- Lee, W. A. & Peacocke, A. R. (1951). *J. chem. Soc.* p. 3361.
- Lucy, J. A. & Kent, P. W. (1953). *Research, Lond.*, **6**, 49s.
- MacDonald, D. L. & Knight, C. A. (1953). *J. biol. Chem.* **202**, 45.
- Markham, R. & Smith, J. D. (1949). *Biochem. J.* **45**, 294.
- Mozingo, R. (1941). *Org. Synth.* **21**, 15.
- Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
- Pasternak, C. A. & Kent, P. W. (1952). *Research, Lond.*, **5**, 486.
- Sinsheimer, R. L. (1954). *J. biol. Chem.* **208**, 445.
- Smith, J. D. & Markham, R. (1952a). *Nature, Lond.*, **170**, 120.
- Smith, J. D. & Markham, R. (1952b). *Biochim. biophys. Acta*, **8**, 350.
- Tamm, C., Hodes, M. D. & Chargaff, E. (1952). *J. biol. Chem.* **195**, 49.
- Tamm, C., Shapiro, H. S., Lipshitz, R. & Chargaff, E. (1953). *J. biol. Chem.* **203**, 673.
- Wyatt, G. R. (1951). *Biochem. J.* **48**, 584.

The N-Terminal and Sulphur-containing Residues of Bacitracin A

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The results of electrometric titrations and of reactions with 1-fluoro-2:4-dinitrobenzene (FDNB) led Newton & Abraham (1953a, b) to conclude that bacitracin A contained a free δ -amino group which was part of an ornithine residue and a free α -amino group which was part of a leucine or isoleucine residue. Other workers failed to demonstrate the presence of an *N*-terminal amino acid in the molecule (Ingram, 1953; Porath, 1953). Lockhart, Newton & Abraham (1954) confirmed the results of earlier experiments and reported that the dinitrophenyl (DNP) derivative of bacitracin A yielded DNP-isoleucine on hydrolysis. However, they stated that the yield of DNP-isoleucine was much less than that to be expected from one *N*-terminal residue per molecule. Some uncertainty remained, therefore, about the nature of the structure carrying the postulated free α -amino group.

Analysis of the amino acids formed on hydrolysis with acid first suggested that bacitracin A contained only two residues of isoleucine (Barry, Gregory & Craig, 1948), but studies of the amino acid sequence showed that isoleucine residues occurred at three positions in the peptide chain (Craig, Hausmann & Weisiger, 1954; Lockhart & Abraham, 1954a; Hausmann, Weisiger & Craig, 1955a; Weisiger, Hausmann & Craig, 1955). One of the isoleucine residues appeared partly as *allo*-isoleucine in acid hydrolysates (Piez, 1954). In the light of earlier experiments, the results of partial hydrolysis could best be accommodated by assuming that bacitracin A contained an *N*-terminal isoleucine residue that was linked through its carboxyl group to a potential cysteine residue. Nevertheless, the complete picture appeared to be more complicated. Both Lockhart & Abraham

(1954a) and Hausmann *et al.* (1955a) suggested that the amino group of this isoleucine was engaged in cross-linkage with a phenylalanine residue, or interacted with the latter during the hydrolytic breakdown of the molecule. This suggestion was made because several peptides containing the sequence Phe.Ileu appeared to be present in partial hydrolysates. The homogeneity of some, at least, of the peptides obtained by Lockhart & Abraham (1954a) was open to question, but the bis-DNP derivative of a peptide Phe.Ileu.CySH.Leu, obtained by Hausmann *et al.* (1955a), appears to have been well characterized.

The sulphur atom in bacitracin A is present as a masked thiol group which appears as part of a cysteine residue on hydrolysis. Newton & Abraham (1953b) suggested that bacitracin A contained a thiazoline ring:

This suggestion was made to account for the ready liberation of a thiol group in hot acid solution and for the behaviour of bacitracin A on hydrogenolysis. After treatment with Raney nickel an alanine residue was present whose α -amino group was free, and two new substances were detected among the products of hydrolysis. One of these substances was thought to be an amino alcohol, because hydrolysates in which it was present yielded ammonia and a volatile aldehyde on treatment with periodate. The changes occurring on hydrogenolysis could be interpreted in terms of the removal of the sulphur atom, followed by the hydrolysis and reduction of a resulting $-\text{CH}=\text{N}-$ grouping.

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The isoleucylcysteine fragment which is found in partial hydrolysates of bacitracin A appears to be derived from a structure hitherto unknown in polypeptides. The present paper describes attempts to obtain more precise information about this structure by using reagents for the free amino groups of peptides and by further study of the products of hydrogenolysis with Raney nickel. Some of the results have already been reported in outline (Lockhart *et al.* 1954; Lockhart & Abraham, 1954*b*). Other interesting evidence about this part of the molecule has been mentioned by Hausmann, Weisiger & Craig (1955*b*), who have concluded that a thiazole derivative is formed when bacitracin A is converted into bacitracin F by oxidation in air.

METHODS

Materials

Bacitracin A. The sample of bacitracin A prepared by Lockhart & Abraham (1954*a*) was used. This material was obtained in the solid state by freeze-drying a solution at pH 4 and was partly in the form of its hydrochloride. On exposure to the laboratory air it rapidly absorbed water. The antibacterial activity of the hydrated product was 72 i.u./mg. Elementary analysis was carried out on the hydrated product. (Found: C, 48.2; H, 7.3; N, 14.2; S, 2.0; Cl, 3.2; loss at 100° *in vacuo* (5 hr.), 9.8. Calc. for

C, 48.3; H, 7.4; N, 14.5; S, 2.0; Cl, 3.3; $9\text{H}_2\text{O}$, 9.9%. Mol. wt., 1638.)

The value 1638 has been used in calculations involving the mol. wt. of this product. Hausmann *et al.* (1955*b*) have reported analytical data that are consistent with the formula $\text{C}_{66}\text{H}_{108}\text{O}_{16}\text{N}_{17}\text{S}$ for bacitracin A.

Commercial bacitracin. Experiments were also carried out with samples of bacitracin kindly supplied by the Commercial Solvents Corporation, Terre Haute, Indiana, U.S.A. (Lot 25; 61 i.u./mg.), and by A/S Apothekernes Laboratorium, Oslo, Norway (7364-F. 9; 65 i.u./mg.).

