When this hydrochloride (0.005 mmole) was dissolved in 50 ml. of 0.01 *M* bicarbonate (pH 8.75), the λ_{max} at 217 mμ (ε 12,100) disappeared after 1 hr., and continuous extraction with methylene chloride gave 5,10-dioxo-1,6-diazacyclodecane.

Treatment of 34 mg. (0.2 mmole) of the cyclodipeptide with 10 ml. of 0.1 *M* carbonate solution (pH 10.7) for 3.5 hr. on the steam bath followed by continuous extraction with methylene chloride gave 22 mg. of 2-pyrrolidinone.

Gramicidin A. V. The Structure of Valine- and Isoleucine-gramicidin A

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Contribution from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education and Welfare, Bethesda, Maryland. *Received October 16, 1964*

Gramicidin A is a linear N-acylated pentadecapeptide ethanolamide. Its N-terminal L-*valine or* L-*isoleucine are blocked by a formyl group which is cleaved by mild methanolysis at room temperature and easily identified as formic acid by gas chromatography or as formaldehyde after reduction by the chromotropic acid assay. Reformylation of desformylgramicidin A, via O-formylgramicidin A, gave back gramicidin A. Desformylgramicidin A was subjected to ten successive Edman degradations under conditions modified to accommodate the insolubility of its residual peptide fragments in water. The phenylthiohydantoins, obtained by cyclization with trifluoroacetic acid, were assayed by gas chromatography up to step 10. In addition, the residual peptides up to step 10 were hydrolyzed and their amino acids determined quantitatively. Selective N-bromosuccinimide (NBS) cleavage of the bonds following the four tryptophans in gramicidin A releases aminoethanol and the NBS-oxidation product of leucyltryptophan but no dioxindolalanine spirolactone fragment expected from a Try-Try sequence. These findings combined with the older observations suggest the sequence HCO-*L-*Val*-Gly-*L-*Ala*-D-*Leu*-L-*Ala*-D-*Val*-L-*Val*-D-*Val*-L-*Try*-D-*Leu*-L-*Try*-D-*Leu*-L-*Try*-D-*Leu*-L-*Try*-NHCH$_2$CH$_2$OH for valine-gramicidin A and HCO-*L-*Ileu . . . for isoleucine-gramicidin A. Thin layer chromatography, optical rotatory properties in various solvents, and molecular weight studies by ultracentrifugation and osmometry are discussed in terms of easy association of at least two molecules of gramicidin A whose unprecedented alternating sequence of (mostly hydrophobic)* L- *and* D-*amino acids permits a characteristic secondary structure dependent on the nature of the solvent.*

Gramicidin was isolated in 1941 by Hotchkiss and Dubos.[2] A cyclic structure was assumed for this peptide antibiotic because of the absence of basic and acidic functions, and because initially only neutral amino acids, but no alcohols or fatty acids were found in hydrolysates of gramicidin.[3] Later the discovery of aminoethanol[4] in gramicidin hydrolysates prompted Synge to propose an "ortho" peptide bond[5] for the aminoethanol linkage (Figure 1) in order to explain both the neutral character of gramicidin and the single hydroxyl function present in the antibiotic. However, the work of Griot and Frey[6] limits the existence of stable cyclols to systems containing a diacylimide structure. The elimination of a cyclol structure as well as results obtained from NBS oxidation of gramicidin supported a structure in which tryptophan is linked to the amino group of ethanolamine whose hydroxyl group is free. Such a linear structure can be written only if one assumes the presence of an N-terminal blocking group whose nature was of such a kind that it had escaped the attention of the previous investigators.

A. The N-Formyl Terminal Blocking Group.[7] Gramicidin A[8] represents a mixture of valine- and isoleucine-gramicidin A.[9-11] It releases a volatile acid on hydrolysis with 50% sulfuric acid. This acid was isolated by low-temperature distillation and identified as formic acid either by gas chromatography[12] or by the chromotropic acid test[13] after reduction to formaldehyde. Quantitative experiments indicated a liberation of 0.8–1 mole of formic acid per 1850 g. of gramicidin A. No other acid was found by gas chromatography except acetic acid in a sample which had been freeze dried from acetic acid and still retained 7% of this acid. However, acetic acid was no longer found after recrystallization of this sample from ethanol–water or after drying at 100° and 10^{-2} mm. Neither deformylated gramicidin A nor tryptophan liberated any trace of formic acid in control experiments. The n.m.r. spectrum[14] of gramicidin A in deuteriomethanol shows a broad peak at 8.45 p.p.m. (tetramethylsilane as internal standard), the peak area being one-twelfth to

(1) Public Health Service Fellow 1962–1964.
(2) R. D. Hotchkiss and R. J. Dubos, *J. Biol. Chem.*, **141**, 155 (1941).
(3) R. D. Hotchkiss, *ibid.*, **141**, 171 (1941).
(4) R. L. M. Synge, *Biochem. J.*, **39**, 355 (1945).
(5) R. L. M. Synge, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 191 (1950).
(6) R. G. Griot and A. J. Frey, *Tetrahedron*, **19**, 1661 (1963); *cf.* G. I. Glover and H. Rapoport, *J. Am. Chem. Soc.*, **86**, 3397 (1964).
(7) R. Sarges and B. Witkop, *ibid.*, **86**, 1861 (1964).
(8) J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, **172**, 839 (1948).
(9) S. Ishii and B. Witkop, *J. Am. Chem. Soc.*, **85**, 1832 (1963).
(10) L. K. Ramachandran, *Biochemistry*, **2**, 1138 (1963).
(11) E. Gross, S. Ishii, and B. Witkop, unpublished results.
(12) E. S. Erwin, G. J. Marco, and E. M. Emery, *J. Dairy Sci.*, **44**, 1768 (1961); *cf.* H. J. Langner, *Angew. Chem.*, **77**, 95 (1965).
(13) W. M. Grant, *Anal. Chem.*, **20**, 267 (1948).
(14) We are greatly obliged to Dr. J. W. Daly for the determination and interpretation of the n.m.r. data.

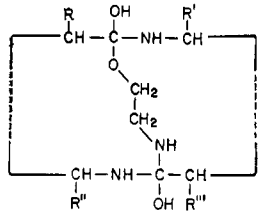


Figure 1. Original "cyclol structure" of gramicidin A which accommodates aminoethanol in a transannular orthopeptide arrangement.

one-fourteenth that of the aromatic indole protons at 5.2–6.0 p.p.m. The addition of formic acid to the gramicidin solution gives rise to a sharp peak at 8.32 p.p.m. By comparison the formyl proton of formyl-L-valine appears at 8.40 p.p.m.

B. Desformylgramicidin A.[15] Treatment of gramicidin A with 1.5 *N* hydrochloric acid in methanol at room temperature for 1 hr. releases the N-formyl group[16] and yields desformylgramicidin A, a compound previously called secogramicidin A.[15] With the comparable formyl-L-valine this cleavage proceeds in more than 95% yield. N-Acetyldesformylgramicidin is recovered unchanged after methanolysis under comparable conditions. Desformylgramicidin A is basic and can be separated from unreacted gramicidin A (up to 6%) by chromatography on a Dowex 50W-X2 column. It contains 1.1 ± 0.1 moles of free NH_2-terminal L-valine and L-isoleucine per 1850 g. Reformylation of desformylgramicidin A with acetic anhydride–formic acid[16] leads to N,O-diformyldesformylgramicidin A (O-formylgramicidin A) which, after base treatment, regenerates gramicidin A characterized by thin layer chromatography (Figure 2).

