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Effect of Phospholipase A_2 and Cholesterol Oxidase on Perazine and Promethazine Penetration into Human Erythrocytes and on Fluidity of the Membrane

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To clarify the mode of action of drugs on human erythrocyte membrane and the role of lipids in transmembrane transport of drugs, the cell and membrane were treated with phospholipase A_2 and cholesterol oxidase respectively, and the penetration of perazine and promethazine into the membrane and the membrane fluidity were estimated. It was found that 58.3% of phosphatidylcholine in the membrane was hydrolyzed. The fluidity of the membrane, estimated by the electron spin resonance method, was significantly enhanced by phospholipase A_2 treatment. When lysophosphatidylcholine and fatty acids produced were extracted by using albumin, the fluidity was greatly decreased. Although the treatment with cholesterol oxidase induced degradation of 24% of the cholesterol in the membrane, the fluidity was not altered. The amounts of these drugs that penetrated into the erythrocytes were significantly increased after phospholipase A_2 treatment, but dramatically decreased after the extraction of the products formed by phospholipase A_2, while the amounts were not significantly increased by cholesterol oxidase treatment. These resulted led us to postulate that phospholipids in the erythrocyte membrane are probably involved in the penetration of the two drugs, whereas the involvement of cholesterol is far less, and that the increased fluidity of the membrane lipids certainly contributes to the enhancement of penetration.

Keywords——erythrocyte membrane; perazine penetration; promethazine penetration; membrane fluidity; phospholipase A_2 treatment; cholesterol oxidase treatment; human erythrocyte

It is now well established that phospholipid distribution across the erythrocyte membrane bilayer is asymmetrical.[1–4] Sphingomyelin (SM) and phosphatidylcholine (PC) are predominantly present in the outer bilayer and all of the phosphatidylserine (PS) and most of the phosphatidylethanolamine (PE) are present on the inner side of the membrane bilayer. During the whole 120-d lifespan of the human erythrocyte, the overall phospholipid composition remains rather constant,[5,6] and upon extreme variations in dietary fats, the alterations in fatty acid composition are less pronounced than the changes in lipid composition of the plasma.[7–9] The lipid composition of the erythrocyte thus appears to be highly conserved throughout. It seems probable that changes in phospholipid asymmetry affect specific membrane properties such as permeability, fluidity and resistance to mechanical or osmotic stress.

It is widely accepted that various drugs penetrate into erythrocyte membrane in a manner that is dependent on the concentration of un-ionized or free drug.[10] The penetration of drugs is probably affected by alterations in the lipid organization.

We have been studying the mechanism of drug-induced hemolysis of human eryth-

rocytes.[11-13] To further characterize the mode of action of some drugs on the erythrocyte membrane, our attention has been directed to the penetration of perazine and promethazine into the membrane treated with phospholipase A_2 [EC 3.1.1.4], into the membrane from which lysoPC has been removed by albumin and into the membrane treated with cholesterol oxidase [EC 1.1.3.6]. In addition, the fluidity of the erythrocyte membrane treated with these enzymes was estimated in relation to the perturbation of membrane structure.

Materials and Methods

Materials——Perazine dimalonate (Morishita Pharmaceutical Co., Osaka) and promethazine hydrochloride (Shionogi Pharmaceutical Co., Osaka) were used throughout this experiment. Phospholipase A_2 (800 IU/mg protein) from bee venom and bovine serum albumin (fatty acid-free) were obtained from Sigma Chemical Co. Cholesterol oxidase (16.7 units/mg protein) from *Pseudomonas* species was kindly supplied by Amano Pharmaceutical Co., Nagoya). The spin labels, 2-(14-carboxytetradecyl)-2-ethyl- and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl (abbreviated as I (1,14) and I (12,3), respectively) were purchased from Aldrich Chemical Co.

