Brain (Na+,K+)-ATPase

OPPOSITE EFFECTS OF ETHANOL AND DIMETHYL SULFOXIDE ON TEMPERATURE DEPENDENCE OF ENZYME CONFORMATION AND UNIVALENT CATION BINDING*

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We examined effects of ethanol and dimethyl sulfoxide on the regulation and apparent thermodynamic properties of moderate affinity Na+ and K+ binding that regulates the K+-dependent phosphorylase activity of (Na+,K+)-ATPase. Ethanol and other alcohols reduced the apparent affinity for Na+ and K+ at their moderate affinity sites and increased the negative ΔH and ΔS of cation binding. Dimethyl sulfoxide had the opposite effects. Inhibition by ethanol was favored by high temperature or low K+. Ethanol potentiated inhibition of K+ binding by ATP or Mg2+. Ethanol also shifted the equilibrium between K+-sensitive and insensitive forms of (Na+,K+)-ATPase toward the K+-sensitive form; in this case, it reduced the negative ΔH and ΔS for the transition to K+-sensitive enzyme. Again, dimethyl sulfoxide had the opposite effects. These data indicate that ethanol and other agents considered to affect membrane fluidity act by a combination of membrane (on cation binding) and solvent (on conformation) effects. The most important effect of ethanol and similar agents on the enzyme is to prevent the formation of K+-sensitive enzyme by cation binding and to destabilize K+-sensitive enzyme in the presence of ATP. These results also add further evidence that the sites by which Na+ and K+ produce K+-sensitive enzyme are similar or identical.

The changes in conformation that regulate (Na+,K+)-ATPase activity appear to be sensitive to membrane and solvent structure (1). The transition from the E1 (ATP-sensitive) to the E2 (K+-sensitive) conformation has negative enthalpy and entropy (2), as does the binding of K+ and its congeners to moderate affinity "regulatory" sites (3). Changes in the amount of order in the membrane or the solvent may therefore be important in physiologic and pharmacologic effects on (Na+,K+)-ATPase. The amount of order in the cell membrane is decreased by a number of "depressant" drugs including ethanol (4). Consistent with the negative apparent entropy of K+ binding described above, ethanol has been reported to reduce the apparent affinity of (Na+,K+)-ATPase for K+ (4, 5).

The reduction in K+ affinity by ethanol could be interpreted either as a stabilization of E1, the (Na+,K+)-ATPase conformation with high ATP and low K+ affinity (3), or a direct effect on K+ binding. Previous studies of ethanol effects on (Na+,K+)-ATPase did not completely distinguish between the possibilities. In fact, Kalant and Rangaraj (6) suggested that ethanol stabilized the E2 conformation, since it prevented dephosphorylation. Cation regulation of the K+-p-nitrophenylphosphatase associated with (Na+,K+)-ATPase provides a possible means for resolving these questions, because it is possible to distinguish Na+ and K+ binding involved in phosphorylation and dephosphorylation from that involved in the regulation of enzyme conformation (3), and because K+, ATP, and Na+ effects can be studied independently (7).

It has generally been assumed that the effects of ethanol are mediated by increased fluidity of membrane lipids. Changes in solvent structure are also a possibility, however. While ethanol decreases order in the cell membrane, it may increase order in aqueous solution due to the formation of additional H2O-H2O hydrogen bonds around the ethyl moiety (8). This would be expected to increase the solvation of poorly solvated cations, such as K+ (9), possibly reducing the driving force for K+ binding. K+ binding has a negative apparent ΔS (3), as does the conformational change to E2 (2). Decreased order in the cell membrane would be expected to increase this negative entropy change, while increased order in the solvent would decrease it.

