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EFFECTS OF ETHANOL AND ACETALDEHYDE ON HEPATIC PLASMA MEMBRANE ATPases

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Abstract—To elucidate possible causes of the hepatocyte swelling and necrosis found in alcoholic liver disease, the effects of ethanol and acetaldehyde on the activities of two hepatic plasma membrane ATPases—(Na^+K^+) ATPase and Mg^{2+} ATPase—were investigated. The activity of another plasma membrane-bound enzyme, 5′ nucleotidase, was also determined to assess the specificity of these effects. Over concentrations ranging from 8 to 90 mM ethanol did not cause significant inhibition of any of the three enzymes. At 120 mM ethanol (Na^+K^+) ATPase activity was inhibited by 20% ($P < 0.01$) and at higher concentrations there was progressive inhibition of all three enzymes that was non-competitive in type. Acetaldehyde produced non-competitive inhibition of (Na^+K^+) ATPase and Mg^{2+} ATPase at concentrations of 6 and 56 mM respectively and 5′ nucleotidase activity was also inhibited at these concentrations. We conclude that ethanol and acetaldehyde inhibit (Na^+K^+) ATPase and Mg^{2+} ATPase activities as part of a generalised effect on the liver plasma membrane. Because the inhibitory concentrations of both substances are higher than are usually found in alcoholic subjects or in experimental animals after alcohol feeding, it seems unlikely that direct suppression of ATPase activity by ethanol or acetaldehyde is responsible for the morphological abnormalities of alcohol-induced liver disease. It could, however, be implicated in the development of hepatocellular necrosis in severe ethanol poisoning.

Plasma membrane ATPases are concerned with transmembrane ion transport and maintenance of the normal intracellular electrolyte and water content. In liver there appear to be two such enzymes: (Na^+K^+) ATPase which transports K^+ into the cell up its electrochemical gradient, causing simultaneous efflux of Na^+ [1], and Mg^{2+}-dependent ATPase which is concerned with calcium and magnesium transport and maintains the normally low intracellular concentration of Ca^{2+} [2, 3]. Because of the stoichiometric relationship of $3Na^+:2K^+$ ions transported via (Na^+K^+) ATPase, inhibition of this enzyme would result in net accumulation of univalent cations in the cell and hence an increase in cell volume. Inhibition of Mg^{2+} ATPase, by reducing the rate of Ca^{2+} efflux would lead to an increase in intracellular Ca^{2+} concentration; the importance of this is emphasised by recent evidence that ionised calcium is a mediator of cell lysis [4].

These ATPases play a central role in maintaining the integrity of the liver cell, and are of interest in the pathogenesis of alcohol-induced liver disease because one of the characteristic early features of this condition is hepatocyte swelling [5, 6], which is often followed by cell necrosis [7]. The increase in cell size is partly caused by accumulation of triglycerides and retention of secretory proteins [8] but a 50% increase in intracellular water content has been demonstrated recently [9]. Inhibition of plasma membrane ATPases by ethanol or acetaldehyde could therefore be responsible for some of these morphological abnormalities. At concentrations of 100 mM and above, ethanol has been shown to inhibit (Na^+K^+) ATPase activity in several tissues including heart [10], brain [11] and intestinal mucosa [12]. In a hepatic plasma membrane preparation a dual response to ethanol was reported, with an increase in (Na^+K^+) ATPase activity at low concentrations (30–80 mM) and inhibition at higher levels [13].

The initial metabolite of ethanol, acetaldehyde, is a far more reactive and toxic substance that its parent compound, and has been implicated in the damage to subcellular organelles that occurs in alcoholic liver disease. It is known to inhibit (Na^+K^+) ATPase activity in brain [14, 15] and myocardium [10], and at much lower concentrations than ethanol. There is no information about its effects on hepatic plasma membrane (Na^+K^+) ATPase activity and the effects of ethanol or acetaldehyde on Mg^{2+} ATPase activity have not been systematically investigated.

