10. PRODUCTION OF AMINES BY BACTERIA

4. THE DECARBOXYLATION OF AMINO-ACIDS BY ORGANISMS OF THE GROUPS *CLOSTRIDIUM* AND *PROTEUS*

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THE previous papers of this series have dealt with the production of amines by strains of *Bact. coli* [Gale, 1940, 1] and *Strep. faecalis* [Gale, 1940, 2, 3] and with the properties of the amino-acid decarboxylases involved. The present communication continues the study for members of the strictly anaerobic group of *Clostridia*. Results obtained with members of the *Proteus* group are also included, but it will appear that these organisms are not important amine-producers.

Kendall & Schmidt [1926] found that cultures of *Cl. welchii*, when grown in a medium containing glucose, produce a substance in the medium which has the physiological reactions of histamine. Later Kendall & Gebauer [1930] isolated a substance from a bulk culture which had the chemical properties of histamine. Eggerth [1939], in his investigations on the production of histamine by bacteria in culture, obtained positive results with four strains of *Cl. welchii* and most accounts of the toxins of this organism now mention the formation of a 'non-specific histamine-like toxin'. Apart from these instances there appears to be no mention of amine-production by *Clostridia* in the literature. The amino-acid metabolism of certain of the strict anaerobes has been studied: *Cl. sporogenes* by Stickland [1934; 1935], Woods [1936], Kocholaty & Hoogerheide [1938]; *Cl. tetanomorphum* by Woods & Clifton [1937; 1938], and *Cl. botulinum* by Clifton [1940].

Methods

The general technique of preparation of washed suspensions, determination of their dry weight and the manometric investigation of their power to decarboxylate amino-acids has been described fully in the previous papers [Gale, 1940, 1, 2]. In the present survey, the organisms were grown overnight in 800 ml. 2% glucose broth (tryptic digest of casein), the total organisms obtained made into washed suspension and tested against the 16 amino-acids previously described; for the first experiment in each case, washed suspensions containing 20–30 mg. bacteria/ml. were used so that small activities could be detected, and the suspensions later diluted to extents indicated by the preliminary test. The optimum pH was determined in every case and the experiment carried out, unless otherwise stated, at a thermostat temperature of 30°. Decarboxylase activity is measured in terms of Q_{CO_2} as before and values <1 are regarded as not significant.

The *Clostridia* were kept in most cases in tubes of broth and heart muscle. In the case of *Cl. welchii* a 'cellar' of alkaline egg medium was also maintained, the organism being subcultured into meat-broth before use. It was found,

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however, that the organism lost activity after 5-6 serial cultivations in broth and so cultures were taken afresh from the egg medium once a week. For experimental purposes the medium used consisted of tryptic digest of casein with a little heart muscle and 2% additional glucose. This was inoculated with the contents of a cultured tube and incubated at 37° overnight in a Fildes and MacIntosh anaerobic jar. In all cases the organisms were examined microscopically before use to determine the extent to which sporing had occurred; in most cases cultures of 12-15 hr. consisted almost entirely of vegetative cells, exceptions being *Cl. septique* and *Cl. tetanomorphum* where considerable numbers of spores were sometimes encountered. Most of the *Clostridia* grow very thickly in the medium described, growth ceasing after 8-12 hr. when the culture has reached 0.75-0.95 mg. dry weight of bacteria/ml. medium in many cases. *Cl. septique* again forms an exception in that the onset of sporing seems to limit the growth to approx. 0.3 mg./ml. On harvesting, the culture was poured through glass wool to remove fragments of meat and then centrifuged and washed as usual. A few strains of *Cl. welchii* proved difficult to centrifuge cleanly owing to 'roping' of the culture, but this difficulty was only marked with the strains S.R. 9 and Adams, in which cases it was necessary to rely on serial concentration of the suspension rather than on clean separation of the organisms. All washed suspensions were used on the day on which they were prepared, but it was not found necessary to take any special measures to keep them active with regard to the decarboxylases over a period of 4-7 hr.

Organisms and strains used

Cl. welchii S.R. 9. Classical strain used for toxin production. Isolated from war wound. Toxic type A.

Cl. welchii T.W. 14. Isolated from case of puerperal fever. Type A.

Cl. welchii Chelsea. Probably atoxic strain isolated from the urine of a pregnant woman.

Cl. welchii Fisher. Isolated from war wound. Type A.

Cl. welchii Adams. Very toxic strain from war wound. Type A.

Cl. welchii B.W. 21. Isolated from war wound by Prof. MacIntosh. Type A.

Cl. welchii S. 107. Isolated from sheep faeces. Type A.

Cl. paludis Wye 4. *Welchii*-type organism causing sheep disease. Type B.

Lamb dysentery U. 15. *Welchii*-type organism. Type C.

Cl. septique Pasteur III. Original strain isolated by Pasteur.

All the above organisms and also *Cl. septique* Stokes and Jerral were obtained from Dr M. Robertson to whom my thanks are due for advice on the maintenance of these cultures. I am indebted to Dr Carruthers of the Emergency Public Health Laboratory Service for strains of *Cl. septique*, the non-toxigenic strain of *Cl. tetanum* Fildes and for the various *Proteus* organisms mentioned. All other organisms mentioned below were obtained from the National Collection of Type Cultures and are referred to by their collection number.

