dency of any bond in the original molecule to acquire increased stability to acid or alkali once it is embodied in a dipeptide molecule should be definitely advantageous.

SUMMARY

1. The hydrolysis velocity constants for a number of dipeptides in acid at 37° have been determined, and are compared with the data of Levene *et al.* (1932) for similar dipeptides in alkali.

2. The course of hydrolysis of gramicidin by acid under the same conditions has been followed by determination of the evolution of amino groups, free amino-acids and 'hydroxyamino' groups.

3. The nature of the process of hydrolysis by acid of long peptide chains is discussed in relation to the available data.

I wish to thank Mr W. Wolff for his technical assistance.

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The Hydroxyamino Component of Gramicidin Hydrolysates

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The presence of a compound in acid hydrolysates of gramicidin, containing the groupings

was inferred by Hotchkiss (1941) from the fact that periodate liberates formaldehyde and NH_3 from such hydrolysates (cf. Christensen, Edwards & Piersma, 1941; Synge, 1944a). Hotchkiss further showed that the material responsible for these reactions with periodate was not an α -amino-acid, since the α -amino-acid content of hydrolysates (determined by the ninhydrin- CO_2 procedure) was unaffected by treating them with periodate. Serine

was not therefore responsible for the reaction (cf. Gordon, Martin & Synge, 1943b). Hotchkiss rejected the idea that the material might be isoserine or hydroxylysine, and noted its tendency to decompose, with liberation of NH_3 , during isolative manipulations. Failure to isolate even small amounts of aldehyde-acid as acidic hydrazone after oxidation with periodate led Hotchkiss later (1944) to doubt that the hydroxyamino component was a carboxylic acid. As a result of chromatographic experiments with acetylated hydrolysates of gramicidin, the same doubt had occurred to the present author, and experiments were undertaken to test for the presence in gramicidin hydrolysates of volatile amino-alcohols.

Occurrence of ethanolamine in gramicidin hydrolysates

It was found that on azeotropic distillation with toluene of an acid hydrolysate of gramicidin which had been rendered alkaline with excess of K_2CO_3 , ethanolamine (2-aminoethanol-1) was recovered in the distillate; control experiments showed that this could not be derived as a simple artefact from the known amino-acid constituents of gramicidin. A short note of this result has already been published (Synge, 1944b). The ethanolamine has been identified by: (1) its elementary composition; (2) its content of basic groups and amino-N; (3) the yield of NH_3 , acetaldehyde (none) and formaldehyde (2 molecules) obtained on treatment with periodate; (4) its volatility, optical inactivity, and ionophoretic behaviour; (5) the preparation from it of a chloroaurate which has been found crystallographically identical with authentic ethanolamine chloroaurate.

Since the procedure first employed for isolating the ethanolamine was rather violent, it was desirable to isolate it by a milder treatment, not involving high temperatures or rendering the hydrolysate alkaline. This was achieved by a new ionophoretic technique devised by Dr A. J. P. Martin and his colleagues (Conden, Gordon & Martin, 1946; see Martin & Synge, 1945). By this technique ethanolamine was isolated from acid-hydrolysates of gramicidin after simple neutralization to pH 6.

The yields of ethanolamine obtained by both the distillation and ionophoretic procedures do not suggest the presence of other hydroxyamino compounds, there being sufficient ethanolamine present to account for all of the NH_3 liberated by periodate treatment of the same unfractonated acid hydrolysates of gramicidin. No CO_2 was liberated during acid hydrolysis of gramicidin.

Ethanolamine was also isolated ionophoretically from partial hydrolysates of gramicidin obtained by the action of acid for 2 days at 37° . At the same time, a basic fraction, having a lower migration rate and yielding ethanolamine and amino-acids on further acid hydrolysis, was isolated. The nature of this fraction is discussed at greater length below.

The above data may be taken to indicate that the ethanolamine is not derived by decarboxylation of an amino-acid, and that ethanolamine residues possibly occur as such in the undegraded structure of gramicidin.

On heating with ninhydrin, ethanolamine gives a colour considerably weaker than that due to an equivalent of amino-acid. More acute observation of paper strip chromatograms with gramicidin hydrolysates (Synge, 1944a) would have led to its recognition as a faint band moving more slowly than the glycine band with butanol- H_2O . The

chromatographic experiments of Gordon *et al.* (1943b) could not, however, have been expected to detect its neutral *N*-acetyl derivative.

