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ETHANOL EFFECTS ON ACTIVE Na^+ and K^+ TRANSPORT IN CULTURED FETAL RAT HEPATOCYTES*

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Abstract—To define further the influence of ethanol on membranes, its effects on Na^+ pump function were studied in monolayer cultures of fetal rat hepatocytes. The effects of ethanol (2 and 4 mg/ml) on total K^+ influx, ouabain-sensitive K^+ influx, Na^+ pump density (from specific [^{3}H]ouabain binding), pump turnover rates and intracellular Na^+ were measured following exposure of the cells to ethanol for 1–24 hr. In parallel studies, the effects of ethanol (2 mg/ml) on cell water content and membrane fluidity were measured. Ethanol had no immediate effect on K^+ influx, but after 1 hr ethanol in concentrations of 2 and 4 mg/ml decreased the total K^+ influx (μmol/10^{11} cells/sec) from a control of 8.5 ± 0.64 to 4.46 ± 0.50 and 4.09 ± 0.26 respectively (N = 6 for each experiment; $P < 0.001$). This represented the maximum effect of ethanol since after 6 and 24 hr of ethanol treatment the K^+ influx had increased towards control levels but remained significantly ($P < 0.01$ for 2 mg/ml and $P < 0.001$ for 4 mg/ml) below that in control cells even at 24 hr. The decrease in K^+ influx reflected a decrease in mean ouabain-sensitive K^+ influx from a control of 5.87 to 3.24 and 2.70 (μmol/10^{11} cells/sec) after a 1-hr treatment with 2 and 4 mg ethanol/ml medium respectively. Ethanol (2 mg/ml) treatment for 1 hr decreased Na^+ pump density ($\times 10^5$ molecules ouabain per cell) from a control of 2.80 ± 0.30 to 1.70 ± 0.11 ($P < 0.001$). At 6 and 24 hr [^{3}H]ouabain binding showed a pattern similar to that seen with the K^+ influx, tending to return to pretreatment levels. There was no change in individual pump turnover rates in the presence of ethanol. Following exposure to ethanol, cellular Na^+ content steadily increased over the first 6 hr and then returned to control levels. When corrected for parallel changes in cell volume, however, intracellular Na^+ concentration increased by 17% ($P < 0.01$) after 1 hr and thereafter remained at this higher level throughout the 24-hr period. Measurements of membrane fluidity showed that it was increased markedly by ethanol at a concentration of 2 mg/ml and that the effect bore a close temporal relationship to the changes in active K^+ influx and Na^+ pump density. We conclude that ethanol has a depressant effect on hepatic Na^+ pump function, resulting in an increase in intracellular Na^+ and an eventual gain in cell water. The results suggest that this is due to a decrease in the number of functional Na^+ pumps, possibly resulting from the solubilizing effect of ethanol on the plasma membrane.

Both acute and, especially, chronic ingestion of alcohol (ethanol) are associated with altered hepatic function. This may be reflected in metabolic [1], structural [2, 3] and ultrastructural changes [3], the former thought to be largely responsible for the production of portal hypertension [4, 5]. A key concept, common to many cells, is an alteration of plasma membrane structure and fluidity [6–9]. This may result in increased cellular permeability and leakage of intracellular molecules to the extracellular space [10, 11]. Changes in membrane permeability [12] could also result in an alteration of intracellular electrolytes following exposure to alcohol. For example, it has been shown in the myocardium of rats fed a diet containing ethanol that Na^+, K^+, Ca^{2+} and Mg^{2+} tend to be displaced down their respective concentration gradients with a resulting increase in intracellular Na^+ and Ca^{2+} and a decrease in cell K^+ and Mg^{2+} [12]. Similar findings have been shown in the liver of rats exposed acutely to alcohol [13].

While often ascribed to a reversible, non-specific increase in membrane permeability [12], these changes may also represent altered active transport of Na^+ via the Na^+ pump. Ethanol has been shown to inhibit ($Na^+ + K^+$)ATPase activity in many tissues *in vitro* [14]. The tissues include liver [15, 16], cardiac sarcolemmal microsomes [17], brain [18], muscle [19], intestine [20], erythrocytes [21] and leukocytes [22]. Most of these studies, however, were carried out using the purified enzyme or membrane fragments, and there is only indirect evidence that such changes occur in intact hepatocytes or *in vivo* [13]. One form of evidence is the demonstration that low doses of ethanol inhibit active $^{86}Rb^+$ influx into rat hepatocytes prepared by enzymatic digestion [16].

The present study was carried out to evaluate further the effects of ethanol on Na^+ pump function in hepatocytes. Fetal rat liver cells were chosen for study since (a) they were available and well-characterized as to purity and function in our unit, (b) they had been shown previously to accumulate water on exposure to ethanol, and (c) they do not metabolize ethanol to acetaldehyde to any degree, permitting an assessment of the effect of ethanol *per se* on the Na^+ pump. The studies were designed to evaluate only the effects of acute exposure (up to 24 hr) of the cells to ethanol in concentrations of 2 and 4 mg/ml. Specifically, the effects of ethanol on pump-mediated fluxes,

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Na^+ pump density, and pump turnover rates were evaluated and correlated with measurements of cell water volume and cell membrane fluidity.

