

Hydrophobic interactions of phenoxazine modulators with bovine serum albumin

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Abstract. The interaction of 10-(3'-N-morpholinopropyl)phenoxazine [MPP], 10-(4'-N-morpholinobutyl)phenoxazine [MBP], 10-(3'-N-morpholinopropyl)-2-chlorophenoxazine [MPCP], 10-(3'-N-piperidinopropyl)-2-chlorophenoxazine [PPCP] or 10-(3'-N-morpholinopropyl)-2-trifluoromethylphenoxazine [MPTP] with bovine serum albumin (BSA) has been studied by gel filtration and equilibrium dialysis methods. The binding of these modulators, based on dialysis experiments, has been characterized using the following parameters: percentage of bound drug (β), the association constant (K_1), the apparent binding constant (k) and the free energy change (ΔF°). The binding of phenoxazine derivatives to serum transporter protein, BSA, is correlated with their octanol-water partition coefficient, $\log_{10} P$. In addition, effect of the displacing activities of hydroxyzine and acetylsalicylic acid on the binding of phenoxazine derivatives to albumin has been studied. Results of the displacement experiments show that phenoxazine benzene rings and tertiary amines attached to the side chain of the phenoxazine moiety are bound to a hydrophobic area on the albumin molecule.

Keywords. Phenoxazine; hydrophobic interaction; displacement experiments; bovine serum albumin.

1. Introduction

Investigation involving the binding of drugs to albumin has been described in numerous publications because of its pharmacokinetic importance¹. In addition, the drug–albumin complex may be considered a model for gaining general fundamental insights into drug–protein binding. General rules of protein binding gained from this model could apply at least partially to the drug–receptor complex, provided that the receptor has a protein structure. Determination of the albumin binding of several structurally related compounds is a valuable tool for identifying those groups of a drug molecule that are involved in binding and for characterizing the binding forces concerned with the interaction of drugs with protein.

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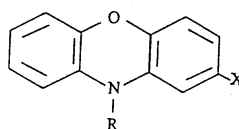
The binding of phenothiazine drugs to bovine serum albumin (BSA) has been studied¹. Although most of the authors obtained total binding constants of the same order of magnitude, the number of binding sites varied considerably. It has been found² that the number of binding sites on BSA for promazine and chlorpromazine changed with the concentration of the drugs, higher numbers being obtained at higher drug concentrations. These results suggest that phenothiazine derivatives are bound by hydrophobic interaction to the aromatic amino acids of the BSA molecule and that, under the influence of high drug concentrations, the number of available sites increase by swelling and unfolding of the BSA molecules in solution. Glasser and Krieglstein³ correlated the $\log_{10}P$ of some phenothiazine drugs and related compounds to their $\log(\beta/\alpha)$ values, β and α being the fractions of bound drug and free drug respectively. A fairly good linear correlation ($r = 0.969$) for five 10-dimethylaminopropyl derivatives of phenothiazine was obtained. However, when other drugs were included, the correlation deteriorated.






Compounds of pharmacological interest have been found among phenoxazine derivatives⁴ and have been claimed to be nervous system depressants, in particular with sedative, antiepileptic, tranquillising, spasmolytic, antitubercular and anthelmintic activities. Drug resistance has become a major obstacle in the clinical treatment of cancer as well as some microbial diseases. A variety of small molecules capable of modulating drug resistance has been prepared and examined. While a number of pharmacological agents have been shown to reverse drug resistance *in vitro*, there is still a need to identify more potent, more specific and less toxic modulators for clinical use. In an attempt to search for more potent and less toxic drug reversal agents, Thimmaiah *et al*⁵ have reported that the parent phenoxazine facilitated the uptake of vincristine (VCR) and vinblastine (VLB) in multidrug resistant (MDR) GC₃/cl and KBCh^R-8-5 cells to a greater extent than verapamil. In a subsequent study^{4,6}, twenty N¹⁰-substituted phenoxazines were synthesized and examined for their ability to enhance the uptake of VLB and VCR in GC₃/cl and KBCh^R-8-5 cells. Recently, Thimmaiah *et al*⁷ have demonstrated that 2-chlorophenoxazines are able to partially reverse VLB resistance in MDR colon carcinoma cell line GC₃/cl and completely reverse 86-fold VLB resistance in the MDR1 overexpressing breast carcinoma cell line BC 19/3. The same agents can partially sensitize BC 19/3 cells to taxol and doxorubicin, suggesting that the chlorophenoxazines show some specificity for modulating VLB resistance. The results show that substitution on the phenoxazine ring at position N¹⁰ is associated with an increase in antiproliferative and anti-MDR activities. Nevertheless, the exact mechanism of pharmacological action of phenoxazines remains unknown. Since these molecules are found to be useful as potential anti-MDR agents, studies have been undertaken to elucidate the nature of interactions with serum transporter protein, BSA.

