Interaction of the Fluorescent Probe 2-*p*-Toluidinylnaphthalene-6-sulfonate with Peptides. Structural Requirements for Binding and Fluorescence Enhancement†

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ABSTRACT: The interaction of the fluorescent dye 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) with peptides has been studied by thin film dialysis and fluorescence techniques in order to determine the structural requirements for binding and fluorescence enhancement of TNS. The dialysis experiments showed that TNS binds to tyrocidines A, B, and C, gramicidin S-A, bacitracin A, an open-chain derivative of tyrocidine B, and bradykinin, but not to a derivative of tyrocidine B in which the δ-amino group of the ornithine residue was succinylated. However, fluorescence enhancement of TNS was observed only in the presence of the three tyrocidines. The tyrocidines are known to be extensively self-associated under the conditions used, whereas the other peptides, with the exception of succinyltyrocidine B, do not self-associate. From these results we suggest that there are two separate processes involved in the binding and fluorescence enhancement of TNS with these peptides. The first process is ionic interaction between the sulfonate group of TNS and a positively charged group of the peptide, such as the ornithine δ-amino group of the tyrocidines and gramicidin S-A. This ionic interaction seems to be primarily responsible for the binding of TNS to the peptides as revealed in the dialysis experiments. TNS does not bind to succinyltyrocidine B because the ornithine δ-amino group has been succinylated. The binding of TNS to the peptides by ionic forces does not result in fluorescence enhancement. Fluorescence enhancement requires a second condition, namely, the positioning of the aromatic system of TNS in a suitable environment. This second condition also requires peptide self-association, since fluorescence enhancement is observed only when TNS can bind ionically to the peptide and the peptide self-associates.

The fluorescent dye 2-*p*-toluidinylnaphthalene-6-sulfonate[1] and related molecules have been widely used as conformational probes of proteins and membranes (Edelman and McClure, 1968; Stryer, 1968; Chance, 1970). They are attractive for this purpose because they are practically nonfluorescent in water, but highly fluorescent when dissolved in organic solvents or when bound to appropriate sites on proteins (Weber and Laurence, 1954) and membranes. There is considerable evidence which indicates that the fluorescence enhancement of TNS and related compounds is due to a local environment at the binding site of decreased polarity (Stryer, 1965; McClure and Edelman, 1966; Daniel and Weber, 1966; Turner and Brand, 1968). Other factors, such as increased rigidity of the probe molecule when bound (Ainsworth and Flanagan, 1969; Penzer, 1972), may also play a role.

Despite their widespread use as probes of macromolecules and supramolecular structures, the nature of the sites where TNS and related molecules bind, and the forces which govern this binding, are not well defined. Hydrophobic interaction between the aromatic system of TNS and regions of low polarity of the target structure is often suggested to be the major binding force, but there are indications that ionic interactions may also be significant (Flanagan and Ainsworth, 1968).

We have attempted to characterize further the requirements for binding and fluorescence enhancement of TNS by studying its interaction with naturally occurring small peptides of known primary structure, especially the cyclic decapeptide antibiotic tyrocidine B. This peptide self-associates extensively in aqueous solution by hydrophobic interactions (Ruttenberg *et al.*, 1966; Laiken, 1970; Williams *et al.*, 1972; Stern *et al.*, 1969), and thus provides an interesting model system having some properties in common with both proteins and lipids.

From the experiments reported here, the structural requirements for the binding of TNS to peptides and for fluorescence enhancement of TNS with peptides have been clarified. In addition, the fluorescence of TNS bound to aggregated tyrocidine B has been found to be sensitive to the extent of aggregation of the peptide, and these results have been interpreted by use of a model for the conformation of tyrocidine B.

Experimental Procedure

Crude tyrocidine hydrochloride was separated into its components by CCD[1] in the solvent system chloroform–methanol–0.01 M HCl (2:2:1, v/v/v) (Ruttenberg *et al.*, 1965). Gramicidin S-A and bacitracin A were also prepared by CCD (Craig *et al.*, 1949, 1969). Bradykinin was a commerical sample (Nutritional Biochemical Co.). The *N*-succinyl derivative of tyrocidine B was synthesized according to the method of Ruttenberg *et al.* (1966) and purified by CCD. Linear tyrocidine B was obtained by reductive cleavage of the phenylalanyl–proline bond (Ruttenberg *et al.*, 1964). The primary structures of these peptides are given in Figure 1.

The potassium salt of TNS was prepared by the method of McClure and Edelman (1966). Its purity was checked by thin layer chromatography on silica gel G (Merck) using the solvent *sec*-butyl alcohol saturated with 1% aqueous ammonia. A single zone, with R_F 0.52, was obtained. The shape and wavelength maximum of the fluorescence emission spectrum of this material dissolved in absolute ethanol were independent of the excitation wavelength over the range 260–

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[1] Abbreviations used are: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; CCD, countercurrent distribution.

