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A KINETIC DESCRIPTION FOR SODIUM AND POTASSIUM EFFECTS ON $(Na^+ + K^+)$-ADENOSINE TRIPHOSPHATASE: A MODEL FOR A TWO-NONEQUIVALENT SITE POTASSIUM ACTIVATION AND AN ANALYSIS OF MULTIEQUIVALENT SITE MODELS FOR SODIUM ACTIVATION

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SUMMARY

1. Dissociation constants for sodium and potassium of a site that modulates the rate of ouabain-$(Na^+ + K^+)$-ATPase interaction were applied to models for potassium activation of $(Na^+ + K^+)$-ATPase. The constants for potassium (0·213 mM) and for sodium (13·7 mM) were defined, respectively, as activation constant, K_a and inhibitory constant, K_i.

2. Tests of the one- and the two-equivalent site models, that describe sodium and potassium competition, revealed that neither model adequately predicts the activation effects of potassium in the presence of 100 or 200 mM sodium.

3. The potassium-activation data, obtained at low potassium and high sodium, were explained by a two-nonequivalent site model where the dissociation constants of the first site are 0·213 mM for potassium and 13·7 mM for sodium. The second site was characterized by dissociation constants of 0·091 mM for potassium and 74·1 mM for sodium.

4. The two-nonequivalent site model adequately predicted the responses to concentrations of potassium between 0·25 and 5 mM in the presence of 100–500 mM sodium. At lower sodium concentrations the predicted responses formed an upper limit for the function of observed activities. This limit was reached at lower concentrations of potassium

and higher concentrations of sodium, which inferred saturation of the sodium-activation sites with sodium.

5. Sodium-activation data were corrected for sodium interaction with potassium-activation sites by use of the two-nonequivalent site model for potassium activation. Tests of equivalent site models suggested that the corrected data for sodium activation may be most consistent with a model that has three-equivalent sites. Other multiequivalent site models (n = 2, 4, 5 or 6), however, cannot be statistically eliminated as possibilities. The three-equivalent site activation model was characterized by dissociation constants of 1·39 mM for sodium and 11·7 mM for potassium. The system theoretically would be half-maximally activated by 5·35 mM sodium in the absence of potassium.

6. Derivation of the model for sodium activation assumed that the affinities of these sites for sodium and potassium are independent of cation interactions with the potassium-activation sites. Therefore, the kinetic descriptions for sodium and potassium effects form a composite model that is consistent with simultaneous transport of sodium and potassium.

7. Predictions of the composite equation are in reasonable agreement with data obtained by variation of sodium (potassium = 10 mM), variation of potassium (sodium = 100 mM) and by simultaneous variation of sodium and potassium (sodium:potassium = 10). Sodium-activation data (2·5–20 mM sodium) also agree with predictions of the model in the presence of potassium concentrations which are thought to be present at the sodium-activation sites *in vivo*.

8. The kinetic description for sodium (three-equivalent sites) and potassium (two-nonequivalent sites) activation of the transport-ATPase is in accord with the probable stoichiometric requirements of the sodium pump. The model is also in general agreement with other studies on intact transporting systems and $(Na^+ + K^+)$-ATPase in fragmented membrane preparations with respect to potassium activation, although there is a quantitative disagreement. The model for sodium activation, though consistent with data obtained by other studies on fragmented $(Na^+ + K^+)$-ATPase preparations, is in apparent variance with much of the data obtained for intact transporting systems. The description for potassium activation suggests that the rates of ouabain binding to $(Na^+ + K^+)$-ATPase are modulated by competition between sodium and potassium for one of the two potassium-activation sites.

INTRODUCTION

The kinetics of sodium and potassium activation of $(Na^+ + K^+)$-ATPase and the sodium pump have been interpreted in terms of two species of interaction sites on the membrane (Skou, 1957, 1960; Post & Jolly, 1957; Post, Merritt, Kinsolving & Albright, 1960; Glynn, 1962; Whittam, 1962; Laris & Letchworth, 1962; Whittam & Ager, 1964; Baker, 1965). One site is part of the internal surface of the membrane, possesses high affinity for sodium and low affinity for potassium, and activates catalysis when occupied by sodium but inhibits when occupied by potassium. The second site is part of the external surface of the membrane, manifests high affinity for potassium and low affinity for sodium, and activates ATP hydrolysis when occupied by potassium and inhibits when occupied by sodium. The former site is referred to as the 'sodium-activation site' and the latter as the 'potassium-activation site'.

The purpose of this paper is to present kinetic models that are consistent with the responses of $(Na^+ + K^+)$-ATPase to sodium and potassium. Previous studies have shown that when one ligand activator is maintained at a constant and high concentration, $(Na^+ + K^+)$-ATPase activity responds in a sigmoidal manner to increasing concentrations of the second ligand activator (Skou, 1957; Post *et al.* 1960; Green & Taylor, 1964; Squires, 1965). It is generally agreed that allosteric (Squires, 1965; Robinson, 1967, 1970) or multiple-'independent'-site models (Garrahan & Glynn, 1967*b*; Sachs & Welt, 1967; Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt 1969*b*; Priestland & Whittam, 1968) explain these data.

In fragmented membrane preparations, competition between the cations for the two types of activation sites constitutes the greatest obstacle to derivation of kinetic models for $(Na^+ + K^+)$-ATPase. In the study reported here, we use a somewhat different approach in that the apparent dissociation constants for one activation site were derived by inhibitor binding experiments. We have recently shown that sodium and potassium, in the presence of magnesium and ATP, compete for a common site that modulates the rate of [^{3}H]ouabain binding (Lindenmayer & Schwartz, 1973*a*; see also Baker & Willis, 1970). The relative dissociation constants for the two cationic species (13·7 mM for sodium and 0·213 mM for potassium) suggest that this local membrane 'environment' may be equivalent to a potassium-activation site. (These constants were derived under conditions which presumably induced near-maximal saturation of the sodium-activation sites with sodium.)

We have explored this hypothesis by testing kinetic models, which included these constants, against potassium-activation data. This analysis yielded a two-nonequivalent site model for activation by potassium.

The model was subsequently applied to sodium-activation data in order to correct for sodium interaction with the potassium-activation sites. The corrected data are consistent with a three-equivalent site model for sodium activation. A composite model for sodium and potassium activation applied to a wide range of sodium and potassium concentrations.

