TABLE I.
Summarized Analytical Data.

Materials*	Nicotinic acid: HCl extraction: Samples used	Avg γ/g	Range γ/g	Nicotinic acid: NaOH extraction: Samples used	Avg γ/g	Range γ/g	Pantothenic acid: Samples used	Avg γ/g	Range γ/g
Sunflower seed meal	7	184	173-198	5	312	285-330	8	43	33-58
Wheat germ meal (A)	2	77	72-82	3	67	63-72	8	30	23-35
" " " (B)	2	40	38-42	3	39	38-40			
Corn germ meal	5	38	35-40	5	35	34-36	8	25	24-26
Soybean meal	4	27	26-29	4	24	23-26	9	43	34-59
" " (expeller)	2	17	16-19	2	17	17-18	10	26	19-32

* Furnished by Archer-Daniels-Midland Company, Minneapolis, Minn.; Central Soya Company, Decatur, Ind.; and VioBin Corporation, Monticello, Ill.

proximately 60% greater than that of soybean meal produced by an expeller process. The corn germ and wheat germ have less pantothenic acid than the sunflower seed meal. This is probably due to inherent differences between these materials and not to differences in the manufacturing processes because the corn germ, one lot of wheat germ, and the sunflower seed were subjected to the same conditions in the removal of the oil and in the subsequent treatment.

Summary. Sunflower seed meal is an excellent source of nicotinic acid. The content of pantothenic acid is equal or superior to that of soybean meal, wheat germ or corn germ.

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Chemical Derivatives of Gramicidin.

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Gramicidin has been found to react readily with formaldehyde to yield a product of improved pharmaceutical properties.[1] A study of the reaction mechanism indicated that formaldehyde was added in the form of hydroxymethyl groups quantitatively to the indole rings of the tryptophane,[2] which occurs to the extent of 40% in gramicidin. It was the object of the present investigation to search for other reagents which might, like formaldehyde, reduce the hemolytic and toxic but not the antibiotic activity of gramicidin, and at the same time render the derivative more water-soluble.

Reaction with Various Aldehydes. Representative results obtained with a series of aldehydes are summarized in Table I, Section A. Nitrogen analyses indicated that all aldehydes used combined with gramicidin, though to a variable extent. Acetal and glucose did not react appreciably. In contrast to the selective effect of formaldehyde, which decreases the hemolytic but not the antibacterial activity, all other aldehydes appeared to affect both activities to a similar degree. The aliphatic aldehydes generally caused a slight reduction, but glyoxal and

[1] Lewis, J. C., Dimick, K. P., Feustel, I. C., Fevold, H. L., Olcott, H. S., and Fraenkel-Conrat, H., *Science*, 1945, **102**, 274.

[2] Fraenkel-Conrat, H., Brandon, B. A., and Olcott, H. S., *Fed. Proc.*, 1946, **5**, 134; in press.

TABLE I.
Physical and Biological Properties of Gramicidin Derivatives.

Treatment	Nitrogen %	Equivalent of reagent introduced*	Solubility mg/100 ml†	Activity, % of original	
				Antibacterial	Hemolytic
		Section A.			
None	14.5	—	0.6	100	100
Formaldehyde	13.7	19	2.6	96	13
Acetaldehyde	13.7	13	1.3	64	88
Glyoxal (at 23°)	13.2	14	1.7	36	40
Glyoxylic acid	13.5	10	6.5 (>25)	79	82
Same, followed by formaldehyde	13.6	—	2.8	76	25
Tigaldehyde§	13.6	8	2.5	82	100
Benzaldehyde§	12.9	10	3.0	23	31
p-Dimethylamino benzaldehyde§	13.7	4	1.8	15	10
Acetal§	14.0	—	1.5	97	100
Glucose§	14.2	1	0.6	97	100
		Section B.			
Sulfuric acid conc.§	10.8	5		12	10
Pyridine-chlorosulfonic acid	9.9	18	(9.1)	41	14
		Section C.			
Acetic anhydride in acetic acid‡	13.7	10	0.1	104	100
Same, after formaldehyde	12.6	19.5	0.0	38	60
Acetic anhydride in pyridine	13.9	6	0.6	59	92
Same, after formaldehyde	12.2	24	0.5	22	15
Succinic anhydride in pyridine	13.6	7	1.1 (3.8)‖	85	40
Same, after formaldehyde	11.0	25	1.0 (>25)	24	4
Same, followed by formaldehyde	13.4	2.2-4.1	2.3 (5.6)	75	10

* Per 10^4 g of the original gramicidin, calculated from the nitrogen content for aldehydes, determined analytically for other derivatives.

