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RABBIT ERYTHROCYTE MEMBRANE INTERACTION WITH PROMAZINE AND CALCIUM

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Abstract—Promazine behaved as a competitive antagonist to the association of calcium with hemoglobin-free erythrocyte membrane when the interactions were characterized by membrane fluorescence quenching or by using a fluorescent probe. The promazine-induced quenching of membrane fluorescence could be reversed by the addition of calcium. Addition of calcium to mineral cation-free membrane in the presence of the drug resulted in an increased fluorescence in the region of 300–320 nm. The calcium-induced spectral change was interpreted as being due to increased tyrosine emission. The association of drug possibly induced structural modifications of the erythrocyte membrane, as suggested by light scattering. The fluorescence quenching of membrane was only slightly altered by variation in pH. It is suggested that the drug may serve as a stabilizer of bound calcium through the formation of coordinated complexes.

Since the review of phenothiazine derivatives by Guth and Spirtes [1] in which they noted that the alteration of membrane properties is of primary importance, little conclusive evidence has emerged to suggest the exact mechanism by which the phenothiazines produce their manifold effects. They suggested that metal complexation is involved.

Uzunov and Weiss [2], who worked with the membrane-bound adenylcyclase system, observed that the normal stimulating effect of sympathomimetic compounds on the enzyme was blocked by phenothiazines. Phenothiazine drugs were also reported to alter 3′,5′-cyclic AMP and adrenocorticotropic hormone-stimulated corticosterone synthesis [3, 4]. The inhibition of hormonally stimulated adenylcyclase activity by the phenothiazines has also been reported by Wolff and Jones [5], who concluded that the effect was due to the surface properties of these drugs.

Despopoulos [6] compared several phenothiazine derivatives on the basis of their abilities to prevent hypotonic lysis of erythrocytes and noted that the phenothiazines might interact with sites normally associated with calcium. Kwant and Seeman [7] and Seeman *et al.* [8] suggested that the phenothiazines compete for Ca^{2+} binding sites on the erythrocyte membrane. The ability of phenothiazine derivatives to stabilize the conformation of membrane protein has also been reported by Holmes and Piette [9]. Several other observations involving phenothiazine–membrane interactions have also been recently reported [10–12].

The present study was undertaken to investigate further the interaction of phenothiazine derivatives on membranes and the influence of calcium on such interactions. The principal method employed in this study was the alteration of absorption and fluorescence emission properties of the interactants. Wallach *et al.* [13] have suggested that the phenothiazines might be suitable acceptor molecules for the transfer of excitation energy by a nonradiative dipole–dipole interaction from the amino acid tryptophan and, as such, would provide a means of studying the alteration of macromolecular properties associated with these compounds. This possibility was investigated (J. D. Keeler, unpublished, 1972) and was not found to be a satisfactory explanation.

Promazine [10(3′-dimethylamino-1′-propyl)-phenothiazine], a prototype phenothiazine derivative and a clinically used tranquilizer, was used in this study. The findings of Forstner and Manery [14, 15] that the high affinity binding sites for Ca^{2+} are proteins on the erythrocyte membrane, led to the choice of the rabbit erythrocyte membrane as the model membrane system employed. In order to corroborate the observed changes in membrane fluorescence caused by both promazine and calcium, the fluorescent probe, 1-anilino-8-naphthalene sulfonate (ANS), was also used. Rubalcava *et al.* [16] and Gomperts *et al.* [17] utilized this fluorescent probe to study the cation–membrane interactions.

MATERIALS AND METHODS

Chemicals. Promazine hydrochloride was a gift from Smith, Kline & French (Philadelphia, Pa.). The fluorescent probe, 1-anilino-8-naphthalene sulfonate (ANS), as the magnesium salt was purchased from Eastman Kodak (Rochester, N.Y.). All other chemicals used were of reagent grade. Triple glass-distilled or deionized water was used for all procedures.

