Biochimica et Biophysica Acta, 732 (1983) 256–265
Elsevier

BBA 71718

EFFECTS OF ETHANOL IN VITRO ON RAT INTESTINAL BRUSH-BORDER MEMBRANES

CAROLEE K. HUNTER [a], L. LOCKIE TREANOR [a], J. PATRICK GRAY [a], SUSAN A. HALTER [b], ANASTACIO HOYUMPA, Jr. [a] and FREDERICK A. WILSON [a,*]

Departments of [a] Medicine and [b] Pathology, Vanderbilt University School of Medicine, Nashville, TN 37232 (U.S.A.)

(Received December 15th, 1982)
(Revised manuscript received March 8th, 1983)

Key words: Ethanol; Glucose transport; Taurocholic acid, Membrane fluidity; EPR; Morphometric analysis; (Rat intestine)

Ethanol, at concentrations found in the intestinal lumen after moderate drinking, has been shown to inhibit carrier-mediated intestinal transport processes. This inhibition could occur by direct interaction with membrane transporters, dissipation of the energy producing Na^+ electrochemical gradient and/or non-specific alteration of membrane integrity. The latter alteration may be reflected by changes in membrane fluidity, chemical composition or vesicular size. These possibilities were examined with studies in purified brush border membrane vesicles of rat intestine. Ethanol inhibited concentrative Na^+-dependent D-glucose uptake in a dose-dependent manner. In contrast, ethanol did not inhibit concentrative D-glucose uptake under conditions of D-glucose trans-stimulation in the absence of a Na^+ electrochemical gradient. Ethanol also inhibited initial, concentrative Na^+-dependent taurocholic acid uptake, as well as equilibrium uptake. That ethanol exerted a dual effect on transport by increasing membrane conductance for Na^+ while decreasing intravesicular space was supported by direct studies of Na^+ uptake. Morphometric analysis confirmed that ethanol-treated membranes had a decreased intravesicular size when compared to untreated membranes. Finally, membrane fluidity measured by EPR showed that ethanol had a significant fluidizing effect without producing qualitative changes in membrane proteins, as determined by SDS gel electrophoresis. These results suggest that ethanol inhibits carrier-mediated transport by dissipation of the Na^+ electrochemical gradient and alteration of membrane integrity rather than by direct interaction with membrane transporters.

Introduction

Ethanol administration has been shown to decrease the intestinal absorption of substances that require a carrier-mediated process. Evidence is accumulating to indicate that ethanol exerts its effects by altering membrane properties through a nonspecific physical interaction rather than specific drug-membrane receptor interaction [1]. The intestinal microvillous (brush border) membrane is the site of Na^+-coupled active transport systems and is the component of the enterocyte that receives the greatest exposure to ethanol. Purified brush-border membrane vesicles have been found to be particularly suitable for studying the effects of ethanol under a variety of defined experimental conditions. These studies demonstrated that ethanol depresses Na^+ gradient-dependent D-glucose uptake by membrane vesicles in a dose- and time-dependent manner [2]. Ethanol-induced in-

* Present address: Division of Gastroenterology, The Milton S. Hershey Medical Center, The Pennsylvania State University, P.O. Box 850, Hershey, PA 17033, U.S.A.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

hibition of D-glucose uptake could occur by (1) specific inactivation of the Na^+-dependent solute transport system, (2) collapse of the inwardly directed Na^+ gradient, and/or (3) alteration in size or shape of vesicles. Previous studies suggested that ethanol acted to perturb membrane integrity and inhibit glucose uptake by allowing the Na^+ gradient to dissipate [3]. The objective of the present study was to examine more closely the effects of ethanol on Na^+-dependent solute transport and relate these effects to changes in permeability, fluidity, chemical composition, size and shape of intestinal brush-border membrane vesicles. The results confirm that ethanol inhibits Na^+-dependent solute uptake while increasing membrane conductance for Na^+. The additional findings of altered membrane fluidity and vesicle size also suggest that ethanol-induced inhibition of transport may occur by more than one mechanism involving the integrity of the microvillous membrane.

Materials and Methods

Materials. [G-^{3}H]Taurocholic acid (3.58 Ci/mmol), D-[U-^{14}C]glucose (310 mCi/mmol) and sodium-22 were purchased from New England Nuclear Corp. (Boston, MA). Unlabeled taurocholic acid was obtained from Steraloids, Inc., Pawling, NY. Enzymes and substrates for leucine aminopeptidase (EC 3.4.11.2) were obtained from Boehringer (Indianapolis, IN). The I(12,3) spin label (*N*-oxyl-4′,4′-dimethyloxazolidine derivative of 5-ketostearic acid), which probes and monitors the region of the polar heads close to the surface of the lipid bilayer, was purchased from Syva Corp., Palo Alto, CA. All other chemicals were purchased as the highest purity available.

