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EXAMINATION OF PHENOTHIAZINE–ALBUMIN INTERACTION BY ULTRAVIOLET DIFFERENCE SPECTROPHOTOMETRY

PING C. HUANG and SABIT GABAY

Biochemical Research Laboratory, Veterans Administration Hospital, Brockton, Mass. 02401, and Harvard School of Dental Medicine, Boston, Mass., U.S.A.

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Abstract—The binding properties of several phenothiazines to both human and bovine serum albumin have been systematically examined by ultraviolet difference spectrophotometric methods. The difference spectra of their interaction at pH 7·4 showed characteristically two positive and two negative absorption peaks at the 330, 260 and 290, 250 nm regions respectively. The difference spectra, which were derived mostly from perturbation of the phenothiazine chromophores in a hydrophobic environment, were quite specific, as structurally similar chlorpromazine sulfoxide, imipramine and chlorprothixene gave entirely different patterns of much weaker absorption. This may be taken as evidence that the latter compounds were bound at sites on the protein surface quite different from those of the phenothiazines. Contrary to the conclusion reached in recent publications which advocated that only one of the phenothiazine benzene rings was involved in the binding, the present studies with substituted derivatives on various positions show that this is not the case, but rather the whole phenothiazine nucleus takes part in the binding process. Furthermore, the effect of acetylsalicylic acid on the binding of phenothiazines demonstrated that the interaction is a noncompetitive interference which does not involve the same binding sites. The significance of these results is discussed in relation to various types of binding forces.

PLASMA protein binding is generally considered as an important factor in the transportation, distribution and elimination of drugs. The free unbound drug molecules are presumed to be available for transport to the receptor sites and hence protein binding controls the diffusion rate and the apparent distribution volume. Recently, the binding of phenothiazine drugs to serum proteins has been a subject of renewed interest. Several papers have appeared using gel filtration on Sephadex column,[1–4] equilibrium dialysis[1,2,5] or the batch method.[6] In the present study, the binding of several phenothiazine derivatives as well as some structurally similar drugs to both human (HSA) and bovine (BSA) serum albumin was examined using the ultraviolet difference spectrophotometric method. While some of the results are complementary to those obtained by other methods, additional information concerning the nature of the binding sites and some distinct binding parameters of phenothiazine–albumin interaction was derived.

A preliminary communication of part of these findings was previously presented.[7]

MATERIALS AND METHODS

Reagents and preparations

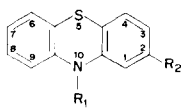
Pretreatment of albumins. Crystalline human albumin and bovine serum albumin were obtained from Schwarz/Mann Research. Fatty acids were removed from the albumins by the charcoal treatment method of Chen[8] at acid pH. The activated charcoal (Mallinckrodt) was washed with distilled water, filtered and dried prior to its use. Acetylation of HSA and BSA by acetylsalicylic acid was performed at pH 7·4 following the procedure of Hawkins *et al.*[9] The concentrations of HSA and BSA used throughout these studies were estimated on the basis of their molar extinction coefficient at 280 nm.[10] The values of $E_{1\,cm}^{1\%}$ 5·30 for HSA and 6·67 for BSA were used.

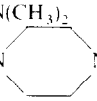
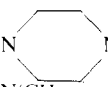
Phenothiazine derivatives. Chlorpromazine (CPZ) hydrochloride, CPZ sulfoxide hydrochloride, trifluoperazine dihydrochloride and trimeprazine tartrate were kindly supplied by Smith, Kline & French Laboratories; promazine hydrochloride and promethazine hydrochloride by Wyeth Laboratories; fluphenazine dihydrochloride and triflupromazine hydrochloride by Squibb Inc.; perphenazine and acetophenazine maleate by Schering Corp.; imipramine by Geigy Pharmaceuticals, and chlorprothixene hydrochloride by Hoffmann–La-Roche, Inc. Samples of 2-bromopromazine, 6-hydroxychlorpromazine, 7-hydroxychlorpromazine, 8-hydroxychlorpromazine, 7-methoxychlorpromazine hydrochloride, 7,8-dimethoxychlorpromazine hydrochloride, 3,7-dimethoxychlorpromazine hydrochloride, 7-hydroxychlorpromazine sulfoxide, chlorpromazine *N*-oxide-2 H_2O and 3,7-dichlorpromazine were kindly provided by Dr. A. A. Manian of the National Institute of Mental Health, Psychopharmacology Research Branch. The compounds were used without further purification. The CPZ free base[11] was prepared by alkaline conversion from the hydrochloride in water followed by extraction with ether and recrystallization from 95% ethanol, m.p. 56–57°. Aqueous solution of the samples was prepared by first dissolving the sample in small amounts of doubly glass-distilled water or, in the case of free base, in 0·1 N hydrochloride, then made up with appropriate buffer. Solutions were always freshly prepared prior to their use and screened from exposure to direct light at all times. Table 1 presents the structures of some of the phenothiazine derivatives which have been investigated.

All other materials were of analytical or reagent grade.

General methodology. Ultraviolet absorption spectra were recorded on a Cary 15 spectrophotometer. Difference absorption spectra were obtained by using a pair of split-compartment-tandem mixing cells[12,13] (Pyrocell 5014) with a 2 × 4 mm light path and 1 ml vol. in each compartment. Solutions of albumin and drugs were placed separately in each compartment in both the sample and reference cell. Since the contents of both cells were equal, a straight baseline was obtained. The albumin and drug solution in the sample cell was then mixed by covering the cell with a small piece of parafilm paper and inverting it several times. The difference spectra thus recorded are expressed in terms of difference absorbance (ΔA). The solvent perturbation of difference spectra of drugs was obtained using regular 10 mm light path rectangular cells. Calculated amounts of drugs were first dissolved in 95% ethanol, and equal portions of 0·01 ml were placed in each of the sample and reference cells. The solvents were carefully evaporated to dryness under a stream of

