pH 2 with concentrated HCl, and evaporated. Recrystallization of the residue from EtOH–Et_2O afforded 1.25 g (43%) of **40**·HCl as white plates, mp 199.5–201 °C. After repeated recrystallizations from EtOH–Et_2O, a purified sample melted at 209–211.5 °C.

3-(α,α,β,β-Tetrafluorophenethyl)-*o*-xylene α,α′-Dibromide (41). A stirred mixture of 6.13 g (0.022 mol) of **2h**, 7.85 g (0.044 mol) of NBS, a trace of benzoyl peroxide, and 175 ml of CCl_4 was refluxed for 6 h and filtered, and the filtrate was evaporated to dryness. Recrystallization of the oily solid residue from petroleum ether gave 4.3 g of white crystals, mp 88–92 °C. The concentrated mother liquor deposited a second crop that was recrystallized from petroleum ether to obtain 1.0 g: mp 85–91 °C; combined yield, 54%. A sample for analysis was sublimed at 85 °C (0.02 mm): mp 90–92 °C; NMR ($CDCl_3$) 4.44 (2 H, br s with fine splitting, 2-CH_2Br), 4.70 (2 H, s, 1-CH_2Br). Anal. ($C_{16}H_{12}Br_2F_4$) C, H, Br.

***N,N′*-Dimethyl-3-(α,α,β,β-tetrafluorophenethyl)-*o*-xylene-α,α′-diamine (42).** The dibromide **41**, 6.0 g (0.0136 mol), was added to 100 ml of $MeNH_2$(l) cooled in a dry ice–Me_2CO bath. After 45 min, the bath was removed and the solution allowed to evaporate. Trituration of the residue with C_6H_6 followed by filtration and evaporation of the filtrate left crude **42** as a gummy solid. A solution of the base in EtOH treated with HBr(g) and diluted with Et_2O yielded the dihydrobromide salt that was recrystallized twice from EtOH: yield, 3.8 g (55%); mp 250–252 °C.

***N,N′*-Dibenzyl-3-(α,α,β,β-tetrafluorophenethyl)-*o*-xylene-α,α′-diamine Hydrobromide (44) and *N*-Benzyl-4-(α,α,β,β-tetrafluorophenethyl)isoindoline (43).** A solution of 10.46 g (0.0238 mol) of **41** and 7.65 g (0.0715 mol) of benzylamine in 240 ml of C_6H_6 was stirred at room temperature for 15 min and at reflux for 3 h. The precipitate of benzylamine hydrobromide was collected and the filtrate evaporated. Trituration of the residual oil with Et_2O yielded crystalline **44**·HBr that was recrystallized from C_6H_6–EtOH–Et_2O to obtain 3.5 g (26%), mp 173–176 °C. Recrystallization from Me_2CO gave mp 176–177.5 °C.

The Et_2O filtrate from the trituration of the diamine **44**·HBr was evaporated. A solution of the residual oil in 25 ml of MeOH–2.5 ml of 48% HBr was diluted with Et_2O to precipitate **43**·HBr that was recrystallized from EtOH: yield, 1.29 g (11%); mp 235–238 °C. Recrystallization from MeOH–Et_2O using charcoal gave white crystals, mp 236.5–238.5 °C. Anal. ($C_{23}H_{19}F_4N$·HBr) C, H, N.

4-(α,α,β,β-Tetrafluorophenethyl)isoindoline (45). A solution of 1.63 g (0.0035 mol) of **43**·HBr in 160 ml of EtOH–14 ml of MeOH was hydrogenated at 1 atm and 25 °C over 5% Pd/C (320 mg) until uptake ceased. The mixture was filtered and evaporated, and the residual solid was recrystallized from EtOH–Et_2O using charcoal to obtain 1.0 g (78%) of **45**·HBr, mp 190–192 °C. Recrystallization from *i*-PrOH gave mp 192–194 °C.

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References and Notes

(1) Presented at the 167th National Meeting of the American Chemical Society, Los Angeles, Calif., April 1974, MEDI 40.
(2) W. R. Hasek, W. C. Smith, and V. A. Engelhardt, *J. Am. Chem. Soc.*, **82**, 543 (1960).
(3) L. A. Bigelow and R. S. Hanslick, "Organic Syntheses", Collect. Vol. II, Wiley, New York, N.Y., 1943, p 244.
(4) (a) G. Ascanio, F. Barrera, E. V. Lantsch, and M. J. Oppenheimer, *Am. J. Physiol.*, **209**, 1081 (1965); (b) F. Barrera, G. Ascanio, J. H. Boutwell, M. P. Panis, and M. J. Oppenheimer, *Am. J. Med. Sci.*, **252**, 177 (1966).
(5) Details of the testing method have been described previously.[6]
(6) M. L. Torchiana, C. A. Stone, H. C. Wenger, R. Evans, B. Lagerquist, and T. O'Malley, *J. Pharmacol. Exp. Ther.*, **194**, 415 (1975).
(7) M. Koral and E. I. Becker, *J. Org. Chem.*, **27**, 1038 (1962).
(8) (a) E. P. Kohler and W. D. Peterson, *J. Am. Chem. Soc.*, **55**, 1080 (1933); (b) J. H. Speer and A. J. Hill, *J. Org. Chem.*, **2**, 142 (1937).
(9) H. H. Hatt, A. Pilgrim, and W. J. Hurran, *J. Chem. Soc.*, 93 (1936).
(10) M. Friedman, *J. Org. Chem.*, **30**, 859 (1965).
(11) R. T. Arnold and R. C. Fuson, *J. Am. Chem. Soc.*, **58**, 1295 (1936).
(12) H. T. Clarke and E. E. Dreger, "Organic Syntheses", Collect. Vol. I, Wiley, New York, N.Y., 1943, p 87.
(13) H. L. Haller, P. D. Barglett, N. L. Drake, M. S. Newman, S. J. Cristol, C. M. Baker, R. A. Hayes, G. W. Kilmer, B. Magerlein, G. P. Mueller, A. Schneider, and W. Wheatley, *J. Am. Chem. Soc.*, **67**, 1591 (1945).

Quantitative Correlations between Albumin Binding Constants and Chromatographic R_M Values of Phenothiazine Derivatives

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The binding constants of 15 phenothiazine derivatives to bovine serum albumin were obtained by a circular dichroic probe technique. The lipophilicity of the drugs, measured by a reversed-phase thin-layer technique using oleyl alcohol and methanol–water mixtures as the solvents, is expressed as R_{M_w}. The binding constants were of the same order of magnitude as the literature values, and the R_{M_w} values correlated well with log $P_{octanol}$ values from the literature. Correlations of log K with R_{M_w} were found to be more satisfactory when corrections for the state of ionization of the phenothiazines were made, the nonprotonated species accounting for the bulk of the binding. A better correlation was obtained when contributions from both species were taken into account. Similar correlations were attempted between R_{M_w} values and enzyme inhibitory activities of these phenothiazines taken from the literature.