Membrane preparation. Hemoglobin-free rabbit erythrocyte membranes (REM) were prepared by the method of Dodge *et al.* [18] with Tris–Cl buffer (pH 7·4) substituted in the lytic step. After the last wash, the pellet was resuspended in an equal volume of Tris buffer and refrigerated to be used within 48 hr. The protein concentration of the erythrocyte membrane was determined by the method of Lowry *et al.* [19] with human serum albumin (Nutritional Biochemicals, Cleveland, Ohio) used as the standard.

Instrumentation. The Aminco-Bowman model 4-8202 spectrophotofluorometer (American Instrument Co., Silver Springs, Md.) was calibrated by the method of Chen [20] and used for all fluorescence measurements. A Coleman 124 double-beam spectrophotometer was used for the ultraviolet absorption experiments. The titration apparatus used was a Beckman/Spinco 153 micro-titrator burette. All titrations were performed at $25 \pm 0{\cdot}1°$ using a thermostatic water bath.

Fluorescence quenching. The concentration of the membrane was adjusted to 0·15 mg protein/ml in 0·01 M Tris–Cl buffer, pH 7·4. Two ml of the solution was placed in a 1-cm^2 cuvette and the emission at 350 nm light was determined upon excitation with 280 nm light. Sub-microliter quantities of 1×10^{-2} M promazine–HCl in 0·01 M Tris–Cl buffer were added, mixed, and the emission was redetermined. The quantity of drug introduced was controlled so as to produce at least 1 per cent change in the fluorescence emission. Drug was titrated until the change in fluorescence became linear with further additions of drug. The titrations were repeated several times and the representative data used for calculations. Inner filter effect was estimated by replacing the 1-cm light path with a 0·3-cm path at the various concentrations of the drug employed. From the fluorescence quenching using the two path lengths, the inner filtering could be estimated. The correction was negligible below 5×10^{-5} M promazine; all other data presented here were corrected in this manner.

Fluorescent probe titrations. Methods and conditions were similar to those used above in the quenching experiments. The probe, ANS, was added to the membrane suspension at a concentration of 5×10^{-5} M. The emission from bound probe was observed at 490 nm when excited at 390 nm. The 390 nm excitation was employed to prevent the excitation of promazine, which absorbed significantly from 200–320 nm.

Analysis of fluorometric titration data. Double-reciprocal plots of the corrected fluorescence change versus drug concentration were constructed after the method of Gomperts *et al.* [17]. The negative value of the reciprocal concentration extrapolated from such plots was used as a measure of the relative affinity constant. The reciprocal plots had more than one linear phase, indicating the intercepts for different affinities. The constants within a linear range were computed by regression analysis of the data in the form of double-reciprocal quantities. These constants for different binding sites were also verified by Scatchard plots [21].

Determination of maximum binding of promazine to membranes. The maximum concentration of drug bound was determined by measuring the change in fluorescence produced by a fixed drug concentration, with various concentrations of membrane. Double-reciprocal plots constructed from the change in fluorescence against the membrane concentration were linear and their intercepts yielded the maximum change in fluorescence produced by that concentration of drug when all bound [22]. These were obtained at various drug concentrations. The maximal binding was also determined by using fluorescent probe and by obtaining the fraction of drug bound at various promazine concentrations by separating the membrane vesicles by centrifugation (5000 *g* for 40 min).

pH dependence of the promazine-induced fluorescence quenching and membrane suspension light scattering. The pH of the erythrocyte suspension in 0·01 M Tris buffer was adjusted with NaOH or HCl in the range of 2 10. The quenching produced by 5×10^{-6} M promazine at the various pH values was determined. The same membrane suspensions were used to determine the pH influence on the change in light scatter signal produced by promazine. The excitation and emission monochromators were set at 400 nm and the intensity of the scatter signal was recorded with and without the drug added. The difference in scatter signals with and without the drug was plotted against the corresponding pH.

