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Na^+-DEPENDENT PHOSPHORYLATION OF THE RAT BRAIN $(Na^+ + K^+)$-ATPase POSSIBLE NON-EQUIVALENT ACTIVATION SITES FOR Na^+

DONALD FOSTER and KHALIL AHMED

*Toxicology Research Laboratory, Minneapolis Veterans Hospital, Minneapolis, Minn. 55417 *, and the Department of Laboratory Medicine and Pathology, University of Minnesota 55455 (U.S.A.)*

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Summary

1. The steady state levels of Na^+-dependent phosphoenzyme (E-P) in the $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) of rat brain, obtained from a time course study of phosphoenzyme formation at 4° C, were dependent on the concentration of Na^+ in the reaction and were maximal in the presence of 64 mM Na^+. The plot of phosphoenzyme vs. Na^+ concentration gave a curve which on conversion to a double reciprocal plot (1/E-P vs. $1/Na^+$) gave a line with two breaks, yielding apparently three linear segments. This may be taken to indicate the presence of multiple Na^+ sites for the formation of the phosphoenzyme. To test this hypothesis further, the following approach was taken. By making the assumption that the phosphoenzyme may represent bound Na^+, it was possible to subject the data to rigorous multiple-site analysis by utilizing steady-state binding equations described by Klotz and Hunston (1971) (Biochemistry 10, 3065—3069), and by Scatchard (1949) (Ann. N.Y. Acad. Sci. 51, 660—672). The analysis of the data by these methods suggests that there may be three non-equivalent Na^+ activation sites for the formation of Na^+-dependent phosphoenzyme in the $(Na^+ + K^+)$-ATPase. The estimated intrinsic association constants (K_a) for activation by Na^+ at each of the three sites were 3.4, 0.295, and 0.025 mM^{-1}, respectively.

2. The steady-state level of Na^+-dependent phosphoenzyme was reduced by ^2H_2O (deuterated water) and Me_2SO (dimethylsulfoxide). This inhibition was reversed by increasing the concentration of Na^+ in the reaction but remained constant over a time course at any given Na^+ concentration. An analysis of the effect of ^2H_2O on Na^+-dependent phosphoenzyme formation in the presence

* Please address all communications to this address.

Abbreviations: ^2H_2O, deuterated water; Me_2SO, dimethyl sulfoxide; $(Na^+ + K^+)$-ATPase, $(Na^+ + K^+)$-stimulated adenosine triphosphatase.

of gradually increasing Na^+ concentration also revealed the presence of three non-equivalent sites for Na^+. The intrinsic association constants (K_a) for the activation of Na^+ at each of the three sites in the presence of ^2H_2O were changed to 1.4, 0.232, and 0.033 mM^{-1}, respectively, which suggests a differential effect of ^2H_2O on the three non-equivalent Na^+ sites.

3. On statistical grounds (±2 S.E. of mean) a two non-equivalent site model also fits the data. In this case, the intrinsic association constants (K_a) were 2.44 and 0.041 mM^{-1} in H_2O medium, and 1.062 and 0.048 mM^{-1}, in ^2H_2O medium, respectively, showing a differential effect of ^2H_2O on the two non-equivalent sites.

4. The inhibitory effect of ^2H_2O and Me_2SO on the formation of Na^+-dependent phosphoenzyme was maximal when the enzyme was allowed a contact with these agents prior to the addition of Na^+ in the reaction. On the other hand, prior contact of the enzyme with Na^+ reduced or abolished the inhibitory effect of ^2H_2O or Me_2SO. Prior contact of the enzyme with ATP also abolished the inhibition of Me_2SO. These results support the view that H_2O plays a regulatory role in the active center of the (Na^+ + K^+)-ATPase so that its presence tends to favor the E_2 (K^+-accepting) form of the enzyme whereas Na^+ binds to at least two of its activation sites, in an apparently competitive manner with respect to H_2O, yielding the conformation suitable for the phosphorylation (E_1). At the lowest affinity Na^+ activation site for either the 2 or 3 non-equivalent site case the presence of H_2O may facilitate its binding. The results also suggest that both Na^+ or ATP can independently shift the enzyme conformation to E_1.

Introduction

(Na^+ + K^+)-stimulated transport ATPase (EC 3.6.1.3) present in most mammalian plasma cell membranes, is considered to represent the enzymic basis for the movement of cations across the cell membrane [1]. Evidence from several laboratories has suggested that the operation of this enzyme proceeds through a series of intermediary steps involving conformational changes related to Na^+-dependent phosphorylation of the enzyme and the K^+-dependent breakdown of the phosphoenzyme. In this context, the use of modifiers or inhibitors of the partial reactions of this enzyme system has provided a considerable amount of information on the ionic interactions in the ATPase, and possible mechanism(s) of its operation (for recent reviews see e.g. refs. 1—3). In previous work, we demonstrated that substitution of ^2H_2O (deuterated water) for H_2O in the ATPase reaction resulted in an inhibition of the Na^+ activation of the ATPase in an apparently competitive manner, while the associated K^+-dependent *p*-nitrophenyl phosphatase was stimulated by ^2H_2O [4]. These observations based on a study of the kinetics of (Na^+ + K^+)-ATPase led us to postulate that the binding of Na^+ to the (Na^+ + K^+)-ATPase produced a conformation which was required for the initiation of the phosphoenzyme formation in the presence of ATP, and further, that H_2O played a role in this interaction between Na^+ and the enzyme at this site. Several studies on the effects of a variety of solvents on

the (Na^+ + K^+)-ATPase have been reported recently [5—9]. Among the various solvents examined, dimethylsulfoxide (Me_2SO) appears to have some features in common with ^2H_2O, in that it inhibits the Na^+-dependent activation of the enzyme while concomitantly stimulating the K^+-dependent *p*-nitrophenyl phosphatase [6,7]. However, the results reported on the effect of Me_2SO on the Na^+-dependent phosphoenzyme formation in the (Na^+ + K^+)-ATPase are at variance [6,8]. We have extended these studies and have examined in detail the action of ^2H_2O and Me_2SO on the formation of Na^+-dependent phosphoenzyme in the (Na^+ + K^+)-ATPase complex. These experiments offer some clues to the possible nature of the interaction of Na^+ and H_2O at the phosphorylation sites in the (Na^+ + K^+)-ATPase. Further, we have measured the steady-state levels of phosphoenzyme formed when the concentrations of Na^+ are gradually increased (in K^+-free reaction media), and have devised an approach to analyze and relate these data in terms of the possible activation sites for Na^+ for the formation of Na^+-dependent phosphoenzyme in the ATPase complex. On the basis of these studies, we suggest the presence of three non-equivalent sites for Na^+ interaction with the (Na^+ + K^+)-ATPase. A preliminary account of this work has been given [10].