† In 25% alcohol, 0.125 *M* sodium chloride; values in parenthesis represent solubility of the sodium salts.

‡ Reacted for 4 hours at 23°C.

§ These reactions were performed only once; all others at least twice. The data represent average values.

‖ (>25 in the absence of salt.)

the aromatic aldehydes caused marked losses. Determinations of the solubility in 25% alcohol indicated that all derivatives were more soluble than gramicidin, the glyoxylic acid-treated product exceeding in solubility the formaldehyde derivative, methylol-gramicidin. Furthermore, the glyoxylic acid derivative which contained up to 8 free acid groups per 10^4 g, yielded a neutral sodium salt which was not precipitated from concentrated alcoholic solution by the addition of much water. This product thus would appear to be of great potential usefulness except for the fact that its hemolytic and, presumably, its toxic activity was almost as high as that of unmodified gramicidin. Repeated attempts to reduce the hemolytic activity by treatment with formaldehyde before or after treatment with glyoxylic acid were not successful. It appeared that the glyoxylic acid residues were replaced by methylol groups, since the products had the desired low hemolytic activity, but only about one-fifth of the original acid groups. They were consequently less soluble in water at neutrality. The retention of hemolytic potency by the glyoxylic acid derivative was attributed to incomplete substitution of its indole groups. In view of the instability and commercial unavailability of glyoxylic acid, attempts were made to prepare more completely reacted glyoxylic acid derivatives by treating gramicidin with glyoxal, and subsequently oxidizing any free aldehyde groups with hydrogen peroxide, but without success.

Reaction with Sulfuric Acid and Pyridine-Chlorosulfonic Acid. Attempts were then made to obtain water-soluble derivatives of gramicidin by means of sulfation reactions (Table I, Section B). Concentrated sulfuric acid had been found to yield acid sulfate

esters with hydroxyl groups of proteins,[3] and the addition product of chlorosulfonic acid with pyridine introduced sulfate groups into the indole groups as well.[4] Gramicidin had been used as a model substance in the elucidation of these reactions. When the derivative obtained with cold concentrated sulfuric acid was assayed, it was found to be low both in hemolytic and antibacterial activity; sulfation of methylol-gramicidin by this technic gave, against expectation, a highly insoluble product.

Pyridine-chlorosulfonic acid, on the other hand, yielded derivatives, which, isolated as the barium or sodium salts, were usually soluble in dilute aqueous alcohol in the absence of salt, but not in water. These products retained varying fractions of the original antibacterial activity, and from 10 to 38% of the hemolytic activity. Thus it appears that the introduction of sulfate radicals into the indole groups of gramicidin, while being somewhat harder to control, affects the 2 biological activities in the same direction as does the introduction of methylol groups. On the other hand sulfation may cause more marked increases in solubility of gramicidin. Sulfation of methylol-gramicidin by the same technic gave a product of low activity and solubility in all solvents.

Reaction with Acid Anhydrides (Table I, Section C). Treatment of gramicidin with acetic anhydride in *glacial acetic acid* at temperatures up to 40° and for reaction periods up to 3 days led to the introduction of increasing amounts of acetyl. The highest value (18 equivalents per 10^4 g material, after correction for the blank values of 5.0 equivalents of volatile acid liberated from gramicidin) was of the same order as the total number of indole groups (19.4) and considerably in excess of the hydroxyl groups (4.5) present in gramicidin. Thus it appears that at least part of the indole groups are acetylated under these conditions. Similar treatment of methylol-gramicidin led to the introduction of up to 23 equivalents of acetyl. Assays of the acetylated derivatives obtained at room temperature showed no loss in activity for that derived directly from gramicidin; and some decrease in antibacterial and regeneration of hemolytic activity for the derivative of methylol-gramicidin. In contrast to gramicidin, most simple indoles cannot readily be N-acetylated; 5-methoxyindole is an exception.[5] However, Toennies and Kolb have indicated that acetic anhydride in the presence of perchloric acid reacts readily with tryptophane to form the indole-N-acetyl derivative.[6]

