TABLE I

DEHYDROALANINE, LYSINE, AND NEW AMINO ACID CONTENT OF MODIFIED RNase

Compound	Moles of dehydroalanine per mole of protein[a] Calcd.	Found	Moles of lysine Calcd.	Found	Moles of compound III
S-DNP RNase	8	5.5	10	6.5	3
Acetylated S-DNP RNase	8	7.6	10	9.4	0
Native RNase	...	2.2	10	8.5	2.1
Oxidized RNase[9]	...	0.2	10	9.6	0

[a] The amount of dehydroalanine present was determined by estimation of the pyruvic acid formed upon acid hydrolysis.

Table I). This protein, as do cystine derivatives,[10] at high pH yields dehydroalanine which can undergo the reaction described above. Oxidized RNase, which does not undergo β-elimination of its cysteic acid residues, yields almost no dehydroalanine and no LAL. The finding[11] that alkali-treated wool is no longer soluble in cuprammonium–sulfite–urea mixture may be due to the fact that the above reaction between the ϵ-group of lysine and dehydroalanine residues results in extensive cross linking.

Application of the specific scission of the dehydroalanine residues in peptide chains makes it possible to determine at which points of the peptide chain the above reaction occurs. Evidently only the residues next to the carboxyl group of unreacted dehydroalanine residues will appear as N-terminal end groups after the specific fragmentation. A comparison of N-terminal end groups appearing after cleavage of acetylated protein will, therefore, indicate the dehydroalanine residues which reacted with ϵ-amino groups of lysine. The N-terminal groups[12] present after cleavage of RNase derivatives are listed in Table II.

TABLE II

YIELDS OF N-TERMINAL RESIDUES AFTER CLEAVAGE OF RNase DERIVATIVES

Amino acid	Mole/mole of S-DNP RNase (I)	Mole/mole of acetylated S-DNP RNase (IV)	Mole/mole of S-DNP "S-protein"[13]
Lysine	0.07	0.95	0.02
Arginine[a]	0.16	0.15	..
Aspartic acid	0.31	0.34	0.34
Serine	0.47	0.42	1.34
Glutamic acid	0.20	0.52	0.40
Alanine[b]	0.26	0.24	0.23
Glycine[b]	0.42	0.36	0.36

[a] Arginine was found to give low yields by the cyanate method as found with arginine amide. [b] Values corrected for the nonspecific formation of additional quantities of these amino acids in the cyanate method.[8]

The N-terminal amino groups appearing after cleavage of IV have been described previously.[2] It can be seen that the only differences between the acetylated and nonacetylated derivative are in the lysine and glutamic acid values.

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(12) The N-terminal groups were identified by the cyanate method [G. H. Stark and D. G. Smyth, *J. Biol. Chem.*, **238**, 214 (1963)].

(13) F. M. Richards and P. J. Vithayathil, *ibid.*, **234**, 1459 (1959).

Subtilisin ↓ (between Ala-20 and Ser-21)

NH_2-Lys(1)······Ala(20) ↓ Ser(21)······Cys(26) AspNH_2(27)····Cys(40) Lys(41)····Cys(58) Ser(59)····

Cys(65) Lys(66)······Cys(72) Gly[2](73)······Cys(84) Arg(85)·····Cys(95) Ala(96)·····Cys(110) Glu(111)·····Va(124)

The nonappearance of Lys-41 and Lys-66 (see abbreviated formula of reduced RNase) and the decrease in the value for Glu-111 indicate that the dehydroalanine residues derived from Cys-40, Cys-65, and Cys-110 were involved in the reaction described. The dissappearance of Lys-1 also implicates this residue in the reaction. It seems probable that, because of spatial proximity, the pairs 40–41 and 65–66, respectively, reacted (*cf.* the reaction of N-acetyl-S-DNP-L-cysteinyl-L-lysine methyl ester hydrobromide quoted above). The third pair would then be Lys-1 and dehydroalanine-110. A specific interaction of two residues so remote from each other may be due to a specific spatial configuration. This view is supported by the finding that reduced and S-dinitrophenylated "S-protein," in which residues 1 to 20 are absent, gave a yield of N-terminal Glu-111 similar to that obtained from IV (see Table II; in this case an additional serine group due to residue 21 is found).

The approach described here might be of possible value as a tool in the determination of the chemical topography within a protein molecule.

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Formyl, a Novel NH_2-Terminal Blocking Group in a Naturally Occurring Peptide. The Identity of *seco*-Gramicidin with Desformylgramicidin[1,2]

Sir:

Gramicidin, a peptide antibiotic which contains no free amino or carboxyl group, was isolated in 1941 by Hotchkiss and Dubos,[3] who initially assumed a cyclic structure on the basis that hydrolysates of gramicidin contained only neutral amino acids, but no fatty acids or alcohols.[4] Later the discovery of ethanolamine in gramicidin hydrolysates prompted Synge[5] to propose an *ortho*-peptide bond[6] for the linkage of ethanolamine to explain the neutral character of gramicidin and the free hydroxyl, the only functional group present.

We have now found that gramicidin A[7] contains an N-formyl group. Two-hour hydrolysis of gramicidin at 110° in 50% sulfuric acid, followed by distillation at room temperature under high vacuum into a trap cooled to −80°, yielded a solution of formic acid in water as distillate. The formic acid was identified

(1) Gramidicin A. III. Preceding papers: S. Ishii and B. Witkop, *J. Am. Chem. Soc.*, **85**, 1832 (1963); **86**, 1848 (1964).

(2) Presented in part at the IUPAC Meeting, Kyoto, April, 1964.

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(4) R. D. Hotchkiss, *ibid.*, **141**, 171 (1941).

