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The Distribution of Glutamic Acid Decarboxylase in the Family Enterobacteriaceae, examined by a Simple Chromatographic Method

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SUMMARY: By a simple chromatographic technique with filter-paper disks the distribution of glutamic acid decarboxylase in the family Enterobacteriaceae was surveyed. The decarboxylase occurred irregularly among 230 strains belonging to this family. The test for glutamic acid decarboxylase may prove to be of value in the classification of organisms related to the genus *Salmonella*, in the classification of paracolon types and in the identification of sub-groups within the genera *Escherichia* and *Aerobacter*.

We have shown (Proom & Woiwod, 1949) that when bacteria are grown on an acid-hydrolysed casein medium changes may occur in the culture fluids, revealed chromatographically, which are peculiar to certain species or groups of bacteria; and we suggested that they might be of value in bacterial classification. We have now surveyed the distribution of glutamic acid decarboxylase (GAD) among strains and species of the family Enterobacteriaceae and have devised a very simple chromatographic technique for demonstrating its presence.

METHODS

The production, by some strains of bacteria, of γ -aminobutyric acid by specific decarboxylation of glutamic acid can be demonstrated by the chromatographic technique of Woiwod (1949). It was apparent, however, that for routine use in a bacteriological laboratory a much simpler method was required, and a filter-paper disk technique was devised which is a considerable modification of that described by Rutter (1950).

Filter-paper disk chromatography

The apparatus for the filter-paper disk chromatographic method is illustrated in Fig. 1 and the method is as follows. The lid (A) of a large Petri dish (c. 14 cm. diam.) is placed on the bench with its edges uppermost, and in it is placed a sheet of filter paper (F), which covers the bottom and is saturated with water. Centrally on the wet filter paper is placed the lid (B) of a smaller Petri dish (c. 9 cm. diam.) containing a layer (D) of *m*-cresol saturated with water. This inner dish (B) can be covered by the bottom of the larger Petri dish (G). Provided the filter paper is kept saturated with water the *m*-cresol/water equilibrium is maintained in the chamber formed by the two parts A and G of the larger Petri dish.

The chromatogram disks used are 12.5 cm. Whatman No. 4 filter papers. A paper is marked with a pencil, eight marks being made and numbered, equidistant around the circumference of a circle of radius 1 cm. from the centre of the paper. This can be conveniently done with the aid of a circular plastic disk of the same diameter as the filter paper and with holes drilled at the appropriate positions.

A small hole is made in the centre of the filter paper (C) to take a 'wick' (E) made of a loosely-rolled cylinder of Whatman No. 4 paper cut to the required length. The samples to be chromatographed are spotted on to the filter-paper disk, at the marked positions, using a fine glass capillary (Woiwod, 1949). The filter-paper wick (E) is then pushed through the hole in the centre of the

Fig. 1

Fig. 2

Fig. 1. Diagram of apparatus for filter-paper disk chromatography. A, Inverted 14 cm. Petri lid; B, 9 cm. Petri dish; C, Filter-paper disk; D, *m*-cresol saturated with water; E, Filter-paper wick; F, Filter paper saturated with water; G, Inverted bottom of larger Petri dish.

Fig. 2. Diagram of biscuit-tin drying oven. A, 9-in. cube biscuit tin; B, Electric lamp fixed in lid; C, Rod for suspending filter papers; D, Outlet for connexion to water pump; E, Suspended filter paper.

paper disk (C) and the disk placed on the top of the smaller Petri dish (B), ensuring that the wick dips into the layer (D) of *m*-cresol. The Petri dish (B) and disk (C) are then covered with the inverted bottom (G) of the larger Petri dish. The *m*-cresol is drawn up the wick by capillarity and resolution of the constituents of the sample occurs as the solvent front moves as an expanding circle across the filter-paper disk.

The apparatus is left on the bench at room temperature. It takes about 1 hr. for the solvent front to reach the circumference of the filter-paper disk. The filter-paper disk is then removed by a clip attached to the edge of the paper and is suspended and dried in a hot-air oven. The oven can be simply made from a biscuit tin (A), Fig. 2 (c. 9 in. cube) heated with a 60-W electric lamp; the fumes are removed from the tin by a rubber tube connected to a water pump. Such an apparatus is illustrated in Fig. 2 and was satisfactory in routine use. After drying in the oven, which takes about 30 min., the disks are dipped in a solution of 0.1 % (w/v) ninhydrin in chloroform and are heated

for a few minutes in the oven to develop the chromatogram. Very occasionally a batch of filter papers is encountered in which the solvent front moves in an irregular fashion from the centre of the paper. Although it is usually possible to obtain results from such papers they are not satisfactory.