Whether the effects on ATPases are specific or are manifestations of a more generalised change in plasma membrane function has also not been established. Ethanol is known to intercalate into membrane lipids and to produce conformational changes in biomembranes with alterations in their physical characteristics [16]; acetaldehyde also produces generalised changes in membrane structure. In the present study we have investigated the effects of ethanol and acetaldehyde *in vitro* on the activities and kinetics properties of (Na^+K^+) ATPase and Mg^{2+} ATPase in purified plasma membrane fractions derived from rat liver. We have also assessed the specificity of these effects by comparing changes in activity of another plasma membrane bound enzyme, 5′ nucleotidase.

MATERIALS AND METHODS

Preparation of liver plasma membrane. Male Wistar albino rats weighing 180–270 g, and allowed free

Table 1. Enzyme specific activities in liver plasma membranes and homogenates

	(Na^+K^+) ATPase	Mg^{2+} ATPase	5′ Nucleotidase	Glucose-6 phosphatase	Succinate dehydrogenate
Plasma membrane preparation	24.7 ± 1.7 $n = 10$	70.8 ± 3.4 $n = 10$	40.2 ± 1.5 $n = 7$	0.39 ± 0.04 $n = 6$	0.0155 ± 0.002 $n = 6$
Homogenate	1.09 ± 0.13	8.06 ± 1.3	1.98 ± 0.4	0.8 ± 0.1	0.0464 ± 0.006
Relative enrichment	26.2 ± 3.7	9.0 ± 1.5	20.6 ± 1.3	0.48 ± 0.14	0.33 ± 0.04

The activities of (Na^+K^+) ATPase and Mg^{2+} ATPase were determined at a substrate concentration of 5 mM ATP, and values (means ± S.E.) are expressed as μmoles Pi/mg protein/hr. 5′ nucleotidase activity was determined with a concentration of 0.2 mM AMP and is expressed as μmoles Pi/mg protein/hr. Glucose-6-phosphatase and succinate dehydrogenase were determined with substrate concentrations of 0.1 M glucose-6-phosphate and 4 mM 2.6 dichlorophenol indophenol and are expressed as μmoles Pi/mg protein/hr and μmoles indophenol reduced/mg protein/hr respectively.

access to standard laboratory chow and water, were the source of liver plasma membrane fractions. Animals were killed, in the non-fasted state, by cervical dislocation and the liver immediately removed and immersed in 0.5 mM $CaCl_2$/1 mM $NaHCO_3$ buffer (pH 7.5) at 4°. The homogenisation was performed as described by Ray [17], the rest of the purification procedure following the aqueous two-phase polymer method of Brunette and Till [18] as modified by Lesko *et al.* [19]. Approximately 4 g of liver was homogenised in 50 ml 0.5 mM $CaCl_2$/1 mM $NaHCO_3$ buffer, and then filtered through fine muslin; the filtrate was centrifuged at 1000 *g* for 30 min at 4°. The pellet was resuspended in buffer, and centrifuged at 1400 *g* for 15 min at 4°; this was repeated twice. The final pellet was suspended in 30 ml top phase of the two-polymer mixture of Dextran 500 (Pharmacia, Sweden) and Polyethyleneglycol 6000 (Sigma Chemical Co., London). Ten milliliter aliquots of this suspension were added to 10 ml of the bottom phase of the polymer mixture which was then centrifuged at 1100 *g* for 15 min at 4°. The plasma membranes were removed from the interface and resuspended in 10 ml of fresh top phase. The procedure was repeated three times. The plasma membranes were washed with 30 ml 50 mM Tris buffer (pH 7.5) and centrifuged at 2400 *g* for 20 min at 4°. The washing procedure was repeated four times and the final pellet was resuspended in Tris buffer.

Enzyme assays. Activities of (Na^+K^+) ATPase and Mg^{2+} ATPase were determined in homogenates and plasma membrane preparations in triplicate by the method of Ismail-Beigi and Edelman [20], with the modification that the reaction was started by addition of substrate (ATP, disodium salt, "vanadium-free"; Sigma Chemical Co., London) instead of plasma membrane. The incubation medium consisted of 25 mM Tris buffer, 5 mM $MgCl_2$, 120 mM NaCl and 12.5 mM KCl (pH 7.4); 100 μl plasma membrane suspension (containing 60 μg protein) was added to give a total volume of 1 ml. After five minutes' incubation at 37°, the reaction was terminated by addition of 0.5 ml 15% (w/v) ice-cold trichloroacetic acid. The inorganic phosphate released during conversion of ATP to ADP was measured by the method of Fiske and Subbarow [21]. Mg^{2+} ATPase activity was determined after addition of 1 mM ouabain (Sigma Chemical Co., London), a specific inhibitor of (Na^+K^+) ATPase, to the reaction mixture. The difference between total and Mg^{2+} ATPase activities represents (Na^+K^+) ATPase activity. (Na^+K^+) ATPase and Mg^{2+} ATPase activities were linear with protein concentrations over a range of 25–150 μg/ml and up to seven minutes' incubation.

