Gramicidin. IX. Preparation of Gramicidin A, B, and C*

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ABSTRACT: By countercurrent distribution and redistribution the commercially available peptide antibiotic, gramicidin, was resolved on a preparative scale. The group of lipophilic peptides contained gramicidins A, B, and C, each of which consisted of a pair of congeners, *i.e.*, valine–gramicidin (80–95%) and an isoleucine–gramicidin (5–20%). The sum of aromatic amino acids was always four, namely, four tryptophan in gramicidin A, three tryptophan plus one phenylalanine in gramicidin B, and three tryptophan plus one tyrosine in gramicidin C. A more hydrophilic, strongly antibiotic group of peptides, designated gramicidin D, contained five to six additional amino acids. Complete amino analyses, including time-dependence studies of hydrolyses, are given over the entire range of 999 transfers.

The primary sequence of valine– and isoleucine–gramicidin A (Sarges and Witkop, 1964a,b, 1965a) and its confirmation by synthesis (Sarges and Witkop, 1965b) have been reported previously. The structure of the major congeners, gramicidin B (Sarges and Witkop, 1965c) and gramicidin C (Sarges and Witkop, 1965d) has also been established. This paper describes in some detail the resolution of commercial gramicidin on a preparative scale into its components by the use and extension of the original method of Craig (Gregory and Craig, 1948).[1]

Experimental Section

Countercurrent Distribution. Commercially available gramicidin (60 g, Penick, lot 514-RTF) was dissolved in 1200 ml of lower phase of the solvent system: benzene–chloroform–methanol–water, 15:15:23:7 (v/v) (Gregory and Craig, 1948), and spread over 30 cells of a 500-tube countercurrent distribution machine,[2] the units of which had a lower phase volume of 40 ml. Upper phase volumes were also adjusted to 40 ml. At the completion of 500 transfers, the upper phase was withdrawn at each transfer and directed to an automatic fraction collector until a total transfer number of 999 had been reached. The fractions were located by measuring the ultraviolet absorption at 280 mμ of the individual tubes.

The distribution pattern (Figure 1) indicates the presence of (at least) four distinct components which are designated (Gregory and Craig, 1948) gramicidin A, B, and C; the minor component at the far right of the distribution pattern will be referred to as gramicidin D.

Results and Discussion

Theoretical curves (Craig and Craig, 1950) were calculated for the four peaks and plotted in the distribution pattern (Figure 1). For gramicidin A the left-hand slopes of the experimental and theoretical curve are superimposable. The discrepancy in the right-hand portion of the curves indicates the presence of additional components different from gramicidin A. When the material isolated from tubes 381–438 was redistributed it was shown to contain isoleucine–gramicidin and valine–gramicidin, which separate on extensive distribution with the number of transfers exceeding 2000 (*cf.* Ramachandran, 1963). The experimental and theoretical curve of gramicidin B closely resembles the pattern found for gramicidin A. Gramicidin C was present in the upper phase volumes which had been withdrawn from the machine and had spread over a large number of tubes; its separation from tailing substances related to gramicidin A after 999 transfers is still incomplete. The amino acid composition of the hydrophilic component D is different from that of gramicidin A, B, and C.

Gramicidin A constitutes the major part of commercial gramicidin. The amount of gramicidin B is only one-tenth of that of gramicidin A. Gramicidin C amounts to approximately two-thirds of the weight of gramicidin B (see Table I). Gramicidin D is indeed a minor component.