C. Edman Degradation of Desformylgramicidin A.[17] Gramicidin A is not attacked by enzymes such as chymotrypsin, pepsin, nagarse, and pronase.[15] Partial hydrolysis and methanolysis does not yield useful fragments beyond the level of di- and tripeptides. We therefore resorted to a modified Edman degradation[18] of desformylgramicidin A. Because of the insolubility of desformylgramicidin A as well as of the degraded peptides in water, phenyl isothiocyanate had to be used in a medium of triethylamine–pyridine[19] in the absence of water. The neutral phenylthiohydantoin (PTH) derivatives of the respective NH_2-terminal amino acids were separated from the basic residual peptides by fractionation on a Dowex 50W-X2 column with methanol and methanol–ammonia. For the cyclization step trifluoroacetic acid[20] was used. This method, although comparatively gentle, is not mild enough to prevent the gradual destruction of tryptophan during the course of the multiple Edman degradation, which was therefore not pursued beyond the tenth step.[21–23]

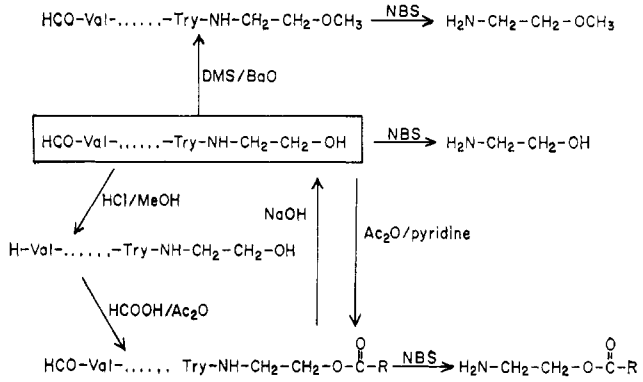


Figure 2. Interconversions of valine-gramicidin A involving methanolysis to desformylvaline-gramicidin A and oxidative cleavage of gramicidin A, its O-acetyl and O-methyl derivative to aminoethanol, its O-acetate, and O-methyl ether.

The PTH derivatives of the amino acids were characterized by thin layer chromatography[24] and by gas chromatography.[25] Aliquots of the residual peptides were hydrolyzed and analyzed on an automatic amino acid analyzer.[26] These methods gave consistent results, suggestive of the sequence H-Val-Gly-Ala-Leu-Ala-Val-Val-Val-Try-Leu-(Try_3,Leu_2,aminoethanol) for valine-gramicidin A and the analogous sequence H-Ileu-Gly. . . . .for isoleucine-gramicidin A, assuming that the only difference between valine- and isoleucine-gramicidin A lies in the N-terminal amino acids. Of the gramicidin A examined 18% consisted of isoleucine-gramicidin A. PTH-tryptophan was identified after the ninth degradation step by thin layer chromatography, PTH-leucine after the tenth step by gas chromatography.

D. N-Bromosuccinimide Oxidation. Oxidation of gramicidin A with NBS, which cleaves the peptide bond next to a tryptophan carboxyl group,[27] leads to several fragments. Since there are four tryptophan residues in gramicidin A, four NH_2-terminal fragments should be liberated per mole (1880 g.) of gramicidin A. Increasing amounts of NBS per mole of gramicidin A linearly increase the release of amino groups and of aminoethanol up to 6–8 moles of NBS per mole of

(15) S. Ishii and B. Witkop, *J. Am. Chem. Soc.*, **86**, 1848 (1964).
(16) J. C. Sheehan and D. D. H. Yang, *ibid.*, **80**, 1154 (1958).
(17) R. Sarges and B. Witkop, *ibid.*, **86**, 1862 (1964).
(18) P. Edman, *Acta Chem. Scand.*, **7**, 700 (1953).
(19) S. Ericson and J. Sjoquist, *Biochim. Biophys. Acta*, **45**, 290 (1960).
(20) W. Konigsberg and R. J. Hill, *J. Biol. Chem.*, **237**, 2547 (1962).
(21) In our hands a degradation attempt by catalytic reduction of 2-nitro-4-methanesulfonylphenyldesformylgramicidin A in methanolic solution containing triethylamine failed. This degradation method would have been advantageous, since cyclization to the 3-substituted 2-hydroxy-7-methanesulfonyldihydroquinoxaline occurs on warming at pH 5.[22]
(22) P. de la Llosa, M. Justisz, and E. Scoffone, *Bull. soc. chim.* France, 1621 (1960). We are greatly indebted to Dr. E. Scoffone, University of Padua, for a generous supply of 2-nitro-4-methanesulfonylphenyl derivatives of valine, leucine, alanine, and tryptophan.
(23) We also tried to utilize the ozonization of (bound) tryptophan to (bound) formylkynurenine [B. Witkop, *Ann.*, **556**, 103 (1944); *cf.* E. Scoffone, *et al.*, *Gazz. chim. ital.*, **93**, 841 (1963)] in order to decrease the sensitivity to acid. Supposedly kynurenine withstands refluxing for 10 hr. in 25% sulfuric acid [Y. Kotake and M. Kiyakawa, *Z. physiol. Chem.*, **195**, 147 (1931)]. While the ozonization of model N-acetyltryptophan gave a clearly recognizable end-point and a good yield of N'-formyl-N^{α}-acetylkynurenine [R. R. Brown and J. M. Price, *J. Am. Chem. Soc.*, **77**, 4158 (1955)], no definable end point [resorcinol added, *cf.* A. Previero, M. A. Coletti, and L. Galzigna, *Biochem. Biophys. Res. Commun.*, **16**, 195 (1964)] and only 1.4–1.7 moles of kynurenine (amino acid analyzer), instead of the expected 4 moles, were observed in the ozonolysis and subsequent hydrolysis of gramicidin A [*cf.* L. Galzigna, A. Previero, A. Reggiani, and M. A. Coletti, *Experientia*, **20**, 669 (1964)].
(24) M. Brenner, A. Niederwieser, and G. Pataki, *ibid.*, **17**, 145 (1961).
(25) J. J. Pisano, W. J. A. VandenHeuvel, and E. C. Horning, *Biochem. Biophys. Res. Commun.*, **7**, 82 (1962).
(26) We are greatly indebted to Dr. E. Gross for the amino acid analyses.
(27) A. Patchornik, W. B. Lawson, E. Gross, and B. Witkop, *J. Am. Chem. Soc.*, **82**, 5923 (1960); *cf.* E. Gross and B. Witkop, *Advan. Protein Chem.*, **16**, 291 (1961).

gramicidin A (1.5–2 moles of NBS per mole of tryptophan) when an end value of 1.3–1.5 moles of amino group per mole of gramicidin A is reached. Although the tryptophan is completely oxidized, only 35% of the theoretical cleavage is observed. The cleavage mixture therefore contains, in addition to aminoethanol, a variety of complex peptides of oxidized tryptophan. When the reaction mixture is chromatographed on a Sephadex column in 50% acetic acid, several peaks with ultraviolet absorbance are found. The last peak eluted, which should contain the fragment with lowest molecular weight, has all the properties of the NBS oxidation product of leucyltryptophan, whereas the oxidation products of tryptophan and of carbobenzyloxytryptophyltryptophan are eluted later. This is a good indication that no 5-bromodioxindolalanine spirolactone, expected from a Try-Try sequence, is liberated during NBS oxidation of gramicidin A. It is safe to conclude that the remaining tryptophan and leucine residues should occur in an alternating sequence.

The liberated aminoethanol was assayed and identified by paper electrophoresis (Table I) and, after N,O-trifluoroacetylation, by gas chromatography (Table II). NBS oxidation of O-methyl-[28] and O-acetyl-[3] gramicidin A releases 2-methoxyethylamine and 2-acetoxyethylamine,[29] respectively (Table III.) From O-succinyl-gramicidin A[30] a ninhydrin-positive zwitterionic fragment was obtained which in its electrophoretic behavior was identical with O-succinylethanolamine (Tables II and III).

Table I. Relative Migration of Aminoethanol Derivatives on High Voltage Paper Electrophoresis

$NH_2CH_2CH_2OR$	pH 6.5	pH 3.6
R = H	1.00	1.00
R = CH_3	0.97	0.97
R = $CH_2CH_2CH_2CH_3$	0.70	0.69
R = $COCH_3$	0.81	0.81
R = $COCH_2CH_2COOH$	0.0	0.51
R = CHO		0.95
Ethylenimine	1.30	1.35

Table II. Gas Chromatography of Trifluoroacetylated (TFA) Amino Alcohols

	Rel. retention time[a]	Rel. peak area, molar basis
TFA-$NHCH_2$-$CH(CH_3)O$-TFA	1.0	1.00
TFA-$NHCH_2CH_2O$-TFA	1.5	0.64
TFA-$NHCH_2CH_2OCH_3$	0.18	
TFA-$NHCH_2CH_2O$-*n*-Bu	0.86	1.22
TFA-$NHCH_2CH_2OCOCH_3$	2.1	1.06
TFA-$NHCH_2CH_2OCHO$	2.3	
$CH_3CONHCH_2CH_2O$-TFA	0.7	
$HCONHCH_2CH_2O$-TFA	0.54	
Succinimide	9.7	

[a] 4% neopentyl glycol succinate on Chromosorb W, flame ionization detector.

