The pig is not known to form deoxycholic or cholic acids, or any bile acid hydroxylated at C-12. No other mammal has so far been shown to make (normally) any bile salt hydroxylated at C-6; the only known examples of creatures having this ability are certain teleostean fish and the domestic hen (Ohta, 1939; Isaka, 1940; Isaka & Azato, 1940; Mabuti, 1941; Yamasaki, 1951).

Thus pig bile salts are unique in a special way. This uniqueness must be due to (a) mutation occurring in ancestors having already the cholesterol $\rightarrow$ cholic acid sequence (or part of it), or (b) descent from a very primitive form originally making C-6-hydroxylated bile salts. A decision between possibilities (a) and (b) obviously cannot be made except by consideration of all the available evidence, but when the evolutionary position of the pig is ascertained its relationship to that of other vertebrates will have to account for the remarkable chemical nature of pig's bile salts. In considering this question, it should be noted that the general trend of the present work suggests that the chemical nature of bile salts is of a very conservative character.

SUMMARY

1. Hyocholic acid, $C_{24}H_{40}O_5$, from pig bile has been shown to be 3 α :6 α :7-trihydroxycholanic acid (II); the hydroxyl group at C-7 is probably α -orientated.

2. This substance appears to be the first authentic example of a 6:7-dihydroxycholane: the presence of the hydroxyl group at C-6 in this cholane derivative causes marked hindrance in the reactions of the C-7 hydroxyl group and of the ketone derived from it.

3. Pig bile contains at least one unidentified substance, giving a spot on paper chromatograms of the ethylated 'bile acids'.

4. Attention is drawn to the remarkable chemical nature of pig bile salts, and it is suggested that this unique nature must be taken into consideration in studies of pig evolution.

The author wishes to thank Mr A. Baker for collections of fresh pig bile, and Dr I. D. P. Wootton for the infrared spectral examination.

REFERENCES

- Anchel, M. & Schoenheimer, M. (1938). *J. biol. Chem.* **124**, 609.
 Barton, D. H. R. & Klyne, W. (1948). *Chem. & Ind.* p. 755.
 Bush, I. E. (1952). *Biochem. J.* **50**, 370.
 Dane, E. & Wulle, H. (1941). *Hoppe-Seyl. Z.* **267**, 1.
 Ekdahl, P.-H. & Sjövall, J. (1955). *Acta physiol. scand.* **34**, 329.
 Fernholz, E. (1935). *Hoppe-Seyl. Z.* **232**, 202.
 Haslewood, G. A. D. (1943). *Biochem. J.* **37**, 109.
 Haslewood, G. A. D. (1954a). *Biochem. J.* **56**, xxxviii.
 Haslewood, G. A. D. (1954b). *Biochem. J.* **56**, 581.
 Haslewood, G. A. D. (1955). *Physiol. Rev.* **35**, 178.
 Haslewood, G. A. D. & Sjövall, J. (1954). *Biochem. J.* **57**, 126.
 Haslewood, G. A. D. & Wootton, V. (1950). *Biochem. J.* **47**, 584.
 Haslewood, G. A. D. & Wootton, V. M. (1951). *Biochem. J.* **49**, 67.
 Hoehn, W. M., Linsk, J. & Moffett, R. B. (1946). *J. Amer. chem. Soc.* **68**, 1855.
 Hoehn, W. M., Schmidt, L. H. & Hughes, H. B. (1944). *J. biol. Chem.* **152**, 59.
 Ido, T. & Sakurai, R. (1939). *J. Biochem., Tokyo*, **29**, 51.
 Isaka, H. (1940). *Hoppe-Seyl. Z.* **266**, 117.
 Isaka, H. & Azato, M. (1940). *J. Biochem., Tokyo*, **32**, 241.
 Kimura, T. (1937). *Hoppe-Seyl. Z.* **248**, 280.
 Lardon, A. (1947). *Helv. chim. acta*, **30**, 597.
 Mabuti, H. (1941). *J. Biochem., Tokyo*, **33**, 143.
 Ohta, K. (1939). *Hoppe-Seyl. Z.* **259**, 53.
 Shimizu, K. (1953). *J. Biochem., Tokyo*, **40**, 69.
 Shoppee, C. W. (1949). *J. chem. Soc.* p. 1671.
 Takeda, K., Igarashi, K. & Komeno, T. (1954a). *Pharm. Bull., Tokyo*, **2**, 348.
 Takeda, K., Komeno, T. & Igarashi, K. (1954b). *Pharm. Bull., Tokyo*, **2**, 352.
 Trickey, E. B. (1950). *J. Amer. chem. Soc.* **72**, 3474.
 Wieland, H. & Dane, E. (1932). *Hoppe-Seyl. Z.* **210**, 268.
 Windaus, A. (1926). *Liebigs Ann.* **447**, 233.
 Windaus, A. & Bohne, A. (1923). *Liebigs Ann.* **433**, 278.
 Yamasaki, K. (1951). *J. Biochem., Tokyo*, **38**, 93.

