Identification of Characteristic Extracellular Ninhydrin-Positive Substances Produced by some Bacteria

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SUMMARY: When certain species of bacteria are grown in an acid-hydrolysate of casein medium, ninhydrin-positive substances which were not present in the uninoculated medium appear on chromatograms of the culture filtrates. *Shigella paradysenteriae* and *Escherichia coli* produce γ -aminobutyric acid by decarboxylation of glutamic acid. The substance produced by *Serratia marcescens* resists acid hydrolysis, gives a positive Sakaguchi reaction and matches arginine on two-dimensional chromatography. The substances produced by *Clostridium sporogenes*, *Cl. bifermentans* and *Cl. sordellii*, but not by any of the other species of Clostridia examined, are δ -aminopentanoic acid, probably derived from proline, and α - and γ -aminobutyric acids produced by unknown mechanisms. *Proteus vulgaris* and *Clostridium tetani* each produce two polypeptides containing a high proportion of amino-acids in the groups valine/methionine and leucine/isoleucine. These two pairs of polypeptides are similar in R_F value and gross amino-acid composition. *Staphylococcus aureus* produces α -aminobutyric acid which may be derived from threonine.

By partition paper chromatography we showed previously (Proom & Woiwod, 1949) that certain species of bacteria, grown in an acid-hydrolysed casein medium, produced extracellular ninhydrin-positive substances. The production of a given substance was often associated with a particular group or species of bacteria and its appearance was concurrent with other changes on the chromatogram.

The substances produced by *Serratia marcescens*, *Shigella paradysenteriae*, *Proteus vulgaris* and *Clostridium tetani* were further examined and identified as arginine, γ -aminobutyric acid and polypeptides respectively (Woiwod & Proom, 1949). The present paper reports more fully the identification and characterization of these substances together with those produced by *Escherichia coli*, *Clostridium bifermentans* and *Staphylococcus aureus*. The possible value of these observations in bacterial classification will be discussed in a subsequent paper.

METHODS

Cultures in acid-hydrolysed casein medium were grown at 37° as previously described (Proom & Woiwod, 1949) and the culture filtrates were examined by the chromatographic technique of Woiwod (1949*a*). Single-dimensional chromatograms were run on sheets of Whatman No. 4 paper (c. 22 in. $\times$ 18 in.) with *n*-butanol + acetic acid as the solvent.

Usually four sheets were run with each culture filtrate, each sheet having thirty-five channels, with 0.015 ml. of filtrate for each channel put on the paper by means of a micrometer syringe. After running and drying the chromatograms the ninhydrin-positive material to be investigated was located by means of its fluorescence under ultra-violet light. Strips of filter-paper with

the material so located were cut from the papers and soaked overnight in water. The solutions thus obtained were pooled and the pool divided into two equal portions. Each portion was placed in a 10 ml. round-bottom flask having a ground-glass neck, and evaporated to dryness on a water-bath under reduced pressure. One portion was then hydrolysed by refluxing at atmospheric pressure with about 5 ml. of *c.* 6*N*-HCl for 70 hr. using an air condenser. The acid was then removed by drying-down repeatedly with distilled water *in vacuo* on a water-bath. The residue was dissolved in a small volume of water. The dried but unhydrolysed portion of material was redissolved in the same volume. A strip of blank paper of approximately the same size was also extracted with water and the extract evaporated to dryness and hydrolysed. This was to control effects due to the possible presence of polypeptide impurities in the paper (Wynn, 1949). The solutions were then further investigated.

RESULTS

The polypeptides produced by Proteus vulgaris and Clostridium tetani

In the culture filtrates from all the strains of *Cl. tetani* and *Pr. vulgaris* examined, ninhydrin-positive material was observed which moved faster than leucine on the single-dimensional chromatogram with *n*-butanol + acetic acid as solvent. Two such spots were observed with both organisms, the slower-moving spot giving a considerably weaker colour reaction with ninhydrin than did the faster-moving spot. It was sometimes necessary to load the chromatogram heavily with culture-filtrate in order to demonstrate the presence of this slower-moving material. This weaker reaction with ninhydrin does not, of course, necessarily reflect the actual concentration of material on the paper.

