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INTERACTION OF PROMAZINE WITH BOVINE SERUM ALBUMIN AND HEXADECYLTRIMETHYLAMMONIUM BROMIDE USING LIGHT ABSORPTION AND FLUOROMETRIC TECHNIQUES

JESS D. KEELER and RAGHUBIR P. SHARMA

Toxicology Curriculum, Department of Veterinary Science, Utah State University, Logan, Utah 84322, U.S.A.

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Abstract—The association of promazine with bovine serum albumin and ammonium detergent micelle was measured using the changes in the u.v. absorption and the fluorescence emission of the interactants. The endogenous fluorescent properties of promazine were not altered upon binding to bovine serum albumin. The association of promazine with protein could be determined by measuring the drug-induced fluorescence quenching of tryptophan emission. Such fluorescence-quenching reaction was pH sensitive. In bovine serum albumin binding of promazine, there was an increase in the extinction coefficient of the drug when associated with protein. Promazine binding to serum protein produced a hyperchromic change in the drug's absorption spectrum which paralleled the fluorescence-quenching reaction. The involvement of carboxyl groups in binding of promazine to protein molecules and the effect of the carboxyl groups on the emissions of both tyrosine and tryptophan are suggested. A slight increase in both absorption and fluorescence of promazine was observed when associated with detergent micelle. The addition of the detergent caused an increase in the extinction coefficient of promazine and a shift to longer wavelength of the absorption maximum.

THE INTERACTIONS of drugs with biological macromolecules have been the subject of considerable investigation. To appreciate the complexity of obtaining relative or statistical indices about the drug macromolecular interactions, the determination of such processes can be considered in the general framework of binding processes carried out with molecules less complicated than biological systems.

This study was undertaken to characterize the effect of phenothiazines on certain molecules that can serve as a model for complex biological systems, e.g. membranes, where such drugs are believed to have pharmacological effects.[1,2] The principal method employed in this study was the alteration of absorption and fluorescent emission properties of the interactants. Wallach *et al.*[3] suggested that the phenothiazines might be suitable acceptor molecules for the transfer of excitation energy by a non-radiative dipole–dipole interaction from the amino acid tryptophan and, as such, would provide a means for studying the alteration of protein properties associated with these compounds. Bovine serum albumin was chosen as a model protein with which to determine whether the transfer mechanism would provide useful understanding of the interactions of promazine with biological membranes. Promazine [10(3′-dimethyl-amino-1′-propyl)phenothiazine] hydrochloride, a prototype of pharmacologi-

cally active phenothiazine derivatives,[4] was used in the current study. Promazine has been known to associate with bovine serum albumin.[5]

The effect of environment polarity on the absorption and emission properties of the drug was also investigated by employing a positively charged micellular suspension of hexadecyltrimethylammonium bromide. This was undertaken since the hydrophobic interactions of the phenothiazines were viewed as being of importance to the compound's biological activities.[6]

Fluorescent spectroscopy has been widely used to study the association of the phenothiazine derivatives with biological macromolecules. Teller *et al.*[7] and Levine *et al.*[8] used the quenching of protein fluorescence to determine the amount of drug associated with several proteins and/or membrane. Seghatchian[9] used uncorrected fluorescent excitation and emission spectra to determine the association of chlorpromazine with the metal-containing enzyme aldolase.

METHODS

Materials. Promazine-HCl was a gift from Smith, Kline & French (Philadelphia, Pa.). Electrophoretically 100% pure bovine serum albumin was purchased from Swartz-Mann (Orangeburg, N.Y.). Hexadecyltrimethylammonium bromide, practical grade, was purchased from Eastman-Kodak (Rochester, N.Y.). All other chemicals were reagent grade. Triple glass distilled or deionized water was used for all procedures.