2. Experimental

The synthesis and chemical characterization of MPP, MBP, MPCP, PPCP and MPTP have been carried out as per the literature^{4,8,9}. The structures of the compounds are given in table 1.

Acetylsalicylic acid, bovine serum albumin and hydroxyzine were purchased from Sigma Chemical Company, USA. All chemicals were of reagent grade. All binding measurements were made in the presence of 0.02M phosphate buffer, pH 6.8, containing 0.15M NaCl and 3 mM sodium thiosulphate¹. Just before protein binding of a drug was

Table 1. Name, molecular structure, molecular weight and $\log_{10}P$ values of the phenoxazine derivatives.

Compound	Name	R	X	MW	$\log_{10}P$
MPP	10-(3'-N-morpholinopropyl)-phenoxazine	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}$ 	-H	346.50	1.48
MBP	10-(3'-N-morpholinobutyl)-phenoxazine	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}$ 	-H	360.50	1.72
MPCP	10-(3'-N-morpholinopropyl)-2-chlorophenoxazine	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}$ 	-Cl	381.00	1.90
PPCP	10-[4'-N-piperidinopropyl]-2-chlorophenoxazine	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}$ 	-Cl	379.00	2.40
MPTP	10-(3'-N-morpholinopropyl)-2-trifluoromethylphenoxazine	$-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{N}$ 	$-\text{CF}_3$	414.50	2.50

determined, the pH of the sample solution was measured and, wherever necessary, adjusted to pH 6.8 with 0.1N HCl or 0.1N NaOH.

2.1 Separation of phenoxazine-albumin complex by gel-filtration experiment

Binding of phenoxazine derivatives to albumin was studied using gel filtration experiments. The experiments were performed on a 20×1.3 cm column of sephadex^R G-50 fine (Pharmacia) at 22°C, equilibrated with standard buffer solution. The flow rate was maintained at 25 ml/h. BSA solution (20 ml, 1%) containing 1.0×10^{-4} M phenoxazine modulator, after incubation at 37°C for 6 h, was loaded on to the column and washed with standard buffer. The effluent from the column was collected in fractions of 3 ml. Of these, 1.5 ml each was used to determine protein concentration and the remaining 1.5 ml for phenoxazine modulator estimation. Albumin content was determined by the 'biuret' method, while phenoxazine modulator was estimated colorimetrically after extracting with *n*-heptane in 50% sulphuric acid containing 10 mg % FeCl_3 .

2.2 Study of interaction of phenoxazines with BSA by equilibrium dialysis method

Sample solution (20 ml) containing BSA (1%) and one of the modulators (MPP, MBP, MPCP, PPCP or MPTP) in the concentration range $0.1-5 \times 10^{-4}$ M was taken in a 50 ml centrifuge tube and incubated at 37°C for 6 h in a shaking water-bath incubator. Into each of four dialysis tubes ($3/4$ " or 1.9 cm diameter), 5 ml of the above reaction mixture was pipetted out. The dialysis tubes were then closed and immersed in a standard buffer solution taken in a measuring jar. The samples were dialysed for 12 h at 22°C. At the end

of the dialysis experiment, free phenoxazine modulator from the buffer medium was extracted into *n*-heptane. The organic layer was then evaporated and phenoxazine concentration measured calorimetrically using 4 ml of 50% sulphuric acid containing 10 mg % FeCl₃.

