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Gramicidin A. I. Determination of Composition and Amino Acid Configuration by Enzymatic and Gas Chromatographic Methods[1]

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A combination of enzymatic degradation with quantitative gas chromatographic analysis has made possible the complete assignment of configuration to four of the six amino acids present in hydrolysates of gramicidin A, a cyclopeptide antibiotic recently shown to be heterogeneous and consisting of a mixture of *valine-gramicidin* and *isoleucine-gramicidine*. The new method is rapid, sensitive, quantitative and generally applicable.

The amino acid analysis of gramicidin A, as reported previously[1] and in paper II of this series,[3] revealed its heterogeneity. Isoleucine was found to be present as a new minor component in a slightly variable but non-stoichiometric amount. The sum of valine and isoleucine in all samples was constant. This suggested that isoleucine substituted for valine in a new species of gramicidin which may be called isoleucine-gramicidin. Gramicidin A, therefore, in our case could be a mixture of 60–80% of valine-gramicidin and 40–20% of isoleucine-gramicidin, a ratio which is probably dependent on the number of transfers used in the purification by countercurrent distribution technique.[4] This paper describes the optical configuration of the constituent amino acids, especially of isoleucine, by using a novel combination of enzymatic and gas chromatographic methods. The amino acids in the hydrolysate remaining after separate treatment with D- and L-amino acid oxidases were assayed by quantitative gas chromatography as the methyl esters of their DNP[5] derivatives. This adaptation of an originally qualitative technique[6] permits the rapid quantitative assay of amino acid mixtures with an accuracy of 2%. The systems of Weygand, Saroff and Zomzely (alkyl esters of trifluoroacetyl derivatives)[7,8a,8b] and of Meister (amyl esters of acetyl derivatives)[9] did not lend themselves as readily to quantitative analyses as the one developed by Pisano, *et al.*[6] Thin-layer chromatography was also used as a supplemental method.

Experimental

Materials.—The experiments were performed with the specimen of gramicidin A described in a subsequent paper.[3]

D-Amino acid oxidase, a relatively crude preparation from hog kidney (Lot 602), L-amino acid oxidase, a highly purified preparation from *Crotalus adamanteus* venom (Lot LAO 27), and crystalline beef liver catalase (Lot 5542) were obtained from the Worthington Biochemical Corporation. FAD (Lot F 31B-062) was purchased from the Sigma Chemical Co.

DNP-Amino acids were obtained as a kit (Lot G1068–G1085) from the Mann Research Laboratories. FDNB was obtained from the Pierce Chemical Co.

The solution of diazomethane was the ether layer obtained when 7.3 g. of N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Co.) was added in small portions to an ice-cooled mixture of 100 ml. of ether and 30 ml. of 50% aqueous potassium hydroxide.

Analytical Methods.—For thin-layer chromatography, the Desaga apparatus and silica gel G (both purchased through Brinkmann Instruments Inc.) were used. Thin-layer plates (approximately 250 μ in thickness, 200 × 200 mm.) were dried *at room temperature* overnight. The solvent system composed of chloroform, methanol and acetic acid (95:5:1, v./v.)[10] was used as the developer for DNP-amino acids, the solvent system of toluene, pyridine and acetic acid (80:10:1, v./v.) for DNP-amino acid methyl esters.

Gas chromatographic analyses were carried out on a model 60-1A compact chromatograph analysis unit of the Research Specialties Co., equipped with a flame ionization detector. The column packing, 1% SE-30 coated on Gas-Chrom P (100-120 mesh), was donated by Dr. J. Pisano. It was packed in a 6-foot stainless steel tube (internal diameter, $^1/_8$ inch). During the analyses the column was maintained at 175° and the rate of flow of the carrier gas, N_2, was adjusted to 10 ml. per minute as measured at the inlet.

