Racker, E. (1950). *J. biol. Chem.* **184**, 313.
Sacktor, B. (1954). *J. gen. Physiol.* **37**, 343.
Siekivitz, P. & Potter, V. R. (1955). *J. biol. Chem.* **215**, 221.
Siekivitz, P. & Watson, M. L. (1956). *J. biophys. biochem. Cytol.* **2**, 653.
Slater, E. C. & Holton, F. A. (1953). *Biochem. J.* **55**, 530.
Slater, E. C. & Holton, F. A. (1954). *Biochem. J.* **56**, 28.
Wade, H. E. & Morgan, D. M. (1954). *Biochem. J.* **56**, 41.
Wade, H. E. & Morgan, D. M. (1955). *Biochem. J.* **60**, 264.
Watanabe, M. I. & Williams, C. M. (1951). *J. gen. Physiol.* **34**, 675.
Winteringham, F. P. W., Bridges, P. M. & Hellyer, G. C. (1955). *Biochem. J.* **59**, 13.
Winteringham, F. P. W., Harrison, A. & Bridges, R. G. (1952). *Nucleonics*, **10**, 52.

# Observations on the Molecular Weight and Chemical Composition of Nisin A

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(*Received* 17 *March* 1958)

Nisin is an antibiotic normally made up of a mixture of peptides containing the following amino acids: alanine, glycine, serine, aspartic acid, valine, methionine, leucine, isoleucine, histidine, lysine, proline, lanthionine and β-methyl-lanthionine.

Some of the single peptides received a preliminary investigation by Berridge, Newton & Abraham (1952), who worked with commercial material prepared at great cost for therapeutic experiments. Cheeseman & Berridge (1957) described a more economical method of preparation of material which was intended for structural investigations. This report describes the separation of the major peptide by countercurrent distribution, a determination of its molecular weight and the derivation of some possible amino acid sequences.

The determination of amino acids by elution from paper chromatograms was used to derive a minimum molecular weight. This was in good agreement with the maximum molecular weight calculated from diffusion coefficients. Thus a reasonable estimate for actual molecular weight could be made.

The molecule as a whole proved too complex for a total determination of the amino acid sequence at this stage, but the structures of several small portions of the polypeptide chain isolated by partial hydrolysis in acid were determined, end-group analysis being used.

## EXPERIMENTAL

*Solvents for countercurrent distribution.* Unless otherwise stated, all solvents employed were of A.R. grade (British Drug Houses Ltd.). The butan-1-ol was tested before use for the presence of peroxides (Newton & Abraham, 1950), and was considered free of peroxides if no red colour developed when the alcohol was added to a freshly prepared solution of pure KSCN and $FeSO_4,(NH_4)SO_4,6H_2O$. Any positive samples found were redistilled over zinc powder before use.

Berridge *et al.* (1952) had used a solvent system consisting of methanol, butan-1-ol and acetate buffer at pH 3·0, in which nisin had an overall partition coefficient close to unity. This system had two advantages for its use. First, the partition coefficient at the beginning was close to unity; and other things being equal the maximum resolving power is obtained from a limited number of transfers when the geometric mean of the partition coefficients of the components of a mixture approaches unity (Craig & Craig, 1956). Secondly, at the pH used, some of the groups in the material appeared to be partly ionized and differences in solubility due to differences in the dissociation constants of the ionizable groups may occur under such conditions. The system had, however, a serious disadvantage from the preparative aspect which was that the nisin was not very soluble. It was therefore necessary to produce a new solvent which did not have this disadvantage, but retained the two advantages mentioned above. A mixture consisting of butan-1-ol, water, acetic acid and aq. $NH_3$ soln., with the pH of the aqueous phase at 2·8 to 3·0, was found to give satisfactory results. It was made by mixing 5 l. of water, 3·75 l. of butan-1-ol and 1·24 l. of acetic acid. The pH of the aqueous phase was adjusted by addition of conc. aq. $NH_3$ soln. to the mixture, and the solvent was kept overnight to reach equilibrium.

*Apparatus.* This consisted of approximately 100 units of normal design (cf. Craig, Hausmann, Ahrens & Harfenist, 1951), except that the second or decantation chamber (*B*) was similar to the first (*A*), having the same volume and having a decantation tube also at the same height (Fig. 1). Thus it was also possible to have bottom phase in chamber *B*. A complete cycle of operations therefore involved the addition of top phase to *A*, equilibration, separation, decantation of top phase into *B*, equilibration, separation and decantation. For one-half of the equilibrations the bottom phase in *B* was alone. This system had the advantage that for a given capital outlay and space practically double the usual number of units could be obtained, but there was the disadvantage that more transfers were required to effect a given separation than would be

necessary with the double number of Craig tubes. The apparatus gave a theoretical result with phthalic acid prepared from A.R. potassium hydrogen phthalate.

### *Preparation of nisin A*

The sample of nisin used for this work was a solution of the bulked acetone precipitates, described in scheme 2 in the paper by Cheeseman & Berridge (1957). The nisin was in solution in 0·05 N-HCl and had a total activity of $87 \times 10^6$ units with a specific activity of 28 units/$\mu$g.

A portion of this solution, sufficient for three chambers of the countercurrent-distribution apparatus, was adjusted to contain the same concentration of butan-1-ol, aq. $NH_3$ soln., acetic acid and water as the bottom phase of the solvent mixture described above. The first three chambers of the apparatus were loaded and transfers begun, but it was necessary after each of the first ten transfers to centrifuge the contents of the chambers containing two layers to break the emulsion which proved difficult to separate. Interfacial precipitates which occurred during these initial stages were also removed after each centrifuging. After ten transfers the material remaining in the bottom layers of the first three chambers was removed and replaced by fresh bottom layer. The cycle of operations was continued for 336 transfers by which time the major peak of material was in chamber 85, giving a partition coefficient of $K$ 0·68. The equation of Williamson & Craig (1947) for determining the partition coefficient was modified to make allowance for the double-chamber unit:

$$\frac{Tr}{Tr+2} = \frac{(r+2)(r+1)}{(n-r)(n+r+2)} \cdot \frac{4(K+1)}{K^2};$$

for example, in a given case, $A = r$ and $B = r+1$ (Fig. 1).