DNP-bacitracin A. Bacitracin A (9.7 ml.) was dissolved in 0.1 ml. of 8% (w/v) NaHCO_3 and 5 ml. of water. FDNB (13 mg.) in ethanol (10 ml.) was added and the solution was adjusted to pH 9 with 8% (w/v) NaHCO_3 . The single-phase reaction mixture was allowed to stand in the dark for 70 min., when the optical density at 350 μ reached a constant value relative to that of a similar solution from which the bacitracin had been omitted. Ethanol was removed by distillation *in vacuo*, the aqueous solution was adjusted to pH 8.5, and excess of FDNB was removed by three extractions with an equal vol. of ether. After removal of excess of ether, the aqueous phase was adjusted to pH 1.5 with conc. HCl, when DNP-bacitracin A was precipitated. The precipitate was separated by centrifuging, washed with 0.01 N-HCl and dried in a desiccator (9.8 mg.). The optical density of this material in 1% (w/v) NaHCO_3 (0.026 mg./ml.) was 0.360 at 350 μ . It presumably consisted of the tri-substituted derivative mixed with some of the mono- and di-substituted derivatives (cf. Craig, Hausmann & Weisiger, 1953). In calculations involving the mol. wt. of this product an approximate value of 1900 has been used.

Deaminated bacitracin A. Bacitracin A (10 mg.) was dissolved in 0.5 ml. of 50% (v/v) aqueous acetic acid. A solution of 128 mg. of barium nitrite in 0.5 ml. of 50% (v/v) aqueous acetic acid was added and the mixture was shaken at 37° for 0.5 hr. The solution was cooled and the supernatant separated from a small amount of white precipitate and evaporated to dryness in a vacuum desiccator. The residue was dissolved in a small volume of 2N-HCl and the solution evaporated to dryness. This process was repeated and the residue was then stirred with ethanol (4 ml.). The pale-yellow extract was evaporated to dryness, yielding 7 mg. of crude deaminated bacitracin A.

Desulphurized bacitracin A. This was prepared in the manner described by Newton & Abraham (1953*b*).

Other compounds. D-Isoleucinol was prepared by the method of Karrer, Portmann & Suter (1948) from D-isoleucine. The chloroaurate, crystallized from water, had m.p. 158°.

N-DNP-isoleucinol was prepared by a modification of the method of Grassmann, Hörmann & Endres (1953). D-Isoleucinol hydrochloride (4.9 mg.) was dissolved in 0.1 ml. of 1.5% (w/v) trimethylamine carbonate (pH 9.3), FDNB (0.01 ml.) in 0.2 ml. of ethanol was added and a few further drops of ethanol were added to effect solution. After the mixture had stood for 2 hr. in the dark, 15 mg. of glycine were added and the mixture was shaken for 2 hr. to convert any excess of FDNB into DNP-glycine. Ethanol was removed by evaporation *in vacuo*, 0.24 ml. of 1% (w/v) trimethylamine carbonate was added, and the aqueous solution was extracted three times with ether. Evaporation of the ethereal extracts yielded 8.9 mg. of DNP-isoleucinol as a yellow oil.

1-Amino-2-methyl-*n*-butane was prepared by heating DL-isoleucine (252 mg.) to 200°, as described by Ehrlich (1907). The product was distilled in steam from aqueous K_2CO_3 and collected in 2N-HCl, to yield the hydrochloride (75 mg.). The chloroplatinate melted at 192–195°. (Found: C, 20.3; H, 4.8. $(\text{C}_6\text{H}_{13}\text{N})_2\cdot\text{H}_2\text{PtCl}_6$ requires C, 20.6; H, 4.8%.) Ehrlich (1907), using L-isoleucine, obtained the chloroplatinate of a partially racemized product whose m.p. was given as 240°.

N-Ethyl-2,4-dinitroaniline was prepared from ethylamine and FDNB. It was obtained as a crystalline solid, m.p. 111°. Dermer & Hutcheson (1943) gave m.p. 111°. On a microgram scale this compound was best prepared by the method used for obtaining DNP-isoleucinol.

Hydrolysis of bacitracin A and its derivatives

Hydrolysis for amino acid analysis. Samples of bacitracin A (4.6 mg.) were hydrolysed in sealed tubes under N_2 with 0.5 ml. of 6N-HCl at 105° for 24 and 90 hr. The hydrolysates were evaporated to dryness in a vacuum desiccator in the presence of P_2O_5 and moist NaOH pellets. Deaminated bacitracin A (5.2 mg.) was similarly hydrolysed with 1 ml. of 6N-HCl at 105° for 24 hr.

Hydrolysis of DNP-bacitracin A. A sample (3.9 mg.) of DNP-bacitracin A was heated in a sealed tube under N_2 with 1 ml. of 6N-HCl at 110° for 10 hr. The hydrolysate was evaporated to dryness as described above. The residue was dissolved in 2 ml. of water and was extracted three times with 2 ml. of ether. The aqueous solution was evaporated to dryness in a vacuum desiccator. The ethereal solution was shaken with three separate lots (2 ml.) of 1% (w/v) NaHCO_3 in water. The ethereal solution retained some

yellow colour due to neutral material. The NaHCO_3 solution was adjusted to pH 1.5 with conc. HCl , shaken with three lots of ether, and the ethereal extract evaporated to dryness. The residue contained acidic ether-soluble DNP derivatives.

Analysis of products

Ninhydrin colour densities were measured in an Evans Electroselenium Ltd. (E.E.L.) portable colorimeter. Other optical densities were measured in a Beckman model DU spectrophotometer or in a Hilger Uvispek photoelectric spectrophotometer (M. 700/302). The cells used were 1 cm.

Amino acids. Qualitative analysis of amino acid mixtures was carried out by chromatography and ionophoresis on Whatman no. 1 paper. Butanol-acetic acid (Woiwod, 1949) and *tert.*-pentanol-water (Work, 1949) were used for developing the chromatograms and spots were coloured with ninhydrin. In the latter solvent system leucine was separated from isoleucine. Ionophoresis was carried out in the manner described by Newton & Abraham (1954) and Lockhart & Abraham (1954a), with sheets of paper 36 cm. long and collidine acetate buffer, pH 7.0. In most cases a potential of 500 v was applied to the ends of the paper for 2.5 hr.

Quantitative analyses were made on the columns (100×0.9 cm.) of Dowex 50-X8 (200-400 mesh), as described by Moore & Stein (1951). The photometric ninhydrin method of Moore & Stein (1948) was used, in the first experiments, for analysis of the effluent fractions. Later, the modified procedure of Moore & Stein (1954) was adopted.

