Biochimica et Biophysica Acta, 436 (1976) 686–695

BBA 77345

THE EFFECT OF ETHANOL ON ION TRANSPORT IN FROG SKIN

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(Received May 20th, 1975)
(Revised manuscript received January 26th, 1976)

SUMMARY

Frog skin has been used as a model epithelial sodium-transporting system to study the effect of ethanol on ion transport. Treatment of the outside of frog skin with ethanol decreased the net sodium transport due to inhibition of $^{22}Na^+$ influx. Ethanol did not alter sodium outflux when bathing the outside of the skin. The inhibition was in proportion to the concentration of ethanol, 0.25 M resulting in 50 % inhibition. The chloride permeability of the skin was increased several-fold when the skin was exposed to ethanol in either bathing solution. With 0.4 M ethanol in the inner bathing solution, all the unidirectional fluxes of Na^+ and Cl^- were increased. The movement of Cl^- was evaluated by comparison of Cl^- flux with urea flux, since urea is thought to move passively across frog skin via an extracellular (shunt) pathway. Chloride flux was increased to a greater extent than urea flux. These experiments indicate that ethanol affects chloride permeability beyond an increase in extracellular ion flow and independent of its effect on Na^+ transport.

INTRODUCTION

The purpose of this study is to define the effects of ethanol on specific ion fluxes in frog skin in order to determine if ethanol alters ion movement through changes in membrane permeability or through an effect on active ion transport. Järnefelt [1] has shown that aliphatic alcohols inhibit the Na^+-stimulated ATPase of rat brain membranes. The effect is a general phenomenon, since cation transport and the $(Na^+ + K^+)$-ATPase are inhibited by ethanol in a variety of tissues and species [2, 3]. Recent studies with purified plasma membrane preparations containing $(Na^+ + K^+)$-ATPase indicate that ethanol inhibits the enzyme at the dephosphorylation step without affecting phosphorylation [4, 5].

Ethanol is classified with the general anesthetics, a large group of drugs whose

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effects correlate well with their solubility properties and structure [6]. That the diverse effects of ethanol on biological systems are produced by roughly equivalent concentrations of the alcohol, are rapid, and easily reversible suggests that its action is related to the physical properties of the molecule. Ethanol alters the ion permeability characteristics of simple black lipid membranes [7]. Several recent studies on the effects of alcohols on lipid bilayers, bacterial membranes, and red cell membranes have not been able to distinguish between mechanisms of action involving the hydrocarbon chains of the membrane phospholipids, or protein components, or both [8–10]. Whatever the mechanism, the action of ethanol on membrane systems can result in changes in (1) enzymatic properties of membrane proteins, (2) ion-binding properties of the proteins or lipids, (3) changes in ion permeability of the membranes, or a combination of these changes.

We have used the frog skin as a model of the epithelial Na^+-transporting system and have concluded that ethanol directly inhibits Na^+ transport in frog skin and at the same time alters the ion permeability properties. The dynamics of ion movement are discussed in relation to current models of Na^+ transport in frog skin.

MATERIALS AND METHODS

Experiments were performed with half pieces of abdominal frog skin (*Rana pipiens*) mounted as a barrier between Lucite half-chambers fitted with "O" rings to minimize edge damage. The chambers were stirred with magnetic stirring bars. The chamber volumes were 1.8–2.2 ml and the skin area exposed was 1.2–1.4 cm^2. All experiments were done at room temperature. The composition of the Ringer bathing solution was 110 mM NaCl, 2 mM KCl, and 2.5 mM $CaCl_2$ buffered with 10 mM Tris · HCl, pH 8.1. Vasopressin (Pitressin, Parke-Davis, Detroit, Mich.), 0.4 I.U./ml, was routinely added to the serosal bathing solution to enhance ion flux and to maintain the skin in stimulated condition for the duration of each experiment.

