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Formation of ϵ -(Aminosuccinyl)-lysine from ϵ -Aspartyl-lysine from Bacitracin A, and from the Cell Walls of Lactobacilli

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Work in two Laboratories has led to the conclusion that the polypeptide antibiotic bacitracin A contains the sequence $L\text{-Asp} \xrightarrow{\epsilon} L\text{-Lys} \xleftarrow{\alpha} L\text{-Ileu}$, in which $\rightarrow$ denotes a C-N bond (Lockhart & Abraham, 1954*a, b*, 1956; Craig, Hausmann & Weisiger, 1954; Theodoropoulos & Craig, 1956). Part of the evidence for this sequence is based on the analysis of two products of hydrolysis of bacitracin A which are strikingly resistant to acid. One of these products appeared to consist only of lysine and aspartic acid residues and the other of lysine, aspartic acid and isoleucine residues (Lockhart & Abraham, 1956). Nevertheless, both products were more basic than was to be expected if they were peptides containing two amino groups and two carboxyl groups. Further information about their structures was therefore desirable.

Experiments recorded in this paper provide an explanation of the anomalous behaviour of the products containing lysine and aspartic acid which are formed from bacitracin A. Synthetic ϵ -(α -L-aspartyl)-L-lysine and ϵ -(β -L-aspartyl)-L-lysine (Swallow, Lockhart & Abraham, 1958) are both different from the 'Asp.Lys' found in hydrolysates of bacitracin A. However, both synthetic peptides yield a derivative of aminosuccinimide, ϵ -(aminosuccinyl)-lysine (I), which appears to be identical with the product from the antibiotic, on treatment with concentrated hydrochloric acid at 80°. A similar type of cyclization has been found to occur with the α - and β -isohexylamides of aspartic acid (Swallow *et al.* 1958) and (less readily) with α -(α -L-aspartyl)-L-lysine and α -(β -L-aspartyl)-L-lysine which have been synthesized by Pimlott & Young (unpublished work).

After this work had begun Cummins & Harris (1956) reported that hydrolysis of the cell walls of certain lactobacilli yielded a substance which was composed largely of lysine and aspartic acid but which resisted treatment with 6*N*-hydrochloric acid at 100°. The properties of this substance raised the question whether it was related to the cyclic compound from bacitracin A and from the ϵ -aspartyl-lysines (Swallow & Abraham, 1957). A direct comparison of the two products has now indicated that they are identical or differ only in optical configuration.

Brief accounts of some of the work described here have already been given (Swallow & Abraham, 1957; Abraham, 1957).

METHODS

Materials

ϵ -Aspartyl-lysines. The α - and β -isomers were prepared as described by Swallow *et al.* (1958).

α -Aspartyl-lysines. The two isomers were kindly provided by Miss P. Pimlott and Dr G. T. Young.

N-Benzoyloxycarbonyl-L-aspartic anhydride. This was prepared by the method of John & Young (1954).

α - and β -isohexylamides of aspartic acid. These were prepared by the method of Swallow *et al.* (1958).

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

Partial hydrolysate of bacitracin. A solution of bacitracin in 11*N*-HCl (40 ml./g.) was kept at 80° for 43 hr. in a sealed flask. The hydrolysate was evaporated to dryness in a rotary evaporator (Craig, Gregory & Hausmann, 1950). The residue was dissolved in water and evaporation repeated twice to remove HCl. The pattern obtained when

this hydrolysate was subjected to electrophoresis on paper in 0.05M-collidine acetate (pH 7) was similar to that obtained earlier with a partial hydrolysate of bacitracin A (Lockhart & Abraham, 1956).

Partial hydrolysate of ϵ -aspartyl-lysine. A solution of ϵ -aspartyl-lysine in 11N-HCl (20 ml./g.) was kept at 80° for 24 hr. The hydrolysate was treated in a similar manner to the partial hydrolysate of bacitracin.

Licheniformin. We are grateful to Dr R. K. Callow and Dr T. S. Work for samples of licheniformins A, B and C.

Bacterial cell walls. We have to thank Dr C. S. Cummins and Dr H. Harris for a sample of the cell walls of *Lactobacillus brevis* and Mrs K. Crawford for the preparation of cell walls of *Bacillus licheniformis* by the method of Cummins & Harris (1956).

Hydrolysis and identification of peptides

Peptides were hydrolysed in sealed Pyrex tubes, usually in 0.5 ml. of acid under conditions described in the text. Acid hydrolysates were evaporated to dryness *in vacuo* at room temperature over P_2O_5 in the presence of solid NaOH. In some cases hydrolysis was carried out in 0.3N-Ba(OH)₂. Most of the Ba²⁺ ions were precipitated from these solutions, as BaCO₃, before evaporation *in vacuo*.

Chromatography and electrophoresis on Whatman no. 1 paper were carried out in the systems described by Swallow *et al.* (1958). In general, 20–40 μ g. of peptide was applied to the paper.

2,4-Dinitrophenyl (DNP) derivatives were prepared as described by Lockhart & Abraham (1956).

Electrometric titrations were carried out in the manner described by Newton & Abraham (1953).

