Pharmacology Biochemistry & Behavior, Vol. 18, Suppl. 1, pp. 7–13, 1983. © Ankho International. Printed in the U.S.A.

Ethanol and Biological Membranes: Injury and Adaptation[1]

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RUBIN, E. AND H. ROTTENBERG. *Ethanol and biological membranes: Injury and adaptation.* PHARMACOL BIOCHEM BEHAV **18**: Suppl. 1, 7–13, 1983.—Ethanol intoxication affects the protein and lipid constituents of biological membranes. Mitochondria exhibit specific decreases in components of the electron transport chain and in protein synthesis. *In vitro* ethanol reduces the transition temperatures of membrane-bound enzyme activities and decreases the order parameter. On the other hand, both are increased after chronic ethanol administration. After chronic ethanol treatment membranes are resistant to disordering by ethanol, possibly owing to an increased saturation of mitochondrial phospholipids, particularly cardiolipin. The increased rigidity of mitochondrial and synaptosomal membranes is associated with reduced binding of ethanol and of the general anesthetic halothane. The data suggest that initially ethanol increases the fluidity of all biological membranes. If continued chronically, this effect is balanced by a change in the lipid composition of the membranes, which increases their rigidity and makes them resistant to disordering by ethanol (homeoviscous adaptation). The change in molecular order reduces the binding of ethanol and other compounds, but also impairs a variety of membrane-bound functions. These changes may play a role in tolerance to ethanol and cross-tolerance to anesthetics, and in the pathogenesis of maladies associated with alcohol abuse.

Ethanol Membranes Homeoviscous adaptation

MOST toxic agents, when administered for prolonged periods of time, produce disease in a highly specific manner. For example, chronic carbon tetrachloride intoxication results in cirrhosis of the liver, cobalt and adriamycin cause cardiomyopathies, and many carcinogens produce tumors of only one or two organs. By contrast chronic alcoholism is associated with disorders of many organs, including the liver, pancreas, heart, skeletal muscle and brain. It seemed to us unlikely that ethanol produces injury in these widely different organs by completely different mechanism. For instance the type of metabolic changes which occur in the liver can hardly be responsible for injury to the heart or brain, organs which do not metabolize ethanol. In view of the demonstration that ethanol fluidizes biological membranes [8,21], and that chronic ethanol administration increases the resistance of the membranes to the fluidizing effect of ethanol in synaptosomes [5], it appeared likely that chronic ethanol administration exerts effects in all biological membranes. If such effects can be shown to be instrumental in causing tissue damage, the variations in alcohol-related maladies of various organs may reflect the differentiation of that organ. Thus, homeoviscous adaptation of membranes, defined as alterations in structure which tend to maintain normal fluidity, represents a unified theory of organ damage produced by chronic alcoholism.

MITOCHONDRIAL ELECTRON TRANSPORT

The correlation of functional and structural changes produced by ethanol has been best elucidated in mitochondrial membranes. Morphologic changes in hepatic mitochondria produced by chronic ethanol ingestion are well described [7, 9, 11, 12, 15, 17, 19]. Mitochondria are enlarged and distorted, exhibit disoriented cristae, and often contain paracrystalline inclusions. Other organs in which mitochondria display the same morphologic changes include the small intestine [20], skeletal muscle [18], heart [1], and pancreas [6]. These ultrastructural changes are accompanied by impairment of mitochondrial respiration with a variety of substrates, including NADH, succinate, glutamate-malate, and fatty acids [3, 4, 23, 24] (Table 1). These functional defects stimulated studies of the effects of ethanol on mitochondrial bioenergetics, particularly factors related to the electron transport chain and energy transduction. In hepatic mitochondria from animals treated chronically with ethanol, steady-state rates of ATP synthesis during oxidative phosphorylation are conspicuously decreased, as is the maximal rate of ATP hydrolysis [23]. The contents of substrate-reducible cytochromes aa_3 and b are each decreased about 50%, whereas the amounts of cytochrome $c+c_1$, extractable flavin and ubiquinone are unchanged [24] (Table 2). Interestingly during ascorbate oxidation cytochrome c is maintained at a higher level of steady state reduction in mitochondria from ethanol-treated rats [24]. It is likely that under some circumstances, particularly with ascorbate as the substrate, the electron transfer reaction between cytochromes c and a may become rate limiting for the flow of

[1]This work was supported in part by Grants AA3442 and AA5662 from the National Institute of Alcohol Abuse and Alcoholism and also by Grant GM 28173 from the National Institutes of Health.