Instruments. The Aminco-Bowman model 4-8202 spectrophotofluorometer, calibrated by the method of Chen,[10] was used for all fluorescent measurements. A Coleman 124 double-beam spectrophotometer was used for ultraviolet absorption experiments. The titration apparatus used was a Beckman/Spinco 153 micro-titrator burette.

Fluorescence-quenching experiments. For bovine serum albumin, a molecular weight of 67,000 was assumed.[11] A typical serum albumin concentration employed was $2{\cdot}5 \times 10^{-6}$ M in Tris-Cl buffer at pH 7·4. The solution was stored overnight in a refrigerator to stabilize its fluorescence and equilibrated to 22° just prior to its use. Two ml of the solution was placed in a cuvette and the protein emission at 350 nm was determined upon excitation with 280 nm light. Submicroliter quantities of 1×10^{-2} M promazine–HCl in 0·01 M Tris-Cl buffer were added, mixed, and protein emission was redetermined. The quantity of drug introduced was controlled so as to produce at least a 1 per cent change in the intensity measurement. Drug was titrated until the change in fluorescence became constant and linear with further addition of the drug. All titrations were repeated several times and representative data used for the plots. All determinations were made at a constant temperature of $22 \pm 0{\cdot}3°$. The light absorption by promazine at the excitation and emission wavelengths used was minimal and linear in the entire range of concentrations, indicating an absence of any inner filter effect.

Double-reciprocal plots of the absolute values of the fluorescent change (corrected for dilution and slight light absorption from the final linear change) vs the concentration of drug were constructed after the method of Gomperts *et al.*[12] The negative values of the reciprocal concentration extrapolated from these reciprocal plots were used as a measure of the relative or apparent affinity constant. The reciprocal plots had more than one linear phase, and the extrapolated intercept for the different phases was interpreted as being due to binding sites of different affinities.

The resonance transfer distance between the bovine serum albumin tryptophan residue and promazine was estimated using the method described by Latt *et al.*[13]

Binding of promazine determined by difference absorption spectroscopy. The difference in ultraviolet absorption of promazine when bound to serum protein was determined by difference absorption spectroscopy. The optical density of the protein was determined initially at 301 nm and then promazine was titrated into both the sample and reference cuvettes. The difference in the optical densities, after subtracting the protein absorption, was taken as the measure of the binding. All measurements were made at 22°.

pH dependence of the promazine-induced quenching of protein emission. The pH of the protein solution was adjusted to the values of 3, 5, 7·4 and 9, using concentrated NaOH or HCl. Fluorescent-quenching titrations were then performed as outlined above.

Detergent effect on promazine optical properties. To a fixed concentration of promazine in Tris-Cl buffer at pH 7·4, variable concentrations of hexadecyltrimethylammonium bromide solution were added. The increase in the fluorescence emission from the drug was determined at 450 nm, when excitation took place at 301 nm. To determine if the increased fluorescence was due to an increased quantum yield, the fluorescent quantum yield of promazine was first determined relative to quinine sulfate by the method of Chen.[14] The change in the u.v. absorption of the drug in the presence of the ammonium detergent at micellular concentrations ($1{\cdot}8 \times 10^{-2}$ M in 0·01 M Tris-Cl buffer, pH 7·4) was determined.

RESULTS

The absence of an appreciable enhancement of fluorescence from the drug when interacted with the serum protein prevented the direct use of the fluorescence of promazine in determining the association of the interactants.

The double-reciprocal plots of the fluorescence quenching of serum albumin vs promazine concentration were biphasic in nature. A representative plot is shown in Fig. 1. The character of the fluorescence quenching was dependent on the pH of the medium. The fluorescence-quenching curves of the bovine serum albumin by varying

