Fluorescent Probe Study of Sulfonamide Binding to Povidone

C. H. HSIAO, H. J. RHODES †, and M. I. BLAKE ×

Abstract □ The possibility of using a fluorescent probe technique for the study of drug–povidone (I) interactions was investigated. 1-Anilino-8-naphthalenesulfonate (II) was used as the probe. Sulfanilamide, sulfacetamide, and sulfabenzamide were used as the binding competitors. Both sulfacetamide and sulfabenzamide decreased the fluorescence intensity of the I–II complex, while sulfanilamide increased the intensity. The fluorescence depression was greater with sulfabenzamide than with sulfacetamide, indicating that the former is more strongly bound to povidone. Since sulfabenzamide has a greater hydrophobic group (phenyl) than sulfacetamide (methyl), the binding of these sulfonamides to povidone is probably at least partially hydrophobic in nature. The enhanced fluorescence intensity of the I–II complex in the presence of sulfanilamide is believed to involve hydrogen bonding in which the sulfanilamide acts as an intermediary between I and II. Double reciprocal plots for the I–II and sulfonamide–I interactions were employed to obtain a binding constant of $3.2 \times 10^4\ M^{-1}$ for the I–II interaction. The association constants for sulfacetamide and sulfabenzamide were calculated by means of the Klotz equation to be 13.4 and 56.8 M^{-1}, respectively. The povidone molecules appear to have 1.28 binding sites for these compounds under the experimental conditions.

Keyphrases □ Sulfonamides, various—binding to povidone, fluorescent probe study □ Povidone—binding to various sulfonamides, fluorescent probe study □ Fluorescent probes—study of binding of various sulfonamides to povidone □ Binding—various sulfonamides to povidone, fluorescent probe study □ Antibacterials—various sulfonamides, binding to povidone, fluorescent probe study

The interaction of povidone (I) with drugs is of theoretical interest and some practical importance inasmuch as it may influence their availability, efficacy, and transport. The fluorescence probe technique is used widely to study the interaction between drugs and macromolecules. The concept of a fluorescent probe was first introduced in 1952 (1). Fluorescent probes are defined (2) as compounds whose spectral behavior (such as fluorescence, excitation, emission, and quantum yield) is influenced by their physical and chemical environment.

In some cases, the fluorescence properties of both ligand molecules and polymers may be changed by interactions (3–5). Some fluorescent compounds become quenched whereas others are only fluorescent when bound (6). Certain dyes, referred to as fluorescent probes, fluoresce weakly in aqueous solutions but fluoresce strongly when bound to proteins (1) or other polymers (7). They have been used for the indirect determination of the protein binding parameters of other drugs by competitive binding (8–12). The large increase in fluorescence emission and hypsochromic shift in the presence of protein has been attributed to the binding of the probe at hydrophobic sites (13, 14).

The fluorescent probe 1-anilino-8-naphthalenesulfonate (II) also fluoresces strongly when bound to I (7). The basis of this investigation was to explore the possibility of using this fluorescent probe technique to study the interaction of I with the sulfonamide drugs sulfanilamide, sulfacetamide, and sulfabenzamide.

EXPERIMENTAL

Materials—Povidone[1] (I), pharmaceutical grade, average molecular weight 40,000, was purified by passing its aqueous solution (5%) through a mixed-bed ion-exchange resin[2], then dialyzed against distilled water in dialysis tubing[3], and finally lyophilized. The fluorescent probe 1-anilino-8-naphthalenesulfonate (II) was recrystallized twice from distilled water.

Sulfanilamide[1], sulfacetamide[4], and sulfabenzamide[5] were used without further purification. Phosphate buffer, pH 7.4, was prepared according to a literature method (15). Other chemicals and solvents were reagent grade and were used without further purification.

Apparatus—All fluorescence measurements were made on a spectrophotofluorometer[6].

Methods—The binding of the probe, II, to I was determined by measuring the increase in fluorescence following the titration of the I solution with the probe. The fluorescence intensity was measured on the fluorescence spectrophotometer with excitation at 365 nm and emission at 480 nm. Solutions of I were prepared in pH 7.4 phosphate buffer. Aliquots, 3 ml of low ($2.5 \times 10^{-5}\ M$) and high (6.25×10^{-4} and $12.5 \times 10^{-4}\ M$) concentrations in a 1-cm cell, were titrated by successive additions of 2 μl of $6 \times 10^{-3}\ M$ II, delivered from a microsyringe[7]. Experiments were carried out at room temperature (21 ± 2°).

Titrations of the low concentration I solution were repeated in the presence of $2 \times 10^{-2}\ M$ sulfanilamide, sulfacetamide, and sulfabenzamide, individually. The drugs were added to the solution prior to titration. All titrations were run in triplicate.