Culture-filtrates were chromatographed as previously described, and strips of paper containing the individual spots were cut and eluted with water. The hydrolysed and unhydrolysed materials were run on single-dimensional chromatograms; the results are shown in Pl. 1, fig. 1. It is evident that on hydrolysis the materials of both the faster- and the slower-moving spots gave a number of amino-acids. The exact amino-acid composition of each hydrolysate was not ascertained, but the acids present were mainly in the leucine and valine groups (leucine, *isoleucine*, phenylalanine, methionine and valine). There was also a similarity in the gross amino-acid composition of all four hydrolysates. It can also be seen from Pl. 1, fig. 1 that the slower-moving spot from *Pr. vulgaris* was no longer visible on the chromatogram after elution from the paper. After hydrolysis of this spot, however, besides the amino-acids arising from hydrolysis of the original polypeptide, unchanged polypeptide was also again visible. This effect was observed on a number of occasions, but the reason for this behaviour is not known. It may perhaps be that the substances are rendered insoluble during the evaporation procedure after elution from the paper. Alternatively, some chemical change may occur when the chromatogram is dried before elution, which does not necessarily affect the solubility but only the reaction of the eluted material with ninhydrin. This latter mechanism would explain the difficulty of demonstrating the slower-moving spot on two-dimensional chromatograms.

In view of the similarity in R_F values and gross amino-acid composition of the corresponding pairs of polypeptides produced by both organisms, attempts were made by means of two-dimensional chromatography to establish their identity. Culture filtrates from *Pr. vulgaris* and *Cl. tetani* were freeze-dried and concentrated by redissolving in one-tenth of their original volume of distilled water. They were examined by two-dimensional chromatography on No. 4 Whatman paper using *n*-butanol + acetic acid followed by *m*-cresol and also benzyl alcohol. The faster-moving spot from both organisms occupied the same position on all the chromatograms. This fact, in conjunction with the similarity in the gross amino-acid composition of their hydrolysates, supports the view that the faster-moving polypeptides produced by *Pr. vulgaris* and *Cl. tetani* are chemically identical. The slower-moving spots, however, could not be detected on the two-dimensional chromatograms, and it was not possible further to load the chromatograms without smearing due to increased salt content. Culture-filtrates were therefore de-salted by the method of Consden, Gordon & Martin (1947). They were then concentrated by freeze-drying and redissolved in a small volume of water. It was still, however, not possible to demonstrate the presence of the slower-moving material on the two-dimensional chromatogram, although it was visible at the end of the initial *n*-butanol + acetic acid run. This supported the view that heating the chromatogram caused a change either in ninhydrin reactivity or solubility, at least in the case of the slower-moving material.

The production of γ -aminobutyric acid by Shigella paradysenteriae and Escherichia coli

Chromatograms of filtrates from cultures of *Sh. paradysenteriae* and *Esch. coli*, after being kept for 7 days at pH 4.5, showed a strong ninhydrin-positive spot in approximately the same position as tyrosine on the *n*-butanol + acetic acid chromatogram. The intensity of this spot was inversely related to the intensity of the glutamic acid spot (Proom & Woiwod, 1949). Culture filtrates were chromatographed as previously described, and strips of paper containing the unidentified material were cut out and eluted with water. The hydrolysed and unhydrolysed materials were further investigated. In searching for possible amino-acids to correspond in position with the unhydrolysed material, it was observed that its position on single-dimensional chromatograms could be closely matched with γ -aminobutyric acid. Two-dimensional chromatography with *n*-butanol + acetic acid followed by *m*-cresol separated the unhydrolysed material into a ninhydrin-positive substance and a fluorescent ninhydrin-negative substance which apparently had the same R_F values in *n*-butanol + acetic acid, although the latter material preceded the ninhydrin-positive material in *m*-cresol. Two samples of synthetic γ -aminobutyric acid from different sources also showed the presence of the fluorescent ninhydrin-negative material (Pl. 1, fig. 2). This suggests that it is not an impurity. A possible mechanism for its formation is ring-closure with the formation of a lactam. Whether the material is, in fact, the lactam of γ -aminobutyric acid is being investigated. The material produced by the organism, after hydrolysis and

two-dimensional chromatography (*n*-butanol+acetic acid followed by *m*-cresol) showed these two spots, together with two further fluorescent spots, only one of which was ninhydrin-positive. A similar picture was obtained when synthetic γ -aminobutyric acid was subjected to the same acid treatment. The chemistry of the production of these two spots has not yet been elucidated. However, it is clear that the material from *Sh. paradysenteriae* and synthetic γ -aminobutyric acid are identical. Confirmatory evidence was obtained by the procedure of Crumpler & Dent (1949), in which the paper chromatogram is dusted with basic copper carbonate along the path the amino-acids will travel during chromatography. Those which form copper salts, and this includes all α -amino-acids (Woiwod, 1949*b*), will run to one side and fail to react with ninhydrin, whereas non α -amino-acids appear in their normal positions on the chromatogram. It was found simpler to treat the paper by spraying with a 0.05 % (w/v) solution of cupric acetate (A.R.) in ethanol instead of with dry copper carbonate, and allowing the paper to dry before putting on the samples for chromatography in the usual way. Between 40 and 50 ml. of this solution per sheet of Whatman No. 4 paper (c. 22 $\times$ 18 in.) gave the best results. All such copper-treated single-dimensional chromatograms were run with *m*-cresol as solvent, since no copper complexes are formed when *n*-butanol+acetic acid is used. The results obtained with *Sh. paradysenteriae* are shown in Pl. 2, fig. 3; it can be seen that γ -aminobutyric acid and the unknown material are the only substances to run on the chromatogram and their positions are identical. Similar results are obtained with *Esch. coli*. This evidence, together with that already given, can be taken as reasonable proof that the material produced by *Sh. paradysenteriae* and *Esch. coli* is γ -aminobutyric acid.