2.3 Displacement of phenoxazine modulator from BSA binding site by equilibrium dialysis

The displacement of phenoxazines by hydroxyzine or acetylsalicylic acid was studied by means of equilibrium dialysis. In these experiments, binding of the phenoxazine modulator to BSA was determined after a displacing agent, either hydroxyzine or acetylsalicylic acid, was added to the incubation mixture.

2.4 Measurement of lipophilicity

Relative lipophilicity at *pH* 7.2 of each of the compounds used in this study was assessed using an adaptation of the method of Zamora *et al*¹⁰. This method involves measuring the partitioning of modulator between 1-octanol and phosphate buffer solution (PBS) (*pH* 7.2). High pressure liquid chromatography (HPLC) grade 1-octanol was pre-saturated with aqueous phase buffer and, conversely, buffered aqueous phase was pre-saturated with HPLC grade 1-octanol before use. The modulator was dissolved in aqueous phase buffer/octanol at a final concentration of 1×10^{-4} M, an equal volume of 1-octanol/buffer was added and the tubes were then continuously inverted for 15 min (experiments carried out over time intervals ranging from 5 to 60 min confirmed that equilibration was reached within 15 min). The final concentration of modulator in both aqueous and octanol fractions was assessed by measuring the UV absorbance of these experimental fractions. The partition coefficient, *P*, was determined by dividing the concentration of modulator in the 1-octanol phase by the concentration in the aqueous phase. Log₁₀*P* was used as a measure of lipophilicity.

3. Results and discussion

3.1 Evaluation of binding parameters

The binding of phenoxazine modulators (MPP, MBP, MPCP, PPCP and MPTP) to BSA, based on dialysis experiments, was characterized by the parameters, percentage of bound modulator (β), association constant (K_1), apparent binding constant (*k*) and free energy change (ΔF°) (table 2). The symbols, dimensions and methods of analysis of the values used to characterize the protein binding and the hydrophobic character of the modulators are summarized in table 3. Results of the gel filtration experiment show that the bound phenoxazine modulator moves with the velocity of BSA. When experiments were performed in series for one substance, only the fractions after the protein zone have been assayed for phenoxazine modulator.

The effect of concentration of phenoxazine modulator (MPP, MBP, MPCP, PPCP or MPTP) on the binding to BSA was studied by dialysis experiments. In these studies, concentrations of the modulator were varied in the range $0.1-5 \times 10^{-4}$ M, keeping the protein concentration constant (1%). The data obtained are shown in figure 1. Examination of the data shows that binding increases with increasing concentration of

phenoxazine at low modulator/protein ratios. The amount of free drug remains the same in spite of the fact that the concentration of the modulator is further increased (figure 1), thereby suggesting more binding sites on BSA, an observation similar to that made in the case of binding of phenothiazines to BSA¹. The apparent binding constant k and regression coefficient m were calculated by plotting concentration of free-phenoxazine modulator vs. concentration of bound-phenoxazine modulator (figure 2). In order to calculate the total binding constant, K_1 , the Scatchard plot for the binding of the

Table 2. Binding of several phenoxazine derivatives to bovine serum albumin.

Phenoxazine derivative	Percentage of bound drug (β) ^a	Regression coefficient (m) ^b	Apparent binding constant (k)	Associated constant $K_1 \times 10^{-4}$ (M^{-1}) ^c	Free energy of binding $-\Delta F^{0d}$ (cal M^{-1})	Log (β/α)
10-(3'-N-morpholinopropyl)-phenoxazine (MPP)	31.78	0.38	0.48	1.3	5553	-0.3317
10-(3'-N-morpholinobutyl)-phenoxazine (MBP)	54.40	1.11	1.28	3.1	6063	0.0766
10-(3'-N-morpholinopropyl)-2-chlorophenoxazine (MPCP)	66.64	1.73	1.99	4.2	6241	0.3005
10-(3'-N-piperidinopropyl)-2-chlorophenoxazine (PPCP)	77.68	3.29	3.99	9.9	6701	0.5416
10-(3'-N-morpholinopropyl)-2-trifluoromethyl phenoxazine (MPTP)	73.60	2.58	2.91	5.8	6430	0.4453

