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Non-peptide Linkages in Gramicidin

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Gregory & Craig (1948; cf. Craig, Gregory & Barry, 1950) showed by counter-current distribution that crystalline gramicidin as usually prepared is a mixture of at least three closely related substances. That present in largest amount (*A*) contained the residues previously reported, whereas *B* contained in addition phenylalanine and *C* tyrosine. The work described in the present paper was done with samples of gramicidin obtained from tyrothricin by simple solvent extraction and crystallization, and hence contained at least these three components. Despite this complication it is felt that the information gained should cast some light on structural features that may be common to members of the group of closely related substances known as gramicidin.

We undertook this work in the hope of gaining information as to how gramicidin, apparently made up entirely from residues of monoamino-monocarboxylic acids and ethanolamine, is nevertheless devoid of basic properties and ionizing groups. A general review of previous data on the structure of gramicidin has been given by Synge (1950; cf. Hodgkin, 1950), and this includes a brief statement of the problem with a preliminary account of some of our present data. Some of the information given in that review needs modification in view of the discovery by Hinman, Caron & Christensen (1950)

that the racemic material obtained on partial acid hydrolysis which was thought by Christensen (1943, 1944) to be an equimolecular mixture of the peptides D-valyl-D-valine and L-valyl-L-valine is more probably a mixture of D-valyl-L-valine and L-valyl-D-valine. While the experiments now described do not lead to any rigorous conclusions as to structure they at least rule out a number of possible solutions of the problem.

It has been established by a number of methods that the gramicidins are neutral substances possessing no free amino or carboxyl groups. No free ionizing groups were found on electrometric titration in 60% (v/v) aqueous ethanol by Christensen, Edwards & Piersma (1941; confirmed in present paper, cf. Fig. 1), on titration in ethanol and in acetone by Hotchkiss (1941) or on titration in glacial acetic acid with perchloric acid (present paper). No amino groups were detected by Hotchkiss (1941) either by reaction with nitrous acid according to Van Slyke or by the colour reaction with ninhydrin. Similarly, we could obtain no stable yellow *N*-dinitrophenyl (*N*-DNP) derivative by reaction with 1-fluoro-2:4-dinitrobenzene (FDNB), and the migration behaviour of gramicidin in an electric field at pH 6 was no different from that of the neutral substance *N*-DNP-ethanolamine under the same conditions.

The only free functional groups of gramicidin other than indole groups seem to be hydroxyl groups possessing an alcoholic function. Gramicidin

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is readily acetylated by acetic anhydride (Hotchkiss, 1941; Fraenkel-Conrat, Brandon & Olcott, 1947); it gives a succinyl derivative with succinic anhydride (Olcott, Lewis, Dimick, Fevold & Fraenkel-Conrat, 1946), a sulphuric half ester with cold concentrated sulphuric acid (Reitz, Ferrel, Fraenkel-Conrat & Olcott, 1946) and a phosphoric ester with phosphorus pentoxide in phosphoric acid (Ferrel, Olcott & Fraenkel-Conrat, 1948). It has now been shown that these hydroxyl groups can be methylated by means of methyl iodide and silver oxide. Table 1 gives a comparison of the number of gram residues of each of these groups introduced into gramicidin together with the number of gram residues of ethanolamine present in acid hydrolysates of the

Table 1. *Relation of ethanolamine content to number of hydroxyl groups of gramicidin*

Group introduced	g. residues/10 ⁴ g. original gramicidin	Reference
Acetyl	8.2	Hotchkiss (1941)
Acetyl	6	Fraenkel-Conrat <i>et al.</i> (1947)
Succinyl	6.6	Olcott <i>et al.</i> (1946)
Sulphuric ester	5.9	Reitz <i>et al.</i> (1946)
Phosphoric ester	6.1	Ferrel <i>et al.</i> (1948)
Methoxyl	5.4	This paper
Ethanolamine liberated on acid hydrolysis (after correction for estimated destruction)	6.1	Synge (1945)

same amount of gramicidin. The agreement between these sets of figures suggests that the number of free hydroxyl groups is equal to the number of ethanolamine residues, and hence that the former derive from the latter. This would imply that ethanolamine residues are incorporated in the gramicidin molecule by a peptide bond on the amino group, thus: $R \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot OH$, where R signifies the remainder of the gramicidin molecule. Methylation of gramicidin would then be expected to give the derivative $R \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot OCH_3$. Hydrolysis of the methylated product with an acid that would not split the ether linkage should then give rise not to ethanolamine but to *O*-methylethanolamine, $NH_2 \cdot CH_2 \cdot CH_2 \cdot OCH_3$. However, this was not found; complete hydrolysis of methylated gramicidin with sulphuric acid (cf. Gordon, Martin & Synge, 1943) under conditions that do not lead to destruction of *O*-methylethanolamine did not liberate any volatile amino compound. The non-volatile residue from such a hydrolysis contained only 39% of the original methoxyl content of the methylated gramicidin, and chromatography on paper failed to reveal any trace of *O*-methylethanolamine. Clearly the methoxyl groups in methylated gramicidin are much more acid-labile than would be expected were they present in the ether linkage $-CH_2 \cdot CH_2 \cdot OCH_3$.

