striatus and *B. hottentotta minax*). Fresh venom when required was collected by the method of Jousset de Bellesme (1872), in which the gland is stimulated electrically and the venom collected through a capillary tube fitted to the sting. Venom so collected was milky in appearance, was soluble in 0.9 % saline and clotted on standing. Data on the absorption spectra are recorded in Table 1, from which it is seen that the toxins prepared from the four species all show the same absorption band at 278m μ . The two venoms both showed a band at 260m μ , the shift to 278m μ apparently occurring during the process of purification.

spectra. With 0.5 % formalin toxicity decreased from M.L.D. $\equiv$ 0.05–0.08 mg. in 15 days; with 1.0 % formalin the respective values were 0.05–0.10 mg. in 15 days, without significant change in the absorption band. Even with 5 % formalin the M.L.D./100 g. rat had increased only from 0.05 to 0.16 mg. during 15 days; the absorption band was then affected.

The action of formalin at 55° was much more rapid, the M.L.D. increasing from 0.05 to 0.5 mg./100 g. rat in 24 hr. with 0.5 % formalin, or within 1 hr. with 5 % formalin, the characteristic absorption band becoming inflected.

Table 1. *Absorption spectra data on scorpion toxins and venoms*

Species	Substance examined	Cell (mm.)	$\lambda_{\max}$ (m μ)	$E_{\max}$	$\lambda_{\min}$	$E_{\min}$	M.L.D./100 g. rat (μ g.)
<i>B. quinque striatus</i>	Toxin HCl 0.2 %	5	278	1.16	253	0.67	25
<i>B. leptochelys</i>	"	5	278	1.44	253	0.97	33
<i>B. acute carinatus</i>	"	5	278	1.43	253	0.96	27
<i>B. hottentotta minax</i>	"	5	278	1.10	253	0.80	20
<i>B. quinque striatus</i>	Venom 0.5 ml. + 1.5 ml. saline	10	262.5	0.6	—	—	(0.3 ml.)
<i>B. hottentotta minax</i>	Venom 1 drop + 0.75 ml. saline	5	262.5	1.0	219	0.2	(0.1 ml.)

Effects of pH and of temperature. The toxins were stable when kept in solution in 0.1N-HCl at 0° for at least 6 days, neither the absorption band nor the M.L.D. altering during this time. They were unstable in 0.1N-NaOH, the M.L.D. being at least 10 times greater after 24 hr. at 0° and the characteristic absorption band becoming inflected. Toxin in distilled water at 55° was unaltered in 24 hr., but after 6 days had only one-tenth its former activity and the absorption band had changed.

Effect of formalin. Solutions (0.2 %) of toxin in water with different concentrations of formalin were allowed to stand at 0° and examined at intervals for toxicity and for changes in the absorption

Effect of acetaldehyde. When acetaldehyde (5 %) was used instead of the formalin at 55° the potency of the toxin decreased from 0.05 to 0.08 mg./100 g. rat in 1 hr. without alteration of the absorption band, but after 24 hr. under these conditions the M.L.D. had increased to 0.5 mg. and the characteristic absorption band was no longer present.

It appears from the parallelism in the changes in toxicity with the changes in the absorption band produced by various treatments that this absorption band is related to the toxin.

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Analysis of a Partial Hydrolysate of Gramicidin by Partition Chromatography with Starch

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Following the analysis of the constituent amino-acids of gramicidin previously reported (Gordon, Martin & Synge, 1943b), a beginning has been made with a study of the peptides arising from the partial hydrolysis of gramicidin by acid. In the present paper are described the use of a new technique (partition chromatography employing starch) which

has proved valuable for this work and may have wider applicability, and the isolation of the dipeptide *l*-valylglycine from gramicidin by partial hydrolysis with acid. Some further evidence concerning the amino-acid composition of gramicidin and the optical nature of the constituent valine residues has been obtained.

COMPARISON OF AMINO-ACID COMPOSITION AND OTHER PROPERTIES OF GRAMICIDIN FROM TWO SOURCES

The Wallerstein gramicidin previously studied was insufficient for the present work; it seemed best to employ it in a detailed comparison with a new batch obtained from Dr R. D. Hotchkiss. The two specimens were compared in respect of melting-point, rotation, N content and amino-acid composition, properties which did not differ significantly from one specimen to another nor after recrystallization (Table 2). For the purposes of this discussion they are regarded as identical. It has not proved possible to ascertain whether both specimens were obtained from the same strain of organism.

In determining the amino-acid composition somewhat milder conditions of acid hydrolysis were employed than formerly (Gordon *et al.* 1943*b*), since control experiments on known amino-acid mixtures had shown (Table 1) that a better tryptophan recovery could be obtained in this way. With gramicidin itself, the tryptophan figures were indeed more accurate than those previously found, but the values for other amino-acids were consistently and significantly different from those originally published. Subsequent to these analyses, Christensen (1943) reported the occurrence of valylvaline in acid hydrolysates of gramicidin, and valylglycine has now been isolated as described below. The presence of these peptides, which are unusually resistant to acid hydrolysis, sufficiently explains the deviations from results previously obtained. It is therefore justifiable to attribute to gramicidin the composition determined after more vigorous hydrolysis (Gordon *et al.* 1943*b*), viz.: 6 leucine, 6 tryptophan, 5 valine, 3 alanine and 2 glycine residues and 2 residues of an unknown hydroxyamino compound per assumed molecule. Experiments employing the 'paper strip' partition chromatographic technique of Consden, Gordon & Martin (1944) were undertaken with gramicidin hydrolysates, in the hope of detecting this unknown compound, but strips developed with *n*-butanol- H_2O or phenol- H_2O mixtures on treating with ninhydrin revealed pictures which could be simulated in every respect by mixtures of the known components of gramicidin. (This provided an independent check on the qualitative amino-acid composition of gramicidin.) Determination of the NH_3 liberated by periodate from gramicidin hydrolysates (Table 2) confirmed Hotchkiss's (1941) results quantitatively, whereas a mixture of the known constituents of gramicidin treated in the same way yielded negligible NH_3 . The reality of this hydroxyamino component is thus not in doubt, but it is possible that it is not an amino-acid at all. It is clearly important to determine the nature of this ultimate hydrolysis product before proceeding further in the study of partial hydrolysis.