Electrical connections were made via Ringer-agar bridges. Tips of the potential bridges (1.6 mm internal diameter) were apposed near the surface of the membrane at the center. The potential difference (PD) was measured with matched Ag/AgCl electrodes (Annex Instruments, Santa Ana, Calif.). The electrode and bridge junction potentials measured in Ringer's solution did not exceed 0.2 mV. External current, for voltage clamping, was applied from Ag electrodes in wells of Ringer's via Ringer-agar bridges (2.7 mm internal diameter). PD and short-circuit current (I_{sc}) were measured with automatic voltage clamp equipment developed here or with a modified Wenking Potentiostat (Model PCA 72L, Brinkman Instruments, Burlingame, Calif.). No correction was made for the specific resistance of the solution, which was 60 Ω cm.

From each frog, the abdominal skin was bisected laterally and one half-skin served as the control. Skins were discarded if PD and I_{sc} of the pair differed by more than 5 %. In most experiments, absolute ethanol was added directly to the bathing solutions in the chambers to produce the appropriate concentrations. Ethanol was used at a number of concentrations, but even at the maximum of 0.4 M, it did not alter the resistance of the bathing solution.

Fluxes of $^{22}Na^+$, $^{36}Cl^-$, and $^{86}Rb^+$ and ^{14}C-labeled solutes were determined from unidirectional measurements of tracer flow during the steady state when there was no significant back-flux of tracer. Chamber-mounted half-skins having equal PD

were short-circuited and allowed to equilibrate about 10 min to determine that currents were equal and stable. One half-skin served as control while ethanol was added to the appropriate chamber solution of the other half-skin. 15 min was sufficient for the I_{sc} to stabilize at a new level after the addition of alcohol. All chamber solutions were replaced at the time tracer amounts of radioactively labeled solute was introduced. 20 min was sufficient time for all tracer solutes (except rubidium) to reach a steady-state flux. The flux data are reported in $\mu mol \cdot cm^{-2} \cdot h^{-1}$ as the average of three 20-min samples. Wash-out experiments were done with open-circuit skins after preloading with radioactive tracer. Loading solutions were removed, chambers rinsed and wash-out started within 15 s. Then, the chamber solutions were removed and replaced every 2 min for an appropriate time. At the end of the experiment, the skin was assayed for remaining tracer. For the determination of tracer uptake or tracer remaining after wash-out, exposed pieces of skin were hydrolyzed in 1 M NaOH with gentle warming. Aliquots of hydrolysate were mixed with BBS-2 solubilizer (Beckman Instruments, Inc., Fullerton, Calif.) for assay. Radioassay was by liquid and crystal scintillation spectrometry (Packard Instruments, Co., Downers Grove, Ill.) with a counting error of $< 3\%$. When appropriate, quenching was corrected for by internal standardization.

The frogs were obtained from E. G. Hoffman, Oshkosh, Wisc., and were maintained in wet/dry holding tanks and fed live crickets. Solutions were prepared with reagent grade chemicals. Ethanol was absolute, USP. High specific activity $^{22}NaCl$, $^{36}Cl^-$, and $^{86}RbCl$ were obtained from International Chemical and Nuclear Corp., Irvine, Calif. Extracellular space was estimated with hydroxy[^{14}C]methyl inulin (mol. wt. 5200) from Amersham/Searle, Arlington Heights, Ill. New England Nuclear, Boston, Mass., supplied [^{14}C]urea.

The Student's t-test was used to evaluate the differences between mean values.

RESULTS

The response of frog skin I_{sc} to the addition of ethanol to the bathing solutions was proportional to the concentration of ethanol. When ethanol was added to the outer bathing solution, I_{sc} decreased immediately to a new level and remained steady for several hours. 50 % inhibition of I_{sc} was obtained with 0.25 M ethanol, although the variation in response of individual skins was quite large. During the winter months, the skin was much less responsive and a 50 % inhibition of I_{sc} required 1.0 M ethanol in the outer bathing solution. In order to assure a good response, a concentration of ethanol of 0.4 M was used for the ion flux studies reported here. The skin resistance, estimated from PD and I_{sc}, was not changed significantly by ethanol on the outside of the skin; that is, the PD was decreased to about the same extent as the I_{sc}. Removing ethanol by rinsing the skin and replacing the chamber solutions with fresh buffer without ethanol promptly restored the PD and I_{sc} of the skin to control levels. Once the skins were mounted in the chambers, vasopressin was always present in the serosal bathing solution in order to maximize the tracer ion fluxes.

