The Amide and Carboxyl Groups of Bacitracin A

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The polypeptide antibiotic, bacitracin A, has been assigned the provisional structure I, in which → represents a C–N bond (Weisiger, Hausmann & Craig, 1955*a*, *b*; Lockhart & Abraham, 1954; Lockhart, Abraham & Newton, 1955).

The precise nature of the linkages in several parts of this structure remained to be established. Bacitracin A contains two titratable acidic groups and yields one equivalent of ammonia on treatment with hot dilute acid. An amide group was therefore assumed to be present in the molecule, but its location was uncertain. Weisiger *et al.* (1955*a*) obtained a peptide from dinitrophenyl-bacitracin A which gave lysine and aspartic acid on hydrolysis but migrated as a base when subjected to electrophoresis on paper at pH 5·6, and they suggested that the aspartic acid in this peptide was present as an amide. However, Swallow & Abraham (1957, 1958) showed that a basic substance derived from the ε-aspartyl-lysine sequence in the molecule was not an amide but ε-(aminosuccinyl)lysine. It was not known whether L-aspartic acid was α- or β-linked to the ε-amino group of lysine and whether D-glutamic acid was α- or γ-linked to L-isoleucine. Moreover, the position of the D-aspartic acid residue had not been determined with certainty. The properties of certain peptides formed on partial hydrolysis of bacitracin A suggested that this residue projected, as shown, from a ring of six residues; but the alternative sequence

$$\text{L-His} \rightarrow \text{D-Asp} \rightarrow \text{L-Asp} \xrightarrow{\varepsilon} \text{L-Lys},$$

forming part of a ring of seven residues, was not rigidly excluded.

The present paper describes an attempt to obtain more information about these parts of the molecule from a study of the product obtained when esterified bacitracin A was reduced with lithium borohydride. Nystrom, Chaikin & Brown (1949) found that esters could be reduced to alcohols with lithium borohydride whereas amides were unaffected. The first attempt to apply the reaction to an esterified protein (insulin) gave promising results (Chibnall & Rees, 1951), but later studies indicated that the reaction might not have the desired specificity, since some reductive cleavage of peptide bonds could occur (Crawhall & Elliott, 1955). Soon after the present work had begun, Chibnall & Rees and their colleagues reported the results of a careful and detailed investigation into the use of lithium borohydride for the characterization of amide and *C*-terminal residues in proteins (Chibnall & Rees, 1958). They concluded that the reductive cleavage of peptide bonds was not a serious complication with proteins of relatively low molecular weight. Some of their procedures were closely followed in subsequent experiments with bacitracin.

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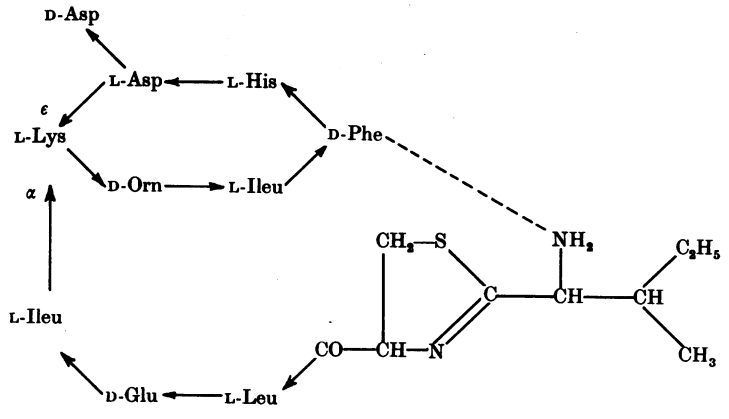


(I)

EXPERIMENTAL

Materials

Tetrahydrofuran. This was refluxed over K for 24 hr. and then distilled. It was stored over Na wire in the dark.

Lithium borohydride. The product from L. Light and Co. Ltd. was recrystallized shortly before use in the manner described by Chibnall & Rees (1958). The reducing power of this preparation was estimated by the iodate method of Lyttle, Jensen & Struck (1952).