Quantitative determination of ethanolamine in gramicidin

An estimate of the number of ethanolamine residues in the stoichiometric minimum unit of gramicidin has been complicated somewhat by the incomplete yield of NH_3 obtained on treating ethanolamine with periodate, and more seriously by the instability of ethanolamine under the conditions employed for hydrolyzing gramicidin. The results of applying the periodate- NH_3 reaction to ethanolamine obtained by other workers are given in the experimental section; the method employed in the present work gave sufficiently constant recoveries (90%) to permit a correction factor to be applied with some confidence to all determinations.

When gramicidin was hydrolyzed for 24 hr. at 110 – 115° in a 1:1 mixture of glacial acetic acid and 10N-HCl, the ethanolamine-N in the hydrolysate was found to be 3.0–3.5% of the total-N of gramicidin. Higher values (4.7–5.1%) were found with hydrolysates prepared at a slightly lower temperature and containing less acetic acid (Hotchkiss, 1941; Synge, 1944a and present work). Control experiments showed that ethanolamine heated by itself in these acid solutions is considerably destroyed, and that in the presence of a mixture of the known amino-acid constituents of gramicidin the destruction is still greater. As with hydrolysates of gramicidin itself, higher yields were obtained from the mixtures subjected to the lower temperatures and lower acetic acid concentrations. When the ethanolamine figures for gramicidin hydrolysates are corrected by the recoveries obtained in these control experiments, they are found to comprise, as ethanolamine-N, 5.5–6.0% of the total-N of gramicidin. The figure 6.7% would represent two residues of ethanolamine in the postulated stoichiometric minimum unit of gramicidin, and it is not unreasonable to suppose that in fact two such residues are present, and that the destruction of ethanolamine residues during their liberation from chemical linkage is slightly greater than the destruction observed with free ethanolamine.

The observations of Synge (1945) on the course of liberation of ethanolamine in low temperature hydrolysis of gramicidin give direct evidence of destruction, which is as great in the 1:1 acetic acid-10N-HCl mixture at 37° as at 110 – 115° . It is suggested that in the high concentrations of acetic acid, there may be a significant equilibrium Fischer-Speyer esterification to *O*-acetyethanolamine, which might be expected, under the alkaline conditions of the periodate- NH_3 determination, not to saponify

simply, but to rearrange to *N*-acetyethanolamine (Bergmann & Brand, 1923; Bergmann, Brand & Weinmann, 1923) and thus escape reaction. At the higher temperatures, destruction of ethanolamine probably occurs in other ways, since liberation of NH_3 is observed. The breakdown of serine in hot acid provides known analogies, and the same reaction which produces pyruvic acid from serine (Abderhalden & Broich, 1933; Damodaran & Ramachandran, 1941) would explain the formation from ethanolamine of the small quantities of acetaldehyde noticed by Hotchkiss (1944) in a hydrolysate of gramicidin.

The ethanolamine yield from different preparations of gramicidin hydrolyzed in the same way appeared to be the same. The small quantities of fatty acid and P present in gramicidin, as well as varying in quantity from one preparation to another, were in every case inadequate to account for the ethanolamine being derived from contaminating phospholipid (cephalin) (cf. Table 2).

Mode of incorporation of ethanolamine residues in gramicidin

The evidence now presented, taken with the conclusions of Gordon *et al.* (1943*b*) and Synge (1944*a*) leads to the postulate that the stoichiometric minimum unit of gramicidin embodies residues of amino-acids, etc., as follows, which account completely for the N of gramicidin: 6 tryptophan (*l*); 6 leucine (*d*); 5 valine (*d* and *l*); 3 alanine (*l*); 2 glycine; 2 ethanolamine.

If the ethanolamine residues are involved in peptide linkage through the amino group, and have the hydroxyl group free, the hydroxyl groups of gramicidin (Hotchkiss, 1941) are then adequately accounted for.

Such a stoichiometric minimum unit, independent of its degree of condensation, hydration, etc. would have 144 C atoms per 30 N atoms. The data of Hotchkiss & Dubos (1941) on the elementary composition of gramicidin give the somewhat higher ratio of 148 C per 30 N, suggesting that some non-nitrogenous carbon-containing residue may still remain to be found. The possibility of firmly bound acetone of crystallization has not been excluded; acetone of crystallization was noticed in preparing acetyl-*dl*-leucylglycine (Gordon, Martin & Synge, 1943*a*).

