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Membrane solubility of ethanol in chronic alcoholism. The effect of ethanol feeding and its withdrawal on the protection by alcohol of rat red blood cells from hypotonic hemolysis

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Membranes from ethanol-fed rats are resistant to the in vitro effects of ethanol on membrane structure and function. We have proposed that the resistance arises from adaptive changes in membrane composition which lower the solubility (partition coefficient) of ethanol in these membranes. The partition of ethanol (and other alcohols and anesthetics) into red blood cells protects the cells from hypotonic hemolysis. Here, we show that the protection by alcohols and anesthetics of red blood cells from ethanol-fed rats is greatly attenuated. This finding indicates that the membrane solubility of these agents is lowered in chronic alcoholism and thus explains the resistance to the acute effects of ethanol. The protection from hemolysis decreases over 2 weeks of ethanol-feeding and returns to normal values within 1 day after ethanol withdrawal. These changes are associated with a parallel increase in total and free serum cholesterol during ethanol feeding and a return to normal values within a day after withdrawal. However, we find only a slight increase in the cholesterol/phospholipid ratio of the red blood cell membranes during the development of ethanol tolerance. In rats fed a cholesterol and saturated fat diet, the increase in serum cholesterol is also associated with an attenuation of the protection from hypotonic hemolysis.

Introduction

Chronic alcoholism is associated with the development of tolerance to and dependence on ethanol, which in turn leads to a severe withdrawal syndrome when ethanol is withdrawn abruptly [1,2]. Although the mechanism(s) of the acute effect(s) of ethanol on behavior is not fully understood, there is little doubt that it is closely related to the pharmacological effects elicited by general anesthetics [3]. Therefore, it is clear that the site(s) of the acute behavioral effects of ethanol is located in the plasma membrane of the central nervous system. However, alcohol distributes in all organs of the body and partitions into all cell membranes, as well as the membranes of cell organelles. While the presence of alcohol in these sites may not be responsible for the acute behavioral effects, it may, nevertheless, contribute to the long-term effects observed in chronic alcoholism. Indeed, pathological manifestations of chronic alcoholism are observed in various other organs, such as the liver, heart and skeletal muscle. These may be related to the effect of alcohol on the function of the membranes of these organs [4]. In addition, and unlike other drugs, alcohol is a caloric-rich metabolite of intermediate metabolism; its consumption in quantities that lead to chronic alcoholism has profound effects on intermediate metabolism in the liver [5].

Recent studies with animal models have indicated that in parallel to the development of toler-

ance and dependence there are changes in the structure and function of various membranes [6–12]. One of the most compelling observations is that membranes isolated from animals that were fed or inhaled ethanol over an extended period of time become resistant to the in vitro effects of ethanol on membrane function and its' physical properties [8–12].

These observations are compatible with the suggested existence of a biological adaptation process in which the organism modulates its membrane composition to compensate for the effects of the drugs [13]. It has been reported that the resistance to the fluidizing effect of ethanol in plasma membranes of chronic alcoholic mice or rats is associated with increased cholesterol content [12,14,15]. Other changes in membrane composition were reported, but no clear pattern was observed [7,11,16–18]. We have previously shown that liver mitochondria from ethanol-fed rats are resistant to the uncoupling effect of ethanol on membrane enzymes [10] and also to the fluidizing effect of ethanol [11]. This resistance is associated with a major change in the fatty acid composition of the phospholipid cardiolipin [11]. We have also shown that the resistance to the effect of ethanol on mitochondrial, as well as synaptosomal membranes, is associated with a decrease in the partition coefficients of various ampiphilic compounds, including alcohol and halothane [19]. Moreover, the decreased partition coefficient of halothane is, in turn, associated with a resistance of these membranes to the strong fluidizing effect of halothane [19].

These observations offered a simple mechanism for the development of tolerance and dependence in these membranes. Accordingly, tolerance is the result of an alcohol-induced change in membrane composition which leads to a decreased partition of ethanol into the membrane. Since alcohol effects, as well as those of other anesthetics, depend strongly on their partition coefficient [20], the reduced partition would lead to apparent tolerance and resistance to acute effects of ethanol [4,19]. Dependence is postulated to be the result of the changed lipid composition which is adapted for optimal membrane function in the presence of ethanol. To confirm the generality of this hypothesis, it is necessary to determine accurately the partition coefficient of ethanol in purified plasma membranes from control and ethanol-fed animals. Unfortunately, the partition coefficient of ethanol in plasma membranes is very low and it is difficult to determine accurately by a direct method. We have previously studied the partition of halothane in purified red blood cell membranes [21]. In these plasma membranes, the partition coefficient of halothane was significantly reduced in ethanol-fed rats, as determined both by a direct tracer distribution measurement and indirectly from the extent of quenching by halothane of a membrane-embedded fluorescent dye [21]. However, our attempts to estimate, with sufficient accuracy, the partition coefficient of ethanol in red blood cells by the tracer distribution method have failed because of its inherently low value.

It is, however, well-established that the partition of drugs and other ampiphiles into red blood cell membranes is strongly correlated with the protection of the red blood cells from hypotonic hemolysis [20,22]. Since the mechanism of this effect is fairly well understood and clearly depends on the volume of drugs incorporated at the membrane surface, we decided to utilize this phenomenon for the estimation of the relative partition of ethanol in membranes from control and ethanol-fed rats. We found that red blood cells from ethanol-fed rats are protected from hemolysis by ethanol (and other alcohols) to a lesser extent than controls, indicating a lower partition coefficient. Since it was claimed that membrane resistance is the result of increased cholesterol, we have used this new assay (the extent of ethanol protection from hemolysis) to investigate the time-course of the development of membrane changes due to ethanol feeding and its withdrawal, and its relationship to serum and membrane cholesterol.