A moderately plausible alternative would be to

suppose that the glycine and ethanolamine found in roughly equimolecular proportions in acid hydrolysates of gramicidin (Synge, 1945) arise by a sort of Cannizzarro reaction from residues of aminoacetaldehyde present in the intact gramicidin (cf. Trier, 1911). These could be conceivably linked in a normal peptide chain thus:

giving a site for substitution of acid-labile *O*-methyl groups. We synthesized a model compound in which aminoacetaldehyde residues occurred in peptide linkage, namely the diethylacetal of carbo-benzyloxy-L-valylaminoacetaldehyde. On treating this compound or the parent aminoacetal with hot acid neither glycine nor ethanolamine could be found among the reaction products. We were accordingly less inclined to regard the ethanolamine of gramicidin hydrolysates as an artifact, and endeavoured to obtain products by partial hydrolysis of gramicidin that might throw light on the mode of linkage of its precursor residues.

Partial hydrolysis studies

It was felt likely that the bonds linking ethanolamine or its precursor in the gramicidin molecule would be more labile to hydrolysis than normal peptide bonds, and therefore a study of partial hydrolysates should give some information on the mode of incorporation of ethanolamine.

It was found that gramicidin was surprisingly resistant to ethanolic alkali; no titratable groups were detected after treatment with 0.08N-ethanolic sodium hydroxide at 37° for periods up to 1 week.

Acid hydrolysis using 10N-hydrochloric acid in dioxan was found to be more effective—titration of the hydrolysate showed that 0.5 equivalents of acidic groups and 0.75 equivalents of basic groups/3800 g. (the most probable molecular weight of gramicidin given by X-ray analysis, see Hodgkin, 1950) were liberated after 16 hr. at 20°. Increasing the time of hydrolysis to 24 hr. liberated 1.1 equivalents of acidic groups and 1.75 equivalents of basic groups/3800 g. (Fig. 3).

The low-temperature hydrolysis of gramicidin using aqueous dioxan-10N-hydrochloric acid mixtures was also studied and found to give a similar result after 9 days at 2° to that given by hydrolysis for 1 day at 20°. The relatively more rapid liberation of basic groups points to the presence of basic peptides that would be expected to be either *O*-acyl or *N*-acyl derivatives of ethanolamine (as no basic amino-acids are present in gramicidin). Such basic peptides were also found by Synge (1945) after 2 days' hydrolysis at 37° in 10N-aqueous hydrochloric acid-acetic acid (1:1, v/v).

It was noticed that whenever these hydrolysates of gramicidin, dissolved in aqueous ethanol, were made more alkaline than pH 4, a copious white precipitate formed that would not dissolve at any pH above 4. Chromatography on paper showed that this precipitate differed from the material remaining in solution. Both low-temperature and room-temperature hydrolyses gave rise to this insoluble peptide material which, under the conditions used, invariably accounted for at least half of the original gramicidin. Two fractions were thus obtained by adjusting the hydrolysate to pH 6, denoted ethanol-soluble peptides and ethanol-insoluble peptides respectively. Substitution with the dinitrophenyl group indicated that both these peptide fractions contained tryptophan as the only amino-acid with an unsubstituted amino group. It does not seem previously to have been noted that DNP-tryptophan is unstable to acid hydrolysis and breaks down to give dinitroaniline and a yellow product which runs at the same rate as DNP-tryptophan on silica-gel chromatograms, but no longer gives any colour with the Ehrlich reagent.

Further fractionation of the ethanol-soluble peptides was carried out by electrophoresis in an ethanolic gel of silicic acid at pH 6. Two diffuse bands were obtained both moving towards the cathode. The fastest band consisted of tryptophan-free peptides giving a strong colour with ninhydrin and the slower band consisted of tryptophan-rich peptides that gave only a weak colour with ninhydrin. Poor recoveries of the peptides from the gel prevented this technique being used on a larger scale for the isolation of the different peptide fractions.

Chromatography on starch was found to be a more efficient method of fractionation of the ethanol-soluble peptides. By use of the solvent system *cyclohexane-isopropanol-water*, gramicidin and gramicidin-like peptides (fraction 1) could be rapidly eluted from the column. Successive elution with ethanol and 60% (v/v) aqueous ethanol gave two further fractions (fractions 2 and 3 respectively).

All these fractions were run on paper chromatograms using the *cyclohexane-isopropanol* system. Fraction 1 contained apparently unchanged gramicidin and tryptophan-rich peptides, fraction 2 consisted of long-chain peptides containing tryptophan and giving a weak colour with ninhydrin and fraction 3 consisted of shorter peptides containing no tryptophan but giving a strong colour with ninhydrin. The marked inhomogeneity of each of these fractions was demonstrated by the paper chromatograms shown in Figs. 4 and 5.

The partial acid hydrolysis of the ethanol-insoluble peptide material was next studied, as it was felt likely that this represented an initial hydrolysis product of gramicidin. Hydrolysis with an

aqueous dioxan-hydrochloric acid mixture showed that short-chain peptides were produced; these ran at the same rate on paper chromatograms as the short-chain peptides in fraction 3. It is concluded that peptides arise in the following stages: gramicidin $\rightarrow$ ethanol-insoluble peptide material $\rightarrow$ short-chain peptides.

Further analytical work was carried out on the fractions obtained from the starch column, each fraction being analysed for tryptophan and ethanolamine. The results are summarized in Table 2. It is clear that the material in fraction 3 consists of short-chain peptides rich in ethanolamine but containing no tryptophan. A similar fraction was obtained ionophoretically by Synge (1945) from partial hydrolysates of gramicidin. A qualitative amino-acid analysis, carried out on complete hydrolysates of each fraction by paper chromatography, showed that glycine, alanine, valine, leucine and tryptophan were present in all fractions except fraction 3 which contained no tryptophan and only a small amount of leucine.