Experiments on the Lysine and Aspartic Acid Residues in Bacitracin A

BY I. M. LOCKHART AND E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford

(Received 19 September 1955)

In several structural features the bacitracin family appears to be unique among polypeptides that have so far been subjected to detailed chemical study. The *N*-terminal and sulphur-containing residues in bacitracin A have been discussed in an earlier paper (Lockhart, Abraham & Newton, 1955). In addition to this portion of the molecule, the lysine residue is a centre of interest.

The earliest experiments on the reaction of bacitracin A with 1-fluoro-2:4-dinitrobenzene (FDNB) revealed that neither of the two amino groups of the lysine residue in the molecule was free (Craig, Hausmann & Weisiger, 1952; Newton & Abraham, 1953). Studies of the amino acid sequence in bacitracin A showed that the probable arrangement of residues in the vicinity of the lysine was

that shown below (I), in which $\rightarrow$ denotes a C-N bond (Craig, Haussmann & Weisiger, 1954; Lockhart & Abraham, 1954a).

Some of the simple peptides containing lysine that were obtained from bacitracin A were remarkably resistant to hydrolysis, surviving prolonged treatment with 11N hydrochloric acid at 80° (Lockhart & Abraham, 1954b). The results of experiments that were made possible by this stability are described in the present paper.

METHODS

Bacitracin used for hydrolysis

Bacitracin A. The sample of bacitracin A was that described by Lockhart & Abraham (1954a).

Commercial bacitracin. Commercial bacitracin (lot 25, 61 i.u./mg. and lot B-55-13, 68 i.u./mg.) was supplied by the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A.

Deaminated bacitracin A. This was prepared by treating bacitracin A with nitrous acid as described by Lockhart *et al.* (1955).

Desulphurized bacitracin. This was prepared by treating commercial bacitracin with Raney nickel as described by Lockhart *et al.* (1955).

Partial hydrolysis and separation of peptides

Hydrolysis. Samples of bacitracin were hydrolysed in 11N-HCl (20-40 mg./ml.) in sealed tubes at 80° for times varying from 21 to 91 hr. The hydrolysates were then evaporated to dryness *in vacuo*.

Ionophoresis in a four-compartment cell. In some experiments a preliminary separation of the acidic, neutral and basic material in hydrolysates was made in a four-compartment cell similar to that described by Synge (1951).

Ionophoresis on paper. Peptides were separated by ionophoresis on sheets of Whatman no. 1 paper (36 cm. long) and eluted from the paper in the manner described by Lockhart & Abraham (1954a). Collidine-acetate buffer, pH 7, was used (Newton & Abraham, 1954) and the potential gradient was 14 v/cm. Ionophoresis was generally carried out for 8 hr., but in some analytical experiments the time was shortened to 2.5 hr. The spots were located by spraying with ninhydrin.

Paper chromatograms. Peptides were also separated by chromatography on Whatman no. 1 paper, with butanol-acetic acid (Woiwod, 1949) as solvent system. The peptides were eluted from the paper in the manner described by Lockhart & Abraham (1954a).

Analysis of peptides

Peptides which appeared to be homogeneous were hydrolysed in 6N-HCl at 110° for 20 hr. Qualitative analysis of the resulting amino acids was made by chro-

matography and ionophoresis on paper. Isoleucine was distinguished from leucine on chromatograms run with the *tert.*-pentanol-water system of Work (1949). Lysine was distinguished from ornithine by ionophoresis under the conditions described by Lockhart & Abraham (1954a). The other amino acids encountered could be distinguished on chromatograms run with butanol-acetic acid (Woiwod, 1949).