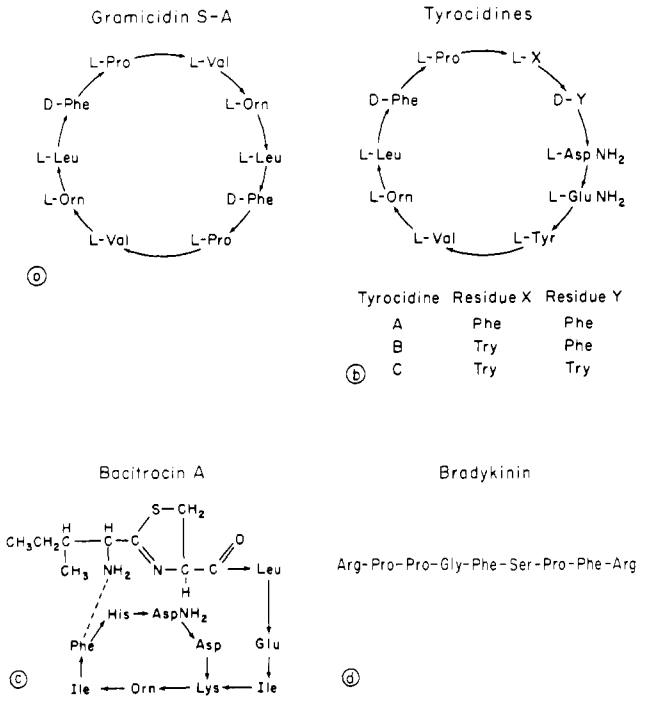

FIGURE 1: Primary structures of the peptides used in this study. Linear tyrocidine B was obtained by cleavage of the phenylalanyl–proline bond of tyrocidine B. Succinyltyrocidine B was prepared from tyrocidine B by succinylation of the ornithine δ-amino group.

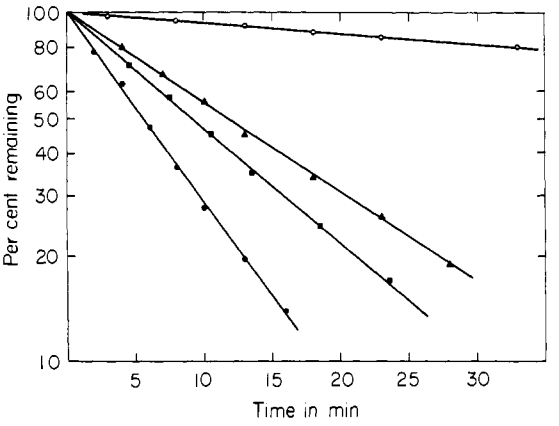

FIGURE 2: Thin film dialysis escape curves: (●) escape of TNS in the absence of any peptide; (■) escape of TNS in the presence of linear tyrocidine B; (▲) escape of TNS in the presence of tyrocidine B; initial peptide and TNS concentrations were 5.0×10^{-5} M; (○) escape of bacitracin A, initial concentration 1.0×10^{-3} M.

375 nm. This is further evidence that the TNS preparation contained only one fluorescent species (Weber, 1961). The material was stored in the dark at 4°.

Concentrated stock solutions of tyrocidine B and TNS were prepared in 0.1 M acetic acid. These were kept at room temperature in the dark. Solutions for fluorescence experiments were made by using Hamilton microsyringes to deliver small volumes of the stock solutions into 1 cm square cuvets containing 2.0 ml of 0.1 M acetic acid.

Fluorescence measurements were done on a Turner Model 210 spectrofluorometer operating in the uncorrected luminescence mode. Fluorescence intensity was taken as the height of the emission spectrum, in arbitrary units, at the wavelength of maximal emission. Excitation was at 375 nm. The excitation bandwidth was 10 nm and the emission bandwidth was 25 nm in all cases. The temperature of the sample was controlled by water circulating through the cuvet holder. Most measurements were taken at 25 ± 1°. At higher temperatures, the cuvet was tightly sealed to prevent solvent evaporation. The temperature was increased slowly and held at each level until there was no further change in fluorescence. During temperature equilibration, the slits were closed and the sample was kept in darkness.

When the absorbance of the solutions used for fluorescence measurements exceeded 0.1 at the wavelength of excitation, a correction for self-absorption of incident and emitted light was applied to the emission intensities. In order to be appropriate for the geometry of the fluorescence instrument used, the correction was empirically determined by obtaining the fluorescence intensity of TNS in absolute ethanol at the wavelength of maximal emission as a function of TNS concentration. This relation was linear until self-absorption became significant. A correction factor for each TNS concentration was calculated from the ratio of the intensity from the extrapolated linear portion of the graph to the observed intensity.

Absorption spectra were obtained on a Cary Model 14B recording spectrophotometer.

