactory extraction is obtained only from cells which have been grown under standard conditions for longer than 22 hr. It has been shown before (Gale, 1940) that cultures harvested early in the growth phase have little activity, but here we find that the enzyme, although developed, cannot be extracted satisfactorily from the dried cells unless growth is allowed to continue for about 24 hr. The standard conditions of growth adopted as a result of these findings consist of 24 hr. growth at 25° in 2% glucose-casein-tryptic-digest medium. To prepare a batch of enzyme we normally culture 15 l. of medium under these conditions, centrifuge down the organisms in a Sharples supercentrifuge, wash the cells once with water and make them into a thick cream with water ready for treatment with acetone.

Table 1. *Optimal growth conditions for the production and extraction of* l(+)-*lysine decarboxylase* (Esch. coli)

	Q_{CO_2} (lysine)	
A. *pH of medium* (during growth at 27°)	Washed suspension	Acetone powder
7·0	118	218
6·0	250	426
5·0	410	721
2% glucose broth (final pH = 5·2)	254	430
B. *Growth temperature* (in 2% glucose broth)		
37°	55	156
27°	205	337
Room temp. (*c.* 20°)	252	421

C. *Effect of age of culture on extractability*

Age of culture at 25° in 2% glucose broth (hr.)	Suspension of acetone powder (units/ml.)	Supernatant (units/ml. extract)	Extraction (%)
14	2·82	0·48	17
18	3·16	1·26	40
22	3·10	2·04	66

PRELIMINARY STUDIES ON THE PREPARATION OF THE ENZYME

Preparation of acetone powder and extraction of enzyme therefrom

The thick cream of washed organisms is poured rapidly into 5 vol. acetone and then stirred vigorously until coagulation occurs. The coagulum is allowed to settle and the supernatant liquid, which should be nearly or quite clear, is poured off. The rest of the material is filtered on a Büchner funnel (capacity *c.* 100 ml.) with slight suction, the filtrate being passed through again if necessary. When the cake is almost dry and beginning to crack, it is flooded with acetone and the whole worked up to a cream before suction is again applied. When the

acetone has been removed, this operation is repeated with *c.* 100 ml. dry ether. Suction is maintained until the powder is dry, when it is crumbled and spread out on a glass dish in the air to dry. Powders prepared in this way show an apparent increase in lysine decarboxylase activity, estimated on a carbon basis (Table 1), and retain this undiminished for a long period; but preparations from *Esch. coli* are best preserved by drying and storing *in vacuo*.

Preliminary experiments indicated that it is possible to extract the lysine decarboxylase from the acetone-powdered organism by incubation with buffer. Table 2 shows the degree of extraction obtained in 2 hr. at 27° with various buffer solutions: in each case 70 mg. of the powder were suspended in 2·5 ml. buffer in a centrifuge tube, the suspension incubated for 2 hr. at 27° and the sediment then spun off; 0·5 ml. of the clear supernatant was taken for test at pH 6·0. Table 2 shows that extraction is favoured by mildly alkaline conditions, being optimal between pH 8·0 and 9·0. Investigation of the effect of time and temperature on the extraction by M/45 borate buffer pH 8·5 shows that the process is rather more efficient at 37°, and for further experiments the powder was extracted for 2 hr. at 37° in M/45 borate buffer pH 8·5, with 1 ml. buffer/20 mg. powder.

Table 2. *Variation of extraction of enzyme with* pH

(Contents of tubes: 70 mg. acetone powder + 2·5 ml. buffer. Incubated for 2 hr. at 27° and sediment centrifuged off; test carried out at pH 6·0 on 0·5 ml. clear supernatant in each case.)

Buffer	pH	Activity of extract (μl. CO_2/ 0·5 ml./10 min.)
M/45 phthalate	4·0	4
	5·0	23
M/45 phosphate	6·0	139
	7·0	141
M/45 borate	8·0	220
	9·0	202
	10·0	34
	11·0	14

It has been shown above that extraction takes place more readily from cells grown for 22–24 hr. but there are other, as yet unknown, factors controlling the ease of extraction from the acetone powder and considerable variations are encountered from preparation to preparation made under apparently identical conditions. If the percentage extraction is less than 20, a further yield can sometimes be obtained by repeating the extraction process.

Preliminary experiments on the purification of the extracted enzyme

Acidification. The extract obtained in this manner is an opalescent, faintly yellow solution. On acidification a precipitate is formed which increases in bulk as the pH falls. Attempts were made to effect a purification in this way, but it was found that precipitation in buffers of pH below 5·0 resulted in complete loss of activity.

Adsorption on to and elution from alumina C_γ. Aliquots of the crude enzyme extract were adjusted by means of buffer solutions to pH 5–10, and an equal volume of alumina C_γ suspension (16 mg./ml.; pH 7·0) added. After standing for 10 min., the alumina was centrifuged off and the activity of the supernatant tested. It was found that the enzyme is adsorbed at mildly acid or alkaline values and that the enzyme is active in the adsorbed state on the alumina. Accordingly a more accurate estimate of the amount adsorbed can be made by suspending the alumina in the initial volume of water and testing the activity of this suspension. Table 3, A, shows the results of one such experiment, in which optimal adsorption occurred when the extract was adjusted to pH 6·0 before the addition of the alumina C_γ. A further experiment was carried out in which the proportion of alumina suspension to extract was varied, after preliminary adjustment to pH 6·0. Table 3, B, shows the amount of activity

Table 3. *Adsorption of the enzyme on alumina* C_γ

A. *Optimum* pH *for adsorption*

(1 ml. crude extract + 1 ml. buffer + 1 ml. alumina C_γ suspension: left standing 10 min. and alumina centrifuged off; alumina resuspended in 3 ml. phosphate at pH 6·0 and 1 ml. taken for test.)

pH during adsorption	Activity of suspension (μl. CO_2/ml./5 min.)
5·0	90
6·0	135
7·0	32
8·0	129
9·0	114
10·0	29
Total activity before centrifuging	151

B. *Proportion of alumina suspension*

(Tubes set up with 1 ml. crude enzyme extract adjusted to pH 6·0. Various amounts of alumina C_γ suspension (16 mg./ml.) added and volume made up to 2 ml. with water; after 10 min. alumina centrifuged off and 0·5 ml. supernatant taken for test at pH 6·0.)