TECHNIQUE OF SEPARATION OF PARTIAL HYDROLYSIS PRODUCTS

Preliminary experiments done in collaboration with Dr A. H. Gordon at Leeds showed:

(1) About half of the peptide bonds in gramicidin are split by treatment with 10*N*-HCl and acetic acid (1:1 v/v) at 37° for 10 days, as judged from the Van Slyke amino-N determination. All partial hydrolysates discussed here have been prepared in this way.

(2) Partition chromatography on silica gel of acetylated partial hydrolysates gave a complicated series of bands, some of which 'tailed' so excessively on the columns that separations were obviously less effective than those obtained in our study of gelatin (Gordon *et al.* 1943*c*) by this technique. Quantitative amino-acid analysis of the resulting fractions confirmed the ineffectiveness of the separations. It has already been shown (Gordon *et al.* 1944) that acetyl-tryptophan 'tails' on silica gel partition chromatograms, and it is to be expected that acetylpeptides containing more than one tryptophan residue would show excessive 'tailing'.

(3) On paper strip partition chromatograms (Consden *et al.* 1944) a number of free peptides travel as reasonably narrow bands whose presence can be revealed by treatment with ninhydrin. Table 3 shows the relative band-rates of some of these in the *n*-butanol- H_2O system. The 'strip' and 'two-dimensional' paper chromatograms with gramicidin partial hydrolysates revealed an array of fairly well-separated compounds greater in number, and in many cases differing in position from those obtained with complete hydrolysates.

Further experiments have now been conducted with gramicidin partial hydrolysates on *n*-butanol- H_2O paper strip chromatograms. Colouring these by spraying with Ehrlich's reagent in acid solution revealed two well-marked bands (presumably of tryptophan peptides) faster than the band corresponding to free tryptophan, but none slower than it. 'Tailing' of tryptophan-containing substances appeared to be negligible. Attempts were therefore made to reproduce this type of chromatogram on the preparative scale.

Use of starch chromatograms. Working with Dr S. R. Elsden, and on his suggestion, we employed raw potato starch, and found it entirely suitable for the purpose (Elsden & Syngé, 1944). It was found that amino-acids travel as quite sharp bands in column chromatograms made up with starch and developed with *n*-butanol saturated with water. The presence of the amino-acid bands was revealed by passing an ethereal solution of ninhydrin through the column after development (Consden *et al.* 1944).

Comparison of the partition coefficients of simple monoamino-acids as calculated from their band-rates and the chromatogram constants (Table 4)

shows fair agreement with the direct determinations of England & Cohn (1935). The calculations are similar to those of Consden *et al.* for paper chromatograms, and suggest that the phenomena are essentially the same, as would be expected on chemical grounds. In practice it was found that gramicidin partial hydrolysates gave an identical sequence of bands (examined by fluorescence, ninhydrin and Ehrlich's reagent) on paper or starch chromatograms, and it seems probable that most of the separations described by Consden *et al.* will be reproducible on the preparative scale using starch. Preliminary experiments have shown that the glutamic acid-aspartic acid separation can be done on starch as well as on paper with phenol-H₂O.

There has been no indication in these experiments that optical antipodes or racemates of any compound travel at different rates on the chromatograms. Resolution of optical antipodes is a theoretical possibility on account of the asymmetry of starch and cellulose; that it does not occur provides further evidence that the latter substances act as inert supports for a liquid phase.

The experience with gramicidin partial hydrolysates on starch chromatograms is discussed later. Here it is only necessary to draw attention to the fact that when this mixture was applied as the hydrochlorides of its components, valylglycine travelled as a band (as hydrochloride) at about twice the rate of alanine (which emerged from the chromatogram almost free from HCl). These two substances in the free state travel as bands having closely similar rates in *n*-butanol-H₂O and would be difficult to separate. The presence of HCl apparently exerts a differential effect. This observation has not yet been followed up, but taken with the data of Consden *et al.* on the effects of varying pH, it suggests that new and useful separations may be effected by control of this factor.

Throughout this work the qualitative ultra-micro paper-strip techniques described by Consden *et al.* have proved very useful for controlling analytical operations, checking purity, and confirming identity while sacrificing only a few μ g. of material.

RESULTS OF CHROMATOGRAPHIC ANALYSIS OF GRAMICIDIN PARTIAL HYDROLYSATES

It was found that the colour reaction with ninhydrin in ether on starch chromatograms was useful for distinguishing free amino-acids from peptides, which gave no colour until heated. Partial hydrolysates of gramicidin, in contrast to complete hydrolysates, did not give bands corresponding to free valine or glycine. The leucine band, the tryptophan band (slightly behind the leucine and imperfectly separated from it) and the alanine band were always observed. The absence of free valine is confirmed by

the quantitative analyses given below. The absence of these amino-acids in the free state fits in with the finding of large yields of peptides embodying them both by Christensen (1943) and in the present work.

After pilot chromatograms had been studied, a large-scale chromatographic analysis was done on a starch chromatogram employing *n*-butanol-H₂O as developing solvent. Fractions were collected 'blind' from the effluent in pre-arranged volumes, but examination of the fluorescent bands of tryptophan peptides, etc., under ultra-violet irradiation showed that flow was quite even and that all parts of each band-front arrived at the bottom of the chromatogram simultaneously.

Fig. 1. Properties of fractions of gramicidin partial hydrolysate.