The effect of 0.4 M ethanol in the outer bathing solution on tracer Na^+ flux across the skin agreed with the I_{sc} response. The results of experiments in which Na^+ influx ($^{Na^+}J_{in}$) was measured for 1 h, followed by Na^+ outflux ($^{Na^+}J_{out}$) are shown in Table I. The Na^+ influx was decreased while the outflux was not changed. The correla-

TABLE I

EFFECT OF 0.4 M ETHANOL IN OUTER SOLUTION ON Na^+ FLUX

The values shown are the mean flux ±S.D. The number of experiments is in parentheses. Results are expressed in $\mu mol \cdot cm^{-2} \cdot h^{-1}$.

	I_{sc}	$^{Na^+}J_{in}$	$^{Na^+}J_{out}$	$^{Na^+}J_{net}$
Control (6)	1.58±0.44	1.60±0.52	0.18±0.14	1.42±0.50
Ethanol (6)	0.62±0.42	0.73±0.36	0.17±0.14	0.56±0.37
	$P < 0.001$	$P < 0.001$	n.s.	$P < 0.001$

n.s., not significant.

tion of net sodium flux ($^{Na^+}J_{net}$) calculated from unidirectional tracer fluxes with I_{sc} was the same whether or not ethanol was present in the outer bathing solution, as shown in Fig. 1. The regression coefficient for the correlation of the calculated net sodium flux with short-circuit current (I_{sc}) is 0.91 for pooled control and ethanol data.

Table II shows the effect of 0.4 M ethanol on chloride permeability. Lines 1 and 2 indicate that in control short-circuited skins $^{Cl^-}J_{in}$ and $^{Cl^-}J_{out}$ are similar and there is no net flux of Cl^-. Lines 3 and 4 demonstrate that ethanol in the outer bath alters $^{Cl^-}J_{in}$ and $^{Cl^-}J_{out}$ to the same extent; that is, there is still no net flux of Cl^-. It is not possible to compare the values of Cl^- flux in lines 1 and 2 with those in lines 3 and 4 in order to determine the effect of ethanol. These experiments were done with groups of frogs with markedly different permeability properties as reflected in the 2-fold

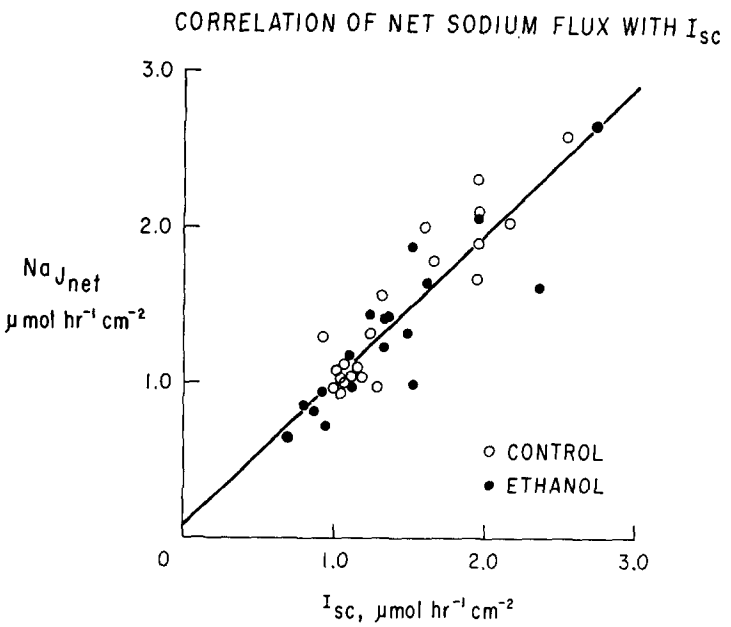

Fig. 1. Correlation of net sodium flux with short-circuit current. Points represent 20-min flux samples. The line was determined by least squares. Analysis of pooled control and ethanol data.