5′ nucleotidase activity was measured by the method of Michell and Hawthane [22]; it was linear with protein concentrations up to at least 150 μg/ml for up to 30 min. Activities of glucose-6-phosphatase and succinate dehydrogenase were measured by standard methods [23, 24]. The mean (±S.E.M.) specific activities are shown in Table 1.

Protein concentrations were determined by the method of Lowry *et al.* [25] using bovine serum albumin as standard.

Assessment of purity of plasma membrane preparation. The purity of the plasma membrane fraction was assessed by comparing the relative enrichment (the ratio between specific activity in plasma membrane and homogenate) of the surface membrane enzymes, (Na^+K^+) ATPase (sinusoidal and lateral membranes), Mg^{2+} ATPase (canalicular surface) and 5′ nucleotidase (sinusoidal and canalicular surface) [26–28] to that of glucose-6-phosphatase (a microsomal enzyme) and succinate dehydrogenase (a mitochondrial enzyme). The relative enrichment was 26.2 ± 3.7 for (Na^+K^+) ATPase, 9.0 ± 1.5 for Mg^{2+} ATPase and 20.6 ± 1.3 for 5′ nucleotidase (Table 1), indicating that all hepatocyte surfaces were present. In contrast, relative enrichment for glucose-6-phosphatase and succinate dehydrogenase was 0.48 ± 0.14 and 0.33 ± 0.04 respectively. Thus there was little contamination of the plasma membrane preparation by other intracellular organelles.

Measurement of the kinetic parameters. V_{max} and K_m were determined from the reaction rates (V) obtained with several different substrate concentrations (S) over a range from 0.2 to 5 mM ATP for (Na^+K^+) ATPase and Mg^{2+} ATPase and from 0.022 to 0.2 mM AMP for 5′ nucleotidase. Values for V and S were plotted on a double reciprocal plot and the linear regression equations calculated using least-squares analysis.

Mean V_{max} and K_m for (Na^+K^+) ATPase were 26.6 ± 0.66 μmoles Pi/mg protein/hr and 0.53 ± 0.047 mM ATP respectively. The corresponding par-

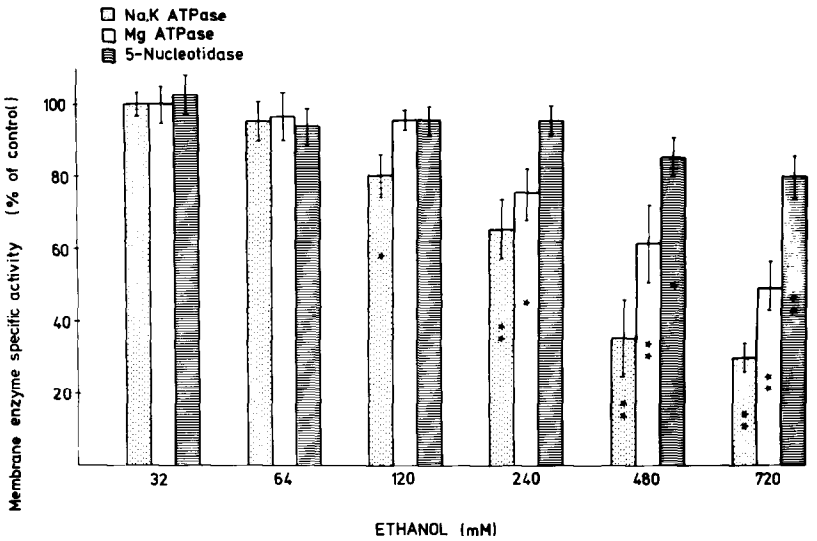

Fig. 1. Effect of ethanol (32–720 mM) on activities of (Na^+K^+) ATPase, Mg^{2+} ATPase and 5′ nucleotidase in purified liver plasma membrane fractions. Enzyme specific activities are expressed as the percentage of the control values and represent the mean ± standard error of 8–10 incubations at each concentration. *P < 0.01 compared with values in control experiments. **P < 0.001 compared with values in control experiments.