RESULTS AND DISCUSSION

The fluorometric titration results are shown in Fig. 1. In the absence of I, the fluorescence of II was not significant (curve a); but when I was added, the intensity was greatly enhanced (curves b and c). At the two high I concentrations (6.25×10^{-4} and $12.5 \times 10^{-4}\ M$), the titration gave essentially identical fluorescence intensity (curve c), indicating that all II added was fully bound under these conditions. At low I concentrations, II was only partially bound (curve b).

The fraction of II bound (X) is usually determined according to:

$$X = \frac{F_p - F_0}{F_b - F_0} \qquad \text{(Eq. 1)}$$

where F_p and F_0 are the fluorescence intensities of a given concentration of II in a solution of low I concentration and in a solution without I, respectively; and F_b is the fluorescence intensity of the same concentration of II fully bound to a high concentration of I. Such treatment yields true values of X, provided the fluorescence intensity of the bound species, I–II, is a linear function of the concentration. However, the linearity of F_b with the concentration of the I–II complex can only be realized when the absorbance of I–II at the exciting wavelength is low (<0.02) (16). This can be seen from the fluorescence–absorbance relationship equation (17):

$$F_b - F_0 = \phi I_0 \left[2.3A - \frac{(2.3A)^2}{2!} + \frac{(2.3A)^3}{3!} - \frac{(2.3A)^4}{4!} + \ldots \right] \qquad \text{(Eq. 2)}$$

where ϕ is the quantum yield of the emitting species, I_0 is the intensity of the exciting radiation, and A is the absorbance. When the absorbance is low, the higher power terms in the equation become negligible. When

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Fisher Scientific Co., Pittsburgh, Pa.
[3] Scientific Products Co., McGraw Park, Ill.
[4] K & K Laboratories, Plainview, N.Y.
[5] Matheson, Coleman and Bell, Norwood, Ohio.
[6] Perkin-Elmer fluorescence spectrophotometer model 203, Perkin-Elmer Corp., Norwalk, Conn.
[7] Hamilton.

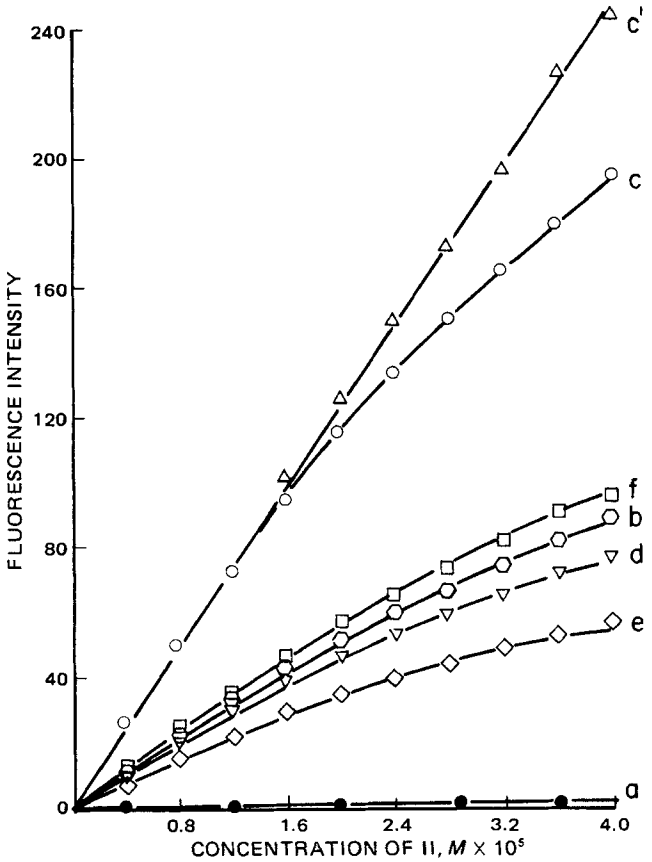

Figure 1—*Fluorescence titration curves of I with II in the absence of I (a), at high I concentrations (6.25×10^{-4} and 12.5×10^{-4} M) uncorrected and corrected for absorbance effect (c and c'), and at low I concentration (2.5×10^{-5} M) alone (b) and in the presence of 2×10^{-2} M sulfacetamide (d), 2×10^{-2} M sulfabenzamide (e), and 2×10^{-2} M sulfanilamide (f), individually.*