METHODS

Experimental design

Membrane preparations that contain ($Na^+ + K^+$)-ATPase were isolated from beef brain as previously described (Lindenmayer & Schwartz, 1970), treated with NaI by the method of Matsui & Schwartz (1966) and stored at $-15°$ C until use. Specific activities were measured at 37° C in the presence of NaCl and KCl as indicated, 50 mM-Tris-Cl, pH 7·4, 2·5 mM-$MgCl_2$, 20–40 μg protein and 2·5 mM-Tris-ATP. The reactions were terminated after 10 min by addition of ice-cold trichloroacetic acid (10 % w/v). Linearity of phosphate production was maintained during the assay period. Blanks for the reactions contained no added sodium and/or potassium. The ouabain-insensitive component was 0–5 % of total ATPase activity. Inorganic phosphate was assayed by the procedure of Fiske & SubbaRow (1929) and protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Derivation of a kinetic description for potassium activation of ($Na^+ + K^+$)-ATPase. Plots of ($Na^+ + K^+$)-ATPase activity *vs.* potassium (Fig. 1*a*) and double-reciprocal plots of some of these data (Fig. 1*b*) reveal the complexities of the enzyme's response to potasssium. In the presence of low sodium (i.e. 20 mM or less) the increase in activity with increasing potassium appears to approximate a rectangular hyperbola (Fig. 1*a*) until high potassium levels are reached where double-reciprocal plots bend upward (Fig. 1*b*). At high levels of sodium the potassium-activation curves clearly become sigmoidal (Fig. 1*a*, *b*). These complexities preclude an accurate assessment of catalytic activity when potassium saturates its activation sites (i.e. at V_{max}). This problem may be circumvented by the procedure detailed in the test of the following model.

The simplest model for potassium activation, assuming Michaelis–Menten kinetics, is the one-site model for which potassium (activation) and sodium (inhibition) compete. This model with the dissociation constants for the site that modulates cardiac glycoside binding (Lindenmayer & Schwartz, 1973*a*) is shown in eqn. (1), where $n = 1$.

$$v = \frac{V_{max}}{\left[1 + \frac{0{\cdot}213}{[K^+]}\left(1 + \frac{[Na^+]}{13{\cdot}7}\right)\right]^n} \quad (1)$$

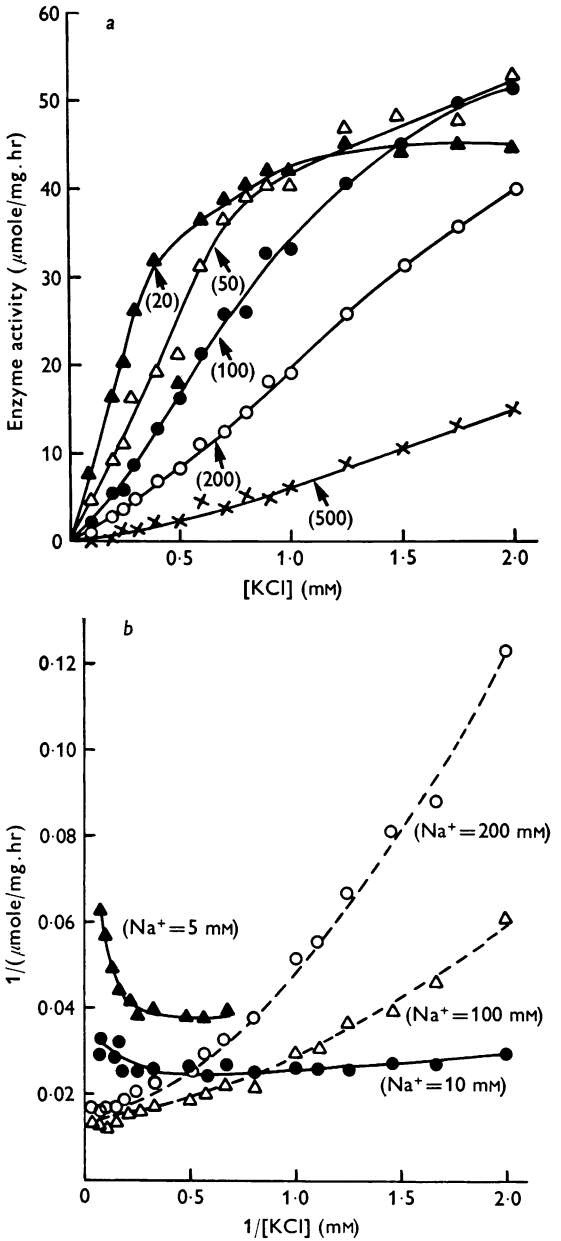

Fig. 1. The effect of sodium on potassium activation of $(Na^+ + K^+)$-ATPase activities. Enzyme activities were determined from 10-min assays at 37° C in the presence of 50 mM-Tris-Cl, pH 7·4, 2·5 mM-$MgCl_2$, potassium as indicated on the abscissa, sodium as shown in parentheses (mM) and 2·5 mM-Tris-ATP. *a*, enzyme activities *vs.* potassium concentrations; *b*, double reciprocal plots of activities *vs.* potassium concentrations. In this and subsequent Figures, 'enzyme activities' are expressed as μmole inorganic phosphate/mg protein.hr.

V_{max} can be calculated by use of this relationship and velocities observed for particular sodium and potassium concentrations.

$$(V_{max})_{cal} = v_{obs}\left[1+\frac{0{\cdot}213}{[K^+]}\left(1+\frac{[Na^+]}{13{\cdot}7}\right)\right]^n \qquad (2)$$

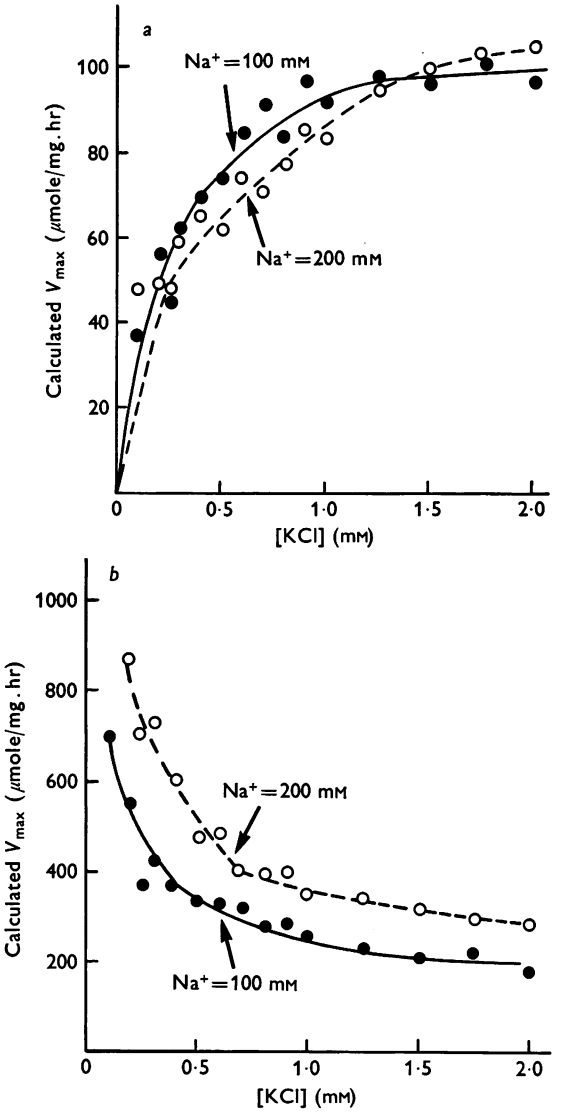

Fig. 2. $(V_{max})_{cal}$ *vs.* potassium concentration at 100 and 200 mM sodium. Values for $(V_{max})_{cal}$ were obtained through use of eqn. (2) and observed enzyme activities. *a*, values derived by use of the one-site model (n = 1; eqn. (2)); *b*, values derived by use of the two-equivalent site model (n = 2; eqn. (2)).