The 40 fractions collected were analyzed for tryptophan by a modification of the method of May & Rose (1922), and estimates of fluorescence and the colour on heating with ninhydrin were also made. Results are illustrated in Fig. 1. No tryptophan peptides appeared to move more slowly than free tryptophan, and the present paper deals with

the bands slower than, and free from tryptophan, which represent a small, relatively simple portion of the mixture. The faster moving components await further investigation.

No material moving more slowly than alanine and giving colour with ninhydrin had been detected in the preliminary experiments, so the large-scale chromatogram was discontinued when the alanine band had run out.

Small samples of the fractions following the tryptophan were hydrolyzed with acid, and the constituent amino-acids determined qualitatively on paper-strip chromatograms, with the results shown in Fig. 2. On the basis of this information, the fractions were placed in five groups (A, B, C, D, E, Fig. 2) and bulked samples from all the fractions in

Fig. 2. Amino-acid composition and grouping of slower-moving fractions.

each group were hydrolyzed and subjected to quantitative amino-acid analysis (Table 5). Groups B and D contained negligible quantities. The other groups are discussed below.

Group A. That this fraction consisted mainly of free alanine is established by (1) its band-rate on the starch chromatograms and its reaction with ninhydrin in the cold; (2) qualitative (on paper) and quantitative (on silica gel) analyses of its constituent amino-acids after hydrolysis; (3) the fact that direct acetylation of the material in this fraction gave a good yield (on the alanine content of the fraction) of material agreeing in properties with acetylalanine and yielding on hydrolysis nearly one molecule of alanine per $-\text{COOH}$ group. Were a peptide such as alanylalanine present, hydrolysis of its acetyl derivative would yield two or more molecules of alanine per $-\text{COOH}$ group.

Group C. This fraction consisted mainly of *l*-valylglycine. For the quantitative analysis of its amino-acid composition, hydrolysis by 6*N*-HCl at 100° for 24 hr. was employed. The resulting analysis, in addition to equimolar quantities of acetylglycine and acetylvaline, gave a strong additional band agreeing in rate with acetylalanine. Since, however, the strip experiments excluded the presence of this quantity of alanine, it seemed possible that the acetyl derivative of unhydrolyzed dipeptide might

be present. Glycyl-*dl*-valine and *dl*-valylglycine were prepared and subjected to the same conditions of hydrolysis and subsequent analysis. It was found that while glycylvaline gave the expected yield of valine and glycine only, valylglycine gave a result corresponding qualitatively and quantitatively with that from the natural product. It was then established that the natural product: (1) on electrometric titration agreed quantitatively in behaviour with valylglycine; (2) on acetylation gave a good yield of material agreeing in chromatogram band-rate with acetylvalylglycine, having the expected acid equivalent weight, and yielding on hydrolysis one molecule of valine and one of glycine per $-\text{COOH}$ group; (3) had a strong positive optical rotation consistent with that found by Fischer & Scheibler (1908*a*) for synthetic *l*-valylglycine, and on acid hydrolysis yielded *l*-valine of high optical activity (as found by Fischer & Scheibler (1908*a*) with the synthetic material). This third observation provides additional evidence for the order of the amino-acid residues, since Fischer & Scheibler (1908*a*) found that synthetic glycyl-*l*-valine has a negative rotation.

The techniques employed for establishing the structure of the compound were chosen to avoid destruction of the component residues, since it was desired to conserve as much of the valine as possible for the determination of its optical form. It was further necessary to avoid all fractionation depending on crystallization, in order to conserve optical character. Chromatographic purifications were therefore employed throughout.

Group E. Similar evidence to that adduced in the previous section suggests that the 'alanine' of the amino-acid analysis of this fraction is an artefact of incomplete hydrolysis. The material may contain a tripeptide embodying leucine, valine and glycine, but the total amount so far available has not permitted more detailed study.

From these experiments it is apparent that the partial hydrolysate yields about 0.75 mol. of free alanine per assumed gramicidin molecule, and about 1.15 mol. of *l*-valylglycine, or more if it be assumed that fraction E embodies this peptide attached to leucine at one or other end. It is therefore probable that both the glycine residues of the assumed molecule occur linked through their $-\text{NH}_2$ groups to *l*-valine. This result serves as a sort of control of the 'partial hydrolysis' approach to molecular structure. Gordon *et al.* (1941) stressed the desirability of obtaining a check of this kind on the method.

The high yield of *l*-valylglycine is readily explained by the resistance of *dl*-valylglycine to acid hydrolysis whilst Christensen's (1943) isolation of valylvaline suggests that valyl peptides as a class may be particularly resistant. Fischer & Abderhalden (1907), moreover, obtained evidence for the occurrence of a dipeptide of glycine and valine in

partial acid hydrolysates of elastin. The study by Levene, Steiger & Rothen (1932) of the hydrolysis of a number of dipeptides by alkali at 37° showed valylglycine to be the most resistant of these. No systematic experiments on the kinetics of acid hydrolysis of simple peptides seem to have been published. It is hoped shortly to undertake such a study in relation to the kinetics of acid hydrolysis of gramicidin. The results so far available, together with Steiger's (1934*b*) study of the hydrolysis of a number of acetyl peptides by the acidity of their own aqueous solutions, suggest considerable parallelism between the resistance of peptides of aliphatic monoamino-acids to acid and to alkali. If this be established, it will be unfortunate for partial hydrolysis studies, since alkali will give breakdown products similar to those obtained with acid.

THE OPTICAL CHARACTER OF THE VALINE RESIDUES IN GRAMICIDIN

Gordon *et al.* (1943*b*) suggested two possible explanations for the racemic character of the valine isolated from acid hydrolysates of gramicidin, and the isolation of *l*-valylglycine from partial hydrolysates of gramicidin now permits a hoped-for experimental approach to this problem. (In the following discussion the possibility of Walden inversion during hydrolysis, acetylation, etc., is ignored, since it seems unlikely that it can occur. It should be noted, however, that Fischer & Scheibler (1908*b*) have described some anomalous behaviour by valine derivatives in respect of Walden inversion.)