Bacitracin. The bacitracin used was kindly supplied by the Commercial Solvents Corp., Terre Haute, Ind., U.S.A. (lot B55–13, 68 i.u./mg.). The main component of this material was bacitracin A, but some bacitracin B and other bacitracins were also present. Bacitracin A (100 i.u./mg.) was prepared from this material by the method of Newton & Abraham (1950), an 11-tube, 29-transfer, countercurrent distribution being used.

Amino alcohols and hydroxyamino acids. Samples of aspartidiol, γ-hydroxy-β-aminobutyric acid and δ-hydroxy-γ-aminovaleric acid were kindly provided by Dr A. C. Chibnall and Mr M. W. Rees. γ-Hydroxy-α-aminobutyric acid (homoserine) was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. δ-Hydroxy-α-aminovaleric acid was synthesized from γ-ethyl glutamate.

Synthesis of δ-hydroxy-α-aminovaleric acid. γ-Ethyl glutamate (0·59 g.), prepared by the method of King & Spensley (1950), and tetrahydrofuran (15 ml.) were placed in an apparatus similar to that described by Chibnall & Rees (1958). An eightfold molar excess of recrystallized $LiBH_4$ (0·52 g.) in tetrahydrofuran (20 ml.) was added, when the ester rapidly dissolved. The solution was heated (oil bath, 90°) so that it refluxed gently for 2 hr. It was then allowed to cool and a small excess of methanolic HCl added to decompose excess of $LiBH_4$ and any borane complexes. The product and inorganic material were precipitated by the addition of dry ether (100 ml.), the solid was washed twice with ether and finally freed from ether *in vacuo*. An aqueous solution of this product (0·5 g.) was desalted by electrophoresis in a four-compartment cell similar to that described by Synge (1951). When the current had fallen to a minimum of 20 mA at 500 v the contents of the specimen compartment were evaporated to dryness. The residue (0·36 g.) was recrystallized from a small volume of water. δ-Hydroxy-α-aminovaleric acid had m.p. 225° (decomp.) and ran with R_F 0·26 when subjected to chromatography on paper in butanol–acetic acid–water (Woiwod, 1949) (Found: C, 44·6; H, 8·2; N, 10·3. Calc. for $C_5H_{11}O_3N$: C, 45·1; H, 8·3; N, 10·5%).

δ-Hydroxy-α-aminovaleric acid was first prepared by Sörensen (1905) from the sodium derivative of phthalimidomalonic ester and αγ-dibromopropane.

Procedures

Esterification of bacitracin. A solution of bacitracin or bacitracin A in dry methanolic 0·1 N-HCl (1 g./100 ml.) was kept at 25° for 24 hr. in a stoppered flask (cf. Chibnall, Mangan & Rees, 1958*b*). The solvent was removed *in vacuo* in a rotary evaporator (Craig, Gregory & Hausmann, 1950), the residue was dissolved in a little dry methanol and reprecipitated by the addition of dry ether (100 ml.). The solid ester hydrochloride was separated by centrifuging, washed once with dry ether and freed from ether *in vacuo*.

Estimation of ammonia nitrogen in peptides. The amount of NH_3 nitrogen in bacitracin and bacitracin A was estimated by the method of Chibnall, Mangan & Rees (1958*a*). This method depends on the fact that a polypeptide may be precipitated from methanolic HCl by the addition of ether while ammonium salts remain in solution. The peptide (0·2 g.) was dissolved in methanolic 0·1 N-HCl (10 ml.) and immediately precipitated with ether (40 ml.). The precipitate was separated by centrifuging and the operation repeated twice. The final precipitate was washed with dry ether (50 ml.) at the centrifuge and freed from ether *in vacuo*. The recovery of peptide was 96%. The combined supernatants were evaporated to dryness and the residue was dissolved in water and introduced into a Markham still (Markham, 1942). After the addition of 4 ml. of M-Na_2HPO_4–NaOH buffer, pH 11·0, any NH_3 present was distilled over into 5 ml. of Conway reagent (Conway, 1935). The residual acid in the resulting solution was titrated with 0·01 N-$Ba(OH)_2$.

