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Aggregation of Tyrocidine in Aqueous Solutions

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Summary: The formation of aggregates of tyrocidine B at 4°C and 20°C in aqueous solutions was studied by means of light scattering and fluorescent techniques. The apparent weight molecular weight of tyrocidine B aggregates was found to be 36,000 at 4°C and 28,800 at 20°C. Fluorescence titration experiments with dansyl-chloride resulted in an aggregational number of 31 (4°) and 28 (20°) indicating that one molecule of dye is bound per monomer of molecular weight 1,200. From a Scatchard plot apparent association constants of 1.22×10^5 M (4°) and 0.95×10^5 M (20°) were calculated. From the angular dependence of scattered intensity the radii of gyration were determined to be 60 Å and 58 Å, respectively.

Aminoacyl adenylates are intermediates in the biosynthesis of gramicidin S and tyrocidines which are nucleic acid independent (1). Gramicidin S and tyrocidines are cyclic decapeptides composed of two identical sequences of five different amino acids (2), but tyrocidines are composed of up to nine different amino acids joined together in a cyclic ring (3). The tyrocidines are usually mixtures of decapeptides resulting from a lack of enzyme specificity in Bacillus brevis permitting substitution of tryptophan for phenylalanine and of either tryptophan or phenylalanine for tyrocine which leads to variants (4,5).

Recently is has been shown that tyrocidine in aqueous solution can inhibit RNA synthesis *in vitro* due to formation of a

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DNA·tyrocidine complex (6,7). The inhibition of RNA synthesis in vivo is partially compensated by gramicidin D which restores RNA synthesis of tyrocidine inhibited transcription (7). One outstanding physical property of tyrocidines is their strong tendency to associate (8,9) whereas gramicidin S does not. This tendency is of great interest since the mechanistic action of the tyrocidine with DNA could be that of an aggregate of certain size. Moreover, the aggregational state of tyrocidine in aqueous medium (up to 40% water) in ethanol (methanol) has not been investigated and should be relevant to the inhibition studies of RNA synthesis (7).

Materials and Methods

Tyrocidine was obtained from United States Biochemical Corp., Cleveland, Ohio, and further purified by countercurrent distribution according to Ruttenberg et al. (10) from which the fractions containing tyrocidine B were pooled and further purified (11). Dansyl chloride (DNS) and 1-anilino sulfonate (ANS) were obtained from Serva (Heidelberg, W. Germany). Fluorescence measurements were made in a Perkin Elmer Model MPF III fluorimeter, equipped with a Hitachi 018-0054 polarization accessory. The fluorescent emission was converted for DNS (ANS) absorption (12) and for dilution effects. The amount of fluorescent dye bound to tyrocidine B was calculated by plotting 1/F, the fluorescence of a 1.15×10^{-5} M solution of ANS in the presence of a 10 mg/ml tyrocidine B solution (20-40% H_2O/80-60% ethanol w/w), or titrating a 1.15×10^{-5} M ANS solution (DNS) with tyrocidine B. The intercept yields E_{max} at the ordinate with E_{max} the maximum fluorescence occurring for a quantity of totally bound label. The bound label (ANS_B or DNS_B) was then calculated according to $(ANS_B) = F/(E_{max}/1.15 \times 10^{-5})$ with F the observed fluorescence for any given label concentration.

Light scattering. Experiments were performed using a Fica 50 photogoniodiffusiometer with unpolarized light of $\lambda = 5465$ Å from a helium-argon laser (10 mW). The data were collected at two temperatures (4° and 20°C) in 20-40% H_2O/80-60% ethanol (w/w). The molecular weights were determined according to

$$\frac{Kc}{\Delta R} = M_W^{-1} + 2A_2c + ... = [M_W^{-1}P(\theta)]^{-1} = M_W^{-1}(1 + \tfrac{1}{3}h^2R_g^2 + ...)$$

