# Binding Study of Tetracyclines to Human Serum Albumin Using Difference Spectrophotometry

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**Abstract** □ The binding of several tetracyclines to human serum albumin was studied using difference spectrophotometry and a spectrophotometric probe, 2-(4′-hydroxybenzeneazo)benzoic acid. Difference spectra observed for the interaction between the probe and human serum albumin were similar to probe–bovine serum albumin spectra but were less intense for a given concentration of probe and did not reach saturation as quickly. Difference spectra for the tetracyclines were dependent on the characteristics of the ring substituents. More hydrophobic substituents on the D and C rings tended to give more intense difference spectra, but charge-transfer complexing may also have been involved since methacycline with a methylene group in the 6-position showed the most intense spectra of the compounds studied. Solvent perturbation, pH, and urea studies tended to confirm that something other than hydrophobic binding of the tetracyclines was involved. Drug–probe displacement studies showed that methacycline gave the greatest probe displacement followed by doxycycline, chlortetracycline, oxytetracycline, and tetracycline. This order of displacement of the anionic probe indicates that both hydrophobic and charge-transfer binding are involved. Experiments with calcium ion and ethylenediaminetetraacetic acid showed that the difference spectra obtained with the tetracyclines and human serum albumin were not the result of metallic bridge–chelate formation.

**Keyphrases** □ Tetracyclines—binding to human serum albumin, related to ring substituents, difference spectrophotometry, spectrophotometric probe □ Albumin, human serum—binding to tetracyclines, related to ring substituents, difference spectrophotometry, spectrophotometric probe □ Difference spectrophotometry—study of binding of tetracyclines to human serum albumin □ Spectrophotometric probe—2-(4′-hydroxybenzeneazo)benzoic acid, study of binding of tetracyclines to human serum albumin

The interaction of drugs with plasma proteins has been the subject of many investigations (1). The antibiotics have received considerable attention because of the involvement of binding in events of pharmacological importance. Various mechanisms such as chelation, adsorption, and ionic and hydrophobic binding have been proposed for the binding of tetracyclines to serum albumins (2–5).

Recent studies established difference spectrophotometry as a powerful tool for detecting small changes in the environment of a chromophore (6) and for elucidating conformational changes of globular proteins in solutions (7). In a preceding article, the binding of sulfonylureas and phenothiazines to bovine serum albumin, using difference spectrophotometry and 2-(4′-hydroxybenzeneazo)benzoic acid as a probe, was reported (8). The purpose of the present study was to use 2-(4′-hydroxybenzeneazo)benzoic acid as a spectrophotometric probe with difference spectrophotometry to study the binding of selected tetracyclines to human serum albumin.

## EXPERIMENTAL

**Materials**—Human serum albumin[1] (crystalline, 4×), 2-(4′-hydroxybenzeneazo)benzoic acid[2], oxytetracycline hydrochloride[3], doxycycline hyclate[3], methacycline hydrochloride[3], chlortetracycline hydrochloride[4], and tetracycline hydrochloride[5] were used as obtained without further purification. Solvents were spectral grade, and all other chemicals were reagent grade.

**Apparatus**—Absorption spectra and difference absorbance measurements were made with a double-beam spectrophotometer[6] equipped with tandem cell holders.

**Absorbance Difference Titrations**—The absorbance difference titrations in the presence and absence of tetracyclines were carried out manually with microsyringes[7]. The 2-(4′-hydroxybenzeneazo)benzoic acid probe was dissolved in methanol at a concentration of $1 \times 10^{-2}$ *M*. The human serum albumin solutions were prepared in 0.05 *M* tromethamine buffer (pH 7.4). Concentrations of the albumin solutions were estimated by measuring the absorbance at 280 nm (9) and using $E^{1\%}_{1\,cm} = 5.30$; the molecular weight of 69,000 was used to calculate the molar concentrations.

Two 300-ml quantities of albumin solution were pipetted into two other cells and were placed in the reference and sample beams in such a manner that a buffer and a protein solution cell were in tandem in each beam. After running a baseline, the contents of the buffer solution cell in the reference beam and the protein solution cell in the sample beam were titrated with successive additions of 6 μl of probe solution. Absorbance differences then were measured.

