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Gramicidin S: the Sequence of the Amino-acid Residues

By R. CONSDEN, A. H. GORDON, A. J. P. MARTIN, *The Wool Industries Research Association, Torridon, Headingley, Leeds 6* AND R. L. M. SYNGE, *The Lister Institute of Preventive Medicine, London*

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We report here a study of the products of partial hydrolysis of gramicidin S by mineral acid acting at low temperature. Identification of these products has permitted an unequivocal formulation of the sequence of the amino-acid residues in this compound. A preliminary report of this work has been published (Consdén, Gordon, Martin & Synge, 1946).

Gramicidin S seemed a particularly favourable object for study, since it had been shown to consist of the residues of the five amino-acids, *l*-valine (*V*), *l*-ornithine (*O*), *l*-proline (*P*), *l*-leucine (*L*) and *d*-phenylalanine (*Φ*) condensed together in equimolecular proportions; the presence of one free amino group/stoichiometric minimum unit, and the absence of free carboxyl groups, as well as the high optical rotation, were taken to indicate a closed peptide-chain (*cyclopeptide*) structure (Synge, 1945b). Subsequently Sanger (1946) established that the free amino group is the δ -amino group of the ornithine residue and the peptide chain therefore consists of amino-acid residues in α -linkage to one another. The crystallographic studies of Crowfoot & Schmidt (to be published) indicate that the molecule comprises either five or ten amino-acid residues; if the latter, the molecule also must possess a twofold axis of symmetry. This could occur only by twofold repetition of a particular sequence of the five different residues. It would be difficult, from studies of the later products of hydrolysis by acid, to distinguish between a given *cyclopentapeptide* and the corresponding *cyclodecapeptide* structure, and we have not attempted to do so. The hydrolysis products which we have identified could all arise by the breakdown, without rearrangement, of the sequence of residues of the structure:

occurring either once or more often in a closed peptide chain. The fact that a high proportion of the possible hydrolysis products have been identified (some of them by more than one technique) gives a firm basis for assigning this structure to gramicidin S. That no products have been noted that are inconsistent with this structure, fits with the simple picture of the structure of gramicidin S which the crystallographic data suggest. A decision between the *cyclopentapeptide* and *cyclodecapeptide* structures could perhaps be obtained by a study of the physical properties of gramicidin S in the dissolved state.

Knowledge of the structure of gramicidin S is likely to prove helpful for the more difficult problem of the nature of tyrocidine, which embodies the same peculiar amino-acid residues (Gordon, Martin & Synge, 1943c), and in which the δ -amino group of the ornithine is likewise free (Christensen, 1945). The present demonstration of the occurrence in nature of a *cyclopeptide* raises the question whether such structures may be embodied in proteins as sub-units (Chibnall, 1942, 1946). Such sub-units would have no characteristic terminal groups available for recognition, and would thus escape detection by methods employing this approach (Fox, 1945; Sanger, 1945).

The unequivocal results of the present study give increased confidence in the use of partial hydrolysis products (at least those formed by acid acting at low temperature) for the study of protein structure. The sort of control that is needed for such work was discussed by some of us six years ago (Gordon, Martin & Synge, 1941; cf. Synge, 1943); work with glutathione (reviewed by Fox, 1945) and gramicidin (Synge, 1944) and the results presented here provide further evidence for the assumption that no rearrangement of linkages occurs during acid hydrolysis. The rather

random character of the attack by acid on peptide structures leads to the conservation (as a variety of lower peptides) of all or most of the peptide bonds present in the original structure. This is an advantage in the study of molecules of relatively simple structure, such as gramicidin S. In the study of more complicated molecules the splitting of particular bonds and separation of the few resulting hydrolysis products seems more desirable as a first step. For this purpose proteolytic enzymes are promising agents, if it can be shown that they do not effect structural rearrangements by synthesizing new peptide bonds. Detailed studies of the action of enzymes on peptides of known structure should throw light on the question.

