

**Drug Tolerance in Biomembranes:**

**A Spin Label Study of the Effects of Ethanol**

*Abstract. Ethanol in vitro increased the fluidity of spin-labeled membranes from normal mice. Membranes from mice that had been subjected to long-term ethanol treatment were relatively resistant to this fluidizing effect. The data suggest that the membranes themselves had adapted to the drug, a novel form of drug tolerance.*

Ethanol is one of a large group of drugs that can produce general anesthesia and (at much higher concentrations) can block nerve conduction. Most of these drugs lack the structural complexity expected of a drug that acts by combining with a specific receptor. Because the anesthetic potencies of such drugs correlate with their lipid solubilities, they are thought to exert their biological effects by entering the lipid portion of biomembranes and disrupting membrane function. Cell membranes and model membranes expand (1) and become more fluid (2, 3) when immersed in nerve-blocking concentrations of such drugs. We have recently shown (4) that the fluidizing effects of ethanol in biomembranes can be measured at very low concentrations with a sensitive electron paramagnetic resonance (EPR) technique. We report here that tolerance to this effect develops in mice, which suggests that mammalian cells can control the physical properties of their membranes in response to drugs.

Male DBA/2J mice were maintained for 8 days on a liquid diet (Slender, Carnation Company) to which ethanol was added to provide 33 percent of the calories. Controls were pair-fed the same diet with sucrose replacing ethanol calories. We used membranes from the animals whose ethanol intake patterns met our previously defined criteria for development of physical dependence (5). They had consumed an average of 18 g of ethanol per kilogram of body weight per day during the last 4 days. Their mean blood ethanol concentration at the time they were killed was  $3.4 \pm 0.87$  mg/ml (mean  $\pm$  standard deviation).

Since these animals were known to be physically dependent, we considered them to be in a "tolerant-dependent" state. Although we did not make numerical estimates of the extent of functional tolerance, we did note that none of the mice had lost their righting reflex at blood ethanol concentrations that would be hypnotic for most normal animals.

Cardiac blood from five to seven mice was pooled in each of three replicate experiments. Erythrocyte membranes were prepared under conditions that minimize membrane disruption (4, 6). From the combined whole-brain homogenates of the same mice, we isolated myelin, mitochondrial membranes, and synaptosomal plasma membranes by a flotation-sedimentation technique (4, 7). Each of these four membrane preparations was then spin-labeled by incubation for 30 minutes at 37°C with 5-doxytstearic acid (*N*-oxyl-4',4'-dimethyl-oxazolidine derivative of 5-ketostearic acid, SYVA Company, Palo Alto). This fatty acid spin label tends to align itself with the fatty acid chains of phospholipids in the membrane bilayer. Its EPR spectrum is affected by its motion; that is, by the fluidity of its environment. The EPR spectrum of a rapidly tumbling spin label consists of three evenly spaced peaks. When the spin label is immobilized, the peaks become broader and farther apart. The spectrum can be characterized by an order parameter, *S* (8), determined from the separation of the peaks, along with reference data from crystals oriented in the magnetic field. In model membranes, the order parameter can vary between the limits of 1.0 (completely ordered) and 0 (completely fluid). In natural biomembranes, order parameters indicate intermediate fluidity: *S* = .6. Our EPR spectra were obtained at 37°C with a modified Varian EM-500 spectrometer and a PDP8/c computer (9).

Spectra were obtained for portions of each of the four kinds of spin-labeled preparations in the presence of various concentrations of ethanol (tested in random order). Measurements of the baseline order parameter with no ethanol added to the membranes were interspersed among the measurements where ethanol was added. The last column in Table 1 shows that the baseline order parameter differed among the various membrane types; mitochondrial membranes were the most fluid, the erythrocyte and synaptosomal membranes were of intermediate fluidity, and myelin was the most ordered. These results confirm those of our previous study (4). There were small differences between the three replicate experiments, reflecting the variability in spectral characteristics seen in similar preparations to which the spin label is separately added. We could not detect consistent differences in the baseline order parameter between membranes from groups receiving long-term ethanol treatment and the corresponding membranes from their sucrose-control mates.

When the same membrane preparations were tested in the presence of eth-

anol, tolerance to this effect develops in mice, which suggests that mammalian cells can control the physical properties of their membranes in response to drugs. inhalation anesthetics. Anesthetic effects are governed by the Meyer-Overton rule, which in its original formulation almost a century ago held that the potency of a general anesthetic is directly correlated with its lipid solubility (i.e., how readily it dissolves in tissues containing fats). Thus, the more soluble an anesthetic is in lipids, the more potent are its effects. With the development of cell biology in the 1960's, it became clear that the lipids of the Meyer-Overton law are the phospholipids (i.e., a type of lipid, or fat) that forms the structural basis of cell membranes. Further progress in this area came in the late 1960's, when Hubbell and McConnell (1969) demonstrated that general anesthetics, including alcohol, cause molecular disordering of biological membranes. This article and subsequently those of other researchers (e.g., Trudell et al. 1973) suggested that alcohol exerts its intoxicating effects by dissolving in the membrane and "fluidizing" that structure, thereby in some way altering the function of the cell.

