Wilcox, *ibid.*, p. 25; *Science* **192**, 745 (1976).
20. J. King and D. Willis, *NASA Spec. Publ.* **366**, 39 (1973); L. Svalgaard, *ibid.*, p. 119.
21. I thank S. A. Gauthreaux, Jr. for making available his ceilometer and moon-watch data and, especially, for his advice and encouragement throughout this study. I also thank S. Emlen, W. Keeton, and R. Larkin for their helpful and critical comments on an earlier draft. J. Waide also reviewed the manuscript and offered statistical advice, as did J. Holman. C. W. Helms made available financial support for the study through the Department of Zoology, Clemson University. This work was completed while I held a research assistantship under U.S. Air Force grant AFOSR-75-2787.

1 November 1976; revised 7 December 1976

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 ± 0.87 mg/ml (mean ± 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-doxylstearic 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: $S \simeq .6$. Our EPR spectra were obtained at 37°C with a modified Varian EM-500 spectrometer and a PDP 8/e 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-

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-doxylstearic 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	Mean of membrance type ± SEM
		1	2	3		
Erythrocyte	Sucrose	.589	.588	.597	.591	.589 ± .0038
	Ethanol	.573	.600	.589	.587	
Synaptosome	Sucrose	.590	.592	.604	.595	.595 ± .0023
	Ethanol	.590	.593	.599	.594	
Mitochondria	Sucrose	.570	.579	.571	.573	.574 ± .0017
	Ethanol	.570	.573	.579	.574	
Myelin	Sucrose	.607	.624	.631	.621	.617 ± .0044
	Ethanol	.602	.620	.618	.613	

anol, however, we did observe a difference between the ethanol and control groups. Erythrocyte and synaptosomal membranes from ethanol-treated mice were less responsive to ethanol in vitro than membranes from the control animals. The results of one of these experiments are shown in Figs. 1 and 2. The data show a concentration-related increase in fluidity (decrease in S) on addition of ethanol to synaptosomal membranes. The small decreases of .005 in the order parameter caused by ethanol at 4 mg/ml in these experiments agree with the changes in fluidity predicted for the same concentration of anesthetic agents in phospholipid model membranes (*3*). Figures 1 and 2 show that ethanol was consistently less effective in membranes from ethanol-treated animals than those from control mice. This decreased effect of a particular concentration of a drug in animals receiving long-term treatment is, by definition, tolerance. Two additional replications of this experiment also showed a difference between the ethanol-treated and sucrose-control groups in the same direction as above. The magnitude of the difference was greater in one experiment and less in the other than in the experiment represented in Figs. 1 and 2. An analysis of variance, using data from all three experiments, confirmed the differences between the two groups (*10*). No clear effect of long-term ethanol treatment was seen in the myelin or mitochondrial fractions.

These experiments were designed to test the hypothesis (*11*) that mammalian cells can regulate the fluidity of their membranes in response to pharmacological conditions. The results were partially in accord with our predictions. We did observe that the cell membranes of tolerant-dependent mice exhibited less change in their fluidity in the presence of ethanol than those of control mice (tolerance). However, the hypothesis predicts that the ethanol-adapted membranes tested in the absence of ethanol should be more ordered than normal (dependence). We saw no evidence of an increased order parameter in the alcohol-free membranes of the ethanol-treated group (Table 1). On the other hand, it is possible that our methods are sensitive enough to detect tolerance but not dependence because we estimated tolerance by adding different concentrations of ethanol to a single spin-labeled preparation, whereas dependence data (difference between baseline order parameters) required comparisons between different preparations that were separately spin-labeled. The dependence data, therefore, have greater variability than the tolerance data. Traynor *et al.* (*12*) recently reported that tolerance develops to a neurophysiological effect of ethanol in *Aplysia* without any detectable abnormality in the ethanol-free state.

An optimal range of membrane fluidity appears to be important for many biological functions (*13*), and evidence is accumulating that there are physiological regulatory mechanisms to control membrane fluidity. Homeostatic control of membrane composition in response to environmental temperature has been shown in bacteria (*13, 14*) and in poikilothermic vertebrates (*15*). Mammalian cells, which seldom encounter changes in temperature, may still have occasion to adjust their membrane fluidity. The continuous presence of a fluidizing agent like ethanol might provide the stimulus for an adaptive response in the membrane itself. Our experimental data are consistent with this prediction.

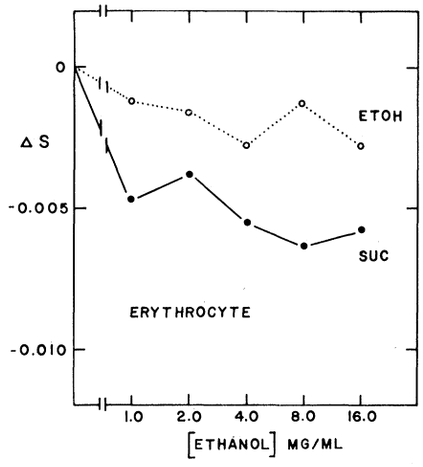

Fig. 1. Changes in order parameter in erythrocyte membranes on addition of ethanol in vitro in one experiment. The ordinate shows the change in order parameter (ΔS) from baseline at each of the ethanol concentrations shown on the abscissa (plotted on a logarithmic scale). Open circles represent a spin-labeled preparation of erythrocyte membranes from five ethanol-treated mice; filled circles represent a corresponding preparation from their sucrose-fed partners in the same experiment. The baseline order parameter values are listed in Table 1 (experiment 1). A decrease in the order parameter reflects an increase in membrane fluidity.