The mixed peptides of ethanolamine contained in fraction 3 might be of two types: (a) *O*-acyl derivatives of ethanolamine: $\text{RCO} \cdot \text{OCH}_2\text{CH}_2\text{NH}_2$, or (b) *N*-acyl derivatives of ethanolamine:

It is known that *O*-acyl derivatives of β -hydroxy-amino compounds rearrange spontaneously in alkaline solution to give *N*-acyl derivatives, the reverse change in acid solution being very much slower (Bergmann & Brand, 1923; Bergmann, Brand & Weinmann, 1923; Phillips & Baltzly, 1947):

Titration of an amino-acid ester of ethanolamine would therefore show loss of one basic group on back titrating from an alkaline pH. Loss of basic groups on titration thus provides a means of detecting the presence of amino-acid esters of ethanolamine in gramicidin hydrolysates. This loss of basic groups in alkaline solution explains the failure to detect any free amino groups of ethanolamine in gramicidin hydrolysates by the FDNB technique, as the reaction was carried out under mildly alkaline conditions. The failure of the tryptophan-free material of fraction 3 to yield any DNP-amino-acid, despite its high amino N content, could be attributed to destruction of DNP-glycine under the hydrolysis conditions, or to other yet unknown causes.

Back titration of a low-temperature partial hydrolysate of gramicidin, prepared as described earlier, showed the expected loss of basic groups (Fig. 6). Similar titrations carried out on the fractions obtained from such a hydrolysate show that with the exception of the insoluble peptide fraction, all showed some fall in basic groups on treatment with

alkali. In Table 3 are shown the figures obtained from the titration curves and comparative figures obtained by measurement of free amino groups by the Van Slyke technique. The agreement between the two sets of figures is not good, the reason probably being inaccuracy in estimation of basic

groups by the titration procedure owing to the difficulty in correcting the curves for the effects of the solvent on the titration. Nevertheless, the trend of the figures is in the same direction. A rather smaller loss of amino groups in fraction 3 than would be expected on the basis of its ethanolamine content might be due either to an insufficient reaction time being given in the Van Slyke apparatus or to ion-exchange effects on the column used for the fractionation causing rearrangement of the amino-acid esters of ethanolamine.

It is reasonable to assume from this evidence and that of Syngé (1945) that the partial acid hydrolysis of gramicidin leads to the production of ethanolamine esterified with peptides of glycine, alanine, valine and leucine, together with long-chain peptides having tryptophan as the end residue carrying a free amino group.

Conclusions

Any structures put forward must account for the following facts:

(i) The only substances so far found in acid hydrolysates of gramicidin are the monoamino-monocarboxylic acids, glycine, alanine, valine, leucine, tryptophan, phenylalanine and tyrosine and the base ethanolamine. These account for all or nearly all of the carbon and nitrogen of gramicidin.

(ii) The free hydroxyl groups of gramicidin are not the primary alcoholic groups of ethanolamine residues.

(iii) The intact molecule possesses no free amino or carboxylic groups.

(iv) No volatile or non-volatile fatty acids or other ether-extractable carboxylic acids are present.

(v) No carbon dioxide is evolved during acid hydrolysis of gramicidin, suggesting that no ureido structure is present.

It is tentatively suggested that the ethanolamine residue is incorporated as such in the gramicidin molecule, being linked with a peptide bond by its hydroxyl group and linked with either an indole group of tryptophan or an enolized peptide bond by its amino group:

Methylation of the free hydroxyl group of the postulated structure would lead to formation of an acid-labile methoxyl compound. This structure is similar to that put forward by Phillips & Baltzly (1947) to explain the *O*-acyl rearrangement of esters of ethanolamine, and by Isbell & Frush (1949) to explain rearrangements of sugar acetates with ammonia.

EXPERIMENTAL

Materials

Melting points are given uncorrected. Optical rotations were measured in a 0.5 dm. tube. C, H and N (Dumas) determinations were by Drs Weiler and Strauss, Oxford.

Gramicidin. Commercial tyrothricin (Wallerstein Laboratories Inc., New York, Batch L30-13941) was extracted with a mixture of equal volumes of acetone and ether as described by Hotchkiss & Dubos (1941). The product obtained was repeatedly crystallized from dry acetone until the rotation was constant: $[\alpha]_D^{20} + 3^\circ$ in 95% (v/v) aqueous ethanol (c, 0.17).

β -Methoxyethylamine was prepared by two methods, both simpler than that described by Traube & Peiser (1920). (a) β -Methoxyethanol (0.5 g.mol.) was treated with PBr_3 as described by Karvonen (1912) and the mixture was distilled. The fraction boiling up to 140° was collected in water, the oil separated, dried over CaCl_2 and fractionally redistilled. The fraction boiling over the range $102\text{--}104^\circ$ was collected giving β -methoxyethyl bromide in 60% yield (21 g.). This was refluxed with potassium phthalimide (13 g.) on a bath at 120° for 4 hr. The solution was cooled, filtered, evaporated to dryness *in vacuo* and the residue crystallized from water, aqueous ethanol and finally from dioxan affording β -phthalimidoethyl methyl ether (2 g.) as colourless needles, m.p. $147\text{--}148^\circ$. (Found: N (Kjeldahl) 6.0; OCH_3 , 16.0. $\text{C}_{11}\text{H}_{11}\text{O}_3\text{N}$ requires N, 6.8; OCH_3 , 15.1%.) The β -phthalimidoethyl methyl ether was then hydrolysed with hot 6N-NaOH and the liberated β -methoxyethylamine distilled and collected in water. The aqueous solution of the amine was titrated to neutrality with 0.1 N-acetic acid and made up to 50 ml. This solution of the amine acetate was analysed for

methoxyl content and N (Kjeldahl). The concentration of amine acetate given by the methoxyl estimation was 3.8 mg./ml. and by total nitrogen content 4.0 mg./ml. The identity of the amine was confirmed by preparation of the *N*-DNP-derivative, which was identical with the product described in (b) below.