The *N*-terminal amino acids in the peptides were determined by the method of Sanger & Thompson (1953), the experiments being carried out under the conditions used by Lockhart & Abraham (1954a). The ether-soluble dinitrophenyl (DNP)-amino acids were run on paper chromatograms, the system of Blackburn & Lowther (1951) being used.

Separation of α -DNP-lysine and ϵ -DNP-lysine. The water-soluble α -DNP-lysine and ϵ -DNP-lysine could easily be distinguished by ionophoresis on paper in 0.02M sodium borate buffer, pH 9.1. A potential gradient of 14 v/cm. was applied to the paper for 16 hr. At the end of the experiment α -DNP-lysine had migrated 9.3 cm. towards the cathode and ϵ -DNP-lysine 4.9 cm. towards the cathode. At pH 9.1 both these substances would be expected to carry a net negative charge. Their migration towards the cathode is presumably due to the endosmotic flow in the system used. However, the *pK* of the free amino group of α -DNP-lysine should be higher than that of ϵ -DNP-lysine, because the amino group in the former compound is further removed from the influence of the carboxyl group. Hence the net negative charge of α -DNP-lysine should be less than that of ϵ -DNP-lysine and the former should be carried further towards the cathode. A similar phenomenon was demonstrated with leucine and 6-aminohexanoic acid in the same system. Leucine migrated 8 cm. and 6-aminohexanoic acid 10 cm. towards the cathode.

Optical configuration of aspartic acid residues

Estimation of aspartic and glutamic acids. The amounts of free aspartic and glutamic acids in bacitracin A that had been hydrolysed with 11N-HCl at 80° for 43 hr. were determined on a column of Dowex 50 $\times$ 8 (100 cm. $\times$ 0.9 cm.) by the procedure of Moore & Stein (1951). About 4.5 mg. of hydrolysate was applied to the column. The experiment was terminated after the emergence of glutamic acid.

D-Aspartic acid oxidase. This was prepared as described by Still, Buell, Knox & Green (1949). The kidneys from three rabbits yielded 2.5 g. of an acetone-dried powder. The enzyme solution used in the following experiments was prepared by stirring the powder with 10 parts by weight of 0.1M sodium phosphate buffer, pH 7.2, and removing the solid by centrifuging.

Uptake of oxygen by aspartic acid in the presence of D-aspartic acid oxidase. This was measured with a Warburg respirometer at 37°. The oxidation was carried out in the presence of ethanol, since under these conditions the rate of O₂ uptake is about doubled (Keilin & Hartree, 1936; Still *et al.* 1949). The main compartment of each vessel contained 1 ml. of enzyme solution, 0.4 ml. of 0.1M sodium phosphate buffer, pH 7.2, and 0.4 ml. of 4% (v/v) ethanol. The centre well contained 0.2 ml. of 10N-NaOH. The sample of the amino acid (1-3 mg.) in 0.4 ml. of 0.1M sodium phosphate buffer, pH 7.2, was placed in the side arm. The vessels were filled with O₂.

Under the conditions used about 50 μ l. of O_2 was absorbed in 70 min. in the presence of 1 mg. of DL-aspartic acid, 100 μ l. in the presence of 2.0 mg. and 140 μ l. in the presence of 3.0 mg. The O_2 uptake after 70 min. was used to estimate the amount of D-aspartic acid in mixtures of aspartic acid (1.5–3.0 mg.) and glutamic acid (0–1.5 mg.) obtained from bacitracin. No absorption of oxygen occurred in the presence of L-aspartic acid (3 mg.) or DL-glutamic acid (3 mg.).

Isolation of aspartic and glutamic acids. Aspartic and glutamic acids were isolated from hydrolysates obtained by treating bacitracin (5 g.) with 11N-HCl at 80° and 6N-HCl at 110°. After removal of the HCl *in vacuo* a preliminary fractionation of the hydrolysate was carried out by ionophoresis in the four-compartment cell. The acidic fraction (200–700 mg.) was chromatographed on a column (1.5 cm. $\times$ 34 cm.) of Dowex 1 $\times$ 10 acetate in 0.5N acetic acid (cf. Hirs, Moore & Stein, 1954). The effluent was collected in 2 ml. fractions with a flow rate of 8 ml./hr. Samples (10 μ l.) were removed from every third fraction for the determination of ninhydrin colour densities (Moore & Stein, 1954). The fractions containing glutamic acid (usually 22–36) were combined and concentrated in a rotary evaporator (Craig, Gregory & Hausmann, 1950) to about 2 ml., when the product crystallized. Ethanol (6 ml.) was added, the mixture cooled in ice and the glutamic acid removed by filtration. The fractions containing aspartic acid (usually 90–130) were treated similarly. The products behaved like pure glutamic acid and aspartic acid respectively when chromatographed on paper in butanol-acetic acid.