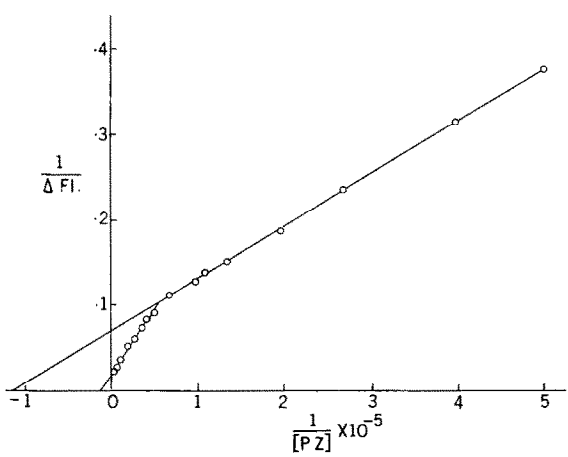

FIG. 1. Double-reciprocal plot of bovine serum albumin (BSA) fluorescence vs fluorescence quenching (arbitrary units) produced by the addition of promazine. BSA, $2{\cdot}5 \times 10^{-6}$ M in Tris-Cl 0·01 M buffer at pH 7·4. Excitation and emission recorded at 280 and 350 nm respectively.

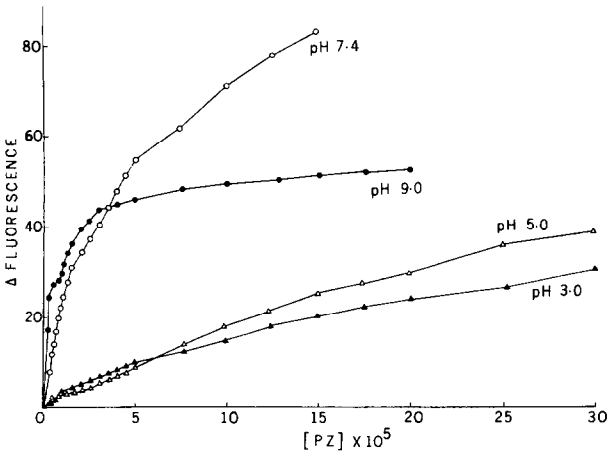

FIG. 2. pH dependence of promazine-quenching reaction for bovine serum albumin. Conditions are similar to those in Fig. 1, with the pH adjusted by the addition of HCl or NaOH. The affinity constants derived from double-reciprocal plots are given in Table 1. The intensity of protein fluorescence in the absence of added ligand was adjusted to be 100, and all other readings are relative to this value.

concentrations of promazine at different pH values are given in Fig. 2. The apparent affinity constants obtained from these curves are listed in Table 1.

In order to determine the effect of the concentration of ionized carboxyl groups on promazine's ability to quench protein fluorescence, the following calculations were carried out. The ratio of the observed protein fluorescence at the different concentrations of promazine to the initial protein was divided by the ratio of protein carboxyl groups ionized at different pH values to the concentration of the drug in the solution. The resulting numbers were plotted against the drug concentrations, and such a plot is shown in Fig. 3. The number of carboxyl groups in bovine serum albumin was taken to be 100 with an average p*K*a of 3·95.[11]

The u.v. absorption spectrum of bovine serum albumin alone was obtained, and the drug then titrated into both the sample and reference cells. Figure 4 is the comparison of the change in the absorption of the drug with the fluorescence quenching of bovine serum albumin by promazine.

If the mechanism by which promazine quenched protein fluorescence was by resonance transfer, then the distance of separation between the donor and acceptor

TABLE 1. EXTRAPOLATED APPARENT AFFINITY CONSTANTS AT DIFFERENT pH VALUES*

	Relative apparent affinity constants at two promazine concentrations	
pH	$<2 \times 10^{-5}$ M (M)	$> 2 \times 10^{-5}$ M (M)
3	$0{\cdot}03 \times 10^5$	$0{\cdot}4 \times 10^5$
5	$1{\cdot}0 \times 10^5$	$0{\cdot}06 \times 10^5$
7·4	$1{\cdot}1 \times 10^5$	$0{\cdot}2 \times 10^5$
9	$11{\cdot}0 \times 10^5$	$1{\cdot}5 \times 10^5$

* Constants were obtained from double-reciprocal plots of the data shown in Fig. 2.