TABLE 1. CHEMICAL STRUCTURES OF SOME OF THE PHENOTHIAZINES



Name	R_1	R_2
Promazine	$CH_2—CH_2—CH_2N(CH_3)_2$	H
Promethazine	$CH_2—CH(CH_3)—N(CH_3)_2$	H
Trimeprazine	$CH_2—CH(CH_3)CH_2—N(CH_3)_2$	H
Chlorpromazine	$CH_2—CH_2—CH_2N(CH_3)_2$	Cl
2-Bromopromazine	$CH_2CH_2CH_2N(CH_3)_2$	Br
Perphenazine	$CH_2CH_2CH_2N$  NCH_2CH_2OH	Cl
Acetophenazine	$CH_2CH_2CH_2N$  NCH_2CH_2OH	$—\overset{O}{\overset{\|}{C}}—CH_3$
Triflupromazine	$CH_2CH_2CH_2N(CH_3)_2$	CF_3
Fluphenazine	$CH_2CH_2CH_2N$  NCH_2CH_2OH	CF_3
Trifluoperazine	$CH_2CH_2CH_2N$  NCH_3	CF_3
Chlorpromazine *N*-oxide	$CH_2CH_2CH_2NO(CH_3)_2$	Cl
Chlorpromazine sulfoxide (S → O)	$CH_2CH_2CH_2N(CH_3)_2$	Cl

nitrogen; the reference cell was then filled with 2 ml buffer and the sample cell with 2 ml of the perturbing solvent used. It should be noted that CPZ hydrochloride by itself is not soluble in cyclohexane, but the trace amount of ethanol which remained in the cell wall brought it into solution. Control experiments show that ethanol did not have any effect on the difference spectra.

RESULTS

Characteristics of albumin–phenothiazine interaction versus structurally similar compounds

Specificity of CPZ *difference spectra.* The difference absorption spectrum for the interaction of HSA and CPZ in phosphate buffer, pH 7·4, as presented in Fig. 1A, is characterized by two positive (263 and 335 nm) and two negative (248 and 294 nm) absorption peaks. The difference spectra obtained from structurally related compounds under identical conditions are also depicted in Fig. 1—they are (1) CPZ sulfoxide, a major metabolite of CPZ (curve B); (2) imipramine, an iminodibenzyl derivative and an antidepressant (curve C); and (3) chlorprothixene, a thioxanthene derivative (curve D). It is noteworthy that the last three compounds showed an entirely different absorption pattern from that of CPZ.

7-Hydroxychlorpromazine-sulfoxide showed a difference spectrum indistinguishable from that of the parent compound, CPZ sulfoxide. Acetophenazine with an acetyl group at carbon-2 and a slightly different side chain at N-10 showed one positive peak at 275 nm and two negative peaks at 236 and 264 nm (the intensities

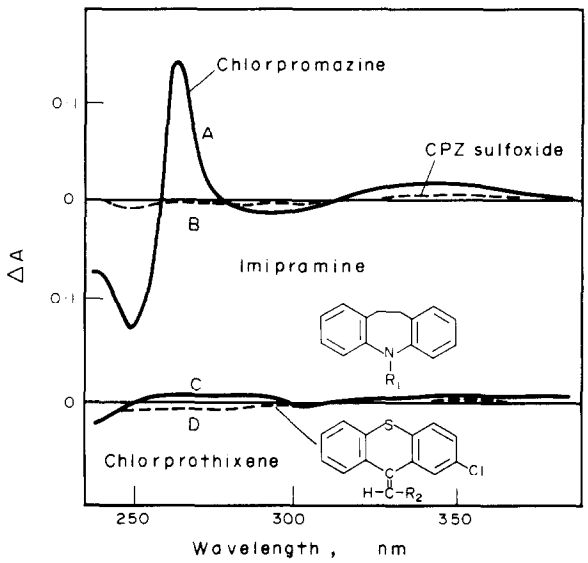


FIG. 1. Difference spectra of the interaction of HSA with CPZ and structurally related compounds. HSA ($1{\cdot}8 \times 10^{-5}$ M) and drug (5×10^{-5} M) in 0·05 M phosphate buffer at pH 7·4, 25°. Curve A, CPZ; B, CPZ sulfoxide; C, imipramine [R_1:$(CH_2)_3$·$N(CH_3)_2$]; D, chlorprothixene [R_2:$(CH_2)_2$·$N(CH_3)_2$].

were of just about the same magnitude as that of CPZ sulfoxide), whereas perphenazine, a 2-chloro substituent compound showed a spectrum nearly identical to that obtained from CPZ. When all of the above compounds were interacted with BSA, spectra almost identical to those obtained from HSA were observed.

Effect of carbon-2 substitution on the ultraviolet difference absorption. As shown in Fig. 2, it is evident that the difference absorption spectra resulting from the interaction of phenothiazine derivatives with both HSA and BSA were markedly influenced by carbon-2 substitutions. The intensity as well as the absorption maxima was found to be related to the substitution on carbon-2 in the following decreasing order: CF_3 > Cl > H. This was accompanied by a slight red shift of the absorption maxima. Promazine, the unsubstituted derivative, showed two positive peaks at 260 and 325 nm, while CPZ moved to 263 and 335 nm, and trifluoperazine, in turn, shifted to 266 and 340 nm. Various side chain substitution at the N-10 position of the phenothiazine nucleus slightly altered the peak intensities, but the maxima remained essentially the same.

Difference perturbation spectra in cyclohexane and buffer. The generation of the difference absorption peaks could be attributed either to the perturbation of the phenothiazine absorption bands by a different environment at the protein binding sites or to the perturbation of the protein aromatic amino acid residues as the result of the interaction, or to a combination of both. Fig. 3 allows comparison of the protein interaction difference spectrum of HSA and CPZ as opposed to solvent perturbation difference spectra of CPZ in cyclohexane and buffer. Curve 3A shows the difference spectrum of CPZ hydrochloride going from buffer to cyclohexane. Curve 3B is that of CPZ free base going from buffer to cyclohexane and curve 3C is the HSA and CPZ difference spectrum reproduced from Fig. 1A for convenience.