We have examined the effects of ethanol on the conformational change between E1 and E2 and on apparent thermodynamic constants describing cation interactions with (Na+,K+)-ATPase. We compared these effects to those of dimethyl sulfoxide because that compound, opposite to ethanol, has been shown to increase apparent affinity for K+ (7) and to reduce membrane fluidity (10, 11). Regulation of K+-p-nitrophenylphosphatase activity was examined because K+ and Na+ effects can be studied independently and effects of K+ and ATP binding can be distinguished (7). The experiments in this paper 1) examined effects of ethanol and dimethyl sulfoxide on the apparent thermodynamic properties of K+ binding to its regulatory site and on K+-ATP interactions, 2) compared effects on K+ and Na+ binding, and 3) examined effects on the apparent thermodynamic parameters describing the E1→E2 transition.

MATERIALS AND METHODS

Source of Tissue and Materials—Frozen beef brains were obtained from Pel-Freeze Biologicals (Rogers, AR). Tris salts of ATP (vanadium-free grade) and p-nitrophenylphosphate were obtained from Sigma. Deoxycholate and NaI-treated microsomes were prepared as described by Nakao et al. (12) and modified by us (7). Protein was determined by the method of Lowry et al. (13).

Assays of K+-p-nitrophenylphosphatase Activity—About 5–10 μg of protein were incubated in a 60-μl total volume containing 50 mM imidazole HCl, pH 7.5, 5 mM MgCl2, 10 mM Tris p-nitrophenylphosphate, 5 mM dithiothreitol, and other ligands as described in the text. Incubation was stopped with 0.6 ml of cold 0.1 M NaOH and p...
nitrophenol determined by optical absorption at 410 nm. All assays were in triplicate. K⁺-p-nitrophenylphosphatase activity associated with (Na⁺,K⁺)-ATPase was taken as the activity with K⁺ less than without added K⁺, generally about 80% of total activity. Incubation with (Na⁺,K⁺)-ATPase was taken as the activity with K⁺ less than were in triplicate.

the concentration of ethanol was 0.25 or less of the p-nitrophenylphosphate added. Unless stated otherwise, reactions were equilibrated to each temperature for 30 min, and enzyme suspensions were equilibrated for 10 min before reactions were started. Complete activation curves were carried out at each temperature.

Analysis of Data—Apparent maximal velocity and half-maximal effector concentrations were estimated by least squares curve fitting to the equation

\[ v = \frac{V((K_m/M)^n + 1)^{-n}(M/K_m)^n + 1)}{X} \]

as discussed previously (14). In this equation, M is the concentration of effector, Kₘ is the concentration giving half-maximal activation, n is the Hill coefficient, and, in the case of biphasic activation curves, Kₘ is the concentration of M causing half-maximal inhibition.

Effect of Temperature and KC1 concentration on inhibition of K⁺-p-nitrophenylphosphatase by ethanol. The effect of temperature may reflect effects of ATP or Mg²⁺ producing a half-maximal increase in Kᵢ, and AHᵢ from the Arrhenius equation (15, 16). Apparent constants for cation activation were calculated using ΔG = -RT ln Kᵢ, and ΔS = (ΔH - ΔS)/T (16). Concentrations were expressed in moles/liter. These must be considered apparent constants because Kᵢ cannot be assumed to be a simple equilibrium constant and because of possible variations of AH with temperature due to changes in heat capacity (17), as we have discussed previously (3).

Significance of differences in parameters were determined by dividing the difference by the sum of the standard errors of the estimate and comparing to the two-tailed t distribution for the appropriate number of degrees of freedom (18).

RESULTS

Effect of Temperature and KC1 on Inhibition of K⁺-p-nitrophenylphosphatase by Ethanol—Fig. 1 shows the effect of ethanol on K⁺-p-nitrophenylphosphatase activity at high and low temperature and KC1 concentration. High temperature and low KC1 increased the sensitivity of the enzyme to inhibition by ethanol. The effect of KC1 is consistent with previous reports that KC1 prevented inhibition of (Na⁺,K⁺)-ATPase by ethanol (5). The effect of temperature may reflect the temperature-dependent relationship that we have reported between temperature and apparent K⁺ affinity (3). These results can also be interpreted as suggesting that ethanol is a more effective inhibitor under conditions favoring the existence of El enzyme (2).