Decarboxylation of amino-acids by organisms of the groups Clostridium and Proteus

The general conditions under which certain amines are produced by bacteria have been elucidated in the previous papers of this series. In the present work it has been the custom first to undertake a survey of the decarboxylating activities of several strains of each organism when grown in glucose broth and then to choose the most active strain for detailed investigation. As a result of this survey with the two groups of organisms it has been found that only four amino-

acids are attacked under the usual experimental conditions and Table 1 shows the activities of the various positive organisms. In each case the Q_{CO_2} value is given for the activity at the optimal pH (see below) and 30° and represents the initial steady velocity of simple decarboxylation of the amino-acid in question. The decarboxylation is quantitative in every case since the theoretical volume of CO_2 is obtained within experimental error on completion; the decarboxylation is not accompanied by any other reaction so that the product is the amine corresponding to the amino-acid substrate and this is confirmed by isolation and identification of the product as shown later. For this preliminary work the organisms were grown overnight in the 2% glucose broth medium described and the pH of the medium measured potentiometrically before harvesting as a measure of the saccharolytic activity of the organisms concerned.

Table 1. *Decarboxylation of amino-acids by various Clostridium and Proteus organisms grown in 2% glucose broth at 37°. Activities expressed as Q_{CO_2} obtained with washed suspensions at 30° and the optimum pH in each case*

Organism	Strain	Final pH in medium	Q_{CO_2}			
			Histidine	Ornithine	Tyrosine	Glutamic acid
<i>Cl. welchii</i>	S.R. 9	4.6	34	—	—	70
<i>Cl. welchii</i>	T.W. 14	4.5	3	—	—	237
<i>Cl. welchii</i>	Chelsea	4.5	30	—	—	368
<i>Cl. welchii</i>	Fisher	4.6	29	—	—	100
<i>Cl. welchii</i>	Adams	4.6	27	—	—	116
<i>Cl. welchii</i>	B.W. 21	4.1	60	—	—	132
<i>Cl. welchii</i>	S. 107	4.2	23	—	—	50
<i>Cl. paludis</i>	Wye 4	4.6	26	—	—	36
Lamb dysentery	U. 15	4.6	6	—	—	40
<i>Cl. septique</i>	Carr.	5.3	—	180	—	—
<i>Cl. septique</i>	Pasteur	5.3	—	270	—	—
<i>Cl. septique</i>	Stokes	6.0	—	176	—	—
<i>Cl. septique</i>	Jerral	5.75	—	70	—	—
<i>Cl. aerofetidum</i>	505	5.3	—	—	50	325
<i>Cl. fallax</i>	Morcom	5.1	14	—	—	—
<i>Cl. fallax</i>	2907	5.05	—	—	—	—
<i>Cl. bifermentans</i>	2914	5.2	8	—	—	80
<i>Cl. bifermentans</i>	536	5.4	—	—	—	30
<i>Pr. vulgaris</i>	Fildes	5.25	—	—	—	324
<i>Pr. vulgaris</i>	Crook	5.63	—	9	—	112
<i>Pr. vulgaris</i>	Carr.	4.85	—	2	—	46
<i>Pr. morganii</i>	Carr.	5.25	—	12	—	134
<i>Pr. morganii</i>	Carr.	5.8	—	16	—	—

Proteus organisms HX 2, HXL 11, HX 19, etc., also attacked glutamic acid, while Kingsbury strains apparently possessed no decarboxylating enzymes. The following members of the *Clostridium* group showed no decarboxylating activities under the usual experimental conditions:

- Cl. sporogenes* Bellette 533 and 2911.
- Cl. histolyticum* Weinberg and 6282 (American).
- Cl. chauvoei* 287 and 2721.
- Cl. oedematiens (novyi)* 2908 and Cossard 277.
- Cl. tetanum* Fildes.
- Cl. butyricum* Weizmann 619.
- Cl. tertium* 336.
- Cl. multifementans-tenalbus* 2032.
- Cl. tetanomorphum* 500 and 2909.
- Cl. sphenoides* 507.

The organisms chosen for detailed investigation were *Cl. welchii* B.W. 21, *Cl. septique* Pasteur III, *Cl. aerofetidum* 505, *Cl. fallax* Morcom, *Cl. bifermentans* 2914 and *Proteus morganii*; the results set out below were obtained chiefly with these selected strains.