(b) *N*-DNP-ethanolamine (3 g.) (for preparation see below) was dissolved in 20 ml. of dioxan and treated with a solution of 1 g. of dimethyl sulphate in 10 ml. of dioxan, 10 ml. of 10*N*-NaOH were added and the two-phase system formed was shaken repeatedly and then left for 3 hr. at 37°. By the end of this time crystals had appeared in the dioxan layer. This layer was separated, evaporated to dryness *in vacuo* and the resulting oil crystallized from ethanol-acetone and then from CHCl_3 to give yellow prisms of *N*-DNP- β -methoxyethylamine (2.5 g.), m.p. 145.5°. (Found: C, 44.7; H, 4.6; N, 17.4. $\text{C}_8\text{H}_{11}\text{O}_5\text{N}_3$ requires C, 44.8; H, 4.6; N, 17.4%.)

The product (2.5 g.) was refluxed with 20 ml. of 10*N*-NaOH until dissolution was complete. The solution was distilled and the distillate titrated to neutrality with 0.1 *N*-acetic acid and made up to 50 ml. This solution was analysed for methoxyl content. The amine concentration from the titration was 5.5 mg./ml. and from the methoxyl estimation 5.3 mg./ml. The amine was identified by treating 2 ml. of the amine acetate solution with 80 mg. of fluorodinitrobenzene (FDNB) in 2 ml. of ethanol in the presence of 100 mg. of NaHCO_3 . On standing, fine yellow prisms separated, which after crystallization from aqueous ethanol, had m.p. 144°. The mixed m.p. with an authentic sample of *N*-DNP- β -methoxyethylamine was identical.

N-2,4-Dinitrophenylethanolamine. Ethanolamine (0.01 g.mol.) was heated in ethanolic solution with 1-chloro-2,4-dinitrobenzene (0.005 g.mol.) for 3 hr. The solution was taken to dryness *in vacuo* and the residue crystallized from ethyl acetate-light petroleum to give orange-yellow needles (900 mg.), m.p. 91°. Recrystallization from aqueous ethanol afforded *N*-DNP-ethanolamine as needles, m.p. 96°. Waldkötter (1938) gives the m.p. as 90°. (Found: C, 42.9; H, 3.9; N, 18.3. Calc. for $\text{C}_8\text{H}_9\text{O}_5\text{N}_3$: C, 42.3; H, 3.9; N, 18.5%.)

DNP-L-Tryptophan. *L*-Tryptophan (55 mg.) was dissolved in water (2 ml.) together with NaHCO_3 (200 mg.). FDNB (93 mg.) in ethanol (3 ml.) was added and the solution shaken for 3 hr. The ethanol was removed *in vacuo*, water was added and the solution was extracted three times with ether. The aqueous layer was acidified and the oily precipitate was crystallized from aqueous methanol to give *DNP-L*-tryptophan (70 mg.), m.p. 178° (decomp.). Porter & Sanger (1948) give m.p. 175°. (Found: C, 55.2; H, 3.2. Calc. for $\text{C}_{17}\text{H}_{18}\text{O}_6\text{N}_4$: C, 55.0; H, 3.8%.)

Carbobenzyl-L-valylaminoacetate. Carbobenzyl-L-valyl chloride (Synge, 1948) prepared from 0.39 g. of carbobenzyl-L-valine in 4 ml. of ether after washing with ice water was allowed to react with 0.16 ml. of the diethylacetal of aminoacetaldehyde. On addition of saturated aqueous KHCO_3 solution and shaking carbobenzyl-L-valylamino-diethylacetal crystallized in the ether layer, was filtered off, washed with ether and dried (130 mg.). On recrystallization from ether m.p. was unchanged (133–134°). $[\alpha]_D^{25} = -28^\circ$ in ethanol (c, 1.5). (Found: C, 62.4; H, 8.4; N (Kjeldahl) 7.2. $\text{C}_{19}\text{H}_{20}\text{O}_5\text{N}_2$ requires C, 62.3; H, 8.2; N, 7.6%.)

Analytical methods

Methoxyl determinations were carried out by the micro-Zeisel procedure using the apparatus of Steyermark (1948)

modified in one respect. The reaction flask of the apparatus, instead of being sealed on was connected by a ground-glass joint so that the flask could be removed and solutions whose methoxyl content was required could then be evaporated to dryness *in situ*. The volumetric procedure (Grant, 1945) was preferred to the gravimetric estimation of silver iodide used by Steyermark. The method was standardized by means of pure vanillin; consistent recoveries of 95–97% were obtained.

Kjeldahl nitrogen determinations on materials containing tryptophan residues were made after preliminary treatment with red P and HI according to Friedrich (see Grant, 1945; Hotchkiss & Dubos, 1941).

Tryptophan. The colorimetric procedure of May & Rose (1922) was employed. A series of standards were prepared using a pure sample of acetyl-L-tryptophan. The tubes were allowed to stand for 24 hr. at 37° followed by 24 hr. at room temperature in the dark to develop the full colour before readings were taken.

Ethanolamine. Each fraction was hydrolysed in a sealed evacuated tube using a mixture consisting of glacial acetic acid (1 vol.) and 5*N*-HCl (4 vol.) for 24 hr. at 125°. A determination of the ethanolamine content of each fraction was carried out in Conway units by determining the ammonia liberated by periodate oxidation as described by Synge (1945).*

Acid-base titration of gramicidin

In aqueous ethanol. Gramicidin (40 mg.) was dissolved in 2 ml. of 60% (v/v) aqueous ethanol and titrated over the range pH 2–12 with 0.08*N*-HCl and 0.08*N*-NaOH using a glass electrode. The titration curve obtained was not significantly different from that given by titration of the solvent alone (Fig. 1).

