MOLECULAR PHARMACOLOGY, 16, 181-188

Interaction of Phenothiazines and Lower Aliphatic Alcohols with Erythrocyte Membranes: A Scanning Calorimetric Study

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(Received July 17, 1978)
(Accepted March 5, 1979)

SUMMARY

KRISHNAN, K. S. AND JOHN F. BRANDTS: Interaction of phenothiazines and lower aliphatic alcohols with erythrocyte membranes: A scanning calorimetric study. *Mol. Pharmacol.* **16,** 181-188 (1979).

Human erythrocyte membranes at pH 7.4 in 310 imosm sodium phosphate buffer show several distinct endothermic transitions when studied by differential heat capacity calorimetry. Various tranquilizers in the phenothiazine family shift one of these major transitions, normally at 67°, down to about 60° when present at concentrations of the order of 40 μM or less. The lower aliphatic alcohols in dilute concentrations affect two of these transitions and at higher concentrations shift all the transitions down, by temperatures as much as 30°. The concentrations of alcohols needed to cause similar effects decrease with increasing number of carbon atoms. The effect of alcohols and phenothiazines below 150 μM can be reversed by washing the membranes in sodium phosphate buffer. The common effect of all these compounds at anesthetic concentrations seems to be mainly on one transition, which has been tentatively identified as involving a lipid domain on the membrane which has previously been shown to function in anion transport.

INTRODUCTION

The great interest in phenothiazines, resulting particularly from their use as major tranquilizers, is reflected in the number of reviews that have appeared in recent years (1-5). It has been postulated that one of the major effects, and conceivably the primary mode of action of these drugs, is the alteration of membrane properties. Domino *et al.* (6), for example, stress that membranes may be involved in the action of phenothiazines in view of the marked surface activity of these drugs. It has also been known for some time that ethanol and other small aliphatic alcohols exert a general depressant effect upon membrane-linked functions of a variety of organisms (7) and inhibit such membrane-linked phenomena as the motility of leukocytes, spermatozoa and parameceum, the luminiscence of bacteria and contractility of rat atrium (8). Wright (9) has shown that ethanol also inhibits the conduction of nerve impulses, and it has been suggested that alcohols function at the cellular level primarily by altering membrane permeability and ion flux machinery (10). Thus, alcohols also apparently have their site of action in membranes and thereby affect impulse transmission (11-13).

There have been a number of studies on the interaction of both phenothiazines and alcohols with numerous isolated membrane systems (14-25), with a strong emphasis on the use of fluorescent and EPR[2] probes.

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[2] The abbreviation used is: EPR, electron paramagnetic resonance.

0026-895X/79/040181-08$02.00/0

Although it has uniformly been found that both phenothiazines and alcohols cause perturbation of membrane-bound probes, these studies have not yet led to any consensus concerning their mode of action. Various suggestions include 1) effects that cause increased rigidity of the protein phase; 2) increased mobility in the lipid phase; and 3) expansion of the membrane. Alcohols, in addition, show a biphasic nature since they cause a second large effect on membranes (17, 25, 26) at above-lytic concentrations, which has been suggested to result from disruption of protein-lipid interactions (18).

Thus far, there have been no studies which point to *specific* membrane components as the site of action of these and other membrane-active drugs. The present studies utilize scanning calorimetry as a means of focusing attention on specific structural domains within the erythrocyte membrane. Using a highly-sensitive differential heat capacity calorimeter, five well-defined structural transitions can be observed for human erythrocyte ghosts over the temperature region from 40° to 80° (27, 28). Each of these transitions is largely irreversible and appear to result from the cooperative disruption of a specific local region of the membrane which is highly-ordered at physiological temperature. Earlier studies have been successful to a degree in identifying the membrane components which participate in each of these transitions (28–32).[3]

It will be seen from the results presented in this paper that the phenothiazines and alcohols interact most strongly with one of the five transitions when concentrations are low, i.e., with the C transition. Thus, the "C region" of the native membranes appears to have high specificity for these drugs. This is all the more interesting since it is known that this region of the membrane is also functional in anion transport (31), and thereby the results suggest a potential relationship between membrane permeability and the presence of anesthetics.