Properties of the amino-acid decarboxylases

l(-)-Histidine decarboxylase. Kendall & Schmidt [1926] reported that 66 strains out of 72 of *Cl. welchii* tested produced a histamine-like substance when grown in the presence of glucose. In Table 1 it can be seen that all the strains used in washed suspension by the author produce histamine under the appropriate conditions. One strain, not included in Table 1, attacked glutamic acid rapidly but failed to decarboxylate histidine, but since this strain has also proved abnormally erratic in its power to produce specific toxin, it has been omitted. Kendall & Schmidt state that certain of their strains lost the power to produce histamine on continued subcultivation and it has been mentioned above that the author found it necessary to return to the alkaline egg medium once a week in order to maintain activity. Histidine is also decarboxylated, but at a slower rate, by some strains of *Cl. fallax* and *Cl. bifermentans*. Gale [1940, 1] showed that strains of *Bact. coli* in washed suspension will decarboxylate histidine quantitatively to histamine at an optimal pH of 4.0. When testing the power of *Cl. welchii* to carry out this reaction at various pH values, it became obvious that 4.0 is not the optimum pH in this case. Fig. 1 shows the variation of histidine decarboxylase activity with reaction pH for strains of *Cl. welchii*, *Cl. fallax* and *Cl. bifermentans*. The optimal activity is obtained between pH 2.5 and 3.0 in all cases: *Cl. welchii* B.W. 21 appears to have a well-defined optimum at 2.5 while that of the S. 107 strain lies nearer 3.0. The Q_{CO_2} figures for pH values < 2 can only be regarded as approximate as the HCl-phthalate mixtures used are not very effective buffers over this part of the range and calculations were accordingly made over a short period of activity, the pH being measured immediately before and after this period. The pH-activity curve is markedly different from that obtained for the decarboxylation of histidine by *Bact. coli* [Gale, 1940, 1] which has an optimum at pH 4.0 in all cases tested. The curve obtained with the coliform organisms falls steeply on the acid side of pH 4.0, the enzyme being almost inactive at pH 3.5 and it has been suggested that this is due to denaturation of the enzyme protein while in actual practice it is often found that the organisms coagulate below pH 4.0. This is not the case with *Cl. welchii* where coagulation of the organisms does not take place even at pH 1 and the pH-activity curve for the histidine decarboxylase is approximately symmetrical. Thus the difference in the apparent properties of the two enzymes may be due to the greater resistance to denaturation of the proteins of *Cl. welchii* over those of the coliform organisms, but the question is not likely to be solved until both enzymes are obtained in a cell-free state.

l(+)-Ornithine decarboxylase. Ornithine is decarboxylated rapidly by all four of the *Cl. septique* strains tested and much more slowly by *Proteus morganii* and some strains of *Pr. vulgaris*. Fig. 2 shows the variation of ornithine decarboxylase activity of some of these three organisms with reaction pH. The *Cl. septique* strains show a well-marked optimum at pH 5.5, the most alkaline value obtained for an amino-acid decarboxylase up to the present. The ornithine decarboxylase of *Bact. coli* has an optimum at pH 5.0 [Gale, 1940, 1].

l(-)-Tyrosine decarboxylase. Tyrosine is decarboxylated comparatively slowly by *Cl. aerofetidum* at an optimum pH of 5.0 as shown by Fig. 3. Un-

fortunately it has been possible to obtain only one strain of this organism. The pH optimum for this one strain is the same as that found for *Strep. faecalis* [Gale, 1940, 2], but whereas that organism attacked only tyrosine, *Cl. aerofetidum* also decarboxylates glutamic acid.

Fig. 1.

Fig. 1. Variation of histidine decarboxylase activity (Q_{CO_2}) with pH. 1, *Cl. welchii* B.W. 21. 2, *Cl. welchii* Chelsea. 3, *Cl. welchii* S. 107. 4, *Cl. welchii* S.R. 9. 5, *Cl. fallax* Morcom. 6, *Cl. bifermentans*.

Fig. 2.

Fig. 2. Variation of ornithine decarboxylase activity (Q_{CO_2}) with pH. 1, *Cl. septicum* Pasteur III. 2, *Cl. septicum* Carruthers. 3, *Cl. septicum* Jerral. 4, *Pr. morganii*.

l(+)-Glutamic acid decarboxylase. Glutamic acid is attacked by all the strains of *Cl. welchii*, *Cl. bifermentans* and *Cl. aerofetidum* tested and also by the majority of *Proteus* and coliform organisms. Fig. 4 gives the variation of the decarboxylase activity with pH in a selection of these cases; the pH optimum lies between 3.5 and 4.5, usually at 4.0. In all cases the pH-activity curve shows a rapid drop on the acid side of the optimum. In these studies of the decarboxylating powers of various bacteria, the glutamic acid enzyme has been the most frequently encountered and, in general, the most active of the amino-acid decarboxylases investigated. A quantitative study of the CO_2 liberated shows that only one of the $-COOH$ groups of the glutamic acid molecule is removed and, as is shown below, the product has always proved to be γ -aminobutyric acid. It may be that γ -aminobutyric acid plays some important part in the cell metabolism.

Thermolability. In the original investigations with *Bact. coli* it was found that the activity of the washed suspensions fell off rapidly and that this loss was increased with rise of temperature. This thermolability affects all these investigations but it varies greatly from organism to organism and is not constant for any one enzyme; for instance, the glutamic acid decarboxylase of *Bact. coli* is found to be very sensitive to temperature, but the similar enzyme of *P. morganii* is far

less sensitive and, when working at the experimental temperature of 30°, a linear decarboxylation is often obtained lasting over an hour with this organism. Similarly the histidine decarboxylase of *Cl. fallax* Morcom maintains a steady rate at 30°, whereas in most other cases the rate of decarboxylation begins to fall off within 15 min. after mixing. For the experiments involving isolation of the product, quantities of washed suspension etc. were arranged so that the decarboxylation should be complete within an hour and such large scale experiments were checked manometrically.