Fig. 1. The titration of gramicidin in 60% (v/v) aqueous ethanol. Δ , Solvent only; $\circ$, solvent + 40 mg. gramicidin.

In glacial acetic acid. Gramicidin (40 mg.) was titrated in glacial acetic acid with perchloric acid using crystal violet as indicator, by the technique of Nadeau & Branchen (1935). Despite a slight muddiness of the end point due to the presence of a small amount of water, a negligible amount of perchloric acid was necessary to make the solution acid.

Treatment of gramicidin with FDNB

Gramicidin (20 mg.) in 2 ml. of 60% (v/v) aqueous ethanol was treated with 10 mg. of FDNB in 1 ml. of ethanol in the presence of 100 mg. of NaHCO_3 at 20°. After standing for

2 hr. the ethanol was removed *in vacuo* and the pale-yellow solution acidified with HCl. The colour of the solution disappeared and no coloured DNP derivative of gramicidin could be obtained.

Electrophoretic behaviour of gramicidin

The aqueous gels of silicic acid used by Consden, Gordon & Martin (1946) in their ionophoretic studies cannot be used for water-insoluble materials such as gramicidin. Ethanolic gels were prepared as follows and used instead. A mixture of ethyl silicate (Albright and Wilson Ltd., Oldbury) (100 ml.), ethanol (50 ml.), water (25 ml.) and 5% (w/v) aqueous HCl (1 ml.) was shaken until a homogeneous system was obtained. This solution was stable for periods up to 3 weeks. When diluted (1:10) with 0.5M-ammonium acetate in ethanol the solution gave a firm gel after 30–60 min. having pH 5–6. 120 ml. of gel were set in a trough 30 × 15 cm.

The brass cathode of the cell was bathed continuously with 0.5M-ethanolic ammonium acetate kept acid by the addition of glacial acetic acid (final concentration 0.1M). The carbon anode was bathed with 0.5M-ethanolic ammonium acetate. The starting gutter in which the solution of gramicidin in the gel solution was set was displaced off-centre towards the anode. When a potential of 28 V./cm. was applied, the gel passed a current of 70 mA. The gel was kept cool by a water jacket on the bottom of the apparatus. The direction of electroendosmotic flow was shown to be towards the cathode by using a solution of *N*-DNP-ethanolamine in the gutter; the yellow band moved slowly towards the cathode. Gramicidin (10 mg.) was dissolved in 10 ml. of the gel-forming solution which was allowed to set in the gutter of the ionophoresis apparatus. A potential of 5 V./cm. was applied and a filter-paper print of the gel was taken after 5 hr. The paper was dried and sprayed with Ehrlich reagent. The gramicidin band was shown to have moved only 4 mm. towards the cathode.

Methylation of gramicidin

With dimethyl sulphate. Gramicidin (40 mg.) was dissolved in 3 ml. of dioxan and a solution of 0.5 g. of dimethyl sulphate in 3 ml. of dioxan was added. This solution was then treated with 1 ml. of saturated aqueous Ba(OH)₂, and the mixture shaken continuously for 1 hr. A further 1 ml. of the baryta was added and the shaking continued for 1 hr. The precipitated barium sulphate was then removed and the solution adjusted to pH 6 with a 0.01N solution of H₂SO₄ in aqueous dioxan. The solution was filtered, evaporated to dryness *in vacuo*, the residue taken up in ethanol, precipitated with water and dried. The white powder thus obtained slowly turned purple on standing in air. This material contained 3.51% OMe.

With methyl iodide. Gramicidin (40 mg.) was dissolved in 3 ml. of dioxan and 0.5 ml. of methyl iodide was added; 200 mg. of freshly prepared moist Ag₂O were added and the mixture warmed on the water bath to 60°. After 15 min. the solution was filtered, evaporated *in vacuo*, the residue was dissolved in ethanol, and precipitated with water; a stable white powder was formed. Methoxyl estimation of the product showed it to contain 1.68% OMe. A number of other samples of gramicidin were treated in the same way and were shown to contain 1.54, 1.90, 1.75 and 1.74% OMe corresponding to an average figure of 5.4 OMe groups/10⁴ g. gramicidin. The nitrogen content (Kjeldahl-Friedrich) of one

sample of gramicidin fell from 14.0% N to 10.9% on prolonged (35 min.) methylation with methyl iodide suggesting the possible occurrence of *N*-methylation, this big fall in percentage N being greater than could be accounted for by *O*-methylation.

Hydrolysis of methylated gramicidin

Gramicidin methylated by methyl iodide (200 mg.) was dissolved in 1.5 ml. of glacial acetic acid and 2 ml. of 10N-H₂SO₄ were added. This mixture was heated in a sealed evacuated tube at 110–115° for 24 hr. The tube was opened, most of the acetic acid was removed *in vacuo* and the residual solution was transferred to the flask of a liquid-liquid extraction apparatus of the type used by Syngé (1945) for the extraction of ethanolamine from a hydrolysate of gramicidin. Toluene (150 ml.) and 10 ml. of a saturated solution of NaHCO₃ were added, and the flask was then attached to the extraction apparatus and heated at such a rate that the toluene boiled vigorously. The condensed toluene dropped back through a funnel and passed upwards through a layer of water 12 cm. deep in the extraction tube. Any β -methoxyethylamine present would distil with the toluene and be extracted by the water layer. At the end of 3 hr. the extraction was considered complete and the aqueous layer in the apparatus was removed. The layer was titrated to neutrality with 0.001N-HCl and required only four drops of acid. The solution was evaporated to dryness *in vacuo* below 40° and the residue was dissolved in water, and made up to 5 ml. Portions (1 ml.) of this solution were analysed for N and OMe. None of either was detected.

