The Chemistry of Tyrocidine. VII. Studies on Association Behavior and Implications Regarding Conformation*

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ABSTRACT: The tyrocidines, cyclic decapeptides of known primary structure, are known to associate extensively. Studies were made in order to examine this property and its possible implications on the conformations of the peptides. The techniques of thin film dialysis, equilibrium ultracentrifugation, and rotatory dispersion were applied to the tyrocidines and to gramicidin S, a peptide of similar primary structure. In addition, several derivatives of these peptides were prepared and examined with respect to their physical properties and their antibiotic activities. For the tyrocidines, only those derivatives which retained the ability to associate showed activity as antibiotics. Studies on the peptides were performed in a variety of solvent mixtures and tentative conclusions concerning association and conformation were drawn from the results.

Association is apparently due to intermolecular hydrophobic bonding without the participation of so-called π bonding, despite the fact that the tyrocidines contain 40% aromatic residues. Whereas the residue side chains are probably at the surface of the molecule, the peptide backbone seems buried and is presumably tied up in intramolecular hydrogen bonding, possibly in the mode of an antiparallel pleated-sheet structure.

Because of their structural simplicity, their relative ease of manipulation, and their greater stability, small peptides have been useful as models for studying some of the properties of proteins. In this regard, we have been interested in the tyrocidines which are antibiotic cyclic decapeptides of known primary structure (Paladini and Craig, 1954; King and Craig, 1955; Ruttenberg *et al.*, 1965a). The amino acid sequences of the three homologous tyrocidines and of the related peptide gramicidin S[1] (Consden *et al.*, 1947; Battersby and Craig, 1951) are given in Figure 1.

One physical property of the tyrocidines which is uncommon for polypeptides of their size is their strong tendency to associate (Pedersen and Synge, 1948; Craig and King, 1955). This tendency is of interest as it is also often found with proteins. Generally, two types of interactions are considered responsible for the association of proteins, namely, (1) interactions through interchain hydrogen bonds of a β-polypeptide structure and (2) the side-chain interactions of electrostatic and hydrophobic origins (Schellman and Schellman, 1964). However, the large number of interacting groups in a protein molecule present a formidable obstacle for a systematic study of these interactions. The fact that the tyrocidines are small peptides made them particularly attractive for such a study. Furthermore, it may be expected that the results of such a study could aid in defining the probable conformations of the tyrocidines.

With these thoughts in mind, thin film dialysis, equilibrium ultracentrifugation, and optical rotatory dispersion studies were carried out with the tyrocidines and several of their derivatives. Mainly tyrocidine B was used for these studies as it was present in more plentiful supply than the A and C homologs. The conclusions which were drawn concerning the intermolecular association and the conformation of tyrocidine were based on: (1) the effects of modification of tyrocidine functional groups on its solubility and its rate of dialysis, and (2) the solvent effect on its properties as measured by thin film dialysis and by equilibrium ultracentrifugation.

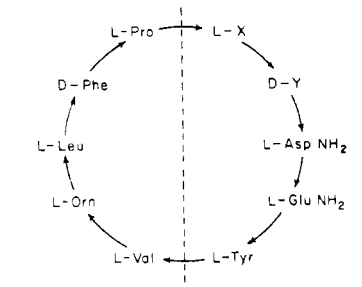
Experimental Section

Materials. The tyrocidine B was isolated by countercurrent distribution (Ruttenberg *et al.*, 1965a) from crude tyrocidine, Wallerstein No. ON 13554. We wish to thank the Wallerstein Co. for a gift of the sample. The gramicidin S was isolated from crude gramicidin S (Sharp and Dohme) as described by Craig *et al.* (1949). All solvents used were redistilled. The dialysis tubing was Visking dialysis casing size 18/32.

Methods. For the thin film dialysis experiments, the diffusion cell was the same as that described by Craig and Konigsberg (1961); the volumes of the retentate and the diffusate were 0.5 and 3.5 ml, respectively. The equilibrium ultracentrifugation studies were made in a Spinco Model E analytical ultracentrifuge in cells

* From The Rockefeller University, New York, New York 10021. *Received March 3, 1966; revised June 17, 1966.* Portions of this work have appeared in a Communication to the Editor (Ruttenberg *et al.*, 1965b). This work was supported in part by U. S. Public Health Service Grant A.M. 02493.

[1] Crude gramicidin S actually consists of several peptides. We will use this term to refer to the major component, gramicidin SA (Craig *et al.*, 1949).