All difference spectra were obtained by using a pair of split-compartment tandem cells of 4-mm light path except where otherwise noted. All absorbance difference measurements were made at ambient temperature.

**Data Treatment for Probe Studies**—The absorbance difference observed upon addition of the probe to human serum albumin was measured for each successive addition of the probe in the absence and presence of the competitive drugs at 484 nm. These data were used to calculate the fraction of bound probe, *X*, in a manner similar to that reported previously (8) using the following equation:

$$X = \frac{\Delta A_l}{\Delta A_h} \qquad \text{(Eq. 1)}$$

where $\Delta A_l$ and $\Delta A_h$ refer to the absorbance difference of a given concentration of probe in solutions of low and high protein concentration or to the absorbance difference in the absence and presence of excess sites, respectively.

To determine the value of $\Delta A_h$ for a given concentration of probe, absorbance difference titrations were carried out at several different protein concentrations and the values of $\Delta A_h$ were taken to be values extrapolated to the intercepts of plots of $1/\Delta A$ *versus* $1/[P]$, where $[P]$ represents the total concentration of protein.

After values of *X* were found for all points along the titration curve, the data were treated according to the Scatchard (10) equation:

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

where $\bar{V}$ is the number of moles of bound probe per mole of protein, $[D]$ is the concentration of free probe, *n* is the number of binding sites on the protein, and $K_a$ is the intrinsic association constant of the complex.

The competitive binding of tetracyclines was studied using 2-(4′-hydroxybenzeneazo)benzoic acid as an indicating probe. The binding of probe to albumin was determined in the presence and

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.

[2] Aldrich, Milwaukee, Wis.
[3] Pfizer, Brooklyn, N.Y.
[4] American Cyanamid Co., Pearl River, N.Y.
[5] Bristol Laboratories, Syracuse, N.Y.
[6] Cary 118c, Varian Associates, Palo Alto, Calif.
[7] Hamilton Co., Reno, Nev.

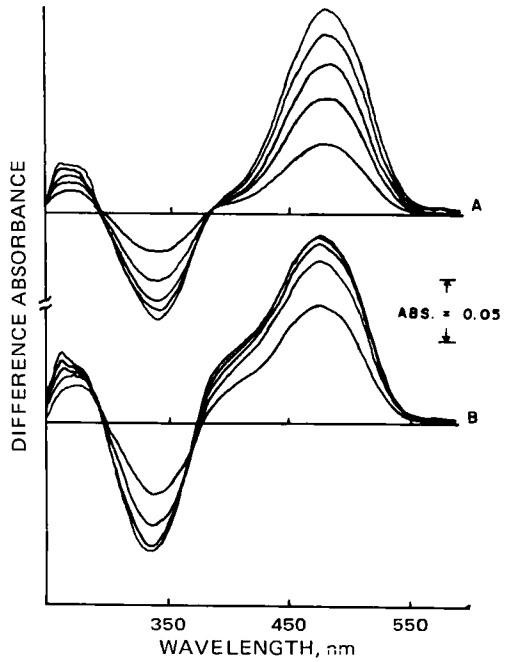



**Figure 1**—*Difference spectra of serum albumin solutions ($1.38 \times 10^{-5}$ M) with successive ($2 \times 10^{-5}$ M) increments of 2-(4'-hydroxybenzeneazo)benzoic acid in 0.05 M tromethamine buffer, pH 7.4. Absorbance difference increased with increasing probe concentration. (Tandem 10-mm light path cells were used.) Key: A, human serum albumin–probe system; and B, bovine serum albumin–probe system.*