Alumina C_γ suspension added (ml.)	Activity of supernatant (μl. CO_2/ml./5 min.)
0·0	189
0·1	127
0·2	48
0·4	19
0·6	17
0·8	11

left in the supernatant after the alumina has been removed in such an experiment; most of the enzyme is adsorbed if the acidified extract is treated with half its volume of standard alumina C_γ suspension. Elution was tested with phosphate buffers at various pH levels and was found to be complete in M/5 phosphate buffer at pH 7·0: on a larger scale, elution is not always complete in one operation and it has been the custom to elute twice, each elution being made with a volume of M/5 phosphate pH 7·0 equal to that of alumina suspension originally used for adsorption.

Separation of decarboxylases. Washed suspensions of *Esch. coli* grown and prepared as above contain the decarboxylases of arginine, lysine, histidine and ornithine. The ornithine enzyme is destroyed by the acetone-drying process, whereas the other three enzymes are unaffected. These latter have very similar properties, and the extraction process, adsorption with, and elution from alumina C_γ take place under the same conditions for all three; hence the procedures described above do not bring about any separation of the three enzymes. During the early investigations on the possibility of acid precipitation of the crude extract, it appeared that the lysine enzyme was rather more stable to mild acidity than those of arginine and histidine. Accordingly, attempts were made to effect a separation of the enzymes by acid precipitation in the presence of ethanol. After various trials it was found that if the pH of the crude extract is adjusted to 5·5 or 6·0 in the presence of 20 % ethanol, a precipitate is formed which contains the bulk of the lysine decarboxylase activity but little arginine activity (*vide* Table 4, A). It is important that the ethanol be added before and not after acidification, otherwise general loss of activity occurs. The histidine enzyme is destroyed to the same extent as the arginine decarboxylase by this procedure.

It was found that the lysine decarboxylase activity can be stabilized under these conditions if adsorption on to alumina C_γ is combined with the precipitation by acid in the presence of 20 % ethanol. Table 4, B, shows that if the crude extract is adjusted to pH 6·0 with 3 % acetic acid in the presence of 20 % ethanol, and one-half volume of alumina C_γ suspension is then added, the supernatant left after removal of the alumina contains little lysine or arginine activity, while a suspension of the alumina in the original volume of water carries the greater part of the lysine enzyme in an adsorbed state. Elution of the alumina with M/5 phosphate at pH 7·0 results in a preparation very active with respect to lysine and containing little arginine enzyme.

Table 4. *Acid-ethanol precipitation and adsorption on to alumina* C_γ

	Activity (μl. CO_2/0·5 ml./5 min.) with substrate	
	Arginine	Lysine
A. Preparation process		
1. Crude enzyme extract	30	137
2. Adjust to pH 5·5 with acetic acid; add ethanol to 20%; remove and dissolve precipitate in original volume	4	8
3. Add ethanol to 15%; adjust to pH 6·0 with acetic acid; remove and dissolve precipitate in original volume	6	89
4. Add ethanol to 20%; adjust to pH 6·0 with acetic acid; remove and dissolve precipitate in original volume	2	99
B. 1. Crude enzyme extract	34	204
2. Add ethanol to 20%; adjust to pH 6·0 with acetic acid; remove and dissolve precipitate in original volume	6	169
3. Add ethanol to 20%; adjust to pH 6·0 with acetic acid; add half vol. alumina C_γ suspension; leave 10 min.; remove alumina; test supernatant	5	6
4. Suspend alumina from (3) in original volume of water	3	136
5. Eluate in M/5 phosphate from alumina (4)	2	131
6. Eluate corresponding to (5) from alumina on which adsorption occurred in absence of ethanol	26	156

Bact. cadaveris possesses a very active lysine decarboxylase, a very weak arginine decarboxylase and no other enzymes of this group. When the above procedure is followed in the case of extracts prepared from acetone-dried *Bact. cadaveris*, the arginine enzyme is completely destroyed and the eluate is specific for lysine.

Adsorption will occur on to calcium phosphate under much the same conditions as those discussed for alumina C_γ, but elution is more difficult and will only occur satisfactorily in the presence of 15 % ammonium sulphate. It will be shown below that the presence in the preparations of ammonium sulphate has an inhibitory effect and consequently the use of calcium phosphate as an adsorbent was discarded.