Preparation of Erythrocyte Suspension and Hemoglobin-free Erythrocyte Ghosts——Human erythrocytes from fresh blood were isolated by centrifugation and the buffy coat was removed by aspiration. The cells were washed three times with isotonic NaCl–phosphate buffer (NaCl 8.5, $NaH_2PO_4 \cdot 2H_2O$ 0.343 and $Na_2HPO_4 \cdot 12H_2O$ 3.443 g/l, pH 7.4) and resuspended in Medium A[14] (KCl 90 mmol, NaCl 45 mmol, Na_2HPO_4/NaH_2PO_4 10 mmol and sucrose 44 mmol/l, pH 8.0) at $10 \pm 0.2\%$ hematocrit. Iodoacetate was added to the erythrocyte suspension at a final concentration of 5 mmol/l and the cells were incubated at 37 °C for 15 min. After three washings, the cells were incubated for 15 min at 37 °C with 10 volumes of Medium A containing 5 mmol/l diamide (Sigma Chemical Co.) and washed three times with Medium A. Iodoacetate and diamide were used for the blockage of SH groups of intracellular glutathione and the suppression of shape changes[14] during experiments, respectively. Human erythrocyte ghosts were prepared by the method of Dodge *et al.*[15]

Phospholipase A_2 Treatment and Extraction of LysoPC and Fatty Acids——The treatment of erythrocyte suspension with phospholipase A_2 and the extraction (35 mg albumin/ml cells) of lysoPC and fatty acids produced (phospholipase A_2/albumin) were carried out by the procedures described by Haest *et al.*[14]

Cholesterol Oxidase Treatment——Since no oxidation of cholesterol by cholesterol oxidase occurred in intact cells, ghost membranes were used in this experiment. A mixture of ghost suspension (1.0 ml, 5 mg protein/ml) and isotonic NaCl–20 mM phosphate buffer (0.4 ml, pH 6.5) was incubated with 20 units (0.2 ml) of cholesterol oxidase for 1 or 2 h at 37 °C. The membrane were quickly washed twice with the cold isotonic NaCl–phosphate buffer, pH 7.4, and were resuspended in the isotonic buffer at 5 mg protein/ml.

Quantification of Phospholipids and Cholesterol——Erythrocyte lipids were extracted by the method of Rose and Oklander[16] and phospholipids were separated by two-dimensional thin-layer chromatography (2D-TLC) on silica plates as described by Roelofsen and Zwaal.[17] Thin-layer plates were developed in the first direction with $CHCl_3 : CH_3OH : NH_3$ (25%): H_2O (90 : 54 : 5.5 : 5.5, by volume) and the second direction with $CHCl_3$: $CH_3OH : CH_3COOH : H_2O$ (90 : 40 : 12 : 2, by volume) at room temperature. The content of phospholipid phosphorus was determined by the method of Ames.[18] Cholesterol content was determined by the ferric chloride–acetic acid method of Zak and Henly.[19]

Preparation of Spin-Labeled Erythrocytes or Ghosts and Electron Spin Resonance (ESR) Measurement——The preparation of the spin-labeled erythrocytes or ghosts and ESR measurement were carried out by the methods described previously.[20] The rotational correlation time, τ_c, and the order parameter, S, were calculated as described in the previous paper.[20]

Electron Microscopy——Erythrocytes treated with phospholipase A_2 were fixed with 1.5% glutaraldehyde in isotonic phosphate buffer, pH 7.2. The cells were washed three times with the same buffer and dried with increasing concentrations of acetone (60 to 100%, v/v). The specimens were coated with platinum and viewed with a Hitachi scanning electron microscope, model S-700.

Measurement of Perazine and Promethazine Penetrated into Cell or Membrane——A cell (2 or 3 ml, hematocrit value $10 \pm 0.2\%$) or ghost suspension (1 ml, 5 mg protein/ml) was added to 10 ml of drug solution, below the hemolytic concentrations, in isotonic NaCl–phosphate buffer, and the mixture was incubated for 30 min at 37 °C. The cells or membranes were carefully but quickly washed twice with 20 ml of the isotonic buffer. The drug in the suspension was extracted with ethyl acetate by the method described previously.[13]

Protein Determination——Protein concentration was determined by the procedure described by Lowry *et al.*[21] with bovine serum albumin, fraction V, as a standard.

Statistical Analysis——The means of all data are presented with their standard deviation (mean $\pm$ S.D.). Statistical analysis was performed using the non-paired Student's t-test, and a p value of 0.05 or less was considered to be significant.

