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Tetracycline Binding to Bovine Serum Albumin Studied by Fluorescent Techniques

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Abstract □ Binding of demeclocycline and oxytetracycline to bovine serum albumin was studied using fluorescent methods. The mechanism of binding for tetracyclines is shown to be hydrophobic. The number of binding sites and the equilibrium constants were calculated over a range of protein concentrations. Two strong binding sites, at or near the tryptophan residues of bovine serum albumin, were found for both tetracyclines. The equilibrium constants for tetracyclines increased with increasing protein concentration, and the number of binding sites on the protein decreased with increasing protein concentration. These findings suggest the possibility of a sharing of one tetracycline molecule by more than one protein molecule at relatively high protein concentrations.

Keyphrases □ Demeclocycline—mechanism of binding to bovine serum albumin studied using fluorescent techniques □ Oxytetracycline—mechanism of binding to bovine serum albumin studied using fluorescent techniques □ Tetracyclines, demeclocycline and oxytetracycline—binding to bovine serum albumin, fluorescent techniques □ Serum protein binding—demeclocycline and oxytetracycline, fluorescent techniques □ Fluorescent techniques, probe and quenching titration—used to study mechanism of binding of demeclocycline and oxytetracycline to bovine serum albumin

The binding of drugs by plasma proteins has been recognized as an important factor in drug availability, drug efficacy, and drug transport for many years (1). Many experimental techniques have been employed to study drug–protein interactions (2–4). The fluorescence probe technique (5, 6) has recently been employed to study the mechanism of drug–protein binding and the nature of binding sites. This technique has also been very useful in biochemical research related to binding (7–9). The number of probes available at this time is limited; to explore the use of probes in binding studies more fully, it is necessary to search for new probe compounds. Therefore, the fluorescence studies of tetracyclines and bovine serum albumin were carried out in this laboratory.

Tetracyclines are fluorescent compounds with the excitation and emission maxima in the range of 390 and 520 nm., respectively. Ibsen and Urist (10) reported that the excitation maximum of tetracyclines varies with the presence of metal ions. They found that increasing metal concentration produced a bathochromic shift, indicating the formation of metal complexes. The quantum yields of several tetracyclines in various solvents and of their protein mixtures were reported by Popov *et al.* (11, 12). In a recent paper (13), they also studied the interaction of tetracyclines with bovine serum albumin using a fluorescence technique. The increase of the fluorescence of tetracyclines accompanied by an auxochromic shift of the fluorescence maxima in the presence of protein and in nonpolar solvents suggests that the tetracyclines exhibit the main features of fluorescence probes.

Fluorescence quenching titration is another sensitive method for the study of drug–protein molecular interactions. The procedure has been applied to many systems (14–16) and was best described by Chignell (17). It was found that the native fluorescence of bovine serum albumin was quenched by most of the tetracyclines. To examine the possible use of tetracyclines as probe compounds for drug–protein binding studies, the nature of binding of demeclocycline and oxytetracycline to bovine serum albumin was studied by both methods.

EXPERIMENTAL

Materials—Demeclocycline hydrochloride[1], oxytetracycline hydrochloride[2], and crystalline bovine serum albumin[3] were obtained from commercial sources. Methanol[4] was spectroscopic grade, and all other chemicals were reagent grade or of special purity. All chemicals were used without further purification.

Apparatus—Fluorescence measurements were made with a spectrophotofluorometer[5] equipped with a 150-w. xenon lamp and 1P21 photomultiplier tube. The relative fluorescence intensities were recorded directly from fluorometer readings.

Fluorescent Titration—The fluorescence and quenching titrations were performed manually with microsyringes[6]. Two milliliters of

[1] Lot 48151-915, American Cyanamid Co.
[2] Lot 48175-939, American Cyanamid Co.
[3] Control No. 1322, Nutritional Biochemicals Corp.
[4] Matheson, Coleman & Bell, Norwood, Ohio.
[5] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[6] Hamilton.