PREPARATION OF *l*(+)-LYSINE DECARBOXYLASE

Table 5 shows the steps in the purification of lysine decarboxylase worked out on the basis of the preliminary findings described above. As each step of

Table 5. *Purification of* l(+)*-lysine decarboxylase from* Esch. coli *and* Bact. cadaveris

Source of enzyme ...		*Esch. coli*					*Bact. cadaveris*				
Stage	Preparation	ml.	Activity/ml.	Units	mg. C/ml.	$Q^{C}_{CO_2}$	ml.	Activity/ml.	Units	mg. C/ml.	$Q^{C}_{CO_2}$
1	Intact organism suspension	—	196	—	4·12	508	—	—	—	—	963
2	Acetone powder suspension	230	744	1610	10·4	860	90	1100	990	7·2	1,830
3	Crude extract	225	455	1022	2·95	1,850	84	1100	924	6·1	2,170
4	Combined eluates from alumina C_γ	204	348	710	1·02	4,100	84	885	745	—	—
5	Protein precipitated with 50 g. $(NH_4)_2SO_4$/100 ml.	100	571	571	1·63	4,210	54	1125	607	3·28	4,500
6	First $(NH_4)_2SO_4$ fraction:										
	(*a*) 0–40% saturated $(NH_4)_2SO_4$	25	170	42	1·50	1,360	20	500	100	1·74	3,450
	(*b*) 40–56% saturated $(NH_4)_2SO_4$	25	1700	420	0·97	21,100	25	1890	472	2·97	7,650
	(*c*) 56–66% saturated $(NH_4)_2SO_4$	25	111	23	1·15	1,160	20	90	18	—	—
7	Second fractionation of 6 *b*:										
	(*a*) 0–40% saturated $(NH_4)_2SO_4$	15	880	132	0·869	12,200	15	475	71	—	—
	(*b*) 40–47% saturated $(NH_4)_2SO_4$	15	1540	232	0·448	41,300	15	2600	390	1·78	17,500
	(*c*) 47–52% saturated $(NH_4)_2SO_4$	15	112	17	0·353	3,780	15	20	3	—	—
8	Third fractionation of 7 *b*:										
	(*a*) 0–41% saturated $(NH_4)_2SO_4$	10	Negligible	—	—	—	10	370	37	—	—
	(*b*) 41–47% saturated $(NH_4)_2SO_4$	*10*	*800*	*80*	*0·209*	*46,000*	*10*	*3120*	*312*	*1·12*	*33,500*
	(*c*) 47–52% saturated $(NH_4)_2SO_4$	10	1310	131	0·49	32,000	10	92	9	—	—
9	Fraction 8 *b* adsorbed on to and eluted from alumina C_γ	7	578	41	0·269	25,700	—	—	—	—	—

the purification was carried out, the following measurements were made: volume of dissolved preparation, the lysine decarboxylase activity of a suitable measured fraction, expressed as μl. CO_2 liberated from *l*(+)-lysine/5 min./ml. preparation, and the C content of a suitable fraction. From these values the number of enzyme units and $Q^{C}_{CO_2}$ were calculated as a measure of the yield of material and degree of purification respectively. The table shows figures obtained for preparations starting from washed suspensions of both *Esch. coli* and *Bact. cadaveris*. The stages in the preparation are as follows.

1. Thick washed suspension of organism grown for 24 hr. at 25° in 15 l. 2% glucose-casein-tryptic-digest broth.

2. The washed suspension is treated with 5 vol. acetone, filtered off on a Büchner funnel, washed with acetone and then with ether, and dried in air. The dry powder is broken up, weighed and suspended in M/45 borate buffer, pH 8·5, using 1 ml. buffer/20 mg. powder.

3. The suspension of acetone powder in buffer is incubated, with occasional stirring, for 2 hr. at 37°. The suspension is then centrifuged at 3000 r.p.m. for 15 min. to remove the inactive sediment. At this stage the degree of extraction should be tested. Generally good yields and clean fractionation are obtained in the later stages of the preparation only if the extraction exceeds 50% at this stage.

4. Ethanol is added to a final concentration of 20%, and 3% acetic acid then added with stirring until the pH lies between 5·5 and 6·0 (yellow to bromothymol-blue; blue to bromocresol-green). A volume of alumina C_γ suspension (16–20 mg./ml.), equal to half the volume of extract at stage 3, is then added and the whole left to stand for 10 min. The alumina is centrifuged and the supernatant discarded. The alumina is resuspended in M/5 phosphate, pH 7·0, of the same volume as the amount of alumina C_γ suspension taken for adsorption. After 10 min. the alumina is again centrifuged, the supernatant collected, and the elution repeated under the same conditions. The eluates are combined. Yields over this stage vary between 70 and 80%, with a purification of 200–250%.

5. The volume of the combined eluates is measured and 50 g. solid ammonium sulphate added/100 ml. The ammonium sulphate is stirred in until dissolved and the solution left to stand for 10 min. The precipitate is then centrifuged for *c.* 30 min. at 3000 r.p.m. The supernatant solution is discarded, the precipitate drained as far as possible and then dissolved in a convenient amount of water. There is usually a small loss of material at this stage, but the further fractionation is more constant and precipitates centrifuge more easily after this bulk precipitation. On solution in water there is usually an insoluble residue which can be removed by centrifuging if the material is to be used at this stage without further purification. The preparation from *Bact. cadaveris* is specific for lysine at this stage and can be used for the estimation of lysine, protein analysis, etc.

6. Further purification is carried out by progressive ammonium sulphate fractionations. The volume of the preparation, stage 5, is measured and sufficient saturated ammonium sulphate solution is added to bring the final concentration to 40 % saturation. After standing 5 min., the precipitate is centrifuged off, 15 min. at 3000 r.p.m. being sufficient to pack the material in the tube. The ammonium sulphate concentration in the supernatant is then raised to 56 % by the addition of the calculated quantity of saturated solution and the second fraction centrifuged down separately. The third fraction is precipitated by raising the concentration to 66 % saturation. Each fraction is dissolved in water for test, and the bulk of the enzyme is found in the middle fraction.

