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Binding Study of Sulfonylureas and Phenothiazines to Bovine Serum Albumin Using Difference Spectrophotometry

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Abstract □ 2-(4′-Hydroxybenzeneazo)benzoic acid is a spectrophotometric probe which shows absorption spectrum changes upon binding to protein. Difference absorption spectra of this probe were used as an indirect measurement of the binding of selected sulfonylurea and phenothiazine drugs to bovine serum albumin. The results obtained using the spectrophotometric probe were similar to data obtained from other methods, especially fluorescent methods. Of the four sulfonylureas studied, tolbutamide showed the highest binding affinity, followed by glyburide, glipizide, and acetohexamide, in that order. The data collected for phenothiazine drugs indicated that chlorpromazine has the highest affinity, followed in order by trifluoperazine, perphenazine, fluphenazine, and promazine. Correlation of these results with chemical composition indicated that the interaction of phenothiazine drugs with bovine serum albumin was of a hydrophobic nature.

Keyphrases □ Sulfonylureas and phenothiazines—binding to bovine serum albumin, difference spectrophotometry using 2-(4′-hydroxybenzeneazo)benzoic acid □ Phenothiazines and sulfonylureas—binding to bovine serum albumin, difference spectrophotometry using 2-(4′-hydroxybenzeneazo)benzoic acid □ Spectrophotometry, difference—determination, binding of sulfonylureas and phenothiazines to bovine serum albumin using 2-(4′-hydroxybenzeneazo)benzoic acid □ 2-(4′-Hydroxybenzeneazo)benzoic acid—used to determine binding of sulfonylureas and phenothiazines to bovine serum albumin, difference spectrophotometry

The phenomena of drug–protein binding and of competitive binding of drugs for available protein sites have been the subjects of many investigations and have been reviewed (1). Various experimental procedures and analysis methods have been used to study drug–protein interactions. These include equilibrium dialysis (2), ultrafiltration (3), gel filtration (4), NMR rate measurements (5), and fluorescence techniques (6).

Compared to dialysis or ultrafiltration techniques, spectrophotometric and/or fluorescence probe techniques are capable of providing data similar to what could be obtained through dialysis or ultrafiltration studies but are less time consuming, simpler, and more reproducible. It also may be possible to estimate the nature of binding and binding sites from the chemical structure and spectral properties of the probes used (7).

In spite of their usefulness in protein binding studies, fluorescence probes cannot be used successfully in certain instances such as the lack of a fluorescence change upon binding and/or a change of fluorescence due to a mechanism other than binding (*e.g.*, photoxidation).

Although fluorescence probes have been explored in recent years, little information has been generated concerning spectrophotometric probes. In 1968, Moriguchi and coworkers (8–11) studied the binding of the 2-(4′-hydroxybenzeneazo)benzoic acid probe to bovine serum albumin. Recently, Nazareth *et al.* (12) used this probe as an agent that reflects the binding of L-thyroxine to serum albumin. However, in this study they employed ultrafiltration to separate the free probe from serum albumin to calculate the ratio of bound drug to protein.

The usefulness of spectrophotometric probes in the study of the binding of drugs to protein has not been adequately explored. In addition, certain procedures and methods of data treatment reported for fluorescence probes (13, 14) appear to be useful for the spectrophotometric probe technique. This study was made to explore the usefulness of 2-(4′-hydroxybenzeneazo)benzoic acid as a spectrophotometric probe in studying the interaction of sulfonylurea and phenothiazine drugs with bovine serum albumin and to define, if possible, the binding parameters of some of these drugs.

EXPERIMENTAL

Materials—Bovine serum albumin[1] (crystalline), 2-(4′-hydroxybenzeneazo)benzoic acid[2], tolbutamide[3], acetohexamide[4], glipizide[5], glyburide (glibenclamide)[3], chlorpromazine hydrochloride[6], trifluoperazine hydrochloride[6], promazine hydrochloride[7], perphenazine[8], and fluphenazine hydrochloride[8] were used as obtained without further purification. The solvents used were spec-

[1] Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Aldrich, Milwaukee, Wis.
[3] The Upjohn Co.
[4] Eli Lilly and Co.
[5] Instituto Carlo Erba Per Richerche Therapeutiche, Italy.
[6] Smith Kline and French Corp.
[7] Wyeth Laboratories.
[8] Schering Co.