Procedure.—Hydrolysis of gramicidin A was performed in the following two ways: A solution of 22.1 mg. of gramicidin A in 2.0 ml. of glacial acetic acid plus 10 ml. of constant boiling HCl was sealed in an evacuated glass tube and heated at 110° for 48 hours. The hydrolysate was evaporated to dryness in a flash evaporator and the residue was dissolved in 4 ml. of water. After neutralization to *p*H 6 by the addition of 2.0 *N* NaOH, the solution was diluted to a total volume of 5.00 ml. (*hydrolysate I*).

A solution of 17.0 mg. of gramicidin A in 1.0 ml. of glacial acetic acid plus 1.0 ml. of 12.0 *N* HCl was sealed in an evacuated glass tube and kept at 37° for 10 days followed by heating at 110° for 38 hours. The hydrolysate was evaporated to dryness, and 29.8 μmoles of glycine in 2.5 ml. of water was added as an internal standard for the quantitative analysis. The solution was neutralized to *p*H 7 with 2.0 *N* NaOH and the total volume was adjusted to 5.00 ml. (*hydrolysate II*).

Enzymatic oxidations with amino acid oxidases were carried out in a Warburg apparatus at 38° with the use of air as the gas phase.

D-Amino Acid Oxidase Reaction.—The main compartment of the flask contained 1.00 ml. of hydrolysate I or II, 1.0 ml. of sodium pyrophosphate buffer (0.1 *M*, *p*H 8.2), 30 units of catalase and 4×10^{-3} μmole of FAD in a total volume of 2.14 ml. The sidearm contained 8.0 mg. of D-amino acid oxidase in 0.2 ml. of water.

L-Amino Acid Oxidase Reaction.—The main compartment contained 0.60 or 0.80 ml. of hydrolysate I or II, respectively, 1.0 ml. of 2-amino-2-(hydroxymethyl)-1,3-propanediol–HCl buffer (0.1 *M*, *p*H 7.7) and 0.1 ml. of 1 *M* KCl in a total volume of 2.10 ml. The side arm contained 1.3 mg. of the enzyme in 0.2 ml. of 0.1 *M* KCl. The center well contained 0.1 ml. of 20% KOH.

Control experiments were run by incubating the enzymes (separately) with an identical volume of water in place of the hydrolysate. The flasks were tipped after a 10-minute equilibration period and readings were taken at 10- or 20-minute intervals for 6 hours until the oxygen uptake ceased. The *p*H of the reaction products was adjusted to 9 by the addition of 90 mg. of $NaHCO_3$ and 4 drops of 2.0 *N* NaOH.

Dinitrophenylation was carried out by the addition of 0.2 ml. each of FDNB to the products and by agitation at 40° for 3 hours. After excess FDNB had been extracted with ether, the solutions were acidified with 10 drops of 6 *N* HCl. The DNP-amino acids were then extracted with ether. The ether was evaporated and the residues were dissolved in 1.0 ml. of dimethoxyethane. DNP derivatives of the original hydrolysates were also prepared under the same condition. For thin-layer chromatography, 2- to 3-μl. aliquots from the sample solutions were applied on a plate.

The remaining parts of the solutions were mixed with 2 ml. of diazomethane solution in ether and allowed to stand for 20 to 30 minutes at room temperature. The resulting solutions of DNP-amino acid methyl esters were evaporated to dryness and redissolved in appropriate volumes of dimethoxyethane. Aliquots of the solutions containing the amounts corresponding to 2.2–5.0 μg. of gramicidin A were introduced into a gas chromatograph column with a 10-μl. Hamilton syringe. Thin-layer chroma-

(1) Presented in part at the 142nd National Meeting of the American Chemical Society, Atlantic City, N. J., September, 1962; Abstracts p. 57C.

(2) Visiting Scientist of the USPHS, 1960–1963.

(3) E. Gross, S. Ishii and B. Witkop, in preparation.

(4) J. D. Gregory and L. C. Craig, *J. Biol. Chem.*, **172**, 839 (1948); *cf.* L. K. Ramachandran, *Biochemistry*, in preparation.