(28) A. T. James and R. L. M. Synge, *Biochem. J.*, **50**, 109 (1951).
(29) C. W. Crane and H. N. Rydon, *J. Chem. Soc.*, 527 (1947).
(30) H. S. Olcott, J. C. Lewis, K. P. Dimick, H. L. Fevold, and H. Fraenkel-Conrat, *Arch. Biochem.*, **10**, 553 (1946).

Table III. Liberation of (Volatile) Fragments in the Oxidation of Gramicidin A Derivatives (HCO-Val(Ileu)-...Try-NH-CH_2CH_2OR) with 8 Moles of NBS per Mole of Gramicidin A

Substituent R	Total primary amine,[a] moles	H_2NCH_2-CH_2OH, moles	H_2NCH_2-CH_2OR, moles
H	1.32	0.39	0.39
CH_3	1.43	0.28[b]	0.17
$COCH_3$	1.42	0.04	0.45
$COCH_2CH_2COOH$	1.38	0.04	Amount not determined
SO_2CH_3	1.38	0.15	*c*
$SO_2C_6H_7CH_3$	0.80	0.0	*c*

[a] Determined by trinitrophenylation. [b] The difference between O-methyl- and O-acetylgramicidin A in this respect is unexpected. [c] The liberated fragment migrates as fast as ethyleneimine on paper electrophoresis.

These findings support a linear pentadecapeptide structure for gramicidin A in which the amino group of aminoethanol is linked to the carboxyl end of tryptophan and the hydroxyl group is free.

At this point it is appropriate to re-evaluate the evidence that led to the postulated attachment of aminoethanol in a cyclol structure.[5] The main support for the cyclol hypothesis had been the observation[28] that hydrolysis of O-methylated gramicidin gives rise to aminoethanol, but not to 2-methoxyethylamine. We could confirm this observation but found that, contrary to Synge's findings, 2-methoxyethylamine itself is not acid stable and on hydrolysis in 0.3 ml. of acetic acid and 1.5 ml. of 6.0 *N* hydrochloric acid at 110° for 20 hr. is converted to 2-acetoxyethylamine and aminoethanol, ascertained by gas chromatography and amino acid analysis. 2-*n*-Butoxyethylamine[31] is only partially converted to these products. Hydrolysis of O-*n*-butylgramicidin A leads to a mixture of 2-*n*-butoxyethylamine, 2-acetoxyethylamine, and aminoethanol, determined by paper electrophoresis and gas chromatography.

These results taken together suggest the following structure for *valine-gramicidin A:* HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-$NHCH_2CH_2OH$, and HCO-L-Ileu-Gly-. . . . for *isoleucine-gramicidin A.*

The optical configuration of the individual amino acids has been determined earlier.[9] The placement of two L- and two D-valine residues follows from the studies of Synge[32] and Christensen,[33] who isolated the following peptides by partial hydrolysis: L-Val-Gly, L-Ala-D-Leu, L-Ala-D-Val, D-Val-L-Val, L-Val-D-Val. A D-Leu-D-Leu peptide has never been isolated, a fact which supports the alternating sequence of three leucine and four tryptophan residues in positions 9–15 of gramicidin A. The peptide D-Leu-Gly, reported by Synge,[32] gave rise to speculations about the molecular weight of gramicidin which then should contain two glycine residues.[34] There is no room for such a peptide according to our results. We suspect that it is L-Ileu-Gly.[35] The loss of all glycine after the second

(31) W. R. Boon, *J. Chem. Soc.*, 307 (1947).
(32) R. L. M. Synge, *Biochem. J.*, **44**, 542 (1949).
(33) J. W. Hinman, E. L. Caron, and H. N. Christensen, *J. Am. Chem. Soc.*, **72**, 1620 (1950).
(34) K. Okuda, C. Lin, and T. Winnick, *Nature*, **195**, 1067 (1962).
(35) R. L. M. Synge, private communication.

Edman degradation is unequivocal proof for the molecular weight of 1882 for valine-gramicidin A, a value independently confirmed by other methods (section E). The alternating pattern of L- and D-amino acids as well as the water insolubility of gramicidin readily explain the resistance of gramicidin A to attack by enzymes. The N-formyl residue is a novel blocking group for a naturally occurring peptide. N-Formylmethionyl-s-RNA has recently been implied in the protein synthesis of *Escherichia coli*.[36]

E. Derivatives of Gramicidin A. Several derivatives of gramicidin in which the hydroxyl function was substituted by methoxy or acyloxy residues have been described.[3,28,30] We have prepared O-methylated gramicidin A with methyl iodide–silver oxide[28] (*Anal.* Found: 53% methoxyl of the theoretical value), with diazomethane–fluoroboric acid[37] (*Anal.* Found: 70% methoxyl), and with dimethyl sulfate–barium oxide[38] in dimethyl sulfoxide and dimethylformamide (*Anal.* Found: 92% methoxyl). O-*n*-Butylated gramicidin A was prepared with *n*-butyl iodide and silver oxide. Gramicidin can be tritylated and the O-trityl group is easily removed by heating with acetic acid, giving back gramicidin.

Treatment of desformylgramicidin with anhydrides such as acetic, succinic, and *o*-carboxybenzenesulfonic anhydride leads to N,O-diacylated desformylgramicidin, which can be saponified by base to N-acyldesformylgramicidin. The biological activities of some of these derivatives are listed in Table IV.

Table IV. Antibiotic Activities of Derivatives of Gramicidin against Various Microorganisms at a Concentration of 10 μg. per ml

Microbial strain	A	B	C	D	E
B. subtilis	−	+	+[b]	+	+
S. faecalis	−	−	+	−	−
B. megaterium	−	−	+	−	−
B. cereus	−	−	+	−	−
M. flavus	−	−	+	−	−
S. aureus	±	−	+	−	−
S. fradiae	−	−	+	−	±
S. griseus	−	−	+	−	−
R. nigricans	+	+	+	±	−
S. epidermus	−	−	+	−	−

[a] We are indebted to S. B. Penick and Co., especially to Dr. W. G. Bywater, for these assays. The plus signs signify growth, the minus signs denote absence of growth as a result of antibiotic activity. The column headed A represents gramicidin A; B, N,O-diacetyldesformylgramicidin; C, N,O-dibenzoyldesformylgramicidin-O-sulfonic acid; D, N-acetyldesformylgramicidin; and E, N,O-disuccinyldesformylgramicidin. [b] The absence of antibiotic activity for the most water-soluble representative in this group of gramicidin derivatives is interesting. For a correlation of lipophilic residues with antibiotic activity, *cf.* K. Vogler, *et al.*, *Helv. Chim. Acta*, **47**, 526 (1964).

F. Association Phenomena and Secondary Structure. The establishment of the primary sequence of gramicidin A still leaves many of its unusual properties unexplained. Before more is known about the secondary architecture of the gramicidin molecule it may suffice to enumerate briefly these phenomena.