DNP-amino acids. Paper chromatography of DNP-amino acids was carried out on Whatman no. 4 or 3 MM paper in the system of Blackburn & Lowther (1951). Quantitative analysis of DNP-isoleucine (from 3.9 mg. DNP-bacitracin A) was made on a column of acid-washed kieselguhr (16.5×0.9 cm.), using water-saturated chloroform-ethyl methyl ketone (11:9 by vol.) as developing solvent (Mills, 1952). Fractions containing DNP-isoleucine were combined and evaporated to dryness. The residue was dissolved in acetic acid (4 ml.) and the optical density of the solution measured at $340 \text{ m}\mu$. (Schroeder & LeGette, 1953).

DNP-isoleucine and DNP-leucine were not separated on paper chromatograms or on the kieselguhr column. To distinguish between them the DNP-derivative was heated with $0.3 \text{ N-Ba}(\text{OH})_2$ (1 ml.) in a sealed tube at 105° for 1 hr. (Mills, 1950). After CO_2 had been passed through the solution (and the excess of CO_2 removed *in vacuo*) to precipitate Ba^{2+} as BaCO_3 , the supernatant was evaporated to dryness and the residue examined on paper chromatograms run with *tert.*-pentanol-water (Work, 1949). In some experiments hydrolysis was carried out with NH_3 (Lowther, 1951) instead of with $\text{Ba}(\text{OH})_2$. After hydrolysis a spot in the position of leucine or isoleucine was obtained from the corresponding DNP-amino acid.

Separation and identification of volatile bases. On a small scale, volatile bases found to be present in desulphurized bacitracin were separated by ionophoresis on paper in collidine acetate at pH 7 and eluted with water. The experimental procedure was similar to that described by Lockhart & Abraham (1954a). About 50 mg. of the desulphurized bacitracin was applied to the paper as a series of spots (each 1 mg.). A potential gradient of 14 v/cm. was

applied for 2 hr. On a larger scale, the bases were extracted into ether, or distilled in steam, from an alkaline solution (see Results). Distillation in steam was carried out in the apparatus of Markham (1942).

The volatile bases were identified on paper chromatograms developed with butanol-acetic acid (Woiwod, 1949) and with butanol-pyridine-water (Chargaff, Levine & Green, 1948), and by paper ionophoresis in collidine acetate (pH 7.0). DNP-derivatives were identified on paper chromatograms developed with *tert.*-pentanol-phthalate buffer (pH 6) (Blackburn & Lowther, 1951), or with decalin-acetic acid-isopentanol (15:10:4 by vol.) (Grassmann *et al.* 1953).

Oxidation of isoleucinol to isoleucine. Mixtures containing isoleucinol and other volatile bases were treated with silver oxide to convert the isoleucinol into isoleucine (cf. Chibnall & Rees, 1953). In a typical experiment, a mixture (57 mg.) containing about 10 mg. of isoleucinol hydrochloride was dissolved in 5 ml. of water, freshly prepared moist silver oxide (from 100 mg. of AgNO_3) was added and the mixture stirred continuously at 80° for 3 hr. The precipitate was removed by centrifuging and washed three times with water. The aqueous solution was acidified with conc. HCl , the supernatant was separated by centrifuging and the last traces of Ag^+ were removed with H_2S . The resulting solution was freeze-dried.

Desalting. Isoleucine obtained by oxidation of mixtures of volatile bases (containing inorganic salts) was desalted in the following way (J. M. Barry, private communication). An aqueous solution of the preparation (1 ml.) was added to a column (diam. 1.3 cm. and ht. 1 cm.) of the hydrogen form of Dowex 50. The column was washed with 6 ml. of water and the resin was then suspended in water and the pH adjusted to 8 with 2 N-NH_3 . The resin was separated by centrifuging and washed with water and the supernatant was freeze-dried.

RESULTS

Isoleucine content of bacitracin A. The amounts of isoleucine, *allo*isoleucine, and some of the other amino acids found in hydrolysates of bacitracin A by analysis on columns of Dowex 50 are shown in Table 1. Hydrolysis was carried out in 6 N-HCl at 105° for 24 and 90 hr. respectively. In the first case, aspartic and glutamic acids were not determined and the experiment was terminated when leucine had emerged from the column. In the second case, the experiment was terminated after the emergence of phenylalanine.

Table 1. Amounts of certain amino acids in hydrolysates of bacitracin A

Hydrolysis was carried out in 6 N-HCl at 105° .

Amino acid	Molecules/molecule of bacitracin A after		Molecules/molecule of leucine after	
	24 hr.	90 hr.	24 hr.	90 hr.
Aspartic acid	—	1.66	—	1.90
Glutamic acid	—	0.78	—	0.89
Cystine	0.43	0.23	0.50	0.26
<i>allo</i> Isoleucine	0.44	0.44	0.51	0.51
Isoleucine	1.75	1.96	2.00	2.25
Leucine	0.87	0.87	1.00	1.00
Phenylalanine	—	0.89	—	1.02

The N-terminal residue of bacitracin A

Ninhydrin colour density given by bacitracin A. The colour density obtained when bacitracin A was treated with ninhydrin (Moore & Stein, 1948), compared with that obtained from leucine, acid-inactivated bacitracin A, 6-aminohexanoic acid, lysine, gramicidin S and α -(L-valyl)-L-ornithine under similar conditions is shown in Table 2. A solution of acid-inactivated bacitracin A was prepared by treating bacitracin A with N-HCl at 100° for 20 min. The colour densities per mole are relative to that given by leucine, which has been assigned an arbitrary value of 1.

Table 2. *Ninhydrin colour densities given by bacitracin A and other substances*

The sample (in 0.1 ml. of water) was heated with 0.5 ml. of the ninhydrin reagent (Moore & Stein, 1948) in colorimeter tubes, 1.4 cm. in internal diam. The solution was diluted with 7.5 ml. of propanol-water (1:1 by vol.) and the colour density was measured in an E.E.L. portable colorimeter (Evans Electroselenium Ltd., Harlow, Essex), Filter 626 being used.

