leucine isomers, valine, methionine, proline, alanine and tyrosine in the hydrolysate of a single sample of protein.

4. Determinations of these constituents of wool and cow-hide gelatin are reported.

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The Amino-Acid Composition of Gramicidin

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The bactericidal substance gramicidin, isolated by Dubos *et al.* from cultures of various bacteria [for bibliography cf. Dubos & Hotchkiss, 1942] is of polypeptide character, yielding on acid hydrolysis a mixture of amino-acids, some of which possess the 'unnatural' *d*-configuration [Hotchkiss, 1941; Lipmann, Hotchkiss & Dubos, 1941; Christensen, Edwards & Piersma, 1941].

We have subjected acid hydrolysates of gramicidin to analysis by our new techniques [Gordon, Martin & Synge, 1943a] and have been able to confirm and extend most of the data of these authors.

Taken in conjunction with the analytical results of Hotchkiss [1941], our analyses suggest that the gramicidin molecule contains 24 amino-acid residues or some integral multiple thereof. These are made up as shown in Table 1.

The presence of leucine and tryptophan residues in gramicidin has already been established by isolation and characterization [Hotchkiss, 1941; Christensen *et al.* 1941]. Christensen *et al.* also claim to have isolated alanine through its dioxypyridate and

Table 1. *Suggested composition of minimum molecule of gramicidin*

	No. of residues	No. of atoms N
Leucine	6	6
Tryptophan	6	12
Valine	5	5
Alanine	3	3
Glycine	2	2
Hydroxyamino compound	2	2
Total	24	30

phosphotungstate, although no experimental details are given. They obtained a negative result for glycine, using the colorimetric method of Patton [1934]. We have established the occurrence in the hydrolysate of alanine, valine and glycine by isolation and characterization as the acetyl derivatives, and have confirmed in the same way the presence of leucine and tryptophan.

Our technique was not suitable for the isolation of the hydroxyamino compound [Hotchkiss, 1941]. We have, however, established by control experiments (see Experimental Section) that serine is

absent from gramicidin hydrolysates. Our methods would probably also reveal higher aliphatic hydroxyamino-acids, and on the evidence presented by Hotchkiss, *isoserine* would seem to be the substance whose properties agree best with what is known at present of this constituent of gramicidin.

Although our isolative procedure leads to slight racemization [cf. Martin & Synge, 1941], we have been able to confirm that the leucine of the hydrolysate is largely of the *d*-configuration, while the tryptophan has the *l*-configuration. The alanine is also largely in the *l*-form. We find, however, that the valine isolated is racemic, and since this method of isolation enabled us to prepare from wool [Martin & Synge, 1941] and from gelatin [Gordon *et al.* 1943c] acetylvaline retaining a large part of its optical activity, we conclude either that the valine residues of gramicidin are very readily racemized during hydrolysis, or that both *d*- and *l*-valine residues co-exist in the gramicidin molecule. It is impossible at present to choose between these alternatives. We have previously noted a similar phenomenon, viz. the occurrence of racemic phenylalanine in wool hydrolysates, and the possibility that selective racemization occurs during acid hydrolysis has been discussed in that connexion [Martin & Synge, 1941] (cf. Gordon *et al.* [1943a] for isolation of optically active acetylphenylalanine by a similar procedure).

Since glycine does not react with the *d*-amino-acid oxidase [Krebs, 1935], the α -amino-acids oxidized by the latter [Lipmann *et al.* 1941] must consist largely of leucine and of half the valine residues. These amount to 38.6% of the total α -amino-acid residues in the postulated molecule, in fair agreement with the figure 43–46% determined by Lipmann *et al.*

In Table 2 we correlate our experimental data with those of Hotchkiss [1941] and with the values calculated for the postulated minimum molecule containing 30 atoms of nitrogen.

Table 2. *Constituents of gramicidin hydrolysate*

	(N as % of total N)		
	Present detn.	Hotchkiss [1941]	Calc.
Leucine	20.2	16.1*	20.0
Tryptophan	40–45	37.3	40.0
Valine	16.6	—	16.7
Alanine	10.1	(25–35)	10.0
Glycine	5.3–6.6	—	6.7
Hydroxyamino N + NH ₂ -N	—	6.0	6.7
—OH groups (before hydrolysis)	—	7.3	6.7
(equivs. as N)			
α -Amino N (ninhydrin)	—	72.6	73.3
Amino-N (Van Slyke)	—	80.4	80.0
Basic groups by titration	—	80.5	80.0
(equivs. as N)			
Acidic groups by titration	—	84.7	80.0
(equivs. as N)			

* By isolation.