Materials and Methods

Animals. Male, Sprague-Dawley rats weighing 130–150 g were fed a nutritionally adequate, totally liquid diet [23], which contained 35% of the total calories as ethanol (obtained from Bioserve, Inc.). Pair-fed littermate controls were given the same diet, except that ethanol was replaced by an isocaloric amount of carbohydrates. Daily ethanol consumption was 14–16 g/kg body weight. The

rats were killed by decaptitation after having been fed the previous night. After ethanol withdrawal, experimental animals were given the same diet as control. In experiments which were designed to raise the serum cholesterol level (Fig. 4), the same protocol was followed, except that the alcohol liquid diet was replaced with a low-fat diet (Bio-serve, Inc.) which was supplemented with cholesterol (0.5%) and coconut oil (70% of calories).

Preparation of red blood cells and erythrocyte ghosts. Blood was collected from decapitated rats into heparinized tubes and erythrocytes were separated from plasma by centrifugation for 10 min at $1000 \times g$ (4°C). The serum was saved for cholesterol determination and the red blood cell pellet was washed three times with an isotonic buffer solution (0.172 M Tris-HCl, pH 7.6) and finally, suspended in a small volume of the same buffer until used for the experiments. For lipid analysis of plasma membrane, we prepared erythrocyte ghosts by suspending the pellet in hypotonic buffer (0.011 M Tris-HCl, pH 7.6). The ghosts were collected by centrifugation at $20\,000 \times g$ for 30 min and washed 4–5 times in the same buffer until no hemoglobin was visible. The washed pellet was frozen at −20°C [24].

Lipid extraction and analysis. Membrane lipids were extracted by a modified Bligh-Dyer procedure [25]. The extract was dissolved in chloroform. Phospholipids were determined as phosphate after hydrolysis [26] and cholesterol was determined by the cholesterol oxidase-peroxidase assay as previously described [27]. Serum total cholesterol was determined by the enzymatic assay using the Boehringer Kit, and serum-free cholesterol was determined by the cholesterol oxidase-peroxidase assay, as described [27].

Determination of the extent of erythrocyte hemolysis. The assay was performed essentially as described [22]. Red blood cells were mixed with the test solution, incubated for 15 min at 25°C and centrifuged in Eppendorff microfuge ($11\,000 \times g$) for 5 min. The supernatant hemoglobin content was determined from the absorbance at 540 nm. In each set of experiments, the hemolysis was calculated as a percentage of complete hemolysis obtained by incubating the same amount of cells in hypotonic medium (0.011 M Tris-HCl) as described above.

All enzymes were obtained from Boehringer, other reagents were of analytical grade. Statistical analysis of paired animal experiments are based on the paired Student's *t*-test. Correlation analysis is based on a linear-regression program.

Results

The protection of erythrocytes from hypotonic hemolysis by alcohols was tested by the following procedure: first, the maximal extent of hemolysis of small volumes of the stock red blood cell suspension was determined by incubating with hypotonic medium. A volume yielding an optimal value for the spectroscopic determination of hemoglobin was selected. Then, the selected volume was incubated with hypotonic buffer solutions of various osmolarities to determine the osmolarity required for 30% hemolysis. Finally, cells were incubated in solutions of hypotonic buffer that produce 30% hemolysis containing increasing concentrations of alcohols.

Fig. 1 shows typical results from an experiment with red blood cells from an ethanol-fed rat (35 days) and its pair-fed control. Fig. 1A shows that at a concentration range of 1–10% (0.172–1.72 M), ethanol protected up to 53% of cells from hypothonic hemolysis in control rats. Maximal protection is obtained at 8%. In sharp contrast, blood cells from ethanol-fed rats are much less protected. Maximal protection is observed at 5% alcohol with only 20% of cells protected. Increasing concentrations of ethanol lead to increased hemolysis. At 10% ethanol, blood cells from ethanol-fed rats are hemolyzed to a larger extent (120%) than without ethanol. As demonstrated below, the increased hemolysis at high ethanol concentrations is due to a significant dilution of the buffer at these ethanol concentrations, which reduces the osmolarity of the medium, since ethanol itself does not produce appreciable osmotic pressure. This effect is observed both in ethanol-fed and controls. However, in controls the decrease in osmolarity, which increases hemolysis, is balanced by increased protection leading to flattening of the curve, whereas in alcohol-fed rats the protection is much weaker resulting in net increase of hemolysis. This experiment was repeated with 15 pairs, which were fed ethanol for 35–50 days, and the