Inhibition of K⁺-p-nitrophenylphosphatase by Alcohols—

The abbreviation used is: Me₂SO, dimethyl sulfoxide.

![Fig. 1. Effects of temperature and KCl concentration on inhibition of K⁺-p-nitrophenylphosphatase by ethanol. The x axis shows the concentration of ethanol; the y axis, the fraction of activity compared to that without ethanol. Circles are for 40 °C and squares for 6 °C; solid lines represent 20 mM KCl, and dotted lines 2 mM KCl.](http://www.jbc.org/)

![Fig. 2. Inhibition of K⁺-p-nitrophenylphosphatase by alcohols.](http://www.jbc.org/)
we have shown that Na\(^+\) can stimulate \(\rho\)-nitrophenylphosphatase activity in a manner similar to K\(^+\) (7, 14). Fig. 4 shows that ethanol reduced the apparent affinity for this effect of Na\(^+\), similar to its effects on K\(^+\).

**Effects of Dimethyl Sulfoxide on K\(^+\) Activation**—We have previously shown that Me\(_2\)SO increased K\(^+\) affinity at the moderate affinity site (7). Me\(_2\)SO has been reported to promote K\(^+\)-dependent phosphatase activity and phosphoryl enzyme hydrolysis (20) and to reduce membrane fluidity (10, 11). As shown in Fig. 5, Me\(_2\)SO increased apparent K\(^+\) affinity at each temperature studied and appeared to decrease the slope of the regression line, opposite to the effects of ethanol.

**Summary of Effects on Temperature Dependency of K\(^+\) Activation**—Estimates of apparent \(\Delta H\) and \(\Delta S\) for cation binding can be obtained from the temperature dependence of \(K_a\) as described under “Materials and Methods.” Table II summarizes the effects of ethanol and Me\(_2\)SO on these parameters. Ethanol increased, and Me\(_2\)SO decreased, \(\Delta H\) and \(\Delta S\).

**TABLE II**

| Conditions      | \(\Delta H\) (kcal/mol) | \(\Delta S\) (cal/mol) |
|-----------------|-------------------------|------------------------|
| K\(^+\), control | -18                     | 36                     |
| K\(^+\) + ethanol| -36                     | -70                    |
| K\(^+\) + Me\(_2\)SO | -29                    | 25                     |
| Na\(^+\), control | 8                       | -32                    |
| Na\(^+\) + ethanol| -31                    | 13                     |

**Fig. 3. Effects of ethanol on temperature dependence of K\(^+\)-\(\rho\)-nitrophenylphosphatase.** Solid circles are without and open circles with 0.25 M ethanol. The y axis shows \(\ln K_a\) for K\(^+\) activation at each temperature.

**Fig. 4. Effect of ethanol on temperature dependence of Na\(^+\) activation of K\(^+\)-\(\rho\)-nitrophenylphosphatase.** Reactions were carried out as described under “Materials and Methods” with 0.5 mM Tris/ATP and 0.5 mM KCl. Solid circles are without and open circles are with ethanol. The y axis shows \(\ln K_a\) for Na\(^+\) activation at each temperature.

**Fig. 5. Effect of dimethyl sulfoxide on temperature dependence of K\(^+\) activation of K\(^+\)-\(\rho\)-nitrophenylphosphatase.** Open circles are without and closed circles are with 20% dimethyl sulfoxide. Otherwise, data are presented described as in the legend to Fig. 3.

Effect was greatest at high temperature. Ethanol did not alter affinity of the high affinity K\(^+\) site (\(K_a = 0.114\) mM with and 0.100 mM without ethanol; data not shown).

In the presence of ATP and very low K\(^+\) concentrations,
Ethanol had similar effects on $\Delta H$ and $\Delta S$ for Na$^+$ activation. 