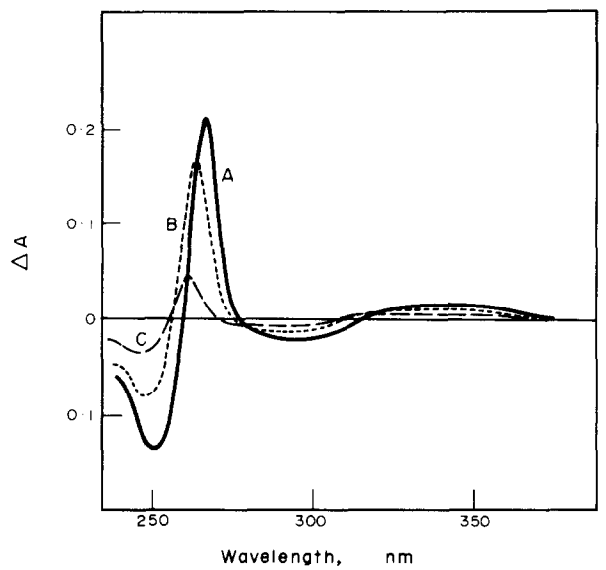


FIG. 2. Effect of substitution on the difference spectra of HSA and phenothiazines. HSA ($1{\cdot}8 \times 10^{-5}$ M) and drug (5×10^{-5} M) in 0·05 M phosphate buffer at pH 7·4. 25°. Curve A. triflupromazine; B. CPZ; C. promazine.

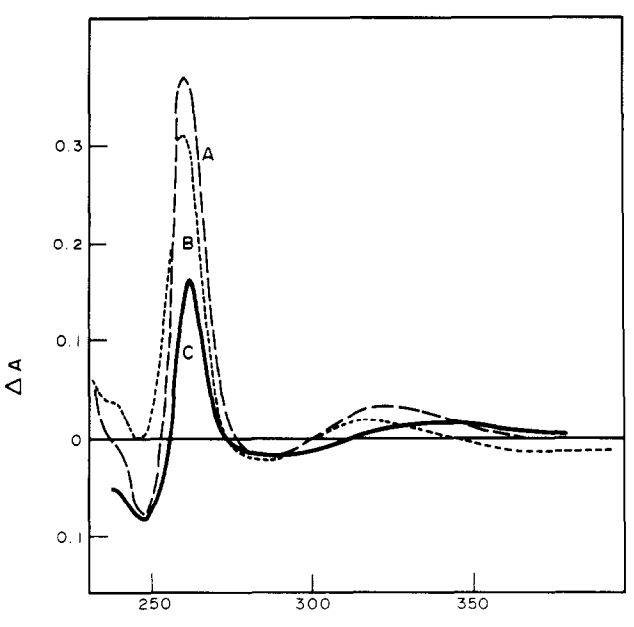


FIG. 3. Difference spectrum of HSA–CPZ and solvent perturbation spectra of CPZ in cyclohexane vs 0·05 M phosphate buffer, pH 7·4. Curve A. CPZ·HCl ($2{\cdot}5 \times 10^{-5}$ M) in cyclohexane vs in buffer; B. CPZ ($2{\cdot}5 \times 10^{-5}$ M) in cyclohexane vs in buffer; C. HSA ($1{\cdot}8 \times 10^{-5}$ M) and CPZ (5×10^{-5} M) in 0·05 M phosphate, pH 7·4.

It can be seen that both solvent perturbation spectra of CPZ hydrochloride and CPZ free base were quite similar to the HSA and CPZ difference spectrum, particularly in the 260 nm region. On the other hand, when CPZ sulfoxide, imipramine and chlorprothixene were examined under identical conditions, the solvent perturbation difference spectra revealed entirely different peaks from those of their respective protein interaction difference spectra, as shown in Fig. 4.

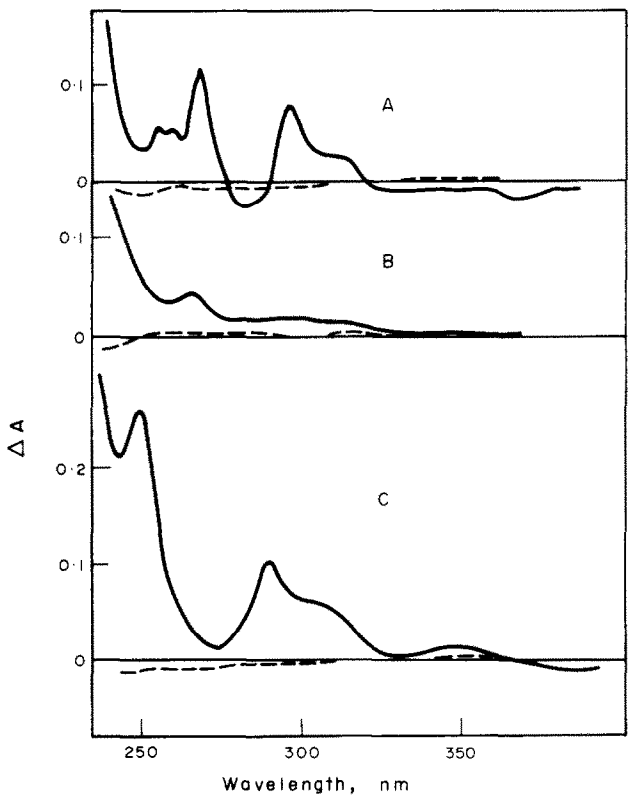


FIG. 4. Comparison of solvent perturbation and protein interaction difference spectra of the structurally related compounds. Experimental conditions and concentrations were identical to those in Fig. 3. A, CPZ-sulfoxide; B, imipramine; C, chlorprothixene. (Dotted lines were reproduced from Fig. 1 for convenience.)