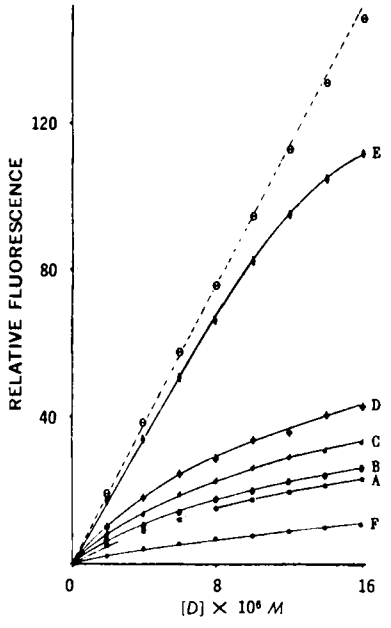

Figure 1—*Plots of demeclocycline fluorescence intensities as a function of demeclocycline concentrations,* [D], *at (A) 5.095 × 10⁻⁶ M, (B) 7.99 × 10⁻⁶ M, (C) 13.61 × 10⁻⁶ M, (D) 19.55 × 10⁻⁶ M, and (E) 136.1 × 10⁻⁶ M bovine serum albumin, and (F) without bovine serum albumin. The values of* I_b *are plotted in dashed line.*

each protein solution was titrated with successive additions of 2 μl. of drug solution (2×10^{-3} *M*) in methanol. Methanol did not affect the binding of the drug in the concentration range used. The protein solutions were prepared in pH 7.4 phosphate buffer. Concentrations of protein solutions were determined by measuring the absorbance at 280 nm. and using $a = 0.66$ (18); the molecular weight of 69,000 was used to determine the molar concentrations. All titrations were carried out at 25 ± 0.1°. The temperature was controlled by the use of thermostated cell compartments.

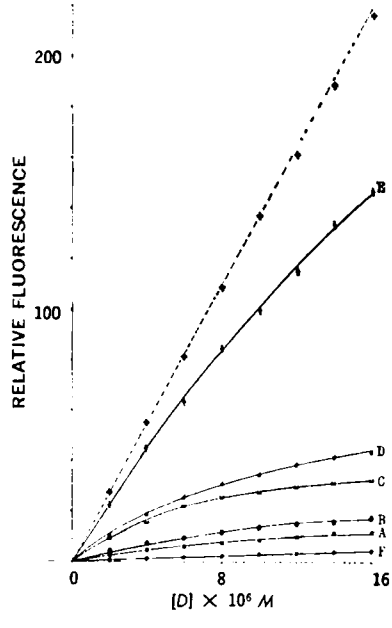

Figure 2—*Plots of oxytetracycline fluorescence intensities as function of oxytetracycline concentrations,* [D], *at (A) 1.436 × 10⁻⁶ M, (B) 7.436 × 10⁻⁶ M, (C) 14.18 × 10⁻⁶ M, (D) 21.62 × 10⁻⁶ M, and (E) 143.6 × 10⁻⁶ M bovine serum albumin, and (F) without bovine serum albumin. The values of* I_b *are plotted in dashed line.*

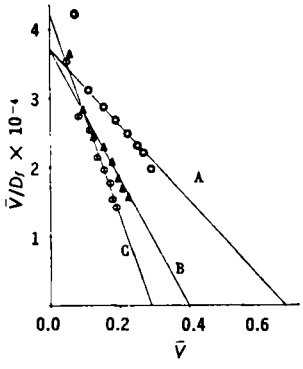

Figure 3—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the demeclocycline-bovine serum albumin system in the demeclocycline concentration range of 2–16 × 10⁻⁶ M at (A) 5.095 × 10⁻⁶ M, (B) 7.99 × 10⁻⁶ M, and (C) 13.61 × 10⁻⁶ M bovine serum albumin.*

Treatment of Data—The fluorescence enhancement of the drug, upon addition to bovine serum albumin, was determined, and these data were used to calculate the binding constants for the albumin–drug complexes. The fraction of bound drug, X, was calculated from the following equation (7):

$$X = (I - I_f)/(I_b - I_f) \qquad \text{(Eq. 1)}$$

where I and I_f refer to the fluorescence intensities of a given concentration of drug in solutions of low protein concentration and in solutions without protein, respectively; I_b refers to the fluorescence intensity of the same concentration of drug in solutions of high protein concentration, which gives the fluorescence intensity of the drug in the presence of excess binding sites. In this study, the fluorescence titrations were carried out at several different protein concentrations, and the values of I_b were taken to be the extrapolated values of the plots of $1/I$ *versus* $1/[P]$, where $[P]$ represents the concentration of protein. After the value X is found for each point along the titration curve, the Scatchard equation (19) may be applied to determine the association constant of the drug:

$$\bar{V}/D_f = nK_a - \bar{V}K_a \qquad \text{(Eq. 2)}$$

where $\bar{V}$ is the number of moles of bound drug per mole of protein, D_f is the concentration of free drug, n is the number of binding sites on the protein, and K_a is the association constant of drug. The $\bar{V}$ is determined by multiplying the value for X by the ratio of the total drug concentration to the total protein concentration in solution. When $\bar{V}/D_f$ is plotted against $\bar{V}$, a straight line is obtained with a slope equal to $-K_a$. The ordinate and abscissa intercepts of this line give nK_a and n, respectively. The Scatchard equation assumes that the intrinsic association constants for all binding sites are equal.

In the fluorescence quenching titration method, titrations are made over a range of protein concentrations. The intensities, expressed as a percentage of the initial fluorescence of the protein, are then plotted as a function of the drug–protein ratio. Above a certain critical protein concentration, the titration curves are superimpos-

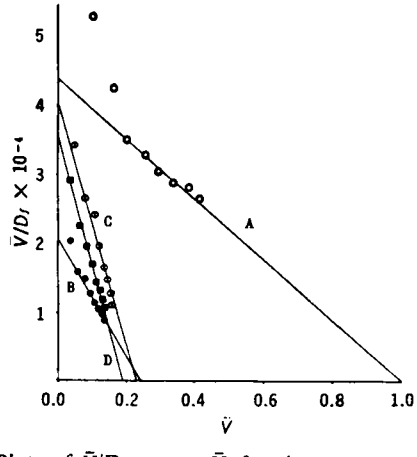

Figure 4—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the oxytetracycline-bovine serum albumin system in the oxytetracycline concentration range of 2–16 × 10⁻⁶ M at (A) 1.436 × 10⁻⁶ M, (B) 7.436 × 10⁻⁶ M, (C) 14.18 × 10⁻⁶ M, and (D) 21.62 × 10⁻⁶ M bovine serum albumin.*

Table I—Equilibrium Constants and n Values for Binding of Demeclocycline and Oxytetracycline to Bovine Serum Albumin

$[P] \times 10^6$ M	$[D] \times 10^6$ M	K_a	n
Demeclocycline–Bovine Serum Albumin System			
5.095	2–16	5.456×10^4	0.68
7.99	2–16	9.200×10^4	0.40
13.61	2–16	14.450×10^4	0.29
1.377[a]	8–80	0.781×10^4	2.00
11.02[a]	8–80	0.858×10^4	2.00
13.77[a]	8–80	0.858×10^4	2.00
27.54[a]	8–80	0.858×10^4	2.00
40.65[a]	8–80	1.130×10^4	1.96
68.87[a]	8–80	1.620×10^4	1.80
Oxytetracycline–Bovine Serum Albumin System			
1.436	2–16	4.390×10^4	1.00
7.436	2–16	8.360×10^4	0.24
14.180	2–16	17.320×10^4	0.23
21.620	2–16	18.740×10^4	0.19
1.377[a]	8–80	0.743×10^4	2.00
14.060[a]	8–80	0.775×10^4	2.00
25.340[a]	8–80	0.775×10^4	2.00
44.330[a]	8–80	0.950×10^4	1.79

[a] Calculated from quenching data.

able since all the drug is bound. The concentration of bound drug (D_b) for a solution containing the protein below this critical concentration can be calculated by the equation:

$$D_b = (D_t)(R_h)/(R_l) \qquad \text{(Eq. 3)}$$

where D_t is the total drug concentration of the solution, and R_h and R_l are the drug–protein ratios at the same intensity for high and low protein solutions, respectively. The results can then be graphically treated using the Scatchard equation.

RESULTS AND DISCUSSION

The excitation and emission maxima, λ_{ex} and λ_{em}, for demeclocycline and oxytetracycline were found to be 382 and 520 nm. and 375 and 515 nm., respectively, in the concentration range used in this study. In the presence of bovine serum albumin, an auxochromic shift of about 20 nm. for the emission maxima was observed for both tetracyclines. Increasing protein concentration also produced a bathochromic shift for the excitation maxima. The fluorescence titrations were carried out over a range of protein concentrations, and the excitation and emission maxima used for demeclocycline and oxytetracycline were 390 and 504 nm. and 384 and 490 nm., respectively. The fluorescence data of the demeclocycline–bovine serum albumin and the oxytetracycline–bovine serum albumin systems, in which the fluorescence intensities are plotted as a function of drug concentration, are shown in Figs. 1 and 2, respectively. The dashed lines represent the I_b values.