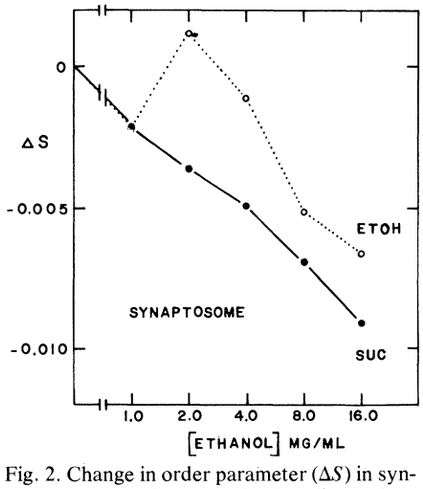

Fig. 2. Change in order parameter (ΔS) in synaptosomal membranes. The baseline values are listed in Table 1 (experiment 1). Changes in S are shown as in Fig. 1.

JANE H. CHIN
DORA B. GOLDSTEIN
Department of Pharmacology, Stanford University Medical Center, Stanford, California 94305

References and Notes

1. P. Seeman, *Pharmacol. Rev.* **24**, 583 (1972); *Experientia* **30**, 759 (1974).
2. W. L. Hubbell and H. M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.* **61**, 12 (1968); J. C. Metcalfe, P. Seeman, A. S. V. Burgen, *Mol. Pharmacol.* **4**, 87 (1968); S. J. Paterson, K. W. Butler, P. Huang, J. Labelle, I. C. P. Smith, H. Schneider, *Biochim. Biophys. Acta* **266**, 597 (1972).
3. J. R. Trudell, W. L. Hubbell, E. N. Cohen, *Biochim. Biophys. Acta* **291**, 321 (1973).
4. J. H. Chin and D. B. Goldstein, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 707 (1976); *Adv. Exp. Med. Biol.*, in press; *Mol. Pharmacol.*, in press.
5. We analyzed the daily intake record for each mouse and calculated its "effective intake" of ethanol as previously defined [D. B. Goldstein and V. W. Arnold, *J. Pharmacol. Exp. Ther.* **199**, 408 (1976)]. The total intake was corrected by subtracting two components that appear not to contribute to physical dependence: (i) the 10 g kg^{-1} day^{-1} that is cleared by the liver and does not appear in the venous blood, and (ii) any intake prior to a day when the intake dropped below the 10-g/kg cutoff. A 24-hour break appears to allow recovery from previously acquired physical dependence. Mice with effective intakes of 20 to 60 g/kg reliably show moderate to severe signs of physical dependence, as scored by convulsions on handling [D. B. Goldstein, *ibid.* **180**, 203 (1972)]. The mice used here had a mean effective intake of 35 g/kg of ethanol.
6. P. G. Kury and H. M. McConnell, *Biochemistry* **14**, 2798 (1975).
7. D. H. Jones and A. I. Matus, *Biochim. Biophys. Acta* **356**, 276 (1974).
8. W. L. Hubbell and H. M. McConnell, *J. Am. Chem. Soc.* **93**, 314 (1971).
9. J. H. Chin, J. R. Trudell, E. N. Cohen, *Life Sci.* **18**, 489 (1976).
10. Two-way analysis of variance was computed for erythrocyte membranes and separately for synaptosomal membranes, using three replications of five ethanol concentrations and two long-term treatment groups. The change in order parameter from baseline in response to added ethanol was significantly less in ethanol-treated animals than in controls in both types of membrane (erythrocyte, $F = 19.2$, d.f. = 1,24, $P < .001$; synaptosomal, $F = 21.4$, d.f. = 1,24, $P < .001$). The fluidity changes in synaptosomal membranes were also different at the various concentrations of ethanol ($F = 18.51$, d.f. = 4,24, $P < .001$). The pattern of the response to ethanol in each type of membrane was the same in both ethanol-treated and sucrose-control mice (that is, the interaction term in the analysis of variance was not significant).
11. M. W. Hill and A. D. Bangham, *Adv. Exp. Med. Biol.* **59**, 1 (1975).
12. M. E. Traynor, P. B. J. Woodson, W. T. Schlapfer, S. H. Barondes, *Science* **193**, 510 (1976).
13. D. Chapman, *Q. Rev. Biophys.* **8**, 185 (1975); J. E. Cronan and E. P. Gelmann, *Bacteriol. Rev.* **39**, 232 (1975); D. L. Melchior and J. M. Steim, *Annu. Rev. Biophys. Bioeng.* **5**, 205 (1976).
14. M. Sinensky, *J. Bacteriol.* **106**, 449 (1971).
15. J. R. Hazel and C. L. Prosser, *Physiol. Rev.* **54**, 620 (1974).

16. We thank E. N. Cohen and J. R. Trudell in the Department of Anesthesiology for extensive use of their EPR system. We also thank J. R. Trudell for helpful suggestions and encouragement during the progress of this work, B. Cox for critical reading of the manuscript, and V. Arnold for technical assistance. Supported by PHS grant AA01066.

8 November 1976