On the evidence now available it appears that in the gramicidin molecule two *l*-valine residues occur linked through their carboxyl groups to glycine, and further that on acid hydrolysis for 10 days at 37°, followed by that for 36 hr. at 115°, these will give nearly 2 mol. of *l*-valine in the hydrolysate.

The optical activity of the valine resulting from gramicidin hydrolyzed in this way was determined by acetylating the hydrolysate, isolating the valine chromatographically as its acetyl derivative, hydrolyzing this fraction, and determining its rotation in 6*N*-HCl. By eliminating crystallization, fractionation of optical isomers was avoided. The only known optically active contaminant, *l*-tryptophan, has a negligible rotation in HCl (Abderhalden & Kempe, 1907*a*; Lutz & Jirgensons, 1931). The valine resulting was found to have $(\alpha)_D$, $-10^\circ \pm 3^\circ$. *l*-Valine has $(\alpha)_D$, $+28.8^\circ$ (Fischer, 1906).

Since two of the five valine molecules obtained in this way must be *l*-valine, it is necessary to postulate that the other three are *d*-valine and thus evidence is provided for the co-existence at different points in the gramicidin molecule of both *d*- and *l*-valine residues.

When the gramicidin was hydrolyzed at 115°

without preliminary treatment at 37°, the valine isolated in the same way had $(\alpha)_D$, $0^\circ \pm 3^\circ$. This suggests that racemization may result from the use of hot acid in the early stages of hydrolysis. Racemization of residues in peptide linkage by hot acid has been demonstrated by Levene & Steiger (1930), and this seems the most plausible explanation of the racemic character of the valine obtained by Christensen (1943) from valylvaline resulting from hot acid hydrolysis of gramicidin.*

It is therefore to be expected that peptides embodying *d*-valine will be found on further investigation of partial hydrolysates of gramicidin. In their preparation, hot acid should be avoided in the early stages of hydrolysis.

Gramicidin seems to be the first protein-like material with which there is evidence for the occurrence of both *d*- and *l*- residues of the same amino-acid in the molecule. To demonstrate this conclusively the isolation of peptides of *d*-valine will be necessary. In other cases, where partially or completely racemic amino-acids have been reported from acid hydrolysates of proteins (e.g. Chibnall, Rees, Williams & Boyland (1940); Martin & Synge (1941*a*)), it seems preferable to continue to regard these as arising by selective racemization of the *l*-isomer during hydrolysis until evidence to the contrary is advanced, e.g. by isolation of partial hydrolysis products. There is some evidence that selective racemization of this kind occurs in the early stages of hydrolysis of gramicidin by hot acid.

EXPERIMENTAL

Methods

Partition chromatography of acetyl derivatives on silica gel. Batch γ of silica gel (Gordon *et al.* 1944) was employed throughout this work. Unless otherwise stated, material was acetylated and prepared for chromatography according to Gordon *et al.* (1943*a*); 3 g. silica gel were made up in columns having 1 cm. internal diameter. Peonin chloride (Gordon *et al.* 1944) was employed as indicator for all chromatograms except those initially made up with 3% *n*-butanol-chloroform; in these the '*R*-NH₄' indicator described by Liddell & Rydon (1944) was used.

The 'separating funnel' extraction procedure in preparing material for chromatography was retained for rapidity of working in spite of selective losses of alanine and glycine and some inaccuracy, which could have been obviated by using a 'column' extraction procedure (Gordon *et al.* 1943*d*, 1944). In control experiments, recoveries of alanine have averaged 96%, of glycine 75%, and the figures for these acids given below have been corrected accordingly. Chro-

* (Note added in proof.) H. N. Christensen (*J. biol. Chem.* 154, 427 (1944)), has now shown that the valylvaline isolated by him is the *dd*-*ll* and not the *dl*-*ld* racemic modification. This cannot, he considers, arise by racemization during hydrolysis. Nevertheless, it might be that steric factors necessitate the racemization of a *d*-valyl-*d*-valyl- grouping by epimerization at both asymmetric centres simultaneously or not at all.

matographic manipulation and titration of the resulting fractions was as described by Gordon *et al.* (1943*a*).

Partition chromatography of free amino-acids and peptides on filter-paper strips. This was done according to the general directions of Consden *et al.* (1944). Where material was applied to the paper as its hydrochloride, it was not neutralized with NH_3 before development of the chromatogram. Whatman no. 1 filter paper was employed throughout.

Miscellaneous. Optical rotations were determined in a 0.5 dm. tube. Melting-points are recorded uncorrected. All organic solvents were redistilled in the laboratory through a fractionating column. Glacial acetic acid was distilled from $\text{K}_2\text{Cr}_2\text{O}_7$ at atmospheric pressure. Unless otherwise stated, evaporations were *in vacuo* below 40° .

Preparation of peptides, etc. studied

dl-Valylglycine was prepared according to Fischer & Schenkel (1907). Satisfactory amination was not achieved by following the directions of Levene *et al.* (1932).

Glycyl-dl-valine was prepared according to Levene *et al.* (1932). In contrast to their data, the air-dry product lost only 2.8% of its weight on drying to constant weight over P_2O_5 at 100° . There is thus little evidence for water of crystallization; Abderhalden, Rindtorff & Schmitz (1928) noticed none.

dl-3-isoPropylpiperazine-2:5-dione was prepared according to Fischer & Schenkel (1907) from dl-valylglycine. The product had m.p. $244-245^\circ$.

l-Leucyl-l-tryptophan was prepared (with Dr A. H. Gordon) according to Abderhalden & Kempe (1907*b*).

Glycylglycine, glycyl-dl-leucine, dl-leucylglycine and dl-leucyl-dl-leucine 'A' were the preparations referred to by Gordon *et al.* (1943*a*). d-Leucylglycylglycine was obtained from Roche Products Ltd.