An essentially similar procedure was used to estimate the NH_3 nitrogen formed during esterification of bacitracin (100 mg.) in methanolic 0·1 N-HCl (25 ml.). In this case 150 ml. of ether was used for precipitation, since the ester hydrochloride was more soluble than bacitracin itself in ether–methanol. The recovery of ester hydrochloride was 85–90%.

Estimation of amide nitrogen in peptides. The peptides were first freed from NH_3 nitrogen by precipitation as described above. A sample of peptide (10 mg.) in 2 N-HCl (0·5 ml.) was heated in a sealed Pyrex tube at 100° for 3 hr. to liberate amide nitrogen as NH_4Cl. The tube was opened, two drops of bromocresol green solution added as an internal indicator and the solution adjusted to pH 5·2 with N-NaOH. The contents of the tube were then washed quantitatively into a Markham still and NH_3 was estimated as before.

Reduction of bacitracin ester hydrochloride with lithium borohydride. The sample to be reduced (about 100 mg.) was ground to a fine powder, weighed into a 50 ml. test tube and dried for 2 days *in vacuo* over P_2O_5. A 24-fold molar excess of $LiBH_4$ in tetrahydrofuran (10 ml.) was added and the tube fitted to a three-way adaptor carrying a mercury-sealed stirrer and a reflux condenser fitted with a $CaCl_2$ tube (cf. Chibnall & Rees, 1958). Stirring was begun and the reaction vessel placed in an oil bath at 90° so that the tetrahydrofuran refluxed gently. After 6 hr. the mixture was cooled and a small excess of methanolic HCl added, when the product dissolved. The solution was stirred for a further hour and dry ether (50 ml.) then added. The precipitate, consisting of reduced ester and inorganic material, was centrifuged, washed twice with dry ether and freed from ether *in vacuo*.

Hydrolysis of product of reduction. The product obtained on reduction of bacitracin ester with $LiBH_4$ was hydrolysed in 6 N-HCl at 105° for 24 hr., or in 12 N-HCl at 80° for 43 hr. (concn. about 20 mg./ml.). The resulting solutions were evaporated to dryness *in vacuo* in the presence of solid NaOH.

Desalting and preliminary separation of products of hydrolysis. (*a*) Neutral amino acids and hydroxyamino acids were separated from other products in a small amount of hydrolysate by electrophoresis on a sheet of Whatman no. 1 paper (14 v/cm. for 2·5 hr.) in collidine acetate buffer (0·05 M to acetate), pH 7·0. The apparatus

used was similar to that of Flynn & de Mayo (1951). About 2·5 mg. of hydrolysate was applied as a series of spots to the sheet (16 in. wide) and after electrophoresis the material showing no net charge at pH 7·0 was located and eluted with water as described by Lockhart & Abraham (1954).

(*b*) A larger amount (50 mg.) of hydrolysate was dissolved in about 2 ml. of water and added to a column (10 cm. × 0·9 cm. diam.) of Dowex-50 × 4 in the hydrogen form. Water (15 ml.) was passed through the column to remove inorganic anions. The resin was then stirred in a beaker with water (about 30 ml.) and aq. 2N-NH_3 soln. was added until the pH rose to about 9·5. The resin was separated by centrifuging and the supernatant fluid, containing mainly acidic and neutral amino acids and hydroxyamino acids, was evaporated *in vacuo*. The resin was again stirred with water (30 ml.) and 0·3N-$Ba(OH)_2$ added until the pH rose to about 11·5. The resulting eluate contained basic amino acids and other bases which were ninhydrin-positive. Ammonia was removed from the eluate by distillation *in vacuo*.

(*c*) The eluate obtained from Dowex-50 between pH 9·5 and 11·5 was added to a column (8·5 cm. × 0·9 cm.) of Dowex-2 × 8. The column had been first washed with 2N-NaOH until chloride-free and then with CO_2-free water until the percolate was neutral (cf. Rees, 1958). Bases with no acidic function passed through the column when the latter was washed with CO_2-free water and emerged in the first 14 ml. of percolate. Basic amino acids were subsequently eluted with N-HCl.