Tyrocidine	Residue X	Residue Y
A	Phe	Phe
B	Try	Phe
C	Try	Try

FIGURE 1: Amino acid sequences of tyrocidine A–C. The pentapeptide to the left of the dotted line occurs in the cyclic decapeptide gramicidin S.

described by Yphantis (1960). The rotatory dispersion measurements in the long-wavelength region (from 350 to 700 mμ) were made in a Rudolph spectropolarimeter Model 220; the measurements in the short-wavelength region (200–350 mμ) were made on a Cary recording spectropolarimeter. Amino acid analyses were performed on a Spinco Model 120 amino acid analyzer as described by Spackman *et al.* (1958). The antibiotic activities were measured semiquantitatively by applying the various peptide solutions to agar plates infected with *Bacillus subtilis*.

Iodination. Tyrocidine B (100 mg) was dissolved in 5 ml of 50% methanol containing 0.5 ml of triethylamine.[2] A 0.15 N solution of I_3^- prepared by the method of Gruen *et al.* (1959) was added dropwise over a period of 1 hr until the yellow color persisted (this required approximately 2 ml). The solution was then brought to dryness in a rotary evaporator and the dry residue was applied in 2 ml of 50% acetic acid to a 0.9 × 150 cm column of Sephadex G-25.[3] Elution with 50% acetic acid gave a single peak at 50 ml, the same position as for unmodified tyrocidine. The ultraviolet spectrum of the material from this peak was measured, and comparison of the molar extinction at λ_{max} 311 mμ with that for diiodotyrosine according to Gruen *et al.* (1959) indicated that the yield of diiodotyrocidine B was about 88%. Diiodotyrosine could not be determined by amino acid analysis, as it is labile to acid hydrolysis.

Succinylation. For the succinylation procedure, 140 mg (100 μmoles) of tyrocidine B was dissolved in 7 ml of 30% acetone in water. To this was added 1.4 ml (10 mmoles) of TEA and 100 mg (1 mmole) of succinic anhydride. The reaction was allowed to proceed for 4 hr at room temperature after which the solution was brought to dryness in a rotary evaporator and the residue was dissolved in 2 ml of 50% acetic acid and chromatographed on Sephadex G-25. The complete absence of any ninhydrin color across the band indicated that quantitative succinylation of the δ-amino group of ornithine had occurred.

Condensation of Tyrocidine B and N-Succinyltyrocidine B. Equimolar amounts (28 mg, 20 μmoles) of tyrocidine B and succinyltyrocidine B were dissolved in 2 ml of 50% acetic acid and chromatographed on Sephadex G-25. The result is shown in the first pattern in Figure 2. The ninhydrin color was due entirely to the presence of the unmodified tyrocidine B. The material from this chromatogram was isolated by lyophilization and was dissolved in 5 ml of *t*-butyl alcohol containing 0.5 ml of water. To this was added 2 mg (2 mmoles) of dicyclohexylcarbodimide (DCC), a condensing reagent described by Khorana in 1952 and used by Sheehan and Hess in 1955 for the formation of amide bonds from free amino and carboxyl groups. The reaction was allowed to proceed for 10 hr at 37°. At the end of this time, the material was lyophilized and the residue was extracted with 1-ml portions of 50% acetic acid which were combined and applied to the Sephadex column. The results of chromatography are shown by the second pattern in Figure 2. Absence of ninhydrin color across the band indicated that essentially complete coupling of two tyrocidine B molecules had occurred. The fact that the position of the main peak was 6 ml earlier than the tyrocidine B decapeptide was further evidence in favor of the conclusion that the covalently linked dimer had been synthesized. An ultraviolet spectrum of the material in 50% acetic acid was identical with that for unmodified tyrocidine B.

Dinitrophenylation of the Tyrosine in N-Succinyltyrocidine B. Succinyltyrocidine B (20 mg, 14 μmoles) in 50% methanol (5 ml) was treated with a 60-fold excess of FDNB for 2 hr at 25° in the presence of a 100-fold excess of TEA. The reaction mixture was brought to dryness in a rotary evaporator and the residue was dissolved in 1 ml of glacial acetic acid and chromatographed on Sephadex G-25 in 50% acetic acid. The peak containing the *O*-DNP-*N*-succinyltyrocidine B in quantitative yield emerged in the position of unmodified tyrocidine B.

Methylation of the Tyrosine in N-Succinyltyrocidine B. Succinyltyrocidine B (56 mg, 40 μmoles) was dissolved in 3 ml of 20% methanol and the solution was placed in a 5-ml-capacity water-jacketed reaction vessel maintained at 37°. A glass electrode was lowered into the solution and the pH was maintained at 9.0 throughout the reaction by means of a pH-Stat (Radiometer TTT1). A 200-fold excess of dimethyl sulfate was introduced and the reaction was allowed to proceed for 12 hr. The mixture was then brought to dryness in a rotary evaporator and the residue was dissolved in 2 ml of 50% acetic acid and desalted on Sephadex G-25. The peak position was again identical with that for unmodified tyrocidine B. The material in the peak

[2] Abbreviations used: TEA, triethylamine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; FDNB, fluorodinitrobenzene.