Results

Changes in Phospholipid Composition Following Phospholipase A_2 Treatment

It has been shown that phospholipase A_2 from bee venom attacks a major fraction of the PC located in the outer lipid layer of human erythrocyte membrane[22] and that the enzyme from *Naja naja* cleaves 56% of the PC without changing the discoidal shape of the cells.[14] The lipid extracts after phospholipase A_2 and phospholipase A_2/albumin treatments were separated by 2D-TLC (data not shown). The appearance of a large amount of lysoPC, a decrease in PC and the extraction of lysoPC by albumin were observed. About 58% of PC was hydrolyzed by the enzyme treatment, as shown in Table I. The hemolysis following digestion was less than 3% in all cases.

Fluidity of Erythrocyte Membrane Following Phospholipase A_2 Treatment

Figure 1 shows representative ESR spectra obtained with erythrocytes labeled with spin label I (12,3) or I (1,14), after phospholipase A_2 and the enzyme/albumin treatments. Both treatments produced significant changes in the spectrum of I (1,14); the high-field extreme of the spectrum was markedly enhanced by phospholipase A_2 treatment. The S and τ_c values of both probes, calculated from the ESR spectra, are listed in Table II. The τ_c value after phospholipase A_2 treatment was lowered by 25% compared with that of the untreated cells, whereas phospholipase A_2/albumin treatment significantly increased the τ_c value. It is thus

TABLE I. Hydrolysis of Phospholipids in Erythrocytes by Phospholipase A_2

Cell	Total phospholipid phosphorus (μg/ml 10% hematocrit cell)	PC hydrolyzed (%)
Untreated	11.76 ± 1.04	0
Phospholipase A_2	11.98 ± 1.15	58.3
Phospholipase A_2/albumin	$9.94 \pm 0.96^{a,b)}$	—

Each value represents the mean $\pm$ S.D. of 4 experiments. *a*) $p<0.05$ in untreated *vs.* phospholipase A_2. *b*) $p<0.02$ in phospholipase A_2 *vs.* phospholipase A_2/albumin.

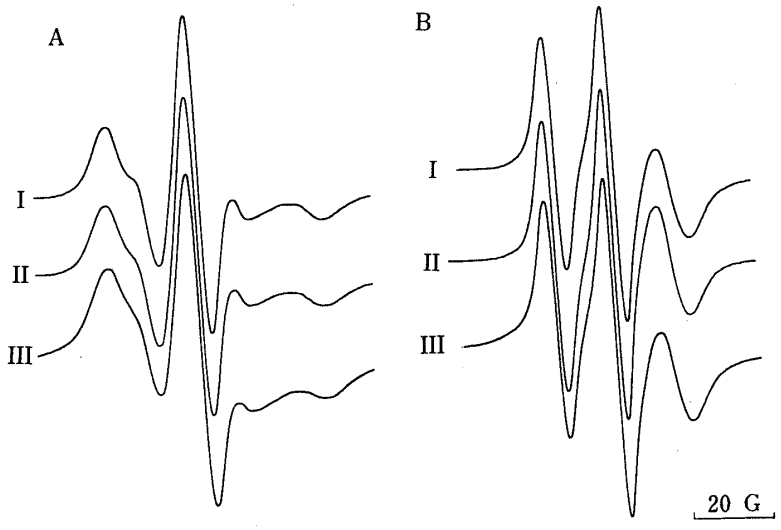

Fig. 1. ESR Spectra of I(12,3)-Labeled and I(1,14)-Labeled Erythrocytes Following Treatment with Phospholipase A_2 or Phospholipase A_2/Albumin

I, untreated; II, phospholipase A_2 treatment; III, phospholipase A_2/albumin treatment; A, I(12,3)-labeled; B, I(1,14)-labeled.

TABLE II. The Freedom of Motion of Lipid Spin Labels I(12,3) and I(1,14) in Erythrocytes Treated with Phospholipase A_2 or Phospholipase A_2/Albumin

Treatment	S 37 °C	τ_c (ns) 25 °C	τ_c (ns) 37 °C
Untreated	0.635±0.008	3.38±0.14	2.99±0.21
Phospholipase A_2	0.635±0.006	2.97±0.28[a)]	2.44±0.31[b)]
Phospholipase A_2/albumin	0.626±0.016	3.81±0.21[c,d)]	3.61±0.11[c,d)]

Each value represents the mean±S.D. of 4—5 experiments. *a*) $p<0.05$ in untreated *vs.* phospholipase A_2. *b*) $p<0.02$ in untreated *vs.* phospholipase A_2. *c*) $p<0.02$ in untreated *vs.* phospholipase A_2/albumin. *d*) $p<0.001$ in phospholipase A_2 *vs.* phospholipase A_2/albumin.