Techniques employed for study of the partial hydrolysates

Although we had an ample supply of gramicidin S, we deliberately developed and employed methods of analysis and identification applicable to smaller quantities of material, since in studies of other peptides and proteins only small quantities may be available. It is also to be expected that in partial hydrolysates the peptides incorporating particular bonds of the original structure will, as a result of lability under the conditions of hydrolysis, be present only in small quantity. Such peptides may, for structural elucidation, be of equal significance with those that are more abundant, and it is important to have methods suitable for their recognition. It should be noted that the significant experiments in the present work have been effected on altogether less than 10 mg. of gramicidin S. If the amino-acid residues present are known, and authentic specimens of peptides are available for comparison, the criteria of identity here employed seem equal or superior to the traditional criteria which require milligram quantities for their establishment. The identification of peptides by partition chromatography on paper is fully discussed in the preceding paper (Consden, Gordon & Martin, 1947). Four out of the five dipeptides expected to arise from partial hydrolysis were rigorously identified by these means, and the nature of two such tripeptides was also indicated.

In applying to simple peptides Sanger's (1945, 1946) methods of end-group assay (substitution of free basic groups with 2:4-dinitrophenyl (DNP-) radicals) it is possible, after the treatment with fluorodinitrobenzene, to extract the substituted peptides with organic solvents away from any material that has not reacted and which remains in the acidified aqueous reaction mixture. With hydrolysates of material that is known to be fully substituted, identification by paper chromatography of the free amino-acids could be expected to provide valuable information complementary to the identification of the DNP-amino-acids by chromatography on silica gel. Unfortunately, more exacting control experiments than those mentioned in our

preliminary note (Consden, Gordon, Martin & Syngé, 1946) have shown that traces of free amino-acids arise by breakdown of the DNP-amino-acids during hydrolysis by acid; the paper chromatograms with hydrolysates of DNP-peptides have therefore only proved of unequivocal value for the identification of δ -DNP-ornithine.

In work with DNP-amino-acids it was found that non-adsorptive silica gels, such as are useful for partition chromatography of acetylamino-acids (e.g. gels γ and ϵ , Gordon, Martin & Syngé, 1944) do not permit useful separation of DNP-amino-acids employing the solvent-systems of Sanger (1945). When such gels are used in chromatograms, the DNP-amino-acids all run together as fast bands. Samples of silica gel provided by Dr Sanger gave, however, the expected separations. Their action must be attributed to the superposition of rather strong adsorption effects upon the effects due to distribution between the two liquid phases. With the structurally similar 2:4-dinitrophenylhydrazones of aliphatic carbonyl compounds, Roberts & Green (1946) have obtained useful separations with silica in simple adsorption chromatograms.

Other special techniques used in the present work, namely ionophoresis in silica jelly (Consden, Gordon & Martin, 1946) and quantitative determination of acetyl-amino-acids by partition chromatography with silica gel (Gordon, Martin & Syngé, 1943*a*, 1944; Tristram, 1946) had already been employed with complete hydrolysates of gramicidin S (Syngé, 1945*b*). In the present work the complete separation of free ornithine from peptides of ornithine by ionophoresis is a technical point worthy of note.

Identification of peptides present in the partial hydrolysates

From the results with two-dimensional partition chromatograms of unfractionated partial hydrolysates, the dipeptides α -(valyl)ornithine (VO), ornithylleucine (OL), leucylphenylalanine (L Φ) and phenylalanylproline (Φ P) were conclusively identified by the methods of Consden, Gordon & Martin (1947). The experiments also provided evidence for the tripeptides VOL and Φ PV, of which the constituent residues and the residues with free amino groups were identified. This evidence was more than sufficient to establish the sequence of the residues as -VOL Φ P-. Experiments of the same type with the ionophoretic fractions showed that the basic peptide fraction (B) contained, as expected, only the peptides containing ornithine, while the other peptides were found in the neutral fraction (A).

Independent corroboration of the structure was obtained by further study of the basic peptide fraction (B) from the ionophoresis. Quantitative determination of the amino-acids in a complete hydrolysate of this fraction gave the molecular proportions to be $O > V > L > P$; Φ was absent. Since each peptide in this fraction must contain an ornithine residue, arguments analogous to those suggested for acetylpeptides by Gordon, Martin & Syngé (1943*b*) can be applied. In this way are

excluded all but eight of the possible sequences, viz.: -OVLP(Φ)-, -POVL(Φ)-, -PLOV(Φ)-, -LOVP(Φ)-, -VOLP(Φ)-, -LVOP(Φ)-, -PVOL(Φ)-, -PLVO(Φ)-.