The binding of phenothiazine derivatives to bovine serum albumin (BSA) has been studied by several authors using a variety of techniques.[1-8] Although most of the authors obtained total binding constants of the same order of magnitude, the number of binding sites varied considerably. Janchen et al.[8] found 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. They suggested that phenothiazine derivatives are bound by hydrophobic interaction with the aromatic amino acids of the BSA molecule and that, under the influence of high drug concentrations, the number of available sites increased by swelling and unfolding of the BSA molecules in solution. In the literature cited,[1-8] the binding of the phenothiazine drugs to BSA is considered to be the result

of hydrophobic interactions, as was proven to be the case for a wide range of other organic compounds.[9] Glasser and Krieglstein[5] correlated the log $P_{octanol}$ values (at pH 7.4) of some phenothiazine drugs and related compounds with their log β/α values, β and α being the fractions of bound drug and free drug, respectively. They obtained a fairly good linear correlation (r = 0.969) for five 10-dimethylaminopropyl derivatives of phenothiazine. When other drugs were included the correlation deteriorated. Kriegelstein et al.[6] found that perazine, a propylpiperazine derivative, did not fit the linear correlation between log $P_{octanol}$ and log β/α, obtained with the other compounds. The aim of the present work was to investigate the interaction with BSA of a larger number of phenothiazine derivatives with different substituents at C_2 and N_{10}, to correlate the binding constants with R_{M_w} values, obtained from reversed-phase thin-layer chromatography (RP TLC) experiments, and to look for possible quantitative correlations between the hydrophobicity of these compounds (as expressed by R_{M_w}) and literature values of their biological activity.

In view of the instability of the phenothiazine derivatives and their tendency to escape the aqueous medium and adsorb at every available surface that is less polar than water, all the experiments in which aqueous solutions of phenothiazine drugs are involved should preferably be of short duration, and contact of the solutions with other materials than glass should be avoided.

In the present work these conditions were met by using circular dichroism (CD) for the determination of the binding constants.[10] The values of these constants were calculated from the reduction of the ellipticity induced into sulfaethidole after its binding to BSA by the presence of the phenothiazine drugs, using the method of Perrin and Nelson.[11,12] Observations on the displacement of basic drugs from BSA by acidic drugs and vice versa have been described in the literature.[1,8,11] Phenothiazine derivatives have been reported to give small induced optical activities following their interaction with BSA;[13] this phenomenon was further investigated.

Experimental Section

1. **Materials.** The phenothiazine derivatives used in this study are listed in Table I and were obtained from drug companies or commercial sources. Sulfaethidole, N^1-(5-ethyl-1,3,4-thiazol-2-yl)sulfanilamide (Smith Kline & French), was recrystallized twice from water. The crystallized BSA was obtained from Sigma Chemical Co. (batch number 10C-8090). Distilled water was used throughout.

2. **pK_a^c Values Determination.**[14] The pK_a^c values in water (25 °C, ionic strength 0.1 M) of propericiazine, perazine, butaperazine, dixyrazine, fluphenazine, and perphenazine were determined by the method as described by Green.[15] After shaking the free base[16] with a borate buffer or a phosphate buffer, and removing the excess of solid compound by centrifugation, the UV absorption of the solution was measured using a Perkin-Elmer UV-VIS spectrophotometer Model 139. The K_a^c values were computed from the solubilities of the drugs at different pH values, measured with a Metrohm Präzisions pH meter (E510) using a Metrohm (EA121) combination glass electrode.

3. **R_{M_w} Values Determination.** Compound 8–10 and 13–16 (Table I) were chromatographed using a reversed-phase thin-layer technique which has been described previously.[17] The stationary nonpolar phase consisted of a Kieselguhr G layer which was directly impregnated with oleyl alcohol by coating the glass plates (20 × 20 cm) with a slurry of Kieselguhr G in a mixture of oleyl alcohol, acetone, and dioxane. The polar mobile phases were represented by methanol–water mixtures, containing ammonia and potassium chloride, of pH[x] 10.5 and ionic strength 0.1 M.[18] The methanol concentrations in the mobile phases ranged from 30 to 55% (w/w). For a given compound 10–12 determinations of the R_M value at each of the different methanol concentrations were performed. Graphs of R_M against the methanol concentration (%, v/v) were extrapolated to yield the R_{M_w} values (=R_M at 0% methanol) of the compounds.

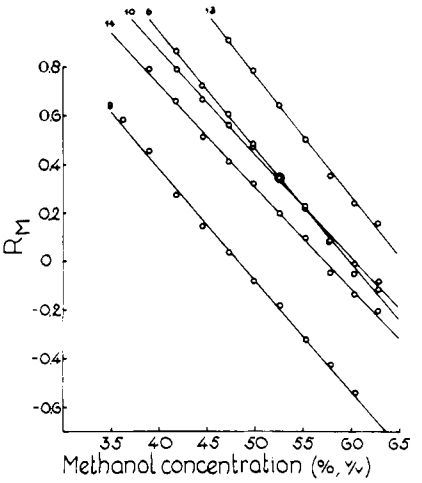


Figure 1. R_M values of some phenothiazine derivatives at different methanol concentrations. The graphs of the compounds are numbered according to Table I.

4. **Determination of the Effective Binding Constants of the Phenothiazine Drugs with BSA.** The method used is essentially the same as has been described by Perrin and Nelson.[11,12] Experimental conditions were such that the phenothiazine derivatives gave minimal induced optical activity in comparison to the large sulfaethidole signal. Little corrections were then necessary. The CD spectra were obtained with a Dichrographe III (Jobin Yvon, Long Jumeau, France) with a slit, programmed for a band-pass of 2 nm.

All solutions were prepared in a 0.054 M sodium phosphate buffer solution, pH 7.44 (25 °C), made isotonic with sodium chloride. The BSA concentration in all of the experiments was 1.45×10^{-5} and 2.11×10^{-5} M. Sulfaethidole solutions were used in the displacement studies. In the displacement experiments the solutions were scanned in 10-mm cells from 420 to 265 nm. The effects of the phenothiazines on the CD spectra of BSA were also investigated through the same wavelength region, and any correction necessary for the small effect of the compounds on the ellipticity was made. Solutions containing 1.45×10^{-5} mol/l. of both the phenothiazine drugs and BSA were scanned, in 50-mm cells, from 420 to 300 nm. (Solutions of butaperazine with BSA and propericiazine with BSA were also investigated at higher wavelengths.)

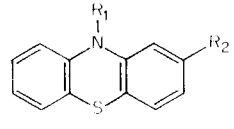
Results and Discussion

The relative partition coefficients of the free bases of the phenothiazine derivatives (compounds **6**, **8–10**, **13–16**, Table I) in the oleyl alcohol–water system were measured using the RP TLC technique as described above. Graphs of the R_M values of the compounds are shown in Figure 1. The resulting straight lines can be represented by[17]

$$R_M = R_{M_w} + bC \quad (1)$$

where C is the methanol concentration (%, v/v), b is a constant, and R_{M_w} is the R_M value at 0% methanol. Since R_{M_w} = log $P_{oleyl\ alcohol}$ + log r [r = phase-volume ratio (constant for a given chromatographic system) and $P_{oleyl\ alcohol}$ = partition coefficient in the oleyl alcohol–water system], and since log $P_{oleyl\ alcohol}$ is linearly correlated with log $P_{octanol}$ (or π)[19] with a slope that is all but equal to one,[17,20] the R_{M_w} values can be used in QSAR studies instead of log $P_{octanol}$, yielding the same type of correlations with only a different value for the intercepts.[21] Cyanopromazine, of which the R_{M_w} value had been determined previously together with the R_{M_w} values of compounds **1–5** (Table I),[17] served as a reference. The R_{M_w} values of compounds **6**, **8–10**, and **13–16** were corrected by adding