An alternate technic of acetylation was suggested by Hotchkiss' observation[7] that acetic anhydride in pyridine reacts exclusively with the hydroxyl groups of gramicidin, thus permitting the use of Stodola's acetylation technic[8] as a measure of its hydroxyl groups. This method was also found applicable in determining the number of methylol groups introduced into gramicidin through formaldehyde treatment.[2] Assays of gramicidin and methylol-gramicidin, O-acetylated by this technic, showed the former to have lost part of its antibiotic, but none of its hemolytic activity, while the latter was greatly lowered in both respects. None of the acetyl derivatives were more water-soluble than the starting material.

The readiness with which this reaction occurred suggested that it might lead to a water-soluble product if succinic anhydride were used instead of acetic anhydride. The extent of combination of succinic anhydride with gramicidin could readily be ascertained by titration of the acid groups introduced into the nonpolar polypeptide. In good agreement with the acetyl values, 10^4 g of gramicidin bound 7 equivalents of succinic anhydride, and 10^4 g of methylol-gramicidin bound about 25 equivalents of succinic anhydride. The succinyl derivative of grami-

[3] Reitz, H. C., Ferrel, R. E., Fraenkel-Conrat, H., and Olcott, H. S., *J. Am. Chem. Soc.*, 1946, **68**, 1024.

[4] Reitz, H. C., Ferrel, R. E., Olcott, H. S., and Fraenkel-Conrat, H., *J. Am. Chem. Soc.*, 1946, **68**, 1031.

[5] Blaikie, K. G., and Perkin, W. H., Jr., *J. Chem. Soc.*, 1924, 296.

[6] Toennies, G., and Kolb, T. T., *J. Biol. Chem.*, 1943, **144**, 219.

[7] Hotchkiss, H. D., *J. Biol. Chem.*, 1941, **141**, 171.

[8] Stodola, F., *Mikrochemie*, 1937, **21**, 180.

cidin retained about 80% of the original antibacterial and 40% of the hemolytic activity; as the sodium salt it gave a colloidal 2% solution in distilled water and a clear solution in 5% ethyl alcohol. However, in the presence of other electrolytes its solubility was only slightly higher than that of the methylol-gramicidin; as the free acid, it had low solubility in water. The hemolytic activity of this product could be lowered by subsequent formaldehyde treatment. This, however, led to partial hydrolysis of the succinic ester groups, even if the reaction was performed under gentle conditions (Table I, Section C).

The succinyl derivative of methylol-gramicidin had 24% of the original antibacterial, and only 1 to 5% of the hemolytic activity. Its sodium salt was freely soluble in water (5% solutions were prepared), even in the presence of sodium chloride. However, hydrolysis of ester bonds occurred at an appreciable rate, and this led to a gelling of concentrated aqueous solutions within a few hours at room temperature.

The observed increases in water solubility and decreases in hemolytic activity achieved by reaction of gramicidin or methylol-gramicidin with succinic anhydride were accompanied by marked decreases (95 to 98%) in the toxicity of the products toward rats. This was particularly surprising in the case of succinic acid-treated gramicidin, which was not toxic when given in dosages ranging up to 40 times the lethal dose of the original gramicidin, notwithstanding the fact that the derivative retained, as stated above, about 40% of the original *in vitro* hemolytic activity. The derivatives obtained by consecutive treatments with formaldehyde and succinic anhydrides and the toxicity data have been the subject of a preliminary note.[9]

Methods and Materials. Gramicidin was kindly supplied by the Wallerstein Laboratories.* All reagents were commercial preparations, with the exception of glyoxylic acid, which was prepared according to Doebner.[10]