Interactions between ATP and $K^+$—We have previously shown that ATP simultaneously decreased $V$ and increased $K_0$ for $K^+$ activation of $K^+$-p-nitrophenylphosphatase (7). These effects have different concentration requirements and represent competition with ATP for the phosphoryl acceptor site and stabilization of the $E_1$ (low $K^+$ affinity) enzyme conformation, respectively (7). As shown in Fig. 6, ethanol markedly enhanced the ability of any concentration of ATP to increase $K_0$ without affecting $V$ appreciably.

Table III summarizes the effects of $K^+$ congeners and ligands that alter apparent affinity for $K^+$ on inhibition of $K^+$-p-nitrophenylphosphatase by ATP. The apparent $K_i$ for ATP varied inversely with $K_0$ for the activating cation.

**TABLE III**

| Ligand      | $K_0$, cation activation, mM | $K_i$, ATP inhibition, mM |
|-------------|------------------------------|----------------------------|
| $K^+$       | 1.82                         | 0.141                      |
| NH$_4^+$    | 8.23                         | 0.069                      |
| Rb$^+$      | 0.87                         | 0.216                      |
| $K^+$ + Me$_2$SO | 1.00                     | 0.240                      |
| $K^+$ + ethanol | 4.00                     | 0.090                      |

a ATP inhibition curves were carried out with 2 mM KCl or RbCl or with 10 mM NH$_4$Cl.

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** Mg$^{2+}$ effects on $K^+$ activation of $K^+$-p-nitrophenylphosphatase. The data in this figure are analogous to those described in the legend to Fig. 6, except that the parameters describe $K^+$ activation curves at a series of fixed Mg$^{2+}$ concentrations with open circles and dashed lines or without closed circles and solid lines 0.15 M ethanol. A, maximal velocity; B, apparent affinity for $K^+$.

Ethanol increased and Me$_2$SO decreased apparent affinity for ATP activation, opposite to their effects on $K^+$ affinity.

Interactions between Mg$^{2+}$ and $K^+$—The apparent affinity for $K^+$ is a function of the Mg$^{2+}$ concentration (14). Therefore, effects on $K^+$ activation may be secondary to effects on affinity for Mg$^{2+}$. Fig. 7 shows the effect of Mg$^{2+}$ on $V$ and $K_0$ for $K^+$ activation with and without 0.15 M ethanol. Ethanol increased the apparent affinity for Mg$^{2+}$ as an activator of $K^+$-p-nitrophenylphosphatase (Fig. 7A), consistent with our previous description of an inverse relationship between $K^+$ binding and Mg$^{2+}$ affinity (14). Ethanol also decreased the concentration of Mg$^{2+}$ required to reduce $K^+$ affinity. Ethanol did not alter the proportionate increase in $K_0$ with increased Mg$^{2+}$, however (Fig. 7B).

Apparent Thermodynamic Constants for the $E_1$-$E_2$ Conversion—Because Li$^+$ does not interact appreciably with the moderate affinity $K^+$ site, Li$^+$-p-nitrophenylphosphatase activity without Na$^+$ or ATP is an index of the amount of enzyme that is spontaneously in the $E_2$ conformation and therefore has accessible catalytic $K^+$ sites (2). Thermodynamic constants describing the $E_1$-$E_2$ conversion can thus be derived from the temperature dependence of the ratio of Li$^+$- to $K^+$-p-nitrophenylphosphatase (Equation 5 under "Materials and Methods; Ref. 2). Fig. 8 shows that ethanol decreased the ratio, or $E_2/(E_1 + E_2)$, at each temperature. The apparent thermodynamic constants are summarized in Table IV. Ethanol decreased apparent $\Delta H$ and $\Delta S$ for the $E_1$-$E_2$ conversion. As we have previously reported (2), these parameters were increased by Me$_2$SO. Ethanol reduced and Me$_2$SO increased the temperature at which $E_1$ and $E_2$ were equal.