Effect of pH

The difference spectrum of the interaction between HSA or BSA and CPZ is highly pH-dependent, as shown in Fig. 5. For example, an increase of only 0·5 unit from 7·4 to 7·9 showed a marked increase (nearly 50 per cent) of the absorption intensity at 263 nm, whereas a decrease in pH from 7·4 to 6·5 reduced the difference spectrum almost to zero. At pH higher than 7·9, CPZ started to precipitate from the buffer and the difference spectrum could not be obtained.

Effect of urea

The effect of urea on the difference spectrum of the interaction between HSA and CPZ is presented in Fig. 6. When the urea concentration is below 8 M, no change is discernible. In the presence of 8 M urea, a slight blue shift of the difference peak and some new feature in the 290 nm region of the spectrum were observed.

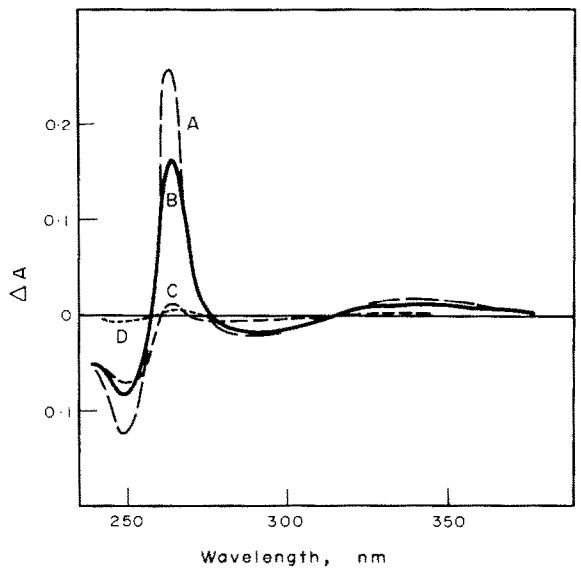


FIG. 5. Effect of pH on the difference spectra of the interaction between HSA and CPZ. Curve A, pH 7·9; B, pH 7·4; C, pH 6·5 all in 0·05 M phosphate buffer at 25°; D, 0·05 M glycine HCl buffer, pH 3·0 at 25°.

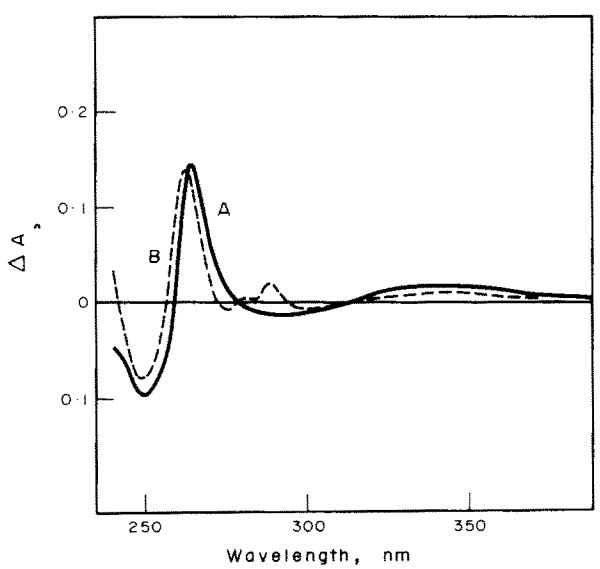


FIG. 6. Effect of urea on the difference spectra of the interaction between HSA and CPZ. Curve A, HSA ($1·8 \times 10^{-5}$ M) + CPZ (5×10^{-5} M), pH 7·4; B, in the presence of 8 M urea in either of the component solutions of both the sample and the blank cells.

Examination of the nature of the binding process

In order to have a better understanding of the functional groups and regions of the phenothiazine molecule involved in the interaction and binding to HSA or BSA, the difference spectra of a series of phenothiazine derivatives were thoroughly examined and some of their relevant properties are listed in Tables 2 and 3. All of

TABLE 2. COMPARISON OF ALBUMIN BINDING OF VARIOUS PHENOTHIAZINE DERIVATIVES FROM DIFFERENCE SPECTRA DATA*

Compound	Human serum albumin			Bovine serum albumin		
	$\Delta\lambda_{max}$†	ΔA‡	$\Delta\int\epsilon\,d\lambda$§	$\Delta\lambda_{max}$†	ΔA‡	$\Delta\int\epsilon\,d\lambda$§
Chlorpromazine	263	100	100	263	100	100
Promazine	259	29·4	25·2	258	42·8	45·5
Promethazine	259	35·3	37·6	258	65·9	71·7
Trimeprazine	260	42·6	46·6	259	69·2	82·2
Perphenazine	263	96·5	95·9	263	83·6	87·7
Triflupromazine	267	141	130	266	126	126
Fluphenazine	267	106	101	267	94·5	94·8
Trifluoperazine	267	112	120	267	108	110

* Conditions: temperature, 25 ; 0·05 M phosphate buffer, pH 7·4; albumin ($1·8 \times 10^{-5}$ M) + phenothiazines ($5·0 \times 10^{-5}$ M).

† The positive absorption maximum of the difference spectra used for comparison.

‡ Normalized difference absorbance at $\Delta\lambda_{max}$ against CPZ as 100.

§ Normalized relative oscillator strength for the difference absorption peak $\Delta\lambda_{max}$ against CPZ as 100.

the 17 phenothiazine derivatives tested under the conditions described in the legends to Tables 2 and 3 exhibited the same characteristics, e.g. two positive and two negative peaks, as with CPZ. Since the positive peaks at the 260 nm region were the predominant ones, they were used as a basis for comparison. The intensities of the difference absorption peaks being a good indication of the degree of interaction or the binding processes for different phenothiazines were expressed in terms of difference absorbance (ΔA) and difference relative oscillator strength ($\Delta\int\epsilon\,d\lambda$). The latter was obtained by integrating the whole area under the difference absorption peak. All data were the average of three runs (mean ± S.E. 2 per cent) and were normalized against CPZ as the standard (100) for comparison.