The contents of chambers 70–100, 101–126 and 127–152 inclusive were bulked to form three solutions. The material was recovered from each by precipitation with a method previously described in the preparation of nisin (Cheeseman & Berridge, 1957). In this method the solution was added to 2 vol. of 0·01 N-HCl and the mixture was then saturated with NaCl. The precipitated nisin separated at the interface between the aqueous and organic layers. This method was used in preference to the method of concentration and subsequent precipitation with acetone, as used by Berridge *et al.* (1952), as experiments had shown that losses of activity may occur when the earlier method is used.

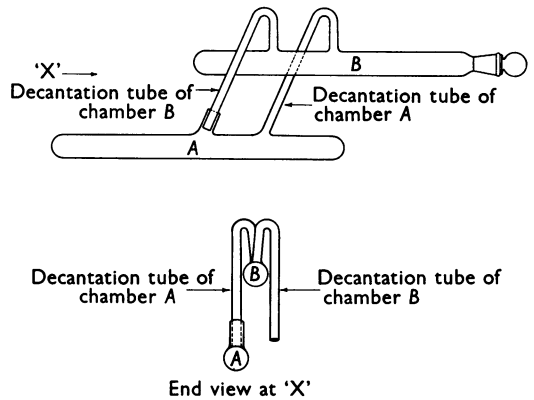



Fig. 1. Modified unit for countercurrent distribution.

The recovered nisin was redissolved in a few millilitres of 0·05 N-HCl and reprecipitated with 3 vol. of ice-cold acetone and centrifuged. The precipitate was resuspended in a few drops of water in order to remove the residual NaCl, nisin being of low solubility in saturated NaCl, and again centrifuged. The supernatant was discarded and the precipitate was dissolved in a minimum quantity of 0·05 N-HCl. This solution was heated to boiling for 1 min. for partial sterilization and stored in the refrigerator. When dry nisin was required it was precipitated with ice-cold acetone, washed with absolute acetone and dried *in vacuo*.

To confirm that the major peak isolated from the preparative experiments was a single component it was necessary to repeat the distribution in a different solvent system. A sample of the peak material was therefore subjected to distribution in the solvent mixture used by Berridge *et al.* (1952).

Two other similar experiments were carried out in order to produce sufficient material for further use.

### *Determination of amino acids*

To afford the minimum destruction and loss of amino acids during hydrolysis it was necessary to use constant-boiling HCl that had been twice-distilled in glass apparatus to remove traces of metals which increase the rate of deamination of the amino acids (Jacobsen, 1949), and to carry out the hydrolysis under vacuum in a sealed tube.

A sample of nisin A weighing 59·8 mg. was hydrolysed in this way for 18 hr. at 105°, with 1 ml. of HCl. This was approximately the minimum time and temperature required for complete hydrolysis as indicated by the absence of peptides from paper chromatograms prepared from the mixture. These limits were chosen to ensure a minimum destruction of the amino acids. It is clear that other compounds with more resistant peptide bonds would need a longer hydrolysis. After drying *in vacuo* over $P_2O_5$ and KOH, the material was extracted five times with 5 ml. of ether to remove traces of ether-soluble material obtained by the breakdown of amino acids during hydrolysis, and the residue was dissolved in 10 ml. of 0·01 N-HCl. Measured portions (0·1 ml.) were spotted by means of an Agla micrometer syringe (Burroughs Wellcome and Co., London) on to Whatman no. 1 filter paper (18½ in. × 22½ in.). Paper chromatography was carried out according to the technique described by Levy & Chung (1953). After running and drying, the papers were heated for 30 min. at 85–90° to develop the fluorescence. The positions of the amino acids revealed under ultraviolet light were marked with pencil. One of the chromatograms from each tank was developed with ninhydrin to act as an additional control.

The marked areas of the amino acids were cut from the paper and each piece was cut into small strips and placed in a test tube. To each test tube was added 2 ml. of 1% $NaHCO_3$ soln., and, after 20 min. at room temperature, 1 ml. of ninhydrin reagent prepared by the method of Moore & Stein (1954*b*). The test tubes were then covered and heated in a boiling-water bath for 15 min. On cooling 5 ml. of ethanol–water (50:50, v/v) was added to each tube. The colour of the solutions was read on a Hilger Spekker instrument with the 606 yellow filter (601 violet for proline). A range of leucine standards was run for each experiment.

A mixture of amino acids of the kinds and quantities present in nisin was subjected to hydrolysis and chromato-

graphy exactly as was the nisin itself. The percentage loss of each amino acid was thus determined and the results obtained from the nisin hydrolysate were adjusted accordingly.

*Determination of the diffusion coefficient*

Diffusion coefficients ($D$) were determined in the Tiselius electrophoresis apparatus (Hilger), in which a cylindrical lens and an inclined knife edge are used to form the optical pattern representing the boundaries.

Nisin hydrochloride (peak A) in the form of a dried powder was dissolved in a solution containing HCl (0·05 M) and KCl (0·2 M), to eliminate diffusion potentials. The solution contained 232 mg. of nisin in 15 ml. and this formed the solution in the lower half of one side of the cell and in the whole of the other side.

Solvent was layered over the nisin on both sides so as partly to fill the sections above the U-tube. The two arms were levelled after the U-tube had been placed in the water bath (temperature 25°, closely controlled with a good toluene regulator and Sun-Vic proportioning head). The bottom section was slid into place, and the apparatus left for 3–4 hr. for temperature equilibration. The junction was then sharpened by the capillary method of Kahn & Polson (1947). Photographs of the boundary, taken at time intervals, were enlarged on to high-contrast paper (Ilford Ltd. Document no. 60) and traced on to transparent graph paper for measurement. The base line was also photographed, but its deviation from straightness was insignificant.

A test run with 0·25 N-KCl under these conditions gave 1·90, 1·91 and $1·94 \times 10^{-5}$ cm.$^2$/sec. for $D$ at different times. Gosting (1950), using more elaborate methods, reported values for $D$ between 1·84 and $1·85 \times 10^{-5}$ at this concentration and temperature. Thus the technique used for nisin would be accurate enough to discriminate between multiples of the minimum molecular weight which had been calculated from its amino acid content.