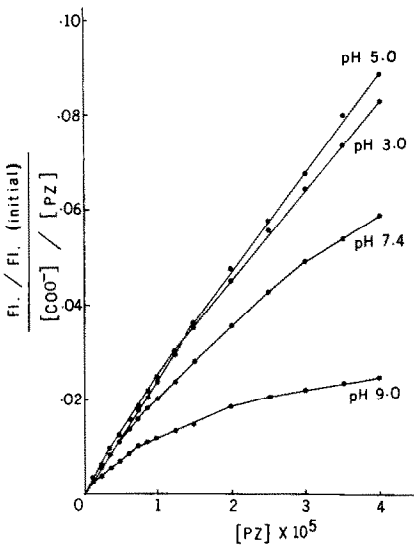

FIG. 3. Influence of carboxyl group ionization on pH dependence of promazine quenching. Data are from the experiments shown in Fig. 2. Details are in text.

would provide a measure of this possibility. The distance of separation was determined for a 50 per cent transfer probability. The normalized fluorescence emission spectrum of bovine serum albumin was determined in the absence of the drug. The u.v. absorption of promazine alone in the region of overlap was also determined. Figure 5 presents the overlap for the two spectra. The overlap integral (J) was evaluated using the numerical integration of Scheid.[15] The calculation of the separation distance was carried out by the method of Latt *et al.*[13] using a quantum yield for tryptophan in bovine serum albumin, $Q = 0{\cdot}259$;[16] a refractive index, $n = 1{\cdot}6$;[17] and the mutual

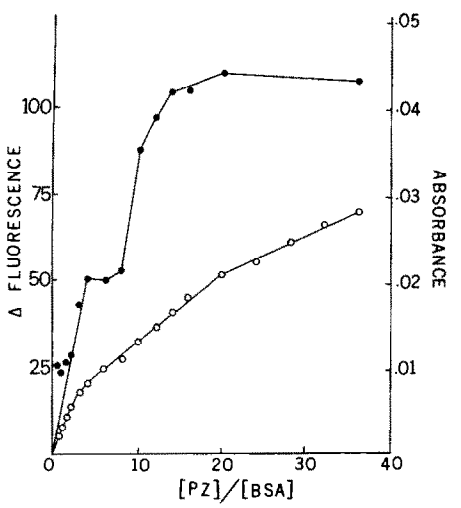

FIG. 4. Comparison of the change in promazine absorption at 301 nm (●—●) with promazine quenching of bovine serum albumin (○—○) at 350 nm (excitation at 280 nm). BSA, $2{\cdot}5 \times 10^{-6}$ M in 0·01 M Tris-Cl buffer, pH 7·4. Initial fluorescence of the protein was adjusted as in Fig. 2.

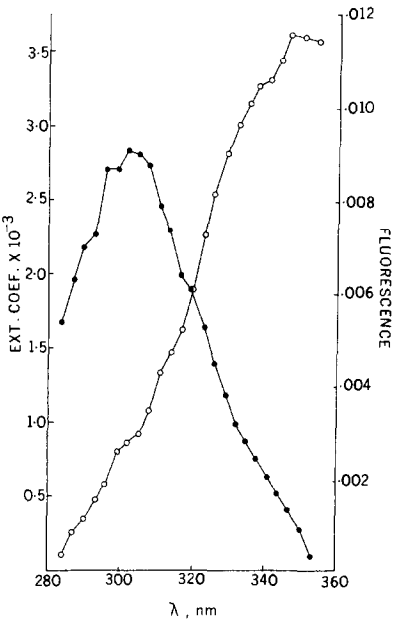

FIG. 5. Overlap spectrum of promazine absorption (●—●) and bovine serum albumin fluorescence emission (○—○), emission normalized to 1, in 0·01 M Tris-Cl buffer, pH 7·4. Absorption at 301 nm and fluorescence excitation and emission at 280 and 350 nm respectively.

orientation factor for transition dipoles of donor and acceptor molecules $K^2 = 2/3$.[13] The distance of separation was calculated to be 15·3 ± 1·0 Å.