Recently, investigators[14] using a gel filtration method have studied the effect of some simple aromatic substances as well as a series of acidic and basic drugs on

TABLE 3. COMPARISON OF THE EFFECTS OF VARIOUS SUBSTITUTIONS ON THE PHENOTHIAZINE RING TOWARD ALBUMIN BINDING FROM DIFFERENCE SPECTRA DATA*

Compound	Human serum albumin			Bovine serum albumin		
	$\Delta\lambda_{max}$†	ΔA‡	$\Delta\int\epsilon\,d\lambda$§	$\Delta\lambda_{max}$†	ΔA‡	$\Delta\int\epsilon\,d\lambda$§
Chlorpromazine	263	100	100	263	100	100
2-Bromopromazine	263	120	122	263	115	118
3,7-Dichlorpromazine	265	135	132	265	138	130
6-Hydroxychlorpromazine	270	76·2	82·3	269	85·1	87·4
7-Hydroxychlorpromazine	266	37·8	50·0	265	47·6	48·1
8-Hydroxychlorpromazine	268	62·6	71·6	266	54·9	61·8
7-Methoxychlorpromazine	266	35·2	40·3	266	42·5	39·9
3,7-Dimethoxychlorpromazine	264	53·3	54·8	264	35·0	37·1
7,8-Dimethoxychlorpromazine	267	59·1	62·0	266	31·8	34·0
Chlorpromazine *N*-oxide	263	41·2	44·5	263	40·1	43·4

* Conditions: temperature, 25 ; 0·05 M phosphate buffer, pH 7·4; albumin ($1·8 \times 10^{-5}$ M) + phenothiazines ($5·0 \times 10^{-5}$ M).

† The positive absorption maximum of the difference spectra used for comparison.

‡ Normalized difference absorbance at $\Delta\lambda_{max}$ against CPZ as 100.

§ Normalized relative oscillator strength for the difference absorption peak $\Delta\lambda_{max}$ against CPZ as 100.

the binding of promazine or CPZ to BSA. They have shown that most of the compounds used could displace phenothiazines from their "binding sites" on the albumin molecule. In a more recent publication,[2] they have reached the conclusion that only one of the phenothiazine rings can possibly be attached to albumin. However, in our opinion, the displacement of one drug by another on albumin does not necessarily involve the same binding sites. Experiments on the competitive binding between acetylsalicylic acid and CPZ to HSA as well as BSA were carried out using ultraviolet difference spectroscopy. These are summarized in Table 4. Preincubation of albumin with acetylsalicylic acid at physiological pH resulted in an

TABLE 4. EFFECT OF ACETYLSALICYLIC ACID ON THE BINDING OF CHLORPROMAZINE TO HUMAN AND BOVINE SERUM ALBUMINS

Conditions*	Human serum albumin			Bovine serum albumin		
	$\Delta\lambda_{max}$†	ΔA‡	$\Delta\int\epsilon\,d\lambda$§	$\Delta\lambda_{max}$†	ΔA‡	$\Delta\int\epsilon\,d\lambda$§
Without acetylsalicylic acid	263	100	100	263	100	100
Preacetylation‖	263	97·8	96·5	263	101	98·5
With acetylsalicylic acid¶	262	77·5	75·0	262	80·2	81·4

* Temperature, 25°; 0·05 M phosphate buffer, pH 7·4. Final concentrations used: albumins ($1{\cdot}8 \times 10^{-5}$ M) and CPZ ($5{\cdot}0 \times 10^{-5}$ M).

† The positive absorption maximum of the difference spectra used for comparison.

‡ Normalized difference absorbance at $\Delta\lambda_{max}$ against CPZ as 100.

§ Normalized relative oscillator strength for the difference absorption peak $\Delta\lambda_{max}$ against CPZ as 100.

‖ Albumins were preacetylated with acetylsalicylic acid according to the method of Hawkins *et al.*[9] at pH 7·4, excess acetylsalicylic acid was removed by dialysis and the concentration of albumin readjusted to $1{\cdot}8 \times 10^{-5}$ M, assuming that the molar extinction coefficient remained unchanged at 280 nm.

¶ Acetylsalicylic acid, 5×10^{-4} M, in CPZ solution.

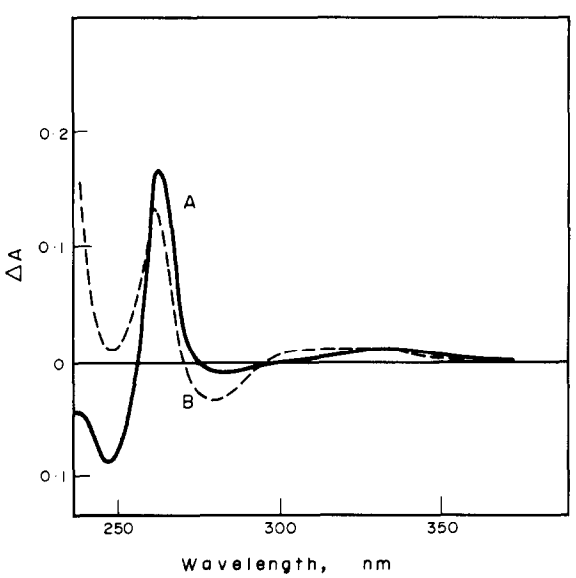


FIG. 7. Influence of acetylsalicylic acid on the difference spectrum of the interaction between BSA and CPZ. Curve A, acetylated BSA ($1{\cdot}8 \times 10^{-5}$ M) and CPZ ($5{\cdot}0 \times 10^{-5}$ M), which was identical to the reaction of the untreated BSA; B, BSA ($1{\cdot}8 \times 10^{-5}$ M) + CPZ ($5{\cdot}0 \times 10^{-5}$ M) and acetylsalicylic acid ($5{\cdot}0 \times 10^{-4}$ M); pH 7·4 at 25° throughout.

irreversible acetylation of the molecule. After the excess acetylsalicylic acid was removed by dialysis, the binding of CPZ with the acetylated HSA or BSA behaved exactly like the native albumins. However, when a large excess of acetylsalicylic acid was present in the CPZ solution, the binding of CPZ to HSA or BSA was markedly affected. The difference spectrum for the interaction of BSA with CPZ in the presence of acetylsalicylic acid is presented in Fig. 7.