with $K = (\partial n/\partial c)_{\mu = const} \times 2\pi^2 n_0^2/\lambda_0^4 N_A$, $h = \frac{4\pi}{\lambda} \sin\theta/2$, and ΔR the Rayleigh ratio $= \frac{\Delta I}{I_B} R_B \left(\frac{n_0}{n_B}\right)^2$, n_0 = the solvent refractive

index and n_B the refractive index of benzene (B), ΔI = the measured scattered intensity of the tyrocidine B solution corrected for solvent at any scattering angle (13). Sedimentation equilibrium experiments were performed at 4° and 20°C using a Beckman-Spinco Model E analytical centrifuge, using rotor speeds of 10,000 rpm and different cell loadings. The determined partial specific volume for the calculations of the weight average molecular weights was 0.793 $ml \cdot g^{-1}$ (11). The optical scanning system has been used throughout the measurements at 280 nm.

Results

Tyrocidine B (11) in a solution of 80% ethanol (methanol) and 20% water shows a variation on the apparent molecular weight with total tyrocidine concentration (Fig. 1). From Fig. 2 the nonideality of such a solution normally used for biochemical inhibition studies is seen, even at low tyrocidine B concentration yielding a second virial coefficient of $A_2 = 6.22 \times 10^{-4}$ mole ml/g^2. Apparent weight average molecular weights and apparent z-average mean square radius of gyration of the uncorrected effects of optical anisotropy were found to be 3,600 at

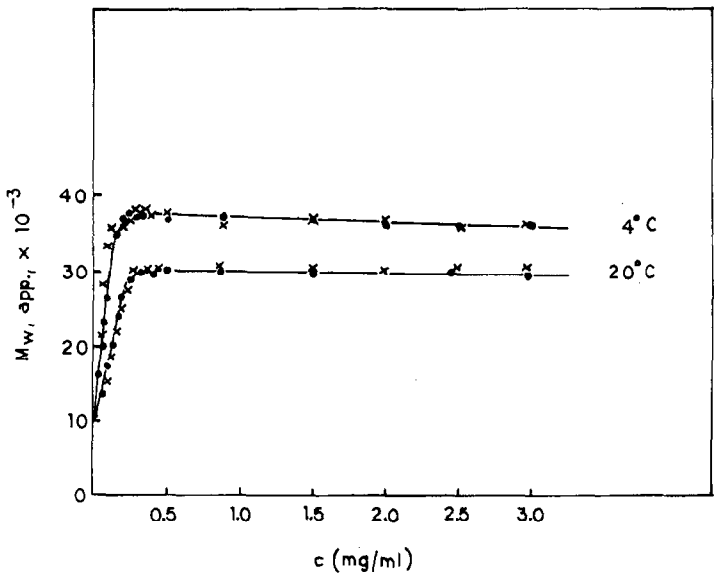

Fig. 1. Apparent weight average molecular weight of tyrocidine B in aqueous solution from sedimentation equilibrium measurements and light scattering experiments. ●—● sedimentation equilibrium; x—x light scattering.