In the hydrolysate of the DNP-peptides derived from this fraction, DNP-valine but no DNP-leucine was found. This establishes the occurrence of the sequence -VOL- and limits the possible structures to -VOLP(Φ)- and -PVOL(Φ)-. Unfortunately, the destruction of DNP-proline during the hydrolysis prevented distinction by this method between the two possibilities. It is inferred that the ornithine peptide fraction contained the peptides VO, OL, VOL and PVO. The isolation of δ -DNP-ornithine from this material after treatment with fluoro-2:4-dinitrobenzene confirms Sanger's (1946) conclusion as to the mode of linkage of the ornithine in gramicidin S.

Course of hydrolysis of gramicidin S by acid at 37°

In the absence of quantitative data as to the amounts of each peptide present at each stage of the hydrolysis, the detailed kinetics of the process cannot be analyzed. However, the following facts appear noteworthy. (1) In the low temperature hydrolysis of gramicidin S the rupture of peptide bonds does not show the initially rapid rate followed by marked slowing-down that is characteristic of ordinary proteins (e.g. Gordon, Martin & Syngé, 1941) and gramicidin (Syngé, 1945*a*). With these, it may be supposed that some bonds in the structure are markedly more labile than others, and undergo preferential attack. With gramicidin S this effect is absent; indeed it may be supposed that the original molecule is more resistant to hydrolysis than are the split products immediately derived from it. Such a difference could be attributed to steric effects present in the relatively rigid cyclopeptide structure which disappear when the ring is opened. The kinetics of the attack by pepsin on native proteins has similar qualities (Tiselius & Eriksson-Quensel, 1939). (2) The proportion of free amino-acids formed during the hydrolysis is at all times less than expected, were the peptide bonds split at random. This agrees with previous data that, in general, dipeptides are more resistant to acid hydrolysis than higher peptides embodying the same residues (cf. Syngé (1945*a*)). Dipeptides are, in fact, the predominant peptides in the later stages of hydrolysis, and four out of the five expected dipeptides have been identified. (3) Nevertheless, the bond P-V in the structure appears to behave in a special way. The dipeptide, PV, was not found in the partial hydrolysates, despite a search for it, controlled with synthetic material. Control experiments showed that had the dipeptide PV been liberated in quantities similar to those of the other dipeptides, a proportion of it, sufficient for detection, would have survived hydrolysis for 60 days. How-

ever, the two tripeptides, Φ PV and PVO, which incorporate this bond, appeared to be present in significant amounts. It must be assumed that both these tripeptides and their precursors break down in such a way as not to liberate a significant proportion of PV. Similar kinetic anomalies must, it seems, be anticipated in future structural investigations. Sufficient data do not yet exist for their prediction or rational interpretation in physical terms.

EXPERIMENTAL

Material. For the present study specimen III of gramicidin S (hydrochloride) was used. This was prepared by crystallization from a crude 4% (w/v) ethanolic solution as issued for clinical use in the U.S.S.R. (series 9-1, Control No. 1857, 17/8/44). The recrystallized specimen had physical, chemical and crystallographic properties (Crowfoot & Schmidt, to be published) providing adequate identification with preparations previously studied (specimens I and II, Syngé, 1945*b*).

Low temperature hydrolysis of gramicidin S

Gramicidin S, unlike gramicidin, is soluble in 10N-HCl. However, unless otherwise stated, hydrolysates employed in this work were prepared, as in previous studies with gramicidin and related peptides (Syngé, 1944, 1945*a*), by keeping a 1.2% (w/v) solution of gramicidin S (hydrochloride) in 1:1 (v/v) glacial acetic acid:10N-HCl at 37°. All glacial acetic acid used was purified by repeated crystallization followed by distillation from $K_2Cr_2O_7$. In view of the absence of tryptophan, no special precautions were taken to exclude air; the solution was kept in the incubator in a glass-stoppered vessel and samples for analysis were withdrawn at intervals. The solution darkened very slowly to a pale brown colour.