MATERIALS AND METHODS

Erythrocyte membranes were prepared by the procedure of Dodge *et al.* (33), b hypotonic hemolysis. The final compositio of the suspending buffer was obtained b washing the membranes two times in th appropriate solvent with a 1:20 excess vo ume. Membrane concentrations were dete mined by drying the samples to constar weight at 105°. The SITS treatment of th red blood cells was done according to pr cedures described by Cabantchik and Roth stein (34), and as previously used in this la (31). DIDS treatment was carried out a described by Cabantchik and Rothstei (35).

Heat capacity measurements were mad on a high-sensitivity, differential scannin calorimeter (36), similar in design to a con mercially-available instrument (MicroC Inc., Amherst, Mass.). Concentrations the membranes were between 1 and 1.5% The noise level of the instrument was typ ically less than 5% of the transition ampl tudes.

Alcohols used were spectroscopic grad Dartal (thiopropazate-2 HCl) was a produ of G. D. Searle & Co. Fluphenazine wa obtained from Squibb. Prochlorperazin and chlorpromazine were obtained fro Smith, Kline and French. Drugs wer freshly dissolved in appropriate sodiu phosphate buffer and these solutions wer used to wash membranes. The washin were performed immediately prior to th start of the calorimetric run. The referen cell of the calorimeter contained the dr at the same concentration as the samp cell, but contained no membrane. Th drugs were supplied in pure salt form an used as such.

RESULTS

Human erythrocyte membranes at p 7.4 in 310 imosm sodium phosphate buff show five distinct endothermic transitio at 50, 55, 62, 66 and 77° (curve a in Fig. 1 These are called the A, B_1, B_2, C and transitions, respectively. Of these, only I is sensitive to pH with an apparent p around 7.5 (30). At lower ionic strengt (77.5 imosm), B_1 and B_2 fuse together form a single B transition. Circular dichr ism experiments from this laboratory ha suggested that only the A and D transitio involve extensive protein unfolding. Extra

[3] Brandts, J. F., R. Carlson, R. D. Traverna, J. Snow, and K. Lysko. Unpublished observations.

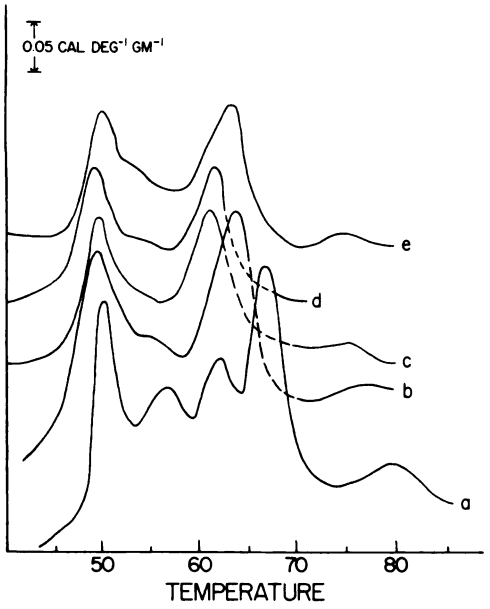


FIG. 1. *Differential heat capacity scans of human ythrocyte membranes in the presence of various enothiazines*

Ghosts were washed twice in 310 imosm sodium osphate buffer at pH 7.4 which contained the drugs the concentration indicated. a. No drugs. b. 40 μM lorpromazine. c. 40 μM prochlorperazine. d. 40 μM phenazine. e. 40 μM Dartal (thiopropazate-2HCl).

on experiments have shown definitely at the A transition, centered near 50°, is ue to the partial unfolding of spectrin (28,). Further experiments have indicated in- lvement of bands IV.1 and IV.2 in the B_1 ansition and band III in the B_2 transition 9).[3] From various chemical and enzymatic odification studies, a strong case has been ade for the C transition being due to some id-dependent region of the membrane 0, 31), located in close proximity to band I.

Curves b–e in Figure 1 show the effect of rious phenothiazines, at a concentration 40 μM, on the human erythrocyte mem- ane transitions. All four drugs shift the C ansition down by 4–7°. The B_2 transition apparently buried underneath the C tran- ion while the B_1 transition is much re- ced in size and perhaps lowered in tem- rature. There seems to be an overall de- ease in the height and sharpness of all aks.