There are more serious difficulties involved in regarding gramicidin as being formed simply by peptide condensation of the above residues. Unless abnormal linkages are assumed, the resulting unit would have a free basic or amino group, and there is evidence that gramicidin does not possess such groups (Hotchkiss, 1941). The fact that it forms a crystalline compound with flavianic acid (Tishler, Stokes, Trenner & Conn, 1941) is scarcely significant,

since such acids are well known to form stoichiometric 'molecular compounds' with neutral substances such as aromatic hydrocarbons.

The 'extra' carbon of gramicidin as well as its neutral character would be readily explained were carboxylic acid residues also present. Hotchkiss (1941) states that carboxylic acids (volatile or non-volatile, ether-extractable) are absent from gramicidin hydrolysates. In the present study likewise, exhaustive extraction with ether, adequate to isolate oxalic acid, failed to reveal the presence of such an acid.

A less plausible explanation of the neutral character of gramicidin could be the presence of carbonic acid residues (ureido groups) linking two amino-acid residues thus:

Such groupings would not, on acid hydrolysis, necessarily liberate CO_2 (cf. Corwin & Damerel, 1943; Gordon, Martin & Synge, 1943*c*) but might be expected to give, on mild acid hydrolysis, nitrogenous products bearing a net negative charge at pH 6 (cf. Locquin & Cerchez, 1931; Cerchez, 1931). However, in the ionophoretic experiment with a 2-day partial hydrolysate described below, no significant quantity of N was found to migrate towards the anode (see Table 5). The possibility of both the glycine and ethanolamine of the hydrolysate arising by Cannizzaro rearrangement of aminoacetaldehyde residues should not be ignored (cf. Trier, 1911).

A satisfactory account of the over-all properties of gramicidin in terms of its constituent residues must await further work. Leaving this problem on one side, it seemed possible to investigate the mode of linkage of ethanolamine residues in gramicidin by examining the basic fractions obtained by ionophoresis from a 2-day low-temperature acid hydrolysate. Here it might be expected that a proportion of ethanolamine residues would be found bound as relatively simple 'peptides'.

The analytical data presented in the experimental section for such a fraction show: (i) that the fraction probably consists of 'peptides' involving ethanolamine, from which ethanolamine can be liberated on acid hydrolysis; (ii) that significant quantities of tryptophan are absent from it; (iii) that the total quantity of ethanolamine found in the fraction corresponds to only $\frac{1}{4}$ – $\frac{1}{3}$ of the ethanolamine that is free in the original hydrolysate. This low yield of bound ethanolamine can be ascribed to the fact that ethanolamine appears to be set free from gramicidin in acid hydrolysis faster than the average peptide bond is broken (Synge, 1945, Fig. 3). A hydrolysate of the 'ethanolamine peptide' fraction was examined by a butanol- H_2O paper strip chromatogram (Consden, Gordon & Martin, 1944; cf. Synge, 1944*a*) and revealed the presence of glycine, alanine,

valine and leucine as well as of ethanolamine. Two-dimensional chromatograms (Consden *et al.* 1944) with the intact 'ethanolamine peptide' fraction showed at least four major components in it, none of which, as control experiments showed, was the simple 'peptide', *N*-alanylethanolamine or *N*-leucylethanolamine. Thus this basic peptide mixture is both low in yield and complex in nature. It may contain artefacts formed by esterification of ethanolamine —OH groups with amino-acids and peptides (see above).

Gramicidin appears to be the first case described of the natural occurrence of ethanolamine (2-amino-ethanol-1) linked through its amino group. The next higher homologue (2-amino-propanol-1) is found linked in this way in ergometrine (for bibliography see Guggenheim, 1940, pp. 119, 158). This is by no means the only point of chemical similarity between the ergot 'alkaloids' and the 'polypeptides' derived from *Bacillus* spp.

A possible significance of the finding of ethanolamine residues in gramicidin for the mechanism of its antibacterial activity should be mentioned. Modern views on the chemical mechanism of antibacterial action lay special stress on the inhibition of enzyme systems by substances of structure related to that of normal substrates, coenzymes, etc. It may prove not merely coincidental that gramicidin (1) possibly contains, according to data presented here, ethanolamine residues linked thus: *R*.CO.NH.CH₂.CH₂OH; (2) interferes with bacterial growth and anabolism and, more specifically, with the metabolism of phosphate (Hotchkiss, 1944); (3) is inhibited in these activities by the cephalins *O*-phosphatidyl-serine and *O*-phosphatidyl-ethanolamine, containing the groupings:

(for references see Hotchkiss (1944))

In this connexion the isolation by Outhouse (1937) of ethanolamine phosphate

from cancerous (growing) tissue may have some significance.