Figure 6 demonstrates the effect the detergent had on the emission of a fixed concentration of the drug. An increase in the fluorescence emission of the drug was observed when various amounts of detergent were added. In order to determine the maximal change in fluorescence, the fluorescence change was plotted against the reciprocal of the detergent concentrations. From the change in fluorescence, the effect of the detergent on the fluorescent quantum yield was determined as follows. The quantum yield for the drug in aqueous buffer was first computed relative to quinine sulfate after the method of Chen.[14] The quantum yield of promazine was calculated to be 0·012 relative to quinine sulfate. The extinction coefficient of the drug in the presence of the detergent at 301 nm, $E_s = 5{\cdot}62 \times 10^3$, was determined by u.v. absorption at the excitation wavelength used for the drug. The quantum yield of promazine in detergent, Q_x, was then computed using the following relationship,[14]

$$Q_x = \frac{E_s F_x Q_s}{E_x F_s}$$

where F_x and F_s are fluorescence intensity in the absence and the presence of the detergent, E_x and E_s are excitation coefficients without or with the detergent, and Q_s is the quantum yield of promazine in the absence of the detergent respectively. The quantum yield of promazine in detergent, $Q_x = 0{\cdot}01$, was obtained using the above formulation.

The addition of the detergent caused an increase in the extinction coefficient of the

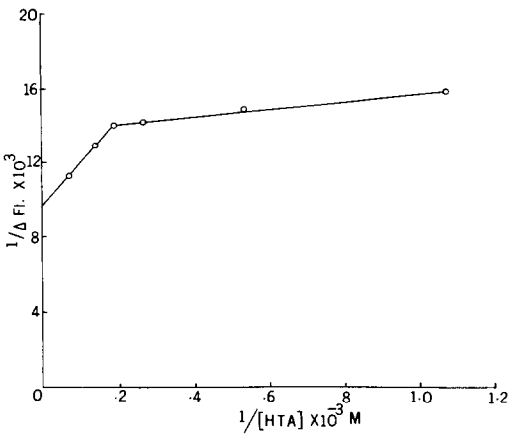

FIG. 6. Reciprocal plot of fluorescence increase of promazine in various concentrations of hexadecyltrimethylammonium bromide, excitation at 350 nm and emission 450 nm, in 0·01 M Tris-Cl buffer, pH 7·4.

drug and a shift to longer wavelengths of the absorption maxima. Table 2 presents the comparison of the absorption properties in different solvents and/or phases.

DISCUSSION

The fluorescence quenching of bovine serum albumin in the region of tryptophan emission appears to be sensitive to the association with promazine. Promazine association with bovine serum albumin allows at least a partial characterization of the affinity of the drug for the protein. The affinity constants for promazine with bovine serum albumin have been determined by other techniques and have been reported to be 0·9 to 1·3 × 10^4 M obtained by gel filtration method,[5] and 8·4 × 10^4 M by equilibrium dialysis method.[20] The fluorescence method for determining the affinity of phenothiazine derivatives to proteins is considerably easier and faster than either of the other two methods. It is apparent that the measure of affinity largely depends on the concentration of the drug.