The model is presumed to fit the data if a constant value for $(V_{max})_{cal}$, greater than zero, is maintained for velocities observed over a range of sodium and potassium concentrations. Fig. 2*a* presents this analysis for the first model (eqn. (1); $n = 1$) and Fig. 2*b* presents the same analysis for a two-site activation model where the sites are equivalent (eqn. (1); $n = 2$). Clearly, the data are not explained by either model.

The data were next tested against a two-nonequivalent site model, where one of the sites is that described by the denominator of eqn. (1) with $n = 1$.

$$v = \frac{V_{max}}{\left[1 + \frac{0{\cdot}213}{[K^+]}\left(1 + \frac{[Na^+]}{13{\cdot}7}\right)\right]\left[1 + \frac{K_a}{[K^+]}\left(1 + \frac{[Na^+]}{K_i}\right)\right]}. \quad (3)$$

Set [A] equal to the term from eqn. (1).

$$[A] = \left[1 + \frac{0{\cdot}213}{[K^+]}\left(1 + \frac{[Na^+]}{13{\cdot}7}\right)\right] \quad (4)$$

whence,

$$v_{obs}[A] = \frac{V_{max}}{\left[1 + \frac{K_a}{[K^+]}\left(1 + \frac{[Na^+]}{K_i}\right)\right]}. \quad (5)$$

The two-nonequivalent site model, therefore, predicts that a plot of $v_{obs}[A]$ *vs.* potassium should yield a rectangular hyperbola. The ordinate of Fig. 2*a* is equivalent to $v_{obs}[A]$ (from eqn. (2)) and the curves in this Figure resemble rectangular hyperbolae. Double-reciprocal plots of the data presented in Fig. 2*a* further support this contention (Fig. 3). The degree of scatter in these plots is somewhat greater than what is usually observed. This is due to the use of low potassium (maximal concentration for the plots was 2 mM) in the presence of high sodium (minimal concentration was 100 mM) in order to minimize the probability for potassium interaction with the sodium-activation sites; these restrictions result in low enzyme activity. In each case, the line was hand drawn by visual inspection and the apparent dissociation constant for potassium, K'_a, was determined by extrapolation to the abscissa. The inset of Fig. 3 demonstrates the reproducibility of these analyses and data and shows that K'_a for the second site is related to the sodium concentration by eqn. (6) where K_i is the dissociation constant of the second site for sodium.

$$K'_a = K_a(1 + [Na^+]/K_i). \quad (6)$$

Least-squares analysis of the data in the inset yielded $0{\cdot}091 \pm 0{\cdot}034$ mM for the K_a. From the slope of the line (K_a/K_i), K_i was estimated to be 74·1 mM. Thus, potassium activation of $(Na^+ + K^+)$-ATPase should be

consistent with a two-nonequivalent site model, where both sites must bind potassium before enzyme catalysis.

$$\left(\frac{v}{V_{max}}\right)_{pred} = \frac{1}{\left[1+\frac{0{\cdot}213}{[K^+]}\left(1+\frac{[Na^+]}{13{\cdot}7}\right)\right]\left[1+\frac{0{\cdot}091}{[K^+]}\left(1+\frac{[Na^+]}{74{\cdot}1}\right)\right]}. \quad (7)$$

Post, Sen & Rosenthal (1965) demonstrated that the K_m for sodium for phosphorylation of $(Na^+ + K^+)$-ATPase by [γ-^{32}P]ATP was low in the absence of potassium (viz. about 1·6 mM). Since this effect is thought to

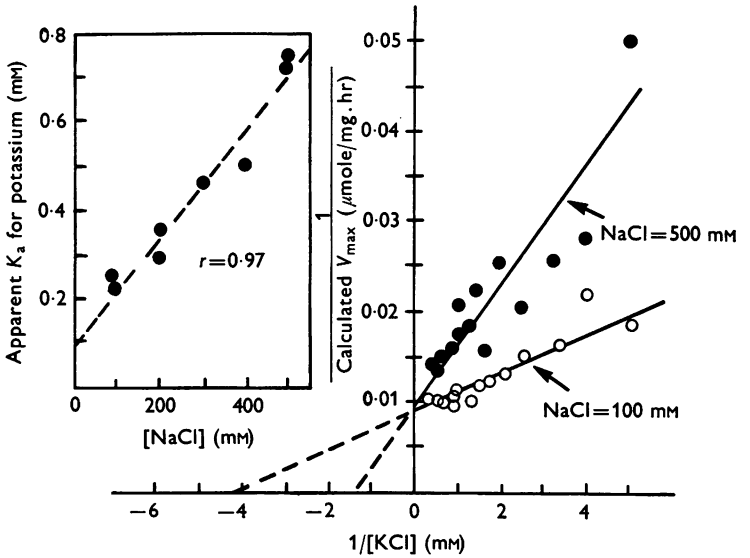

Fig. 3. Double-reciprocal plots of $(V_{max})_{cal}$, obtained by use of the one-site model (n = 1; eq. (2)), *vs.* potassium concentrations at 100 and 500 mM sodium. Extrapolation to the abscissa yielded apparent K_a for potassium. *Inset* – apparent K_a of potassium *vs.* sodium concentrations. Least-squares analysis was used to construct the line.

be mediated through the sodium-activation sites, we assumed that these sites are more than 95% saturated with sodium when free sodium is greater than 100 mM and free potassium is less than 10 mM. If this assumption and the model described by eqn. (7) are correct, constant $(V_{max})_{cal}$ should be obtained from this equation by substituting observed velocities obtained over a range of sodium and potassium concentrations for v.

$$(V_{max})_{cal} = v_{obs}\left[1+\frac{0{\cdot}213}{[K^+]}\left(1+\frac{[Na^+]}{13{\cdot}7}\right)\right]\left[1+\frac{0{\cdot}091}{[K^+]}\left(1+\frac{[Na^+]}{74{\cdot}1}\right)\right]. \quad (8)$$

This analysis (Table 1) supported the validity of the model for concentrations of potassium between 0·25 and 5 mM. The drop in $(V_{max})_{cal}$ at 10 mM

potassium (Table 1) is assumed to be secondary to potassium displacement of sodium from the sodium-activation sites. It may be argued that the data presented in Table 1 actually reflect the balance between a progressive inhibition of activity through ionic-strength effects and an activation effect through increased saturation of the sodium-activation sites with sodium, since free sodium is raised from 100 to 500 mM. A precise balance between these effects, however, is unexpected. The linear relationship between K'_a and sodium (inset of Fig. 3) also indicates that ionic-strength effects, if any, over the range studied are minimal.