^a β is the percentage of bound drug in a 1% BSA solution with a total concentration $c = 10^{-4}M$ of phenoxazine modulator

^b m , the regression coefficient and k , the apparent binding constant, were obtained from figure 2, see also table 3

^c K_1 is the association constant obtained from the Scatchard plot (figure 3)

^d ΔF^0 is the free binding energy calculated from $-RT \ln K$

Table 3. Symbols, dimensions and methods of analysis of the parameters used.

Parameter*	Symbol	Dimension	Method of analysis
Total conc. of deriv.	c	M	By weight, colourimetry
Conc. of free deriv.	c_f	M	Gel filtration, dialysis
Conc. of bound deriv.	c_b	M	$c_b = c - c_f$
Conc. of albumin	c_a	g/100 ml	By weight, biuret method
Percent. of free deriv.	α	%	Gel filtration, dialysis
Percent. of bound deriv.	β	%	$\beta = 100 - \alpha$
Sp. binding capacity	\bar{r}	M/M	$\bar{r} = c_b/c_a$ in moles
Regression coeff.	m	-	Figure 2
Appar. binding const.	k	-	$k = c_b/c_f$ (figure 2)
Assoc. const.	K_1	$10^4 M^{-1}$	Scatchard plot (figure 3)
Free binding energy	ΔF^0	Cal/M	$\Delta F^0 = -RT \ln K_1$
Partition coeff.	P	M/M	Partition between <i>n</i> -octanol and buffer solution, pH 7.2

*Abbreviations: Conc. – concentration; deriv. – derivative; percent. – percentage; sp. – specific; coeff. – coefficient; appar. – apparent; assoc. – association; const. – constant.

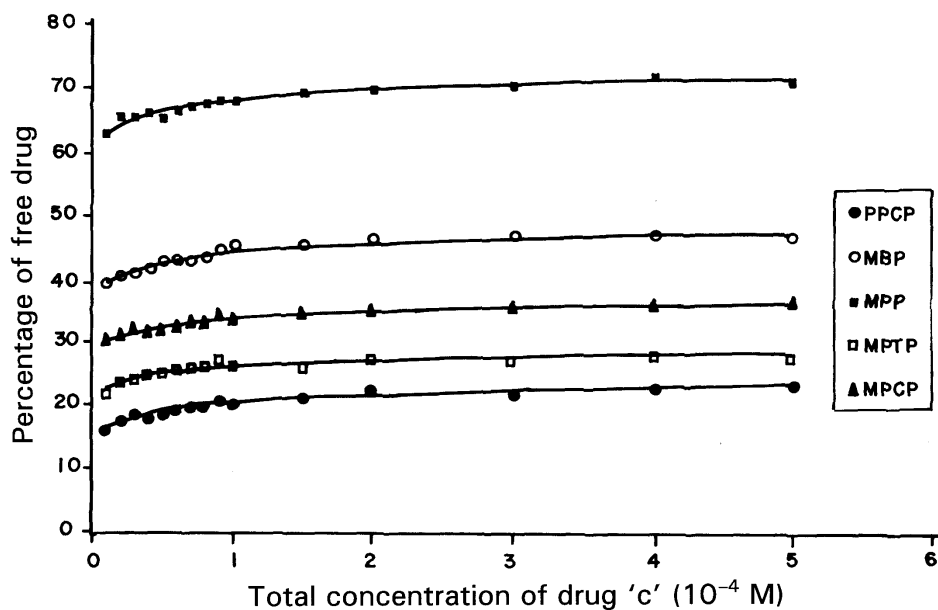


Figure 1. Binding of varying concentrations of the phenoxazine modulators to bovine serum albumin. Plots of percentage of free phenoxazine derivative versus total concentration of phenoxazine derivative (10^{-4} M). Binding measurements were carried out in a 1% BSA solution (pH 6.8, 22°C , incubated at 37°C for 6 h). Each point represents the mean value of two experiments.