Further hydrolysis of the samples was effectively prevented by evaporating the samples to dryness *in vacuo* below 40°, dissolving the residues in water (with the earlier samples the addition of a little acetic acid was required to effect solution) and storing at 0°.

Table 1. *Course of low-temperature acid hydrolysis of gramicidin S*

Days of hydrolysis	(As % of total N of gramicidin S)	
	Amino-N	α -N of free amino-acids
0	15.3	0
2	18.9	3.4
10	28.3	6.2
25	50.3	14.5
40	57.0	23.4
54	61.0	27.8
Theoretical for complete hydrolysis (Syngé, 1945 <i>b</i>)	83.3	83.3

Table 1 shows the course of hydrolysis as indicated by determinations of amino-N (Van Slyke—0.5 hr. reaction time) and free amino-acids (ninhydrin- CO_2 according to Van Slyke, Dillon, MacFadyen & Hamilton (1941)—with 50 mg. ninhydrin at volume 2 ml. and pH 2.5). In the absence of information as to the liberation of the imino groups of

Table 2. Amino-acids formed after hydrolysis and after deamination and hydrolysis of spots from two-dimensional chromatograms

(H signifies hydrolysis only. D signifies deamination followed by hydrolysis. The number of crosses indicate the depth of colour given by each amino-acid spot with ninhydrin. Very roughly, each additional cross indicates two to three times as much material. Cut 3 for deamination missed the position of the peptide on the paper. The proline-found on deaminating cut 1 was, presumably, formed from ornithine (cf. Consden *et al.* 1947). Cuts 1a and 1b (see Fig. 1b) were investigated separately but gave the same result. Cuts 3a and b were combined. See text for nomenclature of peptides.)

Cuts	1a and b		2		3a and b		4		5		6	
Amino-acids found	H	D	H	D	H	D	H	D	H	D	H	D
Ornithine	×	×	×	×	×	×	×	×	×	×	×	×
Proline	.	×	.	.	.	.	×	×	×	×	×	×
Valine	×	×	.	.	×	.	.	.	×	×	×	×
Leucine	.	.	×	×	×	.	.	.	×	.	×	×
Phenylalanine	.	.	.	.	.	.	×	×	×	.	×	×
Peptides presumably originally present:												
Main component	VO		OL		VOL		ΦP		ΦPV		LΦ	
Minor component	.		.		.		.		LΦ		ΦPV	

proline residues it is not possible to calculate the average size of the peptides present in the mixture. The quantitative data in Table 1 are, however, consistent with the evidence from identification studies (below) that the peptides of the 58-day hydrolysates were predominantly dipeptides with a small proportion of higher peptides.

Analysis of 58-day hydrolysate of gramicidin S by chromatography on paper. Two lots of 1 mg. each of the material obtained by hydrolyzing gramicidin S for 58 days, as above, were subjected to two-dimensional chromatography on paper in *s*-collidine-3% NH₃ for 37 hr. followed by benzyl alcohol-HCN for 25 hr. One sheet was coloured with 0.1% ninhydrin in *n*-butanol (Fig. 1a) and using this as

washed off, hydrolyzed and analyzed on paper chromatograms using *s*-collidine-3% NH₃ (Consden *et al.* 1947). As may be seen from Fig. 1a, spots 5 and 6 overlapped and complete separation could not be expected. Using similar amounts of material, two further chromatograms were developed and treated in exactly the same way except that the peptides were deaminated before hydrolysis. The amino-acids thus formed and the peptides which they had presumably constituted are shown in Table 2.

No attempt has been made to gauge the strengths of the peptides on the basis of the amounts of amino-acids set free on hydrolysis. Table 3 records the approximate relative intensities of the peptide spots as observed on the two-dimensional chromatograms serving as guides. Evidence from paper chromatograms for the occurrence of prolyl-valylornithine in a 5-day hydrolysate was obtained.

Table 3. Relative intensities of dipeptide spots obtained from a 58-day hydrolysate of gramicidin S

(The significance of the crosses is the same as in Table 2.)