TABLE 1. $(V_{max})_{cal}$ at various sodium and potassium concentrations obtained by use of the two-nonequivalent site model for potassium activation

NaCl (mM)	$(V_{max})_{cal}$ (μmole P_i/mg protein/hr)	KCl (mM)	$(V_{max})_{cal}$ (μmole P_i/mg protein/hr)
100	97 ± 4 (42)	0·25	97 ± 9 (32)
150	92 ± 4 (42)	0·50	100 ± 4 (32)
200	100 ± 2 (42)	0·75	98 ± 3 (32)
300	102 ± 2 (42)	1·0	103 ± 3 (32)
400	103 ± 4 (28)	2·0	102 ± 2 (32)
500	97 ± 7 (28)	5·0	99 ± 3 (32)
		10·0	88 ± 1 (32)

A series of assays were carried out as described in Methods, at the potassium concentrations shown on the right-hand side of the Table. The series was repeated at the sodium concentrations shown to the left. $(V_{max})_{cal}$ was calculated using observed activities and eqn. (8). Mean $(V_{max})_{cal}$ for all determinations was 98 ± 2 (n = 224) μmole P_i/mg protein/hr. The value presented for each sodium concentration is the mean ± S.E. of mean for values determined at all potassium concentrations. Conversely, the value presented for each potassium concentration is the mean ± S.E. of mean of values determined for all sodium concentrations. Numbers of determinations are shown in parentheses.

A question remains as to whether the two-nonequivalent site model applies for a wider range of potassium and sodium concentrations. In this regard, the model for potassium activation should constitute limits for activities that are reached only when the sodium-activation sites become saturated with sodium. In other words, the model should not *underpredict* activities. The following analysis tested this criterion. A mean value for $(V_{max})_{cal}$ was determined from eqn. (8) by using velocities observed for potassium concentrations of 0·25–2 mM in the presence of 200 mM sodium. These cation concentrations were chosen since they yield observed velocities that are fitted (Table 1) by the two-nonequivalent site activation model (eqn. (7)). Velocities observed for much wider ranges of

potassium (range 0·1–12 mM) and sodium (range 1–200 mM) were expressed as a percentage of $(V_{max})_{cal}$ and plotted against $(v/V_{max})_{pred}$ from eqn. (7) for the appropriate potassium–sodium pairs (Fig. 4). This analysis showed that the model constitutes limits since the function of observed activities (i.e. Observed Percentage of $(V_{max})_{cal}$) is low or equal to predicted activities (i.e. Percentage V_m predicted; Fig. 4). The limits are reached at low potassium and high sodium concentrations, which are presumed to reflect maximal sodium activation. The function of observed activities is never higher than what the model predicts, which is consistent with the concept that the potassium-activation model (eq. (7)) is applicable to much wider ranges of potassium and sodium than those tested by the analysis presented in Table 1.

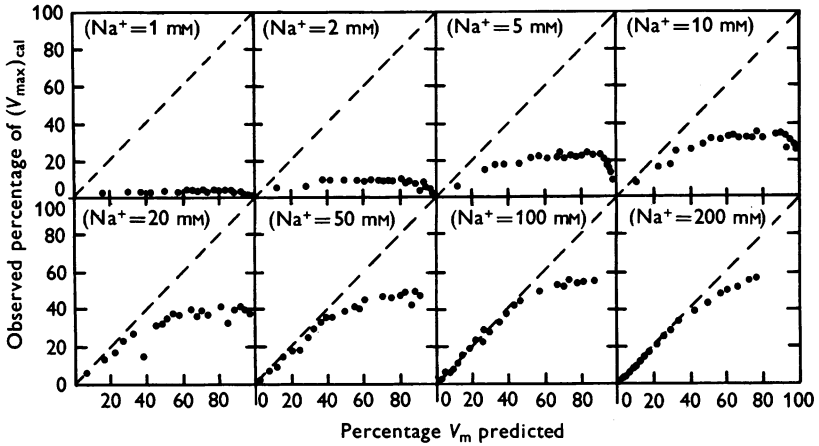

Fig. 4. Observed percentage of $(V_{max})_{cal}$ *vs.* percentage V_m predicted. A value of 117 μmole P_i/mg protein/hr for $(V_{max})_{cal}$ was obtained by use of eqn. (8) and activities observed in the presence of 200 mM sodium and 0·25–2 mM potassium. The dashed line represents perfect prediction by the two-nonequivalent site model which is expressed by eqn. (7). The values (filled circles) that are presented for percentage of $(V_{max})_{cal}$ were obtained by using enzyme activities measured in the presence of the sodium concentrations shown and 0·1–12 mM potassium. Values as presented for a particular sodium concentration reflect those observed for low (to the left) to high (towards the right) potassium.

Derivation of a kinetic description for sodium activation of $(Na^+ + K^+)$-*ATPase.* Fig. 5*a* presents a series of sodium-activation curves obtained at different potassium concentrations and Fig. 5*b* presents double-reciprocal plots of the same data. Some of the complexities apparent in the response to sodium are due to sodium interaction with the potassium-activation sites, which induces inhibition of enzyme activity (eqn. (7)). The data may be corrected for this effect by assuming simultaneous transport of sodium