Preparation of brush-border membrane vesicles. Male 180–220 g Sprague-Dawley rats fed regular chow ad libitum were killed by a blow on the head and bled. The entire small intestine was removed and divided into ten segments of equal length, numbered 1–10, proximal to distal. Segments 2–4 and 7–9 were used for the study of jejunum and ileum, respectively. The brush-border vesicles were prepared from mucosa scraped from these segments using a modified divalent cation precipitation method originally described for kidney brush-border membrane vesicles [4]. Preparations were maintained at approx. 4°C throughout. Mucosal scrapes from 4–6 rats were homogenized using a Sorval Omni-mixer at maximal speed in 150 ml of 60 mM mannitol, 2.4 mM Tris-HCl, pH 7.1. The homogenate was treated with 10 mM $MgCl_2$ for 15 min before centrifugation at $7600 \times g$ for 15 min. The supernatant was then centrifuged at $20\,000 \times g$ for 30 min. The resulting pellet was suspended in 70 ml of 100 mM mannitol, 20 mM Hepes-Tris, pH 7.1, and homogenized in a Potter-Elvehjem apparatus for 10 strokes at highest speed. The homogenate was treated with 10 mM $MgCl_2$ for 15 min prior to centrifugation at $7600 \times g$ for 15 min. The supernatant was then centrifuged at $20\,000 \times g$ for 30 min. The pellet was suspended in 2 ml of 100 mM mannitol, 20 mM Hepes-Tris, pH 7.1, with a 25-gauge needle and syringe. The suspension was then centrifuged at $1900 \times g$ for 5 min. The supernatant was collected and centrifuged at $48\,000 \times g$ for 30 min. The pellet was resuspended in desired volume of transport buffer as described in the figure legends. Purity of the membranes was assessed by the measurement of $(Na^+ + K^+)$-ATPase [5] and leucine aminopeptidase using the Boehringer Kit No. 124869 (Boehringer, Indianapolis, IN) [6]. Protein was determined by the method of Lowry et al. [7] using bovine serum albumin as a standard. The enrichment in specific activity (final pellet per homogenate) of the luminal marker, leucine aminopeptidase was more than 20-fold, whereas enrichment of the basolateral marker, $(Na^+ + K^+)$-ATPase, was less than 1.2-fold.

Transport measurements. Uptake of radiolabeled D-glucose and taurocholic acid by brush-border membrane vesicles was measured by the membrane filtration technique [8,9]. Typically, membrane vesicles were incubated in 300 mM mannitol and 20 mM Tris-Hepes, pH 7.4, at 0°C for 60 min without (control) or with 2% (v/v) ethanol. Transport was initiated by adding 10–20 μl of membrane suspension to 50–100 μl of incubation medium kept in a water bath at 37°C. The composition of the incubation media is given in the figure legends. At the desired time intervals a 20 μl aliquot was removed from the incubation suspension and diluted in 1 ml of ice-cold stop solution. The stop solution contained 100 mM mannitol, 100 mM NaCl, 10 mM K_3PO_4 (pH 7.4), 1 mM taurocholic acid and 0.2 mM phlorizin. The

stop solution containing the vesicles was immediately pipetted onto the middle of a pre-wetted filter (cellulose nitrate, 0.45 μm pore size, Sartorius Filters, Inc., Hayward, CA) and kept under suction. The filter was immediately washed with 4 ml of ice-cold stop solution and then dissolved in Brays solution. The radioactivity remaining on the filters was counted with standard liquid scintillation techniques. All experiments were performed at least in triplicate with freshly prepared membranes and were repeated for a total of six or more determinations. Absolute solute uptake was expressed as picomoles per milligram of protein.

Electron microscopic studies and morphometric analysis. To simulate conditions of equilibrium transport, brush-border membrane vesicles were incubated in transport buffer at 0°C for 60 min, followed by incubation at 37°C for 60 min without or with 2% (v/v) ethanol. Membranes were then fixed for 90 min in 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The suspensions were centrifuged at 100 000 × *g* for 1 h to form a pellet. The pellets were post fixed in 1.5% osmium tetroxide, dehydrated in graded ethanols and en bloc stained with uranyl acetate.

They were embedded in Epon 812, and thin sections were cut and stained with uranyl acetate and lead citrate. To determine whether there was a difference in size between the untreated vesicles and vesicles treated with ethanol, pellets were embedded without respect to orientation. Thin sections were obtained from the approximate center of the pellet and examined at 9000 × and 22 000 × in a Philips 300 electron microscope. Because the vesicles were evenly dispersed over the thin section, random photographs were taken of all pellets to yield a final magnification of 50 750 ×. Measurements were made only on vesicles with the following features: (1) the membrane of the vesicle was visible around the entire structure; (2) the center of the vesicle was not electron dense; and, (3) the vesicle was single and did not have a smaller vesicle inside. Vesicles of any shape were included in the analysis. A Bioquant II (R and M Biometrics, Nashville, TN 37215) computer system was used to make the measurements. Vesicles were analyzed to determine the area, perimeter and length vs. width (or degree of roundness). The observer had no knowledge of the treatment of the vesicles.

