Some Properties of the Bacitracin Polypeptides

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Newton & Abraham (1950) found that crude bacitracin could be resolved into a series of similar polypeptides by counter-current distribution between solvents. Better resolution and larger amounts of the products have now been obtained by using a greater number of transfers and starting with more concentrated solutions than were used in the earlier experiments. It has been possible, by a single distribution, to prepare the various bacitracins in quantities sufficient for some of their chemical and biological properties to be compared.

Hausmann, Ahrens & Harfenist (1951) and containing 101 tubes. The solvent system consisted of *n*-butanol (4 vol.), amyl alcohol (1 vol.), in equilibrium with 0·05 M-potassium phosphate buffer (5 vol.), at pH 7·0 (Newton & Abraham, 1950). Analysis of the distribution curve (see Experimental) shows that the crude bacitracin was resolved, or partly resolved, into at least ten different components. Table 1 shows the proportions in which nine of these components were present, their partition coefficients (K) in the solvent system used, and

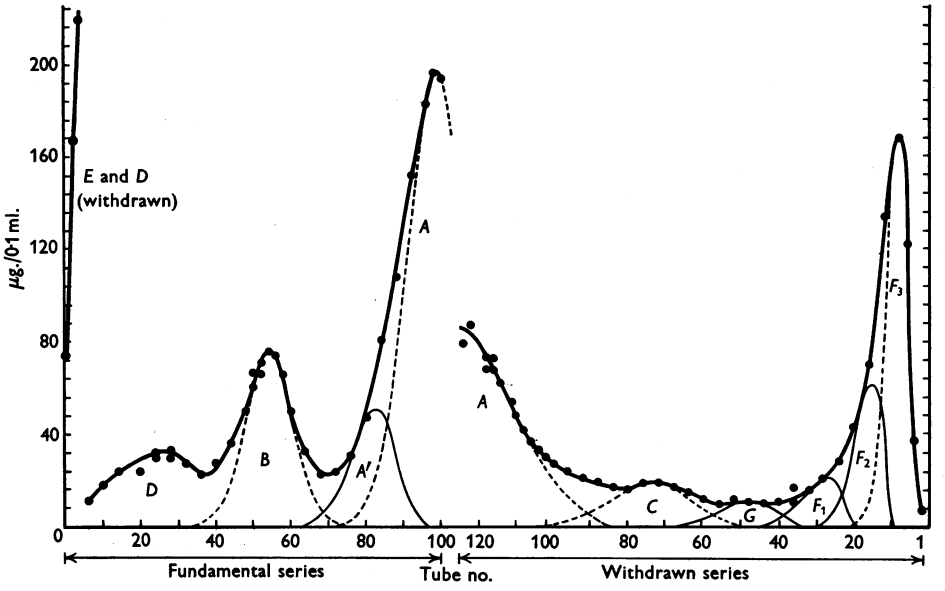

Fig. 1. 221-transfer distribution of crude bacitracin in amyl alcohol-*n*-butanol (4:1); 0·05 M-potassium phosphate buffer, pH 7·0. ●—●, total material in 0·1 ml. samples, taken from both phases in the fundamental series and from the single phase in the withdrawn series; – – –, calculated curves; ——, approximate interpretation.

RESULTS

Resolution of crude bacitracin by counter-current distribution

Fig. 1 shows a distribution curve of a sample of crude bacitracin that was previously described as ayfivin, batch 1 (Arriagada, Savage, Abraham, Heatley & Sharp, 1949). The distribution was carried out in an all-glass machine, similar to that designed by Craig, the fractions from the machine which were pooled and concentrated at the end of the experiment.

The component named bacitracin *A'* (Fig. 1) has not so far been investigated. Evidence for the existence of such a component was obtained from earlier experiments (Newton & Abraham, 1950), and this evidence has been strengthened by the results of the present counter-current distribution. It is possible to obtain a preparation of bacitracin *A*

Table 1. *Components of crude bacitracin obtained by counter-current distribution (see Fig. 1)*

(In the fourth column the numbers carrying a bar represent the numbers of fractions withdrawn from the machine.)

Bacitracin	% of total material	K = (concn. in alcohol phase/ concn. in aqueous phase at pH 7·0)	Fractions selected for concentration
E	17·5	<0·1*	0–4
D		0·1*	20–35
B	12	0·32	48–62
A'	7	0·58*	—
A	37	0·79	90–100; $\overline{124}$–$\overline{100}$
C	5·5	1·4	$\overline{80}$–$\overline{60}$
G	2	2·0*	$\overline{56}$–$\overline{40}$
F_1	2·5	3·9*	$\overline{30}$–$\overline{26}$
F_2	5·5	7·3*	$\overline{20}$–$\overline{16}$
F_3	11	15	$\overline{8}$–$\overline{6}$

* Approximate values.

whose distribution curve corresponds to that of a single substance over nearly its whole range. There is thus reason to believe that the deviation of the curve in the region of the postulated bacitracin A' (Fig. 1) is not really due to a distribution of bacitracin A under non-ideal conditions.

