centrations at equilibrium is essential for assessment of the uncertainty in binding data now available.

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Alcohol-Induced Tolerance in Mitochondrial Membranes

Lieber and his colleagues (*1*) state that they have found no evidence for a correlation between the structure of membrane phospholipids and function of mitochondrial membranes from ethanol-fed rats, or for resistance to disordering by ethanol, which we reported earlier (*2*). However, extensive studies in our laboratory show that the decreased rate of respiration, first described by Cederbaum *et al.* (*3*) in intact mitochondria, can be explained as a direct consequence of decreased content and activity of individual protein components of the respiratory chain in mitochondrial inner membranes (*4*). What causes this decrease is not clear. It might be due to direct inhibition of mitochondrial protein synthesis (*5*) or to interference with membrane assembly. The latter may be influenced by the phospholipid composition of the membranes. Moreover, the respiratory activity indeed may be influenced by the phospholipid composition. We have shown that mitochondrial membranes from ethanol-fed rats display an increased saturation in the acyl chains of cardiolipin (*6*), an essential phospholipid component of the electron transport chain, which may contribute to the regulation of the respiration rate. Therefore, we suggested that "The phospholipid composition . . . probably plays a role in other modulations of membrane structure and function . . . " (*6*). Since Lieber and his colleagues (*1*) analyzed only total fatty acid composition of the mitochondrial membranes, and cardiolipin is a minor component, they could not detect this increased saturation of cardiolipin acyl chains. Nevertheless, their data regarding total fatty acid composition actually confirm our findings of a significant increase in stearic acid and a decrease in palmitic acid in both phosphatidylcholine and phosphatidylethanolamine.

Our evidence for increased resistance to disordering by ethanol is based on studies with electron paramagnetic resonance (EPR) spin probes (*2*). The order parameter, measured by 5-doxyl stearic acid or 12-doxyl stearic acid, is significantly decreased by low concentrations of ethanol in liver mitochondria from normal rats but not in ethanol-fed rats. Similarly, the partition of doxyl-decane is greatly enhanced by ethanol in control rats but not ethanol-fed rats. Similar results were obtained earlier by several other groups in synaptosomal membranes and red blood cells (*7*) and more recently by us in liver microsomes (*8*). We, therefore, believe that this is a general phenomenon relevant to all membranes in all tissues (*9*). It is necessary to explain why Lieber and his colleagues could not confirm this observation in their studies. We have found that the resistance to disordering by ethanol is observed at high temperature (35°C) but not at low temperature (15°C) (*2*). Lieber and his colleagues measured 12-(9-anthroyloxy) stearic acid (12 AS) fluorescence anisotropy at 28°C, where the difference, if it exists, is expected to be small. The sensitivity of 12 AS anisotropy to small structural changes is at least one order of magnitude lower than that of the EPR technique, particularly in highly scattering membranes such as mitochondria. In fact, we suspect that scattering artifacts were not properly corrected for in their studies. Vanderkooi and Chance (*10*) studied fluorescence anisotropy of 12 AS in mitochondria at 20° to 45°C (Fig. 3 in *10*); their measured polarization values ranged from 0.125 to 0.1. This value corresponds to an anisotropy range of 0.087 to 0.069. We measured the fluorescence anisotropy of 12 AS in mitochondria and obtained a value of 0.08 at 28°C. This value is in excellent agreement with those obtained by Vanderkooi and Chance (*10*), but is one-third those reported by Lieber and his colleagues (*1*). Nevertheless, because of the low values of fluorescence anisotropy and the large corrections for light scattering, the effect of low concentrations of ethanol on membrane fluidity cannot be easily detected. We suspect that Lieber and his colleagues, in fact, measured the considerable effect of alcohol on light scattering, which is caused by mitochondrial swelling.

In summary, there is sufficient evidence from studies of rat liver mitochondria and other membrane systems to indicate that chronic alcoholism is associated with changes of membrane structure, composition, and function and that these changes lead to tolerance to the acute effects of ethanol.

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Gordon *et al.* (*1*) examined the basis of a challenging theory proposed by Rottenberg and his colleagues (*2*) that the "chronic consumption of ethanol induces an adaptation of membrane composition causing increased membrane rigidity (decreased fluidity). . . . The increased rigidity impairs normal membrane function . . . but in the presence of moderate concentrations of ethanol the membrane becomes sufficiently fluid to resemble normal membranes (dependence)" (*2, 3*).