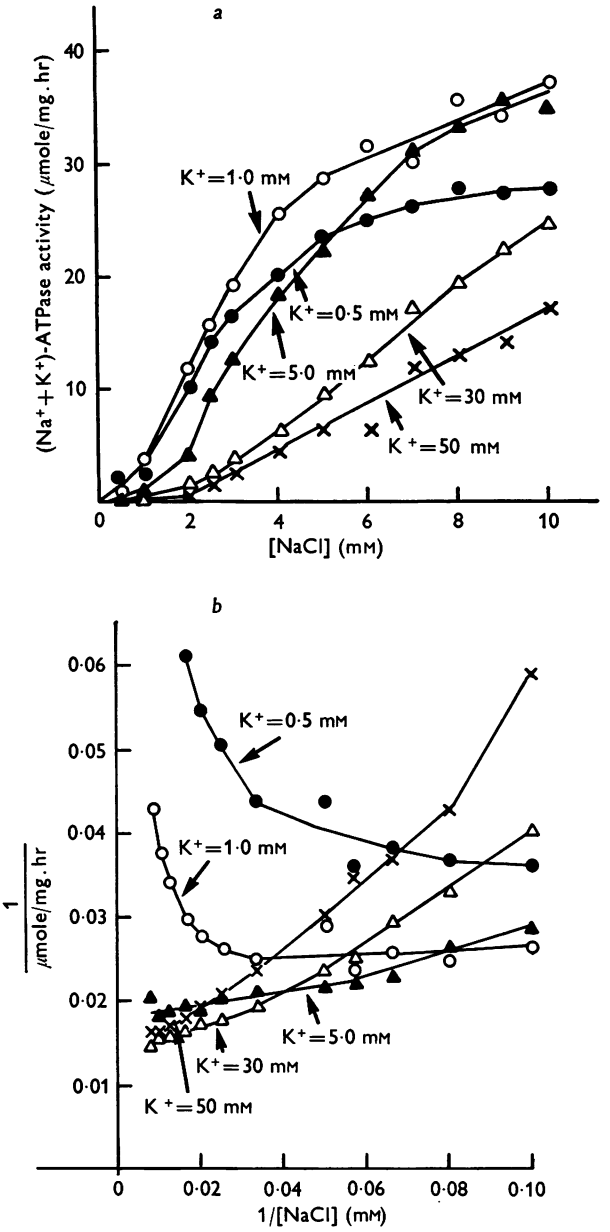

Fig. 5. The effect of potassium on sodium activation of $(Na^+ + K^+)$-ATPase. Enzyme activities were determined from 10-min assays at 37° C in the presence of 50 mM-Tris-Cl, pH 7·4, 2·5 mM-$MgCl_2$, potassium and sodium as shown and 2·5 mM-Tris-ATP. *a*, enzyme activities *vs.* sodium concentrations; *b*, double-reciprocal plots of activities *vs.* sodium concentrations.

and potassium, which implies that cation interactions with the sodium- and potassium-activation sites are independent. Further analysis of the corrected data was limited to the consideration of multiequivalent-site models for sodium activation, in which sodium (activation) and potassium (inhibition) compete for the sites. By setting the term for the second potassium-activation site equal to [B] (from eqn. (7)),

$$[B] = \left[1+\left[\frac{0{\cdot}091}{[K^+]}\left(1+\frac{[Na^+]}{74{\cdot}1}\right)\right]\right], \tag{9}$$

we obtain a composite equation for sodium and potassium activation of $(Na^+ + K^+)$-ATPase, in which [A] is defined by eqn. (4) and n is the number of equivalent sites which must bind sodium to effect catalysis.

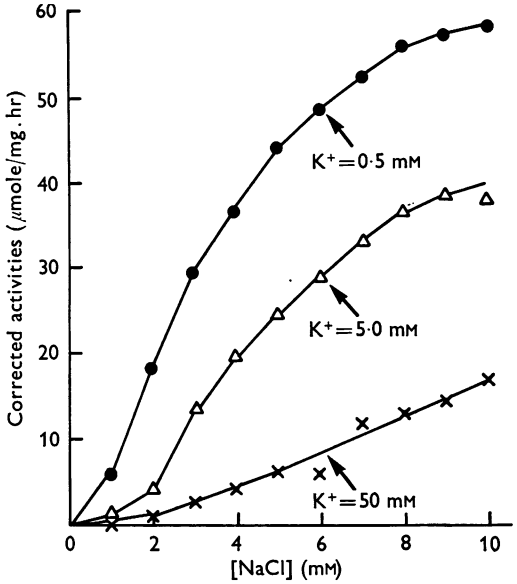

Fig. 6. The effect of potassium on sodium activation of $(Na^+ + K^+)$-ATPase. Observed enzyme activities were obtained at the various sodium and potassium concentrations and were corrected for sodium-induced inhibition of activity through interaction with the potassium-activation sites by use of eqn. (11).

$$v = \frac{V_{max}}{[A][B]\left[1+\frac{K_a}{[Na^+]}\left(1+\frac{[K^+]}{K_i}\right)\right]^n}. \tag{10}$$

The data were corrected for cation interaction with the potassium-activation sites.

$$v_{obs}[A][B] = v_{cor} = \frac{V_{max}}{\left[1+\frac{K_a}{[Na^+]}\left(1+\frac{[K^+]}{K_i}\right)\right]^n}. \tag{11}$$

Fig. 6 presents plots of corrected velocities *vs.* sodium. These plots show that n cannot equal one since the curves are sigmoidal. When n is greater than one, double-reciprocal plots of $(v_{cor})^{1/n}$ *vs.* sodium should yield straight lines for the correct value of n. Fig. 7*a*, *b*, *c* present these plots for $n = 2$, 3 and 4, respectively. These analyses suggest that $n = 3$ for sodium activation since the apparent deviation from straight lines is less obvious for $n = 3$ than for $n = 2$ or 4. The apparent dissociation constant for sodium (obtained by extrapolation to the abscissa, Fig. 7*b*) is linearly related to the potassium concentration (Fig. 8). Least-squares analysis of the data in Fig. 8 yielded $1{\cdot}39 \pm 0{\cdot}29$ mM for the dissociation constant of sodium and 11·7 mM for K_i (calculated from the slope, K_a/K_i; see eqn. (12)).

$$K'_a = 1{\cdot}39\,(1 + [K^+]/11{\cdot}7) \qquad (12)$$

A statistical assessment of various multiequivalent site models for sodium activation (eqn. (11); $n = 2$, 3, 4, 5 and 6) indicated that a

TABLE 2. Statistical comparisons of different multiequivalent site models for sodium activation

	Procedure A		Procedure B	
n	V_{max}	SSD	V_{max}	SSD
2	82·12	16,023	83·72	2861
3	79·21	12,814	81·85	2844
4	77·04	13,524	81·27	2885
5	75·63	14,801	81·04	2931
6	74·59	15,970	80·92	2969

Enzyme activities observed for 124 sodium–potassium pairs were corrected for cation interactions with the potassium-activation sites. Multiequivalent site activation models ($n = 2$, 3, 4, 5 and 6) were then fitted by computer (IBM 360/50) to the data such that values for K_a, K_i and V_{max} (eqn. (11)) were obtained which minimized the sum of squares of the differences (SSD) between observed and predicted activities. *Procedure A*: the minimal SSD between observed and predicted was calculated from

$$\text{SSD} = \Sigma\phi_i^{2n}\left(v_i - \frac{V_{max}}{\phi_i^n}\right)^2,$$

where v_i = observed, corrected velocities for particular sodium–potassium pairs;

$$\phi_i = 1 + \frac{K_a}{[Na^+]}\left(1 + \frac{[K^+]}{K_i}\right)$$

for the sodium–potassium pair and the assumed value of n; n = number of equivalent sites which must bind sodium to hydrolyse one ATP molecule and V_{max} = activity when sodium saturates the sites. *Procedure B*: minimal SSD was calculated from

$$\text{SSD} = \Sigma\left(v_i - \frac{V_{max}}{\phi_i^n}\right)^2.$$

Procedure A places greater weight on velocities obtained at lower sodium and higher potassium. Procedure B treats all velocities as equally important.