TABLE III. The Freedom of Motion of Lipid Spin Labels I(12,3) and I(1,14) in the Membranes of Erythrocytes Treated with Cholesterol Oxidase

Treatment	S 37 °C	τ_c (ns) 25 °C	τ_c (ns) 37 °C
Untreated	0.638±0.006	3.00±0.05	2.56±0.04
Cholesterol oxidase	0.636±0.004	2.97±0.04	2.49±0.05

Each value represents the mean±S.D. of 4—5 experiments.

suggested that the production of lysoPC and fatty acids in the membrane enhances the molecular motion of the probe in the interior of the lipid bilayer and the extraction of them from the membrane decreases the motion. On the other hand, the S value and the width of outer extrema, $2T_{\parallel}$, of I (12,3) were not altered by phospholipase A_2 or the enzyme/albumin treatment. This may indicate less alteration of the fluidity of the outer surface.

Changes in Cholesterol Content and Membrane Fluidity Following Cholesterol Oxidase Treatment

The treatment of erythrocyte membrane with cholesterol oxidase (20 units/ml ghost) resulted in degradation of 24% of the tatal cholesterol in the membrane after a 1-h incubation and 40% after a 2-h incubation. The S and τ_c values calculated from ESR spectra are shown in Table III. Both values were essentially similar for the untreated and the oxidase-treated (for 1 h) membranes, suggesting that the partial oxidation (24%) of cholesterol in the erythrocyte membrane with the enzyme does not drastically affect the membrane fluidity.

Amounts of Perazine and Promethazine Penetrated into Erythrocyte and Membrane Treated with Enzymes

Table IV shows the amounts of perazine and promethazine that penetrated into erythrocytes treated with phospholipase A_2. The amounts were markedly increased after the treatment, while the extraction of lysoPC and fatty acids by albumin obviously decreased the amounts of the drugs in the cells.

Table V depicts the amounts of the two drugs that penetrated into ghost membrane after cholesterol oxidase treatment. The drug penetration into the treated membrane was slightly enhanced compared with that into the untreated membrane. However, the increase after cholesterol oxidase treatment was much smaller than that following phospholipase A_2 treatment and was not significant.

The results of the drug penetration experiment suggest that transport or permeation of

TABLE IV. Amount of Drugs Penetrated into Erythrocytes Treated with Phospholipase A_2 or Phospholipase A_2/Albumin

Treatment	Perazine (mM)		Promethazine (mM)	
	0.1	0.2	0.2	0.5
Untreated	1.620±0.074	3.055±0.078	0.974±0.103	2.393±0.038
Phospholipase A_2	1.999±0.193[a)]	3.418±0.395	1.567±0.113[b)]	3.079±0.224[b)]
Phospholipase A_2/albumin	0.926±0.054[b,c)]	1.865±0.260[b,c)]	0.742±0.092[a,c)]	1.523±0.268[a,c)]

Each value (10^{-8} mol) represents the mean±S.D. of 4—6 experiments. *a)* $p<0.01$ in untreated *vs.* phospholipase A_2 or phospholipase A_2/albumin. *b)* $p<0.001$ in untreated *vs.* phospholipase A_2 or phospholipase A_2/albumin. *c)* $p<0.001$ in phospholipase A_2 *vs.* phospholipase A_2/albumin.

TABLE V. Amount of Drugs Penetrated into Erythrocyte Membrane Treated with Cholesterol Oxidase

Treatment	Perazine (mM)	
	0.1	0.2
Untreated	5.608±0.529	7.756±0.657
Cholesterol oxidase	6.174±0.556	8.176±0.653

Each value (10^{-8} mol) represents the mean±S.D. of 4—5 experiments.