Physical and chemical properties of the bacitracin polypeptides

Partition coefficients of bacitracins A, B and C. Fig. 2 shows the partition coefficients of bacitracins A, B and C at 22°, between amyl alcohol-*n*-butanol (4:1) and aqueous 0·05 M-potassium phosphate buffer at pH values varying from 3·5 to 10·5. The

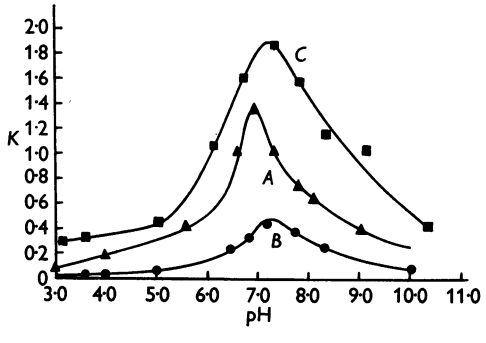

Fig. 2. Partition of bacitracins A, B and C between amyl alcohol-*n*-butanol (4:1); 0·05 M-potassium phosphate at various pH values. K = (concn. in alcohol phase/concn. in aqueous phase).

concentration of material in each layer was measured by the photometric ninhydrin method after hydrolysis (Newton & Abraham, 1950). The pH values were those shown by a glass electrode inserted into the lower phase at equilibrium. With all three substances the partition coefficient rises to a maximum in the region of pH 7. With bacitracin A, at least, the pH at the maximum appears to coincide with the isoelectric point (Sharp, Arriagada, Newton & Abraham, 1949). The steep rise in the partition coefficient between pH 5·5 and 6·9 may be associated with the liberation of a hydrogen ion from the glyoxaline ring of histidine, and the subsequent fall between pH 6·9 and 8·5 with the liberation of a hydrogen ion from an α-amino group (Newton & Abraham, 1953).

In contrast to their behaviour in this solvent system, bacitracins A and B were found to have nearly the same partition coefficient when distributed in a system composed of *sec.*-butanol and 3 % (w/v) aqueous acetic acid. The values of K (= concn. in alcohol phase/concn. in aqueous phase), determined directly by weighing, were 0·42 for bacitracin A and 0·47 for bacitracin B. The system composed of *sec.*-butanol and aqueous acetic acid was previously shown to be of very low efficiency in resolving the bacitracin polypeptides (Newton & Abraham, 1950).

Ultraviolet absorption spectra. The ultraviolet absorption spectra of the bacitracin polypeptides fall into three groups, which are shown in Figs. 3–5. Bacitracins E, D, B and A show a broad maximum at 253 mμ. (Fig. 3). At this wavelength the extinction coefficients of D, B and A are similar, and considerably greater than that of E. Bacitracins C and G absorb more strongly than A and B, and show a sharp maximum at 258 mμ. and a shoulder at 268 mμ. (Fig. 4). Bacitracins F_1, F_2 and F_3 all show a shoulder at 253 mμ. and a broad maximum at 288 mμ., but F_1 absorbs more strongly than F_2, and F_2 more strongly than F_3 (Fig. 5).

After hydrolysis of bacitracin A with 6 N-hydrochloric acid at 110° for 18 hr. its absorption maximum at 253 mμ. disappears.

Ionizable groups. A titration curve of bacitracin A is shown in the following paper (Newton & Abraham, 1953). The titration curves of bacitracins B, C and G are almost identical with that of A between pH 2·5 and 8·5. Between pH 8·5 and 11·5, however, the amounts of ionizable groupings which titrate in C and G are more than twice those in A, and the amount in B is about 1·5 times that in A.

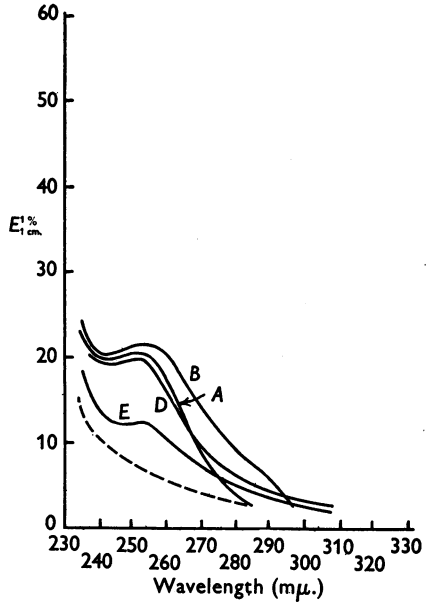

Fig. 3. Ultraviolet absorption spectra of bacitracins *E*, *D*, *B* and *A* in aqueous solution at pH 3·0. ——, bacitracins; – – –, acid hydrolysate of bacitracin *A*.