![Fig. 8](http://www.jbc.org/)

**Fig. 8.** Proportion of $E_2$ enzyme ($E_2/(E_1 + E_2)$ ratio) as a function of temperature. The open circles and dotted lines are with and the closed circles and solid lines are without 0.25 M ethanol. The ratios were calculated using Equation 3 and the maximal velocities from $K^+$ or Li$^+$ activation curves, with or without ethanol, at each temperature.

**TABLE IV**

| Constant | Control | Ethanol | Me$_2$SO |
|----------|---------|---------|----------|
| $\Delta H$ (kcal/mol) | $-24.14 \pm 0.97$ | $-18.2 \pm 0.34^c$ | $-29.54 \pm 1.93$ |
| $\Delta S$ (e.u.) | $-87.9 \pm 3.4$ | $-69.8 \pm 1.25^c$ | $-105.84 \pm 3.81^d$ |
| $T_c$ (°C) | 274.6 | 261 | 281 |
| $K$ (0 °C) | 1.287 | 0.210 | 3.260 |
| $K$ (37 °C) | 0.007 | 0.004 | 0.008 |

*Constants were derived from the direction $E_1$-$E_2$ as described under "Materials and Methods" and in Ref. 2.

*Ethanol different from control, $t = 3.97$, $p = 0.0026$.

*Ethanol different from control, $t = 4.56$, $p = 0.001$.

*Me$_2$SO different from control, $t = 2.56$, $p = 0.035$.

*The temperature at which $E_1 = E_2$.

/ For $E_2/E_1$. 

*For $E_2/E_1$. 

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**DISCUSSION**

**Regulation of Phosphatase Activity by Univalent Cations**—

(Na⁺,K⁺)-ATPase can exist in two conformations or groups of conformations, here called E1 and E2 (1). E1 has high affinity for ATP and low affinity for K⁺, while E2 has the opposite properties. E1 can be converted to E2 by the interaction of either Na⁺ or K⁺ with moderate affinity regulatory sites (7). In the case of K⁺, this corresponds to the α (21) or β (7) site. In the case of Na⁺, apparent affinity of the regulatory site is somewhat lower than that for Na⁺-dependent phosphorylation or ADP-ATP exchange, and the site appears distinct from the site involved in phosphorylation (22). In the E2 state, a high affinity "catalytic" site (β according to Robinson (21) or C in our previous papers (7)), which is involved in p-nitrophenylphosphate hydrolysis, is accessible. The regulatory and catalytic sites differ in catalytic selectivity (2, 21), temperature dependence (3), and, by about 56-fold, apparent K⁺ affinity (7). Ethanol reduced K⁺ affinity of regulatory, but not catalytic sites. The Na⁺ and K⁺ interactions described in this paper involve the regulatory sites. These experiments permit no conclusions on the orientation of the Na⁺ and K⁺-binding sites. Strictly speaking, in fact, the term "site" in these studies can only refer to a saturable kinetic effect, rather than a location on the enzyme. Experiments where sides could be distinguished suggest that the Na⁺ regulatory site is on the inside and the high affinity K⁺ site on the outside of the membrane (1). Results of experiments using cholesterol depletion (23), inhibition of ouabain binding by internal KCl (24), and K⁺-mediated p-nitrophenylphosphate activity in inside-out red blood cell vesicles (25) suggest that the K⁺-regulatory site is on the inner face of the membrane. As the affinity for this site is markedly reduced by ATP (3, 7), it may represent a K⁺-discharge site. K⁺-phosphatase activity could thus be considered as a function of an "occluded K⁺" (26, 27) enzyme form.

**Effects of Temperature and K⁺ on Inhibition of K⁺-p-nitrophenylphosphatase by Ethanol**—High K⁺ and low temperature reduced inhibition of K⁺-p-nitrophenylphosphatase by ethanol (Fig. 1). Because apparent affinity for K⁺ increases at low temperatures (3), this is consistent with suggestions that K⁺ has competitive effects on inhibition by ethanol (5). Ethanol inhibits most effectively under conditions favoring E1 over E2, suggesting that ethanol acts by directly preventing the E1-E2 conversion or by preventing the cation binding that leads to it.