Semiquantitative determinations of amino acids and peptides separated by paper electrophoresis. The components of some hydrolysates were estimated semiquantitatively, after separation by electrophoresis, by a method similar to that of Peart (1956). Samples of hydrolysate (each 1.7 μ l. containing about 30 μ g. of product) were spotted at intervals on a single sheet of paper. Known amounts of leucine (2–16 μ g.) were also spotted on the same sheet. After electrophoresis in 10% (v/v) acetic acid (14v/cm., 2.5 hr.) the paper was dried in air at 60°, sprayed on both sides with a solution of ninhydrin in butanol (0.16%, w/v) and heated for 15 min. at 90°. Squares of paper containing the spots were cut out, together with two blank squares. Each square was cut into strips and placed in a small test-tube containing 5 ml. of 50% (v/v) ethanol. The tubes were shaken occasionally during 1 hr. to elute the pigment, and the optical density of each solution was then measured at 570 m μ in a Beckman spectrophotometer. Provided that the amount of amino acid or peptide was not less than 2 μ g., the accuracy for duplicate runs was $\pm 10\%$. The molar colour yields of ϵ -(α -Asp).Lys, ϵ -(β -Asp).Lys, ϵ (aminosuccinyl)-lysine [ϵ -(Asu).Lys], aspartic acid and lysine were measured against a leucine standard in a similar way and found to be 1.4, 0.9, 0.7, 0.6 and 0.9 respectively. The corresponding values obtained by the usual ninhydrin method in solution (Moore & Stein, 1948) were 1.43, 1.64, 1.08, 0.88 and 1.12 respectively.

Chromatography on ion-exchange resins

Aspartic acid, glutamic acid and phenylalanine were removed from partial hydrolysates of bacitracin by adsorption on a column of Dowex 1 $\times$ 10 (200–400 mesh) in

the acetate form (Fig. 1a) under conditions similar to those used by Hirs, Moore & Stein (1954). Free aspartic acid was removed similarly from the products obtained by treating ϵ -(α -L-aspartyl)-L-lysine with 11N-HCl at 80°. The ninhydrin colour densities given by samples (0.1 ml.) of fractions from the column were determined by the method of Moore & Stein (1954). Colour densities were measured in an Evans Electro Selenium Ltd. (EEL) portable colorimeter.

The fractions containing the basic and neutral amino acids and peptides (except phenylalanine, see Fig. 1a) were bulked and evaporated in a rotary evaporator with a bath temperature at 25°. The residue was chromatographed at 22°, as shown in Fig. 1b, on a column of Dowex 50 $\times$ 4 (200–400 mesh) prepared according to Hirs *et al.* (1954). Elution was begun with 1.0N-HCl. When 185 fractions had been collected, a mixing chamber (250 ml., stirred magnetically) was introduced into the flow system and by means of this the strength of HCl was gradually raised from N to 4N (a further 330 fractions). Ninhydrin colour densities (Moore & Stein, 1954) were determined on samples (0.1 ml.) from every fraction containing ninhydrin-positive material. Further samples (0.5 ml.) were taken from every twentieth fraction for titration of the acid; when the normality of the latter was greater than 2 the samples used for ninhydrin determinations were neutralized with 2N-NaOH.

The fractions within each peak were collected and evaporated to dryness in a rotary evaporator. The identity of the residue was ascertained by electrophoresis on paper in collidine acetate (0.05M to acetate, pH 7.0) and by paper chromatography in butanol-acetic acid-water (Woiwod, 1949).

Continuous paper electrophoresis

In some experiments electrophoresis was carried out on a preparative scale in a Spincor continuous paper electrophoresis apparatus, model C.P. (Beckman Instruments Inc., Spincor Division, California). The buffer used was pyridine acetate (0.05M to acetate, pH 5.0) and the flow down the paper was adjusted so that a spot of acid fuchsin took about 3 hr. to run the length of the paper (33 cm.). A partial hydrolysate of ϵ -aspartyl-lysine (3.7 mg./ml.), mixed with bromocresol green and methyl red as visible indicators, was applied to the paper at a constant rate of 1 drop/50 sec. A potential of 13v/cm. was applied horizontally across the paper (width 35 cm.), and the effluent collected from the 32 drip points at the lower end of the sheet. The drip points were numbered from anode (1) to cathode (32). In a continuous run of 6 days the visible indicators, which ran towards the anode, did not change in position.

The amino acid pattern was ascertained by analysis of samples (0.5 ml.) from each drip point. The samples were evaporated to dryness *in vacuo*, the residues each dissolved in 30 μ l. of water and spots (3.5 μ l.) of the resulting solutions were used for paper electrophoresis at pH 7.0 in 0.05M-collidine acetate.

Synthesis of L-aminosuccinylisohexylamine (II)

Benzoyloxycarbonyl-L-aspartic anhydride (2.0 g., 1 mol. prop.) was heated in a sealed tube at 130° for 3 hr. with isohexylamine (1.07 ml., 1 mol. prop.). The tube was cooled, opened and reheated for 1 hr. to drive off the water

eliminated in the reaction. The clear yellow-brown syrup was dissolved in ethanol (30 ml.), acidified with acetic acid (1.0 ml.) and hydrogenated in the presence of Pd-charcoal (10% Pd; 0.2 g.) until no more CO_2 was evolved. The solution was filtered and evaporated to dryness in a rotary evaporator.

The residual oil was dissolved in ether (8.0 ml.), and the required substance extracted into 2N-HCl (5.0 ml.). The acid solution was extracted six times with ether (5.0 ml. portions) until it was no longer fluorescent in ultraviolet light. The aqueous phase was then adjusted to pH 7.0 with 2N-NaOH and the oil which separated was extracted into ether (5.0 ml.). The whole extraction process was repeated twice more, and the final ethereal solution was shaken with 2N-HCl (2.0 ml.). The aqueous phase was evaporated to dryness *in vacuo* in the presence of solid NaOH.

