'Gramicidin S': Over-all Chemical Characteristics and Amino-acid Composition

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(Received 12 June 1945)

'Gramicidin S' (Soviet gramicidin) is the name given to a crystalline substance of polypeptide character and possessing antibacterial activity, which is elaborated by a strain of *Bacillus* isolated from garden soil. The earlier studies concerning it have been brought together in a publication by Sergiev (1943), of which a short abstract has appeared in English (Gause & Brazhnikova, 1944*a*). More recent data are given by Gause & Brazhnikova (1944*b*), Belozersky & Passhina (1944) and Sergiev (1944).

The present chemical study has extended, and in part confirmed, the preliminary chemical data reported by Belozersky & Passhina. From the data now presented it emerges that gramicidin S is the hydrochloride of a basic peptide, whose stoichiometric minimum unit embodies one residue each of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine and *d*-phenylalanine, possesses one free basic (amino) group and no free carboxyl groups, and is combined with one residue of chloride. The properties and composition of the substance remain constant on recrystallization.

The relatively fast reaction of the amino-N of gramicidin S with HNO_2 reported by Belozersky & Passhina (1944) does not seem an adequate reason for their denial that the amino group is in the side-chain of the ornithine residues, since relatively little is known, so far, of the reaction rates of the amino-N of complicated peptides. Gramicidin S did not evolve CO_2 on heating with ninhydrin in acetic acid. The colour reaction with ninhydrin, noted by Belozersky & Passhina and in the present study, does not necessarily signify that free amino groups in the α -position are present, since a wide variety of nitrogenous compounds gives a colour on heating with ninhydrin. In the present study, electrometric titration revealed no groups ionizing in the pH range usually associated with carboxyl groups. Excessive 'protein error' with the indicator employed prevented determination of carboxyl groups by acetone titration. Belozersky & Passhina state that carboxyl groups are present. They appear not to have noticed the presence of chloride in gramicidin S.

Further physical studies will be necessary before it can be decided whether the stoichiometric minimum unit represents the 'true' molecule, or is some submultiple thereof. But whatever the degree of polymerization, and whatever the location of the

free amino groups established by future chemical study, it is difficult to escape the conclusion that gramicidin S embodies a 'cyclopeptide' structure with an endless closed peptide chain. Its very high optical rotation may be regarded as supporting this view (cf. Kauzmann, Walter & Eyring, 1940).

The analytical data on which the present conclusions rest are summarized in Tables 2, 3, and 4. They suggest that one molecule of 'bound' water is probably present in dried gramicidin S per stoichiometric unit of 5 amino-acid residues formed from the free amino-acids by elimination of 5 molecules of water. The theoretical requirements in Table 2 are calculated for 0, 1 and 2 such 'bound' water molecules.

The amino-acid data (Tables 3 and 5) present a point of analytical interest. In view of Belozersky & Passhina's report that gramicidin S is hydrolyzed rather slowly with acid, vigorous conditions were employed for hydrolysis, and control experiments with known mixtures of the amino-acids under these conditions showed that some destruction ensued. With tyrocidine hydrolysates excessive variability has been noted in the amino-acid yields (Gordon, Martin & Synge, 1943*c*; Christensen, Uzman & Hegsted, 1945). However, with gramicidin S such variations were not found; correction of the yields on the basis of the recoveries found with known mixtures of amino-acids (Table 5) gave stoichiometric results (Table 3). This suggests that destruction of amino-acids during hydrolysis of gramicidin S is not greatly different from that of the free amino-acids and their acetyl derivatives under the same conditions. This difference between the behaviour of tyrocidine and gramicidin S may tentatively be attributed to the presence of tryptophan in the former.

The chemical data now presented make it clear that gramicidin S is very closely related indeed to tyrocidine. The occurrence, as primary hydrolysis products of these two substances, of both *d*-phenylalanine and *l*-ornithine in itself renders them unique among natural products hitherto described. The relation is made clear by reference to Table 1. It is seen that 2 units of gramicidin S could be embodied as such in 1 unit of tyrocidine. The high negative rotations of both substances may be regarded as indicative of common structure.

Table 1. *Comparison of numbers of residues embodied in stoichiometric minimum units of tyrocidine hydrochloride and gramicidin S*

	Tyrocidine HCl*	Gramicidin S
<i>l</i> -Ornithine	2	1
<i>l</i> -Proline	2	1
<i>l</i> -Valine	2	1
<i>l</i> -Leucine	2	1
<i>d</i> -Phenylalanine	3	1
<i>l</i> -Tryptophan	2	0
<i>l</i> -Tyrosine	2	0
<i>l</i> -Aspartic acid	2	0
<i>l</i> -Glutamic acid	2	0
Ammonia	3	0
Chloride	2	1

* Hotchkiss (1944); Christensen *et al.* (1945).

