CHROMATOGRAPHIC INVESTIGATIONS OF AMINO ACIDS FROM MICRO-ORGANISMS

I. THE AMINO ACIDS OF *CORYNEBACTERIUM DIPHTHERIAE*

by

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Study of the biochemistry of micro-organisms has revealed an essential similarity in general chemical composition and in enzymic processes between unicellular organisms and multicellular higher animals. The amino acid content of bacterial cells and their protein has, however, been relatively little studied owing to technical limitations. These limitations have now been largely overcome by the introduction of paper partition chromatography by CONSDEN, GORDON, AND MARTIN (1944), a technique which enables all the amino acids in a few milligrams of any mixture to be characterized and roughly estimated.

The objects of the present study were (a) to characterize the amino acids, both free (soluble) and bound (insoluble), in *C. diphtheriae*, (b) to determine the gross effect of certain variations in the culture medium on the bound amino acid content of the cells and (c) to investigate changes in the amino acids of synthetic media caused by growth therein of *C. diphtheriae*.

Relatively few publications are available recording complete amino acid analyses of bacteria. Early figures for the arginine, histidine, lysine and cystine contents of various bacterial species have been summarized by PORTER (1946) and by CAMIEN, SALLE, AND DUNN (1945). Only two investigations on the amino acids of *C. diphtheriae* have been reported, in both cases only arginine, histidine, lysine and cystine having been determined. TAMURA (1914) examined a protein fraction from the organisms, and HIRSCH (1931) used whole cells.

More complete amino acid analyses, by microbiological assay, of hydrolysates of whole cells of various microorganisms and reports on effects of change in composition of the medium on the levels of individual amino acids were published by CAMIEN, SALLE, AND DUNN (1945) and by STOKES AND GUNNES (1946). POLSON (1948) investigated by paper partition chromatography the amino acids in hydrolysates of *Escherichia coli*. In all of these studies, results are given for whole cells and no distinction is made between free and bound amino acids. FREELAND AND GALE (1947), GALE (1947), and TAYLOR (1947) gave figures for both the free and the combined arginine, lysine, histidine, tyrosine and glutamic acid of various microorganisms, determined by specific amino acid decarboxylases, and examined the combined amino acids of *E. coli*. and *Aerobacter aerogenes*

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grown on a variety of media. The free amino acids were determined after disruption of the cells in boiling water — subtraction of this figure from the total amino acid in the acid hydrolysate gave the bound amino acid.

In the present study, free amino acids and alcohol-soluble peptides were extracted from washed wet *C. diphtheriae* cells by ethanol. The extracts were examined by two dimensional paper partition chromatography, as were also the amino acids present in hydrolysates of the alcohol-insoluble residues.

Chromatograms were also made of two media of known amino acid composition before and after growth of *C. diphtheriae*. Since the work was done, a similar investigation with single-dimensional chromatograms was reported by LINGGOOD AND WOIWOD (1948), who followed changes in peptone broth and casein hydrolysate after growth of *C. diphtheriae*.

METHODS

C. diphtheriae, P.W. 8 (substrain "Toronto") was used throughout this work. It was cultivated at 34° C for 7 days, either on synthetic media of known composition (Table I) or on casein hydrolysates of known iron content containing 0.1 g of casein nitrogen/100 ml supplemented by 0.15 g glycine/100 ml (HOLT, 1948).

TABLE I

COMPOSITION OF SYNTHETIC MEDIA FOR CULTIVATION OF *C. diphtheriae*

	g/100 ml of medium	
	Medium 1	Medium 2
Tryptophan	0.01	0.01
Glycine	0.05	0.1
Valine	0.05	0.05
Alanine	0	0.05
Leucine	0.05	0.1
Glutamic acid	0.1	0.25
Methionine	0.04	0.04
Tyrosine	0.04	0.04
Arginine	0.03	0.03
Histidine	0.02	0.02
Lysine	0.02	0.02
Aspartic acid	0.05	0.05
Proline	0.06	0.06
Phenylalanine	0.04	0.04
Cystine	0.01	0.01
β-Alanine	0.0005	0.0005
Pimelic acid	0.0005	0.0005
Nicotinic acid	0.001	0.001
NaCl	0.5	0.5
KH_2PO_4	0.2	0.2
$MgSO_4 \cdot 7H_2O$	0.03	0.03
$FeSO_4 \cdot 7H_2O$	0.0004	0.0004
Maltose	0.9	0.9

After harvesting, the cells were washed three times by centrifugation from saline containing 0.85 g. NaCl/100 ml, were extracted 3 or 4 times by standing overnight at room temperature under ethanol, and were then extracted 3 times with acetone. The ethanol extracts were pooled and concentrated *in vacuo* to a small bulk; the suspended fat was removed by ether extraction, and the remaining aqueous solution filtered when necessary.

