CHROMATOGRAPHIC INVESTIGATIONS OF AMINO ACIDS FROM MICRO-ORGANISMS

II. ISOLATION OF TWO UNKNOWN SUBSTANCES FROM *CORYNEBACTERIUM DIPHTHERIAE*

by

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Following an investigation on the amino acids present in *C. diphtheriae*, the presence of two unknown ninhydrin-reacting substances was reported WORK[1, 2]. One of these, a basic substance, was found in relatively low concentrations in ethanol extracts of the cells; the other, possibly a neutral α-amino acid, occurred in the insoluble cell residues in approximately the same concentration as the other amino acids; it was also present, to a lesser extent, in ethanol extracts from the cells. The neutral material has since been found by other workers in chromatograms from materials of bacterial origin (see footnote on this page and the paper by ASSELINEAU, CHOUCROUN AND LEDERER[3]). The following communication is an interim report on the properties of the two materials from *C. diphtheriae*, published now in order to accompany the paper of ASSELINEAU *et al.*, the manuscript of which Dr LEDERER kindly sent me.

RESULTS

Fig. 1 represents the positions, in relation to other amino acids, of the two unknown materials (spots 17 and 23) on two-dimensional chromatograms run in phenol/NH_3 and collidine/lutidine. Solutions containing these materials as the only ninhydrin-reacting

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Kjem. Ing. M. KLUNGSØYR (Sildolje- og Sildemelindustriens Forskningsinstitutt, Bergen, Norway) and Dr R. L. M. SYNGE (Rowett Research Institute, Bucksburn, Aberdeenshire, Scotland) write: "In work at the Rowett Research Institute with rumen contents from sheep, we have frequently observed a weak spot colouring with ninhydrin and corresponding in position with your spot 17 on two-dimensional phenol collidine paper chromatograms. The material responsible was for the most part associated with the sedimentable part of the rumen contents, from which it was not removed by extraction with aqueous ethanol. Acid hydrolysis was required to liberate the material. The spot was not found on chromatography of acid hydrolysates of the food; it was therefore thought that the material is a component of ruminal micro-organisms. On ionophoresis in silica jelly at p_H 6 the material migrated with the neutral amino-acids; admixture of the material isolated by yourself from *C. diphtheriae* caused no new spot on the chromatograms, whereas admixture of ethanolamine phosphoric ester (which would be found in the same region of the chromatograms) caused a new spot to appear, close to the spot being studied. Moreover, on ionophoresis at p_H 6, ethanolamine phosphoric ester migrated with the dicarboxylic amino acids. It is hoped that the new substance may prove useful as a marker of microbial activities in the rumen".

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substances gave negative results when tested by Professor HAPPOLD for growth-promoting activity for an exacting strain of *C. diphtheriae* in concentrations up to 0.005 mg/ml (CHATTAWAY, DOLBY, HALL AND HAPPOLD[4]).

Neutral substance (spot 17)

The source of the neutral substance (spot 17, Fig. 1) was the acid-hydrolysed ethanol-insoluble residue from *C. diphtheriae*. The substance could be obtained free from other amino acids by first removing basic amino acids on ion exchange resins, and then running chromatograms using paper squares or columns with collidine or butyl alcohol/acetic acid as solvents; the slowest running band being the unknown material.

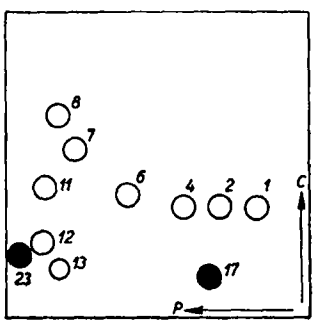

Fig. 1. Two-dimensional chromatogram showing positions of the two unknown ninhydrin-reacting substances 23 and 17, in relation to known amino acids.
Solvents: 1) phenol/NH_3; 2) collidine/lutidine.
1. Aspartic acid; 2. glutamic acid; 4. glycine; 6. alanine; 7. valine; 8. leucine; 11. proline; 12. arginine; 13. lysine; 17. unknown neutral substance; 23. unknown basic substance

