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EFFECT OF ETHANOL ON THE KINETICS OF RAT BRAIN ($Na^+ + K^+$) ATPase AND K^+-DEPENDENT PHOSPHATASE WITH DIFFERENT ALKALI IONS

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Abstract—($Na^+ + K^+$)-dependent ATPase [(Na + K)-ATPase] and K^+-dependent *p*-nitrophenyl phosphatase [pNPPase] activities in rat brain heavy microsomal fractions were studied in the presence of 120 mM Na^+ and varied concentrations of K^+, Rb^+, Cs^+, Li^+ or NH_4^+. Scatchard and Hill plots indicated non-hyperbolicity (cooperativity) with all except Li^+, which supported a considerably lower activity than any of the other ions tested. Addition of 0.22 M ethanol to the incubation mixtures produced a formally competitive inhibition of ATPase activity with K^+, Rb^+ and Cs^+, a non-competitive inhibition with Li^+, and a mixed inhibition with NH_4^+. The changes in pNPPase activity generally followed a similar but less clear-cut pattern. The values of the Hill constants were not changed for either enzyme activity. The findings are interpreted as evidence that ethanol inhibits ATPase activity by inducing conformational changes which alter the consequences of ion binding to the various receptor sites.

It has been shown in earlier studies that ethanol (EtOH), in concentrations which produce *in vivo* mild to severe intoxication, inhibits the active transport of cations in a variety of tissues. This has been demonstrated with isolated frog skin [1], human erythrocytes [2] and guinea pig and rat brain cortex slices [3–5]. The active transport of cations involves the Na^+, K^+, Mg^{2+}-stimulated adenosine triphosphatase [(Na + K)-ATPase] of the cell membrane [6]. EtOH was found to inhibit the activity of this ATPase in beef brain microsomal preparation [7] and synaptosomes from guinea pig brain [8].

An apparent competitive antagonism between EtOH and K^+ with respect to their effects on both enzyme activity and ion transport has been reported previously [9]. Since then, kinetic studies of the effect of Na^+ and K^+ on the binding of ouabain and other inhibitors to the (Na + K)-ATPase have indicated the functional significance of conformational changes of the protein structure [10, 11]. There appear to be at least two K^+-binding sites of non-equivalent nature [12], which interact allosterically [13, 14]. The present study deals with the possibility that the interaction of EtOH and K^+ with the enzyme may also be of an allosteric nature. This possibility has been further explored by replacing K^+ by other monovalent cations which can replace it with varying degrees of efficacy [15].

Brain membrane preparations show in addition a K^+-stimulated *p*-nitrophenyl phosphatase (pNPPase) activity which is believed by many, but not all, investigators to reside in the same enzyme molecule as the (Na + K)-ATPase activity [16]. Since the pNPPase activity is measured in the absence of Na^+, the ability of the other ions to replace K^+ can be studied without the possibility of complicating interactions with Na^+. The effect of ethanol on the pNPPase activity was therefore also studied in the presence of the various replacement ions.

METHOD

Preparation of heavy microsomal ATPase from rat brain. Male albino rats (Wistar strain, Canadian Breeding Laboratories, Montreal) weighing 350–400 g were maintained on Purina rat chow and tap water *ad lib.* For preparation of ATPase, the rat was decapitated, and the brain was immediately removed at room temperature and homogenized in 10 vol. of ice-cold 0.25 M sucrose solution in buffer containing 20 mM NaCl, 5 mM Na_2EDTA, 1 mM $MgCl_2 \cdot 6H_2O$ and 10 mM imidazole at pH 7.2. The heavy microsomal fraction was isolated by the method of Post and Sen [17]. Any required dilution of the enzyme was made just before use, with imidazole buffer (1 N HCl, 0.1 mM H_4EDTA and 10 mM imidazole). The enzyme preparation is stable for about 2 weeks at 2–4°, but no preparation was kept for more than 10 days.