Examination of products by electrophoresis and chromatography on paper. Electrophoresis on Whatman no. 1 paper (14v/cm. for 2·5 hr.) was carried out as described by Newton & Abraham (1954) in collidine acetate buffer (0·05M to acetate), pH 7·0, and in 10% (v/v) acetic acid, pH 2·3. Paper chromatograms were run on Whatman no. 1 paper in the following solvent systems: (*A*) butan-1-ol–acetic acid–water (4:1:4, by vol.) (Woiwod, 1949); (*B*) butan-1-ol–acetic acid–water (4:1:5, by vol.) (Partridge, 1948); (*C*) butan-2-one–propionic acid–water (15:5:6, by vol.) (Clayton & Strong, 1954); (*D*) triethylamine–acetone–water (1:16:3, by vol.) (Wright & Stadtman, 1956); (*E*) AnalaR phenol (75 g.)–water (21 ml.)–aq. NH_3 soln. (sp.gr. 0·88; 4·6 ml.) (Underwood & Rockland, 1954). Systems *A*, *B* and *E* were freshly prepared for each run. In system *C* the solvent began to drip from the end of the paper after 10 hr., but development of the chromatogram was continued for a total of 20 hr. After runs in systems *D* and *E* the papers were dried at room temperature in a stream of air. In general, 5–10 μg. of individual amino acids or hydroxyamino acids and 50–100 μg. of peptide hydrolysates were applied to the paper. Spots were revealed by ninhydrin.

RESULTS

Esterification of bacitracin

When subjected to electrophoresis on paper at pH 7·0, bacitracin A migrated as a single spot 2·2 cm. towards the cathode. (Under similar conditions glycine was carried 1·0 cm. towards the cathode by endosmosis.) The methyl ester of bacitracin A migrated 8·2 cm. towards the cathode at pH 7·0, and showed no evidence of contamination with smaller peptides or amino acids. The ultraviolet-absorption spectrum of the methyl ester hydrochloride of bacitracin A was very similar to that of bacitracin A itself (cf. Newton & Abraham, 1953*b*), indicating that the thiazoline ring had remained intact during the treatment with methanolic HCl.

The sample of commercial bacitracin used separated into two main components when subjected to electrophoresis on paper at pH 7·0. One component was indistinguishable from bacitracin A. The other behaved as though it had no net charge and was probably a mixture of bacitracin E and F (Newton & Abraham, 1953*a*; Craig, Weisiger, Hausmann & Harfenist, 1952). Esterification of commercial bacitracin yielded a product which also gave two main spots on electrophoresis at pH 7·0. One spot was found in the position occupied by the methyl ester of bacitracin A and one had migrated less far (5·5 cm.) towards the cathode.

The amounts of ammonia and amide nitrogen found in the different peptides are given in Table 1. This shows that about one-third of the amide nitrogen was removed during the course of esterification.

Identification of the products of reduction of aspartic acid and glutamic acid residues by chromatography and electrophoresis on paper

An aspartic acid residue that is α-linked in a peptide chain, or present as a *C*-terminal α-amide, should give γ-hydroxy-α-aminobutyric acid (II) on esterification and subsequent reduction with $LiBH_4$. Aspartic acid that is β-linked, or present

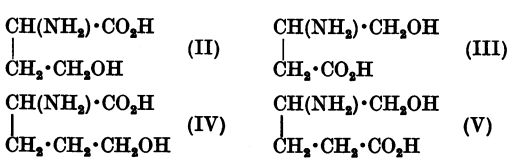
Table 1. *Ammonia and amide nitrogen in bacitracin and its methyl ester*

Estimations of amide nitrogen were done in triplicate on each sample. For methods of estimation see text.