Experimental procedure

Materials

^2H_2O (99.5%, lot TTS) was purchased from Mallinckrodt Chemical works, and was distilled twice in an all-glass apparatus before use. Me_2SO of the analytical grade was purchased from Baker Chemicals. Sucrose and Tris were of the ultrapure grade from Schwartz-Mann, New York; NaCl, KCl, and $MgCl_2$ were spectroscopically pure. Solutions of ATP, including those containing [γ-^{32}P] ATP, were passed through large columns of Tris form of AG-50 × 8 cation exchange resin, to eliminate any contamination from Na^+, K^+ or NH_4^+. EDTA was dissolved in Tris base, and treated the same way as ATP, described above. All other details concerning the materials used have been given previously [4,11—13].

Methods

Preparation of (Na^+ + K^+)-ATPase. The details concerning the preparation of rat brain membrane (Na^+ + K^+)-ATPase and its properties are the same as described in previous work [4,14]. The enzyme preparation was suspended in a medium consisting of 0.25 M sucrose, 10 mM imidazole-HCl and 1 mM EDTA, pH 7.4, following two additional washes in this medium. It was stored frozen in small aliquots and was stable over several weeks. The specific activity of the (Na^+ + K^+)-stimulated ATPase on the average was 125 μmol/mg of protein per h while the basic Mg^{2+}-stimulated component was generally between 5—10% of the total (Mg^{2+} + Na^+ + K^+)-dependent ATPase.

Preparation of phosphoenzyme. The standard reaction medium, in a final volume of 2 ml, maintained at 4° C, consisted of 30 mM Tris · HCl, pH 7.45 (at 4° C), 0.3 mM $MgCl_2$, 8 mM NaCl, 0.05 mM [γ-^{32}P] ATP ($6 \cdot 10^4$ dpm/nmol of ATP) and between 350 to 500 μg of rat brain membrane (Na^+ + K^+)-ATPase preparation. In general, the reaction time was 4 s which gave the steady-state

level phosphorylation, and unless otherwise stated, the reaction was initiated by the addition of [γ-^{32}P] ATP and was terminated by adding 25 ml of ice cold 5% (w/v) trichloroacetic acid containing 15 mM NaH_2PO_4 and 0.6 mM ATP. The radioactivity due to ^{32}P incorporated in the protein was measured as described before [11].

To determine the accuracy of the phosphoenzyme activity measured at low added Na^+ concentrations (e.g. at 0.5 mM Na^+), control experiments were performed to determine the contribution of the endogenous Na^+ in the ATPase. For this, phosphoenzyme formed in the presence of 0.3 mM Mg^{2+} alone, and 0.3 mM Mg^{2+} + 16 mM K^+ was measured. The difference between these two values should represent the amount of phosphoenzyme activity due to the endogenous contamination of the enzyme by Na^+; in several experiments the mean values of phosphoenzyme activity were precisely the same in both cases. Further, by atomic absorption spectroscopy, the enzyme preparation was found to contain no detectable NH_4^+ and K^+, while the amount of Na^+ present was 60 nmol/mg protein, which would contribute negligible amounts of Na^+ in the reaction. The value of the phosphoenzyme obtained in the presence of (Mg^{2+} + K^+) was subtracted from that in the presence of (Mg^{2+} + Na^+), the difference being the Na^+-dependent phosphoenzyme formed in the (Na^+ + K^+)-ATPase system. The value of phosphoenzyme in the presence of (Mg^{2+} + K^+) was no more than 6% of that obtained in the presence of (Mg^{2+} + 64 mM Na^+). All data are calculated as pmol ^{32}P per mg of protein or as percent phosphorylation (compared with maximal phosphoenzyme formed in the presence of 64 mM Na^+) when different enzyme preparations were used.

Other procedures. Methods for the estimation of protein, and preparation of [γ-^{32}P] ATP were the same as given before [11,12]. Preparation of the solutions in ^2H_2O was the same as reported previously [4,11].

Results

Effect of ^2H_2O and Me_2SO on Na^+-dependent phosphoenzyme formation. It has been reported that the concentrations at which ^2H_2O [4] and Me_2SO [6,7] produce maximal, reversible, inhibitory effects on the (Na^+ + K^+)-ATPase are 80—90 and 30%, respectively. The maximal inhibition of Na^+-dependent phosphoenzyme formation by ^2H_2O was also observed at concentrations of 80—90% [11]. In the presence of 80% ^2H_2O or 30% Me_2SO, the inhibition of the formation of Na^+-dependent phosphoenzyme was modified by the level of Na^+ in the reaction. The inhibition at 0.5, 1.0, 8.0, and 64 mM Na^+ in the presence of ^2H_2O was 50, 28, 23, and 7%, respectively. However, even at 128 mM Na^+, a small but reproducible inhibition (about 7%) by ^2H_2O was still apparent. Similarly, inhibition by Me_2SO was reduced from 69% at 8 mM Na^+ to 20% at 64 mM Na^+. It may be mentioned that in previous work, it was established that the inhibition of (Na^+ + K^+)-ATPase [4] or Na^+-dependent phosphoenzyme formation [11] by ^2H_2O was not due to alterations in the pH optima for these activies in the presence of ^2H_2O.