Redistribution of Gramicidin A, B, and C. The homogeneity of gramicidin A, B, and C was tested by redistribution in the same solvent system. For gramicidin A (Figure 2) the left-hand slopes of the theoretical and experimental curve overlap, whereas the right-hand slopes diverge and indicate, as mentioned, that more

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TABLE 1: Components and Their Yield in the Resolution of Commercial Gramicidinby Countercurrent Distribution.[a]

Phase	Tube Number	Weight (g)	Yield (%)	Type of Antibiotic
	0–199	Discarded		
Lower and upper phase in counter-current distri-bution machine	200–221	0.466		
	222–242	1.347	2.2	Gramicidin B
	243–300	1.993		
	301–329	Discarded		
	330–339	2.455		
	340–380	16.448	27.3	Gramicidin A
	381–438	22.561		
	439–460	3.774		
	461–500	4.416		
Upper phase withdrawn	916–999	2.017		
	841–915	1.701		
	800–840	1.016	1.6	Gramicidin C
	700–799	1.307		
	631–699	0.168		
	551–630	0.214		
	537–550	0.042		
	525–536	0.041	0.1	Gramicidin D
	496–524	0.063		
	469–495	0.005		
	Total recovery: 60.034 g = 100%			

[a] For the isolation of components A, B, C, and D the contents of the tubes closest to the theoretical curves were combined. The volatile solvents were removed by evaporation *in vacuo*, and the residues were dissolved in glacial acetic acid and lyophilized.

than one component is present. The same observations were made upon redistribution of gramicidin B and C (Figure 2).

Amino Acid Analyses. The combined and lyophilized fractions from the original distribution (Figure 1) and redistributions (Figure 2) were hydrolyzed and analyzed for the individual amino acids. Despite the high tryptophan contents the recovery of tryptophan was satisfactory when the hydrolyses were carried out *in vacuo* at 110° in the presence of one-fifth (by volume) of glacial acetic acid in 6.0 N hydrochloric acid. This is in contrast to the hydrolysis of most proteins where carbonyl-containing degradation products of amino acids impair the recovery of tryptophan. The neutral amino acids were determined by the conventional method (Spackman *et al.*, 1958) (150-cm columns) and by the accelerated method (Spackman, 1963).

The separation of ethanolamine and ammonia on 15-cm columns (Spackman *et al.*, 1958) was difficult. The problem could be solved by the use of 50-cm columns and resin packings that had previously been used for the separation of basic substances of physiological origin. Instead of the routinely employed buffer of pH 4.26, sodium citrate buffer of pH 5.28 was used. Elution volumes for ethanolamine and ammonia were in the range of 210 and 225 ml, respectively. In the accelerated systems with spherical ion-exchange resins (Beckman Custom Research Resin, Type AA-27, Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) the separation of ethanolamine and ammonia in the meantime has been greatly improved and the elution times have been shortened (E. Gross and J. M. Morell, paper in preparation).

The presence of chloroform in the four-component solvent systems causes the aqueous layer to be the upper phase, which in the course of distribution accumulates the hydrophilic peptides.

The amino acid analyses (Table II) show the presence of *two* distinct groups of peptides in commercial gramicidin: The lipophilic group, which contains gramicidin A, B, and C, is spread over tubes 200–300 and 330–500 and over the upper phase withdrawn from the machine at high transfer numbers. The hydrophilic group consists of peptides with strikingly different amino acid composition. No *free* amino acids were detected upon direct amino acid analysis of the following fractions of the main distribution: 631–699, 551–630, 537–550, 525–536, 496–524, and 469–495.

The highly lipophilic gramicidins A and B were retained in the distribution equipment. Gramicidin C, in

TABLE II: Countercurrent Distribution of Gramicidin Amino Acid Analyses of Individual Fractions (μmoles/mg).