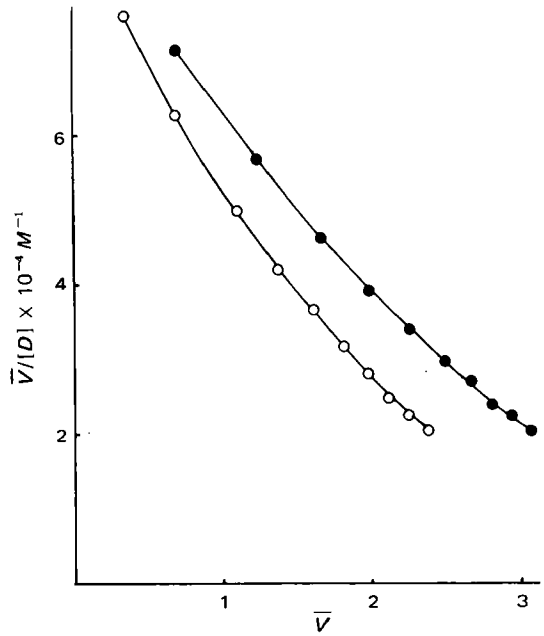



**Figure 2**—*Scatchard plots of 2-(4'-hydroxybenzeneazo)benzoic acid binding to human serum albumin in 0.05 M tromethamine buffer, pH 7.4. $\bar{V}$ = number of moles bound probe per mole of protein, and [D] = concentration of free probe. Key: ●, $1.36 \times 10^{-5}$ M human serum albumin; and ○, $3.45 \times 10^{-5}$ M human serum albumin.*

absence of competitive drugs by varying the concentration of probe at constant albumin and tetracycline concentrations. The binding constants of competitive drugs were calculated by using the Klotz *et al.* (11) equation:

$$K_b = \frac{n[P_t]K_a[D] - K_a[D][PD] - [PD]}{[B_t]K_a[D] - n[P_t]K_a[D] + K_a[D][PD] + [PD]} \times \frac{K_a[D]}{[PD]} \quad \text{(Eq. 3)}$$

where $K_b$ is the association constant for the competitor, $K_a$ is the association constant for the probe, $[D]$ is the concentration of free probe, $n$ is the number of binding sites, $[P_t]$ is the total concentration of protein, $[B_t]$ is the total concentration of competitor, and $[PD]$ is the concentration of bound probe.

## RESULTS AND DISCUSSION

The difference absorption spectra for the interaction of several concentrations of probe and human serum albumin in 0.05 *M* tromethamine buffer at pH 7.4 are presented and compared to the difference spectra of probe–bovine serum albumin in Fig. 1. The spectra were characterized by two positive peaks (484 and 262 nm) and one negative peak (345 nm) in the wavelength range of 250–600 nm. The difference spectra obtained for the interaction between human serum albumin and probe were similar to the difference spectra obtained for bovine serum albumin. However, difference spectra of the human serum albumin–probe system were less intense than the bovine serum albumin system and did not reach saturation as easily upon the increase in concentration of the probe.

Difference absorbance data, measured at 484 nm for human serum albumin–probe system and treated according to the method outlined under *Experimental*, are shown as Scatchard plots for two concentrations of albumin in Fig. 2. The Scatchard plots deviate from linearity. Such deviation has been considered to be due to the heterogeneity of the albumin with respect to its binding properties and was discussed elsewhere (8). It is sufficient to note here that binding parameters for the interaction between the probe and human serum albumin were concentration dependent. The greater probe binding capacity at low albumin concentrations may be due to a conformational change in the protein. Similar ob-