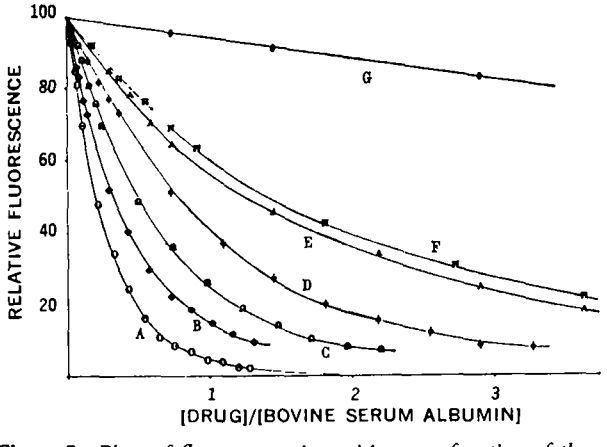

Figure 5—*Plots of fluorescence intensities as a function of the concentration ratios of demeclocycline and bovine serum albumin, where the bovine serum albumin concentrations for curves A, B, C, D, E, F, and G are 137.7 × 10⁻⁶ M, 68.87 × 10⁻⁶ M, 40.65 × 10⁻⁶ M, 27.54 × 10⁻⁶ M, 13.77 × 10⁻⁶ M, 11.02 × 10⁻⁶ M, and 1.377 × 10⁻⁶ M, respectively.*

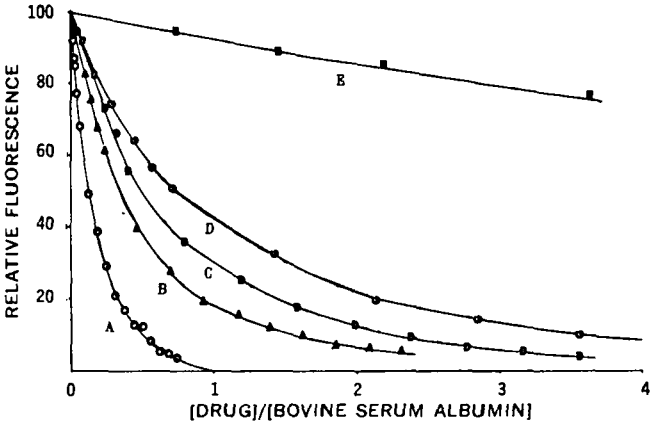

Figure 6—*Plots of fluorescence intensities as a function of the concentration ratios of oxytetracycline and bovine serum albumin where the bovine serum albumin concentrations for curves A, B, C, D, and E are 162.5 × 10⁻⁶ M, 44.33 × 10⁻⁶ M, 25.34 × 10⁻⁶ M, 14.06 × 10⁻⁶ M, and 1.377 × 10⁻⁶ M, respectively.*

The Scatchard plots for the binding of demeclocycline to bovine serum albumin are shown in Fig. 3. At different protein concentrations, the Scatchard plots gave straight lines corresponding to different n and K_a values. As shown in Table I, the equilibrium constant increased consistently with increasing protein concentration, whereas n decreased with increasing protein concentration. The same effect has also been observed in the binding of oxytetracycline to bovine serum albumin. The Scatchard plots of the oxytetracycline–bovine serum albumin system are shown in Fig. 4, with the corresponding n and K_a values listed in Table I.

The native fluorescence of bovine serum albumin was quenched by the addition of demeclocycline and oxytetracycline. The titration curves are shown in Figs. 5 and 6. The intensities, expressed as a percentage of the initial fluorescence of protein, are plotted as a function of the drug–protein ratio. The excitation and fluorescence maxima for all solutions were taken to be 285 and 354 nm., respectively. The concentration of bound drug and other parameters used for the Scatchard plot were calculated by the method previously described.