7. The middle fraction is now refractionated between the limits indicated in Table 5 and most of the activity comes out in the 40–47 % saturated ammonium sulphate fraction, effecting a purification of 2–3 times over stage 6.

8. The middle fraction from stage 7 is again refractionated over approximately the same limits. With the *Bact. cadaveris* preparation this third fractionation results in the removal of more inactive protein, giving a purification of 200 % in the middle fraction, which still separates cleanly. The material from *Esch. coli* begins to be divided among the fractions at this stage and although most of the enzyme comes down in the 47–52 % fraction, the highest activity is still shown by the 41–47 % range. The arginine activity remaining in preparations obtained from *Esch. coli* after stage 4 accompanies the lysine activity until this stage, when separation occurs, most of the arginine activity appearing in the 47–52 % fraction. The 41–47 % fraction always contains some arginine activity when *Esch. coli* is the source, and the $Q^{C}_{CO_2}$ (arginine) lies between 500 and 1500.

9. Further ammonium sulphate fractionation after stage 8 results in division of the enzyme among the fractions, without significant purification. In an attempt to remove the remaining traces of arginine activity from the *Esch. coli* preparation, adsorption on to alumina at pH 6·0 in the presence of 20 % ethanol followed by elution in phosphate was tried again with fraction 8 *b*. This procedure resulted, however, in a loss of activity without significant separation of the enzymes.

For the investigation of the properties of the enzyme, material obtained in fraction 8 *b* has been used, with *Bact. cadaveris* as the source. In a small percentage of preparations a trace of arginine decarboxylase activity survives destruction, but this disappears if the preparation is kept for 3–4 days in the ice-chest. Preparations made from *Bact. cadaveris* and dissolved in M/200 phosphate pH 6·0 can be kept in the ice-chest for 2–3 weeks without serious deterioration. Preparations from *Esch. coli* lose up to 50 % of their activity within 24 hr., partly due to spontaneous resolution of the protein-coenzyme complex (see later). The preparations form clear colourless solutions in water.

A preparation from *Esch. coli*, fraction 8 *b*, was centrifuged on the ultra-centrifuge for us by Dr A. G. Ogston at the Biochemical Department, Oxford. The material was non-homogeneous.

Ultra-violet absorption spectrum. The preparations give typical protein ultra-violet absorption spectra with a peak at 265 mμ. The preparation 8 *b*, $Q^{C}_{CO_2} = 33{,}500$, source *Bact. cadaveris*, gave extinction $\log_{10} I_0/I = 1{\cdot}5$ at 265 mμ ($d = 2$ cm.) for a solution containing 0·28 mg. C/ml.

PROPERTIES OF THE PURIFIED ENZYME

pH *optimum.* Fig. 1 shows the variation of *l*(+)-lysine decarboxylase activity with the reaction pH. The optimum lies at pH 6·0, whether phthalate, phosphate or citrate buffers are used. In most experiments reported, the buffer used was M/5 phosphate, but M/5 citrate buffer was used for the investigation of certain metal inhibitors. The

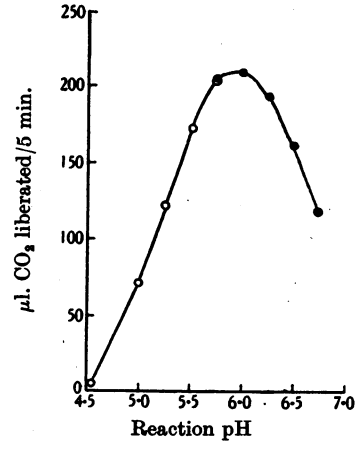

Fig. 1. Activity-pH curve for *l*(+)-lysine decarboxylase. o—o M/10 phthalate buffers. •—• M/5 phosphate buffers. Contents of cups: 0·5 ml. enzyme; 2·0 ml. buffer; 0·5 ml. M/15 *l*-lysine. Temp. 30°.

pH optimum of the purified enzyme differs from that measured with the intact organism, which may lie anywhere between 4·5 and 5·0, varying from culture to culture, the curve showing an abrupt drop on the acid side of the optimum (Gale, 1940). We have suggested that this difference in the pH optimum measured with the intact cell and with the cell-free enzyme is due to the pH difference between the internal and external environments of the cell produced as a result of the neutralizing effect of the decarboxylase action (Gale & Epps, 1942).

Effect of substrate concentration. Fig. 2 shows the variation of velocity of decarboxylation with concentration of substrate for the enzyme preparations from the two sources. The two curves are very similar, though it would appear that the enzyme

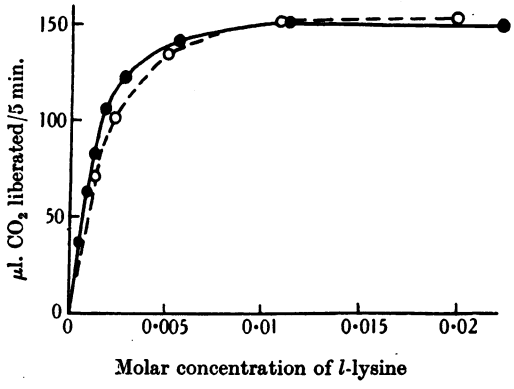

Fig. 2. Effect of lysine concentration on lysine decarboxylase activity. •—• Enzyme from *Bact. cadaveris.* o- - -o Enzyme from *Esch. coli.* Contents of cups: 0·5 ml. enzyme; 2·0 ml. M/5 phosphate pH 6·0; 0·5 ml. substrate solution. Temp. 30°.

from *Bact. cadaveris* has a slightly higher affinity for the substrate than that isolated from *Esch. coli.* In both cases the value of the Michaelis constant falls between 0·0012 and 0·0018 M. In our experimental tests, the final concentration of substrate initially present in the manometer cups is M/90, which is sufficient to saturate the enzyme from either source.

