TABLE I[a]

	Indole, μmole	Phenol, μmole	NBS, μmoles	$\Delta\epsilon_{280} \times 10^{-3}$ No urea	$\Delta\epsilon_{280} \times 10^{-3}$ 8.0 *M* urea	$\Delta\epsilon_{260} \times 10^{-3}$ No urea	$\Delta\epsilon_{260} \times 10^{-3}$ 8.0 *M* urea
N-Acetyltryptophanamide	0.5	0	0.8	−4.0	−4.0	0	0.5
Skatole	0.5	0	2.5	−2.7	−2.5	4.5	3.2
Phloretic acid	0	0.6	2.5	0.05	0	5.5	0
Lysozyme[b]	0.6	0.3	2.5	−3.4	−3.9	3.9	1.4
Insulin	0	1.0	2.5	0.1	0	2.0	0

[a] NBS, in 8.0 *M* urea or in water, was added to the appropriate compound in 2 ml. of 0.2 *M* sodium acetate buffer, pH 3.5, with or without urea. The spectra were scanned immediately and subsequent changes in absorbance at 260 and 280 mμ were followed. The values quoted in the table were those observed after 15 min. Phloretic acid in urea showed slow changes after this time (Fig. 2), but the other compounds gave steady readings. Owing to the varying ratio of NBS to tyrosine and tryptophan in the different compounds, the absorbance changes observed are only comparable on a qualitative basis. [b] Primary structure of lysozyme (Try_6, Tyr_3): R. E. Canfield, *J. Biol. Chem.*, **238**, 2698 (1963); P. Jollès, *Angew. Chem.*, **76**, 20 (1964).

urea had little effect on the decrease in absorbance at 280 but greatly reduced the increase at 260 mμ. Even after several hours, insulin showed no increase in absorbance at 260 mμ (Fig. 4), in contrast to phloretic acid, presumably because the N-bromourea is consumed by other reducing groups (–SS–, R–SR) present in the protein.

In the absence of free SH groups, NBS reacts much more rapidly with tryptophan than with any of the other oxidizable amino acid side chains. Under these circumstances NBS is specific for tryptophan, provided that minimal amounts of reagent are added and the tryptophan is not buried deeply in the protein. However, when NBS is used for cleavage of tryptophyl peptide bonds, it is necessary to use a considerable excess of reagent to obtain adequate yields of cleavage products.[6] Under these circumstances there may be some danger that tyrosyl bonds will also be cleaved. It is therefore advantageous to perform such experiments in urea solutions, both to improve the yield of cleavage products by unfolding the molecule and to reduce nonspecific cleavage of tyrosyl bonds.[7]

(6) L. K. Ramachandran and B. Witkop, *J. Am. Chem. Soc.*, **81**, 4028 (1959).

(7) Bromocarbamide (N-bromourea) has recently been introduced as a highly selective oxidant for the conversion of aldose derivatives to aldonic acid derivatives: J. Kiss, *Chem. Ind.* (London), 73 (1964).

[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA 14, MARYLAND]

Gramicidin A. II. Preparation and Properties of "*seco*-Gramicidin A"

BY SHIN-ICHI ISHII[1] AND BERNHARD WITKOP

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Treatment of "gramicidin A" with 1.5 *N* anhydrous hydrogen chloride in absolute methanol at room temperature for 1 hr. selectively cleaved one peptide bond and led to the liberation of two new NH_2-terminal residues in the same Val:Ileu ratio as the starting material which consisted of valine-gramicidin and isoleucine-gramicidin. "*seco*-Gramicidin A" has no free carboxyl. Further methanolysis of DNP-"*seco*-gramicidin A" at 75° gave a mixture of DNP-L-valylglycine and DNP-L-isoleucylglycine dipeptides.

The absence of free $-NH_2$ and –COOH groups and the mode of incorporation of aminoethanol have been the delaying factors in the structural elucidation of gramicidin A. Synge and James[2] carefully examined partial hydrolysis of gramicidin, assuming that "*ortho*-amide" bonds were involved in the binding of aminoethanol and that they would be more labile to hydrolysis than normal peptide bonds. By the action of a mixture of aqueous 10.0 *N* hydrochloric acid and dioxane on gramicidin at low temperature, various peptide fragments were obtained, most of which were recognized to have NH_2-terminal tryptophan residues. The marked heterogeneity of the products, however, permitted no definite conclusion.

We have now found that "gramicidin A," partially purified by countercurrent distribution, on treatment with anhydrous hydrogen chloride (1.5 *N*) in methanol at room temperature led to rapid and specific cleavage of only one linkage in the molecule. The product, provisionally called "*seco*-gramicidin A" contains all of the building stones of gramicidin A in addition to a new NH_2-terminal group.

(1) Associate in the Visiting Program of the USPHS, 1960–1963.

(2) R. L. M. Synge, *Biochem. J.*, **39**, 355 (1945); A. T. James and R. L. M. Synge, *ibid.*, **60**, 109 (1951).

The Cleavage Reaction.—The cleavage of gramicidin A with methanolic hydrogen chloride at room temperature was followed both by the increase in optical rotation and by the liberation of terminal amino groups. The results are summarized in Fig. 1. The specific rotation of gramicidin increased rapidly and reached a plateau after 2 hr. The concomitant liberation of primary amino groups reached 1 mole of amino group per mole of gramicidin after 80 min. and thereafter proceeded at a markedly slower rate. The free amino groups were determined before and after treatment of the cleavage products with base. As shown in Fig. 1 the NH_2 values were somewhat lower after base treatment, but the difference was insignificant. The free amino groups were determined colorimetrically after trinitrophenylation[3] of the cleavage products under slightly basic condition (pH 7.66).