At lower ionic strengths (77.5 imosm), der conditions where B_1 and B_2 are fused to form a single B transition at 57°, it was found that the same degree of shift of the C transition persists. However, the transitions are crowded together and more difficult to resolve. Figure 2 shows the effect of phenothiazines at ca. 40 μM concentration on the ghosts suspended in the lower salt buffer at the same pH, i.e., 7.4. It can be seen that A and B transitions are virtually unaffected and the C transition is shifted down by several degrees. The effect of Dartal (thiopropazate-2 HCl) under these salt conditions is somewhat unusual. There seems to be small upshift of the A transition and the resulting lack of resolution gives a strange-looking endotherm.

Figure 3 shows the effect of increasing concentrations of prochlorperazine on the endothermic transitions. At concentrations above 150 μM the membranes were aggregated. The drug effects below this concentration show the progressive shift toward lower temperature of the C transition. These effects could be reversed by washing

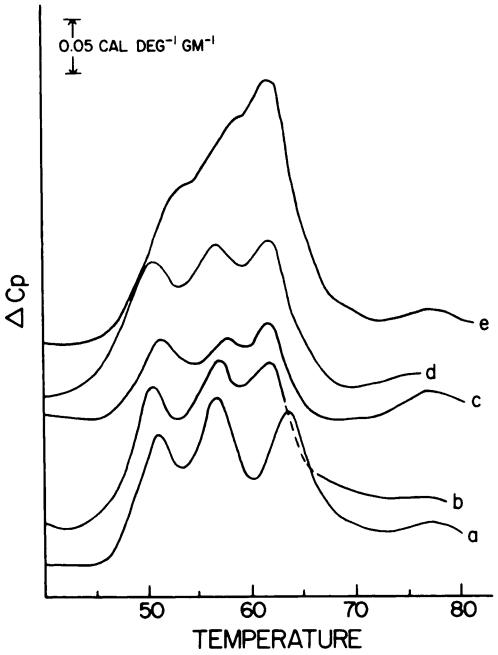


FIG. 2. *Differential heat capacity scans of human erythrocyte ghosts in low salt conditions in the presence of drugs*

The buffer was 77.5 imosm sodium phosphate. Washing protocol was the same as in Figure 1. a) Control (77.5 sodium phosphate pH 7.4, no drugs); b) + 40 μM chlorpromazine; c) + 40 μM fluphenazine; d) + 40 μM prochlorperazine; e) + 40 μM Dartal.

the drug-treated membrane twice with 310 imosm pH 7.4 sodium phosphate buffer. Membranes treated with drugs up to 150 μM and subsequently washed twice in 310 imosm pH 7.4 buffer gave calorimetric scans identical to membranes which had not been exposed to the drug.

Alcohols at low concentrations have an effect similar to the drugs in that they shift the C transition down (Fig. 4). The relative effect of alcohols at the same concentration follows an order propanol > ethanol > methanol. It is also noteworthy that alcohols not only affect the C transition but also shift the B_2 transition substantially down. T_m is virtually unchanged in A and B_1 but B_1 is reduced in size.

At above lytic concentrations, alcohols have a more dramatic effect on the membranes (Fig. 5). At high concentrations (ETOH 10%, MEOH 20% or propanol 4%) all the transitions are shifted down and a pronounced difference in the pattern of the

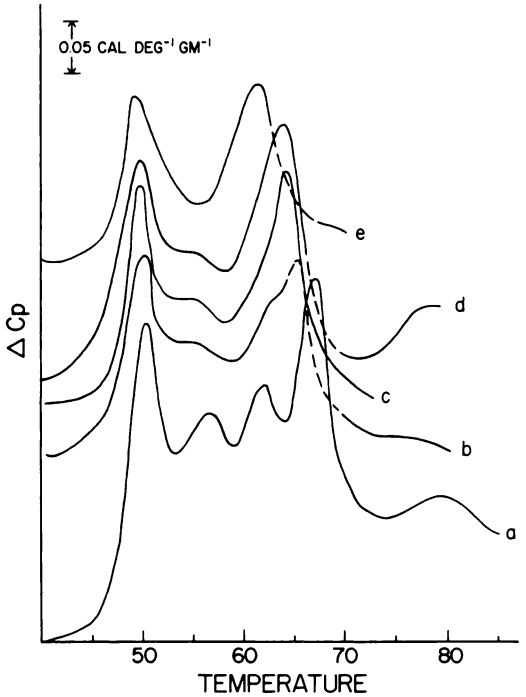


FIG. 3. *Effect of increasing concentrations of prochlorperazine on the human erythrocyte endotherm*

The buffer was 310 imosm sodium phosphate, pH 7.4. a) control; b) 8 μM prochlorperazine; c) 30 μM prochlorperazine; d) 40 μM prochlorperazine; e) 70 μM prochlorperazine.