Electron paramagnetic resonance (EPR) studies. For spin label incorporation into membranes, 5 nmol of I(12,3) spin label were incubated with freshly prepared brush-border membranes, suspended in 300 mM mannitol, 20 mM Hepes-Tris, pH 7.4, for 15 min at 37°C. To avoid probe-probe interactions [10], the probe/membrane ratio was maintained at less than 10 μg probe per mg membrane protein. The spin labeled-membranes were then incubated at 0°C. for 60 min in the absence and presence of 2% (v/v) ethanol. EPR spectra were obtained at 20°C with a Varian E109 Century Series EPR Spectrometer equipped with an E-102-04 microwave bridge and E_{231} rectangular cavity (Varian Instrument Group, Palo Alto, CA). The membrane preparation (400 μl) was placed in a WG-812 quartz flat cell (Wilnad Glass Co., Inc., Buena, NJ) which was aligned in the rectangular cavity perpendicular to the magnetic field. The spectrometer was operated for first harmonic in-phase absorption at 10 mW microwave power, 1.0 gauss modulation amplitude (peak-to-peak) and 100 kHz modulation frequency. The EPR spectra displayed characteristics of fast, anisotropic motion, and the $2T_{11}$ (the separation between the outer hyperfine extrema) was measured as an indicator of the degree of ordering of membrane lipid. A decreased value of $2T_{11}$ was interpreted as resulting from decreased order of membrane lipid, i.e., increased membrane fluidity [11].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Brush-border membranes were treated with or without 2% (v/v) ethanol as described above for transport measurement. They were then dissolved in 25 mM Tris-HCl buffer, pH 8.0, containing 2.5 mM EDTA, 3% sodium dodecyl sulfate, 25% sucrose, 1.5% dithiothreitol and 0.0025% pyroninyl. After heating to 100°C for 2 min and then cooling in ice, 10 μl (50 μg protein) of solubilized membrane was applied to each well of an electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA). Discontinuous SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli [12] using a 1.5 mm thick slab gel with a 5% (w/w) acrylamide running gel. The electrode buffer contained 40 mM Tris, 20 mM sodium acetate, 2 mM Na_2EDTA, and 0.1% SDS, pH 7.4. The gel was run for 4 h at room temperature, applying a constant current of 40

mA/cm^2. Gels were stained overnight with 0.05% Coomassie brilliant blue in destaining solution (250 ml isopropanol, 100 ml acetic acid, 650 ml water). Destaining was achieved by several changes of destaining solution. The gels were then dried between cellophane, and the pattern of bands on the dried gels were quantified by densitometry (Double Beam Recording Microdensitometer, Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, U.K.).

Statistical treatment of data. Analysis of data from transport, morphology and fluidity experiments for significant differences was according to the Student's *t*-test [13].

Results

Effect of ethanol on time course of D-glucose uptake

As shown in Fig. 1, when jejunal brush border membrane vesicles were prepared in a Na^+-free medium and incubated in a Na^+-containing buffer, D-glucose showed a rapid initial uptake which exceeded uptake at 60 min (equilibrium). The initial (20 s) uptake of D-glucose, in the presence of the Na^+ outside $> Na^+$ inside ($Na^+_o > Na^+_i$) gradient, was stimulated more than 10-fold compared with the uptake in the absence of Na^+ ($K^+_o > K^+_i$ gradient). At equilibrium, however, the uptake of D-glucose in the presence of sodium was not significantly different from the uptake without sodium. Fig. 1 further shows that the pretreatment of brush border membranes with 2% (v/v) ethanol for 1 h at 0°C resulted in the significant inhibition of Na^+ gradient-stimulated initial transport of D-glucose but leaves the Na^+-independent uptake unchanged.

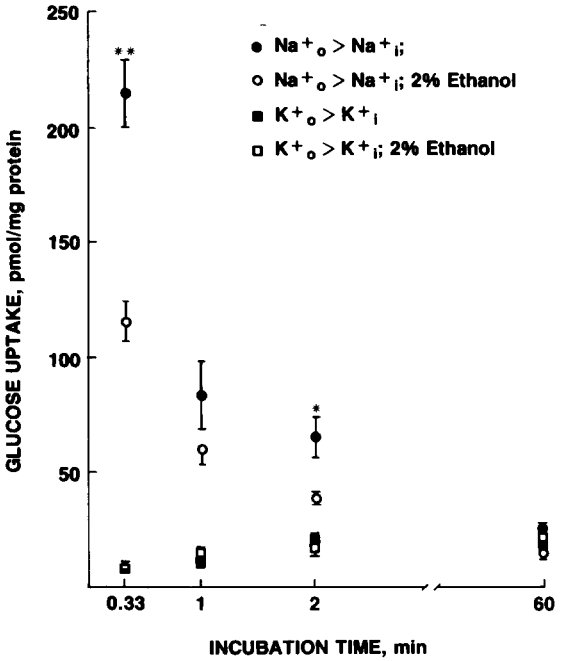

Fig. 1. Effect of ethanol on time course of D-glucose uptake. Jejunal brush-border membrane vesicles were pre-incubated in 300 mM mannitol, 20 mM Hepes-Tris, pH 7.4 for 60 min at 0°C either in the absence, (●, ■) or presence (○, □) of 2% (v/v) ethanol. The pretreated vesicles then were incubated in media at 37°C containing 60 μM D-[^{14}C]glucose, 100 mM mannitol, 20 mM Hepes-Tris, pH 7.4, and either 100 mM NaCl (●, ○) or 100 mM KCl (■, □) and either in the absence (●, ■) or presence (○, □) of 2% (v/v) ethanol. Values represent the mean ± S.E. for nine determinations. * $P < 0.05$, ** $P < 0.01$.