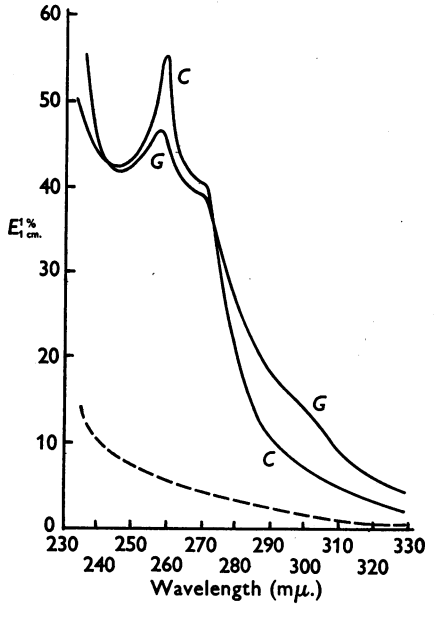

Fig. 4. Ultraviolet absorption spectra of bacitracins *C* and *G* in aqueous solution at pH 3·0. ——, bacitracins; – – –, acid hydrolysate of bacitracin *A*.

Amino-acid composition. On two-dimensional paper chromatograms (see Experimental), acid hydrolysates of bacitracin *A* showed a pattern which closely resembled that given by a mixture of the following amino-acids in simple proportions by weight: cystine, 0·5; ornithine, 1; lysine, 1; histidine, 1; aspartic acid, 2; glutamic acid, 1; phenylalanine, 1; leucine, 1; and isoleucine, 2. This basic pattern also appeared on chromatograms of hydrolysates of the other bacitracins. However, a strong spot in the position occupied by valine was shown, in addition, by bacitracins *B*, *D* and *E*, and a much weaker one by bacitracins *C*, *G*, F_1, F_2 and F_3.

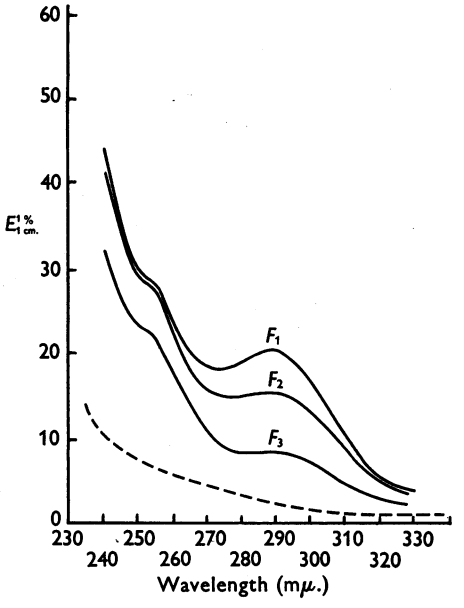

Fig. 5. Ultraviolet absorption spectra of bacitracins F_1, F_2 and F_3 in aqueous solution at pH 3·0. ——, bacitracins; – – –, acid hydrolysate of bacitracin *A*.

A strong spot in the position occupied by glycine was shown by bacitracins *C* and *G*. This spot gave the same reddish colour as that given by glycine when developed with ninhydrin solution to which a small amount of collidine had been added (Woiwod, 1949).

The amount of histidine in some of the bacitracins was determined colorimetrically by the Pauly reaction (Jorpes, 1932). The following quantities of histidine (in moles/100 g. peptide hydrochloride) were estimated to be present by comparing the colours given by the unhydrolysed peptides with those given by standard solutions of histidine: *E*, 0·037; *D*, 0·048; *B*, 0·057; *A*, 0·057; F_2, 0·057; F_3, 0·056. The colour given by bacitracin *A* after hydrolysis with 6N-hydrochloric acid at

110° for 24 hr. indicated the presence of 0·066 mole of histidine/100 g. peptide hydrochloride.

An attempt was made to estimate the amount of ornithine present in acid hydrolysates of various bacitracins by the Chinard method described by Stein & Moore (1951). However, it was found that lysine gave a colour with the Chinard reagent whose density was about one-quarter of that given by ornithine. Hydrolysates of equal weights (estimated by the photometric ninhydrin method) of bacitracins *E*, *D*, *B*, *A*, *C*, and a mixture of F_1, F_2 and F_3, gave very similar Chinard colours. The colour densities were those that would be expected if approximately 0·64 mole of ornithine and 0·64 mole of lysine were present in 100 g. of each peptide hydrochloride.

