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The Affinity of Various Phenothiazine Drugs for Membranes of Intact Human Erythrocytes and Their Membrane-Transforming Activity

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SUMMARY

Membrane shape changes of human erythrocytes induced by each of five cationic and two anionic phenothiazines were observed semiquantitatively, and the amounts of the drugs incorporated into erythrocytes or ghosts were determined. Cationic and anionic phenothiazines induced membrane invagination and exvagination on human erythrocytes, respectively. The former drugs were incorporated mainly into the membrane of cells, whereas only a small portion of the latter drugs was incorporated into the membrane and the remainder into the cytosol. The initial concentrations of cationic phenothiazines needed to induce the same extent of shape change decreased in the order promethazine, chlorpromazine, perazine, prochlorperazine, and trifluoperazine, indicating that their shape-transforming activities increase in the same order. The phenothiazines with a halogen atom at position C-2 of the phenothiazine nucleus exhibited higher affinity for the membrane than those with a hydrogen atom. Phenothiazines with the *N*-methylpiperazinyl group in the side chain showed a stronger effect on membrane shape change than those with the dimethylamino group and with the same nonpolar moiety. The introduction of halogen atom(s) appears to increase the affinity of drugs for the plasma membrane, and the polar head group in the side chain seems mainly responsible for perturbation of the membrane, which leads to induction of a shape change.

INTRODUCTION

It has been reported that membrane exvagination and invagination on human erythrocytes are almost instantly induced by anionic and cationic amphiphilic drugs, respectively, having affinity for the plasma membrane. Although electric charges of their polar head groups have an important role in determining the type of the shape changes to be induced as reported in previous work (1–5), possible influences of the nonpolar portion of the drug molecules do not seem to be sufficiently examined, because the majority of workers have employed drugs with structurally unrelated, nonpolar moieties. Mohandas and Feo (5) successfully used two series of cationic and anionic drugs with the same phenothiazine nucleus. They determined the amount of the drug incorporated into whole erythrocytes and correlated the intensity of the shape changes with the amount of drug incorporated into whole cells. However, it was not revealed how much of the total amount of a drug incorporated into whole cells is actually bound to the plasma membrane, the probable site of drug action. The present work is an extension along this line, and we report a structure-activity relationship of phenothiazine drugs, based on determinations of the shape change induced at various initial concentrations of the drugs added and of the amounts incorporated into whole erythrocytes and membrane "ghosts."

MATERIALS AND METHODS

Erythrocytes. Human erythrocytes from freshly drawn acid citrate dextrose blood, kindly supplied by the Kyoto Prefectural Red Cross Blood Center, were washed three times with 20-fold volumes of 140.5 mM NaCl containing 10 mM phosphate buffer (pH 7.4) and resuspended in phosphate-buffered saline.

Human erythrocyte ghosts. Hemoglobin-free ghosts were prepared by hemolyzing 1 volume of packed erythrocytes in 20 volumes of cold 20 mOsM phosphate buffer (pH 7.4) according to the procedure of Dodge *et al.* (6), and resuspended in this buffer.

Chemicals. Phenothiazine neuroleptics, CPZ,[1] PMZ, PZ, PCPZ, and TFPZ, were kindly supplied by Yoshitomi Pharmaceutical Company (Osaka, Japan). Phenothiazine anti-inflammatory drugs, MA and PA, were presented by Prof. H. Fujimura (Gifu University Medical School, Gifu, Japan). These drugs were dissolved in phosphate-buffered saline and the pH was adjusted to 7.4 by adding HCl or NaOH.

Morphological observation of erythrocytes. To various drug solutions preincubated at 37° was added the eryth-

[1] The abbreviations used are: CPZ, chlorpromazine; PMZ, promethazine; PZ, perazine; PCPZ, prochlorperazine; TFPZ, trifluoperazine; MA, metiazinic acid; PA, protizinic acid.