Dr R. D. Hotchkiss kindly examined the nature of the antibacterial action of a specimen of grami-

cidin S and stated (private communication) that it is similar to that of tyrocidine, and different from that of gramicidin (cf. Hotchkiss, 1944). Great chemical similarity to tyrocidine has now been demonstrated. Hotchkiss (1944) attributes the bactericidal action of tyrocidine to the presence of basic and fatty groups in the same molecule, in other words he concludes that it is similar in nature to that of the cationic detergents. Gause & Brazhnikova (1944b) report that gramicidin S is more effective than tyrothricin against Gram-negative bacteria. Tyrocidine is the major component of tyrothricin and the one known to be active against Gram-negative organisms. A direct comparison in this laboratory of the activity of gramicidin S and tyrocidine hydrochloride against various strains of *Neisseria* spp. and *Aerobacter aerogenes* in broth containing 10% (v/v) of serum has confirmed that

Table 2. *Determinations on unhydrolyzed gramicidin S*

	% of dried. gramicidin S, Specimen I		Required for $C_{30}H_{46}O_6N_6 \cdot HCl - X \cdot H_2O$		
	Present work	Belozersky & Passhina (1944)			
			X=0	X=1	X=2
C	57.5	—	59.3	57.7	56.0
H	7.6	—	7.75	7.86	7.94
N (Kjeldahl)	12.9–13.3	13.0	13.8	13.5	13.1
Cl (Carius)	5.6	—	5.86	5.69	5.53
S (Carius)	Nil	—	Nil	Nil	Nil
Combustion residue	Nil	—	Nil	Nil	Nil
P	0.07	—	Nil	Nil	Nil
OMe (Zeisel)	Nil	—	Nil	Nil	Nil
Chloride (Volhard)	4.8	—	5.86	5.69	5.53
Amino-N (Van Slyke): 5 min.	—	1.64	2.31	2.24	2.18
Amino-N (Van Slyke): 30 min.	—	1.87	2.31	2.24	2.18
Basic groups as N (ethanol titration) (1 equiv. = 1 atom)	—	1.97	2.31	2.24	2.18
Basic groups (electrometric titration)	2.0–2.5	—	2.31	2.24	2.18
Carboxyl groups (electrometric titration)	Nil	Present	Nil	Nil	Nil
α -Amino groups (ninhydrin- CO_2)	Nil	—	Nil	Nil	Nil
Tryptophan (colorimetric)	Nil	Nil	Nil	Nil	Nil

Table 3. *Determinations on acid hydrolysates of gramicidin S*

Determination	As % of total N of gramicidin S			
	Present work		Belozersky & Passhina (1944)	Theoretical
	Gramicidin S Specimen I	Gramicidin S Specimen II		
Amino-N (30 min. Van Slyke)	84.7	—	90.1	83.3
α -Amino-N (ninhydrin-CO ₂)	79.0	—	—	83.3
NH ₂	0.2	—	0.7	Nil
Hydroxyamino-N	0.2	—	—	Nil
Humin-N	—	—	0.3	—
Basic amino-acid N	—	—	27.0	33.3
Ornithine-N	33.0	33.6	29	33.3
Proline-N	16.7	16.8	9-14	16.7
Valine-N	17.1	17.1	—	16.7
Leucine-N	16.8	17.3	About 33*	16.7
Phenylalanine-N	16.6	16.1	Nil	16.7
Tyrosine (colour reaction)	Nil	—	Nil	Nil
Other usual protein constituents (Consden, Gordon & Martin, 1944)	Nil	—	Various tests negative	Nil

* The leucine figure of 70% of Belozersky & Passhina (1944) is a misprint for 40%.

gramicidin S is much the more effective of the two. This may tentatively be attributed to its higher relative content of basic (ornithine) residues.

The name gramicidin S conveys correctly the fact that the new peptide has a similar origin and mode of preparation to the gramicidin of Dubos and Hotchkiss (see Hotchkiss, 1944). However, it is confusing for a number of reasons: chemically the substance contains only one species of residue (*L*-valine) in common with gramicidin; it possesses, unlike gramicidin, considerable antibacterial activity against Gram-negative organisms; it appears to exert its antibacterial activity by virtue of a different mechanism. In all these respects the new peptide has close affinities with tyrocidine. It is to be hoped that the discoverers will consider renaming it in such a way as to bring out these affinities.