TABLE II

THE EFFECT OF 0.4 M ETHANOL IN OUTER BATHING SOLUTION ON Cl^- FLUX

Values are expressed as μmol · cm^{-2} · h^{-1} and represent the mean ±S.D. The number of experiments is in parentheses.

			I_{sc}	$^{Cl^-}J_{in}$	$^{Cl^-}J_{out}$
1	Control	(5)	2.69 ± 0.91	1.07 ± 0.35	
2	Control		2.60 ± 0.95		1.01 ± 0.48
3	Ethanol	(4)	1.02 ± 0.64	0.89 ± 0.22	
4	Ethanol		0.92 ± 0.70		0.92 ± 0.53
5	Control	(5)	2.14 ± 0.29		0.39 ± 0.22
6	Ethanol		0.70 ± 0.48		0.79 ± 0.17
			$P < 0.01$		$P < 0.01$
7	Control	(6)	2.43 ± 0.99	0.96 ± 0.44	
8	Ethanol		1.90 ± 0.75	1.89 ± 0.88	
			$P < 0.05$	$P < 0.05$	

difference in I_{sc}. With paired half-skins, one half serving as non-treated control, ethanol in the outer bathing solution doubled both $^{Cl^-}J_{in}$ and $^{Cl^-}J_{out}$, lines 5 and 6, and 7 and 8. The effect of ethanol on chloride permeability was independent of the extent to which ethanol inhibited the I_{sc} and $^{Na^+}J_{in}$. During winter months, the I_{sc} was not decreased by adding 0.4 M ethanol to the outer bathing solution with some frogs, although $^{Cl^-}J_{in}$ and $^{Cl^-}J_{out}$ were markedly increased.

The response of frog skin was different when ethanol was in the serosal bathing solution. There was a small gradual increase in I_{sc}, then the current returned to control levels within 30 min. Table III shows the effect of 0.4 M ethanol in the serosal bathing solution on Na^+ and Cl^- fluxes. The unidirectional fluxes of both Na^+ and Cl^- were significantly increased by ethanol in the inner bathing solution. This treatment did not change I_{sc} and the net Na^+ isotopic flux is close to that calculated from the electrical current measurement.

Since the action of ethanol on Na^+ and Cl^- fluxes depended on the side of the ethanol treatment, the effect of 0.4 M ethanol in both bathing solutions at the same time was determined. In this experiment with paired half-skins, both bathing solutions of one of the half-skins were replaced with 0.4 M ethanol in Tris-Ringer buffer. The I_{sc} did not change immediately although there was a gradual decline during the subsequent 2 h of the experiment. The outflux or influx of $^{22}Na^+$ and $^{36}Cl^-$ was measured followed by determination of the opposite unidirectional flux. Table IV gives the results of this study. Correlation of the I_{sc} with net Na^+ flux is not appropriate in this type of experiment where one unidirectional flux is measured and then followed by the opposite flux measurement. The I_{sc} and net Na^+ flux did decrease some with bilateral ethanol treatment but these decreases were not statistically significant. Both the influx and the outflux of Cl^- increased, some skins showing as much as a 3-fold increase with the alcohol treatment.