Stability of β -methoxyethylamine to acid hydrolysis. 1 ml. of an aqueous solution of β -methoxyethylamine acetate containing 5.69 mg. of the amine was treated in a sealed evacuated tube with glacial acetic acid (1.5 ml.) and 10N-H₂SO₄ (2 ml.) for 24 hr. at 110–115°. The tube was opened and the contents were treated as described above for the methylated-gramicidin hydrolysate. The aqueous layer was removed from the extraction apparatus and was titrated with 0.012N-HCl. The recovery of β -methoxyethylamine on the basis of the OMe content was 82% and on the basis of the titration 90%.

Counter-current distribution of a hydrolysate of methylated gramicidin

Methylated gramicidin (200 mg.) was hydrolysed in the acetic acid-H₂SO₄ mixture for 24 hr. at 125° and the hydrolysate was treated with saturated Ba(OH)₂ solution to remove excess H₂SO₄, the final pH being adjusted to 5. The filtered solution was then evaporated to dryness in the first tube of a 24-tube counter-current distribution apparatus. A 24-tube distribution was carried out using 30 ml. of the butanol phase and 15 ml. of the aqueous phase of the solvent system *n*-butanol (5 vol.), acetic acid (1 vol.) and water (4 vol.). Qualitative amino-acid analyses of the contents of each tube were carried out by means of *n*-butanol-water paper chromatograms (Fig. 2). Two spots that could not be accounted for showed up, the first running slower than glycine, and the second running slower than valine. A two-dimensional chromatogram (phenol-water-NH₃/collidine-water) of a small volume of the total hydrolysate showed the presence of at least ten spots, suggesting that the methylation with methyl iodide had caused *N*-methylation at the

peptide bonds, giving rise to *N*-methyl amino-acids on hydrolysis, as indicated by the drop in nitrogen content on methylation.

The fractions from the distribution were then combined into groups and each group was analysed for OMe content. The results are shown in Fig. 2. Only 39% of the original OMe content could be found in the hydrolysate of methylated gramicidin.

Fig. 2. 24-Tube counter-current distribution of acid hydrolysate of methylated gramicidin using *n*-butanol-acetic acid-water system. The amino-acids present in each fraction are indicated as well as the methoxyl content of grouped fractions. For details see text.

Behaviour of aminoacetaldehyde derivatives under acid hydrolysis conditions

Aminoacetal (*A*) (25 mg.) and 20 mg. carbobenzyloxy-L-valylaminoacetal (*B*) were heated in sealed evacuated tubes with 1.2 ml. 6*N*-HCl and 0.3 ml. acetic acid for 27 hr. at 105°. The resulting solutions, which contained much brown 'humin' and had the smell characteristic of acid hydrolysates of protein, were evaporated to dryness *in vacuo* and taken up in water, one-fiftieth portions of the solutions (neutralized with NH₃ vapour) were chromatographed on Munkelt OB filter paper using phenol-water. The brown pigment ran fast in each case; *A* gave, on heating after spraying with ninhydrin, two pink spots having *R_F* 0.25 and 0.75. *B* gave, without heating, a fast-moving spot corresponding with valine. However, in neither case was glycine or ethanolamine detected, addition of either or both of these substances giving rise to new spots on the chromatograms (*R_F*, 0.4-0.5).

Partial hydrolysis of gramicidin

Treatment of gramicidin with alkali. Three samples of the same batch of gramicidin (50 mg.) were each dissolved in 2 ml. of 0.08*M*-NaOH in 60% (v/v) aqueous ethanol in a boiling tube. The tubes were evacuated and sealed. The first was allowed to stand at room temperature for 20 hr. and then opened and titrated *in situ* with 0.08*M*-HCl in 60% (v/v) aqueous ethanol, using a glass electrode. The second tube was opened and titrated after 65 hr. at room temperature, and the third after 7 days at 37°. The curves obtained differed in no respect from blanks with the ethanolic NaOH.

Acid hydrolysis of gramicidin. Gramicidin (40 mg.) was dissolved in a mixture of dioxan (2 ml.), purified by the method of Oxford (1934), and 10*N*-HCl (0.5 ml.). The tube was evacuated, sealed and kept overnight at room tempera-

ture. The liquid in the tube separated into two layers; the lower, dioxan-rich layer rapidly developed a pink colour and finally a deep-brown colour, whereas the upper layer remained colourless. After 16 hr. the tube was opened and the contents taken to dryness *in vacuo*, the temperature never being allowed to rise above 20°. The residue was dissolved in 60% (v/v) aqueous ethanol and titrated with 0.08*M*-ethanolic NaOH to pH 9 using a glass electrode. The electrode was then changed for an 'Alki' glass electrode (Cambridge Instrument Company) and the titration continued up to pH 12. A flocculent white precipitate formed during the titration when the pH rose above 4 and would not dissolve at any pH above 4. The experiment was then carried out extending the time of hydrolysis to 24 hr. The titration curves obtained are shown in Fig. 3.

Fig. 3. Comparison of the rate of release of acidic and basic groups from gramicidin on partial hydrolysis by conc. HCl in dioxan solution. The curves are plotted to coincide at pH 7. For experimental details see text. Δ , Solvent blank titration. (a) 24 hr. hydrolysis at 20°; $\circ$, experimental curve; $\square$, curve corrected for solvent titration. (b) 16 hr. hydrolysis at 20°; $\bullet$, experimental curve; $\blacktriangle$, curve corrected for solvent titration.