Fig. 3.

Fig. 4.

Fig. 3. Variation of tyrosine decarboxylase activity (Q_{CO_2}) with pH: *Cl. aerofetidium*.

Fig. 4. Variation of glutamic acid decarboxylase activity (Q_{CO_2}) with pH. 1, *Cl. aerofetidium*. 2, *Cl. welchii* Chelsea. 3, *Cl. welchii* B.W. 21. 4, *Pr. morganii*. 5, *Cl. bifermentans*.

Temperature coefficient. The temperature coefficient has been determined in most cases by carrying out manometric experiments under optimum pH conditions and at four different temperatures in each case. Fig. 5 shows the variation of initial Q_{CO_2} in the various cases with thermostat temperature. In most cases a straight line can be drawn through the points obtained for any one enzyme and organism, an exception being the ornithine decarboxylase of *Cl. septique* Pasteur which shows an 'optimum temperature' of 29–30°. There is a general falling-off of activity in most cases for temperatures above 40°. Glutamic acid decarboxylase of *Pr. morganii* shows an unusual variation with temperature, the Q_{CO_2} increasing very rapidly between 25 and 37° and then falling off almost to zero by 40°; only the early portion of this curve is shown on Fig. 5. Table 2 shows the temperature coefficients calculated from the lines shown in Fig. 5.

Effect of oxygen. With one exception the amino-acid decarboxylases of the *Clostridia* are unaffected by the presence of oxygen, the exception being the glutamic acid decarboxylase of *Cl. welchii*, a 50 % inhibition of which is obtained if air is used in the manometers instead of N_2 .

Fig. 5. Variation of activity with temperature. 1, *Pr. morganii*. 2, *Cl. septicum* (reduced 5 ×). 3, *Cl. aerofaecium* (reduced 10 ×). 4, *Cl. bifermentans*. 5, *Cl. welchii* B.W. 21. 6, *Cl. welchii* B.W. 21. 7, *Cl. aerofaecium*. 8, *Cl. bifermentans*: *Cl. fallax*. 9, *Pr. morganii*.

●—● Glutamic acid. ○—○ Ornithine.
×—× Histidine. ▲—▲ Tyrosine.

Table 2. *Temperature coefficients of amino-acid decarboxylases*

Substrate	Organism	Temp. coeff.
Glutamic acid	<i>Cl. welchii</i> B.W. 21	1.9
	<i>Cl. aerofaecium</i> 505	2.7
	<i>Cl. bifermentans</i> 2914	2.0
	<i>Pr. morganii</i>	4.3
Histidine	<i>Cl. welchii</i> B.W. 21	2.1
	<i>Cl. bifermentans</i> 2914	2.2
	<i>Cl. fallax</i> Morcom	2.1
Ornithine	<i>Cl. septicum</i> Pasteur	2.45
	<i>Pr. morganii</i>	2.45
Tyrosine	<i>Cl. aerofaecium</i> 505	2.1

Growth conditions necessary for the formation of the decarboxylases

Age of culture. 2 l. of the 2 % glucose broth medium were incubated in a flask fitted with a three-hole rubber bung: through the bung three glass tubes were passed, one connected to a hydrogen cylinder and conveying the gas below the level of the medium, a second reaching to the bottom of the flask and bent over outside to act as a sampling jet and the third a short tube connected outside to a Bunsen valve, the whole apparatus being assembled under sterile conditions. After inoculating the medium with the contents of a cultured meat-broth tube as usual, a stream of H_2 was swept through the flask to render the contents anaerobic.