Effect of different ethanol concentrations on D-glucose uptake

Ethanol depressed Na^+ gradient-dependent D-glucose uptake by jejunal brush-border membrane vesicles in a dose-dependent manner. As shown in Table I, the pretreatment of brush-border membranes with increasing concentrations of ethanol

TABLE I

EFFECT OF DIFFERENT ETHANOL CONCENTRATIONS ON D-GLUCOSE UPTAKE

Jejunal brush-border membrane vesicles were pre-incubated at 0°C for 60 min and incubated at 37°C for 0.33 and 60 min (equilibrium) in the presence of 0, 1, 2, 3 or 4% (v/v) ethanol. Except for ethanol concentrations, the conditions of the experiments were similar to those described in the legend of Fig. 1. % equilibrium = 100 (uptake at 0.33 min)/(uptake at 60 min). Values represent the mean ± S.E. for nine determinations.

Ethanol concn. (%)	Glucose uptake (% of equilibrium)	
	$Na^+_o > Na^+_i$	$K^+_o > K^+_i$
0	678.3 ± 54.9	60.9 ± 11.9
1	575.4 ± 59.5	41.3 ± 14.7
2	490.0 ± 62.3 [a]	50.4 ± 12.6
3	423.5 ± 53.2 [b]	49.7 ± 11.2
4	336.0 ± 38.5 [b]	60.9 ± 14.0

[a] Significantly different ($P < 0.05$) from uptake in the absence of ethanol.

[b] Significantly different ($P < 0.01$) from uptake in the absence of ethanol.

TABLE II

EFFECT OF ETHANOL ON TRANS-STIMULATION OF D-GLUCOSE UPTAKE

Jejunal brush-border membrane vesicles were pre-incubated in 100 mM mannitol, 100 mM NaCl, 20 mM Hepes-Tris, pH 7.4, 2 mM D-glucose, 6 μg/ml gramicidin for 60 min at 0°C either in the absence (control) or presence of 2% (v/v) ethanol. The uptake of 0.1 mM D-[^{14}C]glucose was measured after diluting the pre-incubated vesicles 1:20 into incubation medium containing 100 mM NaCl at 37°C either in the absence or presence of 2% (v/v) ethanol. The values represent the mean ± S.E. for nine determinations.

Time (min)	Glucose uptake (pmol/mg protein)	
	Control	Ethanol
0.33	171.8 ± 20.5	171.5 ± 22.7
1	187.9 ± 13.5	197.6 ± 28.2
2	217.7 ± 40.8	227.3 ± 24.5
60	97.8 ± 14.2	86.2 ± 16.9

(0–4%) resulted in a progressive inhibition of Na^+ gradient, but not K^+ gradient, initial uptake of D-glucose.

Effect of ethanol on trans-stimulation of D-glucose uptake

The inhibition of Na^+-glucose uptake with ethanol could occur by specific inactivation of the Na^+-solute co-transporter (putative carrier). To examine co-transporter activity, counter transport experiments were performed under conditions of Na^+ equilibrium [14]. Preloading of jejunal membrane vesicles with unlabeled D-glucose was used to drive the transport of radiolabeled D-glucose against its concentration gradient (the latter in terms of isotope concentration). Gramicidin, an ionophore which increases the cation conductance of membranes [15], was added to nullify the development of an electrical gradient secondary to Na^+/D-glucose efflux from the vesicles. Table II shows that this preparation resulted in 'overshooting' uptake, i.e., the amount of radiolabeled D-glucose present in the vesicles transiently exceeded the equilibrium value. Table II also demonstrates that pretreatment of membranes with 2% ethanol did not abolish the overshoot. These results suggest the lack of a direct inhibitory effect of ethanol on the putative D-glucose carrier.

Effect of ethanol on time course of taurocholic acid uptake

In order to determine whether ethanol inhibits other Na^+ gradient-dependent transport systems, studies were performed on taurocholic acid uptake by ileal brush-border membrane vesicles [16–20]. As shown in Fig. 2, when ileal vesicles were prepared in a Na^+-free medium and incubated in a Na^+-containing buffer, taurocholic acid showed a rapid uptake during the first 2 min. The initial uptake of taurocholic acid in the presence of the $Na^+_o > Na^+_i$ gradient was stimulated more than 2-fold compared with its uptake in the presence of a $K^+_o > K^+_i$ gradient. The significant difference persisted up to 2 min of incubation. At equilibrium (60 min) the uptake of taurocholic acid in the presence of Na^+ was not statistically different from uptake without Na^+. Furthermore, from the data in Fig. 2, it is apparent that the uptake of taurocholic acid at 2 min exceeded significantly

