THE SEPARATION AND CHARACTERIZATION OF BACITRACIN POLYPEPTIDES

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Bacitracin is an antibiotic produced by a certain strain of bacteria now classified as Bacillus licheniformis (1). It was discovered by Johnson, Anker, and Meloney (2) in 1945 and has come to be an antibiotic of considerable promise (3).

Counter-current distribution studies (4, 5) by Barry, Gregory, and Craig conclusively demonstrated that the antibiotic activity was due to one or more polypeptides which yielded phenylalanine, leucine, isoleucine, cystine (or cysteine), glutamic acid, aspartic acid, histidine, lysine, and ornithine on complete hydrolysis in hydrochloric acid. The active principle is, therefore, a substance of considerable complexity, even though it has been reported to diffuse readily through a nitrocellulose membrane (1), a result confirmed in this laboratory.

Aside from its importance as an antibiotic, bacitracin is especially interesting to the protein chemist in that it is one of the few readily available polypeptides of considerable complexity, yet is by no means so complex as a protein. It is, therefore, a valuable model and as such is worthy of the most searching study from the standpoint of purity. Moreover, certain preparations have been disappointing in regard to undesired toxic manifestations when tested clinically (6). Therefore, the question whether a single substance in the chemical sense has been isolated may reasonably be raised. This question has been under study from time to time by us (4) for several years, but a conclusive answer has awaited the development of better equipment and techniques for fractionating this class of substances.

Attempts to purify bacitracin and obtain a crystalline product have been made by a considerable number of workers in different laboratories but without success thus far. A variety of different fractionation procedures have been employed. In our experience, counter-current distribution offers the most promise for isolation of a single chemical individual. Newton and Abraham (7) have reached a similar conclusion with a polypeptide antibiotic called ayfivin, which apparently is identical with bacitracin.
The reliability of counter-current distribution as a criterion of purity depends primarily upon the number of transfers which can be applied and the selectivity of suitable systems. The more recent studies on bacitracin in this laboratory have been made with a 220 tube glass train operated automatically (8) and capable of applying several thousand transfers if this number should be required. The question of the best all round system, however, cannot be disposed of so easily.

From the standpoint of stability and for other technical reasons, mainly those involving the automatic counter-current distribution apparatus available, the 2-butanol-3 per cent acetic acid system originally suggested (4) remains the one of choice in this laboratory in spite of the conclusions of Newton and Abraham (7). They found this system less selective than one of phosphate buffer at a pH approximating 7.0, but apparently overlooked the possibilities inherent in the use of higher concentrations of the peptide with the acetic acid system in order to achieve selectivity. The bacitracins do not give strictly linear partition isotherms at the higher concentrations, and it is possible to produce a slightly skewed pattern deliberately to achieve separation of the major component (9).

Thus a 6 gm. charge of a crude bacitracin kindly supplied by the Commercial Solvents Corporation has given the pattern of Fig. 1, c at 527 transfers. The main peak is designated bacitracin A in conformity with the suggestion of Newton and Abraham. Their C component is probably identical with our bacitracin C. As mentioned later, it is a transformation product of bacitracin A. Bacitracin E crystallized readily on evaporation of the solvent but has not thus far been studied since its antibiotic activity is low. It is almost absent from certain preparations.

That bacitracin B represents a real component and is not the result solely of skewing of the major band was first shown by complete hydrolysis of material occurring in this region and comparison by paper chromatography with a hydrolysate of material from the right half of the major band. Fig. 2 is a drawing of the map given by bacitracin A. The B component gave all these spots plus an additional spot whose position on the paper corresponded to that of valine. No suggestion of this spot was found in component A. As has been stated earlier (5), the so called "valine" spot is not due to valine. It has been isolated in small amount in crystalline form but has not thus far been identified.

Evidence that the material occurring in the peak tubes of the major band is a single component was obtained by rerunning the solute in Tubes 191 to 195 without isolation from the solvent. At 157 transfers the pattern of Fig. 1, a was obtained. Complete absence of bacitracin C is indicated, since this would occur in the region of Tubes 70 to 90. Partition ratios determined directly at three points gave the values placed on the pattern. However, the partition ratio calculated from the position of the peak is
Fig. 1. Distribution patterns showing the fractionation of crude bacitracin A. $K = \text{partition ratio.}$
0.414. The deviation from the calculated is, therefore, due to a non-linear partition isotherm and, at most, there can be only a small amount of bacitracin B in the left limb of the band.

In another run carried approximately to the point of the pattern of Fig. 1, c, bacitracins E and D were removed and the machine was set for "recycling" (8). At 900 transfers the pattern of Fig. 1, b was obtained. Here the shoulder containing the B component is unmistakable and a larger yield of the A component essentially free from B is obtained. Material taken from Tubes 310 to 340 has become our standard preparation of bacitracin A.

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fig. 2. Drawing of the paper chromatography map of the hydrolysate of bacitracin A. Cy = cystine, Or = ornithine, Hi = histidine, Ly = lysine, Asp = aspartic acid, Glu = glutamic acid, φal = phenylalanine, iL = isoleucine, L = leucine.

For recovery the combined solutions were rapidly concentrated in the rotary evaporator (10) under reduced pressure at a temperature never above 25°, until the alcoholic phase had evaporated and the aqueous phase had reached approximately 200 ml. The solution was then lyophilized.

Analysis—Loss on drying at 100° = 9.6 per cent; C 55.20, H 7.30, N (total) 15.8, N (Van Slyke) 2.20, N (amide) 0.98, S 2.2, OCH₃ none, N—CH₃ none.

The white residue has been redistributed in the acetic acid system. A pattern almost identical with that of Fig. 1, a indicated no transformation during isolation. No change was noted in this pattern after storing the bacitracin at 6° for several months, which is contrary to our experience with a crude preparation (5).