Table I. CD Characteristics of the Phenothiazine Derivatives on Binding to BSA



No.	Compd	R_1	R_2	λ region[a] (nm) of negative sign of induced opt act.	λ_{max},[b] nm	10^3-θ_{obsd},[b] deg	λ region[a] (nm) of positive sign of induced opt act.	λ_{max},[b] nm	10^3-θ_{obsd},[b] deg
1	Promazine	$-(CH_2)_3N(CH_3)_2$	$-H$	382–295			295		
2	Triflupromazine	$-(CH_2)_3N(CH_3)_2$	$-CF_3$	388–333 278	351	6.4	333–278	315	7.1
3	Chlorpromazine	$-(CH_2)_3N(CH_3)_2$	$-Cl$	384–320	345	2.0	291		*c*
4	Methopromazine	$-(CH_2)_3N(CH_3)_2$	$-OCH_3$	330–284			365–330 284	340	1.8
5	Methylpromazine	$-(CH_2)_3N(CH_3)_2$	$-CH_3$	382–287	328	1.8	287		
6	Cyanopromazine	$-(CH_2)_3N(CH_3)_2$	$-CN$	338–300					
7	Thioridazine	$-2-(CH_2)_2-c-NC_5H_9-1-CH_3$	$-SCH_3$	388–287	338	4.3	287		
8	Propericiazine	$-(CH_2)_3-c-NC_5H_9-4-OH$	$-CN$	750–440 347–300	600		440–347 300	370	2.0
9	Mesoridazine	$-2-(CH_2)_2-c-NC_5H_9-1-CH_3$	$-SOCH_3$						
10	Perazine	$-(CH_2)_3-c-N(CH_2CH_2)_2N-4-CH_3$	$-H$	365–289	314	2.6	289		
11	Trifluoperazine	$-(CH_2)_3-c-N(CH_2CH_2)_2N-4-CH_3$	$-CF_3$	398–348 318–292	365	1.8	348–318	333	2.2
12	Prochlorperazine	$-(CH_2)_3-c-N(CH_2CH_2)_2N-4-CH_3$	$-Cl$	385–289	330	3.0	289		
13	Butaperazine	$-(CH_2)_3-c-N(CH_2CH_2)_2N-4-CH_3$	$-COC_3H_7$	±750–440 348–327 309–289	590 333	 2.3	440–348 327–309 289	382 317	3.1 3.3
14	Dixyrazine	$-CH_2CHCH_3CH_2-c-N(CH_2CH_2)_2N-4-CH_2CH_2OCH_2(CH_2OH)$	$-H$	350–310			310		
15	Fluphenazine	$-(CH_2)_3-c-N(CH_2CH_2)_2N-4-CH_2CH_2OH$	$-CF_3$	386–358 317–291			358–317 291		
16	Perphenazine	$-(CH_2)_3-c-N(CH_2CH_2)_2N-4-CH_2CH_2OH$	$-Cl$	388					

[a] Values of λ (indicating the end of a wavelength region) lower than 250 nm are not mentioned. [b] Uncertain or inaccurate values of λ_{max} and θ_{obsd} are left out. [c] No measurable induced optical activity between 320 and 291 nm.

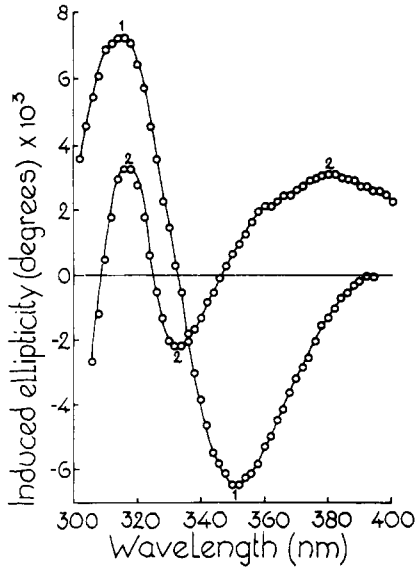


Figure 2. Induced optical activity of triflupromazine (1) and butaperazine (2) in the presence of BSA. Concentrations of triflupromazine, butaperazine, and BSA: 1.45×10^{-5} M (50-mm cell).

to them the difference between the R_{M_w} values of cyanopromazine in both investigations. This difference was found to be 0.06. The resulting corrected R_{M_w} values are shown in Table II. The R_{M_w} values of compounds **1–4**, **10**, **15**, and **16** (Table I) were correlated with the log $P_{octanol}$ values from the literature[22] (Table II). The correlation was found to be

$$R_{M_w} = 1.006\ (\pm 0.105) \log P - 1.277\ (\pm 0.484) \quad (2)$$
$$n = 7;\ r = 0.974;\ s = 0.155$$

Sjöholm and Sjödin[13] found that all of the phenothiazine drugs which were investigated by them showed induced optical activity in the wavelength region between 350 and 250 nm following binding to human serum albumin (HSA) for a drug/HSA ratio of 10:1. They also observed similarities between the UV spectra and the difference CD spectra of these compounds, especially in the wavelength region between 300 and 250 nm. With the current equipment it was possible to investigate the CD spectra of 1.45×10^{-5} M solutions of BSA, to which equimolar concentrations of the phenothiazine derivatives had been added. The CD spectrum of BSA with triflupromazine showed a well-defined biphasic curve (Figure 2) with a negative peak at 351 nm and a positive peak at 315 nm. Scanning solutions of triflupromazine, in different concentrations, with BSA revealed an isobestic point at 333 nm. (It is interesting to notice that the difference UV spectra of some phenothiazine derivatives with BSA displayed characteristically two positive and two negative peaks at 260, 330 and 250, 290 nm, respectively.[23])

The CD characteristics of the phenothiazine drugs–BSA solutions are shown in Table I. Where possible the wavelength of maximal ellipticity, λ_{max}, and the observed ellipticity, θ_{obsd}, have been mentioned. The CD spectra of BSA with butaperazine (Figure 2) and with propericiazine also showed deviations from the BSA spectrum in the region of 800–400 nm. For the dimethylaminopropyl derivatives, the height of the difference CD signals at λ_{max} of the negative peak in the 400–300-nm region decreased in the following order: triflupromazine > chlorpromazine > methylpromazine and promazine > cyanopromazine. This rank order is the same as for the hydrophobicity of these compounds expressed by log $P_{octanol}$ or R_{M_w}.[17] The

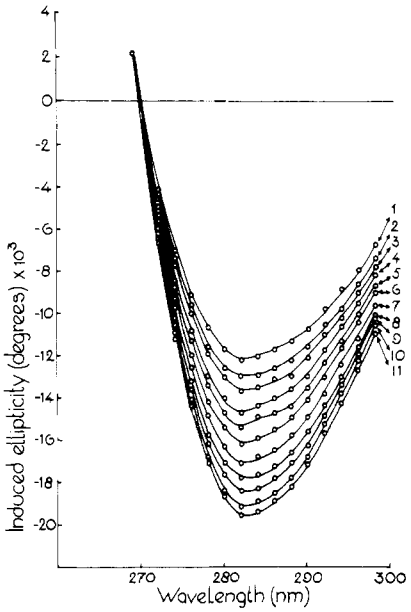


Figure 3. Extrinsic Cotton effect curves for sulfaethidole in the presence of BSA and chlorpromazine. Concentrations: BSA, 1.45×10^{-5} M; sulfaethidole, 2.11×10^{-5} M (11); chlorpromazine, 2.00×10^{-4} M (1), 1.80×10^{-4} M (2), 1.60×10^{-4} M (3), 1.40×10^{-4} M (4), 1.20×10^{-4} M (5), 1.00×10^{-4} M (6), 8.0×10^{-5} M (7), 6.0×10^{-5} M (8), 4.0×10^{-5} M (9), and 2.0×10^{-5} M (10) (10-mm cell).