EXPERIMENTAL

Materials

Three specimens of *gramicidin*, W2, R3 and RM, were employed in the present study. The origin and preparation of W2 and R3 was described by Synge (1944a). RM was the material left in the mother liquors during the preparation there described of R3 from R1.

The authentic ethanolamine employed for identification and control experiments was a commercial sample kindly given by Dr F. A. Robinson for which amino-N (Van Slyke), basic groups (titration with HCl) and the yield of formaldehyde on periodate oxidation bore the theoretical ratios to one another.

Analytical methods

NH₃ liberated by periodate. These determinations were carried out in Conway units, employing the reagents recommended by Van Slyke, Hiller & MacFadyen (1941). The method has been described by Gordon, Martin & Synge (1943d) (cf. Ramsay & Stewart, 1941; Synge, 1944a). In the present work, the dishes were equilibrated for 2 hr. at room temperature without shaking. Allowing the usual correction for incomplete equilibrium absorption of NH₃ in the boric acid, 100% recovery of NH₃ from serine was found as before. On the same basis ethanolamine gave 87–93% recoveries of NH₃ consistently, and the figure of 90% recovery has been employed as a further correction of ethanolamine figures throughout this work. NH₃ blanks were run in parallel with all periodate-NH₃ determinations and periodate-NH₃ figures quoted refer to the difference of the pair of determinations. Van Slyke *et al.* (1941) noted yields of 85% of NH₃ from ethanolamine by their aeration procedure. Artom (1945), by a steam distillation procedure, has obtained theoretical yields.

Acetaldehyde and formaldehyde liberated by periodate. These were both determined in the same oxidation mixture by Mr M. W. Rees, Cambridge, by an unpublished modification of the methods of Shinn & Nicolet (1941) and Nicolet & Shinn (1941). The method gives theoretical yields of the aldehydes from threonine and serine, and control experiments with ethanolamine gave no acetaldehyde and the theoretical yield (2 molecules) of formaldehyde (cf. Jones, 1944).

Other estimations. Tryptophan: the colorimetric procedure of May & Rose (1922) was employed; P was determined colorimetrically after incineration with H₂SO₄ and perhydrol by Dr M. G. Macfarlane; C and H were determined by Dr G. Weiler, Oxford.

Isolation of ethanolamine by azeotropic distillation with toluene

Isolation of ethanolamine from biological material (phospholipid hydrolysates, etc.) has usually been accomplished by the use of precipitants or insoluble derivatives. Blix (1940) took advantage of its volatility, and employed a distillation method. Artom (1945) has employed a chromatographic method. For the present work distillation seemed advantageous, and use was made of toluene as a carrier solvent, since it forms positively azeotropic mixtures with ethanolamine as well as with water, and thus hastens the removal of ethanolamine from the non-volatile residue.

The toluene used was first washed with acid and distilled from soda-lime through a fractionating column at atmospheric pressure. An ordinary continuous liquid extraction apparatus (such as that illustrated (b-4632) in the catalogue of A. Gallenkamp and Co. Ltd.) was employed for the ethanolamine distillation. The HCl hydrolysate from which it was desired to isolate ethanolamine was first evaporated to dryness *in vacuo* in the flask of the apparatus; the residue was dissolved in 1–2 ml. of saturated K₂CO₃ solution in the

flask, which was then half filled with toluene. The apparatus was then assembled; the distilling flask was placed in an oil-bath which was heated so that the toluene boiled briskly. This caused the condensed distillate to bubble through the water at the bottom of the extraction tube before returning to the distilling flask. Ethanolamine was extracted by the water from the distillate. Soon after distillation had begun, the aqueous solution in the distilling flask lost water, and became a hard white crust on the walls of the flask. Distillation was continued for 3 hr. At the end of this time the water layer was collected from the extraction tube, which was rinsed out three times with distilled water. The resulting combined aqueous solution was then evaporated to about one-third its original volume below 40° *in vacuo*. In this way any NH_3 present was removed without significant loss of ethanolamine. Ethanolamine was determined in this solution, or in a suitable sample of it, by titration with 0.01 *N*-HCl, employing the indicator of Ma & Zuazaga (1942). Periodate- NH_3 determinations on the same solutions gave concordant results.