minimal sum of squares of the differences between observed and predicted activities was obtained for $n = 3$ (Table 2). The minimum for $n = 3$ was most apparent when greater weight was placed on activities observed at low sodium and high potassium (i.e. Procedure A *vs.* Procedure B; Table 2). This is consistent with the responses to sodium which are shown in Fig. 6. Thus, the deviations from the Michaelis–Menten case ($n = 1$)

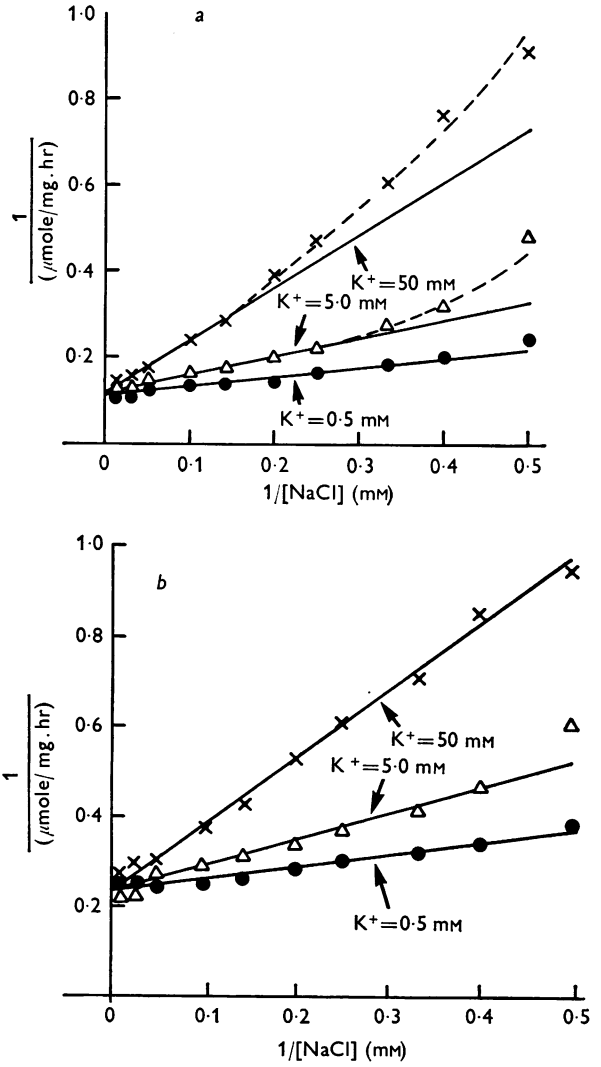

Fig. 7. Double-reciprocal plots of (corrected activities)$^{1/n}$ *vs.* sodium concentrations. Activities were obtained as described in the legend of Fig. 5 and corrected as in Fig. 6 for the various sodium and potassium concentrations. *a*, $n = 2$; *b*, $n = 3$ and *c*, $n = 4$ for eqn. (11).

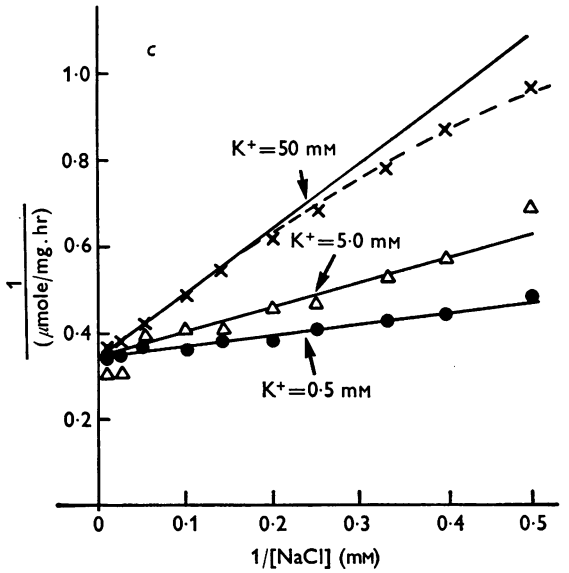

Fig. 7*c*. For legend see facing page.

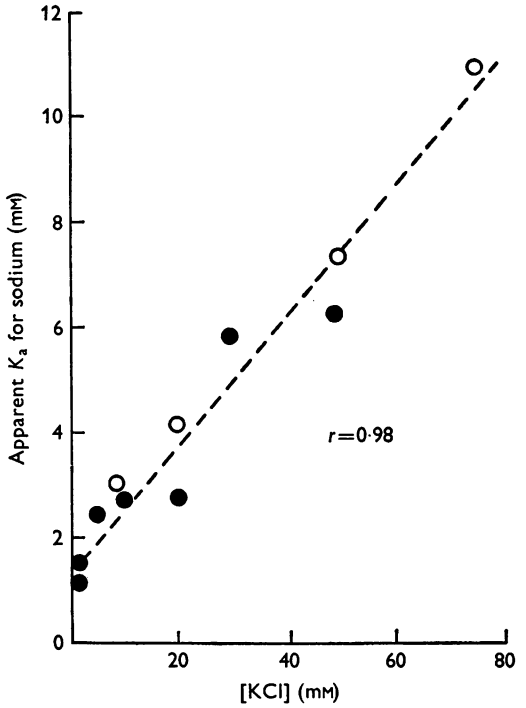

Fig. 8. The effect of potassium on the apparent dissociation constant (K_a') for sodium. Double-reciprocal plots as shown in Fig. 7*b* where $n = 3$ (eqn. (11)) were extrapolated to the abscissa to obtain values for the apparent K_a for sodium at different potassium concentrations. Least-squares analysis was used to construct the line. Filled and open circles represent two different enzyme preparations.

are greatest at low sodium and high potassium. While the data *appear* to be best fit by the model for $n = 3$ (Table 2), models for $n = 2$, 4, 5 or 6 cannot be eliminated statistically as possibilities. However, the stoichiometry of the intact pump is thought to be three sodiums pumped out per ATP consumed (Sen & Post, 1964; Gardos, 1964; Whittam & Ager, 1965; Garrahan & Glynn, 1967*d*; Baker, 1965).