TABLE 1

EFFECT OF CHRONIC ETHANOL CONSUMPTION ON RESPIRATION RATES AND RESPIRATORY CONTROL IN SUBMITOCHONDRIAL PARTICLES FROM RAT LIVER

Substrate	Parameter	Control	Ethanol-treated	% Change	p
NADH [16]	Respiration rate (uncoupled)*	301 ± 14	173 ± 14	−42	<0.001
	Respiratory Control Index (−oligomycin)†	3.36 ± 0.13	3.06 ± 0.14	− 9	<0.05
	Respiratory Control Index (+oligomycin)‡	4.46 ± 0.18	4.16 ± 0.20	− 7	n.s.§
	Respiration rate + cytochrome c¶ / Respiration rate − cytochrome c	1.06 ± 0.02	1.06 ± 0.02	0	n.s.
Succinate [15]	Respiration rate (uncoupled)*	236 ± 14	180 ± 13	−24	<0.001
	Respiratory Control Index (−oligomycin)†	2.84 ± 0.06	3.05 ± 0.06	+ 7	<0.01
	Respiratory Control Index (+oligomycin)‡	3.26 ± 0.12	3.62 ± 0.11	+11	<0.02
	Respiration rate + cytochrome c¶ / Respiration rate − cytochrome c	1.03 ± 0.01	1.07 ± 0.02	+ 4	n.s.

*Nanoatoms of oxygen/min/mg of protein in the presence of carbonylcyanide m-chlorophenylhydrazone (CCCP) (1.25 μM).
†Respiration rate in the presence of CCCP (1.25 μM) divided by respiration rate in the presence of substrate alone.
‡Respiration rate in the presence of CCCP (1.25 μM) divided by respiration rate in the presence of substrate and oligomycin (0.5 μg/mg of protein).
§n.s.=Not significant.
¶Respiration rate in the presence of CCCP (1.25 μM) and added cytochrome c (10 μM) divided by respiration rate in the presence of CCCP alone.

TABLE 2

EFFECT OF CHRONIC ETHANOL TREATMENT ON THE CYTOCHROME CONTENT OF HEPATIC MITOCHONDRIAL MEMBRANES

	Mitochondria			Submitochondrial Particles		
	Control	Ethanol-Treated	% Change	Control	Ethanol-Treated	% Change
Substrate-reducible						
cytochrome aa_3	0.17	0.09	−47	0.33	0.20	−39
cytochrome b	0.10	0.05	−50	0.20	0.11	−45
cytochromes c + c_1	0.32	0.30	− 6*	0.32	0.25	−22
Chemically-reducible						
cytochrome aa_3	0.17	0.09	−47	0.34	0.21	−38
cytochrome b	0.14	0.09	−36	0.24	0.19	−21
cytochromes c + c_1	0.33	0.32	− 3*	0.35	0.29	−17

*Not significant.
Substrate-reducible cytochromes aa_3 and c + c_1 were determined from difference spectra recorded following anaerobiosis achieved by oxidation of succinate or NADH; cytochromes b were measured in the presence of substrate and antimycin (1 μg/mg protein) under aerobic conditions. Chemically-reducible cytochrome content was measured after addition of dithionite (approximately 2 mM) to the same preparations. Values indicate the means for 9 and 12 pairs of rats for mitochondria and submitochondrial particles, respectively. The S. E. was ≤0.01 for all values. Differences between the preparations from ethanol-treated and control rats are statistically significant at $p<0.001$, except where noted.