Amino Acid	Fraction Number																
	200–221	222–242 B	243–300	330–339	340–380 A	381–438[a]	439–460	461–500	916–999	841–915	800–840 C	700–799	631–699	551–630	537–550	525–536 D	496–524	469–495
Aspartic acid							[b]			[b]			0.170	0.450	0.366	0.358	0.316	0.100
Threonine							[b]						0.028	0.038	0.048	0.032	0.012	0.014
Serine							[b]			[b]			0.020	[b]	[b]	[b]	[b]	0.016
Glutamic acid							[b]			[b]			0.168	0.478	0.432	0.372	0.310	0.114
Proline													0.216	0.482	0.444	0.418	0.338	0.080
Glycine	0.508	0.502	0.514	0.482	0.488		0.502	0.506	0.530	0.596	0.580	0.634	0.604	0.158	0.198	0.222	0.162	0.100
Alanine	1.018	1.026	0.994	0.972	1.000		1.006	0.992	1.034	0.952	0.908	0.801	0.410	0.248	0.366	0.402	0.374	0.100
Valine	1.796	2.052	1.940	1.632	2.030		2.104	1.948	2.150	2.098	2.078	1.984	1.604	0.994	1.104	1.198	0.896	0.256
Methionine										[b]			0.090	0.132	0.144	0.100	0.038	[b]
Isoleucine	0.296	0.060	Trace	0.408	0.110		0.022	0.010	0.052	0.064	0.036	0.026	0.064	0.078	0.104	0.076	0.042	0.020
Leucine	2.028	2.028	1.900	1.920	1.972		1.960	1.944	2.028	2.110	1.988	1.966	1.528	0.906	1.020	1.086	0.832	0.258
Tyrosine				Trace			[b]		0.078	0.350	0.344	0.274	0.162	0.204	0.186	0.150	0.142	0.044
Phenylalanine	0.492	0.504	0.446	Trace			[b]		[b]	[b]		0.054	0.372	0.766	0.664	0.600	0.508	0.114
Tryptophan	1.424	1.562	1.556	1.856	2.032		1.836	1.688	2.036	1.698	1.656	1.512	1.992	0.638	0.840	0.658	0.328	0
Lysine													0.142	0.384	0.404	0.340	0.288	0.060
Ethanolamine	0.378	0.426	0.374	0.348	0.386		0.312	0.302	0.352	0.384	0.452	0.394	0.254	0.050	0.104	0.084	0.080	0.036
Ammonia	0.202	0.178	0.170	0.202	0.114		0.172	0.136	0.126	0.152	0.186	0.328	0.428	0.728	0.826	0.766	0.758	0.602

[a] Redistributed for separation of valine– from isoleucine–gramicidin A. [b] Trace amounts in hydrolysate.

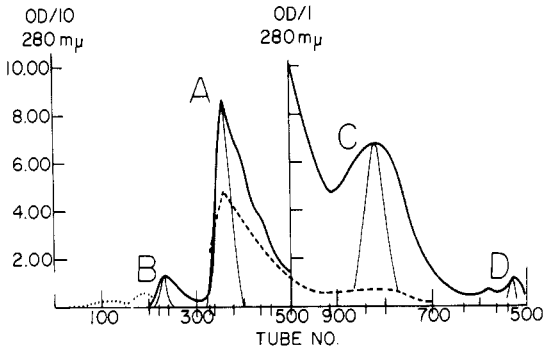

FIGURE 1: Countercurrent distribution of gramicidin (Penick, lot 514-RTF). Solvent system: benzene–chloroform–methanol–water, 15:15:23:7 (v/v); 40-ml lower phase + 40-ml upper phase. Gramicidin (60 g) dissolved in the lower phase volumes of tubes 0–30; forerun, tubes 31–79 lower + upper phase; tubes 80–129, two volumes of lower phase; ———, lower phase analyzed; -----, upper phase analyzed; ———, theoretical curve. (A) Gramicidin A, OD = 8.60, K = 0.56, N = 360. (B) Gramicidin B, OD = 1.35, K = 0.30, N = 232. (C) Gramicidin C, OD = 6.75, K = 1.56, n = 820. (D) Gramicidin D, OD = 1.21, K = 16.65, n = 530.