induced optical activities shown by promazine, cyanopromazine, dixyrazine, fluphenazine, and perphenazine following the binding to BSA are too small to enable binding parameters being measured with sufficient accuracy. For QSAR studies all of the binding constants should be measured by the same method and so it was decided to determine the binding constants from the ability of the compounds to displace sulfaethidole from its primary binding site on BSA. The ellipticity of the sulfaethidole–BSA complex was never less than 20 times that of the phenothiazine–BSA complex at the wavelength of measurement. The ellipticity induced into the sulfaethidole after binding to BSA was reduced in the presence of each of the phenothiazines. The effect of various concentrations of chlorpromazine is shown in Figure 3. The induced curves are shown uncorrected for the small effect of chlorpromazine alone. When corrected, the curves are all of the same shape having a negative peak near 280 nm with an isobestic point at 269 nm. With the exception of thioridazine and butaperazine, all of the other phenothiazines caused similar CD spectra. Butaperazine and thioridazine caused the negative peak to shift to a slightly higher wavelength (about 282 nm). The CD curves in the presence of cyanopromazine and propericiazine were at the higher concentrations of these compounds disturbed by the high light absorption in the wavelength region between 280 and 265 nm. The changes in the induced CD spectra of the sulfaethidole–BSA complex were measured at 286 nm for cyanopromazine, at 282 nm for propericiazine, thioridazine, and butaperazine, and at 280 nm for all of the other compounds. Sulfaethidole was shown to bind with BSA on one single site of high affinity and three secondary sites of lower affinity.[24] The binding constant for the high affinity site, measured by CD, was found to be 2.1×10^5 l. mol^{-1}. The concentrations of BSA and of sulfaethidole were chosen so that 70% of the available primary binding sites on the albumin is filled with sulfaethidole, whereas less than 1% of the total drug concentration is attached to the secondary sites. Since,

Table II. pK_a^c, R_{M_W}, Log $P_{octanol}$, and Log K Values of the Phenothiazine Derivatives

										BSA binding constants from CD measurements				
			Values from RP TLC											
No.	Compd	pK_a^c	R_{M_W}	s^a	n^b	Log P_{oct}^c	Log K	s^a	n^b	Concn range, M	Log K + log $\left(\frac{K_a^c + [H^+]}{K_a^c}\right)$	Log K_D^h	Log K^d	Log K^e
1	Promazine	9.42[f]	3.43[g]	0.03	128	4.68	3.21	0.03	10	5.0×10^{-5}–3.0×10^{-4}	5.32	4.54	3.95, 4.00	3.45
2	Triflupromazine	9.21[f]	4.24[g]	0.04	76	5.32	3.88	0.03	12	1.5×10^{-5}–1.65×10^{-4}	5.78	5.17	4.58	3.99
3	Chlorpromazine	9.36[f]	4.12[g]	0.04	76	5.35	3.66	0.03	10	2.0×10^{-5}–2.0×10^{-4}	5.71	4.98	4.32	3.79
4	Methopromazine	9.41[f]	3.19[g]	0.03	128	4.63	3.33	0.03	10	4.0×10^{-5}–4.0×10^{-4}	5.43	4.66	4.11	
5	Methylpromazine	9.36[f]	3.78[g]	0.02	134		3.37	0.05	10	2.5×10^{-5}–2.5×10^{-4}	5.42	4.69		
6	Cyanopromazine	9.29[f]	2.94[g]	0.02	140		3.15	0.02	8	3.0×10^{-5}–2.4×10^{-4}	5.13	4.46		
7	Thioridazine	9.5[h]	4.51[i]				3.81	0.04	10	1.0×10^{-5}–1.0×10^{-4}	6.00	5.15		
8	Propericiazine	8.5	2.17	0.03	163		3.11	0.08	8	2.5×10^{-5}–1.25×10^{-4}	4.32	4.12		
9	Mesoridazine		2.32	0.02	164									
10	Perazine	8.1	2.67	0.02	140	3.80	3.60	0.02	10	2.5×10^{-5}–2.5×10^{-4}	4.45	4.36	4.32	3.33
11	Trifluoperazine	8.1[h]	3.55[j]				3.91	0.02	10	6.25×10^{-5}–6.25×10^{-5}	4.76	4.67		3.80
12	Prochlorperazine	8.1[h]	3.38[j]				3.83	0.04	10	7.5×10^{-6}–7.5×10^{-5}	4.68	4.59		3.70
13	Butaperazine	8.1	3.37	0.04	106		4.02	0.04	10	1.0×10^{-5}–1.0×10^{-4}	4.87	4.78		
14	Dixyrazine	8.0	2.49	0.02	152		3.52	0.06	9	2.0×10^{-5}–5.0×10^{-5}	4.29	4.21		
15	Fluphenazine	7.9	3.03	0.02	140	4.47	3.79	0.03	8	2.0×10^{-5}–9.0×10^{-5}	4.48	4.41		
16	Perphenazine	7.9	2.74	0.02	140	3.92	3.57	0.03	9	2.0×10^{-5}–1.0×10^{-4}	4.26	4.20		

[a] s = standard deviation. [b] n = number of determinations. [c] Log $P_{octanol}$ values from ref 6 and 22. [d] BSA binding constants from ref 6 and 7. [e] Intrinsic binding constants from ref 3. [f] pK_a^c values from ref 29. [g] Values from ref 17. [h] pK_a^c values from ref 15. [i] Calculated from R_{M_W} values of mesoridazine and π values of ref 38. [j] Calculated from R_{M_W} of perazine and π values of ref 38. Log K_D = log K + log $(K_a^c + [H^+])$ − log $(K_a^c + \phi[H^+])$.

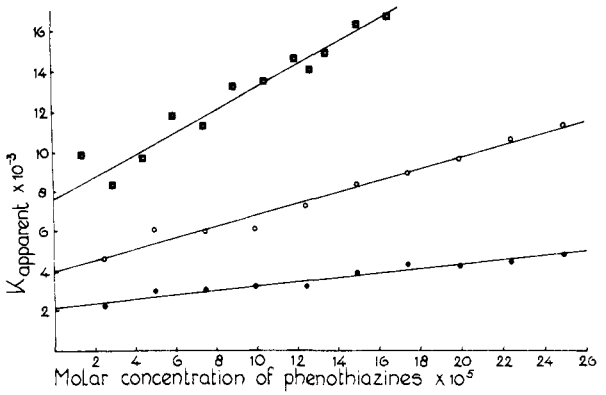


Figure 4. Apparent binding constants of phenothiazine derivatives for the primary sulfaethidole binding site on BSA as a function of concentration: (□) triflupromazine, (○) perazine, (●) methylpromazine.

furthermore, the binding of sulfaethidole to the secondary sites does not cause any induced optical activity, binding to these secondary sites was ignored.

Using the method as previously described,[12] apparent binding constants were calculated for the phenothiazines at different ligand concentrations. Making a correction for the small effect of the phenothiazine derivatives on the BSA spectrum alone, the apparent binding constant, K_{app}, can be calculated with eq 3 (eq 3 results from the equation of Klotz et al.[25] with n, the number of binding sites, equal to 1)

$$K_{app} = \frac{2.1 \times 10^5\left[1.45 \times 10^{-5} - C \times\left[1+\frac{1}{(2.11 \times 10^{-5} - C)2.1 \times 10^5}\right]\right] \times (2.11 \times 10^{-5} - C)}{C\left[C' - (1.45 \times 10^{-5} - C) \times\left[1+\frac{1}{(2.11 \times 10^{-5} - C)2.1 \times 10^5}\right]\right]} \quad (3)$$

where C' is the total concentration of the phenothiazine drug, and C is the concentration of the sulfaethidole bound to BSA

$$C = 1.012 \times 10^{-5}\left(\frac{b + \frac{a-b}{a}\cdot\frac{C'}{C''}\cdot c}{a}\right) \quad (4)$$

in which a is the peak height of the induced CD spectrum of the sulfaethidole–BSA solution alone, b is the peak height after addition of the phenothiazine drug, and c is the difference in the CD signals of the BSA solution alone and of BSA with the phenothiazine drug at a concentration of C''. (b and c are supposed to have opposite signs in eq 4; if not, $-c$ must be inserted in eq 4).