The decrease in net Na^+ influx and in I_{sc} when ethanol is present in the outer solution contrasts with the increase seen in net Na^+ flux with alcohol in the inner solution. The lack of effect with ethanol on both sides suggests that ethanol is, by some mechanism, inhibiting the inward movement of sodium only when the outside

TABLE III

EFFECT OF 0.4 M ETHANOL IN THE INNER BATHING SOLUTION ON Na^+ AND Cl^- FLUXES

The values are the mean ±S.D. in $\mu mol \cdot cm^{-2} \cdot h^{-1}$ with the number of experiments in parentheses. The influx and outflux experiments were done on different half-skins, one half control, the other with 0.4 M ethanol inside. The I_{sc} is the mean of all the experiments.

	I_{sc}		Influx		Outflux	
Na^+ flux						
Control	2.89±1.05	(6)	2.69±0.72	(3)	0.59±0.04	(3)
Ethanol	3.19±0.66		4.67±0.47		1.03±0.04	
	n.s.		$P < 0.001$		$P < 0.001$	
Cl^- flux						
Control	3.11±1.06	(9)	1.36±0.87	(5)	0.87±0.34	(6)
Ethanol	3.12±0.98		3.34±0.62		1.52±0.43	
	n.s.		$P < 0.01$		$P < 0.02$	

n.s., not significant,

of the skin is exposed to the alcohol. At the same time, the permeability to chloride is increased when ethanol is on either or both sides of the skin.

To gain insight into these different effects on Na^+ and Cl^-, we followed the procedure described by Mandel and Curran [11] to determine the effect of ethanol on the flux of chloride through an extracellular or "shunt" pathway. This consisted of the simultaneous measurement of [^{14}C]urea and $^{36}Cl^-$ outfluxes across skins short-circuited or voltage-clamped at −50 or −100 mV. Evaluation of the results of such an experiment requires the assumption that urea moves across the frog skin by way of an extracellular pathway. Analysis of chloride flux in this manner [12] indicated that the flux of chloride is linearly related to urea flux and that the ratio of permeabilities (P_{Cl} vs. P_{urea}) is not changed by the applied voltage. The effects of ethanol in this system are shown in Table V. While there was a linear relationship between chloride and urea fluxes of control skins when clamped at −50 or −100 mV ($r >$ 0.97), there was poor correlation of chloride and urea fluxes with short-circuited control or alcohol-treated skins ($r = 0.68$ and 0.44, respectively). Data for alcohol-

TABLE IV

THE EFFECT OF 0.4 M ETHANOL IN BOTH BATHING SOLUTIONS ON Na^+ AND Cl^- FLUXES

The values are the mean ±S.D. in $\mu mol \cdot cm^{-2} \cdot h^{-1}$ with the number of experiments in parentheses.

		I_{sc}	Na^+ flux		Cl^- flux	
			In	Out	In	Out
Control	(6)	1.89±0.79	3.17±0.81	0.80±0.32	1.26±0.74	1.25±0.37
Ethanol	(6)	1.31±0.87	2.93±0.85	0.86±0.25	3.12±1.40	2.39±1.36
		n.s.	n.s.	n.s.	$P < 0.05$	n.s.

n.s., not significant.

TABLE V

THE EFFECT OF 0.4 M ETHANOL OUTSIDE ON $^{36}Cl^-$ AND [^{14}C]UREA OUTFLUX WITH SKIN CLAMPED AT 0, −50, AND −100 mV

Outflux values are the mean of eight experiments ± S.D. expressed in $\mu mol \cdot cm^{-2} \cdot h^{-1}$. Slope and regression coefficient (r) were determined by least squares.