Product	Equiv./1638 g. of product*	
	Ammonia N	Amide N
Bacitracin A hydrochloride	0·04	0·91
		0·88
		0·90
Bacitracin A methyl ester hydrochloride	0·28	0·59
		0·55
		0·64
Bacitracin	0·16	1·02
		1·08
		1·06
Bacitracin methyl ester hydrochloride	0·37	0·69
		0·68

* 1638 g. has been chosen as being close to the molecular weight of the hydrated bacitracin A hydrochloride isolated after countercurrent distribution (cf. Lockhart, Abraham & Newton, 1955). Provided that the methyl ester hydrochloride has the same degree of hydration, its molecular weight should not differ from this value by more than 3%.

as a *C*-terminal asparagine residue, should give γ-hydroxy-β-aminobutyric acid (III), and a *C*-terminal aspartic acid should give aspartidiol. δ-Hydroxy-α-aminovaleric acid (IV) should be obtained from a glutamic acid residue that is α-linked and δ-hydroxy-γ-aminovaleric acid (V) from one that is γ-linked.



The behaviour of the four hydroxyamino acids on paper chromatograms in different solvent systems (see Experimental section) is shown in Table 2. The compounds were separated into pairs in systems *A*, *B* and *E*, but the resolution of all four compounds was achieved only in system *C*. γ-Hydroxy-α-aminobutyric acid and δ-hydroxy-α-aminovaleric acid were not widely separated in system *C* but could readily be distinguished in system *D*. Distinction of the hydroxyamino acids from the neutral amino acids of bacitracin A presented no difficulty (Table 2).

Chibnall, Haselbach, Mangan & Rees (1958) found that γ-hydroxy-β-aminobutyric acid lactonized readily in acid solution. A sample of each of the four hydroxyamino acids was therefore heated at 100° in 6N-HCl for 1 min., the solution evaporated to dryness *in vacuo* and the residue tested for the presence of a lactone by electrophoresis on paper at pH 7. γ-Hydroxy-β-aminobutyric acid, γ-hydroxy-α-aminobutyric acid and δ-hydroxy-α-aminovaleric acid yielded spots which could be attributed to lactones (Table 2). The finding that the lactone of γ-hydroxy-α-aminobutyric acid migrated towards the cathode much more slowly than the lactone of γ-hydroxy-β-aminobutyric acid and somewhat more slowly than the lactone of δ-hydroxy-α-aminovaleric acid was not surprising, since the pK_a of the amino group (and thus its fractional positive charge at pH 7·0) should be lower the nearer the position of the amino group to the $-CO_2^-$ group. Under the conditions used here, about half of the γ-hydroxy-β-aminobutyric acid was converted into its lactone. The lactone from γ-hydroxy-α-aminobutyric acid was formed in smaller yield and appeared to be less stable. The lactone from δ-hydroxy-α-aminovaleric acid was detected only in trace amounts and appeared to undergo progressive hydrolysis during its migration at pH 7·0.

Products formed on reduction and hydrolysis of esterified bacitracin A

Esterification of bacitracin A and commercial bacitracin respectively, followed by reduction with $LiBH_4$ and hydrolysis, led to products which gave very similar patterns when subjected to electrophoresis or chromatography on paper. In their content of lysine, ornithine, histidine, leucine, isoleucine and phenylalanine these products resembled hydrolysates of bacitracin A itself, but they differed from the latter in containing no detectable amount of cystine. They also differed from hydrolysates of bacitracin A (and from hydrolysates of a sample of bacitracin A which had been treated with $LiBH_4$ without previous esterification) in several other respects.

A notable feature of the pattern obtained from hydrolysates of the reduced peptide ester on electrophoresis at pH 7·0 was the absence of a detectable spot in the position occupied by glutamic acid (Fig. 1). This was correlated with the presence, in the neutral fraction of the hydrolysate, of a substance which behaved as δ-hydroxy-α-aminovaleric acid on chromatography in systems *A*, *B*, *C* and *D*, and on electrophoresis at pH 2·3.

Table 2. *Chromatography and electrophoresis on paper of hydroxyamino acids and of neutral amino acids from bacitracin A*

For details of the solvent systems used in chromatography see text. Electrophoresis was for 2·5 hr. with a potential gradient of 14 v/cm.