ATPase assay. A 0.1-ml portion of suitably diluted enzyme preparation, together with other additions as indicated below, was added to 1.0 ml of buffer–substrate solution to give a final volume of 1.5 ml with a final concentration of 3 mM $Na_2ATP \cdot 3H_2O$, 2.7 mM $MgCl_2 \cdot 6H_2O$, 20 mM imidazole and 20 mM glycylglycine at pH 7.6. In each run, tubes containing 0.17 mM ouabain and no added Na^+ or K^+ were included for measurement of the Mg-ATPase. For measurement of total activity the remaining tubes contained no ouabain, but 120 mM Na^+ was added together with 1–10 mM K^+ or Rb^+, 5–250 mM Li^+, 1–20 mM Cs^+ or 1–100 mM NH_4^+, all added as their chlorides. In a parallel series in each run, sufficient water was replaced by EtOH solution to give a final EtOH concentration of 0.22 M, to study the inhibitory effects of EtOH.

All assays were run in duplicate. All components of the incubation mixture were preincubated at 37° for 3 min and the reaction was started by the ad-

dition of the enzyme. It was stopped after 20 min by 0.5 ml of cold 1.2 M $HClO_4$. Inorganic phosphate was determined by the method of Fiske and Subbarow [18]. The reducing agent, 1-amino-2-naphthol-4-sulfonic acid (ANSA), must be added to the solution before the addition of molybdate in order to prevent formation of a precipitate in the presence of Cs^+. Skou [19] was able to prevent precipitation of Cs^+ in concentrations up to 60 mM or more when he took this precaution, but in our hands the Cs^+ concentration could not exceed 20 mM. Perhaps this is because Skou used amidol instead of ANSA as the reducing agent. The other cations did not interfere with the estimation.

Protein content was determined by the Lowry and Lopez [20] method. Protein stock standard solution was prepared from a solution containing 10 mg protein-N/ml (Armour Pharmaceutical Co.). All protein assays were run in duplicate. (Na + K)-ATPase activity was calculated by subtracting the Mg-ATPase activity from the total activity in the presence of other cations. Specific activity of the (Na + K)-ATPase was expressed in μmoles P_i/mg of protein/hr. Three different enzyme preparations were used for the assay of (Na + K)-ATPase with and without the addition of 220 mM EtOH in the presence of K^+. Identical experimental procedures and enzyme preparations were used when K^+ was replaced by NH_4^+, Rb^+ or Cs^+. Five enzyme preparations were used for the assays in which Li^+ replaced K^+.

pNPPase assay. Heavy microsomal preparations from rat brain were prepared exactly as for ATPase measurement. The reaction mixture for pNPPase assay contained 0.2 ml of suitably diluted enzyme preparation plus 3.75 mM $MgCl_2 \cdot 6\ H_2O$, 1.5 mM Tris·HCl, 7.5 mM *p*-nitrophenylphosphate in a final volume of 2.4 ml at pH 7.6. Each run included tubes containing 1.8 mM ouabain for measurement of non-specific (i.e. K^+ independent) pNPPase activity. For measurement of total activity, the remaining tubes contained no ouabain, but K^+, Rb^+, Cs^+, Li^+ or NH_4^+ in the same concentrations as used in the ATPase study. In addition, each run included a parallel set with 0.22 M EtOH.

All assays were run in triplicate. After a 3-min preincubation of the components at 37°, the reaction was started by addition of the enzyme and continued for 20 min. It was stopped by addition of 0.4 ml of 0.6 M trichloracetic acid. The incubation mixture was centrifuged for 10 min at 6000 rev/min in a Sorvall RC-2 centrifuge at 0°. A 0.2-ml portion of the supernatant fraction was added to 1 ml of 1 M Tris solution (pH 10.7), and the absorbance at 410 nm was measured in a Gilford model 250 spectrophotometer. K^+-dependent activity was obtained from the difference between the total and the non-specific activity. Protein measurement and expression of activity were as described for ATPase assay. Li^+-stimulated activity was measured in four separate preparations, while five preparations were used with each of the other ions.

