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Noncovalent Association of Tyrocidine B*

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ABSTRACT: The noncovalent association of tyrocidine B was investigated by equilibrium ultracentrifugation. In solvents containing several per cent of acetic acid, the association is relatively simple and equilibrium is rapidly attained. The stoichiometry can be adequately represented as an equilibrium between monomer and two polymers of different sizes. In aqueous salt solution, however, the association is complex: the size of the associated units gradually increases after the solution is prepared. This increase can be reversed by warming the solutions. Preliminary investigations of the related polypeptides tyrocidine C, gramicidin S, and an acetylated derivative of gramicidin S suggest a complex relationship between the structure of the peptide and its association behavior.

The tyrocidines, cyclic decapeptides obtainable from cultures of *Bacillus brevis*, are useful molecules for study of the noncovalent association of polypeptides in aqueous solution. They are readily soluble and have high extinction coefficients in the ultraviolet spectrum. Near neutral pH they possess only a single electrostatic charge and thus do not have the large ratio of charge to mass common to most water-soluble small peptides. Their molecular weights and chemical structures are known (Paladini and Craig, 1954; King and Craig, 1955; Ruttenberg *et al.*, 1965). The tendency of tyrocidines to associate has been known since Pedersen and Synge (1948) observed that the diffusion coefficients of partially purified fractions of these peptides were consistent with molecular weights in the range 1900–5100. King and Craig (1955) noted that purified tyrocidine A passed through dialysis membranes unexpectedly slowly. Ruttenberg, King, and Craig (1966) investigated the association of tyrocidine B and several of its derivatives by thin film dialysis (Craig, 1964). They found extensive association of tyrocidine B, diiodotyrocidine B, *N*-succinyltyrocidine B, *O*-methyl-*N*-succinyltyrocidine B, and of a derivative of tyrocidine A in which the aromatic groups had been catalytically reduced. An open-ring derivative of tyrocidine B, prepared by reductive cleavage of the proline–tryptophan bond, did not associate strongly. They concluded that interactions between aromatic groups are not important, but that the cyclic structure of the peptide is necessary for the association. The experiments reported below were conducted in order to elucidate the strength and stoichiometry of the association and to investigate the effects of solvent and temperature upon it.

Experimental Section

Materials. Tyrocidine hydrochloride (Wallerstein Co., lot no. ON 13554) was separated into its components by countercurrent distribution to 3000 transfers in a system composed of chloroform–methanol–0.01 M HCl (2:2:1, v/v) (Ruttenberg, 1965). A detailed analysis of this separation was carried out (Williams and Craig, 1967), with the conclusion that the tyrocidine B fraction contains no more than 3% of the similar peptide, tyrocidine C; and that the tyrocidine C fraction contains a smaller proportion of tyrocidine A. Gramicidin S-A, prepared by countercurrent distribution (Craig *et al.*, 1949), was the gift of Dr. Michael Ruttenberg.

Acetylated gramicidin S-A was prepared by allowing the peptide to react with a limiting amount of acetic anhydride in a solvent of ethanol and triethylamine. Three products were separated from the reaction mixture by countercurrent distribution. Ninhydrin reactions showed that these products possessed zero, one, and two free amino groups per molecule. It was concluded that mono- and di-*N*-acetylgramicidin

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S-A, as well as unmodified starting material, had been recovered.

Methods. Equilibrium ultracentrifugation was performed with Spinco Model E instruments, one of which was equipped with an ultraviolet absorption scanner. The bulk of the work was performed with the Rayleigh interference system, modified so that the double slit was located on the lower collimating lens (Yphantis, 1960, 1964). Short-column experiments were performed in centerpieces (Yphantis, 1960) which accommodate four solution–solvent pairs in columns of 0.9 mm length in the radial direction. Long-column experiments were performed in centerpieces (Yphantis, 1964) which accommodate three solution–solvent pairs in columns of about 3 mm length in the radial direction. Cells of either 12- or 30-mm optical path were employed. The An-D rotor was used at speeds above 12,000 rpm, and the An-J rotor at lower speeds. No difficulty was experienced with rotor vibration or solution convection at speeds as low as 2500 rpm.

Initial concentrations of stock solutions were determined by means of synthetic boundary experiments performed in a capillary type cell at 12,000 rpm. With few exceptions, duplicate runs were made on each stock solution. If the differences between these two determinations exceeded 3% of their mean, the concentration determination was rejected. Dilutions of stock solutions were made by weight. Both equilibrium and synthetic boundary cells were subjected to blank experiments in which the cells were filled with water and run at the speed of the experiment in order to provide correction for residual distortion of the interference fringes.

A liquid fluorocarbon (FC-43, Minnesota Mining and Manufacturing Co.) was used to provide a visible cell bottom in most experiments. There have been reports (Adams and Lewis, 1968) that the properties of proteins are sometimes altered by this liquid. In a number of experiments in which conditions were identical except for the presence or absence of FC-43, the association behavior of tyrocidine B was not affected.

Photographic plates were measured on a Gaertner two-dimensional comparator, Model M2001-RS. Approximately 40 points were measured for each long-column channel. Point values of reduced apparent molecular weights σ_w, and σ_z, were calculated as: $\sigma_w = d(\ln y)/d\xi$ and $\sigma_z = (d^2y/d\xi^2)/(dy/d\xi)$. The quantity y is the concentration in refractive index units, and $\xi = r^2/2$, where r is the radius of the point in question. An early version of a computer program developed by Roark and Yphantis (1969) was used to carry out a fit of a quadratic by the method of least squares to a number of adjacent points. The number of points was adjusted between 5 and 11 to produce a span of at least 300-μ vertical displacement. The values of σ_w obtained from the coefficients of the quadratic were assigned to the central point of each set.