electrons toward oxygen. On the other hand with NADH as the substrate the NADH dehydrogenase reaction may limit the rate of respiration. The diminished activity of NADH dehydrogenase may be related to the decreased content of certain iron-sulfur clusters in the NADH dehydrogenase complex which occurs in mitochondria of ethanol-treated animals [22]. The results suggest that the lower respiratory rates found after chronic ethanol treatment arise, at least in part, from specific alterations in the content of particular electron transfer chain carriers, but that different alterations, and the energy state of the mitochondria, limit the rate of respiration with different substrates. In intact hepatocytes

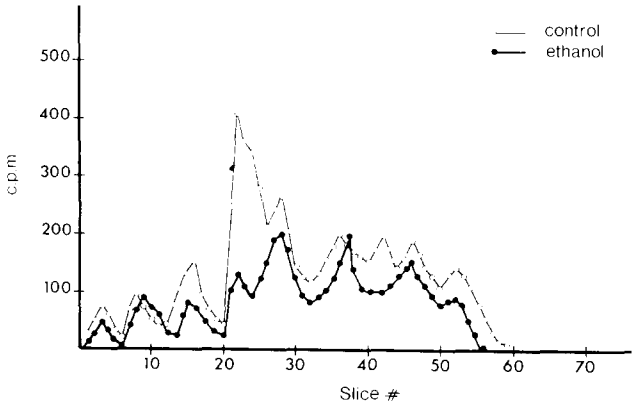

FIG. 1. SDS-gel electrophoresis profile of acetic acid-lubrol pellet from normal mitochondria incubated in the presence and absence of 100 mM of ethanol. Protein (100 μg) was added to each gel. Under these experimental conditions cytochrome c migrated to the area corresponding to slices 47 and 48. Area of greatest inhibition corresponds to slices 21 to 26.

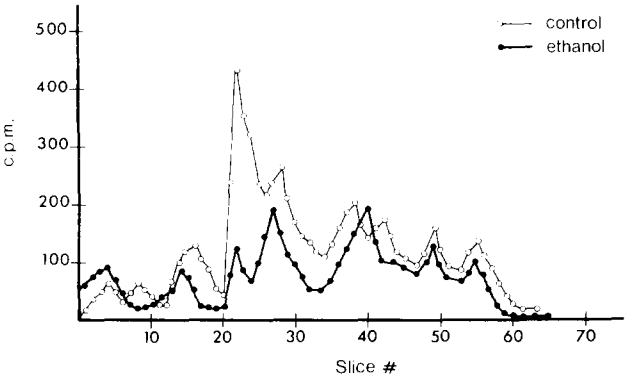

FIG. 2. SDS-gel electrophoresis profile of acetic acid-lubrol pellets derived from mitochondria of an ethanol-treated rat and its corresponding control. Both rats were injected with ^{3}H-leucine (500 μCi) intraperitoneally in the presence of cycloheximide. Protein (150 μg) was applied to each gel. Under these conditions cytochrome c migrated to the area corresponding to slices 48 to 49. Area of greatest inhibition corresponds to slices 21 to 26.

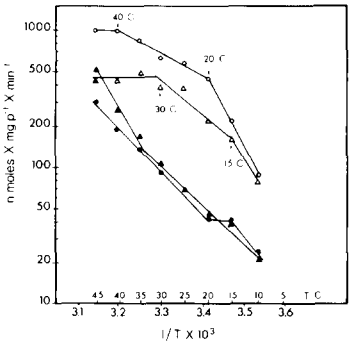

FIG. 3. Arrhenius plots of ATP hydrolysis by normal rat liver mitochondria. Effect of ethanol on ATPase activity between 10°C and 45°C is shown. ATPase activity was followed by acid production with a pH electrode. Reaction medium contained 0.2 M sucrose, 50 mM KCl, 2 mM Tris-HCl (pH 8.0) and 2 mM $MgCl_2$. Mitochondria were added (1.5 mg per ml) and rotenone was added (2 μM) to inhibit endogenous respiration. ATP (2 mM) was added, and the rate of Mg-stimulated ATPase was measured after the steady state was established. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (1 μM) was added; the rate of CCCP-stimulated ATPase is given as the amount of ATP hydrolyzed during the first minute after the addition of CCCP. ●, Mg-stimulated ATPase; ○, CCCP-stimulated ATPase; ▲, Mg-stimulated ATPase in the presence of 1.0 M ethanol; △, CCCP-stimulated ATPase in the presence of 1.0 M ethanol.