The cell residues remaining after ethanol and acetone extraction were dried and hydrolysed at 100° C under reflux for 24 h with 6 *N*-HCl; the HCl was removed *in vacuo*, and the solids were dis-

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solved in sufficient water to give a total N content of about 1 mg/ml. Nitrogen was estimated by the standard micro-kjeldahl procedure, one sample of the cells being taken for dry weight determination at the same time.

CHROMATOGRAPHIC TECHNIQUES

With a few exceptions, the techniques and apparatus were the same as those described by DENT (1948). The troughs for holding solvents were either of glass or of polythene tubing. The filter paper used at the beginning of the work was Whatman No. 1, but this was later changed to No. 4, as the solvents travelled faster over No. 4 grade and better separation was obtained in the "collidine" run.

Water-saturated phenol was used as the first solvent, followed by a mixture referred to in this text as "collidine". This mixture was prepared as suggested by Dr. C. E. DENT (unpublished), from equal parts of *sym*-collidine and a commercial mixture of the 2 : 4 and 2 : 5 lutidine isomers. Great care was taken to ensure that the "collidine" mixture was saturated with water when used, a precaution necessitated by the marked variation in composition of the saturated solution caused by small temperature changes in the region of 18° C; an unsaturated solvent produced poor separation of the amino acids. The ninhydrin colour was developed by heating for 5 minutes at 110° C.

The individual amino acids were generally identifiable by their position in relation to other known amino acids; "the pattern of the spots" (DENT, 1948) being the most satisfactory method of characterization. Where any doubt existed, further tests, advocated by DENT (1948), were performed to establish the identity of the amino acid in question. Although already published by DENT, these tests are considered to be of such importance that they will be listed again.

(1) Matching against the suspected amino acid run under identical conditions.

(2) Specific colour reactions — *e.g.*, PAULY, SAKAGUCHI.

(3) Change of reaction of atmosphere surrounding filter paper during solvent runs; particularly useful in testing basic substances whose speed of running in phenol is markedly slowed on changing from a basic to an acidic atmosphere.

(4) Chemical treatment. This may involve acid or alkaline hydrolysis, oxidation with peroxide, or any other suitable treatment before repeating a chromatographic separation of the amino acid or its reaction products.

(5) Change of solvent. This made possible, for example, the separation of the leucine isomers (see Results) which is impossible with the conventional phenol-collidine two-dimensional chromatograms (DENT, 1948).

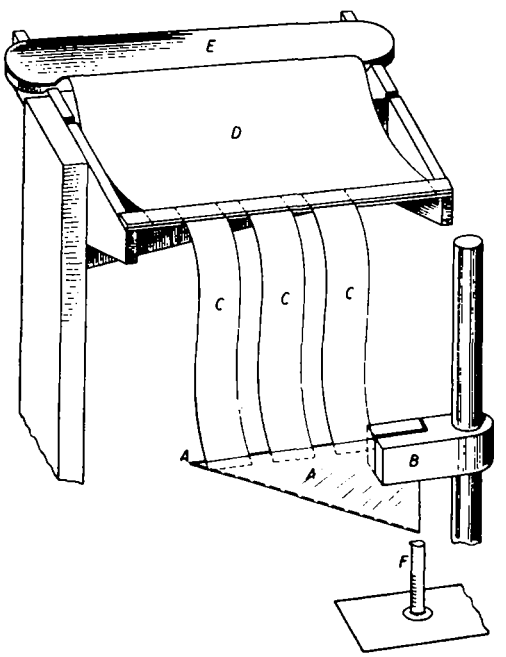

Fig. 1. Diagram of apparatus for washing material off "cuts" from chromatograms on a preparative scale

(6) Isolation. In the case of a completely unknown substance, final characterization will only be achieved after isolation and purification. Provided sufficient quantities of material are available, the substance can be purified by any suitable chemical procedure. Isolation by cutting out and eluting spots or bands from two or one-dimensional chromatograms can be carried out on small quantities of material (DENT, 1947, b AND CONSDEN, GORDON, AND MARTIN, 1947).

In the present study elution was carried out as suggested by CONSDEN, GORDON, AND MARTIN (1947). Sufficient eluate for preparative purposes could be collected by the following arrangement (see Fig. 1). Triangular glass plates A, placed with their right angles uppermost, were stuck to a wooden burette clamp B, so that on closing the clamp the two surfaces made contact. The filter paper strips C, were hung from a sheet of wet filter paper, D, immersed in a trough, E, containing the eluting liquid; their bottom tips being between the two plates, A. The eluting liquid, dripping from the strips, collected between the plates, ran down the inclined edge and finally dripped off the acute angle into a container F. When the elution was completed, the plates were separated by unscrewing the clamp, and the liquid remaining between the plates all ran into the container. It was found that elution proceeded faster if the whole apparatus was placed in a jar saturated with the eluting solvent, but this was not essential.