		Control		Alcohol		P
		Outflux		Outflux		
$^{Cl^-}J_{out}$	0	0.45 ± 0.14		0.73 ± 0.23		< 0.01
	−50	1.08 ± 0.69		3.23 ± 2.62		< 0.01
	−100	3.18 ± 1.80				
$^{urea}J_{out}$	0	4.75 ± 1.88		5.05 ± 1.58		n.s.
	−50	9.00 ± 5.61		17.42 ± 12.7		< 0.05
	−100	16.08 ± 10.30				
		Slope	r	Slope	r	
$^{Cl^-}J$ vs. ^{urea}J	0	50.3	0.68	64.9	0.44	n.s.
	−50	119.8	0.98	198.9	0.99	< 0.001
	−100	172.6	0.99			

n.s., not significant.

treated skins at −100 mV voltage-clamp are not shown since these skins were electrically unstable and the effect of alcohol was no longer reversible. As we have already shown, $^{Cl^-}J_{out}$ sc was nearly doubled in alcohol-treated skins as compared to controls. At the same time, in short-circuited skins, urea outflux was not changed by alcohol. Because of the poor correlation between $^{Cl^-}J$ and ^{urea}J, the increase in the slope of $^{Cl^-}J$ vs. ^{urea}J at I_{sc} in alcohol-treated skins is not statistically significant. At −50 mV voltage-clamp, there is an increase in chloride flux, urea flux, and the slope of $^{Cl^-}J$ vs. ^{urea}J. If, under these experimental conditions, urea flux is a measure of the permeability of extracellular pathways, then these results suggest that while alcohol increases this permeability, this pathway cannot account for all the increase in chloride flux when alcohol is present.

A similar increase in chloride permeability after ethanol treatment was observed when ($Na^+ + K^+$)-ATPase was inhibited with 10^{-4} M ouabain. These experiments were conducted on matched half-skins which were treated with 10^{-4} M ouabain in the inner bathing solution. Sufficient time was allowed for I_{sc} to stabilize near zero before ethanol was added to the outer bathing solution. In twelve experiments, $^{Cl^-}J_{out}$ was increased from 1.16±0.68 to 2.03±1.42 (mean±S.D., $P < 0.05$). This experiment indicates that ethanol enhances the chloride permeability of the frog skin independent of its effect on Na^+ transport.

Israel [2] has proposed that ethanol interferes with the cellular utilization of ATP by competitively inhibiting ($Na^+ + K^+$)-ATPase at the potassium binding site. We have studied the effect of increasing the potassium concentration in Ringer's solution on the inhibition of I_{sc} by ethanol. Increasing K^+ from 2 to 20 mM did not attenuate the inhibitory effect of ethanol. To gain further insight into the K^+ compart-

ment of frog skin, $^{86}RbCl$ in Tris-Ringer's in which RbCl was substituted for KCl was used in uptake and wash-out studies to evaluate the effect of ethanol. Rubidium substitutes well for K^+ in a variety of physiological preparations [13]. Our substitution of RbCl for KCl caused no apparent change in Na^+ or Cl^- fluxes, the correlation of net sodium flux with I_{sc}, or the inhibitory effect of ethanol. We found that 2 h was necessary for isotope equilibration when $^{86}Rb^+$ bathed the inside of short-circuited skin. Less than 2 % of the $^{86}Rb^+$ appeared in the outer bathing solution during 2 h of incubation. The presence of 0.4 M ethanol in the outer bathing solution did not alter the $^{86}Rb^+$ space in four experiments in which the skin was incubated with $^{86}Rb^+$ (0.3 Ci/mol) in Rb^+ Ringer in the inner bathing solution for 2 h. The $^{86}Rb^+$ space calculated from these experiments was 0.38 ± 0.06 ml/cm^2 for control skins and 0.34 ± 0.08 for alcohol-treated skins. This study suggests that the effect of ethanol is not at the level of the uptake or release of $K^+(Rb^+)$ from the inner bathing solution. In addition, in experiments on the wash-out of $^{86}Rb^+$ from prelabeled skin, it was found to be unaffected by the presence of 0.4 M ethanol, during the prelabeling or during wash-out.

DISCUSSION

Some effects of ethanol on the electrical properties of frog skin in vitro have been previously reported. Fuhrman [14] exposed the serosal surface of frog skin to 0.4 M ethanol. He found that there was a large decrease in resistance while the I_{sc} did not vary by more than 10 %. He reported that in 1 h there was a 10-fold increase in chloride outflux. Israel and Kalant [15] reported that ethanol (0.04–0.35 mol/l) added to the solution bathing the outside of frog skin caused an immediate decrease in I_{sc} proportional to the concentration of ethanol. In both of these studies, and in the present study, normal electrical properties of the skin were promptly restored by washing the alcohol from the skin.