For short-column runs, photographic plates were measured at two points equally spaced about the midpoint of the liquid column. Weight-average molecular weights were computed according to the method of Yphantis (1960), with the approximation that the concentration at the midpoint of the channel is equal to the initial concentration.

Attempts to measure the partial specific volumes ($\bar{v}$) of the tyrocidines failed because of difficulties in obtaining reliable dry weights. A value of $\bar{v}$ of 0.746 cm^3/g was calculated for tyrocidine B by the method of Cohn and Edsall (1943). This value includes a covolume correction of 0.001 cm^3/g, and was employed in all calculations of molecular weight. Similarly, the refractive increment of tyrocidine B was not measured, but was taken to be 1.82×10^{-3} dl/g, or 40 fringes for a solution of 1-g/dl concentration in a 12-mm light path.

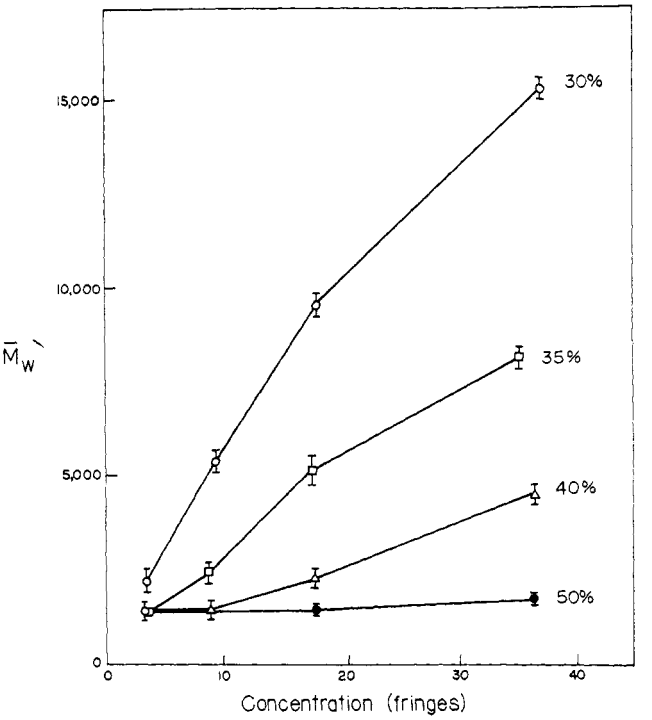

FIGURE 1: Apparent weight-average molecular weight of tyrocidine B as a function of peptide concentration in different solvents. Solvents were 30% acetic acid + 0.1 *m* NaCl (○), 35% acetic acid + 0.1 *m* NaCl (□), 40% acetic acid + 0.1 *m* NaCl (△), and 50% acetic acid + 0.1 *m* NaCl (●). Conditions: 23°; 32,500 rpm; short-column runs.

Results

Association in Acetic Acid–Water–Salt. Acetic acid acts as a solubilizing and dissociating agent for tyrocidine B. Figure 1 presents the results of short-column experiments at four concentrations of acetic acid in water to which a constant molal concentration (0.1 *m*) of NaCl had been added. Below 28% acetic acid and at this ionic strength, tyrocidine B is not soluble at a concentration of 1 g/dl. The peptide is dissociated effectively to monomer by 50% acetic acid. For further investigation, a solvent consisting of 30% aqueous acetic acid, 0.1 *m* in NaCl, was chosen. The peptide dissolves readily in this solvent, the ionic strength is high enough to reduce the second virial coefficient to a manageable magnitude, and association is pronounced enough to be readily measured.

In Figure 2 are shown the results of long-column experiments at four different temperatures in 30% acetic acid + 0.1 *m* NaCl. The overlap of the points from different channels indicates that the association behavior is purely concentration dependent (Squire and Li, 1961; Roark and Yphantis, 1969). This conclusion is reinforced by the fact that the results of short-column equilibrium experiments coincide with the results of these experiments.

Such an association equilibrium can, in principle, be described by a sequential association scheme (eq 1) such as that proposed by Steiner (1952). Here, c_i and m_i are the concentrations (in grams per deciliter and moles per liter,

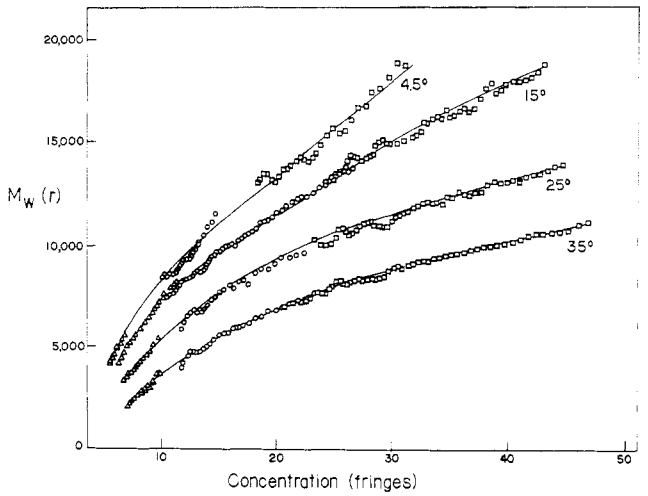

FIGURE 2: Apparent weight-average molecular weight of tyrocidine B as a function of peptide concentration, in 30% acetic acid + 0.1 *m* NaCl. Results of four experiments. Initial concentrations were 0.9 g/dl (□), 0.45 g/dl (○), and 0.23 g/dl (△). Conditions: 14,000 rpm at 4.5°, 15,700 rpm at 15°, 23,560 rpm at 25°, and 27,610 rpm at 35°; long-column runs.