The conditions used for treating gramicidin with the various aldehydes were those found favorable in the case of formaldehyde.[1] Sodium carbonate, instead of sodium hydroxide, was used in part of the experiments as an alkaline buffer. When 100 mg of gramicidin, dissolved in 2.5 ml of 95% ethanol containing 200 mg of sodium carbonate, was treated for 2 days at 53° with 1 ml of 40% aqueous formaldehyde, the isolated product corresponded in all respects to that obtained in the usual manner. Acetaldehyde treatment in the presence of sodium carbonate led to a brown impure product, while a white product was obtained in the presence of sodium hydroxide.

The technics of sulfation with concentrated sulfuric acid and pyridine-chlorosulfonic acid have previously been described.[3,4] Acetylation with acetic anhydride was performed either in pyridine according to Stodola (one hour at 95°),[8] or in 50% solution in glacial

[9] Olcott, H. S., Lewis, J. C., Dimick, K. P., Fevold, H. L., and Fraenkel-Conrat, H., *Arch. Biochem.*, 1946, **10**, 553.

* Part of the gramicidin used in these studies was prepared in this laboratory by a method which depends upon the selective precipitation of tyrocidin and tyrocidin-like peptides from tyrothricin solutions by means of phosphotungstic acid. For example, to 50 g of tyrothricin in 650 ml of absolute alcohol was added 85 ml of a 60% solution of phosphotungstic acid in the same solvent. The precipitate was centrifuged off and washed once with 100 ml of absolute alcohol. The combined soluble fractions were precipitated by the addition of 4 liters of water and sodium chloride, if necessary, to coagulate the suspension. The crude gramicidin precipitate was collected by centrifugation, suspended in about 200 ml of water, brought to pH 10 by the addition of 1 *N* sodium hydroxide, and allowed to stand overnight (to decompose the phosphotungstate). The gramicidin was centrifuged off and washed repeatedly by resuspension and recentrifugation to remove traces of tungsten. Salt or ammonium carbonate was added to coagulate milky suspensions when these could not be separated by centrifugation. The final dried product weighed 10.5 g (21% of the tyrothricin) and appeared to contain approximately 5% tyrocidin by an *in vitro* hemolytic assay. A sample of pure gramicidin treated by this procedure was found to have unchanged chemical and biological properties.

[10] Doebner, O., *Ann. der Chem.*, 1900, **311**, 129.

acetic acid at temperatures of 20 to 40° for time periods up to 3 days. Succinic anhydride was used successfully only in pyridine, because of its limited solubility in glacial acetic acid. Similar products were obtained when the reactions were performed for 18 hours at 40 or 53°, or for one hour at 78 or 95°.

All derivatives, except those sulfated or acetylated on the indole nitrogen, were isolated by precipitation from acid solution with excess water. The products were washed repeatedly with water, redissolved in alcohol and again precipitated with water. Acetic acid served for acidification and in many instances also for flocculation of the products; in other cases, sodium chloride was used in amounts up to 0.1 M. Isolation was easily accomplished because of the fact that gramicidin and all of its derivatives are only very slightly soluble in acid aqueous media. The water-soluble derivatives obtained in the course of this study were all sodium salts, soluble only in neutral aqueous solution. The product obtained with pyridine-chlorosulfonic acid had to be isolated from neutral solution as the sodium or barium salt, because the indole-sulfate bond was unstable in acid solution.[4] This was achieved by removing excess sulfate as barium sulfate, pyridine by evaporation from alkaline solution, and neutral inorganic matter by extraction with water. The gramicidin derivative was subsequently extracted with 50% aqueous alcohol. Nitrogen and tryptophane analyses supplied a reliable measure of the purity of the product. The indole-acetylated derivative also was unstable upon dilution of the acetic acid reaction mixture with water. It was isolated by evaporation of all volatile matter under a high vacuum.