METHODS

Preparation of cultured rat hepatocytes. Primary monolayer cultures of 19-day-old rat fetal hepatocytes were generated as previously described [23]. Hepatocytes were isolated and plated overnight (35 mm Primeria plates, Falcon Plastics, Oxnard, CA) in WE culture medium supplemented with 10% dialyzed fetal calf serum. They were subsequently maintained in defined WE medium (without arginine) supplemented with hydrocortisone sodium succinate (0.3 μg/ml) and epidemal growth factor (2 ng/ml) and refed at 24-hr intervals. Monolayers were grown for 48 hr in defined medium prior to study. Cell counts were determined on a Coulter counter. The resulting monolayers expressed a homogenous parenchymal population arranged in a trabecular architecture with formation of canalicular-like elements. Population doubling times were approximately 30 hr. Kupffer cell contamination was less than 1%. The cells synthesized and secreted albumin, and membrane integrity in both ethanol and control cultures was indicated by exclusion of trypan blue and maintenance of concentrative membrane aminoacid transport capacity [23].

Solutions used. All measurements of ionic content and fluxes were carried out with the cells in a physiologic Balanced Salt Solution (BSS) [24, 25]. This solution, used to minimize the pH changes that would have occurred had growth medium been used, contained (mM): Na^+, 136.8; K^+, 5.35; Ca^{2+}, 2.25; Mg^{2+}, 1.03; Cl^-, 148.22; PO_4^{3+}, 0.43; glucose, 11.1; plus 5% calf serum and 0.0002% phenol red, (pH 7.2). The cells were allowed to equilibrate in this solution for 3 hr after removal of the growth medium before any flux measurements were made.

The ethanol-containing solutions were prepared by adding the desired volume of absolute ethanol, using a micropipette, to either the BSS or growth medium when the latter was necessary for the longer term exposures (24 hr). The ethanol levels in the petri dishes for both the short and long-term (24 hr) ethanol treatments were verified (Stat-Pack Ethyl Alcohol Test, Behring Diagnostics, La Jolla, CA) and were found to remain stable in this system throughout the period of the study.

Measurement of ion fluxes. The methods employed to determine the K^+ influx have been described previously [24–27]. Preliminary studies were carried out to evaluate the characteristics of the K^+ influx in these cells. These preliminary studies showed that the K^+ influx in the monolayers of isolated hepatocytes could be described as a single exponential and that the cells were half-loaded with $^{42}K^+$ in 15 min. This is very similar to the kinetics of K^+ influx previously described for cultured neonatal rat myocytes [24, 25]. Therefore, influx measurements were made by exposing the cells to $^{42}K^+$ for a period of time (2 min) shorter than the half-time of K^+ uptake. Following exposure for 2 min to $^{42}K^+$, the cells were washed free of extracellular tracer using an isotonic Na^+- and K^+-free Ca^{2+}–sorbitol solution [24] at 0°. The cellular content of $^{42}K^+$ was then determined and the influx, expressed as micromoles per 10^{11} cells per second was calculated as previously described [24] using the equation of Keynes and Lewis [28].

The Na^+ and K^+ contents of the cells were determined by equilibration with $^{24}Na^+$ and $^{42}K^+$ respectively. Following equilibration, the cells were washed free of extracellular tracer, as described above, and the contents were calculated as before [24]. For the majority of experiments, the cell ion content was expressed as micromoles per 10^9 cells, but in those experiments in which the volume of cell water was measured, it was possible to express these results as millimoles per liter of cell water. All ion influx and content experiments were carried out at 37°.

Determination of [^{3}H]ouabain binding. The number of Na^+ pump sites per cell, under various experimental conditions, was measured using [^{3}H]ouabain binding [24]. [^{3}H]Ouabain (New England Nuclear, Waltham, MA) was used to prepare a stock solution of 10^{-6} M [^{3}H]ouabain in BSS from which serial dilutions were made to achieve the desired final concentrations. Ouabain uptake was measured by exposing the cells to [^{3}H]ouabain, washing the cells free of extracellular, unbound, isotope and measuring the amount bound by liquid scintillation spectrometry. The amount bound (molecules of [^{3}H]ouabain per cell) was then determined from the cell number, sample counts, and the specific activity of the soak solution.

Preliminary studies were carried out to determine the kinetics of [^{3}H]ouabain binding in the cultured hepatocytes. Duplicate determinations of [^{3}H]ouabain binding were made in the presence and absence of 20 mM K^+ to provide separation of specific from non-specific binding [24], using [^{3}H]ouabain in concentrations from 10^{-9} to 10^{-6} M. These preliminary studies indicated that maximum specific binding in these cells occurred in the presence of 5×10^{-7} M [^{3}H]ouabain in a K^+-free solution. Under these conditions, specific binding accounted for 70% of total [^{3}H]ouabain binding. The preliminary studies also demonstrated that the binding of [^{3}H]ouabain to the cells was rapid (half-time 10 min) and had reached saturation by 30 min. The preliminary studies also demonstrated that the presence of ethanol in concentrations of either 2 or 4 mg/ml did not affect the kinetics of glycoside binding to the cells in this preparation. Based on these preliminary data, maximum specific ouabain binding was determined in control and treated cells by exposing the cells to 5×10^{-7} M [^{3}H]ouabain in a K^+-free solution for 30 min. Where the effect of ethanol on the ouabain binding was being measured, ethanol was included in the [^{3}H]ouabain solution.