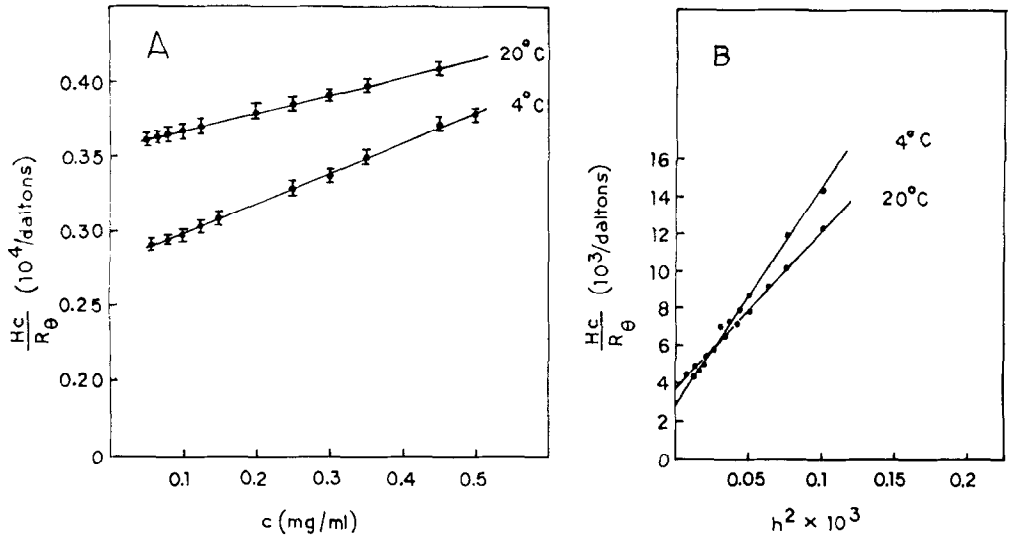

Fig. 2A. Zero angle extrapolation for tyrocidine B at 4°C and 20°C. Extrapolations are by linear least squares fit.

Fig. 2B. Angular dependent extrapolation at zero concentrations for tyrocidine B at 4°C and 20°C at 546 nm. Extrapolations are by least squares fit.

4°C and 3,364 at 20°C, 70 Å (4°C) and 58 Å (20°C). The determined second virial coefficient from light scattering experiments is in fair agreement with the value obtained by sedimentation equilibrium measurements (Table 1). In conjunction with these techniques, an alternate means of determining the aggregation process of tyrocidine B is using fluorescence methods with dansyl chloride (DNS) as label. In aqueous solutions DNS as well as ANS fluoresces only in environments where water moderates quenching conformations cannot be attained. With the determined E_{max} titration of a solution of tyrocidine B with DNS yields the fluorescent data as a function of label concentration from which the required data for a Scatchard plot is obtained (Fig. 3) (14) by plotting L/c versus L, with L the moles DNS bound/mole tyrocidine B as a monomer concentration, and c is the concentration of free DNS. The intercept at L/c = 0 yields the maximum value

Table I

Molecular Weights of Tyrocidine B in Different Solvent Compositions and Temperatures

Concentration	Sedimentation Equilibrium		Light Scattering		Solvent Composition
	4°C	20°C	4°C	20°C	
10 mg/ml	-	-	3,700 (3,600)	- 3,700	10% H_2O; 90% C_2H_5OH
10 mg/ml	36,500	28,000	36,500	28,100	20% H_2O; 80% C_2H_5OH
10 mg/ml	36,300	28,100	36,510	28,200	40% H_2O; 60% C_2H_5OH
20 mg/ml	36,700	28,150	36,550	28,275	40% H_2O; 60% C_2H_5OH
40 mg/ml	36,750	28,250	36,600	28,290	20% H_2O; 80% C_2H_5OH
0.1 mg/ml	18,900	10,900	18,800	11,000	40% H_2O; 80% C_2H_5OH
0.05 mg/ml	12,000	9,700	12,100	10,500	40% H_2O; 80% C_2H_5OH
0.02 mg/ml	10,900	9,600	10,500	9,700	40% H_2O; 80% C_2H_5OH
0.01 mg/ml	10,700	9,500	10,000	9,500	40% H_2O; 80% C_2H_5OH

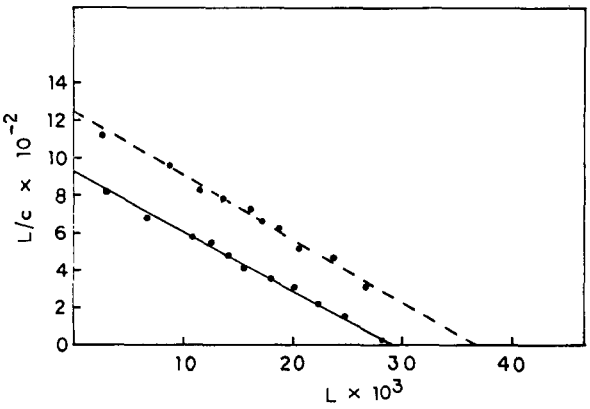

Fig. 3. Scatchard plot resulting from the titration of 1% tyrocidine B by ANS and DNS at 4°C (---) and 20°C (●—●).