Influence of calcium on promazine–membrane interactions. Fluorescence quenching titrations and the fluorescence probe titrations were conducted with and without the presence of calcium. In order to gain insight into the possible interaction between the aromatic amino acid and promazine in the membrane system, the corrected emission spectra of the membrane system were determined in the presence of 10^{-3} M Ca^{2+} or 5×10^{-6} M promazine, or both. The drug and Ca^{2+} were added in a reverse order in different experiments as mentioned in the Results section. Each corrected spectrum was replicated at least three different times. Spectra at other excitation wavelengths and at different Ca^{2+} concentrations were also determined to confirm the results.

RESULTS

Promazine caused a decrease in the emission from the membrane protein (Fig. 1). This quenching was prevented by the addition of Ca^{2+} to the erythrocyte membrane suspension. Figure 2 is the reciprocal plot of the change in fluorescence of the suspension with calcium present and absent. At least two different slopes in the reciprocal plots were discernible, indicating the interactions with different binding sites. The

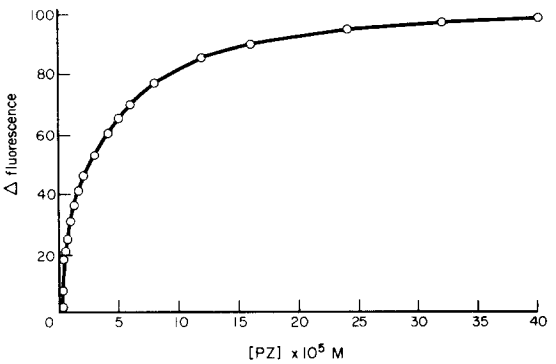

Fig. 1. Per cent of maximal fluorescent change produced in rabbit erythrocyte membranes (REM) by promazine. Excitation and emission were at 280 and 350 nm respectively. REM concentration is 0·15 mg protein/ml in Tris–Cl, 0·01 M, buffer at pH 7·4. After correcting for inner filtering, the maximal change in protein fluorescence produced by the drug was on the order of 60 per cent of the initial value.

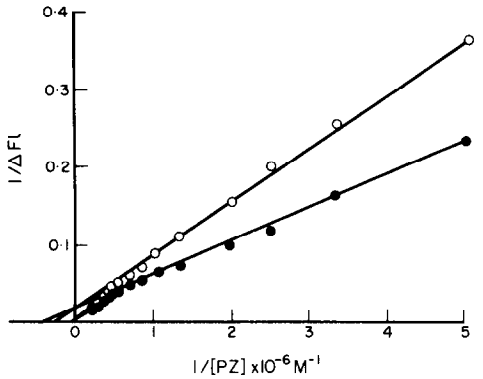

Fig. 2. Reciprocal plots of membrane fluorescence quenching (corrected) by promazine (relative affinity constants in Table 1) with (○——○) and without Ca^{2+} (●——●) added (10^{-3} M). Conditions as in Fig. 1.

negative reciprocal concentration intercepts of these two slopes, considered as a relative measure of the apparent affinity constants at different ranges of drug concentrations, are indicated in Table 1.

Similar findings were observed when the fluorescent probe, ANS, was employed to characterize the interactions. The fluorescence of ANS continuously increased when the drug was added to the membrane suspension, indicating higher binding of the fluorescent probe to membrane with increasing drug concentration. The biphasic reciprocal plots for ANS fluorescence with and without calcium added are shown in Fig. 3 and the respective apparent affinity constants are shown in Table 1.

It is apparent from the data in Figs. 2 and 3 that calcium acts competitively with the drug binding on the membrane. When the amount of calcium is varied in similar experiments and all other conditions are kept constant, the reciprocal plots would give the estimates of the binding affinities of calcium on the membrane sites. Again, a biphasic nature was observed from these reciprocal plots. The two different apparent affinity constants for calcium and membrane, determined by the fluorescence quenching and ANS fluorescence methods, are indicated in Table 2.