Determination of cell number and cell volume. In each experiment randomly selected plates of cells were taken from each treatment group for determination of cell number. The plates for cell counting [23] were treated exactly as those used for other aspects of the experiments.

Hepatocyte water content was determined as previously described [23]. Basically, intracellular water space was defined as the cellular volume occupied by 3-*O*-methyl-D-glucose following incubation to steady state [24, 29]. Total cell volumes were approximated

using a ZM Coulter counter.

Determination of membrane fluidity. For determination of basal membrane fluidity (no ethanol present during measurement), hepatocytes were grown as described above except that the monolayer was on glass coverslips in 60 mm culture dishes. Prior to determinations, the coverslips were incubated for 10 min at 25° in oxygenated Earle's Balanced Salt Solution (EBSS) containing 5 μM TMA-DPH [1-(4-trimethylammonium phenyl-1,3,5-hexatriene)] *p*-tolicenesulfonate (Molecular Probes, Eugene, OR). This cationic derivative of DPH incorporates into the outer hydrophobic region of the outer leaflet of the plasma bilayer, near the aqueous-membrane interface with some incorporation into more fluid intracellular membranes [30, 31]. Readings were made at 25° with the coverslip mounted in a cuvette, held perpendicular to the horizontal plane, and inclined 22° to the incident beam of polarized excitation light. The monolayer was oriented at a 68° angle to the direction of light flow of detected emitted light, the latter monitored at a 90° angle from the excitation source. Fluorescence was measured with a Farrad MK-2 spectrofluorometer (Farrad Corp., Valhalla, NY) equipped with excitation and emission monochromators and polarizers. Excitation and emission wavelengths were 440 and 365 nm, respectively.

Fluorescence intensity was first measured with the emission polarizer parallel ($I_{\parallel}$), then perpendicular ($I_{\perp}$), to the orientation plane of the exciter polarizer (0°). The exciter polarizer was then set at 90°, and readings were taken with emission polarizer initially parallel ($I_{\parallel}$), then perpendicular ($I_{\perp}$), to the new exciter polarizer orientation. Blank values were obtained with the complete system minus (a) probe, (b) cells and (c) probe and cells. The sum of the blanks, for a particular sample, was subtracted from each set of experimental readings. These corrected values were used for calculation of fluorescence anisotropy (*r*).

Materials. $^{24}Na^+$ (1.9 mCi/mg), $^{42}K^+$ (0.2 mCi/mg), and [^{3}H]ouabain (20 Ci/mmol) were purchased from New England Nuclear. Biologicals were from Gibco Laboratories, Grand Island, NY; all chemicals (from the Sigma Chemical Co., St Louis, MO) were of analytical grade.

Statistical analysis. Student's *t*-test for paired and unpaired data were used to test for the significance of differences between groups. Linear regression analyses were done by least-squares fit. All results are expressed as mean ± SE.

RESULTS

Effect of ethanol on K^+ influx. In untreated cells, the K^+ influx (μmol/10^{11} cells/sec) was 8.5 ± 0.64 (mean ± SE, N = 6). Of this total K^+ influx (Fig. 1), the Na^+ pump dependent, or ouabain-inhibitable, portion amounted to 5.87 ± 0.89 μmol/10^{11} cells/sec or some 70% of the total influx. The proportion of the K^+ influx accounted for by the ouabain-sensitive or Na^+ pump dependent component in these cells is, therefore, very similar to that in a variety of other tissues.

Applied acutely to the cells, ethanol in concentrations of 2 and 4 mg/ml had no effect on either the total K^+ influx or the ouabain-sensitive component of that flux (Fig. 1). On the other hand, cells that had been exposed to ethanol (2 and 4 mg/ml) for 1 hr showed a significant reduction in both the total and the ouabain-sensitive K^+ influx (Fig. 1). Cells treated with an ethanol concentration of 2 mg/ml for 1 hr showed a reduction in the K^+ influx (μmol/10^{11} cells/sec) from a control of 8.5 ± 0.64 to 4.46 ± 0.50 ($P < 0.001$; for each N = 6). In cells treated with ethanol at 4 mg/ml for 1 hr, the total K^+ influx was 4.09 ± 0.26 μmol/10^{11} cells/sec, again significantly ($P < 0.001$) less than in controls.