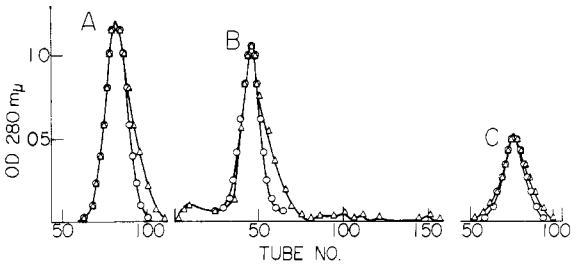

FIGURE 2: Redistribution of gramicidin antibiotics. Solvent system: benzene–chloroform–methanol–water, 15:15:23:7 (v/v); upper phase 10 ml, lower phase 10 ml. △-△-△, experimental curve; O-O-O, theoretical curve. (A) Gramicidin A, 400 mg of fraction 340–380 (Figure 1) in tube No. 0; OD = 1.21, n = 175, N = 81.5. (B) Gramicidin B, 300 mg of fraction 222–242 (Figure 1) in tubes 0–4; OD = 1.04, n = 203, N = 44. (C) Gramicidin C, 300 mg of fraction 800–840 (Figure 1) in tubes 0–4; OD = 0.52, n = 203, N = 126.

which one residue of tryptophan is replaced by tyrosine, is less lipophilic; it was extracted into the upper aqueous phase and moved out of the distribution machine. Peptides composed of amino acids not present in gramicidin would be more hydrophilic and extracted more rapidly; they would not accompany peptides containing leucine, valine, and tryptophan in ratios resembling those of gramicidin A, B, and C (see Table II, tubes 537–699). Therefore gramicidin D and the adjoining fractions are considered to be peptides composed of all of the amino acids found and not mixtures of minor variants of gramicidin A, B, or C and hydrophilic peptides. Except for the amino acid analyses, gramicidin D and the related antibiotics have as yet not been studied in detail.

Gramicidin A. Gramicidin A was isolated from tubes 340–380 (Table I; Figure 1) of the main distribution. The amino acid analysis confirmed the presence of 1 glycine, 2 alanine, 4 valine, 4 leucine, 4 tryptophan, and 1 ethanolamine. Ammonia, found in fractional amounts of 1 molar equiv., is present owing to minor decomposition occurring during hydrolysis. Isoleucine (Ishii and Witkop, 1963) appeared persistently in most analyses in amounts of up to 0.25 mole/mole of gramicidin A. Sequential analysis of gramicidin A (Sarges and Witkop, 1964b, 1965a) (see Ramachandran, 1963) and extensive redistributon of tubes 381–438 have established that gramicidin A represents a mixture of two analogs, namely, valine^1- and isoleucine^1-gramicidin A. Their complete separation requires a high number of transfers. The amount of isoleucine present in tubes 330–500 decreases with higher transfer numbers. The ratios of the other amino acids agree with those of gramicidin A (Table II).

Samples of the material isolated from the redistribution of tubes 340–380 (Figure 2) were hydrolyzed for 24, 48, and 72 hours. The analytical results (Table III) show that longer hydrolysis improves the values for valine which occurs as the resistant tripeptide Val-Val-Val.

TABLE III: Amino Acid Compositon of Gramicidin A after Redistribution as a Function of the Time of Hydrolysis.

	Hydrolysis Times		
Amino Acid	24 Hours (μmoles/mg)	48 Hours (μmoles/mg)	72 Hours (μmoles/mg)
Glycine	0.520	0.518	0.534
Alanine	1.018	1.016	1.040
Valine	1.994	2.024	2.074
Isoleucine	0.106	0.108	0.104
Leucine	2.020	2.100	2.108
Tryptophan	1.813	1.885	1.799
Ethanolamine	0.336	0.340	0.364
Ammonia	0.064	0.068	0.060