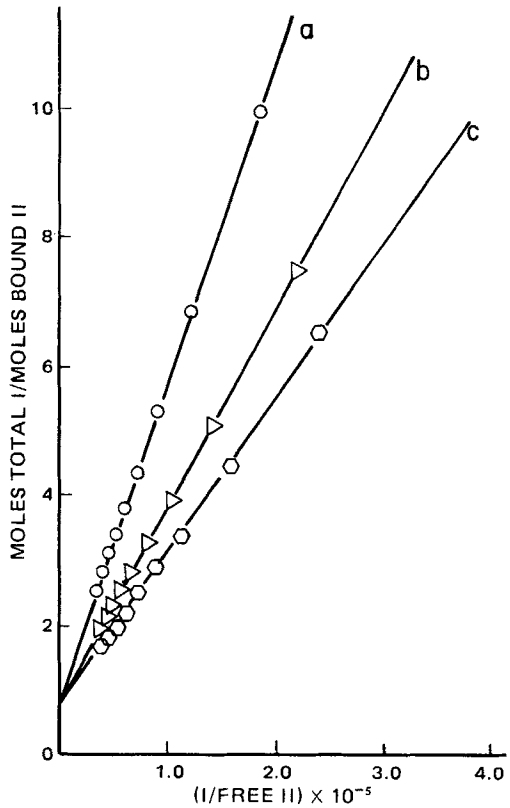

Figure 2—*Double reciprocal plot of the binding of II alone (a) or in the presence of 2×10^{-2} M sulfacetamide (b) or sulfabenzamide (c) to 0.1% I in pH 7.4 phosphate buffer at room temperature.*

the absorbance is high, the second or even the third term must be considered.

At the excitation wavelength, 365 nm, the molar absorptivity for the I–II complex was 5.9×10^3 liters/mole (II) cm. Therefore, at a II concentration greater than 3.4×10^{-5} M ($A = 0.02$), the deviation from linearity will be greater than 2%. It is then necessary to take into account the second term, $(1.63A)^2$, for the titration with the I concentration. Curve c in Fig. 1 was plotted using the observed values of $F_b - F_0$. After the correction to the observed fluorescence intensities was applied, a straight-line plot (line c' in Fig. 1) was obtained, verifying that the deviation from linearity of curve c was indeed due to the absorbance effect.

When the titrations are carried out at low I concentration (curve b), II is only partially bound, and the fluorescence intensity of bound II is approximately a linear function of its concentration. In other words, the absorbance, A, is so small that the second term in Eq. 2 is negligible. Thus, the readings from curve b can be compared directly to the corresponding readings on curve c to obtain the fraction of II bound using Eq. 1. The fluorescence intensity at any point on curve b can be considered as arising from the bound ligand concentration giving the same observed intensity on curve c. From these values, one can also calculate the fraction of II bound as:

$$X = \frac{[\text{II}]_c}{[\text{II}]_b} \qquad \text{(Eq. 3)}$$

where $[\text{II}]_b$ is the concentration of II corresponding to curve b, and $[\text{II}]_c$ is the concentration of II giving the same observed fluorescence intensity in curve c.

Figure 1 shows the titration results of the fluorescence intensity of the I–II complex in the presence of sulfacetamide (curve d) and sulfabenzamide (curve e). Since no change of the pH or the emission maxima was observed, the decrease in fluorescence intensity of the I–II complex could only be attributed to the competition between II and the sulfonamides for the binding sites. The difference in fluorescence intensities with and without sulfonamides is related to the binding of drug and is used to calculate the binding constant for the sulfonamide–I complex.

The fluorescence depression was greater with sulfabenzamide than with sulfacetamide, indicating that the former was more strongly bound to I. Since sulfabenzamide has a greater hydrophobic group (phenyl) than sulfacetamide (methyl), the binding of sulfonamides to I is probably at least partially hydrophobic in nature.

In contrast, the fluorescence intensity of the I–II complex was enhanced in the presence of sulfanilamide (curve f, Fig. 1). One possible explanation for this unexpected phenomenon is that sulfanilamide enhances the I–II binding through some mechanism since I–sulfanilamide or II–sulfanilamide did not show any significant amount of fluorescence at the wavelength employed. Dollery *et al.* (18) observed that chlorothiazide could increase fourfold the binding of the ganglionic blocking agent pempidine (1,2,2,6,6-pentamethylpiperidine) to albumin. It seems likely that the anionic chlorothiazide acts as an intermediary between the albumin and the cationic pempidine. Similarly, sulfanilamide may act, through hydrogen bonding, as an intermediary between I and II.

The binding parameters of the I–II interaction can be obtained by a double reciprocal plot constructed according to:

$$\frac{1}{r} = \left(\frac{1}{N} + \frac{1}{NK}\right)\frac{1}{A} \qquad \text{(Eq. 4)}$$

where r is the average number of moles of II bound per mole of I = X ($[\text{II}]/[\text{I}]$), N is the number of binding sites, K is the binding association constant, and A is the concentration of free II = $(1 - X)[\text{II}]$.