Amide nitrogen. Hydrolysis of the bacitracin polypeptides results in the liberation of volatile base. This base, which is assumed to be ammonia derived from an amide group, is completely freed by treating the peptides for 20 min. at 100° with N-hydrochloric acid, or for 3 hr. at 37° with 0·1N-sodium hydroxide. The following amounts of such amide nitrogen (in g.equiv./100 g. peptide hydrochloride) are liberated from the various bacitracins: *E*, 0·006; *D*, 0·011; *B*, 0·064; *A*, 0·065; *C*, 0·054; *G*, 0·057; F_1, 0·069; F_2, 0·069; F_3, 0·069. It is evident that bacitracins *E* and *D* differ from the other polypeptides in yielding a much smaller quantity of volatile base and it is probable that they do not contain an amide group.

Groups reacting with fluoro-2:4-dinitrobenzene (FDNB). Bacitracins *A*, *B* and *G* were allowed to react with FDNB in sodium bicarbonate solution (Sanger, 1945) and the dinitrophenyl (DNP) derivatives hydrolysed with acid. The ether-soluble material from the hydrolysates was analysed on paper chromatograms, using the procedure of Blackburn & Lowther (1951). The free amino-acids and DNP derivatives in the water-soluble material were analysed on single-dimensional paper chromatograms developed with benzyl alcohol-hydrogen cyanide (Consden, Gordon, Martin, Rosenheim & Synge, 1945) and on two-dimensional paper chromatograms developed first with butanol acetic acid and then with 80 % (w/w) phenol in the presence of the vapour of 50 % (v/v) acetic acid.

The ether-soluble material from bacitracin *A* appeared to contain DNP-leucine or DNP-isoleucine. The leucine-isoleucine spot obtained from the water-soluble material was found to be reduced in size, whereas the phenylalanine spot appeared to be unchanged. The ether-soluble material from bacitracin *B* appeared to contain DNP-leucine, or DNP-isoleucine, and also DNP-valine, while the valine spot on the chromatogram of the water-soluble material was reduced in size. The water-soluble material from bacitracin *G* appeared to contain no glycine. DNP-glycine was not detected in the ether-soluble material, but about 80 % of any DNP-glycine present in the DNP derivative of the peptide would have been destroyed under the conditions that were used for hydrolysis (Porter & Sanger, 1948).

Chromatograms of the water-soluble material from bacitracins *A*, *B* and *G* showed yellow spots in the positions occupied by *gly*-DNP-histidine and δ-DNP-ornithine (*gly*-DNP-histidine is a histidine derivative containing a DNP residue as a substituent on the glyoxaline ring.) They showed the presence of free lysine, but very little ornithine or histidine.

Reduction of ferricyanide. On treatment with 0·5N-hydrochloric acid at 100° for 20 min. bacitracin *A* develops an ability to reduce potassium ferricyanide at room temperature, which is due, at least in part, to the liberation of a thiol group (Newton & Abraham, 1953). After this treatment the various bacitracins showed a positive nitroprusside reaction and reduced the following amounts of ferricyanide (in g.equiv./100 g. peptide): *E*, 0·027; *D*, 0·047; *B*, 0·093; *A*, 0·093; *C*, 0·076; *G*, 0·049; F_1, 0·047; F_2, 0·067; F_3, 0·067.

Antibacterial activity. Activity against *Corynebacterium xerosis* was measured by the cylinder-plate method (Heatley, 1944), the unit of activity being that defined by Arriagada *et al.* (1949). The various bacitracins showed the following activities in units/mg.: *E*, 0·3; *D*, 0·5; *B*, 13·5; *A*, 36·0; *C*, 18·0; *G*, 5·0; F_1, 2·0; F_2, 1·0; F_3, 0·5.

DISCUSSION

The solvent system used in the counter-current distribution described in this paper was tried originally because its pH was one at which the overall partition coefficient of bacitracin activity changed rapidly with hydrogen-ion concentration. It was thought that these were conditions under which the greatest differences in the partition coefficients of the constituents of the mixture might be found. This idea appeared to be borne out by the fact that very poor resolution was obtained in a system at a lower pH, consisting of *sec.*-butanol and aqueous acetic acid (Newton & Abraham, 1950). However, direct measurement of the distribution of bacitracins *A*, *B* and *C* between amyl alcohol-butanol and buffer at various hydrogen-ion concentrations has shown that the partition coefficients of these three substances, at least, show considerable differences over a fairly wide pH range. Some factor other than pH must therefore be responsible for the poor resolving power of *sec.*-butanol and aqueous acetic acid. Possibly the relative inefficiency of the latter can be related to the fact that it is closer than the amyl alcohol butanol-buffer system to the

critical point at which a single phase is formed: the more similar the two phases in their properties the less selective is the system likely to be. The structural features of the various bacitracins which are responsible for the wide differences in their partition coefficients in the amyl alcohol-butanol-buffer system are not yet known.