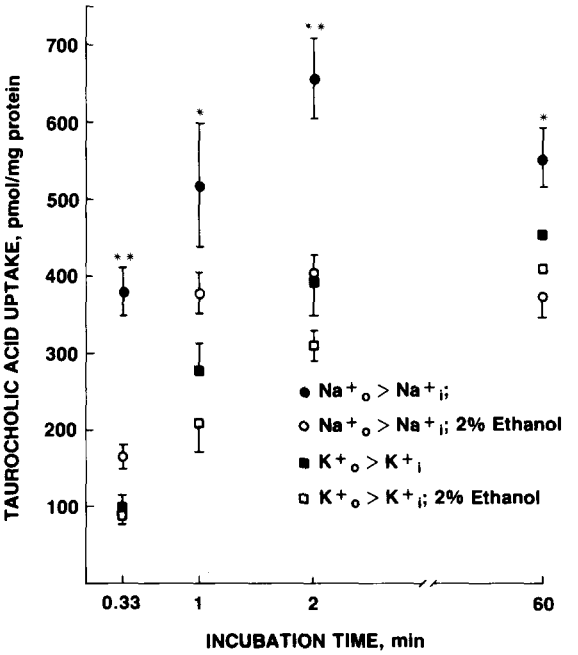

Fig. 2. Effect of ethanol on time course of taurocholic acid uptake. Ileal brush-border membrane vesicles were pre-incubated at 0°C for 60 min and incubated at 37°C either in the absence or presence of 2% (v/v) ethanol. The conditions of the experiments were similar to those described in the legend of Fig. 1 except that 10 μM [^{3}H]taurocholic acid was substituted for D-glucose. Values represent the mean ± S.E. for nine determinations. * $P < 0.05$, ** $P < 0.01$.

the equilibrium value suggesting a transient intravesicular accumulation of the bile acid. The uptake in the absence of Na^+ never significantly exceeded the equilibrium value. Fig. 2 also shows that treatment of membrane vesicles with 2% ethanol significantly inhibited the Na^+ gradient-stimulated initial uptake (0.33–2 min) of taurocholic acid. Finally, a surprising finding was that at equilibrium (60 min) Na^+-dependent taurocholic acid uptake was significantly greater in untreated than ethanol-treated membrane vesicles. These results suggest that the inhibition of Na^+-dependent taurocholic acid transport by ethanol could occur by more rapid dissipation of the energy producing Na^+ gradient and/or alteration of equilibrium (intravesicular) space. These two possibilities were evaluated by the following experiments.

Effect of ethanol on time course of Na^+ uptake

To determine the effect of ethanol on membrane permeability for Na^+, studies were carried out to measure ^{22}sodium uptake by jejunal brush-border membrane vesicles. As shown in Table III, the pretreatment of membranes with 2% ethanol resulted in increased initial (20 s) uptake of Na^+; whereas, equilibrium uptake (60 min) was significantly greater in untreated than ethanol-treated membrane vesicles. The findings support the possibility that ethanol exerts a dual effect on vesicle transport: increasing the membrane conductance for Na^+, thereby more rapidly dissipating the $Na_o^+ > Na_i^+$ gradient, and decreasing the intravesicular space.

Effect of ethanol on the intravesicular space

To test the possibility that ethanol decreases intravesicular space, electron micrographs of vesicles with and without ethanol treatment were analyzed morphometrically. For these studies, vesicles prepared from the ileum were chosen because ethanol inhibited equilibrium uptake of taurocholic acid by vesicles from this site. Table IV shows that the average area of the 258 vesicles not treated with ethanol was 41 165 nm^2 compared to 22 983 nm^2 of the 369 ethanol-treated vesicles ($P < 0.01$). There was no significant differences in shape between the ethanol-treated and untreated vesicles ($P > 0.05$). Studies were carried out to determine whether the differences in vesicle size could be related to their position within a single pellet. Ethanol-treated membranes were embedded in two ways: one in which the top of the pellet was embedded down and examined first and the other in which the bottom of the pellet was embedded down in the block and sampled first. As shown in Table V, a total of 323 vesicles were analyzed. The avarage area of the vesicles in the top of the pellet was not statistically different ($P > 0.05$) from the area of vesicles in the bottom of the pellet. Moreover, there was little variation in shape of the vesicles with the shortest diameter/longest diameter being 0.833 in the top of the pellet and 0.815 at

TABLE III

EFFECT OF ETHANOL ON TIME COURSE OF Na^+ UPTAKE

Jejunal brush-border membrane vesicles were pre-incubated in 300 mM mannitol, 20 mM Hepes-Tris, pH 7.4, for 60 min at 0°C either in the absence (control) or presence of 2% (v/v) ethanol. The pretreated vesicles then were incubated in media at 37°C containing trace quantities of $^{22}Na^+$, 100 mM mannitol, 100 mM NaCl, 20 mM Hepes-Tris, pH 7.4 either in the absence or presence of 2% (v/v) ethanol. The values represent means ± S.E. for six determinations.