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ESTIMATION OF AMINO ACIDS

Rough estimations of the amount of amino acids present in a mixture were made by comparing the colour intensity (matched against an arbitrary scale) and size of the spots with those given by known amounts of pure amino acids run under identical conditions. These criteria as employed gave consistent results, with an accuracy of ± 50%, provided amounts of amino acids were chosen which gave sub-maximal colour intensities. Above a certain amount of amino acid, a maximal colour is reached, and comparison of size of spot alone does not give a satisfactory estimation in two dimensional chromatograms. FISHER, PARSONS, AND MORRISON (1948) claim that with one-dimensional strips the area of the spot is proportional to the logarithm of the amino acid content, but this was not found to be the case with two dimensional chromatograms.

RESULTS

SEPARATION OF LEUCINE ISOMERS

Before investigating the amino acids present in *C. diphtheriae*, it was considered essential to establish a satisfactory method of separating the leucine isomers. EDMAN (1945) and WRETLINE (1947) employed a pyridin-amyl alcohol-water mixture instead of collidine, but obtained poor separation of the isomers. Benzyl alcohol-water and n-butanol-water solvents have been used by CONSDEN, GORDON, AND MARTIN (1944) and by CONSDEN, GORDON, MARTIN, AND ROSENHEIM (1945) in one dimensional chromatograms, but in this laboratory the separation produced by these solvents was not considered satisfactory.

Amyl alcohol-water gave better separation, although all the Rf values were lower than those given by CONSDEN, GORDON, AND MARTIN (1944). The actual distance run by the amino acid down the paper was increased by allowing the solvent to run for several days and to drip from the bottom of the paper. By this means commercial amyl alcohol produced satisfactory separation of leucine, isoleucine and phenylalanine (Table II). However, since the results could not be repeated with different samples of solvent, and since commercial amyl alcohol consists of an unspecified mixture of the different isomers, it was decided to investigate individual isomers. Table II shows the results with 4 isomers, either in the absence or the presence of diethylamine vapour. The presence of this substance was found to increase the distance run by phenylalanine

TABLE II

SEPARATION OF AMINO ACIDS IN AMYL ALCOHOL-WATER ON ONE-DIMENSIONAL FILTER PAPER CHROMATOGRAMS. SOLVENT ALLOWED TO RUN OVER END OF PAPER. (NUMBERS INDICATE DISTANCE TRAVELLED RELATIVE TO LEUCINE)

Amyl alcohol used	Diethylamine vapour	Position of							Shape of spots
		leucine	iso-leucine	nor-leucine	phenyl-alanine	valine	methionine	tyrosine	
Commercial	—	1	0.81		0.46		0.64		Variable, sometimes v. elongated
Normal	—	1	0.86		0.77	0.42	0.52	0.37	Oval
Normal	+	1	0.85		1	0.52	0.52	0.29	Oval
Iso-	—	1	0.82		0.8	0.36	0.53	0.44	Elongated
Iso-	+	1	0.8		0.93	0.39	0.39	0.24	Elongated
Active	—	1	0.84		0.84		0.43		Oval
Tertiary	—	1	0.85		1	0.48	0.43	0.48	Round
Tertiary	+	1	0.86	1.2	1.11	0.51	0.65	0.45	Round

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and methionine, allowing them also to be separated. The correct amount of diethylamine must be determined for each individual apparatus, as an excess causes a general purple background to appear on development of the ninhydrin colour. Considerable variation in the shape of the spots was found with different amyl alcohols, and the final choice, tertiary amyl alcohol, was selected because it produced the least elongated spots and the best separation. By this means, the leucine isomers, phenylalanine, methionine and valine could be separated and identified in a complex amino acid mixture (*e.g.*, protein hydrolysate), since all the other naturally-occurring acids ran considerably more slowly along the paper (Fig. 2). The Rf values for tyrosine, the amino acid with the next highest speed of running are included in Table II. Fig. 3 shows results obtained from hydrolysates of insoluble cell residues of *C. diphtheriae*.

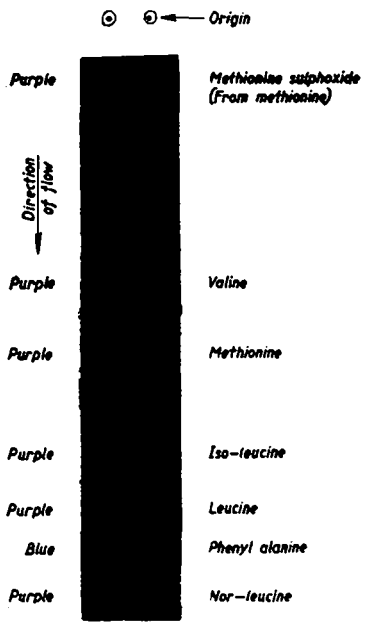

Fig. 2. Amino acid mixtures run on one-dimensional chromatogram for 3 days; solvent, tertiary amyl alcohol-water + diethylamine vapour