The pale-yellow solid remaining was triturated with acetone and gave a mass of white needles, which were collected, washed with acetone and dried. Yield 0.26 g. *L*-Aminosuccinylisohexylamine hydrochloride had m.p. 192–194° and partly sublimed just before melting. The sublimate was in the form of thin plates, but the starting material consisted of tiny needles. (Found: C, 51.1; H, 8.1; N, 12.0; Cl, 15.3. $\text{C}_{10}\text{H}_{19}\text{O}_3\text{N}_2\text{Cl}$ requires: C, 51.2; H, 8.1; N, 12.0; Cl, 15.1%). The substance gave a single spot (R_f 0.78) on paper chromatography in butanol-acetic acid-water, and migrated towards the cathode as a single spot on paper electrophoresis at pH 7.0 (Fig. 2). The spots, developed with ninhydrin, were yellow-orange. On treatment at room temperature with 0.3N-Ba(OH)₂ for 0.5 hr., the substance yielded two products which were separated by electrophoresis on paper at pH 2.3 and corresponded to the α - and β -isohexylamides of aspartic acid (Swallow *et al.* 1958). The β -isomer greatly predominated.

RESULTS

Cyclization of ϵ -aspartyl-lysines to ϵ -(aminosuccinyl)-lysine

Cyclization of ϵ -aspartyl-lysines in acid solution. ϵ -(α -L-Aspartyl)-L-lysine and ϵ -(β -L-aspartyl)-L-lysine behaved as neutral compounds during electrophoresis on paper at pH 7 (Fig. 2A, spot 1). After treatment with 11N-hydrochloric acid at 80° for 30 min. both products contained similar small amounts of free lysine and aspartic acid and a significant quantity of a basic substance which migrated, at pH 7, about half as fast as histidine towards the cathode (Fig. 2A, spot 2). After 10 hr. under the same conditions only a small amount of aspartyl-lysine remained and the intensity of the spots obtained with ninhydrin suggested that the major component of the mixture was the slow-moving base (spot 2). The relative intensities of the spots changed only very slowly when treatment with acid was prolonged further, but a gradual increase in the amounts of lysine and aspartic acid was observed (Table 1).

It seemed likely that the slow-moving base was ϵ -(aminosuccinyl)-lysine (I), whose formation from the neutral ϵ -aspartyl-lysines involved the masking

of a carboxyl group. This view was confirmed when the substance was separated from the other products of the reaction and some of its properties were studied.

Separation of ϵ -(aminosuccinyl)-lysine. Two methods were used for the separation, on a preparative scale, of the products obtained when ϵ -aspartyl-lysines were treated with 11N-HCl at 80° for 24 hr. and the HCl was then removed *in vacuo*. The simplest procedure was electrophoresis in the Spince continuous paper-electrophoresis apparatus, the buffer being pyridine acetate, pH 5 (see Experimental). Under the conditions used both ϵ -aspartyl-lysines were collected from drip points 9 to 11, ϵ -(aminosuccinyl)-lysine from 18 to 24, and lysine from 26 to 30. Aspartic acid migrated off the paper into the buffer circulating over the anode. The appropriate fractions were combined and the solutions freeze-dried. The residue of ϵ -(aminosuccinyl)-lysine was a colourless hygroscopic solid.

The second procedure involved removal of free aspartic acid by adsorption on a column of Dowex 1 $\times$ 10 (acetate form) and chromatography of the combined neutral and basic material on Dowex 50 $\times$ 4 in HCl (Hirs *et al.* 1954). The conditions used were very similar to those described below for the separation of ϵ -(aminosuccinyl)-lysine from hydrolysates of bacitracin (see Fig. 1). The

Fig. 1. (a) Elution from a column of Dowex 1 $\times$ 10 (25 cm. $\times$ 1.2 cm.) of a partial hydrolysate of bacitracin with 0.5N-acetic acid. Load, 250 mg.; flow rate, 4 ml./hr. Fractions each 1.0 ml. (b) Elution from a column of Dowex 50 $\times$ 4 (120 cm. $\times$ 2.0 cm.) of material corresponding to the first peak from (a). Eluent 1.0–4.0N-HCl; load, about 350 mg.; flow rate, 24 ml./hr.; fractions each 6.0 ml. The double peak in the cystine position indicates the presence of *meso*- and D- or L-isomers (cf. Hirs *et al.* 1954).

Table 1. *Hydrolysis under various conditions of ϵ -(α -aspartyl)-lysine, ϵ -(aminosuccinyl)-lysine and α -(α -aspartyl)-lysine*

The number of crosses indicates the approximate strength of amino acid or peptide as estimated visually from the intensities of the spots developed with ninhydrin after paper electrophoresis. The figures in parentheses show the approximate mol. prop. (%) of the components of the hydrolysate (see Experimental). Differences between the visual and numerical results are to be attributed to differences in ninhydrin colour yields. ϵ - and α -(Aminosuccinyl)-lysine are abbreviated to ϵ - and α -(Asu). Lys. α -(β -Asp). Lys and α -(α -Asp). Lys are not distinguished from each other.