[3] All Sephadex chromatography was performed on the same column with the specifications given in Figure 2.

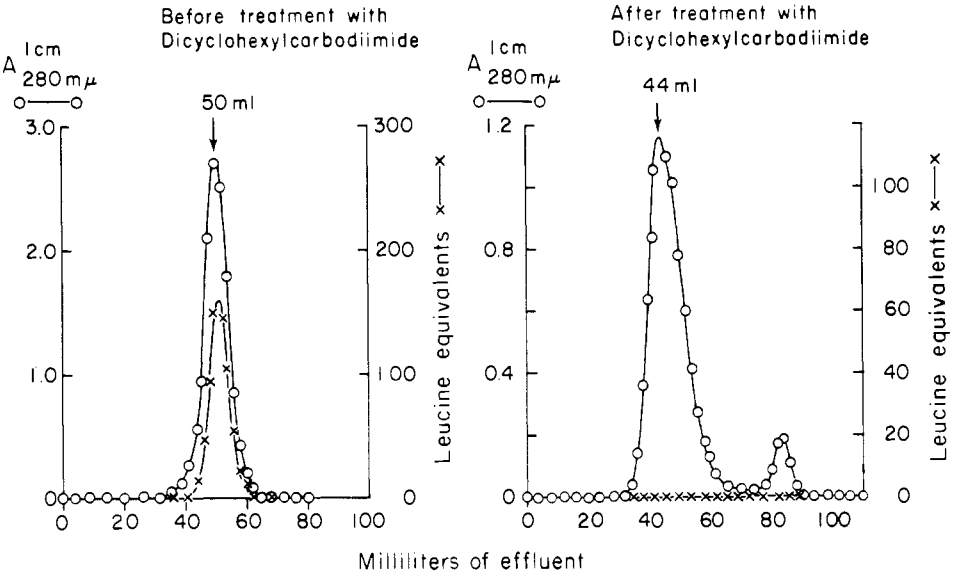


FIGURE 2: Chromatography on a 0.9 × 150 cm column of Sephadex G-25 in 50% acetic acid of a 1:1 mixture of succinyltyrocidine B and unmodified tyrocidine B (20 μmoles of each) before and after treatment with DCC. Aliquots (100 μl) were diluted to 1 ml for the analyses. The absence of ninhydrin color following the reaction indicates that coupling of the two peptides had occurred.

showed no alkaline spectral shift characteristic of the free phenol group, indicating quantitative conversion of the starting material to *O*-methyl-*N*-succinyltyrocidine B. The *O*-methyl group was observed to be hydrolyzed by 6 N HCl to the extent of 75% after 20 hr at 110°; therefore, amino acid analysis could not be used for quantitative estimation of the amount of methylation. However, *O*-methyl analysis on the intact peptide by the Zeisel method showed that the yield of methylation was 94%.

Hydrogenation of Tyrocidine A. Tyrocidine A (140 mg, 100 μmoles) was dissolved in 30 ml of glacial acetic acid. The solution was placed in a 250-ml Parr bomb together with 1.3 g of PtO_2. The bomb was attached to a reciprocating shaker and flushed with hydrogen gas. The hydrogenation reaction was then allowed to proceed for 3 days at ambient temperature at 60 psi. At the end of this time the catalyst was filtered off and the solution was evaporated to dryness. The residue was dissolved in 4 ml of 50% acetic acid and applied to the Sephadex column in order to remove any contaminants which might have come from the catalyst or the apparatus. The peptide emerged as a single peak in the position of unmodified tyrocidine A. It was isolated by lyophilization and a portion was subjected to hydrolysis and amino acid analysis. There was no tyrosine or phenylalanine present in the analysis but a new peak appeared 170 min after leucine. On the analyzer used, leucine appeared at 153 min. The intact peptide had no ultraviolet absorption above 220 mμ. This indicated that complete hydrogenation of the three phenylalanine residues and the tyrosine residue had taken place.