Valylornithine	×	×	×	×	×
Ornithylleucine	×	×	×		
Leucylphenylalanine	×	×	×		
Phenylalanylproline	×	×			

Comparison of the 58-day hydrolysate of gramicidin S with synthetic peptides by chromatography on paper. Two two-dimensional chromatograms were developed as described above, except that no NH₃ was used in the collidine direction. To one sheet, 1 mg. of the 58-day partial hydrolysate of gramicidin S was applied. To the second, was applied 0.5 mg. hydrolysate together with 100 μg. each of the synthetic peptides α-(*l*-valyl)-*l*-ornithine, *l*-ornithyl-*l*-leucine, *l*-leucyl-*d*-phenylalanine, *d*-phenylalanyl-*l*-proline and *l*-prolyl-*l*-valine (Syngé, 1948). Excepting prolylvaline, which was observed as a yellow spot near to and to the right of the valine spot, no extra spots were observed on the sheet carrying the synthetic peptides on colouring with ninhydrin. The *R_F* values of the above peptides were determined on strips and are given in Table 4.

Fig. 1. (a) Diagram of location of amino-acids and peptides on paper chromatogram of 58-day partial acid hydrolysate of gramicidin S. (b) Diagram of cuts made on duplicate chromatogram for isolation of the peptides. Numbers refer to peptide spots. Amino-acid spots are indicated by the following abbreviations: Or, ornithine; P, proline; V, valine; L, leucine; Φ, phenylalanine.

a guide (Consden *et al.* 1947), the amino-acid spots on the other were cut out and also coloured. In the light of this information the peptide spots were cut out (Fig. 1b),

Table 4. R_f values of dipeptides

(The letters after the figures refer to colour given with ninhydrin; PG = pink grey; Y = yellow; without initials = purple.)

Solvent Addition	s-Collidine	s-Collidine NH ₃ (0.3%)	Benzyl alcohol HCN	n-Butanol HCN
α -(Valyl)-ornithine	0.30	0.27	0.00	—
Ornithylleucine	0.38	0.39	0.01	—
Leucylphenylalanine	0.81	0.80	0.62	0.70
Phenylalanylproline	0.85 (PG)	0.83 (PG)	0.35 (PG)	0.56
Prolylvaline	0.60 (Y)	0.52 (Y)	0.11 (Y)	0.10 (Y)

Rate of hydrolysis of prolylvaline. Synthetic *l*-prolyl-*l*-valine (1.2 mg.) was dissolved in 1 ml. glacial acetic acid. 1 ml. 10N-HCl was added and the solution was kept at 37° and tested at intervals by partition chromatography on paper using collidine as solvent; 1.2 mg. ornithylleucine was treated similarly as a control. After 21 days PV and OL both gave strong spots, after 44 days PV was weak while OL was strong, and after 70 days PV was very faint while OL was still strong.

Ionophoretic fractionation of partial hydrolysate. Material resulting from a 58-day hydrolysis of gramicidin S as above was subjected to ionophoresis according to the procedure of Consden, Gordon & Martin (1946, method C). The analysis was carried out in a 4 cm. broad trough containing silica jelly impregnated with 0.1N-ammonium acetate and strengthened by incorporation of paper pulp. The jelly was covered with a glass lid before the beginning of the run. A quantity of material from the hydrolysate containing 6.9 mg. N was dissolved in 0.5 ml. water, and the resulting solution was neutralized (bromothymol blue) with strong ammonia solution (sp.gr. 0.88) and transferred quantitatively to the gutter in a total volume of 1.5 ml. The ionophoresis was allowed to proceed for 18 hr. with 2.3–2.6 V. cm⁻¹. The electrodes were washed with 0.1N-ammonium acetate solution containing for the cathode, additional acetic acid (0.0125N) and, for the anode, additional ammonia (0.0125N). At the end of the run the lid was removed and a print from the jelly was taken on a filter-paper strip. The strip was dried, first in air, and then in a H₂SO₄-soda lime desiccator connected to a Hyvac pump for 1 hr. (to remove ammonium acetate, which is liable to give a background colour in the ninhydrin treatment). It was then sprayed with ninhydrin solution and heated. The resulting pattern is sketched in Fig. 1, together with the cuts decided on. It is of interest that, presumably owing to the presence of copper in the water-glass from which the jelly was made, the bands in cuts B and C in the jelly were coloured faintly blue. The jelly from each cut was dried in a vacuum desiccator, with high vacuum in the later stages to remove ammonium acetate, and the dry material was made into a column in a chromatogram tube (1 cm. internal diameter). H₂S was passed for a few minutes to render the copper insoluble, and then the material was extracted by passing seven columns' length of 1% (v/v) acetic acid. The extracts were evaporated to dryness *in vacuo* and the residues made up to volume in water and stored at 0° until required for study.