Thin film dialysis experiments were done as described by Craig (1967). The solvent was 0.1 M acetic acid, and the temperature was 27 ± 1°. TNS was detected in the dialysate by its absorbance at 315 nm. Since the observed rate of escape of a solute in thin film dialysis is proportional to the concentration of free solute molecules inside the membrane, it is possible to calculate the fraction bound to a nondialyzing or slowly dialyzing component by the decrease in dialysis rate. From the 50% escape times ($t_{1/2}$) of TNS alone and in the presence of various peptides, it was possible to calculate the initial free TNS concentration for each TNS–peptide solution using the equation $S_{free} = [(t_{1/2})_0/(t_{1/2})]S_{total}$, where S_{free} is the initial free TNS concentration, S_{total} is the initial total TNS concentration, and $(t_{1/2})_0$ and $(t_{1/2})$ are the 50% escape times of TNS in the absence and presence of a peptide, respectively.

The porosity of the dialysis membrane was adjusted to give maximum discrimination between TNS and the peptides. During the time intervals required for 50% escape of TNS (less than 15 min) less than 10% of any peptide escaped. In the calculations presented here, this slow escape of the free peptide and peptide–TNS complex has been neglected.

Results

Binding and Fluorescence of TNS with Peptides. The binding of TNS to various peptides was studied by the technique of thin film dialysis. Figure 2 shows some typical escape curves obtained by this method, and Table I summarizes the binding results. The escape curves of TNS both in the presence and absence of any peptide were linear. At higher concentrations they were curved as expected in the presence of tyrocidine B and linear tyrocidine B where a higher fraction is bound. The peptides themselves passed through the membrane much more slowly than TNS; the 50% escape time for bacitracin A, for example, was 110 min. The escape curves of the tyrocidines showed the curvature characteristic of concentration-dependent associating systems (Burachik *et al.*, 1970; Stewart *et al.*, 1970).

When solutions containing TNS and a peptide were dialyzed, the rate of escape of TNS was sometimes slower than the rate for TNS in the absence of the peptide, due to binding

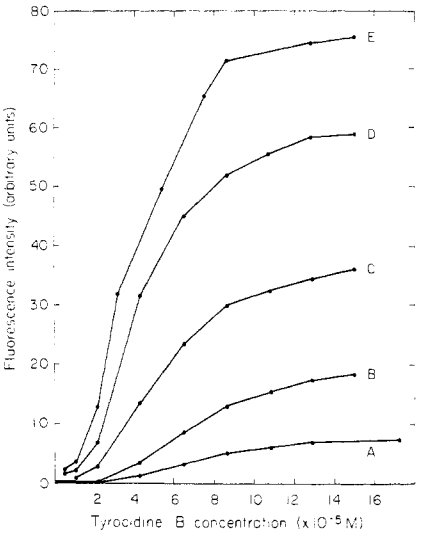

FIGURE 3: Dependence of TNS fluorescence intensity upon tyrocidine B concentration. TNS concentrations were A, 1.0×10^{-6} M; B, 2.5×10^{-6} M; C, 5.0×10^{-6} M; D, 7.5×10^{-6} M; E, 1.0×10^{-5} M.

TABLE I: Binding and Fluorescence Enhancement of TNS with Various Peptides.[a]

Peptide	50% Escape Time of TNS in Presence of Peptide (min)	Approximate Fraction of TNS Bound to Peptide	Fluorescence Enhancement of TNS Obsd in Presence of Peptide
None	5.5		
Tyrocidine A	13.0	0.58	250-fold
Tyrocidine B	12.0	0.54	250-fold
Tyrocidine C	10.5	0.48	200-fold
Linear tyrocidine B	9.0	0.38	None
Succinyl-tyrocidine B	5.5	0	None
Gramicidin S-A	8.5	0.36	None
Bacitracin A	7.5	0.26	None
Bradykinin	6.5	0.10	None

[a] TNS and peptide concentrations were 5×10^{-5} M in 0.1 N acetic acid. Tyrocidines A, B, and C and succinyltyrocidine B were aggregated at these concentrations; the other peptides were monomeric. Fluorescence enhancement is the increase in TNS fluorescence at 440 nm in the presence of the peptide compared to the fluorescence of TNS alone in 0.1 N acetic acid.

of a fraction of the TNS to the much slower dialyzing peptide. TNS was found to bind appreciably to the three aggregated tyrocidines, linear tyrocidine B, gramicidin S-A, and bacitracin A, and more weakly to bradykinin. The latter four peptides are known to be monomeric in the solvent and concentration used, so TNS was presumably binding to monomers of these peptides. There was no decrease in the dialysis rate of TNS in the presence of aggregated succinyltyrocidine B, indicating that TNS did not bind to this peptide.