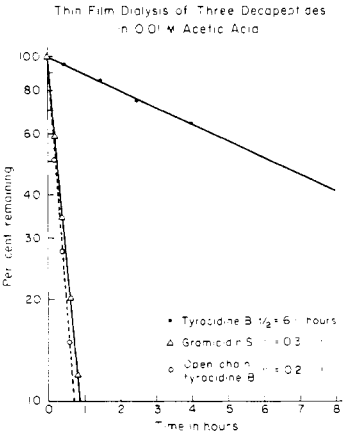


FIGURE 3: Thin film dialysis of three decapeptides in 0.01 M acetic acid at 40°; initial solute concentration, 4 mg/ml.

Results and Discussion

Earlier studies by Craig and King (1955) on the dialysis rate of tyrocidine A and B showed that these peptides dialyzed very slowly as compared to peptides of the same molecular weight, suggesting that association was occurring. In the present work, the dialysis behavior of tyrocidines A–C were found to be almost identical and therefore the studies were confined to the B peptide. The similarity in primary structure between tyrocidine B and gramicidin S suggested

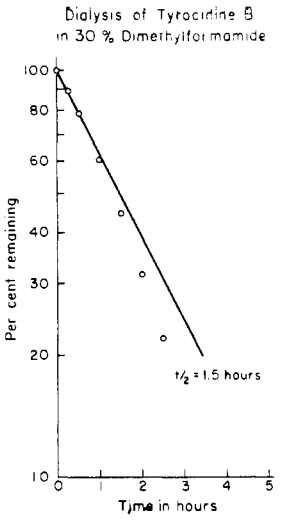


FIGURE 4: Thin film dialysis of tyrocidine B in 30% dimethylformamide which was 0.01 M in ammonium acetate and acetic acid. The experiment was performed at 40°; initial solute concentration, 4 mg/ml.

TABLE I: Escape Times in Dialysis of 0.4% Solutions of Gramicidin S and Tyrocidine B in Various Solvents.[a]

Tyrocidine B			
% Acetic Acid	50% Escape Time (hr)	% DMF	50% Escape Time (hr)
0.05	6.2	10	1.8
5	4.9	30	1.1
10	3.8		
25	3.0		

Gramicidin S	
Solvent	50% Escape Time (hr)
0.05% acetic acid	0.3
30% dimethylformamide	0.6

[a] The measurements in acetic acid and dimethylformamide were performed in different membranes of similar porosity. The diffusates were assayed by absorbance at 280 mμ in the case of tyrocidine and by alkaline hydrolysis followed by ninhydrin assay in the case of gramicidin S. The dimethylformamide solutions were 0.01 M in ammonium acetate and acetic acid.

that a comparison of the dialysis rates of these two peptides would be appropriate. As shown in Figure 3, there was found a most striking difference between the rates of diffusion for these two peptides. In addition, the open-chain tyrocidine B which had been obtained by cleavage at the phenylalanylproline linkage by $LiAlH_4$ (Ruttenberg *et al.*, 1964) dialyzed at the same rate as the gramicidin S. Measurements by equilibrium ultracentrifugation under these conditions (R. C. Williams, Jr., unpublished observations) have confirmed that gramicidin S is a monomer under these conditions, whereas tyrocidine B is highly associated.

If the association of tyrocidine B is reversible, then there should be a measurable concentration dependence. In thin film dialysis (Craig, 1965), one would expect to see an increased dialysis rate as the concentration of peptide inside the membrane decreased. This was observed, as indicated in Figure 4. This experiment employed 30% dimethylformamide as solvent instead of the 0.01 M acetic acid as in Figure 3; the initial rate in this solvent was four times faster than in 0.01 M acetic acid. The change of dialysis rate for tyrocidine B in different concentrations of dimethylformamide and acetic acid is shown in Table I. It is seen that the tyrocidine is less strongly associated in dimethylformamide than in acetic acid.

The tyrocidine B derivatives were studied by dialysis. The results (Table II) indicated that neither the ornithine amino group nor the tyrosine hydroxyl are necessary for the association to occur. The partial ionization of the tyrosine hydroxyl group appeared to have little effect on the state of association, as shown by the similar rates of dialysis for the *N*-succinyl and *O*-methyl-*N*-succinyl derivatives in 0.01 N NH_4OH. These results were an indication that the tyrosine hydroxyl group is not necessary for the association to occur, a fact which was confirmed also by the observation that tyrocidine D, a peptide with no tyrosine, gave exactly the same diffusion curve as tyrocidine B (Ruttenberg and Mach, 1966).

A sample of tyrocidine B was studied by means of equilibrium ultracentrifugation in 10% DMF using the technique developed by Yphantis (1960). The results of these measurements are given in Table III; they indicate that the measurements by dialysis are valid indices of the state of association and its concentration dependence.