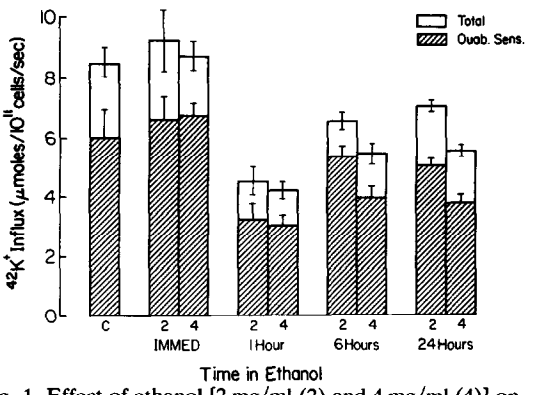

Fig. 1. Effect of ethanol [2 mg/ml (2) and 4 mg/ml (4)] on $^{42}K^+$ influx in cultured hepatocytes. Columns show total $^{42}K^+$ influx and bars represent ±1 SE (N = 6). Shaded portions show ouabain-sensitive influx obtained by subtracting ouabain-insensitive influx from total influx.

With continued exposure to ethanol for either 6 or 24 hr, there was a tendency for the K^+ influx to return toward control values (Fig. 1). After 6 hr of treatment, the K^+ influx (μmol/10^{11} cells/sec) in cells treated with an ethanol concentration of 2 mg/ml was 6.66 ± 0.21 ($P < 0.001$ vs control) and in those cells treated with an ethanol concentration of 4 mg/ml the K^+ influx was 5.74 ± 0.37 ($P < 0.001$ vs control). After 24 hr of treatment, it appeared that the total K^+ influx had returned even further toward control values, (Fig. 1), but the total values at 24 hr were not significantly different from the values at 6 hr for either the 2 mg/ml group or the 4 mg/ml group. At 24 hr, the K^+ influx in those cells exposed to 2 mg ethanol/ml was 7.09 ± 0.15 μmol/10^{11} cells/sec ($P < 0.01$ vs control), and in those cells exposed to 4 mg ethanol/ml a value of 5.57 ± 0.20 was obtained ($P < 0.001$ vs control). These results would indicate that, although ethanol does not produce an immediate decrease in the K^+ influx in cultured hepatocytes, there is a significant depression of this active transport system after 1 hr of treatment and that the effect is sustained throughout a 24-hr period. It would appear, however, that there is some tendency for the active transport to recover to near control levels at the end of the 24-hr treatment period (Fig. 1).

Further characterization of the effects of ethanol on the $^{42}K^+$ influx was obtained from paired measurements of $^{42}K^+$ influx in the presence and absence of 10^{-2} M ouabain which, in preliminary experiments, had been shown to produce maximum inhibition of the K^+ influx. As shown in Fig. 1, the decline in the

$^{42}K^+$ influx in the presence of ethanol, in absolute terms, principally reflected a decrease in the active or ouabain-sensitive component of that flux. In control cells, the ouabain-sensitive K^+ influx measured 5.87 ± 0.89 μmol/10^{11} cells/sec which declined to 3.24 ± 0.47 and 2.70 ± 0.25 after a 1-hr ethanol treatment of 2 and 4 mg/ml, respectively. In those cells treated with 2 mg ethanol/ml the ouabain-sensitive K^+ influx remained considerably below the control levels at 6 and 24 hr measuring 4.87 ± 0.26 and 4.58 ± 0.11 respectively. A similar pattern of inhibition of the ouabain-sensitive influx was noted in those cells treated with 4 mg/ml for either 6 or 24 hr (Fig. 1).

Values for the depression of the K^+ influx produced by ethanol at concentrations of either 2 or 4 mg/ml were very similar at 1 hr, there being no significant difference in the measured K^+ influx between groups at that time (Fig. 1). After 6 hr of treatment, however, the K^+ influx in the presence of 4 mg ethanol/ml was significantly ($P < 0.01$) below that recorded in the presence of 2 mg/ml. Similarly, at 24 hr the K^+ influx measured in the presence of 4 mg ethanol/ml was significantly lower than that in the presence of 2 mg/ml ($P < 0.001$).

It should also be noted that in the presence of ethanol there was a decrease in the ouabain-insensitive component of the K^+ influx. Although, in absolute terms, this was not the major contributor to the decrease in K^+ influx, the percent reduction in the ouabain-insensitive K^+ influx was similar to that seen in the ouabain-sensitive component. This effect on the ouabain-insensitive K^+ influx did reach levels of significance ($P < 0.01$) at 1, 6 and 24 hr in the presence of ethanol at concentrations of both 2 and 4 mg/ml.

Effect of ethanol on [^{3}H]ouabain binding. To characterize further the effects of ethanol on Na^+ pump dependent K^+ fluxes, studies were carried out to define the maximum specific ouabain binding in control and ethanol-treated cells. This was done to permit calculation of Na^+ pump density (Na^+ pump sites per cell) and individual Na^+ pump turnover rates.

Ethanol, in the concentration tested (2 mg/ml), did not affect the kinetics of [^{3}H]ouabain binding. In control and in ethanol-treated cells, [^{3}H]ouabain binding was rapid ($T_{\frac{1}{2}} = 10$ min) and in each case had reached saturation within 30 min. Preliminary studies (see Methods) had shown that maximum specific binding occurred in the presence of 5×10^{-7} M [^{3}H]ouabain in a K^+-free solution.