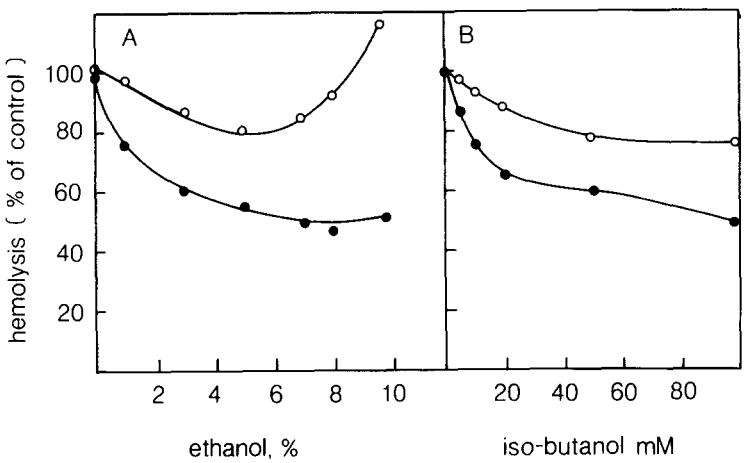

Fig. 1. Protection by alcohols of red blood cells from hypotonic hemolysis in an ethanol-fed rat and its pair-fed control. (A) The effect of ethanol (1–10%) on the relative extent of hemolysis in ethanol-fed (○) and control (●). Procedure as described in Materials and Methods. Ethanol feeding: 35 days. Hemolysis without drugs (taken as 100%) was 32% of total number of cells. (B) The effect of iso-butanol (5–100 mM). Other conditions and symbols as in (A).

decreased protection of ethanol-fed was found to be highly significant ($P < 0.0000$). The relative percentage of hemolysis with 10% ethanol in 15 pairs was $122 \pm 20\%$ (S.D.) for ethanol-fed rats and $74 \pm 13\%$ for controls.

Fig. 1B shows a similar experiment with iso-butanol. Much lower concentrations of iso-butanol are required to obtain similar protection. This is compatible with the correlation between the partition coefficient and extent of protection [20,22]. It is observed that isobutanol protection is also reduced in red blood cells from ethanol-fed rats (Fig. 1B). The difference is similar to differences observed with ethanol, suggesting that the change of the partition coefficients is not specific to ethanol, in agreement with our previous findings [19]. In the experiment of Fig. 1A, 5% ethanol was required in cells from alcohol-fed rats to give a protection (20%) equivalent to 1% ethanol in control rats, indicating a 5-fold decrease of the partition coefficient. Similarly, 50 mM isobutanol was required to give 20% protection in cells from ethanol-fed as compared to 10 mM in control, indicating a similar decrease of partition coefficients. The average difference in 15 pairs was considerably smaller (2.05 ± 0.63) but was highly significant ($P > 0.0001$).

Because the ethanol protection of cells from hemolysis is counter-balanced by a dilution effect in the assay described above, we modified the assay to correct for the dilution effect. Fig. 2A shows an experiment in which the extent of hemolysis was tested as function of medium osmolarity in the presence and absence of 10% ethanol. The calculation of osmolarity in the ethanol-containing suspension is based on the increased volume of the combined water/ethanol solution. It is observed that when the corrected osmolarity of the suspension is taken into account, 10% ethanol protects cells from both ethanol-fed rats and control. Although there were individual differences in the shape of the hemolysis curve, there was no significant difference in osmotic fragility between cells from control and ethanol-fed in the absence of ethanol (not shown). However, the extent of protection by 10% ethanol was always reduced in cells from ethanol-fed rats.

This is more evident in Fig. 2B which shows the protection from hemolysis by 10% ethanol as a function of the extent of hemolysis (data are extrapolated from Fig. 2A). As expected, the protection decreases as the osmolarity decreases. However, the difference between ethanol protection in control and ethanol-fed is relatively independent of osmolarity. Based on these results, we devised an improved assay for protection by ethanol and other alcohols. Aa in the previous assay, we first searched for the osmolarity that resulted in 30% hemolysis. We then compared the hemolysis with 10% ethanol to a buffer solution of the selected osmolarity diluted with water to obtain the same osmolarity as the ethanol/water solution. This resulted in approx. 60% hemolysis in the suspension without ethanol and a lesser hemolysis in the suspension with ethanol. Similar experiments were performed with other alcohols and halothane,

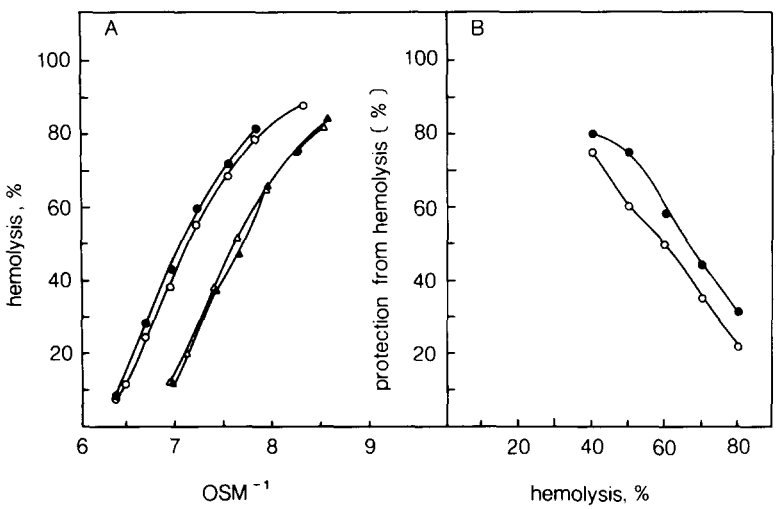

Fig. 2. Hemolysis as a function of medium osmolarity and its inhibition by ethanol in an ethanol-fed rat (35 days) and its control. Procedures as described in Materials and Methods. Ethanol feeding: 35 days. Osmolarity was changed by changing the buffer (Tris-HCl) concentration. Ethanol was 10% by volume. The osmolarity of the ethanol-containing suspension was calculated on the assumption that ethanol does not produce osmotic pressure. (A) Hemolysis (% of total cell number) as a function of reciprocal osmolarity. Cells from ethanol-fed rats were incubated without ethanol (○) or with 10% ethanol (△); controls incubated without ethanol (●) or with 10% ethanol (▲). (B) Extrapolated from (A) showing the percentage of cells protected from hemolysis by ethanol as a function of the extent of hemolysis in ethanol-fed (○) and control rats (●).

where the control was corrected for dilution as necessary (in the cases of methanol, ethanol and propanol).