$$\begin{array}{llll} P_1 + P_1 \rightleftharpoons P_2 & K_2 = c_2/c_1^2 & K_2^m = m_2/m_1^2 \\ P_2 + P_1 \rightleftharpoons P_3 & K_3 = c_3/c_1c_2 & K_3^m = m_3/m_1m_2 \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ P_i + P_1 \rightleftharpoons P_{i+1} & K_{i+1} = c_{i+1}/c_1c_i & K_{i+1}^m = m_{i+1}/m_1m_i \end{array} \quad (1)$$

respectively) of polymer P_i, which is made up of i monomers. Adams (1967) has shown how to extract the first three or four equilibrium constants from data of molecular weight against concentration; but the accuracy of available data will not permit the evaluation of more. Since complexes of at least 12 monomers are present, some simplification of the proposed association stoichiometry must therefore be made in order to describe the formation of large tyrocidine B polymers.

Two simplifications which have been found to describe complicated associations adequately in other cases were tried without success. The first of these was an "isodesmic" association scheme similar to that of Van Holde and Rossetti (1967) for the association of purine. The essential assumptions of this scheme are two. First, it is assumed that all the K_i^m of eq 1 are equal. Second, it is assumed that $\ln y_i = BM_ic$, where y_i is the activity coefficient on a weight concentration scale. One then obtains

$$(M_w^2/M_1^2)/(1 - BM_1M_wc)^2 = 1 + 4Kc \quad (2)$$

where B is a constant second virial coefficient, K is an equilibrium constant on a gram-per-deciliter scale, M_1 is the molecular weight of the monomer, and M_w is the apparent weight-average molecular weight which obtains at the concentration, c. If the assumption of equal values of $K_i(m)$ applies, a plot of the left-hand side of eq 2 against c will yield a straight line when the correct value of B is chosen. Such plots were constructed with the data of Figure 2. Large and systematic deviations from linearity were found for all values of B. It was therefore concluded that the isodesmic scheme is not a good description of the association process of tyrocidine B.

The second simplification attempted was the assumption of the micelle stoichiometry commonly employed to describe the association of detergent molecules. In this scheme it is assumed that only one polymer species, P_n, is present in significant amounts. Then eq 1 may be reduced to

$$nP_1 \rightleftharpoons P_n \qquad K_n = c_n/c_1^n \quad (3)$$

With the assumption that $\ln y_i = BM_ic$, one obtains a modification of an equation of Rao and Kegeles (1958):

$$Kc^{n-1} = \frac{(n-1)^{n-1}[M_w - M_1(1 - BcM_w)][M_1(1 - BcM_w)^{n-1}]}{nM_1(1 - BcM_w) - M_w} \quad (4)$$

Curve fitting was carried out by ordinary methods, with the use of integral values of n. The data could be adequately represented by eq 4 at each temperature, but only by the use of a *negative* second virial coefficient. This result indicates attractive forces between the solute particles over and above those involved in the hypothetical micelle-forming reaction.

In order to account for the negative apparent second virial coefficient, two modifications of the micelle equilibrium were formulated and tested. The first scheme comprises an equilibrium between the monomer and a micelle composed of n monomers, coupled with an equilibrium between the micelles and a "supermicelle," composed of a number, j, of the micelles (an nj-mer). The second scheme comprises an equilibrium between monomer and a micelle composed of n monomers, which in turn is in equilibrium with a series of progressively larger micelles, each formed by the addition of a single monomer to a micelle. The molar equilibrium constants for each of the sequential additions of monomer are assumed to be equal.

The first of these reaction schemes can be formulated as

$$\begin{array}{ll} nP_1 \rightleftharpoons P_n & K_n = c_n/c_1^n \\ jP_n \rightleftharpoons P_{nj} & K_{nj} = c_{nj}/c_n^j \end{array} \quad (5)$$

Combining the two equations on the right, one has

$$c_{nj} = K_jK_n^jc_1^{nj} \quad (6)$$

The total concentration, c, and the weight-average molecular weight, M_w, can then be written in terms of the concentration of monomer as

$$c = c_1 + K_nc_1^n + K_jK_n^jc_1^{nj} \quad (7)$$

$$M_w = (M_1/c)[c_1 + nK_nc_1^n + njK_jK_n^jc_1^{nj}] \quad (8)$$

In order to carry out curve fitting to relation 8, the numbers n and j may be estimated as follows. Let $M^* = M_w/M_1$ and $M^{**} = M_w/M_1n$. Then at low concentrations

$$c \cong c_1 + K_nc_1^n \quad (9)$$

$$M^* - 1 \cong (1/c)K_nc_1^n(n-1) \quad (10)$$

Then

$$\frac{d(M^* - 1)}{dc} \cong \frac{(n-1)}{c} nK_n c_1^{n-1}\left(\frac{dc_1}{dc}\right) - \left(\frac{1}{c^2}\right)K_n c_1^n(n-1) \quad (11)$$