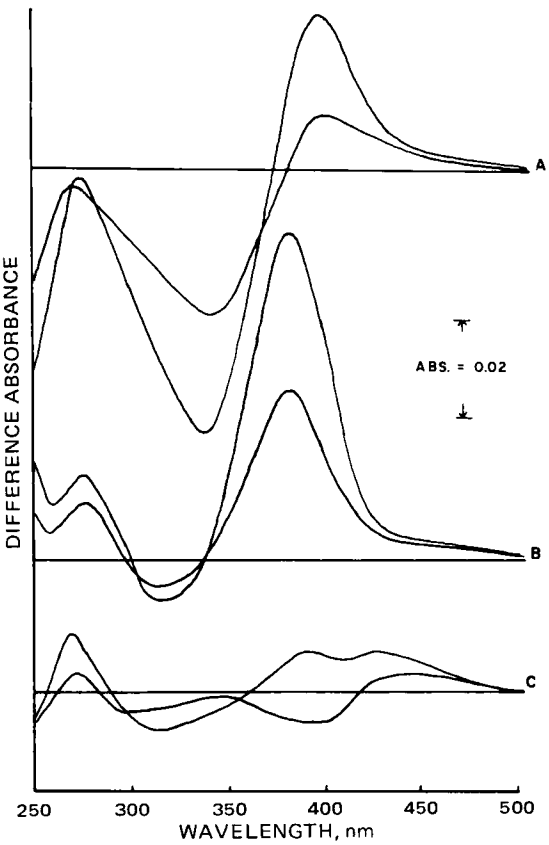



**Figure 3**—*Difference spectra of oxytetracyclines ($1.25 \times 10^{-4}$ and $2.5 \times 10^{-4}$ M) and human serum albumin ($1.38 \times 10^{-5}$ M) in 0.05 M tromethamine buffer, pH 7.4. Absorbance difference increased with the higher drug concentration. Key: A, methacycline; B, doxycycline; and C, oxytetracycline.*

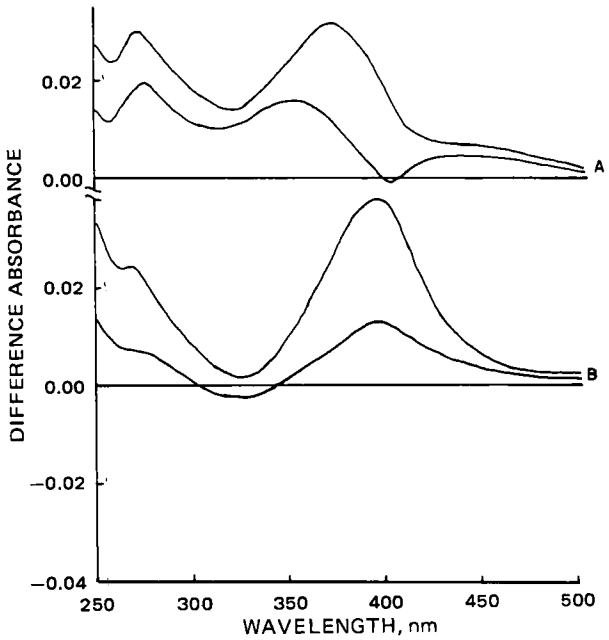



**Figure 4**—*Difference spectra of tetracyclines ($1.25 \times 10^{-4}$ and $2.5 \times 10^{-4}$ M) and human serum albumin ($1.38 \times 10^{-5}$ M) in 0.05 M tromethamine buffer, pH 7.4. Absorbance difference increased with higher drug concentration. Key: A, tetracycline; and B, chlortetracycline.*

servations and interpretations were made for the interaction between methyl red and albumin (12).

Figures 3 and 4 show the difference absorption spectra for the binding of tetracyclines and oxytetracyclines, respectively, to human serum albumin in tromethamine buffer at pH 7.4. The interactions between both groups (tetracyclines and oxytetracyclines) with human serum albumin consistently revealed at least