Control experiments showed that mixtures of the known amino-acids of gramicidin subjected to the same acid hydrolysis procedure as gramicidin, and to this isolation procedure, yielded no basic material; when ethanolamine was added to the mixture immediately before the distillation, 90% of it was recovered.

Characterization of ethanolamine isolated from hydrolysates of gramicidin

Gramicidin preparation RM (212 mg.) was hydrolyzed in a sealed evacuated tube, with 2 ml. of glacial acetic acid and 2 ml. 10*N*-HCl, for 24 hr. at 110–115°. The hydrolysate was treated as above for the isolation of ethanolamine, and samples of the resulting basic aqueous solution, free from NH_3 , were employed for the following determinations: (1) titration of basic groups with HCl (in duplicate as above); (2) periodate- NH_3 determination; (3) formaldehyde and acetaldehyde evolved on oxidation with periodate; (4) *N* (Kjeldahl); (5) amino-*N* (Van Slyke: 4 min.).

Analysis (1) was done in the outer compartment of Conway dishes. The resulting solutions were then evaporated *in situ*, and the residues were employed for analysis (2). Control experiments showed that the presence of indicator did not interfere with the periodate- NH_3 reaction. The results of the analysis are expressed, in Table 1, on the basis of the basic group titration.

Table 1. *Analyses on solution of ethanolamine isolated from gramicidin*

Analysis for	Result (units per basic equiv. as titrated with acid)
Hydroxyamino- <i>N</i> (periodate- NH_3) (atoms)	1.03
Acetaldehyde liberated by periodate (molecules)	0.008
Formaldehyde liberated by periodate (molecules)	2.30
<i>N</i> (Kjeldahl) (atoms)	1.09
Amino- <i>N</i> (atoms)	0.98

On the basis of the previous titrations of samples with HCl, the amount of HCl required for neutralization was

added to the rest of the solution, and it was evaporated to dryness *in vacuo* below 40°. The residue (dried over H_2SO_4 —soda-lime *in vacuo* at room temperature) took the form of deliquescent needle-like crystals arranged in rosettes on the bottom of the flask. Its weight was 113 g./equiv. of HCl employed for neutralization. It had $[\alpha]_D^{18.5}$, $0^\circ \pm 2^\circ$ (water, $l=0.5$, $c=0.9$). Found: C, 26.0; H, 9.6% (calc. for ethanolamine hydrochloride, $\text{C}_2\text{H}_5\text{ON} \cdot \text{HCl}$: equiv. wt. 97.5; C, 24.6; H, 8.2%).

The high periodate-formaldehyde, equivalent weight and C figures suggest that glycerol (derived from fatty material contaminating specimen RM—see below) may have been present. Ethanolamine samples isolated from specimen W2 which contained less fatty contamination (see Table 2) gave formaldehyde: $-\text{NH}_2$ ratios within 2% of the theoretical. There may also be some slight contamination by non-basic nitrogenous material, perhaps derived from indole.

From an ethanolamine HCl sample isolated in the same way from a hydrolysate of gramicidin R3: (1) by periodate oxidation and treatment with dimedone according to Nicolet & Shinn (1941) formaldehyde was isolated and identified as its crystalline dimedone derivative (m.p. 187° uncorr., not depressed on admixture of authentic material having m.p. 189°); (2) a 'gold salt' was prepared by treating with three parts by weight of commercial 'brown gold chloride' in concentrated aqueous solution in the presence of excess HCl. As with authentic ethanolamine, long yellow needles crystallized out readily (cf. Trier, 1911). The crystals, together with a sample of authentic ethanolamine chloraurate, were submitted to Dr D. Crowfoot and Mr G. M. J. Schmidt, Department of Chemical Crystallography, Oxford, who report:

'The two specimens were of somewhat different appearance; the product from natural sources was in the form of thin very long needles, whereas the synthetic sample consisted of thick prisms. Optical and X-ray examination, however, proved them to be identical.