RESULTS

Peptides from bacitracin A

The results obtained when samples of bacitracin A that had been hydrolysed with 11N hydrochloric acid at 80° for periods varying from 21 to 91 hr. were examined by ionophoresis on paper at pH 7 are shown in Fig. 1. It is clear that large amounts of the free acidic and basic amino acids were liberated under the conditions of hydrolysis, with the notable exception of lysine. Even after hydrolysis for 43 hr. there appeared to be no significant quantity of free lysine present. Only three spots which could be assigned to peptides were then detected by ionophoresis on paper (Fig. 1, spots 1–3). A fourth peptide, which was neutral, was revealed by chromatography on paper in butanol-acetic acid, when it showed an R_f value greater than that of leucine.

When the time of hydrolysis varied from 21 to 31 hr., two spots, other than 1–3 and those due to free amino acids, were revealed by ionophoresis on paper (Fig. 1). The positions of these spots (cf. Lockhart & Abraham, 1954a) suggested that the one between histidine and spot 3 was due to Orn.Ileu.Phe, and that the one between the neutral material and glutamic acid was Leu.Glu.

An analysis of peptides 1–4 is given in Table 1, the results being recorded in the manner used by Lockhart & Abraham (1954a). Peptides 1–3 were

separated by ionophoresis on paper and peptide 4 was eluted from a paper chromatogram developed with butanol-acetic acid. The peptides appeared to be Asp (Ileu).Lys, Asp.Lys, [Ileu, Lys] and Ileu.Phe respectively. The finding of Hausmann, Weisiger & Craig (1955) that Ileu.Phe (peptide 4) gives a poor colour yield with ninhydrin was confirmed.

Fig. 1. Ionophoresis on paper at pH 7 for 2.5 hr. of products formed from bacitracin A in 11N-HCl at 80°. The depth of shading represents the approximate intensity of the spots obtained when the paper was sprayed with ninhydrin.

Peptides from deaminated bacitracin A

An experiment in which a sample of deaminated bacitracin A was treated with 11N hydrochloric acid at 80° for 43 hr. yielded the four peptides that were obtained under similar conditions from bacitracin A. The phenylalanine obtained by hydrolysis of peptide 4, however, was accompanied by a small quantity of material which gave an unidentified grey-blue spot (R_f , 0.69 in butanol-acetic acid). The colour of the spot was similar to that given by phenylalanine with ninhydrin. A similar spot was obtained in earlier work on the amino acid sequence in bacitracin A (Lockhart & Abraham, 1954a).

After elution from a chromatogram and hydrolysis with 6*N* hydrochloric acid at 110° the substance responsible for this spot appeared to be unchanged, indicating that it was not a peptide.

Peptides from commercial bacitracin

Commercial bacitracin. Partial hydrolysis of bacitracin (lot 25) in 11*N* hydrochloric acid at 80° for 43 hr. again yielded peptides 1–4. In this experiment, a sufficient amount of peptide 3 (Ileu.Lys) was obtained to carry out an analysis for free amino groups. After hydrolysis of the DNP-peptide with 6*N* hydrochloric acid at 110° for 9 hr. in a sealed tube under nitrogen, ϵ -DNP-lysine was identified in the water-soluble material and the ether-soluble fraction yielded DNP-isoleucine. The ether-soluble fraction, unlike the corresponding fraction from peptides 1 and 2 (Table 1), showed no green fluorescent spot at the origin on paper chromatograms which were run under the conditions described by Blackburn & Lowther (1951) and viewed under ultraviolet light (365 m μ).

the remaining bound aspartic acid, which could be liberated by further hydrolysis with 6*N* hydrochloric acid at 110°, consisted almost entirely of the residue linked directly to lysine. A preliminary experiment was carried out in which D-aspartic acid oxidase was used to determine the proportion of D- and L-isomers in both the aspartic acid liberated by hydrolysis at 80° and in that liberated subsequently by hydrolysis at 110°. In a second experiment two samples of aspartic acid were isolated in crystalline form, and their specific rotations were measured.