The maximal binding for promazine with erythrocyte membranes was determined using membrane

Table 1. Apparent affinity constants for promazine and REM observed by different methods and with or without the addition of Ca^{2+}

Method	Calcium concn (M)	Affinity constants* (M^{-1}) K'	K''
Fluorescence quenching	none	$2{\cdot}6 \times 10^5$	$2{\cdot}0 \times 10^4$
	1×10^{-3}	$1{\cdot}0 \times 10^5$	$1{\cdot}0 \times 10^4$
Using ANS probe	none	$2{\cdot}8 \times 10^5$	$1{\cdot}6 \times 10^4$
	1×10^{-3}	$1{\cdot}0 \times 10^5$	$1{\cdot}1 \times 10^4$

* Apparent affinity constants were obtained from the negative reciprocal concentration intercepts. Figs. 2 and 3. The two constants, K' and K'', represent the affinity in different ranges of promazine concentrations. ANS concentration was 5×10^{-5} M.

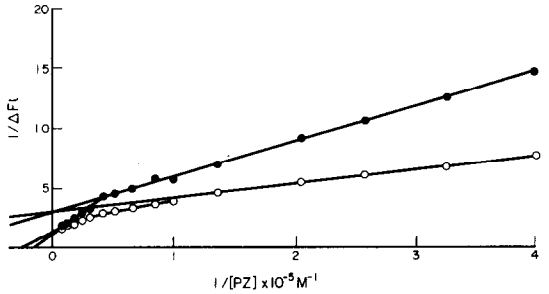

Fig. 3. Reciprocal plots of ANS fluorescence with erythrocyte membrane by promazine. ANS concentration, 5×10^{-5} M. Other conditions are the same as in Fig. 1. Excitation and emission were at 390 and 490 nm respectively. The upper curve (●——●) is in presence of 10^{-3} M Ca^{2+}, while the lower (○——○) is without calcium.

Table 2. Apparent affinity constants for Ca^{2+} and REM observed by different methods*

Method	K_1'	K_1''
Fluorescence quenching	$1{\cdot}6 \times 10^3$ M^{-1}	$1{\cdot}0 \times 10^3$ M^{-1}
Using ANS probe	$1{\cdot}8 \times 10^3$ M^{-1}	$0{\cdot}4 \times 10^3$ M^{-1}

* Apparent affinity constants were obtained from the negative reciprocal concentration intercepts. Fixed promazine concentration of 5×10^{-6} M was used and the amount of Ca^{2+} varied. ANS concentration was 5×10^{-5} M. Other conditions are described in text.

fluorescence quenching, the fluorescent probe, and centrifugation. The results are shown in Fig. 4. It appears that the binding ratios determined using the probe data were more consistent with the values obtained by centrifugation.

The effect of a fixed concentration of promazine on the fluorescence of the membrane at pH 2–10 is shown in Fig. 5. The decrease in the 90° light scatter by the drug was also measured at the same pH values and is included in Fig. 5.

The corrected emission spectra of REM, with and without Ca^{2+}, and with and without the addition of promazine are given in Fig. 6. When promazine was added to mineral cation-free membrane, there was a general decrease in the tryptophan emission. The

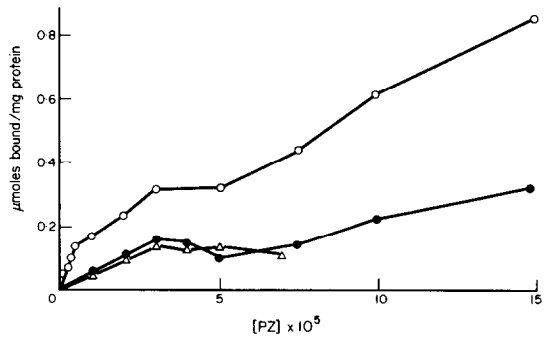

Fig. 4. Binding of promazine to erythrocyte membrane determined by fluorescence quenching (○——○), fluorescent probe, ANS (●——●), and centrifugation (△——△).