ameters for Mg^{2+} ATPase were 71.3 ± 4.3 μmoles Pi/mg protein/hr and 0.52 ± 0.05 mM ATP and for 5′ nucleotidase 29.5 ± 2.2 μmoles Pi/mg protein/hr and 0.030 + 0.002 mM AMP.

Ethanol and acetaldehyde levels in the assay media. Levels of ethanol and acetaldehyde were determined in untreated plasma membrane preparations and also at the end of the enzymatic reaction to ensure that no losses had occurred. One millilitre aliquots of the assay media were mixed with twice the volume of 100 mM chloral hydrate and 10 mM 4-methylpyrazole (to inhibit any aldehyde dehydrogenase and alcohol dehydrogenase respectively) in 0.9% saline contained in screw-capped glass tubes. The samples were deproteinised by addition of 100 μl 60% perchloric acid and 1 ml of 0.1 g/l *n*-propanol was added as internal standard. Ethanol and acetaldehyde concentrations were determined by head-space gas chromatography. After incubation of the tubes at 60° for 30 min, 1 ml of head space was injected on to a 100 m × 0.7 mm capillary column coated with Carbowax 400. The port temperature was 125° and the column was maintained at an oven temperature of 55°. The carrier gas was nitrogen and the flow rate 20 ml/min. Ethanol and acetaldehyde were measured by a flame ionisation detector (at a temperature of 125°) and their concentrations determined by calculating the ratio of their peak heights to that of the *n*-propanol internal standard, and using calibration curves prepared from aqueous standards.

No ethanol or acetaldehyde was detected in untreated plasma membrane preparations (lower limit of sensitivity of assay for ethanol 4 μM and for acetaldehyde 2 μM). No significant losses of ethanol or acetaldehyde occurred in the reaction media. When ethanol was added to the assay media at the beginning of the reaction at concentrations of 16, 32 and 80 mM, mean concentration at the end of the reaction (in three experiments) were 18, 21 and 84 mM respectively. The corresponding levels of acetaldehyde detected were 5, 8 and 31 μM. When acetaldehyde was added at concentrations of 20, 80 and 160 μM at the beginning of the reaction, mean concentrations at the end were 26, 82 and 186 μM respectively.

Statistical analysis. Results are expressed as mean values ± S.E.M. Significance of the differences between groups was assessed by Student's *t*-test.

RESULTS

Effects of ethanol. At concentrations of 8, 16, 32, 64 and 90 mM ethanol there was no significant change in the activity of any of the three enzymes (Fig. 1).

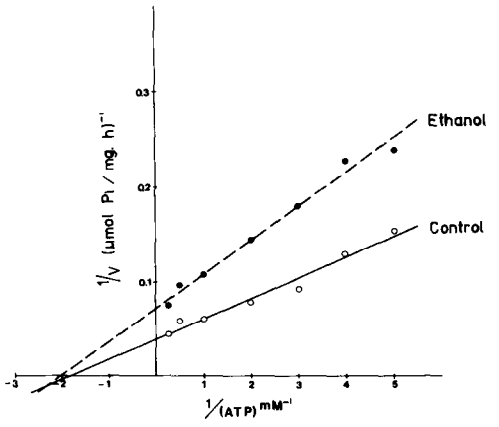

Fig. 2. Effect of ethanol (480 mM) on V_{max} and K_m of (Na^+K^+) ATPase. Results represent the mean of 6 determinations at each substrate concentration.