Fig. 3 shows the results of these experiments with red blood cells from eight pairs (methanol and ethanol) or five pairs (propanol, isobutanol,

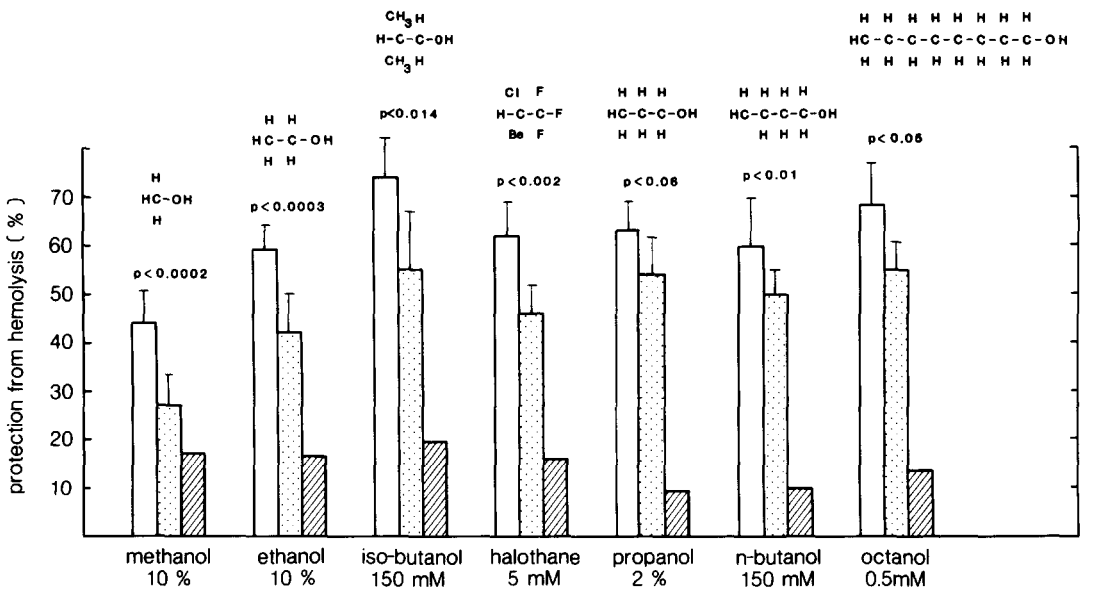

Fig. 3. The protection by alcohols and anesthetics (halothane) from hypotonic hemolysis in ethanol-fed rats and their controls. The figure shows the effect of methanol (10%), ethanol (10%), isobutanol (150 mM), halothane (5 mM), propanol (2%), *n*-butanol (150 mM) and octanol (0.5 mM). The concentrations of the various compounds were selected to give approximately the same protection as ethanol in controls (60%). The open bars show the percentage protection in controls, the stippled bars show the percentage protection in ethanol-fed, and the hatched bars show the averaged pair difference. There were eight pairs in the ethanol and methanol experiments and five pairs in the other experiments. The standard deviation is shown above each average and the probability (paired *t*-test) is indicated as well. Assay as described in Materials and Methods. Ethanol feeding: 35 days.

n-butanol, halothane and *n*-octanol) that were fed ethanol for 35 days. The concentration of each agent was selected to give approximately equal protection in controls. It is observed that with all alcohols and halothane, cells from ethanol-fed rats are less protected than their pair-fed controls. The difference is of approximately equal magnitude for methanol, ethanol, isobutanol and halothane, and somewhat lower for octanol, propanol and *n*-butanol. Thus, it appears that the partition of one and two carbon-chain molecules are somewhat more affected by ethanol-feeding than longer-chain alcohols. Isobutanol is, of course, a 4-carbon compound. However, its structure is of a 2-carbon chain with two methylene side-chains. It is therefore possible that the phospholipid modification is more pronounced near the head-group than at the hydrophobic membrane core.

In parallel with the studies of hemolysis, we have analysed the cholesterol content of the serum obtained from ethanol-fed rats and their controls. For the eight pairs shown in Fig. 3, total cholesterol was 73 ± 18 mg/dl in controls and 112 ± 20 mg/dl in ethanol-fed. This difference was highly significant ($P < 0.0003$). The free serum cholesterol was 20.3 ± 6.2 mg/dl in control and 31.4 ± 6.0 mg/dl in ethanol-fed ($P > 0.0000$), thus showing essentially the same difference as the total cholesterol. To test whether elevated serum cholesterol leads to reduced protection, we fed rats a diet rich in cholesterol and saturated fat (coconut oil) as described in Materials and Methods. After 4 weeks on this diet, there was a significant difference in serum cholesterol between controls, 76 ± 8 mg/dl, and cholesterol-rich diet rats, 89 ± 11 mg/dl ($P < 0.0004$), $n = 6$. The alcoholic protection from hypotonic hemolysis in these animals is shown in Fig. 4. For all alcohols, there was a significant reduction in protection in rats fed with cholesterol-rich diets. Although the differences are not as high as in Fig. 3, the difference in serum cholesterol between the two groups is also smaller, thus indicating a negative correlation between serum cholesterol and protection from hypotonic hemolysis.