Time course of the effect of ^2H_2O on the formation of phosphoenzyme. The data given in Fig. 1 depict the variations of steady-state phosphoenzyme with varying concentrations of Na^+. However, at any given concentration of

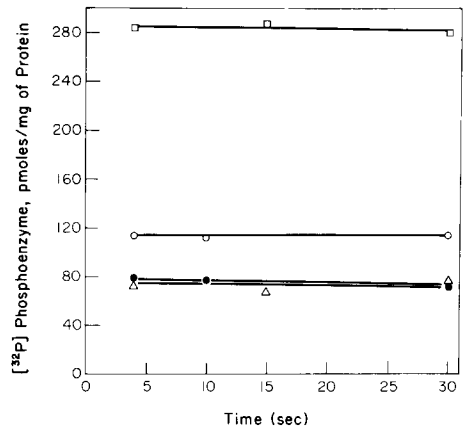

Fig. 1. Time course of Na^+-dependent phosphoenzyme formation in the presence and absence of ^2H_2O. The experimental details are the same as described under Methods, except that the reaction time was varied as shown, in the presence of: △, 0.5 mM Na^+, ○, 8 mM Na^+, and □, 64 mM Na^+, all in H_2O system; and, ●, 8 mM Na^+, in 80% ^2H_2O system.

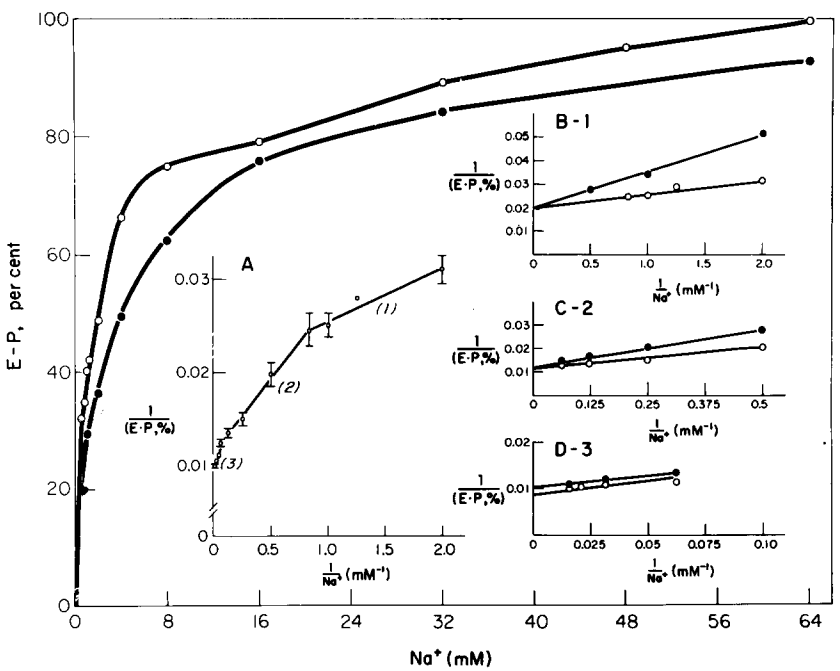

Fig. 2. Effect of varying concentration of Na^+ on the steady-state level of phosphoenzyme in the presence and absence of ^2H_2O. Na^+ concentration was varied as shown. Phosphoenzyme is plotted as the percent of the maximal phosphoenzyme formed (i.e. in the presence of 64 mM Na^+). The actual amount of steady state phosphoenzyme formed in the presence of 64 mM Na^+ was 264.5 pmol/mg of protein in the H_2O system, and 246.1 pmol/mg of protein in the ^2H_2O system. All lines in panels B1, C2 and D3 were fitted by the method of least squares. All other experimental details were as described under Methods. ○, H_2O controls; ●, 80% ^2H_2O. Insets, panel A shows the double reciprocal plot (1/% E-P vs. $1/Na^+$) in the H_2O system; the error bars denote S.E. derived according to the method of Johansen and Lumry [44]; panels B1, C2 and D3 represent the three segments marked at 1, 2 and 3 in panel A in the presence of H_2O (○) or 80% ^2H_2O (●) reaction media.

Na^+, the level of phosphoenzyme formed remains steady over a time course of 30 s as shown in the figure; in other control experiments the steady-state level of phosphoenzyme formed in the presence of 8 mM Na^+ did not change significantly over a period of 80 s (data not shown). The inhibition produced by ^2H_2O in the presence of 8 mM Na^+ was also constant over a reaction period of 30 s.

Formation of phosphoenzyme in the presence of varying Na^+. The effect of gradually increasing concentrations of Na^+ on the formation of steady-state Na^+-dependent phosphoenzyme in the H_2O reaction media is shown in Fig. 2 (open circles). Maximal steady-state levels of Na^+-dependent phosphoenzyme were obtained at some 64 mM Na^+. As is evident from Fig. 2 (inset A), a double reciprocal plot of the data showed a break at two points, yielding three linear segments designated in Fig. 2 inset A, as 1, 2 and 3. These segments are depicted as the expanded plots in the insets B1, C2, and D3 to illustrate that the slopes and the points of intersection on the ordinate are different for each of the three segments. The non-linearity of the plot in Fig. 2, inset A, may possibly be interpreted as empirical evidence for the presence of multiple non-equivalent Na^+ sites for the formation of phosphoenzyme in the (Na^+ + K^+)-ATPase.

However, this plot alone is considered inadequate to determine the number of sites and their constants [15—17]. Therefore, the procedures described by Klotz and Hunston [15] and by Scatchard [18] to determine the number of binding sites and their kinetic constants, were considered. Since the steady-state phosphoenzyme varies with Na^+ concentration (Fig. 1), we let the phosphoenzyme represent Na^+ binding for its formation (see also the Discussion).