(5) The abbreviations used are: DNP, dinitrophenyl; FDNB, 1-fluoro-2,4-dinitrobenzene; FAD, flavin adenine dinucleotide.

(6) J. J. Pisano, W. J. A. VandenHeuvel and E. C. Horning, *Biochem. Biophys. Res. Commun.*, **7**, 82 (1962).

(7) F. Weygand, B. Kolb, A. Prox, M. A. Tilak and I. Tomida, *Z. physiol. Chem.*, **322**, 38 (1960).

(8) (a) H. A. Saroff and A. Karmen, *Anal. Biochem.*, **1**, 344 (1960); (b) C. Zomzely, G. Marco and E. Emery, *Anal. Chem.*, **34**, 1414 (1962).

(9) D. E. Johnson, S. J. Scott and A. Meister, *ibid.*, **33**, 669 (1961).

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

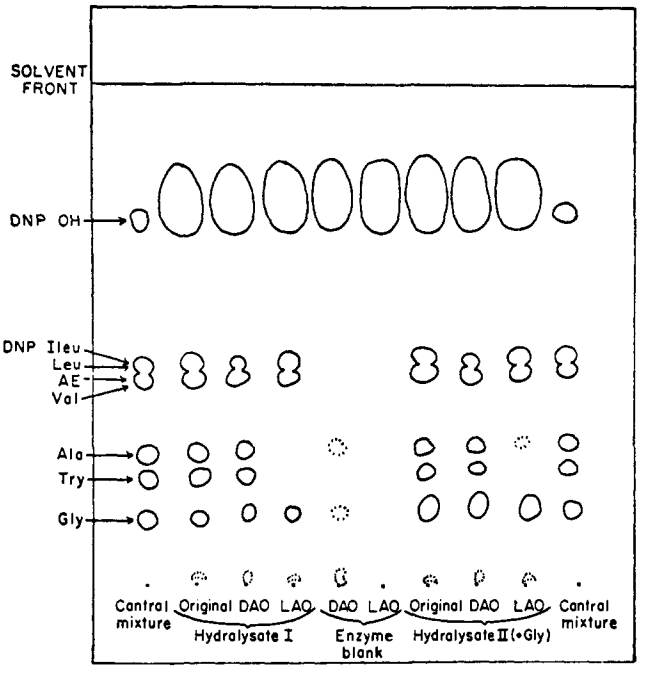

Fig. 1.—Thin-layer chromatogram of the amino acids (as DNP derivatives) present in hydrolysates of gramicidin A before and after the action of D-amino acid oxidase (DAO) or L-amino acid oxidase (LAO). A thin-layer plate of silica gel G was used. The developer was a mixture of chloroform, methanol and acetic acid (95:5:1, v./v.).

tography was used again to check the completeness of the esterification.

Results

Figure 1 shows the thin-layer chromatogram of the DNP derivatives of gramicidin hydrolysates and their digests by oxidases. On the chromatogram, tryptophan and alanine have been eliminated completely, or almost completely, by the action of L-amino acid oxidase, so they must be of the L-configuration. Because of the poor resolution of the leucine–valine area, further information could not be obtained from this chromatogram.

A set of gas chromatograms in Fig. 2 is a typical example of the results in hydrolysate I and its enzymatic digests. As seen in the figure, most of the leucine and about one-half of the valine disappeared after the action of D-amino acid oxidase, while alanine and isoleucine remained intact. L-Amino acid oxidase, on the other hand, consumed alanine, isoleucine and about one-half of valine. DNP-tryptophan methyl ester and DNP-amino ethanol were not detected on this column under these conditions.

Before the quantitative evaluation of these gas chromatographic data, control experiments with authentic amino acid derivatives were run. Linearity was observed quite satisfactorily between amounts of samples and peak areas on the chromatograms for all the amino acids tested. Figure 3 illustrates the relationship in DNP-isoleucine methyl ester which was examined most extensively; the linearity was maintained here in the range of 1 to 8 millimicromoles with the standard deviation (devised by arithmetic means) of 2.0%. The peak areas were measured by weighing the paper cut out of the peaks. The peak heights were also plotted against the amounts of the amino acid as shown in Fig. 3. In this case the calibration curve was somewhat concave.