Substance	R_F in system				R_{Val} in system	Migration (cm.) towards cathode	
	A	*B*	*D*	*E*	*C*	pH 7·0	pH 2·3
γ-Hydroxy-α-aminobutyric acid (γ-α-HAB)	0·24	0·23	0·47	0·56	0·50	1·0	7·4
γ-Hydroxy-β-aminobutyric acid (γ-β-HAB)	0·31	0·28	0·41	0·60	0·71	1·0	15·0
δ-Hydroxy-α-aminovaleric acid (δ-α-HAV)	0·26	0·27	0·35	0·68	0·55	1·0	7·4
δ-Hydroxy-γ-aminovaleric acid (δ-γ-HAV)	0·36	0·35	0·43	0·70	0·96	1·0	15·0
γ-α-HAB lactone*	0·39	—	—	—	—	7·5	20·0
γ-β-HAB lactone†	0·32	0·33	—	—	1·09	13·0	—
δ-α-HAV lactone	—	—	—	—	—	9·0	—
Aspartidiol	0·36	0·35	0·57	—	>1·5	14·8	17·3
Leucine and isoleucine	0·67	0·61	0·48	—	1·32	1·0	7·2
Phenylalanine	0·63	0·56	0·48	—	1·32	1·0	6·6

* Grey colour with ninhydrin. † Yellow colour with ninhydrin.

When the reduced peptide ester was hydrolysed in 12N-HCl for 43 hr. at 80° it gave a product which was shown by electrophoresis at pH 7·0 to contain ε-(aminosuccinyl)lysine but only a trace of aspartic acid. When hydrolysis was carried out in 6N-HCl for 24 hr. at 105° the product contained about half as much aspartic acid as did a similar hydrolysate of bacitracin A (Fig. 1). Corresponding with the missing aspartic acid, a substance was present in the neutral fraction of the hydrolysate which behaved as γ-hydroxy-α-aminobutyric acid on chromatography in systems *A*, *B*, *C* and *D*, and on electrophoresis at pH 2·3. Treatment of the neutral fraction with 6N-HCl at 100° for 1 min. resulted in the formation of a substance which behaved as the lactone of γ-hydroxy-α-aminobutyric acid on electrophoresis at pH 7·0.

The neutral fraction of hydrolysates of reduced bacitracin A ester contained two further substances not found in hydrolysates of bacitracin A. One was indistinguishable from proline in systems *A*, *B*, *C* and *D*, and gave a bright-blue colour identical with that given by proline when the paper was sprayed with a solution of isatin in acetone (Smith, 1953). The other, which showed an R_F value similar to that of alanine in systems *A* and *B* but was distinguished from alanine by chromatography in system *C*, was not identified.

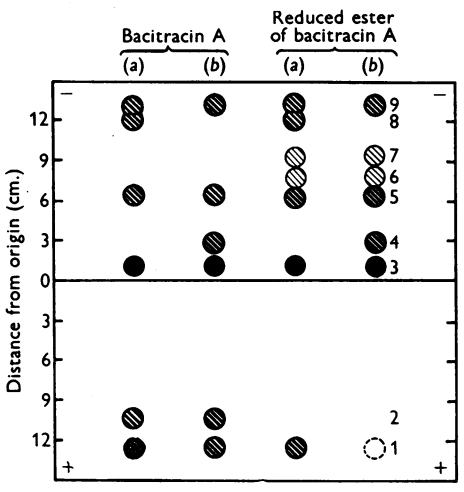


Fig. 1. Electrophoresis on paper at pH 7·0 (14v/cm., 2·5 hr.) of hydrolysates of bacitracin A and the reduced ester of bacitracin A respectively. The depth of shading represents the approximate intensity of the spots revealed by ninhydrin. (*a*) Hydrolysis in 6N-HCl, at 105° for 24 hr.; (*b*) hydrolysis in 12N-HCl at 80° for 43 hr. 1, Aspartic acid; 2, glutamic acid; 3, neutral amino acids and hydroxyamino acids; 4, ε-(aminosuccinyl)lysine; 5, histidine; 6, γ-hydroxy-α-aminobutyric acid lactone; 7, δ-hydroxy-α-aminovaleric acid lactone; 8, lysine; 9, ornithine.