Tryptophane analyses were performed by the Horn and Jones method[11] after hydrolysis; 5 to 10 mg were heated for 20 hours at 100° with 0.8 ml of glacial acetic acid and 3.0 ml of 6 *N* hydrochloric acid in evacuated Thunberg tubes, which had been flushed with CO_2. The tryptophane content of pure gramicidin was found to be 38.1%.† Benzoyl-d,l-tryptophane after similar hydrolysis contained 63.9% tryptophane; calculated 66.1%. Table II shows the apparent tryptophane content of the various derivatives of gramicidin.

Analyses of intact gramicidin in 50% acetic acid solution by the Horn and Jones method indicated about 40% tryptophane, but the value varied from 35 to 45%, depending upon the amount of gramicidin used, and thus had no absolute quantitative significance.

The spectrophotometric analyses occasionally performed did not necessitate hydrolysis; they indicated a tryptophane content of 39.6% for gramicidin.

Hydroxyl groups were determined by quantitative acetylation[8] (Table II). Bound acetyl groups were determined after hydrolysis for 3 days at 100° in evacuated Thunberg tubes with 6 N sulfuric acid, followed by steam distillation and titration of the distillate.[12] Sulfate groups were determined after hydrolysis as barium sulfate.[3] The acid groups introduced through glyoxylic acid or succinic anhydride treatments were titrated in alcoholic solutions with aqueous 0.1 *N* alkali with phenolphthalein as indicator. The Kjeldahl method was found to give the expected tryptophane nitrogen values when mercuric oxide was used as catalyst.[14]

Assays for hemolytic and antibacterial activity were performed by the methods described in the previous publication.[1]

To determine the solubility of the products, samples of 2.5 mg were dissolved in 2.5 ml of absolute alcohol, then treated with 8.25 ml of water and 1.25 ml of 1 *M* sodium chloride. The precipitates were centrifuged off after 15 to 30 minutes. The amount of gramicidin remaining in solution was de-

[11] Horn, M. J., and Jones, D. B., *J. Biol. Chem.*, 1944, **157**, 153.

† Hotchkiss[7] found 37.3%. Synge[13] gave 38.6 and 41.1% as the corrected (by 10%) values for two recrystallized preparations.

[12] Blackburn, S., and Phillips, H., *Biochem. J.*, 1944, **38**, 171.

[13] Synge, R. L. M., *Biochem. J.*, 1944, **38**, 285.

[14] White, L. M., and Secor, G. E., *Ind. Eng. Chem., Anal. Ed.*, 1946, **18**, 457.

TABLE II.
Chemical Properties of Gramicidin Derivatives.

Reagent	Equivalents introduced*		Equivalents of tryptophane found colorimetrically*	
	Reagent	Hydroxyl	With hydrol.	Without hydrol.†
Untreated	—	—	19.4	20
Formaldehyde	19	19	about 5‡§	<5§
Acetaldehyde	13	0	17.2	18
Tigaldehyde	8	2	15.2	
Benzaldehyde	10	3	15.4	18
Glyoxal	14	4	9.7	
Sulfuric acid	5	—	16.9	
Pyridine-chlorosulfonic acid	18	—	16.3	
Acetic anhydride in acetic acid	18	—		20
Acetic anhydride in pyridine	6	—		20
Succinic anhydride in pyridine	7			18
Formaldehyde, then acetic anhydride (in pyridine)	24.5	—		<5§
Formaldehyde, then succinic anhydride	25	none		<5§
Acetic anhydride (in acetic acid), then formaldehyde		16.5		<5§
Succinic anhydride, then formaldehyde		24.8		<5§

* Per 10^4 g of the original gramicidin.
† Approximate values only are obtained without hydrolysis.
‡ Methylol gramicidin, upon hydrolysis, yields acid-insoluble humin.
§ Based on readings of an atypical purple color.

termined by means of spectrophotometric tryptophane analyses. This method alone is sufficiently sensitive to be applicable to the low concentrations; it has the further advantage over the colorimetric method that it appears to give true tryptophane values for substituted indole derivatives, such as methylol-gramicidin, which retains only about 25% of its original colorimetric value.[2] The introduction of benzene rings by means of benzaldehyde, however, led to high apparent tryptophane values for this derivative (127% of that calculated on the basis of nitrogen).