To examine this theory, we designed a controlled nutritional experiment, which included a group of Chow-fed rats (for which respiratory functions have been well defined), ethanol-fed rats, and the pair-fed controls of the latter. Mitochondrial membranes from the Chow-fed animals contained a larger amount of saturated fatty acids than the mitochondrial preparations from the pair-fed controls and were more resistant to the fluidizing effects of ethanol, although respiratory functions in the membranes of the two groups were similar. In contrast, the

amounts of saturated and unsaturated fatty acids in the mitochondrial phospholipids in the ethanol-fed animals were similar to those in their pair-fed controls, yet their respiratory functions were markedly different. The addition of ethanol at concentrations comparable to those present in man and animals consuming ethanol (50 to 130 m*M*) (more appropriate than the 1.0*M* used by Rottenberg *et al.*) caused a progressive decrease in the microviscosity of the membrane that was similar in the two groups. The failure to show a significant difference between these two groups cannot be attributed to a lack of sensitivity in the experimental techniques since, when there was a significant increase in the content of saturated fatty acids, a statistical difference in the response to the fluidizing effects of ethanol was easily detected. No direct correlation was found between the rigidity of the acyl chains of the phospholipid fatty acids and the activity of the respiratory chain. These findings substantiate the earlier studies of Williams *et al.* (*4*) showing that the functions of the respiratory chain are not regulated by the nature and physical state of the acyl chains of the phospholipid fatty acids.

Our data should not be misconstrued as suggesting that membrane phospholipids play no role in maintaining membrane integrity or in regulating the activity of membrane-bound enzymes. The proposition that chronic ethanol consumption may lead to alterations in the interactions of phospholipids and proteins was not disputed or even addressed in our report. The mobility of the spin label probe, *S*-deoxystearic acid, used by Rottenberg *et al.* (*2*) monitors the physical domain of the membrane close to the polar head groups of the phospholipid, whereas the fluorescent probe 12-(9-anthroyloxy)stearic acid (12 AS) used by us (*1*) monitors the physical state of the interior lipid domain of the membrane. The microviscosity of the latter domain is governed in part by the level of saturation of the phospholipid fatty acids. Thus, two different domains of the mitochondrial membrane were investigated and could explain the apparent discrepancies between these two reports. Such differences in response to the effects of chronic ethanol feeding at different depths of the lipid bilayers of synaptosome membrane have been reported (*5*).

I am somewhat puzzled by the remarks made by Rottenberg and his colleagues concerning the role of light scattering in the measurement of anisotropy by steady-state polarization. As shown by Johnson (*6*), Lentz *et al.* (*7*), and Teale (*8*), corrections for turbidity lead to anisotropy values higher than the apparent values. The anisotropy values reported by Rottenberg *et al.* (0.08 at 28°C) are strikingly similar to those widely reported for pure phospholipid vesicles in which the movement of the probe is not restrained by the presence of protein (*9*). In recent studies, I measured the microviscosity of mitochondrial membranes of Chow-fed rats with 12 AS and diphenylhexatriene as the fluorescent probes at probe-to-lipid ratios from approximately 1:3 to 1:200; the corrected fluorescent anisotropy r_s values were 0.16 and 0.19, respectively. The values are in the same range as our previously reported data and those of Kinosita *et al.* (*10*) and Schinitzky and Inbar (*11*). However, these values are not absolute but relative.

Even if one disregards the fluidity issue, the main point of our study still stands. Differences were not detected in the functions of the hepatic mitochondria of the two control groups, despite significant differences in the content of saturated fatty acids, whereas mitochondria from the ethanol-fed animals exhibited decreased capacity to oxidize substrates, despite having a lipid composition similar to that of their pair-fed controls. These data cast doubt on the direct link between functional, compositional (lipid), and fluidity changes of membranes as described by Rottenberg *et al.* (*2*, *3*).

In conclusion, our data and the reports of others (*12*) do not support the view of the adaptive process proposed by Rottenberg *et al.* Further basic research will be required before the link between the physical and functional effects of ethanol can be understood.

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