Preparation of gramicidin specimens studied

(1) *Material received from Dr R. D. Hotchkiss.* The material (R1) as received had not been recrystallized. Recrystallization from acetone, followed by washing of the product with pure ether (British Pharmacopoeia), yielded R2, 21% of material passing into the mother liquors. R3 was obtained in the same way from R2, 13% of the latter passing into the mother liquors.

(2) *Wallerstein material.* Wallerstein tyrothricin (of the batch previously studied by Gordon *et al.* (1943*b*, *d*)) was washed with B.P. ether to remove fatty material, and worked up for gramicidin according to Hotchkiss & Dubos (1941). The first crystalline product (W1) and that obtained after a single recrystallization as above (W2) have been studied.

Comparison of gramicidin specimens

The results of this comparison are shown in Table 2. Material was dried to constant weight *in vacuo* at room temperature. Optical rotation was determined in 94%

alcohol ($c=1.5$). N was determined in duplicate by the Kjeldahl procedure after treatment with red P and HI as previously. The amino-acid contents are based on the N figures thus obtained.

For the *amino-acid analyses* the previous procedure (Gordon *et al.* 1943*b*) was followed with 9–10 mg. samples, except that: (1) hydrolysis was effected in sealed evacuated tubes with 0.8 ml. glacial acetic acid and 3.0 ml. 5*N*-HCl for 48 hr. at 100° ; (2) the leucine fraction obtained from the *n*-propanol-cyclohexane chromatogram was not subjected to refractionation but titrated directly.

The results with a control mixture of five acetamino-acids, hydrolyzed and analyzed in exactly the same way as the gramicidin specimens, are given in Table 1.

Table 1. *Analysis of control mixture of acetamino-acids simulating gramicidin*

Compound	Taken for analysis (mg.)	Recovery (%)
Acetyl-d-leucine	3.75	100.5, 103
Acetyl-l-tryptophan	4.25	85, 98
Acetyl-dl-valine	2.15	103, 108
Acetyl-dl-alanine	1.55	97, 100 (corrected)
Acetyl-glycine	0.84	89, 122 (corrected)

In Table 2, which shows the analytical figures for various gramicidin specimens, the valine and tryptophan figures are corrected, 90% recovery of tryptophan and 105% recovery of valine, as found from Table 1, being assumed. (Control experiments showed that on the 3% butanol-chloroform columns with silica gel batch γ , about 5% of acetyltryptophan 'tails' into the acetylvaline fraction (Gordon *et al.* 1944).) Glycine and alanine figures are given corrected for selective extraction losses (see above).

Hydroxyamino and NH_2 determinations were done according to Gordon *et al.* (1943*e*). The hydrolysate of 5 mg. of material was evaporated in the outer chamber of a Conway dish (whose inner and outer rims had been smeared with vaseline) in a H_2SO_4 -soda lime vacuum desiccator. Water and reagents were added and the determination was carried out as previously. Control experiments with $(\text{NH}_4)_2\text{SO}_4$ and dl-serine again showed 94% recovery, and the results have been corrected accordingly. The hydrolysate of the control mixture shown in Table 1 under these conditions yielded 0.07% of its N as NH_2 and 0.2% as hydroxyamino-N.

The appearances of all specimens during hydrolysis and analysis were indistinguishable. At 100° the flocculated material dissolved more slowly than previously in the hydrolysis mixture. After the first 24 hr. of hydrolysis,

Table 2. *Comparison of gramicidin specimens*

Specimen	m.p.	$(\alpha)_D$ at temp.		N (%)	As % of total N of gramicidin					
					Leucine N	Tryptophan N	Valine N	Alanine N	Glycine N	Hydroxy-amino N
R1	$224-6^\circ$	+ 9.4°	21°	14.1	23.4	36.0	14.0	11.1	6.6	—
R2	—	+ 9.9°	17.5°	14.1	23.3	38.8	15.9	10.7	7.9	—
R3	225°	+ 11.9°	19.5°	14.1	23.5	38.4	13.9	10.1	7.4	4.3
W1	—	+ 8.5°	18°	14.0	25.7	39.1	14.3	10.6	8.4	—
W2	—	+ 8.0°	18°	13.9	25.2	41.1	15.7	11.7	7.8	4.6

there remained undissolved a drop of floating oil, which did not diminish in amount on further heating. No such oil was apparent with hydrolysates of the control mixture. It may have been due to contamination by difficultly removable fatty material, which may also be responsible for the low N content of the gramicidin specimens. The fact that the leucine figures are higher than formerly found is partly due to non-removal of the previously noted anomalous bands by a butanol-chloroform fractionation. These may be formed by the acetyl derivatives of difficultly hydrolyzable peptides.

Partition chromatography of free peptides on filter-paper strips

The band-rate data in Table 3 for the peptides studied with *n*-butanol-H₂O are on the *R_F* scale (Consden *et al.* 1944) and should be compared with the data of those authors for free amino-acids. The bands obtained were similar in width to those with free amino-acids. More vigorous heating than with free amino-acids was required for obtaining a colour with ninhydrin. Colours ranged from dull pink to bluish purple. These experiments simultaneously provided a check on the homogeneity of the peptide preparations studied.