Gramicidin B. The B fraction, isolated from the combined tubes 222–242, contains phenylalanine and three tryptophan residues. The remaining amino acids are the same as in gramicidin A. The results of sequence analysis (Sarges and Witkop, 1965c) show that phenylalanine substitutes for tryptophan in position 11. In the

same way as gramicidin A, gramicidin B is a mixture of valine- and isoleucine-gramicidin (Sarges and Witkop, 1965c), the separation of which, though not carried through, should be possible by a sufficient number of transfers to judge from the analytical data of fractions 200–221, 222–242, and 243–300. Tubes 243–300 contain only traces of isoleucine. At the low K value of gramicidin B in the solvent system employed, this fraction should lend itself to purification and isolation of valine-gramicidin B. Except for the phenylalanine-tryptophan exchange the ratios of the other amino acid residues are identical with those of gramicidin A (Table II). Samples of redistributed gramicidin B (Figure 2) were hydrolyzed for 24, 48, and 72 hours and analyzed. Again, only the values for valine improved with increasing hydrolysis time (Table IV).

TABLE IV: Amino Acid Composition of Gramicidin B after Redistribution as a Function of Time of Hydrolysis.

Amino Acid	Hydrolysis Times		
	24 Hours (μmoles/mg)	48 Hours (μmoles/mg)	72 Hours (μmcles/mg)
Glycine	0.48	0.52	0.50
Alanine	0.98	1.04	1.02
Valine	1.64	1.96	2.02
Isoleucine	0.02	0.02	0.01
Leucine	1.98	2.04	2.04
Phenylalanine	0.48	0.50	0.54
Tryptophan	1.48	1.40	1.44
Ethanolamine	0.38	0.40	0.38
Ammonia	Trace	0.06	0.12

Gramicidin C. Gramicidin C is located in tubes 800–840. Except for one distribution over a low number of transfers, gramicidin C has not been redistributed extensively. Isoleucine decreases with increasing transfer numbers. Like fractions A and B, gramicidin C is a mixture of valine- and isoleucine-gramicidin C (Sarges and Witkop, 1965d). Of the three gramicidins, the C fraction is lowest in isoleucine. The amino acid analyses show that one tyrosine substitutes for one tryptophan. Although the value for tyrosine is low, in analogy to gramicidin A and B, the data (Table II) suggest the following ratios: Gly, Ala_2, $(Val + Ileu)_4$, Leu_4, Tyr, Try_3, ethanolamine. Only the two immediately adjoining fractions (tubes 841–915 and 700–799, respectively) resemble gramicidin C. In fraction 916–999 gramicidin A and gramicidin C overlap. Fraction 631–699 shows the spectrum of amino acids characteristic of gramicidin D.

The experimental and theoretical curves of redistributed gramicidin C are in better agreement (Figure 2) than those of gramicidin A and B, although gramicidin C, which was isolated from upper phase volumes that had been withdrawn from the distribution equipment, went through fewer actual transfers than gramicidin A and B, which were left in the machine. The analytical data (Table V) for hydrolysates after 24, 48, and 72 hours of hydrolysis support the amino acid compositions expressed for all gramicidins (A, B, and C) in residues per mole of antibiotic, as shown in Table VI.

TABLE V: Amino Acid Composition of Gramicidin C after Redistribution as a Function of the Time of Hydrolysis.

Amino Acid	Hydrolysis Times		
	24 Hours (μmoles/mg)	48 Hours (μmoles/mg)	72 Hours (μmoles/mg)
Glycine	0.586	0.578	0.576
Alanine	0.860	0.880	0.858
Valine	2.000	2.156	1.972
Isoleucine	0.034	0.034	0.028
Leucine	2.028	1.946	1.936
Tyrosine	0.340	0.344	0.328
Phenylalanine			
Tryptophan	1.602	1.620	1.440
Ethanolamine	0.418	0.408	0.344
Ammonia	0.116	0.162	0.142

TABLE VI: Amino Acid Composition of Gramicidin A, B, and C (residues/mole of gramicidin).