of L and the slope - K, the apparent association constant. For ten titrations the average value of 1/L was 30.5 ± 1.5 at 4°C and 23.5 ± 2 at 20°C and K = 1.22 x 10^5 at 4°C and 0.95 x 10^5 at 20°C. Performing the same experiments with a mixture of tyrocidines the average values are L = 32 and K = 1.25 x 10^5 M (4°C). Moreover, conducting experiments in the presence of 10% H_2O,

only three sites can be titrated yielding a molecular weight of 3,600 for tyrocidine B, and no concentration dependence of the weight-average molecular weight upon tyrocidine B concentration is observed in this solvent system.

The effect of tyrocidine B concentration on the rate of aggregation in a solution containing 45% H_2O and 55% ethanol (w/w) also was measured. When the peptide concentration in this solvent system is decreased below 0.02 mg/ml, only six binding sites for the fluorescent dye are titrated, whereas above 0.01 mg/ml no concentration dependence was detected. The weight average molecular weight, therefore, attained in a given time is independent of the tyrocidine B concentration above a concentration of 0.02 mg/ml and only dependent on the concentration of water, which is consistent with the results of Ruttenberg et al. (15).

Discussion

Since tyrocidines have inhibitory effects on the transcription rates of DNA, which can be reduced by gramicidin D only to about 50% (7), a study of the size and temperature behavior of tyrocidine is warranted. The data presented show conclusively that tyrocidine B in aqueous solution is a stable aggregate of molecular weight 28,500 at 20° C and 36,500 at 4° C. These results are consistent with the reported values by Ruttenberg et al. (15) who studied tyrocidine B aggregation in a solvent of dimethylformamide/acetic acid/water yielding somewhat lower values ($\sim$ 20,000), possibly due to differences in the partial specific volumes and solvent composition. Furthermore, the relatively high values of the radii of gyration at both temperatures suggest that the molecule is elongated rather than

spherical which is supported by the values of the intrinsic viscosities ($[\eta]$ = 12.5 $ml \cdot g^{-1}$ Å and 11.8 $ml \cdot g^{-1}$ at 20° C), whereas for globular particles intrinsic viscosities of 3-4 $ml \cdot g^{-1}$ are obtained (16). Furthermore, the ratio of $R_g/M^{1/3}$ is 1.5 whereas for most globular particles the value is in the range of 0.6-0.75. This is a further indication for an anisometric or complex particle of these aggregates of tyrocidine.

Preliminary calculations of the free energy changes were performed from the association constants and were found to be ΔG = -0.25 Kcal/mol, ΔH = -9.1 Kcal/mol and ΔS = +30.5 cal/mol·degree. The low ΔH values combined with the positive entropy changes point to hydrophobic interactions between the tyrocidine B molecules at a water concentration of 40%. Whereas during aggregation ΔH is a function of temperature, ΔS is independent of temperature, but ΔS is heavily dependent on the solvent composition in which the antibiotic is dispersed. Perhaps the association of tyrocidines is related to their detergent properties as well, since detergents also form micelles of certain shapes (17).

These reported properties, molecular weights, radiis of gyration, association constants of tyrocidine B in aqueous solutions at different temperatures are indicative that the peptide bonds are possibly buried and tied up in intramolecular hydrogen bonding so that the residue side chains extend toward the surface of the aggregate since chemical modifications, e.g., acetylation of the ornithine iodination of the tyrosine, do not change the observed aggregation process (11).

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