Table 3. *Band-rates of peptides on filter-paper strips developed with n-butanol water*

Compound	Band-rate (<i>R_F</i>)
<i>dl</i> -Leucyl- <i>dl</i> -leucine 'A' }	0.8
<i>l</i> -Leucyl- <i>l</i> -tryptophan }	
<i>dl</i> -Leucylglycine }	0.25
Glycyl- <i>dl</i> -leucine }	
<i>d</i> -Leucylglycylglycine	0.13
<i>dl</i> -Valylglycine	0.08
Glycylglycine	0.03

Preparation of starch for partition chromatography

Raw potato starch, as obtained from Messrs Gordon Slater Ltd., Manchester, contained a significant quantity of material extractable by water, and gave a pale pink colour on heating with ninhydrin. This disappeared after washing by decantation six times with 10 parts by weight of distilled water. Air-dry starch (5 g.), which had been washed in this way, was extracted by keeping at room temperature for 24 hr. with 20 ml. *n*-butanol saturated with water. The extract yielded 0.4 mg. of non-volatile matter. The extracted starch was resuspended in fresh wet *n*-butanol and made into a chromatogram. After 12 ml. of this solvent had passed through the chromatogram, the next 12 ml. of effluent were collected, and yielded on evaporation 0.15 mg. of non-volatile matter. For subsequent work air-dry starch, which had been washed as above with distilled water and dried at 37° to a free-running powder, was made into chromatograms with wet *n*-butanol, and a column's length of solvent was allowed to flow through the chromatogram under gravity before applying the material for analysis. In this way contamination of the effluent by the starch was reduced sufficiently for the present work.

Since chromatograms of this kind ran more slowly than was wished, the largest granules (15% by weight of the original starch) were isolated by sedimentation and made into chromatograms. These flowed about twice as fast as those made with unfractionated starch, but corresponding

amino-acid bands on them appeared nearly twice as broad. Unfractionated starch was therefore employed for all the work described here.

Behaviour of free amino-acids on starch chromatograms developed with n-butanol

Glycine, alanine, valine, leucine and tryptophan ran as bands on starch chromatograms prepared as above. The bands were comparable in width and mutual resolution with those obtained by the paper-strip method.

A quantitative study was made on a starch chromatogram in a tube having internal diameter 1 cm. Half milligram quantities of *dl*-alanine, *dl*-valine and *dl*-leucine were applied in a minimum of wet *n*-butanol, and the chromatogram was developed with the same solvent. When the solvent level in the chromatogram tube had fallen by a total of 9.8 cm. a 0.5% (w/v) solution of ninhydrin in B.P. ether was substituted for solvent above the column. The centres of the resulting blue bands were measured *x* cm. from the top of the column. To calculate *R* values for the bands (Martin & Synge, 1941b) it was assumed that the band was immobilized at the moment when reached by the ether front passing down through the column. Since the solvent in the column moved 2.3 times as fast as in the tube above the column,

$$R = \frac{x}{9.8 + (x/2.3)}$$

From the *R* values, the partition coefficient was calculated by equation (2) of Martin & Synge (1941b), it being assumed that the starch grains held the same volume of aqueous phase as of water when saturated in an atmosphere having relative humidity of 1. Starch saturated in this way (5.73 g.) made a column of length 9.0 cm. The water content of the starch was found to be 38% by weight on the anhydrous starch present. The specific gravity of anhydrous starch was taken to be 1.65. The results of these calculations are compared in Table 4 with the direct determinations of *α* by England & Cohn (1935). The *α* values ascribed to these authors are those corresponding to the lowest concentration of each substance studied by them.

Table 4. *Partition coefficients of amino-acids between n-butanol water phases determined from band-rates on starch chromatograms and directly*

Amino-acid	Movement of band (x cm.)	<i>R</i>	<i>α</i> (from band-rate)	<i>α</i> (England & Cohn, 1935)
<i>dl</i> -Alanine	1.0	0.10	44.0	42.2
<i>dl</i> -Valine	3.2	0.29	13.7	13.9
<i>dl</i> -Leucine	7.0	0.55	6.4	5.5

Quantitative experiments on recovery of amino-acids from starch chromatograms have not yet been carried out.

Chromatographic fractionation of gramicidin partial hydrolysate

Van Slyke amino-N determinations (4 min. reaction time) on various gramicidin specimens hydrolyzed for 10 days as below gave 39, 41 and 45 % of the N as amino-N.

252 mg. gramicidin R3 were dissolved in 12.5 ml. glacial acetic acid, and 12.5 ml. 10 N-HCl were added to the solution. This caused no precipitation. The solution was kept in a sealed evacuated vessel at 37° for 10 days, during which time a slight fluffy precipitate formed and the solution became bluish purple. At the end of this time the mixture was evaporated to dryness *in vacuo* and kept for a further $\frac{1}{2}$ hr. at 45° while evacuating with a water-pump. The gummy residue was entirely soluble in wet *n*-butanol. It was transferred quantitatively in 5 ml. of this solvent (followed by three washings of 5 ml.) to a chromatogram made from 150 g. air-dried starch in a 5.1 cm. tube.

The chromatogram was developed with fresh solvent, and in the course of 48 hr. 40 serially numbered fractions were collected in addition to a preliminary fraction (P) which preceded the fastest-moving material. In the early stages, striking red, green and bluish white fluorescent bands were observed on ultra-violet irradiation of the column. The positions of these bands are shown in Fig. 1. The red fluorescent band was associated with a visible red pigment; behind this band the column appeared quite colourless in ordinary light. The effluent fractions were stored at 0° pending investigation.

A drop of effluent from each fraction was placed as a spot on filter-paper. The paper was sprayed with ninhydrin and heated. The relative intensities of the resulting spots, as judged roughly by eye, are illustrated in Fig. 1. Those corresponding to free amino-acids (noted in Fig. 1) appeared more rapidly than the others.

Tryptophan determinations were done colorimetrically on samples of 1/200 of the volume of each fraction. The butanol was evaporated in a vacuum desiccator containing H₂SO₄; 2.5 ml. glacial acetic acid and 2.5 ml. 0.1 % (w/v) solution of *p*-dimethylaminobenzaldehyde in 10 N-HCl were added to this residue, and the resulting solution was stored in the dark for 24 hr. at 37° followed by 24 hr. at room temperature. The resulting blue colour was compared with that of a series of standards of *l*-leucyl-*l*-tryptophan (containing from 0.2 to 12.2 µg. of tryptophan N) which had been treated in the same way. Control experiments had shown that the tryptophan in gramicidin and in leucyltryptophan yielded the same amount of colour under these conditions, whereas that in acetyltryptophan yielded about 20 % less colour. The colour with leucyltryptophan, acetyltryptophan and the partial hydrolysate fractions began to be obvious immediately the reagents were added, whereas with

intact gramicidin it appeared rather slowly. The results of these determinations are shown in Fig. 1.