Graphs of K_{app} against the drug concentration (Figure 4) showed a linear relationship. Extrapolation to zero drug concentration eliminated problems due to binding of the phenothiazine drugs to sites of lower affinity. The resulting binding constants (at zero concentration) can be considered as the binding constants of the compounds at the primary binding site of sulfaethidole on BSA (Table II). The binding constants found for the compounds are of the same order of magnitude as the values found with other techniques[3,6,7] (Table II). Krieglstein et al.[4] concluded from their experiments on the binding of phenothiazine derivatives to BSA at low drug/protein ratios that there is possibly only one binding site on the albumin molecule with a comparatively high affinity for the phenothiazine derivatives. (At higher total phenothiazine concentrations an increasing number of binding sites was found.[8]) With a few minor exceptions there is no change in the characteristics of the induced CD curves of sulfaethidole upon addition of the phenothiazine drugs. It is therefore unlikely that the reduction in the binding of sulfaethidole to BSA is due to conformational changes in the albumin caused by the presence of the phenothiazine drugs. This reduction in binding can probably be attributed to a competition between the phenothiazine drugs and the sulfaethidole at the primary binding site of sulfaethidole on BSA. This may be a direct competition, or else the same area of the binding site is shared by the competing ligands, as suggested by Tanford.[26] The correlation between the log K values of compounds **1–4** and **10**, obtained from the displacement studies, with the log K_{GF} values from gel filtration (and equilibrium dialysis) experiments[6,7] was found to be

$$\log K = 1.110\ (\pm 0.087) \log K_{GF} - 1.190\ (\pm 0.372) \quad (5)$$
$$n = 5;\ r = 0.991;\ s = 0.042$$

(The numbers in parentheses are the standard deviations.) Three equilibria in the solutions of BSA with the phenothiazine drugs should be considered, assuming the drug causes no conformational changes in the BSA

$$DH^+ \rightleftharpoons D + H^+ \quad (a)$$

$$D + P \rightleftharpoons D\cdot P \quad (b)$$

$$DH^+ + P \rightleftharpoons DH^+\cdot P \quad (c)$$

where D and DH^+ are the free base and the protonated species, respectively, and P represents the BSA. The equilibrium constants are denoted by K_a^c, K_D, and K_{DH^+}, where K_D is the binding constant of the free base and K_{DH^+} is the binding constant of the protonated species. The symbol K is attributed to the observed binding constant.

$$K_a^c = [D][H^+]/[DH^+] \quad (6)$$

$$K_D = [D\cdot P]/[D][P] \quad (7)$$

$$K_{DH^+} = [DH^+\cdot P]/[DH^+][P] \quad (8)$$

$$K = ([D\cdot P] + [DH^+\cdot P])/([D] + [DH^+])[P] \quad (9)$$

Substitution of eq 7 and 8 into eq 9 yields

$$K = \frac{K_D[D] + K_{DH^+}[DH^+]}{[D] + [DH^+]} \quad (10)$$

and substitution of eq 6 into eq 10 results in

$$K = \frac{K_a^c K_D + K_{DH^+}[H^+]}{K_a^c + [H^+]} \quad (11)$$

If only the free base binds to the BSA ($K_{DH^+} = 0$), then eq 11 develops into

$$K_D = K\frac{K_a^c + [H^+]}{K_a^c}$$

or

$$\log K_D = \log K + \log\left(\frac{K_a^c + [H^+]}{K_a^c}\right) \quad (12)$$

An analogous expression for acids was derived by Fujita and Hansch,[27] who assumed that only one form of the

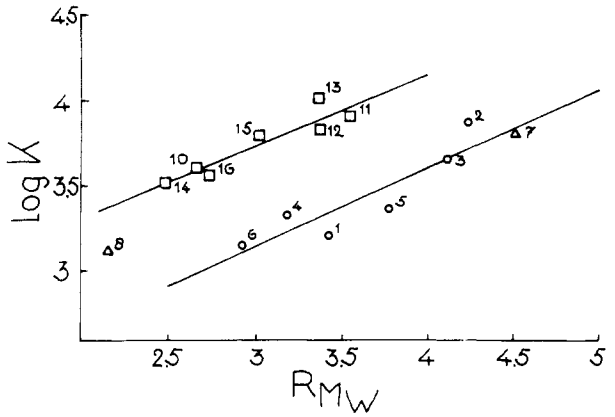


Figure 5. Logarithm of effective binding constants of phenothiazine derivatives as a function of their R_{M_w} values. The compounds are numbered according to Table I: (○) dimethylaminopropyl derivatives 1–6; (△) alkylpiperidyl derivatives 7 and 8; (□) propylpiperazine derivatives 10–16.

compounds (sulfanilamides) was bound to BSA. If only the protonated form binds to BSA ($K_D = 0$), eq 11 becomes

$$K_{DH^+} = K\frac{K_a^c + [H^+]}{[H^+]}$$

or

$$\log K_{DH^+} = \log K + \log\left(\frac{K_a^c + [H^+]}{[H^+]}\right) \quad (13)$$

If there is no difference in the binding capacities of the free base and the protonated species ($K_D = K = K_{DH^+}$), then K is independent of pH. If this were the case for the phenothiazine derivatives, and assuming that the binding of these compounds to BSA is caused by hydrophobic bonding, then a graph of log K against R_{M_w} (Figure 5) should result in a straight line. This is obviously not true for the phenothiazine drugs. The binding of promazine and of chlorpromazine was found to increase with increasing pH.[2,3,23,28] This may suggest that the free base molecules are more strongly bound to BSA than the protonated form. Similar observations on the pH dependence of the binding of promazine to BSA were made by Krieglstein and Kuschinsky,[7] who explained this increased binding at higher pH values by an increase of positive charges on the BSA molecules and by swelling and unfolding of the BSA molecules. Correcting for the degree of ionization (eq 12) yielded values for the binding constants of the free bases as shown in Table II [log K + log $[(K_a^c + [H^+])/K_a^c]$]. These values were correlated with the R_{M_w} values of the compounds.

$$\log K + \log [(K_a^c + [H^+])/K_a^c] = 0.778 (\pm 0.117) R_{M_w} + 2.419 (\pm 0.394) \quad (14)$$
$$n = 15; r = 0.879; s = 0.291$$

Comparing eq 14 with eq 15 and 16

$$\log K + \log [(K_a^c + [H^+])/[H^+]] = 0.180 (\pm 0.121) R_{M_w} + 3.029 (\pm 0.408) \quad (15)$$
$$n = 15; r = 0.381; s = 0.302$$

$$\log K = 0.211 (\pm 0.108) R_{M_w} + 2.886 (\pm 0.362) \quad (16)$$
$$n = 15; r = 0.477; s = 0.268$$

It is obvious that eq 14 gives the best fit, strongly suggesting that the free base molecules are mainly responsible for the BSA binding, as had already been suggested in the literature.[1,3,5] Values of log K + log $[(K_a^c + [H^+])/K_a^c]$ were plotted against R_{M_w} (Figure 6), and it is manifest that compounds **1–7**, having pK_a^c values of 9.2–9.5, and compounds **10–16**, with pK_a^c values of 7.9–8.1, are scattered, as in Figure 5, around two distinctly separated and almost parallel lines. (Similar results were found when the log K and log K_D values from the work of Nambu and Nagai[3] were plotted against R_{M_w}). The correlations of log K + log $[(K_a^c + [H^+])/K_a^c]$ with R_{M_w} for these two series of compounds separately are presented by eq 17 and 18. For compounds **1–7**