Use of D-aspartic acid oxidase. Bacitracin (88 mg. of lot 25) was dissolved in 10 ml. of 11*N* hydrochloric acid and the solution kept at 80° for 43 hr. After removal of the hydrochloric acid *in vacuo* the hydrolysate was subjected to ionophoresis in a four-compartment cell. The acidic portion, consisting largely of free aspartic and glutamic acids, was named fraction A. The rest of the material, containing peptides of aspartic acid and lysine, was hydrolysed with 6*N* hydrochloric acid at 110° for 24 hr. The acidic material in this

Table 1. *Peptides from bacitracin A after hydrolysis with 11N hydrochloric acid at 80° for 43 hr.*

Spot no.	<i>R_F</i> value (butanol-acetic acid)	Migration (pH 7.0, 8 hr.) (cm.)	Amino acids present	Strength of amino acid after		DNP-amino acid identified	Structure
				Hydrolysis	DNP treatment		
1*	0.42	-6.0	Lys	++++	+++	Ileu	Asp.(Ileu).Lys
			Asp	++++	+	Asp†	
			Ileu	++++	.		
2	0.15	-7.75	Asp	++++	+	Asp†	Asp.Lys
			Lys	++++	++†		
3	0.39	-14	Ileu	+	.	.	[Ileu, Lys]
			Lys	+	.		
4	0.72	-2.0	Ileu	+++	+	Ileu	Ileu.Phe
			Phe	+++	+++		

* This spot gave a brown colour with ninhydrin.

† The DNP-Asp was accompanied by material which remained at the origin on paper chromatograms (Blackburn & Lowther, 1951) and showed a bright-green fluorescence in ultraviolet light (365 m μ).

‡ Identified as α -DNP-Lys.

Desulphurized bacitracin. The peptides Asp.(Ileu).Lys and Asp.Lys were also identified in partial hydrolysates of a sample of bacitracin (lot 25) that had been desulphurized by treatment with Raney nickel.

Optical configuration of the two aspartic acid residues in bacitracin

Bacitracin A contains one residue of glutamic acid and two residues of aspartic acid. Analysis on a column of Dowex 50 (Moore & Stein, 1951) of a hydrolysate obtained by treating bacitracin A with 11*N* hydrochloric acid at 80° for 43 hr. showed that at least 0.9 mole of glutamic acid, but only 1.34 moles of aspartic acid, were present in the free state. The work already described indicated that

hydrolysate consisted mainly of aspartic acid. After separation from the neutral and basic material by ionophoresis in the four-compartment cell it was named fraction B.

A further sample of bacitracin (76 mg. of lot 25) was hydrolysed with 3 ml. of 6*N* hydrochloric acid at 110° for 24 hr. The acidic amino acids (known from earlier work to consist of 1 mole of glutamic acid and 2 moles of aspartic acid) were separated from the remainder by ionophoresis in the four-compartment cell and the resulting mixture was named fraction C.

The total amino acid content of each of the fractions A–C was estimated from photometric ninhydrin determinations (Moore & Stein, 1948). When considered, together with the proportions in

which the two acidic amino acids were known to be liberated from bacitracin A on hydrolysis under the conditions used, these determinations indicated that the three fractions had the following composition: fraction A, 1.5 mg. of aspartic acid plus 1.0 mg. of glutamic acid; fraction B, 1.5 mg. of aspartic acid; fraction C, 3 mg. of aspartic acid plus 1.6 mg. of glutamic acid. The amount of D-aspartic acid in each fraction was estimated by comparing its rate of oxidation, in the presence of D-aspartic acid oxidase, with the rates of oxidation of control solutions containing known amounts of D-aspartic acid (see Methods). The results indicated that the aspartic acid in fraction A was $66 \pm 2\%$ D, whereas that in fraction B was $58 \pm 2\%$ L and that in fraction C was racemic.

Isolation of two samples of aspartic acid. Bacitracin (5 g. of lot B55-13) was hydrolysed in 125 ml. of 11N hydrochloric acid. Two fractions containing aspartic acid (corresponding to A and B of the preceding experiment) were isolated by a procedure similar to that already described. In the present case, however, fraction A consisted of acidic material liberated on hydrolysis for 20 hr. (instead of for 43 hr.) at 80°. After removal of fraction A, the remaining neutral material was hydrolysed for a further 24 hr. at 80°. The acidic material formed during this time was removed, by ionophoresis in the four-compartment cell, and discarded. The remainder of the sample was hydrolysed in refluxing 6N hydrochloric acid (190 ml.) for 24 hr., and the acidic material liberated during the hydrolysis represented fraction B. In a parallel experiment, 5 g. of bacitracin was first treated with 11N hydrochloric acid at 80° for 44 hr. and then with refluxing 6N hydrochloric acid for 24 hr. The acidic portion of the final hydrolysate represented fraction C.