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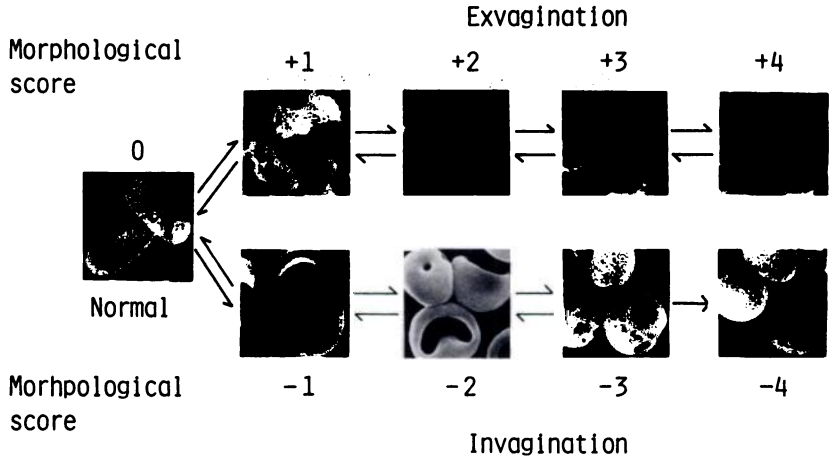


FIG. 1. *Assignment of morphological score to each stage of the shape change of human erythrocytes*

rocyte suspension (also preincubated) to make a final hematocrit of 10%. The mixture was incubated at 37° for 10 min. A portion of the drug-treated erythrocyte suspension (0.5 ml) was added to 2 ml of 0.9% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4). After standing for 60 min at room temperature for fixation, the fixed erythrocytes were thoroughly washed with distilled water, air-dried, and shadowed with carbon and gold. The preparation was then observed under a scanning electron microscope, JEOL Type JSM-35, with an accelerating voltage of 20 kV. In order to express the extent of shape change of a given cell population semiquantitatively, a morphological score with plus or minus sign was assigned to each stage of the shape change as described in Fig. 1, and the morphological index was calculated according to the following formula (4):

$$\text{Morphological index} = \Sigma\,(\text{morphological score}) \times [(\text{no. of transformed cells})/(\text{total cell no.})]$$

Determination of the amount of drug incorporated into erythrocytes or ghosts. Erythrocytes were treated with various drugs as described above and centrifuged at 900 × *g* for 10 min. Ghost suspensions equivalent to a 10% suspension of original washed erythrocytes were also treated with drugs under the same conditions and centrifuged at 20,000 × *g* for 30 min. The amount of drug incorporated into erythrocytes and ghosts was determined by measuring the decrease in uv absorption at 254 nm of the supernatant of the incubation mixture, basically according to the method of Mohandas and Feo (5). In order to remove the interference of hemoglobin, derived from a slight hemolysis of erythrocytes, the amount of hemoglobin in the supernatant was determined by measuring its absorption at 543 nm, and the corresponding uv absorption of this hemoglobin at 254 nm was subtracted from the total absorption of the supernatant.

In this study, the amount of the drug bound to ghost membrane was assumed to represent the amount incorporated into the membrane fraction of the intact erythrocytes, and the amount of the drug incorporated into the cytosol fraction of the intact erythrocytes was calculated by subtracting the amount bound to ghost membranes from the amount bound to intact cells. Although the validity of such an assumption cannot be proved experimentally at present because there is no direct means of measuring the amount of drug bound to the membrane of intact cells, it may be acceptable for the purpose of comparison of relative (not absolute) amounts of analogous drugs bound to cell fractions.

RESULTS

Figure 2 shows the extent of membrane shape change of human erythrocytes as a function of initial concentration of each of five cationic phenothiazines added to medium; PMZ, CPZ, PZ, PCPZ, and TFPZ. The extent of membrane invagination, which was induced instantly