To further investigate the relationship between serum cholesterol and protection from hypotonic hemolysis, we examined the time-course of the changes in serum cholesterol and protection from hypotonic hemolysis during ethanol feeding, and after withdrawal and refeeding. The results of these experiments are shown in Fig. 5. The lower panel shows the protection, by 10% ethanol, from hemolysis in ethanol-fed rats and their controls. The upper panel shows the corresponding total serum cholesterol. It is observed that total serum cholesterol increases and protection decreases in parallel in ethanol-fed rats, reaching a maximum after about 3 weeks. Withdrawal from ethanol leads to complete reversal within 1 day of both the serum cholesterol elevation and the protection decrement. Refeeding of ethanol after 4 days of withdrawal resulted in a slow rise of the serum cholesterol and slow decline in protection reaching stable values after 14 days.

This experiment leads to several conclusions:

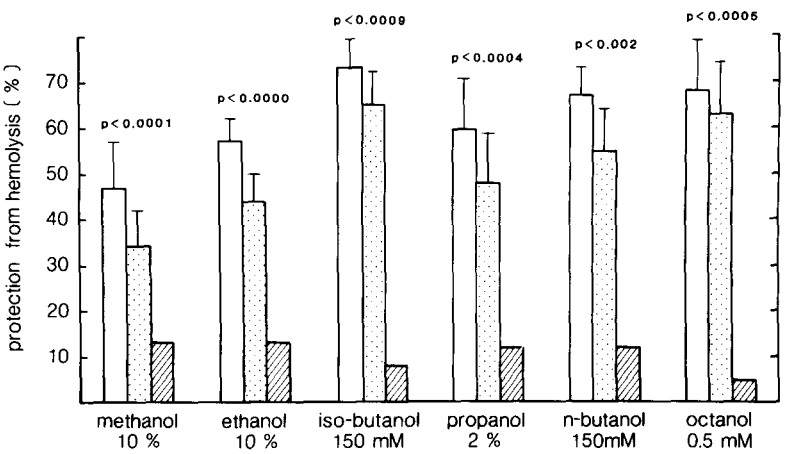

Fig. 4. Protection from hypotonic hemolysis by alcohols in cholesterol/coconut oil-fed rats and their controls. Conditions as in Fig. 3, except that instead of ethanol, experimental animals were fed ad libitum (28 days) a diet rich in saturated fat (70% of total calories as coconut oil) and cholesterol (0.5%), controls were fed the regular liquid diet, ad libitum. Open bars show control, stippled bar shows fat-rich diet animals, and hatched bars show the difference.