Our values for tryptophan and glycine are semi-quantitative only, for reasons discussed in the Experimental Section.

The method of Kendall & Friedemann [1930] used by Hotchkiss for determining alanine gives seriously high values in the presence of valine and leucine.

We have added Hotchkiss's figure for NH₂ in the hydrolysate to his determined hydroxyamino N, since the hydroxyamino compound seems a probable source of the NH₂ liberated during treatment with hot acid [cf. Hotchkiss, 1941; Gordon, Martin & Synge, 1941].

There is reasonable agreement between the experimental data and the figures calculated for the molecule postulated in Table 1.

This minimum molecule deduced by stoichiometric reasoning has the molecular weight 2790, assuming that (i) the unknown hydroxyamino compound is *isoserine*, and (ii) the gramicidin molecule is a cyclopeptide of 24 residues, formed from the free amino-acids by elimination of 24 molecules of water.

This value for the molecular weight of gramicidin is in fair agreement with that found by Tishler, Stokes, Trenner & Conn [1941] by isothermal distillation in methanol, namely, 3100, and also with the value 2500–4500 found by the same authors by diffusion measurements in *n*-butanol. The cryoscopic determinations of these authors and of Hotchkiss [1941] gave lower values. The cryoscopic data of Tishler *et al.* for gramicidin in cyclohexanol showed apparent lowering of the molecular weight at low gramicidin concentrations. If this were due to a reversible dissociation of 'chemically bound' water from the gramicidin molecule, i.e. to water which is not removable by the usual drying procedures, but which is capable of equilibrium dissociation under conditions where it could be substituted by molecules of solvent, the results would be readily explicable. Such dissociated water, while affecting the cryoscopic data, would distil with the methanol in the isothermal distillation experiment, and thus escape detection. (Tishler *et al.* [1941] do not describe their drying procedure in detail.) If this were so the cryoscopic behaviour of gramicidin in cyclohexanol would be profoundly modified by drying it 'azeotropically' in dilute solution in cyclohexanol or similar solvent. Tishler *et al.* [1941] mention that gramicidin is hygroscopic. Abderhalden & Gohdes [1931] have described the binding of water by alanine peptides.

In the absence of further data it seems reasonable to conclude that the minimum molecular weight deduced for gramicidin by stoichiometric reasoning is the true molecular weight, and that we are dealing with a 24-cyclopeptide.

The equivalent weight given by Tishler *et al.* [1941] for gramicidin in combination with flavianic

acid ($2986, 3036 \pm 300$) is in substantial agreement with the molecule postulated.

Assuming that the molecule contains two residues of isoserine and no chemically bound water, and that it is a cyclopeptide without terminal $-\text{NH}_2$ or $-\text{COOH}$ groups, the molecular formula can be deduced, namely, $\text{C}_{146}\text{H}_{202}\text{O}_{26}\text{N}_{30}$, giving a calculated elementary composition of C, 62.8; H, 7.25; N, 15.05%. Hotchkiss & Dubos [1941] record C, 62.7; H, 7.59; N, 14.8%. Tishler *et al.* [1941] agree about the C and N content, but find H, 7.36–7.41%.

In the absence of information on the exact nature of the hydroxyamino compound and in view of possible 'chemically bound' water, one can only say that the composition postulated for gramicidin is not incompatible with its determined elementary composition.

The stoichiometric data obtained in the present study are in striking agreement with the Bergmann-Niemann hypothesis on protein structure, in that the total number of residues in the molecule, and the number of each species of residue except valine, are expressible in the form $2^m \times 3^n$, where m and n are integers. Even with the valine residues, in view of their unascertained optical character, it would be possible to postulate three d -residues and two l -residues or *vice versa* as independent components. Alternatively, gramicidin may not be a homogeneous substance but a 'molecular compound' of two or more similar, but distinct, peptides crystallizing together in definite proportions. In the Experimental Section of this paper we present evidence that substances made up of some of the same amino-acids as are in gramicidin, but present in different proportions, are to be found with it in tyrothricin.