Preparation of cultures for chromatography

The culture medium used consisted of (in %, w/v): glutamic acid, 1; glucose, 3; sodium chloride, 0.5; with added metallic salts and growth factors as in the medium of Proom & Woiwod (1949). The growth factors and metallic salts were not necessary for the majority of the strains examined but were included for the benefit of fastidious organisms. The medium was adjusted to pH 7.4, dispensed in 2 ml. amounts in 1/4 oz. screw-capped bottles and sterilized by autoclaving at 15 lb. for 30 min. These bottles were inoculated with strains to be tested and incubated for 24 hr. at 37°, or at 28° for those species which grew either poorly or not at all at 37°. After 24 hr. incubation a few drops of Universal indicator (British Drug Houses Ltd.) were added to each bottle and the pH adjusted to about 4.5 with sterile N-HCl. To each bottle was then added 0.5 ml. of sterile 0.2 M acetate buffer (pH 4.5) and the cultures incubated for a further 24 hr. The cultures were then steamed for 1 hr. and tested for the presence of γ -aminobutyric acid by the chromatographic method just described.

Examination of culture fluids for glutamic acid decarboxylase

Glutamic acid does not move appreciably from the starting position and γ -aminobutyric acid moves close to the edge of the filter-paper disk. A positive GAD reaction, therefore, is the appearance of a spot, when developed with ninhydrin, near the circumference, indicating the presence of glutamic acid decarboxylase. Cultures of known positive and negative strains or a solution of γ -aminobutyric acid may be included as controls. After a little experience with the test these controls may be omitted. Since the solvent front moves as an expanding circle across the filter paper the γ -aminobutyric acid spot is not circular but appears as a band 1–2 cm. in width. The results obtained with the filter-paper disk method agree completely with those obtained when the same cultures were analysed on larger papers by the method of Woiwod (1949); the two methods are equally sensitive. Provided a culture shows visible growth in 24 hr. it is not necessary to increase the incubation period either for growth or enzyme action at pH 4.5. Occasionally a strain fails to grow in 24 hr. and further incubation is necessary.

RESULTS

The strains examined were from the Wellcome Bacterial Collection supplemented by strains from the National Collection of Type Cultures, the Metropolitan Water Board and the Water Pollution Research Laboratories. Many of the strains from the NCTC were received under names having little more

than historical interest and in order to simplify the expression of the results the *coli* and *aerogenes* strains were classified according to their biochemical reactions, using the nomenclature usually employed in the bacteriological examination of water (see *The Bacteriological Examination of Water Supplies*, Report 71, H.M. Stationery Office, 1939). The paracolon organisms were regarded as variants, being non-fermenters or late-fermenters of lactose, of a parent species; for example strains received under the name of *Bacterium columbense* were classified as non-lactose-fermenting variants of *Bact. coli* type I.

Table 1. *The distribution of glutamic acid decarboxylase in the family Enterobacteriaceae*

Organism	Reactions				Glutamic acid decarboxylase test. No. of strains		
	M.R.	V.P.	Citrate	Indole	Examined	Positive	Negative
<i>Bact. coli</i> type I	+	—	—	+	72	71	1
<i>Bact. coli</i> type II	+	—	—	—	1	0	1
<i>Bact. coli</i> intermediate type I	+	—	+	—	12	0	12
<i>Bact. coli</i> intermediate type II	+	—	+	+	3	2	1
<i>Bact. coli</i> irregular type I	+	—	—	+	3	3	0
<i>Bact. aerogenes</i> type I	—	+	+	—	16	0	16
<i>Bact. aerogenes</i> type II	—	+	+	+	5	2	3
<i>Bact. cloacae</i>	—	+	+	—	4	0	4
Friedländer's bacillus					9	0	9
<i>Paracolon</i> sp. (<i>Bact. coli</i> type I except for lactose)					11	11	0
<i>Paracolon</i> sp. (<i>Bact. coli</i> intermediate type I except for lactose)					4	0	4
<i>Paracolon</i> sp. (<i>Bact. aerogenes</i> type I except for lactose)					4	0	4
Plant pathogens (<i>Bacterium (Erwinia)</i> sp.)					6	0	6
<i>Serratia</i> sp.					7	0	7
<i>Salmonella</i> sp.					43	0	43
'Arizona' group of coliforms					6	0	6
<i>Shigella</i> sp.					24	18	6

M.R., Methyl red test; V.P., Voges-Proskauer test.

The results are summarized in Table 1. Of 72 strains of *Bact. coli* type I and 3 strains of *Bact. coli* irregular type I, all except one strain of *Bact. coli* type I gave a positive GAD test. All these strains were of animal origin (human, ovine, bovine, canine), most of the bovine strains having been isolated from cases of white scour. The correlation between the presence of GAD and the animal origin of the strain is, in this group, nearly complete and rather better than the correlation with the results of the Eijkman reaction.

All of 12 strains of *Bact. coli* intermediate type I, 16 strains of *Bact. aerogenes* type I, and 4 strains of *Bact. cloacae* were GAD negative.