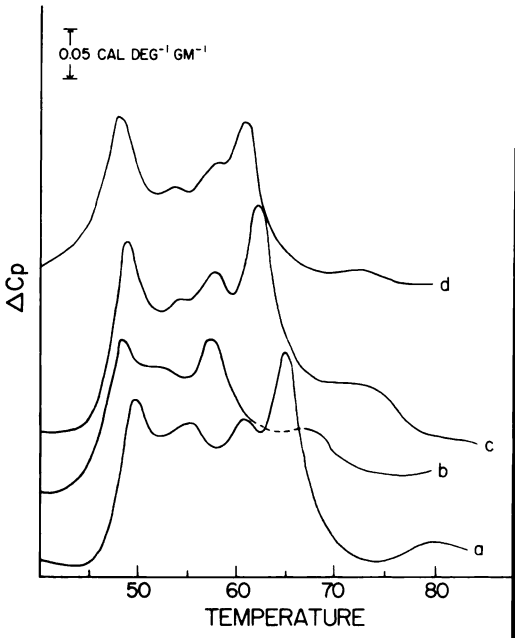


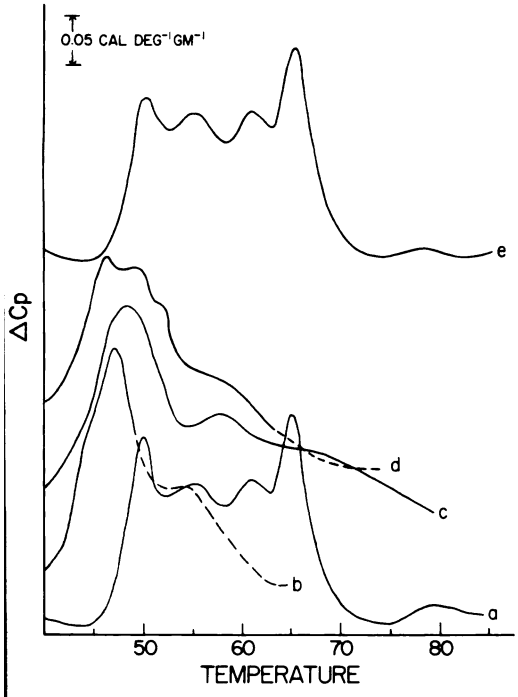
FIG. 4. *Effect of alcohols at below lytic concentrations on human erythrocyte ghosts at 310 imosm, pH 7.4*

a) control; b) 10% MeOH c) 2% EtOH; d) 1% *n*-propanol.

endotherm is obtained. The shifts of the transitions vary from about 5° for the A transition to nearly 20° for the C transition. The effect of alcohols even at these concentrations can be reversed by washing the alcohol treated membranes in 310 imosm sodium phosphate twice. Figure 5 curve e shows the scan for an *n*-propanol (4%) treated sample where the alcohol was subsequently removed before heating by washing in 310 imosm sodium phosphate. The normal calorimetric pattern is observed.

The effect of increasing concentrations of *n*-propanol on the membrane transitions is shown in Figure 6. At low concentrations (1%) the major effect is a shift down of the C and B_2 transitions. At higher concentrations all the peaks including the D transition are shifted down. At 10% propanol (inset in Figure 6) the transitions all occur below 45° and cannot be resolved. Even at this concentration the propanol effect can be reversed by washing the membranes in 310 imosm sodium phosphate buffer.