Time (min)	Sodium uptake (nmol/mg protein)	
	Control	Ethanol
0.33	34.1 ± 2.0	43.7 ± 1.5 [b]
1	63.7 ± 3.0	67.6 ± 3.9
2	76.2 ± 3.0	70.1 ± 4.0
60	69.6 ± 10.2	47.9 ± 5.2 [a]

[a] Significantly different ($P < 0.05$) from control.
[b] Significantly different ($P < 0.01$) from control.

TABLE IV

VESICLE AREA AND SHAPE WITHOUT AND WITH ETHANOL TREATMENT

Treatment	Number of Vesicles	Average area (nm^2)	Average Shape [a]
Without ethanol	258	41 165	0.726 ± 0.038
With ethanol [b]	369	22 983	0.716 ± 0.050

[a] Average shape equals mean value for shortest diameter/longest diameter ± S.E.
[b] Ileal brush-border membrane vesicles were incubated with 2% ethanol (v/v) for 1 h at 0°C followed by 1 h at 37°C to simulate conditions of equilibrium uptake.

TABLE V

VESICLE AREA AND SHAPE WITHIN A SINGLE PELLET

Location in pellet	Number of vesicles	Average area (nm^2)	Average shape [a]
Top	177	21562	0.833 ± 0.020
Bottom	146	17529	0.815 ± 0.027

[a] Average shape equals mean value for shortest diameter/longest diameter ± S.E.

the bottom ($P > 0.05$). Thus, differences in vesicle size cannot be explained by their relative position in a given pellet.

Effect of ethanol on membrane fluidity

Alterations in membrane vesicle permeability may result from changes in structure and/or chemical composition. Such changes may be reflected by alterations in membrane fluidity. Since acute ethanol has been shown to exert a fluidizing effect on membranes in vitro [21–23], studies were performed to related changes in membrane transport with fluidity. Jejunal brush-border membranes were incubated in 300 mM mannitol, 20 mM Hepes-Tris, pH 7.4, for 60 min at 0°C either in the absence (control) or presence of 2% (v/v) ethanol in the same manner as described above for vesicle uptake studies. The degree of membrane fluidity then was determined by EPR with the I(12,3) spin label at 20°C. The $2T_{\parallel}$ was measured