The aspartic and glutamic acids in fractions A and C and the aspartic acid in fraction B (which contained almost no glutamic acid) were isolated after chromatographing the fractions on columns

of Dowex 1 acetate (see Methods). The amounts of crystalline amino acids that were obtained, and the specific rotations of these amino acids in N hydrochloric acid, are shown in Table 2. The results indicate that the aspartic acid in fraction A is 93% D, and that in fraction B is 76% L. The aspartic acid in fraction C is almost completely racemic.

The fact that bacitracin A yields D-glutamic acid on acid hydrolysis (see Table 2) has been reported previously. Glutamic acid which was isolated from bacitracin A that had been hydrolysed with 6N hydrochloric acid at 110° for 24 hr. showed $[\alpha]_D - 25^\circ$ in N-HCl (Craig *et al.* 1952).

DISCUSSION

With the exception of Ileu.Phe, which was first found by Barry, Gregory & Craig (1948) in hydrolysates of bacitracin A, all four peptides that were detected after treating bacitracin A with 11N hydrochloric acid at 80° for 43 hr. contained lysine. The small number of these peptides, and the fact that they differed sharply in isoelectric point or in size, made their separation by ionophoresis on paper at pH 7 a relatively simple matter. The formation of the three peptides containing isoleucine from deaminated bacitracin A, as well as from bacitracin A itself, confirms the conclusion (Lockhart & Abraham, 1954b) that neither the isoleucine residue linked to lysine nor that linked to phenylalanine occupies an N-terminal position.

The three peptides containing lysine behaved as though they were Asp(Ileu).Lys, Ileu.Lys and Asp.Lys respectively. The most resistant to hydrolysis by acid was Asp.Lys. The stability of the peptides may be due to the presence in them of two positively charged amino groups and of amino acids with large side chains. Both factors might hinder the approach of the hydroxonium ion which catalyses the hydrolysis of the peptide bond.

The separation of two dipeptides containing lysine as a C-terminal amino acid enabled the residues linked to each of the two amino groups of lysine to be determined by the use of FDNB. The experiments appeared to show clearly that an isoleucine residue was joined to the α -amino group of lysine and an aspartic acid residue to the ϵ -amino group.

The work of Craig *et al.* (1952) had shown that when all the aspartic acid was liberated from bacitracin A by hydrolysis with 6N hydrochloric acid at 110° the resulting product was racemic. The simplest explanation of this finding was that one residue had the D- and one the L-configuration. The picture obtained when samples of bacitracin A which had been treated with 11N hydrochloric acid at 80° for varying periods of time were subjected to ionophoresis on paper at pH 7 indicated

Table 2. *Specific rotations of aspartic and glutamic acids liberated from bacitracin under various conditions of hydrolysis*

The yields represent the amounts of amino acids isolated from 5 g. of bacitracin. Optical rotations were measured in N-HCl (c, 3.0). For details of hydrolysis and isolation see text. Fischer (1899) gives: D-aspartic acid, $[\alpha]_D^{20} - 25.5^\circ$ in 0.97N-HCl; D-glutamic acid, $[\alpha]_D^{20} - 30.05^\circ$ in 0.37N-HCl.

Fraction from hydrolysate	Aspartic acid		Glutamic acid	
	Yield (mg.)	$[\alpha]_D^{20}$	Yield (mg.)	$[\alpha]_D^{20}$
A	144	$-22 \pm 0.5^\circ$	159	$-29 \pm 0.5^\circ$
B	151	$+13.5 \pm 0.5^\circ$	—	—
C	512	$-1 \pm 0.5^\circ$	274	$-23 \pm 0.5^\circ$