As pointed out by us [Gordon *et al.* 1941], stoichiometric data do not justify conclusions about the relative positions of the various residues in the molecule, and we hope, should material be available, to throw light on the arrangement of the residues by a study of partial hydrolysis products [cf. Gordon *et al.* 1943*b*].

Gramicidin is the first naturally occurring peptide-like substance of this degree of complexity which is probably homogeneous, and for which the amino-acid composition has been ascertained with reasonable accuracy and completeness. Of the protamines, which appear to have similar molecular weights [cf. Svedberg & Pedersen, 1940], clupein, which has been most closely investigated, has been shown to be a mixture of substances fractionable by solvent precipitation of their methyl esters [Felix & Dirr, 1929; Felix & Mager, 1937]. Although it is certain that two-thirds of the residues of clupein are arginine residues, technical difficulties have in the past prevented accurate analysis of the monoamino-acids present. The hormone secretin appears to be

a peptide of similar molecular weight [cf. Ågren, 1939]. Niemann [1939] has shown that, according to Ågren's analyses, some of its amino-acids occur in the proportions postulated by himself and Bergmann.

Apart from tyrocidine, which is found with gramicidin, few naturally occurring polypeptides of this order of molecular size have been accurately characterized. Systematic search will probably reveal more of them, and they may prove, as Kossel [1928] hoped, a valuable bridge between the very complicated proteins and their simple hydrolysis products. The study of such compounds will be particularly fruitful when they display physiological activity. The study of the partial hydrolysis products [cf. Gordon *et al.* 1943*b*] is likely at the present stage to give less equivocal results as to structural sequences in the molecule than the study of proteins containing hundreds of amino-acid residues. Also some of the partial hydrolysis products may retain physiological activity, and thereby throw light on its nature.

Dubos & Hotchkiss [1942] have reviewed biological aspects of the anti-bacterial action of gramicidin. The present work confirms that gramicidin is very unlike the average protein in amino-acid composition—notably in the absence of basic and acidic constituents and in the high proportion of tryptophan, leucine and valine. Lipmann *et al.* [1941] have discussed the possibility that the d -amino-acid residues play a part in its antibacterial action. Possibly the as yet unknown hydroxyamino compound has a special role, or the valine and leucine residues may have special inhibitory effects such as were noticed by Gladstone [1939] with these amino-acids in the free form in the nutrition of *Bacillus anthracis*. Unfortunately, Gladstone does not report experiments employing the d -isomers in the absence of the l -isomers. Perhaps, however, larger and more specific structural features of the molecule are concerned. Gramicidin might act in any of these ways if chemotherapeutic action depends on competitive inhibition of metabolic processes. Further study of its chemical structure along the above lines would seem to be a promising approach to this problem.

EXPERIMENTAL

Preparation of material for analysis— question of homogeneity

All samples of gramicidin were made by us from a Wallerstein preparation of tyrothricin. Four separate specimens were studied, the preparation of which we describe here. Before analysis all samples were dried to constant weight over H_2SO_4 and soda-lime in a vacuum desiccator at room temperature. N determinations were made by the Kjeldahl procedure after pre-treatment with red P and HI as recommended by Hotchkiss & Dubos [1941]. In our hands this procedure gave theoretical N values with acetyl-

tryptophan. Hotchkiss & Dubos record the N content of gramicidin as 14.8% and give the following optical data: $(\alpha)_D^{25} + 2.5^\circ$ (ethanol, $c = 1.5$), $(\alpha)_D^{25} + 5^\circ$ (95% ethanol, $c = 0.4$).

Preparation A. 4 g. of tyrothricin were worked up by the method of Hotchkiss & Dubos [1941]; the crude crystalline material was recrystallized once from acetone. After washing with ether the product weighed 117 mg. The whole preparation was hydrolysed for qualitative investigation of the amino-acids.

Preparation B. 1 g. of tyrothricin was worked up in the same way, the crude product was recrystallized thrice from acetone and washed with ether each time. Yield 19.9 mg. m.p. 226–227° (uncorr.). (Found: N, 14.6%.)