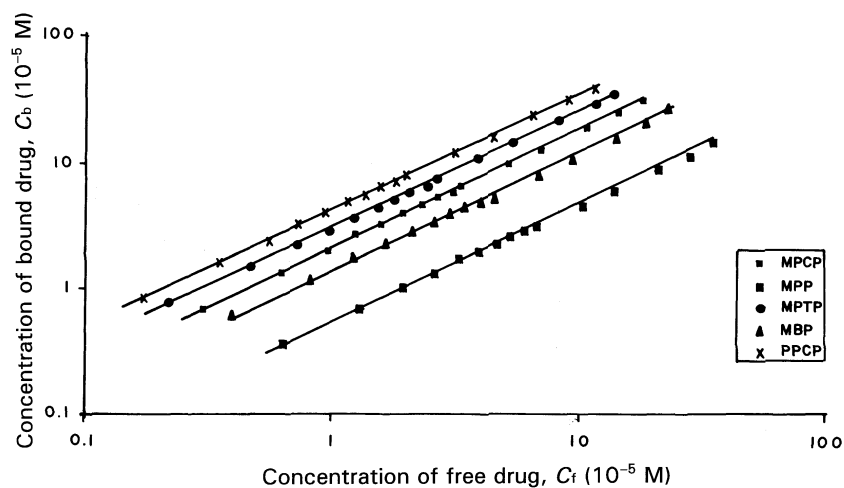


Figure 2. Binding capacity of a 1% albumin solution for phenoxazine derivatives. Plots of concentration C_b of bound phenoxazine derivative (0.1×10^{-5} to 100×10^{-5} M) vs. concentration of free phenoxazine derivative (0.1×10^{-5} to 100×10^{-5} M). Binding measurements were carried out in 1% BSA solution (pH 6.8, 22°C , incubated at 37°C for 6 h). Each point represents a single experiment. Here we obtain the binding constants m and k , see also table 2. For statistical data see table 4.

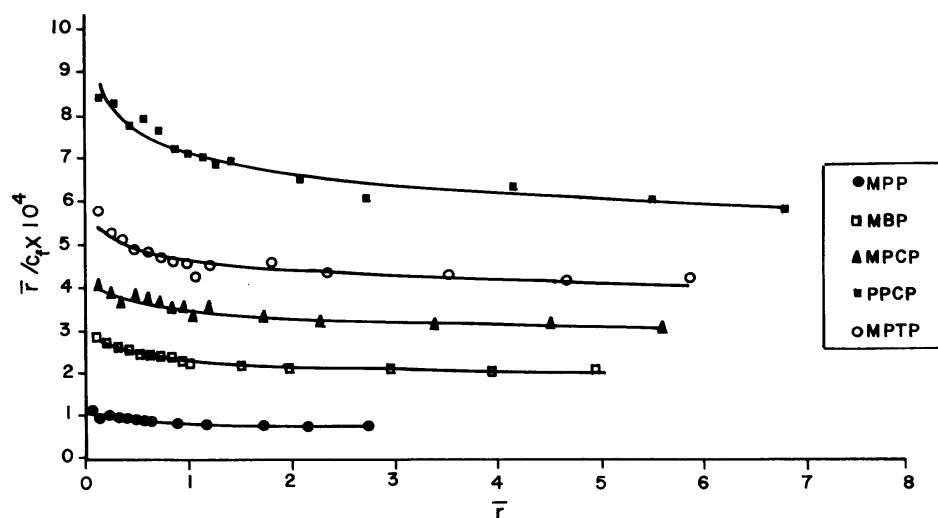


Figure 3. Scatchard plot of the binding of the phenoxazine derivatives to bovine serum albumin. \bar{r}/c_f in 10^{-4} M^{-1} vs. number of moles of phenoxazine per mole of albumin (\bar{r}). All measurements were made in 1% albumin solution ($\text{pH} = 6.8$, 22°C). Each point represents the mean value of two single experiments. For total binding constant K_1 see table 2.

Table 4. Statistical data for the binding of varying concentrations of phenoxazine derivatives with bovine serum albumin.