Table V demonstrates the dependence of the specific rotation of gramicidin A on the nature of the solvent. These variations initially seemed to support a cyclol structure with two new easily epimerizable asymmetric orthopeptide centers. There is no relationship between the specific rotation and the dielectricity constant of the solvent. There is, however, evidence of association to dimers in solvents such as dioxane, ethyl acetate, and methyl ethyl ketone (Table VI). This equilibrium between monomer and dimers is probably the reason for the multiplicity of spots which gramicidin shows on thin layer chromatography and which is dependent on the nature of the solvents.[9]

Table V. Dependence of Specific Rotation of "Gramicidin A" on Solvent and Dielectric Constants

Solvent	$[\alpha]^{20}D$, deg.	$\epsilon_{20°}$
N-Methylacetamide	+14.3 ± 1.0	175.0[a]
N,N-Dimethylformamide	+5.0 ± 1.0	37.6
Methanol	+24.5 ± 1.0	33.5
Ethanol	+5.6 ± 1.0	25.0
1-Propanol	−6.0 ± 1.0	20.8
2-Butanone	−6.2 ± 1.5	18.7
Acetic acid	0	6.2
Ethyl acetate	−10.0 ± 1.0	6.0
Propionic acid	−14.7 ± 1.0[c]	3.15[b]
Dioxane	+6.5 ± 1.0	2.28

[a] 30.5°. [b] Measured at λ 73 cm.; 17°. [c] The rotational values, especially in propionic acid and methanol, are difficult to reproduce and seem to depend on the history (evaporation, lyophilization, drying) of the gramicidin sample.

Table VI. Molecular Weight of Gramicidin A Determined by Osmometry

Solvent	Found
Dimethylformamide	1970
Ethanol	2014
Methyl ethyl ketone	4190
Dioxane	4406
Ethyl acetate	4426
Chloroform	2460[a]

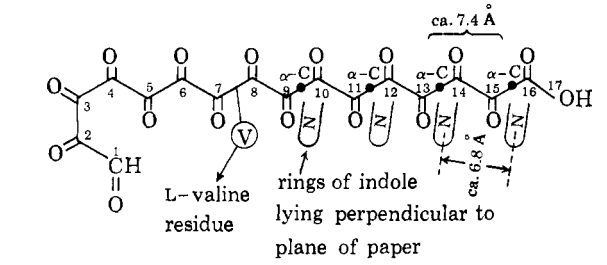
[a] A saturated solution of gramicidin A in pure chloroform contains only 0.1%. The molecular weight was determined in commercial chloroform containing 0.52% ethanol.

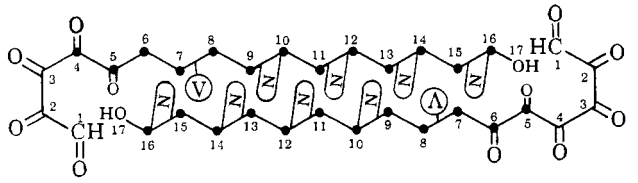
The rotatory dispersion of gramicidin A in chloroethanol or methanol follows a linear Drude plot ($1/[\alpha]_\lambda$ *vs.* λ^2), explicable in terms of a simple summation of the rotations of the individual residues. In chloroform, however, there is a negative Cotton effect which indicates the introduction of a separate rotational factor.[39]

The hydroxyl function of gramicidin is masked in the infrared spectrum. Although it is possible to build a cyclic structure of gramicidin A (Stuart–Briegleb models)[40] in which the hydroxyl adds to the N-terminal formyl, it is more likely that hydrogen bonding between molecules is involved. The amide I band of gramicidin in chloroethanol at 1650 cm.$^{-1}$ is typical of random polypeptides. This band shifts to a sharp peak at 1633 cm.$^{-1}$ in chloroform, indicative of a hydrogen-bonded parallel β-type arrangement.[39,41]

(36) K. Marcker and F. Sanger, *J. Mol. Biol.*, **8**, 835 (1964).
(37) M. Neeman, M. C. Caserio, J. D. Roberts, and W. S. Johnson, *Tetrahedron*, **6**, 36 (1959).
(38) R. Kuhn and H. Trischmann, *Ber.*, **96**, 284 (1963).
(39) S. Ishii, unpublished results.
(40) We are indebted to Dr. Kenneth D. Kopple, General Electric Research Laboratory, Schenectady, N. Y., for this information.
(41) *Cf.* John A. Schellman and C. G. Schellman, *J. Polymer Sci.*, **49**, 129 (1961).

If one assumes *cis*-peptide bonds for the first three peptide linkages, CHO-L-Val-Gly-L-Ala, all subsequent peptide bonds become *trans*. If one builds a model of such a peptide sequence (Catalin models) the following arrangements and spacings of the C=O projections (numbered consecutively 1–16, with 17 representing the terminal hydroxyl of the bound aminoethanol) are possible for monomer and dimer[42]





Preliminary observations on monomolecular layers of gramicidin A in ethyl acetate–benzene (1:1 and 1:2) point to the existence of a compressible film of flat molecules.[43]

The partial specific volume of gramicidin A in dimethylformamide is 0.8205. This value is higher than the usual value for polypeptides and proteins in aqueous solvents. On the one hand this may point to a random structure in this solvent; on the other hand the electrostriction effect would not be expected to be operative in dimethylformamide. The molecular weight in dimethylformamide by the sedimentation equilibrium technique[44] gave an extrapolated value of 2051.[45]

Another interesting aspect probably associated with the secondary structure of gramicidin A is its ability to facilitate the transport of monovalent cations across mitochondrial membranes.[46] Such an effect has previously been observed for the cyclic depsipeptide valinomycin[47] in which two D-residues alternate with two L-residues.

Experimental

The solvent systems for thin layer chromatography are abbreviated as follows: A: AcOH–$CHCl_3$ (2:1); C: butanone–2-pyridine (7:3). The R_f values in these solvent systems are difficult to reproduce and should only be considered as relative guide lines. The R_f values for the derivatives of gramicidin (Table IV) were determined with gramicidin A as reference. In solvent system C the R_f value refers to the strongest and fastest moving spot.

Gramicidin A. The gramicidin A was a mixture of valine- and isoleucine-gramicidin A, obtained by countercurrent distribution of commercial gramicidin.[8] Commercial gramicidin was obtained from Nutritional Biochemical Corp. and from S. B. Penick and Company.

Determination of Molecular Weight of Gramicidin A by Ultracentrifugation.[45] The molecular weight of gramicidin A, dissolved in N,N-dimethylformamide, was determined in a Spinco Model E analytical ultracentrifuge equipped with a Rayleigh interference optical system. The sedimentation equilibrium technique as described by Richards and Schachman[44] was utilized. The experiments were performed at a temperature of 25° and a speed of 35,600 r.p.m., using the An-D rotor and a 12-mm. double sector cell. With an average column length of 1.4 mm., equilibrium was attained within 150 min. This was of importance as prolonged exposure to N,N-dimethylformamide has a deleterious effect on the aluminum-filled epon centerpiece used here. The initial concentration expressed by the number of fringes for the 1% solution was experimentally determined by a synthetic boundary run in the ultracentrifuge. The initial concentrations for the more dilute solutions were calculated from these data. The photographic plates were measured with a Nikon Model 6 comparator and ln c *vs.* r^2 was plotted. The densities of the N,N-dimethylformamide and of the gramicidin A in this solvent were measured pycnometrically. The pycnometer was weighed on a Mettler semimicro balance and appropriate corrections for air bouyancy were applied. The value of the partial specific volume, $\bar{v}$, was calculated by the formula $\bar{v} = (1/\rho_0) - [(1/c)(\rho - \rho_0)]/\rho_0$ where ρ_0 = density of solvent, ρ = density of solution, and c = concentration in grams per 100 cc.

The plots of ln c *vs.* r^2 were linear, indicating that the material was homogeneous and approaching ideal behavior. The values of (d ln c)/dr^2 were calculated from the graphs, and the apparent molecular weights were calculated using the formula $M_{app} = 2RT[(\text{d ln } c)/\text{d}r^2]/(1 - \bar{v}_\rho)\omega^2$, where R = gas constant, T = absolute temperature, c = concentration in fringe numbers, r = radius from the center of rotation, and ω = angular velocity in radians/sec. The reciprocals of the apparent molecular weights were plotted as a function of concentration. In this way an excellent fit to the linear equation $1/M_{app} = 1/M + 2A_2c$ is obtained and the concentration dependence is small. An extrapolated value of 2051 was found for the molecular weight. The second virial coefficient, A_2, was 3.21×10^{-5}. The partial specific volume was found to be 0.8205. The fact that this is higher than the usual values for proteins and polypeptides in aqueous solvents is not surprising as the electrostriction effect would not be operative in a solvent such as N,N-dimethylformamide.