'The crystals are monoclinic needles or prisms elongated along [001], and bounded by (010), (110) and terminal faces not indexed. Their birefringence is very high; their most readily determined optical characteristic is the extinction direction which lies at an angle of 24° to [001] in (010). The unit cell dimensions determined by X-ray methods are $a=7.50$, $b=14.40$, $c=4.42 \text{ \AA}$, $\beta=112.5^\circ$, space group $P2_1$. The crystals agree in morphology and optics with the microphotograph and descriptions given by Knorr (1897) and by Grubenmann in Trier (1911).'

Yields of ethanolamine isolated from different gramicidin preparations

The yields of ethanolamine, determined by titration after azeotropic distillation with toluene as above, are shown in Table 2 for the three gramicidin specimens studied. Hydrolysis was effected in a mixture of equal volumes of glacial acetic acid and 10*N*-HCl for 24 hr. at 110–115° as above. The content of P of the gramicidin specimens and the fatty acid content of the hydrolysates are also given, expressed in units equivalent to the ethanolamine figures. The fatty acid was determined by extraction of aqueous suspensions of evaporated hydrolysates with three successive half-volume portions of B.P. chloroform. The combined chloroform extracts were evaporated and stored over soda-lime

in a vacuum desiccator overnight, dissolved in ethanol, and titrated, using phenolphthalein as indicator, with 0.01N-aqueous $\text{Ba}(\text{OH})_2$. Precipitation, presumably of 'barium soap', occurred during the titrations. The acid equivalent weights of the chloroform extracts lay in the range 200–350.

Table 2. *Ethanolamine, P and fatty acid contents of gramicidin specimens*

Specimen ...	Yield (% of total N of gramicidin)		
	R3	RM	W2
Ethanolamine N	3.2	3.3	3.2
P as N (1 atom P=1 atom N)	0.7	0.2	0.03
Fatty acid as N (1 equiv. acid=1 atom N)	0.8	2.0	0.4

*Recovery of ethanolamine after heating
in HCl-acetic acid mixture*

Ethanolamine was treated (by itself and in the presence, in the appropriate proportions, of the amino-acid constituents of gramicidin) under the different conditions that have been employed for hydrolyzing gramicidin, viz. in sealed evacuated tubes (A) in excess of 1 vol. acetic acid + 4 vol. 5N-HCl for 48 hr. at 100° (cf. Syngé, 1944a); (B) in excess of 1 vol. acetic acid: 1 vol. 10N-HCl for 24 hr. at 110–115° (as in isolative experiments described above).

After evaporation of the acid *in vacuo*, the periodate- NH_3 reaction was carried out on the residue. The results are shown in Table 3. When ethanolamine was added to the amino-acid mixture *after* it had been heated under conditions (B), 102% recovery was given by the analysis.

Table 3. *Recoveries of ethanolamine determined by periodate- NH_3 reaction*

Hydrolysis conditions	Recovery (%)	
	(A)	(B)
Without amino-acids	97	76
With amino-acids	82	57

*Determination of ethanolamine in gramicidin
hydrolysate by periodate- NH_3 reaction*

This was done on gramicidin hydrolysates exactly as with the made-up mixtures above. Specimen R3 hydrolyzed under conditions (A) gave ethanolamine-N 4.7% of its total N. Corresponding figures were obtained, after similar hydrolytic treatment, by Hotchkiss (1941): 5.1% corr.; and Syngé (1944a): 4.8%, 5.1% corr. Under hydrolytic conditions (B), with all three specimens of gramicidin figures lying in the range 3.3–3.5% were obtained, in agreement with the isolative data reported in Table 2. The data for the same determination on hydrolysates of gramicidin prepared by acting with acid at 37° for various times are given by Syngé (1945).

*Carbon dioxide evolved in hydrolysis
of gramicidin*

This was determined according to Van Slyke & Folch (1940), after connecting the sealed tube in which the hydrolysis had been conducted to the Van Slyke-Neill mano-

metric apparatus (cf. Gordon *et al.* 1943c). A blank determination with 2 ml. glacial acetic acid and 2 ml. 10N-HCl heated for 24 hr. at 110–115° gave 0.3 mg. CO_2 , while 212 mg. of gramicidin RM, heated under the same conditions in the same mixture, gave only 0.2 mg. CO_2 . Probably the neutralization of some of the HCl by amino-acids liberated decreased the destruction of acetic acid.