As the production of γ -aminobutyric acid occurs only at acid pH it seemed likely that it arose by decarboxylation of glutamic acid (Gale, 1940). This was shown to be the case, by the examination of filtrates from washed organisms of *Sh. paradysenteriae* and *Esch. coli* suspended in a 1 % solution of glutamic acid and incubated for 7 days at pH 4.5. In most cases the glutamic acid had completely disappeared or was much decreased, whilst γ -aminobutyric acid appeared.

The production of α - and γ -aminobutyric acids and δ -aminopentanoic acid by Clostridium bifermentans, Cl. sordellii and Cl. sporogenes

We have previously shown (Proom & Woiwod, 1949) that culture filtrates from all the strains of *Cl. bifermentans* and *Cl. sordellii* showed an increase in the strength of the valine/methionine spot and the appearance of a strong spot just before tyrosine on the single-dimensional chromatogram with *n*-butanol+acetic acid as solvent. We now find that this effect is also given by filtrates from *Cl. sporogenes* but not by filtrates of any of the other Clostridia examined, i.e. *Cl. perfringens*, *Cl. novyi*, *Cl. histolyticum*, *Cl. tetani*, *Cl. haemolyticum*, *Cl. septicum*, *Cl. capitovale*, *Cl. butyricum*, *Cl. sphenoides* and *Cl. tetanomorphum*.

The intensity of these spots from *Cl. bifermentans* and *Cl. sordellii* was inversely related to the intensity of the proline spot. In view of our experience with *Sh. paradysenteriae* it seemed worth while to consider possible mechanisms

by which ninhydrin-positive substances could be produced from proline. One such mechanism would be the opening of the proline ring and subsequent reduction to give δ -aminopentanoic acid as described by Stickland (1935) for *Cl. sporogenes*. The spot appearing in the valine position was identified as δ -aminopentanoic acid by comparing its position with an authentic sample on a single-dimensional chromatogram using *n*-butanol+acetic acid as solvent, and on a two-dimensional chromatogram developed by *n*-butanol+acetic acid followed by *m*-cresol (Pl. 2, fig. 4). Final proof was obtained by running two-dimensional chromatograms on paper treated with copper acetate before running with the second solvent (*m*-cresol). The suspected δ -aminopentanoic acid appeared in its usual position, being, like γ -aminobutyric acid, unable to form a copper complex. These experiments satisfactorily explained the relationship with proline of one of the ninhydrin-positive metabolic products seen on the chromatogram, but still left the strong spot near tyrosine unidentified. Two-dimensional chromatography of an eluate of this material revealed two substances present which from their positions appeared most likely to be α - and γ -aminobutyric acids. This was confirmed by running artificial mixtures of these substances in *n*-butanol+acetic acid followed by *m*-cresol with alanine, valine and proline as markers. An exact match was obtained. The γ -aminobutyric may have arisen from glutamic acid decarboxylase activity which would be relatively weak at the pH of the culture filtrate (c. pH 8.0). The mechanism of the α -aminobutyric acid formation is at present unknown.

The production of arginine by Serratia marcescens

When culture filtrates of *Ser. marcescens* were examined by paper chromatography a marked increase in the intensity of the ninhydrin reaction in the region of the basic amino-acids lysine, histidine and arginine was observed. Elution and chromatography of the material responsible for this failed to reveal more than one substance present, nor were any new spots observed after acid-hydrolysis. The material was not histidine or lysine, from which it could be separated by two-dimensional chromatography. From its position it appeared to be arginine and this was proved by two-dimensional chromatography using lysine and glycine as markers and a sample of synthetic arginine for comparison, and also by the fact that it gave a strong Sakaguchi reaction. The magnitude of the increase of arginine was not determined, and the mechanism of its formation is at present unknown.