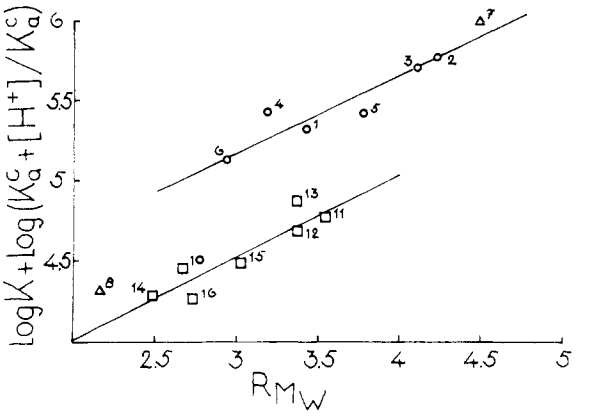


Figure 6. Logarithm of the binding constants of phenothiazine derivatives, corrected for ionization, as a function of the R_{M_w} values. For details see Figure 5 and Table I.

$$\log K + \log [(K_a^c + [H^+])/K_a^c] = 0.489 (\pm 0.075) R_{M_w} + 3.712 (\pm 0.284) \quad (17)$$
$$n = 7; r = 0.946; s = 0.107$$

and for compounds **10–16**

$$\log K + \log[(K_a^c + [H^+])/K_a^c] = 0.517 (\pm 0.107) R_{M_w} + 2.973 (\pm 0.328) \quad (18)$$
$$n = 7; r = 0.907; s = 0.108$$

Both eq 17 and 18 show a reasonably good fit, with about the same slope, but different values for the intercepts. When correlating the log K values with the R_{M_w} values for the two series, we obtained for compounds **1–7**

$$\log K = 0.468 (\pm 0.085) R_{M_w} + 1.736 (\pm 0.323) \quad (19)$$
$$n = 7; r = 0.926; s = 0.122$$

and for compounds **10–16**

$$\log K = 0.428 (\pm 0.076) R_{M_w} + 2.452 (\pm 0.231) \quad (20)$$
$$n = 7; r = 0.930; s = 0.076$$

The intercept of eq 19 is smaller than that of eq 20. However, the intercept of eq 17 is larger than that of eq 18. This can be explained by assuming that the protonated species participates in the protein binding to an extent that cannot be neglected. (This is confirmed by the investigations of Curry,[28] who found that even at pH 1.7 a considerable fraction of chlorpromazine was bound to HSA.) Thioridazine was found to be situated on the graphs of the promazines (compounds **1–6**) which have a different N-substituent but about the same pK_a^c as thioridazine. Propericiazine, which is, like thioridazine,

an alkylpiperidyl derivative, but has a different pK_a^c, did not lie on these graphs (Figures 5 and 6). Also, compounds **10–16** have different substituents at N_{10}, but approximately equal pK_a^c values, and all these compounds are scattered around the same lines. This suggests that the ratio of the free base/protonated species determines the position of the compounds in these graphs, rather than the chemical nature of the N_{10} substituent. For both series of compounds (**1–7** and **10–16**) hydrophobic bonding was found to be predominant (eq 17 and 18). After correction of log K for the degree of protonation, making allowances for the binding due to the protonated species, the resulting log K_D values may be expected to correlate better with R_{M_w} than the log K + log $[(K_a^c + [H^+])/K_a^c]$ values. A correction term should then be added to log K, which reflects the influence of the pK_a^c as well as the difference in binding constants of the protonated species and of the free base. Since only the pK_a^c, and not the type of the N-substituent, had a marked influence on the positions of the compounds in Figures 5 and 6, it was assumed that the relative contribution of the protonated species to the binding of the compounds to BSA was the same for all of the phenothiazines or

$$K_{DH^+} = \phi \cdot K_D \tag{21}$$

where ϕ is a constant. Substitution of eq 21 in eq 11 and rearranging results in

$$K = K_D \frac{K_a^c + \phi[H^+]}{K_a^c + [H^+]}$$

or

$$\log K_D = \log K + \log (K_a^c + [H^+]) - \log (K_a^c + \phi[H^+]) \tag{22}$$

Correlations of these log K_D values with R_{M_w} can be represented by

$$\log K + \log (K_a^c + [H^+]) - \log (K_a^c + \phi[H^+]) = aR_{M_w} + b \tag{23}$$

For the evaluation of ϕ the following procedure was used. Two (theoretical) compounds, A and B, with the same R_{M_w} value but with different log K values were considered. Compound A was supposed to belong to the same group as compounds **1–7** and compound B to that of compounds **10–16**. The R_{M_w} value was chosen to be 3.39, this value being the arithmetic mean of the mean R_{M_w} values of each of the series of compounds. Substitution into eq 19 gives log K = 3.32 for A, and further substitution into eq 17 gives a K_a^c value of 4.38×10^{-10}. This value for K_a^c and the value for $[H^+]$ at pH 7.4 ($[H^+] = 4.84 \times 10^{-8}$) were inserted into eq 23 to give

$$3.32 + \log (4.38 \times 10^{-10} + 4.84 \times 10^{-8}) - \log (4.38 \times 10^{-10} + \phi \times 4.84 \times 10^{-8}) = aR_{M_w} + 3.39 \tag{24}$$

In the same way eq 25 was derived for compound B.

$$3.90 + \log (8.55 \times 10^{-9} + 4.84 \times 10^{-8}) - \log (8.55 \times 10^{-9} + \phi \times 4.84 \times 10^{-8}) = aR_{M_w} + 3.39 \tag{25}$$

ϕ was solved from eq 24 and 25 to yield a value of 3.93×10^{-2}.

The log K_D values, calculated by inserting this value of ϕ into eq 22, are presented in Table II. The correlation between log K_D and R_{M_w} was found to be

$$\log K_D = 0.469\ (\pm 0.038)\ R_{M_w} + 3.049\ (\pm 0.129) \tag{26}$$
$$n = 15;\ r = 0.959;\ s = 0.095$$

Thus, by attributing some of the binding to the protonated species, considerable improvement in the correlation of the albumin binding of the phenothiazine drugs with their lipophilicities is obtained (compare eq 26 with eq 14). Krieglstein et al.[6] found that the amount of promethazine that was bound to BSA at pH 7.4 was higher than the amount of promazine bound to BSA. Both molecules differ only in the position of the dimethylamino group in the propyl chain, and the authors explained this anomaly by supposing that the *n*-propyl chain of promazine is not in a position to contribute to the binding of the drug to BSA, whereas the methyl group in the aliphatic side chain of promethazine may lie sufficiently close to the surface of the albumin molecule to intensify the binding of the drug molecule by hydrophobic interaction. The pK_a^c value of promethazine[15] is 9.1 and the pK_a^c of promazine[29] is 9.42. The higher effective binding constant of promethazine at pH 7.4 can therefore easily be explained by the higher ratio of free base/protonated species for promethazine as compared with promazine. The discrepancy found by Krieglstein et al.[6] between the observed and calculated values of α for perazine can also be explained in the same way.