*Ether-extractable acid in gramicidin
hydrolysates*

A hydrolysate of 54 mg. gramicidin RM made under conditions (A) above, was evaporated to dryness *in vacuo*, dissolved in water, and extracted with chloroform as in the fatty acid determinations above. The aqueous layer was then transferred to the extraction chamber of a continuous liquid extractor, and extracted overnight with ether that had been purified by successive washing with NaHSO_3 and NaOH solutions and distilled wet through a fractionating column. The ether extract, after concentration *in vacuo* and keeping overnight in a vacuum desiccator over soda-lime, required only 0.08 ml. 0.01N- $\text{Ba}(\text{OH})_2$ for neutralization to phenolphthalein. A corresponding mixture of the known components of gramicidin (including ethanolamine), on treatment in the same way, yielded an extract requiring 0.12 ml. for neutralization. When 1.3 mg. of succinic acid were added to this mixture after heating it with acid but before the chloroform extraction, 98% of the succinic acid was titrated in the ether extract. It may be inferred from the data in *International Critical Tables* that at least one-third of any oxalic acid present would have been recovered by this procedure.

*Ionophoretic isolation of ethanolamine from
gramicidin hydrolysates*

The methods employed for this purpose have been developed by Drs R. Consden, A. H. Gordon & A. J. P. Martin (1946), who kindly made them available for the present work. The ionophoresis was carried out in a long slab of dilute silica jelly strengthened with paper pulp and embodying a 0.2M-acetic acid-sodium acetate buffer solution of pH 6. The electrodes applying the potential to the slab were kept continuously washed with a stream of the same buffer solution, to prevent pH changes and carry away electrode reaction products. The solution to be investigated was inlaid in the slab ($22 \times 1\frac{1}{2}$ in., and less than $\frac{1}{4}$ in. deep) in a gutter cut across it about one-third of the distance from anode to cathode. A glass lid lay on the slab.

The factors determining migration and separation of substances in the apparatus cannot be discussed fully here. It must be said that in addition to the net charge and ionic mobilities of the various substances, electroendosmosis of fluid in the pores of the gel and adsorption on the micelles of the gel are two important factors determining migration rates. The electroendosmosis was demonstrated by the migration towards the cathode of monoamino-monocarboxylic acids having no net charge at pH 6; the adsorption by the considerable increase in the migration rates under the same applied voltage of ethanolamine, lysine, etc. on substituting sodium acetate with ammonium acetate buffer. (Dr Martin interprets this effect as due to competitive elution by ammonium ions of the amino compounds adsorbed on the silica micelles.)

In spite of difficulty in interpreting theoretically the effects observed, the procedure has proved useful for effecting preparatively the isolations required in the present work, and the empirically determined migration rates have provided further evidence of the identity of the hydroxy-amino component of the hydrolysates with ethanolamine.

Ionophoretic recognition of ethanolamine in complete hydrolysate of gramicidin

Gramicidin RM (20 mg.) was hydrolyzed by procedure (B) above. After evaporation of excess acid the residue was dissolved in water, the pH adjusted to 6 by addition of NaOH, and 1 mg. each of *DL*-lysine and *L*-aspartic acid added as 'markers'. The resulting mixture was subjected to ionophoresis, with the apparatus running for 12 hr. with 130 V. across the electrodes. The resulting current was about 150 ma. At the end of this time a filter-paper print from the jelly was coloured by spraying with 1% ninhydrin solution in butanol containing 20% (v/v) of acetic acid, followed by careful heating at 70–80°. This revealed four bands, as shown in Table 4.

Table 4. *Results of ionophoresis of gramicidin hydrolysate*

No.	Band corresponding to	Migration of centre of band (cm.)	Migration towards	% of hydroxy-amino-N of gramicidin hydrolysate recovered in corresponding fraction
1	Ethanolamine	20	Cathode	76
2	Lysine	13.5	Cathode	5
3	Monoamino-acids	5.5	Cathode	1
4	Aspartic acid	8	Anode	1

The ethanolamine band on the prints was less strongly coloured than the amino-acid bands; its colour characteristically developed to a maximum more slowly. Control experiments with known mixtures showed that authentic ethanolamine migrated under these conditions at the same rate relative to lysine and the monoamino-monocarboxylic acids. Only two bands (corresponding to ethanolamine and to monoamino-monocarboxylic acids) could be detected in 'complete' hydrolysates of gramicidin examined ionophore-

tically without added 'markers'. When the fractions were worked up (by drying the jelly, and extracting the dry material with 1% (v/v) acetic acid solution), the hydroxy-amino-N (periodate-NH₂) was determined on each. The results expressed in Table 4 show clearly that ethanolamine is responsible for the NH₂ liberated by periodate from gramicidin hydrolysates.