Since the effect of phenothiazines and perhaps alcohols on the C transition might



effectively mask their possible effect on the B_2 transition it was imperative to check under conditions in which overlap of B_2 and C can be avoided. Highly-specific, covalent inhibitors of anion transport, SITS (4 acetamido-4′ isolthiocyano stilbene- 2,2′-disulfonic acid) and DIDS (4,4′-disothiocyano- 2,2′-stilbene disulfonic acid), shift up the C transition by nearly 12° and leave the rest of the transitions unchanged (31). The effect of 2% *n*-propanol on DIDS-treated membranes and the effect of 100 μM prochlorperazine on SITS-treated membranes are shown in Figure 7 and Figure 8, respectively. In comparison with controls it can

FIG. 5. *Effect of alcohols at above lytic concentrations*

a) control; b) 20% MeOH; c) 10% EtOH; d) 4% *n*-propanol; e) Reversal of 4% *n*-propanol. The sample treated in 4% *n*-propanol was washed twice in 310 imosm sodium phosphate buffer, pH 7.4, and then heated.

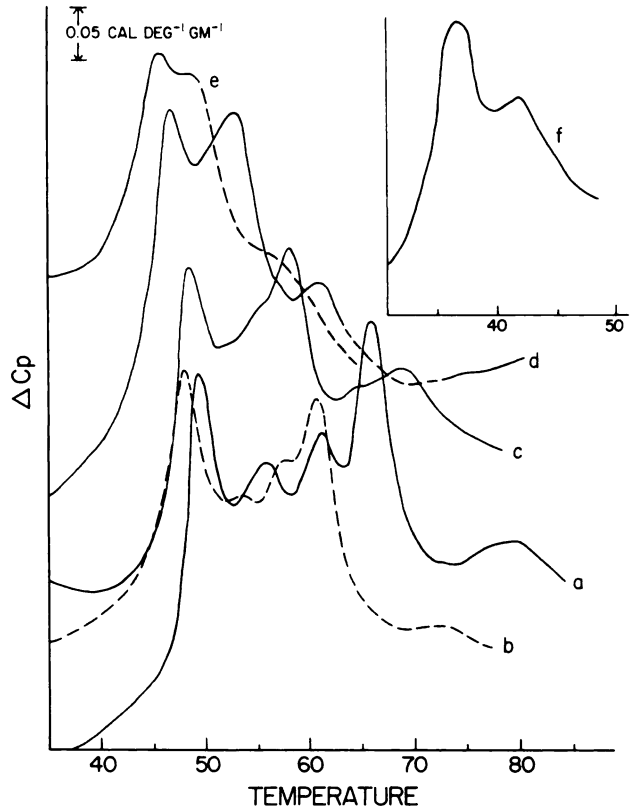


FIG. 6. *Effect of increasing concentrations of n-propanol on human erythrocyte membrane transitions* The buffer was 310 imosm sodium phosphate, pH 7.4. a) control; b) 1%; c) 2%; d) 3%; e) 4%; f) (inset) 10%.

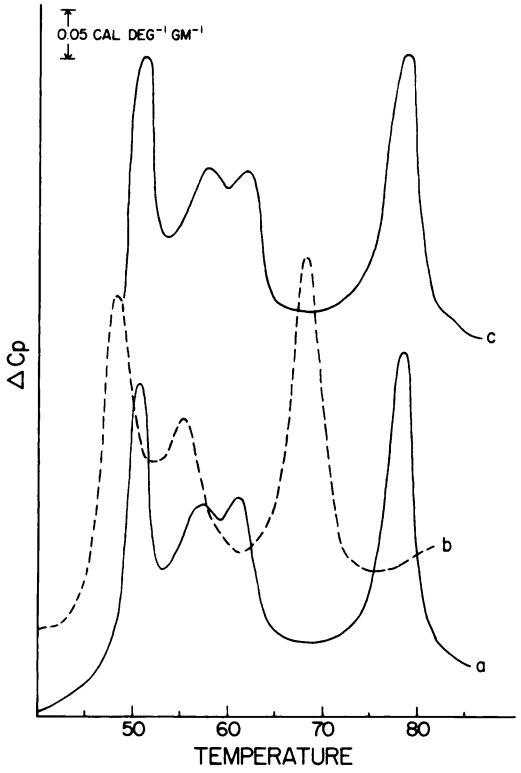


FIG. 7. *Effect of 2% n-propanol on DIDS-treated ghosts*

a) DIDS control, 310 imosm phosphate buffer, pH 7.4; b) DIDS control + 2% *n*-propanol; c) Reversed in 310 imosm phosphate buffer, pH 7.4.

