Existence of either the discocyte or the echinocyte requires accommodation of the cell's cytoskeleton to these two morphologies. In turn, part of the control over the cytoskeleton is mediated by transmembrane proteins, particularly through glycophorin and possibly through a glycophorin-band 3 complex (3). The intruding lipid anions likely provide more than simply the driving force for echinocyte formation. They may interact directly with the transmembrane proteins to relax controls over the cytoskeleton. There are close to $3 \times 10^5$ glycophorin dimers and $6 \times 10^5$ such monomers. Insertion of $2 \times 10^7$ lipids in the human erythrocyte membrane, triggering echinocyte formation, is equivalent to ~ 70 ligands/glycophorin dimer. If the dimer is compact, all ligands are unlikely to be bound to glycophorin. However, they may form a domain in glycophorin's vicinity of the kind Van Zoelen et al (9) describe.

*Received for publication 13 April 1981.*

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# THE MOLECULAR BASIS OF ALCOHOL TOLERANCE AND DRUG CROSS-TOLERANCE IN CHRONIC ALCOHOLISM

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Tolerance to ethanol in chronic alcoholics probably arises from alterations in the membrane properties of the nervous system. (Curran and Seeman, 1977; Chin and Goldstein, 1977). We recently reported that liver mitochondrial membranes from rats chronically fed ethanol are resistant to the uncoupling effect of ethanol, which is associated with a resistance to the disordering of the membrane lipid structure by ethanol. (Rottenberg et al., 1980; Waring et al., 1981). This resistance is caused by altered lipid composition of the mitochondrial membranes. We report now that membrane binding of ethanol, anesthetics, and hydrophobic molecules in brain synaptosomes and liver mitochondria from rats is conspicuously reduced after chronic ethanol consumption. These membranes are resistant to structural disordering by both ethanol and halothane. Tolerance, cross-tolerance, and dependence in chronic alcoholics therefore could result, in part, from membrane alterations which inhibit the binding of ethanol and other drugs.

## RESULTS AND DISCUSSION

To study the effect of ethanol and halothane on membrane structure we employed two spin-labeled membrane probes. We calculate the order parameter, $S$, from the hyperfine splitting of the EPR spectra of 5-doxyl stearate (Gaffney, 1975). The partition coefficient of the nitroxide-labeled decane, 5N10, between the hydrophobic membrane and the medium is calculated from the contribution of the bound and free species to the composite EPR spectrum. (Linden et al., 1973.)

Fig. 1 shows the results of these measurements in liver mitochondria and brain synaptosomes from ethanol-fed rats and their controls. It can be seen that membranes from ethanol-fed rats are more rigid than those from the controls. When titrated with ethanol from 25 mM to 1.0 M, control membranes become much more fluid, as indicated by a decrease in the order parameter and an increase in the partition coefficient. Membranes from ethanol-fed rats are much less affected by the addition of ethanol and maintain relatively rigid membranes even at high concentrations of ethanol. Comparing the order parameters and the probe partition coefficients in membranes from ethanol-fed rats and controls, it can be seen that membranes from ethanol-fed animals in the presence of moderate ethanol concentrations are as fluid as membranes from the control in the absence of ethanol; the latter presumably are



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Volume 37 January 1982 14–16