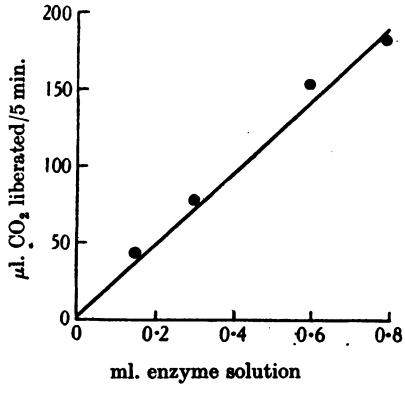

Fig. 3. Proportionality between CO_2 liberation and enzyme concentration.

Effect of enzyme concentration. Fig. 3 shows that a linear relation exists between velocity of reaction and enzyme concentration over the range used in manometric tests.

Effect of salt concentration. In the course of preliminary work, we noticed that the presence of ammonium sulphate in our preparations appeared to have an inhibitory effect. Fig. 4 shows the effect on the velocity, in M/5 phosphate pH 6·0, of the presence of various concentrations of ammonium sulphate (curve 2): concentrations of the order 0·05 M (1 % saturated ammonium sulphate) have a significant effect. Similar results are obtained with Na_2SO_4 or NaCl. Since dialysis of the preparations to remove salts results in irreversible inactivation of the enzyme, methods of elution from adsorbents, etc., involving the use of salts have been avoided.

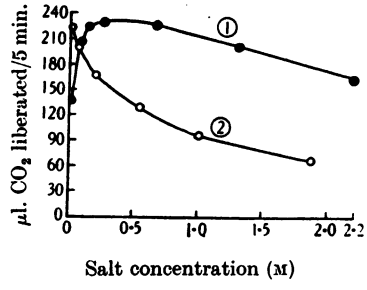

Fig. 4. Effect of salt concentration on lysine decarboxylase activity. (1) •—• Phosphate (pH 6·0). (2) o—o Ammonium sulphate in presence of 0·2 M phosphate pH 6·0.

Curve 1, Fig. 4, shows the effect of varying the concentration of phosphate buffer: there appears to be an optimum concentration of buffer corresponding to 0·1–0·5 M. Below this concentration there is a loss of activity, probably due to inefficient buffering action, while the effect of high concentrations appears to be similar to that obtained with ammonium sulphate. Manometric tests have been carried out in 0·2 M phosphate.

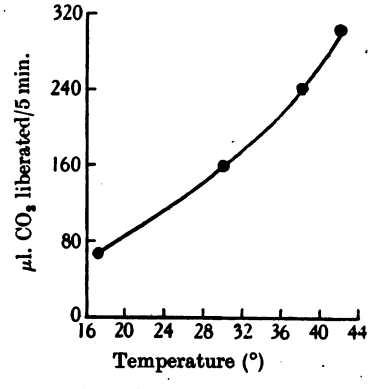

Fig. 5. Effect of temperature on activity of lysine decarboxylase.

Effect of temperature. Fig. 5 shows the variation of velocity with reaction temperature: the temperature coefficient over the range 30–40° is 1·7.

(Experimental tests were always made in a thermostat at 30° and all the rates quoted are for this temperature.)

Substrate specificity. Of the following 15 common amino-acids tested the only one attacked by the purified enzyme preparation (*Bact. cadaveris*) is *l*(+)-lysine: *l*(+)-lysine, *d*(−)-lysine, *l*(+)-arginine, *l*(+)-glutamic acid, *l*(+)-ornithine, *l*(−)-histidine, *dl*-alanine, *dl*-valine, *l*-leucine, *l*(−)-phenylalanine, *l*(−)-tyrosine, *l*(−)-tryptophan, *dl*-serine, *l*(−)-proline, *l*(−)-aspartic acid. The action on α-acetyl, α-methyl, ε-acetyl, ε-methyl-derivatives of lysine, prepared by Dr F. Sanger of this laboratory, was also tested: since no activity was obtained in any of these cases it would seem that the integrity of both α and ε amino-groups in the lysine molecule is necessary for decarboxylase activity. Piperidine-α-carboxylic acid was not attacked. The enzyme is optically specific and will not decarboxylate *d*(−)-lysine. Use has been made of this activity to resolve *dl*-lysine and prepare the unnatural isomer (Neuberger & Sanger, 1944).