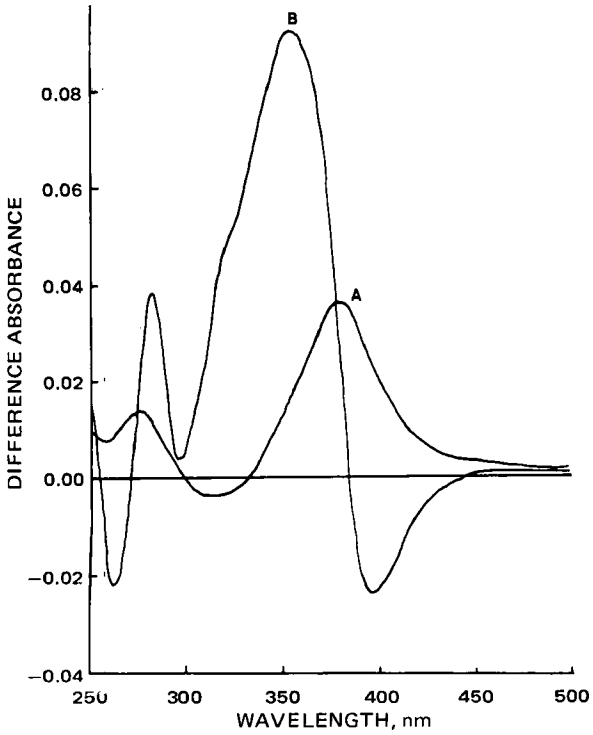



**Figure 5**—*Difference spectrum of doxycycline–human serum albumin and solvent perturbation spectrum of doxycycline in 20% dioxane. Key: A, doxycycline ($1.25 \times 10^{-4}$ M) and human serum albumin ($1.38 \times 10^{-5}$ M) in buffer* versus *buffer; and B, doxycycline ($1.25 \times 10^{-4}$ M) and 20% dioxane in buffer* versus *buffer.*

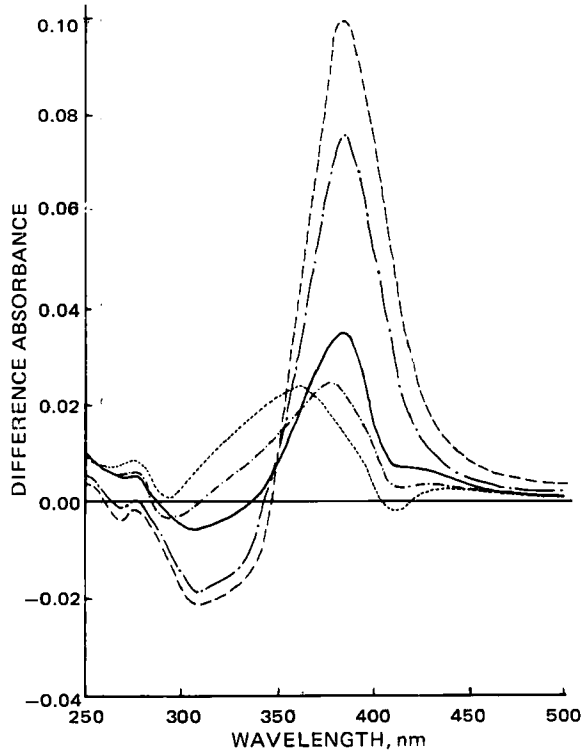



**Figure 6**—*Effect of pH on the difference spectrum of doxycycline ($8.33 \times 10^{-5}$ M) and human serum albumin ($1.38 \times 10^{-5}$ M). (Tandem 10-mm light path cells were used.) Key: – – –, pH 7.95; —·—, pH 7.70; ——, pH 7.40; —··—, pH 7.25; and ····, pH 7.05.*

two positive absorption peaks and one negative peak. The positions of the peaks were concentration dependent.

The increase in the intensity of the difference spectra for the interaction of drugs with human serum albumin depended on the characteristics of the ring substituent groups at various positions of the 2-naphthacenecarboxamide moiety, especially at the 4-, 5-, 6-, and 7- positions. The 7-chloro- and/or 6-deoxytetracyclines induced more intense difference spectra than other tetracyclines. Although this result was consistent with the hydrophobic nature of the interaction as suggested by Ma *et al.* (13) based on fluorescence studies, additional factors such as charge-transfer-type complexing also seem to have been involved. Such indications were the large red shift in the absorption maxima and/or the presence of a new absorption band at about 374–393 nm. Further support of the charge-transfer-type complexing was shown with methacycline with a methylene group at the 6-position. This compound had the most intense difference spectra of any of the compounds studied.

**Solvent Perturbation Spectra**—The generation of the difference spectra in the presence of protein may be attributed either to the perturbation of the drug's chromophores by a different environment at the protein binding sites or to the perturbation of the protein as the result of the binding or to a combination of both. In an attempt to characterize the nature of the difference spectra observed, a study of drugs in buffered 20% dioxane against buffer was performed. Figure 5 shows the interaction difference spectrum of doxycycline and human serum albumin as opposed to solvent perturbation spectra in 20% dioxane and buffer. Comparison of these difference spectra indicates some similarities in their patterns, which may indicate the existence of a nonpolar environment around the albumin binding sites. However, since deviations in the positions of the absorption bands existed between the spectra, the additional contribution from mechanisms other than the nonpolar environment and/or the perturbation of the protein *via* binding with drugs must also be involved.