Maximum specific binding in control cells was reproducible. For week 1 (Fig. 2), maximum specific binding, in terms of molecules [^{3}H]ouabain per cell, was $3.26 \pm 0.31 \times 10^5$ molecules per cell. When the measurement was repeated 1 week later (Fig. 2), a value of $2.8 \pm 0.3 \times 10^5$ molecules per cell was obtained. These values were not significantly different from each other. Treatment with ethanol at 2 mg/ml for 1, 3, 6 and 24 hr resulted in a significant decrease in maximum specific [^{3}H] ouabain binding per cell. After a 1 hr treatment, total specific [^{3}H]ouabain binding ($\times 10^5$ molecules per cell) had decreased to 1.7 ± 0.11 ($P < 0.001$ vs control), and after 3 hr the value was 1.69 ± 0.37 ($P < 0.001$ vs control). After 6 hr, however, as in the case of the K^+ influx, there appeared to be some recovery, and specific

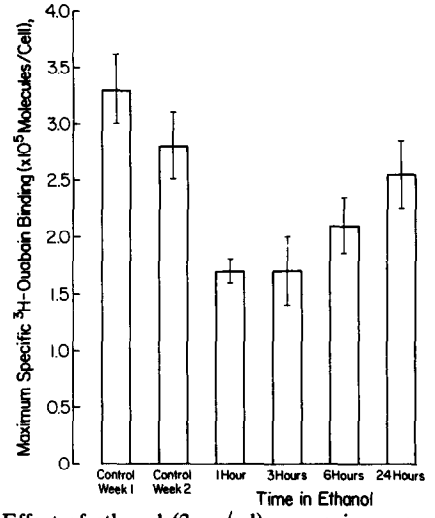

Fig. 2. Effect of ethanol (2 mg/ml) on maximum specific [^{3}H]ouabain binding in cultured hepatocytes. The value for control week 1 was obtained during the preliminary studies to determine the kinetics of ouabain binding on the cells. The value for control week 2 (the week during which the ethanol studies were carried out) was not significantly different, showing the reproducibility of the method. For each column, N = 6 and bars represent ±1 SE.

[^{3}H]ouabain binding had increased to 2.15 ± 0.25 ($P < 0.01$ vs control), and at 24 hr a value of 2.56 ± 0.5 (P = NS vs control) was obtained. The value at 24 hr was markedly higher than the values obtained at 1 and 3 hr of ethanol treatment.

Effect of ethanol on Na^+ pump turnover rate. From the ouabain-sensitive K^+ fluxes and the maximum specific [^{3}H]ouabain binding, it was possible to calculate individual Na^+ pump turnover rates, on the assumption that each Na^+ pump bound 1 molecule of [^{3}H]ouabain. The number of Na^+ pump sites per cell would therefore be represented by the number of molecules of [^{3}H]ouabain bound per cell. It was also assumed that each pump cycle transported 2 molecules of K^+ into the cell, coupled with the extrusion of 3 molecules of Na^+ [21, 24].

In untreated cells, the Na^+ pump turnover rate was calculated at 60 per sec, a value very similar to that reported in a variety of other tissues [24, 27]. Treatment with ethanol in a concentration of 2 mg/ml for up to 24 hr, although associated with a decrease in active K^+ influx, was not associated with any change in Na^+ pump turnover rate. This would suggest that the decrease in the active or ouabain-sensitive K^+ influx or in Na^+ pump activity may be due to a decrease in the number of available functioning Na^+ pumps per cell and not to any alteration in the individual Na^+ pump kinetics. This argument is based entirely on the initial assumption that each Na^+ pump binds only 1 molecule of [^{3}H]ouabain and that this relationship holds true for all conditions.

Effect of ethanol on intracellular Na^+. In view of the apparent inhibitory effect of ethanol on the active K^+ influx and the fact that it appears to decrease the number of available Na^+ pump sites per cell (as measured by specific [^{3}H]ouabain binding), it was felt that the effects on intracellular Na^+ concentration should be

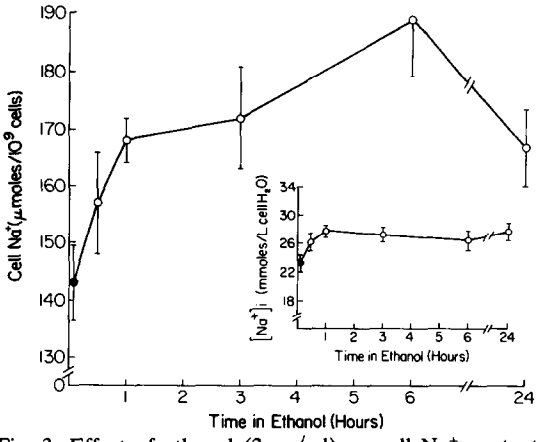

Fig. 3. Effect of ethanol (2 mg/ml) on cell Na^+ content (μmol/10^9 cells). Points shown are the mean ± 1 SE of six observations. Inset shows intracellular Na^+ concentration (mmol/liter cell H_2O) when Na^+ content is corrected for changes in intracellular water.

examined. This would provide confirmatory evidence of the inhibitory effect of ethanol on active transport via the Na^+ pump and permit an evaluation of whether or not ethanol uncoupled active Na^+–K^+ transport.