Fig. 6 shows the course of decarboxylation of a sample of hydroxy-lysine kindly given to us by Dr D. D. Van Slyke. The sample contained about 13% lysine and the analytical data supplied by Dr Van Slyke were: NH_2-N, 14·24; C, 36·89; Cl, 18·34%; hydroxy-lysine, estimated from the ammonia evolved with periodate, 86·5%; $[\alpha]_D$ in

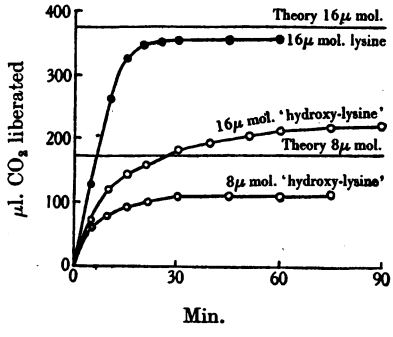

Fig. 6. Decarboxylation of *l*-lysine and hydroxy-lysine (13% lysine)

N-HCl, +4·7°. For test, 16 μmol. *l*(+)-lysine, 16 μmol. and 8 μmol. hydroxy-lysine were shaken with the enzyme preparation. The decarboxylation of the lysine proceeded normally to 92% completion. An initial rapid evolution of CO_2 was obtained with the 'hydroxy-lysine' due to the lysine present in the preparation; this was followed by a slower evolution of CO_2, eventually ceasing at a quantity corresponding to the decarboxylation of approximately half the theoretical amount of hydroxy-lysine present. The CO_2 evolution corresponds to the decarboxylation of something in the preparation other than lysine. It is possible that the hydroxy-lysine is racemized during preparation and that only the natural isomer is attacked or, alternatively, that there may be more than one hydroxy-lysine. Further investigations were not possible owing to lack of material. We have found that tyrosine decarboxylase will decarboxylate dihydroxyphenylalanine (Epps, 1944) and that glutamic acid decarboxylase will attack synthetic β-hydroxyglutamic acid; therefore the introduction of an —OH group into the substrate molecule does not prevent the action of other decarboxylases, although it decreases the rate of decarboxylation in the three cases studied.

Quantitative nature of the decarboxylation of lysine. Using 0·5 ml. M/30 *l*(+)-lysine (Roche Products Ltd.) under the standard manometric conditions described above, a series of 19 tests gave mean CO_2 output = 344 μl., standard deviation 4·7, range 337–354. Theoretical decarboxylation = 373 μl. The experimental output under these conditions is thus 92% of calculated, but working at pH 6·0 there is some retention of CO_2, and if this is estimated at the end of the experiment by the addition of acid with a correction for blanks (Woods & Clifton, 1937), the mean gas output from 10 tests = 366 μl., standard deviation 5·1, range 359–378; this represents 98% of calculated, so that the decarboxylation is quantitative.

If *dl*-lysine is used as substrate, the mean gas output from 0·5 ml. M/30 solution is 164 μl., corresponding to 45% of calculated.

Identification of product. The following quantities were put up in an Erlenmeyer flask: 15 ml. M/30 *l*(+)-lysine, 60 ml. M/5 phosphate pH 6·0, and 7·5 ml. enzyme preparation (70 units lysine decarboxylase). The flask was shaken at 30° for 30 min. and the contents were then deproteinized with colloidal iron. The amine product was isolated as the picrate by the method previously described (Gale, 1940): yield 178 mg. Analysis of the product twice recrystallized from hot water (F. Weiler, Oxford): C, 36·29; H, 3·59; N, 19·75%. Calc. for cadaverine picrate: C, 36·42; H, 3·57; N, 20·0%.

Inhibitors. Table 6 lists the lowest molar concentrations of various common inhibitors which will produce 95–100% inhibition of lysine decarboxylase. The enzyme is very sensitive to silver and mercury, considerably less sensitive to copper and iron. Hydroxylamine and hydrazine are effective inhibitors in concentrations of 10^{-5} and 10^{-4}M respectively; 10^{-3}M-cyanide or azide produce 95% inhibition, which suggests the presence of a metal in the enzyme constitution. However, sodium sulphide, carbon monoxide and the 'copper inhibitors' (8-hydroxy-quinoline, sodium diethyl-dithio-carbamate and α-benzoin-oxime (Stotz, Harrer & King, 1937)) have no significant inhibitory action in

concentrations below 0·01 M. We are indebted to Dr Nicholson for spectrometric examination of ashed enzyme for metals: the only metals found were Ca, Ba and Sr, which were equally present in control portions of the ammonium sulphate used.

Table 6. *Inhibitors of* l(+)-*lysine decarboxylase*

(−log (*I*) = negative log of molar concentration of inhibitor.)

Inhibitor	−log (*I*)	Inhibition (%)
$HgCl_2$	5	100
$AgNO_3$	5	98
NH_4OH	5	100
$NH_2.NH_2$	4	95
$KMnO_4$	4	100
HCN	3	93
NaN_3	3	98
$CuSO_4$	3	89
$FeSO_4$	3	82
Na_2S	2	32
Iodoacetate	2	20
Sulphanilamide	2	15

Substances ineffective in concentrations below 0·01 M: NaF, urethane, chloramine T, 8-hydroxyquinoline, Na diethyl-dithio-carbamate, α-benzoin-oxime, sulphathiazole, carbon monoxide.

That the cyanide inhibition is reversible was demonstrated in the following way. Manometers were put up in duplicate containing final concentrations of 0·001 and 0·0001 M cyanide, together with controls without cyanide. In one of each pair, 0·3 ml. 40 % NaOH was placed in the centre cup, with a fluted filter paper strip dipping into the alkali. The manometers were then shaken in the bath at 30° with their taps closed for 1 hr. At the end of that time the filter papers were removed, the alkali cleaned out and the centre cups acidified. The manometers were then reassembled and, after the usual equilibration period, the substrate added in all manometers. Table 7 shows that cyanide exerts its usual inhibitory effect in those manometers without alkali, whereas the inhibitory effect has been reversed to a large extent in those manometers where alkali in the centre cup has absorbed part of the cyanide.