In all the cases, the number of experiments = 26

Derivative	Equation of the regression line in the double logarithmic system	Correl. coeff. (r)	Significance of r
MPP	$y = 0.0414 + 0.38 x$	0.9970	Significant
MBP	$y = 0.0434 + 1.11 x$	0.9890	Significant
MPCP	$y = 0.0568 + 1.73 x$	0.9990	Significant
PPCP	$y = 0.0900 + 3.20 x$	0.9980	Significant
MPTP	$y = 0.0443 + 2.58 x$	0.9990	Significant

Abbreviations: Correl. – correlation; coeff. – coefficient

Table 5. Statistical data for the displacement of phenoxazine derivatives from their albumin binding sites by hydroxyzine.

Number of parallel experiments in each case = 20

Phenoxazine deriv.	Eqn. of the regression line $y =$	Correlation coefficient (r)	Significance of r
MPP + Hyd.	$65.93 + 0.263x$	0.9183	Significant
MBP + Hyd.	$46.39 + 0.305x$	0.9761	Significant
MPCP + Hyd.	$28.39 + 0.355x$	0.8930	Significant
PPCP + Hyd.	$20.02 + 0.483x$	0.9902	Significant
MPTP + Hyd.	$25.26 + 0.200x$	0.9619	Significant

Abbreviations: Deriv. – derivative; eqn. – equation; Hyd. – hydroxyzine

phenoxazine modulators to BSA was obtained (figure 3). Comparison of the k and K_1 values within the compounds examined, showed that the modulators bind to BSA in the order: PPCP > MPTP > MPCP > MBP > MPP, indicating that phenoxazines containing $-\text{CF}_3$ or $-\text{Cl}$ in position C-2 have greater affinity to the protein than those containing $-\text{H}$ in that position. Binding of these compounds to BSA was supported by ΔF° values (table 2). Further, the compounds at IC_{10} were examined for their ability to modulate the cytotoxicity of VLB against KBCh^R-8-5 cells and the data shows that anti-MDR activity increases in the order PPCP > MPTP \approx MPCP > MBP > MPP^{4,6-9}. This rank order is the same as for the binding efficiency of these compounds with BSA.

3.2 Relationship between bovine serum albumin binding and hydrophobic character of phenoxazine modulators

Several authors have demonstrated a correlation between the hydrophobic character and protein binding of low molecular weight substances¹¹⁻¹⁵. These results suggest that hydrophobic interactions play an important role in protein binding of organic compounds. However, a good correlation between protein binding and partition coefficients can be shown only for substances of structurally related groups. Hence, not only can hydrophobic interactions be present in protein binding of organic molecules, but also other binding mechanisms such as ionic binding, hydrogen bonding or steric effects, etc. must be also involved¹⁶.

The hydrophobic character of phenoxazine modulators ($\log_{10}P$) was determined (table 1) to look for a possible quantitative correlation between the lipid solubility of these compounds and their protein binding ability. Analysis of the relationship between lipid