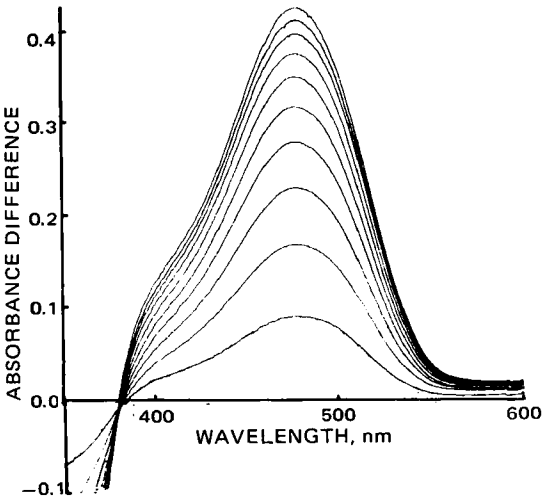


Figure 1—*Typical absorption difference spectra of bovine serum albumin solution (6.9 × 10^{-5} M) with successive increments of 2-(4′-hydroxybenzeneazo)benzoic acid.*

tral grade, and all other chemicals were reagent grade.

Apparatus—Absorption spectra and difference absorbance measurements were made with a double-beam spectrophotometer[9] equipped with tandem cell holders.

Absorbance Difference Titrations—The absorbance difference titrations in the absence and presence of drugs were carried out manually with microsyringes[10]. The probe was dissolved in methanol at a concentration of 1 × 10^{-2} *M*. The bovine serum albumin solutions were buffered at pH 7.4 with 0.05 *M* phosphate for sulfonylureas and 0.05 *M* tromethamine for phenothiazines.

Two 3.00-ml quantities of bovine serum albumin solution were pipetted into two cells and two 3.00-ml quantities of buffer solution were pipetted into two other cells; these cells were placed in the reference and sample beams in such a manner that a buffer cell 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 3 μl of probe solution and absorbance differences were measured.

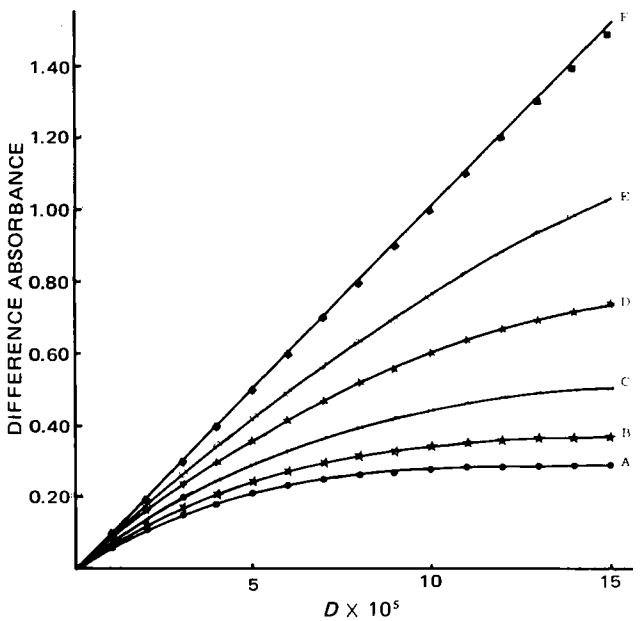


Figure 2—*Absorbance differences as a function of the 2-(4′-hydroxybenzeneazo)benzoic acid concentration, D, at various bovine serum albumin concentrations: A, 3.45 × 10^{-5} M; B, 4.6 × 10^{-5} M; C, 6.9 × 10^{-5} M; D, 13.8 × 10^{-5} M; and E, 55.2 × 10^{-5} M. The theoretical line (F) is based on calculated values of absorbance difference for high concentrations of bovine serum albumin.*

[9] Cary 118C, Varian Associates, Palo Alto, Calif.
[10] Hamilton Co., Reno, Nev.