If "*seco*-gramicidin" arises through acid-catalyzed N → O acyl migration, the possibility must be considered that the basic conditions of the trinitrophenylation method might reverse this process and lead to O → N acyl migration. The detection of free amino

(3) K. Satake, T. Okuyama, M. Ohashi, and T. Shinoda, *J. Biochem.* (Tokyo), **47**, 654 (1960).

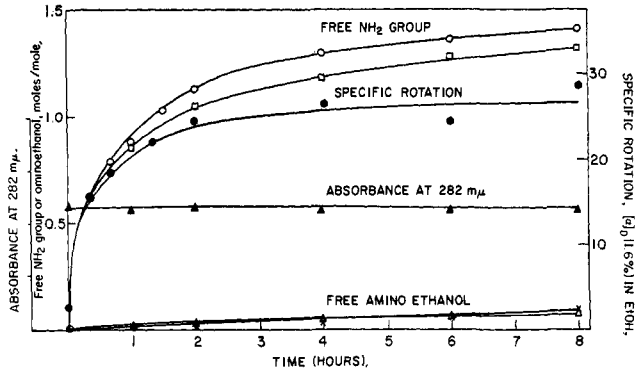


Fig. 1.—Selective cleavage of gramicidin A by the action of 1.5 *N* anhydrous hydrogen chloride in methanol at 26°: —O—O—, free amino group determined before and, —□—□— after, treatment with base; —X—X—, free aminoethanol determined before and, —Δ—Δ— after, treatment with base.

groups, *e.g.*, of aminoethanol, which might have been partially liberated in the cleavage process, might thus be missed by the trinitrophenylation method. In addition, therefore, ninhydrin colorimetry[4] which works at pH 5 was also applied to the products resulting from methanolysis for 100 min. The values were 0.70 and 0.72 valine-equivalents per mole before and after treatment with base, respectively, as compared with a value of 1.13 moles per mole by the trinitrophenylation method. These results minimize the possibility of reversible acyl migration.

Careful consideration was given to the possible liberation of aminoethanol during methanolysis. A sensitive method for the detection of traces of aminoethanol was gas chromatography of the N,O-bistrifluoroacetyl derivative. As shown in Fig. 1, the liberation of aminoethanol, though detectable by this method, is extremely small and did not increase after treatment of the cleavage products with base.

The reaction products were also examined by paper electrophoresis at 1500 v. and 40 amp. for 70 min. in an aqueous formic–acetic acid buffer system of pH 1.9. Only one ninhydrin- and Ehrlich-positive spot was observed at the starting line. Insolubility in the aqueous medium may be the explanation for the immobility of the spot. Other spots were not observed except when the time of methanolysis was extended for longer than 6 hr. In that case an additional weak spot which migrated as fast as aminoethanol was detected by the positive ninhydrin test.

The last information contained in Fig. 1 concerns the stability of the four tryptophan residues in the gramicidin molecule under the conditions of methanolysis. The optical density at 280 mμ of the four indole chromophores remained remarkably constant throughout the reaction.

Analysis of the Amino Terminal Residues.—The cleavage product resulting from methanolysis at room temperature for 1 hr., termed "*seco*-gramicidin A," which had 0.92 mole of free amino group per mole, was chosen for the NH_2-terminal analysis by the DNP[5] procedure.[6] Figure 2 shows the thin-layer chromatogram of the ether-soluble fraction from a hydrolysate of DNP-*seco*-gramicidin A, after esterification with

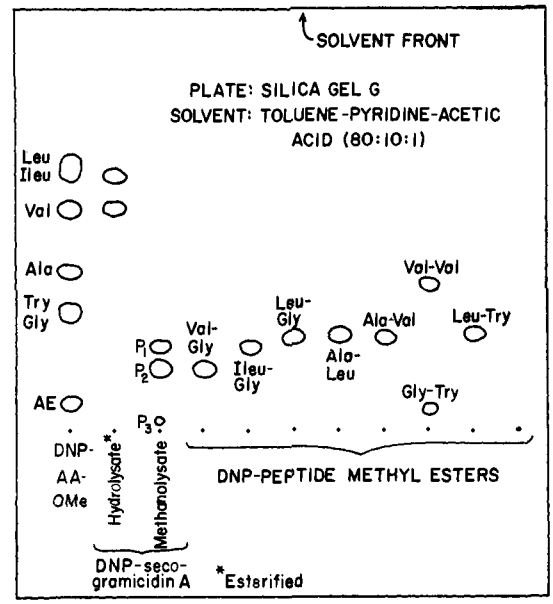


Fig. 2.—Thin-layer chromatogram of the DNP-amino acids and -peptide methyl esters present in a hydrolysate (after esterification) and a methanolysate of DNP-*seco*-gramicidin A in comparison with authentic control samples. A thin-layer plate of silica gel G was used. The developer was a mixture of toluene, pyridine, and acetic acid (80:10:1, v./v.).