Hydrolysis at low temperature using a mixture giving only one phase was then adopted. This was dioxan (10 vol.), water (3 vol.), 10*N*-HCl (3 vol.). It was found that hydrolysis for 9 days at 2° gave a result similar to that obtained as above in 24 hr. at 20°. The low-temperature hydrolysis was used in all subsequent experiments.

End-group determinations on gramicidin hydrolysates

The terminal amino-acids, possessing a free amino group, present in these hydrolysates of gramicidin were determined by the method of Sanger (1945) involving the formation of the *N*-DNP derivatives and their separation on silica-gel columns. All solvent systems and columns used were first standardized by the use of pure preparations of DNP

amino-acids. The DNP-peptides were formed by treatment of solution of the peptide in 80% (v/v) aqueous ethanol with excess FDNB in the presence of NaHCO_3 at 20°. After standing overnight the yellow solution was taken to dryness *in vacuo* and dissolved in water, acidified and extracted with CHCl_3 . The DNP-peptides in CHCl_3 solution were precipitated by the addition of light petroleum, filtered and then hydrolysed *in vacuo* in a mixture of acetic acid (0.8 ml.) and 6N-HCl (3.0 ml.) for 24 hr. at 125°. The hydrolysates were taken to dryness and then run on an ether-water silica-gel column to remove any residual mineral acid.

Fractions soluble in aqueous ethanol at pH's above 4. The DNP-amino-acids obtained from the ether-water column were run on the following series of columns (the solvent systems used were as described by Sanger (1945), except where otherwise specified):

(i) Ethanol-ligroin. Two bands were obtained, the first being a broad yellow band with $R=0.2$, and the second being brown with $R=0.1$. The faster band was not DNP-valine ($R=0.45$) or DNP-leucine ($R=1.0$). The two bands were collected separately and the material in the faster band was run on column (ii).

(ii) 5% *iso*Propanol-cyclohexane. One band was obtained ($R=1.0$). The band could not be DNP-glycine ($R=0.1$) or *N*-DNP-ethanolamine ($R=0.27$). The material in this band could therefore be either DNP-tryptophan or DNP-alanine. To test this point a mixed chromatogram with DNP-alanine was run on an ethanol-ligroin column. Two bands were obtained, the first having the rate of DNP-alanine ($R=0.25$) and the second $R=0.1$. This suggested that the unknown DNP-amino-acid was DNP-tryptophan, and was confirmed when a mixed column with DNP-tryptophan using the 5% *isopropanol-cyclohexane* mixture gave only one band with ($R=0.9-1.0$). However, when the material was tested with the Ehrlich reagent no colour was given; DNP-tryptophan under the same conditions gave a green colour. However, tests on the stability of DNP-tryptophan to acid hydrolysis alone and in the presence of gramicidin under the conditions used for the DNP-peptides, showed that in both cases isolated material behaved in exactly the same manner as the DNP-amino-acid obtained from the DNP-peptides. The material in the slow brown band obtained from column (i) was found to revert to a yellow colour on standing and on running on an *isopropanol-cyclohexane* column two bands were obtained the first with $R=0.86$, the second $R=0.30$. The material in the first band behaved in the same way on an ethanol-ligroin column as did the main band obtained from column (i). The material in the second band was shown not to be *N*-DNP-ethanolamine, DNP-glycine, DNP-valine, DNP-leucine, or DNP-tryptophan by comparison of its R value on ethanol-ligroin, or *isopropanol-cyclohexane* columns. It was concluded that it might be dinitroaniline derived from the breakdown of DNP-tryptophan, as similar material was obtained after treating DNP-tryptophan with acid. The evidence therefore suggests that the products isolated from the DNP-peptides correspond with those resulting from DNP-tryptophan.

Fractions insoluble in aqueous ethanol above pH 4. The material obtained as above from the ether-water column was run on the following columns:

(i) 5% *iso*Propanol-cyclohexane. One broad band obtained ($R=1$).

(ii) Ethanol-ligroin. One slow band obtained, moving at the same rate as DNP-tryptophan.

As only a small amount of material was available no

further tests could be carried out. The aqueous residue from the hydrolysis of the DNP-peptide was run on a paper chromatogram using *n*-butanol-water. The only amino-acids found were glycine, alanine, valine and leucine, whereas the original ethanol-insoluble peptide hydrolysed under the same conditions contained the aliphatic amino-acids and also tryptophan. This suggests that the free amino group of this peptide is that of tryptophan.

No indication of the presence of any other DNP-amino-acids in the hydrolysed DNP-peptides could be obtained. It was hoped that *N*-DNP-ethanolamine would be present, and the solvent system methanol (2 vol.), water (1 vol.), CHCl_3 (5 vol.), and light petroleum (10 vol.) was elaborated in order to separate *N*-DNP-ethanolamine ($R=0.5$) from 2,4-dinitroaniline ($R=0.1$). No DNP-ethanolamine was, however, discovered.

Fig. 4a.

Fig. 4b.

Fig. 5a.

Fig. 5b.

Figs. 4, 5. Paper chromatograms of fractions isolated from partial hydrolysates of gramicidin. Solvents: 60% (v/v) aqueous ethanol (Fig. 4) and ethanol (Fig. 5) run to edge of diagram from starting lines shown: (a) sprayed with ninhydrin; (b) sprayed with Ehrlich's reagent. A, Gramicidin; B (band 1), C (band 2) and D (band 3) are fractions from starch columns; E, ethanol-insoluble peptide.