Preparation C was made from the combined mother liquors from the crystallization of preparations A and B, using the ethanol-ether precipitation of Hotchkiss & Dubos for preliminary purification. The first crop of crystals had $(\alpha)_D^{20} - 8.8^\circ$ (ethanol, $c = 0.7$). On recrystallization from acetone the product had $(\alpha)_D^{20} - 4.1^\circ$ (ethanol, $c = 1.2$). A further recrystallization yielded the final product, 18.5 mg., having $(\alpha)_D^{18.5} + 1.5^\circ$ (ethanol, $c = 0.6$). (Found: N, 13.0%.) In this, and the following preparation, washing of the crystals with ether was omitted, and this may have been responsible for the presence of the fatty material, which appeared as a separated layer after hydrolysis with acid. This fatty contaminant is presumably responsible for the lowered N content of preparations C and D.

Preparation D. This was made from the mother liquors from the recrystallization of preparation C. It did not readily yield crystals even after treatment with ethanol-ether. A purification by partition chromatography was attempted. It was found that gramicidin could be detected visually very readily owing to its fluorescence (presumably due to the tryptophan residues) on exposure to ultraviolet light. Trial experiments with silica gel chromatograms (using no indicator) showed that gramicidin can be made to move as a band. In the solvent system methanol-cyclohexane (the more polar phase being stationary, and absorbed in the gel), gramicidin scarcely moved at all on development. In aqueous systems, using as developing solvents 30% *n*-propanol-cyclohexane, 30% isopropanol-cyclohexane and 15% ethanol-cyclohexane, gramicidin moved as a band having $R = >1$, 0.5 and 0.2 respectively (same terminology as in previous paper [Gordon *et al.* 1943a]). The main preparation was then carried out on about 65 mg. of crude material, using 30% isopropanol-cyclohexane as the developing solvent. This caused the separation of three fluorescent fractions from the main band. One of these moved at $R > 1$ (presumably fatty components), while the other two moved more slowly than the main fraction. This main fraction was run out of the column and collected separately; after evaporation of the solvent *in vacuo*, the preparation was taken up in ethanol and filtered to free it from silica which had escaped with it from the column. The ethanol was removed *in vacuo*, and the product was then allowed to crystallize from acetone. Crystallization was much slower than in previous preparations, taking 24 hr. for completion. The product, weighing 35 mg., had $(\alpha)_D^{20} + 3^\circ$ (ethanol, $c = 1.1$) and on recrystallization (an equally slow process) 18.3 mg. of the final product was obtained having $(\alpha)_D^{20} + 2^\circ$ (ethanol, $c = 0.6$). (Found: N, 12.9%.) Like preparation C, this material yielded a fatty layer on acid hydrolysis.

It seems therefore that some of the fatty material is so closely associated with the gramicidin that it resists chromatographic separation. The chromatographic purification, though eliminating the laevorotatory material noticed in the course of preparation C, does not give a really pure product, since, in addition to its slow crystallization, preparation D differs significantly in amino-acid composition from preparations B and C, having a lower content of valine and tryptophan, and a higher content of glycine, alanine and leucine than they have. This trend in composition of material obtained by working up the mother liquors (already noticeable in preparation C—cf. Table 6) suggests that, in addition to gramicidin, tyrothricin contains other components made up of some of the same amino-acids as the latter. In the analysis of none of these preparations have any qualitative differences in the amino-acids present been noted.

Although the chromatographic procedure used with preparation D did not lead to any dramatic purification, it seems likely that chromatographic methods may be of value for the quantitative extraction of gramicidin from tyrothricin preparations. The results mentioned here justify further study. From the data on simpler compounds given by Gordon *et al.* [1943a], gramicidin appears to distribute itself between solvent phases in a manner not inconsistent with its amino-acid composition.

Our overall yields of material judged to be rich in gramicidin have never amounted to more than 5% of the 5 g. of tyrothricin which we had at our disposal. This is rather less than the yields mentioned by Hotchkiss & Dubos [1941].

Antibacterial activity of the preparations

Dr F. C. Happold of Leeds University has kindly carried out bacterial growth inhibition tests [cf. Dubos & Cattaneo, 1939] on our preparations B, C and D, and reports:

‘The samples of gramicidin have been tested for bactericidal or bacteriostatic action in tubes of beef infusion peptone broth (5 ml.). A 6 hr. culture of *Staphylococcus aureus* was inoculated into the experimental tubes and growth observed at 4, 5 and 6 hr. after inoculation. At 6 hr. a loop of each culture was spread over nutrient agar to give an additional comparison of the degree of growth. The gramicidin used was 200 $\mu\text{g.}/\text{ml.}$ in 2% ethanol and formed a stable suspension. A 1/10 dilution of this was also made in sterile distilled water so that when 0.2, 0.1 and 0.05 ml. of each of these two suspensions were added to the tubes of broth they gave concentrations of 40, 20, 10, 4, 2 and 1 $\mu\text{g.}/5 \text{ ml.}$ medium.