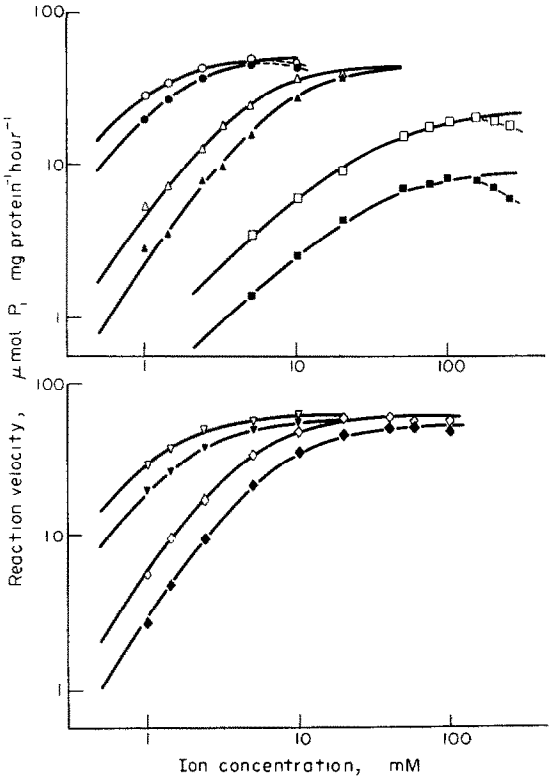

Fig. 1. Double-logarithmic reaction velocity curves for (Na + K)-ATPase activity of rat brain microsomal preparations in the presence of 120 mM Na^+ and varied concentrations of other cations: ▽, K^+; ○, Rb^+; △, Cs^+; ◇, NH_4^+; and □, Li^+. Open symbols indicate the values in the absence of ethanol, and filled symbols indicate values for the same preparation in the presence of 0.22 M ethanol. Each point represents the mean of three to five different preparations. Solid lines are computed rate curves over the concentration ranges indicated, using the computed parameters given in Table 1. Broken lines indicate inhibition encountered at higher ion concentrations.

RESULTS

Figure 1 shows a double-logarithmic plot of the ATPase activity for each cation, in the presence and absence of ethanol. It is clearly evident that the K^+ may be replaced successfully by the other activating monovalent cations tested in the presence of 120 mM Na^+. The effectiveness of the various ions in stimulation of (Na + K)-ATPase activity, in the ascending portions of the respective curves, appears to be in the following order: $Li^+ < Cs^+ < NH_4^+ < Rb^+ < K^+$. This is in agreement with the findings of Skou [6].

Corresponding plots for the pNPPase activity are shown in Fig. 2. The order of relative effectiveness of the various ions is identical to that found with the ATPase activity. This finding is quite similar though not identical to that reported by Bader and Sen [21].

When plotted on an arithmetic scale, the rate curves were all non-sigmoidal. The maximum velocity for NH_4^+ was reached at a concentration of about 30 mM, and was greater than those shown by the other ions. Maximum velocities for Rb^+, K^+, Cs^+ and Li^+ were reached at about 5, 10, 20 and 150

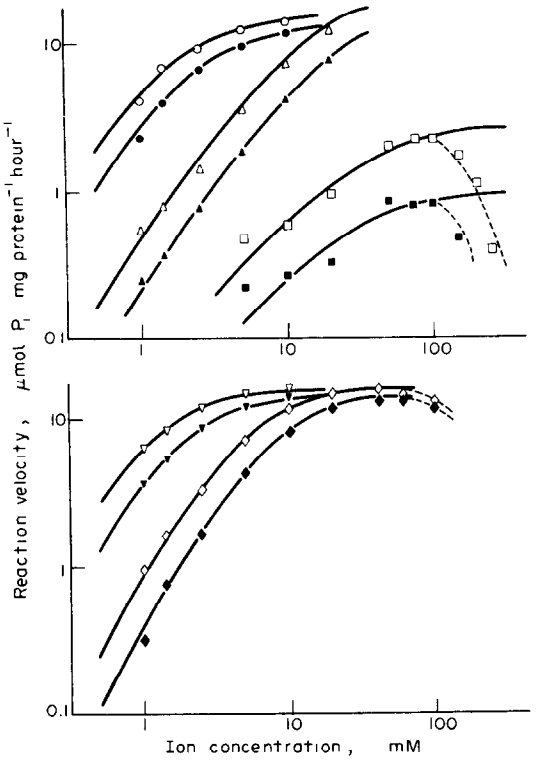

Fig. 2. Double-logarithmic reaction velocity curves for pNPPase activity of rat brain microsomal preparations in the absence of added sodium, but with varied concentrations of other cations. Symbols and lines are as explained in the legend to Fig. 1.

mM respectively. Self-inhibition, or a non-specific inhibitory effect of high ionic strength, was evident at higher cation concentrations. This resulted in an intersection of the Rb^+ and NH_4^+ curves at about 10 mM.