*End-group analysis*

*N*-group analysis with the 1-fluoro-2:4-dinitrobenzene (FDNB) method (Sanger, 1945, 1949) was carried out first on the purified intact nisin. Elliott (1952) suggested that groups which do not react with FDNB when the protein is insoluble may do so when it is soluble, and as nisin has a low solubility at the normal pH of the FDNB experiment, i.e. at pH 8–9, a further reaction was carried out at a pH at which nisin was readily soluble, i.e. about 2·5, the solution being treated with FDNB at room temperature for 72 hr. After hydrolysis of the dinitrophenyl (DNP)-nisin and the separation of the unsubstituted amino acids (Sanger, 1949), the DNP-amino acids were identified by paper chromatography (Blackburn & Lowther, 1951). A short period of hydrolysis (2 hr.) was carried out with one sample so that any acid-labile DNP-amino acids, e.g. DNP-glycine and DNP-proline, could be recognized (Sanger, 1945). *C*-group analysis was carried out with carboxypeptidase (Lens, 1949) from Armour Ltd. The enzyme was dissolved in alkaline solution and the pH was brought to 8·0 as soon as solution was complete (Harris, 1955). Nisin, being insoluble at the pH of the carboxypeptidase reaction, was therefore subjected to action of the enzyme in a solution containing 6 M-urea, the pH being adjusted to 8–9 by the addition of 1% trimethylamine. Under these conditions the nisin was completely soluble. It is probable that the molecule was also modified. Experiments on nisin suspensions in the absence of urea were also made.

Halsey & Neurath (1955) have shown that carboxypeptidase is active in 6 M-urea and that the urea may make the polypeptide or protein more readily available to the action of the enzyme by causing an unfolding of parts of the molecule. The enzyme experiments were carried out at 37° in the presence of di*iso*propyl phosphorofluoridate (DFP) (15 $\mu$l. of 0·1 M-DFP soln./mg. of enzyme) to inhibit any endopeptidase activity (Sanger & Thompson, 1953) and with an enzyme:substrate ratio of 1:50 (w/w).

*Partial hydrolysis, fractionation and analysis*

Nisin A (400 mg.) was hydrolysed in about 5 ml. of 6 N-HCl (glass-distilled constant-boiling mixture) for 5 days at 37°. The acid was then removed *in vacuo* over $P_2O_5$ and NaOH pellets. A second sample of 450 mg. was treated in the same manner, the products being worked up and analysed separately. The resulting amino acids and peptides were separated into fractions by elution chromatography on sulphonated-polystyrene cation-exchange resin (Dowex 50, cross-linked with 4% divinylbenzene and graded 200–400 mesh/in.). A satisfactory flow rate was obtained by removing coarse material with a 40-mesh/in. sieve and by removing fine material with a 270-mesh/in. sieve. The resin was prepared for use by the procedure of Moore & Stein (1951), and packed into a column (1·7 cm. diam., 2 m. long). The method of gradient elution described by Moore & Stein (1954*a*) was used with the same buffers except that it was necessary to add 2 N-NaOH gradually to the citrate–acetate buffer at the end of the run, to remove peptides which resisted elution at pH 5·1. Some of them even remained on the resin until the eluent was almost entirely 2 N-NaOH.

The positions of the various peaks in the eluate diagram (Fig. 4) were determined in the usual way, with the buffered ninhydrin reagent and 0·5 ml. samples from alternate tubes.

Each of the fractions so isolated was desalted and concentrated on a short column of Dowex 50X4 in the $H^+$ form, the amino acids and peptides being eluted with aq. 2 N-$NH_3$ soln. (Boulanger & Biserte, 1951; Partridge & Brimley, 1952).

The desalted fractions were subjected to two-dimensional paper chromatography (Whatman no. 3MM, 18¼ in. × 22½ in.). The solvent used for the first dimension was butan-1-ol–water–acetic acid (4:5:1, by vol.) (Partridge, 1946), and that for the second, phenol–*m*-cresol (1:2, w/w) saturated with borate buffer, pH 8·3 (Levy & Chung, 1953). The paper was not, however, treated with buffer before the run in the second dimension as the presence of the buffer salts in the peptides to be eluted would have interfered with subsequent paper chromatography.

The positions of individual peptides and amino acids were marked after observation by fluorescence in ultraviolet light after heating the paper at 80–95° for 30 min., or, in some cases, by spraying with 0·025% ninhydrin, but this was usually avoided when the peptides were to be subjected to the DNP reaction.

A pointed section of paper containing each marked spot was cut out and the spot was washed towards the apex of the paper with 2% acetic acid. There it was concentrated by evaporation and finally eluted in a small volume. A

portion of the eluted material was dried, redissolved in a little 6N-HCl and hydrolysed in a sealed capillary overnight. The acid was then removed and the amino acids were identified mainly by two-dimensional paper chromatography as above. To reveal the amino acids a modification of the ninhydrin reagent similar to that of Levy & Chung (1953) was used. This consisted of ethanolic 0·2% ninhydrin–2:4:6-collidine–acetic acid (25:1:5, by vol.).

When sufficient peptide was available, amino end-group analyses were also made by the FDNB method of Sanger & Thompson (1953). The DNP-amino acids were identified either by paper chromatography in the *tert.*-amyl alcohol (2-methylbutan-2-ol) system (Blackburn & Lowther, 1951) or by reconversion into the free amino acid by heating with ammonia (Lowther, 1951) and subsequent paper chromatography.

Carboxypeptidase was used to reveal the *C*-terminal amino acids. Samples were taken at intervals and their subsequent investigation by paper chromatography enabled a determination of some *C*-terminal sequences to be made.

## RESULTS

### *Homogeneity of the nisin*

The distribution curves observed during purification (Fig. 2) suggested that only one major component was present. Relatively small changes in pH of the solvent mixture resulted in changes of the partition coefficient ($K$). When the pH was 2·8 $K$ was 0·62, at pH 2·9 $K$ was 0·68 and at pH 3·0 $K$ was 0·72.