‘Preparations C and D were tested with a culture which was less rapidly viable than the one used with B. The result was even more dramatic. The

Table 3. *Antibacterial activity of gramicidin*

Preparation B	Growth at		
	4 hr.	5 hr.	6 hr.
Peptone infusion broth	+++	++++	++++
+ 0.2 ml. 2% ethanol	++	++++	++++
+ 1 μ g. gramicidin	+	+++	+++
2 "	Tr.	++	++
4 "	Nil	?	+
10 "	Nil	Nil	? Tr.
20 "	Nil	Nil	Nil
40 "	Nil	Nil	Nil

alcohol controls and the infusion broth tube alone showed signs of growth at 6 or at 24 hr. When a loop of each tube was spread on agar all the gramicidin tubes were found to be obviously sterile.'

Analytical procedure

Following Hotchkiss [1941] we hydrolysed with HCl in aqueous acetic acid excluding air. After evaporation from alcoholic solution in a vacuum desiccator over H_2SO_4 and soda-lime, all our preparations (in Hysil test-tubes) were dissolved in 0.8 ml. of glacial acetic acid. 3.0 ml. of 6N HCl (which caused some precipitation) was added, the tube was evacuated, sealed, and heated for 24 hr. in a bath of refluxing 6N HCl. The precipitated material dissolved during the first $\frac{1}{2}$ hr. of heating.

The hydrolysate was prepared for chromatography as described by Gordon *et al.* [1943a]. A preliminary investigation, using the analytical technique described there, revealed that a shortened procedure could be used for separating the acetyl derivatives. This is illustrated in Table 4.

Characterization of gramicidin constituents

Preparation A (117 mg.) was fractionated according to the procedure described above with columns of a suitably increased diameter. The resulting fractions were worked up and characterized as described below. All m.p.'s are uncorrected unless otherwise stated. N determinations were made by the Kjeldahl procedure on samples of material in solution, which samples had been titrated with $N/100 \text{ Ba(OH)}_2$ to determine the acid equivalent weights, using the N-free indicator phenolphthalein. For the N determination on acetyltryptophan, the titration mixture was evaporated to dryness *in vacuo* and treated with red P and HI before incineration, as in the N determinations on gramicidin. C, H determinations were carried out by Dr G. Weiler, Oxford.

(1.1) *Acetyl-d-leucine*. Successive recrystallizations from *n*-propanol-cyclohexane and from water yielded 20 mg. of product having m.p. 183° (not depressed on admixture of authentic acetyl-*d*-leucine, but depressed on admixture of acetyl-*l*-leucine), $(\alpha)_D^{19} + 17.2^\circ$ (ethanol, $c=0.7$). Synge [1939] records for acetyl-*d*-leucine m.p. $186\text{--}188^\circ$, $(\alpha)_D^{22} + 23.2^\circ$ (ethanol, $c=3.7$). (Found: C, 55.1; H, 8.61; N, 8.0%; acid equiv. wt. 174. Calc. for $\text{C}_8\text{H}_{15}\text{O}_3\text{N}$: C, 55.5; H, 8.67; N, 8.1%; acid equiv. wt. 173.)

(2.1) *Acetyl-l-tryptophan*. Successive recrystallizations from chloroform-petrol and from water yielded 16 mg. of product having m.p. 178° (not depressed on admixture of authentic acetyl-*l*-tryptophan), $(\alpha)_D^{20} + 23.6^\circ$ (ethanol, $c=0.5$). Du Vigneaud & Sealock [1932] record m.p. $189\text{--}190^\circ$ (corr.), and Gordon *et al.* [1943a] record $(\alpha)_D^{19} + 26.0^\circ$ (ethanol, $c=1.1$). (Found: C, 62.4; H, 5.68%. $\text{C}_{15}\text{H}_{14}\text{O}_3\text{N}_2$ requires: C, 63.3; H, 5.69%.) The material, recrystallized from CHCl_3 , was further analysed as the molecular com-