Determination of the N-Formyl Group. A. Chromotropic Acid Assay. Samples of 25–30 mg. of gramicidin A were transferred to a 3-ml. hydrolysis tube, 1 ml. of concentrated sulfuric acid and 1 ml. of water were added at −80°, and the tube was evacuated at −80°, sealed, and heated at 110° for 2 hr. with oc-

(42) Dr. Donald T. Warner of the Upjohn Co., Kalamazoo, Mich., had the kindness to construct models of gramicidin A and to suggest the above possibilities for the monomer and dimer. We would like to extend our thanks for this assistance and collaboration.

(43) We are indebted to Dr. Harry Sobotka, Mt. Sinai Hospital, New York, N. Y., for this information.

(44) E. G. Richards and H. K. Schachman, *J. Phys. Chem.*, **63**, 1578 (1959).

(45) We are thankful to Dr. Marc S. Lewis for this determination.

(46) A. R. Crofts and J. B. Chappell, personal communication.

(47) C. Moore and B. C. Pressman, *Biochem. Biophys. Res. Commun.*, **15**, 562 (1964).

casional shaking. There was a clear solution after 30 min. at 110°. The hydrolysate was cooled and, after addition of 1 ml. of water, distilled into a receiver (cooled to −80°) at 10^{-2} mm. and room temperature. After 2 hr. the distillate was collected and diluted with water to 3 ml. For the chromotropic acid test[13] a 0.5-ml. aliquot of this solution was cooled to 0° and the formic acid reduced to formaldehyde by the addition of 2 drops of concentrated hydrochloric acid and a sliver of magnesium metal (15 mg.). After 15 min. at 0° one more drop of concentrated hydrochloric acid was added and after 30 min. 1.5 ml. of chromotropic acid reagent, prepared by dissolving 600 mg. of chromotropic acid in 180 ml. of concentrated sulfuric acid and 20 ml. of water. The mixture was heated to 110° for 45 min. whereupon a deep purple color developed whose extinction at 575 mμ was measured against a blank sample. Although the reduction of formic acid by this method is not quantitative, comparison with standard solutions of formic acid indicated a release of 0.6–0.8 mole of formic acid per 1850 g. of gramicidin A. The chromotropic acid test on the distillate before reduction was negative; thus no formaldehyde is present in gramicidin A hydrolysates. In control experiments desformylgramicidin A or tryptophan did not release a trace of formic acid under these hydrolytic conditions.

B. Assay by Gas Chromatography. For the gas chromatographic analysis 1-ml. aliquots of the distillate were made alkaline by the addition of 0.2 ml. of 0.1 *N* sodium hydroxide evaporated to dryness, and the residue was dissolved in 0.1 ml. of 1.0 *N* phosphoric acid. Aliquots of this solution (10 μl.) were injected in a 6-ft. column, $^1/_8$-in. diameter, filled with 20% Tween-80–2% phosphoric acid on Chromosorb W 60/80 mesh. For the detection of formic acid an argon detector was used (180°, 2000 v.). The column temperature was 118° with 20 p.s.i. argon pressure at the column inlet. Under these conditions the formic acid peak appeared after 7 min., the acetic acid peak after 5.5 min. With standard solutions of formic acid the amount of formic acid and the peak area were found to be proportional. A release of 0.8–1.0 mole of formic acid per 1850 g. of gramicidin A was calculated. No other acid was found except acetic acid in a sample of gramicidin A, which had been freeze dried from acetic acid solution and contained 7% of this solvent.

Desformylgramicidin A. To a solution of 500 mg. of gramicidin A (containing 7% acetic acid) in 40 ml. of absolute methanol was added 10 ml. of 7.5 *N* hydrochloric acid in abstract methanol, and the mixture was allowed to stand at room temperature for 1 hr. After evaporation, the residue (482 mg.) was dissolved in 20 ml. of methanol and applied to a column (cooling jacket) filled with 40 ml. of Dowex 50W X2 resin (H^+ form) equilibrated with methanol. From this column 200 ml. of methanol eluted 30 mg. (6%) of unreacted gramicidin A, which was converted to desformylgramicidin A by repetition of the methanolysis. A solution of 400 ml. of 2.0 *N* ammonia in methanol (80 ml. of concentrated ammonia and 400 ml. of methanol) eluted 418 mg. (90%) of desformylgramicidin A, which after precipitation from methanol–water had m.p. 195–205°, and on chromatography on a Sephadex 625 column (4.3 × 110 cm.) gave a single peak. For the determination of free amino groups the modified[15] method of Satake,[38] trinitrophenylation and measurement of the optical density at 340 mμ, was used.

Reformylation of Desformylgramicidin A.[16] To a solution of 40 mg. of desformylgramicidin A [R_f 0.22 (A), 0.10 (C); $[\alpha]^{20}D$ +5.4 ± 1.0° (*c* = 1, propionic acid)] in 1 ml. of 98% formic acid was added 0.3 ml. of acetic anhydride at 0°. After 30 min. the ice bath was removed and the mixture allowed to stand at room temperature for 4 hr. and then evaporated. The reaction product [R_f 0.59 (A); 0.72 (C)], which on thin layer chromatography behaves like O-acetylated gramicidin A [R_f 0.59 (A); 0.71 (C)], was dissolved in 3 ml. of methanol and treated with 0.2 ml. of 1.0 *N* sodium hydroxide at room temperature in order to remove the O-formyl group. After 1 hr. the mixture was poured onto a column containing 3 ml. of Dowex 50 W X2 (H^+ form, methanol), from which methanol (30 ml.) eluted 32 mg. (80%) of a ninhydrin-negative product [R_f 0.52 (A), 0.65 (C); $[\alpha]^{20}D$ −3.6 ± 2.0° (*c* 0.55, propionic acid); *cf.* Table V], whose thin layer chromatograms were identical with those of gramicidin A [R_f 0.52 (A), 0.65 (C)].

Multiple Edman Degradation.[18–20] Pyridine and triethylamine were refluxed for 3 hr. over phthalic anhydride and then distilled; phenyl isothiocyanate was distilled *in vacuo*. The reagent, 100 ml. of pyridine, 3 ml. of triethylamine, and 1 ml. of phenyl isothiocyanate, was stored at −18°. A 100-mg. sample of desformylgramicidin A was dissolved in 20 ml. of this reagent and warmed for 4 hr. on a water bath at 40°. The solvents were then removed by evaporation, finally at 40° for 2 hr. *in vacuo* (10^{-2} mm). The usual procedure of washing with benzene had to be omitted because the reaction product was too lipophilic. For the cyclization the N-phenylthiocarbamyl peptide was dissolved in 5 ml. of anhydrous trifluoroacetic acid (distilled over phosphoric acid and sulfuric acid) and allowed to stand at room temperature for 1 hr. After evaporation the residue was dissolved in methanol and poured onto a water-cooled column filled with 30 ml. of Dowex 50W X2 resin (H^+ form, methanol). The column was washed with 100 ml. of methanol in order to elute the PTH-amino acid (and the neutral by-products) and subsequently with 200 ml. of 2.0 *N* methanolic ammonia which eluted the degraded peptide. It was difficult to remove the degraded peptide quantitatively from the ion-exchange resin. A second fraction was obtained by elution with piperidine–water–methanol (1:2:7, v./v.). Furthermore, there is some loss of peptide probably due to trifluoroacetylation during the cyclization step, since in the neutral fraction there was always some Ehrlich-positive material remaining on the start on thin layer chromatography in chloroform–formic acid (20:1). The next degradation step was performed in the same manner.