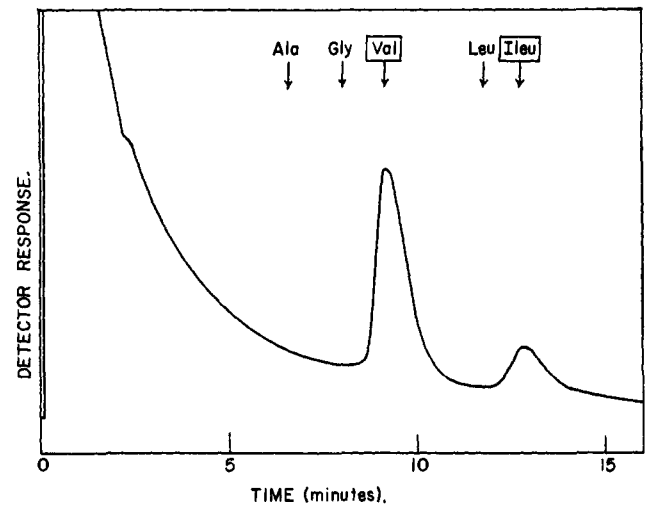


Fig. 3.—Gas chromatogram of the DNP-amino acid (as methyl esters) present in a hydrolysate of DNP-*seco*-gramicidin A.

diazomethane. Two yellow spots were detected: one was identical with the spot of DNP-valine methyl ester and the other with that of DNP-leucine or isoleucine methyl ester in two different solvent systems. Discrimination between leucine and isoleucine was possible by gas chromatography.[6] The gas chromatogram (Fig. 3) showed three well-resolved peaks due to DNP-valine, -isoleucine, and -alanine methyl esters, with the last one as a minor peak only. Quantitative results obtained from the gas chromatography are presented in Table I.

DNP-*seco*-gramicidin A was also methanolyzed at 75° in methanol half-saturated with anhydrous hydrogen chloride for 20 hr. in a glass ampoule which was flushed with nitrogen and sealed. The homogeneous reaction mixture maintained the same tint of light yellow and the same ultraviolet absorption spectrum during the whole operation. This indicates that no destruction of tryptophan occurred during methanoly-

(4) S. Moore and W. H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

(5) The abbreviations used are: DNP, dinitrophenyl; FDNB, 1-fluoro-2,4-dinitrobenzene; TNP, trinitrophenyl; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

(6) *Cf.* S. Ishii and B. Witkop, *J. Am. Chem. Soc.*, **85**, 1832 (1963).

TABLE I

QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF THE DNP-AMINO ACIDS[a] PRESENT IN HYDROLYSATES OF DNP-*seco*-GRAMICIDIN A

Figures are in mole per mole of DNP-*seco*-gramicidin A[b]

Valine	0.53
Isoleucine	.27
Alanine	.05
Leucine	0
Tryptophan	0[c]
Glycine	0
Sum	0.85
Total DNP group	0.87[d]

[a] Analyzed as methyl esters. [b] The molecular weight of DNP-*seco*-gramicidin A was assumed to be 2020 in this calculation. [c] Gas chromatography was not applicable to the analysis of DNP-tryptophan methyl ester. Neither DNP-tryptophan methyl ester nor related dipeptides were detectable on thin layer chromatograms of the methanolysate, in which all tryptophan residues were still intact. [d] Determined by ultraviolet absorption spectroscopy of DNP-*seco*-gramicidin A.

sis. The ether-soluble DNP-derivatives from the methanolysate were analyzed by thin-layer chromatography. As seen in Fig. 2, three yellow spots were detected, P1, P2, and P3, which had the same R_f values as authentic DNP-Ileu-Gly methyl ester, DNP-Val-Gly methyl ester, and DNP-Val-Gly, respectively. There were no Ehrlich-positive yellow spots. The assignments for P1 and P2 were confirmed when these spots were extracted separately from preparative chromatograms and their constituents analyzed after hydrolysis; P3 was also isolated and rechromatographed on a thin layer plate after diazomethane treatment. Since the spots P1 and P2 were observed only after rechromatography of esterified P3, fraction P3 must be a mixture of DNP-Ileu-Gly and DNP-Val-Gly produced by the hydrolysis of their parent methyl esters. Next to the NH_2-terminal valine-isoleucine in "*seco*-gramicidin A" must be glycine. The yields of the DNP-dipeptides as they were recovered from the thin-layer spots were estimated by the measurement of optical densities at 339 mμ as

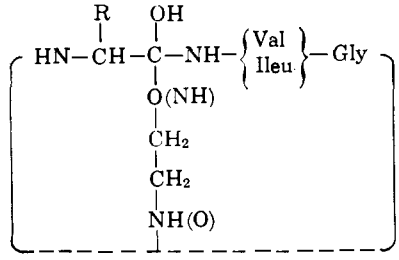
P1 = DNP-Ileu-Gly methyl ester:	0.21 mole/mole of DNP-SG-I
P2 = DNP-Val-Gly methyl ester:	0.35 mole/mole of DNP-SG-I
P3 = DNP-Ileu-Gly + DNP-Val-Gly:	0.08 mole/mole of DNP-SG-I

L-Val-Gly has already been found by Synge[7] in a partial acid hydrolysate of a crude gramicidin preparation. It will be recalled that gramicidin A contains only *one residue of glycine*.[6,8]

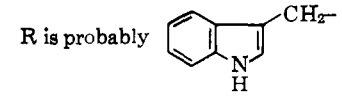
The heterogeneous character of "gramicidin" has already been commented on in papers I[6] and V[8] of this series and by Ramachandran.[9] A nonstoichiometric amount of L-isoleucine, in our case 0.38 mole/mole, was present in the hydrolysate of gramicidin A as a partial substitute for L-valine.[6] It was concluded that one of two residues of L-valine is replaced by L-isoleucine in "isoleucine-gramicidin A," one component of gramicidin A. The results from the analyses of the NH_2-terminal residues of "*seco*-gramicidin A" suggest that the cleavage by hydrogen chloride–methanol occurs exactly at the unique position in the molecule where the Val ⇄ Ileu interchange takes place. This fortunate coincidence facilitates sequence determination work on "*seco*-gramicidin A," because it makes unnecessary the difficult separation of isoleucine-gramicidin from valine-gramicidin, if one makes the simplifying assumption that the two species differ only with regard to substitution but not sequence.