With the few strains available of *Bact. coli* intermediate type II and *Bact. aerogenes* type II the GAD test was variable. There was an interesting

relation between the GAD reaction of the non-lactose-fermenting or paracolon variants, and of the parent strains. Thus 11 strains which had the biochemical reactions of *Bact. coli* type I, except for delayed or no reaction with lactose, were GAD positive whereas 4 non-lactose-fermenting variants of *Bact. aerogenes* type I and 4 non-lactose-fermenting variants of *Bact. coli* intermediate type I, were all negative.

Nine strains of Friedländer's bacillus, including representatives of serological types A, B and C, were all GAD negative. The biochemical reactions of these strains were variable and none of them had the biochemical pattern of a species which is predominantly GAD positive.

Six strains of plant pathogens (2 strains of *Bact. carotovorum* and 1 strain each of *Bact. amylovorum*, *Bact. salicis*, *Bact. tumefaciens* and *Bact. rhizogenes*) and 7 chromogenic actively-proteolytic strains belonging to the genus *Serratia*, were all GAD negative.

Nineteen strains of *Proteus*, including representatives of the species *Pr. vulgaris*, *Pr. mirabilis*, *Pr. morganii*, *Pr. rettgeri* and a number of X19 and XK strains were either weakly GAD positive or negative and there was no correlation between the GAD reaction and a particular species.

The GAD reaction was also variable in the genus *Shigella*. Twenty-four strains were examined; some were strongly positive, others weakly positive or negative and there appeared to be no correlation between the GAD reaction and the particular species.

Forty-three strains of the genus *Salmonella* were tested and were all negative. Six strains of the closely related 'Arizona' group of coliforms were also negative. Four strains of the 'Bethesda' group of coliforms were also negative but the biochemical reactions of these four strains (NCTC Nos. 7820, 7822, 7824, 7829) would suggest a relationship with *Bact. coli* intermediate type I, a group which is essentially GAD negative.

DISCUSSION

In previous work (Proom & Woiwod, 1949) we showed that when bacteria are grown on an acid-hydrolysate of casein medium changes may be seen on the filtrate chromatograms which in some instances were associated with a bacterial group or species. However, before the chromatographic method can be widely used in routine bacteriology the technique should be simple, and it must be possible to test a number of cultures in a short time, with apparatus which is readily available and occupies little space. Within the limitation that it is only applicable to some simple mixtures of amino-acids our method satisfies these conditions. Results are obtained on the second day after inoculation; the setting-up of the chromatograms for 50-100 cultures occupies about 30 min. and the analysis is completed in about 3 hr.

The biochemical reactions used in the identification and classification of bacteria have, in the main, been selected empirically. There seems no reason to suppose, for example, that the decarboxylation products of amino-acids are of greater or less value in classification than the fermentation of particular carbohydrates. The overriding principles governing the use, for classification,

of a particular biochemical reaction are its utilitarian value within the group studied and the ease with which the test can be performed.

From the present results it is apparent that glutamic acid decarboxylase is scattered irregularly among strains and species in the family Enterobacteriaceae as a whole, but that in a few cases the test may be of taxonomic interest.

Sufficient strains of *Salmonella* have been examined to suggest that GAD is not present in members of this genus. Closely related organisms such as the 'Arizona' group of coliforms are also GAD negative. The test may thus be of value in classification of organisms possibly related to *Salmonella*.

The GAD test may also be of value in the classification of the paracolon organisms. If paracolon organisms are regarded as late- or non-lactose-fermenting variants of a parent species our results would suggest that when the parent species is GAD positive the variant would have the corresponding character and vice versa.

King & Fletcher (1950) examined 47 strains of coliform organisms and found that GAD was characteristic of *Bact. coli* type I; they also found a positive correlation between the Eijkman and GAD reactions. We have extended their observations, confirming that *Bact. coli* type I is essentially GAD positive. We have, however, observed the presence of GAD in a minority of strains of *Bact. coli* of other types. The 3 strains of *Bact. coli* irregular type I which were available and 2 of 3 strains of *Bact. coli* intermediate type II, were positive. None of these strains gave a positive Eijkman reaction.

In our hands a few of the strains of *Bact. aerogenes* type II gave a positive Eijkman reaction and in the case of the three strains of *Bact. aerogenes* type II giving a positive Eijkman reaction this was correlated with a positive GAD reaction. The Eijkman tests were performed in MacConkey bile salt lactose broth and incubated in a thermostatically controlled water-bath at 44°, the tests being adequately controlled. We appreciate that the occurrence of Eijkman positive strains in Britain is unusual. However, the positive strains were retested on a number of occasions and the results confirmed.

The results suggest that the relatively simple GAD test may be of value in laboratories routinely testing large numbers of coliform organisms.

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