Table I gives Rf values of this substance in various solvents together with those of other ninhydrin-reacting substances of similar Rf values. It can be seen that the substance behaves differently chromatographically from all the other known peroxide-stable amino acids and also from ethanolamine phosphoric acid; it has already been reported (WORK[1]), to be stable to hydrogen peroxide and to alkaline phosphatase. The ninhydrin colour of spot 17 resembled, when fresh, the typical purple given by most of the amino acids, but after a few days standing in the dark, the colour changed to a greyish-blue colour which distinguished it from other amino acids.

TABLE I

Rf VALUES OF UNKNOWN NEUTRAL SUBSTANCE AND SOME OTHER NINHYDRIN-REACTING SUBSTANCES

Substance	Phenol-Ammonia Atmosphere	Phenol-Acetic acid Atmosphere	Collidine/Lutidine	Butyl alcohol/acetic acid
Unknown substance	0.25	0.16	0.08	0.10
Aspartic acid	0.16	0.18	0.22	0.24
Glutamic acid	0.28	0.34	0.22	0.38
Glycine	0.37	0.34	0.22	0.34
Aminomalonic acid	0.37	—	0.22	—
α-aminoadipic acid	0.30	—	0.22	—
Diaminoglutaric acid	0.16	—	0.22	—
Ethanolaminephosphoric acid	0.26	0.34	0.08	0.19
Hydroxyglutamic acid	0.23	—	0.19	0.30

The material was unchanged by 48 hours hydrolysis with 20% HCl at 160°C or with 30% $Ba(OH)_2$ at 135°C; it also resisted treatment with alkaline periodate. It could not be detected on chromatograms which had been treated with copper acetate prior to running, thus behaving in a similar manner to α-amino acids (CRUMPLER AND DENT[5]). On electrodialysis it remained with the neutral amino acids and it was not adsorbed

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with aspartic and glutamic acids on small amounts of acid-treated alumina (WIELAND[6]) although with a large excess of alumina it was adsorbed. These properties suggest that the unknown substance is a neutral α-amino acid. It behaved similarly to a sample of Dr LEDERER's material, both in respect to Rf values and in its behaviour to copper.

The most concentrated aqueous solutions of the material yielded crystals, which, after repeated recrystallization, were needle shaped and did not melt up to 305° C. The crystals were identified as the material giving spot 17 on the chromatogram; 20 μg produced a single strong spot 17.

Basic Substance

Preparations containing the unknown basic substance (Fig. 1 (23)) as the only ninhydrin-reacting substance were made from acid-hydrolysed ethanolic extracts of *C. diphtheriae* after a preliminary "cleaning up" by phase separation between phenol and 3*N* HCl. The aqueous phase was subjected to fractional precipitation by phosphotungstic acid in 0.25*N* HCl (VAN SLYKE, HILLER, AND DILLON[7]), the unknown basic substance being precipitated at lower concentrations of phosphotungstic acid than the basic amino acids. The first phosphotungstic acid precipitate was reprecipitated and decomposed by VAN SLYKE's method, and the solution so obtained gave only one spot (23) on chromatograms. The base could be eluted with water from filter paper chromatograms and the eluted material had the same properties as the substance obtained directly by phosphotungstic acid precipitation. A pale orange crystalline picrate (m.p. 235°C) was prepared from the eluate and 25 μg of picrate when run on a chromatogram gave a good ninhydrin spot 23, the picric acid splitting off during the phenol run. Decomposition of the picrate with benzene and dilute HCl enabled the free base to be obtained. The basicity of the substance was confirmed by electrodialysis and by adsorption on alkali-treated ion-exchange resins such as Zeocarb. 215 or Amberlite IRC/50; it could be eluted from the latter with *N*/2 HCl.