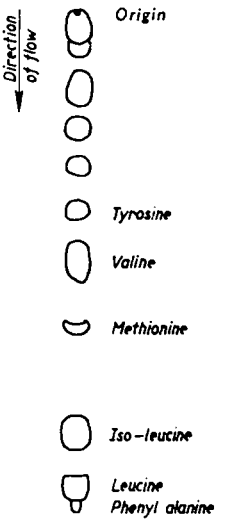

Fig. 3. Hydrolysed ethanol-insoluble residues of *C. diphtheriae* (32 μg N) run on one-dimensional chromatogram for 3 days. Solvent, tertiary amyl alcohol-water + diethylamine vapour

INSOLUBLE CELL RESIDUES OF *C. diphtheriae*

Cells of *C. diphtheriae* were grown in 3 different media, which are described in the Method section. The amino acids identified in the acid hydrolysates of the residues remaining after alcohol and acetone extraction of the specimens examined, are listed in Table III, in which a rough quantitative comparison is included. Fig. 4 shows diagramatically a typical square obtained from an amount of hydrolysate equivalent to 48 μg N.

Although the method of estimation is far from accurate, it shows the constancy in amino acid composition of the cells grown on various media. This constancy was even

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TABLE III

AMINO ACIDS FOUND IN HYDROLYSATES FROM INSOLUBLE CELL RESIDUES OF *C. diphtheriae*

(Figures show approximate concentration of amino acids, expressed as amino acid N × 100/total N. – indicates that no estimation has been made.)

Amino acid	*C. diphtheriae* grown on		
	Casein hydrolysate 0.14 μg Fe/ml	Casein hydrolysate 1.4 μg Fe/ml	Synthetic medium 2 1.4 μg Fe/ml
Aspartic acid	4	4	5
Glutamic acid	9	8	8
Serine	2	2	3
Glycine	5	5	5
Threonine	1	1	1
Alanine	8	8	8
Valine	7	7	7
Leucine	4	4	4
Isoleucine	4	5	4
Phenylalanine	3	3	3
Cystine	–	–	–
Methionine	3	–	–
Tyrosine	2	2	2
Histidine	4	–	–
Proline	5	5	5
Hydroxyproline	1	1	1
Arginine	10	10	10
Lysine	5	–	5
Total N content, percent dry weight	12.9	13.6	7.1

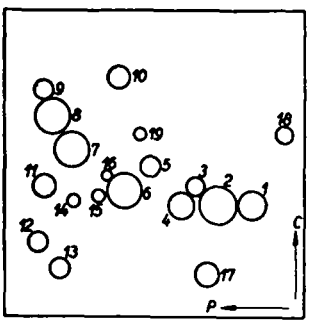

Fig. 4. Acid hydrolysate of insoluble residues of *C. diphtheriae* (48 μg N) run on two dimensional chromatogram. Solvents, phenol NH_3 + collidine. 1. aspartic acid, 2. glutamic acid, 3. serine, 4. glycine, 5. threonine, 6. alanine, 7. valine, 8. leucine and methionine, 9. phenylalanine, 10. tyrosine, 11. proline, 12. arginine, 13. lysine, 14. methionine sulphoxide, 15. histidine, 16. hydroxyproline, 17. unidentified, 18. cysteic acid, 19. unidentified

better appreciated in the comparison of individual squares, which showed remarkable similarity in colour-depth and size of amino acid spots from hydrolysates of different cell residues, taking total nitrogen content as the basis for comparison (12, 18, 32, and 120 μg N).

Besides the known amino acids, an unknown purple spot (17) was consistently found in all fresh hydrolysates in a position corresponding very closely to that of ethanolamine phosphoric acid in the "map of spots" published by DENT (1948). This spot from *C. diphtheriae* has not yet been identified; it is unchanged in position after treatment with alkaline phosphatase in contrast to ethanolamine phosphoric acid; it is also unaffected by hydrogen peroxide and is thus not one of the sulphur-containing amino acids such as cystine, lanthionine or cystathione which are located in about the same position on the square.

A brown spot (19) turning purple on standing was not identified as any known substance; its speed of running was unchanged by using an acid atmosphere, so that it could not have been a basic substance such as glucosamine (DENT, 1948). It did not appear consistently and has not been further investigated.

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ETHANOL EXTRACTS OF *C. diphtheriae*

Fig. 5 shows the spots found in squares obtained from an amount of the ethanol extract of *C. diphtheriae* corresponding to 120 μg N. With the exception of hydroxyproline and histidine, all the known amino acids found in the insoluble cell residues were identified in the ethanol extracts. These extracts also gave, in addition, spots corresponding to α-amino butyric acid (21), γ-amino butyric acid (22), hydroxylysine (20), β-alanine (25) and an unidentified purple spot (23). Hydrolysis of the extracts with 6*N*-HCl produced no change in position of any of these spots, nor was there any gross change in their colour-strength or size.