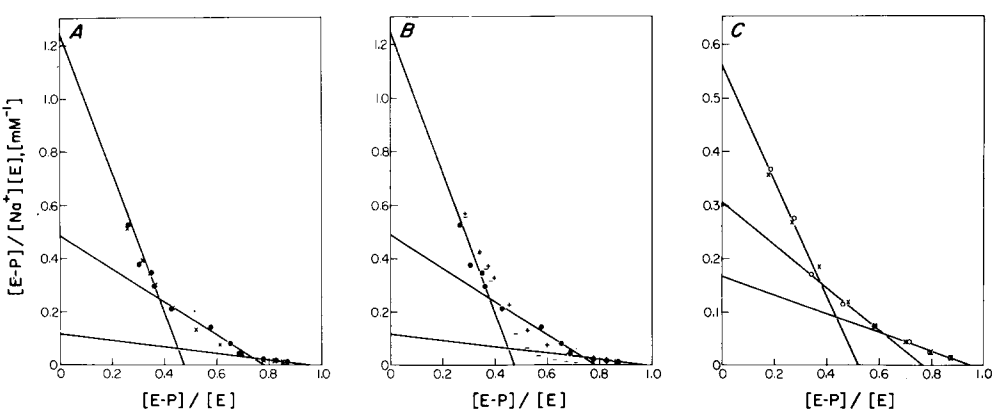

Fig. 3. Scatchard plot analysis of Na^+ activation sites. Panel A, the values for [E-P] and Na^+ are the same as in Table I. E, as defined in the Appendix, is the total concentration of enzyme in the reaction (304 pmol/mg of protein). The theoretical values (X) were calculated as described under the Appendix; •, experimental values, where [E-P]/[E] represents the moles of Na^+ bound per unit of the enzyme which has one covalently linked phosphate. Panel B, a test for two non-equivalent sites is shown. •, experimental values; + or —, two possible theoretical fits which presumably bracket the best fit for this model. Panel C, three non-equivalent site model applied to Na^+-dependent phosphoenzyme formation in the presence of ^2H_2O. The values of the Na^+-dependent phosphoenzyme formed in the presence of ^2H_2O are as shown in Fig. 2; the amount of phosphoenzyme at 64 mM Na^+ in the presence of 80% ^2H_2O was 246.1 pmol/mg of protein. ○, experimental points; X, calculated points on the basis of three non-equivalent sites for Na^+. All lines are drawn to fit the experimental points.

By doing so it is possible to plot the data in terms of a Scatchard plot as shown in Fig. 3 (panel A) to describe Na^+ binding. The actual values of phosphoenzyme activity and standard errors are given in Table I. The three intercepts on the abscissa may suggest three classes of binding sites while the uppermost intercept on the ordinate gives a stoichiometric binding constant designated in the Appendix as $\hat{K}$. Based on the general equation (2) given in the Appendix, models for two and three equivalent and non-equivalent binding sites were tested by plotting $r_t = t[\text{E-P}]/[\text{E}]$ vs. $[Na^+]$, as shown in Fig. 4, where [E-P] represents the phosphoenzyme concentration. The theoretical values for data points in Fig. 4, panels A and B, for the three and two non-equivalent site models were also plotted in Fig. 3, for comparison of experimental values with the theoretical values for three non-equivalent site (Fig. 3, panel A) or two non-equivalent site models (Fig. 3, panel B). As shown in Figs. 3 and 4, the two and three equivalent site models showed considerable deviation between the theoretical and experimental points; the result with a one-site model was so deviant as not to merit inclusion in the figure. The model with two non-equivalent sites also appeared to be incapable of fitting the experimental points within ±1 S.E. on both ends of the curve in Fig. 4. The deviation was minimal (within ±1 S.E.) when the data were fitted to a three non-equivalent site model, and a good agreement was noted between the calculated and the experimental points (Fig. 3, panel A and Fig. 4, panel A). The intrinsic association constants (K_a) for the activation by Na^+ were estimated from the three non-equivalent site model shown in Fig. 4, panel A, as described under the Appendix; the values of these constants in the H_2O system were 3.4, 0.295, and 0.025 mM^{-1}. Other theoretical details for the above plots are given in the Appendix.

Effect of ^2H_2O on the formation of phosphoenzyme in the presence of varying Na^+. The data given in Fig. 2 also show the effect of gradually increas-

TABLE I

VALUES OF DATA POINTS IN FIGS. 2, 3 AND 4

E-P refers to the steady-state Na^+-dependent phosphoenzyme as pmol/mg of protein, in the presence of the various concentrations of Na^+ shown. E refers to the total concentration of enzyme in the reaction as defined in the Appendix, and was 304 pmol/mg of protein. The calculations of the standard error (S.E.) in the various transformations of E-P were based on the procedure of Johansen and Lumry [14] as described in the Appendix.

No. of determinations	Na^+ (mM)	[E-P] ± S.E.	[E-P]/[E] ± S.E.
11	0.5	79.8 ± 3.83	0.2623 ± 0.014
2	0.8	92.0	0.3026
5	1.0	106.0 ± 5.67	0.3486 ± 0.022
4	1.2	109.3 ± 8.20	0.3595 ± 0.031
6	2.0	129.0 ± 8.40	0.4243 ± 0.031
4	4.0	175.0 ± 7.33	0.5756 ± 0.034
6	8.0	198.2 ± 7.97	0.6519 ± 0.034
6	16.0	209.3 ± 7.14	0.6884 ± 0.033
2	32.0	236.5	0.7779
2	48.0	251.8	0.8282
12	64.0	264.5 ± 6.08	0.870 ± 0.028
2	96.0	242.8	