Another control experiment was on the determination of relative response of the flame ionization detector employed to various amino acids. The values (relative peak areas) expressed on a molar basis are: isoleucine = 1.00, leucine = 0.90, valine = 0.97, alanine = 0.66, glycine = 0.27.

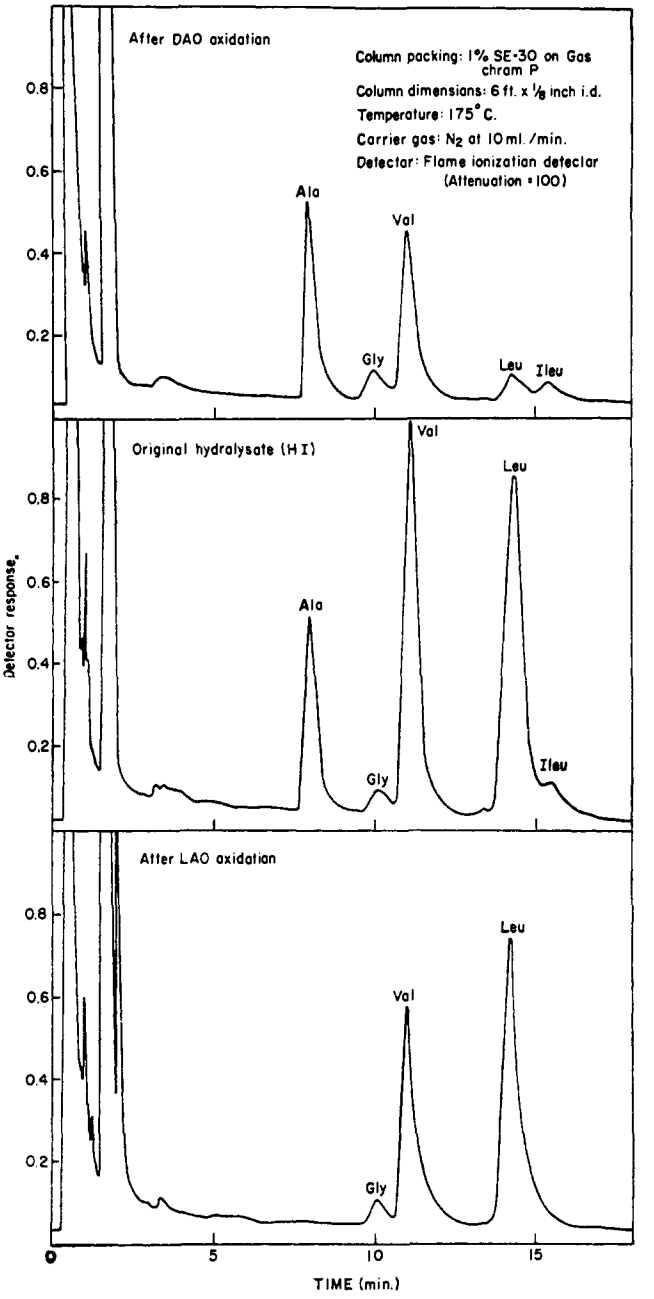

Fig. 2.—Comparison of gas chromatograms of the amino acids (methyl esters of DNP derivatives) present in the hydrolysate (I) of gramicidin A before and after the action of D-amino acid oxidase (DAO) or L-amino acid oxidase (LAO). The amount of each sample injected into the column corresponded to 2.90 μg. of original gramicidin A.

On the basis of these results, the chromatograms in Fig. 2 and others were corroborated by the peak area measurement. The summarized data are presented in Table I. The table also contains the data of the oxygen uptake during the oxidase reactions determined by a Warburg apparatus.