Measurements of intracellular Na^+ concentration using $^{24}Na^+$ as a tracer were made in cells exposed to ethanol (2 mg/ml) for up to 24 hr. In the absence of ethanol, the Na^+ content of the cells (μmol/10^9 cells) was 142.47 ± 6.38 (mean ± SE; N = 6). After 30 min of treatment with ethanol, this increased to 156.88 ± 8.97 (P = NS vs control). The rise in intracellular Na^+ (Fig. 3) continued with time, and values of 168.34 ± 3.97, 172.83 ± 19.71 and 189.32 ± 10.35 were obtained at 1, 3 and 6 hr, respectively (for each, $P < 0.001$ vs control). By 24 hr, however, intracellular Na^+ had dropped from a high at 6 hr of 189.32 ± 10.35 to 167.34 ± 6.97 which although less than the highest level measured was still significantly ($P < 0.001$) greater than control (Fig. 3). Parallel experiments confirmed previous studies [23] showing a significant ($P < 0.05$) increase in cell water (μl/10^6 cells) following a 24-hr exposure to a 2 mg/ml concentration of ethanol [5.69 ± 0.24 (ethanol) vs 4.87 ± 0.10 (control)] with no detectable increase in cell water with 6 hr or less of treatment. Using these values, cytosolic Na^+ concentration was calculated (Fig. 3 inset). This indicated a 17% increase ($P < 0.05$) in the latter by 1 hr of exposure, after which no further change in Na^+ concentration occurred.

These results confirm that ethanol inhibits coupled active Na^+ and K^+ transport, the decrease in active transport being associated with a progressive increase in intracellular Na^+ concentration. Although for many of the studies it was not possible to express the intracellular Na^+ concentration as millimoles per liter of cell water, in those studies in which the volume of cell water was measured the control value for intracellular Na^+ (mmol/liter cell water) was 23.66 ± 1.13 (mean ± SE), a value very similar to that obtained in a variety of other mammalian preparations.

Effect of ethanol on membrane fluidity. The inclusion of ethanol (2 mg/ml) into the cell growth medium resulted initially in a significant decrease in the basal fluorescence anisotropy values of the TMA-DPH probe incorporated into the plasma membranes of rat fetal hepatocytes (Fig. 4). Basal values of r, determined without ethanol being present at the time of fluidity measurement, were decreased significantly ($P < 0.05$) at 3–4 hr, and similarly at 6–7 hr, after ethanol addition. Control cells were paired to the ethanol-exposed cells within the same day's experiment. The fluorescence anisotropy value for TMA-DPH in plasma membranes of all control cells, measured at 25°, was 0.2596 ± 0.0017 (mean ± SE, N = 140). Values obtained for r in ethanol-exposed cells were decreased by 5.0 and 4.5%, at the 3–4 hr, and at the 6–7 hr, time points, respectively, after ethanol addition (Fig. 4). This effect of ethanol on basal membrane fluidity values was seen only with cells that had been exposed to ethanol for less than 8 hr. The effect was lost thereafter, with fluorescence anisotropy values at 8–10 hr, 12–15 hr, and 26–32 hr after ethanol addition not differing ($P > 0.05$) from the corresponding controls.

DISCUSSION

While there are many studies that have shown that ethanol decreases ($Na^+ + K^+$)-ATPase activity in a wide variety of tissues [14], including liver [15, 16], this study represents one of only a few studies that specifically address the issue of the effect of ethanol on Na^+ pump function in intact hepatocytes. The concentrations of ethanol tested (2 and 4 mg/ml) were selected because they correspond to blood alcohol levels associated with moderate to severe inebriation. The model of cultured fetal hepatocytes was selected because many of their basic characteristics are similar to those of other hepatic preparations [23, 32], and they offer all of the advantages of cultured cells for ion flux determinations. Further, preliminary studies demonstrated that active K^+ transport in these cells was very similar to that in other mammalian cells. This study was designed to examine only the effects of acute (<24 hr) ethanol on Na^+ pump function.

There is considerable evidence, which has been elegantly reviewed by Roach [14], that ethanol inhibits ($Na^+ + K^+$)-ATPase in a wide variety of membrane preparations, *in vitro*. Much of this work has examined brain and nerve tissue in an attempt to define the CNS effects of alcohol ingestion. In microsomal preparations from rat cerebral cortex, ethanol inhibited ($Na^+ + K^+$)-ATPase [33] which was later correlated with a decrease in the indirectly measured K^+ transport in the same preparation [34]. With few exceptions [35], other workers [36] have confirmed the inhibitory effect of ethanol on brain-derived ($Na^+ + K^+$)-ATPase.