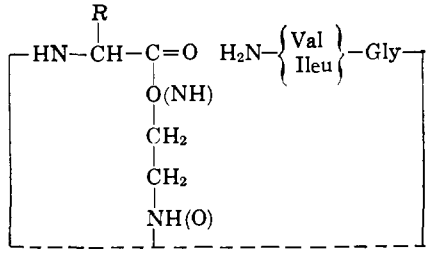
The cleavage leads to "*seco*-gramicidin A" in a highly selective manner.[10] "*seco*-Gramicidin A" behaves like gramicidin A in the process of gel filtration through a Sephadex G-25 (fine) column with 50% acetic acid[11] as the eluent. Both substances emerged as a single peak at the void volume of the column. This behavior indicates equal molecular weights for gramicidin A and "*seco*-gramicidin A."

The gentle conditions, ease and high selectivity of the cleavage excludes the possibility that methanolysis occurred at normal peptide bonds. The *ortho*-peptide structure I involving linkage of aminoethanol either at

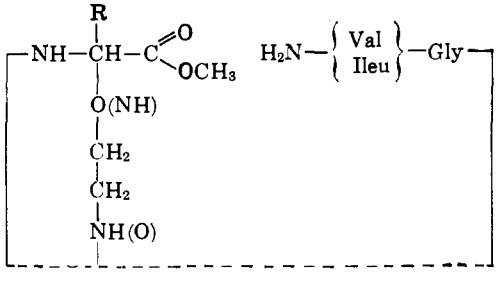


I: Valine– / Isoleucine– } gramicidin A





II



III

the nitrogen or oxygen end would require the assumption of an inner ester II or a methyl ester III for

(7) R. L. M. Synge, *Biochem. J.*, **38**, 385 (1944).
(8) E. Gross, S. Ishii, and B. Witkop, in preparation.
(9) L. K. Ramachandran, *Biochemistry*, **2**, 1138 (1963).

(10) The only minor (3–6%) NH_2-terminal residue found in crude "*seco*-gramicidin A" was alanine which occurs only twice in gramicidin A.

(11) Sephadex G-25 in conjunction with elution by 50% acetic acid acts as a very efficient molecular sieve for relatively small peptides (D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 227 (1963)). Sarges used this method in the fractionation of the lithium borohydride cleavage products of gramicidin A.

"*seco*-gramicidin A." Both structures are unlikely because "*seco*-gramicidin A" does not behave like an ester and has no methoxy group.

An inner ester bond as in II should be cleaved by lithium borohydride to yield, after hydrolysis, an amino alcohol residue.[12] Treatment of "*seco*-gramicidin A" with lithium borohydride, after hydrolysis and trinitrophenylation, led to an amino alcohol. By thin-layer chromatography, mainly TNP-tryptophanol was found besides the expected TNP-aminoethanol. However, TNP-tryptophanol was also found when gramicidin itself was subjected to reduction by lithium aluminum hydride, hydrolysis, and trinitrophenylation. The NH_2-terminal amino acids liberated by the action of $LiBH_4$ were valine, isoleucine, and alanine determined as DNP derivatives. Sarges found that fractionation of $LiBH_4$-treated gramicidin in 50% acetic acid over Sephadex G-25 reveals the presence of at least seven fragments. The $LiBH_4$ method, therefore, is of little use in this case.

In conclusion, the linkage in gramicidin A vulnerable to the action of controlled acidic methanolysis as well as to reductive cleavage by lithium borohydride is probably not an "*ortho*-peptide" bond.[13] It is shown in a subsequent communication[14] that "*seco*-gramicidin A" is in reality *desformylgramicidin A*.

Attempted Enzymatic Cleavage of "*seco*-Gramicidin A."—Since, in contrast to gramicidin A, "*seco*-gramicidin A" is soluble in 0.015 *N* aqueous hydrochloric acid containing 35% ethanol, enzymatic digestion of such a solution with pepsin was tried. No increase of free amino groups was detected even after treatment for 49 hr. at 37°. The action of pronase (*Streptomyces protease*) and nagarse (*Bac. subtilis protease*) was equally unsuccessful.

Biological Activity of "*seco*-Gramicidin A."—In routine assays against *Streptococcus faecalis* "*seco*-gramicidin A" had 60% of the activity of a commercial gramicidin A control sample. It may be recalled that the activity of *cyclic* peptides, such as gramicidin S and phalloidin, is lost on ring fission.[15]

Experimental

All melting points are corrected.

Materials.—Almost all of the experiments were performed with the gramicidin purified by countercurrent distribution.[16] A commercial sample of gramidicin purchased from the Nutritional Biochemicals Corporation was also used in some special cases.

DNP-amino acids were obtained as a kit (lot G1068–G1085) from the Mann Research Laboratories. FDNB was purchased from the Pierce Chemical Co. Most of the DNP-peptides were prepared after Levy's method[17] by dinitrophenylation of the respective peptides, which were obtained from the Mann Research Laboratories. The following DNP-peptides obtained in this way behaved as homogeneous substances on thin-layer chromatography either before or after esterification with diazomethane. The solvent systems employed in the chromatography are described under Analytical Methods.

Dinitrophenyl-L-valylglycine had m.p. 201–202° after recrystallization from acetone–*n*-hexane; λ_{max} (HOAc) 338 mμ (ϵ_{max} 16.1 × 10^3).