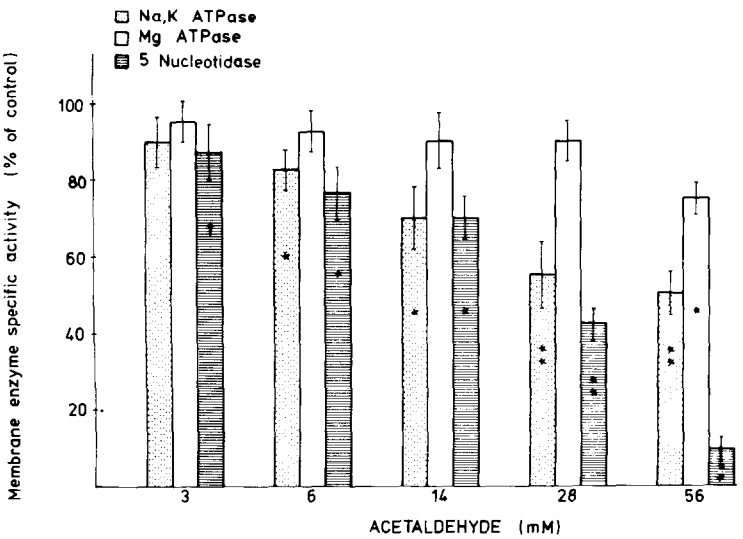

Fig. 3. Effect of acetaldehyde (3–56 mM) on activities on (Na^+K^+) ATPase, Mg^{2+} ATPase and 5′ nucleotidase in purified liver plasma membrane fractions. Enzyme specific activities are expressed as the percentage of the control values and represent the mean ± standard error of 8–10 incubations at each concentration. †$P < 0.05$ compared with values in control experiments. *$P < 0.01$ compared with values in control experiments. **$P < 0.001$ compared with values in control experiments.

At a concentration of 120 mM ethanol (Na^+K^+) ATPase activity was inhibited by 20% ($P < 0.01$). At 240 mM ethanol both (Na^+K^+) ATPase activity and Mg^{2+} ATPase activity were inhibited [by 35% ($P < 0.001$) and 25% ($P < 0.01$) respectively] and at higher concentrations there was progressive inhibition of all three enzymes.

At 480 mM ethanol V_{max} for (Na^+K^+) ATPase was reduced by 49% (Fig. 2) (from 26.6 ± 0.66 to 13.4 ± 0.46 μmoles Pi/mg protein/hr, $P < 0.001$; Fig. 2) and for Mg^{2+} ATPase by 30% (from 71.2 ± 4.3 to 50.2 ± 3.2 μmoles Pi/mg protein/hr, $P < 0.001$). V_{max} for 5′ nucleotidase was reduced by 15% (from 39.5 ± 1.3 to 35.5 ± 1.9 μmoles Pi/mg protein/hr, $P < 0.05$). No significant change in K_m was found for any of the three enzymes (from 0.53 ± 0.047 mM ATP to 0.50 ± 0.030 mM for (Na^+K^+) ATPase; $P > 0.10$; from 0.52 ± 0.05 mM ATP to 0.55 ± 0.042 mM for Mg^{2+} ATPase, $P > 0.10$) and from 0.303 ± 0.0028 to 0.0287 ± 0.031 mM AMP for 5′ nucleotidase $P > 0.10$), indicating a non-competitive type of inhibition.

Effect of acetaldehyde. No significant change in (Na^+K^+) ATPase or Mg^{2+} ATPase activity was observed when acetaldehyde was added to the reaction mixture at concentrations of 10, 20, 40, 80, 120, 240, 480 μM, 1 and 3 mM. At a concentration of 3 mM acetaldehyde, 5′ nucleotidase activity was inhibited by 13% ($P < 0.05$). At 6 mM acetaldehyde (Na^+K^+) ATPase activity was inhibited by 18% ($P < 0.05$) and 5′ nucleotidase by 24% ($P < 0.01$), and at higher concentrations there was progressive inhibition of both enzymes. Mg^{2+} ATPase was the least sensitive enzyme and showed significant inhibition only when the acetaldehyde concentration reached 56 mM.