The results for pNPPase activity were quite similar. The concentrations at which maximal activity was reached were about 10, 10, 20–25, 30 and 100 mM for K^+, Rb^+, Cs^+, NH_4^+ and Li^+ respectively. Self-inhibition was again seen at higher concentrations of NH_4^+ and Li^+ (Fig. 2).

EtOH inhibited both enzyme activities in all the parallel assays of the different preparations. At a cation concentration of 5 mM, the inhibition of ATPase activity is roughly inversely proportional to the degree of activity in the controls: 10 per cent for K^+, 5 per cent for Rb^+, 36 per cent for Cs^+ and NH_4^+, and 88 per cent for Li^+. The corresponding degrees of inhibition for pNPPase activity were 16, 20, 39, 40 and 69 per cent.

Scatchard and Lineweaver–Burk transformations of the results showed marked curvatures which suggested that cooperativity may be present between different ionic-binding sites on the enzyme, and that the inhibitory effect of EtOH may result from an allosteric interaction.

Further analysis of this possibility was based on the Hill equation [22, 23]:

$$v = \frac{Vc^n}{K^n + c^n}$$

where v is the reaction velocity, c the substrate concentration and V the asymptotic maximal velocity, and the parameter K (a Michaelis-type and not a dissociation constant) equals the concentration at $v = V/2$.

The results for control and EtOH groups were evaluated separately by unweighted nonlinear regression. The implied constancy of observational errors was compatible with the known behaviour of the experimental system, and with the results of an analysis of residuals (differences between the observations and the calculated values predicted by regression) [24]. However, this assumption is not critical, since nearly identical conclusions were reached by the use of a weighted nonlinear regression with weights based on the assumption of approximately constant percentage error of the experimental observations. The sets of calculations were then repeated, assuming in turn the identity of V, K or n for the control and EtOH rate curves. The assumption of identity of the investigated

Table 1. Computed kinetic parameters for rat brain (Na + K)-ATPase and p-NPPase activities supported by K^+ and by substitute cations, in the absence and presence of 0.22 M ethanol

	V (μmoles mg^{-1} hr^{-1})		K (mM^{-1})		n	
Ion	Control	Ethanol	Control	Ethanol	Control	Ethanol
(Na + K)-ATPase						
K^+	63.7 ± 2.9*	59.8 ± 0.7	1.13 ± 0.09	1.65 ± 0.04†	1.50 ± 0.28	1.45 ± 0.05
Rb^+	53.2 ± 1.6	51.3 ± 0.3	0.94 ± 0.04	1.38 ± 0.02†	1.29 ± 0.12	1.60 ± 0.02
Cs^+	44.4 ± 2.3	48.4 ± 3.2	4.21 ± 0.42	8.36 ± 1.00†	1.27 ± 0.17	1.39 ± 0.10
NH_4^+	62.8 ± 2.1	54.1 ± 0.5†	4.58 ± 0.36	6.80 ± 0.15†	1.51 ± 0.13	1.50 ± 0.04
Li^+	24.0 ± 1.1	8.9 ± 0.6†	29.5 ± 3.5	20.4 ± 3.8	1.05 ± 0.07	1.13 ± 0.17
K^+-pNPPase						
K^+	15.9 ± 0.2	14.6 ± 0.1†	1.32 ± 0.03	1.99 ± 0.03†	1.66 ± 0.08	1.65 ± 0.08
Rb^+	15.5 ± 0.7	13.6 ± 0.8	1.80 ± 0.15	2.49 ± 0.28	1.52 ± 0.18	1.53 ± 0.18
Cs^+	21.2 ± 1.1	16.3 ± 1.2	14.3 ± 1.1	20.0 ± 1.9	1.44 ± 0.05	1.45 ± 0.05
NH_4^+	16.5 ± 0.3	14.4 ± 0.2†	5.54 ± 0.23	8.06 ± 0.21†	1.72 ± 0.09	1.74 ± 0.05
Li^+	2.9 ± 0.8	1.1 ± 0.6	31.6 ± 18.9	28.5 ± 32.3	1.17 ± 0.39	1.08 ± 0.62

* Mean ± S.E. of computed parameter.

† Significantly different from corresponding control value ($P < 0.05$).