These substances found in the ethanolic extracts but not in the cell residue hydrolysates were all present in low concentration, and were only visible when the chromatogram was overloaded with the more commonly occurring amino acids; for the sake of clarity Fig. 5 does not depict this overloading.

In acid-hydrolysed ethanol extracts, the material responsible for the unidentified spot (23) changed its position on the square to 23A (Fig. 5B) when the phenol run was

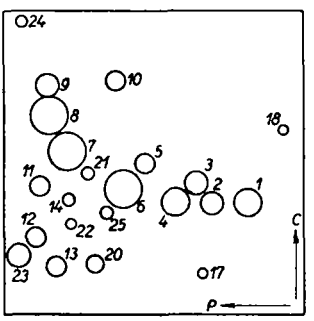

Fig. 5A

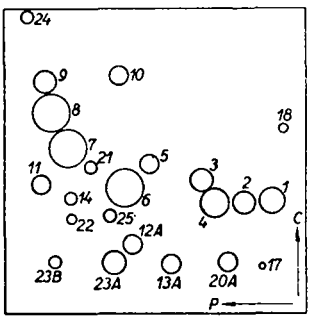

Fig. 5B

Fig. 5. Ethanol extract of *C. diphtheriae* run on two-dimensional chromatograms; solvents: A, phenol-NH_3 and collidine; B, phenol-acetic acid and collidine. No difference was found between chromatograms of hydrolysed or non-hydrolysed extracts, with the exception of spot (23) which gave 23 A and B in phenol-acetic acid before hydrolysis and 23A after hydrolysis. 1 aspartic acid, 2 glutamic acid, 3 serine, 4 glycine, 5 threonine, 6 alanine, 7 valine, 8 leucines and methionine, 9 phenylalanine, 10 tyrosine, 11 proline, 12 arginine (NH_3), 12A arginine (acetic acid), 13 lysine (NH_3), 13A lysine (acetic acid), 14 methionine sulphoxide, 17 unidentified, 18 cysteic acid, 20 hydroxylysine (NH_3), 20A hydroxylysine (acetic acid), 21 α-aminobutyric acid, 22 γ-aminobutyric acid, 23 unidentified bases (NH_3), 23A and B unidentified bases (acetic acid), 24 unidentified, 25 β-alanine

carried out in an acetic acid atmosphere. This slowing by acid was similar to that shown by the basic amino acids arginine, lysine and hydroxylysine, whose acid positions are also shown in Fig. 5B, and it suggests that the unknown material is basic. In this respect the material differs from "fast arginine" (DENT, 1948) which gave a similar spot in phenol/ammonia but was less slowed by acid. The unhydrolysed ethanol extracts from *C. diphtheriae*, which gave spot 23 in phenol/ammonia, produced two spots in phenol/acetic acid, one in position 23A, the other (23B) corresponding to DENT's "fast arginine" (Fig. 5B). Spot 23 gave negative colour reactions with the PAULY or SAKAGUCHI reagents or with EHRLICH's p-dimethylaminobenzaldehyde reagent. The spot obtained after 6*N*-HCl hydrolysis appeared unchanged after additional hydrolysis for 24 h at 100° C with 12 *N*-HCl or with N-ammonia. It was also unaffected by hydrogen peroxide treatment. Further investigations are in progress.

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The supernatant liquid obtained from a large batch of toluene-treated *C. diphtheriae* cells which had stood at room temperature for several months presented similar squares. A yellow-brown spot (24) was also found in squares from this liquid or from its hydrolysate, but has not been further investigated.

CHANGES IN SYNTHETIC MEDIA FOLLOWING GROWTH OF *C. diphtheriae*

Attempts to find a suitable synthetic medium for growth of *C. diphtheriae* had produced a medium (medium 1, see Methods) which maintained only poor growth. Changes

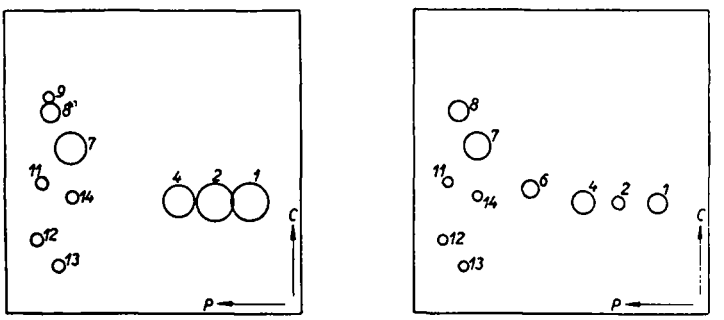

Fig. 6A Fig. 6B

Fig. 6. Synthetic medium I (15 μl). A before growth, B after growth, of *C. diphtheriae*. Run on two-dimensional chromatogram. Solvents, phenol-NH_3, collidine. 1 aspartic acid, 2 glutamic acid, 4 glycine, 6 alanine, 7 valine, 8 leucine and methionine, 9 phenylalanine, 11 proline, 12 arginine, 13 lysine, 14 methionine sulphoxide