As for ethanol, an acetaldehyde concentration that significantly inhibited all three enzymes was used to study the effects on V_{max} and K_m. At a concentration of 56 mM, V_{max} for (Na^+K^+) ATPase was reduced by 57% from 26.6 ± 0.66 to 11.5 ± 0.23 μmoles Pi/mg protein/hr ($P < 0.001$); for Mg^{2+} ATPase it was reduced by 20% (from 71.3 ± 4.3 to 55.6 ± 2.7 μmoles Pi/mg protein/hr; $P < 0.01$), and for 5′ nucleotidase by 84% (from 39.5 ± 1.3 to 6.5 ± 1 μmoles Pi/mg protein/hr; $P < 0.001$). No significant change in K_m was found for any enzyme [from 0.53 ± 0.047 mM ATP to 0.54 ± 0.036 mM for (Na^+K^+) ATPase, $P > 0.10$; from 0.52 ± 0.05 mM ATP to 0.58 ± 0.08 mM for Mg^{2+} ATPase, $P > 0.10$; and from 0.0303 ± 0.028 mM AMP to 0.0307 ± 0.003 mM for 5′ nucleotidase; $P > 0.10$] indicating that acetaldehyde also produces a non-competitive type of inhibition.

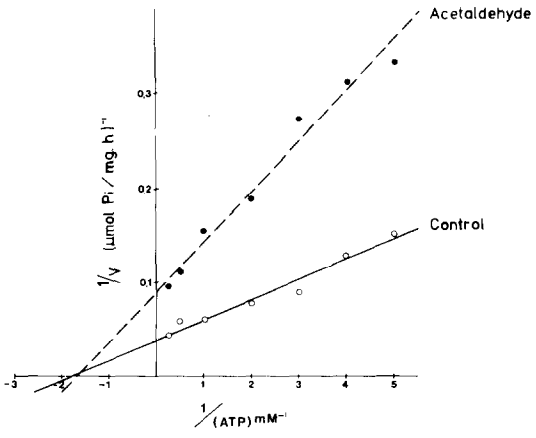

Fig. 4. Effect of acetaldehyde (56 mM) on V_{max} and K_m of (Na^+K^+) ATPase. Results represent the mean of 6 determinations at each substrate concentration.

DISCUSSION

All three enzymes investigated in this purified liver plasma membrane preparation were inhibited non-competitively by ethanol; (Na^+K^+) ATPase was the most sensitive enzyme, showing significant inhibition at an ethanol concentration of 120 mM (550 mg per 100 ml) while Mg^{2+} ATPase was inhibited at twice that concentration. The non-specific nature of the inhibition suggests that ethanol has a generalised effect on plasma membrane function possibly by intercalating into the liquid bilayer and causing conformational changes as a result. Ethanol is known to alter the physical properties of biomembranes [16, 29] and has been shown by spin probes to cause fluidisation at concentrations *in vitro* of 100 mM and above [30]. The differences in sensitivity of the three enzymes to inhibition by ethanol could reflect a difference in their susceptibility to changes in their physical environment—in this respect (Na^+K^+) ATPase which undergoes conformational changes during the process of transporting sodium and potassium ions across the plasma membrane [2] might be relatively more sensitive to alterations in the membrane's physical properties. No enhancement of (Na^+K^+) ATPase activity (or that of any other enzyme) was seen at lower ethanol concentrations in contrast to the findings of Ricci *et al.* [13], who reported a 58% increase at an ethanol concentration of 32 mM. The disparity between these findings is difficult to explain, but enhancement of (Na^+K^+) ATPase activity has not been observed in preparations from other tissues [11, 12, 15].

Acetaldehyde also caused inhibition of all three enzymes but at much lower concentrations, approximately 1/100th those of ethanol. Like ethanol it produced a non-competitive type of inhibition and there was variation in the degree of inhibition produced—(Na^+K^+) ATPase was less sensitive than 5′ nucleotidase while Mg^{2+} ATPase was inhibited only by 56 mM acetaldehyde. In view of the different order of sensitivity of these enzymes to inhibition by acetaldehyde compared with ethanol it is unlikely that the mechanism of action is the same. Acetaldehyde can also intercalate into biomembranes but it is a more chemically reactive compound than ethanol, binding irreversibly to membrane phospholipids and to proteins, forming Schiff's bases with their amino groups [31, 32]. It is possible that covalent binding to the enzymes or a permanent structural alteration of the plasma membrane is responsible, although in a cardiac plasma membrane preparation, acetaldehyde-induced inhibition of (Na^+K^+) ATPase activity was largely reversed by subsequent washing [10].