(5) R. L. M. Synge, *Biochem. J.*, **39**, 355 (1945).

(6) R. L. M. Synge, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 191 (1950).

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

either by reduction to formaldehyde (Mg, HCl) and subsequent reaction with chromotropic acid,[8] or by gas chromatography[9] of the distillate after evaporation of the sodium salt solution and acidification with dilute phosphoric acid (6-ft. column packed with 20% Tween 80 and 2% phosphoric acid on chromosorb W, argon detector). No fatty acid other than formic acid could be detected by gas chromatography, except acetic acid (7%) in a sample of gramicidin A which had been freeze-dried from acetic acid. However, acetic acid was no longer found when gramicidin A was either dried at 100° and 10^{-2} mm. over KOH for 25 hr. or recrystallized from ethanol–water.

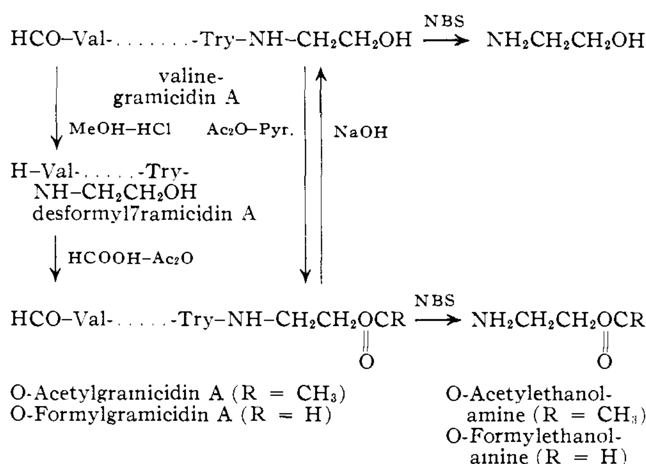
Desformylgramicidin A or tryptophan in control experiments did not liberate any trace of formic acid under the same hydrolytic conditions.

Quantitative results obtained by comparison with standard solutions of formic acid indicate that 0.6–0.7 mole of HCOOH (chromotropic acid reaction, extinction at 575 mμ) or 0.8–1.0 mole of HCOOH (gas chromatography) per 1850 g. of gramicidin A are liberated.

The n.m.r. spectrum of gramicidin A in D_3COD[10] shows a broad peak at 8.45 p.p.m. (internal standard TMS), the peak area being $^1/_{12}$ to $^1/_{14}$ as large as that of the aromatic indole protons at 6.0–5.2 p.p.m. Addition of formic acid to the solution of gramicidin gives rise to a sharp peak at 8.32 p.p.m. By comparison, the formyl proton of formyl-L-valine appears at 8.40 p.p.m.

In the light of these new results, the gentle methanolysis of gramicidin[1] to the so-called *seco*-gramicidin A, a compound with one new NH_2-terminal group per mole of compound (1850 g.), is simply the removal of the N-formyl group to yield desformylgramicidin A, a reaction that was demonstrated to proceed with the comparable N-formyl-L-valine in more than 95% yield under the same conditions. Gramicidin A was treated with 1.5 *N* HCl in methanol at room temperature for 1 hr. After evaporation, the desformylgramicidin A was separated from 6% unreacted gramicidin A by fractionation of the methanolic solution of the reaction product on a Dowex W50 X2 column. The neutral gramicidin A passes through the column, while the basic desformylgramicidin A is eluted with 1.0 *N* NH_4OH in methanol. Treatment of desformylgramicidin A, $[\alpha]^{20}D$ +5.4° (propionic acid), with formic acid–acetic anhydride[11] at room temperature for 4 hr. leads to a ninhydrin-negative substance, presumably O-formylgramicidin, which migrates on thin layer chromatography in two different solvent systems (acetic acid–chloroform, 2:1; pyridine–2-butanone, 3:7) like O-acetylgramicidin A, acetylated with Ac_2O in pyridine.[12] Base treatment of this ester leads in 80% over-all yield to a compound which, on thin layer chromatography, is identical with gramicidin A, $[\alpha]^{20}D$ −5° (propionic acid).

While N-bromosuccinimide (NBS) oxidation of gramicidin A leads to the liberation of aminoethanol,[13] acetylated gramicidin A releases O-acetylethanolamine on treatment with NBS. These compounds were identified by paper electrophoresis and by gas chromatography after trifluoroacetylation (4% neopentylglycol succinate on chromosorb W, flame ionization detector).



In the hydrolysate of O-*n*-butylated gramicidin A (butylation with *n*-BuI–Ag_2O, hydrolysis in 0.3 ml. of AcOH–1.5 ml. of 6.0 *N* HCl at 100° for 20 hr.), 2-*n*-butoxyethylamine, aminoethanol, and aminoethanol acetate are found. 2-*n*-Butoxyethylamine is not stable under these hydrolytic conditions and is partially converted to aminoethanol and 2-aminoethanol acetate.

These results support a linear peptide structure such as HCO-Val-......-Try–NH–CH_2CH_2OH for valine-gramicidin A and the scheme presented in the chart.

The occurrence of an N-formylamino acid[14] in a peptide raises interesting biosynthetic questions.[15]

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Gramicidin A. IV. Primary Sequence of Valine and Isoleucine Gramicidin A

Sir:

The key to the structure of gramicidin A[1] was the selective removal[2] and identification[3] of the formyl group which blocks the NH_2-terminal valine (or isoleucine). This made possible the application of successive Edman degradations and established directly the sequence of the first ten amino acids and indirectly the total sequence of gramicidin A. The solubility of gramicidin A and its degradation products in organic solvents, and their insolubility in water, necessitated a modification of the customary Edman procedure.[4]

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