The value $K$ 1·04 obtained in the analytical experiment (Fig. 3) is in close agreement with the partition coefficient $K$ 1·05 which Berridge *et al.* (1952) obtained for nisin A. From the results in the two solvent mixtures it is probable that the material isolated is a single polypeptide and that it is identical with nisin A.

### *Amino acid composition of nisin*

Table 1 summarizes the results obtained from the quantitative estimation of the amino acids in hydrolysed nisin. The recoveries of β-methyl-lanthionine and lanthionine have been based upon the average for the rest of the amino acids. The colour factors published by Moore & Stein (1954*b*) were used, except for the diamino acids, lanthionine and β-methyl-lanthionine, for which the factor 1·2 was chosen (cf. diaminopimelic acid). It was not necessary to confirm the factors of Moore & Stein (1954*b*) since discrepancies would be automatically 'ironed out' in adjusting the values according to the

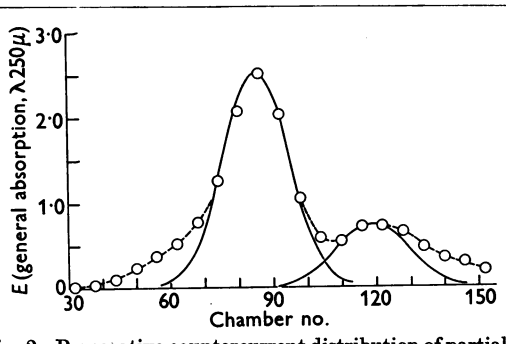



Fig. 2. Preparative countercurrent distribution of partially purified nisin. ○, Experimental results; continuous line, theoretical distribution.

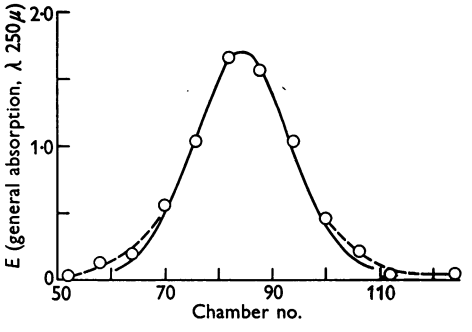



Fig. 3. Analytical countercurrent distribution of nisin A. ○, Experimental points; continuous line, theoretical distribution for $K$ 1·04.

Table 1. *Amino acids and molecular weight of nisin A*

| Amino acid | Concn. (g./100 g. of nisin) | Wt. of nisin to contain 1 mol. | No. of mol./ mol. of nisin (assumed) | Mol. wt. of nisin from columns 3 and 4 |
|---|---|---|---|---|
| Leu } Ileu } | 16·85 | 778 | 9 | — |
| Meth | 6·41 | 2320 | 3 | 6960 |
| Val | 5·07 | 2310 | 3 | 6930 |
| Pro | 3·86 | 2980 | 2 | 5960 |
| Ala | 4·76 | 1870 | 4 | 7480 |
| Gly | 6·62 | 1130 | 6 | 6780 |
| Ser | 1·53 | 6870 | 1 | 6870 |
| Asp | 5·46 | 2440 | 3 | 7320 |
| His | 7·17 | 2170 | 3 | 6510 |
| Lys | 18·95 | 771 | 9 | — |
| β-Lan | 23·30 | 787 | 9 | — |
| Lan | 6·46 | 2550 | 3 | — |
| | 113·44 | | Mean | 6826 |

yields of the whole process of hydrolysis and chromatography carried out on a known mixture simulating nisin.

*Diffusion coefficient*

Tracings from enlarged photographs of the schlieren diagram gave the results in Table 2, which shows the averages of values derived from some duplicate tracings and occasionally from different methods of mensuration.

On the third set of tracings calculations were made by the method of successive analysis at levels corresponding to steps of 0·146 cm. above the original boundary, i.e. on the dilute side. No significant trend in the value of $D$ with dilution was discoverable.

It is clear that the values in Table 2 do not permit Longsworth's (1947) correction for zero time. This merely means that disturbances during the early history of the diffusion produced smaller errors than subsequent uncertainties. The figures may therefore be averaged, giving

$$D = 1{\cdot}63 \times 10^{-6} \text{ cm.}^2/\text{sec.}$$

*Molecular weight*

The average value for the minimum molecular weight, estimated from the quantities of most of the amino acids, is about 7000, the assumed number of residues being 55 (Table 1). As Tristram (1950) has indicated, in order to estimate the number of amino acid molecules in a protein or peptide the percentage error in the analysis of any one amino acid must not exceed $100 \times 0{\cdot}4/R$, where $R$ is the number of residues of that amino acid. As this would require the errors for lysine, β-methyllanthionine and the leucines to be $\leqslant 4{\cdot}4\%$, these results were excluded when the average was taken. That for lanthionine was also excluded because of the uncertain recovery of this amino acid.

The maximum molecular weight of a protein-like molecule with a diffusion coefficient of $1{\cdot}63 \times 10^{-6}$ was calculated in the usual way, assuming the minimum reasonable value for $f/f_0$, namely 1·1 (Svedberg & Pedersen, 1940), and the likely value of 0·75 for the partial specific volume. The value obtained was 8500, which is near enough to 7000 to indicate that the molecule contains only one of the chemically determined units. A change of $f/f_0$ to 1·2 would more than compensate for the difference. We may therefore conclude that the molecular weight of nisin is approximately 7000, and that the molecule is approximately spherical in acid solution.

Table 2. *Diffusion coefficients of nisin A*

The values were obtained with a Tiselius electrophoresis apparatus (see text).

| Diffusion time (sec.) | $10^{-6} \times D$ (cm.²/sec.) |
|---|---|
| 15 840 | 1·56 |
| 26 730 | 1·60 |
| 43 590 | 1·69 |
| 86 220 | 1·65 |
| 117 060 | 1·67 |

*End groups*

No ether-soluble DNP-amino acids were obtained from DNP-nisin hydrolysed overnight, nor from a sample that had been hydrolysed for 2 hr. Increased time of treatment with FDNB and treatment at the lower pH still failed to produce any ether-soluble DNP-amino acids. The water-soluble DNP-amino acids were ε-DNP-lysine and imino-DNP-histidine. No free lysine would be detected in the hydrolysate. The reaction with histidine proceeded slowly but it was accelerated by prior treatment of the nisin with alkali at pH 8–9 for 2–4 hr. An increase in amino nitrogen (ninhydrin colour) also occurs whenever nisin is treated with alkali.