Substance	Amount in sample (μ mole)	Colorimeter reading	Relative colour density/mole
Leucine	0.153	50.5	1.00
6-Aminohexanoic acid	0.153	49.2	0.98
Lysine	0.153	58.7	1.16
Bacitracin A	0.155	78.4	1.53
Acid-inactivated bacitracin A	0.087	76.0	2.61
Gramicidin S	0.077	31.6	1.25
α -(L-Valyl)-L-ornithine	0.153	56.6	1.12

The solution of acid-inactivated bacitracin A contained ammonium chloride (1 mole/mole of bacitracin A) liberated by hydrolysis of the labile amide grouping in bacitracin A (Newton & Abraham, 1953a). The increase in ninhydrin colour density on acid inactivation was completely accounted for by the amount of ammonium chloride formed. This indicated that the labile amide grouping in bacitracin A did not contribute to its ninhydrin colour density. The major component of gramicidin S is a decapeptide (mol.wt. 1142) containing two free δ -amino groups (Synge, 1945; Battersby & Craig, 1951). The dipeptide α -(L-valyl)-L-ornithine contains one free α -amino and one free δ -amino group and is comparable, in this respect, with bacitracin A.

DNP-isoleucine and other products from DNP-bacitracin A. A sample of the acidic ether-soluble material obtained from the hydrolysis of 3.86 mg. of DNP-bacitracin A gave a spot corresponding to isoleucine, or *alloisoleucine*, after treatment with a solution of barium hydroxide at 105°, on a paper chromatogram run with *tert*-pentanol-water. A

further sample gave a solution in sodium bicarbonate solution whose optical density at 350 m μ . could be accounted for by the presence of 143 μ g. of DNP-isoleucine in the total material. Further purification of this material, however, showed that substances other than isoleucine were present.

A paper chromatogram of a sample of the material, with the system of Blackburn & Lowther (1951), showed a yellow spot in the position of DNP-isoleucine (R_F , 0.48), and a small yellow spot in the position of 2:4-dinitrophenol (R_F , 0.29). A bright-green fluorescent spot appeared at the origin when the paper was viewed under ultraviolet light (365 m μ .). Visual comparison of the intensity of the DNP-isoleucine spot with that of spots obtained from known amounts of authentic DNP-isoleucine suggested that about 50 μ g. of DNP-isoleucine were present in the total material.

When the remainder of the material was chromatographed on kieselguhr (Mills, 1952) in chloroform-ethyl methyl ketone (11:9), two yellow bands were observed to move down the column. A faint fast-moving one was 2:4-dinitrophenol, and a slower-moving one, which began to emerge after 14 ml. of solvent had passed through the column, was DNP-isoleucine. The optical densities, at 340 m μ ., of solutions of the substances from the two bands in acetic acid corresponded to the presence of 8.8 μ g. of 2:4-dinitrophenol and 48 μ g. of DNP-isoleucine in the whole of the original material. If 1 molecule of DNP-bacitracin A had yielded 1 molecule of DNP-isoleucine, the amount of the latter obtained in this experiment would have been about 600 μ g. If the small amount of 2:4-dinitrophenol were assumed to be formed by decomposition of DNP-isoleucine during hydrolysis, the yield would be raised from 8 to 10.4%.

Amino acids from deaminated bacitracin A. After bacitracin A had been treated with nitrous acid and hydrolysed with 6N-HCl at 105° for 24 hr., one sample of the hydrolysate (200 μ g.) was studied on a paper chromatogram run with butanol-acetic acid and another (70 μ g. in 2 μ l.) was subjected to ionophoresis on paper in collidine acetate buffer, pH 7. The spots were coloured with ninhydrin. Ionophoresis showed that the spot due to ornithine in hydrolysates of bacitracin A had disappeared. On the chromatogram, the cystine spot was replaced by a spot in the position of cysteic acid. There were faint unidentified spots between the glutamic acid and valine positions and also a spot slightly beyond the valine position. In addition there was a faint-yellow spot in the position of proline (R_F , 0.35). Consden, Gordon & Martin (1947) reported that proline was formed from ornithine when peptides containing the latter were treated with nitrous acid.

Another sample of the hydrolysate (from 5.2 mg. of deaminated bacitracin A) was examined on a

column of Dowex 50. Table 3 shows the relative molar quantities of various amino acids found in the effluent from the column, assuming the value for leucine to be 1.

It is evident that ornithine and *alloisoleucine*, which are both present in hydrolysates of bacitracin A, were absent from the hydrolysate of the material that had been treated with nitrous acid. Fig. 1 shows the part of the effluent curve in which isoleucine and leucine emerged from the column, together with a comparable curve from a hydrolysate of bacitracin A.

Table 3. *Relative amounts of amino acids in a hydrolysate of deaminated bacitracin A*

Hydrolysis was carried out in 6N-HCl at 105° for 24 hr.

Amino acid	Molecules/molecule of leucine
Cysteic acid	0.31
Aspartic acid	1.78
Glutamic acid	0.89
<i>alloisoleucine</i>	0.00
Isoleucine	1.93
Leucine	1.00
Phenylalanine	1.00
Histidine	0.83
Ornithine	0.00
Lysine	0.71

In addition to the amino acids shown in Table 3, there were four small peaks due to unidentified ninhydrin-positive substances, one emerging just before glutamic acid and the other three between glutamic acid and isoleucine. One of the last three may have been due to cystine which had not been oxidized to cysteic acid. No analysis was made for proline.

Products of hydrogenolysis of bacitracin A

The finding of Newton & Abraham (1953*b*) that the DNP derivative of desulphurized bacitracin A yielded some DNP-alanine on hydrolysis was confirmed.

Properties of volatile bases. When desulphurized bacitracin A (1 mg.) was subjected to ionophoresis on paper in collidine acetate at pH 7 (14v/cm. for 2 hr.) and the paper sprayed with ninhydrin, there were a strong spot due to neutral material and three weaker spots due to substances that had migrated rapidly towards the cathode. These basic substances will be referred to as α , β and γ respectively, in order of increasing mobility. The mobility of α was almost the same as that of ornithine. The spot due to β was stronger than that due to α or γ . All three substances could be distilled in steam, from an aqueous solution made

Fig. 1. Elution from a column of Dowex 50 (0.9 cm. $\times$ 100 cm.) of the *alloisoleucine*, isoleucine and leucine present in hydrolysates of (a) bacitracin A, and (b) deaminated bacitracin A. The conditions were those of Moore & Stein (1951).

alkaline with potassium carbonate, and collected in dilute hydrochloric acid; in this way they could be separated from the neutral peptide material which comprised the main bulk of desulphurized bacitracin A.