Test of a composite model for sodium and potassium activation of ($Na^+ + K^+$)-*ATPase.* Assuming that the number of equivalent sodium-activation sites is three, the combined model for sodium and potassium activation is given by eqn. 10, where $K_a = 1{\cdot}39$ mM and $K_i = 11{\cdot}7$ mM (Fig. 8). $(V_{max})_{cal}$ can be calculated from eqn. (13).

$$(V_{max})_{cal} = v_{obs}[A][B]\left[1+\frac{1{\cdot}39}{[Na^+]}\left(1+\frac{[K^+]}{11{\cdot}7}\right)\right]^3 \qquad (13)$$

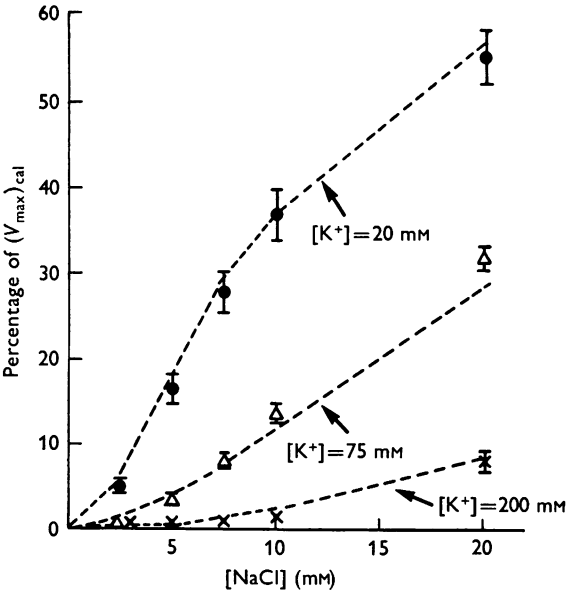

Fig. 9. Percentage of $(V_{max})_{cal}$ *vs.* sodium at different potassium concentrations. $(V_{max})_{cal}$ was calculated for activities observed in the presence of 10 mM potassium and 2·5, 5, 7·5, 10 and 20 mM sodium by use of eqn. (13). The mean value of $(V_{max})_{cal}$ was 94 μmole/mg protein.hr. Percentage of $(V_{max})_{cal}$, on the ordinate, is [corrected activities/$(V_{max})_{cal}$] × 100 and is plotted against sodium for 20 (●), 75 (Δ) and 200 mM (×) potassium. Corrected activities were calculated by eqn. (11) for $n = 3$, $K_a = 1{\cdot}39$ mM and $K_i = 11{\cdot}7$ mM. Interrupted lines represent predictions of this equation.

The hypothesis that a constant $(V_{max})_{cal}$ should be obtained for different v_{obs} was tested by three additional sets of experimental data (obtained with separate enzyme preparations) which employed wide ranges of sodium

and potassium. In the first experiment sodium was varied between 1 and 200 mM in the presence of 10 mM potassium. In the second, potassium was varied between 0·1 and 20 mM in the presence of 100 mM sodium. In the third experiment sodium/potassium was held at 10 with a sodium range of 1–200 mM and a potassium range of 0·1–20 mM. Mean values ± S.E. of mean for $(V_{max})_{cal}$ were 83·8 ± 2·3, 89·2 ± 3·0 and 139·2 ± 11·0 for the respective experiments. Thus, the model predicts, satisfactorily, the responses of $(Na^+ + K^+)$-ATPase to sodium at constant potassium and to potassium at constant sodium. The data obtained by simultaneous variation of sodium and potassium appear to be in reasonable agreement with the model, although the variation in $(V_{max})_{cal}$ was greater than that for the other two experiments.

The final experiment tests the composite model under conditions that mimic the presumed *in vivo* environment of the sodium-activation sites (i.e. high potassium). The data for sodium activation (Fig. 9) observed in the presence of 20, 75 and 200 mM potassium agree closely with the predictions of the model.

DISCUSSION

Derivation of the cation-activation models for $(Na^+ + K^+)$-ATPase employed four major assumptions: (*a*) sodium and potassium effects on enzyme activity are mediated only through the sodium- and potassium-activation sites; (*b*) sodium and potassium are transported simultaneously and the two species of activation sites are independent; (*c*) equilibria exist between free cations, unbound sites and cation-bound sites; thus, the cation interactions are not rate-limiting for any step in the enzyme turnover cycle; and (*d*) the cation-interaction site that modulates cardiac glycoside–enzyme interaction (Lindenmayer & Schwartz, 1973*a*) is a potassium-activation site. The derivation and tests of these models were restricted to the case where the enzyme is incubated in the presence of saturating amounts of magnesium.ATP at pH 7·4 and 37° C in the absence of calcium. It is apparent that the models will involve some quantitative changes as magnesium, calcium, ATP, ADP, inorganic phosphate, pH and temperature are varied (Post, Sen & Rosenthal, 1965; Baker & Stone, 1966; Fujita, Nagano, Mizuno, Tashima, Nakao & Nakao, 1967; Garrahan & Glynn, 1967*a–e*; Lindenmayer & Schwartz, 1973*b*).

The models presented here predict the responses of beef brain $(Na^+ + K^+)$-ATPase to wide ranges of sodium and potassium. No proof as to their uniqueness, however, has been presented and it is clear that the arbitrary incorporation of constants for one of the two potassium-activation sites severely limits the models which might be derived to explain the data. Nonetheless, the models presented here are of interest in two

respects. The first is that they predict responses to potassium and sodium that are consistent with some of the characteristics observed for the sodium pump of intact preparations. The second is that the models suggest molecular characteristics of the pump-enzyme system.

Activation of the sodium pump responds in a sigmoidal and saturable manner to external potassium in erythrocyte (Hoffman, 1962*a*; Sachs & Welt, 1967; Garrahan & Glynn, 1967*b*) and squid axon (Baker *et al.* 1969*b*) preparations. The erythrocyte pump is half-maximally activated by 0·14–0·4 mM external potassium in the absence of external sodium

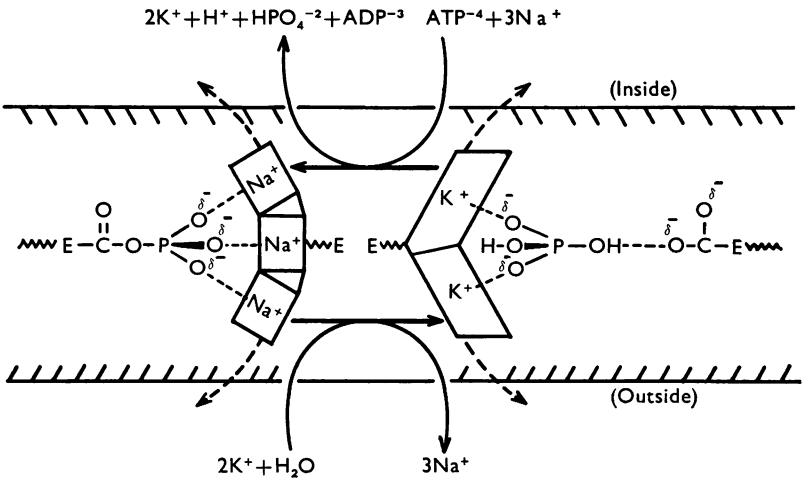

Fig. 10. For legend see facing page.