The counter-current distribution was designed to obtain the various bacitracins as pure as possible in a single experiment. After about 200 transfers the distribution curve of bacitracin *A* resembled closely the curve that would be expected for a single substance over more than half of its range. In weighing the evidence provided by the distribution for the homogeneity of bacitracin *A*, however, it must be remembered that nearly 3000 transfers have been used for the separation of leucine and isoleucine (Craig *et al.* 1951). If bacitracin *A* were really a mixture of polypeptides which differed, for example, only in the relative amounts of leucine and isoleucine that they contained, it would not be surprising to find that the mixture was not resolved by the procedure used here.

The purity of the bacitracins which are found at either end of the distribution curve is even more questionable. If two substances have partition coefficients K_1 and K_2, and if the volumes of the upper and lower phases are equal, the best separation after a given number of transfers with a fixed ratio of K_1/K_2 is obtained when $\sqrt{K_1K_2}=1$ (Craig & Craig, 1950). In other words, substances near the centre of a distribution curve will be more easily resolved than substances at the two ends. Relatively little information about the purity of bacitracins *E* and *F* is provided by the work described here.

In spite of these uncertainties, it is clear that crude bacitracin has been resolved at least into groups of very closely related polypeptides, and it appears from their amino-acid compositions that bacitracins *E*, *D*, *B* or *C* cannot have been formed from bacitracin *A* during the process of purification.

The similarities in the amino-acid patterns shown by hydrolysates of the various bacitracins make it justifiable to describe the latter as members of a single family of polypeptides. Clear-cut differences, however, have been demonstrated in their chemical as well as in their physical and biological properties.

Hydrolysates of bacitracins *E*, *D* and *B* differ sharply from the hydrolysate of *A* in showing a spot on paper chromatograms in the same position as that occupied by valine. The same spot is also visible, though much less intense, in chromatograms of hydrolysates of bacitracins *C*, *G*, F_1, F_2 and F_3. Craig, Gregory & Barry (1949) found a substance in hydrolysates of bacitracin which behaved like valine on chromatography. They reported that the substance appeared to be absent from the purest material that they obtained by counter-current distribution, so that this material presumably consisted of bacitracin *A*. Craig *et al.* (1949) isolated the valine-like substance, however, and stated that it was not valine. In view of this it is surprising that a hydrolysate of the crude bacitracin used in the present experiments behaved as though it contained valine on microbiological assay (see Experimental). Further investigation of the valine-like substance is clearly necessary.

Bacitracins *C* and *G* differ from the other polypeptides in yielding on hydrolysis a substance which behaves like glycine. They also differ in their ultraviolet absorption spectra, both substances showing a strong absorption peak at 258 mμ. and a shoulder at 268 mμ. The possibility must be considered that a glycine residue is not present as such in the polypeptides, but that it is formed from a more complicated structure during the hydrolysis with concentrated hydrochloric acid. For example, certain purines are known to yield glycine when hydrolysed with mineral acids at temperatures above 100° (Smith & Markham, 1950; Frick, 1952).

Carboxyl groups in polypeptides are commonly titrated between pH 2·5 and 5·5, histidine residues between pH 5·5 and 7·0, α-amino groups between pH 7·0 and 8·5, and δ- or ϵ-amino groups between pH 9·5 and 11·5. The close similarity between the titration curves of bacitracins *B*, *A*, *C* and *G* over the pH range 2·5–8·5 therefore suggests that all three substances contain the same proportion of free carboxyl, α-amino, and glyoxaline groups. Reaction with FDNB indicates that bacitracin *A* contains a free α-amino group which is part of a leucine or an isoleucine residue. But bacitracin *B* reacts with FDNB to give a DNP-leucine (or isoleucine) and also a DNP-'valine' derivative, and bacitracin *G* can react to give a DNP-leucine (or isoleucine) and probably a DNP-glycine derivative. Moreover, bacitracins *B*, *C* and *G* show more titratable groups than bacitracin *A* in the pH range 8·5–11·5. This raises the question as to whether the 'valine' in *B*, as well as the glycine in *C* and *G*, is really present as a simple α-amino-acid residue with the amino group free, or whether it is part of a structure whose nature has not yet been determined.