Table 4. *Scheme of fractionation*

In the propanol-cyclohexane column fraction 1 was run out with 5% propanol-cyclohexane. The remaining fractions were run out by development of the chromatogram with 30% propanol-cyclohexane. The qualitative appearance of the bands during the analysis of all four preparations was the same. Initial experiments indicated that fraction 1 required refractionation on a butanol-chloroform column to eliminate two components appearing as faint bands, running a little ahead of, and a little behind, the main acetyl-leucine band. Fractions 3 (alanine) and 4 (glycine) did not require further fractionation with butanol-chloroform.

pound with CHCl_3 [cf. Gordon *et al.* 1943a]. (Found: N, 7.4%; acid equiv. wt. 358. Calc. for $\text{C}_{13}\text{H}_{14}\text{O}_3\text{N}_2 \cdot \text{CHCl}_3$: N, 7.7%; acid equiv. wt. 365.5.)

(2.2) *Acetyl-dl-valine*. A determination of optical rotation on the whole fraction (44 mg.) gave $(\alpha)_D^{20} + 0.3^\circ$ (ethanol, $c=1.5$). Gordon *et al.* [1943a] give for acetyl-*l*-valine $(\alpha)_D^{19} + 4.0^\circ$ (ethanol, $c=2$). On this basis the present material contains $>92.5\%$ of the racemic compound.

Two recrystallizations from CHCl_3 yielded a product having m.p. 145° (not depressed on admixture of authentic acetyl-*dl*-valine for which Synge [1939] records m.p. $144\text{--}146^\circ$). Acetyl-*dl*-norvaline [cf. Gordon *et al.* 1943a] appears

to have a much lower m.p. (Found: C, 52.2; H, 7.97; N, 8.6%; acid equiv. wt. 163. Calc. for $C_7H_{13}O_3N$: C, 52.8; H, 8.17; N, 8.8%; acid equiv. wt. 159.)

(3) *Acetyl-L-alanine*. Crystallization from ethyl acetate-benzene yielded 12 mg. of a product having m.p. 125° (not depressed on admixture of acetyl-L-alanine derived from gelatin), $(\alpha)_D^{18} - 37.2^\circ$ (ethanol, $c=0.4$). For the product from gelatin Gordon *et al.* [1943*b*] record m.p. 126–127°, $(\alpha)_D^{18} - 36.9^\circ$ (ethanol, $c=0.4$). (Found: C, 46.4; H, 6.92; N, 10.6%; acid equiv. wt. 130. Calc. for $C_5H_9O_3N$: C, 45.8; H, 6.87; N, 10.7%; acid equiv. wt. 131.)

(4) *Acetylglycine*. Crystallization from ethyl acetate yielded 2 mg. of a product having m.p. 200° (not depressed on admixture of authentic acetylglycine, m.p. 205–206° [cf. Curtius, 1884]. The m.p. was depressed to 180° on admixture of acetyl-L-glutamic acid). There was barely sufficient material for an accurate determination of N and of acid equivalent weight, so no C, H determination was carried out. Together with the m.p. and chromatographic data, however, these analyses provide reasonable identification of the substance. (Found: N, 11.2%; acid equiv. wt. 123. Calc. for $C_2H_5O_3N$: N, 12.0%; acid equiv. wt. 117.)

Absence of serine from gramicidin

Serine was used in the known mixture employed as a control to the quantitative experiments described below. It always gave a distinct band during analysis, travelling at 0.55 time the rate of band 4 on the 30% propanol-cyclohexane column. This band was never observed during analyses of gramicidin. With the latter a much fainter band moving at 0.3–0.4 time the rate of band 4 was occasionally observed. Whether this was due to the hydroxy-amino compound of gramicidin further investigation will show.

Fraction X (Table 4) was regularly observed in the control experiments as well as in those with gramicidin; separate experiments showed it to be a product derived from tryptophan.

The two minor components of fraction 1, shown as occurring in gramicidin hydrolysates, were not observed during analysis of the known mixture.

Quantitative analysis of gramicidin hydrolysate

6–9 mg. of gramicidin were used for a single analysis, on chromatogram tubes of 1 cm. internal diameter. 3 g. of silica was used in each column.

After an estimate of the relative proportions of the various constituents had been formed, control analyses were carried out on a known mixture of acetamino-acids and serine, containing an amount of each constituent nearly equal to that present in the samples of gramicidin analysed, the same hydrolysis conditions and analytical procedure being used. Apart from the differences mentioned above, the chromatograms were indistinguishable from those obtained with gramicidin. The constituents of the mixture and the percentage recoveries are shown in Table 5. The acetyl derivatives were used in preference to the free amino-acids since, in addition to

being of ascertained purity, their hydrolysis simulated more closely the hydrolysis undergone by gramicidin.