**Temperature Dependence of K⁺ Activation**—Ethanol increased Kₐ, ΔH, and ΔS for K⁺ activation via the K⁺ regulatory site, while Me₂SO had the opposite effects on these parameters. These effects on apparent affinity for K⁺ confirm earlier work performed at a single temperature (5, 7). The relationship between changes in Kₐ and in ΔH and ΔS was opposite to that previously reported among univalent cations between K⁺ and the thermodynamic constants, where ΔH and ΔS increased as Kₐ decreased (3).

**Comparison of Effects on Na⁺ and K⁺**—Ethanol reduced the apparent Na⁺ affinity in the presence of low concentrations of ATP and K⁺, similar to its effects of K⁺ affinity. Apparent ΔH and ΔS were increased, also similar to effects on K⁺.

By using the K⁺-p-nitrophenylphosphate assay, it is possible to study interactions of Na⁺ with its regulatory site in the presence of very low K⁺ concentrations (0.2 mM), essentially dissociating Na⁺ and K⁺ effects. This concentration of K⁺, which is small compared to the Kₐ for K⁺ on Na⁺ activation, is required for binding to the high affinity catalytic site (7).

We have previously shown that, in the presence of low K⁺ and ATP, Na⁺ stimulated K⁺-p-nitrophenylphosphatase activity (7). This effect had similar concentration dependence (7), Hill coefficient (7), and Mg²⁺ interactions (14) to the moderate affinity stimulation of K⁺-p-nitrophenylphosphatase activity by K⁺. Me₂SO also increased the affinity for both Na⁺ and K⁺ (7). Na⁺ and K⁺ activation also is similar with respect to temperature dependence, with decreasing Kₐ as temperature decreases (3). In terms of the relationship among alkali metal cations between K⁺ and apparent thermodynamic parameters for activation, Na⁺ is intermediate between K⁺ and Li⁺ (3). These findings all suggest that properties of the "regulatory" site by which Na⁺ and K⁺ produce K⁺-sensitive enzyme (7) are analogous.

**Interactions between K⁺ and ATP or Mg²⁺—K⁺ and ATP are antagonistic in (Na⁺,K⁺)-ATPase regulation, each stabilizing an enzyme conformation with low affinity for the other (7). Ethanol increased the ability of ATP to reduce K⁺ affinity (Fig. 6) and increased the apparent affinity for ATP as an inhibitor of K⁺-p-nitrophenylphosphatase (Table III). These effects of ethanol were limited to the partial competition between ATP and K⁺, with little effect on competition between ATP and p-nitrophenylphosphate (Fig. 6).

In addition to ethanol, Me₂SO had inverse effects on apparent K⁺ and ATP affinity. K⁺ for the ATP also varied inversely with K⁺ for p-nitrophenylphosphatase activation among univalent cations (Table III). Kₐ for activation of p-nitrophenylphosphatase by K⁺ or Na⁺ is a saturable function of Mg²⁺ concentration (14). Ethanol increased the apparent affinity for Mg²⁺ for both reduction of K⁺ affinity and activation of p-nitrophenylphosphatase (Fig. 7). The latter is reduced by K⁺, with concentration dependence resembling that of the K⁺ regulatory site (14). Kₐ, which increased about 10-fold when [Mg²⁺] was increased from 0.125 to 5 mM, with or without ethanol. Effects of ethanol on Mg²⁺ and ATP thus appear to be secondary to reduction in K⁺ affinity at its regulatory site or to enhancement by ethanol of an enzyme conformation with low affinity for K⁺.

**Effects of Ethanol on Enzyme Conformation**—Ethanol reduced the proportion of E2 at all temperatures (Figs. 8 and Table IV). At 37 °C this effect was not quantitatively important because, even without ethanol, less than 1% of the enzyme was E2. While ethanol cannot reduce E1 directly, its more important effects therefore appear to be the prevention of the E1-E2 transition by univalent cations and destabilization of E2 in the presence of ATP.