It was immediately established by paper chromatography (with s-collidine) that cut C contained free ornithine (apparently unaccompanied by other amino-acids and peptides) and that free ornithine was absent from cut B. It was inferred on theoretical grounds that cut B contained pep-

tides of ornithine and cut A the 'neutral' amino-acids and peptides composed solely of them. The experiments described below show that this was indeed the case, and detailed confirmatory evidence was also obtained by paper chromatography.

Fig. 2. Fractions resulting from ionophoresis of partial hydrolysate of gramicidin S.

Quantitative amino-acid analysis of the ornithine peptide fraction. One-fifth of the material from cut B of the ionophoretic fractionation described above was heated for 48 hr. at 110–115° in 3 ml. 6N-HCl in a sealed evacuated tube. The hydrolysate was acetylated and subjected to chromatographic analysis on silica gel exactly as in the quantitative determination of the amino-acid composition of gramicidin S itself (Synge, 1945b). Applying the same percentage corrections as were then used, the amino-acid composition of the fraction was found to be as given in Table 5. The absence of phenylalanine was further demonstrated by hydrolyzing with acid the material in the 'phenylalanine' and 'leucine' fractions and subjecting the hydrolysate to partition chromatography with benzyl alcohol on paper.

Table 5. Amino-acid composition of the ornithine peptide fraction

(Figures given are calculated for the whole fraction B resulting from the ionophoresis as % of the total N of the partial hydrolysate that was subjected to ionophoresis. No correction has been made for incomplete recovery from the ionophoretic fractionation; figures are therefore minimal.)

	Amino-acid N (as % of total N of gramicidin S)
Phenylalanine	0
Leucine	3.5
Valine	9.2
Proline	1.6
Ornithine	19.2

Investigation of the N-2:4-dinitrophenyl-(DNP)-derivatives of the ornithine-peptide fraction

DNP-amino-acids used in control experiments. DNP-*l*-proline, DNP-*dl*-leucine (Sanger, 1945), α -DNP-*dl*-ornithine, δ -DNP-*dl*-ornithine, δ -DNP-*l*-ornithine HCl·H₂O (Sanger, 1946) were made available by Dr F. Sanger. DNP-*dl*-valine was prepared from *dl*-valine in 90% yield by the procedure used by Sanger (1945) in making DNP-*dl*-alanine. The product had m.p. 182–184° (uncorr.; constant on recrystallization from methanol-H₂O). Abderhalden & Blumberg (1910) record 185°.

$\alpha\delta$ -Di(DNP)-*l*-ornithine. 0.1 g. *l*-ornithine HCl was kept for 24 hr. with 0.4 g. NaHCO₃, 5 ml. water, 10 ml. ethanol, and 0.28 ml. fluorodinitrobenzene. The mixture was then evaporated *in vacuo* at room temperature. An orange gum separated, which later solidified. This was presumed to be the Na salt of di(DNP)-ornithine. The solid was triturated thoroughly with ether, to remove fluorodinitrobenzene, and the residue was then suspended in aqueous acetone and treated with excess 10N-HCl. Pale yellow crystals precipitated. Acetone was removed by partial evaporation *in vacuo* and after keeping overnight at 0° the crystals were filtered off, washed with water and dried in a vacuum desiccator. Yield was 0.29 g. Recrystallization was effected from hot acetic acid. The yield of crystals which formed on cooling was increased by diluting the mixture with water, in which the crystals were very insoluble. The material after drying in a desiccator had m.p. 155–157° (uncorr.; constant on recrystallization) and lost weight on further drying at 100° over P₂O₅ *in vacuo*, indicating the presence of water of crystallization. The material dried at 100° *in vacuo* appeared from the analytical data (Weiler, Oxford) to contain still at least one molecule of water of crystallization. (Found: C, 42.6; H, 3.42; N (Dumas), 17.2. C₁₇H₁₈O₁₀N₆·H₂O requires C, 42.3; H, 3.74; N, 17.4%.)