Because of low solubility in water, all sulfonylureas were dissolved in 0.1 *N* NaOH solution with the exception of glyburide, which was dissolved in methanol. The addition of the microliter quantities of the sodium hydroxide solutions of the drugs did not change the final pH of the cell solutions more than 0.05 pH unit. The phenothiazine drugs were dissolved in tromethamine buffer with the exception of perphenazine, which was dissolved in methanol. All absorbance difference measurements were made at ambient temperature.

Data Treatment—The absorbance difference observed upon addition of the probe to bovine serum albumin was measured for each successive addition in the absence and presence of the competitive drugs at 482 nm. These data were used to calculate the fraction of bound probe, *X*, using the following equation:

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

where ΔA_l and ΔA_h refer to the absorbance difference of a given concentration of probe in solutions of low and high protein concentration, or the absorbance difference in the absence and presence of excess sites, respectively. To determine the value of ΔA_h for a given concentration of probe, absorbance difference titrations were carried out at several different protein concentrations and the values of ΔA_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 for the fraction of bound probe were found for all points along the titration curve, the data were treated according to the Scatchard (15) 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. In addition, plots of $\log \bar{V}/(n - \bar{V})[D]$ *versus* $\bar{V}$ were drawn (13).

The competitive binding of two different groups of drugs, sulfonylureas and phenothiazines, were studied using 2-(4′-hydroxybenzeneazo)benzoic acid as the indicating probe. The binding of the probe to bovine serum albumin was determined in the presence and absence of competitive drugs by varying the concentration of the probe at constant albumin and drug concentrations. The binding constants of competitive drugs were calculated by using the equation of Klotz *et al.* (16):

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

where:

K_b = association constant for competitor
K_a = association constant for probe
$[D]$ = concentration of free probe
$[PD]$ = concentration of bound probe
n = number of binding sites
$[P_t]$ = total concentration of protein
$[B_t]$ = total concentration of competitor

RESULTS AND DISCUSSION

The usefulness of a dye probe for the study of drug–protein binding comes from the fact that the absorption spectrum of the dye changes as a result of binding to the albumin. Figure 1 shows typical absorption difference spectra at constant serum albumin concentration with several concentrations of 2-(4′-hydroxybenzeneazo)benzoic acid. These spectra have a maximum at 482 nm,

Table I—Association Constants of Sulfonylureas to Serum Albumins

Compound	Blood Fraction	Method	Association Constants, M^{-1}	Reference
Acetohexamide	HSA[a]	Dialysis	$K^b = 3.39 \times 10^4$	19
	BSA[c]	Fluorescence	$K^d = 3.76 \times 10^4$	20
	BSA	Spectrophotometry	$K = 1.60 \times 10^4$	This study
Glipizide	BSA	Fluorescence	$K^d = 6.88 \times 10^4$	20
	BSA	Fluorescence	$K^d = 2.45 \times 10^4$	20
	BSA	Spectrophotometry	$K = 4.90 \times 10^4$	This study
Glyburide	BSA	Fluorescence	$K^d = 3.26 \times 10^5$	20
	BSA	Spectrophotometry	$K = 6.13 \times 10^4$	This study
Tolbutamide	HSA	Dialysis	$K^b = 4.05 \times 10^4$	19
	HSA	Dynamic	$K_1^e = 2.16 \times 10^5$	21
		Dialysis	$K_2^d = 1.71 \times 10^2$	
	BSA	Fluorescence	$K^d = 9.04 \times 10^4$	20
	BSA	Spectrophotometry	$K = 1.65 \times 10^5$	This study

[a] HSA = human serum albumin. [b] The pH and temperature of the solution were 7.4 and 40°. [c] BSA = bovine serum albumin. [d] The pH and temperature of the solution were 7.4 and 25°. [e] The pH and temperature of the solution were 7.4 and 37°.

and the absorbance difference increases as the concentration of the probe increases.

The absorbance difference data measured at 482 nm for probe–bovine serum albumin are plotted as a function of probe concentration in Fig. 2. The values of the points on the theoretical line are taken from the extrapolated values of the plots of $1/\Delta A$ *versus* $1/[P]$ (see *Data Treatment*).