The base was stable to treatment on filter paper with copper carbonate (CRUMPLER AND DENT[5]), and is thus not an α-amino acid. Although stable to acid hydrolysis at 100°C (WORK[1, 2]), more rigorous hydrolysis with 20% HCl or 30% $Ba(OH)_2$ produced partial decomposition with appearance of a few amino acids. In view of this fact, the substance may be a stable peptide; CRUMPLER AND DENT did not test the stability of peptides on their copper chromatograms, and a peptide terminating with a non α-amino acid might be resistant.

EXPERIMENTAL

Neutral substance

Dry, alcohol-extracted residues from *C. diphtheriae* were hydrolysed with 8 volumes of 6*N* HCl under reflux for 24 hours; HCl was removed by vacuum distillation, and humin by filtration. The basic amino acids were removed by running the solution through either ammonia-treated Zeocarb. 215 or Amberlite IRC/50 buffered at pH 4.7.

One-dimensional chromatograms were run in collidine on filter paper squares. The position of the slowest running band was located by spraying test strips with ninhydrin; cuts were made in the position of this band and they were eluted with water (WORK[1]). For larger scale preparations, powdered paper pulp (Solca Floc BW Grade 100 mesh) was used in columns after previous washing with boiling *N*/10 acetic acid. The solvent, in this case the butyl alcohol/acetic acid mixture of PARTRIDGE[8], was run through until the percolate contained no ninhydrin-reacting substances; the last fractions contained only the unknown substance, but the greater part of this substance remained on the column and was eluted with water. Eluates from both filter paper sheets and columns showed only spot 17 on paper chromatograms.

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Stability to copper. This treatment, carried out according to the method of CRUMPLER AND DENT[5] consisted in running on pairs of small squares (15 × 15 cm) chromatograms of the substance under investigation together with a number of known amino acids. The control square was untreated, but the other was brushed over with dry copper carbonate in the direction of the phenol run along a line beginning at the spot of application. After running in the usual solvents except that no ammonia was present in the phenol run, spot 17 and those of the control α-amino acids were not found on the copper-treated paper.

Periodate treatment. A solution of the unknown substance, mixed with valine and threonine for control purposes, was treated with an equal volume of 1% periodic acid, made alkaline with NaOH, and allowed to stand for 10 minutes at room temperature. The mixture was desalted (CONSDEN, GORDON, AND MARTIN[9]) and a fraction run on a single-dimensional phenol chromatogram with the untreated mixture as a control. Only threonine was destroyed.

Acid-treated alumina. The crude neutralized hydrolysate of the cells was run through a column of acid-treated alumina, prepared according to WIELAND[6]. If the proportion of alumina to hydrolysate was in the region of 10 g of alumina to an amount of hydrolysate equivalent to 0.3 g dry cells, the percolate was found to contain all the original amino acids (including the unknown substance) with the exception of aspartic and glutamic acids. If, however, the amount of alumina was trebled, the unknown substance was adsorbed with aspartic and glutamic acids while the other amino acids remained in the percolate. Elution with *N*/2 ammonia removed the acidic amino acids and the unknown substance from the column.

Electrodialysis. The apparatus consisted of 4 cells, made from Perspex tubing, separated by parchment membranes previously hardened by soaking in 10% formalin for several hours. Rubber gaskets ensured water-tight joints and each cell was cooled by water. Both electrodes were of platinum foil. The solution to be electrodialysed, for example the crude hydrolysate of the cell residues, was placed in the compartment next to the cathode and maintained at about p_H 5 throughout the experiment. The chloride, usually present in the mixture, migrated to the anode compartment which became too acid to allow the acidic amino acids to enter; these amino acids migrated only to the cell next to the anode. The unknown amino acid was found with the neutral amino acids, mostly in the compartment where the mixture was originally put, but also in the cathode compartment with the basic amino acids from which it could be separated by further electrodialysis.