Procedure for identification of DNP-amino-acids in acid hydrolysates of DNP-peptides. It was required to demonstrate the presence or absence of $\alpha\delta$ -di(DNP)-ornithine, δ -DNP-ornithine, DNP-valine, DNP-leucine and DNP-proline. Preliminary experiments with the pure substances and with mixtures of them showed that the following procedure was satisfactory. For reasons already given, the silica gel used in this work was a preparation by Dr Sanger 'batch 7'. Each silica gel partition chromatogram employed 3 g. of this material made up with the appropriate solvent-system in a tube of 1 cm. internal diameter. The fractionation was based on the data of Sanger (1945), and followed his general procedure.

The mixture for study in aqueous 3.5N-HCl was extracted thrice with equal volumes of ether. The combined ether extracts contained all the DNP-amino-acids other than δ -DNP-ornithine which remained in the aqueous layer and was reserved for paper chromatography (see below). The combined ether extracts were evaporated to dryness *in vacuo*, and chromatographed on an ether-H₂O column. All the yellow material, which moved as a single fast band, was collected in the effluent from the chromatogram and evaporated to dryness *in vacuo*. It was then chromatographed on a chloroform-H₂O column (solvent, B.P. chloroform freed from ethanol by extracting it four times with equal volumes of water). The DNP-derivatives of leucine, valine and proline ran fast, and were collected as before; $\alpha\delta$ -di(DNP)-ornithine moved as a yellow band having

R, 0.1–0.2. After the other DNP-amino-acids had left the column the di(DNP)-ornithine could be accelerated (*R* = 1) by developing with 1% *n*-butanol-chloroform. The fast moving fraction from the chloroform column was then analyzed on an ethanol-ligroin-water column (Sanger, 1945). Three separate bands were obtained; DNP-leucine (*R* = 1), DNP-valine (*R* = 0.4–0.6) and DNP-proline (*R* = 0.2–0.3).

The aqueous residue from the hydrolysate, after ether extraction, was evaporated to dryness *in vacuo*, the residue was dissolved in water and a suitable sample was applied to a paper strip and neutralized with NH₃-vapour; the strip was then developed as a chromatogram with *n*-butanol-H₂O according to the procedure of Consden, Gordon & Martin (1944). Under these conditions δ -DNP-ornithine ran as a yellow band (ahead of leucine, *R_F* = 0.6 approx.). Unlike α -DNP-ornithine, the yellow band of δ -DNP-ornithine became purple on treatment with ninhydrin, and was thus readily recognized (cf. Sanger, 1946 for further data on the evolution of CO₂ by these compounds in the reaction with ninhydrin).

Control experiments. The following mixtures were heated in 4 ml. 5N-HCl for 48 hr. at 110–115° in sealed evacuated tubes: (X) 1.7 mg. $\alpha\delta$ -di(DNP)-*l*-ornithine hydrate, 0.1 mg. *l*-proline, 0.33 mg. *dl*-leucine and 0.45 mg. *dl*-valine; (Y) 0.96 mg. δ -DNP-*dl*-ornithine, 0.78 mg. DNP-*dl*-valine, 0.41 mg. DNP-*dl*-leucine, 0.5 mg. DNP-*l*-proline, 0.1 mg. *dl*-leucine and 0.1 mg. *dl*-valine. The resulting mixtures were subjected to analysis as above. Much of the di(DNP)-*l*-ornithine in (X) had remained undissolved, and was filtered off before conducting the analysis. (X) yielded no coloured material in the aqueous residue from ether extraction, and revealed only one yellow band on the chloroform chromatogram, agreeing in rate with $\alpha\delta$ -di(DNP)-ornithine. (Y) yielded a strongly yellow aqueous residue and paper chromatography showed a single yellow band, agreeing with δ -DNP-ornithine both in rate and in colouring with ninhydrin. Chromatography with silica gel revealed, on the ethanol-ligroin-H₂O column, only the bands of DNP-leucine and DNP-valine. These experiments indicate that, with the vigorous hydrolysis employed, DNP-proline is largely destroyed. $\alpha\delta$ -Di(DNP)-ornithine, δ -(DNP)-ornithine, DNP-valine and DNP-leucine survive hydrolysis well, and no mono-(DNP)-ornithines are formed from $\alpha\delta$ -di(DNP)-ornithine.