By contrast, studies of the effect of ethanol on hepatic ($Na^+ + K^+$)-ATPase have yielded conflicting results. Several years ago it was noted [37] that there is increased oxygen consumption in liver slices obtained from rats fed ethanol for 21–27 days. Further studies [37] showed that the increased O_2 consumption was explained by an increase in ($Na^+ + K^+$)-ATPase activity in the preparation and there was increased active transport of $^{86}Rb^+$ in the livers of the ethanol-fed rats [37]. Subsequently, an increase in intracellular

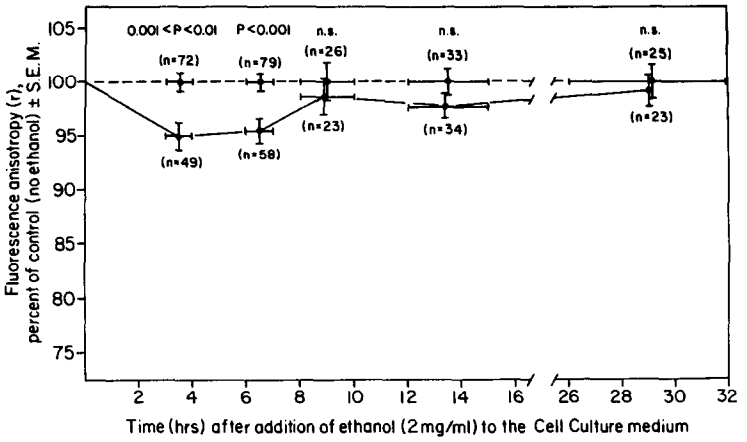

Fig. 4. Effect of ethanol (2 mg/ml) on membrane fluidity of cultured hepatocytes as measured by fluorescence anisotropy. Points are mean ± 1 SE of the number of observations shown in parentheses.

K^+ in the livers of ethanol-fed rats appeared to confirm the stimulatory effect of chronic ethanol on hepatic ($Na^+ + K^+$)-ATPase activity [4, 5]. It was felt that the increased intracellular K^+, resulting from enhanced Na^+ pump activity, osmotically accounted for 40–50% of the hepatocyte swelling seen with chronic ethanol consumption [4, 5]. These studies [4, 5, 37], however, are not strictly comparable to ours, since they deal with chronic ethanol treatment. They are consistent with increased ($Na^+ + K^+$)-ATPase activity seen following chronic ethanol treatment in a wide variety of tissues [14].

Studies that attempt to define the effects of acute ethanol exposure on hepatic Na^+ pump function have also yielded varying results. In rats given ethanol 1 hr prior to being killed there was an increase in hepatic ($Na^+ + K^+$)-ATPase activity to 138% of control at a serum alcohol level of 35.4 mM [15]. Similarly, when 10 to 35 mM ethanol concentrations were added to hepatic membrane preparations *in vitro*, there was a progressive, dose-dependent increase in ($Na^+ + K^+$)-ATPase activity [15]. Further increases in ethanol concentrations towards 80 mM resulted in a decrease in ATPase activity [15] but at none of the concentrations tested was the activity less than in control preparations. On the other hand, both ethanol and acetaldehyde inhibit the ($Na^+ + K^+$)-ATPase activity in hepatic plasma membrane preparations *in vitro* [38]. However, high concentrations ($\geq$120 mM) of ethanol were required to produce measurable inhibition of the enzyme system [38]. This is in sharp contrast to studies [16] which showed that up to 60% of the active $^{86}Rb^+$ influx in isolated adult rat hepatocytes was inhibited by low concentration of ethanol, and the IC_{50} of ethanol on the ouabain-sensitive $^{86}Rb^+$ influx was around 1.4 mM. The present study provides evidence of inhibition of Na^+ pump function in hepatocytes by ethanol in concentrations between 43 and 86 mM. The present studies, therefore, confirm the work of others [14] showing that, acutely, ethanol produces a decrease in the active Na^+ and K^+ transport system. This inhibitory effect of ethanol on active K^+ transport did not occur immediately upon addition of ethanol to the cells, but appeared to be fully developed after 1 hr of ethanol treatment. This time-dependence is very similar to that seen in cultured myocardial cells [27], and suggests that the decrease in K^+ influx is not due to simple competition between ethanol and K^+, since even the highest concentration of ethanol tested had no immediate effect on the cells. The increase in intracellular Na^+ seen in the ethanol-treated cells, suggesting a parallel decrease in Na^+ efflux, further supports Na^+ pump inhibition rather than ethanol:K^+ competition.