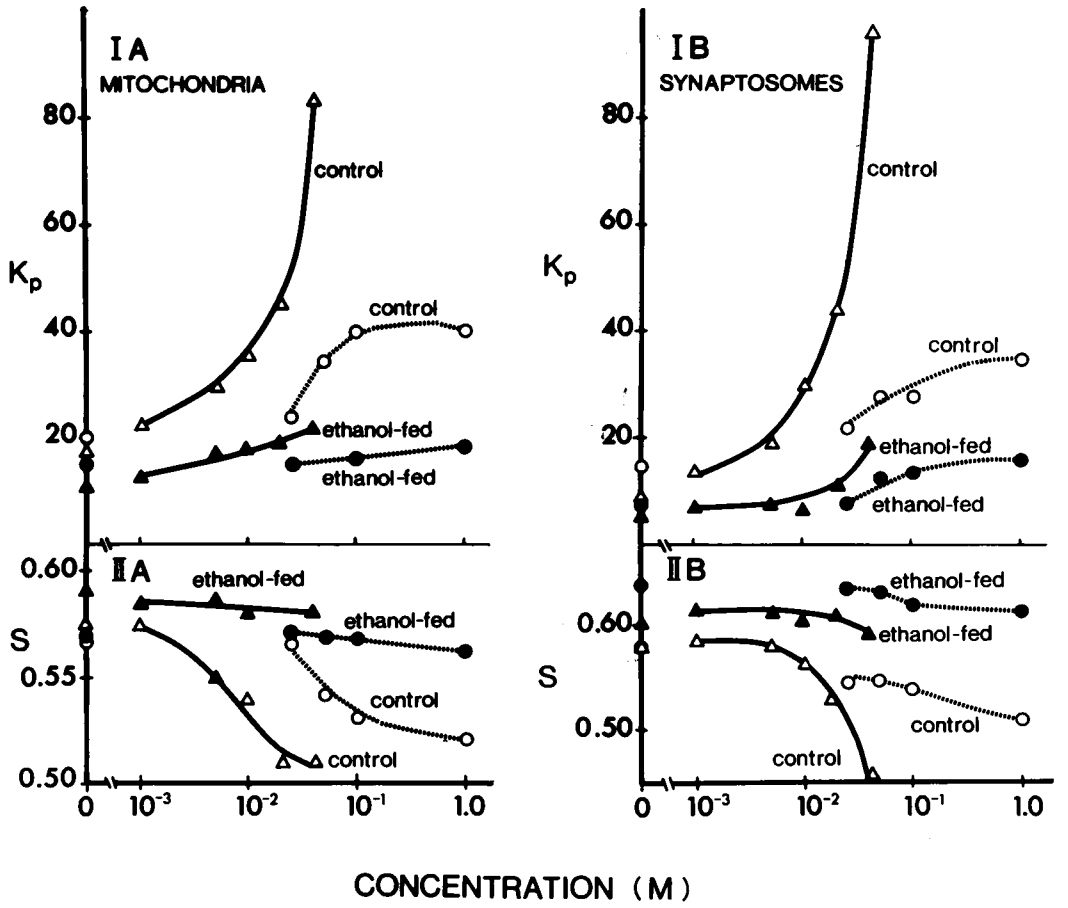


FIGURE 1 The effect of ethanol (O, ●) and halothane (△, ▲) on the 5N10 partition coefficient, $K_p$, (panels I*A,B*) and the 5-doxyl stearate order parameter (panels II*A,B*) in liver mitochondrial membranes, *A*, and brain synaptosomal membranes, *B*, from ethanol-fed rats (▲, ●) and their pair-fed controls (△, O). EPR spectra were obtained with a Varian E-109 (Varian Associates, Inc., Palo Alto, CA) at 35°C ± 0.2°C. Rats were pair-fed for 35 days with ethanol (14 g ethanol/kg body wt/day) or with isocaloric carbohydrate replacing ethanol in controls, as done previously. Liver mitochondrial and brain synaptosomal membranes were prepared by conventional procedures. For EPR measurements, the membranes were suspended in 0.25 M sucrose, 20 mM Tris-Cl (pH 7.4), 2 μM Rotenone, and 10 mM ferricyanide.

in a state which is optimal for membrane function. Surprisingly, there are similar differences in the response to halothane. Halothane is ~ 50 times more potent than ethanol in disordering memembranes. In a titration from 1 mM to 40 mM there is strong disordering in control membranes, as indicated both by the lowering of the order parameter and by the increase in probe partitioning. However, in this case too, membranes from ethanol-fed rats are much less affected, even at high concentrations of halothane. While the response of synaptosomes and mitochondria is quite similar, usually synaptosomal membranes are considerably more rigid than mitochondrial membranes.