Noting that $dc/dc_1 = cM^*/c_1$, one finds that

$$n = M^*\left[1 + \frac{d \ln (M^* - 1)}{d \ln c}\right] \quad (12)$$

Similarly, at high total concentrations

$$c = c_n + K_j c_n^j \quad (13)$$

$$M^{**} - 1 = (1/c)(jK_j c_n^j - K_j c_n^j) \quad (14)$$

so that

$$j = M^{**}\left[1 + \frac{d \ln (M^{**} - 1)}{d \ln c}\right] \quad (15)$$

In practice, estimates of n and j may be found by plotting the right-hand sides of eq 12 and 15, respectively, against concentration. As concentration decreases the *apparent* value of n should decrease to a fixed value. As concentration increases, the *apparent* values of j should decrease to a fixed value.

The second scheme can be formulated as

$$\begin{array}{lll} nP_1 \rightleftharpoons P_n & K_n = c_n/c_1^n & K_n^m = m_n/m_i^n \\ P_1 + P_n \rightleftharpoons P_{n+1} & K_{n+1} = c_{n+1}/c_1 c_n & K_{n+1}^m = m_{n+1}/m_1 m_n \\ P_1 + P_{n+1} \rightleftharpoons P_{n+2} & K_{n+2} = c_{n+2}/c_1 c_{n+1} & K_{n+2}^m = m_{n+2} m_1 m_{n+1} \\ \vdots & \vdots & \vdots \end{array} \quad (16)$$

If $K_{n+1}^m = K_{n+2}^m = \ldots = K^*$, we may then define a constant $k = 10\ K^*/M_1$ in weight concentration units.

It can then be shown that

$$c = c_1 + \frac{K_n c_1^n}{(1 - kc_1)} + \frac{K_n k c_1^{n+1}}{n(1 - kc_1)^2} \quad (17)$$

and that

$$M_w = (M_1/c)\left[c_1 + \frac{nK_n c_1^n}{1 - kc_1} + \frac{2kK_n c_1^{n+1}}{(1 - kc_1)^2} + \frac{kK_n c_1^{n+1}(1 + kc_1)}{n(1 - kc_1)^3}\right] \quad (18)$$

In this case, an estimate of n can be obtained by the same extrapolation as in the supermicelle scheme above. However, if the quantity

$$M^{**}[1 + d \ln (M^{**} - 1)/d \ln c]$$

is calculated, it will not decrease to a fixed value, but rather will *increase* without limit as concentration increases. Thus, by the behavior of the apparent value of j, one may distinguish between these two hypothetical stoichiometries.

In order to test the applicability of the two hypothetical schemes to the data, the observed curves were first adjusted by the application of a second virial coefficient correction. The magnitude of the correction was estimated from an equation due to Scatchard (Tanford, 1961): $B = (250 v_1 z^2)/(m_3 M_2^2)$, where v_1 is the specific volume of the solvent, z is the electrostatic charge on the macromolecular solute, M_2 its molecular weight, and m_3 is the molality of added electrolyte. In the case at hand, B was estimated to be 1.15×10^{-6} (l. moles)/g^2. *Apparent* values of the quantities n and j were calculated from the corrected data. They are shown in Figure 3, plotted against total concentration. The apparent values of n approach well-defined limits. The apparent values of j decrease with increasing concentration, but still have significant slope at the highest concentrations measured, probably because the fraction of the larger polymer is still relatively small at these concentrations. The decrease in the apparent value of j with increasing concentration rules out the scheme involving micelle formation followed by sequential addition of monomers with the same association constant for each addition.

TABLE I: Numerical Values of Variables Used in Curve Fitting.[a]

Temp (°C)	n	j	ln K_n	ΔG_n^0	ln K_j	ΔG_j^0
4.5	11	4	+71.1	−3.6	+16.0	−2.2
15	11	4	+69.6	−3.7	+15.2	−2.2
25	9	3	+54.3	−3.6	+10.0	−2.0
35	8	3	+45.6	−3.5	+9.4	−1.9

[a] The values of n and j are consistent with the lower and upper limits set by the curves in Figure 3. The standard state is 1 mole/l. The apparent free energies correspond to the formation of n-mer and j-mer, in units of kcal/(mole of monomer) and kcal/(mole of n-mer), respectively.

The supermicelle scheme yields a satisfactory fit to the observed data, as shown in Figure 4. The integral values of n and j which provide the best fit, together with the best numerical values of the equilibrium constants, are shown in Table I. The satisfactory nature of the fit does not demonstrate that the supermicelle scheme is a unique description of the observed association behavior of tyrocidine B. This scheme, however, does provide the best description of the four schemes considered here.