Aliquots of the degraded peptides (1–2 mg.) were hydrolyzed in an evacuated, sealed tube at 110° for 49 hr. in a mixture of 0.3 ml. of acetic acid and 1.5 ml. of 6.0 *N* hydrochloric acid. The hydrolysate was analyzed on a Phoenix automatic amino acid analyzer. After hydrolysis for 49 hr. the full value for valine (3.8 moles per 4 moles of leucine) is not reached under these

Table VII. Amino Acid Composition of Desformylgramicidin A (DGA) and of the Peptides Remaining after the Removal of the NH_2-Terminal Amino Acids in the Course of the Edman Degradation[a]

	Gly	Ala	Val	Ileu	Leu	Try	NH_3	2-Amino-ethanol	Hydrolysis time, hr.
DGA	0.90	2.00	2.80	0.18	4.00	3.18	0.44	0.97	24
DGA	0.93	2.07	2.80	0.18	4.00	3.86	2.88	0.88	24[b]
DGA	0.90	1.88	3.32	0.19	3.98	3.20	1.26	0.88	49
DGA	0.98	1.96	3.58	0.17	4.00	1.05	9.08	0.82	72
After the									
1st step	0.90	2.00	2.65	0.00	4.00	1.79	0.85	0.82	49
2nd	0.12	1.90	2.70		4.00	1.12	0.62	0.80	49
3rd	0.07	1.13	2.76		4.00	0.47	0.59	0.78	49
4th	0.00	1.00	2.68		3.10	1.09	0.56	0.79	49
5th		0.10	2.70		3.00	..	2.69	0.97	49
6th		0.09	1.93		3.00	0.53	1.58	0.73	49
7th		0.05	1.24		3.00	..	2.14	0.77	49
8th			0.39		3.00		3.64	0.77	49
9th			0.17		3.00		5.30	0.88	49

[a] The hydrolysis was carried out at 110° in a mixture of 0.3 ml. of acetic acid and 1.5 ml. of 6.0 *N* hydrochloric acid. [b] Without AcOH.

conditions. The following valine values are obtained as a function of time of hydrolysis: 2.80 moles (24 hr.); 3.38 moles (49 hr.); and 3.58 moles of valine (72 hr.). The results are given in Table VII.

The neutral fractions were dissolved in 0.1 ml. of dimethoxyethane and aliquots were chromatographed on silica gel thin layer plates[24] in the system chloroform–formic acid (20:1, v./v.). Under ultraviolet light the PTH-amino acids were visible as dark spots after spraying with fluoresceine (1% solution in methanol). After the ninth degradation step PTH-tryptophan was also detected by its positive reaction with Ehrlich reagent (a solution of 1 g. of *p*-dimethylaminobenzaldehyde in a mixture of 50 ml. of concentrated hydrochloric acid and 250 ml. of ethanol). For the gas chromatographic analysis[25] 1–5-μl. samples were injected into a 6-ft. column, 1/6-in. diameter, filled with 1% SE-30 on Gaschrom P, 100-120 mesh. A flame ionization detector was used. At a column temperature of 216° with 19 ml. of nitrogen per minute as carrier gas, PTH-alanine, valine, leucine, and isoleucine were eluted as sharp peaks (after 2.5, 3.1, 4.1, and 4.1 min., respectively), whereas PTH-glycine gave a broad tailing peak (with a maximum at 3 min.). PTH-tryptophan is not eluted under these conditions.

Attempted Degradation of Desformylgramicidin A via 2-Nitro-4-methanesulfonylphenyl Derivative. To a solution of 56 mg. of desformylgramicidin A in 15 ml. of dimethoxyethane containing 5 ml. of water was added 10 μl. of triethylamine and 110 mg. of 2-nitro-4-methanesulfonylfluorobenzene.[48] The solution was stirred overnight at room temperature and then evaporated. The residue was dissolved in ethyl acetate and washed with 1.0 *N* hydrochloric acid and 5% aqueous bicarbonate and water, dried, and evaporated. The residue was dissolved in chloroform and poured onto a 30-ml. silicic acid column which was eluted with 100 ml. of chloroform. The yellow zone that remained on top of the column was separated mechanically and eluted with methanol. The methanol extract was filtered through a Dowex 50W X2 (H^+, methanol) column and the filtrate evaporated to give 56 mg. of solid 2-nitro-4-methanesulfonylphenyl- (NMSP) desformylgramicidin A. A 1-mg. sample of this material was hydrolyzed in 0.3 ml. of acetic acid and 1.5 ml. of 6.0 *N* hydrochloric acid at 110° for 24 hr. The hydrolysate was evaporated and the residue was dissolved in water and extracted with ether. Preparative thin layer chromatography in butanone-2–benzene–pyridine–acetic acid (120:80:30:3, v./v.) showed the ether layer to contain NMSP-valine and NMSP-isoleucine in a ratio of 0.83:0.17, based on the optical density at 405 mμ. For the hydrogenation 55 mg. of NMSP-desformylgramicidin A was dissolved in 25 ml. of methanol and 0.5 ml. of triethylamine, and 32 mg. of 5% platinum on aluminum (Englehard Ind., Inc.) was added. After 20 min. the solution was colorless, but developed a brownish color on contact with air when the catalyst was filtered off. On repeated hydrogenation this color disappeared again. Acetic acid (1 ml.) was added and the solution was filtered and kept at pH 5 for 23 hr. at 40°. The solution was evaporated and the residue dissolved in methanol and applied to a Dowex 50W X2 (H^+, methanol) column. Elution with methanol gave a neutral fraction in which no 2-hydroxy-3-isopropyl-7-methanesulfonyldihydroquinoxaline (MSQ-valine) could be identified by thin layer chromatography. A basic fraction (40 mg.) was obtained by eluting the column with 2.0 *N* methanolic ammonia. This fraction was treated as above with 2-nitro-4-methanesulfonylfluorobenzene, but no yellow NMSP-peptide was obtained after analogous purification steps.

Oxidative Cleavages with N-Bromosuccinimide. The NBS oxidation of gramicidin A and its derivatives was carried out on 10-mg. samples (5–6 μmoles) dissolved in 4 ml. of ethanol–water (6:4) after the addition of 2 drops of 0.10 *N* sulfuric acid (pH 3–4) with the calculated amount of NBS (8 moles per mole of gramicidin A for complete oxidation). After standing at room temperature for 1 hr. aliquots of this solution (0.15 ml.) were diluted with ethanol to 4 ml. to determine the decrease in optical density at 282 mμ, whereby the established correction factor[27] of 1.31 was used for the absorption of the oxidized tryptophan. For the amino group determination 0.2-ml. aliquots were incubated with 0.5 ml. of an aqueous 0.2% trinitrobenzenesulfonic acid solution together with 0.5

(48) H. Zahn and H. Zuber, *Ber.*, **86**, 172 (1953).

ml. of sodium phosphate buffer (pH 7.66) and 1 ml. of 2-methoxyethanol at 40° for 3 hr. After acidification with 2 ml. of 1.0 *N* hydrochloric acid in 2-methoxyethanol the optical density at 340 mμ was measured against a blank sample. The value 10.5 × 10^3 for the molar extinction of the TNP-peptides was used for the calculation.

For the determination of aminoethanol and its derivatives by gas chromatography 0.2-ml. aliquots were mixed with 0.25 ml. of an aqueous solution containing 50 μg. of 1-amino-2-hydroxypropane as an internal standard and two drops of 2.0 *N* hydrochloric acid in order to form nonvolatile salts. The mixture was evaporated and dried over phosphorus pentoxide. To the solution of the residue in 0.5 ml. of dimethoxyethane was added 10 μl. of trifluoroacetic anhydride. After standing at room temperature for 5 min. 5–10 μl. aliquots of this reaction mixture were injected into a gas chromatographic column filled with 4% neopentyl glycol succinate on Chromosorb W, 60/80 mesh (6-ft., $^1/_8$-in. diameter). The gas chromatograph was equipped with a flame ionization detector. Nitrogen served as the carrier gas (15–20 ml./min.). At column temperatures of 120–160° the trifluoroacetylated aminoalcohols are eluted as sharp peaks (Table II).

N-Trifluoroacetyl-2-methoxyethylamine is not well separated from the solvent peak. However, 2-methoxyethylamine can be detected easily on the 50-cm. column of the amino acid analyzer from which it is eluted after the ammonia peak.

For paper electrophoresis a high voltage apparatus with 40 v./cm. on Schleicher + Schuell paper No. 589 was used (Table III). The results are summarized in Table IV.