Interrelationship of difference absorbance (ΔA), binding stoichiometry (N) and equilibrium association constant (k)

The equilibrium between albumin (P) with a single binding site and phenothiazine (C) is as follows:

$$P + C \rightleftharpoons PC$$

The association constant (k) relates the concentration of the components at equilibrium

$$k = \frac{[PC]}{[P][C]} \tag{1}$$

Let ϕ be the fractional saturation of the albumin.

$$\phi = \frac{\text{moles of phenothiazine bound}}{\text{moles of total albumin}} = \frac{[PC]}{[P_0]} = \frac{[P]}{[P] + [PC]} = \frac{k[P][C]}{[P] + k[P][C]} \tag{2}$$

or

$$\phi = \frac{kC}{1 + kC}$$

where C is the molar concentration of the unbound phenothiazine. The difference absorption peaks derive only from the perturbation of PC[15,16]

$$\phi = \frac{\Delta A}{\Delta A_{max}} \tag{3}$$

where ΔA_{max} is the maximum difference absorbance at P. If the albumin has N independent binding sites for phenothiazines,

$$r = \phi N \tag{4}$$

or

$$= \frac{NkC}{1 + kC} \tag{5}$$

where r is the average number of binding sites bound, and

$$C = C_0 - rP_0 \tag{6}$$

equation (5) could be rearranged to the familiar Klotz' equation

$$1/r = 1/N + 1/Nk \cdot 1/C \tag{7}$$

or Scatchard's equation

$$r/C = kN - kr \tag{8}$$

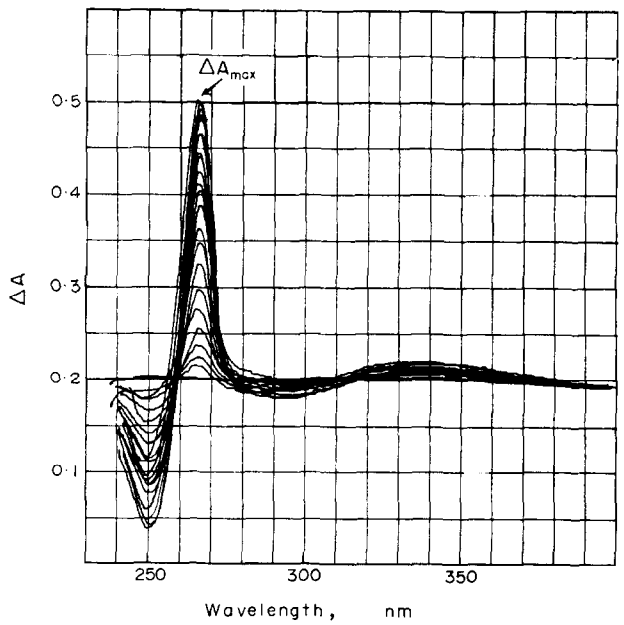


FIG. 8. Difference spectrophotometry study for the binding between HSA and triflupromazine. HSA ($1{\cdot}8 \times 10^{-5}$ M), triflupromazine ($3{\cdot}65 \times 10^{-6}$ to $1{\cdot}46 \times 10^{-4}$ M), pH 7·4, 25°, 0·05 M phosphate buffer.

A typical binding equilibrium study using the difference spectrophotometry method for the binding between HSA and triflupromazine is shown in Fig. 8. The concentration of HSA was kept constant at $1{\cdot}8 \times 10^{-5}$ M and the concentration of triflupromazine varied between $3{\cdot}65 \times 10^{-6}$ and $1{\cdot}46 \times 10^{-4}$ M. Each separate difference spectrum represents a set of experiments under identical conditions in

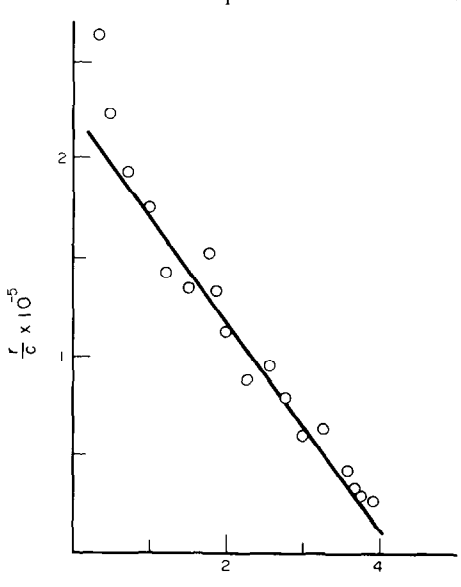


FIG. 9. Scatchard plot for the binding of triflupromazine to HSA. Data calculated from Fig. 8.

0·05 M phosphate buffer, pH 7·4, 25°. The stoichiometry (N) was obtained from a plot of ΔA vs C_0. The data were then compiled from equations 3, 4 and 6 and subjected to a Scatchard's plot as shown in Fig. 9. Similar binding equilibrium studies with a few representative phenothiazines were performed and the results are listed in Table 5. When these experiments were repeated with BSA, under identical conditions, the same stoichiometric relationship was obtained.