Substance	Conditions of hydrolysis			Composition of hydrolysate						
	Reagent	Time (hr.)	Temp.	Asp	ϵ -(β -Asp). Lys	ϵ -(α -Asp). Lys	ϵ -(Asu). Lys	Lys		
ϵ -(α -Asp). Lys	11 N-HCl	24	20°	.	.	+	+	.		
		0.5	80	+	+	+	+	+		
		2	80	+	+	+	+	+		
		4	80	+	+	+	+	+		
		24	80	+	+	+	+	+		
	0.1 N-HCl	70	80	+	+	+	+	+	(10)	+
		105	80	+	+	+	+	+	(77)	+
		3	80	.	+	+	+	.	+	+
		24	80	+	+	+	+	+	+	+
		72	80	+	+	+	+	+	+	+
ϵ -(Asu). Lys	0.1 N-HCl	24	100	+	+	+	+	+	+	+
		24	105	+	+	+	+	+	+	+
	0.25 N-Acetic acid	1	100	+	+	+	+	+	+	+
		8	100	+	+	+	+	+	+	+
		24	100	+	+	+	+	+	+	+
	11 N-HCl	4	80	.	+	+	+	.	+	+
		24	80	+	+	+	+	+	+	+
		43	80	+	+	+	+	+	+	+
		24	80	±	+	+	+	±	(3.5)	+
		0.5	18	.	+	+	+	.	±	(1.6)
α -(α -Asp). Lys	11 N-HCl	0.5	80	±	+	+	+	±	+	+
		5	80	+	+	+	+	+	+	+
		24	80	+	+	+	+	+	+	+
		43	80	+	+	+	+	+	+	+
		0.5	18	.	+	+	+	.	±	+

aspartyl-lysine remaining in the preparation emerged from the column of Dowex 50 as a small band immediately preceding lysine and the ϵ -(aminosuccinyl)-lysine as a relatively large band closely following lysine. The molecular ratio of ϵ -(aminosuccinyl)-lysine to lysine (calculated from ninhydrin colour densities) was 4 to 1. The fractions containing the ϵ -(aminosuccinyl)-lysine were evaporated *in vacuo* to yield a colourless hygroscopic glass.

Preparations of ϵ -(aminosuccinyl)-lysine obtained by these procedures appeared to be almost homogeneous when subjected to ionophoresis on paper at pH 2.3, 5 or 7. Only trace amounts of one other component, which appeared to be ϵ -aspartyl-lysine, could be detected with ninhydrin. However, attempts to obtain the compound in crystalline form, as a free base, a monohydrochloride, a picrate or a copper complex, were unsuccessful.

Properties of ϵ -(aminosuccinyl)-lysine. The infrared spectrum of ϵ -(aminosuccinyl)-lysine, unlike the spectra of the ϵ -aspartyl-lysines, contained a medium band at 1785 cm^{-1} and a strong band at 1705 cm^{-1} . These bands could be assigned to the carbonyl groups of a succinimide ring (Randall, Fowler, Fuson & Dangel, 1949). The spectra of the ϵ -aspartyl-lysines showed a strong band at 3300 cm^{-1} which could be assigned to the NH

stretching mode of the peptides. This band was absent from the spectrum of ϵ -(aminosuccinyl)-lysine.

On paper chromatograms run with butanol-acetic acid-water ϵ -(aminosuccinyl)-lysine was not separated from lysine or from the two ϵ -aspartyl-lysines. These substances were readily distinguished, however, by electrophoresis on paper in collidine acetate (pH 7.0), pyridine acetate (pH 5.0) or aqueous acetic acid (pH 2.3) (Fig. 2A-C).

The fact that ϵ -(aminosuccinyl)-lysine migrated towards the cathode much more rapidly at pH 5 than at pH 7 (Fig. 2A and B) indicated that one of its functional groups ionized in this pH region. Electrometric titration (Fig. 3) showed that it contained three ionizable groups whose pK_a values were 2.5, 6.0 and 9.5 respectively. The pK_a of less than 2.5 could be assigned to a carboxyl group, that of 6.0 to the amino group of an aminosuccinimide ring and that of 9.5 to the α -amino group of a lysine residue.

When the titration was carried to a pH higher than 10 an irreversible change began. Back-titration of a solution which had been kept at pH 11 for 30 min. showed that the group with pK_a 6.0 had disappeared. The resulting curve (Fig. 3) suggested that hydrolysis had occurred with the formation of a mixture of ϵ -(α -aspartyl)- and ϵ -(β -aspartyl)-lysine (cf. Swallow *et al.* 1958). The product obtained when hydrolysis was allowed to occur in 0.3N-barium hydroxide at 20° for 30 min. gave a single spot in a position corresponding to both ϵ -aspartyl-lysines when subjected to electro-

Fig. 2. Electrophoresis on paper (14v/cm. for 2.5 hr.) in (A) collidine acetate (pH 7.0), (B) pyridine acetate (pH 5.0), (C) aqueous acetic acid (pH 2.3), and (D) sodium borate (pH 9.1). Letters refer to single substances and numbers to groups of substances. In systems A, B and C glucose was carried 0.5 cm. towards the cathode by electroendosmosis, and in system D DNP-ethanolamine was carried 1.6 cm. towards the cathode. Group 1: a, ϵ -(α -aspartyl)-lysine; b, ϵ -(β -aspartyl)-lysine; c, α -(α -aspartyl)-lysine; d, α -(β -aspartyl)-lysine. Group 2: e, ϵ -(aminosuccinyl)-lysine (synthetic); f, ϵ -(aminosuccinyl)-lysine (from bacitracin A); g, α -(aminosuccinyl)-lysine; h, N-aspartoyl-isohexylamine. Group 3: i, α -isohexylamide of aspartic acid; k, β -isohexylamide of aspartic acid; l, isohexylamine; m, lysine; n, glutamic acid; o, aspartic acid.