Scatchard plots for the binding of probe to bovine serum albumin are shown in Fig. 3. At various concentrations of protein, the Scatchard plots gave lines that, like fluorescence results, are not truly linear in all ranges of concentration (13). The curvatures of Scatchard plots are generally considered to indicate that probe molecules bind to more than one class of sites on the protein. Recently, based on fluorescence results, it was suggested that the intrinsic association constants may not be equal even in the same class of binding sites (13).

Although these considerations are likely in some instances, another possibility is suggested by the spectrophotometric data collected here. It can be postulated that, in the range of concentration studied, the spectral changes are due to the formation of a charge transfer complex of only one probe molecule with one serum albumin molecule. This possibility is indicated by the fact that the value of $\bar{V}$ in the range of concentration studied never exceeded 1 and, as seen in Fig. 3, that line *C* with the lowest concentration of albumin starts to fall off as $\bar{V}$ exceeds the value of 0.85.

It has been reported from equilibrium dialysis studies that dyestuffs may bind to as many as 11 and/or 22 sites on serum albumin (17, 18). Although physical interpretation of these sites is questionable, it is possible that there are additional binding sites for probe molecules that do not cause spectral changes and that cause negative deviations from linearity of Scatchard-type data plots.

Figure 4 shows the results as plotted according to a modified Scatchard equation (13). In this figure, the values of log $\bar{V}/(n - \bar{V})[D]$, where n is assumed to be 1, are plotted against $\bar{V}$. The lines are linear except at the extremes of concentration.

For the purpose of competitive binding, the fact that the curves of Fig. 3 are not completely linear in all ranges of concentration need cause little concern, because the probe molecules are only used as a relative measure of the binding of drugs and the albumin may not be molecularly homogeneous with respect to its binding properties. It may only be necessary either to select a range of concentration within which the binding data can be mathematically treated or to substitute corresponding values of K for each point along the Scatchard plot into Eq. 3 to calculate the binding constants of the competitor drugs. In fact, for fluorescence data where probe molecules are said to bind to one class of sites, the low values of $\bar{V}$ have been disregarded to obtain a straight line with a high significance factor (13, 16). It is evident that the same treatment can be applied to the data in Fig. 3 to find the values of K and n for the range of concentration within the linear portion of the plot.

Moriguchi *et al.* (8) studied the binding of 2-(4′-hydroxybenzeneazo)benzoic acid to bovine serum albumin Fraction V and expressed their results by a modified Langmuir-type equation:

$$\log [\bar{V}(n - \bar{V})] = m \log [D] + m \log K \quad \text{(Eq. 4)}$$

where m is an empirical parameter and varies with factors such as protein concentration, buffering agent, and temperature.

By this method and by using an estimated value of molar absorptivity that is known to be dependent on factors such as solvent and temperature, the value of n was determined by trial and error so that the plot of log $[\bar{V}(n - \bar{V})]$ *versus* log $[D]$ was linear. In this way, the authors found the values of n and log K to be equal to 2 and 4.2 ± 0.1, respectively, while the value of m varied from 0.96 to 0.86 for albumin concentrations of 0.25×10^{-4}–1×10^{-4} *M*.

Nazareth *et al.* (12) used the same probe to reflect the binding

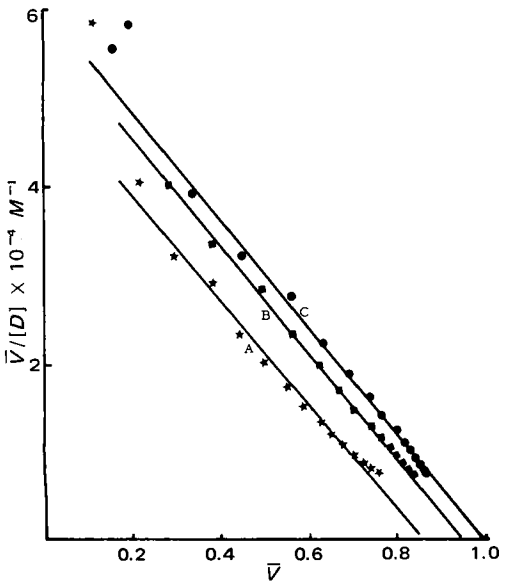


Figure 3—*Scatchard plots of 2-(4′-hydroxybenzeneazo)benzoic acid binding to various bovine serum albumin concentrations in 0.05* M *phosphate buffer, pH 7.4. Key:* ●, 3.45×10^{-5} M; ■, 4.6×10^{-5} M; *and* ★, 6.9×10^{-5} M.