Anal. Calcd. for $C_{13}H_{16}N_4O_7$: C, 45.88; H, 4.74; N, 16.47. Found: C, 46.04; H, 5.10; N, 16.52.

Dinitrophenyl-L-valyl-L-valine had m.p. 178-179° after recrystallization from acetone–*n*-hexane.

Anal. Calcd. for $C_{16}H_{22}N_4O_7$: C, 50.26; H, 5.80; N, 14.65. Found: C, 50.44; H, 5.89; N, 14.37.

Dinitrophenyl-L-leucyl-L-tryptophan had m.p. 200–207° after recrystallization from ethyl acetate–*n*-hexane; λ_{max} (glacial HOAc) 339 mμ (ϵ_{max} 14.9 × 10^3).

Anal. Calcd. for $C_{23}H_{25}N_5O_7$: C, 57.13; H, 5.21; N, 14.49. Found: C, 56.95; H, 5.69; N, 14.48.

Dinitrophenylglycyl-L-tryptophan had m.p. 226–227° dec. after recrystallization from ethyl acetate–*n*-hexane; λ_{max} (in HOAc) 340 mμ (ϵ_{max} 14.7 × 10^3).

Anal. Calcd. for $C_{19}H_{17}N_5O_7$: C, 53.39; H, 4.00; N, 16.39. Found: C, 53.50; H, 4.19; N, 16.23.

Dinitrophenyl-L-isoleucylglycine Methyl Ester.—DNP-L-Ileu-Gly methyl ester was synthesized by the coupling of DNP-L-isoleucine and glycine methyl ester hydrochloride by means of N,N¹-dicyclohexylcarbodiimide in the presence of triethylamine in dichloromethane. The ester had m.p. 148–149° after recrystallization from methanol–water and ethyl acetate–petroleum ether; λ_{max} (EtOH) 341 mμ (ϵ_{max} 16.7 × 10^3); λ_{max}(HOAc) 339 mμ (ϵ_{max} 14.9 × 10^3).

Anal. Calcd. for $C_{15}H_{20}N_4O_7$: C, 48.91; H, 5.47; N, 15.21. Found: C, 49.24; H, 5.60; N, 14.96.

While DNP-DL-Ala-L-Leu could not be obtained in crystalline form, its methyl ester, prepared by the action of diazomethane, crystallized easily from methanol–water (155–156°). It showed a single spot on thin layer chromatograms. The spot of DNP-DL-Ala-DL-Val or its methyl ester on the chromatograms accompanied a minor spot, probably derived from an impurity in the original peptide.

L-Tryptophanol oxalate and DL-valinol were prepared according to the procedures of Karrer, *et al.*,[18] and Vogl, *et al.*,[19] respectively. Aminoethanol was an Eastman Organic chemical, DL-alaninol was from the Mann Research Laboratories, and leucinol hydrochloride and isoleucinol hydrochloride were from the California Corporation for Biochemical Research.

Analytical Methods.—Free amino groups were determined colorimetrically by the TNP-method of Satake, *et al.*[3] The directions for trinitrophenylation were modified as follows, because the samples were insoluble in water: 1 ml. of the sample solution containing 0.2 to 0.5 μmole of amino groups in Methyl Cellosolve, 0.5 ml. of 0.2 *M* sodium phosphate buffer, pH 7.66, and 0.5 ml. of 0.2% TNBS in water were mixed and kept in a dark place at 40° for 3 hr. After addition of 1 ml. of 12.0 *N* HCl–Methyl Cellosolve (1:11, v./v.) to the resulting solution, the optical density was read at 340 mμ. A similar mixture, containing 1 ml. of Methyl Cellosolve instead of the sample solution, was used as a blank. The value of the molar extinction coefficient, 10.5 × 10^3, reported on TNP-peptides, was used for the calculation of the amount of amino groups.

All the gas chromatographic analyses were carried out by Dr. R. Sarges on a Gas Chromatograph Model 600, Research Specialties Co., with a flame ionization detector, Model 660. The condition for quantitative analysis of free aminoethanol was as follows. The sample derived from 5.63 mg. of gramicidin A (containing 0.018–0.28 μmole of aminoethanol) plus 10 μg. (0.133 μmole) of aminoisopropyl alcohol as an internal standard was dissolved in 0.2 ml. of dimethoxyethane, and 10 μl. of trifluoroacetic anhydride was added to the solution. Trifluoroacetylation was completed within 20 min. at room temperature. A 5-μl. aliquot of the resulting solution was injected into a chromatographic column (6-ft. glass tube, 1/6-in. diameter, packed with 2% neopentyl glycol succinate coated on Gas-Chrom P). During the analysis the column was maintained at 154–155° and the rate of flow of the carrier gas, nitrogen, was adjusted to 15 ml. per min. as measured at the inlet. The N,O-bistrifluoroacetyl derivatives of aminoisopropyl alcohol and aminoethanol emerged as well-separated sharp peaks after retention times of 6.3 and 9.4 min., respectively. The areas of both peaks were measured and yielded their relative quantities. With a standard sample mixture, the relative response of the flame ionization detector to both aminoalcohol derivatives was determined. The values (on a

(12) J. L. Bailey, *Biochem. J.*, **60**, 170 (1955); J. C. Crawhall and D. F. Elliot, *ibid.*, **61**, 264 (1955); A. C. Chibnall and M. W. Rees, *ibid.*, **68**, 105 (1958).

(13) *ortho*-Peptide bonds may show varying degrees of reactivity. The conditions of mild methanolysis leading to mono (and bis) "*seco*-gramicidin A" are insufficient to cleave bacitracin, whose postulated structure contains one *ortho*-peptide bond (L. C. Craig, personal communication).