Fig. 3. Titration of ϵ -(aminosuccinyl)-lysine hydrochloride (1.6 mg./ml.) in water at 20° . x, Forward titration with 0.1N-NaOH; ●, back titration with 0.1N-HCl of a solution kept at pH 11 for 30 min.

phoresis on paper at pH 7 or at pH 5 (Fig. 2A, B, spot 1). After electrophoresis at pH 2.3 it showed a major spot in the position corresponding to ϵ -(β -aspartyl)-lysine and a minor spot in that corresponding to ϵ -(α -aspartyl)-lysine (Fig. 2C, spots b and a). In contrast with its lability in alkaline solution, ϵ -(aminosuccinyl)-lysine was remarkably resistant to hydrolysis in 11N-hydrochloric acid at 80° (Table 1).

The DNP derivative of ϵ -(aminosuccinyl)-lysine yielded DNP-aspartic acid and α -DNP-lysine on hydrolysis and also an ether-soluble product which remained at the origin on paper chromatograms (Blackburn & Lowther, 1951) and showed a bright-green fluorescence in ultraviolet light (365 m μ). A similar product was reported by Lockhart & Abraham (1956) to be formed on reaction of the 'Asp. Lys' from bacitracin A with 1-fluoro-2:4-dinitrobenzene.

*Interconversion of ϵ -(α -aspartyl)- and
 ϵ -(β -aspartyl)-lysine*

Electrophoresis on paper at pH 2.3 showed that ϵ -(α -aspartyl)-lysine was converted largely into ϵ -(aminosuccinyl)-lysine by treatment with 11N-hydrochloric acid for 10 hr. at 80°. In addition to this product and some free lysine and aspartic acid, however, a small spot corresponding to the original peptide was discernible, and also a second spot, of similar intensity, corresponding to ϵ -(β -aspartyl)-lysine. The concentrations of the two ϵ -aspartyl-lysines then appeared to undergo relatively little change when treatment with acid was prolonged to 70 hr. (Table 1). Similar results were obtained with ϵ -(β -aspartyl)-lysine, which was converted into a mixture containing small amounts of both aspartyl-lysines, as well as larger amounts of lysine, aspartic acid, and ϵ -(aminosuccinyl)-lysine, on prolonged treatment with 11N-hydrochloric acid at 80°. A similar conversion of ϵ -(α -aspartyl)-lysine into the β -aspartyl isomer was observed in 0.25N-acetic acid at 100°. In this case, however, the intensity of the spot due to the β -isomer was considerably greater than that due to the α -isomer after 24 hr. (Table 1).

*Cyclization of ϵ -(α -L-aspartyl)-L-lysine
under other conditions*

In 0.1N-hydrochloric acid at 80° both the rate of hydrolysis of ϵ -(α -L-aspartyl)-L-lysine to aspartic acid and lysine and its cyclization to the succinimide were much slower than in 11N-acid. After 72 hr. a considerable proportion of the original peptide remained unchanged (Table 1). However, the reduction in the rate of hydrolysis was less than the reduction in the rate of imide formation, and the amounts of lysine and aspartic acid formed in the early stages of the reaction were relatively greater than the amount of imide. After electrophoresis on

paper (pH 2.3) of the reaction products at times varying from 1 to 24 hr. the spots corresponding to imide, lysine and aspartic acid were all of comparable intensity. The imide itself underwent very little hydrolysis during 24 hr. in 0.1N-hydrochloric acid at 80°, but it yielded a small amount of the β -aspartyl peptide and a trace of α -isomer.

In 0.1N- or N-hydrochloric acid at 100° the rate of formation of aspartic acid and lysine was large compared with the rate of accumulation of imide. This probably implies that the ring-opening of the imide is associated with a higher energy of activation than is the hydrolysis of the dipeptide. Although the imide is relatively stable in 6N-hydrochloric acid at 80° it is completely hydrolysed in 24 hr. at 105°. In 11N-hydrochloric acid at 20° ϵ -(α -L-aspartyl)-L-lysine yielded a significant amount of imide in 24 hr. but no detectable quantity of lysine or aspartic acid.

ϵ -(Aminosuccinyl)-lysine from bacitracin A

The compound detected by Lockhart & Abraham (1956) in hydrolysates of bacitracin A, and provisionally described as Asp. Lys, was separated by ion-exchange chromatography from other products formed when bacitracin was treated with 11N-hydrochloric acid at 80°. Free aspartic and glutamic acids were first removed by adsorption on a column of Dowex 1 $\times$ 10 acetate. Under the conditions used, phenylalanine was also removed at this stage, since it emerged from the column as a sharp peak immediately following a mixture of the basic and remaining neutral products (Fig. 1a). The latter was chromatographed on Dowex 50 $\times$ 4 in hydrochloric acid whose concentration was raised from 1.0 to 4.0N (Fig. 1b). The compound 'Asp. Lys' emerged from the column after histidine (fractions 480-500). It was followed by Ileu. Lys and then by the compound described provisionally by Lockhart & Abraham (1956) as Asp(Ileu). Lys.

Evaporation of fractions 480-495 *in vacuo* yielded an amorphous residue. This product migrated towards the cathode at the same rate as ϵ -(aminosuccinyl)-lysine (obtained from ϵ -aspartyl-lysine) when subjected to electrophoresis on paper at pH 7. The product also migrated at the same rate as ϵ -(aminosuccinyl)-lysine on electrophoresis at pH 5.0 and 2.3 (Fig. 2, spot 2), and was not separated from ϵ -(aminosuccinyl)-lysine by chromatography on paper in butanol-acetic acid-water. On treatment with 0.3N-barium hydroxide at room temperature for 1 hr. it was converted entirely into material which showed no net charge at pH 7. Electrophoresis on paper at pH 2.3 showed that this material consisted of two ninhydrin-positive components, the major component migrating towards the cathode at the same rate as ϵ -(β -aspartyl)-lysine and the minor component at

the same rate as ϵ -(α -aspartyl)-lysine. In this behaviour, also, the compound from bacitracin A was indistinguishable from ϵ -(aminosuccinyl)-lysine.