Gramicidin S provides an interesting addition to our knowledge of the polypeptides synthesized by *Bacillus* spp. Structurally, it may turn out to be simpler than gramicidin and tyrocidine, and study of it, apart from its intrinsic interest, may prove helpful for structural studies of tyrocidine.

EXPERIMENTAL

Material used and constancy of properties on recrystallization

The first specimen of gramicidin S studied was crude or 'clinical' material in aqueous-ethanolic solution, received through the Medical Research Council; from this, crystalline material was readily prepared by crystallization from aqueous ethanol after removal of fat (cf. Sergiev, 1943). The once recrystallized product cannot have been pure, since two-dimensional partition chromatograms on paper with an acid hydrolysate (Consden, Gordon & Martin, 1944) revealed the presence of faint spots corresponding to amino-acids other than those eventually found in the pure material.

The specimen employed for most of the studies reported here was crystalline material, as received from Prof. P. G. Sergiev, Institute of Tropical Medicine, Moscow. This material is here designated I. Specimen II was obtained from I by dissolving it in 70% (v/v) aqueous ethanol, and allowing crystallization to occur as the solution evaporated at room temperature. A loss of 60% was permitted in the recrystallization. Specimen II had the same N content, optical rotation (see below) and amino-acid composition (Table 3) as specimen I. Gramicidin S is notably more soluble in aqueous than in absolute ethanol.

Optical rotation. This was determined in 70% (v/v) aqueous ethanol ($l=0.5$, $c=1.5$):

Specimen I: $[\alpha]_D^{18}$, -292° . Specimen II: $[\alpha]_D^{20}$, -295° .

Chemical analyses on intact gramicidin S

The results are shown in Table 2. Air-dry gramicidin S loses 3–5% by weight of moisture on drying over P_2O_5 *in vacuo* at 100° . Analyses on intact gramicidin S are referred to material dried in this way. C, H, S, Cl (Carius) and OMe determinations were by Dr G. Weiler, Oxford. P was determined by Dr M. G. Macfarlane colorimetrically

after incineration with H_2SO_4 and perhydrol. N (Kjeldahl) determinations were done with 6 hr. incineration in the mixture of Campbell & Hanna (1937).

Chloride (Volhard) titrations. 5–10 mg. gramicidin S (dissolved in 1.5 ml. 70% ethanol) were treated with 2 ml. 0.02N- $AgNO_3$ (made up in approx. 2N- HNO_3) at room temperature, and back-titrated with 0.02N-KCNS in the presence of iron alum. The rather low results may be attributed to partial precipitation of gramicidin S with Ag under these conditions.

Tryptophan. This was determined colorimetrically with *p*-dimethylaminobenzaldehyde in acetic acid-HCl mixtures (cf. Synge, 1944).

Ninhydrin- CO_2 determination. This was done according to Van Slyke, Dillon, MacFadyen & Hamilton (1941) in 1 ml. glacial acetic acid with 50 mg. ninhydrin, and heating at 100° for 5 min. Gramicidin S (16 mg.), on being heated under these conditions, gave a bright red colour. The CO_2 resulting was not significantly different from the reagent blank.

Electrometric titration. This was done with a Cambridge pH meter, fitted with glass electrode. 100 mg. gramicidin S were suspended in 10 ml. water. On titrating with 0.1N-HCl, the pH curve was not significantly different from that for the water blank. The solution had initial pH 3.7. The addition of NaOH equivalent to the HCl added brought the pH back to this value. Some crystallization from the solution of gramicidin S was noted during the addition of HCl. Presumably this was a solubility product effect. When more 0.1N-NaOH was added, the pH rose above 6 with the first drop; maximum buffering seemed to occur at about pH 8.5. The material in suspension swelled and became gelatinous and lumpy, in such a way that the centres of the lumps could not react with the alkali. Repeated neutralization of the alkali with 1 equiv. HCl (which brought the pH back to its original value) was followed by rendering alkaline again with stirring. This broke up the lumps and appeared to bring all the material into a reactive condition; by comparison with the water blank an estimate could be formed of the free basic groups of gramicidin S (see Table 2).

Acid hydrolysis of gramicidin S

Hydrolysis was effected in sealed evacuated tubes with excess of a 1:1 (v/v) mixture of glacial acetic acid and 10N-HCl for 48 hr. at 110 – 115° . The chromatographic studies of hydrolysates prepared in this way gave no indications of peptides that had escaped hydrolysis. The hydrolysates contained a little humin, which was absent from mixtures of the known amino-acid constituents of gramicidin S treated in the same way. Before further study, hydrolysates were evaporated to dryness *in vacuo*, made up to known volume with water, and stored at 0° .