Discussion

Previously optical configurations have been reported[11,12] for the main constituent amino acids iso-

(11) R. D. Hotchkiss, *J. Biol. Chem.*, **141**, 171 (1941); R. J. Dubos and R. D. Hotchkiss, *Trans. Coli. Physicians Phila.*, [4] **10**, 11 (1942).

(12) H. N. Christensen, R. R. Edwards and H. D. Piersma, *J. Biol. Chem.*, **141**, 187 (1941).

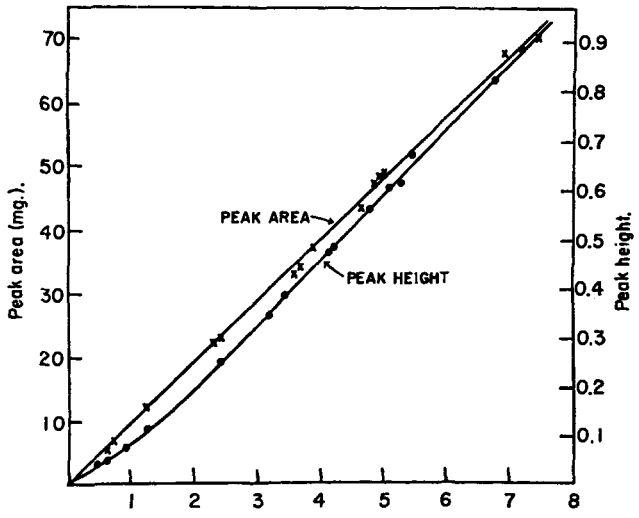

Fig. 3.—Calibration curve for isoleucine. The abscissa shows the amount of isoleucine, the ordinate shows either the peak area or the peak height. The peak areas were measured by weighing the paper cut out of the peaks.

lated from hydrolysates of insufficiently purified gramicidin. Such samples were obtained by simple solvent extraction and crystallization from tyrothricin and contained several other congeners besides gramicidin A. The data in Table I establish the optical configuration of the new minor component, isoleucine. Combined with the data from complete amino acid analyses[3] the results presented in Table I lead to the following amino acid configuration and composition expressed in the nearest integers: L-tryptophan = 4, D-leucine = 4, D-valine = 2, L-valine (1.6) + L-isoleucine (0.4) = 2, L-alanine = 2, glycine = 1, aminoethanol = 1. The figures before adjustment reflect to some extent minor racemization during hydrolysis or incomplete oxidation by the enzymes.

These results confirm the assumption which has by now been reached independently by Ramachandran (personal communication) that gramicidin A is heterogeneous and consists of a valine-gramicidin (60%) and a novel isoleucine-gramicidin (40%). It appears that L-isoleucine substitutes for one rather than two residues of L-valine.[12a] The homogeneity of both valine as well as isoleucine-gramicidin remains to be demonstrated.

According to Synge[13] the valine isolated after hydrolysis with HCl for 48 hours at 110° was racemic, whereas hydrolysis for 10 days at 37°, followed by 36 or 48 hours at 110°, was claimed to produce predominantly D-valine. This finding could not be confirmed. The data in Table I show that both methods of hydrolysis lead to equal amounts of D-valine.

In developing quantitative gas chromatographic technique from available qualitative methods we were limited in our choice. The requirements for a rapid quantitative procedure as well as for accuracy and

(12a) The frequent interchangeability and substitution of valine and isoleucine in *proteins* as a function of species (*cf.* H. Matsubara and E. L. Smith, *J. Biol. Chem.*, **237**, PC 3576 (1962)) may reflect the close similarity of the two code words, *viz.*, UUG for valine and UUA (or UAA?) for isoleucine (O. W. Jones and M. W. Nirenberg, *Proc. Natl. Acad. Sci.*, **48**, 2115 (1962)). The biosynthesis of bacterial peptides does not necessarily follow the pattern of protein synthesis. The *val–ileu* interchange in gramicidin raises an interesting biosynthetic point.

(13) R. L. M. Synge, *Biochem. J.*, **38**, 285 (1944); **44**, 542 (1949).