Amino Acid	Gramicidin A	Gramicidin B	Gramicidin C
Glycine	1	1	1
Alanine	2	2	2
Valine	4	4	4
Isoleucine			
Leucine	4	4	4
Tyrosine	0	0	1
Phenylalanine	0	1	0
Tryptophan	4	3	3
Ethanolamine	1	1	1

Gramicidin D. Gramicidin D (tubes 525–536) and the fractions located in its immediate vicinity contain additional amino acids not found in gramicidin A, B, and C. The absence of free amino acids was confirmed by

TABLE VII: Antibiotic Activity[a] of Gramicidin A–D as Expressed in Millimeter Diameter of Zone of Growth Inhibition for *Streptococcus faecalis*.

Phase	Tube Number	Activity	Antibiotic
Lower plus upper phase in countercurrent distribution machine (containing hydrophobic peptides)	200–221	0	
	222–242	8	Gramicidin B
	243–300	8	
	330–339	8	
	340–380	9	Gramicidin A
	381–438	8	
	439–460	9	
	461–500	8	
Upper (aqueous) phase withdrawn from machine (containing more hydrophilic peptides)	916–999	9–10	
	841–915	10–11	
	800–840	11	Gramicidin C
	700–799	10–11	
	631–699	11	
	551–630	10	
	537–550	10	Gramicidin D
	525–536	10	
	496–524	8	

[a] Dr. M. Weinstein of the Schering Corp., Bloomfield, N. J., conducted the activity tests. We are most appreciative of his kind cooperation.

passing the pooled fractions isolated from tubes 469–699 over Amberlite IR-120 ion-exchange columns. The ratios of leucine: valine: tryptophan in most fractions are reminiscent of those in gramicidin A, B, and C. The amino acid composition of fractions which are not discussed here are recorded in Table II. The designation "gramicidin D" has been used by Ramachandran (1963) for the isoleucine analog of gramicidin A.

Gramicidin D and the related fractions contain only traces of serine. This observation has some bearing on the origin of ethanolamine, which could arise from serine by decarboxylation. Not present in gramicidin D are cystine and the basic amino acids histidine and arginine. While lysine occurs in the D fractions, its lower homolog ornithine, which forms part of the peptide antibiotic tyrocidin, produced by the same *Bacillus brevis*, was not detectable. The absence of ornithine and the presence of glycine and alanine, which do not occur in tyrocidin, rule out the possibility that tyrocidin-like polypeptides are contaminants of our gramicidin sample.

The inhibition of *Streptococcus faecalis* appears to be slightly stronger with the hydrophilic C and D fractions than with the hydrophobic A and B fractions (Table VII). A more detailed assay of fractions close to gramicidin C and D (Table VIII) confirmed this and established activities for gramicidin D which are 6–30 times (*Staphylococcus aureus*) and 3–25 times (*Streptococcus pyogenes*) higher than those of gramicidin C.

Biosynthesis of the Gramicidins. Gramicidin A, B, and C differ in the content of aromatic amino acids: Gramicidin A contains *four* residues of tryptophan, gramicidin B *three* residues of tryptophan and *one* residue of phenylalanine, gramicidin C *three* residues of tryptophan and *one* residue of tyrosine. Analogous interchanges of tryptophan and phenylalanine occur in tyrocidins A–D (Mach *et al.*, 1963).

The biosynthesis of the tyrocidins is influenced by the level and nature of available amino acids (Mach and Tatum, 1964). In the presence of high concentrations of tryptophan a new tyrocidin, designated D, which does not occur naturally, is formed. In this

TABLE VIII: *In Vitro* Comparison of Fractions Containing Gramicidin C and D.[a]

Tube No.	*Staphylococcus aureus* 209P		*Streptococcus pyrogenes* C203	
800–840 (C)	3.1	(3.1)[b]	0.6	(0.6)[b]
700–799	1.56	(3.1)	0.39	(0.14)
631–699	0.19	(0.28)	0.14	(0.032)
551–630	1.2	(2.4)	0.79	(0.19)
537–550 (D)	0.15	(0.16)	0.062	(0.012)
525–536	0.09	(0.25)	0.032	(0.032)
496–524	0.09	(0.17)	0.07	(0.032)

[a] Values (minimal inhibitory concentrations in μg/ml) obtained through the courtesy of Dr. James D. Dutcher from the Squibb Institute for Medical Research. [b] Numbers in parentheses represent repeat assays.

antibiotic three residues of tryptophan have replaced three phenylalanines. The influence of controlled levels of environmental amino acids on the biosynthesis of gramicidin A, B, C, and D is still to be tested.