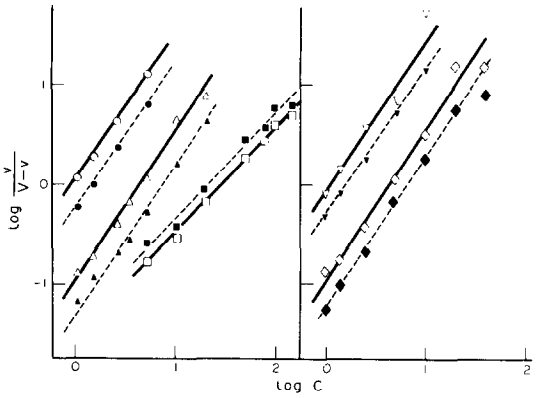

Fig. 3. Hill plots corresponding to the plotted points in Fig. 1 and computed parameters for ATPase in Table 1. Symbols are explained in the legend to Fig. 1.

parameter was then accepted or rejected on the basis of the extra sum of squares principle [25]. The calculated parameters for the two enzyme activities are shown in Table 1.

The values for n for control and EtOH groups did not differ significantly in any instance. Therefore, a pooled value was calculated for each ion with each enzyme. With these final estimated Hill constants, and the V and K values in Table 1, the predicted rate curves for the various ions, with and without EtOH, were computed for ion concentrations below the point at which inhibition became evident. As seen in Figs. 1 and 2, the computed curves, plotted on a double-logarithmic graph, fit the observed values very well.

The corresponding Hill plots based on the same parameters (Table 1) are shown in Figs. 3 and 4. Inspection of Table 1 reveals a few apparent differences between the findings for the two enzymes. In the presence of K^+, Rb^+ and Cs^+ the maximal velocities and Hill coefficients for ATPase activity in the control and the EtOH group are not significantly different. In each case, the Hill constant significantly exceeds unity, thereby indicating positive non-hyperbolicity [26–28]. The Michaelis-type constants are significantly higher in the presence of EtOH, the increments being 46, 47 and 98 per cent for K^+, Rb^+ and Cs^+ respectively.

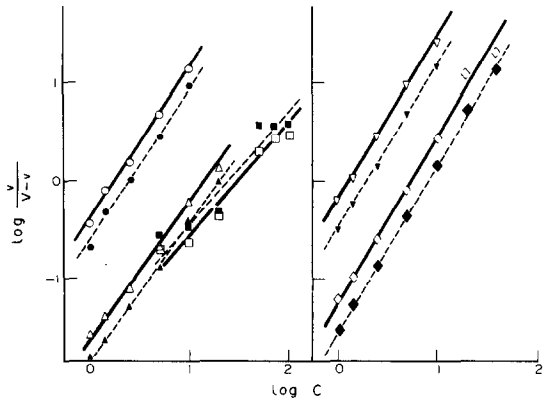

Fig. 4. Hill plots corresponding to the plotted points in Fig. 2 and computed parameters for pNPPase in Table 1. Symbols are explained in the legend to Fig. 1.

The findings were slightly different for pNPPase activity, in that V_{max} for K^+ was slightly but significantly reduced by EtOH, while the increases in K_m (51, 38 and 40 per cent for K^+, Rb^+ and Cs^+ respectively), though similar, were not significant for Rb^+ and Cs^+. However, the Hill plots (Fig. 4) were almost identical to those for ATPase (Fig. 3).

With NH_4^+, the V_{max} was significantly decreased by EtOH for both enzyme activities, and K_m was significantly increased, while n remained unchanged.

Li^+ differed from the other ions in that EtOH reduced V_{max} (though this was significant only for ATPase) but did not alter K_m or n for either enzyme activity.

More detailed scrutiny of Table 1, however, suggests that the discrepancies between the findings with the two enzymes may be attributed to the differing error magnitudes in the various experiments. In the case of K^+, for example, the ratio of V values (EtOH:control) was 0.94 in the ATPase and 0.92 in the pNPPase experiments. However, the very small observational error in the pNPPase studies resulted in a statistically significant EtOH effect, while with the ATPase activity the somewhat larger error prevented the ethanol effect from attaining significance. Similarly, the EtOH:control ratios for K values were about 1.4 or higher for both enzymes, with K^+, Rb^+, Cs^+ and NH_4^+, yet this effect was significant for ATPase in all cases, but not for pNPPase with Rb^+ and Cs^+ because of larger error.