Association of Peptides Related to Tyrocidine B. Each

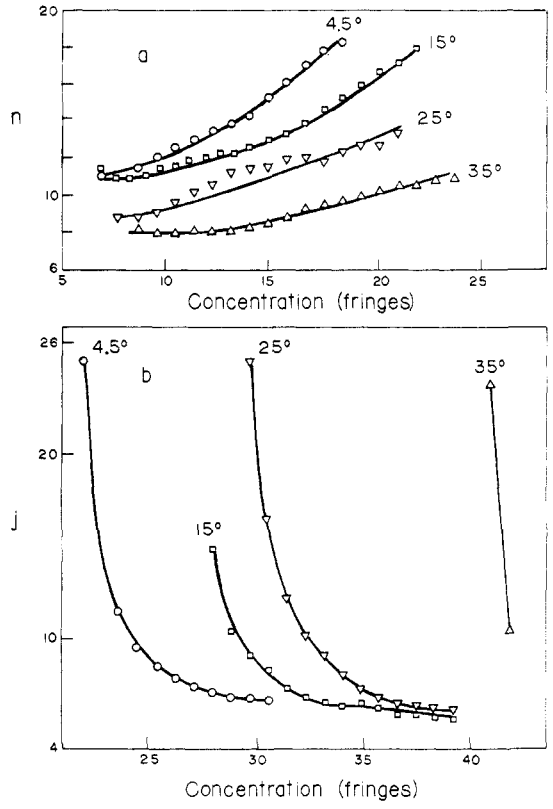

FIGURE 3: *Apparent* values of micelle numbers, n and j, as functions of concentration, for tyrocidine B in 30% acetic acid + 0.1 m NaCl. Temperatures as indicated. See text for definition of these quantities.

of the three tyrocidines undergoes association in solution similar to that displayed by tyrocidine B, although the details of stoichiometry and association constant have been investigated only for tyrocidine B. To assess the ability of two of these peptides to form association *heteropolymers*, a mixture of 50% each by weight of tyrocidine B and C was examined in 30% acetic acid + 0.1 m NaCl. The results

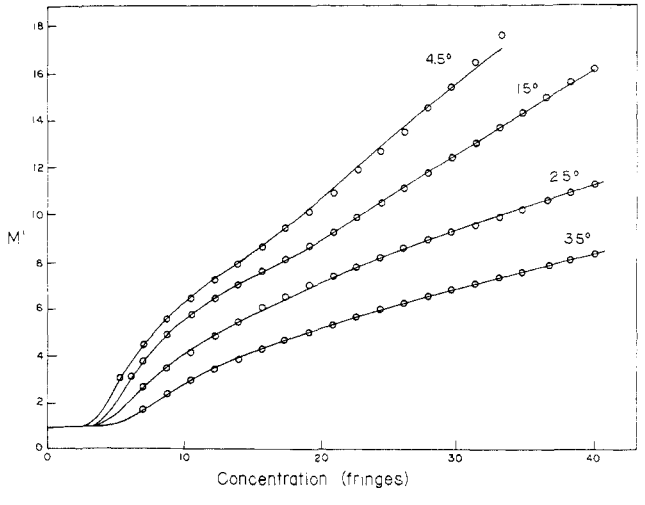

FIGURE 4: Fit of experimental data to supermicelle stoichiometry. Lines are calculated as described in text. Points are smoothed data of Figure 2, corrected for electrostatic second virial coefficient. M' is weight-average molecular weight divided by monomer molecular weight.

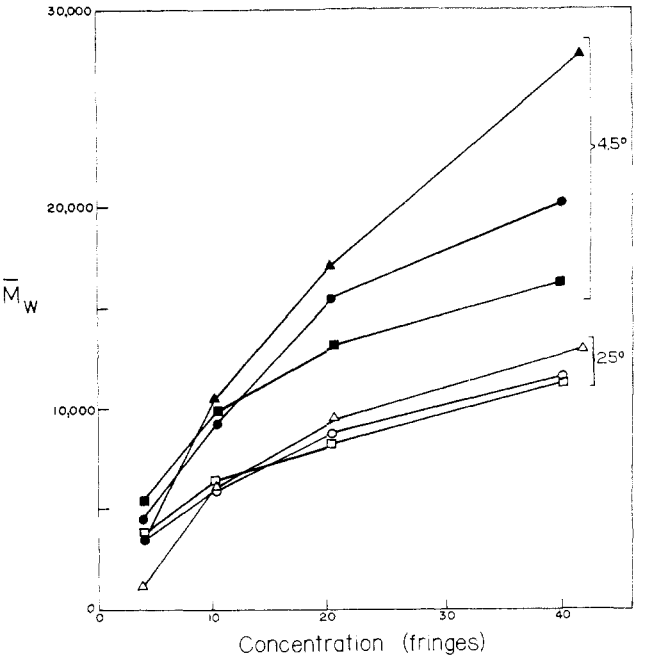

FIGURE 5: Apparent weight-average molecular weights of tyrocidines B and C, singly and mixed, as a function of total peptide concentration. The solvent was 30% acetic acid + 0.1 m NaCl. At 4.5°: tyrocidine B (▲), tyrocidine C (■), and 50% of each (●). At 25°: tyrocidine B (△), tyrocidine C (□), and 50% of each (○). Conditions: 12,065 rpm at 4.5° and 17,980 rpm at 25°; short-column runs.

in Figure 5 make clear that the association reactions of tyrocidines B and C are not altogether specific. If B associated only with B, and C only with C, the M_w of the mixture at a given concentration would be expected to be equal to the mean M_w of the two peptides at half that concentration. Since the M_w of the mixture lies close to or between the values of M_w for each of the components, there is little or no preference shown for homo- or heteropolymer formation by either peptide.