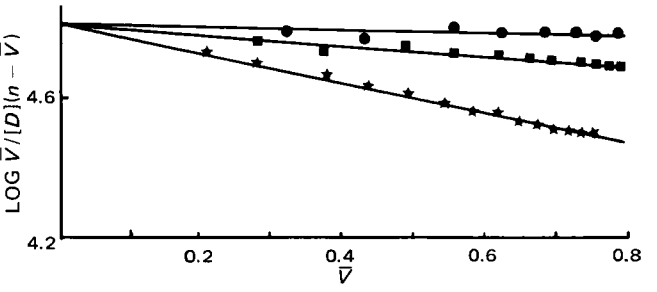
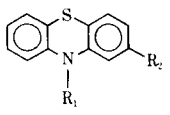


Figure 4—*Plots of* $\bar{V}/[D](n - \bar{V})$ versus $\bar{V}$ *for the binding of 2-(4′-hydroxybenzeneazo)benzoic acid to various bovine serum albumin concentrations in 0.05* M *phosphate buffer, pH 7.4. Key:* ●, 3.45×10^{-5} M; ■, 4.6×10^{-5} M; *and* ★, 6.9×10^{-5} M.

Table II—Association Constants of Selected Phenothiazines to Bovine Serum Albumin



Generic Name	R_1	R_2	Association Constant $\times 10^{-4}\ M^{-1}$
Chlorpromazine hydrochloride	Cl	$-CH_2CH_2CH_2N(CH_3)_2$	2.57
Trifluoperazine hydrochloride	CF_3	$-CH_2CH_2CH_2N\langle\rangle NCH_3$	2.32
Perphenazine	Cl	$-CH_2CH_2CH_2N\langle\rangle NCH_2CH_2OH$	1.96
Fluphenazine hydrochloride	CF_3	$-CH_2CH_2CH_2N\langle\rangle NCH_2CH_2OH$	1.82
Promazine hydrochloride	H	$-CH_2CH_2CH_2N(CH_3)_2$	1.04

of L-thyroxine to serum albumin. Ultrafiltration was used to determine the ratio of bound drug to protein, and low values of $\bar{V}$ were utilized to draw reciprocal plots to show the binding and displacement of probe to and from primary sites of albumin.

A comparison of the spectrophotometric method reported here with the methods of Moriguchi *et al.* (8) and/or Nazareth *et al.* (12) together with the results obtained from each method illustrates the usefulness and simplicity of the spectrophotometric method. It is recognized that the determination of binding parameters may depend on the experimental methods used. For some classes of components, the results obtained from spectrophotometric and fluorescence techniques are different from those of other methods, probably because only the primary (strong) binding sites causing spectral changes are detected, whereas the results obtained from other methods such as dialysis often show larger numbers of binding sites.

Competitive Binding between 2-(4′-Hydroxybenzeneazo)benzoic Acid and Sulfonylureas—A typical difference absorption spectrum in the presence and absence of a drug (glyburide) is shown in Fig. 5; the addition of competitive drug decreased the absorbance differences, presumably because the bound probe was displaced from the protein. The results of difference absorbance measurement at 482 nm in the presence and absence of fixed amounts of each sulfonylurea are shown as Scatchard plots in Fig. 6. The bovine serum albumin and sulfonylurea concentrations were held constant at 6.9×10^{-5} and 5×10^{-5} *M*, respectively, while the concentration of probe was varied from 1×10^{-5} to 11×10^{-5} *M*.