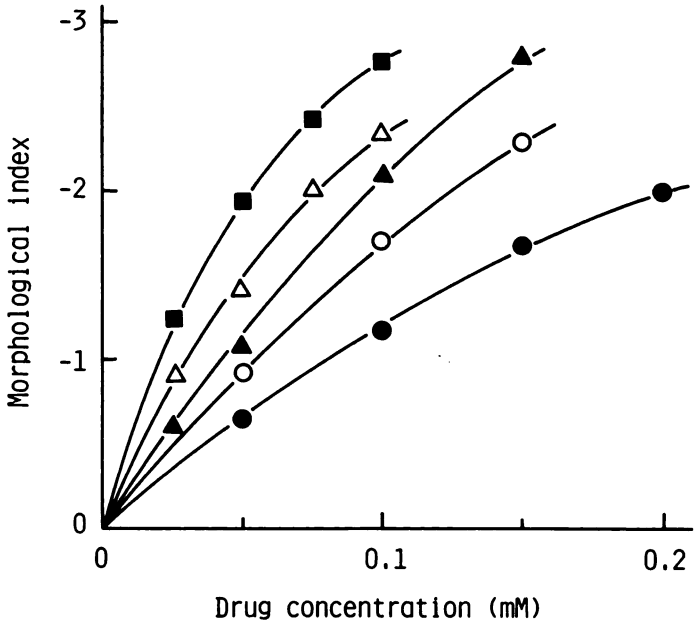


FIG. 2. *Membrane shape change of human erythrocytes induced by cationic phenothiazines*

Human erythrocyte suspension was incubated with PMZ (●), CPZ (○), PZ (▲), PCPZ (△), and TFPZ (■), respectively, in the concentrations indicated at 37° for 10 min.

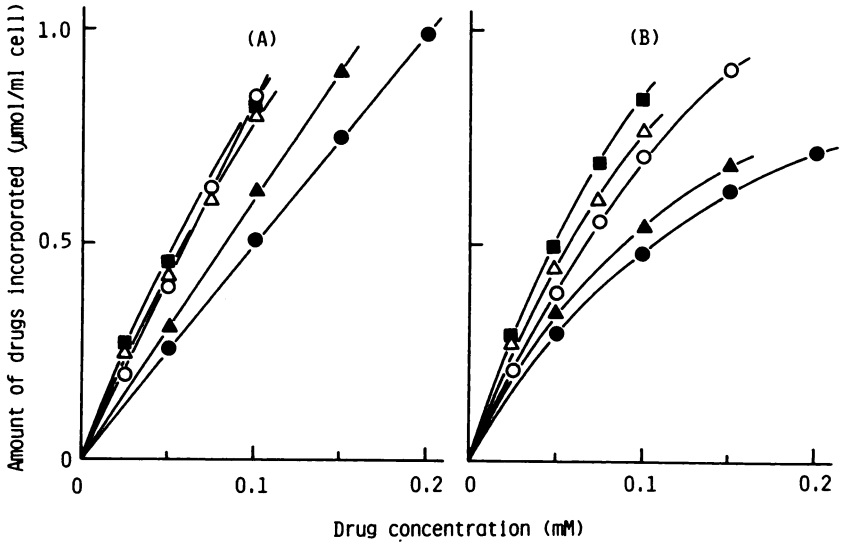


FIG. 3. *Amounts of cationic phenothiazines incorporated into human erythrocytes or ghosts*

Human erythrocyte (A) or ghost (B) suspension was incubated with each of the drugs described in Fig. 2 in the concentrations indicated at 37° for 10 min. The symbols are the same as in Fig. 2.

by the drugs and was independent of temperature in a range of 0–37°, progressed with increasing concentrations of drug added to the external medium. At a given concentration, the intensity of the shape change induced by each drug decreased in the order of TFPZ > PCPZ > PZ > CPZ > PMZ.

Figure 3 shows the amounts of cationic phenothiazines incorporated into erythrocytes (A) or ghosts (B) as a function of their initial concentration added to medium. Incorporation of all of the drugs tested here into erythrocytes or ghosts occurred instantly and was independent of temperature in the range cited above. The amounts incorporated into whole cells increased almost linearly with increasing drug concentrations, whereas at a given concentration they decreased in the following order: TFPZ ≃ PCPZ ≃ CPZ > PZ > PMZ. At lower concentrations, the amounts incorporated into ghosts were almost identical with those incorporated into whole cells, whereas at higher concentrations the amounts incorporated into ghosts were slightly less than those incorporated into whole cells.