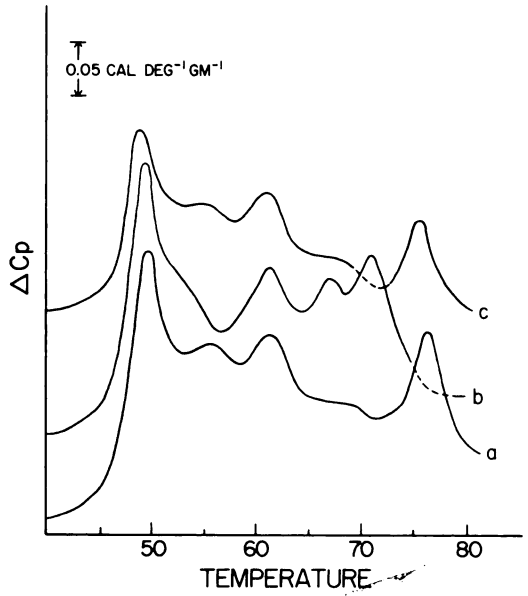


FIG. 8. *Effect of 70 M Prochlorperazine on SITS-treated ghosts in 310 imosm phosphate buffer, pH 7.4*

a) SITS control; b) + 70 μM prochlorperazine; c) reversal.

be seen that the phenothiazine affects only the C transition whereas the alcohol shifts down the B_2 transition as well as the C transition.

Model membrane systems of phosphatidyl choline vesicles behave very similarly to the C transition in the presence of alcohols and phenothiazines. Their effects on the gel-to-liquid crystal transition of liposomes of dipalmitoyl phosphatidyl choline have been determined (data not shown). Ethanol shifts the main transition down by nearly 2° at 2% concentration and by a greater extent the pretransition. The effect of 60 μM prochloroperazine is similar to that found for ethanol, but the latter also causes considerable broadening of the endotherm.

DISCUSSION

Both the phenothiazines and the alcohols interact most strongly with the C transition of the erythrocyte membrane, although the alcohols also cause a significant downward shift of the B_2 transition. Both cause some shift and loss of resolution for the B_1 transition. The two transitions, which appear to involve large amounts of protein unfolding (i.e., the A and D transition) on the basis of CD changes (28), are relatively insensitive to low concentrations of both types of anesthetics.

The primary advantage of the calorimetric method for this type of study is that it permits one to focus attention on particular local structural regions in the membrane. Thus, it is apparent from this study that both the phenothiazines and the alcohols do interact strongly with at least one common region of the native membrane, i.e., the C region, and in so doing they act to depress the temperature of the order → disorder transition.

There is a large amount of evidence that the C region of the membrane has a definite lipid character. The evidence in favor of this idea includes (30): 1) The lack of CD change (223 nm) in the C transition argues against protein unfolding as the primary structural change. 2) The C transition

shows a high sensitivity to many amphipathic additives known to shift lipid melting transitions. 3) It is the only one of the erythrocyte transitions that is insensitive to extensive bilateral proteolysis. 4) The C transition shifts down in temperature and disappears progressively as a function of phospholipid cleavage using either phospholipase C or A. 5) The C transition is the only transition which can be "extracted" from the membrane by brief exposure to lipid-extracting solvents such as *n*-butanol.

Although definitely involving lipid to some degree, the C region also either includes or is regulated to some extent by band III, the principal transport protein of the red cell. That this is true was indicated by earlier work (31) showing that the upward shift of the C transition occurs simultaneously with the binding of DIDS to band III and the concomitant inhibition of anion transport. Thus, the C transition is possibly occurring in a local domain of the membrane which contains phospholipids as well as perhaps some portions of band III that are inaccessible to bilateral proteolysis. It is possible, although highly speculative, that the C transition corresponds to the melting of a small crystalline phospholipid region of the membrane that is modulated strongly by interaction with band III.

The use of scanning calorimetry can therefore be of considerable value in identifying specific regions of a membrane which interact with different drugs, and might thereby be important for determinations of mechanisms of action. As far as these two family of anesthetics are concerned, both interact strongly with a structure known to be part of the anion "channel." Whether or not this interaction with transport sites is related to anesthetic properties will be known only after further studies. However, it has frequently been suggested (1-5) in the past that anesthetic action is related to permeability changes in membranes and our results are supportive of that idea.

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