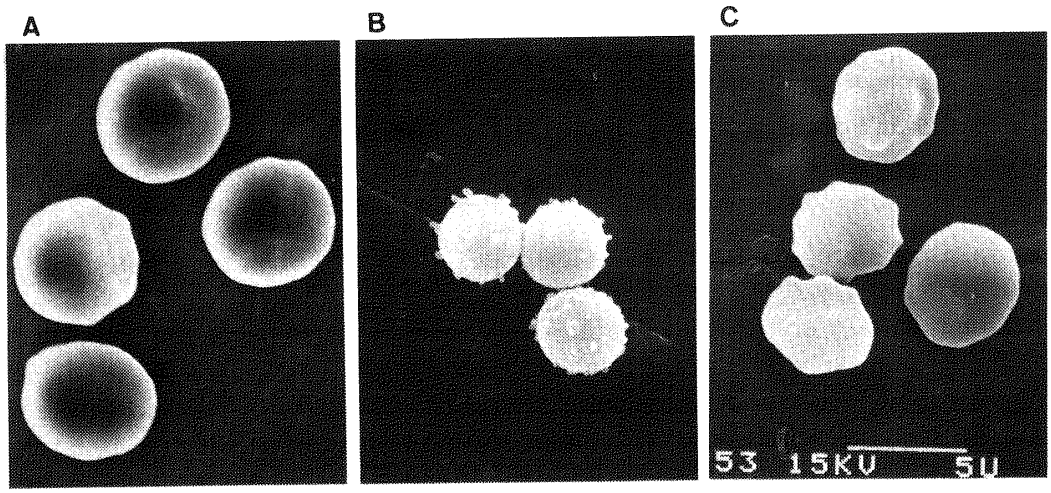

Fig. 2. Scanning Electron Micrographs of Erythrocytes Treated with Phospholipase A_2 or Phospholipase A_2/Albumin

A, untreated; B, phospholipase A_2 treatment; C, phospholipase A_2/albumin treatment. Magnification 5000×.

the two drugs across the erythrocyte membrane probably proceeds through phospholipids, and much less through cholesterol.

Scanning Electron Microscopic Observations

The results of microscopic observations of cells treated with phospholipase A_2 and phospholipase A_2/albumin is shown in Fig. 2. After phospholipase A_2 treatment, erythrocytes rapidly underwent echinocytic transformation, as demonstrated by Fujii and Tamura.[23)] When most of the lysophospholipids and fatty acids produced were extracted by albumin, the cells appeared as slightly irregular discs, although the cell shape was roughly similar to the normal one.

Discussion

A remarkably high fraction of outer layer phospholipids can be removed from the erythrocyte membrane without loss of membrane integrity.[24] Cholesterol is present in abundance in both leaflets of the bilayer and the cholesterol content in the membrane affects the membrane fluidity.[25] However, there is little knowledge about the effect of loss of lipid asymmetry on drug penetration into the erythrocyte membrane. In the present experiments, 58.3% of the PC was cleaved by phospholipase A_2 (Table I). The extent of PC hydrolyzed was similar to the data (55.9 and 41.3% respectively) of Haest *et al.*[14] and Fujii and Tamura.[23] Since treatment with phospholipase A_2 has shown the presence of the majority of PC in the outer bilayer of erythrocyte membrane,[26,27] our results indicate that a large amount of PC in the outer layer was hydrolyzed under the conditions used. Haest *et al.* show that removal of outer layer lipids is not compensated in the normal, shear-deformable membrane by a translocation of inner layer phospholipids to the outer layer.[24] The changed organization of lipids would be maintained during our experiment.

The fact that the τ_c value of the probe was decreased after phospholipase A_2 treatment and increased after phospholipase A_2/albumin treatment (Table II) suggests that large amounts of lysoPC and fatty acids, remaining in the membrane, induced perturbation of the lipid bilayer structure and enhanced the fluidity of the interior of membrane lipids and that the removal of the products from the membrane made the lipid environment more rigid than in the normal membrane. However, the decreased fluidity after the extraction of the hydrolysis products may be in part owing to an absolute loss of phospholipids in the outer layer and the decrease in the outer surface area. Haest *et al.* have shown that 26% of the outer phospholipids are extracted by albumin from phospholipase A_2-treated cells and about 17% of the outer surface area is lost after the extraction with albumin.[14] The observations by scanning electron microscopy demonstrated the partial loss of the outer surface area; the mean diameters of intact cells and phospholipase A_2/albumin-treated cells were 5.38 ± 0.22 and 4.63 ± 0.37 μm, respectively (Fig. 2). After treatment with phospholipase A_2, erythrocytes underwent echinocytic transformation. This phenomenon is probably due to the presence of lysophospholipids primarily in the outer leaflet of the membrane.