The data of Fig. 1 suggest that the altered membranes of the organism are the origin of physical dependence, tolerance and cross-tolerance. Dependence may arise if the membranes of ethanol-fed rats are too rigid for optimal function in the absence of ethanol, since they are similar in rigidity to normal membranes in the presence of ethanol. Tolerance is clearly suggested by the resistance of the membrane from ethanol-fed animals to disordering by high concentrations of ethanol, whereas cross-tolerance to anesthetics is indicated by the membrane resistance to disordering by halothane. While the partition probe is used here to monitor membrane structure, the results also suggest the mechanism for the acquired resistance. If the increased membrane rigidity results in decreased binding of ethanol and anesthetics, then resistance to these compounds is expected. Table I shows the results of experiments in which the partition coefficients of ethanol and halothane were determined in mitochondrial membranes and synaptosomes. In both membranes the partition coefficient of ethanol and halothane is lower in membranes from ethanol-fed rats. In mitochondria the partition coefficient of ethanol in controls is more than four times higher than in ethanol-fed rats. Reduced binding of phenobarbital was also observed in mitochondria from ethanol-fed rats. The differences in the partition coefficients of ethanol and halothane between control and ethanol-fed rats are similar in magnitude to the differences

TABLE I

PARTITION COEFFICIENTS OF ETHANOL AND HALOTHANE IN BRAIN SYNAPTOSOMES AND LIVER MITOCHONDRIA FROM ETHANOL-FED RATS AND CONTROL

| | Ethanol, $K_p$ | Halothane, $K_p$ |
|---|---|---|
| *Mitochondria* | | |
| Ethanol-fed | (8)1.17 ± 0.634 | (5)21.4 ± 2.71 |
| Control | 3.60 ± 0.740 | 28.6 ± 5.58 |
| Control/ethanol-fed | 4.24 | 1.35 |
| *Synaptosomes* | | |
| Ethanol-fed | (5) 0.33 ± 0.11 | (7) 21.07 ± 10.2 |
| Control | (5) 1.00 ± 0.42 | 27.5 ± 11.3 |
| Control/ethanol-fed | 3.07 | 1.38 |

Partition coefficients were determined by incubating the membranes with a $^{14}C$-labeled compound and $^3H_2O$.

in the partition coefficients of the decane spin-probe (1.4 to fourfold difference). Since the latter are strongly correlated with the membrane order parameter, it is apparent that the changes in membrane rigidity are related to the binding of alcohol and anesthetics. Whether the reduced membrane binding is sufficient to explain in full the acquired resistance to the disordering effects of these compounds can only be answered by further refinement of these measurements. However, these data provide sufficient evidence to suggest that tolerance and cross-tolerance result in part from increased membrane rigidity, which decreases the partitioning of ethanol and other drugs into the membranes of chronic alcoholics. A preliminary short report of this work has been published (Rottenberg et al., 1981).

*Received for publication 1 May 1981.*

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# MEMBRANE FLUIDITY CHANGES ACCOMPANY ACTIVATION OF SEA URCHIN EGGS

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The fertilization of sea urchin eggs is a visually dramatic example of cell activation. The resonance spectra of spin-labeled eggs have been used in several ways to analyze the membrane fluidity changes that accompany egg activation. We have consistently observed a decrease in order parameter, $S$, of eggs labeled with 5-doxylstearate associated with fertilization (1, 2). The average decrease in $S$ was 2.1% for *Lytechinus pictus* and 2.5% for *Strongylocentrotus purpuratus*. This is a large change for a biological system. In pure phospholipid systems a change of ~ 20% is associated with the gel to liquid-crystal phase transition, but in biological systems changes of > 1% are quite rare.

## RESULTS AND DISCUSSION

The change in $S$ suggested that membrane fluidity increases upon activation. We have carried out a series of experiments to rule out likely sources of artifacts (2, 3). One potential source of artifacts concerns the changes in the surface structure of the egg at fertilization. In the first minute after fertilization the topography of the plasma membrane is drastically altered by fusion of cortical granules. Furthermore, a layer of protein, the vitelline layer, is removed from the exterior of the egg. Ammonia-activated eggs do not show these surface changes yet they still undergo the fluidity change. Experimental removal of the vitelline layer with dithiothreitol or protease had no effect on $S$. Indeed we found that the order parameter of eggs labeled with 5-doxylstearate did not respond to several treatments designed to alter the surface of the egg (2, 3).

Any interpretation of the spectral change induced by fertilization would certainly depend on the location of the spin probe within the egg. We approached the problem of localizing the probe in two stages. First we sought evidence that the probe might find its way to nonbilayer sites, such as neutral lipid droplets or protein binding sites. Knowing of no prior studies on the behavior of fatty acid spin labels in neutral lipid, we first turned to model systems. Here we found that the partitioning of spin label into neutral lipid



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Volume 37 January 1982 16–17