The production of α -aminobutyric acid by Staphylococcus aureus

Culture filtrates of *Staph. aureus* showed a spot moving slightly slower than tyrosine on chromatography in *n*-butanol+acetic acid (Proom & Woiwod, 1949). This spot was identified as α -aminobutyric acid by two-dimensional chromatography of the eluate from single-dimensional chromatograms. This amino-acid has also been demonstrated inside the cells of this species by Dr B. A. Fry (personal communication) who washed the organisms, disrupted the cells in boiling-water and chromatographed the supernatant after centrifuging-off the cell debris. As with the anaerobic organisms, where we found

this amino-acid in the culture filtrates, it is not yet known how α -aminobutyric acid is formed. When washed suspensions of staphylococci were incubated with threonine and chromatographed, ninhydrin-positive material was observed which appeared to be α -aminobutyric acid. It is possible, therefore, that this acid is formed by removal of the hydroxyl group from threonine.

DISCUSSION

A number of the ninhydrin-positive materials seen on paper chromatograms of culture filtrates, and identified in the present work, have already been demonstrated by other techniques. The production of γ -aminobutyric acid by *Esch. coli* and *Sh. paradysenteriae* are examples of the specific decarboxylation of glutamic acid demonstrated by Gale (1940). Similarly, the production of δ -aminopentanoic acid by anaerobic organisms was demonstrated for *Cl. sporogenes* by Stickland (1935).

The production of polypeptides by *Cl. tetani* and *Pr. vulgaris* and of arginine by *Ser. marcesens* does not appear to have been observed previously. It is interesting to speculate whether the production of similar polypeptides by two such dissimilar organisms as *Cl. tetani* and *Pr. vulgaris* comes by decomposition of bacterial protein by similar proteolytic enzymes or whether these materials are first synthesized and then excreted by the organism.

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Fig. 1

Fig. 2

A. J. WOIWOD & H. PROOM—IDENTIFICATION OF CHARACTERISTIC EXTRACELLULAR NINHYDRIN-POSITIVE SUBSTANCES PRODUCED BY SOME BACTERIA. PLATE 1

Fig. 3

Fig. 4

A. J. WOIWOD & H. PROOM—IDENTIFICATION OF CHARACTERISTIC EXTRACELLULAR NINHYDRIN-POSITIVE SUBSTANCES PRODUCED BY SOME BACTERIA. PLATE 2

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Single-dimensional chromatograms of peptides isolated from culture filtrates of *Pr. vulgaris* and *Cl. tetani*, before and after acid hydrolysis. 1, 5 and 9, *Pr. vulgaris* culture filtrate; 2, fast-moving peptide, unhydrolysed (*Pr. vulgaris*); 3, slow-moving peptide, unhydrolysed (*Pr. vulgaris*); 4, paper blank, unhydrolysed; 6, fast-moving peptide, hydrolysed (*Pr. vulgaris*); 7, slow-moving peptide, hydrolysed (*Pr. vulgaris*); 8, paper blank, hydrolysed; 13, 17, *Cl. tetani* culture filtrate; 10, slow-moving peptide, unhydrolysed (*Cl. tetani*); 11, fast-moving peptide, unhydrolysed (*Cl. tetani*); 12, paper blank, unhydrolysed; 14, slow-moving peptide, hydrolysed (*Cl. tetani*); 15, fast-moving peptide, hydrolysed (*Cl. tetani*); 16, paper blank, hydrolysed. A, lysine, histidine, arginine; B, aspartic acid; C, glycine, serine; D, glutamic acid, threonine; E, alanine; F, proline; G, tyrosine; H, methionine, valine; J, phenylalanine; K, leucine, isoleucine; L, slow-moving peptide; M, fast-moving peptide.

Fig. 2. Two-dimensional chromatograms of material isolated from a culture filtrate of *Sh. paradysenteriae* compared with synthetic γ -aminobutyric acid. Solvents: horizontal, right to left, *n*-butanol+acetic acid; vertical, *m*-cresol.

PLATE 2

Fig. 3. Confirmation of γ -aminobutyric acid in culture filtrates of *Sh. paradysenteriae* by means of single-dimensional chromatograms run on filter-paper with and without copper treatment. 1, 5, 6 and 10, uninoculated medium; 2, 7, valine; 3, 8, synthetic γ -aminobutyric acid; 4, 9, *Sh. paradysenteriae* culture filtrate. Solvent, *m*-cresol.

Fig. 4. Two-dimensional chromatograms of material isolated from culture filtrate of *Cl. bifementans* compared with a synthetic sample of δ -aminopentanoic acid. Solvents: horizontal, right to left, *n*-butanol+acetic acid; vertical, benzyl alcohol.

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