Fractionation of partly-hydrolysed gramicidin by electrophoresis

The peptides were dissolved in a small volume of the ethanolic gel already described and set in the gutter of the apparatus. A potential of 28 V./cm. was applied and the apparatus allowed to run for 5 hr. The position of the peptide bands in the gel was found by printing a filter-paper strip on the gel. One half of the paper strip was sprayed with the Ehrlich reagent in order to detect the position of the tryptophan-containing peptides, the other half was dried

and warmed *in vacuo* to remove ammonium acetate and then sprayed with ninhydrin, in order to detect the short-chain peptides. The faster, tryptophan-free band had moved 4 cm., the slower, tryptophan-rich band 2 cm. towards the cathode.

Peptide fractionation by partition chromatography on starch

Gramicidin (40 mg.) was partially hydrolysed at low temperature, and a solution of the hydrolysate was adjusted to pH 7 as described above. The insoluble peptide material (17 mg.) was removed by filtration and the filtrate was taken to dryness *in vacuo*. The residue was then run on a starch column (washed raw potato starch) using the solvent system water (1 vol.), isopropanol (3 vol.), cyclohexane (7 vol.); the starch took up one-third of its weight of the aqueous phase.

Fig. 6. Titration curves of partial hydrolysate of gramicidin showing loss of basic groups in back titration from pH 12. $\circ$, Titration from pH 2 to 12; $\triangle$, titration from pH 12 to 2; $\square$, blank titration of solvent.

The column was examined in a dark room with an ultra-violet lamp. A fluorescent band ran rapidly through the column and was collected (fraction 1, 1 mg.), leaving an

intensely fluorescent band at the top of the column that could be partly eluted by means of ethanol (fraction 2, 18.0 mg.) leaving a narrow fluorescent band at the top of the column. This band (fraction 3, 7 mg.) was finally eluted with 60% (v/v) aqueous ethanol and was contaminated with the NaCl resulting from the original neutralization of the hydrolysate. The components present in each of these three fractions were investigated by the use of paper chromatograms using as solvents ethanol and 60% (v/v) aqueous ethanol (Figs. 4 and 5).

Analysis of the fractions. Each fraction was analysed as follows: (1) tryptophan; (2) ethanolamine; (3) qualitative amino-acid analyses were carried out on hydrolysates of each fraction by using paper chromatograms with the solvent system *n*-butanol (5 vol.)-acetic acid-(1 vol.)-water (4 vol.); (4) the free amino N of each fraction was measured by the Van Slyke method (4 min. reaction time); (5) titration curves (Fig. 6) were obtained for the unfractionated acid hydrolysate of gramicidin and for each fraction by titrating first from pH 2 to 12 and then back to pH 7 in 60% (v/v) aqueous ethanol and using glass electrodes with a stream of nitrogen gas as a stirrer, as described above (the titration mixtures were above pH 7 for about 10 min.); (6) the total N of each fraction was determined (Kjeldahl-Friedrich). The results are given in Tables 2 and 3. The results of the qualitative amino-acid analyses are given above (p. 111).

Table 2. *Ethanolamine and tryptophan content of fractions isolated from partial hydrolysates of gramicidin*

Fraction	Tryptophan (% by wt.)	Ethanolamine (% by wt.)
Gramicidin	23.8	3.7
Ethanol-insoluble peptides	21.0	4.8
Ethanol-soluble peptides:		
Fraction 1	18.0	3.7
Fraction 2	6.3	3.5
Fraction 3	0	21.4

SUMMARY

1. The absence of basic or acidic groups in gramicidin has been confirmed by titrations in aqueous ethanol and in glacial acetic acid, by its failure to form a coloured dinitrophenyl derivative and by its failure to move as a charged molecule in electrophoresis.

2. The presence of hydroxyl groups in the molecule has been confirmed by methylation with methyl iodide.

Table 3. *Change in amino-nitrogen content of fractions from partially hydrolysed gramicidin after treatment with alkali*

Fraction	Before treatment		After treatment with alkali	
	Van Slyke amino N	Titration amino N	Van Slyke amino N	Titration amino N
Unfractionated hydrolysate	8.0	6.0	4.8	3.7
Ethanol-insoluble peptides	3.8	2.8	3.2	2.8
Ethanol-soluble peptides:				
Fraction 2	2.7	3.1	3.6	2.3
Fraction 3	21	—	15.8	—

3. Hydrolysis of such methylated gramicidin fails to liberate β -methoxyethylamine as would be expected if the methylated hydroxyl groups were those of ethanolamine residues.

4. The methoxyl groups of methylated gramicidin are shown to be acid-labile.

5. Residues of aminoacetaldehyde probably do not serve as precursors of the ethanolamine found in acid hydrolysates of gramicidin.

6. The relative rate of release of basic and acidic groups in the partial acid hydrolysis of gramicidin has been followed by means of titration curves. Gramicidin is not readily hydrolysed by alkali in the cold.

7. The free amino groups of the peptides produced on acid hydrolysis have been recognized as those of

tryptophan residues. Dinitrophenyl-tryptophan is not stable to the acid hydrolysis conditions used.

8. Evidence is presented for the existence of amino-acid and peptide esters of ethanolamine in partial acid hydrolysates of gramicidin.

9. A partial structure for gramicidin is proposed.

10. The new compounds β -phthalimidoethyl methyl ether, *N*-2:4-dinitrophenyl- β -methoxyethylamine and carbobenzyloxy-L-valylaminoacetal have been synthesized, and improved procedures are described for preparing β -methoxyethylamine.

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