Gramicidin S does not undergo association in aqueous solution (Ruttenberg, 1965). Near neutral pH, it carries two positive electrostatic charges, while tyrocidines carry only a single charge. It has been proposed (Ruttenberg, 1965) that repulsive electrostatic forces effectively prevent association of gramicidin S. To test this idea, the molecular weight of the singly charged *N*-acetylgramicidin S was measured by short-column experiments at four concentrations in 30% acetic acid + 0.1 m NaCl. The results, shown in Figure 6, indicate the presence of some weak association of small degree which increases with decreasing temperature. The difference in behavior between gramicidin S and the tyrocidines is distinct, however, and evidently does not depend solely on the electrostatic charge.

Association in Aqueous Salt Solutions. When prepared as a lyophilized powder, tyrocidine B dissolves over a period of many weeks in water at room temperature. The time required to form a solution drops precipitously, however, between 53 and 58°, and at 60° a solution can be prepared in about 5 min. The peptide is soluble at concentrations greater than 0.5 g/dl in NaCl–water over the range of NaCl concentration from 0 to 0.024 M. Aqueous solutions of tyrocidine B were prepared in 0.02 M NaCl by heating to 70° for 10 min in a sealed ampoule. Preparations made in this manner at peptide concentrations below 3 g/dl remain

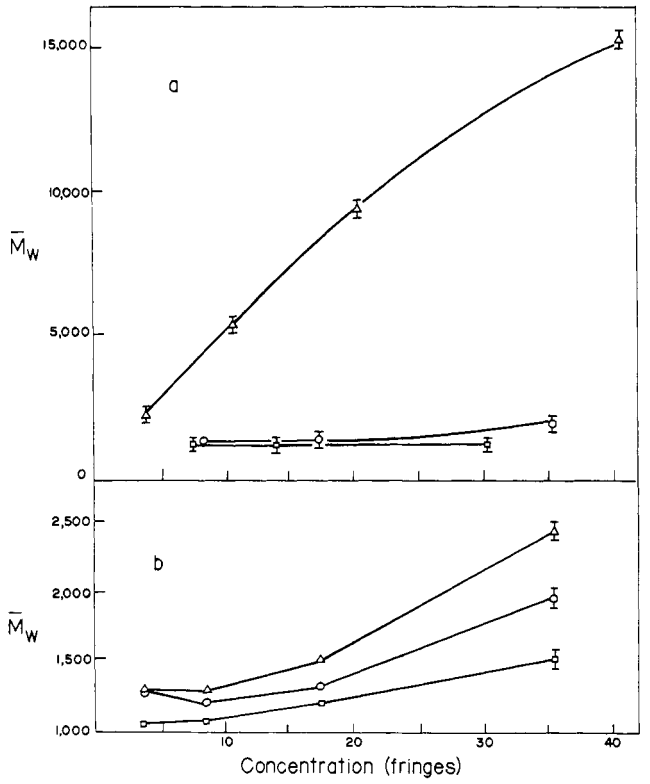

FIGURE 6: Apparent weight-average molecular weights of three peptides as functions of concentration in 30% acetic acid + 0.1 *m* NaCl. (a) Tyrocidine B (△), *N*-acetylgramicidin S (○), gramicidin S (□). Conditions: 24.1°, 34,000 rpm. (b) *N*-Acetylgramicidin S at 4.6° (△), 24.1° (○), and 38.7° (□); all at 34,000 rpm. Short-column runs.

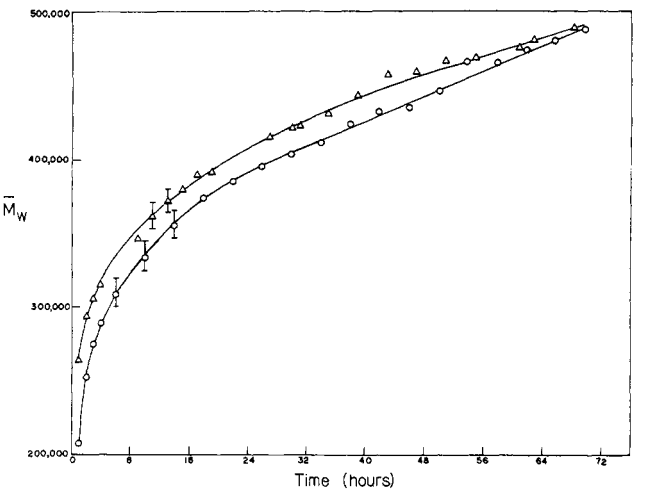

FIGURE 7: Time dependence of apparent weight-average molecular weight of tyrocidine B in 0.02 *m* NaCl. Sample was warmed to 70° before experiments. First experiment (○), second experiment (△). Conditions: 25°, 4000 rpm; short-column runs.

clear and stable for times in excess of 1 year, and can probably be regarded as true solutions.

The apparent weight-average molecular weight of tyrocidine B in these solutions depends upon the time after their preparation. A 0.3-g/dl solution of tyrocidine B in 0.02 *m* NaCl was placed in a short-column centrifuge cell. The filled cell was warmed to 70° for 30 min and then observed at 25° for 72 hr in the centrifuge. The circular points in Figure 7 show the dependence of the apparent weight-average molecular weight of this solution on the time after warming. The time required for attainment of physical equilibrium in the centrifuge was found to be about 5 hr, both by calculation (Van Holde and Baldwin, 1958) and by experiment. Thus the observed increase in apparent molecular weight beyond this time reflects a change in the association of the solute. The increase in molecular weight may be reversed by warming the solution again. The triangular points in Figure 7 show the the results of observations at 25° of the same solution after it had been warmed again in the centrifuge cell to 70° for 30 min. The systematic difference of about 4% in molecular weight between the two curves is probably due to a difference in the manner of warming or cooling the solution, and is not considered significant. It may thus be concluded that that the formation of at least some polymers by tyrocidine B in aqueous solution is a slow process, and that it may be reversed by warming the solution.