Table 3. *Variation of decarboxylase activity with age of culture*

Age of culture hr.	Medium pH	mg. bact./ml. medium	Q _{CO₂}			
			Histidine	Ornithine	Tyrosine	Glutamic acid
<i>Cl. welchii</i> B.W. 21						
0	6.85	—	—	—	—	—
3	6.15	0.181	1.5	—	—	47
4	5.60	0.424	2.5	—	—	107
5	4.83	0.565	4.5	—	—	112
6	4.46	0.755	13	—	—	103
7	4.31	0.830	24	—	—	97
8	4.31	0.890	26	—	—	92
9	4.21	0.960	28	—	—	69
24	4.10	0.965	9	—	—	13
<i>Cl. septicum</i> Pasteur III						
0	7.59	—	—	—	—	—
2	7.40	0.030	—	167	—	—
3	7.23	0.081	—	474	—	—
4	6.92	0.134	—	251	—	—
5	6.59	0.185	—	194	—	—
6	6.37	0.226	—	150	—	—
7½	6.03	0.256	—	166	—	—
8½	5.90	0.270	—	201	—	—
10	5.82	0.282	—	183	—	—
26	5.53	0.311	—	159	—	—
<i>Cl. aerofetidium</i> 505						
0	7.23	—	—	—	—	—
3½	7.21	0.061	—	—	33	8.5
4½	6.92	0.122	—	—	30	7
5½	6.62	0.240	—	—	27	17
7	6.26	0.352	—	—	48	43
8	5.88	0.428	—	—	56	113
9	5.63	0.855	—	—	50	266
10	5.46	0.870	—	—	52	325
26	4.70	0.970	—	—	36	264
<i>Cl. bifermentans</i> 2914						
0	7.04	—	—	—	—	—
3	6.35	0.168	2	—	—	12
4	5.98	0.396	3.5	—	—	92
5	5.79	0.528	7	—	—	125
6½	5.69	0.600	8	—	—	138
7½	5.64	0.660	10	—	—	132
8½	5.58	0.752	8	—	—	131
26	5.22	0.755	5	—	—	94
<i>Cl. fallax</i> Morcom						
0	7.1	—	—	—	—	—
3	6.88	0.048	1	—	—	—
4	6.68	0.165	2	—	—	—
5	6.29	0.333	3.5	—	—	—
6½	5.77	0.480	5	—	—	—
7½	5.47	0.616	5	—	—	—
8½	5.32	0.720	6	—	—	—
9½	5.15	0.800	9	—	—	—
10½	5.12	0.780	12	—	—	—
26	4.63	0.765	6	—	—	—
<i>Pr. morganii</i>						
0	7.19	—	—	—	—	—
2½	6.76	0.061	—	3	—	—
3½	6.55	0.146	—	8	—	—
4½	6.32	0.218	—	16	—	—
6½	5.81	0.346	—	12	—	—
7½	5.70	0.385	—	10	—	—
8½	5.67	0.400	—	7	—	—
26	5.19	0.400	—	6	—	—

In order to take samples at intervals the Bunsen valve was clipped off and the sampling jet opened, when samples of any desired volume could be removed aseptically by slightly increasing the H_2 pressure. In this manner the variation of the decarboxylase activities of the various organisms with 'age of culture' has been followed. The *Clostridia* grow extremely rapidly and turbidity is usually marked 2 hr. after inoculation so that sufficient organisms for the preparation of washed suspensions can be obtained from 300–400 ml. medium after 3 hr. Under these conditions active cell division ceases after 7–11 hr. depending on the organism used, *Cl. bifermentans* 2914 growing the most rapidly of all the organisms tested. With each sample the following determinations were made: pH, dry weight of organism present per ml. medium, and decarboxylase activity against the substrates concerned. The results obtained in representative cases are set out in Table 3. They show that, as usual, early cultures display little activity and that the decarboxylases appear at various stages in the growth cycle, reach maximum activity before growth ceases, and then fall off slowly in activity as the culture ages.

It is obvious that the appearance of the decarboxylases is not conditioned only by the age of the culture, as some enzymes, notably the histidine decarboxylase, appear much later in the growth period than others such as the ornithine decarboxylase. Fig. 6 shows the variation of the decarboxylase activity plotted not against the age of the culture but against the pH of the medium during growth. From this it appears that the enzymes develop as the pH of the medium approaches their optimum pH values. Thus it appears that the main factor in this variation of activity with age of culture is due to the variation in medium pH during growth. A point of possible theoretical interest is that the medium pH at which maximum activity is developed for each enzyme is approximately 1 pH unit to the alkaline side of the optimum pH for that enzyme. This seems to indicate that the actual pH in the vicinity of the enzyme surface within the actively fermenting organism may be below that measured in the medium as a whole.

The case of the ornithine decarboxylase of *Cl. septicum* Pasteur is different from the others. A 2 hr. culture has little activity and there is a rapid increase of activity by the 3rd hr. followed by an almost equally rapid fall to a Q_{CO_2} of 150–180 at which level the activity stabilizes for 24 hr. Growth however continues for 8–9 hr. so that in this case there is a marked oscillation of activity during the early phase of growth and it appears on examination of stained films that the organism undergoes a structural change between 3 and 6 hr. The organisms obtained during the first 2.5 hr. stain homogeneously but as growth proceeds organisms appear which stain darkly at their ends and exhibit a granular structure, while spores are first seen at about the 4th hr. The fall in activity after 3 hr. is thus probably related to some structural change in the organism.

Effect of medium pH during growth. In the previous studies of amine production by bacteria [Gale, 1940, 1, 2] it has been shown that the amino-acid decarboxylases are produced by the organisms in response to an acid growth environment. That the same is true in the present studies has been indicated by Fig. 6. Flasks have been set up containing broth (without glucose) at adjusted pH values, inoculated with the organism, filled with H_2 and then incubated at 37° until growth reached a maximum. The pH of the media after growth was measured and the cultures made up into washed suspensions. It was very noticeable in the work with the *Clostridia* that whereas media adjusted before incubation to pH 8, 7 or 6 remained approximately at the adjusted values, those adjusted to values below 6 altered considerably during the growth of the

organism; thus a medium adjusted to pH 5.07 before incubation was found to have pH 5.91 on harvesting (*Cl. welchii* B.W. 21). This effect may be due to the greater formation and activity of the glutamic acid decarboxylase at low pH values. Fig. 7 shows the amino-acid decarboxylase activity of washed suspensions so prepared plotted against the average pH of the medium during growth. The *Clostridia* do not grow very well below pH 5.5 in the absence of carbohydrate. The results show, as before, that the enzymes are formed in response to an acid growth environment. In many cases, also as before, the organism growing at pH 5 shows as much or greater activity than when grown in glucose broth, showing that the function of the glucose in promoting amine production is to act as a source of fermentation acid. An apparent exception is the histidine decarboxylase of *Cl. welchii*, *Cl. fallax* and *Cl. bifermentans*. In the case of the histidine decarboxylase of *Bact. coli* [Gale, 1940, 1] the enzyme, having an

Fig. 6.