that one of the aspartic acid residues rapidly appeared as free aspartic acid. Since the second aspartic acid residue was found to be linked to lysine by an unusually stable bond it seemed possible that two aspartic acids could readily be separated from each other. In the first experiment that was made, in which the time of the primary hydrolysis was 43 hr., the amount of free aspartic acid formed was 66% of the total amount in bacitracin A. The easily liberated aspartic acid obtained under these conditions was therefore mixed with some of the aspartic acid linked directly to lysine, a finding in accordance with the fact that the peptide Ileu.Lys was present in the hydrolysate. The behaviour of the material towards D-aspartic acid oxidase indicated that about 66% of the aspartic acid present had the D-configuration. This suggested that the easily liberated residue was D-aspartic acid. The second fraction containing aspartic acid was obtained by further hydrolysis of peptides that remained after the first aspartic acid had been removed. The behaviour of the second fraction to D-aspartic acid oxidase, which indicated that about 58% of the aspartic acid present had the L-configuration, favoured the view that the aspartic acid linked to lysine was derived from an L-aspartic acid residue. However, since the amount of racemization which appeared to have occurred was unexpectedly high, this view was thought to require confirmation. A second experiment was therefore carried out in which a more direct procedure was adopted.

In the second experiment, in which the time of the primary hydrolysis at 80° was reduced to 20 hr., the first aspartic acid to be liberated did not amount to more than one mole per mole of bacitracin. After isolation it was found to contain 93% of the D-isomer. A further sample of aspartic acid, isolated from peptides which had survived hydrolysis at 80° for 43 hr., was found to contain 76% of the L-isomer. These results leave no doubt that the easily liberated aspartic acid has the D-configuration and that the aspartic acid derived from the residue next to lysine has the L-configuration. The finding that the second aspartic acid had undergone more racemization than the first is consistent with the fact that more vigorous conditions are required for its liberation. When bacitracin is hydrolysed under conditions used for liberating both aspartic acid residues from the molecule, further racemization of the easily liberated aspartic acid is presumably responsible for the fact that the final product is almost completely racemic.

The peptide Asp.Lys gave a purple spot with the ninhydrin reagent used by LeQuessne & Young (1952), which has been reported to give a purple colour with α -aspartyl peptides and a blue colour

with β -aspartyl peptides. If this test is reliable in the present case, the arrangement around the lysine residue in bacitracin A would now appear to be as shown in (II). An α -aspartyl linkage cannot

be accepted, however, without supporting evidence. Moreover, there are other properties of the peptides Asp.Lys and Asp(Ileu).Lys which are not yet understood and which raise the question whether they are more complicated than appears at first sight. Both these peptides behaved as though they had a small net negative charge during ionophoresis on paper at pH 7, although the structures assigned to them contain two free carboxyl groups and two free amino groups. When the DNP derivatives of Asp.Lys and Asp(Ileu).Lys were hydrolysed, the ether-soluble DNP-aspartic acid was accompanied by material which remained at the origin on paper chromatograms developed with the solvent system of Blackburn & Lowther (1951) and which appeared as a bright-green fluorescent spot when viewed under ultraviolet light. Similar material was obtained (together with DNP-isoleucine) by hydrolysing the DNP-derivative of bacitracin A, but its significance is at present uncertain. Weisiger, Hausmann & Craig (1955) also obtained a peptide containing aspartic and lysine from bacitracin A whose structure did not appear to be straightforward. The isolation from bacitracin A of the peptide Asp.Lys in amounts sufficient for more detailed study, and the synthesis of aspartyl-lysine peptides for comparative purposes, might help to throw more light on these questions.

SUMMARY

1. When bacitracin A or deaminated bacitracin A was treated with 11N hydrochloric acid at 80° for 43 hr. most of the amino acids were liberated in the free state, but four peptides containing isoleucine or lysine resisted hydrolysis.
2. The surviving peptides were separated by ionophoresis and chromatography on paper and appeared to be Ileu.Phe, Ileu.Lys, Ileu(Asp)Lys, and Asp.Lys. The last was the most stable to further hydrolysis.
3. The DNP derivative of Ileu.Lys yielded DNP-isoleucine and ϵ -DNP-lysine on hydrolysis, and the DNP-derivative of Asp.Lys yielded DNP-aspartic acid and α -DNP-lysine.
4. It is concluded that an isoleucine residue is linked to the α -amino group of the lysine residue in bacitracin A and that an aspartic acid residue is

linked to the ϵ -amino group. However, certain properties of a substance containing lysine and aspartic acid raise the question whether its structure is more complicated than that of a simple dipeptide.