Irrespective of the nature of the glycine and 'valine' residues, however, there is evidence that the bacitracins contain special structural features. A structure responsible for the liberation of a thiol group on mild treatment with acid appears to be common to all the bacitracins, and at least two other special structures, one present in bacitracins *C* and *G*, and the other in bacitracins F_1, F_2 and F_3, seem to be necessary to account for the ultraviolet absorption spectra.

EXPERIMENTAL

Counter-current distributions

Solvents. *n*-Butanol and amyl alcohol were purified in the manner described by Newton & Abraham (1950). A mixture of 1 l. of wet butanol and 4 l. of wet amyl alcohol was shaken with 5 l. of 0·05 M-potassium phosphate buffer, pH 6·8, which had been made up in glass-distilled water. The mixture was allowed to stand in a constant-temperature room for 24 hr. before use.

Distribution procedure. In order to obtain enough of the various bacitracins for chemical and biological investigation the first four tubes of the distribution machine were loaded with the starting material at as high a concentration as possible. This was done in the following manner: 2·9 g. of a sample of crude bacitracin hydrochloride (previously referred to as crude ayfivin hydrochloride batch 1 by Sharp *et al.* 1949) were dissolved in 60 ml. glass-distilled water and the solution adjusted to pH 5·0 with 10 N-KOH. This solution was extracted twice with 60 ml. of a solution of 8-hydroxyquinoline in ether, the first ethereal solution containing 50 mg. of 8-hydroxyquinoline and the second 20 mg. It was then extracted four times with 60 ml. quantities of ether alone. The first two extracts were dark green and the last two were colourless. Ether was removed from the aqueous solution *in vacuo* and the latter equilibrated with the solvent system and saturated with the crude bacitracin as follows. Sufficient potassium phosphate buffer (pH 5·0) was added to make 80 ml. of 0·05 M buffer and the solution just saturated with both amyl and *n*-butyl alcohols. The volume was then made up to 80 ml. with water just saturated with amyl and butyl alcohols, 80 ml. of the upper phase of the solvent system were added, and 10 N-KOH was added to the lower layer until the 'pH' (glass electrode) after equilibration was 7·0. A small amount of an oil came out of solution and was removed by centrifuging. Portions (20 ml.) of each layer of the resulting clear solutions were loaded into tubes 0–3 of the distribution machine.

The distribution was begun with 46 transfers, using the 'fundamental' procedure (Craig & Craig, 1950). Single withdrawal of lower layer from the trailing edge of the band was then carried out until the contents of tubes 0–4, which were forming stable emulsions, had been removed. The material withdrawn in these fractions consisted of a mixture of bacitracins *E* and *D*. The fundamental procedure was then continued until 97 transfers had been completed and this was followed by 124 single withdrawals of upper layer, made from the leading edge of the band. All the material left in the machine had been subjected to 221 transfers.

Analysis of distribution curve. The distribution curve shown in Fig. 1 was obtained by measuring the total amounts of polypeptide in samples of the contents of the even-numbered tubes, using the method described by Newton & Abraham (1950). Partition coefficients (K) were determined over the whole curve from the ratios of the amounts of material in successive even-numbered tubes (T_r/T_{r+2}). This was done by using equation (1) for the fundamental series and equation (2) for the withdrawn series (Berridge, Newton & Abraham, 1952; Newton & Abraham, 1952).

$$T_r/T_{r+2} = F'_r F'_{r+1} \frac{1}{K^2}, \quad (1)$$

$$T'_n/T'_{n-2} = F_w F_{w-1} \frac{1}{(K+1)^2}, \quad (2)$$

where
$$F'_r = \frac{(r+1-\frac{1}{2}[x-1])}{(n-r+\frac{1}{2}[x-1])},$$

$$F_w = \frac{(n-1)}{(n-R-\frac{1}{2}[x+1]-1)},$$

n = number of transfers, r = tube number, $R+1$ = total number of tubes in the machine and x = number of tubes initially filled with an equal amount of material.

The values obtained for the partition coefficients of the various polypeptides were: $K_B = 0{\cdot}316$, $K_A = 0{\cdot}788$, $K_C = 1{\cdot}37$ and $K_{F_3} = 15{\cdot}0$ (approx.). No precise data were obtained for the other polypeptides by this analysis. Theoretical curves were calculated for bacitracins *A*, *B*, *C* and F_3 using the values of K found above. The calculated curves were then fitted to the maxima of the experimental curve for these compounds. Material from tubes adjacent to these maxima had already been found to have practically constant partition coefficients. The interpretation of the remainder of the curve is only approximate.