Fig. 7.

Fig. 6. Variation of decarboxylase activity with medium pH during growth in glucose broth. 1, *Cl. septique*. 2, *Strep. faecalis* [Gale, 1940, 2]. 3 and 6, *Cl. aerofotidum*. 4 and 8, *Cl. bifermentans*. 5 and 10, *Cl. welchii* B.W. 21. 7, *Pr. morganii*. 9, *Cl. fallax*.

Fig. 7. Effect of growth pH on decarboxylase activity of washed suspensions. 1 and 7, *Cl. bifermentans*. 2 and 3, *Cl. aerofotidum*. 4 and 6, *Cl. welchii* B.W. 21. 5, *Cl. septique* (reduced 10 ×).

●—● Glutamic acid. ○—○ Ornithine.
×—× Histidine. ▲—▲ Tyrosine.

optimum pH at 4.0, behaved in this respect like the other decarboxylases, but as a result of the lower pH optimum of the *Cl. welchii* histidine decarboxylase, the enzyme in this case requires more acid conditions for its formation than are necessary with *Bact. coli*. Thus in glucose broth the histidine enzyme only appears late in the growth period when the medium pH has fallen below 5 (see Fig. 6). When *Cl. welchii* is grown in non-carbohydrate media it is found that the lower the growth pH, the more active the decarboxylase, but the activity of a culture grown at pH 5 has $Q_{CO_2} = 10$ compared with a value of 60–70 for the same strain

(B.W. 21) grown in the presence of glucose. It is not possible to obtain cultures from non-carbohydrate media at pH values much less than 5 so that it appears either that glucose plays a definite part in promoting the formation of this particular enzyme, or that a strongly saccharolytic organism like *Cl. welchii* is able to produce a pH in its immediate vicinity during fermentation below that of the medium in general. Whatever may be the reason, it is true to say that *Cl. welchii* will not form the histidine decarboxylase or produce histamine to any extent *in vivo* unless it is actively fermenting carbohydrate.

As has been noted in the case of the ornithine decarboxylase of *Bact. coli*, the ornithine enzyme of *Cl. septique* shows a very marked response to acid growth conditions; the ornithine decarboxylase activity is scaled down 10 times in Fig. 7 in order to compare with the other curves.

Growth temperature. The decarboxylases of *Bact. coli* are formed more actively when the organisms are grown at 27° than at 37°. This does not seem to be the case with either the *Clostridia* or *Proteus*, growth temperature having little effect in most cases while the histidine enzyme is better formed at the higher temperature as, presumably, fermentation occurs more rapidly under such a growth condition.

Isolation and identification of the amines produced

The products of the decarboxylation processes have been isolated in every case from large-scale experiments based on and controlled by manometric work. The manometric experiments indicate in every case that the decarboxylation under optimum conditions proceeds to completion since the theoretical volume of CO₂ is liberated within experimental error. In most cases the entire organisms obtained from 800 ml. glucose broth have been used for each isolation and the concentration of the washed suspension, together with the quantities of buffer and amino-acid, have been arranged so that the decarboxylation should go to completion within 1 hr. The full details of the experimental procedure and isolation are the same as previously described [Gale, 1940, 1, 2]. The yields on isolation of the products of decarboxylation usually amount to 60–70 % of theory. Analyses are by Weiler.

Histidine decarboxylase: product histamine isolated as dipicrate. *Cl. welchii* B.W. 21. Quantities: 20 ml. washed suspension, 15 mg./ml.; 20 ml. 0.1 M phthalate buffer pH 2.5; 20 ml. M/60 l(–)-histidine monohydrochloride (neutral). Yield 135 mg.—70 % theory. m.p. 239° (corr.). (Found: C, 35.95; H, 2.67; N, 22.25 %. Calc.: C, 35.97; H, 2.64; N, 22.2 %.)

Similar experiments were carried out with:

Cl. welchii S.R. 9 (pH 3.0). (Found: N, 22.08 %. m.p. 238°. Yield 63 %.

Cl. welchii Chelsea (pH 2.5). (Found: N, 22.00 %. m.p. 241°. Yield 58 %.

Cl. bifermentans 2914 (pH 2.5). (Found: C, 36.1; H, 2.68; N, 22.05 %. m.p. 240°. Yield, 83 %.

Cl. fallax Morcom (pH 2.5). (Found: C, 36.2; H, 2.62; N, 21.9 %. m.p. 240°. Yield, 68 %.

Tyrosine decarboxylase: product tyramine isolated as dibenzoyl deriv. *Cl. aerofetidum* 505. Quantities: 15 ml. washed suspension 20 mg./ml.; 15 ml. 0.1 M phthalate buffer pH 5.0; 15 ml. M/60 l(–)-tyrosine. Yield 65 mg.—74 % theory. m.p. 171°. (Found: C, 76.10; H, 5.47; N, 3.89 %. Calc.: C, 76.50; H, 5.54; N, 4.06 %.)