The values of the albumin (BSA) binding constants, $K_{dial.}$, of compounds **1–3** and **10–12** have been determined by Nambu and Nagai,[3] using a dialysis method at 10 °C and pH 7.0. The constants (Table II) are the values of the intrinsic binding constant for the first class of sites. The correlations between log $K_{dial.}$ and R_{M_w}, and between log $K_{dial.}$ + log $[(K_a^c + [H^+])/K_a^c]$ and R_{M_w}, were found to be

$$\log K_{dial.} = 0.377\ (\pm 0.103)\ R_{M_w} + 2.334\ (\pm 0.372) \tag{27}$$
$$n = 6;\ r = 0.877;\ s = 0.131$$

$$\log K_{dial.} + \log\left(\frac{K_a^c + [H^+]}{K_a^c}\right) = 1.149\ (\pm 0.343)\ R_{M_w} + 1.437\ (\pm 1.234) \tag{28}$$
$$n = 6;\ r = 0.859;\ s = 0.436$$

Although these correlations are significant ($\alpha < 0.05$) it is obvious from the values of the slopes that eq 27 and 28 do not give a realistic picture of the BSA binding process.[9] However, when applying the same procedure as described above, the correlation became, with $\phi = 5.6 \times 10^{-2}$

$$(\log K_D)_{dial.} = \log K_{dial.} + \log (K_a^c + [H^+]) - \log (K_a^c + \phi[H^+]) = 0.555\ (\pm 0.036)\ R_{M_w} + 2.791\ (\pm 0.128) \tag{29}$$
$$n = 6;\ r = 0.992;\ s = 0.045$$

Apart from the improved statistics of eq 29 as compared with eq 28 and 27, the slope of eq 29 is now within the range of 0.60 ± 0.13, a value suggested by Helmer et al.[9] for this type of correlation. Correlation of these $(\log K_D)_{dial.}$ values with log K_D from the CD experiments (Table II) resulted in eq 30.

$$(\log K_D)_{dial.} = 1.034\ (\pm 0.116)\ \log K_D - 0.108\ (\pm 0.547) \tag{30}$$
$$n = 6;\ r = 0.976;\ s = 0.078$$

Thus the same log K_D values were found for the free bases

of the phenothiazine drugs using two different methods and solutions of different pH. The above explanation of the binding in terms of predominantly hydrophobic interaction is in contrast to the recent publication of Sharples[30] who suggested that the interaction between some phenothiazines and BSA was predominantly electronic rather than hydrophobic in origin, following fluorescence quenching and partition coefficient measurements. For these measurements a solvent of 90% acetone in water was used as the aqueous phase and some pairs of molecules were found to have R_M values reversed from those expected.[31] These differences in experimental design may account for the discrepancies between the work of Sharples and that reported here.

It is of interest to see if the same type of correlation is suitable for establishing quantitative structure–activity correlations for other biological processes, using biological activity data from the literature. Using the numbers of Frisk-Holmberg and van der Kleyn[22] for the histamine-releasing activity of six phenothiazine drugs (compounds **1**, **3**, **11**, **12**, **15**, **16**), the following correlations were derived (ED_{50} is the drug concentration giving 50% release of the total histamine content of rat mast cells at pH 7.0)

$$\log (1/ED_{50} \times 10^{-3}) = -0.078\ (\pm 0.314)\ R_{M_w} + 2.858\ (\pm 1.068) \quad (31)$$
$$n = 6;\ r = 0.123;\ s = 0.331$$

$$\log (1/ED_{50} \times 10^{-3}) + \log [(K_a^c + [H^+])/K_a^c] = 0.986\ (\pm 0.228)\ R_{M_w} + 0.879\ (\pm 0.777) \quad (32)$$
$$n = 6;\ r = 0.907;\ s = 0.241$$

$$\log (1/ED_{50} \times 10^{-3}) + \log (K_a^c + [H^+]) - \log (K_a^c + \phi[H^+]) = 0.563\ (\pm 0.048)\ R_{M_w} + 2.049\ (\pm 0.163) \quad (33)$$
$$n = 6;\ r = 0.986;\ s = 0.051$$

(with $\phi = 1.1 \times 10^{-2}$). Although, contrary to the statements of Frisk-Holmberg and van der Kleyn, the histamine releasing activity (expressed by $\log 1/ED_{50} \times 10^{-3}$) shows a significant relationship ($\alpha < 0.02$, correlation coefficient 0.907, eq 32) with the partition coefficient of the free bases (R_{M_w}), the correlation was further improved (eq 33) by assigning about 1% of the activity to the protonated molecules using the procedure as described above. This caused the slope of the correlation to become within the range which is usually found for the correlations between $\log P_{octanol}$ and the binding to macromolecules.[9]

Next, the antihemolytic activity of eight phenothiazine drugs[32] (compounds **1–3**, **7**, **11**, **12**, **15**, **16**) could be correlated with the partitioning behavior in the same way. The activity is expressed as $\log 1/C$, where C is the concentration of the drug which causes 50% antihemolysis of dog erythrocytes. The correlations are

$$\log 1/C = 0.105\ (\pm 1.419)\ R_{M_w} + 4.592\ (\pm 0.629) \quad (34)$$
$$n = 8;\ r = 0.243;\ s = 0.279$$

$$\log 1/C + \log [(K_a^c + [H^+])/K_a^c] = 1.058\ (\pm 0.164)\ R_{M_w} + 2.967\ (\pm 0.600) \quad (35)$$
$$n = 8;\ r = 0.935;\ s = 0.266$$

With the values for promazine excluded, eq 35 turned into

$$\log 1/C + \log [(K_a^c + [H^+])/K_a^c] = 1.102\ (\pm 0.095)\ R_{M_w} + 2.732\ (\pm 0.351) \quad (36)$$
$$n = 7;\ r = 0.982;\ s = 0.153$$

Equations 35 and 36 could not be improved by attributing some of the activity to the protonated species.

Hansch and Glave[33] investigated the membrane-perturbing properties of compounds as a function of their lipophilicities. $pC - \log P_{octanol}$ correlations (pC is the negative logarithm of the concentration necessary for a certain biological action) showed slopes in the range of 0.93 ± 0.17. The slopes of eq 35 and 36 are in agreement with this value and also with the slopes of the correlations found between $\log P_{red\ cell\ ghost}$ and $\log P_{octanol}$. Evidently the ionic species of the phenothiazines does not participate in the antihemolytic activity at pH 7.4.

Murthy and Zografi[34] found that chlorpromazine did not partition as an ion pair at pH values higher than pH 7 in the octanol–water system. There seems to be a close parallel between the antihemolysis activity of the phenothiazines and their octanol (or oleyl alcohol)–water partitioning behavior.

Another action of the phenothiazine drugs, for which only the free bases appear to be responsible, is the inhibition of Na^+K^+-activated adenosine triphosphatase (Na^+K^+ATPase) activity at pH 7.4 in rat brain.[35] For compounds **1–3**, **7**, **11**, **12**, **15**, and **16**, the following correlations were obtained (BR is the percentage of inhibition caused by 10^{-4} M solutions of the phenothiazine drugs).

$$\log (BR) = 0.047\ (\pm 0.256)\ R_{M_w} + 1.542\ (\pm 0.935) \quad (37)$$
$$n = 8;\ r = 0.075;\ s = 0.418$$

$$\log (BR) + \log [(K_a^c + [H^+])/K_a^c] = 0.976\ (\pm 0.066)\ R_{M_w} - 0.358\ (\pm 0.241) \quad (38)$$
$$n = 8;\ r = 0.987;\ s = 0.108$$

In view of the slope of eq 38, the in vitro inhibition of Na^+K^+ATPase by the phenothiazine drugs seems to be another process which is closely correlated with the octanol–water partitioning behavior of these compounds. There is no clear pharmacological significance of this correlation, particularly as Akera and Brodie have shown that the in vitro inhibiting activity of chlorpromazine toward this same enzyme was greatly enhanced by UV irradiation.[36,37] However, the correlations of the biological activities of these phenothiazine derivatives, when corrected for the state of ionization, with their partitioning behavior cannot be ignored. Apart from correcting for the degree of ionization, it may also be necessary to take into account the small activity of the protonated species to obtain better quantitative correlations.