Although carboxypeptidase gave rapid and complete hydrolysis of the control peptide, glycylphenylalanine, no hydrolysis occurred with nisin. The enzyme–substrate mixture was subsequently kept for 72 hr., and the enzyme : substrate ratio was increased to 1 : 10 (w/w), but no free amino acids were observed on chromatograms of samples taken up to that time, and complete hydrolysis of the control peptide had occurred within 6 hr. The use of 6 M-urea as solvent, whilst affording increased solubility to the nisin, did not lead to the production of any *C*-terminal amino acids.

*Composition of the partial hydrolysate*

Table 3 indicates some of the peptides observed upon investigation of the 93 individual ninhydrin-positive regions found after two-dimensional chromatography of each of the 15 fractions obtained from the resin column (Fig. 4). Only those peptides which have been used for the derivation of amino acid sequences are noted in Table 3. A complete record is given by Cheeseman (1957). Two-dimensional chromatography was capable in most instances of separating the components of each fraction from the column into apparently pure amino acids and peptides. The sequence of the free amino acids from the column was the same as that described by Moore & Stein (1951). Aspartic acid, serine, glycine, alanine, valine, methionine, leucine, isoleucine and lysine were found as free amino acids in relatively large quantities. Proline, histidine, lanthionine and β-methyl-lanthionine were found free in small quantities only.

The material in fraction 14 was eluted from the column as the pH increased during the addition of

Table 3. *Analysis of peptides from partial hydrolysates*

| Column, fraction no. | Paper subfraction no. | Amino acids after complete hydrolysis | Peptide structure deduced | Remarks |
|---|---|---|---|---|
| 5 | (2) | Asp +<br>Gly +<br>Val + | Gly.(Asp, Val) | By FDNB |
| 5 | (3) | Asp + +<br>Gly + +<br>Val + + | Gly.(Asp, Val) | By FDNB. Same $R_F$ in phenol–cresol as 5 (2) but streaked in butanol–acetic acid. Probably same compound |
| 5 | (4) | Gly + +<br>Ala + + | Gly.Ala | By FDNB |
| 5 | (6) | Asp +<br>Gly + +<br>Ala + | Ala.(Gly, Asp) | By FDNB |
| 6 | (2) | Meth + +<br>Val +<br>Gly +<br>Ala +<br>Lys + | (Gly, Ala, Lys) | Position of free Meth and Val, which therefore may or may not be included in the peptide |
| 6 | (4) | Lys +<br>Gly ±<br>Asp ±<br>Ala ±<br>Leu ± | Gly.(Lys, Asp, Ala, Leu) | By FDNB. The spot for DNP-Gly was weak. ε-DNP-Lys was positive |
| 6 | (7) | Gly + + +<br>Ala + + + | Gly.Ala | By FDNB |
| 6 | (10)<br>(11) | β-Lan + + +<br>Gly + + + | β-Lan.Gly | Mono-DNP-β-Lan was observed |
| 6 | (12) | Lys +<br>Asp +<br>Ala + | (Lys, Asp, Ala) | Too little material for end-group analysis |
| 6 | (13) | Gly + +<br>Ala +<br>Meth + +<br>Ileu + + | Ala.Gly.Ileu.Meth | By FDNB and carboxypeptidase |
| 7 | (3) | Asp + +<br>Meth ± | Asp.Meth | By FDNB |
| 7 | (4) | Asp ±<br>β-Lan +<br>Gly ± | β-Lan.(Asp, Gly) | By FDNB |
| 8 | (1) | Ala + + +<br>Meth + + +<br>Leus + + + | Meth.Ala.Leus | By FDNB and carboxypeptidase |
| 8 | (4) | Gly + + + +<br>Val +<br>Meth + + + | Meth.Gly<br>Val.Gly | Probably two peptides because both DNP-Meth and DNP-Val were obtained |
| 10 | (3) | Gly + + +<br>Meth + + +<br>Leu + + +<br>Ala + | Leu.(Gly, Meth).Ala (?) | By FDNB but yield of DNP-Leu was very low. Ala was disregarded because of its relatively small quantity |
| 14 | (2) | Gly +<br>Ser ±<br>Asp ±<br>Lan ±<br>His ±<br>Val ± | (Gly, Ser, Lan, His, Val) | Too little material for end-group analysis |
| 14 | (4*c*) | His + + +<br>Val + + +<br>Ileu + + +<br>Ser + + + | (Ser, Ileu).His.Val | By carboxypeptidase |
| *Z*3 | (4) | Asp + + +<br>Ala + + + | Asp.Ala | From the second hydrolysate |
| *Z*6 | (5) | Asp + +<br>Lys + +<br>Gly + + | (Asp, Lys).Gly | From the second hydrolysate |

2N-NaOH to the citrate–acetate buffer. This material consisted mainly of large peptides containing the basic amino acids and the lanthionines. Fraction 15, which comprised the last part of the eluate, yielded only free amino acids. This was possibly because of the hydrolysis of residual peptides by the alkali. In some cases, for example in fraction 11, regions giving a faint ninhydrin reaction could be seen on paper chromatograms but on elution and hydrolysis the quantities of amino acids obtained were insufficient to permit confident recognition.