Concentration of solutions. The fractions selected from the counter-current distribution (Table 1) were concentrated in the manner described by Newton & Abraham (1950).

Acid hydrolysis. A solution containing 2–5 mg. of the bacitracin hydrochloride was evaporated to dryness in a small Pyrex test tube; 1 ml. of 6 N-HCl was then added and the tube was sealed and heated at 110° for 24 hr. After hydrolysis the HCl was removed in a stream of air at 100°.

Ultraviolet absorption spectra. These were measured in a Beckman spectrophotometer in aqueous solution at pH 3.

Electrometric titrations. These were carried out at 20° as described by Newton & Abraham (1953).

Amide N. This was measured as described by Newton & Abraham (1953).

Reduction of ferricyanide. This was measured as described by Newton & Abraham (1953).

Paper chromatography

The chromatograms were run on Whatman no. 1 paper (18·5 × 22·25 in.) as described by Dent (1948). The hydrolysate from 200 μg. of polypeptide was used when chromatograms were run in a single dimension, from 400 μg. when they were run in two dimensions.

Single-dimension chromatograms were developed with the butanol-acetic acid mixture of Woiwod (1949). For two-dimensional chromatograms the second solvent was 80% (w/w) phenol, and the atmosphere was saturated with 50% (v/v) acetic acid (Dent, 1948). This solvent gave tolerably good separations of ornithine, lysine and histidine. The phenol-saturated papers were dried at 37° for 4 or 5 hr. (Brush, Boutwell, Barton & Heidelberger, 1951). Butanol-acetic acid-saturated papers were dried at 80° for 30 min. The papers were sprayed with 0·2% (w/v) ninhydrin dissolved in wet butanol containing 0·2% (v/v) of collidine (Woiwod, 1949).

Microbiological assay of valine

Valine was estimated microbiologically with *Leuconostoc mesenteroides* (American Type Culture Collection, no. 7881). The medium and procedure were based on those of Steele, Sauberlich, Reynolds & Baumann (1949). The tubes were incubated for 44 hr. at 37° and growth was estimated turbidimetrically. The response of the organism to DL-valine and to an acid hydrolysate of crude bacitracin coincided

over a wide range of concentrations. 4200 μg. of crude bacitracin produced the same response as 117 μg. of DL-valine. Norvaline did not replace valine for growth under the conditions used.

DNP-bacitracin derivatives

Bacitracin (10 mg.) was mixed with 6·5 mg. of $NaHCO_3$ in 5 ml. water. A solution of 10 mg. of FDNB in aqueous ethanol was added, and the mixture was shaken at room temperature for 3 hr. The contents of the flask were then evaporated almost to dryness and the residue was suspended in about 5 ml. of water. The suspension was extracted twice with an equal volume of ether. The DNP-bacitracin was precipitated from the aqueous solution by excess acid after first removing any residual ether. The yield of DNP-bacitracin was usually between 60 and 80 %, assuming that it contained one DNP residue for each 400–500 g. of peptide. Both DNP-bacitracin *A* and DNP-bacitracin *B* had sharp melting points close to 185°.

Hydrolysis of DNP-bacitracins. About 5 mg. of DNP bacitracin were heated in a sealed tube with 1 ml. of a mixture of equal volumes of glacial acetic acid and conc. HCl. After 12–14 hr. at 105° the acid was evaporated and the residue taken up in a small volume of water and extracted several times with ether. The ether extracts were transferred to a weighed tube and evaporated to dryness. The aqueous residue was also evaporated to dryness.

Paper chromatography of DNP-amino-acids

Ether-soluble fraction. The weighed residue remaining after evaporation of the ether was dissolved in ethanol to give a solution of 75 μg./5 μl. This solution (5 μl.) was applied to Whatman no. 4 paper which had been previously soaked in potassium phthalate buffer of pH 6·3 and dried at room temperature (Blackburn & Lowther, 1951). The chromatograms were usually developed with *tert.*-amyl alcohol saturated with the same phthalate buffer. Benzyl alcohol to which 10 % of ethanol (v/v) had been added before just saturating with buffer was found particularly useful for resolving DNP-valine from a mixture of the DNP derivatives of leucine, isoleucine and phenylalanine. DNP-glycine, DNP-alanine, DNP-valine and DNP-leucine were easily distinguished using these systems, but the systems did not resolve the DNP derivatives of leucine, isoleucine and phenylalanine.