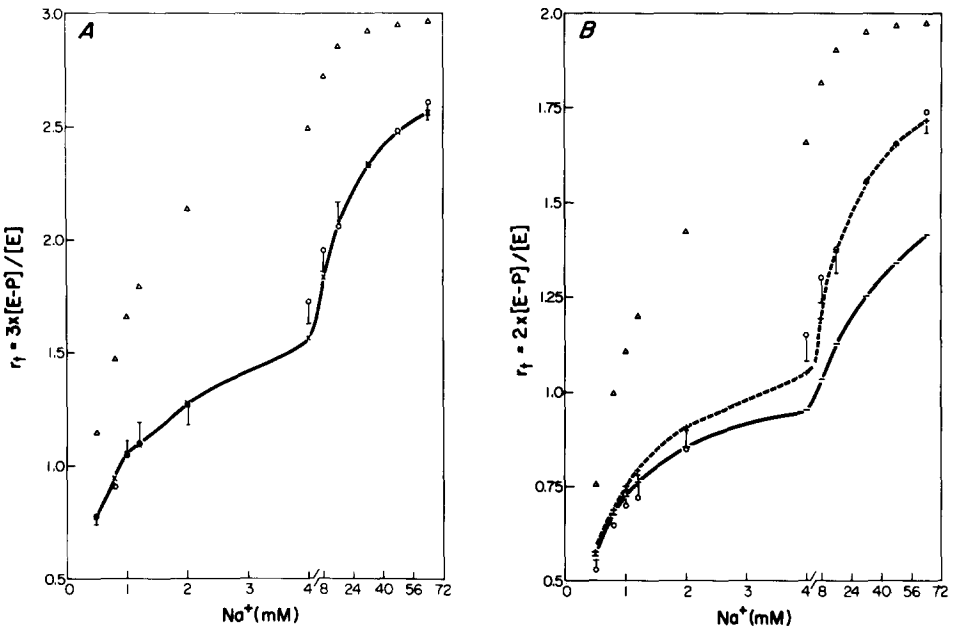

Fig. 4. Multiple site analysis of Na^+ activation of phosphoenzyme formation. Panel A, the concentration of Na^+, values of [E], [E-P], and calculated [E-P]/[E] are those given in Table I and Fig. 3. The equation $r_t = \Sigma_{i=1}^{m} N_i K_i [Na^+]/1 + K_i[Na^+]$, (m = 3), was solved for three equivalent (△) or non-equivalent (X) association constants for Na^+ (see the Appendix for details); ○, experimental points for t X [E-P]/[E]. Panel B, the same equation as in panel A except that m = 2 was solved for the two equivalent (△) or non-equivalent (+ or —) association constants for Na^+; ○, experimental points. All lines are drawn to connect the theoretical points. The apparent intrinsic association constants (K_a) for the activation by Na^+ at each of the three non-equivalent sites were 3.4, 0.295, and 0.025 mM^{-1}, respectively, in the presence of H_2O, and were 1.4, 0.232, and 0.033 mM^{-1}, respectively, in the presence of 80% ^2H_2O.

ing concentrations of Na^+ on the formation of steady-state Na^+-dependent phosphoenzyme in the reaction media containing 80% ^2H_2O. A double reciprocal plot of the data (i.e. 1/E-P vs. $1/Na^+$) gave three linear segments as in the case of the plot obtained in the presence of H_2O (not shown in the inset A, but shown in the expanded scale segments B1, C2, and D3 of Fig. 2). Further, it appeared that the segments designated as no. 1 and as no. 2 in both H_2O and ^2H_2O intercepted the ordinate at the same point in each case, whereas the segments designated as no. 3 did not meet at the same point on the ordinate in the presence of H_2O compared with ^2H_2O. The result suggests that over the range of concentration of Na^+ given in the insets B1 and C2, the maximal phosphoenzyme activity attainable is essentially the same in the presence or absence of ^2H_2O, and that the reduction in the phosphoenzyme levels in the presence of ^2H_2O at these concentrations of Na^+ may be due to a reduced affinity of Na^+. We further subjected the Na^+-dependent phosphoenzyme data obtained in the presence of ^2H_2O to multiple-site analysis as in the case of the data obtained in the presence of H_2O described above (Fig. 3, panel C), which also indicated three Na^+ activation sites. Again, using the intercept on the ordinate of Fig. 3, panel C, and assuming different ratios of Na^+ binding per phosphoenzyme molecule, tests of the number of Na^+ sites and their equiva-

lency showed that an equivalent site model would not fit the data (plots not included in Fig. 4). The intrinsic association constants for Na^+ (K_a) in the presence of ^2H_2O for a three non-equivalent site model were estimated as 1.4, 0.232 and 0.033 mM^{-1}, respectively. These values, compared with the constants obtained from the experiments with H_2O reaction media, indicate a differential effect of ^2H_2O on the three sites. The decrease in the first two constants (K_a) by ^2H_2O was 59 and 21%, respectively, while for the third one there was an increase of 32%. Further, a comparison of the intercepts on the [E-P]/[Na^+][E] axis for H_2O (Fig. 3, panel A) and ^2H_2O (Fig. 3, panel C) shows an overall reduction of 47% in the affinity of Na^+. It may be recalled that 80% ^2H_2O gives an average of 40% inhibition of the (Na^+ + K^+)-ATPase [4].

Consideration of two non-equivalent site models. We have proposed above that a three non-equivalent Na^+ activation site model shows the best fit to our data when all points are considered within ±1 S.E. of each mean. However, on the basis of ±2 S.E. of each mean, the two non-equivalent site model (Fig. 4, panel B, broken line) is not distinguishable from the three non-equivalent site model. If this were the case the K_a values (derived by the same procedures as described above) for Na^+ activation of the enzyme in the presence of H_2O medium were estimated at 2.440 and 0.041 mM^{-1}, respectively. In the presence of ^2H_2O, these values were changed to 1.062 and 0.048 mM^{-1}, respectively. The relative inhibitory effect of ^2H_2O on the high affinity non-equivalent site would thus be 56% with an increase of 17% on the low affinity site.

Effect of the order of addition of ligands on the inhibition by ^2H_2O and Me_2SO. Recently, Albers and Koval [6] showed that Me_2SO did not inhibit the Na^+-dependent phosphoenzyme formation, whereas Kaniike et al. [8] were

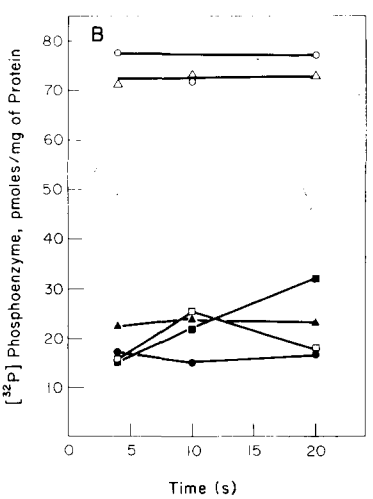

Fig. 5. Time course of the effect of Me_2SO on Na^+-dependent phosphoenzyme formation and the effect of the order of the addition of various components. The enzyme was allowed a contact with Na^+ or K^+, and Me_2SO in the following order prior to the start of the reaction: ○, enzyme + Na^+ in H_2O system; △, enzyme + Na^+, followed by Me_2SO; ▲, enzyme + K^+, followed by Me_2SO; ●, enzyme + K^+ in H_2O system; □, Na^+ + Me_2SO, followed by the enzyme; ■, K^+ + Me_2SO, followed by the enzyme. All other experimental details were the same as for experiments no. 1—6 in Table II.