Licheniformins A, B and C, which, like bacitracin, are produced by strains of *Bacillus licheniformis* (Callow & Work, 1952), yielded no detectable amount of ϵ -(aminosuccinyl)-lysine in 11N-hydrochloric acid at 80°.

*ϵ -(Aminosuccinyl)-lysine from cell walls
of Lactobacillus brevis*

Cell walls of *Lactobacillus brevis* were treated with 11N-hydrochloric acid at 80° for 43 hr. When the product was subjected to electrophoresis on paper at pH 7, a neutral spot, two acidic spots and three basic spots were observed. A major acidic spot corresponded to glutamic acid and a minor one to aspartic acid. Two of the basic spots corresponded to lysine and glucosamine respectively, the former migrating slightly faster than the latter. The third basic spot, which was the most intense of the three, migrated to the same position as ϵ -(aminosuccinyl)-lysine (Fig. 2A).

The material corresponding to the third basic spot was eluted from the paper after electrophoresis at pH 7 on a larger scale. This material migrated towards the cathode at the same rate as ϵ -(aminosuccinyl)-lysine on electrophoresis at pH 5.0 and 2.3. On hydrolysis with 6N-hydrochloric acid at 110° for 24 hr. it yielded equivalent amounts of lysine and aspartic acid. On treatment with 0.3N-barium hydroxide at 20° for 1 hr. it yielded two products which were separated by electrophoresis on paper at pH 2.3. The major product migrated at the same rate as ϵ -(β -aspartyl)-lysine and the minor one at the same rate as ϵ -(α -aspartyl)-lysine (Fig. 2C, spots *a* and *b*).

These results left little doubt that the third basic product from the cell walls of *Lactobacillus brevis* was ϵ -(aminosuccinyl)-lysine. The possibility that it was α -(aminosuccinyl)-lysine was later eliminated when it was shown that this substance was much less stable to acid than the ϵ -isomer, and that ϵ -(β -aspartyl)-lysine was readily distinguishable from α -(β -aspartyl)-lysine by electrophoresis on paper (Fig. 2C and D).

No ϵ -(aminosuccinyl)-lysine was detected in the cell walls of a strain of *Bacillus licheniformis* which produces bacitracin.

*Cyclization of α - and β -isohexylamides
of aspartic acid*

When either the α - or β -isohexylamide of aspartic acid (Swallow *et al.* 1958) was treated with 11N-hydrochloric acid at 80° for 20 hr. some free aspartic acid and isohexylamine were formed and also a considerable amount of a basic product

which showed a mobility similar to that of ϵ -(aminosuccinyl)-lysine on paper electrophoresis at pH 7 (Fig. 2A). This product was revealed as a yellow-orange spot when the paper was sprayed with ninhydrin. It was isolated as a crystalline hydrochloride (m.p. 192–194°) by extraction into ether from an aqueous solution at pH 7, re-extraction into 2N-hydrochloric acid, removal of the acid *in vacuo*, and trituration of the remaining solid with acetone. When treated with 0.3N-barium hydroxide at 20° it was converted into two compounds which were indistinguishable from the α - and β -isohexylamides of aspartic acid respectively on electrophoresis at pH 2.3 (Fig. 2C). The relative intensities of the spots showed that the yield of the β -isomer was considerably higher than that of the α -isomer.

These properties indicated that the basic product was aminosuccinylisohexylamine (II).

Structure (II) was supported by the fact that the product was indistinguishable in its electrophoretic mobility at pH 7, and its behaviour in alkaline solution, from a compound, $\text{C}_{10}\text{H}_{18}\text{O}_2\text{N}_2$, synthesized from benzyloxycarbonyl-L-aspartic anhydride and isohexylamine (see Experimental). The compound $\text{C}_{10}\text{H}_{18}\text{O}_2\text{N}_2$ showed an infrared spectrum containing two strong bands at 1785 and 1710 cm^{-1} respectively which could be attributed to the carbonyl groups of a succinimide ring (Randall *et al.* 1949).

*Cyclization of α -(α -L-aspartyl)-L-lysine and
 α -(β -L-aspartyl)-L-lysine*

α -(α -L-Aspartyl)-L-lysine and α -(β -L-aspartyl)-L-lysine behaved as neutral compounds on paper electrophoresis at pH 7, but were separated by electrophoresis at pH 2.3 or 9.1. The α -(α -aspartyl) isomer was distinguished from ϵ -(α -aspartyl)-lysine by its greater mobility (towards the cathode) at pH 2.3, and the α -(β -aspartyl) isomer was distinguished from ϵ -(α -aspartyl)-lysine by its smaller mobility (towards the anode) at pH 9.2 (Fig. 2C and D).

When α -(α -L-aspartyl)- and α -(β -L-aspartyl)-L-lysine were treated with 11N-hydrochloric acid at 80° a basic product was formed, in addition to free lysine and aspartic acid, which showed the same mobility as ϵ -(aminosuccinyl)-lysine on paper electrophoresis at pH 7 (Fig. 2A). This product was undoubtedly α -(aminosuccinyl)-lysine. It was considerably less stable to acid, however, than the

ϵ -isomer, reaching a maximal and relatively low concentration after 5 hr. and thereafter rapidly diminishing. After 48 hr. only a trace of the compound remained and hydrolysis to aspartic acid and lysine approached completion.