The observation that there is no detectable difference in the dialysis rates for tyrocidines A–C despite the fact that these peptides differ in their degree of aromaticity was an indication that aromatic interactions are not responsible for the association. This was shown also when it was found that fully hydrogenated tyrocidine A associated even to a greater extent than the unmodified peptide. The diffusion half-time for hydrogenated tyrocidine A in 10% acetic acid was 4.8 hr, while that for the unmodified peptide was 3.8 hr. The reason for selecting tyrocidine A for hydrogenation was that the other tyrocidines contain tryptophan which would have been converted by hydrogenation to a secondary amine and therefore another charged group would have been introduced into the molecule.

Measurements of antibiotic activity have shown that all of the tyrocidine derivatives are active except the open-chain peptide. This linear peptide is also the only derivative which does not associate. It is

TABLE II: Escape Times in Thin Film Dialysis for Derivatives of Tyrocidine and Gramicidin S.

Derivative	Solvent (M)	50% Escape Time (hr)
Unmodified tyrocidine B	Acetic acid (0.01)	6.2
N-Succinyltyrocidine B	Ammonium hydroxide (0.01)	5.7
O-Methyl-*N*-succinyltyrocidine B	Ammonium hydroxide (0.01)	7.0
Hydrogenated tyrocidine A	10% acetic acid	4.8
Unmodified gramicidin S	Acetic acid (0.01)	0.3
Di-*N*-succinylgramicidin S[a]	Ammonium hydroxide (0.01)	0.9

[a] Prepared by the same method used with tyrocidine B. It can be seen that the dialysis rate for unmodified gramicidin S does not differ appreciably in 0.01 M acetic acid from the rate for di-*N*-succinylgramicidin S in 0.01 M ammonium hydroxide. This lends justification to the interpretation of the dialysis rates for modified tyrocidine B in 0.01 M ammonium hydroxide.

TABLE III: Summary of the Weight-Average Molecular Weights for Tyrocidine B Obtained by Equilibrium Ultracentrifugation.[a]

Concn of Tyrocidine (%)	Temperature (°C) 25	4
0.5	15,900	19,800
0.2	14,200	18,100
0.1	10,500	13,800

[a] The values for molecular weights are obtained from both schlieren and interference measurements except for the 0.1% solution which is from interference only. The error in the values given is of the order of 5%. The value taken for $1 - \bar{v}\rho$ was 0.25. The solvent was 10% dimethylformamide which was 0.01 *M* in ammonium acetate and acetic acid.

believed that the mechanism of tyrocidine as an antibiotic is that of a detergent (Hotchkiss, 1945). Since detergents also aggregate to form micelles, the association of the tyrocidines may be related to their detergent property. Therefore, the loss of the ability to aggregate may also mean a loss in detergent properties, which in turn would explain the failure of the linear peptide to have antibiotic activity. Surface film studies (A. Rothen, unpublished observations) have confirmed that the intact tyrocidines are good detergents while the open-chain derivative is not. On the other hand, gramicidin S shows little tendency to associate, yet is an antibiotic.

The dialysis and ultracentrifugation data on the association may be summarized as follows. (1) Open-chain tyrocidine does not associate. (2) Gramicidin S, a cyclic decapeptide of very similar primary structure to tyrocidine, does not associate. These two observations indicate that the ring structure is a necessary condition, but it alone is not sufficient to cause association. This in turn suggests that the aggregation requires the proper spatial alignment of several amino acid residues. The failure of gramicidin S to associate may be due to the fact that it has two symmetrically placed charges. (3) Hydrogenated tyrocidine A associates more strongly than tyrocidine A, as indicated by their dialysis rates. Therefore, aromatic interactions alone are not responsible for the association. (4) Chemical modification of the δ-amino group on ornithine or the hydroxyl group on tyrosine apparently does not prevent or inhibit the association. (5) The association is greatest in water, a strong hydrogen-bond breaker, and is less in solvent mixtures with lower dielectric constant such as acetic acid–water or dimethylformamide–water.

The above observations suggest that the association of tyrocidine is not due to intermolecular hydrogen bonding but is due to hydrophobic interactions. These observations are also in agreement with the solubility properties of tyrocidine and its derivatives. They are quite nonpolar and dissolve readily in nonaqueous solvents and in aqueous detergent solution. These physical properties are indicative that the peptide bonds are buried and probably tied up in intramolecular hydrogen bonding, with the result that the residue side chains extend toward the surface of the molecule. This in turn is in agreement with the observations that the δ-amino group of ornithine and the tyrosine hydroxyl group are readily accessible to bulky alkylating and acylating reagents and that the tyrosine residue could react with two molecules of iodine and the ornithine residue could be used to join quantitatively two tyrocidine molecules through a succinyl linkage. The detergent property of tyrocidine can be explained by the proper spatial arrangement of the polar and nonpolar side chains.