Virtually all the α , β and γ formed on hydrogenolysis were present in desulphurized bacitracin A in the free state. After removal of the volatile free bases from a sample of the latter (5.2 mg.) by steam distillation of a solution made alkaline with barium hydroxide (pH approx. 12), barium was removed from the residual solution by precipitation as barium sulphate. The supernatant was evaporated to dryness and the residue hydrolysed with 6*N*-HCl at 105° for 24 hr. The hydrolysate was evaporated to dryness and the product distilled in steam from a solution made alkaline with barium hydroxide. The ratio of the ninhydrin colour density (Moore & Stein, 1954) of a sample of the first distillate to that of a comparable sample of the second was 6.5:1.0. When a sample of the second distillate was subjected to ionophoresis on paper (pH 7), traces of α and β were revealed, but the intensity of the spots was less than that expected from the ninhydrin colour density of the distillate. The latter probably contained ammonia that had been formed during the hydrolysis with hydrochloric acid.

Samples of desulphurized commercial bacitracin (from lot 25, lot 311 and 7364-F. 9) were all found to contain three steam-volatile bases which were indistinguishable from α , β and γ respectively by ionophoresis at pH 7. Further experiments were therefore carried out with the more readily available commercial material. These experiments led to the isolation of the three bases (see below).

Table 4. Volatile bases from desulphurized bacitracin A

Substance	Migration (pH 7) towards cathode (cm.)	R_F value in		Approx. yield (μ g./mg.)
		Butanol- acetic acid	Butanol- pyridine- water	
α	8.5	0.71	0.40	30
β	9.75	0.79	0.65	13
γ	14	0.49	0.22	2

The behaviour of the bases when studied separately by ionophoresis on paper at pH 7 (14 v/cm. for 2 hr.) and on paper chromatograms developed with butanol-acetic acid and with butanol-pyridine-water, is shown in Table 4. The bases were applied to the paper in the form of their hydrochlorides. In all the properties listed, α was indistinguishable from isoleucinol, β from 1-amino-2-methyl-*n*-butane, and γ from ethylamine. The DNP derivatives of α and γ showed the same R_F value as

DNP-isoleucinol (0.88) and *N*-ethyl-2:4-dinitro-aniline (0.81) respectively on paper chromatograms developed with decalin-acetic acid-isopentanol. The identity of α and isoleucinol was confirmed by oxidizing the α with silver oxide. The product behaved as a neutral substance during ionophoresis on paper at pH 7 and showed the same R_F value as isoleucine on a chromatogram run with *tert*-pentanol-water.

Table 4 also shows the approximate amounts of α , β and γ which appeared to be present in 1 mg. of desulphurized bacitracin A. The values were estimated by visual comparison of the intensities of the spots obtained when desulphurized bacitracin A was subjected to ionophoresis on paper with those obtained from known amounts of the hydrochlorides of isoleucinol, 1-amino-2-methyl-*n*-butane and ethylamine.

Isolation of volatile bases. A solution of α , free from β and γ , was obtained by subjecting desulphurized bacitracin (50 mg. from lot 25) to ionophoresis on paper. On a larger scale use was made of the fact that α and β could be extracted from an aqueous alkaline solution with ether, whereas γ remained almost entirely in the aqueous phase. After treatment of the mixture of α and β with silver oxide the unchanged β could be readily separated from the isoleucinol formed from α .

A solution of desulphurized bacitracin (6.2 g. of 7364-F. 9) in 36 ml. of water and 6 ml. of saturated aqueous K_2CO_3 was shaken five times with 40 ml. of ether. The combined ethereal extracts were shaken three times with 2*N*-HCl (40 ml.) and the aqueous solution was freeze-dried. The residue was dissolved in water (4.5 ml.), saturated aqueous K_2CO_3 (4 ml.) was added and the volatile bases were distilled in steam into 2 ml. of 5*N*-HCl. The distillate (40 ml.) was freeze-dried, yielding 57 mg. of residue. The latter was oxidized in aqueous solution with silver oxide. The product was dissolved in water (3 ml.), 0.3*N*-Ba(OH)₂ (1 ml.) added and the volatile bases were removed by distillation in steam into 1 ml. of 5*N*-HCl. The distillate (40 ml.) was freeze-dried, yielding 26 mg. of residue. When a sample of the latter was subjected to ionophoresis on paper (pH 7), the main spot corresponded to β and there was only a trace of unchanged α .

The pH of the alkaline solution of the non-volatile material was adjusted to 5 with 2*N*-H₂SO₄. After removal of the precipitated BaSO₄, the supernatant yielded 43 mg. of a brown residue which consisted partly of BaCl₂. Comparison of a sample of this material with known amounts of isoleucine on a paper chromatogram run with butanol-acetic acid indicated that it contained about 3.5 mg. of isoleucine. It also contained unidentified material, since it showed an absorption maximum ($E_{1\text{ cm.}}^{1\%}$ 6.3) at 352 m μ . When the material was desalted on a short column of Dowex 50, the product weighed 5.1 mg. Measurement of its ninhydrin colour density indicated that it contained 3.1 mg. of isoleucine.

The fraction containing β was dissolved in water (1 ml.), aqueous saturated K_2CO_3 (0.25 ml.) was added and the solution shaken four times with an equal volume of light petroleum (b.p. 60–80°). The extract was shaken with three lots (each 2 ml.) of 2*N*-HCl and the aqueous solution

evaporated to dryness, yielding 12.3 mg. of residue. By this procedure β was separated from traces of α and γ . However, examination of samples of the product by ionophoresis on paper and determination of its ninhydrin colour density indicated that it could contain only about 25% of the hydrochloride of 1-amino-2-methyl-*n*-butane. Its ultraviolet absorption spectrum showed a weak maximum ($E_{1\text{ cm}}^{1\%}$ 3.6) at 260 μ . The unidentified material in the product appeared to remain in the supernatant when β was precipitated as its chloroplatinate. The product (12 mg.) was dissolved in one drop of water and a solution containing 8 mg. of platinum chloride in 0.1 ml. of *N*-HCl was added. The chloroplatinate of β separated as yellow platelets. After recrystallization from hot water it melted at 192–195° (micro hot stage). After mixture with an authentic sample of the chloroplatinate of 1-amino-2-methyl-*n*-butane (m.p. 192–195°) the m.p. was 192–194°. Treatment of the chloroplatinate with aqueous K_2CO_3 , distillation in steam and collection of the distillate in dilute HCl yielded a hydrochloride which showed the typical behaviour of β when subjected to chromatography or ionophoresis on paper.