O-Succinylethanolamine. For the preparation of 2-aminoethanol succinate 390 mg. of N-carbobenzyloxyethanolamine[49] was treated with 1 g. of succinic anhydride in 10 ml. of pyridine at 40° for 21 hr. To the solution was added 30 ml. of ice–water, the mixture was filtered, and the filtrate was adjusted to pH 3 with 2.0 *N* sulfuric acid. This solution was extracted twice with 150 ml. of benzene, and the benzene extract was washed with water, dried over magnesium sulfate, and evaporated. The residue was crystallized from ethyl acetate–petroleum ether to give 431 mg. (73%) of N-carbobenzyloxy-2-aminoethanol succinate, m.p. 72–73°. An analytical sample had m.p. 73–74°.

Anal. Calcd. for $C_{14}H_{17}NO_6$: C, 56.94; H, 5.80; N, 4.74. Found: C, 57.00; H, 5.90; N, 4.78.

Hydrogenation of 200 mg. of N-carbobenzyloxy-2-aminoethanol succinate in methanol with palladium black catalyst gave 108 mg. (98%) of 2-aminoethanol succinate in wax-like needles.

O-Formylethanolamine. 2-Aminoethanol formate was prepared in analogy to 2-aminoethanol acetate.[50] A solution of 12.2 g. of aminoethanol in 50 ml. of formic acid was saturated at 0° with hydrogen chloride gas. The mixture was kept at room temperature overnight and then evaporated. The residue was crystallized from ethanol–acetone to give 17 g. (68%) of the hygroscopic hydrochloride, m.p. 83–84°.

(49) W. G. Rose, *J. Am. Chem. Soc.*, **69**, 1384 (1947).
(50) C. Crane and H. M. Rydon, *J. Chem. Soc.*, 527 (1947).

Anal. Calcd. for $C_3H_7NO_2 \cdot HCl$: C, 28.68; H, 6.42; N, 11.21. Found: C, 28.97; H, 6.44; N, 11.17.

Ozonization of Gramicidin A.[23] Through a solution of 160 mg. of gramicidin A in 50 ml. of 98% formic acid was bubbled a stream of oxygen, containing *ca.* 2% of ozone. The solution developed a dark red color which changed to light yellow after 30 min., at which time an aliquot of the solution showed the complete disappearance of the tryptophan ultraviolet spectrum. The solution was evaporated and the residue was treated with 1.5 *N* hydrochloric acid in methanol at room temperature for 1 hr. to deformylate the N-formylkynurenine residues and the N-terminal amino acid. The reaction mixture was evaporated, dissolved in methanol, and applied to a column of a Dowex 50W X2 (H^+, methanol), which was washed with 60 ml. of methanol and eluted with 100 ml. of 2.0 *N* methanolic ammonia. This basic fraction gave after evaporation 102 mg. of a solid product, aliquots of which were hydrolyzed with 6.0 *N* hydrochloric acid at 110° for 22 and 72 hr. On the amino acid analyzer 1.39 and 1.32 moles of kynurenine, respectively, were found per 4 moles of leucine. The retention time of kynurenine on the 150-cm. column was twice that of valine.

Ozonization of 100 mg. of gramicidin A at 0° in the presence of 100 mg. of resorcinol[23] and treatment as above gave a product containing 1.67 moles of kynurenine after hydrolysis for 22 hr.

Release and Characterization of Peptide Fragments. A sample of 14.7 mg. of gramicidin A was oxidized by 13.3 mg. of NBS in 1.5 ml. of 60% acetic acid containing two drops of 0.1 *N* sulfuric acid. After 20 min. at room temperature the mixture was poured onto a column, 1.5 × 140 cm., filled with Sephadex (G 25, fine) and developed with 50% acetic acid. The ultraviolet absorbance of the eluted fractions (5 ml.) was recorded automatically. At least seven peaks were eluted in fractions 20–40. When 4.8 mg. of L-leucyl-L-tryptophan (Mann) was similarly oxidized with 7 mg. of NBS and chromatographed a major peak appeared in fractions 34–40. Oxidation of 2.7 mg. of L-tryptophan (NBC) with 6 mg. of NBS gave two peaks in fractions 46–49 and 50–54. NBS oxidation of 8.1 mg. of Cbz-L-tryptophyl-L-tryptophan (Cyclo) with 13.8 mg. of NBS gave peaks in fractions 41–45 and 46–54. Among the fragments released by oxidative cleavage of gramicidin A with NBS, therefore, 5-bromospirooxindole lactone resulting from the sequence Try-Try may be excluded, whereas the presence of the dipeptide resulting from oxidative cleavage of the sequence L-Try D-Leu-L-Try is very likely.

O-Methylgramicidin A. Methylation with Methyl Iodide and Silver Oxide. To a solution of 85 mg. of gramicidin A in 6 ml. of dioxane containing 1 ml. of methyl iodide[28] was added 400 mg. of freshly prepared silver oxide and the mixture was kept at 60° for 15 min. After filtration and evaporation of the filtrate the residue was precipitated from ethanol with water to give 41 mg. (48%) of a colorless product whose methoxyl content was 0.90%, *i.e.* 0.54 of the theoretical 1.0 equiv.

Methylation with the Diazomethane–Boron Trifluoride Complex. To a solution of 180 mg. of gramicidin A

in 20 ml. of methylene chloride and 3 ml. of dimethoxyethane was added 0.05 ml. of 20.0 *N* fluoroboric acid.[16] The mixture was cooled to 0° and 40 ml. of diazomethane in methylene chloride was added. The reaction mixture was stirred for 20 min., filtered, washed with 5% bicarbonate solution and water, dried over sodium sulfate, and evaporated. The residue was precipitated from methanol with water to give 77 mg. (43%) of a colorless product containing 1.17% (0.7 equiv.) methoxyl.

Methylation with Dimethyl Sulfate in Dimethyl Sulfoxide. To 300 mg. of gramicidin dissolved in 10 ml. of dimethyl sulfoxide and 10 ml. of dimethylformamide was added 1.5 g. of baryta and then dropwise 2 ml. of dimethyl sulfate.[37] The mixture was stirred at room temperature for 5 hr. and then 2 ml. of concentrated ammonia added, followed by agitation for another 30 min. After the addition of 200 ml. of chloroform and 100 ml. of water the lower layer was washed five times with 100 ml. of water, dried over magnesium sulfate, and evaporated. Precipitation from methanol with water gave 277 mg. (92%) of a colorless product with a methoxyl content of 1.49% (0.92 equiv.). This product [R_f 0.44 (A), 0.92 (C), only 1 spot; gramicidin A 0.42 (A), 0.65 (C)] which was free of unmethylated gramicidin according to t.l.c. was used for all further experiments. Hydrolysis of this O-methylgramicidin with 0.3 ml. of acetic acid containing 1.5 ml. of 6.0 *N* hydrochloric acid at 110° for 24 hr. gave only ethanolamine and its O-acetyl derivative as assayed by paper electrophoresis and gas chromatography after trifluoroacetylation. Only a trace of 2-methoxyethylamine was found on the amino acid analyzer (50-cm. column).

O-Acetylgramicidin A. A solution of 150 mg. of gramicidin A in 3 ml. of pyridine and 0.75 ml. of acetic anhydride was kept at 40° for 18 hr. Precipitation with water at 0°, followed by centrifugation and reprecipitation from ethanol–water (containing some sodium chloride to destroy the colloidal solution), gave 139 mg. (91%) of a colorless product with an acetyl content of 2.34% (1.05 equiv.) [R_f 0.57 (A), 0.78 (C); gramacidin A 0.50 (A), 0.67 (C)]. On hydrolysis with 0.1 *N* alkali at pH 12 (pH-Stat) in ethanol–water for 3 hr., O-acetylgramicidin is reconverted to gramicidin (t.l.c.).

O-n-Butylgramicidin A. A solution of 82 mg. of gramicidin A in 6 ml. of dioxane was alkylated with 1.5 ml. of *n*-butyl iodide and 400 mg. of freshly prepared silver oxide at 60° for 2 hr. The mixture was filtered and evaporated, and the residue was precipitated from ethanol with water to give 86 mg. (100%) of a colorless product [R_f 0.84 (C), only 1 spot; gramicidin A 0.64 (C)] with a butoxyl content of 7.11% (1.85 equiv.).