References and Notes

(1) H. Zia and J. C. Price, *J. Pharm. Sci.*, **64**, 1177 (1975).
(2) P. C. Huang and S. Gabay, *Biochem. Pharmacol.*, **23**, 957 (1974).
(3) N. Nambu and T. Nagai, *Chem. Pharm. Bull.*, **20**, 2463 (1972).
(4) J. Krieglstein, F. Lier, and J. Michaelis, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **272**, 121 (1972).
(5) H. Glasser and J. Krieglstein, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **265**, 321 (1970).
(6) J. Krieglstein, W. Meiler, and J. Staab, *Biochem. Pharmacol.*, **21**, 985 (1972).
(7) J. Krieglstein and G. Kuschinsky, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **262**, 1 (1969).
(8) F. Jänchen, J. Krieglstein, and G. Kuschinsky, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **263**, 375 (1969).
(9) F. Helmer, K. Kiehs, and C. Hansch, *Biochemistry*, **7**, 2858 (1968).
(10) J. H. Perrin and P. A. Hart, *J. Pharm. Sci.*, **596**, 431 (1970).
(11) J. H. Perrin and D. A. Nelson, *Biochem. Pharmacol.*, **23**, 3139 (1974).
(12) J. H. Perrin and D. A. Nelson, *J. Pharm. Pharmacol.*, **25**, 125 (1973).

(13) I. Sjöholm and T. Sjödin, *Biochem. Pharmacol.*, **21**, 3041 (1972).
(14) K_a^c is the concentration-dependent acid dissociation constant.
(15) A. L. Green, *J. Pharm. Pharmacol.*, **19**, 10 (1966).
(16) If the drug was a salt, the free base was prepared by extracting an alkaline suspension of the drug with dichloromethane (DCM) and by evaporating the washed DCM layer.
(17) A. Hulshoff and J. H. Perrin, *J. Chromatogr.*, **120**, 65 (1976).
(18) pH meter readouts of measurements in methanol–water mixtures, after standardizing the meter against a methanol–water buffer solution of the same methanol content, are denoted by the symbol pH^x.
(19) R. Collander, *Acta Chem. Scand.*, **5**, 774 (1951).
(20) A. Leo, C. Hansch, and D. Elkins, *Chem. Rev.*, **71**, 525 (1971).
(21) Care should be taken that adsorption of the compounds onto the support phase is excluded.
(22) M. Frisk-Holmberg and E. van der Kleyn, *Eur. J. Pharmacol.*, **18**, 139 (1972).
(23) S. Gabay and P. C. Huang, *Adv. Biochem. Psychopharmacol.*, **9**, 175 (1974).
(24) H. B. Kostenbauder, M. J. Awad, J. H. Perrin, and V. Averhart, *J. Pharm. Sci.*, **60**, 1658 (1971).
(25) I. M. Klotz, H. Triwush, and F. M. Walker, *J. Am. Chem. Soc.*, **70**, 2935 (1948).
(26) C. Tanford, "The Hydrophobic Effect", Wiley, New York, N.Y., 1973, p 134.
(27) T. Fujita and C. Hansch, *J. Med. Chem.*, **10**, 991 (1967).
(28) S. H. Curry, *J. Pharm. Pharmacol.*, **22**, 197 (1970).
(29) A. Hulshoff and J. H. Perrin, *Pharm. Acta Helv.*, **51**, 65 (1976).
(30) O. Sharples, *J. Pharm. Pharmacol.*, **28**, 100 (1976).
(31) J. H. Perrin and A. Hulshoff, *J. Pharm. Pharmacol.*, **28**, 793 (1976).
(32) P. Seeman, *Pharmacol. Rev.*, **24**, 583 (1972).
(33) C. Hansch and W. R. Glave, *Mol. Pharmacol.*, **7**, 337 (1971).
(34) K. S. Murthy and G. Zografi, *J. Pharm. Sci.*, **59**, 1281 (1970).
(35) P. W. Davis and T. M. Brody, *Biochem. Pharmacol.*, **15**, 703 (1966).
(36) T. Akera and T. M. Brody, *Mol. Pharmacol.*, **4**, 600 (1968).
(37) T. Akera and T. M. Brody, *Mol. Pharmacol.*, **5**, 605 (1969).
(38) C. Hansch, A. Leo, S. H. Unger, K. H. Kim, D. Nikaitani, and E. J. Lien, *J. Med. Chem.*, **16**, 1207 (1973).

p-Aminobenzoic Acid Derivatives. Mode of Action and Structure–Activity Relationships in a Cell-Free System (*Escherichia coli*)

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The agonistic and antagonistic effects of nuclearly substituted *p*-aminobenzoic acids (PABA) on the folate-synthesizing system of *E. coli* have been studied in whole cell and cell-free systems. All studied derivatives form dihydropteroic acid analogues in the presence of a cell-free folate-synthesizing enzyme system. A thin-layer chromatographic system has been elaborated to determine the rate of analogue formation in the cell-free system. Physicochemical parameters of the PABA derivatives, such as pK_a, π, and R_m values, have been determined. These values have been used in a structure–activity analysis which revealed that the rate of analogue formation in the absence of PABA is independent of the lipophilic properties. Ionization seems to be the decisive factor for the incorporation. As all studied PABA derivatives are totally ionized under the experimental conditions, the rates of analogue formation are very similar with the exception of compounds bearing bulky groups in the 2 position. The variance in inhibitory power may therefore either be due to differences in the ability of the analogues to serve as metabolites or to competition with PABA.

The general pathway of pteroic acid and folate synthesis, especially in *E. coli*, has been evaluated during the last decade by Brown,[2a] Jaenicke and Chan,[2b] and Shiota et al.[3] It has been shown that bacterial or plant cell-free folate-synthesizing extracts are inhibited by sulfonamides.[4,5] Bock et al.[6] have demonstrated that sulfonamides, in addition to competing with PABA for the active site of 7,8-dihydropteroate synthetase, also compete for the 7,8-dihydro-6-hydroxymethylpterin (H_2PtCH_2OH), the natural substrate, thereby forming a pteroic acid analogue that results in a diminished synthesis of pteroic acid per se (see Scheme I, A and B). More complicated and contradictory are the results reported on growth-promoting or inhibitory effects of PABA derivatives on different microorganisms. The complication may arise from the possibility that PABA derivatives incorporated into a folate analogue may or may not perform the function of the natural folate in various species,[7,8] whereas in case of the sulfonamides (SA) the reaction product of SA with phosphorylated 7,8-dihydro-6-hydroxymethylpterin (H_2PtCH_2OPP) cannot act as a folate metabolite.[6] Ariens and co-workers[9] reported the gradual change from growth factor to growth inhibitor for certain PABA derivatives for an *E. coli* strain which required PABA for growth. For 2-amino-5-carboxypyridine experimental results are published which make the incorporation of 2-amino-5-carboxypyridine into a folic acid analogue probable (for example, see ref 8). Wacker et al.[10] described the incorporation of *p*-aminosalicylic acid into a folate analogue by the folate-synthesizing enzyme system of enterococci (see Scheme I, C). The folate analogue could take over the function of the normal folate in this bacterial strain.

More recent work on the inhibition of folate-synthesizing enzyme extracts from *E. coli* by PABA derivatives has been published by Thijssen.[11,12] Using [^{14}C]-PABA the degree of inhibition of PABA incorporation into pteroic acid in the presence of PABA derivatives was determined by extraction of the [^{14}C]-PABA that had not reacted after a certain time interval. From these data Thijssen tried to derive some structure–activity correlations. With this technique, however, one is not able to decide whether the PABA derivatives are only competing with PABA for the active site of the enzyme or if there is also competition for the precursor H_2PtCH_2OH and resultant formation of dihydropteroic acid derivatives.

To decide this question we have developed techniques