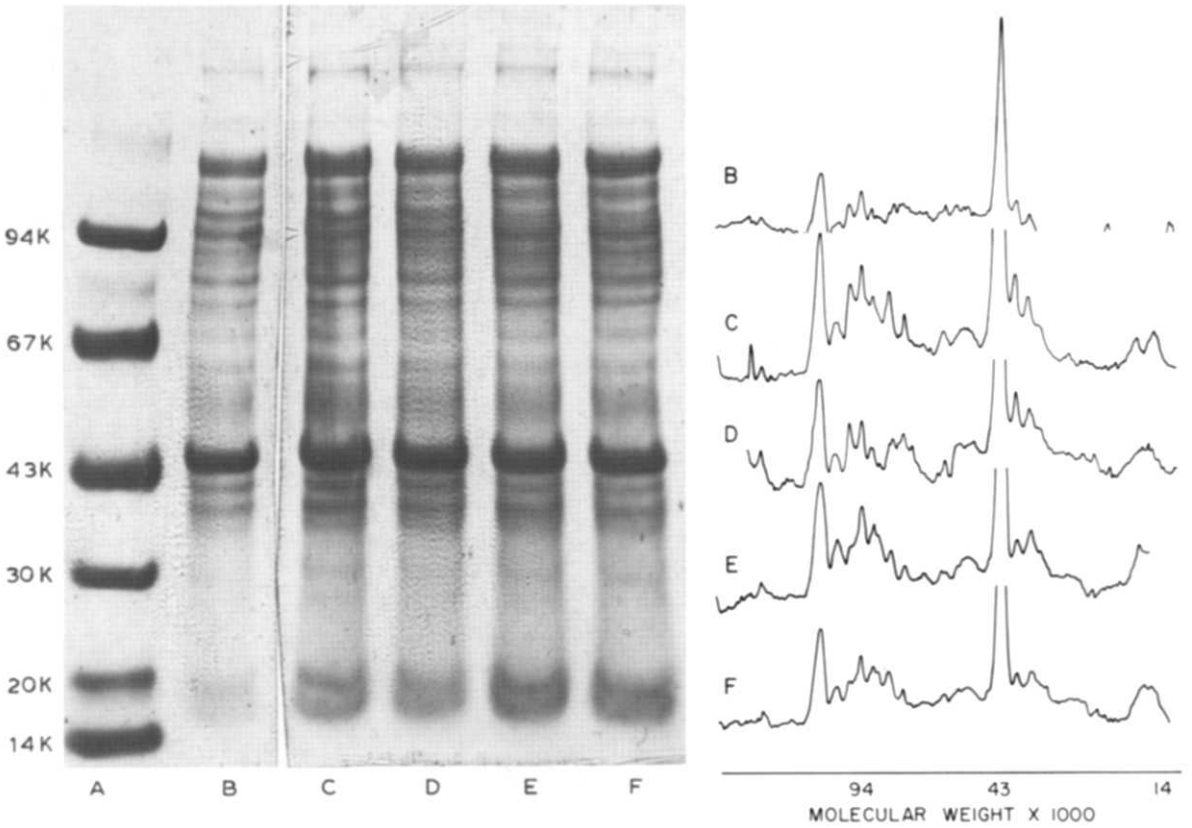

Fig. 3. Left panel. SDS-polyacrylamide gel electrophoretogram of jejunal brush-border membrane vesicles as follows: lane A, protein standards; lane B, freshly prepared vesicles; lanes C and D, vesicles pre-incubated for 60 min at 0°C in the absence and presence of 2% (v/v) ethanol, respectively; lanes E and F, vesicles pre-incubated for 60 min at 0°C followed by incubation for 60 min at 37°C in the absence and presence of 2% (v/v) ethanol, respectively. Protein standards used were phosphorylase B 94 K, bovine serum albumin 67 K, ovalbumin 43 K, carbonic anhydrase 30 K, soybean trypsin inhibitor 20 K, and lysozyme 14 K. Right panel. Densitometry tracings from the SDS-polyacrylamide gel electrophoretogram corresponding to lanes B–F in left panel.

as an indicator of the degree of membrane ordering. Pretreatment of ileal brush-border membranes with ethanol resulted in a significant decrease in $2T_{11}$ from a control value of 57.78 ± 0.08 to 57.50 ± 0.09 gauss ($P < 0.02$, $n = 10$), indicating a greater fluidity of ethanol-treated membranes than untreated membranes.

Effect of ethanol on membrane polypeptides

To further explore the possibility that ethanol inhibits vesicle uptake by altering membrane composition, the effect of ethanol on the integrity of membrane protein was examined by SDS-polyacrylamide gel electrophoresis. Ileal brush border membrane vesicles were treated with 2% ethanol in the same manner as described above for transport and then dissolved in sodium dodecyl sulfate. As shown in Fig. 3, left panel, SDS-polyacrylamide gel electrophoresis revealed more than 20 discrete polypeptide bands with the greatest concentration seen for the 43 kDa polypeptide. When the slab gels were subjected to densitometry (Fig. 3, right panel) no obvious qualitative or quantitative changes were seen between ethanol-treated and untreated membrane vesicles.

Discussion

The present study was undertaken to more closely examine the effects of ethanol on transport processes in purified intestinal brush border membrane vesicles. These studies confirm the results of earlier studies which demonstrated that ethanol inhibits D-glucose uptake in a dose-dependent manner at concentrations within the range of those found in human jejunum during moderate ethanol intake [2]. The results of the present study also support the previous suggestion that ethanol inhibits glucose transport by dissipating the energy-producing Na^+ electrochemical gradient [3]. First, ethanol only affected concentrative D-glucose transport under conditions of an internally directed Na^+ gradient ($Na_o^+ > Na_i^+$). Second, ethanol did not affect concentrative D-glucose transport under conditions in which the vesicles were energized by preloading with glucose (tracer exchange or counter transport). In this experiment, concentrative D-glucose uptake was not occurring secondary to a Na^+ electrochemical gradient because of the presence of initial Na^+ equilibrium and gramicidin to rapidly dissipate any ion gradient that was attained secondary to glucose efflux. Inasmuch as counter-transport is felt to be a carrier-mediated process [14], ethanol did not appear to affect transport via a specific interaction with the brush-border membrane glucose transporter. Third, ethanol inhibited another Na^+-dependent transport system, the concentrative uptake of taurocholic acid by ileal brush-border membrane vesicles. It is unlikely that ethanol specifically interacts with carriers of two distinct transport processes but rather alters the energy-producing Na^+ gradient which drives both active transport systems. Taurocholic acid uptake under tracer exchange conditions was increased only by 5–10% (data not shown). As this difference was not significant, the influence of ethanol on taurocholic acid counter-transport could not be studied. Fourth, direct measurement of Na^+ flux indicated that treatment of membranes with ethanol increased the sodium conductance of the small intestinal brush border membrane.

The electron paramagnetic resonance studies demonstrated that ethanol increased membrane fluidity, as indicated by a decrease in the order parameter T_{11}. Previous studies from our laboratory showed a dose-dependent increase in fluidity of a non-vesiculated brush-border membrane preparation with 0.1–1.5 M ethanol [23]. A similar increase in fluidity after acute ethanol exposure has been noted in other tissue membranes such as erythrocytes, synaptosomes and mitochondria in mice [21]. Moreover, the present finding with the I(13,3) spin label that ethanol disturbs the area of the membrane close to the membrane-water interface agrees with the observation of Vanderkooi [22]. Tillotson et al. [3] failed to demonstrate such a fluidizing effect of ethanol on intestinal brush-border membranes using fluorescence polarization and a diphenylhexatriene probe which localizes in the hydrophobic interior of the membrane. A greater sensitivity of EPR and differences in the membrane localization of the probes may account for the disparity in the results.