Ethanol reduced the apparent ΔH and ΔS for the transition from E1 to E2, opposite to its effects on these parameters for K⁺ activation. This suggests that the mechanism of the effect of ethanol on enzyme conformation is, at least in part, different from that of its effects on K⁺ binding. Me₂SO, in contrast to ethanol, decreased Kₐ, ΔH, and ΔS for K⁺ binding and increased apparent ΔH and ΔS for the E1-E2 transition (2).

**Thermodynamic Properties of K⁺ Binding**—Apparent affinity for K⁺ was decreased by increased temperature and by alcohols. K⁺ binding had appreciable negative ΔH and ΔS, which were increased by alcohols. In contrast, we have shown that K⁺ affinity of the high affinity K⁺ catalytic site was independent of temperature (3). At low temperatures, apparent affinity and other properties of the regulatory site were shown to approach those of the catalytic site (3).

Poorly hydrated cations, such as K⁺, have lower activity coefficients in more structured solvents (28). Therefore, the relationship between K⁺ affinity and temperature could depend on whether the water in the K⁺ site was ordered by the surrounding protein structure (7) or was similar to the surrounding water. Increased order in the solvent, such as from a hydrophobic solute (8), would be expected to reduce the negative ΔS associated with cation binding.
The increase in $\Delta S$ by ethanol therefore appeared not to be a solvent effect. By contrast, $\Delta S$ and apparent affinity increased in parallel among univalent cations (3), suggesting that this relationship is in part due to differences in hydration. The effects of Me$_2$SO were opposite to those of ethanol and were consistent with decreased membrane fluidity. Me$_2$SO has previously been reported to reduce the apparent fluidity of nerve cell myelin (10) and of phospholipid vesicles (11).

Ethanol Effects on Thermodynamic Properties of the E1–E2 Transition—Because the E1–E2 transition has negative $\Delta H$ and $\Delta S$, E2 is stabilized by forces that require a decrease in entropy (2, 3). Ethanol reduced apparent $\Delta H$ and $\Delta S$, opposite to its effects on cation binding. One would expect reduction of membrane order by ethanol to increase the $\Delta S$ for the E1–E2 transition. This effect of ethanol is therefore more consistent with a solvent effect. The same is true of Me$_2$SO, which we have previously shown (2) to increase $\Delta S$, $\Delta S$, and $\Delta H$.

Relationship to Pharmacologic Effects of Ethanol—Ethanol has been shown to affect a large number of interrelated membrane systems, and it would be unrealistic to implicate any single effect in its acute or chronic behavioral actions. The primary purpose of these studies was to use ethanol as a probe for effects of membrane fluidizers on (Na',K')-ATPase regulation. Despite the relatively high concentration required for effects on (Na',K')-ATPase, however, there is evidence suggesting that effects on this enzyme may be among the behaviorally relevant actions of ethanol, including partial reversal of behavioral effects by K$^+$ administration (29), sensitization of (Na',K')-ATPase to ethanol by norepinephrine (6), reports that ethanol tolerance involves changes in membrane lipids that reduce the sensitivity of (Na',K')-ATPase to ethanol (30), and reports of altered (Na',K')-ATPase activity in ethanol withdrawal (31, 32).

An additional question raised by these studies is that of why ethanol did not increase (Na',K')-ATPase activity. Increased membrane fluidity caused by increased temperature (3) or by alterations in the cholesterol/phospholipid ratio (23), like ethanol, decrease apparent affinity for K$^+$, but increase enzyme activity. While the experiments reported here do not address this apparent paradox directly, they suggest the possible explanation that, while high concentrations of ethanol decrease order in the cell membrane, they may increase order in the surrounding solvent (8), which might reduce enzyme turnover.