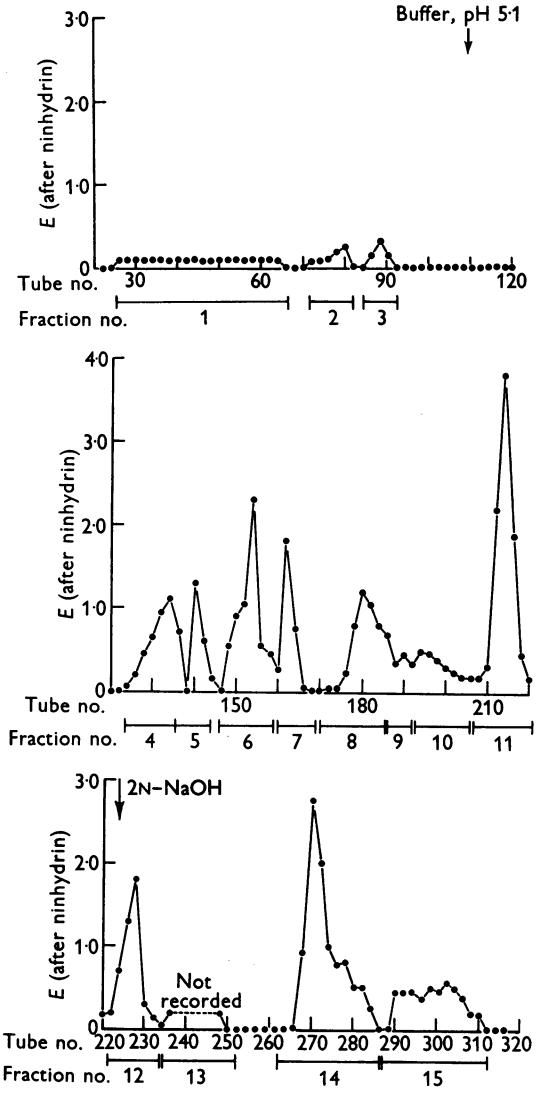



Fig. 4. Fractionation of partial hydrolysate on Dowex 50X4.

*Derivation of sequences of amino acids*

In deriving the sequences below it has been assumed that the β-carboxyl group in aspartic acid is not concerned in chain formation. No account has been taken of possible resynthesis of peptides during partial hydrolysis and subsequent separation. Except for one case in which the identity of a lanthionine spot is doubted, the results have been taken at their face value. We have arrived therefore at a few possible sequences. They are not contradicted by any of the numerous peptides which could be analysed for amino acids only, and not for sequence.

The sequences deduced represent only the first tentative step in unravelling a structure which could well be more complex even than that of bacitracin A, the molecule of which is less than a quarter of the size of nisin. Moreover, the bacitracin molecule has only three points of branching (Abraham, 1957), whereas in nisin the nine lanthionine and three β-methyl-lanthionine residues could give rise to 24 branches. The β-carboxyl groups of aspartic acid could increase this number by three.

The customary abbreviations (Brand & Edsall, 1947) for amino acids and peptides have been used. The abbreviations used for lanthionine and β-methyl-lanthionine are Lan and β-Lan. Leus is used to indicate that Leu or Ileu or both may be present.

From Table 3 we have the peptides

| | |
|---|---|
| $Asp_a$.Meth | 7 (3) |
| $Asp_b$.Ala | *Z* 3 (4) |
| ($Asp_c$, Lys).Gly | *Z* 6 (5) |

As the aspartic acid residues can be distinguished by the amino acids which follow them, it is possible to label them as shown. There are only three of them in the nisin molecule. Therefore all sequences containing aspartic acid must agree with these three.

The peptide 5 (6) (Table 3) is either

Ala.Gly.Asp or Ala.Asp.Gly 5 (6)

whereas peptide *Z* 6 (5) is either

$Asp_c$.Lys.Gly or Lys.$Asp_c$.Gly *Z* 6 (5)

Thus 5 (6) may be part of Ala.Gly.$Asp_c$.Lys.Gly, this being the only way $Asp_c$ could be included in peptide 5 (6). The same order is required for $Asp_a$ and $Asp_b$. Therefore 5 (6) must be

Ala.Gly.Asp 5 (6)

The peptide 5 (2) is Gly.(Asp, Val), but as no Asp may be followed by Val, it must be

Gly.Val.Asp 5 (2)

We also have

β-Lan.(Gly, Asp) 7 (4)

By the argument applied to 5 (6) this cannot be β-Lan.Asp.Gly, but owing to the two carboxyl groups of β-Lan it may be

either β-Lan(Gly, Asp) or β-Lan(Gly.Asp)

which will be abbreviated to

β-Lan, Gly ≈ Asp 7 (4)

Thus we also have the three aspartic acid residues accounted for in

Ala.Gly.Asp.[5 (6)], Gly.Val.Asp.[5 (2)]

and β-Lan, Gly ≈ Asp [7 (4)]

$Asp_c$ must occur in one of these three peptides. Therefore lysine cannot precede aspartic acid and the only permissible arrangement for ($Asp_c$, Lys). Gly [*Z* 6 (5)] is

$Asp_c$.Lys.Gly *Z* 6 (5)

The peptide

Gly.(Lys, Asp, Ala, Leu) 6 (4)

contains an Asp residue, therefore one of the fragments Ala.Gly.Asp [5 (6)], Gly.Val.Asp [5 (2)]

or β-Lan, Gly ≈ Asp [7 (4)]

must be present, at least partly, but Gly.Val.Asp 5 (2) cannot be present because Val cannot precede Asp in Gly.(Lys, Asp, Ala, Leu) [6 (4)]. If the Asp belongs to Ala.Gly.Asp [5 (6)], Ala must have been removed during hydrolysis and 6 (4) must have been part of the sequence

Ala.Gly.Asp.(Ala, Leu, Lys)

into which only $Asp_b$ will fit, giving

Ala.Gly.$Asp_b$.Ala.(Leu, Lys) **A**

If now the other alternative that Asp in 6 (4) belongs to 7 (4) be considered, we arrive at

β-Lan.Gly.$Asp_b$.Ala.(Leu, Lys) **B**

by the same arguments as before. We have therefore these two possibilities for $Asp_b$.

It is possible to label the four alanine residues according to their positions in different fragments as follows:

$Ala_a$.Gly.Ileu.Meth 6 (13)

$Ala_b$.Gly.Asp 5 (6)

Meth.$Ala_c$.Leus [probably Ileu from 7 (1)] 8 (1)

$Asp_b$.$Ala_d$ *Z* 3 (4)

$Ala_d$ being known to be different from the rest because it occurs in **A** or **B**.

Out of the six possible orders for the three amino acids in the peptide

(Lys, Asp, Ala) 6 (12)

the only one which agrees with sequences already deduced for aspartic acid is Asp.Ala.Lys, for Asp can be followed by Ala or Lys but cannot be preceded by [5 (6) 5 (2) or 7 (4)], and if Asp is followed by Lys the next residue must be Gly [*Z* 6 (5)], not Ala. This shows therefore that Asp in 6 (12) is $Asp_b$, and also gives the order of the bracketed components of **A** or **B**.