By calculating the distribution of the drugs in three compartments (i.e., in the plasma membrane, extracellular medium, and intracellular fluid) at a fixed amount of the drug bound (0.5 μmole/ml of cells), using the data indicated in Fig. 3A and B, the following values of the membrane-bound drug percentages were obtained; for TFPZ, PCPZ, CPZ, PZ, and PMZ, 91, 86, 77, 57, and 50%, respectively. These values correspond to 100, 100, 94, 90, and 98% of the entire amounts incorporated into whole cells, respectively. This result indicates that almost all of

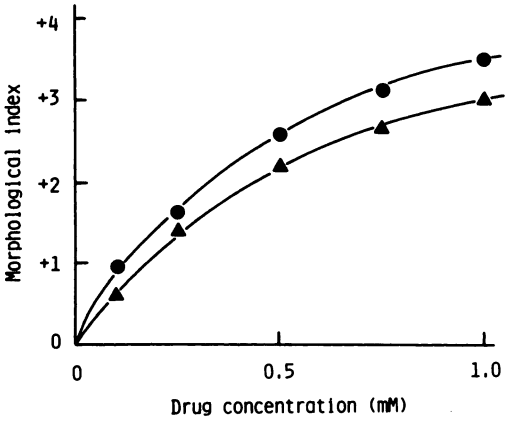


FIG. 4. *Membrane shape change of human erythrocytes induced by anionic phenothiazines*

Human erythrocyte suspension was incubated with PA (●) and MA (▲), respectively, in the concentrations indicated at 37° for 10 min.

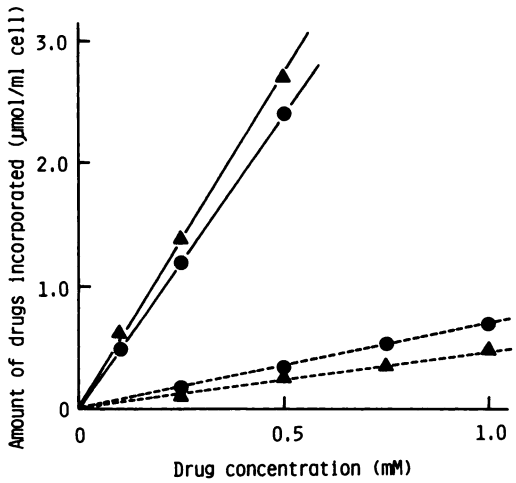


FIG. 5. *Amounts of anionic phenothiazines incorporated into human erythrocytes or ghosts*

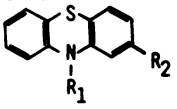
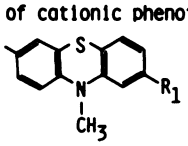
Human erythrocyte (——) or ghost (– – –) suspension was incubated with each of drugs described in Fig. 4 in the concentrations indicated at 37° for 10 min. The symbols are the same as in Fig. 4.

TABLE 1

Extracellular concentrations of phenothiazine derivatives and their amount incorporated into human erythrocytes or ghosts to induce membrane shape change of the index −2 or +2[a]

Drugs	Initial concentration (μM)	Amount incorporated into Erythrocytes (nmol/ml cell)	Amount incorporated into Ghosts (nmol/ml cell)	Side chain[b) R_1	Side chain[b) R_2
Cationic phenothiazines					
Promethazine (PMZ)	194	960	710	$-CH_2-CH(CH_3)-N(CH_3)_2$	-H
Chlorpromazine (CPZ)	123	950	790	$-CH_2-CH_2-CH_2-N(CH_3)_2$	-Cl
Perazine (PZ)	91	570	510	$-CH_2-CH_2-CH_2-N\square N-CH_3$	-H
Prochlorperazine (PCPZ)	75	620	590	$-CH_2-CH_2-CH_2-N\square N-CH_3$	-Cl
Trifluoperazine (TFPZ)	52	440	440	$-CH_2-CH_2-CH_2-N\square -CH_3$	$-CF_3$
Anionic phenothiazines					
Metiazinic acid (MA)	474	2,310	226	$-CH_2-COOH$	-H
Protizinic acid (PA)	363	1,520	231	$-CH(CH_3)-COOH$	$-OCH_3$

[a]The data are derived from the results in Figs 2-5.

[b]General structure of cationic phenothiazines,  and of anionic phenothiazines, 

the drugs incorporated into intact erythrocytes should be present in the membrane.

Similar experiments were carried out on the anionic phenothiazines, MA and PA. Figure 4 shows their effect on membrane shape change of human erythrocytes as a function of initial concentrations added to medium. These drugs instantly induced membrane exvagination of human erythrocytes, and the extent of the shape change progressed with increasing concentrations in medium. At a given concentration in the medium, PA showed a slightly stronger effect than that of MA.