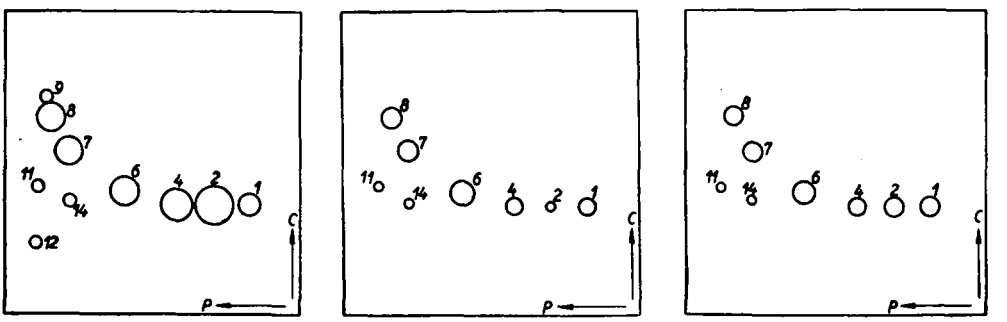

Fig. 7A Fig. 7B Fig. 7C

Fig. 7. Synthetic medium II (10 μl). A before growth, B after growth of *C. diphtheriae*, C after growth hydrolysed 6 N-HCl. Solvents and numbers as in Fig. 6

in amino acid content caused by growth of the organism were followed by comparing chromatograms made from equal volumes (15 μl) of the medium before and after growth. There was, as expected, a general fall in concentration of all amino acids, but aspartic acid and particularly glutamic acid were preferentially removed during growth of the organism (see Fig. 6). Alanine appeared in the culture filtrate, although not originally present in the medium.

Following this result, the basal medium was changed by alteration of the proportion of various amino acids, notably by addition of alanine and of more glutamic acid

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(medium 2). Better growth of the organism was maintained, and the chromatograms obtained from 10 μl (Fig. 7 A and B) show that the drop in glutamic acid was even more striking than in the case of medium 1. Aspartic acid was relatively unchanged, but glycine showed a considerable fall in strength, while alanine in this case showed a slight fall. Acid hydrolysis of a sample of the culture filtrate (Fig. 7C) produced a slight rise in the strength of the glutamic acid spot, but the spot remained far from its original size and intensity, showing that all the glutamic acid lost during growth could not be accounted for as glutamine or any other hydrolysable derivatives still present in the medium. Application of larger amounts (90 μl) of culture filtrate to squares did not show the presence of any ninhydrin-reacting substances other than those composing the medium.

DISCUSSION

Changes in media following the growth of *C. diphtheriae* have been investigated by LINGGOOD AND WOIWOD (1948). Using one-dimensional chromatograms, they obtained results with casein hydrolysate essentially similar to those reported in this paper with synthetic medium 1 — namely, preferential utilization of aspartic acid and glutamic acid and appearance of alanine. The glutamic acid content of this synthetic medium was of the same order as that of the casein hydrolysate used by LINGGOOD AND WOIWOD, but alanine was not present. Raising the glutamic acid level and adding alanine in amount approximately double that present in the casein hydrolysate, resulted in much improved bacterial growth. Some alanine was used up, aspartic acid showed a normal drop in concentration but even more glutamic acid was used than previously. At present it is not possible to say which variation in composition of the modified medium was responsible for the improved growth, nor is it possible to account for the assimilation of such comparatively large amounts of glutamic acid. In medium 1, glutamic acid might have been converted to alanine by transamination, but as sufficient alanine appeared to be present in the modified medium, this explanation is unlikely. The glutamic acid content of the cell residues (8% of total N as glutamic acid N) is certainly higher than that reported for other bacteria by FREELAND AND GALE (1947) whose average value for combined glutamic acid N of 9 types of bacteria was 5.3% of total N. The amount of free glutamic acid in the internal environment (as shown by chromatographic analysis of the ethanol extracts) was not unduly high, and showed no indication of a process of concentration such as was reported by TAYLOR (1947) for Gram-positive organisms. GALE AND MITCHELL (1947), during their studies on glutamic acid assimilation of *Strep. faecalis*, found a disappearance of glutamic acid from the internal environment of the organism which could not be accounted for by protein or peptide synthesis. Possibly glutamic acid is involved in some metabolic processes as yet unknown.