Tosylation of Gramicidin. Treatment of 304 mg. of gramicidin in 5 ml. of pyridine with 320 mg. of tosyl chloride at room temperature for 22 hr. after precipitation with water at 0°, centrifugation, and reprecipitation from ethanol–ether and ethanol–water gave 220 mg. of a slightly colored product. On t.l.c. in C it showed a strong spot with R_f 0.86 (gramicidin A 0.66) trailing from the starting point.

Mesylation of Gramicidin. To a solution of 300 mg. of gramicidin in 5 ml. of pyridine was added 0.125 ml. of methanesulfonyl chloride and the mixture kept at room temperature for 0.75 hr. Precipitation with water at 0°, centrifugation, and reprecipitation from ethanol with water gave 300 mg. of a colorless product which showed a t.l.c. similar to that of the tosylated product.

O-Tritylgramicidin. A solution of 100 mg. of gramicidin in 10 ml. of pyridine was treated with 700 mg. of trityl chloride and kept at 40° for 41 hr. After precipitation with water at 0° and centrifugation the precipitate was dried and then thoroughly washed with ether. The ether-insoluble part was collected to give 40 mg. of product [R_f 0.84 (C); gramicidin A 0.51 (C)], which according to t.l.c. was free of gramicidin. O-Tritylgramicidin gives a green color with Ehrlich reagent and after heating with 60% acetic acid for 5 min. to 100° regenerates triphenylcarbinol and gramicidin (t.l.c.).

N,O-Diacetyldesformylgramicidin. A solution of 200 mg. of desformylgramicidin in 3 ml. of pyridine and 1 ml. of acetic anhydride was kept at 40° for 7 hr. The reaction mixture was evaporated and the residue was dissolved in methanol and filtered over a column of Dowex 50W X2 (H^+ form, methanol). The filtrate was evaporated and the residue precipitated from methanol with water to give 182 mg. (88%) of ninhydrin-negative N,O-diacetyldesformylgramicidin [R_f 0.57 (A); gramicidin A 0.40 (A)].

N-Acetyldesformylgramicidin A ("*Homogramicidin A*"). When 90 mg. of the N,O-diacetyl product was dissolved in methanol and saponified for 9 hr. at room temperature with 1 ml. of 2.0 *N* alkali, filtered over a column of Dowex 50W X2 (H^+, methanol), and precipitated from methanol with water there was obtained 77 mg. (87%) of N-acetyldesformylgramicidin [R_f 0.38 (A); gramicidin A 0.40 (A)]. In contrast to gramicidin A this compound is not changed on treatment with 1.5 *N* hydrochloric acid in methanol at room temperature for 1 hr.

N,O-Disuccinylgramicidin A. A solution of 108 mg. of desformylgramicidin in 3 ml. of pyridine was allowed to react with 1 g. of succinic anhydride at 40° for 14 hr. The mixture was evaporated, dissolved in methanol, and filtered over a column of Dowex 50W X2. Evaporation of the filtrate and precipitation of the residue from methanol with water gave 102 mg. (85%) of N,O-disuccinyldesformylgramicidin A [R_f 0.55 (A); gramicidin A 0.41 (A)].

N,O-Di(benzoyl-2-sulfonic acid)gramicidin A. A solution of 117 mg. of desformylgramicidin in 3 ml. of pyridine was treated with 1 g. of freshly distilled *o*-sulfobenzoic anhydride (recrystallized from benzene) at 40° for 11 hr. The mixture was filtered, the filtrate evaporated, and the residue dissolved in 2 ml. of 50% acetic acid and chromatographed on a column of Sephadex (G 25, fine, 1.5 × 60 cm.) in 50% acetic acid. The substance eluted in the first peak (optical density at 280 mμ) was collected, dried, dissolved in methanol, and filtered through a column of Dowex 50W X2 (H^+, methanol). After evaporation of the filtrate and precipitation of the residue from methanol with ether there was obtained 128 mg. (91%) of N,O-

dibenzoyl(*o*-sulfonic acid)desformylgramicidin [R_f 0.41 (A); desformylgramicidin A 0.12 (A); gramicidin A 0.41 (A)]. This product, when dissolved in a few drops of ethanol, is not precipitated by a large excess of water; such a solution is not degraded by pronase.[51]

(51) We are indebted to Dr. N. M. Green for this test.

Gramicidin A. VI. The Synthesis of Valine- and Isoleucine-gramicidin A

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Coupling of the octapeptide derivative Z-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-N_3 with the heptapeptide ethanolamide L-(H)Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-$NHCH_2CH_2OH$ or, more conveniently, of the pentapeptide Z-L-Val(or L-Ileu)-Gly-L-Ala-D-Leu-L-Ala-OH with the decapeptide ethanolamide D-(H)Val-L-Val-D-Val-L-Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-$NHCH_2CH_2OH$ gave the decapentapeptide ethanolamide R-L-Val(or L-Ileu)-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-$NHCH_2CH_2OH$, having all the properties of N-carbobenzyloxydesformyl (R = Z), desformyl- (R = H), and (R = CHO) valine- or isoleucine-gramicidin.

The structures HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-$NHCH_2CH_2OH$ and HCO-L-Ileu-Gly-. . . were proposed for valine- and isoleucine-gramicidin A.[2] This paper describes the synthesis of these two formyl decapentapeptide ethanolamides and the comparison of their properties with those of the natural antibiotics.

No particular difficulties were expected in the synthesis of these linear peptides since the building stones are neutral amino acids. However, the acid sensitivity of tryptophan put certain limitations on the procedures to be used. The decapentapeptide was to be constructed from two moieties since the over-all yield in a stepwise synthesis of the entire decapentapeptide would be expected to fall off rapidly. There is always the danger of racemization in joining two peptides[3] except when the azide method is employed. Therefore, the peptide moiety containing COOH-terminal glycine would be convenient for such a connection and would remove the issue of racemization. Unfortunately, this approach is not feasible because glycine happens to be the second amino acid in the sequence of gramicidin A.

We planned on joining the octapeptide derivative Z-(or For-)L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-R (I) and the heptapeptide ethanolamide L-(H)Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-AE (II) (Z = carbobenzyloxy, For = HCO, AE = $NHCH_2CH_2OH$) either by the azide method or by coupling with dicyclohexylcarbodiimide in tetrahydrofuran at low temperature in the absence of salts.[4] When the formyl peptides were found to have undesirable solubility properties the carbobenzyloxy group was used throughout this work as the protecting group of choice. If successful, the coupling of I and II should lead to a carbobenzyloxy decapentapeptide ethanolamide (III), identical with N-carbobenzyloxydesformylgramicidin A. Removal of the blocking group should then yield desformylgramicidin A whose reformylation to gramicidin A has been described previously.[2]

The heptapeptide II was prepared by stepwise synthesis from the carboxyl end by the mixed anhydride method, which is fast, convenient, and, when used properly, minimizes the danger of racemization.[5] The hydroxyl group of ethanolamine was not protected. The octapeptide derivative Z-L-Val-. . . .D-Val-OMe (Ia) was prepared in an analogous manner. Unfortunately saponification of this ester to the required fragment I was impossible. The ester was always recovered unchanged after treatment with base. The ester Ia was therefore converted to the hydrazide and coupled, *via* the azide, with the heptapeptide ethanolamide II to a product with the properties of N-carbobenzyloxydesformylgramicidin A where a new difficulty arose: all attempts to remove the protecting group by hydrogenolysis in acetic acid over palladium on charcoal failed, and it was not possible to isolate any tryptophan-containing peptide with the small amount of material available. The same was true for authentic N-carbobenzyloxydesformylgramicidin A. It was found subsequently that hydrogenolysis over palladium black proceeds very smoothly to give desformylgramicidin A when methanol is used as the solvent.

A second more convenient synthetic approach consisted in coupling the pentapeptide derivative Z-L-Val-Gly-L-Ala-D-Leu-L-Ala-OH (IV) with the decapeptide ethanolamide D-(H)Val-L-Val-D-Val-L-Try-D-Leu-L-Try-D-Leu-L-Try-D-Leu-L-Try-AE (V, Chart I). The pentapeptide IV, synthesized by stepwise coupling from the carboxyl end, was obtained as the methyl ester, which was saponified without difficulty. The decapeptide V was prepared by stepwise prolongation of II. With dicyclohexylcarbodiimide, IV and V were

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