In the light of these data, we may consider the possible conformations of tyrocidines. Since tyrocidines are cyclic peptides, they cannot assume helical or

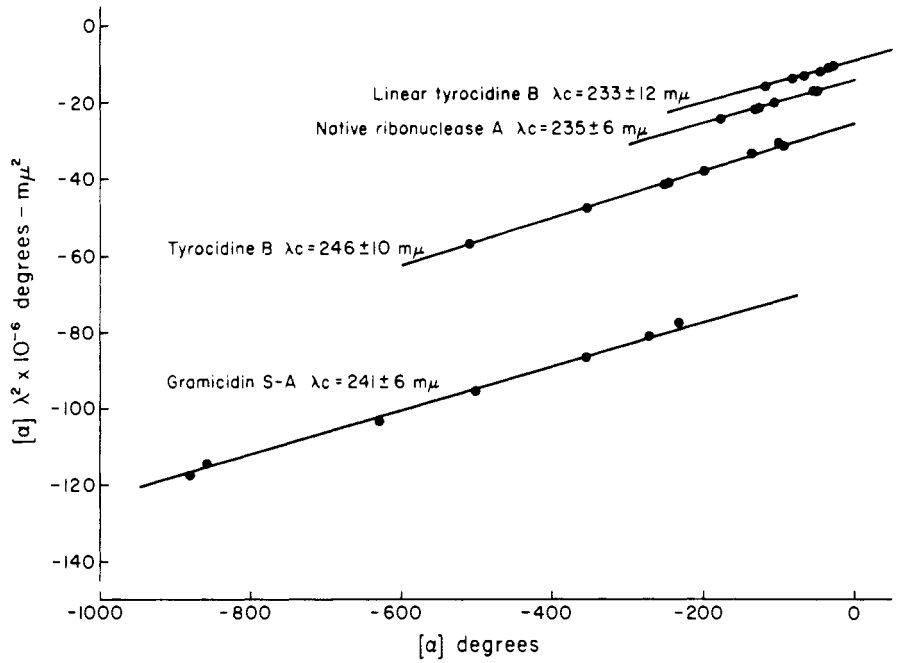


FIGURE 5: Treatment of dispersion data by means of the Drude equation. The measurements were made with a mercury arc source on aqueous solutions in a 1-dm cell at ambient temperature. The concentrations were: linear tyrocidine B, 5 mg/ml; ribonuclease, 10 mg/ml; tyrocidine B, 2 mg/ml; gramicidin S, 5 mg/ml.

parallel-chain pleated-sheet structures. A likely type is the antiparallel pleated-sheet structure (Marsh *et al.*, 1955). As applied to tyrocidines, there can be five possible conformations depending on the amino acid residues selected to link the ends of the two antiparallel chains. In these conformations, the peptide bonds face inward and the residue side chains face outward from the ring. Such conformations for cyclic peptides are not without precedent as other authors have assigned a similar conformation to gramicidin S on the basis of X-ray diffraction studies and its ease of formation from the corresponding pentapeptide (Hodgkin and Oughton, 1957; Schwyzer, 1958).

The optical rotatory dispersion properties of these cyclic peptides are of interest since neither the tyrocidines nor gramicidin S can assume helical structures. When the dispersion data in the region of 350–700 mμ for tyrocidine B, its open-chain derivative, and gramicidin S were handled according to the Drude and the Moffit–Yang (1956) treatments the plots in Figures 5 and 6 were obtained. The results obtained with bovine pancreatic ribonuclease A were included also for the purpose of comparison. The complete dispersion data in the region of 200–350 mμ have been reported earlier (Ruttenberg *et al.*, 1965b). Here it was observed that tyrocidine B and gramicidin S showed distinct troughs at 229 mμ with [α] −9500° and at 233 mμ with [α] −16,500°, respectively, while the open-chain tyrocidine B showed two shallow troughs at 208 mμ with [α] −4700 and at 218 mμ with [α] −4000°.

The dispersion characteristics of the open-chain tyrocidine B are in accord with the currently accepted interpretation of these data, that is, a peptide with a random-coil conformation has a low b_0 value and a diminished optical rotation at 233 mμ (Schellman and Schellman, 1964). It is likely that this open-chain decapeptide has a random-coil conformation as it contains both L- and D-amino acids. However, the large negative b_0 values and the deep dispersion troughs in the 230-mμ region of tyrocidine B and gramicidin S cannot be taken to indicate the presence of a helical conformation since it is not possible for these peptides to have such a structure. It is possible then that these dispersion characteristics may reflect an ordered structure of the antiparallel sheet type, though other investigations have proposed b_0 values ranging from 0 to +800 for the β conformation (see Urnes and Doty, 1961). In the present case, it is unlikely that this ordered structure arises from the intermolecular association of these peptides. Both tyrocidine B and gramicidin S give similar dispersion curves but only tyrocidine shows strong association. In fact, gramicidin S has a higher specific rotation than tyrocidine B.