The rough composition put forward for the insoluble cell residues presents nothing of particular interest, except for the relatively high glutamic acid content. Possibly the arginine content (10% of total N as arginine N) should also be commented on in the light of the findings of FREELAND AND GALE (1947), who suggest that Gram-positive organisms have bound arginine levels of approximately half this figure; the average value for Gram-negative organisms is, on the other hand, the same as that found for *C. diphtheriae*. This organism does not exhibit many of the characteristic properties of Gram-positive organisms, and has in fact been classified by DUBOS (1945) as Gram-variable.

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Comparison of these figures with those previously published for *C. diphtheriae* is difficult. The values given by HIRSCH (1931) were determined on whole cells and show no agreement with the present findings (Table IV). This is not surprising; the figures of FREELAND AND GALE (1947) for 6 micro-organisms show very variable agreement between the total amino acid contents estimated on hydrolysates of whole cells and the figures corrected for free amino acids in the cells. The analyses given by TAMURA (1914) certainly do not refer to whole cells; they were made on the insoluble residue remaining after extraction with alcohol and acid.

The method of estimation used is admittedly rough, and the results are only presented as the nearest whole number; the constancy in the amino acid composition obtained in cells grown on various media is however worthy of note. The media were

TABLE IV

COMPARISON OF LEVELS OF ARGININE, LYSINE AND HISTIDINE FOUND IN *C. diphtheriae* BY PARTITION CHROMATOGRAPHY WITH THOSE GIVEN BY HIRSCH (1931) AND TAMURA (1914)

	% of total N		
	By chromatography	HIRSCH	TAMURA
Arginine N	10	15.9	10.6
Lysine N	5	9.7	4.9
Histidine N	4	6.0	1.0

chosen according to their ability to cause toxin formation by the organism. The casein hydrolysate containing 1.4 μg of iron/ml, produces maximum growth of the organism, with no toxin formation. Reduction of the iron content by 1/10th causes the organism to excrete large amounts of toxin into the medium — at the same time coproporphyrin is also excreted, and there occurs a fall in the intracellular haem pigments, notably of cytochrome b (PAPPENHEIMER AND HENDEE, 1947; RAWLINSON AND HALE, 1949). PAPPENHEIMER AND HENDEE suggested that the toxin is a portion of the cytochrome molecule and, in the absence of sufficient iron for cytochrome formation, both toxin and porphyrin are excreted. Preliminary investigations on a sample of partially purified toxin (kindly provided by Dr. G. R. TRISTRAM) show that its amino acid composition is quite different from that of the insoluble cell residues. The loss by the cell of large quantities of a highly specific protein in the form of toxin might be expected to be reflected in a considerable change in amino-acid composition of the residual protein, but such was not the case. Changing the source of amino acids from a casein hydrolysate to a synthetic mixture of amino acids, present in different proportions from those of the casein hydrolysate, also made no difference to the amino acid composition of the cell residues. These results conform with those of other authors, who find no difference in the amino acid composition of bacterial cells grown on different media (CAMIEN, SALLE, AND DUNN, 1945; STOKES AND GUNNESS, 1946; FREELAND AND GALE, 1947).

The ethanol extracts of *C. diphtheriae* contain 6 ninhydrin-reacting substances not present in the cell proteins. Four of these are known amino acids, of which two, α- and γ-amino butyric acids, were found by DENT (1947 a and b; 1948) in plant extracts and in fluids such as blood and urine, but they have not been found in any protein. A spot corresponding to α-amino butyric acid was found by POLSON (1948) in hydroly-

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sates of whole *E. coli;* ACKERMANN AND KIRBY (1948) have not agreed with its identity and found that it behaved like pantonine.

No evidence has been obtained as to the identity of the other ninhydrin-reacting substances. The basic material mainly responsible for spot (23) is not identical with the basic substance "fast arginine" found by DENT (1948) in pathological blood and urines, as it is slowed to a greater extent in phenol/acetic acid than is DENT's material. Many basic substances, including cadaverine, give spots in approximately this position when run in phenol-ammonia. In the original unhydrolysed alcoholic extracts there was evidently a mixture of basic substances, one of which was similar to "fast arginine", but this disappeared on hydrolysis.

The presence of unidentified ninhydrin-reacting substances in both the ethanol extracts and insoluble cell residues of *C. diphtheriae* is not surprising. The discovery of hitherto unknown amino acids in various antibiotics has shown that microorganisms can produce a variety of amino acids combined in various forms and not usually known to be present in proteins.

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SUMMARY

The approximate amino-acid composition of hydrolysates of ethanol-extracted *C. diphtheriae* cells was determined by paper partition chromatography.

Change of culture medium had no effect on the composition of the cell residues.

A new single dimensional technique for separation of the leucine isomers is described.

Two unidentified ninhydrin reacting substances were found in hydrolysates from the insoluble cell residues.