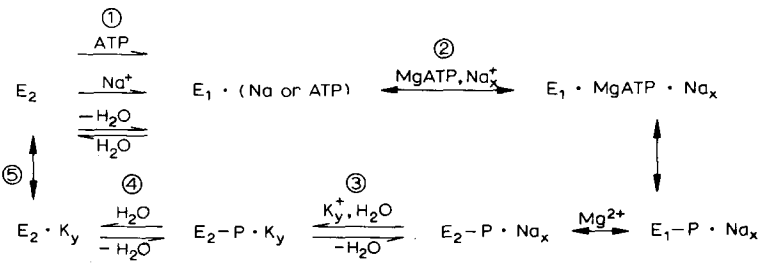

Fig. 6. Sites of action of solvents and H_2O in the operation of the $(Na^+ + K^+)$-ATPase. (1) Na^+ or ATP shift the equilibrium with respect to the enzyme conformation from E_2 to E_1, whereas H_2O, ^2H_2O, and Me_2SO will reverse it; organic solvents such as acetone, ether, alcohols [9] antagonize the action of H_2O, and may shift the equilibrium to the right; (2) E_1Na or E_1ATP reacts with substrate MgATP and Na_x^+ to form the intermediate $[E_1 \cdot MgATP \cdot Na_x]$ which leads to the formation of E_2-P · Na_x; (3) the interaction of K_y^+ is facilitated by H_2O, ^2H_2O and Me_2SO, and antagonized by organic solvents; (4) the interaction of H_2O on the breakdown of K^+-mediated phosphoenzyme is evidenced by stimulatory effect of ^2H_2O or Me_2SO, and inhibitory effect of organic solvents; (5) the presence of Na^+ or ATP leads to the dissociation of $E_2 \cdot K_y$ because of the action of these agents at step 1, above.

able to demonstrate an inhibition of the phosphoenzyme formation by Me_2SO. The results summarized in Table II clearly illustrate that the prior contact of the enzyme with Na^+ or ATP considerably alters the inhibitory effect of Me_2SO or ^2H_2O on the steady-state level of phosphoenzyme. When Me_2SO and Na^+, or ^2H_2O and Na^+, are added together, a large inhibition is observed which is even greater when Me_2SO or ^2H_2O are in contact with the enzyme

TABLE II

EFFECT OF THE ORDER OF ADDITION OF LIGANDS ON THE INHIBITION BY ^2H_2O OR Me_2SO

Additions in the sequence listed with each experiment were made to the standard reaction medium (as given under Methods). For experiment nos. 1—6, where indicated, cations and enzyme were in contact for 10 min followed by a 60-min contact with Me_2SO or ^2H_2O. When cations and Me_2SO or ^2H_2O were added together to the enzyme, the incubation was carried out for 60 min. When Me_2SO and ^2H_2O were added prior to cations, the contact with the enzyme was for 10 min, then an additional 50 min following cations. Following these treatments, the reaction was started by the addition of $[\gamma\text{-}^{32}P]$ ATP and was terminated 4 s later. In experiment no. 7, the enzyme was in contact with ATP (containing $[\gamma\text{-}^{32}P]$ ATP) for 10 s followed by 10 s with Me_2SO, and then the reaction was initiated by the addition of Na^+ and was terminated 10 s later. In experiment 8, Me_2SO was in contact with the enzyme for 10 s followed by $[\gamma\text{-}^{32}P]$-ATP and Na^+ or K^+ for an additional 10 s. The amount of enzyme (E) was 50 μg, Me_2SO, ^2H_2O, and Na^+ were present at a concentration of 30%, 80%, and 8 mM, respectively. Values for Na^+-independent phosphoenzyme (i.e. in the presence of Mg^{2+} + 16 mM K^+) were subtracted from experimental values, to obtain Na^+-dependent phosphoenzyme, and other appropriate controls were included throughout. All other details were the same as given under Methods.

Exp. no.	Sequence of additions	Percent inhibition of phosphoenzyme
1	^2H_2O + E + Na^+	44
2	^2H_2O + Na^+ + E	36
3	Na^+ + E + ^2H_2O	24
4	Me_2SO + E + Na^+	77
5	Me_2SO + Na^+ + E	69
6	Na^+ + E + Me_2SO	2
7	ATP + E + Me_2SO	1
8	Me_2SO + E + ATP	34

prior to the addition of Na^+. This suggests that Me_2SO and ^2H_2O block the interaction of Na^+ with its activation sites.

Further, if ATP is present on the enzyme, Na^+ can also bind to its sites without interference from Me_2SO. This experiment gives clear evidence that the random mechanism works in the $(Na^+ + K^+)$-ATPase since either Na^+ or ATP may get on to their sites independently and that with both present, phosphoenzyme formation will take place. The fact that prior contact of the enzyme with ATP leads to the formation of phosphoenzyme in the presence of Na^+ and Me_2SO suggests that ATP bound to the enzyme assists Na^+ to attach to its active sites. The data in Fig. 5 show that the effect of the order of addition of ligands is not modified over a time course in the presence of Me_2SO. Similar results were observed with ^2H_2O (Fig. 2, and other data not shown).

Discussion

Previous studies utilizing the analyses of the kinetics of activation of the $(Na^+ + K^+)$-ATPase by varying concentrations of Na^+ in the presence of varied or fixed concentrations of K^+ [14,19] have indicated two or three equivalent Na^+ activation sites. These models do not clearly distinguish between the equivalent and non-equivalent activation sites [19]. This type of approach is also complicated by complex interaction of cations at extremes of concentration ratios. ATP-dependent binding of Na^+ to the ATPase has been investigated, but has not been utilized for binding-site analysis [20—22]. The experimental design in the present work relies on the formation of Na^+-dependent phosphoenzyme (the breakdown of which is sensitive to K^+), whereby the complicated interactions of Na^+ and K^+ are avoided, since the reaction system contains only Na^+, and only the Na^+-dependent steady-state phosphoenzyme formed is examined.