The enhancement of TNS fluorescence intensity in the presence of these peptides did not correlate with binding as determined by dialysis (Table I). A large fluorescence enhancement was observed only for aggregated tyrocidine B and the closely related tyrocidines A and C. Little or no enhancement was observed with gramicidin S-A, bacitracin A, linear tyrocidine B or bradykinin, to which TNS binds, or with *N*-succinyltyrocidine B, to which TNS does not bind.

The difference between the associating and nonassociating peptides with relation to the TNS binding is further shown by a concentration study. Thus the apparent association constant of TNS with linear tyrocidine B was calculated from Table I to be 2×10^4 at a molar concentration level of 5.0×10^{-5} for both TNS and the peptide at 25°. At a 15-fold higher concentration level the apparent association constant was found to be essentially unchanged. On the other hand the apparent association constant of tyrocidine B–TNS at the lower concentration was 5×10^4 but 2×10^5 at the higher concentration level.

Further Studies of TNS Fluorescence with Tyrocidine B. Since appreciable fluorescence enhancement of TNS was observed only with the tyrocidines, we investigated the fluorescence characteristics of TNS in the presence of tyrocidine B more fully in order to determine the factors which caused fluorescence enhancement in this case.

The fluorescence intensity of TNS in 0.1 N acetic acid solutions of tyrocidine B was enhanced about 400-fold over that of TNS alone for a solution containing 10^{-5} M TNS and 10^{-4} M tyrocidine B. The quantum yield of TNS in the presence of a large excess of tyrocidine B, where a very high percentage or all of the TNS is bound, was determined by comparing the emission intensity in this case with the intensity of TNS alone at the same concentration in absolute ethanol. From the known quantum yield of TNS in ethanol (McClure and Edelman, 1966), the quantum yield of TNS in the presence of tyrocidine B was calculated to be about 0.33.

The dialysis experiments described above clearly indicated that TNS bound to tyrocidine B aggregates. To determine whether aggregation of the peptide was necessary for fluorescence enhancement, the experiments in Figure 3 were performed. These showed that enhancement of TNS fluorescence in the presence of tyrocidine B did not occur until the tyrocidine B concentration exceeded a minimum value. This behavior, very similar to what is observed when dyes of various kinds are used to determine the critical micelle concentrations of detergents (Shinoda *et al.*, 1963), correlates well with the fact that an apparent critical micelle concentration also exists for tyrocidine B. Ultracentrifugation studies (Laiken, 1970) have shown that aggregation does not begin until peptide concentration reaches a minimum value. The sharp, S-shaped rise in TNS fluorescence which occurs over a narrow peptide concentration range implies that tyrocidine B aggregation is a prerequisite for TNS fluorescence enhancement.

Besides the enhancement of fluorescence, a change in the wavelength of the emission maximum also occurred in TNS–tyrocidine B mixtures. Furthermore, the exact wavelength of maximal emission was dependent upon the extent of aggregation of tyrocidine B, which was varied by changing the temperature, adding NaCl to the solution, or increasing the concentration of tyrocidine B (Williams, 1968). In Figure 4 is shown the wavelength of maximal emission and fluorescence intensity of TNS–tyrocidine B solutions as a function of NaCl concentration. The increasing aggregation of tyrocidine B as the NaCl concentration increased resulted in shorter

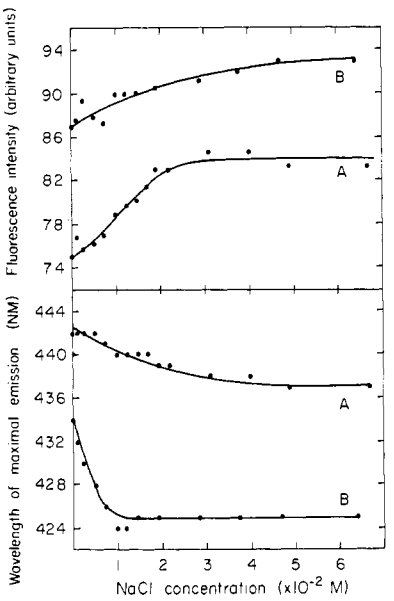

FIGURE 4: Dependence of TNS fluorescence intensity and wavelength of maximal emission in the presence of tyrocidine B upon sodium chloride concentration: A, 5.0×10^{-5} M tyrocidine B, 1.0×10^{-5} M TNS; B, 1.0×10^{-4} M tyrocidine B, 1.0×10^{-5} M TNS.