Ornithine decarboxylase: product putrescine isolated as dipicrate. *Cl. septique* Pasteur III. Quantities: 30 ml. washed suspension 6 mg./ml.; 30 ml. 0.01 M phosphate buffer pH 5.5; 30 ml. M/60 l(+) -ornithine dihydrochloride (neutral).

Yield 197 mg.—72 % theory. Decomposes at 250°. (Found: C, 35.69; H, 3.67; N, 20.00 %. Calc.: C, 35.03; H, 3.65; N, 20.44 %.)

Glutamic acid decarboxylase: product γ -aminobutyric acid. Neuberger [1937] states that the dissociation constants of γ -aminobutyric acid are $pK_1 = 4.23$ and $pK_2 = 10.43$ whereas the author has shown [1940, 1] that the dissociation constants for the α -aminobutyric acid are very different from these, particularly pK_1 which lies in the region of 2. Accordingly it is a simple matter to distinguish between the two possible products of the glutamic acid decarboxylation by carrying out a titration curve on the experimental material and so determining the value of pK_1 . The complete isolation has been carried out in the case of *Bact. coli*.

Found: *Cl. welchii* B.W. 21 (pH 4.0). $pK_1 = 4.15$.

Cl. aerofaecium 505 (pH 4.0). $pK_1 = 4.25$.

Cl. bifermentans 2914 (pH 4.0). $pK_1 = 4.30$.

Pr. morganii (pH 4.0). $pK_1 = 4.25$.

Thus the product in all cases of glutamic acid decarboxylation studied has proved to be the γ -aminobutyric acid.

The production of histamine by Cl. welchii in vivo

It has been shown that whenever *Cl. welchii* grows in the presence of fermentable carbohydrate, conditions are such that histamine is produced from free histidine. *Cl. welchii* ferments muscle glycogen with the production of acid and gas almost as rapidly as it attacks glucose, the glycogen fermentation being strictly adaptive. *Cl. welchii* B.W. 21 grown in the presence of glycogen possesses a histidine decarboxylase Q_{CO_2} of 30–50 determined under the usual conditions. Thus it seems probable that when *Cl. welchii* invades muscle tissue conditions arise which are favourable for the production of histamine, as the fermentation of muscle glycogen would produce the necessary low pH, particularly in 'pockets' in such a heterogeneous medium, and the proteolytic activities of the organism would liberate histidine. It seems unlikely that sufficient histamine would be formed under these conditions to produce any symptoms unless in mixed or massive gangrene infections. This aspect of the investigation is dealt with in the Addendum to this paper. Since *Cl. paludis* and the Lamb dysentery organism behave as other *welchii* strains in producing histamine, this may play a part in the aetiology of diarrhoea in lambs.

Inhibitors of histamine production by Cl. welchii

The production of histamine by *Cl. welchii* can be checked either by inhibition of histidine decarboxylase or by inhibition of fermentation during growth. Of a range of substances tested on washed suspensions, $KMnO_4$ and chloramine T prove to be the most active inhibitors, $M/1000$ solutions of either completely inhibiting the histidine decarboxylase and the fermentation of either glucose or glycogen under optimal conditions. $M/1000$ iodoacetate inhibits fermentation reactions but has no effect on histidine decarboxylase. Acriflavin, though very effective as a disinfecting agent as far as *Cl. welchii* is concerned, does not affect the enzymes in question at concentrations less than $M/10$. The decarboxylase is unaffected by concentrations less than $M/100$ of the following: cyanide, urethane, urea, salicylic acid, fluoride, sulphanilic acid, hydrazine, H_2O_2 , etc., or by bubbling with O_2 .

DISCUSSION

The work presented above gives a general idea of the decarboxylating powers of the *Clostridium* and *Proteus* organisms and the conditions under which such activities can be expected to function. It cannot be regarded as a comprehensive survey as it may well be that some strains of the organisms reported as not showing decarboxylating activities will possess such enzymes, just as only one strain of *Cl. fallax* tested can produce histamine. Those members of the *Clostridium* group which do possess decarboxylases do not appear to have any other marked property in common that distinguishes them from the remainder; they are all strongly saccharolytic, but others of the most saccharolytic of the group, e.g. *Cl. butyricum*, are inactive as far as can be decided at present. *Cl. welchii*, *Cl. septique*, *Cl. bifermentans* and *Cl. aérofoetidum* are all classified in the saccharolytic and proteolytic group of the M.R.C. classification whereas *Cl. fallax* is stated to possess no proteolytic activity. Strains of all these organisms have been reported in the contents of the intestine. Of 9 strains of *Proteus* organisms tested, 7 attacked glutamic acid and 3 ornithine so that the amine-producing activities of this group do not appear to be marked.

SUMMARY

1. Washed suspensions have been prepared of organisms from the groups *Clostridium* and *Proteus* grown in a 2% glucose casein-digest broth and these have been tested for their power to decarboxylate amino-acids.