TABLE I

QUANTITATIVE GAS CHROMATOGRAPHIC ANALYSIS OF THE AMINO ACIDS PRESENT IN HYDROLYSATES OF GRAMICIDIN A BEFORE AND AFTER THE ACTION OF AMINO ACID OXIDASES[a]

	Hydrolysate I			Hydrolysate II		
		Remaining after the action of			Remaining after the action of	
	Original hydrolysate	D-Amino acid oxidase	L-Amino acid oxidase	Original hydrolysate	D-Amino acid oxidase	L-Amino acid oxidase
Glycine[b]	0.9	0.9	0.9	1.0[d]	1.0[d]	1.0[d]
Alanine[b]	2.0	1.8	0	2.1	1.8	0.3
Valine[b]	3.3	1.5	2.0	3.6	1.6	2.0
Isoleucine[b]	0.4	0.3	0	0.4	0.3	0
Leucine[b]	3.9	0.3	3.4	4.0	0.8	3.3
Tryptophan[c]	+	+	−	+	+	−
		Moles of amino acids oxidized[f] by			Moles of amino acids oxidized[f] by	
	Original hydrolysate[e]	D-Amino acid oxidase	L-Amino acid oxidase	Original hydrolysate[e]	D-Amino acid oxidase	L-Amino acid oxidase
Total	13.8	5.6	7.3	13.1[d]	5.6	6.0

[a] Figures are in moles per mole of gramicidin A. The molecular weight of gramicidin A was assumed to be 1850 in this calculation. [b] Determined by quantitative gas chromatography (averages of two analyses). Appreciable amounts of glycine, alanine and valine were detected in the control experiments with D-amino acid oxidase and no substrates, in contrast to the control experiments with L-amino acid oxidase where this was not the case. The figures of the digests from D-amino acid oxidase, therefore, were corrected accordingly. [c] Assayed by thin-layer chromatography. [d] Net values after deduction of the amount of glycine added as an internal standard (see the Experimental part). [e] Total amino groups as determined by the trinitrophenylation method (K. Satake, T. Okuyama, M. Ohashi and T. Shinoda, *J. Biochem.* (Tokyo), **47**, 654 (1960). [f] Calculated from the consumption of oxygen as determined by manometric methods.

reproducibility were met by the method adopted. Both dinitrophenylation as well as esterification of the DNP-amino acids with diazomethane proceeded smoothly and quantitatively. Additional proof for the completeness of these reactions was adduced by thin-layer chromatographic analysis of the products. The reaction mixture of DNP-alanine with diazomethane was also analyzed by gas chromatography and the area of the peak was compared with that from an authentic sample[14] of DNP-alanine methyl ester. The result indicated 100% yield of the methyl ester in the esterification mixture.

The method used here has recently been extended to the detection of hydroxy DNP-amino acid methyl esters, such as serine, threonine, the four hydroxyprolines (*cis*- and *trans*-3- and 4-hydroxyprolines[15,16]) and aminoethanol.[17] The successful chromatography of these hitherto unsuitable hydroxyamino acids depended on trifluoroacetylation of their hydroxy groups with trifluoroacetic anhydride. Gas chromatography can now be used for esters of all of the usual DNP-amino acids belonging to the "ether-soluble group" except DNP-tryptophan and di-DNP-amino acids.

(14) Two authentic specimens of crystalline DNP-alanine methyl ester were prepared, one from the reaction of DNP-L-alanine and diazomethane, the other from the reaction of DL-alanine methyl ester and FDNB. They could not be distinguished by comparison of the behaviors on gas chromatography and thin-layer chromatography and of infrared spectra.

(15) F. Irreverre, K. Morita, A. V. Robertson and B. Witkop, *Biochem. Biophys. Res. Commun.*, **8**, 453 (1962).

(16) F. Irreverre, K. Morita, S. Ishii and B. Witkop, *ibid.*, **9**, 69 (1962).

(17) Unpublished observations of S. Ishii.