(14) R. Sarges and B. Witkop, *J. Am. Chem. Soc.*, **86**, 1861 (1964).

(15) We are greatly indebted to Dr. James D. Dutcher, The Squibb Institute for Medical Research, for arranging for these tests.

(16) J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, **172**, 839 (1948).

(17) H. Fraenkel-Conrat, J. I. Harris, and A. L. Levy, "Methods of Biochemical Analysis," Vol. II, Interscience, Inc., New York, N. Y., 1955, p. 381.

(18) P. Karrer and P. Portmann, *Helv. Chim. Acta*, **32**, 1034 (1949).

(19) O. Vogl and M. Pöhm, *Monatsh.*, **83**, 541 (1952).

molar basis) are aminoethanol = 1.00, aminoisopropyl alcohol = 1.56.

Quantitative gas chromatography of DNP-amino acid methyl esters was done as reported previously.[6] The only difference from the previous procedure consisted in the type of equipment used. The relative responses[20] of the flame ionization detector to various amino acids were determined again in this experiment, because the values were found to vary from one detector to another within small ranges, probably due to differences in the geometry of the detectors. The values expressed as relative peak areas on a molar basis were: isoleucine = 1.00, leucine = 0.83, Val = 0.91, alanine = 0.56.

Silica gel G plates (250 μ, 20 × 20 cm.) for thin-layer chromatography were prepared as described before.[5] The solvent system composed of chloroform, methanol, and acetic acid (95:5:1, v./v.[21]; solvent system I) was used as the developer for DNP-amino acids and peptides; the systems of toluene, pyridine, and acetic acid (80:10:1, v./v.[6]; solvent system II) and of benzene and ethyl acetate (3:1, v./v.; solvent system III) were used for their methyl esters. The development was carried out by ascending technique.

Development of the Cleavage Reaction at a Function of Time.—The solution of 490 mg. of gramicidin A in 13 ml. of methanol was mixed with 13 ml. of a freshly prepared solution of 2.9 *N* HCl (anhydrous) in absolute methanol. The concentration of the methanolic HCl was determined by titration. From the resulting solution which was kept at room temperature (26°), eight 3-ml. aliquots were withdrawn at various time intervals (see Fig. 1) and immediately evaporated to dryness. Each of the residues was dissolved in 6 ml. of ethanol and the solution was divided for the following analyses: TNP colorimetry for amino group determination (before and after base treatment), gas chromatography for free aminoethanol determination (before and after base treatment), ultraviolet absorption spectrum (in isopropyl alcohol), optical rotation measurement (in ethanol, *c* 1.6), and paper electrophoresis. The condition of the base treatment was: 0.4 ml. of 0.25 *N* aqueous KOH was added to 5.63 mg. of the product in 0.6 ml. of ethanol. The resulting homogeneous solution was allowed to stand overnight at room temperature, then acidified with 0.1 ml. of 2.0 *N* HCl, and evaporated to dryness before the analyses.

Preparation of "*seco*-Gramicidin A."—The solution of 188 mg. of gramicidin A in 5 ml. of methanol containing 5 ml. of freshly prepared 2.9 *N* HCl (anhydrous) in methanol was allowed to stand at room temperature for 1 hr. and evaporated to dryness with a flash evaporator. The residue was dissolved (not completely) in 15 ml. of methanol and evaporated again to dryness. The residue was then dissolved in 5 ml. of glacial acetic acid and freeze dried. The colorless powder obtained was dried completely in a vacuum desiccator over P_2O_5 and NaOH. The yield of "*seco*-gramicidin A" was 185 mg. The free amino group content was 0.92 mole/1850 g. (= 1 mole) and the methoxy content was 0.0%.

Preparation and Analysis of DNP-*seco*-gramicidin A.—To the solution of 90 mg. of "*seco*-gramicidin A" in 4 ml. of ethanol, 90 mg. of FDNB and 2 ml. of 10% triethylamine were added. After standing for 12 hr. at room temperature in a dark place, the solvent and triethylamine were removed by evaporation. The residue was washed with ether (4 × 5 ml.) to remove excess FDNB and dinitrophenol and dissolved in 3 ml. of ethanol. DNP-*seco*-gramicidin is insoluble in ether and soluble in ethyl acetate. Ten ml. of 0.1 *N* aqueous salt solution was added to precipitate the product. The precipitate (90 mg.) was collected, washed with water and dried. Because it still contained a small amount (about 7%) of dinitrophenol, further purification for analysis was carried out by preparative thin-layer chromatography with solvent system I. The purified DNP-*seco*-gramicidin A showed an ultraviolet absorption spectrum typical of DNP-peptides, λ_{max} 340 mμ in glacial acetic acid. The CNP content of the product was calculated to be 0.87 mole/mole from the optical density at 340 mμ. In this calculation, the molecular weight of DNP-*seco*-gramicidin A was assumed to be 2020. The molar extinction value, 14.9×10^3, was that of authentic DNP-L-Ileu-Gly methyl ester in acetic acid.