Figure 2 shows the results of the double reciprocal plot for the I–II interaction (curve a) and for sulfonamides–I interactions (curves b and c). The straight line with a common ordinate intercept indicates the competitive nature of the binding interaction (13). Based on the graphs, the binding constant for the I–II interaction was 3.2×10^4 M^{-1}.

The association constants for sulfacetamide and sulfabenzamide can be calculated according to the Klotz equation (19):

$$K_b = \left(\frac{N[P_0]KA - K[A][PA] - [PA]}{CK[A] - [P_0]K[A] + K[A][PA] + [PA]}\right)\frac{K[A]}{[PA]} \qquad \text{(Eq. 5)}$$

where K_b is the association constant for the competitor, K is the association constant for the probe, A is the concentration of free probe, PA is the concentration of bound probe, N is the number of binding sites, P_0 is the total polymer concentration, and C is the total competitor concentration. By means of this equation, the association constants for sul-

facetamide and sulfabenzamide were estimated to be 13.4 and 56.8 M^{-1}, respectively. The I molecules appear to have 1.28 binding sites for these compounds under the described conditions.

In conclusion, the fluorescent probe technique can provide a simple, sensitive, and rapid determination of the extent and nature of binding between sulfonamides and I. The technique, however, may not be applicable for some drugs such as sulfanilamide.

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Rate and Proposed Mechanism of Anhydrotetracycline Epimerization in Acid Solution

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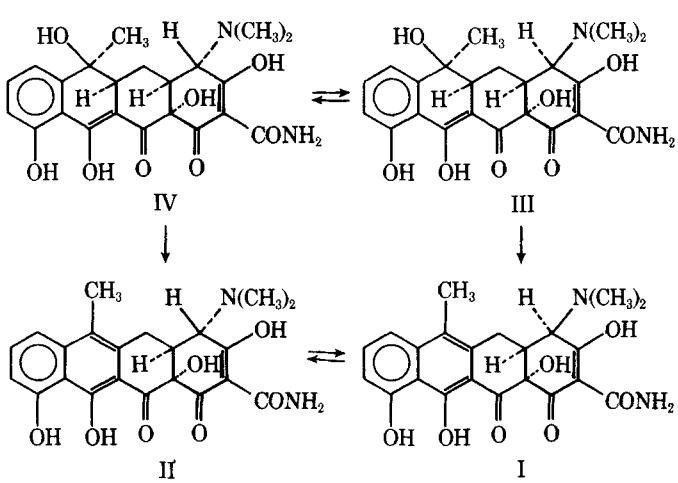
Abstract □ The pathway through which the toxic tetracycline degradation product epianhydrotetracycline forms in solution was studied using high-performance liquid chromatography and circular dichroism, taking advantage of the large difference in ellipticity between the reactant and the product at 285 nm. The epimerization of anhydrotetracycline followed a reversible first-order process, and both analytical methods yielded the same rate constants. The rate constants indicate that anhydrotetracycline epimerization is faster than tetracycline epimerization. The equilibrium favored anhydrotetracycline, and the activation energies for the forward and reverse rates were almost the same as those for tetracycline epimerization. The epimerization was catalyzed by phosphate. Activation energies in 0.1 and 1 *M* phosphate were essentially the same. The equilibrium constants for both anhydrotetracycline and tetracycline favored the natural configuration rather than the epi series. Possible rationalization based on conformational and hydrogen bonding effects is presented.

Keyphrases □ Anhydrotetracycline—epimerization, kinetics and mechanism in acid solution, high-performance liquid chromatographic and circular dichroism study □ Tetracycline degradation—kinetics and mechanism of anhydrotetracycline epimerization in acid solution, high-performance liquid chromatographic and circular dichroism study □ Epimerization—anhydrotetracycline in acid solution, kinetics and mechanism, high-performance liquid chromatographic and circular dichroism study □ High-performance liquid chromatography—study of epimerization of anhydrotetracycline in acid solution □ Circular dichroism—study of epimerization of anhydrotetracycline in acid solution □ Antibacterials—tetracycline degradation, epimerization of anhydrotetracycline in acid solution

Among the degradative pathways of the tetracyclines, those leading to the toxic epianhydrotetracycline by epimerization and dehydration are of special concern. Formation of epianhydrotetracycline (I) from tetracycline (IV) occurs along two routes (Scheme I). Epimerization of tetracycline occurs *via* a reversible first-order process between pH 3 and 5 (1–4). Dehydration of tetracycline to anhydrotetracycline (II) and of epitetracycline (III) to epianhydrotetracycline occurs *via* a pseudo-first-order process at low pH (5–7).

The epimerization of anhydrotetracycline is the only reaction of Scheme I whose kinetics have not yet been reported, probably because of a lack of a suitable method of



Scheme I