Alcohol Effects and the (Na',K')-ATPase Reaction Cycle—Fig. 9 summarizes the overall (Na',K')-ATPase reaction sequence. Due to mutual inhibition between and partially analogous roles of Na$^+$ and K$^+$, mutual inhibition between K$^+$ and ATP, increased Na$^+$ and decreased K$^+$ affinity by ATP (14), and the presence of two nonequivalent K$^+$ sites (7, 21), the (Na',K')-ATPase reaction presents several interacting variables simultaneously. By using K$^+$-p-nitrophenylphosphatase activity, we have been able to separate these variables partially. The data in this paper agree with several earlier reports that ethanol and K$^+$ have antagonistic effects on (Na',K')-ATPase (4–6, 29, 32, 34). Furthermore, they show that ethanol appears to enhance ATP and Mg$^{2+}$ binding through its effects on apparent K$^+$ affinity or on conformational changes mediated by K$^+$. In addition, ethanol reduced the apparent affinity of the regulatory site for Na$^+$, analogous to its effects on K$^+$.

These results appear to conflict with an earlier report by Israel and Salazar (4) that ethanol inhibition of (Na',K')-ATPase activity was enhanced by Na$^+$ and that ethanol enhanced the reduction of K$^+$ affinity by Na$^+$. A possible explanation for this conflict is based on the fact that ATP reduces K$^+$ affinity and increases Na$^+$ affinity (7). When Na$^+$, K$^+$, and ATP are all present, K$^+$ must dissociate before re-binding of ATP (3, 27); this is enhanced by ethanol (Table III and Fig. 6). Thus, ethanol would potentiate the effect of ATP on relative affinity for Na$^+$ and K$^+$ by increasing ATP binding. Similarly, the lack of ethanol effect on $K_c$ for Na$^+$ for the overall (Na',K')-ATPase reaction reported by Lin (33) is consistent with offsetting effects of ethanol on Na$^+$ and ATP (via K$^+$) binding.

Ethanol favors E1 over E2 forms of enzyme, both by shifting the equilibrium toward E1 (Table IV and Fig. 8) and by inhibiting the conversion of E1 to E2 by univalent cation binding. K$^+$ binding leading to the E1–E2 transition is distinct from that directly involved in dephosphorylation of E2P (3, 7, 21), and these can be distinguished using K$^+$-p-nitrophenylphosphatase activity. Ethanol reduced binding of K$^+$ to the moderate affinity site involved in conformational change but had no effect on apparent affinity for the high affinity site, which represents K$^+$ binding to E2 (2, 3, 7, 21). The data in this paper thus show that ethanol both prevents E1–E2 and enhances E2–E1 (Table III), rather than stabilizing E2 as suggested by Kalant and Rangaraj (6). K$^+$-sensitive and -insensitive forms of E2P exist (22, 35). Ethanol appears to stabilize phosphoryl enzyme (6) by inhibiting formation of K$^+$-sensitive phosphoryl enzyme rather than preventing its K$^+$-mediated hydrolysis.

General Conclusions—Increased membrane fluidity, whether produced by temperature or ethanol, was associated with decreased affinity for K$^+$ and increased affinity for ATP and Mg$^{2+}$. Agents that increase membrane fluidity also increased the apparent $\Delta H$ and $\Delta S$ for cation binding. Me$_2$SO, which appears to decrease membrane fluidity (10), had the opposite effects. Effects on the regulatory site for Na$^+$ were analogous to those for K$^+$.

The proportion of enzyme in the E2 state was also altered by ethanol, consistent with an inverse relationship between E2 and membrane fluidity. In this case, the effects of ethanol and Me$_2$SO on $\Delta H$ and $\Delta S$ were opposite to their effects on these parameters for cation binding. These data suggest that the effects of ethanol and Me$_2$SO on cation binding resulted from membrane, rather than solvent, effects. The previously reported variation in affinity and thermodynamic binding parameters among cations (3), however, appeared to result from variations in degree of hydration. The direct effects of ethanol or Me$_2$SO on enzyme conformation also appear to be, at least in part, effects of increased solvent order rather than decreased membrane order.

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