2. *l*(-)-Histidine is decarboxylated to histamine by strains of *Cl. welchii*, *Cl. bifermentans* and *Cl. fallax*; *l*(-)-tyrosine is decarboxylated to tyramine by *Cl. aérofoetidum*; *l*(+)-ornithine is decarboxylated to putrescine by *Cl. septique* and *l*(+)-glutamic acid is decarboxylated to γ -aminobutyric acid by *Cl. welchii*; *Cl. aérofoetidum* and *Cl. bifermentans*. Of 9 strains of *Proteus* organisms tested, 7 decarboxylated glutamic acid and 3 ornithine.

3. The pH optimum for histidine decarboxylase lies between 2.5 and 3.0; for ornithine decarboxylase at 5.5; for tyrosine decarboxylase at 5.0 and for glutamic acid decarboxylase at 4.0.

4. The enzymes appear to vary in thermolability from organism to organism but all have temperature coefficients of the order of 2.

5. When the organisms are grown in glucose broth, early cultures have little activity, the decarboxylases developing later in the growth period as the pH of the medium approaches their optimum pH. The ornithine decarboxylase of *Cl. septique* is exceptional in that its activity reaches a maximum and then falls again within the first 3 hr. of growth; the loss of activity before growth ceases appears to be associated with a change in structure of the organism.

6. In the absence of carbohydrate the decarboxylase activity of the organism depends upon the pH of the medium during growth, low growth pH promoting high decarboxylase activity. The histidine decarboxylase activity of *Cl. welchii* is considerably higher when the organism is grown in the presence of fermentable carbohydrate than when it is grown in a non-carbohydrate broth at pH 5.

7. The possible pathogenic significance of histamine production by *Cl. welchii* is discussed and methods for its prevention noted.

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ADDENDUM

Plasma histamine in gas gangrene

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If the symptoms of *Cl. welchii* infection are in part due to histamine intoxication, the histamine concentration of the plasma should be significantly increased. Information about changes in the histamine content of the whole blood is of doubtful value, since, as Code [1937] has shown, most of the blood histamine is in the formed elements. Cat's arterial blood, treated with heparin and centrifuged without delay, yields a plasma whose histamine equivalent may be determined directly upon the atropinized guinea-pig's ileum. Normal cat's plasma, obtained under 'dial' anaesthesia, has a histamine equivalent of not more than 0.01 $\mu\text{g.}/\text{ml.}$ We have observed that the histamine equivalent of the plasma may be raised to 0.45 $\mu\text{g.}/\text{ml.}$ by the intravenous injection of 0.5 mg. of histamine per kg. of body weight, or to 0.04 $\mu\text{g.}/\text{ml.}$ by the subcutaneous injection of the same dose. No obvious symptoms are produced in a cat given 0.5 mg./kg. of histamine subcutaneously.

We have infected cats with a histamine-producing strain of *Cl. welchii* provided by Dr Gale. They were anaesthetized with 'dial', and were maintained under the anaesthesia throughout the experiment. They received an injection into the soleus or gastrocnemius muscle of one leg of 2–6 ml. of a meat-broth culture aged 15–20 hr., and the plasma was examined 2–6 days later. In those animals in which the infection became fully developed, the limb was swollen and crepitant, and the skin covering it discoloured: the muscles of the calf immediately surrounding the site of injection were broken down to a pulpy mass, and in certain instances there was a considerable collection of thick creamy pus which yielded only *Cl. welchii* on culture. Some of the animals died, but the cat appears able to withstand an extensive involvement of one limb without succumbing to the effects of toxæmia. In no instance could *Cl. welchii* be recovered from the blood.

The histamine equivalent of the plasma from the infected animals has in no case been greater than $0.02 \mu\text{g./ml.}$, and in two animals in which the infection was advanced and the assay particularly satisfactory, values of 0.008 and $0.007 \mu\text{g./ml.}$ were obtained. We took the occasion, in four animals, to determine the histamine equivalent of normal and infected muscles. The histamine equivalent of the infected muscles was greater, by 30 to 230 %. If the normal histamine content of cat's muscle is taken to be $1 \mu\text{g./g.}$, it can be calculated that at least 300 g. of infected tissue would be required to yield 1 mg. of histamine. The histamine equivalent of the purulent exudate in one animal was about the same as that of normal muscle.

Clinical experience shows, and we have found in the cat, that an infection with *Cl. welchii* proceeds more rapidly if other anaerobes such as *Cl. sporogenes* or *Cl. histolyticum* are present. These organisms, although not themselves histamine producers, are actively proteolytic, and might be expected, by increasing the amount of histidine available, to accelerate the liberation of histamine by *Cl. welchii*. In a cat which had received a mixture of 20 hr. broth cultures of *Cl. welchii* (1 ml.) and *Cl. histolyticum* (0.5 ml.), the infection was well developed in 28 hr., involving the thigh muscles as well as the gastrocnemius. The histamine equivalent of the plasma was less than $0.005 \mu\text{g./ml.}$ The histamine equivalent of the infected and pultaceous muscle was about 30 % higher than that of the normal side.

It would appear, therefore, that in cats infected with *Cl. welchii*, no significant pathological effect is produced by the absorption of histamine into the blood stream from the infected tissue.

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