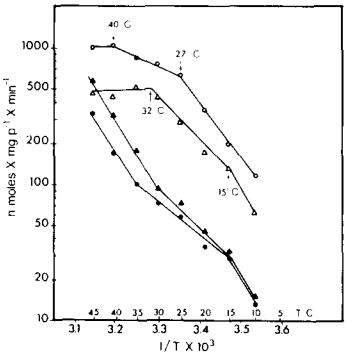

FIG. 4. Arrhenius plots of ATP hydrolysis by liver mitochondria from ethanol-fed rats. Effect of ethanol on ATPase activity between 10° and 45°C is shown. Conditions are the same as in Fig. 3, except that the mitochondria are from ethanol-fed rats. Designations of symbols is the same as in Fig. 3. Results of Figs. 3 and 4 are from pair-fed rats.

we have demonstrated molecular alterations in the cytochrome b region, comparable to those found in submitochondrial particles [25]. However in such cells substrate accessibility, rather than the respiratory chain, may limit the rate of oxygen utilization.

The decreased content of various components of the electron transport chain may be related to inhibition of mitochondrial protein synthesis by ethanol. The presence of ethanol interferes with mitochondrial protein synthesis *in vitro* and *in vivo*, while hepatic mitochondria from rats chronically treated with ethanol display impairment of protein synthesis in the absence of ethanol [2,16]. SDS-polyacrylamide gel electrophoresis of mitochondrial membranes showed that both the presence of ethanol and chronic ethanol intoxication are associated with a decrease in the membrane incorporation of proteins in the 36,000–40,000 dalton range (Figs. 1,2), a region which includes subunits of cytochrome oxidase and ATPase [2]. The mechanism for the inhibition of mitochondrial protein synthesis is not clear. Ethanol may impair protein synthesis at the level of transcription or translation. However in view of the effects of acute and chronic ethanol intoxication on the structure and composition of biological membranes, the assembly of preformed proteins or their subunits into the membrane may be impaired.

STUDIES OF TEMPERATURE DEPENDENCE OF MEMBRANE-BOUND ENZYMES

While decreased mitochondrial respiration may be attributed to defects in the electron transport chain and AT-

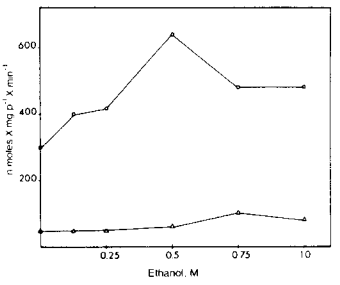

FIG. 5. Effects of ethanol on ATPase activity at 45°C. Conditions are the same as in Fig. 3, except that the ethanol concentration is as indicated. Only Mg-stimulated ATPase activity (in the absence of CCCP) is shown. ○, control rat; △, rat pair-fed with ethanol.

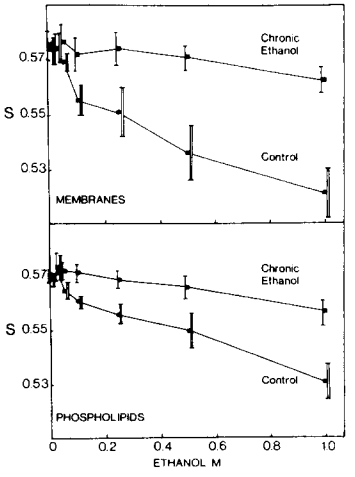

FIG. 7. Effect of ethanol, *in vitro*, on the order parameter of 5-doxylstearic acid in membranes and vesicles of extracted phospholipids (20 mg of phospholipid per ml) from rat liver mitochondria at 35°C. Points represent the mean±SD. Spectral scan, 100 G; midfield line, 3250 G; microwave power, 5 mW; modulation amplitude, 0.63 G; average of four scans.