If, as suggested by a large body of evidence, the phosphoenzyme is the intermediate in the operation of the $(Na^+ + K^+)$-ATPase and may be regarded as the Na^+-carrier in the pump cycle [2,3,23—26] (see also 1), then it may be possible to use the phosphoenzyme as a measure of Na^+ binding. In this case, the steady state equations and graphical methods for studying the binding of small molecules to macromolecules may be applied to analyse Na^+ bound to phosphoenzyme (see e.g. 15,18,27, and the Appendix). By assuming that cation binding sites are independant with fixed affinities and without defining the physical nature of the macromolecular state of the carrier, the steady-state binding equations would apply whether the enzyme were present as different species each having a distinct affinity constant or as a single species with all of the distinct binding sites on a single macromolecule.

The present model attempts to relate the stoichiometry of Na^+ binding per phosphoenzyme molecule based on the observed correspondence between Na^+ concentration and steady-state level of phosphoenzyme. The binding of Na^+ to its sites would be a consequence of an equilibrium involving reaction 1 as shown in Fig. 6. It would be implied from the law of mass action as discussed by Scatchard [18] that the probability of occupancy of multiple Na^+ binding sites would be dependent upon the Na^+ concentration. The result from experiments 4 and 6 in Table II suggests that phosphorylation would occur only

when the conditions of reaction 2 (Fig. 6) are met, including a sufficient amount of bound Na^+ per potential phosphorylation site. Accordingly, the steady-state level of phosphorylation may be used as a measure of Na^+ that was bound prior to phosphorylation. This is in agreement with the concept that the concentrations of Na^+ and K^+ may shift the steady-state concentrations of carrier moieties to control the rates of cation transport [28]. Our suggestion of three non-equivalent Na^+ activation sites for (Na^+ + K^+)-ATPase is compatible with the data on the kinetics of transport in nerve where it was shown that three sites with different Na^+ affinities were necessary to account for the Na^+ activation of Na^+-Na^+ exchange [29]. However, in red cells, three sites of equal apparent affinity for Na^+ are involved in Na^+-Na^+ exchange [30]. It would be interesting to determine if the nature of Na^+ activation sites (i.e. equivalent or non-equivalent) varies with tissues.

The possibility of interaction between the multiple non-equivalent Na^+ sites postulated above cannot be ruled out at the present. Nor can we rule out that our results are due to the possible inhomogeneity of the enzyme system used. Further, based on strict statistical considerations, the two non-equivalent site model may also fit the data.

The results in Table II have several implications. If bound H_2O plays a role at the Na^+ sites [4,11], it would seem reasonable that the agents such as Me_2SO and ^2H_2O which form stronger hydrogen (or deuterium) bonding by replacing H_2O [32,33] would tend to block the interaction of Na^+ with its activation sites. We propose that H_2O shifts the conformation of the enzyme from E_1 to E_2, while Na^+ reverses this trend (Fig. 6). This would imply that Na^+ must expel the bound H_2O from its activation sites in order to bind to these sites, although this does not seem to be the case at one of the Na^+ sites (i.e. the weak Na^+ binding site with the lowest K_a). Removal of H_2O from the higher affinity Na^+ sites could increase the hydrophobicity in the region, thereby preventing the spontaneous breakdown of the phosphoenzyme [9]. However, H_2O and K^+ will tend to move the [K-E_2-P] complex back to a hydrophilic region where the phosphoenzyme will be hydrolyzed. Thus ^2H_2O and Me_2SO would act more effectively than H_2O to favor the shift of E_1 to the E_2 form of the enzyme, which would result in reduced steady-state level of Na^+-dependent phosphoenzyme formation in the presence of ^2H_2O or Me_2SO. This will also be commensurate with increased affinity of K^+ for the K^+ stimulated *p*-nitrophenyl phosphatase activity in the presence of ^2H_2O [4,11] and Me_2SO [6,7], and a stimulation of K^+-mediated breakdown of phosphoenzyme [34,35]. The result in Table II suggests that ATP may act to cause a dissociation of the K-E_2 complex [24] to release K^+, by producing a shift of E_2 conformation to E_1 conformation (Fig. 6). Further, the removal of H_2O from Na^+ sites in the ATPase by solvents such as alcohols, acetone, ether, etc. would facilitate the binding of Na^+ (E_1 conformation, Fig. 6), and increase the phosphoenzyme formed due to stabilization of the complex [9], whereas K^+-mediated hydrolytic steps would be inhibited [5,9]. The increased affinity of K^+ for the ATPase at reduced temperatures [41,42] may also be explained if the enzyme complex favors the E_2 conformation at lower temperatures due to an increase in bound H_2O and/or stronger H bondings, while the reverse is the case at higher temperatures, i.e. the E_1 conformation is favored.

To conclude, the effects of ^2H_2O and Me_2SO on the $(Na^+ + K^+)$-ATPase, and possibly other membrane-associated functions [36—40], may be due to conformational changes caused by replacement of H_2O in the membrane structure and formation of stronger H (or ^2H) bonding. Further, the affinity of Na^+ and K^+ for their sites in the operation of the $(Na^+ + K^+)$-ATPase may be regulated by the bound H_2O at the active center. The points that favor this conclusion, and argue against a simple effect of ^2H_2O on cation hydration alone to explain the present and the previous work by us are as follows: (1) ^2H_2O inhibits $(Na^+ + K^+)$-ATPase, but stimulates K^+-stimulated *p*-nitrophenyl phosphatase; the stimulated activity is blocked by oligomycin [4,11]; (2) Inhibition of phosphoenzyme formation by ^2H_2O or Me_2SO is not altered over a time course, but changes with Na^+ concentration; (3) ^2H_2O acts differentially on multiple nonequivalent Na^+ sites; (4) The order of addition of reactants influences the effect of solvents; (5) ^2H_2O (and Me_2SO) action on K^+-stimulated *p*-nitrophenyl phosphatase is reflected in the stimulation of a K^+-mediated breakdown of a slow component of phosphoenzyme by these solvents [34,35]; and, (6) ^2H_2O stimulates the rate, and steady-state level of $Mg^{2+} + P_i$ or Mn^{2+}-supported ouabain binding to the ATPase, whereas it inhibits the rate, but not the steady-state level, of $(Na^+ + ATP)$-dependent ouabain binding [45].