Table 7. *Reversibility of cyanide inhibition*

Manometer no. ...	1	2	3	4	5	6
Enzyme solution (ml.)	0·2	0·2	0·2	0·2	0·2	0·2
M/5 phosphate pH 6·0 (ml.)	2·0	2·0	2·0	2·0	2·0	2·0
M/15 *l*(+)-lysine (ml., in side-bulb)	0·3	0·3	0·3	0·3	0·3	—
6 M/1000 cyanide (ml.)	—	0·5	0·5	—	—	—
6 M/10,000 cyanide (ml.)	—	—	—	0·5	0·5	—
40 % NaOH (ml., in centre cup)	—	—	0·3	—	0·3	—
Water (ml.)	0·5	—	—	—	—	0·8
Incubate 1 hr. at 30°; remove NaOH from nos. 3 and 5; equilibrate; tip in the substrate:						
Gas evolution (μl. CO_2/5 min.)	163	8	63	53	135	0
Inhibition (%)	—	95	61·5	69	17	—

REVERSIBLE RESOLUTION INTO SPECIFIC PROTEIN AND COENZYME

Dialysis of the purified enzyme preparations results in rapid and irreversible inactivation; preparations from *Esch. coli* are completely inactivated by 12 hr. dialysis against distilled water; preparations from *Bact. cadaveris* lose approx. 50 % of their activity in 24 hr. dialysis. Previous studies with washed suspensions of *Esch. coli* have shown that the lysine decarboxylase activity can be increased by the addition of a boiled suspension of yeast to the bacteria during test. These findings suggest the presence of a dialyzable coenzyme which could not, in the case of intact organisms, be replaced by aneurin diphosphate (Gale, 1940).

Table 8 shows that repeated precipitation of a lysine decarboxylase preparation (stage 7 *b*, source *Esch. coli*) with 66 % saturated ammonium sulphate at pH 9·0 results in the preparation of a protein with little activity towards lysine, but whose activity is considerably increased by the addition of boiled yeast suspension. Further work along these lines has shown that the following is the most satisfactory method of preparing the inactive apo-enzyme.

When *Esch. coli* is the source of the enzyme, the preparation is followed as described as far as stage 5. The precipitate obtained at this stage is dissolved in water and insoluble material centrifuged off. The enzyme is then precipitated once with 66 % saturated ammonium sulphate, dissolved in water and again precipitated with 66 % saturated ammonium sulphate solution containing 10 % ammonia. The precipitate does not centrifuge easily and requires 30 min. at 3000 r.p.m. to pack down. The precipitate is again dissolved and reprecipitated with ammoniacal ammonium sulphate as before; the resolution is aided if the material is left to stand for 15–20 min. before centrifuging. The precipitate is now dissolved in a suitable amount of M/5 phosphate pH 6·0 and tested with and without sufficient coenzyme (co-decarboxylase) to saturate the protein. Considerable loss of material usually accompanies this resolution, sometimes amounting to 60–70 % of the

enzyme. The activity towards lysine of the apo-enzyme preparation, in the absence of added coenzyme, is very small and can be still further reduced by a third alkaline precipitation if desired, although the resolution is best completed by allowing the preparation to stand in solution in the ice-chest for several days. The solution of apo-enzyme can be kept for several weeks in the ice-chest. Table 8 also shows the effect of adding boiled yeast suspension to apo-enzyme prepared in this way from enzymes isolated from *Esch. coli* and *Bact. cadaveris*. The resolution of the enzyme from *Esch. coli* is accomplished far more easily than that of the enzyme from *Bact. cadaveris* and this difference seems to be correlated with the greater stability on keeping and on dialysis of the latter preparation.

Table 8. *Reversible resolution by precipitation with alkaline 66 % saturated ammonium sulphate*

(Preparations made up to original volume after each precipitation.)

Enzyme preparation: lysine decarboxylase preparation stage 7 *b*; source, *Esch. coli*	Activity (μl. CO_2/0·5 ml./10 min.) Alone	+ boiled yeast	Increase due to yeast (%)
Precipitated once at pH 7·0	282	332	18
Precipitated once at pH 9·0	124	265	113
Precipitated twice at pH 9·0	81	204	152
Precipitated thrice at pH 9·0	35	198	465
Twice precipitated with ammoniacal ammonium sulphate as described in text:			
(*a*) source, *Esch. coli*	14	208	1385
(*b*) source, *Bact. cadaveris*	150	223	49

Boiled yeast suspension forms a useful source of coenzyme for general testing purposes but the apo-enzyme can also be reactivated by boiled suspensions of *Esch. coli* or *Bact. cadaveris*, or by boiled preparations of purified lysine decarboxylase itself.

By the use of the decarboxylation of lysine by apo-enzyme preparation in the presence and absence of various substances under the usual conditions, the following substances have been tested for coenzyme activity with negative results: coenzyme I, coenzyme II, aneurin, aneurin diphosphate, riboflavin-adenine-dinucleotide, flavin-mononucleotide, riboflavin, adenylic acid, adenosine, inosine, hypoxanthine, glutathione, yeast nucleic acid, yeast nucleic acid hydrolyzed with NH_4OH, nicotinic acid, nicotinic amide, uracil, pantothenic acid, biotin, pyridoxin, *p*-amino-benzoic acid, inositol, thiochrome: aneurin diphosphate + ashed yeast, or Mg, Ca, Zn, Fe, Cu, Mn, Co, etc.; aneurin diphosphate + riboflavin-adenine-dinucleotide or riboflavin-mononucleotide or riboflavin; aneurin or thiochrome + riboflavin-adenine-dinucleotide or riboflavin-mononucleotide or riboflavin.

The preparation of the codecarboxylase from brewer's yeast is described in a further paper of this series (Gale & Epps, 1944). Table 9 gives the results of an experiment designed to show the recombination of apo-enzyme and codecarboxylase.