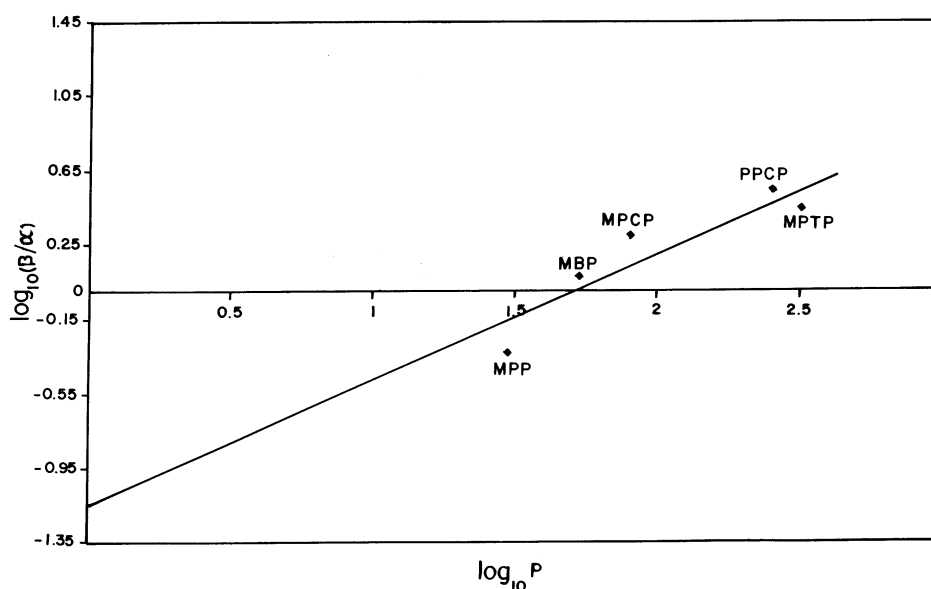


Figure 4. Relation between albumin binding and partition coefficients of phenoxazine derivatives. $\log(\beta/\alpha)$ vs. $\log_{10}P$. The equation of the regression line is $\log(\beta/\alpha) = -1.251 + 0.729 \log_{10}P$.

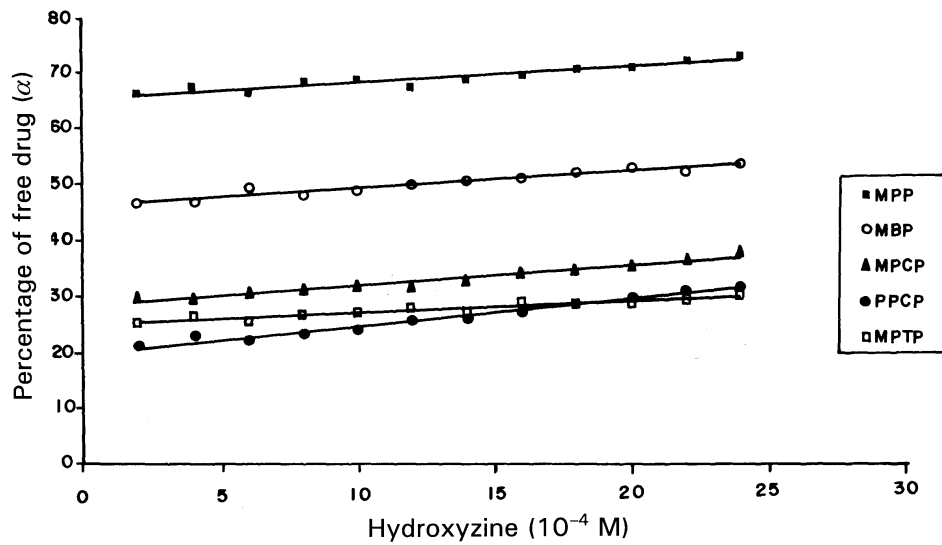


Figure 5. Influence of hydroxyzine on the binding of phenoxazine derivatives to bovine serum albumin. Percentage of free phenoxazine derivative in the albumin solution vs. total concentration of hydroxyzine (10^{-4} M). All measurements were made in 1% albumin solution containing 10^{-4} M, phenoxazine derivative and varying concentrations of hydroxyzine. Each point represents the mean value of two single experiments. For statistical evaluations see table 5.