The molecular weight of tyrocidine B is not purely concentration dependent in 0.02 *m* NaCl. In Figure 8a is shown a plot of the reduced *z*-average molecular weight against the concentration gradient for experiments at three initial concentrations. If the molecular weight of the solute were purely dependent on concentration, the three curves would be coincident. It is seen that they are not, and thus it is concluded that the molecular weight of tyrocidine B is not purely concentration dependent in this solvent. For contrast, Figure 8b shows an equivalent plot of three experiments in 0.02 *m* NaCl to which was added 10% of acetic acid. It is apparent that this modification of the solvent produces

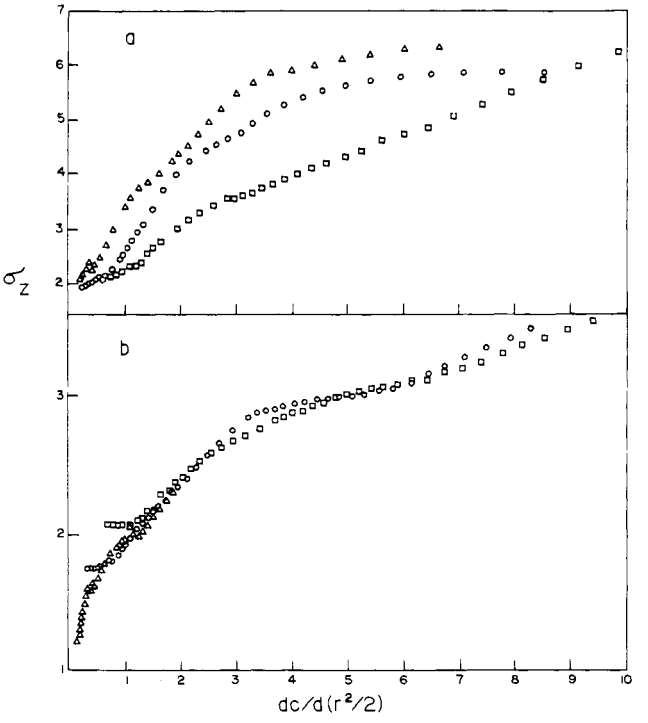

FIGURE 8: Reduced *z*-average molecular weight as a function of the quantity $dc/d(r^2/2)$ (see text for details). (Top) Tyrocidine B in 0.02 *m* NaCl at initial concentrations of 0.06 g/dl (△), 0.12 g/dl (○), and 0.23 g/dl (□). Conditions: 25°, 12,000 rpm. (Bottom) Tyrocidine B in 90% 0.02 *m* NaCl + 10% acetic acid at initial concentrations of 0.06 g/dl (△), 0.12 g/dl (○), and 0.23 g/dl (□). Conditions: 25°, 30,000 rpm.

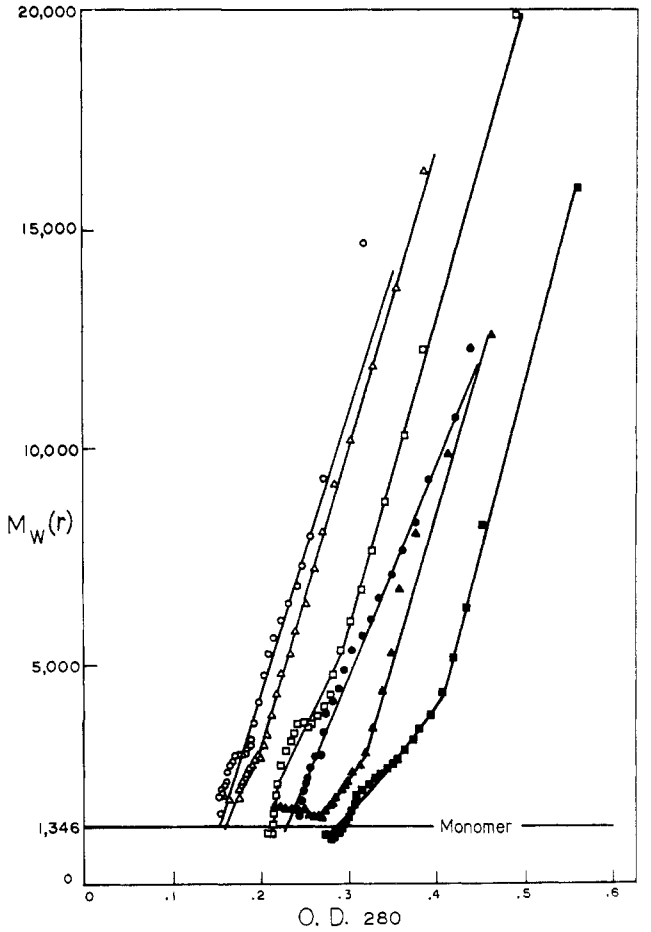

FIGURE 9: Apparent weight-average molecular weight of tyrocidine B, as a function of its concentration in 0.02 *m* NaCl. Initial concentrations: 0.008 g/dl (○), 0.012 g/dl (△), 0.025 g/dl (□), 0.05 g/dl (●), 0.1 g/dl (▲), and 0.3 g/dl (■). Conditions: 24.1° and 34,280 rpm for first four concentrations, 40,370 rpm for last two; long-column runs; measurements performed with absorption scanner.

a purely concentration dependent solute, as observed previously in the case of 30% acetic acid + 0.1 *m* NaCl.