Although the aforementioned studies were relevant to the study of anesthesia and alcohol intoxication, they did not address the problem of behavioral tolerance to alcohol and thus seemed distant from the concerns of alcoholism or chronic alcohol abuse. It was in this context that Chin and Goldstein (1977) were able to link, for the first time, the induction of a "tolerant-dependent" state to changes in a physical parameter. These authors demonstrated that portions of the membranes of nerve terminals (i.e., synaptosomes) from mice treated chronically with alcohol until they had reached a state of alcohol

tolerance and physical dependence were resistant to the fluidizing effect of alcohol in vitro. In Chin and Goldstein's study, the molecular order, or "fluidity," of the isolated membranes in vitro was unchanged by chronic alcohol intake as long as alcohol was not present in the solution. However, when alcohol was added to the medium, the expected fluidizing response was blunted.

This seminal article actually had wider implications than the link established between a behavioral phenomenon and a biological alteration. It also demonstrated in general that mammalian cells can adapt to perturbing conditions by modulating the "fluidity" (and by inference the composition) of their membranes.

The study by Chin and Goldstein stimulated numerous other studies of "membrane tolerance." The resistance to fluidization was later extended to include other membranes as well as com-

Table 1. Baseline order parameters. Membrane fractions were prepared from cardiac blood and from whole-brain homogenates pooled for five to seven ethanol-treated animals in each of three experiments, and from their sucrose-treated partners in the pair-feeding experiments. The fractions were spin-labeled with 5-doxytstearic acid and a portion of each preparation was analyzed by EPR to determine the order parameter in the absence of added ethanol. Since the ethanol and sucrose groups were not significantly different, the data for the six preparations were combined to allow comparison of membrane types (last column); SEM, standard error of the mean.

Source of membrane	Long-term treatment	Order parameter			
		Experiment			Mean of treatment type $\pm$ SEM
		1	2	3	
Erythrocyte	Sucrose	.589	.588	.597	.591
	Ethanol	.573	.600	.589	.587
Synaptosome	Sucrose	.590	.592	.604	.595
	Ethanol	.590	.593	.599	.594
Mitochondria	Sucrose	.570	.579	.571	.573
	Ethanol	.570	.573	.579	.574
Myelin	Sucrose	.607	.624	.631	.621
	Ethanol	.602	.620	.618	.613

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**DRUG TOLERANCE IN BIOMEMBRANES**

Commentary by Emanuel Rubin, M.D.

*Key words: AOD tolerance; cell membrane, AOD effects (AODE); membrane fluidity*

**B**ehavioral tolerance to drugs, particularly alcohol, has been part of folklore for most of recorded history; it is common wisdom that some who drink heartily and frequently are better able to "hold their liquor" than are others who consume a thimbleful of sacramental wine during the year. The mechanism(s) of alcoholic intoxication, let alone tolerance, still are not understood, although many researchers have thought that alcohol affects the central nervous system in a manner similar to that of

ponents of liver and pancreatic cells (Taraschi and Rubin 1985). The resistance also was found to be associated with a decrease in the solubility of alcohol and other anesthetics in membranes (Rottenberg et al. 1981). It was further demonstrated that artificial membranes composed of phospholipids purified from cells of chronically intoxicated rats also are resistant to disordering by alcohol and that the property of "membrane tolerance" seems to reside particularly in anionic (negatively charged) phospholipids (such as phosphatidylinositol, phosphatidylserine, and cardiolipin) (Taraschi et al. 1986).

As is the case in many fields, the original concept that anesthetics (and alcohol) produce their effects on the central nervous system solely by interacting with membrane lipids now appears too simplistic. Increasing evidence exists that alcohol interacts directly with proteins embedded in or associated with cell membranes (Franks and Lieb 1994; Slater et al. 1993; Li et al. 1994). These interactions also follow the Meyer-Overton rule, which can be reinterpreted to substitute interactions between alcohol and these proteins for lipid solubility. Yet direct alcohol-protein interactions do not (at least currently) explain behavioral tolerance, and modulations of such interactions by membrane lipids have been demonstrated (Slater et al. 1993). Thus, lipid-protein interactions remain to be explored, and the adaptive lipid response demonstrated by Chin and Goldstein may yet prove to regulate the development of behavioral tolerance to alcohol. ■

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