The binding constants calculated using the equation of Klotz *et al.* (16) are shown along with literature data in Table I. It can be seen from Table I and Fig. 6 that, as the association constants of the competitor sulfonylureas increase, the slopes of Scatchard plots decline. These findings, together with the fact that binding of probe decreases when it is in competition with sulfonylureas, appear to indicate that these drugs bind to the bovine serum albumin *via* the same site or closely located sites on the protein.

The conclusion to be drawn from Table I is that the binding constants found by the spectrophotometric probe technique are comparable to fluorescence data but are different from dynamic dialysis where more than one class of sites is detected. Among the four sulfonylureas studied, tolbutamide had the highest binding affinity followed in order by glyburide, glipizide, and acetohexamide.

Displacement of 2-(4′-Hydroxybenzeneazo)benzoic Acid by Phenothiazines—The determination of binding constants of phenothiazine drugs to serum albumin by the usual fluorescence probe technique is somewhat difficult because of rapid photoxidation and the consequent rapid change in fluorescence. Equilibrium dialysis is also ruled out because of the length of time required during which drugs are oxidized. Since the spectrophotometric probe technique utilizes very low light levels (compared to the fluorescence technique) and the analysis time is short, it has strong advantages over the other methods for this class of drugs.

In this study, the bovine serum albumin and phenothiazine concentrations were held constant at 4.6×10^{-5} and 1.5×10^{-4} *M*, respectively, while the concentration of the probe was varied from 1×10^{-5} to 15×10^{-5} *M*. Figure 7 shows Scatchard plots of the ef-

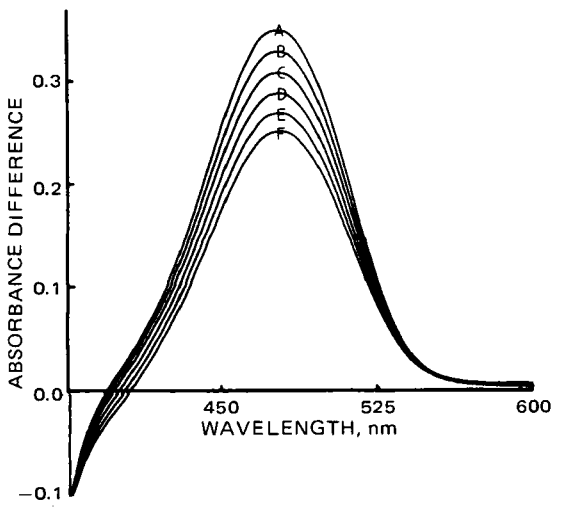


Figure 5—*Absorbance difference spectra for solutions containing bovine serum albumin (6.9×10^{-5} M) and 2-(4′-hydroxybenzeneazo)benzoic acid (6.5×10^{-5} M) in absence (curve A) and presence (curves B, C, D, E, and F) of 1.25×10^{-5} M increments of glyburide.*

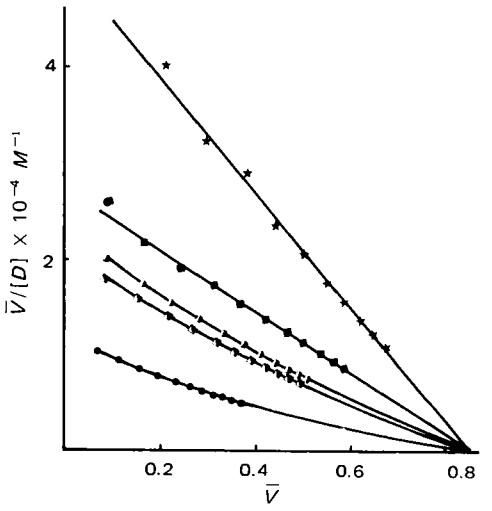


Figure 6—*Scatchard plots of 2-(4′-hydroxybenzeneazo)benzoic acid binding to bovine serum albumin in 0.05 M phosphate buffer, pH 7.4. Key: ★, in absence of drug; ■, in presence of 5×10^{-5} M acetohexamide; ◑, in presence of 5×10^{-5} M glipizide; ◪, in presence of 5×10^{-5} M glyburide; and ●, in presence of 5×10^{-5} M tolbutamide.*