The residue contained sufficient acetic acid to give pH 4 to 5 when dissolved in water. This could be removed by solution of the product in
a small volume of methanol and precipitation of the peptide by addition of the methanol solution to acetone. After two or three precipitations a product was obtained which appeared to be only slightly soluble in water. This treatment did not influence the antibiotic activity. It gave a higher activity per mg. of weight, but this was due to the smaller amount of solvent in the residue. When corrected to the dry weight basis (drying at 100° and 0.2 mm. pressure), activities approximating 50 to 60 units per mg. were regularly obtained. However, this material would be expected to lose activity gradually, owing to the spontaneous formation of bacitracin C.

![Graph](image)

**Fig. 3. Redistribution of bacitracin A in the system of Newton and Abraham**

The yield of the material obtained by lyophilization of a cut of Tubes 310 to 340 from the pattern of Fig. 1, b approximated 2 gm. This standard preparation has been studied further in other systems, including the isoamyl alcohol-\(n\)-butanol-phosphate buffer system of Newton and Abraham (7). We have not reached the same conclusions, however, in regard to the effect of the system.

A run with 200 mg. of our standard bacitracin A preparation gave the pattern of Fig. 3 at 106 transfers. No discrete band occurred on the right but only a drawn out tail, which seemed to be typical of a skewed distribution or of transformation during the run. That the latter possibility is responsible for the effect became evident when partition ratios (\(K\)) were measured on the solute contained in Tubes 70 to 100. The \(K\) values were roughly constant throughout this band but were of the order of 10 or more. This is much too high a value for the position of the band on the pattern. Moreover, material recovered from this portion of the pattern
showed low antibiotic activity when it was assayed. The antibiotic activity approximated that of bacitracin C. In a later contribution it will be shown that, if bacitracin A is permitted to stand at room temperature for several days in a phosphate solution at pH 7 or slightly above, it is converted partially to bacitracin C. In view of these results, we do not consider the phosphate system as suitable for precise characterization of bacitracin A.

Bacitracin B appears not to be derived from A, since it contains an additional amino acid residue as shown by paper chromatography. The hope that our standard preparation of bacitracin A does approach a working degree of chemical purity has now been supported by extensive hydrolysis experiments and quantitative determination of the amino acid residues and by molecular weight studies with the partial substitution method of Battersby and Craig (11). These results will be given in separate communications. The time would now appear ripe for reinvestigation of the problem of the supposed inherent toxicity of the antibiotic. Such studies are in progress.

Absorption Spectrum Measurements—An aqueous solution of the standard preparation containing 1 mg. per ml. in water gave Curve 1 of Fig. 4. All measurements were made with the Beckman quartz spectrophotometer. A weak maximum is apparent in the region of 250 to 255 μm.

As experience with the material was gained, it soon became obvious that this characteristic absorption was caused by a chemically reactive arrangement of unknown linkages, since almost any transformation demonstrable by distribution characteristics, antibiotic activity, etc., resulted in the loss of the characteristic type of absorption.

Thus if bacitracin A was dissolved in 0.1 M Na₂CO₃ at a concentration of 1 mg. per ml. and the absorption measured immediately, the same curve was obtained as when water was the solvent. But on standing several days at 24°, the absorption spectrum changed to that shown in Curve 2 of Fig. 4.

A different type of shift was observed in acid solution. A solution of 1 mg. per ml. in 0.1 N HCl measured immediately did not differ from Curve 1. But on standing several days at 24°, Curve 3 was obtained. The maximum was eliminated entirely.

Bacitracin C, 1 mg. per ml. in water, gave Curve 4. Here the maximum is strikingly shifted to the region of 290 μm. Obviously the transformation which takes place in the more alkaline medium, such as in sodium carbonate, is different from that which produces bacitracin C.

Bacitracin B gave a curve almost identical with A in water, but the shoulder was somewhat lower, as Curve 5 of Fig. 4 shows. The lower

1 Weisiger, J. R., to be published.
intensity is consistent with an additional residue in bacitracin B and a correspondingly higher molecular weight. The absorption spectrum of commercial bacitracin is consistent with its being a mixture of A, B, and C types.

Bacitracins A and B apparently contain linkages which are very reactive to formaldehyde in aqueous solution at pH 7. With an excess of formaldehyde, the absorption of bacitracin A was found to shift quickly from that of the unchanged peptide, Curve 1 of Fig. 4, to one similar to Curve 3. Here again, the characteristic shoulder was eliminated. Such a change was inhibited when the pH was reduced to 4.

It has been suspected that the antibiotic potency of bacitracin is affected by the presence of aldehydes or ketones in solvents used for extraction of the peptide. This may be connected with the type of condensation noted here and may, therefore, have a logical basis.

Bacitracin C apparently is not affected by formaldehyde or, if it is, the absorption spectrum is not altered. Since, as discussed elsewhere, the...
transition from A to C appears to take place with loss of the cysteine spot on paper chromatography of the hydrolysate, reactive linkages surrounding the sulfur atom are probably responsible for the formaldehyde effect and probably also for the characteristic absorption of bacitracin A.

**SUMMARY**

Commercial bacitracin has been shown by counter-current distribution studies to be a mixture comprising a main component and varying amounts of four minor components. The major component has been separated and its ultraviolet absorption spectrum studied in several solutions. It has been found to be labile to alkali, strong acid, and formaldehyde.

**Addendum**—Since this manuscript was submitted, a personal communication has been received from Dr. Abraham, reporting some properties of their bacitracin C which are distinctly different from our C. We, therefore, propose to change the name of our component and hereafter refer to it as "bacitracin F" so that confusion with the component C of Newton and Abraham will be avoided.

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