Study of acid hydrolysates of gramicidin S

The results are shown in Tables 3 and 4.

Amino-N was determined by the Van Slyke manometric procedure with 30 min. reaction time. No yellow colour was noted in the reaction mixture, the absence of tyrosine from the hydrolysate thus being confirmed.

Ninhydrin- CO_2 . The method of Van Slyke *et al.* (1941) was employed. With both pH 2.5 and 4.7 citrate buffers the same result was obtained, and the low value may have its origin in the destruction of amino-acids during the

hydrolysis, noted in the determinations of individual amino-acids.

NH_3 - and hydroxyamino-N. These determinations were carried out by the Conway modification of Van Slyke's procedure described in the preceding paper (Synge, 1945).

Identification of individual amino-acids in hydrolysate. Drs Consden, Gordon & Martin examined the hydrolysate of gramicidin S (specimen I) for amino-acids by their (1944) two-dimensional partition chromatographic method with paper, employing phenol and collidine for development. Only five components were noticed, corresponding in rates to the five amino-acids subsequently shown to be present. Special control experiments with butanol-benzyl alcohol mixtures on one-dimensional paper chromatograms showed that the leucine component was free from isoleucine. The results with paper chromatograms were confirmed by preliminary partition-chromatographic fractionation of acetylated hydrolysates on silica gel, done in this laboratory and employing cyclohexane-propanol- H_2O and chloroform-butanol- H_2O systems successively.

The isolation of the five amino-acid components was then undertaken. An acid hydrolysate of 100 mg. gramicidin S (I) was first fractionated by the ionophoretic procedure of Consden, Gordon & Martin (which is mentioned in the preceding paper (Synge, 1945)). Sodium acetate jelly was employed at pH 6. Two well-defined fractions resulted, the ornithine migrating towards the cathode faster than the other amino-acids. The two fractions were worked up and subjected separately to the acetylation and extraction procedure described by Gordon, Martin & Synge (1943*a*). In the case of the ornithine fraction, the evaporated extract yielded crystals of diacetylornithine without further treatment. The product from the other ionophoretic fraction was submitted to partition chromatographic fractionation on silica gel chromatograms. With 5% propanol-cyclohexane the 'leucine-phenylalanine' fraction was allowed to run out of the chromatogram. The solvent was then changed to 30% propanol-cyclohexane and the 'valine' and 'proline' fractions were run out and collected separately. After evaporation, the 'valine' and 'proline' residues were freed from indicator (peonin) by filtration, in 17% butanol-chloroform, through a little water-saturated silica gel (cf. Gordon *et al.* 1943*c*). The 'leucine-phenylalanine' residue was resolved by chromatographic fractionation with butanol-chloroform on silica gel employing the ' $R\text{-NH}_2$ ' indicator of Liddell & Rydon (1944). All the resulting fractions were evaporated to dryness, giving the corresponding acetylamino-acids in approximately the expected yields.

The optical rotation of each of the five fractions containing the acetylamino-acids was then determined. It seemed preferable, departing from previous practice (cf. Gordon, Martin & Synge, 1943*b, c*; Synge, 1944), to determine this before crystallizing, since the process of crystallization might lead to fractionation of optical isomers. The amount of acetylamino-acid, on which the calculation of specific rotation was based, was determined by titrating a sample of the solution used for polarimetry with 0.01 N -Ba(OH)₂ to bromthymol blue in the usual way. The residues from the optical determinations were then used for crystallizing and characterizing the acetylamino-acids with the results shown in Table 4. Data on the authentic reference compounds and on corresponding fractions obtained from tyrocidine are given by Gordon *et al.* (1943*c*). Reference to

Table 4. Characteristics of acetylamino-acids isolated from hydrolysate of gramicidin S