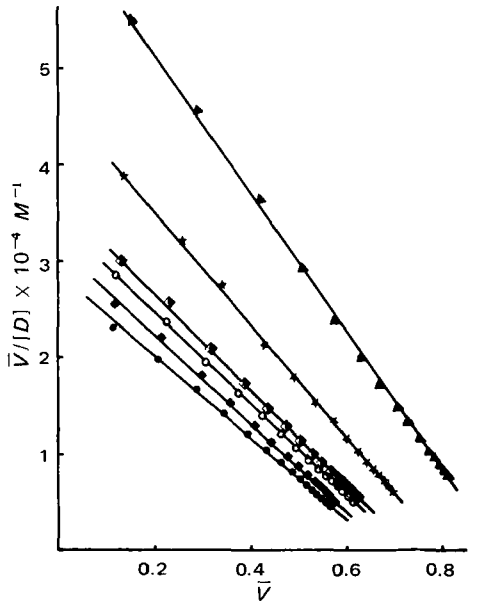


Figure 7—*Scatchard plots of 2-(4'-hydroxybenzeneazo)benzoic acid binding to bovine serum albumin in 0.05 M tromethamine buffer, pH 7.4. Key: ▲, in absence of drug; ★, in presence of 1.5 × 10^{-4} M promazine; ◪, in presence of 1.5 × 10^{-4} M fluphenazine; ○, in presence of 1.5 × 10^{-4} M perphenazine; ■, in presence of 1.5 × 10^{-4} M trifluoperazine; and ●, in presence of 1.5 × 10^{-4} M chlorpromazine.*

fect of adding different phenothiazine drugs to probe–bovine serum albumin solutions, and Table II shows calculated association constants. Chlorpromazine showed the greatest displacement of probe, followed by trifluoperazine, perphenazine, fluphenazine, and promazine. Furthermore, the binding was enhanced by direct or indirect halogen substitution on the phenothiazine moiety. Promazine, with no substitution at the 2-position, had the lowest value of K; chlorpromazine, having one chloride in the 2-position, had the highest value of K.

In addition, the side chain substitution at the 10-position also has considerable effect on the binding of these drugs. This is shown by trifluoperazine and fluphenazine, which have the same substitution at the 2-position but differ by their side chain at the 10-position. The hydroxy group on the fluphenazine appears to reduce the binding capacities. These results seem to indicate that interaction of phenothiazine drugs with bovine serum albumin is hydrophobic in nature. This conclusion is supported by the fact that the greater the hydrophobicity of the drug molecule, the greater is the value of the association constant.

The fact that Scatchard plots in the presence of phenothiazine drugs do not converge at the same intercept probably indicates that the site of binding for these drugs is not identical to that of the probe. It is likely that the binding of these drugs alters the conformation of the protein, thereby causing displacement of the probe. It is hoped that further work with these drugs will help explain the significance of these results.

SUMMARY

Difference spectrophotometry was used to show the displacement of a spectrophotometric probe [2-(4'-hydroxybenzeneazo)-benzoic acid] from bovine serum albumin by two classes of drugs, sulfonylureas and phenothiazines. It was shown that the difference absorbance data obtained could be treated in a manner similar to those obtained from fluorescence probe measurements.

Among the four sulfonylureas studied, tolbutamide showed the largest displacement of probe and the highest calculated binding affinity followed in order by glyburide, glipizide, and acetohexamide. These results closely paralleled reported results obtained by other techniques.

Among the phenothiazines, chlorpromazine showed the highest probe displacement and calculated binding affinity followed in order by trifluoperazine, perphenazine, fluphenazine, and promazine. These results indicated that the order of binding affinity was based on the hydrophobicity of the phenothiazine, with the more hydrophobic molecules being more strongly bound.

It is concluded that the use of difference spectroscopy to study indirectly the binding of drugs by measuring the displacement of a spectrophotometric probe offers several advantages over other methods. These advantages include convenience and fewer problems of oxidation and decomposition because of the short analysis time required and the low light intensities used. Interference from the fluorescence of drug molecules is also less of a problem than when fluorescent probes are used.

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