Appendix

Theory for data expressed in Figs. 3 and 4

We make the following identities and assumptions: [E-P] represents specific Na^+ binding; r_t = moles Na^+ bound/unit of enzyme = t[E-P]/[E]; [E] = total enzyme concentration = $[E\text{-}P]_{max} + 0.15 \times [E\text{-}P]_{max}$ in the absence of K^+ [43]; further, the value for [E] in 80% ^2H_2O was taken as 93% of that in H_2O since a 7% inhibition of the phosphoenzyme at 64 mM Na^+ was observed in the presence of 80% ^2H_2O; [Na] = free Na^+ concentration = $[Na]_T - t[E\text{-}P] \simeq [Na]_T$, the total concentration, since $[Na]_T$ is in units of mM and t[E-P] is in units of nM; t = total number of Na^+ bound to the phosphoenzyme; [E-P] = $[Na_t \cdot E\text{-}P]$, the Na-phosphoenzyme complex; ϵ = free enzyme concentration = [E] — [E-P]; K_a = [E-P]/([Na] [ϵ]), the intrinsic association constant. Therefore,

$$r_t = \frac{t[E\text{-}P]}{[E\text{-}P] + [\epsilon]} = \frac{tK_a[Na]}{1 + K_a[Na]} \tag{1}$$

In general,

$$r_t = \sum_{i=1}^{m} \frac{N_i K_i[Na]}{1 + K_i[Na]} \tag{2}$$

where $t = \Sigma_{i=1}^{m} N_i$ = the total number of sites, and where N_i and K_i are the number of identical sites and the intrinsic association constant, respectively, for each class of sites i. By holding the total enzyme concentration [E] constant, the only dependent variable is [E-P] and the only independent variable is [Na]. For equation (2) it is appropriate to plot r_t vs. $[Na^+]$ (Fig. 4). Further, the

following form of Scatchard equation [17,18] is employed to construct a graph of $r_t/[\mathrm{Na}]$ vs. r_t (Fig. 3).

$$r_t/[\mathrm{Na}] = tK - r_tK \tag{3}$$

In plotting the experimental data by this method, it was first assumed that $t = 1$; therefore, $r_t = [\mathrm{E\text{-}P}]/[\mathrm{E}]$. The general solution of equation (3) states that the intercept on the abscissa gives the total number of binding sites, t, and the intercept on the ordinate gives the product of t and the average of all the association constants, $\langle K \rangle$ [15]. In Fig. 3, the intercept on the $[\mathrm{E\text{-}P}]/[\mathrm{E}]$ axis is essentially 1, which, in terms of phosphoenzyme formation as expressed in equation (3), means that as $\mathrm{Na}^+ \rightarrow \infty$, all of the enzyme is converted into phosphoenzyme. However, by letting the phosphoenzyme represent bound Na^+, the intercept on the abscissa simply gives the value of t that would be the assumed stoichiometry between the phosphoenzyme and Na^+ binding. The uppermost intercept on the ordinate as shown in Fig. 4a, gives the constant $\hat{K}$, such that

$$\hat{K} = t\langle K \rangle \text{ where } \langle K \rangle = \sum_{i=1}^{m} N_iK_i / \sum_{i=1}^{m} N_i \tag{4}$$

and where the K_i values are the intrinsic association constants as given in equation (2). For $t = 1$, $\hat{K} = 1.24 = N_1K_1$ as shown in Fig. 3A; for $t = 2$, $\hat{K} = 2.48 = N_1K_1 + N_2K_2$; and for $t = 3$, $\hat{K} = 3.72 = N_1K_1 + N_2K_2 + N_3K_3$, etc. By assuming different values for t, N_i and K_i, and using the experimental values of Na^+ concentration employed, theoretical values of r_t for 1-, 2- and 3-site models were generated from equation (2), with the appropriate values of $\hat{K}$ as a constraint. For equivalent site models $\hat{K}$ was divided equally among all the K_i values. Initial estimates for the K_i values in the non-equivalent site models may be obtained by substituting experimental values of r_t, $[\mathrm{Na}]$, $\langle K \rangle$, and assumed values of N_i and t into equations (2) and (4). These equations are solved for a value of K_i which is substituted in the equations and the process is repeated until estimates of all the K_i values are obtained. These initial estimates of K_i values are then varied by trial and error until the best fit is obtained. Since the values of r_t calculated from equation (2) represent theoretical values for $[\mathrm{E\text{-}P}]/[\mathrm{E}]$, theoretical values for $[\mathrm{E\text{-}P}]/[\mathrm{Na}][\mathrm{E}]$ were obtained by dividing the calculated values of r_t by the experimental values of $[\mathrm{Na}]$, and are used to test the various models in a Scatchard plot as shown in Fig. 3. The K_i values that gave the best fit for a three non-equivalent site model (Fig. 4A) were designated as the intrinsic association constants (K_a) for Na^+. When plotting points from Fig. 3 to Fig. 4 and vice versa, the obvious division or multiplication by t was performed.

Calculation of the standard error

The experimental values of $[\mathrm{E\text{-}P}]/[\mathrm{E}]$ and the error associated with each are given in Table I. From equation (9) of Johansen and Lumry [44], we define

the variance for $r_t = t[\text{E-P}]/[\text{E}]$ as follows:

$$\sigma_{r_t}^2 = t^2\left[\frac{1}{[\text{E}]^2}\cdot\sigma_{\text{E-P}}^2 + \frac{[\text{E-P}]^2}{[\text{E}]^4}\cdot\sigma_{\text{E}}^2\right] \tag{5}$$

from which S.E. = $\sigma_{r_t}/n^{1/2}$ was calculated, based on the assumption that the experimental determination of [E-P] and [E] are stochastically independent. Although [E] is calculated from $[\text{E-P}]_{max}$, the measurement of [E-P] made at one concentration of Na^+ is independent of that made at another.

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