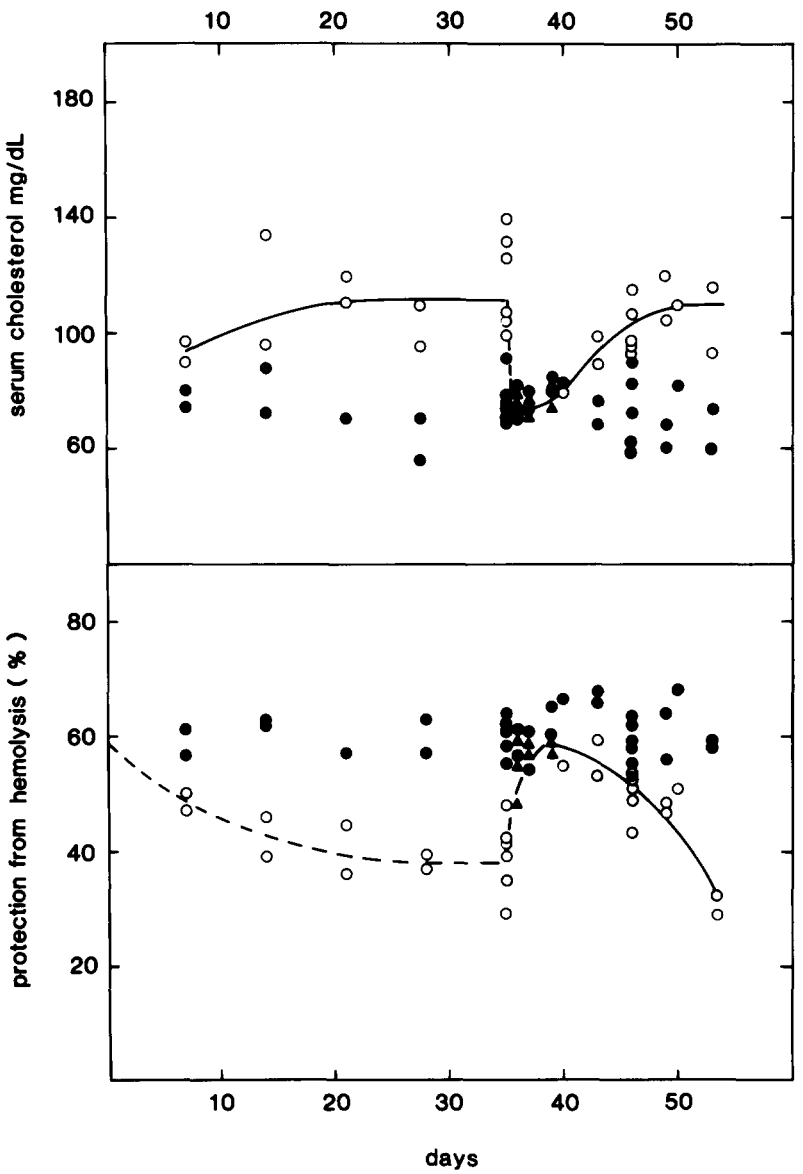

Fig. 5. Time-course of ethanol protection from hypotonic hemolysis (bottom) and total serum cholesterol (top) in ethanol feeding, its withdrawal and refeeding. Assay conditions as in Fig. 3. Ethanol concentration was 10%. ●, control; ○, ethanol-fed during ethanol feeding; ▲, ethanol-fed after withdrawal (ethanol was fed for up to 35 days, then withdrawn for up to 4 days and refed for up to 14 days).

(a) there is a close correlation in the time-course of the changes in serum cholesterol levels and loss of alcohol protection from hemolysis; (b) withdrawal results in very quick restoration of normal serum cholesterol levels and normal alcohol protection; (c) the development of the alcoholic state is slow, extending over a 2-week period. We consider the time-course of development in alcohol refeeding after withdrawal a more accurate measure of the rate of development, since in the initial alcohol feeding period (day 1–7) the alcohol intake is lower than in the following days.

The correlation between changes in cholesterol levels and protection from hemolysis is further demonstrated in Fig. 6 which shows the data of Figs. 4 and 5 plotted as serum cholesterol vs. protection by 10% ethanol from hemolysis. It is observed that the controls and the rats withdrawn from alcohol are clustered at the right-side bottom of the curve (low cholesterol, high protection) while ethanol-fed and cholesterol-fed animals are spread over towards the left-side top (high cholesterol,

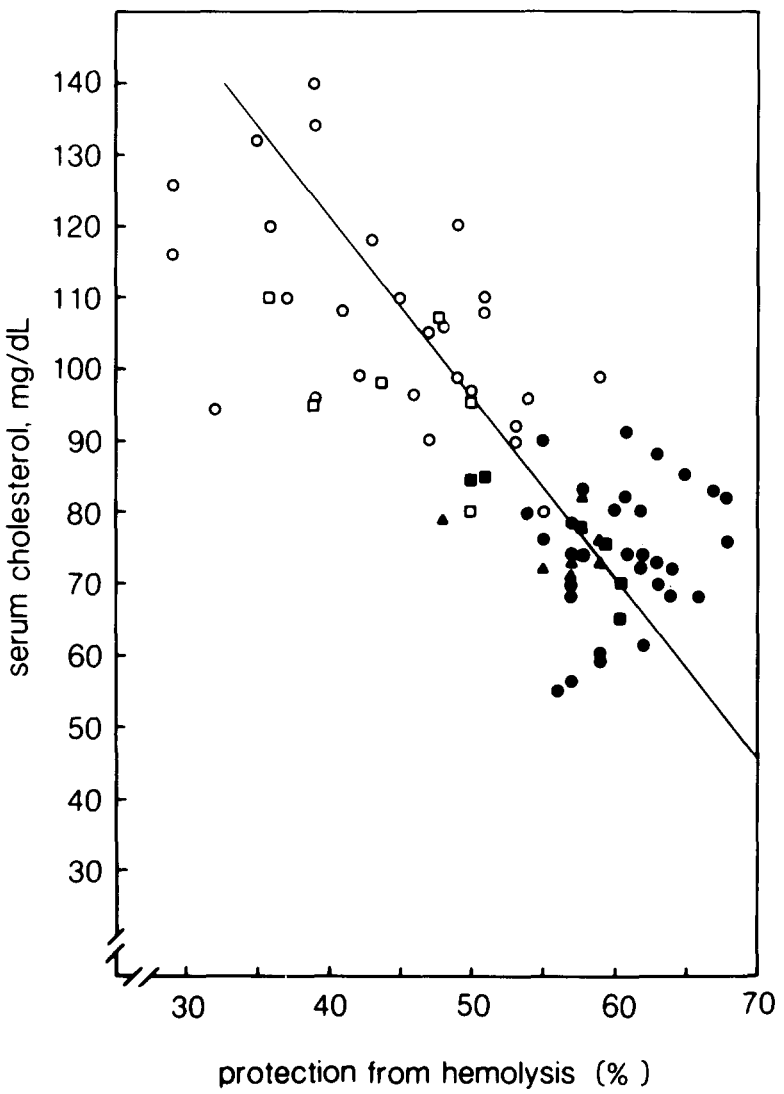

Fig. 6. The correlation between total serum cholesterol and protection from hypotonic hemolysis by 10% alcohol. Data are from Fig. 5 and also include the six pairs from Fig. 4. ●, controls; ○, ethanol-fed; ▲, ethanol withdrawn; ■, controls of Fig. 4; and □, fat-rich diet (Fig. 4).

low protection). The correlation coefficient by linear-regression analysis of the data in this figure is 0.77 ($P < 0.0000$). Interestingly, for the control group alone, there is no correlation between serum cholesterol and protection. Thus, the large variation in protection within the control group is not associated with different cholesterol levels. However, the changes within the ethanol-fed group and the cholesterol-fed group and in the combined group are highly correlated with serum cholesterol levels, indicating an underlying connection between changes in serum cholesterol and ethanol protection from hypotonic hemolysis in ethanol-fed rats. The difference in serum cholesterol between controls and ethanol-fed rats is not due to differences in feeding patterns. In pair feeding experiments, controls consume their diets within a few hours, while ethanol-fed drink slowly all night. However, the controls in Fig. 4 were fed liquid diet ad libitum but show essentially the same cholesterol level as those of Fig. 3. Moreover, with three pairs, controls were given their diet in small portions over the entire feeding period. Their total cholesterol level (72 mg/dl) was also the same as regular controls.