The association and the rotatory dispersion studies both demonstrate that the tyrocidines have a unique conformation. These findings strengthen our initial objective that they can serve as models for the understanding of some of the properties of proteins. The tyrocidines will be particularly valuable as models for learning the chemical and the spatial requirements in the formation of a hydrophobic interaction site.

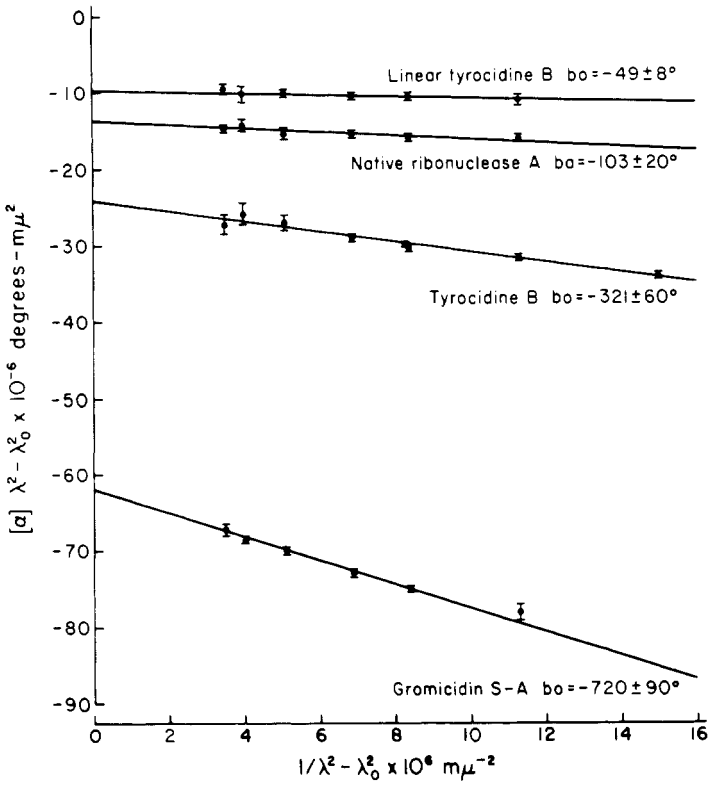


FIGURE 6: Treatment of dispersion data by the Moffit–Yang (1956) method. The same solutions and conditions as in Figure 5 were employed.

After the submission of this article, two papers on the optical rotatory properties of the β configuration in polypeptides and proteins were published by Sarkar and Doty (1966) and Davidson *et al.* (1966). These authors showed that the ORD of the β form of poly-L-lysine has a trough at 230 mμ and has a b_0 value of -147 in the visible region.

Acknowledgments

The authors wish to thank Dr. Bernard Mach for performing the measurements of antibiotic activity, and Dr. David Yphantis for his advice in the performance of ultracentrifuge experiments.

References

Battersby, A. R., and Craig, L. C. (1951), *J. Am. Chem. Soc. 73*, 1887.

Consden, R., Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1947), *Biochem. J. 41*, 596.

Craig, L. C. (1965), *in* Advances in Analytical Chemistry and Instrumentation, Vol. 4, Reilley, C. N., Ed., New York, N. Y., Interscience, p 66.

Craig, L. C., Gregory, J. D., and Barry, G. T. (1949), *Cold Spring Harbor Symp. Quant. Biol. 14*, 24.

Craig, L. C., and King, T. P. (1955), *J. Am. Chem. Soc. 77*, 6620.

Craig, L. C., and Konigsberg, W. (1961), *J. Phys. Chem. 65*, 166.

Davidson, B., Tooney, N., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 156.

Gruen, L., Laskowski, M., and Scheraga, H. A. (1959), *J. Biol. Chem. 234*, 2050.

Hodgkin, D. C., and Oughton, B. M. (1957), *Biochem. J. 65*, 752.

Hotchkiss, R. D. (1945), *Ann. N. Y. Acad. Sci. 46*, 479.

Khorana, H. G. (1952), *J. Chem. Soc.*, 2081.

King, T. P., and Craig, L. C. (1955), *J. Am. Chem. Soc. 77*, 6627.