Amounts of these drugs incorporated both into erythrocytes and ghosts increased linearly with increasing medium concentrations (Fig. 5). Their amounts incorporated into whole cells were about 6–12 times higher than those incorporated into ghosts in the range of concentrations cited. Calculation of the membrane-bound drug percentages, performed as described for cationic drugs, gave the values of 4.9 and 6.8% for MA and PA, respectively. These values represent 9.5 and 14.7% of the entire amount incorporated into whole cells, respectively. This result suggests that the amounts of the drugs incorporated into ghosts are only a fraction of those incorporated into whole cells, and that most of the amounts incorporated into whole cells may be present in the cytosol.

DISCUSSION

There are several studies concerned with the action of phenothiazine derivatives on the membrane, including those on their protective and stimulative effect on the hypotonic hemolysis of human erythrocytes and the increasing effect of the permeability of liposomes (7–9). It was also reported that cationic and anionic phenothiazine drugs induced membrane shape changes on human erythrocytes, cup formation, and crenation, respectively, as a result of their action on the plasma membrane (1, 5). Attempts were made in this study to correlate the action of phenothiazine derivatives on the erythrocyte membrane with their chemical structure by observing the membrane shape change induced as a sensitive indicator of the membrane perturbation by the drugs and to analyze their effect in relation not only to the initial concentration of drugs in the medium but also to their amounts incorporated into the whole cells and their plasma membrane.

In order to correlate the membrane-perturbing effect of the drugs tested here with their structure, calculations were made from the data used in constructing Figs. 2–5 and the results are collected in Table 1. In Table 1 the initial concentration of the drug added to medium and the amount of the drug incorporated into whole cells and into ghosts (membrane) to induce a given intensity of the shape change (−2 or +2 as the morphological index, in this case) are indicated. From Table 1, the following conclusions may be drawn.

First, remarkably higher concentrations of anionic phenothiazine drugs added to the medium or in whole erythrocytes are required to induce the membrane exvagination of +2 than the concentrations of cationic drugs required to induce the invagination of −2.[2] This result is virtually consistent with that reported by Mohandas and Feo (5). However, if we refer their effects to the amounts bound to the plasma membrane, the differences become much less marked, and rather smaller quantities of the anionic drugs are required than the cationic ones. This is

[2] Strict comparison of the membrane-perturbing effect underlying the different types of shape change may be impossible. However, rough comparison between the effects at +2 and −2 stages seems to be possible from our data on the shape change caused by splitting the phospholipid molecules in the outer leaflet of the lipid bilayer (10).

because the affinity of the anionic drugs for the membrane is remarkably lower than that of the cationic drugs (5–7% versus 50–91%, as already discussed). Interestingly, the nature (sign) of the electric charge of the polar side-chain groups seems to determine both the affinity for the membrane and the nature of the membrane-perturbing effects of these drugs, leading to different types of the membrane shape change.

Second, as for the five cationic drugs tested, although their initial concentrations to induce a given degree of shape change vary considerably, the amounts bound to the ghosts to induce the same shape change do not show great variations. Actually, very similar amounts of three analogous drugs with the *N*-methylpiperazinyl groups (PZ, PCPZ, and TFPZ) were found to induce the same intensity of the shape change (440–590 nmoles/ml of cells), and it is also true for two kinds of drugs with the dimethylamino group in common (PZ and CPZ) (710–790 nmoles/ml of cells). This implies that replacement of the dimethylamino group with the *N*-methylpiperazinyl group brought about an increase in the degree of the shape change to be induced, and the latter type of the cationic group should give a stronger membrane-perturbing effect than the former tertiary amine group. This fact may also indicate that the intensity of the shape change induced by the same functional group in the drug molecules is almost exclusively determined by the amount of the drug bound to the plasma membrane, and that the apparent big differences between the initial concentrations of the drugs added to medium to induce the same shape change result from differences in the affinity of the drugs for the membrane.