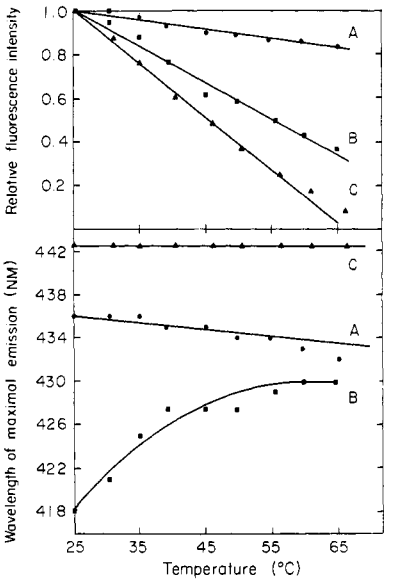

FIGURE 6: Dependence of TNS fluorescence intensity and wavelength of maximal emission upon temperature: A, 2.0×10^{-6} M TNS in ethanol; B, 2.0×10^{-4} M tyrocidine B, 5.0×10^{-6} M TNS in 0.1 N acetic acid; C, 5.0×10^{-5} M tyrocidine B, 1.0×10^{-5} M TNS in 0.1 N acetic acid.

wavelengths of emission and slightly increased fluorescence intensity. Addition of NaCl had no effect on the fluorescence of TNS alone in 0.1 N acetic acid. Shorter wavelengths of emission were also observed if aggregation was increased by increasing the tyrocidine B concentration (Figure 5). On the other hand, if aggregation was decreased by raising the temperature, the wavelength of maximal emission became longer (Figure 6) provided that the tyrocidine B concentration was high enough to give a short wavelength emission maximum at 25°. If the emission maximum at 25° was already near the upper limit for TNS–tyrocidine B solutions, no increase occurred as the temperature was raised. In either case, however, the fluorescence intensity decreased greatly with increasing temperature. For TNS alone in ethanol, increasing temperature leads to shorter emission wavelengths. These results indicate that the emission maximum of TNS as well as the emission intensity are sensitive to the aggregation state of tyrocidine B.

The absorption spectrum of TNS was perturbed when TNS was bound to either aggregated tyrocidine B or monomeric linear tyrocidine B (Figure 7). However, the spectral changes were different in the two cases. The changes in the presence of tyrocidine B were similar to those observed when TNS is dissolved in organic solvents (McClure and Edelman, 1966). When bound to linear tyrocidine B, the changes in the TNS spectrum were much less marked. The absorption spectra suggest that, even though TNS binds to both tyrocidine B and linear tyrocidine B, the environments at the binding sites of the two peptides are quite distinct. By analogy with the absorption changes which occur in organic solvents, TNS seems to be in a more hydrophobic environment when bound to aggregated tyrocidine B.

Fluorescence titrations of tyrocidine B with TNS are shown in Figure 8. Two unusual features should be noted. First, the initial portions of the curves are S-shaped rather than

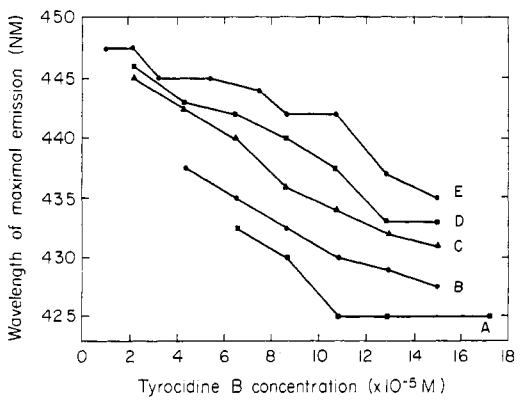

FIGURE 5: Dependence of the wavelength of maximal emission of TNS upon tyrocidine B concentration. TNS concentrations were: A, 1.0×10^{-6} M; B, 2.5×10^{-6} M; C, 5.0×10^{-6} M; D, 7.5×10^{-6} M; E, 1.0×10^{-5} M.

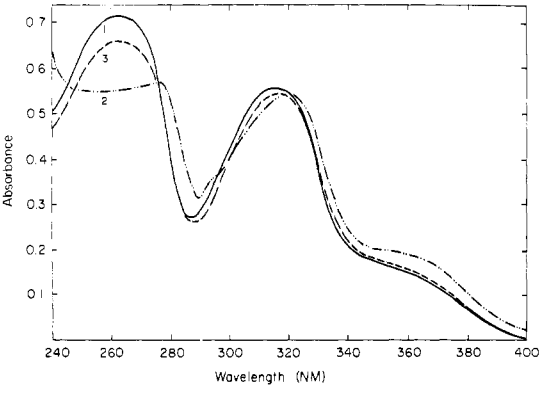

FIGURE 7: Absorption spectra of TNS. 1, 3.0×10^{-5} M TNS *vs.* solvent; 2, 3.0×10^{-5} M TNS in the presence of 1.1×10^{-4} M tyrocidine B *vs.* 1.1×10^{-4} tyrocidine B; 3, 3.0×10^{-5} M TNS in the presence of 1.0×10^{-4} M linear tyrocidine B *vs.* 1.0×10^{-4} M linear tyrocidine B. The solvent was 0.1 N acetic acid.