We have therefore

Ala.Gly.$Asp_b$.$Ala_d$.Lys.Leu

or β-Lan.Gly.$Asp_b$.$Ala_d$.Lys.Leu **C**

The six glycine molecules in nisin may be distinguished from one another by their adjacent amino acids, as follows:

$Ala_a$.$Gly_a$.Ileu.Meth 6 (13)

$Ala_b$.$Gly_b$.Asp 5 (6)

Meth.$Gly_c$ 8 (4), 8 (6)

β-Lan.$Gly_d$ 6 (10), 6 (11)

Val.$Gly_e$ 8 (4), 8 (6)

$Asp_c$.Lys.$Gly_f$ *Z* 6 (5)

Similarly, with the three methionine residues:

$Ala_a$.$Gly_a$.Ileu.$Meth_a$ 6 (13)

$Asp_a$.$Meth_b$ 7 (3)

Leu.$Meth_c$.$Gly_c$ **D**

peptide **D** being derived from

Leu.(Meth, Gly) 10 (3)

since Gly may have Meth preceding it but not Leu.

We have also the peptide

Meth.$Ala_c$.Leus, 8 (1)

which must therefore contain $Meth_a$ or $Meth_b$ and can extend $Ala_a$.$Gly_a$.Ileu.$Meth_a$ [6 (13)] or $Asp_a$.$Meth_b$ [7 (3)]. We may express this as

$Ala_a$.$Gly_a$.Ileu.$Meth_a$, $Asp_a$.$Meth_b$ ≈ $Ala_c$.Leus **E**

The peptides

Gly.Ala 5 (4), 6 (7)

and (Gly, Ala, Lys) 6 (2)

cannot include $Ala_c$ or $Ala_d$, as these are preceded by Meth and $Asp_b$ respectively and if Ala comes first in 6 (2) the other amino acids would not agree with 8 (1) ($Ala_c$) or **A** or **B** ($Ala_d$). It follows that $Ala_a$ or $Ala_b$, or both, are preceded by Gly. The Lys may then be directly before one Ala or before a Gly preceding Ala.

The single serine molecule occurs in the sequence

(Ser, Ileu).His.Val 14 (4*c*)

and in the peptide

(Gly, Ser, Asp, Lan, His, Val) 14 (2)

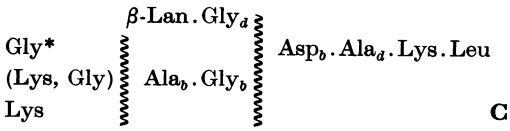
It can be shown that this peptide contradicts the sequences already deduced unless it be assumed

that in interpreting the chromatogram the faint spot designated lanthionine was in fact β-methyllanthionine. In this case the possible structures are numerous. The simplest which would agree with the previous deductions is Ileu.Ser.His.Val. β-Lan.Gly.Asp with a carboxyl and an amino group on β-Lan in unknown linkages. Leaving this as a mere possibility, we may summarize the sequences built up on definite evidence as follows, using sinuous lines to denote alternative sequences between which the choice cannot yet be made.

(1) (Ser, Ileu).His.Val 14 (4*c*)

(2) Leu.Meth$_c$.Gly$_c$ **D**

(3)

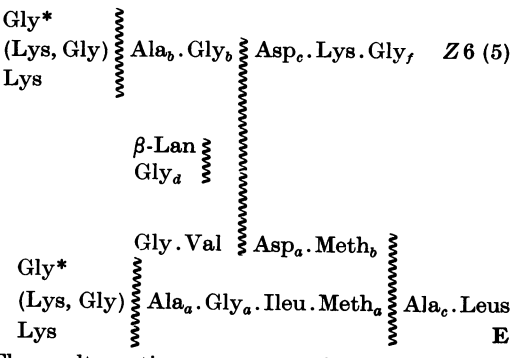



**C**

When the choice indicated by the right-hand sinuous line in (3) can be decided the other alternative can be used in the sequence below, but at present both alternatives must be indicated in both (3) and (4). It is to be noted that the argument indicating the incorporation of β-Lan into Gly

**C** implies the linkage β-Lan.Gly, but this is not so if those amino acids precede Asp$_a$ or Asp$_c$ as in (4) below.

(4)




*Z* 6 (5)

**E**

These alternatives must remain open, because we know the three dipeptides which precede the three aspartic acid residues but, except that Gly.Val does not precede Asp$_b$ [see argument concerning peptide 6 (4)], we do not know which dipeptide belongs to which aspartic acid.

The trace of alanine found in peptide 10 (3) was dismissed as too small to be significant. If it really was a constituent of the peptide the argument above is still valid and **D** would become

Leu.Meth$_c$.Gly$_c$.Ala$_{a\ or\ b}$

which would extend the sequence (4) in the left-hand direction.

* See 5 (4), 6 (7), 6 (2) just above and subsequent argument.

## DISCUSSION

The partially purified nisin had a different composition from that obtained by Berridge *et al.* (1952). Of the two major peaks isolated by the previous authors only one was recovered as a major peak from the nisin distributed in these experiments. The possibility that the other part of the partially purified nisin, obtained as in scheme I of Cheeseman & Berridge (1957), could contain differing ratios of peaks, owing to differences in purification and extraction, has not been investigated.

Other biologically active material separated during the purification of the major peak confirms previous observations that the name nisin must be regarded as a collective one for a number of closely related polypeptides (Berridge, 1949, 1952; Hirsch, 1951; Bavin, Beach, Falconer & Friedmann, 1952; Berridge *et al.* 1952). In this respect nisin is similar to other polypeptide antibiotics which have also been found to occur as families, e.g. gramicidins, tyrocidines (Craig, Gregory & Barry, 1949), licheniformins (Callow & Work, 1952) and bacitracins (Newton & Abraham, 1950, 1953).

Within the limits of the methods employed the total quantities of amino acids recovered are in agreement with the estimated total weight. This would indicate that the molecule is comprised solely of amino acids or that if another compound is present it can only be so in relatively small amounts.