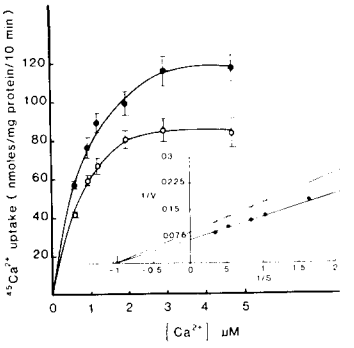

FIG. 6. Effect of free calcium concentration on $^{45}Ca^{2+}$ uptake by liver microsomes from control (○—○) and ethanol-fed (●—●) rats. Free calcium concentration was varied by using a Ca^{2+}-EGTA buffer system, with different concentrations of EGTA. At the end of 10 min, uptake was terminated by the addition of 4 ml of ice-cold washing medium, immediately followed by filtration and a 4 ml rinse of the filters. Each point is the mean±S.E., n=5 ($p<0.01$ for each concentration of Ca^{2+}). Insert shows the Lineweaver-Burke plots for the microsomal $^{45}Ca^{2+}$ uptake for control (○—○) and ethanol-fed (●—●) groups.

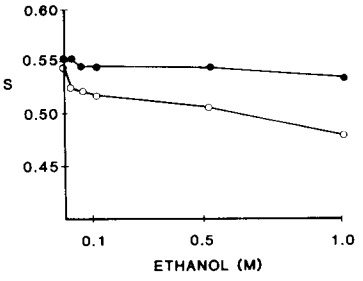

FIG. 8. Effect of *in vitro* ethanol titration, on the molecular order parameter of 5-doxylstearic acid in hepatic microsomal membranes from control (○—○) and ethanol-fed (●—●) groups. Experiments were carried out at 37°C. Points indicate means for 8 scans.

Pase, alterations in the physical state of the lipid bilayer may influence the temperature dependence of enzyme activity. Breaks in Arrhenius plots of membrane-bound enzyme activities are often strongly dependent on phase transitions of the membrane lipids [13]. In the high temperature range, ethanol *in vitro* reduces the transition temperature of NADH-linked respiration and ATPase activity (Fig. 3). On the other hand, after chronic ethanol ingestion the transition temperatures for these activities are increased in hepatic mitochondria (Fig. 4). The addition of ethanol *in vitro* to mitochondria from rats fed ethanol chronically, the situation which obtains in chronic alcoholism, leads to transition temperatures closer to those of normal mitochondria. Thus, the increase in the transition temperature after chronic ethanol intoxication may be an adaptive response to the fluidizing effects of ethanol on the mitochondrial membrane. This concept is supported by the fact that the uncoupling effect of ethanol on mitochondrial ATPase at high temperature is almost absent in mitochondria from ethanol-fed rats (Fig. 5). The effects of ethanol on temperature dependence of enzyme activities has also been studied in hepatic microsomes. Calcium uptake is increased by 30% in microsomes from ethanol-fed animals, the V_{max} being increased and the K_m unchanged [10] (Fig. 6). Arrhenius plots of calcium uptake show that the transition temperature in the mid-temperature range is higher in microsomes from ethanol-fed rats. Ethanol, *in vitro*, inhibits microsomal calcium uptake, the effect being greater in control rats than in those chronically fed ethanol. As in studies of mitochondrial enzymes the rate of calcium uptake by microsomes from ethanol-treated animals in the presence of ethanol is similar to that in microsomes from controls in its absence.

MOLECULAR ORDERING

The breaks in activities of membrane-bound enzymes in Arrhenius plots suggested alterations in the physical state of membrane phospholipids, the breaks probably reflecting lipid phase transitions. By using the spin labels 5-doxyl and 12-doxyl stearic acid as probes, it was found that ethanol *in vitro* decreases the order parameter, S, in hepatic mitochondrial and microsomal membranes [10,27] (Figs.