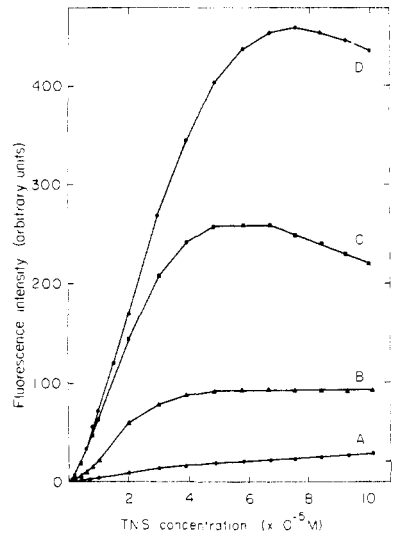

FIGURE 8: Fluorescence titrations of TNS with tyrocidine B. Tyrocidine B concentrations were: A, 1.1×10^{-5} M; B, 2.7×10^{-5} M; C, 5.4×10^{-5} M; D, 8.1×10^{-5} M.

linear, suggesting that there could be cooperative effects in the TNS–tyrocidine B interaction; second, at higher tyrocidine levels, the fluorescence intensity first rises and then declines as more TNS is added. Maximum fluorescence intensity occurs at a probe to peptide ratio of about one. These titration curves indicate that the association of TNS with tyrocidine B is complex. The fluorescence titation data did not fit a simple binding scheme such as that given by the Scatchard equation (Scatchard, 1949). Hence, it was not possible to obtain stoichiometries or association constants from the fluorescence data.

Discussion

Requirements for Binding and Fluorescence Enhancement of TNS with Peptides. Although the fluorescence enhancement of naphthalenesulfonate dyes such as TNS is thought to be caused by an environment of decreased polarity and perhaps increased rigidity (Turner and Brand, 1968; Penzer, 1972; Ainsworth and Flanagan, 1969), there is evidence that the actual binding of such dyes may require more than hydrophobic interactions (Laurence, 1952; Flanagan and Ainsworth, 1968). Our results indicate that binding of TNS without fluorescence enhancement does occur, as with linear tyrocidine B, bacitracin A, gramicidin S-A, and bradykinin. The dialysis experiments showed that TNS binds to these peptides, but virtually no fluorescence enhancement was observed. As pointed out before, none of these peptides self-associates. Succinyltyrocidine B, on the other hand, does aggregate, but TNS does not bind to it. A third situation is illustrated by the tyrocidines, which bind TNS and cause fluorescence enhancement. All of these observations may be explained by assuming that two separate processes are involved in binding and fluorescence enhancement of TNS with these peptides. The first process is ionic interaction of the sulfonate group of TNS with a positively charged residue of the peptide, such as the δ-amino group of ornithine in tyrocidine B, leading to binding of TNS to the peptide. This process results in binding but not fluorescence enhancement. The second process is the positioning of the aromatic system of TNS in an appropriate environment for fluorescence enhancement. This second process requires that the peptide self-associate to form a hydrophobic region where TNS may additionally interact, perhaps resulting in stronger binding through hydrophobic forces. With linear tyrocidine B, gramicidin S-A, bacitracin A, and bradykinin, the first process, ionic binding of TNS, can occur, but since there is no peptide aggregation, a suitable environment for TNS fluorescence enhancement is not formed. With succinyltyrocidine B, in which the δ-amino group of ornithine is succinylated, ionic attraction between TNS and the peptide cannot occur; in fact, there is now electrostatic repulsion between the two molecules. Thus, even though the peptide is highly aggregated, no fluorescence enhancement is seen.

These two processes are also reflected in the absorption spectra of TNS. Ionic attraction between TNS and linear tyrocidine B causes only small changes in the TNS spectrum, but binding to aggregated tyrocidine B, presumably through additional hydrophobic interactions, causes large perturbations in the spectrum.

TNS Fluorescence in the Presence of Tyrocidine B. DESCRIPTION OF TYROCIDINE B AGGREGATION. From several chemical and physicochemical studies, the conclusion has been reached that hydrophobic interactions are the primary driving force for aggregation of tyrocidine B (Laiken, 1970). Organic solvents, such as alcohols, carboxylic acids, dioxane, dimethyl sulfoxide, and pyridine, which are hydrophobic bond-breaking reagents, diminish or prevent aggregation (Williams, 1968; Stern *et al.*, 1969). Succinylation of the ornithine δ-amino group, methylation of the tyrosine phenolic hydroxyl, or complete hydrogenation of the phenylalanines of tyrocidine A does not prevent aggregation (Ruttenberg *et al.*, 1966). Ultracentrifugation studies of tyrocidine B in 30% acetic acid–0.1 M sodium chloride have revealed that, at low to moderate peptide concentrations, the association is adequately described by assuming that at a critical concentration, n peptide monomers come together to form an n-mer; subsequently, as the concentration of n-mers increases, j of these associate to form an nj-mer ($n = 8$ and $j = 3$ in this solvent at 20°). The ΔS of n-mer formation is positive, which is consistent with hydrophobic interaction (Laiken, 1970). In 30% acetic acid–0.1 M sodium chloride at higher peptide concentrations, and in solvents which are more favorable for aggregation, such as the one used in the studies reported here, very large aggregates form, perhaps by a continuation of the process of merging smaller aggregates. Thus it appears that the aggregation behavior of tyrocidine B is similar to micelle formation of detergents, and the conclusion has been drawn that tyrocidine B self-associates by hydrophobic forces to form micelle-like aggregates.