Marsh, R. E., Corey, R. B., and Pauling, L. (1955), *Biochim. Biophys. Acta 16*, 1.

Moffit, W., and Yang, J. T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.

Paladini, A., and Craig, L. C. (1954), *J. Am. Chem. Soc. 76*, 688.

Pedersen, K. O., and Synge, R. L. M. (1948), *Acta Chem. Scand. 2*, 408.

Ruttenberg, M. A., King, T. P., and Craig, L. C. (1964), *Biochemistry 3*, 758.

Ruttenberg, M. A., King, T. P., and Craig, L. C. (1965a), *Biochemistry 4*, 11.

Ruttenberg, M. A., King, T. P., and Craig, L. C. (1965b),

J. Am. Chem. Soc. 87, 4169.
Ruttenberg, M. A., and Mach, B. (1966), *Biochemistry 5*, 2864.
Sarkar, P. K., and Doty, P. (1966), *Proc. Natl. Acad. Sci. 55*, 981.
Schellman, J. A., and Schellman, C. (1964), *Proteins 2*, 1.
Schwyzer, R. (1958), Ciba Foundation Symposium on Amino Acids and Peptides with Antimetabolic Activity, Boston, Mass., Little-Brown, p 171.
Sheehan, J. C., and Hess, G. P. (1955), *J. Am. Chem. Soc. 77*, 1067.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 402.
Yphantis, D. A. (1960), *Ann. N. Y. Acad. Sci. 88*, 586.

Studies on Amino Acid Substitution in the Biosynthesis of the Antibiotic Polypeptide Tyrocidine*

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ABSTRACT: The tyrocidines, a series of antibiotic cyclic decapeptides synthesized by *Bacillus brevis*, have been found to consist of three homologous peptides containing both L- and D-amino acid residues. These peptides, tyrocidines A–C [Battersby, A. R., and Craig, L. C. (1952), *J. Am. Chem. Soc. 74*, 4019] differ by a single amino acid replacement of tryptophan for phenylalanine. By introducing large amounts of tryptophan into the growth medium, it has been possible to alter the relative amounts of these three homologs and also to induce the incorporation of L-tryptophan in place of L-tyrosine, thereby producing a hitherto unobserved peptide which has been designated tyrocidine D. Moreover, it has been possible to induce the incorporation of alloisoleucine and isoleucine, amino acids not normally found in tyrocidine, by adding them to the growth medium. These amino acids have been found to replace nonselectively the residues of valine and leucine. These observations on the lack of specificity in tyrocidine biosynthesis give added support to the earlier hypothesis that tyrocidine biosynthesis proceeds by a mechanism different from that for proteins [Mach, B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 263; Mach, B., and Tatum, E. L. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 876]. The procedures for the induced biosynthesis and chemical characterization of these new tyrocidine peptides are given in this report.

Although tyrocidine was discovered more than 25 years ago (Dubos, 1939; Hotchkiss and Dubos, 1940) it was not until the development of countercurrent distribution that this substance was shown to be a mixture of three closely related polypeptides designated tyrocidines A–C (Battersby and Craig, 1952). Chemical degradation studies elucidated the amino acid sequences for the three peptides (Paladini and Craig, 1954; King and Craig, 1955; Ruttenberg *et al.*, 1965) and revealed that they were cyclic decapeptides which differed by a single amino acid replacement. Such heterogeneity has subsequently been found with other small peptides (Craig, 1956) to an extent which led to the suggestion that a biosynthetic mechanism different from that for proteins might be involved. In view of the current interest in protein biosynthesis, it seemed appropriate to investigate the biosynthesis of tyrocidine. Recent observations in this regard have confirmed the hypothesis that the synthetic mechanism is different from that for proteins (Mach, 1963; Mach and Tatum, 1964). Extension of these studies in the present work has shown that the introduction of large amounts of various natural amino acids into the growth medium can result in their incorporation into tyrocidine. Since such amino acid replacements are unknown in protein biosynthesis, this result reinforces the conviction that tyrocidine biosynthesis proceeds by a mechanism different from protein biosynthesis.

Experimental Section

Methods. The *Bacillus brevis* cultures were grown as previously described (Mach and Tatum, 1964). The reagents used were of the highest purity obtainable. All solvents were redistilled. The dialysis experiments were performed in cells described by Craig and Konigsberg (1961). Amino acid analyses were

* Contribution from the Rockefeller University, New York, New York. *Received March 3, 1966; revised June 20, 1966.* This work was supported in part by U. S. Public Health Service Grants AM 02493-07 and CA 03610-09, and by a fellowship from the Helen Hay Whitney Foundation.

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