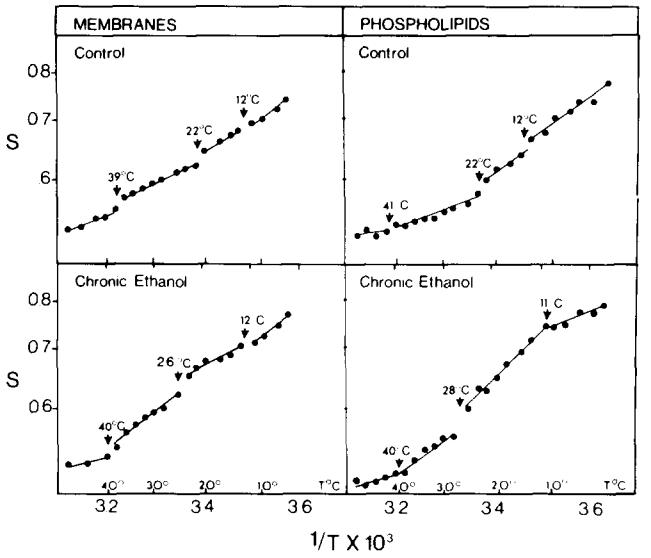

FIG. 9. Temperature dependence of the order parameter(s) in membranes and phospholipid liposomes obtained from a typical pair of control and ethanol-fed rats and labeled with 5-doxylstearic acid. Each point represents the mean of four scans. Order parameter(s) was calculated from the outermost and innermost hyperfine extrema. Membrane suspensions contained 2 μM rotenone and 10 mM ferricyanide to prevent reduction of the spin probe by the sample. Arrows point to breaks in the linearity. In experiments with five pairs the mean±S.E. of the temperature of the discontinuity in the mid temperature range was 19.6±1.0 and 25.8±0.7°C for mitochondrial membranes from control and ethanol-fed rats, respectively. Mean paired difference was 6.2±0.8°C ($p<0.001$). For mitochondrial phospholipids the mean discontinuity was at 22.0±1.4 and 26.4±1.0°C for preparations from control and ethanol-fed rats, respectively, with the average paired difference being 4.4±0.5°C ($p<0.002$). Differences in the other discontinuities were not significant.

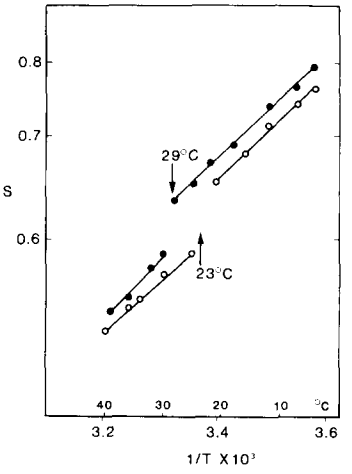

FIG. 10. Temperature dependence of the order parameter(s) for the spin label 5-doxylstearic acid in the liver microsomal membranes of the representative pair of control (○−○) and ethanol-fed (●−●) rats. Each point represents the mean of eight scans. The order parameter was estimated from the innermost and outermost hyperfine splittings. EPR measurements were made with membrane suspensions having a protein concentration of 10 mg/ml and the probe to lipid ration of 1:200. In experiments with six pairs, the mean temperature of the discontinuity was 24.2±1.9°C and 28.9±1.9°C for microsomal membranes from control and ethanol-fed rats, respectively, with a mean paired difference of 4.74±2.6°C ($p<0.002$).

7,8). The same membranes exhibit a pronounced resistance to this fluidizing effect of ethanol after chronic intoxication (Figs. 7,8). Even in the absence of ethanol, *in vitro*, hepatic mitochondrial and microsomal membranes from ethanol-fed rats are more rigid. Moreover when S is plotted as a function of temperature, mitochondria and microsomes from ethanol-fed rats show discontinuities at a higher temperature than those from controls [10,26] (Figs. 9, 10).