One possible explanation of the correlation is

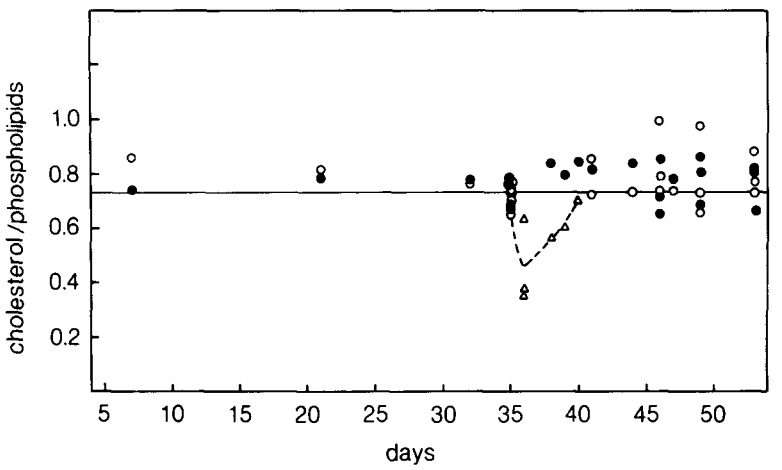

Fig. 7. Membrane cholesterol/phospholipid ratio as a function of ethanol feeding and its withdrawal. Membranes were prepared from pairs shown in Fig. 5. Lipid extraction, cholesterol and phosholipid analysis as described in Materials and Methods. ●, controls; ○, ethanol-fed; △, withdrawn from ethanol.

that increased serum cholesterol leads to an increased cholesterol/phospholipid ratio in the membrane. Cholesterol was shown to reduce the partition coefficient of ampiphiles in liposomes [28] and was reported to be elevated in membranes of red blood cells from ethanol-fed mice [14–16]. To examine whether changes in the membrane cholesterol/phospholipid ratio can explain our results, we extracted the lipids from some of the pairs shown in Fig. 5 and determined the cholesterol/phospholipid ratio. Fig. 7 shows the results of these determinations. Suprisingly, there are only slight differences between the cholesterol level in control and ethanol-fed rats. In 21 pairs, the average ratio for controls was 0.72 ± 0.17 and for ethanol-fed 0.75 ± 0.19 ($P < 0.075$). This slight, and relatively insignificant difference cannot explain the large difference in protection from hypotonic hemolysis between control and ethanol-fed rats. Occasionally, the cholesterol/phospholipid ratio appears to drop 1 day after ethanol withdrawal, rapidly increasing to its normal value over the 4-day withdrawal period. Thus, the decreased protection by ethanol in ethanol-fed rats cannot be attributed to an increased membrane cholesterol/phospholipid ratio. Since total and free serum cholesterol also drop after withdrawal, it appears likely that the occasional swift drop in membrane cholesterol/phospholipid ratio is a direct response to the reduced serum cholesterol and the subsequent increased ratio is due to slower return to normal lipid composition which leads to increased partition of cholesterol. These findings point to a complicated relationship between serum cholesterol, membrane cholesterol and alcohol partition into red blood cell membranes during ethanol feeding and particularly after withdrawal (see Discussion).

Discussion

Alcohol and anesthetics partition in plasma membranes during ethanol feeding and its withdrawal

The results of our studies on the protection of red blood cells by alcohol from hypotonic hemolysis in ethanol-fed rats are compatible with the suggestion [19] that the partition coefficient of ethanol is reduced in chronic alcoholism. The correlation between protection from hemolysis and partition coefficients of a large number of drugs, alcohols, detergents and other chemicals is well-established [20]. It is also quite clear that this effect is due to the expansion of the membrane surface area which, in turn, increases the critical volume red blood cells can assume in hypotonic swelling before hemolysis begins. While there is some uncertainty as to the exact extent of surface extension, it has no bearing on the empircal correlation between protection and partition, or on the validity of the mechanism of the protection.

Therefore, within the context of our experiments there seems to be little doubt that the partition coefficient of ethanol is reduced in red blood cell membranes from ethanol-fed rats. The

magnitude of this reduction may be sufficient to explain the relative resistance of these membranes to the fluidizing effect of ethanol [19,4,12]. We have already demonstrated that these membranes are also resistant to the fluidizing effects of the general anesthetic, halothane, and that halothane partition is reduced, as determined both by direct determination and by a fluorescent quenching assay [21]. Here, we further corroborated these results by showing that the protection of hemolysis by halothane in ethanol-fed rats is reduced by a magnitude similar to the reduction of the partition coefficient. These latter results lend further support to our interpretation of the reduced protection from hemolysis by ethanol as an indication of reduced ethanol partition.