To determine the half-saturation concentration of codecarboxylase, manometer cups were assembled with the following contents: 0·25 ml. apo-enzyme preparation equivalent to 2 units of enzyme, 1·75 ml. M/5 phosphate buffer pH 6·0, in the main part, and 0·5 ml. M/15 *l*(+)-lysine in the side-bulb. To a series of such cups was added 0·5 ml. phosphate buffer containing amounts of codecarboxylase corresponding to 0·01–2·5 μg. carbon, the preparation used being of the highest purity so far prepared (Gale & Epps, 1944). The manometers were equilibrated at

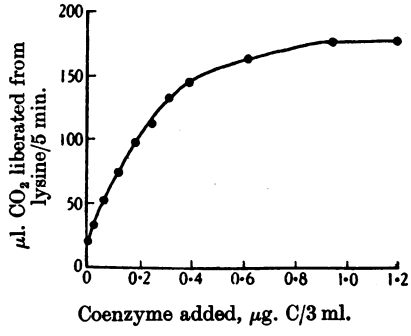

Fig. 7. Effect of coenzyme concentration on rate of decarboxylation of *l*-lysine by apo-enzyme. Contents of cups: 0·25 ml. apo-enzyme solution; 0·5 ml. M/15 *l*-lysine; 2·0 ml. M/5 phosphate buffer pH 6·0; 0·25 ml. Codecarboxylase solution as above. Temp. 30°.

30° for 15 min. before the substrate was added and the steady rate of decarboxylation was then studied for a further 15 min. Fig. 7 shows the dependence of the velocity of decarboxylation on the concentration of codecarboxylase present. Half-saturation of

Table 9. *Recombination of apo-enzyme and codecarboxylase*

Manometer no. ...	1	2	3	4	5	6
M/5 phosphate pH 6·0 (ml.)	1·75	1·75	1·75	1·75	1·75	1·75
Apo-carboxylase preparation (ml.)	0·25	0·25	0·25	0·25	—	—
M/15 *l*(+)-lysine (ml., in side-bulb)	0·5	0·5	—	—	0·5	—
Codecarboxylase preparation (ml.)	—	0·5	0·5	—	0·5	0·5
Water (ml.)	0·5	—	0·5	1·0	0·25	0·75
Gas evolution (μl. CO_2/5 min.)	14	213	0	0	0	0

the apo-enzyme is obtained with an amount of codecarboxylase corresponding to 0·06 μg. C/ml. Conversion of these values into molecular concentrations must await the final purification and characterization of the coenzyme molecule. The shape of the velocity-coenzyme concentration curve would suggest that the non-protein moiety of the enzyme behaves as a coenzyme rather than as a prosthetic group.

SUMMARY

1. The lysine decarboxylase activity of coliform organisms grown in 2 % glucose-casein-digest-broth withstands drying of the organisms in acetone.

2. The enzyme can be extracted from the acetone-dried organisms by incubation with borate buffer at pH 8·5.

3. The partial purification of the enzyme is described; when *Bact. cadaveris* is used as a source, the preparation is specific for *l*(+)-lysine.

4. The enzyme is optimally active at pH 6·0, has a Michaelis constant of approximately 0·0015 M and will attack only *l*(+)-lysine, and possibly hydroxylysine, among the common amino-acids. The integrity of both amino groups in the lysine molecule is essential for activity.

5. The decarboxylation is quantitative and the product is cadaverine.

6. The enzyme can be resolved into specific apo-enzyme and a hitherto undescribed codecarboxylase by repeated precipitation with ammoniacal ammonium sulphate (66 % saturation).

7. The apo-enzyme can be reactivated by codecarboxylase preparations but by no other coenzyme or prosthetic group.

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Studies on Bacterial Amino-acid Decarboxylases

2. *l*(−)-TYROSINE DECARBOXYLASE FROM *STREPTOCOCCUS FAECALIS*

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This paper deals with the isolation, partial purification and some of the properties of *l*(−)-tyrosine decarboxylase from *Streptococcus faecalis*. Gale (1940*b*) showed that *l*(−)-tyrosine is the only one of 16 naturally occurring amino-acids to be decarboxylated by this organism, although it was found later that *l*-3:4-dihydroxyphenylalanine is also decarboxylated (Gale, personal communication). In the present paper an attempt is made to discover whether *l*-3:4-dihydroxyphenylalanine acts as substrate for *l*(−)-tyrosine decarboxylase or whether a separate enzyme is involved.

METHODS

Organisms. Of the faecal streptococci used in this study, one strain was isolated from human stools by Dr Crowley of the Cambridge University Pathology Department; one by Dr Cruickshank of the Group Laboratory, the North-Western Hospital; one was the laboratory stock strain; the rest were isolated by the author.

Substrates. The *l*(−)-tyrosine used for the quantitative experiments was prepared by Merck and Co.; for all other experiments it was isolated from a tryptic digest of casein and thrice recrystallized: *l*(−)- and *dl*-3:4-dihydroxyphenylalanine were supplied by Roche Products Ltd. The purification of the enzyme was worked out with *l*(−)-tyrosine as substrate, but in some cases parallel experiments were made with *l*-3:4-dihydroxyphenylalanine. The enzyme is referred to as *l*(−)-tyrosine decarboxylase throughout; data supporting the theory that a single enzyme is responsible for the decarboxylation of both these amino-acids are presented later.

Method of estimating and expressing enzyme activity. The activity of the enzyme preparations was estimated as for