Figure 9 shows the concentration dependence of the molecular weight of tyrocidine B at very low concentrations in 0.02 M NaCl. These results were obtained at 280 nm with the ultraviolet absorption scanner. The lack of pure concentration dependence is again evident from the lack of overlap of the curves obtained at different initial concentrations. Nevertheless it is clear that for each of the solutions there is a concentration below which association is negligible and above which the apparent molecular weight increases rapidly with concentration, phenomena usually associated with micelle formation. Because of the lack of a true equilibrium, shown by the lack of coincidence of curves of M_w against c, it is not possible to specify a true critical micelle concentration, although it is clear that the bulk of the peptide exists in associated form above a concentration of 0.003 g/dl.

In another experiment, a solution of about 0.002 g/dl was prepared by dilution of a 0.5-g/dl stock solution and observed with the scanner. Only monomer was seen. It may be concluded, therefore, that dissociation of the peptide is rapid in contrast to the slow, time-dependent increase in association described above, and that the peptide exists as monomer at concentration below 0.002 g/dl.

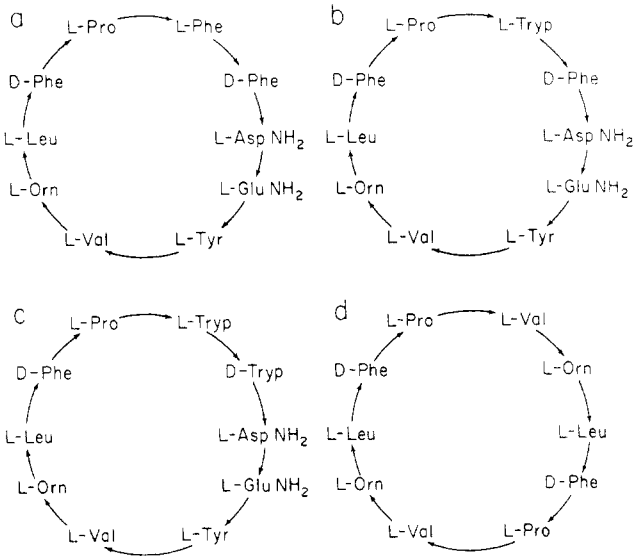

FIGURE 10: Structures of the tyrocidines and gramicidin S. (a) Tyrocidine A (Paladini and Craig, 1954); (b) tyrocidine B (King and Craig, 1955); (*c*) tyrocidine C (Ruttenberg *et al.*, 1965); (*d*) gramicidin S (Consden *et al.*, 1947; Battersby and Craig, 1951).

Discussion

Tyrocidine B in solvents containing acetic acid displays a relatively simple and rapidly reversible association equilibrium. The "supermicelle" scheme describes the data well and constitutes a possible description of the stoichiometry of the association reactions. It is a better description than any of the three other simplifications considered. It is probably not unique: other schemes could be found which would describe the equilibrium equally well within the precision of the data. In particular, the existence of polymers larger than the reported supermicelle size clearly is not excluded by these experiments.

The large numbers of monomers in the polymers seem to be the result of the relative structural simplicity of the monomer. It is evidently not complex enough to provide the limited number of sites or regions of association which must be invoked to account for the small numbers of monomers present, for instance, in most multisubunit enzymes. The variation with temperature of the number of monomers in a tyrocidine B polymer, as well as the capacity of tyrocidine B and C to form heteropolymers, serves to emphasize this point.

Qualitatively, the association of tyrocidine B must depend in a rather delicate way upon its conformation and amino acid composition. Ruttenberg *et al.* (1966) have shown that if the peptide ring (Figure 10b) is opened, association no longer occurs. The present work shows that gramicidin S (Figure 10d), which has a pentapeptide sequence in common with the tyrocidines and approximately the same number of hydrophobic amino acids, does not associate strongly, even when one of its charged groups is blocked. Tyrocidines A and C (Figure 10a,c) each form homopolymers, and tyrocidine B forms heteropolymers with tyrocidine C. These facts indicate that great differences in association behavior may be observed among peptides of similar structure, but that small structural alterations do not always imply large differences in association.

Not all the association phenomena observed are rapidly reversible. It seems unlikely that gross contamination of the

solute is responsible. It seems equally unlikely that slow covalent changes in the molecule are responsible, since both the time dependence and the apparent heterogeneity may be removed by the addition of a small amount of acetic acid to the solvent. Furthermore, the time course of the molecular weight increases may be reversed and repeated simply by heating the solution. Even simple dilution appears to reverse all association. One possible interpretation of these observations is that more than one form of monomer is present in solution, and that the different forms possess distinct association properties. Conversion of one form to the other would occur when the solution is warmed. The observed time dependence of the molecular weight would then be a reflection of the conversion of a weakly associating to a strongly associating form. It seems reasonable to identify the multiple forms with metastable conformations of the molecule. The conversion from one such conformation to another could easily be associated with a large enough activation energy to produce the observed slow rate of change of apparent molecular weight. The observed homogeneity and rapidly reversible equilibrium in the presence of acetic acid would then be a result of the lowering of the activation energy barrier between the forms, leading to the conversion of all of the peptide to one form.

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