Two kinds of hydrolysates were prepared from DNP-*seco*-gramicidin A: one from 3.70 mg. of DNP-*seco*-gramicidin containing 0.580 μmole of DNP-L-alanine as an internal standard, the other from 3.70 mg. of DNP-*seco*-gramicidin alone. In both cases hydrolysis was performed with 0.8 ml. of glacial acetic acid and 0.8 ml. of 12.0 *N* HCl in evacuated ampoules at 110° for 18 hr. Each of the hydrolysates was evaporated to dryness and the residue dissolved in 3.5 ml. of 0.5 *N* aqueous $NaHCO_3$ solution. The solution was washed with 3 × 2 ml. of ether to remove decomposition products of tryptophan which interfere with the gas chromatographic analysis and on thin-layer chromatograms give various fluorescent spots. When the combined ether washings were concentrated and analyzed by thin-layer chromatography, only a trace of DNP-aminoethanol was detected. The aqueous layer was acidified with 0.5 ml. of 6.0 *N* HCl and extracted with 3 × 2 ml. of ether. The ether extract containing DNP-amino acids was dissolved in a small amount of dimethoxyethane and 1 ml. of ethereal diazomethane was added. After the removal of excess diazomethane and solvent, the residue, containing DNP-amino acid methyl esters, was dissolved in 0.1 ml. of dimethoxyethane. Thin-layer and gas chromatographic analyses were carried out with both hydrolysates. Aliquots of 2–4 μl. of the sample solution prepared above were used for the former analysis and 1.0-μl aliquots for the latter. Figures 2 and 3 represent the results from the hydrolysate without internal standard. The gas chromatogram (Fig. 3) permitted the calculation of the relative yields of NH_2-terminal residues. The data, combined with the values from the other hydrolysate, were corrected to afford the absolute values shown in Table I. Because the internal standard was added to the sample before hydrolysis, losses of DNP-amino acids due to destruction during hydrolysis do not affect absolute values obtained in this fashion.

Methanolysis of 4.0 mg. of DNP-*seco*-gramicidin was carried out at 75° for 20 hr. in 2 ml. of methanol half-saturated with hydrogen chloride at 0° (freshly prepared) in a glass ampoule which was flushed with nitrogen and sealed. The methanolysate was taken to dryness and the residue distributed between 3 ml. of ether and 4 ml. of 0.1 *N* HCl. All the yellow material went into the ether layer. The colorless aqueous layer was further washed with 2 × 2 ml. of ether and the combined ether layers were evaporated to dryness. Thin-layer chromatography of this sample showed three spots, P1, P2, and P3, as seen in Fig. 2. The peptides of the three spots were separated by preparative thin-layer chromatography with solvent system II on two silica gel plates. Three yellow bands were excised from the chromatograms and eluted, each with 2 × 6 ml. of methanol. Each of the methanol eluates was evaporated to dryness and the DNP content determined spectrophotometrically after redissolution in ethanol. For the calculation of the amounts, the observed molar extinction value, 16.7×10^3, of DNP-L-Ileu-Gly methyl ester was used. The homogeneity of the preparations was confirmed by rechromatography on thin-layer plates. The substances, P1 and P2, were hydrolyzed at 110° in 0.5 ml. of 6.0 *N* HCl plus 0.1 ml. of acetic acid for 19 hr., evacuated, and sealed in glass ampoules. DNP-isoleucine and DNP-valine were identified in the ether-extractable fractions of the hydrolysates from P1 and P2, respectively, by means of thin-layer chromatography and gas chromatography. In the aqueous layer of both of the hydrolysates, glycine was identified as its DNP-derivative by thin-layer chromatography.

Lithium Borohydride Reduction of "*seco*-Gramacidin A" and Gramicidin A.—Both *seco*- and original gramicidin A were reductively cleaved in the same way: To the solution of 8.0 mg. of the sample in 1 ml. of dimethoxyethane, approximately 50 μmoles of lithium borohydride in 50 λ of dimethoxyethane was added. The resulting turbid solution was allowed to stand overnight (17 hr.) at room temperature. The reaction mixture was adjusted to pH 3.0 with 1 ml. of dilute acetic acid in order to destroy excess reagent. The residue was evaporated to dryness and dissolved in 5 ml. of 1-butanol containing 5 ml. of water. The aqueous layer was extracted with 3 × 3 ml. of 1-butanol and the butanol layer and washings were combined and taken to dryness. The product was hydrolyzed at 110° in 2 ml. of 6.0 *N* HCl and 0.4 ml. of acetic acid in an evacuated ampoule. The hydrolysate was evaporated and dissolved in 0.4 ml. of water. A one-fourth aliquot was trinitrophenylated with 5 mg. of TNBS in 1 ml. of 0.5 *N* aqueous sodium bicarbonate solution at 40° for 2 hr. The resulting solution was extracted with 2 × 2 ml. of ether and

(20) Recently, Landowne and Lipsky [*Fed. Proc.*, **22**, abstract No. 449 (1963)] reported on the usefulness of an electron capture detector for the gas chromatography of DNP-amino acids. According to these authors all common DNP-amino acids could be assayed with the same high sensitivities indicating that response was strictly a function of the DNP group and was independent of the rest of the molecule.

(21) M. Brenner, A. Niederwieser, and G. Pataki, *Experientia*, **17**, 145 (1961).

the ether extract analyzed by thin-layer chromatography in solvent systems II and III. As chromatographic controls, various amino alcohols were trinitrophenylated under the same conditions. With the exception of TNP-leucinol and -isoleucinol all of these TNP-amino alcohols were well separable in both solvent systems. TNP-tryptophanol showed up as the major Ehrlich-positive neutral product besides TNP-aminoethanol, picramide, and one or two minor components.