DISCUSSION

As explained in the Appendix to this paper, the use of double-reciprocal plots for illustrating the EtOH effects offers certain advantages over the more conventional Hill plot, since changes in all three kinetic parameters (V_{max}, K_m and n) can be observed simultaneously. The parallelism of the EtOH and non-EtOH curves for each ion with each enzyme (Figs. 1 and 2) confirms the principal finding in Figs. 3 and 4. The reductions in asymptotes correspond to the decreases in V_{max} shown in Table 1, while the horizontal separations correspond to changes in K_m.

The shape of the Scatchard plots, and the values of the Hill constant significantly greater than 1.0, are consistent with the hypothesis that K^+, Rb^+, Cs^+ and NH_4^+ all combine with more than one site on the protein(s) responsible for the (Na + K)-ATPase and pNPPase activities, and that cooperativity exists between these binding sites. EtOH does not appear to change the degree of cooperativity for ATPase action, but it does produce a formal competitive inhibition of enzyme activation by K^+, Rb^+ and Cs^+. This is consistent with earlier findings concerning EtOH and K^+ [9], and with the view [6] that these three ions react in an essentially similar manner with the enzyme. The only difference with respect to pNPPase activity is the significant reduction by EtOH of the V_{max} for K^+ (Table 1). NH_4^+ differs slightly from K^+, Rb^+ and Cs^+ in that V_{max} is slightly but significantly decreased with both enzymes.

Clearly Li^+ behaves quite differently, even though

it can substitute for K^+ in various preparations [6, 29, 30]. It does not show non-hyperbolicity, so that one cannot infer more than one binding site. In addition, it has a much lower maximum reaction velocity than the other ions, and the inhibition by EtOH is formally non-competitive, at least for ATPase. Hegyvary and Post [15] have found that Li^+ differs from all the other ions tested, in being unable to displace bound ATP from the enzyme. These findings suggest that Li^+ binding may not occur at the same principal site as K^+, or that the additional K^+-binding sites may differ sufficiently from the principal site that they cannot accomodate Li^+. The effect of Li^+ on pNPPase activity shows quite similar features. The only difference is that EtOH did not reduce V_{max} significantly, but this may be due to the unusually large error of estimate due to the very low activities with Li^+.

The order of effectiveness of the ions tested, in terms of the relative velocities of both enzyme activities at 5 mM concentration of each ion, is parallel with the order of selectivity of the same ions for oxyanion groups in the pK_a range of 2–4 [31]. This is compatible with the idea that the order of effectiveness reflects the relative energy changes involved in transfer of the various ions from their hydrated forms in solution to anion-bound forms at the active sites. The fact that addition of EtOH did not alter the relative order of effectiveness, even though it tended to increase K for both activities and for all the ions except Li^+ (Table 1), suggests that EtOH does not alter the basic binding mechanism. Rather, it appears to alter the consequences of ion binding, possibly by inducing conformational changes in the enzyme, or in the membrane in which it is located (cf. Ref. 32).

The double-logarithmic plots in Figs 1 and 2, together with the computed values in Table 1, indicate the principal kinetic conclusions of this study: EtOH affects comparably the kinetic parameters for the two enzymes; for K^+, Rb^+, Cs^+ and NH_4^+, EtOH lowers V_{max} by about 10 per cent, increases K_m by about 50 per cent and does not affect n; for Li^+, EtOH reduces V_{max} by about 60 per cent and does not increase K_m; the Hill coefficient is about 1.0 for Li^+ and 1.5 for the other ions. These findings are consistent with the interpretation that the two activities are carried out by the same enzyme, and that the EtOH effects on the ATPase reflect an action only on the K^+ binding sites.

These findings bear a number of resemblances to those reported by Roufogalis [33], who studied the inhibition of beef brain (Na + K)-ATPase by a variety of depressant drugs of differing lipophilicity. He reported that highly lipophilic drugs (e.g. chlorpromazine) competitively inhibit Na^+ activation of the enzyme, while drugs of lower lipophilicity (e.g. tetracaine) competitively inhibit K^+ activation. The very low lipid–water partition coefficient of EtOH [34] and its competitive inhibition of K^+ activation are consistent with his contention. Our findings are compatible with the suggestion [33, 35] that EtOH, tetracaine, and other preferentially water-soluble membrane depressants interact with relatively superficial sites in the cell membrane, leading to allosteric lipid–protein perturbations close to the externally oriented K^+ site of the ATPase. Essentially the same mechanism has been suggested for the action of EtOH on a membrane-bound lipoxygenase [32].