Krieglstein *et al.*[21] considered the binding of phenothiazines to serum protein as

TABLE 2. COMPARISON OF ULTRAVIOLET ABSORPTION OF PROMAZINE IN ENVIRONMENTS OF DIFFERENT POLARITY

Solvent or addition	Absorption maxima (nm)	Log extinction coefficient
95% Ethanol*	306	3·64
	254	4·53
Methanol†	304	3·637
	254	4·426
Aqueous 0·01 M	301	3·45
Tris-Cl buffer	252	4·36
Plus detergent	309	3·75
	257	4·64

* Warren *et al.*[18]
† Kračmar and Blazék.[19]

having two components, an electrostatic interaction of the protonated amine on the alkyl side chain, and an apolar component which is enhanced by substitution on the ring. The pH dependence of the quenching process (Fig. 2) supports the view of electrostatic interaction. The pH dependence of tryptophan quenching could reflect differences in the binding of promazine, since at the higher pH values more anionic sites would be available for interaction with the drug. In addition, the observation of an increase in the u.v. absorption of the drug when bound (Fig. 4) is consistent with the aromatic portion of the drug being associated with a phase of less polarity than the solvent.

The mechanism for the fluorescent quenching of bovine serum albumin emission by promazine was of interest because a similar quenching was observed with the membrane proteins (unpublished data). The overlap integral was evaluated to determine the distance of separation at a 50 per cent efficient transfer of energy from protein tryptophan to promazine. The computed separation distance, R_o, was 15·3 ± 1·0 Å This distance indicates moderate overlap when compared with the R_o value for tyrosine-tryptophan, reported to be 15 Å by Seliger and McElroy.[22]

If any appreciable energy was transferred from tryptophan to promazine by the resonance mechanism, an excitation band due to tryptophan should have appeared in the promazine excitation spectrum. No such band was observed.

The involvement of the resonance energy transfer cannot be determined on the basis of fluorescence experiments only. The triplet states of promazine may be the route for the transferred energy loss. The absence of an enhanced fluorescence of the drug when interacted with the serum protein hampered the evaluation of the association of the drug by this technique. The fluorescence quantum yield of promazine in aqueous solution was determined to be only 0·012. No increase was detected when the drug was associated with the protein.

Unionized carboxyl groups are known quenchers of tryptophan emission.[23,24] The electrostatic attraction of the ionized carboxyl groups to the charged side-chain amine of promazine, if involved in the binding, would alter the inductive effect of the carboxylic groups on the tryptophan. The pH dependence of bovine serum albumin fluorescence is, in part, due to the change in the concentration of ionized carboxyl groups.[23] To determine the effect of free carboxyl on the promazine quenching of the protein emission, the pH of the protein was varied from 3 to 9 (Figs. 2 and 3). The linear dependence of the fluorescence change on the concentration of the drug to carboxyl ions at acid pH is consistent with their involvement in the quenching reaction (Fig. 3). At alkaline pH, the relation of quenching to carboxyl groups is more complicated. These findings may serve as a basis for further experimentation to more critically define the role of the protein anions and cations on the fluorescent properties of the protein and its affinity for the drug.

The surface activity of the phenothiazine derivatives has been suggested as being involved in the drug–membrane effects.[25] In order to determine the effect of partitioning of the drug between phases of different polarity, the micellular detergent model was used. The choice of the ammonium detergent was based on the observed changes in the optical properties of the drug when associated with the detergent above the critical micellular concentrations. It was interesting that neither neutral nor anionic detergents had detectable effects on the absorption or emission of the drug. A slight increase in both absorption and fluorescence was observed for the drug below the

published critical micellular concentrations of 0·018 moles/kg[26] (Fig. 6 and Table 2) for hexadecyltrimethylammonium bromide.

The presence of the ammonium detergent increased the extinction coefficient and a shift of absorption maximum to a longer wavelength (Table 2). If the change in extinction and a shift were functions of apolar interactions only, they would have been consistent with the properties of the drug to have produced similar changes upon addition to the other detergent micelles investigated. The change in the extinction coefficient observed with the cationic detergent was consistent with the observed change in absorption of the drug seen with the serum protein (Fig. 4); however, the position of the maximum absorption did not change in the case of the protein. The quantum efficiency for the promazine fluorescence was essentially unchanged, $Q_{aqueous} = 0{\cdot}012$ to $Q_{detergent} = 0{\cdot}010$, indicating that the effect was on the ground state.

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