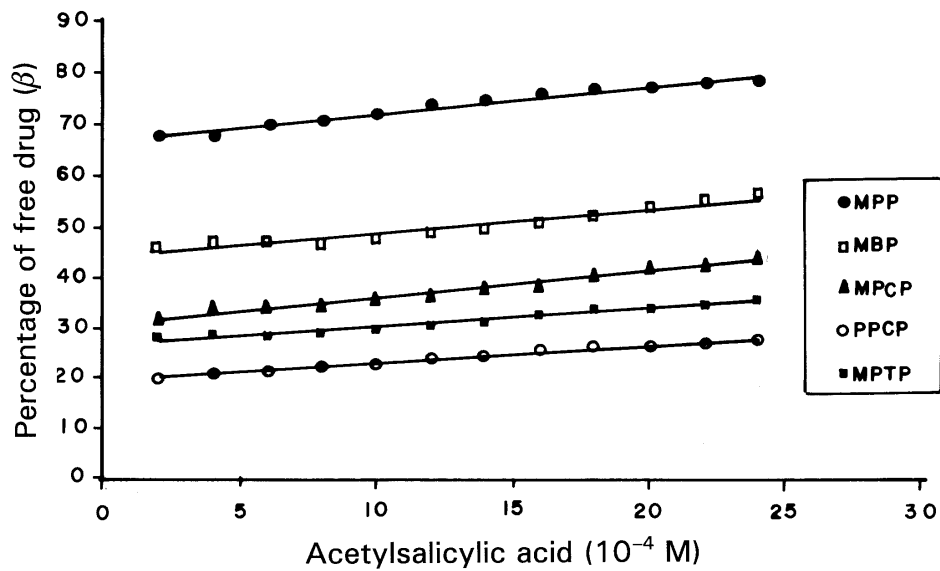


Figure 6. Displacement of phenoxazine derivatives by acetylsalicylic acid from binding to bovine serum albumin. Percentage of free phenoxazine derivative vs. total concentration of acetylsalicylic acid (10^{-4} M). All measurements were made in a 1% albumin solution containing one of the phenoxazine derivatives 10^{-4} M and varying concentrations of acetylsalicylic acid. Each point represents the mean value of two single experiments.

solubility of modulators used in this study and the fraction, $\log(\beta/\alpha)$, where β is the percentage of bound phenoxazine and α is the percentage of free phenoxazine modulator showed a good correlation (figure 4). (β/α) function is preferred for this type of correlation because it is directly analogous to the organic solvent-buffer partition coefficient. The following equation was arrived at from the $\log(\beta/\alpha)$ and $\log_{10}P$ data for five phenoxazine derivatives substituted in position C-2 of the phenoxazine nucleus: $\log(\beta/\alpha) = -1.251 + 0.729 \log_{10} P$ (correlation coefficient $r = 0.91784$). For the five phenoxazine modulators, hydrophobicity decreased in the order: MPTP > PPCP > MPCP > MBP > MPP. Hence, it is apparent from these results that the BSA binding of the phenoxazine derivatives with substituents in position C-2 of the phenoxazine nucleus increases with their hydrophobic character.

3.3 Displacement of phenoxazine modulators from their bovine serum albumin binding sites

Simple aromatic substances like benzoic acid or aniline are able to displace phenothiazines from their binding sites on the albumin molecule^{17,18}. In order to understand the BSA binding moieties of the phenoxazine modulators (MPP, MBP, MPCP, PPCP and MPTP), the authors have studied the displacement experiments by the dialysis method using hydroxyzine and acetylsalicylic acid. In these experiments, the binding of the phenoxazine modulators to BSA was determined after the displacing agent, hydroxyzine or acetylsalicylic acid, was added to the incubation mixture.

Participation of the side chain in the total binding of phenoxazine derivatives is indicated in displacement experiments with hydroxyzine and the data are shown in figure 5. Examination of the data shows that hydroxyzine displaces MPTP, MPP and MBP by about 5–6% and PPCP and MPCP by about 10–12% from their BSA binding sites. This might be explained by assuming that the aliphatic side chain between the N¹⁰- of the phenoxazine nucleus and the nitrogen atom of the side-chain tertiary amine is not in a position to contribute significantly to the binding of the phenoxazine modulators used.

Acetylsalicylic acid competes with the benzene rings of the phenothiazine ring system for binding to BSA¹. Since acetylsalicylic acid competes with the phenothiazine ring system for binding to BSA, the authors have examined the effect of acetylsalicylic acid as displacing agent to determine whether the benzene rings of phenoxazine modulator are involved in binding to BSA. These experimental data are shown in figure 6. Examination of the data from figure 6 has highlighted that acetylsalicylic acid displaces phenoxazine modulators (PPCP and MPTP by 6–7%, MPCP, MBP and MPP by 9–10%) from their BSA binding sites.

In summary, the results of the investigation suggest that phenoxazines are bound to albumin possibly by hydrophobic interactions.

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