It is possible that ethanol increased sodium permeability by an effect on the sealing process of the vesicles. However, the finding of increased fluidity suggests that ethanol alters the intrinsic

physical state of the brush-border membrane. It is not clear how alteration in membrane physical state can be translated directly into a specific mechanism by which ethanol affects membrane permeability for Na^+. Alcohol may interact directly with transport proteins to alter their position or conformation in the membrane. Alternatively, alcohol may penetrate into nearby regions of membrane proteins that are normally occupied by lipid. The displacement of such lipid by lipophilic agents can then indirectly induce loss of protein function [10]. Neither situation, however, requires that ethanol produce qualitative changes in membrane protein composition, which is in agreement with the present studies using SDS gel electrophoresis.

It is apparent that ethanol was exerting more than one effect on membrane vesicles to explain the inhibition of transport processes. This was best demonstrated by a decreased 60 min uptake of Na^+ and taurocholic acid in ethanol-treated jejunal and ileal membranes, respectively. Since 60 min was sufficient time to achieve equilibrium uptake for both substrates [17,24], the lowered uptake could be explained by a decrease in equilibrium intravesicular space. The electron microscopic study indicates that ethanol decreased the size of the vesicles to 56% of the control vesicles. This could account for the change in the equilibrium space, as for the same membrane preparation, the taurocholic acid accessible volume of ethanol-treated vesicles was 62% of the control vesicles. The lack of a clear-cut effect of ethanol on equilibrium uptake of D-glucose in jejunal vesicles is less readily explained. It is possible that Na^+ and taurocholic acid equilibrate into a different space(s) than D-glucose. Under standard osmotic conditions 24–40% of bile acid uptake by brush-border vesicles may not be found in the intravesicular space suggesting binding to the inner or outer membrane surface of the vesicles or transport into a compartment that is not osmotically sensitive [17,20]. Moreover, equilibrium uptake, in pmol/mg protein, of taurocholic acid and Na^+ was 22 and 2800-fold higher, respectively, than D-glucose, suggesting a greater sensitivity of these solutes to changes in intravesicular space. Whatever the mechanism, it is unlikely that a decrease in intravesicular space resulted from osmotic shrinkage of the membrane vesicles. Brush border membrane vesicles are osmotically sensitive as shown by reduction of their intravesicular space when exposed to hypertonic media containing an impermeant solute [17,20]. However, ethanol could not exert such an osmotic pressure gradient because it is a highly permeant substance. Previous studies have shown that the Staverman reflection coefficient of ethanol in jejunum is zero and that ethanol did not produce an osmotic pressure over the mucosa of the hamster jejunum [2].

Acknowledgment

This research was supported in part by Grant 2R01 AM 21696 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

References

1 Wilson, F.A. and Hoyumpa, A.M. (1979) Gastroenterology 76, 388–403
2 Dinda, P.K. and Beck, I.T. (1981) Dig. Dis. Sci. 26, 23–32
3 Tillotson, L.G., Carter, E.A., Inui, K. and Isselbacher, K.J. (1981) Arch. Biochem. Biophys. 207, 360–370
4 Biber, J., Stieger, B., Haase, W. and Murer, H. (1981) Biochim. Biophys. Acta 647, 169–176
5 Berner, W. and Kinne, R. (1976) Pflügers Arch. 361, 269–277
6 Nagel, W., Willeg, F. and Schmidt, F.H. (1964) Klin. Wochenschr. 42, 447–449
7 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
8 Hopfer, U., Nelson, K., Perotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32
9 Berner, W., Kinne, R. and Murer, H. (1976) Biochem. J. 160, 467–474
10 Gordon, L.M., Sauerheber, R.D., Esgate, J.A., Dipple, I., Marchmont, R.J. and Houslay, M.D. (1980) J. Biol. Chem. 255, 4519–4527
11 Swift, L.L., Atkinson, J.B., Perkins, R.C., Dalton, L.R. and LeQuire, V.S. (1980) J. Membrane Biol. 52, 165–172
12 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
13 Snedecor, G.W. and Cochran, W.G. (1967) Statistical Methods, 6th, Edn., pp. 1–593, Iowa State University Press, Ames, IA
14 Will, P.C. and Hopfer, U. (1979) J. Biol. Chem. 254, 3806–3811
15 Urban, B.W., Hladky, S.B. and Haydon, D.A. (1978) Fed. Proc. 37, 2628–2632
16 Lack, L., Walker, J.T. and Hsu, C.H. (1977) Life Sci. 20, 1607–1612
17 Lücke, H.G., Stange, G., Kinne, R. and Murer, H. (1978) Biochem. J. 174, 951–958

18 Beesley, R.C. and Faust, R.G. (1979) Biochem. J. 178, 299–303
19 Rouse, D.J. and Lack, L. (1979) Life Sci. 25, 45–52
20 Wilson, F.A. and Treanor, L.L. (1979) Biochim. Biophys. Acta 554, 430–440
21 Chin, J.H. and Goldstein, D.B. (1977) Mol. Pharmacol. 13, 435–441
22 Vanderkooi, J.M. (1979) Clin. Exp. Res. 3, 60–63
23 Gray, J.P., Hoyumpa, A.M., Dunn, G.D., Henderson, G.I., Wilson, F.A. and Swift, L.L. (1980) Inserm 95, 469–476
24 Lücke, H., Berner, W. and Murer, H. (1978) Pflügers Arch. 373, 243–248