TNS FLUORESCENCE IS SENSITIVE TO THE EXTENT OF TYROCIDINE B AGGREGATION. When TNS or related molecules are bound to proteins, their fluorescence spectra show characteristic wavelength shifts, and quantum yield increases from the probe molecules in water (Weber and Laurence, 1954; McClure and Edelman, 1966; Stryer, 1968). In TNS–tyrocidine B solutions, however, the emission maximum and the fluorescence intensity are dependent upon other factors, such as sodium chloride concentration, tyrocidine B concentration, and temperature. Since these factors are known to cause changes in the extent of aggregation of tyrocidine B (Burachik *et al.*, 1970), it is reasonable to conclude that these fluorescence characteristics of TNS are sensitive to the aggregation state of the peptide, which indicates that the environment of bound TNS changes as the extent of aggregation changes.

The changes in emission maximum shown in Figure 5 are similar to changes observed for TNS and related compounds

n various organic solvents (McClure and Edelman, 1966; ıtryer, 1965). In an extensive study of the emission charcteristics of TNS and related compounds in many solvents, urner and Brand (1968) found that the emission maximum vas a function of the empirical solvent polarity scale suggested y Kosower (1958). Turner and Brand (1968) concluded hat the emission maximum of probe molecules bound to roteins could be used to estimate the polarity of the binding ite. If changing polarity is the cause of the wavelength shift f TNS emission in the presence of tyrocidine B, then it would ppear that the area of the tyrocidine B aggregate to which NS is bound exhibits a continuous change in polarity hroughout at least a part of the aggregation range. In this espect, the TNS binding site formed by aggregated tyrocidine B simulates organic solvent–water mixtures.

COMPARISONS WITH OTHER MICELLAR SYSTEMS AND SMALL OLECULES. Because of the evidence indicating that tyrocidine B aggregation is similar to micelle formation, it is interesting o compare our results with others for the binding of ANS to etergent and phospholipid micelles (Flanagan and Ainsvorth, 1968; Rubalcava *et al.*, 1969; Vanderkooi and Marnosi, 1969; Wallach *et al.*, 1970; Feinstein *et al.*, 1970). NS shows fluorescence enhancement in the presence of ositively charged and neutral micelles, but not in the presence f negatively charged micelles unless the ionic strength is fficiently high. This indicates the importance of charge nteractions in probe fluorescence enhancement. At cetyltrimethylammonium bromide and Triton X-100 concentraions above their respective critical micelle concentrations, ubalcava *et al.* (1969) found that data from fluorescence itrations with ANS indicated simple binding behavior, and Vallach *et al.* (1970) obtained similar results for ANS bound o lecithin or lysolecithin micelles. Our fluorescence data for NS and tyrocidine B do not indicate simple binding beavior. This difference may be due to the fact that detergents uch as cetyltrimethylammonium bromide form micelles ontaining a fairly specific number of individual molecules. olutions of these detergents consist primarily of monomers nd one size of micelle over a wide range of total detergent oncentration (Shinoda *et al.*, 1963). Tyrocidine B solutions, n the other hand, probably contain significant amounts of ggregates of many sizes (under our conditions of extensive ggregation). If the fluorescence characteristics of TNS and imilar probes are sensitive to micelle size, then complex luorescence titrations are to be expected with tyrocidine B.

Another example of fluorescent probe interaction with small olecules is the detection of complex formation between onophorous antibiotics and cations by enhancement of ANS r TNS fluorescence (Feinstein and Felsenfeld, 1971). In the ase of valinomycin, it was found that the emission maximum f ANS was dependent on KCl concentration. Fluorescence itrations gave linear plots of fluorescence *vs.* fluorescence/ NS concentration at two KCl levels. However, it was not lear whether distinct ternary complexes formed among alinomycin, cation, and ANS, or whether the probe was nteracting in some less specific way with cation–antibiotic ggregates. Feinstein and Felsenfeld (1971) also reported hat ANS fluorescence was enhanced in the presence of the yclic peptide antibiotic alamethicin, with a concomitant blue hift of the emission maximum, even in the absence of salt. ubsequent addition of salts led to increased fluorescence and further blue shift of the emission. These results appear to be imilar to our data for TNS and tyrocidine B and perhaps ndicate association.