Compound	Formula	Optical rotation			Crystallized from	Uncorrected			% C		% H		% N		Acid equiv. wt.	
		[α] _D	° C	l=0.5 c =		m.p.	Mixed m.p.	m.p. of authentic material used in mixed	Found	Calc.	Found	Calc.	Found	Calc.	Found	Calc.
<i>NN'</i> -Diacetyl- <i>L</i> -ornithine	C ₈ H ₁₆ O ₄ N ₂	+ 3°	20.5	1.8	Ethanol	156°	157°	157°	50.6	50.0	7.6	7.4	13.0	13.0	216	216
Acetyl- <i>L</i> -proline	C ₇ H ₁₁ O ₃ N	-118°	22	1.0	Water	83°*	82°*	81°*	54.1	53.5	7.2	7.0	7.7*	8.0*	174*	175*
Acetyl- <i>L</i> -valine	C ₇ H ₁₃ O ₃ N	+ 4°	21.5	1.2	Ethanol	163°	165°	168°	54.2	52.8	7.9	8.2	8.5	8.8	156	159
Acetyl- <i>L</i> -leucine	C ₉ H ₁₉ O ₃ N	- 28°	20	1.2	"	182°	185°	186°	54.8	55.5	8.6	8.7	7.7	8.1	175	173
Acetyl- <i>D</i> -phenylalanine	C ₁₁ H ₁₅ O ₃ N	- 48°	20	1.3	"	167°	165°	164°†	64.8	63.7	6.8	6.3	6.6	6.8	206	207

* Determined with air-dry hydrate, C₂H₅O₂N·H₂O.

† Material isolated from tyrocidine (Gordon *et al.* 1943*c*).

* Determined with air-dry hydrate, $\text{C}_7\text{H}_{11}\text{O}_3\text{N} \cdot \text{H}_2\text{O}$.

† Material isolated from tyrocidine (Gordon *et al.* 1943*c*).

this paper will show that the acetyl-amino-acids other than diacetylornithine isolated in the present study possessed fairly high optical rotations indicating that little racemization could have occurred during hydrolysis and isolation. The configuration of two of the acetyl-amino-acids from gramicidin S (acetyl-*d*-phenylalanine and acetyl-*l*-leucine) was further checked, since admixture with authentic specimens of their optical antipodes caused considerable (about 20°) depression of m.p.

Quantitative determination of amino-acids in hydrolysate. Quantitative analyses were conducted on hydrolysates of approximately 10 mg. gramicidin S by the general procedure of Gordon *et al.* (1943 *a*). The 'separating funnel' acetylation and extraction procedure was employed. This gave low but reproducible recoveries of ornithine. The scheme of fractionation shown in Fig. 1 was employed with the acetylated extract.

- * Fraction run out in 5% propanol-*cyclohexane*.
 † Fraction run out in 30% propanol-*cyclohexane*.
 ‡ Fraction run out in 3% butanol-chloroform.

Fig. 1.

For the chromatograms, silica gel batch γ was used throughout; with the *cyclohexane* chromatograms, peonin chloride was used as indicator, and with the chloroform chromatograms, methyl orange (Gordon, Martin & Synge, 1944).

The analyses were controlled by subjecting a mixture, in the correct quantities, of acetyl-amino-acids to the same hydrolytic and analytical procedures. Percentage recoveries in two separate experiments are shown in Table 5.

The values shown in Table 5 have been used as the basis for correcting the yields in the analyses of gramicidin S

Table 5. *Recovery of amino-acids in control experiments*

Material hydrolyzed and analyzed	Percentage recovery	
	(a)	(b)
Acetyl- <i>dl</i> -phenylalanine	93	92
Acetyl- <i>dl</i> -leucine	95	94
Acetyl- <i>dl</i> -valine	95	95
Acetyl- <i>l</i> -proline	103	101
<i>NN'</i> -Diacetyl- <i>l</i> -ornithine	70	72

hydrolysates; the corrected figures for analyses of specimens I and II appear in Table 3.

Direct chromatography of the mixture of acetyl-amino-acids used in the control experiments led to recoveries within 3% of theory; it is therefore to be assumed that destruction occurs during the hydrolytic treatment, since the acetylation and extraction procedure gives quantitative recovery of the amino-acids other than ornithine (Gordon *et al.* 1943 *a, b*; Synge, 1944).

SUMMARY

1. It is probable that the antibacterial substance gramicidin S is a cyclopeptide hydrochloride having a stoichiometric minimum unit formed from one residue each of *l*-ornithine, *l*-proline, *l*-valine, *l*-leucine and *d*-phenylalanine. The unit possesses one free amino-group, no free carboxyl groups and one residue of chloride.

2. The relationship of gramicidin S to tyrocidine hydrochloride is emphasized, and a plea is made for renaming gramicidin S to bring out this relation.

I wish to thank Prof. P. G. Sergiev, Moscow, and the Soviet Red Cross for a supply of gramicidin S and for information about it; Drs R. Consden, A. H. Gordon, and A. J. P. Martin, Leeds, for carrying out the chromatographic experiments with paper; and Dr M. G. Macfarlane for P determinations. I am grateful to Mr W. Wolff for his technical assistance.

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