The volatile base remaining in the alkaline aqueous solution after α and β had been extracted from desulphurized bacitracin with ether was distilled in steam and the distillate (100 ml.) collected in 20 ml. of 2*N*-HCl. The resulting solution was freeze-dried, yielding 200 mg. of a crystalline product which was found to be a mixture of the hydrochloride of γ and NH_4Cl . The mixture was shaken twice with 1 ml. of ethanol, which dissolved the γ . The colourless crystalline residue was dried and analysed by the micro-Kjeldahl method. (Found: N, 24.7. Calc. for NH_4Cl : N, 26.2%.) When mixed with an aqueous solution of sodium picrate it yielded yellow needles whose appearance under crossed nicols was identical with that of crystals of ammonium picrate (Richter, 1937).

The material from the ethanolic extract, consisting of γ and NH_4Cl , was subjected to ionophoresis in 0.05*M* potassium phosphate buffer, pH 6.7, on four sheets of Whatman no. 3MM paper, each sheet carrying 20 spots. A potential of 5.6 v/cm. was applied for 4 hr. After γ had been eluted from the paper with water the eluate was mixed with 4 ml. of a saturated solution of K_2CO_3 , distilled in steam, and the distillate collected in 2 ml. of *N*-HCl. Evaporation of the distillate (20 ml.) to dryness yielded 6 mg. of residue. A sample of the latter (2.4 mg.) was dissolved in two drops of water and 0.5 ml. of a saturated aqueous solution of picric acid was added. On concentrating in a vacuum desiccator yellow crystals separated, which were filtered, recrystallized from hot water and dried *in vacuo* (2.5 mg.). The picrate had m.p. 163–166°. When mixed with an authentic sample of ethylamine picrate (m.p. 166–167°), the m.p. was 166–167°.

Analysis on Dowex 50 of isoleucine formed by oxidation of the isoleucinol from desulphurized bacitracin

A sample (0.5 mg.) of the isoleucine obtained as described above was mixed with authentic DL-leucine (0.25 mg.) and the mixture analysed on a column of Dowex 50. The leucine had previously been shown to yield a single peak when chromatographed under these conditions. The results are

shown in Fig. 2. The product from desulphurized bacitracin consisted of 60% of isoleucine and 40% of *allo*isoleucine. A sample (0.18 mg.) of isoleucine formed by oxidation of isoleucinol that had been obtained in another experiment with desulphurized

Fig. 2. Elution from a column of Dowex 50 (0.9 cm. $\times$ 100 cm.) of a mixture of leucine and the product of oxidation of isoleucinol from desulphurized bacitracin. The conditions were those of Moore & Stein (1951).

Fig. 3. Elution from a column of Dowex 50 (0.9 cm. $\times$ 100 cm.) of *allo*isoleucine, isoleucine and leucine. —, Hydrolysate of bacitracin A; ---, hydrolysate of bacitracin A mixed with the product of oxidation of isoleucinol from desulphurized bacitracin. The conditions were those of Moore & Stein (1951).

bacitracin was mixed with one-half of the product obtained by hydrolysing bacitracin A (9.74 mg.) with 6N-HCl at 105° for 24 hr. The mixture was analysed on a column of Dowex 50, and the second half of the hydrolysate of bacitracin A was analysed separately. The results are shown in Fig. 3. The material added to the first half of the hydrolysate appeared to consist of 67% of isoleucine and 33% of *allo*isoleucine. The isoleucine used in this case had been prepared by a process which was similar to the one described above, with the exception that distillation in steam was replaced by extraction with ether. Steam distillation under alkaline conditions was avoided in order to minimize the possibility of racemization during the course of purification.

DISCUSSION

Analysis of the amino acids present after bacitracin A had been hydrolysed in 6N hydrochloric acid at 105° for 24 hr. indicated that there were 2 mol. of isoleucine and 0.5 mol. of *allo*isoleucine for each molecule of leucine. The amount of isoleucine was somewhat larger when hydrolysis was continued for 90 hr.

The value for isoleucine and *allo*isoleucine combined, which is higher than that previously recorded (Hausmann *et al.* 1955*b*), is consistent with the assumption that the isoleucine-*allo*isoleucine mixture in hydrolysates is derived from three residues in the molecule of bacitracin A. Harfenist (1953) and Hirs, Stein & Moore (1954) pointed out that prolonged hydrolysis was required to liberate all the isoleucine from certain peptides. One of the isoleucine residues of bacitracin A appears in partial hydrolysates in the peptide Ileu.Phe and another in the peptides Ileu.Lys and Ileu(Asp).Lys. All these peptides have been found to be particularly resistant to further hydrolysis (Hausmann *et al.* 1955*b*; Lockhart & Abraham, 1954*b*). It would therefore be understandable if the yield of isoleucine were less than theoretical.

Assuming that bacitracin A does not consist of two peptides with different *N*-terminal residues, the value 0.5 for the quotient *allo*isoleucine/leucine must be accounted for by racemization of the α -carbon atom of either an isoleucine residue or an *allo*isoleucine residue. However, only the former explanation, which has been suggested by Hausmann *et al.* (1955*b*), seems to be consistent with the results of other experiments.

The ninhydrin colour density given by bacitracin A, which is 1.5 times as large per mole as that given by leucine, supports the view that the molecule contains a free amino group other than the δ -amino group of ornithine. Among the compounds used for comparison, 6-aminohexanoic acid showed a

colour density per mole which was almost as high as that shown by leucine. The corresponding values for the compounds containing two free amino groups were greater than the value for leucine but less than that for bacitracin A. It is possible that the ninhydrin colour given by a large molecule containing two free amino groups will depend partly on the distance of one amino group from the other.

There is no doubt that the dinitrophenyl derivative of bacitracin A yields DNP-isoleucine (and/or DNP-*allo*isoleucine) on hydrolysis. The derivatives of isoleucine are the only identified products that could have been derived from an *N*-terminal residue; but the poor yield in which these derivatives were obtained, and the fact that the DNP-isoleucine was accompanied by uncharacterized ether-soluble material, indicate that there are unknown factors in the formation or hydrolysis of the DNP-derivatives of the polypeptide. Porath (1954) has stated that samples of commercial bacitracin contain isoleucine. He has suggested that the latter is still present in preparations of bacitracin A and may be responsible for reports that isoleucine occurs as an *N*-terminal amino acid. However, in the solvent system used here for its isolation, bacitracin A has a partition coefficient which is more than ten times as large as that of isoleucine. No significant amount of isoleucine could therefore have remained in the band from which the final product was isolated.