Too much significance should not be attached to the absolute values, in view of the differences in final colour given by different tryptophan compounds. Summation of the values for all the fractions gives a recovery of about 50 % of the tryptophan originally present.

Amino-acid composition of slower-moving bands

Samples (1/200 of the volume) of each of the tryptophan-free fractions nos. 18–40 inclusive were evaporated to dryness in 'Hysil' test-tubes (Baird and Tatlock, Ltd.) in a vacuum desiccator containing H₂SO₄. The residues were dissolved in 0.12 ml. 6 N-HCl; the tubes were evacuated, sealed and heated overnight at 115°. After the tubes were opened, the acid was removed in a vacuum desiccator containing soda-lime, and a high proportion of the residue in each tube was transferred to a filter-paper strip in distilled water. The strips were developed with *n*-butanol-H₂O, and on colouring with ninhydrin gave the data embodied in Fig. 2, which records relative intensities of amino-acid bands judged by eye. The experiment was repeated with development of the strips with phenol-H₂O, which permitted easier distinction between glycine and alanine, and confirmed the previous result.

On the basis of this information, the fractions were placed in groups A, B, C, D and E as shown in Fig. 2. Samples (1/5 volume) from all the fractions of each group were bulked, and evaporated to dryness below 40° (with a Hyvac pump and an acetone-solid CO₂ bath for condensation). The residues were hydrolyzed in 6 N-HCl at 100° for 24 hr. under a reflux condenser, and analyzed quantitatively on silica gel chromatograms after acetylation, as described above. For each analysis a single 5 % *n*-propanol-cyclohexane column was employed, the

Table 5. *Amino-acid composition of fractions obtained from gramicidin partial hydrolysate*

Fraction-group	As % of total N of original gramicidin			
	Leucine N	Valine N	Alanine N	Glycine N
A	← 0.22* →		2.50	0.17*
B	← 0.14* →		0.15	0.19
C	0.07*	2.46	1.32	2.49
D	← 0.22* →		← 0.40* →	
E	0.49	0.67	0.23	0.53

Figures marked with asterisks were obtained from 'blind' fractions, no corresponding acid band being visible on the chromatograms.

The small quantities of 'background' material in the asterisked fractions may account for some of the material giving fluorescence and colour with ninhydrin (cf. Fig. 1). These substances were not detected in the qualitative amino-acid analysis shown in Fig. 2.

valine, alanine and glycine fractions being run out with 30% *n*-propanol-cyclohexane. The results of these analyses are given in Table 5.

Further investigation of fraction-group A

A further sample (1/5 volume) of this material was evaporated to dryness *in vacuo* below 60°, yielding a crystalline residue. Dissolved in water, the material yielded a slight precipitate on addition of 1 drop of 0.1 *N*-AgNO₃, but required only 0.27 ml. 0.01 *N*-Ba(OH)₂ to render the solution neutral to bromthymol blue, suggesting that mainly free alanine, and not its hydrochloride, was present. The resulting solution was evaporated, acetylated, extracted and analyzed chromatographically, employing 17% *n*-butanol-chloroform as developing solvent. A strong band, having the characteristic rate for acetylalanine, was collected, and was found on titration to account for 90% (corrected) of the alanine content of the fraction (Table 5). The band was followed by a much fainter band, having *R* = 0.1, which was not further studied. The material of the main band, after titration, was hydrolyzed in 6*N*-HCl for 24 hr. at 100°, and subjected to quantitative amino-acid analysis with a single *n*-butanol-chloroform chromatogram. Only alanine was observed and this amounted to 86% (corrected) of a molecule per —COOH group present in the material analyzed.

Further investigation of fraction-group C

Control experiments with synthetic peptides. 3.66 mg. glycyl-*dl*-valine were hydrolyzed and subjected to amino-acid analysis in exactly the same way as in the analyses of Table 5. It yielded 98% of the theoretical valine and 90% (corrected) of the theoretical glycine. No other acid bands were noted in the chromatogram.

3.62 mg. *dl*-valylglycine treated in the same way gave exactly the same appearance on the chromatogram as fraction-group C had done, yielding 63.5% of an equivalent of acetylvaline and 57% (corrected) of an equivalent of acetylvaline per molecule. The band corresponding in rate to acetylalanine (i.e. acetyl-*dl*-valylglycine) yielded 31% (corrected by alanine factor) of an equivalent per molecule.

Optical rotation. All that remained of fraction-group C (77% of the volume) was evaporated to dryness *in vacuo* below 40°, yielding an amorphous product, soluble in ethanol and precipitated from this solution as an oil on addition of ether. On keeping at 0° the oil deposited a few spherulitic crystal growths, which did not increase in size on further keeping. The 23 mg. of amorphous product initially obtained were freed from traces of fatty material by precipitation of a very strong ethanolic solution with excess of ether. 3 mg. were lost in this way, and the product was entirely soluble in water. It gave $(\alpha)_D^{18.5^\circ}$, +58° (water, *c* = 1.25). The rotation is calculated for *valylglycine*, the amount being that estimated from the valine content of the fraction, determined as valine and 'alanine' in Table 5. The highest activity recorded for *l*-valylglycine by Fischer & Scheibler (1908*a*), is $(\alpha)_D^{20^\circ}$, +93.6° (water, *c* = 10); $(\alpha)_D^{20^\circ}$, +39.4° (10% HCl, *c* = 2.8).