**Effect of pH on Difference Spectra**—Figure 6 shows the effect of pH on difference spectra of the interaction between doxycycline and human serum albumin. As pH decreased (from 8 to 7), a slight blue shift along with a marked decrease of the intensity of the spectra was seen. The effect of pH on the difference spectra indicates that, although hydrophobic binding has some role, the

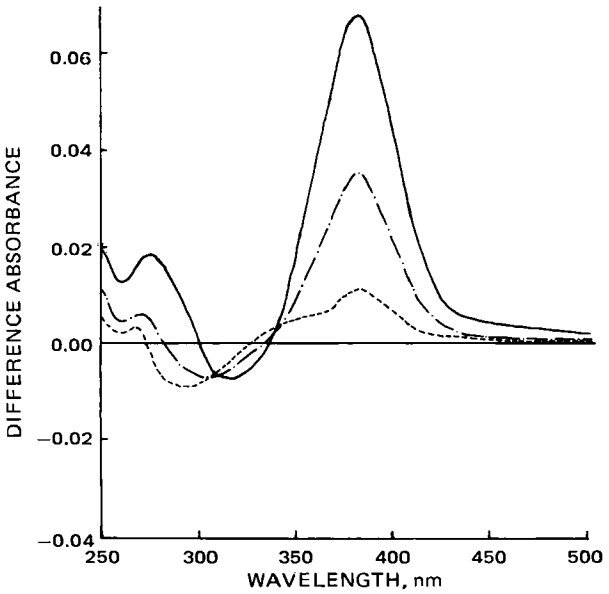



**Figure 7**—*Effect of urea on the difference spectrum of doxycycline (2.5 × $10^{-4}$ M) and human serum albumin (1.38 × $10^{-5}$ M). Key: ——, in the absence of urea; —·—·, in the presence of 2 M urea; and ----, in the presence of 4 M urea.*

ionic interaction cannot be ignored.

The ability of acidic groups on the doxycycline molecule to donate hydrogen was in order of tricarbonylmethane (pKa = 3.4), phenol diketone (pKa = 7.7), and the dimethylammonium group (pKa = 9.7) (14). Thus, neither the dimethylammonium group nor the tricarbonylmethane group underwent significant changes in the pH 7–8 range, and changes in the difference spectra in this range can be attributed to changes in the degree of ionization of the phenol diketone moiety.

As the pH was decreased from 7.95 to 7.7, good correlation was shown between the absorbance difference decrease (21% decrease) and the decrease in calculated amount of ionized phenol diketone

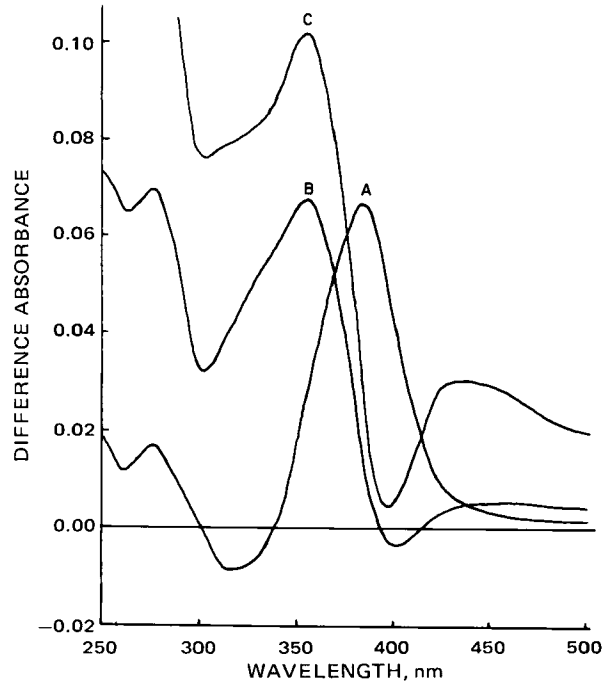



**Figure 8**—*Effect of calcium ion on difference spectrum of doxycycline (2.5 × $10^{-4}$ M) and human serum albumin (1.38 × $10^{-5}$ M). Key: A, in the absence of calcium ion; B, in the presence of 1.25 × $10^{-4}$ M $CaCl_2$; and C, in the presence of 2.5 × $10^{-4}$ M $CaCl_2$.*