The reaction of bacitracin A with nitrous acid, which yields a product that gives no *allo*isoleucine on hydrolysis, appears to have produced clear evidence that the *allo*isoleucine is derived from an *N*-terminal residue. The latter is presumably adjacent to the potential cysteine residue, since Lockhart & Abraham (1954*b*) reported that isoleucine residues in two other positions in the molecule remained intact in the deaminated product.

Additional evidence for this view has come from the study of the products of hydrogenolysis of bacitracin A. Preliminary experiments showed that the amino alcohol first found in hydrolysates of desulphurized bacitracin A might be isoleucinol, and that the aldehyde formed on oxidation with periodate might be α -methylbutyraldehyde. An indication that the amino alcohol was isoleucinol was obtained from its behaviour on two-dimensional paper chromatograms, run first with butanol-acetic acid and then with phenol in the presence of 5N hydrochloric acid. This view gained support when the 2:4-dinitrophenylhydrazone of the aldehyde was shown to have the same R_F value as α -methylbutyraldehyde 2:4-dinitrophenylhydrazone on paper chromatograms (Burton, 1954), and it was consistent with the later finding that the sequence Ileu.CyS- occurred in the molecule. The work described here has shown conclusively that

isoleucinol is a product of hydrogenolysis of bacitracin A, and also that the isoleucinol appears in the free state before hydrolysis of the peptide chain with hydrochloric acid. From this behaviour it is reasonable to conclude that the isoleucinol is derived from an *N*-terminal isoleucine residue. The finding that free 1-amino-2-methyl-*n*-butane is formed on hydrogenolysis may be interpreted in a similar way. Among the amino acid residues known to be present in the molecule, isoleucine is the only conceivable source of this substance.

Reactions which appear to occur when bacitracin A is treated with Raney nickel, and when it is inactivated with hot dilute acid, are illustrated by the scheme shown in Fig. 4. The interrupted lines show the bonds broken on hydrogenolysis. The

and that the resulting isoleucinol might therefore be obtained largely in one optical form. After oxidation of the isoleucinol to isoleucine, analysis on columns of Dowex 50 made it possible to determine whether the isoleucine was a mixture of diastereoisomers. The results showed that considerable racemization had, in fact, occurred, but that isoleucine, and not *allo*isoleucine, was the major product. They were therefore consistent with the assumption that an isoleucine residue occupies the *N*-terminal position in the intact molecule of bacitracin A, and that the *allo*isoleucine in hydrolysates is derived from this residue. It may be mentioned that Brockmann, Grubhofer, Kass & Kalbe (1951) isolated *D-allo*isoleucine from the products of hydrolysis of actinomycin C. In this

Fig. 4

formation of 1-amino-2-methyl-*n*-butane and of ethylamine involves the breaking of carbon-carbon bonds. This is known to occur, to a minor degree, during the action of Raney nickel on penicillin (Kaczka & Folkers, 1949).

Ehrlich (1907) found that *L*-isoleucine yielded a mixture of *L*-isoleucine and *D-allo*isoleucine when heated with barium hydroxide solution at 180° . Assuming that the *allo*isoleucine found in acid hydrolysates of bacitracin A had been formed by racemization from an *N*-terminal isoleucine, it was thought that this change might not take place readily during hydrogenolysis with Raney nickel

case, however, racemization does not seem to be involved, since no other isomer of isoleucine was reported to be present.

The racemization of α -amino acids is generally thought to be a consequence of the ionization of a hydrogen on the α -carbon atom (Neuberger, 1948). In the 2-(1-amino-2-methyl-*n*-butyl)thiazoline which appears to form the terminal part of the bacitracin A molecule, the ionization of hydrogen on the carbon carrying the amino group should be facilitated, under strongly acid conditions, by the positive charges on the nitrogen atoms and by the possibility of resonance in the resulting structure.

Under these conditions the rate of racemization might be sufficiently rapid to compete with reactions that result in the opening of the thiazoline ring and the formation of a normal peptide bond (Fig. 5).

Racemization of the isoleucine obtained by oxidation of the isoleucinol from desulphurized bacitracin may have occurred partly during the process of isolation, but, in view of the relatively

SUMMARY

1. The isoleucine in hydrolysates of bacitracin A has been analysed on columns of Dowex 50. After hydrolysis with 6*N* hydrochloric acid for 24 hr., about 2 mol. of isoleucine and 0.5 mol. of *allo*isoleucine were found to be present for each molecule of leucine. The amount of isoleucine was somewhat larger after hydrolysis for 90 hr.

Fig. 5

mild conditions that were used, it appears more likely to have taken place during hydrogenolysis. However, the possibility that some racemization may occur during the isolation of bacitracin A itself, without fission of the sulphur-containing ring, cannot be ignored.

The results presented in this paper seem to be best explained by the assumption that a thiazoline ring is present in bacitracin A. Moreover, two properties of bacitracin A whose interpretation was previously uncertain now appear to be consistent with the thiazoline hypothesis. The failure to detect a basic group in the molecule which could be attributed to the nitrogen of a thiazoline ring (Newton & Abraham, 1953*b*) is understandable if the thiazoline is substituted by an *N*-terminal residue. The presence of a positively charged amino group in this residue, in addition to the CO of the amide linkage through which the residue is linked to the peptide chain, might well bring the *pK* of the thiazoline nitrogen below the range that was covered by electrometric titration. The decrease in molecular extinction coefficient at 254 mμ., which occurs when bacitracin A is inactivated by acid (Newton & Abraham, 1953*b*), is similar to the molecular extinction coefficient that Kuhn & Drawert (1954) have reported for 2-methylthiazoline in this region. Nevertheless, there are clearly properties of the thiazoline structure which need further exploration. The reasons for the low values obtained when the *N*-terminal residue is estimated by the use of FDNB remain uncertain. At least one product that has not yet been identified appears to be formed during hydrogenolysis with Raney nickel. The mechanism whereby the amino group of the *N*-terminal residue may interact with a phenylalanine residue in the peptide chain, and the possibility, mentioned by Hausmann *et al.* (1955*a*), that acyl migration between sulphur and nitrogen plays a part in the chemistry of bacitracin A, are among other interesting problems for future investigation.