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APPENDIX

A Note on the Use of the Double-Logarithmic Plot for Representation of Enzyme Kinetic Observations

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The conventional representations of enzyme kinetic data, such as the Lineweaver–Burk double-reciprocal plot, the Hill plot and the Scatchard plot, have the disadvantage that each allows the direct evaluation of only some of the parameters. For example, the direct plot of velocity (v) against substrate concentration (c) yields estimates of the asymptotic velocity (V) and the Michaelis-type constant (K) but not of the Hill constant (n). Furthermore, this plot permits the direct evaluation of differences resulting from different treatments, but not of the more meaningful ratios of the parameters V and K observed for two rate curves.

In contrast, a double-logarithmic plot permits the rapid visual demonstration and quantitative comparison of all three parameters for two or more Hill-type rate or binding equations of the form

$$v = Vc^n/(K^n + c^n).$$

In the double-logarithmic plot, the slope of the curve at any given concentration is

$$d(\log v)/d(\log c) = nK^n/(K^n + c^n).$$

Consequently, at low concentrations ($c \ll K$) the slope is approximately equal to n.

For a comparison of two rate curves at the same substrate concentration,

$$\frac{v_1}{v_2} = \frac{V_1}{V_2}\,\frac{K_2^{n_2} + c_2^{n_2}}{K_1^{n_1} + C_1^{n_1}}.$$

Therefore, at low concentrations, this becomes approximately

$$v_1/v_2 = (V_1/K_1^{n_1})/(V_2/K_2^{n_2})$$

while at high concentrations ($c \gg K$)

$$v_1/v_2 = V_1/V_2.$$

Consequently, the double-logarithmic curves are approximately linear at low substrate concentrations, with a slope equaling the Hill coefficient, while at higher concentrations they approach the asymptotic velocity. Therefore, the vertical separation between two curves at the high-concentration end is

$$\log v_1 - \log v_2 = \log(V_1/V_2).$$

At low concentrations, the vertical separation becomes approximately

$$\log v_1 - \log v_2 = \log(V_1/V_2) - \log(K_1^{n_1}/K_2^{n_2}).$$

Thus, the difference between the vertical separations at high and low concentrations corresponds to $\log\text{-}(K_1^{n_1}/K_2^{n_2})$. When the two Hill coefficients are equal, as in the present investigation, this quantity becomes $n \log (K_1/K_2)$ and therefore yields an estimate of the K-ratio.

These features are illustrated in Fig. 5. The heavy line

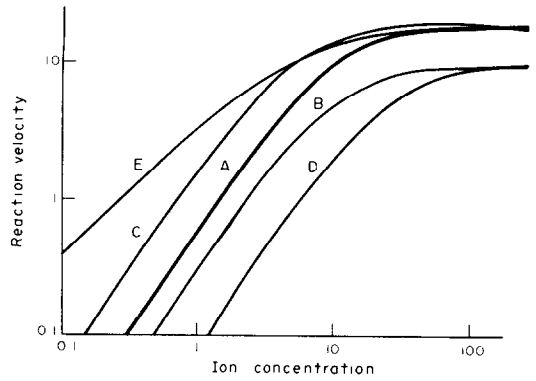

Fig. 5. Theoretical curves to illustrate the properties of the double-reciprocal plot for Hill-type rate equations. The heavy line (A) is the basic computed curve for parameters $n = 1.50$, $V = 20$ and $K = 10$. Lines B and C show the effects of changing a single parameter: for B, $V = 10$; for C, $K = 5$. Lines D and E illustrate the effects of changing two parameters simultaneously: for D, $V = 10$ and $K = 25$; for E, $n = 1.00$ and $K = 5$.

represents a theoretical curve with the parameters $n = 1.50$, $V = 20$ and $K = 10$. The light lines indicate the changes resulting from variation of these parameters singly or in combination. As shown there, a change in V produces a vertical displacement of the whole curve. A change in K results in vertical displacement primarily of the linear (low concentration) segment, while a change of n alters the slope of the linear segment. Change in two parameters simultaneously yields a summation of these effects, which is easily recognizable at a glance.

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