Careful evaluation of the influence of ethanol on tracer Na^+ flux has confirmed that the observed inhibition of I_{sc} reflects inhibition of sodium influx when frog skin is bathed with ethanol. These studies suggest that the presence of ethanol is affecting the $(Na^+ + K^+)$-ATPase responsible for Na^+ transport in frog skin. However, it is not clear from our experiments if the action of ethanol is to decrease the availability of Na^+ to the enzyme or to directly inhibit the enzyme.

Apparently, independent of its effect on Na^+ transport, ethanol markedly alters chloride movement across frog skin. The fact that, when skin is short-circuited and 0.4 M ethanol is present in the outer bathing solution, neither sodium nor urea outflux is significantly altered argues against alcohol causing a general increase in skin conductance due to the hyperosmolarity of the outer bathing solution. In addition, treatment of the outside of the skin with hyperosmolar 0.4 M urea in Tris-Ringer's increased several-fold the fluxes of sodium and chloride across the skin (unpublished observation). This is the expected response of frog skin to hyperosmolarity of the outer bathing solution. Recently, Mandel [12] has suggested that in the short-circuited condition chloride moves intracellularly rather than via a shunt pathway. The effect of ethanol on I_{sc} is to increase chloride but not urea flux and the greater effect of alcohol on chloride than on urea flux at -50 mV voltage-clamp suggests that alcohol is altering the movement of chloride through the cells.

In the frog skin, K^+ is not so important as return cargo for the $(Na^+ + K^+)$-ATPase as in other transport systems. Our failure to find any alteration in K^+ transport caused by ethanol or any antagonism between ethanol and K^+ may be peculiar to the frog skin system. One might expect that a purified membrane preparation would be more sensitive to the effects of hydrophobic solvents than intact frog skin with its protective cornified layer.

In a recent study with a partially purified $(Na^+ + K^+)$-ATPase preparation [4], ethanol, along with other hydrophobic solvents, affected the hydrolysis step directly, not by indirectly antagonizing K^+, as suggested by Israel [2]. The proposal was made [4] that ethanol was altering the hydrophobic region around the phosphate acceptor site where an antagonism between K^+ and water appears to play a role in activation of the enzyme.

The results of our study do not distinguish between an effect of ethanol as an inhibitor of the $(Na^+ + K^+)$-ATPase or on the availability of Na^+ to the transporting site. Ethanol does alter the structural properties of water [16] but in intact frog skin. the enzyme may be structurally protected from the influence of changes in bulk water structure. Cereijido and Rotunno [17] have addressed the role of surface migration of ions in the mechanism of Na^+ transport across frog skin. In accordance with the non-trans-cellular model of sodium transport [18], the diffusion of Na^+ along the outer epithelial surface was calculated to be faster than its diffusion through the bulk solution. The suggestion was made that this surface diffusion could be important in supplying ions to the transport site. Our results are consistent with the view that alcohol in the outer bathing solution alters the availability of Na^+ to the transporting site, perhaps by decreasing the diffusivity of the ion on the surface of the skin.

ACKNOWLEDGEMENTS

Portions of this study were presented at the meeting of the Biophysical Society, Columbus, Ohio, February 27 through March 2, 1973. This work was supported by United States Public Health Service Research Grant (5 RO 1 AA 00294) from the National Institute on Alcohol Abuse and Alcoholism of the National Institute of Mental Health, Research Contract AT (45-1)-1754 from the United States Atomic Energy Commission, and a Grant-in-aid from the Oregon Heart Association. The authors wish to acknowledge the enthusiastic technical assistance of Lisa Snook during the course of this study.

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