2. The ninhydrin colour density given by bacitracin A indicated that a free amino group other than the δ-amino group of ornithine was present in the molecule. In hydrolysates of DNP-bacitracin A, DNP-isoleucine (and/or DNP-*allo*isoleucine) was the only identified product that could have been derived from an *N*-terminal residue.

3. Deaminated bacitracin A, obtained by treating bacitracin A with nitrous acid, yielded no *allo*-isoleucine on hydrolysis. Ornithine was also absent from the hydrolysate and cysteine had been partly oxidized to cysteic acid. The remaining amino acids appeared to be unchanged.

4. Hydrogenolysis of bacitracin A with Raney nickel resulted in the formation of an *N*-terminal alanine residue and three volatile bases. The latter were identified as isoleucinol, 1-amino-2-methyl-*n*-butane and ethylamine respectively. Using commercial bacitracin, 1-amino-2-methyl-*n*-butane was isolated as a chloroplatinate and ethylamine as a picrate.

5. The isoleucinol obtained by hydrogenolysis of commercial bacitracin was oxidized to isoleucine. Analysis of the latter on columns of Dowex 50 showed that it was a mixture of isoleucine and *allo*isoleucine, the isoleucine being the major component.

6. These results are thought to be consistent with the view that bacitracin A contains three isoleucine residues, that an *N*-terminal isoleucine residue is condensed with the cysteine residue to form a thiazoline ring, and that racemization of this isoleucine residue is responsible for the *allo*-isoleucine that appears in hydrolysates.

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REFERENCES

- Barry, G. T., Gregory, J. D. & Craig, L. C. (1948). *J. biol. Chem.* **175**, 485.
- Battersby, A. R. & Craig, L. C. (1951). *J. Amer. chem. Soc.* **73**, 1887.
- Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
- Brockmann, H., Grubhofer, N., Kass, W. & Kalbe, H. (1951). *Ber. dtsch. chem. Ges.* **84**, 260.
- Burton, H. S. (1954). *Chem. & Ind.* p. 576.
- Chargaff, E., Levine, C. & Green, C. (1948). *J. biol. Chem.* **175**, 67.
- Chibnall, A. C. & Rees, M. W. (1953). In *Chemical Structure of Proteins*, p. 79. London: J. and A. Churchill Ltd.
- Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
- Craig, L. C., Hausmann, W. & Weisiger, J. R. (1953). *J. biol. Chem.* **200**, 765.
- Craig, L. C., Hausmann, W. & Weisiger, J. R. (1954). *J. Amer. chem. Soc.* **76**, 2839.
- Dermer, O. C. & Hutcheson, J. W. (1943). *Proc. Okla. Acad. Sci.* **23**, 60.
- Ehrlich, F. (1907). *Ber. dtsch. chem. Ges.* **40**, 2538.
- Grassmann, W., Hörmann, H. & Endres, H. (1953). *Ber. dtsch. chem. Ges.* **86**, 1477.
- Harfenist, E. J. (1953). *J. Amer. chem. Soc.* **75**, 5528.
- Hausmann, W., Weisiger, J. R. & Craig, L. C. (1955a). *J. Amer. chem. Soc.* **77**, 723.
- Hausmann, W., Weisiger, J. R. & Craig, L. C. (1955b). *J. Amer. chem. Soc.* **77**, 721.
- Hirs, C. H. W., Stein, W. H. & Moore, S. (1954). *J. biol. Chem.* **211**, 941.
- Ingram, V. M. (1953). *J. biol. Chem.* **202**, 193.
- Kaczka, E. & Folkers, K. (1949). In *The Chemistry of Penicillin*, p. 244. Princeton University Press.
- Karrer, P., Portmann, P. & Suter, M. (1948). *Helv. chim. acta*, **31**, 1617.
- Kuhn, R. & Drawert, F. (1954). *Liebigs Ann.* **590**, 55.
- 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., Newton, G. G. F. & Abraham, E. P. (1954). *Nature, Lond.*, **173**, 536.
- Lowther, A. G. (1951). *Nature, Lond.*, **167**, 767.
- Markham, R. (1942). *Biochem. J.* **36**, 790.
- Mills, G. L. (1950). *Nature, Lond.*, **165**, 403.
- Mills, G. L. (1952). *Biochem. J.* **50**, 707.
- 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.
- Neuberger, A. (1948). *Advanc. Protein Chem.* **4**, 297.
- Newton, G. G. F. & Abraham, E. P. (1953a). *Biochem. J.* **53**, 597.
- Newton, G. G. F. & Abraham, E. P. (1953b). *Biochem. J.* **53**, 604.
- Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
- Piez, K. A. (1954). *J. biol. Chem.* **207**, 77.
- Porath, J. (1953). *Nature, Lond.*, **172**, 871.
- Porath, J. (1954). *Acta chem. scand.* **8**, 1813.
- Richter, D. (1937). *Biochem. J.* **31**, 2022.
- Schroeder, W. A. & LeGettê, J. (1953). *J. Amer. chem. Soc.* **75**, 4612.
- Synge, R. L. M. (1945). *Biochem. J.* **39**, 363.
- 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.

Nitrogen Excretion in the Tadpoles of *Xenopus laevis* Daudin

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As was pointed out by Delaunay (1931), and emphasized by Needham (1931), the excretion of ammonia as the predominant end-product of protein metabolism is characteristic of aquatic animals as a whole but is supplanted by that of urea (amphibia; chelonian reptiles; mammals) or uric acid (saurian reptiles; birds) in amphibious and terrestrial animals. Needham (1931) collected numerous data in support of this general proposition.

The amphibia are of particular interest from this point of view since they are, in general, wholly

aquatic during the larval period and go on the land only after metamorphosis. Munro (1939) found that the tadpoles of *Rana temporaria* are wholly ammoniotelic, and that the transition to ureotelism takes place in the course of metamorphosis. Thus this animal recapitulates its chemical as well as its evolutionary past in the course of its larval development.

In *Xenopus laevis* we have an amphibian which passes both its larval and adult lives without leaving the water. Unlike *Rana* and the other frogs and toads that have been studied, the adult *Xenopus* continues to excrete the bulk of its waste nitrogen as ammonia (Munro, 1953; Cragg, 1953).

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