The sensitivity of hepatic plasma membrane (Na^+K^+) ATPase to inhibition by ethanol or acetaldehyde is similar to that previously reported in preparations from other sites such as brain and cardiac muscle [14–16]. The physiological importance of these effects remains to be established. In man and in experimental animals systemic blood ethanol concentrations of 120 mM (550 mg per 100 ml) and above are accompanied by signs of severe alcohol intoxication and are potentially fatal. Concentrations in the portal vein, which perfuses the liver, may be two or three fold higher than systemic concentrations during absorption of alcohol but this is a transient phenomenon. Acetaldehyde concentrations of 6 mM and above [such as inhibited (Na^+K^+) ATPase] are also higher than can normally be detected during ethanol oxidation. In a group of alcoholics with early liver damage, peripheral venous blood acetaldehyde levels of 10 μM were found following ingestion of 1 ml ethanol per kg body weight (J. B. Saunders, B. R. Ricciardi, C. J. Dickenson and R. Williams, unpublished observations). After oral administration of similar doses to rats, peak blood acetaldehyde levels averaged 25 μM and hepatic levels rarely exceeded 150 μM [33, 34]. Only when aldehyde dehydrogenase is inhibited by a drug such as disulfiram, do blood concentrations approach millimolar levels [35].

The lack of inhibitory effect of acetaldehyde at micromolar concentrations was not due to loss in the reaction mixture by oxidation to acetate: acetaldehyde levels at the end of the incubation period were in close agreement with the calculated initial concentrations. Although acetaldehyde concentrations that can be detected in whole liver preparations during ethanol oxidation do not have a significant effect *in vitro* on plasma membrane enzymes, it is possible that plasma membranes in close proximity to sites of high alcohol dehydrogenase activity or low aldehyde dehydrogenase activity could be exposed to concentrations of acetaldehyde sufficient to cause direct inhibition of enzyme activity. It is also possible that present techniques for assaying acetaldehyde in tissues are not able to detect that bound to membrane constituents or cellular macromolecules and thus could give spuriously low values for the concentrations present.

The effects of ethanol administration *in vivo* on enzyme activities in purified hepatic plasma membrane preparations have recently been described [13, 36] but interpretation of these findings is difficult. In preparations from rats treated with 6 g ethanol/kg body weight per day for four days and then withdrawn from ethanol for 14 hr before sacrifice, (Na^+K^+) ATPase activity was reduced by 40% compared with preparations from rates not exposed to ethanol [13]. However, when rats were kept on ethanol up to the moment of sacrifice (Na^+K^+) ATPase activity was similar to that in control animals. The authors suggested their findings might be explained on the basis of an adaptive response to an increase in (Na^+K^+) ATPase activity that they observed at low concentrations (32–80 mM) of ethanol, but such an interpretation would not be in accord with our findings that ethanol at concentrations below 120 mM did not affect (Na^+K^+) ATPase activity. In another study of plasma membrane bound enzymes [36], administration of ethanol for six weeks resulted in a significant reduction in 5′ nucleotidase activity, an increase in γ-glutamyltransferase activity and no change in alkaline phosphatase activity. The reasons for these differing effects remain to be elucidated. It is possible that they reflect differences in relative sensitivity to the enzyme-inducing and inhibitory effects of ethanol.

The results in the present study suggest that direct inhibition of plasma membrane ATPases by ethanol

or acetaldehyde is unlikely to be responsible for the intracellular swelling and necrosis found in alcohol-induced liver disease. It must however be emphasised that inhibitory concentrations of acetaldehyde may be attained in localised subcellular compartments and that secondary metabolic changes occurring after prolonged ethanol ingestion may increase the sensitivity of plasma membrane enzymes to inhibition by ethanol or acetaldehyde. Further work is required to define the *in vivo* effects of ethanol on ATPase activity and the levels of enzyme activity at various stages of alcohol-induced liver disease. In acute ethanol poisoning when blood ethanol concentrations can reach 200–250 mM, inhibition of these enzymes may well result in hepatocellular ballooning and lysis, as well as causing disturbances in intracellular metabolism resulting from inhibition of solute transport; similar disturbances may also occur in myocardial and neuronal cells.

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