TNS INFLUENCES TYROCIDINE B AGGREGATION. The possibility that a probe molecule may modify or interfere in some way with the phenomenon it is designed to probe must always be considered. In our experiments such modification was apparent. TNS is an ionic species, and tyrocidine B solubility is known to depend upon salt concentration (Williams, 1968). It was observed that TNS concentrations greater than about 10^{-4} M were very effective in salting out tyrocidine B. It is reasonable to presume that TNS, at concentrations below 10^{-4} M, also increased tyrocidine B aggregation.

In addition, there was some time dependence in the fluorescence spectra obtained from TNS–tyrocidine B solutions. Tyrocidine B is known to show time-dependent aggregation changes by itself (Williams, 1968); this may be the cause of the time-dependent fluorescence changes. Typically, such fluorescence changes consisted of small wavelength shifts toward the blue, and small decreases in fluorescence intensity. All fluorescence measurements were done on solutions prepared immediately beforehand, in order to eliminate as much as possible this time dependency.

Structural Model of TNS–Tyrocidine B Interaction. Tyrocidine B contains the pentapeptide unit which is found in gramicidin S-A. Both are cyclic decapeptides, and circular dichroism (Laiken, 1970) and preliminary nuclear magnetic resonance data (Stern, 1970) suggest that the conformations of the pentapeptide unit of the two peptides are similar. We have built a Corey–Pauling–Koltun (CPK) molecular model of tyrocidine B utilizing the backbone structure of gramicidin S-A, which was determined by nuclear magnetic resonance (Stern *et al.*, 1968). This model reveals several interesting features of the molecule. The ornithine side chain is constrained to extend from one side of the plane formed by the backbone ring. All the other side chains are either in the plane of the ring, or below it, except that of D-phenylalanine between leucine and proline. This side chain, according to the CPK model, may be either in the plane of the ring or above it, on the ornithine side. The presence of a second D-phenylalanine between asparagine and tryptophan ensures that this side chain extends from the plane of the ring opposite the ornithine side chain. Thus, the model reveals a distinct dichotomy between the two sides of the ring formed by the peptide backbone: on one side is the charged ornithine chain and perhaps one phenylalanine benzene ring, while on the other is a large cluster of hydrophobic side chains. This structure helps to explain the micelle-like behavior of the tyrocidines. In analogy with fatty acids and detergents, the ornithine δ-amino group may play the role of the polar head group. The other side of the peptide ring comprises the hydrophobic "tail" of the molecule. The two processes, which we have suggested may be involved in fluorescence enhancement when TNS interacts with tyrocidine B, can be given a plausible molecular basis from an examination of the tyrocidine B CPK model. Ionic binding of TNS to the tyrocidine B ornithine could occur with the aromatic system of TNS still fully exposed to solvent molecules. As additional peptide molecules came together to form an aggregate, the aromatic system of TNS could be partially tucked under the hydrophobic side of the peptide ring, causing an enhancement of fluorescence.

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Physical Studies on Proinsulin. A Comparison of the Titration Behavior of the Tyrosine Residues in Insulin and Proinsulin†

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ABSTRACT: The titration behavior of the tyrosine residues in proinsulin and insulin has been determined. The tyrosines in proinsulin titrate in the same manner as those in insulin. The titration behavior of these residues in both proteins is a function of protein concentration and thus the association state of the protein. In the absence of zinc, the tyrosine ionization is not time dependent, while in the presence of zinc a time-dependent tyrosine ionization is observed. In addition, the presence of zinc, which binds to both proteins and forms metal–protein complexes of each, decreases the amount of tyrosine ionized at equilibrium. The tyrosines affected are tentatively identified based on the X-ray crystal structure (Blundell, T. L., Dodson, G. G., Dodson, E., Hodgkin, D. C., and Vijayan, M. (1971), *Progr. Hormone Res. 27*, 1). The equivalency in the results for the two proteins lends further support to our proposal that the insulin moiety in proinsulin has the same conformation as insulin itself (Frank, B. H., and Veros, A. J. (1968), *Biochem Biophys. Res. Commun. 32*, 155).

We have been examining the physical and chemical properties of proinsulin in order to gain information on its conformation in solution. Among the properties of proinsulin that can be readily examined is the titration behavior of the tyrosine residues in the molecule. Proinsulin contains four tyrosines which are located in the same positions in the insulin portion of proinsulin as in insulin itself (Chance *et al.*, 1968). Therefore, a study of the titration behavior of the tyrosines should increase our knowledge of the state of these residues in the proinsulin molecule.

Upon examining the earlier studies on the titration of the tyrosine residues in insulin (Crammer and Neuberger, 1943; Tanford and Epstein, 1954a,b; Fredericq, 1954; Inada, 1961; Morris *et al.*, 1970), we concluded that the potential effects of factors such as ionic strength, disulfide bond cleavage, protein concentration, and zinc ion interaction had not been defined. Therefore, each of these factors has been carefully

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