Electrometric titration. This was done with 0.1 *N*-HCl and NaOH on 12–13 mg. samples of an initial volume of 5 ml. and at 22°. A Cambridge pH meter with glass electrode was used. The solutions were first taken to pH 2.2 with HCl, then to about pH 10 with NaOH, and then further HCl was added to make the total addition equivalent to the NaOH used. Both the curves of descending pH agreed closely with the curves of ascending pH, and the final pH in each case lay within 0.1 unit of the initial pH. In view of the coincidence of the curves and for clarity of the diagram, only the curves of ascending pH are given (Fig. 3). *dl*-Valylglycine and its diketopiperazine (*dl*-3-*isopropyl*-piperazine-2:5-dione) were compared with the natural product recovered from the determination of optical rotation. The *dl*-valylglycine and its anhydride gave initial pH's of 5.7 and 6.0 respectively, whereas the natural product had an initial pH 2.8 and its solution gave a strong precipitation with AgNO₃, demonstrating that it had emerged from the chromatogram largely as the hydrochloride.

Fig. 3. Comparison by electrometric titration of material in fraction-group C with synthetic materials. +—+ *dl*-3-*isopropyl*piperazine-2:5-dione. □—□ *dl*-Valylglycine. ○—○ Natural product.

In Fig. 3 the pH is plotted against equivalents of NaOH per unit of substance embodying one valine residue. With the natural product the same valine figure is employed as in the optical measurement above.

Acetyl derivative. The solution from the electrometric experiment was evaporated to dryness *in vacuo* below 40°, acetylated and extracted as for a quantitative amino-acid analysis. With this material on a 17% *n*-butanol-chloroform chromatogram of suitably enlarged diameter, a single acid band was observed, having the same rate as acetylalanine. The material from this band was divided in solution into three equal portions—I, II and III.

Solution I was evaporated and stored for reference. The residue crystallized readily.

Solution II was evaporated and weighed after drying to constant weight in a vacuum desiccator over H₂SO₄ and soda-lime. The material was then dissolved in water and titrated to bromthymol blue with 0.01 *N*-Ba(OH)₂. It was thus found to have acid equivalent weight 220. *Acetyl-l*-valylglycine, C₉H₁₆O₄N₂, requires acid equivalent weight 216 (synthetic preparation of acetyl-*dl*-valylglycine, Steiger, 1934*a*). The yield of acetylvalylglycine after acetylation

and extraction was 80% of the theoretical, based on the estimated valine content of the original material.

The material from this titration was hydrolyzed in 6*N*-HCl in a sealed evacuated tube for 36 hr. at 115°. On quantitative amino-acid analysis, employing a *n*-propanol-cyclohexane column, it yielded 95% of a molecule of valine and 88% (corrected) of a molecule of glycine per —COOH group initially present. No other acid bands were observed on the chromatogram. A small sample was employed for paper-strip chromatograms (*n*-butanol-H₂O), in which the hydrolysate was compared by the 'mixed chromatogram' technique with a hydrolysate of synthetic *dl*-valylglycine and with a made-up mixture of *dl*-valine and glycine applied as the hydrochlorides. No divergences were detected in this way.

Optical activity of valine resulting from acid hydrolysis of dipeptide

Solution III (see previous paragraph) was evaporated, and the residue treated with 6*N*-HCl in a sealed evacuated tube at 115° for 36 hr. The hydrolysate was acetylated and extracted in the usual way. To the extract was added the acetylvaline obtained in the analysis of the previous paragraph, and of fraction-group C (Table 5). It was liberated from its Ba salt by addition of the calculated quantity of HCl.

The combined material was then subjected to chromatographic purification on a 3% *n*-butanol-chloroform silica gel column. The acetylvaline fraction thus obtained was hydrolyzed overnight in 6*N*-HCl at 100° and evaporated to dryness. The optical activity of the residue in 6*N*-HCl was $(\alpha)_D^{18.5}, +22^\circ \pm 3^\circ$ ($c=0.9$) (calc. for valine, the amount present being known from the above analyses). This is a high activity in view of the numerous manipulations that might have caused racemization.

Optical activity of valine resulting from hydrolysis of gramicidin

A. 34.6 mg. gramicidin W2 were hydrolyzed in a mixture of 1.5 ml. glacial acetic acid and 1.5 ml. 10*N*-HCl in a sealed evacuated tube for 10 days at 37°. The mixture was

then kept at 115° for 36 hr. Acetylvaline was then prepared from the hydrolysate by exactly the procedure used in quantitative amino-acid analyses of gramicidin (above), using chromatogram tubes of enlarged diameter. The product was hydrolyzed in 6*N*-HCl in a sealed evacuated tube at 100° overnight; this hydrolysate was evaporated to dryness. The optical activity of the residue in 6*N*-HCl was $(\alpha)_D^{20}, -10^\circ \pm 3^\circ$ ($c=1.0$) (calc. for valine with the figure of Gordon *et al.* (1943*b*) for the valine content of gramicidin, 100% recovery being assumed).

B. 34.1 mg. gramicidin W2 were worked up in exactly the same way, except that it was hydrolyzed immediately at 115°, omitting the treatment at 37°. For the valine fraction, $(\alpha)_D^{20}$ was $0^\circ \pm 3^\circ$ (6*N*-HCl, $c=1.0$), calculated on the same basis.

SUMMARY

1. Specimens of gramicidin from two different sources have been compared in respect of a number of properties and further information has been obtained about the ultimate hydrolysis products.

2. Preliminary data are provided on the use of raw potato starch as a medium for partition chromatography of free amino-acids and peptides.

3. Some results are given of the analysis of a partial hydrolysate of gramicidin by this method. From one of the fractions studied the peptide *l*-valylglycine has been obtained in a yield embodying more than half of the glycine of gramicidin.

4. The optical form of the valine residues of gramicidin is discussed in the light of new evidence. It is probable that *d*-valine residues will be discovered to be structural components of gramicidin.

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