addition of Ca^{2+} (1×10^{-3} M) partially reversed the quenching, particularly in the 300–320 nm region. In fact, the fluorescence in that region was increased above that present in the membrane alone. The appearance of a shoulder at 300–320 nm in the corrected emission spectrum of the rabbit erythrocyte membrane suspension with drug after the addition of calcium was at first assumed to be due to Raman scatter. However, the observation that this peak did not change position when the excitation wavelength was changed (intensity did vary markedly, however) suggested that this was not the case. The appearance of a shoulder at 315 nm appeared to be due to tyrosine fluorescence [23]. This was further supported by the observation that the 315 nm shoulder was not seen when the activation wavelength of 290 nm (absorption wavelength of tryptophan) was used. The reversal of quenching in this region was proportional to the concentration of Ca^{2+} in the system.

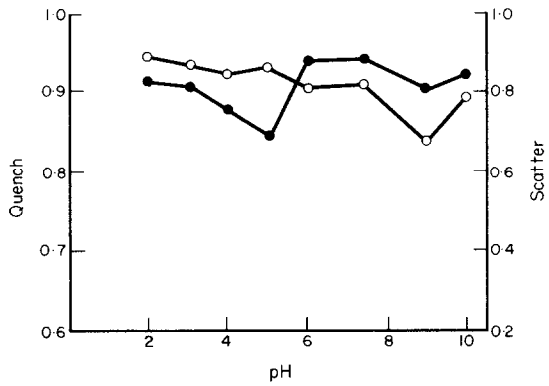

Fig. 5. pH dependence of quenching of rabbit erythrocyte membrane emission (○——○) by promazine (5×10^{-6} M) and the decrease in light scattering at 90° (●——●). Membrane concentration, 0·15 mg protein/ml.

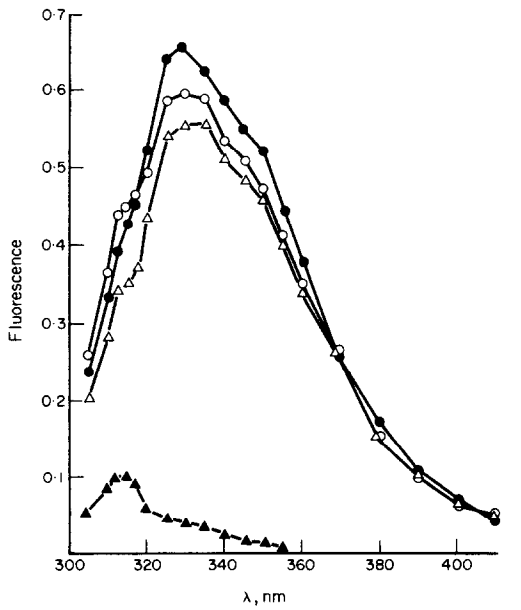

Fig. 6. Corrected emission spectrum of rabbit erythrocyte membrane alone (●——●), and in the presence of 5×10^{-6} M promazine (△——△). The spectrum in between (○——○) is after adding 1×10^{-3} M Ca^{2+}. Excitation was at 280 nm in 0·01 M Tris-Cl, pH 7·4. The lower curve (▲——▲) is the difference between the spectra of REM plus promazine with and without Ca^{2+}.