Roth and Seeman (11) demonstrated that the membrane-buffer partition coefficients, $P_{m/b}$, of anesthetics, measured on erythrocyte ghosts, can be a simple and valid model for studying the action of anesthetics. The $P_{m/b}$ for CPZ, calculated from the data in Fig. 3B, was in a range of 998–3387. Although it is not possible to compare precisely the value obtained here with that reported by Roth and Seeman (11) because of the variation of $P_{m/b}$ with free concentration of drug, the NaCl concentration, and pH of the medium (12, 13), the value calculated by them ($P_{m/b}$ 1600) falls in the range cited above. This implies that the amount of drug bound to ghost membranes obtained here roughly agrees with that obtained by Seeman and co-workers (11–13).

In the case of cationic phenothiazines with halogen atom(s) at position C-2 of the nucleus, the affinity percentage values were in a range of 77–91%, whereas the two phenothiazines without such halogen substitution gave the values of 50 and 57%. These results clearly show that the introduction of halogen atom(s) to the phenothiazine nucleus results in the significant increase in the affinity of the drug to the membrane of human erythrocytes. Zografi and Munshi (14) reported that such halogen substitution on the phenothiazine ring enhanced the surface activity of the drugs, reflecting their hydrophobic characteristics in a decreasing order of $CF_3 \gg Cl > H$. This result is consistent with the order of the affinity percentage values of cationic phenothiazines obtained here.

From the results and interpretations discussed above, it seems possible that the effect of a drug on erythrocyte membranes, and hence probably on plasma membranes in general, could be divided into two phases: one is concerned with the selective transference of drugs from the external medium to the membrane (namely, the affinity of the drug for the membrane), and the other is concerned with the intensity of the membrane-perturbing action of the drug molecule once incorporated (bound) to the membrane. In drug-design problems, for example, such a consideration will be useful since structural modification may be attempted from two different viewpoints.

REFERENCES

1. Deuticke, B. Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. *Biochim. Biophys. Acta* **163**:494–500 (1968).
2. Fujii, T., T. Sato, and K. Nakanishi. *In vitro* shape changes of human erythrocyte membranes. *Physiol. Chem. Physics* **5**:423–430 (1974).
3. Sheetz, M. P., and S. J. Singer. Biological membranes as bilayer couples: a molecular mechanism of drug-erythrocyte interaction. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4457–4461 (1974).
4. Fujii, T., T. Sato, A. Tamura, M. Wakatsuki, and Y. Kanaho. Shape changes of human erythrocytes induced by various amphipathic drugs acting on the membrane of the intact cells. *Biochem. Pharmacol.* **28**:613–620 (1979).
5. Mohandas, N., and C. Feo. A quantitative study of the red cell shape changes produced by anionic and cationic derivatives of phenothiazine. *Blood Cells* **1**:375–384 (1975).
6. Dodge, J. T., C. Mitchell, and D. J. Hanahan. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119–130 (1963).
7. Mao, T. S. S., and J. J. Noval. Some relationships concerning the chemical structure, hemolytic activity, and therapeutic potency of phenothiazines. *Biochem. Pharmacol.* **15**:501–504 (1966).
8. Naoi, M., T. Suzuki, and K. Yagi. Effects of chlorpromazine and other phenothiazine derivatives on the permeability of liposomes. *Biochem. Pharmacol.* **28**:295–299 (1979).
9. Seeman, P., and J. Weinstein. I. Erythrocyte membrane stabilization by tranquilizers and antihistamines. *Biochem. Pharmacol.* **15**:1737–1752 (1966).
10. Fujii, T., and A. Tamura. Asymmetric manipulation of the membrane lipid bilayer of intact human erythrocytes with phospholipase A, C, or D induces a change in cell shape. *J. Biochem. (Tokyo)* **86**:1345–1352 (1979).
11. Roth, S., and P. Seeman. The membrane concentrations of neutral and positive anesthetics (alcohols, chlorpromazine, morphine) fit the Meyer-Overton rule of anesthesis; negative narcotics do not. *Biochim. Biophys. Acta* **255**: 207–219 (1972).
12. Kwant, W. O., and P. Seeman. The membrane concentration of a local anesthetic (chlorpromazine). *Biochim. Biophys. Acta* **183**:530–543 (1969).
13. Seeman, P. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583–655 (1972).
14. Zografi, G., and M. V. Munshi. Effect of chemical modification on the surface activity of some phenothiazine derivatives. *J. Pharm. Sci.* **59**:819–822 (1970).

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