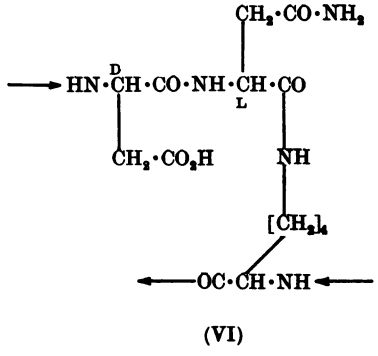
Small amounts of several new basic substances were revealed when hydrolysates of reduced bacitracin A ester were subjected to electrophoresis at pH 7·0. Two of these substances, which migrated less rapidly than lysine towards the cathode, corresponded to the lactones of γ-hydroxy-α-aminobutyric acid and δ-hydroxy-α-aminovaleric acid respectively (Fig. 1). The remaining substances, which migrated further than ornithine, could have consisted of some aspartidiol together with products of the reductive fission of the thiazoline ring and perhaps of peptide bonds. Basic substances with no acidic function were separated from other components of the hydrolysate by passage through Dowex-2. These substances, however, were obtained only in small yield and no definite conclusions about their identity could be reached after they had been examined by chromatography or electrophoresis on paper.

DISCUSSION

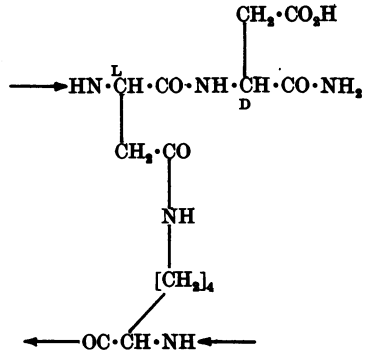
The absence of glutamic acid from hydrolysates of reduced bacitracin A ester indicates that the D-glutamic acid residue in bacitracin A does not itself carry the single amide group of the bacitracin A molecule, while the presence of δ-hydroxy-α-aminovaleric acid and small amounts of proline in these hydrolysates indicates that the glutamic acid residue is α-linked in the peptide chain and that its γ-carboxyl group is free. Proline was shown by Sörensen, Höyrup & Andersen (1911) to be formed from δ-hydroxy-α-aminovaleric acid in the presence of hot concentrated hydrochloric acid.

The presence of ε-(aminosuccinyl)lysine in the product obtained by hydrolysing reduced bacitracin A ester in 12N-hydrochloric acid at 80° indicates that neither of the two carboxyl groups of the L-aspartic acid residue in bacitracin A is free. One of these groups is involved in peptide linkage with the ε-amino group of lysine; the other could therefore be present as an amide or be joined to the residue of D-aspartic acid as shown in I and VII. In either case, one carboxyl group of the D-aspartic acid residue in bacitracin A would be free, and the finding that no significant amount of aspartic acid is liberated when the reduced ester is treated with 12N-hydrochloric acid at 80° would be understandable. The formation of γ-hydroxy-α-aminobutyric acid on hydrolysis of reduced bacitracin A ester thus leads to the conclusion that the β-carboxyl group of the D-aspartic acid residue in bacitracin A is free.

These findings can be accommodated by VI or VII, or by one of two corresponding structures in which the β- and α-carboxyl groups respectively of L-aspartic acid are linked to the ε-amino group of lysine. Thus in a structure of type VI the L-aspartic



(VI)



(VII)

acid would carry the amide group and could be either asparagine or *iso*asparagine. In a structure of type VII the D-aspartic acid would carry the amide group and be present as *iso*asparagine.

The esterification of bacitracin was accompanied by a significant loss of amide nitrogen and it therefore seemed likely that some of the amide group had been converted into an ester. Reduction of an ester grouping formed in this way and hydrolysis of the product should have yielded additional γ-hydroxy-α-aminobutyric acid from VI but some γ-hydroxy-β-aminobutyric acid from the corresponding structure in which the L-aspartic acid was β-linked to lysine. No γ-hydroxy-β-aminobutyric acid was in fact detected. With VII, or the corresponding structure in which the L-aspartic acid was α-linked to lysine, reduction of the new ester should have resulted in the formation of some aspartidiol. No clear evidence for or against the presence of significant amounts of aspartidiol in hydrolysates of the reduced peptide was obtained. Further information about this part of bacitracin A might be gained by hydrolysis of the amide group before esterification and reduction of the peptide, but it is not certain that the amide nitrogen can be removed without the occurrence of a transformation in the molecule (Craig, Konigsberg & Hill, 1958) and such a procedure would be unlikely to facilitate a decision between VII and the corresponding structure in which L-aspartic acid is α-linked to lysine.