Ionophoretic analysis of a partial hydrolysate of gramicidin

The data of Synge (1945, Fig. 3) indicated that simple peptides embodying ethanolamine residues might best be sought in a 2-day partial hydrolysate of gramicidin at 37°. Accordingly, after pilot experiments, 80 mg. of gramicidin RM were subjected to hydrolysis under these conditions, and the hydrolysate was evaporated to dryness below 40°. Addition of water to the residue caused separation of much friable, partly crystalline insoluble matter, and on adjusting this suspension to pH 6 with NaOH further precipitation occurred. The resulting suspension was transferred fairly completely to the ionophoresis apparatus, and subjected to the same treatment as above. On 'printing' and colouring with ninhydrin, three bands were observed: (1) corresponding to ethanolamine, (3) to the compounds bearing no net charge at pH 6, and (2) a weak band separate from (3) having its centre about one-third of the distance from (1) to (3). Only band (3) showed a blue colour on spraying a print with 0.1% *p*-dimethylaminobenzaldehyde in 10N-HCl without subsequent heating. This indicated that bands (1) and (2) were substantially free from tryptophan. The jelly of the ionophoresis apparatus was accordingly cut into fractions, as shown in Table 5, which were worked up and analyzed with the results there shown.

Fraction B (containing water-insoluble material) was extracted, after drying, with glacial acetic acid, and hydrolyzed by procedure (A) above before analysis. Hydrolysis of the other fractions was effected on samples in 6N-HCl for 24 hr. at 100°.

It is seen that the quantities available for analysis were near the lower limits of the analytical methods, which were therefore not as accurate as could be wished. However, the analyses justify the main conclusions drawn in the discussion part of this paper. The relatively large quantity of ammonia liberated in the acid hydrolysis of fraction D should be noted.

For the preliminary chromatographic studies mentioned in the discussion part of this paper, a fraction corresponding

Table 5. *Ionophoretic analysis of a partial hydrolysate of gramicidin*

Frac-tion	Comprising	N found (% of total-N in original gramicidin)							
		Total-N (Kjeldahl)	Trypto-phan-N	Amino-N (4 min. Van Slyke)		NH ₂ -N		Hydroxyamino-N	
				Before hydro-lysis	After hydro-lysis	Before hydro-lysis	After hydro-lysis	Before hydro-lysis	After hydro-lysis
A	Jelly on anode-side of trench	0.35	—	—	—	—	—	—	—
B	Insoluble material in trench (widely excised)	—	—	—	—	—	0.7	—	0.8
C	Band (3)	—	1.6	4.1	—	0.2	0.4	0.0	0.5
D	Band (2)	—	<0.06	0.6	0.8	0.1	0.7	0.0	0.4
E	Intermediate fraction	—	<0.06	0.0	0.1	0.1	0.3	0.0	0.1
F	Band (1)	—	<0.06	1.7	1.8	0.3	0.6	1.9	1.7

to Band (2) was obtained, employing in the ionophoresis a jelly made from waterglass with HCl and from which NaCl had been completely displaced by allowing a slow stream of 0.1N-ammonium acetate to flow over its surface. The ammonium acetate present in the fractions resulting was completely removed by drying them in a high vacuum at room temperature over H_2SO_4 and soda-lime. This procedure, evolved by Dr Martin and his colleagues, yields a product substantially free from salt and therefore more suitable for chromatographic study. Migration in the ammonium acetate gel was 2-3 times as rapid as in the sodium acetate gel with the same applied voltage; the bands observed in the prints, however, had much the same positions relative to one another.

SUMMARY

1. Ethanolamine (2-aminoethanol-1) has been identified as the component of gramicidin hydrolysates which reacts with periodate to yield formaldehyde and NH_3 .

2. Two ethanolamine residues may occur in the

postulated stoichiometric minimum unit of gramicidin. Destruction of ethanolamine during acid hydrolysis of gramicidin is considerable.

3. Attention is drawn to a number of difficulties which arise in relating the over-all properties of intact gramicidin to those which would be expected from study of the products of acid hydrolysis.

4. Preliminary data are given on a basic fraction isolated from a partial acid hydrolysate of gramicidin and thought to contain 'peptides' of ethanolamine.

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