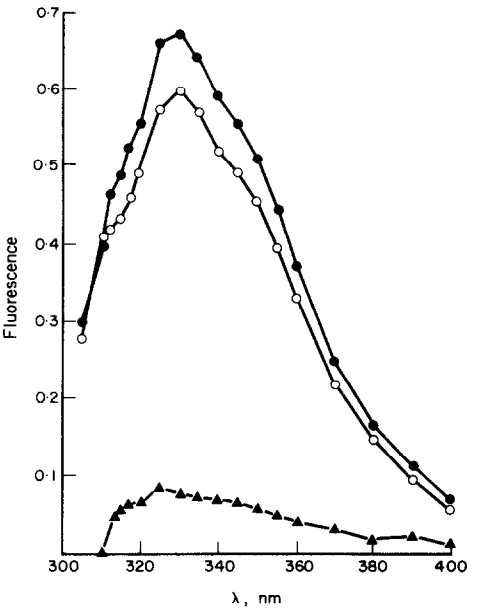

Fig. 7. Corrected emission spectrum of rabbit erythrocyte membrane with Ca^{2+} added before promazine. The upper curve (●——●) is the spectrum before promazine was added, and the other (○——○) after the addition of 5×10^{-6} M promazine. The lower curve (▲——▲) is the differential spectrum between the two. Conditions are the same as in Fig. 6.

Addition of Ca^{2+} before promazine to the erythrocytes produced a difference spectrum markedly different from that produced when promazine was added before calcium (Fig. 7). The quenching efficiency was quite constant over the entire emission spectrum when Ca^{2+} was added first.

DISCUSSION

The hemoglobin-free rabbit erythrocyte membrane was employed as a prototype membrane with which the phenothiazine derivatives should associate. Kwant and Seeman [7] working with human erythrocyte membranes, and Despopoulos [6] working with dog erythrocytes demonstrated that these drugs associated with such membranes.

The competitive nature of the calcium and promazine was demonstrated in this study using low concentrations of the drug (10^{-6} to 10^{-4} M). The competitive nature was revealed both by fluorescence quenching and fluorescent probe titrations. Gomperts *et al.* [17] used the same probe to demonstrate the competitive antagonism of procaine to potassium binding with endoplasmic reticulum.

The pH dependence of the erythrocyte membrane quenching was not pronounced. The decrease at 90° in the light scatter signal (Fig. 5) of the membrane suspension upon addition of the drug is consistent with a change in the activity of the drug. The decrease in the scatter signal is suggestive of an expansion of the membrane by the drug [24] and is in agreement

with the findings of Seeman *et al.* [8]. However, a change in light scatter may also be caused by the aggregation of membrane components.

The reasonableness of the competitive antagonism of promazine and Ca^{2+} to the binding sites was investigated by considering the change produced in the membrane fluorescence emission spectrum by the added drug and Ca^{2+} (Figs. 6 and 7). Addition of Ca^{2+} was able to reverse the fluorescence quenching produced by the drug when promazine was added first. The appearance of tyrosine fluorescence due to addition of Ca^{2+} in this system indicated the involvement of phenolic groups of tyrosine in the drug–membrane interaction. Van Eard and Kawasaki [23] demonstrated in a class A (tryptophan-free) protein prepared from skeletal muscle that the binding of Ca^{2+} to the protein produces an increase in the fluorescence of tyrosine. They interpreted the increase as being due to an increased helicity of the protein and presented optical rotatory data to support that idea. They did not consider the effect of hydrogen bonding of the phenolic hydrogen to ionized carboxyl groups. Such hydrogen bonding of tyrosine would be altered by association of Ca^{2+} with the carboxyl groups involved in binding the Ca^{2+}. The effect of hydrogen bonding on tyrosine emission is to quench its fluorescence [25], the opposite of the effect of carboxyl ion on tryptophan emission.

When Ca^{2+} was added to the membrane system before promazine, the change was different from that produced when the drug was added first (Fig. 7). It may be suggested that promazine not only competes for alkaline earth binding sites, but may also participate by stabilizing ions already bound, by serving as a mono- or bi-dentate ligand to the bound cation.

Forstner and Manery [14, 15] working with calcium binding of human erythrocyte membranes determined that the high affinity binding involved ionized carboxyl groups. The association of Ca^{2+} with high affinity binding sites on membranes may be rationalized on the basis of analogy of this cation complexing with ethylenediaminetetraacetate [26].

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