Basic Substance

Hydrolysates of ethanolic extracts from *C. diphtheriae* were prepared as described (WORK[1]). To a solution of the hydrolysate containing approximately 10 mg of total N per ml sufficient solid phenol was added to produce 2 layers of approximately equal volume. Sufficient HCl was added to the aqueous top layer to bring its normality to 3*N*, and the mixture was shaken well. The phenol layer was shaken with two further batches of 3*N* HCl, and the phenol and HCl were removed from the combined aqueous layers *in vacuo*. Most of the amino acids, with the exception of part of the phenylalanine, valine and leucine, were found in the aqueous layer, which had been considerably purified by this treatment. The residue after vacuum distillation was dissolved in sufficient 0.25 *N* HCl to give a final concentration of total nitrogen of 1 mg/ml. To every 100 ml of solution was added 10.5 ml of a 10% solution of phosphotungstic acid in 0.25 *N* HCl; after standing 2 hours, the precipitate was collected by filtration, washed with acid phosphotungstate solution and dissolved in just enough normal NaOH to give a clear solution. Normal HCl, exactly equivalent to the amount of NaOH present, was used to reprecipitate, and after standing overnight the precipitate was collected by filtration, suspended in 3*N* HCl and stirred for about ¼ hour with a mixture of 125 parts ether, 100 parts amyl alcohol and 5 parts ether (VAN SLYKE, HILLER, AND DILLON[7]). The aqueous layer, after removal of HCl *in vacuo*, gave the single spot 23 when run on a 2-dimensional chromatogram (Fig. 2a). Precipitates were also collected after adding further amounts of acid phosphotungstate to the original filtrate, and were treated in the same way. Chromatograms from successive precipitates, shown in Fig. 2, indicated that with increasing amounts of phosphotungstic acid, the basic amino acids and even non-basic amino acids were precipitated.

One-dimensional chromatograms on filter paper squares were run in phenol with amounts of ammonia in the atmosphere insufficient to allow spot 17 to run to the end as it usually did, so separating it from the phenol-soluble impurities. The eluates of the material, after concentration, showed only the original spot on chromatograms.

Aliquots of the eluates were hydrolysed with 20% HCl at 160° C for 48 hours, or with 30% $Ba(OH)_2$ at 135°C for 72 hours, or 5*N* NaOH for 48 hours at 105°C. After removal of inorganic ions by appropriate methods, the mixtures on chromatography showed some weakening, but not complete disappearance, of spot 23, and also the presence of varying amounts of alanine, glycine, arginine or lysine, glutamic acid and spot 17.

Preparation of picrate. A concentrated solution of the eluted material was treated with an equal volume of saturated sodium picrate solution, a copious crystalline material appeared immediately which could be redissolved by heating. The crystals, recrystallized twice from hot water, melted at 235°C. They were insoluble in all the common organic solvents except acetone.

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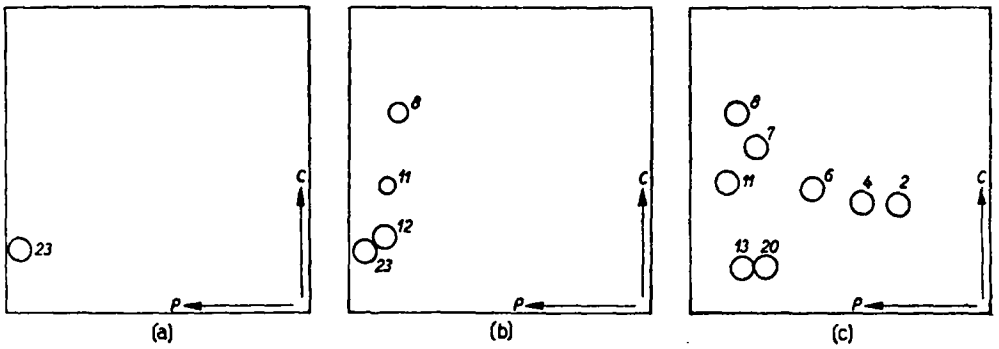