Discussion. The present investigation reveals the remarkable fact that a highly active natural product may be extensively substituted with a variety of chemical agents without undergoing great changes in some or all of its biological activities. Thus, the antibacterial activity of gramicidin is largely retained after substitution of all of its hydroxyl groups, and of all of its indole groups, either on the nitrogen or the carbon atoms, with alcoholic, acidic, or nonpolar side chains. This is even more astonishing, in view of the fact that the hydroxyl and indole groups appear to be the only reactive or polar centers of the gramicidin molecule. The extent of substitution achieved with the various classes of reagents approaches, but never exceeds, the number of equivalents expected to react. The foreign groups introduced may amount to as much as 20% of the weight of the gramicidin for some of the promising derivatives.

The above results might have been of greater general significance if systematic studies of the most favorable reagents and conditions, and complete bioassays, including therapeutic and toxicity studies, had not been outside of the scope of this brief survey of possibilities. The preliminary findings with succinyl derivatives showed that their *in vitro* hemolytic activities were not necessarily indicative of their *in vivo* toxicity. Since only one type of antibacterial and the hemolytic test, both *in vitro*, were used as primary criteria for successful modification, the possibility definitely exists that potentially useful products have remained unrecognized and were bypassed in the course of this study.

The mode of combination of the various reagents with gramicidin has in part been elucidated and demonstrated in previous

studies.[2-4] All aldehydes might be assumed to combine with the indole groups, as does formaldehyde—that is, by addition to the nitrogen atom. However, far fewer hydroxyl groups seemed to be formed, or could be detected by acetylation, than corresponded to the amounts of aldehyde combined. The apparent tryptophane content, as determined colorimetrically, showed decreases of only 50% after glyoxal treatment, with values more closely approaching the original for other aldehydes. In contrast, combination with formaldehyde led to formation of an equivalent number of hydroxyl groups, and to a decrease of the apparent tryptophane content by 75%. The derivative obtained with pyridine chlorosulfonic acid had the expected full chromogenic value. This finding confirmed the conclusion that the sulfate had combined with the nitrogen, rather than with the 2-carbon atom.[2,4] The same was the case for the acetyl groups introduced into the indoles by means of acetic anhydride in acetic acid. The chromogenic value of the derivatives obtained with concentrated sulfuric acid or with acyl anhydrides in pyridine, reagents which combine only with the hydroxyl groups,[3,7] was similarly unchanged, when calculated on the basis of their nitrogen contents.

Summary. Gramicidin was treated with a number of reagents which were known, or could be shown to combine with, either its indole, or its hydroxyl groups, or both. The products were prepared and assayed in search of a water-soluble derivative of low hemolytic and high antibacterial activity. Formaldehyde had previously been shown to decrease selectively the hemolytic activity, but to render gramicidin only slightly less water-insoluble.

1. A glyoxylic acid derivative was obtained, the sodium salt of which was water-soluble. The product, however, retained most of the original hemolytic activity, and attempts to find experimental conditions under which the advantages of the glyoxylic and formaldehyde treatment could be combined were not successful.

2. An indole sulfamate of gramicidin was obtained with pyridine-chlorosulfonic acid, the salts of which were readily soluble in aqueous alcohol. The product showed somewhat greater decreases in hemolytic, than in antibacterial activity.

3. The succinic half esters of gramicidin and methylol gramicidin showed the greatest promise. The succinyl derivative of gramicidin, containing one succinyl group for each ethanolamine residue, gave a water-soluble sodium salt. Its hemolytic activity was slightly decreased and could be further lowered by subsequent formaldehyde treatment. Notwithstanding the *in vitro* hemolytic activity, the derivative appeared to be remarkably nontoxic by intravenous injection. The succinic anhydride derivative of methylol-gramicidin, containing one succinyl for each indole-methylol plus each original hydroxyl group, was lowered in all, but markedly more in hemolytic and toxic than in antibacterial activities. Its sodium salt was water-soluble even in the presence of other ions, but not very stable at neutrality.

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