The resistance to the fluidizing effect of ethanol produced by chronic ethanol intoxication is also found in vesicles composed of phospholipids isolated from mitochondria [27] (Fig. 7). Thus the actue and chronic effects of ethanol on biological membranes reflect changes in the phospholipid bilayer, and the resistance of these membranes from chronically intoxicated animals is caused by alterations in the phospholipid composition of the membranes. In phospholipids extracted from mitochondria derived from ethanol-fed rats, a major change in fatty acid composition of cardiolipin, a negatively charged phospholipid unique to mitochondria, is seen [27]. The fatty acyl residues of this phospholipid are more saturated in mitochondria from ethanol-fed rats than in controls. Since cardiolipin is closely associated with the electron transport complex and the AT-Pase, these changes may play a role in the reduced activity and content of these mitochondrial components.

A lowering of temperature from 35° to 15°C leads to an increase in order parameter in membranes and extracted phospholipids from mitochondria of control and ethanol-treated animals [26]. In addition, after chronic ethanol feeding, the plot of the temperature dependence of the partition coefficient for the decane analog, 5N10, for both membranes and phospholipids, shows an increase in the transition temperature similar to that observed in Arrhenius plots of enzyme activities. It is, therefore, likely that the shifts in the temperature transitions of enzyme activities in membranes correspond to shifts in the lipid phase transitions.

PARTITION OF ETHANOL AND HALOTHANE IN MEMBRANES

The change in the partition coefficient of the spin probe, 5N10, suggested that chronic ethanol intoxication might also change the partition of other lipophilic compounds into membranes. We found that chronic ethanol treatment did indeed reduce the binding of both ethanol and the general anesthetic, halothane, by both liver mitochondrial and synaptosomal membranes [14] (Table 3). The presence of ethanol and halothane also increases the partitioning of 5N10 into the membranes, the effect being diminished in membranes extracted from chronically intoxicated animals. We have obtained similar data using erythrocyte ghosts as a model membrane.

The data presented here are consistent with a simple, unified explanation for a number of phenomena observed in chronic alcoholics: (1) Acute ethanol intake decreases molecular order of membranes. (2) As a result of continued perturbation of the membrane by the binding of ethanol, chronic ethanol consumption leads to an adaptation of membrane composition, such that the molecular order is increased. (3) Increased molecular order is associated with a

TABLE 3

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

	Ethanol	Halothane	Phenobarbital
Mitochondrial membranes			
Ethanol-fed	(8) 1.17 ± 0.634	(5) 21.4 ± 2.71	(5) 22.1 ± 3.3
Control	3.60 ± 0.740	28.6 ± 5.58	33.0 ± 1.5
Control/Ethanol-fed*	4.24	1.35	1.54
Synaptosomal membranes			
Ethanol-fed	(5) 0.33 ± 0.11	(7) 21.7 ± 10.2	
Control	1.00 ± 0.42	27.5 ± 11.3	
Control/Ethanol-fed	3.07	1.38	

Coefficients were determined by incubating the membranes with a ^{14}C-labeled compound and $^{3}H_2O$. The $^{14}C/^{3}H$ ratio in the supernatant and the pellet was determined after sedimentation of the membranes. Partition coefficients were calculated on the assumption that the amount of ^{14}C-labeled compound in the pellet in excess of the amount dissolved in the pellet water is dissolved in the membrane lipids. The number of pairs of rats used for each determination is given in parentheses. Values are means ± standard deviations. All the experimental values are significantly different from the corresponding control values at $p<0.01$ (paired t-test).

*Means of individual pair ratios.

reduction of the binding of ethanol to the membrane, a phenomenon which may bear upon the development of tolerance. (4) In the absence of ethanol the increased molecular order impairs normal membrane function, but the presence of ethanol fluidizes the more rigid membrane so that it resembles normal membranes; hence the development of dependence. (5) The decreased binding of anesthetics to the more rigid membrane may explain, at least in part, cross-tolerance to anesthetics. (6) Cross-tolerance may also result from reduced binding of other drugs that depend in some way on binding to the membrane. (7) The increased partition of lipophilic compounds in the presence of ethanol may increase drug binding or permeability, and may thus be a factor in acute interactions of ethanol and some other drugs. Clearly, further work is required to establish the relation of ethanol-induced membrane alterations to these phenomena.

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