Preparation and study of DNP-derivatives of peptides in ionophoretic fraction B (ornithine peptides)

One-tenth of the extract from cut B of the ionophoresis experiment (see above) was allowed to react at room temperature (intermittent shaking) in 1.5 ml. 70% (v/v) aqueous ethanol with 0.04 g. NaHCO₃ and 0.03 ml. fluorodinitrobenzene. The mixture slowly turned yellow. After 48 hr. the mixture was evaporated *in vacuo* at room temperature, and the resulting aqueous emulsion, diluted to 7 ml. with water, was extracted thrice with equal volumes of ether to remove unchanged fluorodinitrobenzene. Very little yellow colour passed into the ether extracts, which were discarded. Residual ether was removed from the aqueous layer by evaporation *in vacuo* at room temperature. On acidification with 1 ml. 0.85N-HCl a partly crystalline yellow oil separated. On extracting the mixture with ether, most of the yellow colour passed into the extract, and after three such extractions the aqueous residue was colourless.

On evaporation of the combined ether extracts *in vacuo* the residue obtained was a yellow oil, containing some pale yellow dendritic needles, interspersed with more orange spherulites. The whole was readily soluble in ether, and all the yellow material was recovered as a fast-moving band from an ether-H₂O chromatogram. Attempts at analyzing the mixture on a chloroform-H₂O column were unsuccessful, since the yellow material, apart from a very small amount with $R=0.6$, moved as a band whose front had $R=0.1$, but which tailed excessively without resolving. All the yellow material was accordingly washed from the chromatogram with 1% *n*-butanol-chloroform. The extract was evaporated to dryness *in vacuo* at 60° and the residue was hydrolyzed with 4 ml. 5*N*-HCl in a sealed evacuated tube at 110–115°. The material dissolved only slowly; heating was continued for 48 hr. after solution was complete (16 hr.). At the end of this period there was only a very slight separation of crystals of $\alpha\delta$ -di(DNP)-ornithine on cooling, and these dissolved during the subsequent extraction with ether. The analysis was continued as described above. Both the ethereal and aqueous solutions resulting from the initial extraction were strongly yellow. Silica gel chromatography indicated the presence of $\alpha\delta$ -di(DNP)-ornithine and of DNP-valine, but DNP-leucine and DNP-proline were absent. δ -DNP-ornithine was identified by paper chromatography as the yellow constituent of the aqueous layer.

SUMMARY

1. Gramicidin S has been shown to yield, on hydrolysis with acid at 37°, the dipeptides α -(valyl)-ornithine, ornithylleucine, leucylphenylalanine and

phenylalanylproline. The tripeptides α -(prolylvalyl)-ornithine, α -(valyl)-ornithylleucine and phenylalanylprolylvaline are also probably present. A variety of techniques employed for the identifications has yielded concordant results.

2. It is inferred that these peptides arise by the breakdown, without rearrangement, of the structure (α -(*l*-valyl)-*l*-ornithyl-*l*-leucyl-*d*-phenylalanyl-*l*-prolyl-), occurring once or twice in a closed peptide chain. Gramicidin S, on the basis of the physical information so far available, is best formulated as the cyclodecapeptide or cyclopentapeptide possessing this structure.

3. The fact that hydrolysis by acid at low temperature produces many peptides, all derived without rearrangement by the breakdown of a single structure, enables the identification of peptides in such hydrolysates to be used with increased confidence for studies of the sequence of amino-acid residues in peptide structures generally.

4. Some peculiarities in the kinetics of hydrolysis of gramicidin S are noted.

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