Water-soluble fraction. The aqueous residue from the ether extraction was evaporated to dryness and dissolved in water to give a solution containing 150 μg./5 μl. This solution (5 μl.) was applied to Whatman no. 1 paper and the chromatogram was developed with a butanol-acetic acid mixture (Woiwod, 1949). Experiments with model mixtures of amino-acids and DNP derivatives showed that in this system ϵ-DNP-lysine and δ-DNP-ornithine could be seen as distinct yellow spots, and that *gly*-DNP-histidine and *S*-DNP-cysteine gave very pale yellow spots, which could be easily seen in ultraviolet light. ϵ-DNP-lysine occupied a position just above the leucine-isoleucine-phenylalanine spot, while δ-DNP-ornithine was above, and just resolved from, ϵ-DNP-lysine. *S*-DNP-cysteine was not resolved from δ-DNP-ornithine; *gly*-DNP-histidine ran to a position just below glutamic acid.

After marking the yellow spots the paper was sprayed with ninhydrin and the unchanged amino-acids were revealed in the normal way.

SUMMARY

1. A sample of crude bacitracin has been resolved by counter-current distribution between solvents into at least ten polypeptides. These substances have been named bacitracins *E*, *D*, *B*, *A'*, *A*, *C*, *G*, F_1, F_2 and F_3. Their partition coefficients (= concn. in alcohol phase/concn. in aqueous phase) at pH 7·0 increase in this order.

2. The ultraviolet absorption spectra of the bacitracins fall into three groups. Bacitracins *E*, *D*, *B* and *A* show broad maxima at 253 mμ. Bacitracins *C* and *G* absorb more strongly and show a sharp maximum at 250 mμ. and a shoulder at 268 mμ. Bacitracins F_1, F_2 and F_3 all show a shoulder at 253 mμ. and a broad maximum at 288 mμ.

3. All the bacitracins contain cysteine, ornithine, lysine, histidine, aspartic acid, glutamic acid, leucine and/or isoleucine and phenylalanine residues. However, bacitracins *B*, *D* and *E* differ from *A* in yielding on acid hydrolysis a substance which behaves like valine on paper chromatograms, and bacitracin *C* differs from *A* in yielding a substance which behaves like glycine. Smaller amounts of the valine-like substance are obtained from bacitracins *C*, *G*, F_1, F_2 and F_3.

4. The bacitracins contain no free thiol group, but a thiol group appears to be liberated in all of them on mild acid hydrolysis. At the same time ammonia appears to be liberated from an amide group in bacitracins *B*, *A*, *C*, *G*, F_1, F_2 and F_3, bacitracins *E* and *D* containing very little, if any, amide nitrogen.

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Observations on the Nature of Bacitracin *A*

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Bacitracin *A*, the main active member of the bacitracin family of polypeptides, was obtained by counter-current distribution between solvents in amounts sufficient for some of its properties to be investigated in detail (Newton & Abraham, 1953). Previous work with bacitracin (Sharp, Arriagada, Newton & Abraham, 1949; Craig, Gregory & Barry, 1949; Newton & Abraham, 1950) indicated that the substance was a good deal less stable than would be expected if it consisted only of amino-acids joined by normal peptide linkages. The investigations described in this paper were designed largely to throw light on the nature of the more labile portions of the molecule of bacitracin *A*.

RESULTS

Composition and molecular weight of bacitracin A

Barry, Gregory & Craig (1948) reported the results of an analysis made by Moore & Stein, on a starch column, of the amino-acids present in purified bacitracin. Using these results as a basis it was found that paper chromatograms of an acid hydrolysate of bacitracin *A* appeared very similar to chromatograms of the following mixture of amino-acids in the molecular proportions shown (Newton & Abraham, 1953): cystine, 0·5; ornithine, 1; lysine, 1; histidine, 1; aspartic acid, 2; glutamic acid, 1; phenylalanine, 1; leucine, 1; isoleucine, 2.

The relative values obtained by Moore & Stein for the amino-acids other than cystine and ornithine (which was not determined) are in agreement with the molecular proportions of the amino-acids in this mixture. The value suggested here for cystine is based on the sulphur content of bacitracin *A* hydrochloride, which was found by elementary analysis to be 1·82%.

Sharp *et al.* (1949) concluded from experiments with D-amino-acid oxidase that some of the amino-acids in crude bacitracin (ayfivin) had the D-configuration. Craig *et al.* (1949) isolated DL-phenylalanine, DL-aspartic acid and partially racemic D-isoleucine from purified bacitracin.

If the weights of the residues of the amino-acids shown above are combined, and if the weight of three molecules of hydrochloric acid is added, a figure of approximately 1500 is arrived at for the weight of the minimum stoicheiometric unit of bacitracin *A* hydrochloride. The presence of only one atom of sulphur in the molecule is possible because, although cystine is finally obtained on hydrolysis, it is derived from a cysteine residue (see below).