Fig. 2. Two-dimensional chromatograms of decomposed phosphotungstic acid precipitates from hydrolysed alcohol-soluble material from *C. diphtheriae*.
Solvents and numbers as in Fig. 1. 20 = hydroxylysine.
a) Precipitate from 1.05 g phosphotungstic acid per 100 mg N; b) Precipitate from additional 0.5 g phosphotungstic acid per 100 mg N; c) Precipitate from excess phosphotungstic acid

Dinitrophenyl derivative. A solution of the basic substance in 70% ethanol was treated with a mixture of equal weights of fluorodinitrobenzene and sodium bicarbonate (SANGER[10]). A precipitate appeared immediately and was washed with benzene. This precipitate contained a yellow material with Rf 0.61 in phenol/ammonia, in addition to another material (not fluorodinitrobenzene) running the full extent of the phenol run.

Copper treatment. This was carried out as already described; spot 23 which was considerably slowed in phenol by the absence of ammonia (see WORK, 1949[1]) was still present on the chromatogram after copper treatment.

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SUMMARY

1. The preparation of concentrates of two unknown ninhydrin-reacting substances from *C. diphtheriae* is described.

2. One of the substances has properties which suggest it is a neutral α-amino acid. It has been isolated in crystalline form.

3. The second unknown substance, which is basic, may be a peptide which is stable to hydrolysis with 6*N* HCl. It has been isolated as a crystalline picrate.

RÉSUMÉ

1. L'auteur décrit la préparation de concentrés de deux substances inconnues réagissant avec la ninhydrine à partir de *C. diphtheriae*.

2. L'une de ces substances qui a été isolée à l'état crystallisé, semble être, d'après ses propriétés, un acide α-aminé neutre.

3. L'autre substance inconnue, qui est basique, pourrait être un peptide résistant à l'hydrolyse par HCl 6*N*. Elle a été isolé à l'état de picrate cristallin.

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ZUSAMMENFASSUNG

1. Die Herstellung von Konzentraten zweier unbekannter mit Ninhydrin reagierenden Substanzen aus *C. dyphtheriae* wird beschrieben.

2. Die eine dieser Substanzen scheint auf Grund ihrer Eigenschaften eine α-Aminosäure zu sein. Sie wurde in kristallisiertem Zustande isoliert.

3. Die andere unbekannte Substanz ist basisch und könnte ein Peptid sein, dass gegen Hydrolyse mit 6 *N* HCl beständig ist. Sie wurde als kristallisiertes Pikrat isoliert.

REFERENCES

[1] E. WORK, *Biochem. Biophys. Acta*, 3 (1949) 400.
[2] E. WORK, *Bull. soc. chim. biol.*, 31 (1949) 138.
[3] J. ASSELINEAU, N. CHOUCROUN, AND E. LEDERER, *Biochim. Biophys. Acta*, 5 (1950) 197.
[4] F. W. CHATTAWAY, D. E. DOLBY, D. A. HALL, AND F. C. HAPPOLD, *Biochem. J.*, 43 (1948) lix.
[5] H. R. CRUMPLER AND C. E. DENT, *Nature*, 164 (1949) 441.
[6] TH. WIELAND, *Z. physiol. Chem.*, 273 (1942) 24.
[7] D. D. VAN SLYKE, A. HILLER, AND R. T. DILLON, *J. Biol. Chem.*, 146 (1942) 137.
[8] S. M. PARTRIDGE, *Biochem. J.*, 42 (1948) 238.
[9] R. CONSDEN, A. H. GORDEN, AND A. J. P. MARTIN, *Biochem. J.*, 41 (1947) 590.
[10] F. SANGER, *Biochem. J.*, 39 (1945) 507.

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