Although the recoveries by quantitative paper chromatography may be as low as 60 % for some amino acids, the variations between the replicates of each estimation are less than 10 %, permitting a confident approximate analysis. This is good enough when the number of residues per molecule of peptide is low. Similar techniques have been used before (e.g. Smith & Agiza, 1951). The small spread of the molecular weights deduced in Table 1 reflects the small variations in the analyses.

End-group analysis did not produce any evidence of *N*- or *C*-terminal amino acids. The ε-amino groups of all the lysine residues are free, but the slowness of the reaction of FDNB with the imino group of histidine suggests that this group is available only under alkaline conditions.

The lack of hydrolysis by carboxypeptidase does not necessarily indicate the absence of *C*-terminal groups. For example, the *C*-terminal amino acid may be present as proline, or an amide, e.g. glycine amide occurs in oxytocin (du Vigneaud, Ressler & Trippett, 1953), or it may have the D configuration (Neurath & Schwert, 1950) and so not be susceptible to enzyme action. The absence of both *N*- and *C*-terminal residues does, however, suggest the possibility of a closed polypeptide ring.

The amino acid sequences given above need confirmation since in some cases the results depend on single faint spots on chromatograms. Many peptides were present in quantities too small for end-group analysis. These have not been reported here, but only a little more information is needed to permit a choice between the alternatives indicated and to extend the sequences considerably. The branching of the polypeptide chain which may result from the presence of lanthionine and β-methyl-lanthionine could become very complex, as there are 12 of these molecules. This, the centre of interest in nisin, as it is possibly the centre of its antibiotic properties, has not been touched by the techniques of partial hydrolysis and end-group determination. An approach via the C–S–C linkages would probably be more fruitful.

## SUMMARY

1. Nisin A has been purified and analysed for amino acids.

2. Its diffusion coefficient has been determined.

3. The agreement between the molecular weight of nisin calculated from its diffusion coefficient and that calculated from the proportion of various amino acids in the hydrolysate indicates that the molecule is approximately spherical in acid solution and that its weight is about 7000.

4. No amino or carboxyl end groups could be found, save the ε-group of lysine and the imino group of histidine.

5. From the analysis of partial hydrolysates a few possible amino acid sequences have been determined.

## REFERENCES

Abraham, E. P. (1957). *Biochemistry of Some Peptide and Steroid Antibiotics.* CIBA Lect. in Microbial Biochem. New York: John Wiley and Sons Inc.

Bavin, E. M., Beach, A. S., Falconer, R. & Friedmann, R. (1952). *Lancet*, i, 127.

Berridge, N. J. (1949). *Biochem. J.* **45**, 486.

Berridge, N. J. (1952). *Nature, Lond.*, **169**, 707.

Berridge, N. J., Newton, G. G. F. & Abraham, E. P. (1952). *Biochem. J.* **52**, 529.

Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.

Boulanger, P. & Biserte, G.( 1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1930

Brand, E. & Edsall, J. T. (1947). *Annu. Rev. Biochem.* **16**, 224.

Callow, R. K. & Work, T. S. (1952). *Biochem. J.* **51**, 558.

Cheeseman, G. C. (1957). Ph.D. Thesis: University of Reading.

Cheeseman, G. C. & Berridge, N. J. (1957). *Biochem. J.* **65**, 603.

Craig, L. C. & Craig, D. (1956). *Technique of Organic Chemistry.* 2nd ed., vol. 3, part 1, p. 146. Ed. by Weissberger, A. New York: Interscience Publishers Inc.

Craig, L. C., Gregory, J. D. & Barry, G. T. (1949). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 24.

Craig, L. C., Hausmann, W., Ahrens, E. H. & Harfenist, E. (1951). *Analyt. Chem.* **23**, 1236.

du Vigneaud, V., Ressler, C. & Trippett, S. (1953). *J. biol. Chem.* **205**, 949.

Elliott, D. F. (1952). *Biochem. J.* **50**, 542.

Gosting, L. J. (1950). *J. Amer. chem. Soc.* **72**, 4418.

Halsey, Y. D. & Neurath, H. (1955). *J. biol. Chem.* **217**, 247.

Harris, J. I. (1955). *Methods of Biochemical Analysis.* vol. 2, p. 406. Ed. by Glick, D. New York: Interscience Publishers Inc.

Hirsch, A. (1951). *Nature, Lond.*, **167**, 1031.

Jacobsen, C. F. (1949). *C.R. Lab. Carlsberg, Sér. chim.* **26**, 455.

Kahn, D. S. & Polson, A. (1947). *J. phys. Chem.* **51**, 816.

Lens, J. (1949). *Biochim. biophys. Acta*, **3**, 367.

Levy, A. L. & Chung, D. (1953). *Analyt. Chem.* **25**, 396.

Longsworth, L. G. (1947). *J. Amer. chem. Soc.* **69**, 2510.

Lowther, A. G. (1951). *Nature, Lond.*, **167**, 767.

Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.

Moore, S. & Stein, W. H. (1954*a*). *J. biol. Chem.* **211**, 893.

Moore, S. & Stein, W. H. (1954*b*). *J. biol. Chem.* **211**, 907.

Neurath, H. & Schwert, G. W. (1950). *Chem. Rev.* **46**, 69.

Newton, G. G. F. & Abraham, E. P. (1950). *Biochem. J.* **47**, 257.

Newton, G. G. F. & Abraham, E. P. (1953). *Biochem. J.* **53**, 597.

Partridge, S. M. (1946). *Nature, Lond.*, **158**, 270.

Partridge, S. M. & Brimley, R. C. (1952). *Biochem. J.* **51**, 628.

Sanger, F. (1945). *Biochem. J.* **39**, 507.

Sanger, F. (1949). *Biochem. J.* **45**, 563.

Sanger, F. & Thompson, E. O. P. (1953). *Biochem. J.* **53**, 366.

Smith, A. M. & Agiza, A. H. (1951). *J. Sci. Fd Agric.* **2**, 503.

Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*, p. 60. Oxford: Clarendon Press.

Tristram, G. R. (1950). *Chem. & Ind.* **53** (s), 868.

Williamson, B. & Craig, L. C. (1947). *J. biol. Chem.* **168**, 687.