Table 5. *Control experiments on gramicidin analyses*

Component of mixture	Recovery (%)
Acetyl- <i>D</i> -leucine	101, 101
Acetyl- <i>L</i> -tryptophan	66, 73
Acetyl- <i>DL</i> -valine	104.5
Acetyl- <i>DL</i> -alanine	98, 101
Acetylglycine	78, 63
<i>DL</i> -Serine	—

Control experiments showed the high valine figure to be due to contamination by acetyltryptophan which 'tailed' during the separation of fractions 2.1 and 2.2 with 3% butanol-chloroform. This was caused by using, throughout these experiments, a batch of gel which had been aged for too long under acid [cf. Gordon *et al.* 1943*a*]. No such 'tailing' was noticed on the propanol-cyclohexane columns made with the same gel. Accordingly, in the analyses shown in Table 6, the valine figures have been calculated from the titrations for fraction 2.2 *less* 6.8% of the titration for fraction 2.1. No correction has been applied to the leucine and alanine figures.

The low tryptophan recoveries are due to destruction of tryptophan in the course of hydrolysis, etc.; the low glycine recoveries presumably to incomplete extraction of acetylglycine from the acidified acetylation mixture by the 250 ml. of 17% butanol-chloroform used. The figures for glycine and tryptophan in Table 6 have accordingly been corrected assuming these recoveries, and must be regarded as semi-quantitative only. Reserve is particularly necessary in assessing the tryptophan figures, since the destruction during hydrolysis of tryptophan residues in combination in gramicidin may be different from that occurring when they are present in acetyltryptophan.

Table 6 shows the analytical results for preparations B, C and D corrected in this way.

Table 6. *Amino-acid composition of gramicidin preparations*

Preparation ...	(N as % of total N)		
	B	C	D
Leucine	20.0	20.4	21.6
Tryptophan	40.1–44.8	40.1–44.8	33.5–37.2
Valine	17.0*	16.6	15.6
Alanine	9.8	10.4	11.5
Glycine	5.0–6.2	5.7–7.0	6.6–8.0

* Maximum figure, owing to accidental contamination of the material for analysis by acetylvaline. This figure has not been incorporated in the mean values given in Table 2.

In compiling Table 2, the mean values of the determinations on preparations B and C have been used.

SUMMARY

Gramicidin has been subjected to acid hydrolysis, and the presence in the hydrolysate of *d*-leucine, *l*-tryptophan, *dl*-valine, *l*-alanine and glycine has been established.

A quantitative determination of these components has been made, and from this, in conjunction with the data of other authors, it seems probable that the gramicidin molecule contains the following 24 residues: 6 leucine, 6 tryptophan, 5 valine,

3 alanine, 2 glycine and 2 of an unknown hydroxy-amino compound.

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A Study of the Partial Acid Hydrolysis of Cow-hide Gelatin

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In a recent paper [Gordon, Martin & Synge, 1941] we described preliminary experiments aiming at the characterization of products of partial hydrolysis of proteins and through this at the elucidation of structural sequences in the intact protein molecule.

In the present paper we report further work in this direction, using partition chromatography [cf. Martin & Synge, 1941*b*; Gordon *et al.* 1943*a, b*]. This procedure has made possible the semi-quantitative isolation of a number of peptides in the form of their acetyl derivatives. Peptide isolations have usually depended on the chance insolubility of a particular peptide or its derivative, and have given unknown yields. The new, more refined methods make possible a study of all the peptides of a given class occurring in a partial hydrolysate of a protein,

and thus provide a more extensive picture than was formerly possible. The chromatographic procedure has been used simultaneously for rapid determination, on the micro scale, of the amino-acid composition of the peptide fractions isolated, an essential step too frequently neglected by workers in this field.

Apart from its value for the study of protein structure, this work may have applications in the field of serology, since Landsteiner [1942] has shown that the products of the partial hydrolysis of an antigenic protein can specifically inhibit its precipitin reactions with a homologous antiserum.

The present work was carried out with gelatin, since the absence of tryptophan, tyrosine and cystine from this material considerably simplifies the isola-