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References

Craig, L. C., and Craig, D. (1950), *Tech. Org. Chem. 3*, 171.

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

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

Mach, B., Reich, E., and Tatum, E. L. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 175.

Mach, B., and Tatum, E. L. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 876.

Ramachandran, L. K. (1963), *Biochemistry 2*, 1138.

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

Sarges, R., and Witkop, B. (1964b), *J. Am. Chem. Soc. 86*, 1862.

Sarges, R., and Witkop, B. (1965a), *J. Am. Chem. Soc. 87*, 2011.

Sarges, R., and Witkop, B. (1965b), *J. Am. Chem. Soc. 87*, 2020.

Sarges, R., and Witkop, B. (1965c), *J. Am. Chem. Soc. 87*, 2027.

Sarges, R., and Witkop, B. (1965d), *Biochemistry 4*, 2491 (this issue; preceding paper).

Spackman, D. H. (1963), *Federation Proc.* (Abstract) *22*, 244. [*Cf.* Spackman, D. H. (1964), in Serum Proteins and the Dysproteinemias, Sunderman, F. W., and Sunderman, F. W., Jr., eds., Philadelphia, Lippincott, p. 166.]

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

The Synthesis of *erythro*-γ-Hydroxy-L-lysine and Its Nonoccurrence in Collagen*

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ABSTRACT: *threo*-γ-Hydroxy-L-lysine (II), prepared *via* γ-chloro-L-lysine (I) by photochlorination of L-lysine, was converted to the dicarbobenzyloxy lactone IV, opened to the amide V, and oxidized to the γ-keto derivative VI. Catalytic hydrogenation and debenzylation yielded, after hydrolysis of the amide, 72% of the *erythro* acid–lactone mixture III ⇄ VIII and 28% of the *threo* pair II ⇄ VII, which were separated by ion-exchange chromatography.

Catalytic hydrogenation of ε-diazo-δ-oxo-L-norleucine (DON) gave a mixture of 25% *erythro*- and 75% *threo*-δ-hydroxy-L-lysine. By reaction with *S*-methylisothiourea, *erythro*-γ-hydroxy-L-homoarginine lactone (IX), the diastereoisomer of the natural *threo* amino acid from *Lathyrus*, was prepared. Unlike *trans*-3-hydroxy-L-proline, the position isomer of natural 4-hydroxy-L-proline, neither *erythro*-γ-hydroxy-L-lysine, the position isomer of natural *erythro*-δ-hydroxy-L-lysine, nor its *threo* isomer are regular building stones of collagen.

The photochlorination of L-lysine in strong sulfuric acid (Kollonitsch *et al.*, 1964) makes *threo*-γ-hydroxy-L-lysine (II) easily accessible *via* γ-chloro-L-lysine (I) (Chart I) (Fujita *et al.*, 1965). The γ-hydroxy-L-lysine obtained directly from γ-chloro-L-lysine by treatment with 4 equiv of silver acetate at 90° consisted of 97% *threo*- and 3% *erythro*-γ-hydroxy-L-lysine (III). Pure *threo*, II, is easily obtained by recrystallization of the monohydrochloride from aqueous ethanol. The use of base and silver oxide failed to raise the yield of *erythro* isomer III.

Most of γ- and δ-hydroxyamino acids undergo acid-catalyzed epimerization at the α-carbon (Hamilton and

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