The plots of $\bar{V}/D_f$ *versus* $\bar{V}$ for the demeclocycline–bovine serum albumin system are shown in Fig. 7. Again, the equilibrium constant increased with increasing protein concentration, whereas the number of binding sites decreased with increasing protein concentration. The one exception is that a group of straight lines is superimposed

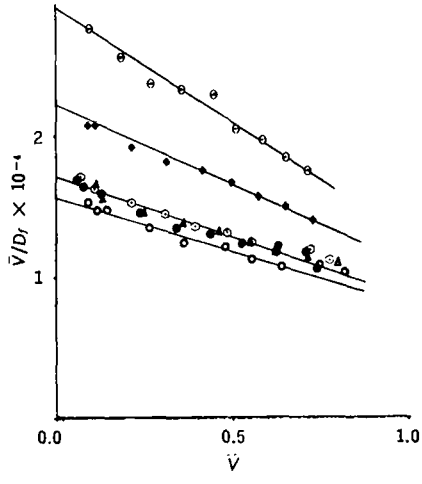

Figure 7—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the demeclocycline–bovine serum albumin system in the demeclocycline concentration range of 8–80 × 10⁻⁶ M at various bovine serum albumin concentrations:* ○, *1.377 × 10⁻⁶ M;* △, *11.02 × 10⁻⁶ M;* ○, *13.77 × 10⁻⁶ M;* ○, *27.54 × 10⁻⁶ M;* Φ, *40.65 × 10⁻⁶ M; and* ⊙, *68.87 × 10⁻⁶ M.*

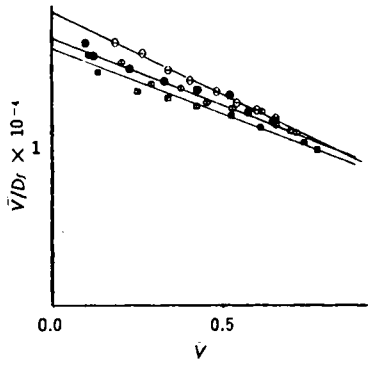

Figure 8—*Plots of* $\bar{V}/D_f$ versus $\bar{V}$ *for the oxytetracycline–bovine serum albumin system in the oxytetracycline concentration range of 8–80* $\times$ 10^{-6} M *at various bovine serum albumin concentrations:* ⊡, *1.377* $\times$ 10^{-6} M; ⊕, *14.06* $\times$ 10^{-6} M; ○, *25.34* $\times$ 10^{-6} M; *and* ⊙, *44.33* $\times$ 10^{-6} M.

upon each other in the protein concentration range of 11.02–27.54 $\times$ 10^{-6} *M*. Similar results were also found in the binding of tetracycline to bovine serum albumin. The values of n and K_a calculated from Figs. 7 and 8 are also shown in Table I; Fig. 8 shows the plots of $\bar{V}/D_f$ *versus* $\bar{V}$ for the binding of oxytetracycline to bovine serum albumin.

The binding information obtained from this study and the results recently reported by Popov *et al.* (13) show that the mechanism of binding for tetracyclines to bovine serum albumin is of a hydrophobic nature. The number of binding sites varies with varying tetracycline and protein concentrations, and the maximum number of strong or high affinity binding sites appears to be two. A quenching of tryptophan fluorescence, which is primarily responsible for the fluorescence of bovine serum albumin, by tetracyclines was also observed. Since bovine serum albumin is known to have two tryptophan binding sites per molecule (20), the quenching studies indicate that the binding sites are at or near the tryptophan residues. This is possible, since previous investigators (21, 22) showed that serum albumin has binding sites for small organic molecules which are located in the vicinity of the tryptophan residue. It is claimed that this is due to a clustering of hydrophobic apolar amino acids in the environment of tryptophan residues of these protein molecules.

The fact that the equilibrium constant is increasing with increasing protein concentration, together with the fact that n is concentration dependent, may suggest a sharing of one tetracycline molecule by more than one protein molecule in the binding. This observation is further substantiated by examination of Figs. 5 and 6, which show that at a certain drug–protein concentration ratio, the native fluorescence of protein is quenched more strongly at high protein concentration than at low protein concentration. In other words, the ratio of bound drug and total drug concentrations at high protein concentration is larger than that at low protein concentration.

The present studies show that the tetracyclines represent a class of compounds that can be used as probes for both techniques used in this investigation. The results also show that investigations at different protein concentrations are necessary to understand further the nature of binding.

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