The finding that the reduction of partition is not specific to ethanol but shared by general anesthetics, alcohols and other ampiphilic compounds raises the question of the mechanism underlying the reduced partition. The evidence suggests that the change is located closer to the membrane surface than the membrane core, but the specificity is not pronounced. It is obvious that membrane composition must change in order to bring about a change in the partition coefficients. Indeed, membrane composition does affect the partition of alcohols and other compounds into model membranes [28,29]. This raises two questions: (1) what are the specific changes that are responsible for the reduced partition? and (2) what is the process that leads to these changes? To a large extent, these questions remain unanswered. However, our results provide several suprising clues that may lead to an understanding of the underlying mechanism. The first clue is to be found in the time-course of these changes. The changes developed relatively slowly over a 2-week period of alcohol feeding, but reverse abruptly within 24 h of withdrawal. The quick reversal may indicate that the mechanism of reversal of the reduced ethanol partition is quite different from the mechanism of acquisition of the changed membrane composition (see below for further discussion of this issue).

Membrane cholesterol, serum cholesterol and the partition of alcohols in ethanol-fed rats

The most unexpected findings of this study are the relationships between the serum cholesterol level, the membrane cholesterol/phospholipid ratio and the partition of ethanol in ethanol-fed rats. It is known that ethanol feeding leads to fatty liver and elevated levels of serum triacylglycerols [30]. These are also some of the manifestations of chronic alcoholism and indeed were used as a criterion for the selection of animal models for chronic alcoholism [23]. It has been shown previously that serum cholesterol also is elevated, in both human and some animal models [30]. In rats, these changes appear to be associated with elevation of high-density lipoprotein [31]. Many factors may determine the level of liver and serum cholesterol. Alcohol is metabolized in the liver to acetyl-CoA, which is a precursor of cholesterol biosynthesis. Also, it is quite evident that changes in fat metabolism, sterol metabolism and hormonal regulation of many processes occur in chronic alcoholism [30].

Since the results indicated an identical time-course for changes in serum cholesterol levels and membrane property changes, we first thought that the membrane property changes were due to a serum cholesterol-induced increase in the membrane cholesterol/phospholipid ratio as reported by others [12,14–16] and demonstrated with model membranes [32]. This simple explanation, however, was found to be inadequate in our model. During the development of tolerance, there is only a relatively small increase in cholesterol/phospholipid ratio. Since changes in serum-free cholesterol are very large, it follows that there are changes in membrane lipid composition which reduce the partition coefficient of cholesterol into the membrane. It is known that cholesterol/phospholipid ratios of biological membranes in the same organism can range over the value 0–1.0, despite a constant serum cholesterol level. This variation is apparently determined by the lipid composition of each membrane. Phosphatidylcholine and sphingomyelin appear to increase cholesterol binding, while phosphatidylethanolamine and possibly cardiolipin decrease cholesterol binding. Fatty acid saturation and the location of the double bonds also appear to influence cholesterol binding [32–35].

Therefore, our finding that membrane cholesterol/phospholipid ratio is essentially unchanged,

suggests a hypothetical mechanism for the induced change in membrane lipid composition: the primary effect of ethanol is to elevate the serum cholesterol level; membrane changes are in response to elevated serum cholesterol and are apparently intended to protect the membrane from excess cholesterol [36]; the reduced partition of ethanol and other agents is a secondary consequence of the changed lipid composition.

An alternative explanation is that the changes in membrane lipid composition are a direct response to elevated membrane ethanol serving to protect the membrane from the effects of ethanol [13]. In this scheme, the elevation of serum cholesterol level can be considered a secondary response to the modified membrane composition which may reduce cholesterol partition and is intended to maintain the normal level of the cholesterol/phospholipid ratio. It is not possible, based on our data alone, to make a clear choice between these two alternative explanations; it is also possible that both mechanisms operate simultaneously. However, the fact that cholesterol feeding leads to reduced ethanol partition is more compatible with the first hypothesis. The results of the withdrawal experiments also favor this hypothesis.

The results of these studies suggest the following rates for the various processes associated with ethanol tolerance and dependence. The increase in serum cholesterol following ethanol feeding is slow (about 2 weeks for development of maximum effect), but the decrease is very fast after withdrawal of ethanol (24 h). Changes in phospholipid composition of red blood cells in response to serum cholesterol takes 3–4 days for completion, while the equilibrium partition of cholesterol between the serum and the membrane is very fast (less than 24 h).

In conclusion, the results of this study confirm our suggestion that membrane tolerance of alcohol results from reduced membrane solubility. They also suggest that in the acquisition by red blood cells of membrane alcohol tolerance in ethanol-fed rats, the primary effect may be due to the elevation of serum cholesterol and that the apparent membrane adaptation to ethanol is, in fact, an adaptation to increased serum cholesterol intended to reduce the partition of cholesterol into the membrane. Since there are elaborate control mechanisms for the adaptation of membrane composition to elevated serum cholesterol, the acquired alcohol tolerance and anesthetic cross-tolerance may be an accidental results of the fundamental control process [36,37]. If this interpretation is valid, we expect the reduced partition of ethanol (and the resistance to ethanol effects) to be retained by isolated phospholipids of membranes from ethanol-fed rats, as we observed previously for mitochondrial phospholipids [19]. Finally, it remains to be seen whether these processes are typical of human chronic alcoholism or limited to our specific animal model.

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