The reason for the slow onset of the inhibition by ethanol is unclear. It is possible that the effect may be mediated by metabolites of ethanol such as fatty acid ethyl esters [39] which can compromise integrity of the phospholipid bilayer of the cell membrane. Alternatively, it has been shown that the assembly of glycoproteins into hepatic plasma membranes is inhibited by ethanol [9] and, therefore, the inhibitory effect of ethanol on active transport may represent impaired assembly of the component proteins of the transport system. Finally, decreased active transport may result from decreased cellular ATP, but this has usually only been seen following chronic ethanol administration [37]. It is uncertain whether the time-courses of ethanol-induced Na^+ pump inhibition in these cells and in cultured myocardial cells [27] are different from those in other tissues, since in those instances [33, 34, 36] ethanol was present in the reaction mixture for at least 20–30 min. In only one study [35] was the effect of ethanol on microsomal ($Na^+ + K^+$)-ATPase activity measured over a period of less than 15 min, and no inhibitory effect of ethanol on ($Na^+ + K^+$)-ATPase activity was seen. Therefore, although no direct comparisons can be made of the inhibitory effect of ethanol on active transport in this tissue with that in others, indirect evidence suggests that it may be a time-dependent function.

Whatever mechanism is involved, the present study shows a close temporal relationship between membrane fluidity and the decrease in both active transport of K^+ and of Na^+ pump density, as measured by [^{3}H]ouabain binding. Previous studies [40, 41] have shown that hepatic ($Na^+ + K^+$)-ATPase activity is sensitive to changes in membrane fluidity, the activity

decreasing as membrane fluidity increases. This study confirms at least a temporal relationship between increasing membrane fluidity and decreased active K^+ transport in the presence of ethanol. Although it is well accepted that acutely administered ethanol increases membrane fluidity [6], it has also been shown that, with chronic exposure to ethanol, membrane fluidity returns toward normal [42–44]. The restoration of normal membrane fluidity is due apparently [42–44] to compensatory restructuring of the lipid components of the membrane, and, at least in nerve tissue [42, 43], is thought to contribute to the development of tolerance to ethanol. It is of interest, in the present study, that the return of normal membrane fluidity in the continued presence of alcohol bears a close temporal relationship to the recovery of active transport.

In the present study, it was noted that both concentrations of ethanol, in addition to inhibiting active K^+ influx, produced a significant reduction in the ouabain-insensitive influx of that ion. Similar findings have been reported in human leucocytes and erythrocytes [22] and cultured myocardial cells [27], although in these studies [22, 27] much higher concentrations (>160 mM) of ethanol were required to produce a significant effect. The reasons for this effect of ethanol on passive K^+ influx are unclear. The effect may be due to a decrease in passive exchange diffusion of the ion about a semipermeable membrane which is known to be dependent on the concentrations of the ion on either side of the membrane [45]. With inhibition of the active K^+ influx by ethanol, intracellular K^+ will be decreased and the gradient favoring exchange diffusion of K^+ will be decreased [45]. Inhibition of the Na^+ pump by ethanol, resulting in changes in intracellular Na^+ and K^+ concentrations, may affect the membrane potential, which in turn may influence passive K^+ influx. It should be pointed out, however, that although significant, the changes in passive K^+ influx are very small and unlikely to be of clinical relevance.

From the [^{3}H]ouabain binding studies it appears that the decreased active transport in the presence of ethanol was due to a decrease in the number of available pump sites per cell. The decreased specific [^{3}H]ouabain binding seen in the presence of ethanol could represent competitive inhibition of glycoside binding by ethanol. However, in confirmatory studies, it was shown that no further increase in specific binding occurred, in the presence of ethanol, at a concentration of 10^{-5} M [^{3}H]ouabain. The decrease in functional Na^+ pump sites, therefore, appears to be real and similar to the finding in cultured myocardial cells [27]. The reason for this decrease in Na^+ pump density remains unclear. It may reflect loss of functioning pump sites due to the solubilizing effect of ethanol on the plasma membrane. On the other hand, there may be impaired assembly of the constituent proteins [9] for ion pumps. Regardless of the mechanism, the decrease in Na^+ pump density is sufficient to account for the decreased active K^+ transport in ethanol-treated cells.

Previous studies [2, 4, 5] have suggested that at least part of alcohol-induced hepatocyte enlargement is due to an increase in intracellular water osmotically induced by an accumulation of intracellular K^+. It should be pointed out, however, that these studies [2, 4, 5] dealt with chronic ethanol administration which appears to stimulate rather than inhibit Na^+ pump function. The present studies indicate that acutely administered ethanol results in an accumulation of intracellular Na^+ rather than K^+. In myocardial cells [27] the increase in intracellular Na^+ is closely related to cell swelling [27] or accumulation of intracellular water, and this appears to be the case in the present study. These results, therefore,suggest that the hepatocyte enlargement due to acute alcoholic injury is more likely due to increases in intracellular Na^+, because of Na^+ pump inhibition.

In conclusion, therefore, ethanol in clinically relevant concentrations suppressed active transport by the Na^+ pump in cultured rat hepatocytes. The effect was time dependent and appeared to be mediated by a decrease in the density of Na^+ pump in the plasma membrane which, in turn, appeared to be related to changes in membrane fluidity. The observation that concentrations of ethanol encountered during acute alcoholic intoxication acutely depressed Na^+ pump function in hepatocytes may have considerable clinical relevance. Ethanol-induced decreases in active transport with concomitant changes in intracellular Na^+ and K^+ may be contributory factors to cell swelling and disordered hepatic metabolism seen in acute alcoholic intoxication.

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