Transbilayer movement of cholesterol in fresh erythrocytes has been demonstrated to be very fast; the half-time of the transmembrane movement (flip-flop) of cholesterol is 3 s at 37 °C.[28] Transbilayer mobility of cholesterol thus may be expected to contribute to drug transport from the outer to the inner layer. However, since the partial oxidation of cholesterol in the membrane did not significantly affect the drug penetration, the drug transport accompanying cholesterol movement may be much less than that in the case of phospholipids. The fact that the lipid fluidity of the membrane was scarcely changed after treatment with cholesterol oxidase demonstrated that the effect of the enzyme treatment on the membrane organization is small.

The concentrations of drugs used in this study were below the hemolytic level, because drug-induced hemolysis would have made it impossible to estimate exactly the amount of drug that had penetrated into membrane. When the amounts of perazine and promethazine that penetrated into erythrocytes and the membrane treated with phospholipase A_2 and cholesterol oxidase, respectively, were estimated, much greater amounts of drug had penetrated into the cells treated with phospholipase A_2 than into untreated cells. On the other hand, the amounts of the two drugs that penetrated into the cells treated with phospholipase A_2/albumin were very much less as compared with the untreated cells. These results indicate that the increased fluidity or perturbation of membrane lipids enhanced drug penetration into cells, whereas decreased fluidity reduced it. This concept is strengthened by the result of cholesterol oxidase treatment, which did not affect the membrane fluidity and did not

TABLE VI. Physicochemical Parameters of Drugs Used

Parameter	Perazine	Promethazine
$P^{a)}$	102.5	123.6
$P_{m/b}{}^{b)}$	10.1	3.0
Activation energy[c] (cal)	1067±268	3125±1333
$V^{d)}$	195.71	164.12
pK_a	8.8	9.1

Each value is the mean of 4 experiments. *a*) Octanol/water partition coefficient. *b*) Membrane partition ratio of drug at pH 7.4. *c*) Activation energy for drug penetration into the membrane. *d*) van der Waals volume of drug.

significantly increase the drug penetration into the membrane. Judging from the results obtained, it would be reasonable to assume that phospholipids in erythrocyte membrane are mainly involved in the penetration or transport of perazine and promethazine, which are lipophilic drugs. The translocation of PC at 37 °C has been found to be quite slow.[29] Therefore the contribution of flip-flop to the drug penetration seems to be ruled out. It is suggested that cholesterol modulates thiopental partitioning into erythrocyte ghosts and an increase in cholesterol content decreases the partition coefficient.[30] However, the decrease in cholesterol content below the normal value may not be related to the drug penetration into the membrane.

We have previously reported that basic drugs including perazine and promethazine have a high membrane/buffer partition ratio ($P_{m/b}$) and the drug penetration is enhanced with increasing drug concentration.[12] In addition, these drugs at 0.2 mM increase the membrane fluidity, as indicated by a decrease in τ_c value.[12] Therefore, the fluidizing action of these drugs may additively contribute to the membrane perturbation induced by phospholipase A_2 and may assist in part the drug penetration into the membrane. However, the relative contribution made by these drugs themselves to the overall penetration remains to be clarified. The physicochemical parameters of the two drugs calculated in our previous studies[12,13] are shown in Table VI. A significant difference is found in the $P_{m/b}$ and activation energy for drug penetration, but not in the partition coefficient or pK_a. As a result, it is suggested that perazine penetrates into the membrane more easily than promethazine. This is consistent with the result of the present study. This phenomenon may be related to a bulky piperazinyl ring in the molecule and the larger van der Waals volume, as suggested in our previous paper.[12]

The conditions (treatment with iodoacetate and diamide) used were rather far from physiological. Despite this feature of the study, it seems possible to draw some limited conclusions. Phospholipase A_2 from bee venom cleaved a larger amount of PC located in the outer lipid layer of erythrocyte membrane and increased the fluidity in the interior of the lipid bilayer. This alteration of membrane organization induced the increase in the penetration of perazine and promethazine into the cells. The partial loss of cholesterol in the membrane scarcely affected the lipid fluidity or drug penetration.

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