Ethanol extracts from the organisms contained most of the known amino acids found in the insoluble residues, but in addition contained α-amino butyric acid, γ-amino butyric acid, hydroxylysine, β-alanine and two unidentified acid-stable ninhydrin-reacting substances.

RÉSUMÉ

La composition approximative en acides aminés d'hydrolysates de cellules de *C. diphteriae* extraites à l'alcool a été déterminée par chromatographie de partage sur papier. Le changement de la nature du milieu de culture n'a pas d'influence sur la composition des résidus cellulaires. Une nouvelle technique de séparation unidimensionelle des isomères de la leucine est décrite. Deux substances réagissant avec la ninhydrine et non encore identifiées ont été trouvées dans les hydrolysats des cellules extraites à l'éthanol.

Les extraits éthanoliques contiennent la plupart des acides aminés connus que l'on rencontre dans la partie insoluble, mais renferment en outre l'acide α-aminobutyrique, l'acide γ-aminobutyrique, l'hydroxylysine, β-alanine et deux substances stables vis-à-vis des acides réagissant à la ninhydrine et encore non identifiées.

ZUSAMMENFASSUNG

Zellen von *C. diphtheriae* wurden mit Äthanol extrahiert, hydrolysiert und die Zusammensetzung des Hydrolysates annähernd durch "Partition Chromatography" auf Papier bestimmt.

Eine Veränderung der Nährlösung hatte keinen Einfluss auf die Zusammensetzung der Zellreste.

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Eine neue eindimensionale Arbeitsweise zur Trennung der Isomeren des Leucins wurde beschrieben.

In Hydrolysaten von unlöslichen Zellresten wurden zwei Substanzen gefunden, die mit Ninhydrin reagieren, aber bis jetzt nicht identifiziert wurden.

Die Äthanolauszüge der Organismen enthielten die meisten der bekannten Aminosäuren, die auch in den unlöslichen Rückständen gefunden worden waren, ausserdem aber noch α-Aminobuttersäure, γ-Aminobuttersäure, Hydroxylysin, β-Alanin und zwei säurebeständige, mit Ninhydrin reagierende Substanzen, die nicht identifiziert wurden.

REFERENCES

W. W. ACKERMANN AND H. KIRBY, *J. Biol. Chem.*, 175 (1948) 483.
M. N. CAMIEN, A. J. SALLE, AND M. S. DUNN, *Arch. Biochem.*, 8 (1945) 67.
R. CONSDEN, A. H. GORDON, AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
R. CONSDEN, A. H. GORDON, AND A. J. P. MARTIN, *Biochem. J.*, 41 (1947) 590.
R. CONSDEN, A. H. GORDON, A. J. P. MARTIN, O. ROSENHEIM, AND R. L. M. SYNGE, *Biochem. J.*, 39 (1945) 251.
C. E. DENT, *Science*, 105 (1947, a) 335.
C. E. DENT, *Biochem. J.*, 41 (1947, b) 240.
C. E. DENT, *Biochem. J.*, 43 (1948) 169.
R. DUBOS, *The bacterial cell*, Harvard Univ. Press., U.S.A. (1945)
P. EDMAN, *Arkiv. Kemi Mineral, Geol.*, 22 (1945) A. 3. 1.
R. B. FISHER, D. S. PARSONS, AND G. A. MORRISON, *Nature*, 161 (1948) 764.
J. C. FREELAND AND E. F. GALE, *Biochem. J.*, 41 (1947) 135.
E. F. GALE, *J. Gen. Microbiol.*, 1 (1947) 53.
E. F. GALE AND P. D. MITCHELL, *J. Gen. Microbiol.*, 1 (1947) 299.
E. F. GALE AND E. S. TAYLOR, *J. Gen. Microbiol.*, 1 (1947) 314.
J. HIRSCH, *Z. Hyg. Infektionskr.*, 112 (1931) 660.
L. B. HOLT, *Brit. J. Exptl Path.*, 29 (1948) 335.
F. V. LINGGOOD AND A. J. WOIWOD, *Brit. J. Exptl Path.*, 29 (1948) 283.
A. M. PAPPENHEIMER AND E. D. HENDEE, *J. Biol. Chem.*, 171 (1947) 701.
A. POLSON, *Nature*, Lond., 161 (1948) 351.
J. R. PORTER, *Bacterial Chemistry and Physiology*, Wiley, N.Y. (1946),
W. A. RAWLINSON AND J. H. HALE, *Biochem. J.* (1949) (In press).
J. L. STOKES AND M. GUNNES, *J. Bact.*, 52 (1946) 195.
S. TAMURA, *Z. physiol. Chem.*, 89 (1914) 289.
E. S. TAYLOR, *J. Gen. Microbiol.*, 1 (1947) 86.
K. A. J. WRETLIND, *Acta Physiol. Scand.*, 13 (1947) 45.

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