TABLE 5. BINDING OF SEVERAL PHENOTHIAZINE DERIVATIVES TO HUMAN SERUM ALBUMIN FROM DIFFERENCE SPECTRA DATA*

Compound	ΔA†	N‡	k§ $\times 10^{-4}$ 1/M	$-\Delta F^\circ$ ‖
Chlorpromazine	100	4	4·2	6270
Triflupromazine	141	4	5·5	6430
6-Hydroxychlorpromazine	76·2	4	3·0	6080
3,7-Dimethoxychlorpromazine	53·3	4	2·2	5890

* Temperature, 25°; 0·05 M phosphate buffer, pH 7·4. HSA ($1·8 \times 10^{-5}$ M).
† Normalized difference absorbance taken from Tables 2 and 3.
‡ Stoichiometry of drug binding sites per albumin molecule.
§ Binding association constant.
‖ Binding free energy calculated from $\Delta F^\circ = -RT\ln k$.

DISCUSSION

As is evident from the difference spectra, the interaction between both HSA and BSA and a series of phenothiazine derivatives revealed consistently, as with CPZ, two positive and two negative absorption peaks. In contrast, several of the structurally related compounds, such as imipramine, chlorprothixene and CPZ sulfoxide, failed to show this characteristic pattern.

Comparison of the difference spectra of the interaction between HSA, as well as BSA, and CPZ with the solvent perturbation difference spectra of CPZ in cyclohexane versus buffer showed close similarities, which is a direct indication of the existence of a hydrophobic environment around the albumin binding sites. The difference spectra, however, did not rule out additional contributions from perturbation of the protein aromatic amino acid residues by CPZ, since minor deviations (see in Fig. 3) exist between the spectra. Imipramine, chlorprothixene and CPZ sulfoxide have nevertheless been shown[4,17] to bind albumin based on data deriving from gel filtration and equilibrium dialysis techniques; in fact, chlorprothixene was found to bind albumin even more strongly than CPZ.[4] The negligible appearance of their difference spectra with albumin and the large discrepancy between the solvent perturbation difference spectra in cyclohexane versus buffer indicate that their binding sites on albumin are quite different from that of phenothiazines. Accordingly, their binding sites are most likely not in a hydrophobic environment, and only very weak perturbation could be observed between the absorption chromophores of these molecules and the protein.

The increase in the intensity of the induced difference spectra for the interaction of albumin with promazine, CPZ and trifluprazine is consistent with the increase of the hydrophobic character of the ring substituent group at the 2-position of these molecules. A similar conclusion ascribable to hydrophobic binding has been obtained through comparison of other parameters from gel filtration and equilibrium dialysis

studies.[2,18] In keeping with the same rationale, in our studies, 2-bromopromazine was found to bind albumin more strongly than CPZ, and 3,7-dichlorpromazine (which has an additional chloride group at the 7-position) was even stronger than either CPZ or 2-bromopromazine.

The forces of hydrophobic interaction appeared to exert a certain influence also in the N-10 substituted side chain moieties. For instance, trimeprazine was bound to albumin more strongly than promethazine, which in turn was better than promazine. In the latter case, the effectiveness may perhaps be attributed to the difference in the preferential conformational forms of the *N*-alkyl side chain in extending the methyl or methylene group into the hydrophobic region on the albumin surface. In the case of the triflupromazine, fluphenazine and trifluoperazine series, the shorter *N*,*N*-dimethylpropylamino group was stronger than either the methylpiperazinylpropyl or 2-hydroxyethylpiperazinylpropyl side chain. Steric effects must have played an important role here, and the binding probably involves only part of the piperazine moiety, the remainder being either in a chair or boat form stretching outside of the binding surface. In the case of perphenazine and fluphenazine, however, the degree of binding decreased further due to the introduction of a hydrophilic group (—OH) at the end of the side chain.

Although the nature of the binding force between CPZ and albumin is predominantly hydrophobic, there could also be a combination of other factors, such as donor–acceptor type complexing, which might play a role in this binding. CPZ has been shown[19] to have a most unusual anti-bonding highest filled molecular orbital in its ordinary stable state, and is thus an exceedingly strong electron donor. In the case of acetophenazine, an electron-withdrawing acetyl group replaced the 2-chlorine and consequently the difference spectrum for the interaction with albumin changed completely. In contrast to trifluomethyl, which is also an electron-withdrawing group, the resonance and conjugation effect of the acetyl group probably altered the orbital energy and electron distribution of the ring to such an extent that it changed the nature of the binding. Again, it should be pointed out that from gel filtration studies on Sephadex columns,[18] acetophenazine was found to bind BSA to about the same extent as promazine, indicating that the binding takes place at a different site.

By using the cyclohexane–buffer system as a reference for modeling the hydrophobic effect and the similarity between the solvent perturbation spectra and the protein interaction difference spectra, it is evident that neither hydrogen bonding nor dipolar interaction was a major factor contributing to the difference spectra. The contribution from hydrogen bonding forces is indeed insignificant, as the difference spectrum between CPZ and albumin remained essentially unchanged in up to 8 M urea, suggesting that considerable bonding structure remains, even in the binding region of the 8 M urea-treated albumin.

The great sensitivity of the difference spectra to pH changes gave firm evidence that, although hydrophobic bonding was the predominant force, ionic interactions should not be ignored. Since the p*K*a of CPZ is about 9·3, most of the species are protonated in aqueous solution below pH 7·9, and the ionization of the binding sites might be of major importance if some weakly acidic groups which were only partially ionized at pH 7·4 were involved; these should be the binding region for the charged side chain dimethyl amino group. At pH 6·5, the conformation of the

albumin does not differ much from that at pH 7·4, yet at this pH the intensity of the difference spectra of CPZ and albumin decreased more than 95 per cent, indicating that a charge repulsion might have occurred. At pH 3·0, albumin becomes unfolded and no significant binding can be expected.