DISCUSSION

Cherbuliez & Chambers (1924) reported that benzamidossuccinimide could be obtained by the action of heat on *N*-benzoylasparagine. Benzyl-oxy-carbonylamino-succinimide was later prepared by the saponification of *N*-benzyloxycarbonyl-L-asparagine methyl ester under mild conditions and yielded L-aminosuccinimide itself on removal of the benzyloxycarbonyl group (Sondheimer & Holley, 1954*a, b*). The cyclization in alkaline solution was assumed to be initiated by the removal of a proton from the amide nitrogen. Battersby & Robinson (1955) showed that the esters of *N*-benzoyl- α -aspartyl peptides and the corresponding β -aspartyl peptides were partly converted into derivatives of aminosuccinimide on treatment with less than one equivalent of dilute sodium hydroxide. Treatment of the imide with an excess of alkali resulted in ring-opening and the formation of a mixture of *N*-benzoylaspartyl peptides, the β -isomer predominating.

The ϵ -(aminosuccinyl)-lysine encountered here has not been obtained in a crystalline form, but its structure appears to have been clearly established. The pK_a of its first amino group (6.0) is very close to that reported for the amino group of aminosuccinimide (5.9). It is formed under conditions similar to those under which the well-characterized aminosuccinylisohexylamine is produced from the isohexylamides of aspartic acid, and its infrared spectrum shows two bands which can be attributed to the CO groups of the succinimide ring. Like the derivatives of aminosuccinimide described previously, it is readily converted by dilute alkali into a mixture of α - and β -aspartyl peptides. The predominance of the β -isomer is to be expected, since the reaction is presumably initiated by the nucleophilic attack of a hydroxyl ion on the two carbonyl groups of the ring, and the carbon derived from the α -carboxyl group of aspartic acid will have the greater fractional positive charge.

John & Young (1954) reported that α -L-aspartyl-L-valine was partly converted into β -L-aspartyl-L-valine when heated in aqueous solution at 100° for 6 hr., but they did not detect any cyclic intermediate. Detection of the formation of ϵ -(aminosuccinyl)-lysine [ϵ -(Asu).Lys] was facilitated by the characteristic behaviour of this compound on electrophoresis and by its great stability in concentrated hydrochloric acid. When ϵ -(α -aspartyl)-lysine [ϵ -(α -Asp).Lys] or its isomer [ϵ -(β -Asp).Lys]

is heated in acid solution the following reactions occur, the velocity constants being represented by k :

The intensities of spots obtained after electrophoresis on paper indicated that the values of k_1 , k_2 and k_3 were of the same order as those of k'_1 , k'_2 and k'_3 respectively, although k'_2 was somewhat greater than k_2 . After 10 hr. in 11*N*-hydrochloric acid ϵ -(α -Asp).Lys and ϵ -(β -Asp).Lys reached almost stationary concentrations which were very low in comparison with that of ϵ -(Asu).Lys. Thus $k_2 + k'_2 \ll (k_1 + k'_1) + (k_3 + k'_3)$. It also appeared that $k_3 \ll k_1$ and $k'_3 \ll k'_1$, so that $(k_2 + k'_2) \ll (k_1 + k'_1)$. In other words, the rate of cyclization under these conditions was much greater than the rate of ring-opening.

In 0.1*N*-hydrochloric acid at 80° the values of k_1 , k_2 and k_3 were all lower than the corresponding values in 11*N*-acid. However, the quotient k_3/k_1 was considerably larger in the dilute acid than in the concentrated, and in the dilute acid hydrolysis competed with imide formation on similar terms.

These results may be explicable in terms of changes in the activity of water. The hydrolysis of amides in acid solution is believed to involve protonization of the amide nitrogen and separation of this nitrogen from the cationoid carbon with concomitant attack on the latter by the oxygen of water (Meloche & Laidler, 1951; Bender & Ginger, 1955):

The rate of hydrolysis rises to a maximum as the concentration of acid is increased (to 6*N*-HCl with formamide) and then declines. The rise is attributed to the conversion of the amide into the conjugate acid, and the subsequent decline to a fall in the activity of water. In the cyclization leading to aminosuccinimides a molecule of water is eliminated and the activity of water in the solution may play a dominant role. Once the imide is formed the resistance of its very weakly basic nitrogen to the addition of a proton may account for its high stability in acid solution.

When α -(α -aspartyl)-lysine was kept at 80° in 11N-hydrochloric acid the values of k_1 and k_2 appeared to be similar to those for the corresponding ϵ -isomer, but the value of $k_2 + k'_2$ was much larger. The reason for the lower stability of the imide (III)

from α -(α -aspartyl)-lysine is not clear, since the proximity of the carboxyl group to the ring nitrogen should make the latter more difficult to protonate. Possibly steric factors are involved, or the carboxyl group itself plays a part in ring-opening. Partridge & Davis (1950) showed that there was a preferential liberation of aspartic acid from certain proteins on hydrolysis with weak acids and suggested that the β -carboxyl group of aspartic acid was acting, under these conditions, as a proton donor. The α -amino and carboxyl groups of ϵ -(aminosuccinyl)-lysine do not appear to be concerned in its resistance to ring-opening, because *N*-aspartoyl*iso*hexylamine shows a similar stability.

It follows from the results obtained here that a mixture of α - and β -aspartyl peptides may be formed on partial hydrolysis, in concentrated hydrochloric acid, of polypeptide chains containing the sequence Asp.Lys. Whether extensive inversion may occur under such conditions when aspartic acid is linked to other amino acids has not been established, but the finding of an α - or β -aspartyl peptide in acid hydrolysates must clearly be interpreted with caution.