The present work indicates therefore that the amide group in bacitracin A is associated with an aspartic acid residue and reduces the number of possible arrangements in this portion of the molecule from 18 to four, but it does not give positive support to any single structure of the four. It can be seen, however, that if either VI or VII was correct bacitracin A could be formed by two condensations in a linear dodecapeptide whose amino acids were linked by classical α-peptide bonds: condensation in an *N*-terminal isoleucyl-cysteinyl fragment could lead to the thiazoline ring and condensation of an aspartic acid carboxyl group with the ϵ-amino group of lysine could yield a cyclopeptide structure of six or seven residues.

SUMMARY

1. Bacitracin A and commercial bacitracin have been esterified by treatment with methanolic hydrogen chloride. About one-third of the amide nitrogen was removed during esterification.

2. The esterified peptides have been reduced with lithium borohydride and the products hydrolysed. The composition of the hydrolysates has been compared with that of similar hydrolysates of bacitracin A by chromatography and electrophoresis on paper.

3. Hydrolysates of the reduced peptide esters contained δ-hydroxy-α-aminovaleric acid and small amounts of proline, but no glutamic acid.

4. Hydrolysis of the reduced peptide esters with 12N-hydrochloric acid at 80° yielded ϵ-(amino-succinyl)lysine but only traces of aspartic acid. Hydrolysis with 6N-hydrochloric acid at 105° yielded aspartic acid equivalent to one residue and γ-hydroxy-α-aminobutyric acid.

5. It is concluded from these results that the glutamic acid residue in bacitracin A is α-linked and that its γ-carboxyl group is free. It is also concluded that one of the aspartic acid residues in bacitracin A is present as an amide and that both carboxyl groups of the aspartic acid directly linked to the ϵ-amino group of lysine are bound. Alternative structures consistent with these conclusions are discussed.

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Studies in the Biochemistry of Micro-organisms

106. METABOLITES OF *ALTERNARIA TENUIS* AUCT.: THE STRUCTURE OF TENUAZONIC ACID*

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Rosett, Sankhala, Stickings, Taylor & Thomas (1957) described the isolation, from culture filtrates of *Alternaria tenuis* auct. grown on a glucose medium, of a number of metabolic products. Several of these appeared to be related to alternariol, a mycelial product formed by the same strains, and shown by Raistrick, Stickings & Thomas (1953) to be 3:4′:5-trihydroxy-6′-methyldibenzo-α-pyrone. The exception was tenuazonic acid, $C_{10}H_{15}O_3N$, the only nitrogen-containing metabolite among the new products; this was quite different in properties. This paper describes the work carried out on this acid and advances a structural formula. Rosett *et al.* (1957) described tenuazonic acid as an optically active viscous liquid, giving a strong orange–red ferric reaction and a green complex copper salt. It behaved as a monoketone and a monobasic acid, and was converted into an optical isomer on boiling with aqueous alkali, or on long keeping.

It has now been shown that hydrolysis of tenuazonic acid with 0·1 N-mineral acid gives good yields of acetic acid and a colourless crystalline monobasic acid, m.p. 117·5–119·0°, which has the formula $C_8H_{13}O_2N$. This substance, shown below to be an epimeric mixture, will be called the deacetyl compound; it yields a mono-2:4-dinitrophenylhydrazone. In addition, tenuazonic acid gives iodoform with alkaline iodine. These reactions show the presence of both a $CH_3 \cdot CO$ and a CO group in the molecule.

Hydrolysis of tenuazonic acid with 2 N-mineral acid gives high yields of acetic acid, carbon dioxide and a basic compound, isolated as a benzenesulphonyl derivative, $C_7H_{14}ON \cdot SO_2C_6H_5$, m.p. 123·5–125·0°, which also has ketonic properties.

* Part 105: Birkinshaw & Chaplen (1958).