It is obvious that the hydrophobic interaction is of major importance for the maintenance of a rigid CPZ–albumin complex, since the presence of any hydrophilic groups on the CPZ molecule, either on the ring nucleus or side chain, caused considerable reduction in the magnitude of the induced difference spectra. For example, CPZ-*N*-oxide was bound to albumin less than half that of CPZ. With introduction of a hydroxyl group into the CPZ ring moiety, such as the 7-hydroxyl analogue of CPZ, its binding to albumin was poorer than that of CPZ. However, the substituent effect appears to differ according to the ring position, the 6-position being least sensitive, while the 7-position has the most influence. Introduction of a methoxyl group into the CPZ ring moiety also resulted in lowering the binding ability of CPZ to albumin. The effect of a 7-methoxyl group was quite similar to that of a 7-hydroxyl group. However, by introducing a second methoxyl group, such as 3,7- or 7,8-dimethoxyl, a significant difference in the binding affinity between HSA and BSA was observed.

In the case of HSA, a second methoxyl group in the 3- or 8-position restored some of the binding from 7-methoxychlorpromazine, while in BSA, further deterioration resulted. The difference in binding between HSA and BSA occurred also in promazine, promethazine and trimeprazine where BSA was much more effective (Table 2). Although the modes of interaction between phenothiazines and HSA or BSA were very similar, these differences probably reflect small alterations of the amino acid sequence near the binding sites.

While numerous studies on the binding of acidic drugs to plasma protein have been reported,[20] publications on basic drugs are relatively scanty. It is generally conceded that albumin has different binding sites for acidic and basic drugs.[21] Although some acidic drugs can displace another acidic drug in a particular binding site, basic drugs never replace acidic drugs in the same site.[22] Thus, the conclusion reached by some authors[1,2] that the displacement of CPZ from its binding site by simple aromatic substances such as benzoic acid or acetylsalicylic acid is due to competition between the benzene rings, which are the integral part of the structure of all substances used, is open to question. Fundamentally, acetylsalicylic acid and CPZ belong to two different charge ionic groups at physiological pH and, therefore, should have separate binding sites on albumin. The observed displacement of CPZ by acetylsalicylic acid on albumin from gel filtration studies[1] as well as from the present difference spectrophotometric studies must stem from noncompetitive interference[23,24] which, as opposed to competitive interference, is not directly involved in the same binding sites. Acetylation of HSA with acetylsalicylic acid at physiological pH results in an average of 1·2 acetyl groups covalently bound to a lysine residue.[25,26] In the present study, it was found that the difference spectra of acetylated HSA or BSA with CPZ were essentially the same as those with the untreated albumins (Table 4). If the alleged lysine residue binding sites for anionic drug were the same for the phenothiazines, as has been proposed,[1,2] introduction of an acetyl group would certainly have a noticeable effect on phenothiazine binding. Since we have been able to demonstrate that substitution of an acetyl, hydroxyl or methoxyl group on the

phenothiazine molecule would alter the protein interaction difference spectra, substitution of an acetyl group on the protein side for the same interaction would be expected to show a similar effect. On the other hand, in the presence of excess acetylsalicylic acid, the difference spectra of CPZ with HSA or BSA not only showed diminishing intensity in the 263 nm region, but the characteristic distinguishing peak changed as well. The first step in the acetylation of albumin involves the binding of acetylsalicylic acid to their binding sites. A simple displacement of CPZ should only decrease the intensity but not change the characteristics of the difference spectra between CPZ and albumin. During the initial phase of the acetylation, interaction with excess acetylsalicylic acid and the subsequent binding of acetylsalicylic acid to their cationic sites must have slightly changed the conformation of the protein and the CPZ binding sites as well. After the departure of the salicylate anion leaving group and the final removal of the excess acetylsalicylic acid, the proper conformation of the CPZ-binding sites on albumin is restored. More detailed description of such changes using a fluorescence polarization method will be discussed elsewhere.

As the difference spectroscopic studies herein reported have determined, the ability of various substituents (particularly in the 6-, 7- and 8-positions) on the phenothiazine ring to give rise to marked differences in the degree of binding, and the inability of some of the structurally related compounds to do so, conclusively point out that all three rings of the phenothiazine nucleus are essential to the binding of CPZ and its congeners to the hydrophobic binding sites.

The three-dimensional crystalline and molecular structure of CPZ has recently been resolved.[27] The molecule is folded about the S—N axis and the angle between the best planes for the two benzene rings was found to be 139·4°. The folding of the molecule enables the sulfur atom to retain its natural valency angle. For the same reason, the conformation of the phenothiazine ring in solution would probably remain the same, which would thus fit in some hollowed contour area as dictated by the binding sites in the hydrophobic region of albumin. Any alteration of this geometric requirement, as in the case of CPZ sulfoxide, imipramine or chlorprothixene, excludes such molecules from this particular albumin binding site.

Binding equilibrium studies of a few selected phenothiazines toward human serum albumin indicated that the binding constants obtained from Scatchard plots corresponded quite well to the normalized difference absorbance between HSA in a proper uniform phenothiazine concentration (5×10^{-5} M). It is thus shown that, under identical experimental conditions, the intensities of the difference absorption peaks are indeed a good indication of the degree of the interaction and that the various phenothiazines studied do involve a common binding process.

The stoichiometry for the binding of phenothiazine to albumin has been reported for BSA and HSA values ranging from 1 to 23.[3,5,18] In our opinion, the stoichiometric relationship depends on the albumin concentration. Several other drugs have also been shown to vary their binding capacity according to the albumin concentration.[28,29] Our studies showed that at $1{\cdot}8 \times 10^{-5}$ M, each mole of HSA or BSA binds four moles of CPZ. This value was verified by intrinsic protein fluorescence quenching methods.[7]

The interaction between phenothiazines and albumin has been a subject of renewed interest. A better understanding of the binding process will provide further insights into the general area of drug–protein interaction, especially with basic drugs. We have

carried out a systematic examination by difference spectrophotometry which has enabled us to demonstrate the structure and hydrophobic nature of one of the albumin binding sites for phenothiazines which excluded structurally related CPZ sulfoxide, imipramine and chlorprothixene. It is thus proposed that difference spectrophotometry could be a complementary method for providing insight into the stoichiometry and affinity of the binding process as distinguished from the widely used equilibrium dialysis or gel filtration techniques.

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