The formation of ϵ -(aminosuccinyl)-lysine from bacitracin A confirms the conclusion of Lockhart & Abraham (1956) that one of the two aspartic acid residues in the antibiotic is linked to the ϵ -amino group of lysine. The product of hydrolysis of bacitracin A which was provisionally described as Asp(Ileu).Lys (Lockhart & Abraham, 1956) is doubtless ϵ -(aminosuccinyl)- α -isoleucyl-lysine. There is as yet no evidence that the aminosuccinimide ring occurs in bacitracin A itself, and it would be difficult to reconcile its presence with other evidence about this portion of the molecule (Lockhart & Abraham, 1954*a*; Hausmann, Weisiger & Craig, 1955). However, the formation of the ring and interconversion of the aspartyl-lysines make it impossible to determine, from the products of hydrolysis, whether the original sequence consists of an α - or β -aspartyl peptide.

The lysine residues in proteins normally have their ϵ -amino groups free, although a peptide in

which an ϵ -amino group of a lysine residue is bound has recently been obtained from collagen (Mechanic & Levy, 1957). It is therefore of interest that the unusual ϵ -aspartyl-lysine sequence occurs in the cell walls of *Lactobacillus brevis*. This sequence is also present in the cell walls of *L. acidophilus*, *L. helveticus*, *L. fermenti*, *L. casei*, *L. delbrueckii*, and some strains of *Actinomyces bovis*, since Cummins & Harris (1956, 1958) have detected in acid hydrolysates of these walls a substance with similar properties to that shown here to be ϵ -(aminosuccinyl)-lysine. The sequence is probably responsible, too, for a substance with similar properties detected by Powell & Strange (1957) in peptides released from the cell walls of *Bacillus sphaericus* by lysozyme. Whether the ϵ -(Asp).Lys sequence in bacterial cell walls contains aspartic acid and lysine residues with the same optical configuration as the corresponding residues in bacitracin remains to be determined.

Extracellular polypeptides may well be synthesized near the cytoplasmic membrane of the bacterial cell and it seemed possible that the ϵ -aspartyl-lysine fragment of bacitracin A was produced by a mechanism which was also involved in the synthesis of a fragment of the cell wall. However, ϵ -(aminosuccinyl)-lysine has not been detected in hydrolysates of the cell wall of a strain of *Bacillus licheniformis* used for producing bacitracin. It has also not been detected in hydrolysates of the cell walls of two strains of *L. bifidus*, although the walls of these organisms contain lysine and aspartic acid residues (Cummins, Glendenning & Harris, 1957). Possibly in those bacterial cell walls which yield ϵ -(aminosuccinyl)-lysine on hydrolysis a lysine residue is the site of a branch in a peptide chain, as it is in bacitracin A.

SUMMARY

1. ϵ -(α -L-Aspartyl)-L-lysine and ϵ -(β -L-aspartyl)-L-lysine cyclize to ϵ -(L-aminosuccinyl)-L-lysine in 11N-hydrochloric acid at 80°. Cyclization occurs more rapidly than hydrolysis to lysine and aspartic acid. The α - and β -*iso*hexylamides of aspartic acid cyclize in a similar manner.

2. ϵ -(Aminosuccinyl)-lysine shows considerable stability to 11N-hydrochloric acid at 80°, but it is slowly hydrolysed to lysine and aspartic acid with the intermediate formation of the two ϵ -aspartyl-lysines. Both aspartyl-lysines reach only a relatively low stationary concentration, but that of the β -isomer is somewhat higher than that of the α -isomer.

3. ϵ -(Aminosuccinyl)-lysine is rapidly hydrolysed in cold dilute alkali to a mixture of α - and β -aspartyl-lysines in which the β -isomer greatly predominates.

4. α -(α -L-Aspartyl)-L-lysine and α -(β -L-aspartyl)-L-lysine cyclize to α -(L-aminosuccinyl)-L-lysine in 11N-hydrochloric acid at 80°, but this derivative of aminosuccinimide is much less stable than that formed from the corresponding ϵ -aspartyl-lysines.

5. ϵ -(Aminosuccinyl)-lysine is formed when bacitracin A is treated with 11N-hydrochloric acid at 80°. It is also formed during acid hydrolysis of the cell walls of certain strains of lactobacilli. The cell walls of other strains of lactobacilli do not yield this compound, although they contain lysine and aspartic acid residues.

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The Production of an Esterase Inhibitor from Schradan in the Fat Body of the Desert Locust

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The oxidative conversion of the systemic insecticides schradan (bisdimethylaminophosphonous anhydride) and dimefox (bisdimethylaminofluorophosphine oxide) into powerful anticholinesterases has been studied in mammalian liver homogenates (Davison, 1954, 1955; O'Brien, 1956, 1957; Fenwick, Barron & Watson, 1957), and shown to be dependent on the presence of Mg^{2+} or Ca^{2+} ions, nicotinamide and diphosphopyridine nucleotide. The microsome fraction of liver cells could effect the conversion if fortified with reduced diphospho-

pyridine nucleotide. O'Brien & Spencer (1953, 1955) reported that many intact insect tissues could produce the same schradan metabolite as did liver, but all attempts to make an active tissue homogenate were unsuccessful. Cockroach-gut homogenates fortified with nicotinamide, diphosphopyridine nucleotide and Mg^{2+} ions failed to convert schradan (O'Brien, 1957) and dimefox (Fenwick *et al.* 1957) into such compounds, and it was suggested that a different mechanism might be involved.