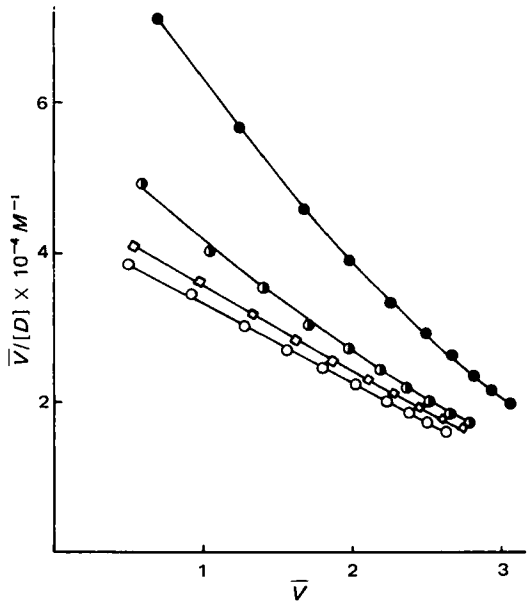



**Figure 9**—*Scatchard plots of 2-(4′-hydroxybenzeneazo)benzoic acid binding to human serum albumin in 0.05 M tromethamine buffer, pH 7.4. $\bar{V}$ = number of moles bound probe per mole of protein, and [D] = concentration of free probe. Key: ●, in the absence of drug; ◑, in the presence of 1.5 × $10^{-4}$ M oxytetracycline; □, in the presence of 1.5 × $10^{-4}$ M doxycycline; and ○, in the presence of 1.5 × $10^{-4}$ M methacycline.*

(22% calculated decrease). As the pH was further decreased to 7.25, a blue shift was observed in the spectrum and the correlation between absorbance difference change and ionized group concentration correlation was lessened, indicating that the proportion of unionized phenol diketone had increased sufficiently to contribute to the spectrum through hydrophobic interaction with the protein. A further decrease in pH to 7.05 caused little change in absorbance difference but resulted in a rather marked blue shift in the spectrum from increased hydrophobic bonding.

**Effect of Urea on Difference Spectra**—The effect of urea on the difference spectrum of the interaction between doxycycline

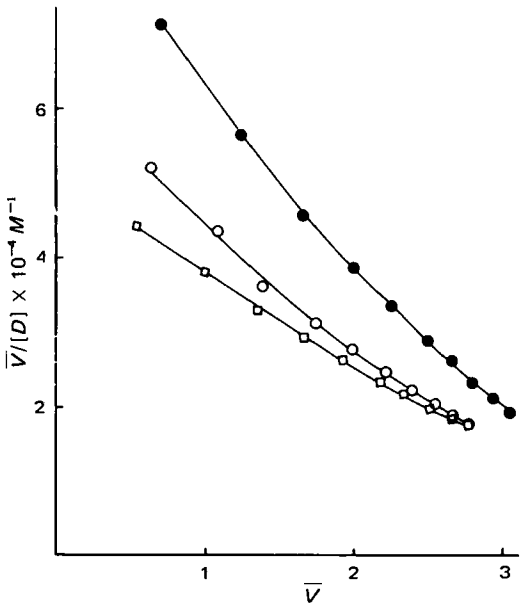
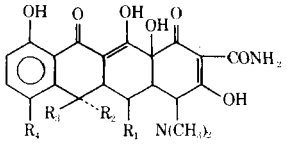