COMMUNICATIONS TO THE EDITOR

Correlation of Solvolysis Rates and Estimation of Rate Enhancements

Sir:

The acetolysis rates of many arenesulfonates ($RR'CHOSO_2Ar$) are quantitatively correlated with the infrared carbonyl stretching frequencies of the corresponding ketones ($RCOR'$). Table I lists rates and frequencies of twenty compounds for which reliable data are available.[1]

TABLE I

Arenesulfonate	No.	log k_{rel}	$\nu_{C=O}$ for ketone, cm.$^{-1}$
Cyclohexyl	1	(0.00)[a]	1716
Cycloheptyl	2	1.78[b]	1705[c]
Cyclooctyl	3	2.76[b]	1703
Cyclononyl	4	2.70[b]	1703[d]
Cyclodecyl	5	2.98[b]	1704
Cycloundecyl	6	2.05[b]	1709[e]
Cyclododecyl	7	0.50[b]	1713[d]
Cyclotridecyl	8	0.66[b]	1713[d]
Cyclotetradecyl	9	0.08[b]	1714[d]
Cyclopentadecyl	10	0.42[b]	1715[d]
Isopropyl	11	0.15[f]	1718
2-Butyl	12	0.53[f]	1721
Methylisopropylcarbinyl	13	0.93[f]	1718
Methyl-*t*-butylcarbinyl	14	0.62[f]	1710
7-Norbornyl	15	−7.00[g,h]	1773
endo-8-Bicyclo[3.2.1]octyl	16	−4.11[i]	1752
2-Adamantyl	17	−1.18[c]	1727[c]
α-Nopinyl	18	−0.73[j]	1717
β-Nopinyl	19	0.04[j]	1717
1,4-α-5,8-β-Dimethanoperhydro-9-anthracyl	20	2.67[k]	1696

[a] S. Winstein, B. K. Morse, E. Grunwald, H. W. Jones, J. Corse, D. Trifan, and H. Marshall, *J. Am. Chem. Soc.*, **74**, 1127 (1952). [b] H. C. Brown and G. Ham, *ibid.*, **78**, 2735 (1956). [c] See ref. 2. [d] T. Bürer and H. H. Günthard, *Helv. Chim. Acta*, **39**, 356 (1956). [e] N. J. Leonard and F. H. Owens, *J. Am. Chem. Soc.*, **80**, 6039 (1958). [f] S. Winstein and H. Marshall, *ibid.*, **74**, 1120 (1952). [g] S. Winstein, M. Shatavsky, C. Norton, and R. B. Woodward, *ibid.*, **77**, 4183 (1955). [h] C. J. Norton, Ph.D. Thesis, Harvard, 1955. [i] See ref. 3. [j] S. Winstein and N. J. Holness, *J. Am. Chem. Soc.*, **77**, 3054 (1955). [k] S. Winstein and L. deVries, unpublished work, quoted in R. Piccolini, Ph.D. Thesis, U.C.L.A., 1960.

Representation in the table has been limited to saturated, secondary arenesulfonates without heterosubstituents; in addition, the following types were specifically excluded: (1) compounds in which ground-state eclipsing interactions are relieved in the solvolytic transition state (for example, cyclopentyl and *endo*-2-norbornyl derivatives), and (2) compounds which have been shown to undergo anchimerically accelerated solvolysis (for example, *exo*-2-norbornyl and cyclobutyl derivatives).

Figure 1 is a plot of the data from Table I. The least-squares straight line through the points obeys the equation $\log k$ (relative to cyclohexyl, 25°) $= -0.132 \cdot (\nu_{C=O} - 1720)$; the correlation coefficient is −0.97.

A qualitative relationship between ketone frequency and solvolysis rate was observed by Schleyer and Nicholas[2] and would, indeed, be expected, since both carbonyl frequency and solvolysis rate are sensitive to bond angle and hybridization.[2-4] It is surprising, however, that the correlation should be so excellent for so many dissimilar compounds; the acetolysis rates cover a range of ten powers of ten, yet no rate varies from the line by much more than about one power of ten.

The correlation provides an extremely useful semi-empirical relationship for the prediction of solvolysis rates. It also allows the magnitude of the combined effects of anchimeric acceleration and other interactions to be estimated by providing a "model" rate from which the effects of angle strain have been factored out.

As an example of the predictive usefulness of the correlation, Table II lists experimental and calculated data for several compounds which were not included in the calculation of the least-squares line, either because they were of slightly different type from those in Table I, or because the infrared data were considered somewhat less reliable. The calculated acetolysis rates agree extremely well with the experimental, even though several of the compounds are primary or unsaturated. It should be noted that although both polar substituents and conjugation affect rate and carbonyl frequency in the same way as angle strain (increased rates corresponding to decreased carbonyl frequencies), it is not clear that the relative effect would necessarily be of the same magnitude as for angle strain. Further testing of this point would be desirable.

Table III lists data for a number of compounds which were excluded from Table I because their acetolysis

(1) Infrared spectra for which no literature reference is cited were measured in dilute solution (CCl_4) on a calibrated Perkin–Elmer 421 grating spectrograph by Mr. Donald Steele. Expanded scale, reduced slit width, and nitrogen sweep were used; frequencies are believed accurate to ±1 cm.$^{-1}$. Other infrared data were chosen from sources which reported similar measurement conditions. Many absorptions were doublets or multiplets; in these cases, weighted average peak positions are given. All acetolysis rates of toluenesulfonates or bromobenzenesulfonates are relative to cyclohexyl toluenesulfonate or bromobenzenesulfonate, respectively, at 25°.

(2) P. von R. Schleyer and R. D. Nicholas, *J. Am. Chem. Soc.*, **83**, 182 (1961).

(3) C. S. Foote and R. B. Woodward, *Tetrahedron*, in press.

(4) (a) J. O. Halford, *J. Chem. Phys.*, **24**, 830 (1956); (b) R. Zbinden and H. K. Hall, Jr., *J. Am. Chem. Soc.*, **82**, 1215 (1960); (c) H. C. Brown, *J. Chem. Soc.*, 1248 (1956).