5. One of the aspartic acid residues in bacitracin A is readily liberated by hydrolysis in the free state. The second residue is linked to lysine by a bond which is unusually resistant to hydrolysis. A fraction which contained mainly the first aspartic acid was separated from a fraction which contained mainly the second. In one experiment the two fractions were subjected separately to the action of D-aspartic acid oxidase. In another the aspartic acid in each fraction was isolated and its specific rotation measured. The results indicate that the easily liberated aspartic acid is derived from a residue with the D-configuration and that the aspartic acid residue next to lysine has the L-configuration.

We wish to thank Dr F. Sanger, F.R.S., for a sample of α -DNP-lysine, and the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A., for generous gifts of bacitracin. We are grateful to Miss M. Read and Mrs A. Talboys for expert technical assistance. One of us (I.M.L.) is indebted to the Medical Research Council for a Scholarship.

REFERENCES

- Barry, G. T., Gregory, J. D. & Craig, L. C. (1948). *J. biol. Chem.* **175**, 485.
- Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
- Craig, L. C., Gregory, J. D. & Hausmann, W. (1950). *Analyt. Chem.* **22**, 1462.
- Craig, L. C., Hausmann, W. & Weisiger, J. R. (1952). *J. biol. Chem.* **199**, 865.
- Craig, L. C., Hausmann, W. & Weisiger, J. R. (1954). *J. Amer. chem. Soc.* **76**, 2839.
- Fischer, E. (1899). *Ber. dtsh. chem. Ges.* **32**, 2451.
- Hausmann, W., Weisiger, J. R. & Craig, L. C. (1955). *J. Amer. chem. Soc.* **77**, 723.
- Hirs, C. H. W., Moore, S. & Stein, W. H. (1954). *J. Amer. chem. Soc.* **76**, 6063.
- Keilin, D. & Hartree, E. F. (1936). *Proc. Roy. Soc. B*, **119**, 141.
- Lequesne, W. J. & Young, G. T. (1952). *J. chem. Soc.* p. 24.
- Lockhart, I. M. & Abraham, E. P. (1954a). *Biochem. J.* **58**, 633.
- Lockhart, I. M. & Abraham, E. P. (1954b). *Biochem. J.* **58**, xlvii.
- Lockhart, I. M., Abraham, E. P. & Newton, G. G. F. (1955). *Biochem. J.* **61**, 534.
- Moore, S. & Stein, W. H. (1948). *J. biol. Chem.* **176**, 367.
- Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
- Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
- Newton, G. G. F. & Abraham, E. P. (1953). *Biochem. J.* **53**, 604.
- Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
- Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 353.
- Still, J. L., Buell, H. V., Knox, W. E. & Green, D. E. (1949). *J. biol. Chem.* **179**, 831.
- Synge, R. L. M. (1951). *Biochem. J.* **49**, 642.
- Weisiger, J. R., Hausmann, W. & Craig, L. C. (1955). *J. Amer. chem. Soc.* **77**, 731.
- Woiwod, A. J. (1949). *J. gen. Microbiol.* **3**, 312.
- Work, E. (1949). *Biochim. biophys. Acta*, **3**, 400.

Isolation of Cephalosporin C, a Penicillin-like Antibiotic Containing D- α -Aminoadipic Acid

BY G. G. F. NEWTON* AND E. P. ABRAHAM
Sir William Dunn School of Pathology, University of Oxford

(Received 26 September 1955)

Abraham, Newton & Hale (1954) described the purification of a hydrophilic antibiotic, cephalosporin N, which was produced by a species of *Cephalosporium* (I.M.I. 49137). Cephalosporin N yielded D- α -aminoadipic acid on hydrolysis, and the results of degradative experiments showed that it was probably (D-4-amino-4-carboxy-n-butyl)-penicillin (Newton & Abraham, 1954).

Crude preparations of cephalosporin N were reported to contain a second antibiotic named cephalosporin C (Newton & Abraham, 1955). The

latter contained sulphur and gave D- α -aminoadipic acid on hydrolysis. Unlike cephalosporin N, it was stable in aqueous solution at pH 3 and showed an absorption band in ultraviolet light with a maximum at 260 m μ . This paper describes the isolation of cephalosporin C, in the form of a crystalline sodium salt, and gives some of its properties.

Cephalosporin C was first encountered as a distinct entity during the purification of crude cephalosporin N penillic acid by chromatography on an anion-exchange resin, when it followed the penillic acid from the column as a well-defined band. After isolation it was found to show

* Member of the Scientific Staff, Medical Research Council.