**Figure 10**—*Scatchard plots of 2-(4′-hydroxybenzeneazo)benzoic acid binding to human serum albumin in 0.05 M tromethamine buffer, pH 7.4. $\bar{V}$ = number of moles bound probe per mole of protein, and [D] = concentration of free probe. Key: ●, in the absence of drug; ○, in the presence of 1.5 × $10^{-4}$ M tetracycline; and □, in the presence of 1.5 × $10^{-4}$ M chlortetracycline.*

Table I—Association Constants of Selected Tetracyclines to Human Serum Albumin



| Generic Name | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Association Constant × $10^{-4}$ $M^{-1}$ |
|---|---|---|---|---|---|
| Oxytetracycline | OH | $CH_3$ | OH | H | 3.53 |
| Doxycycline | OH | $CH_3$ | H | H | 5.32 |
| Methacycline | OH | $=CH_2$ | — | H | 6.28 |
| Tetracycline | H | $CH_3$ | OH | H | 3.15[a] |
| Chlortetracycline | H | $CH_3$ | OH | Cl | 4.03 |

[a] Powis (17) reported two classes of binding sites for tetracycline on human serum albumin and calculated a binding constant of 4.38 × $10^4$ for the first class of sites (dialysis method).

and human serum albumin is shown in Fig. 7. Use of 2 *M* urea markedly decreased the absorption intensity of positive bands and caused a blue shift on the negative band. The fact that the difference spectrum for doxycycline–albumin interaction decreased significantly in the 2 *M* or higher urea-treated albumin strongly indicates that hydrophobic binding is not the only mode of the interaction and, as was concluded earlier, that additional factors must be involved. However, since many aspects of the action of urea on small molecule–protein interaction are still obscure, the conclusion that such observed change in the difference spectrum is due to: (*a*) conformational change of albumin (conversion of the N form to the F form and/or increase of levorotation) (15), (*b*) breakage of hydrogen bonds of the tetracycline–albumin complex (16), (*c*) true competition between tetracycline and urea for the same sites (11), or (*d*) a combination of these factors may be made only with ambiguity.

**Effect of Calcium Ion and Ethylenediaminetetraacetic Acid on Difference Spectra**—It is known that tetracycline–divalent ion complexes are capable of forming metal–bridge chelates with albumins (2). To examine whether such chelates are the cause of the observed difference spectra, ethylenediaminetetraacetic acid, calcium ion, and ethylenediaminetetraacetic acid plus calcium ion were added to the solutions of doxycycline and human serum albumin.

It was found that the addition of either chelating agent alone or an equilvalent amount of chelating agent and calcium ion had no effect on the difference spectra. However, the addition of calcium ion alone changed difference spectra considerably (Fig. 8). The difference spectra generated from solutions of tetracycline–human serum albumin obviously were not the result of a metal–bridge chelate formation. Similar results were reported for fluorescence spectra (5).

**Displacement of 2-(4′-Hydroxybenzeneazo)benzoic Acid by Tetracycline**—To have a better understanding of the functional groups and the nature of the binding force, the displacement of tetracyclines by the spectrophotometric probe was examined. The binding of probe to human serum albumin was determined in the presence and absence of tetracyclines by varying the probe concentration from 2 × $10^{-5}$ to 2 × $10^{-4}$ *M* at constant albumin (1.38 × $10^{-5}$ *M*) and tetracycline (1.5 × $10^{-4}$ *M*) concentrations.

The results of difference absorbance measurements at 484 nm in the presence and absence of each tetracycline are shown as Scatchard plots in Figs. 9 and 10. The binding constants calculated using the Klotz equation (11), with $n = 4$ and probe $K_a = 1.95 \times 10^{-4}\ M^{-1}$, are shown in Table I. Methacycline showed the greatest displacement of the probe followed by doxycycline, chlortetracycline, oxytetracycline, and tetracycline. The increased displacement ability of chlortetracycline compared to tetracycline and/or deoxytetracyclines (methacycline and doxycycline) compared to oxytetracycline may be attributed to the increased hydrophobicity of the molecules in the D and C ring region. The fact that tetracycline had a lesser affinity in comparison to the oxytetracyclines, along with the observed displacement of the anionic probe by these drugs, reinforces the earlier conclusion that hydrophobic binding is not the only mode of the interaction of tetracyclines with human serum albumin.

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