and by 0·9–2·1 mM potassium in the presence of 130–160 mM external sodium (Post *et al.* 1960; Whittam & Ager, 1964; Garrahan & Glynn, 1967*b*; Priestland & Whittam, 1968). The pump in squid axon preparations is half-maximally activated by 0·61 mM external potassium in the absence of sodium and 5·5 mM when 160 mM external sodium is present (Baker *et al.* 1969*b*). The potassium-activation model for $(Na^+ + K^+)$-ATPase (eqn. (7)) predicts a sigmoidal-saturable response to potassium with half-maximal activation at 0·36 mM potassium in the absence of sodium and at 3·3 mM in the presence of 160 mM sodium. Sjodin & Beaugé (1968) and Sjodin (1971) found in muscle cells that sodium efflux rates were sigmoidal with respect to external potassium in 120 mM external sodium but were rectangularly hyperbolic in zero sodium (K_m = 3·3 mM potassium in zero sodium). The lowest external potassium employed in the absence of external sodium, however, was 0·25 mM (Sjodin, 1971), and eqn. (7) predicts that the inflexion for potassium activation

of $(Na^+ + K^+)$-ATPase in the absence of sodium is much lower (i.e. between 10 and 20 μM potassium). Rectangular hyperbolic responses to potassium have been observed in crab nerve (Baker & Connelly, 1966) and in mammalian non-myelinated nerve preparations (Rang & Ritchie, 1968). This type of response, however, may reflect the presence of up to

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Fig. 10. Possible mechanism of sodium pump. Two conformations of a carrier are present. The three-compartment conformer has high affinity for sodium and low affinity for potassium, whereas the two-compartment conformer has low affinity for sodium and high affinity for potassium. In addition, both conformers of the carrier have an inside and an outside orientation with respect to the membrane surfaces (depicted by dashed lines). Each compartment of the carrier is assumed to represent an environment which partially substitutes for the hydration sphere of the cation (i.e. as in ionophores; Pressman, 1968). The partial charges on the free oxygens of orthophosphate (sp^3 hybridized P—O bonds) also interact with the cation to further stabilize their dehydrated states and to partially stabilize dehydrated orthophosphate. Further stabilization of the latter may be realized through interactions with other enzyme residues. ATP and/or the ATP.magnesium complex initiates the pump turnover cycle by converting the two-compartment conformer to the three-compartment conformer, which favours potassium release. Sodium interaction with the latter is associated with transfer of the terminal phosphate from ATP to the enzyme acyl group. These reactions take place on the internal surface of the membrane and are followed by reorientation of the carrier towards the outside. Addition of water splits the acyl phosphate and protonates two of the oxygens of orthophosphate, such that the three-compartment conformer is converted to the two-compartment conformer. (The assignment of the protons to orthophosphate is arbitrary; if the acyl group remains close to the phosphate, the protons may also interact with this group.) Interaction of potassium with the two-compartment conformer and reorientation towards the inner surface allows release of inorganic phosphate to complete the turnover cycle. The stoichiometry and cation affinities of the carrier are dependent upon the state of orthophosphate. For the three-compartment conformer, two charges are equally distributed over the three free oxygens of orthophosphate and distances between these oxygens and the compartments are fixed by the enzyme. For the two-compartment conformer, one charge may be distributed over two of the free oxygens of orthophosphate, but the distances between oxygens and compartments are not restrained by covalent linkage of orthophosphate to the acyl group. Thus, these distances are those of maximal stabilization by non-covalent forces. Cardiac glycosides interact with and stabilize the pump form characterized by the two-compartment conformer of the carrier (Lindenmayer & Schwartz, 1970; 1973*a*, *b*). Formation of an acylphosphate (Chignell & Titus, 1969; Siegel, Koval & Albers, 1969) in the presence of the two-compartment conformer and cardiac glycoside may reflect competition between the closed lactone ring of the drug and the protonated orthophosphate (plus acyl group?) for the molecule of water (suggested by Dr Earl T. Wallick, personal communication, 1973).

2 mM more potassium in the mesaxon and periaxon spaces than in the bulk solution (Baker *et al.* 1969*b*).

Sodium efflux in squid axon (Baker *et al.* 1969*b*) and potassium influx and sodium efflux in erythrocyte preparations (Sachs & Welt, 1967) respond to external potassium in a manner explained by two-equivalent site models for potassium interaction. In this regard, a two-equivalent-site model for potassium activation of $(Na^+ + K^+)$-ATPase was derived, which yields responses to sodium and potassium that are similar, though not identical, to those predicted by eqn. (7).

$$\frac{v}{V_{max}} = \frac{1}{\left[1 + \frac{0{\cdot}139}{[K^+]}\left(1 + \frac{[Na^+]}{31{\cdot}8}\right)\right]^2}. \tag{14}$$

The dissociation constants for potassium and sodium were obtained from eqn. (7) (i.e. $0{\cdot}139 = \sqrt{[0{\cdot}213 \times 0{\cdot}091]}$ and $31{\cdot}8 = \sqrt{[13{\cdot}7 \times 74{\cdot}1]}$. Thus equivalent site models can mimic non-equivalent-site models (and vice versa).

Activation of the sodium pump was reported to be a saturable function of the internal sodium concentration in erythrocytes (Post *et al.* 1960) and in an isolated muscle of the barnacle (Brinley, 1968). Hoffman (1962*b*) demonstrated that internal potassium appears to competitively inhibit activation of the pump by internal sodium in resealed erythrocyte ghosts. In striated muscle, sodium was found to be proportional to a cubed or higher function of internal sodium (Keynes & Swan, 1959; Mullins & Frumento, 1963; Keynes, 1965; Harris, 1965). The multiequivalent site model for sodium activation of $(Na^+ + K^+)$-ATPase appears to be consistent with these observations. The model, however, is inconsistent with data concerning activation of sodium efflux in squid axons, which appears to be a linear function of internal sodium (Hodgkin & Keynes, 1956; Sjodin & Beaugé, 1967; Brinley & Mullins, 1968; Baker *et al.* 1969*a*). Similarly, Thomas (1972) found a linear relationship between sodium efflux and internal sodium in snail neurone although this relationship required a threshold of about 1 mM sodium. It is of interest that Mullins & Brinley (1969) found *potassium influx* to be a nonlinear function of internal sodium in squid axon. They suggested that the difference in responses of sodium efflux and potassium influx to internal sodium may reflect differences in the coupling of sodium efflux to potassium influx by the pump.

Ahmed, Judah & Scholefield (1966) reported that a two-equivalent site model predicts responses of a rat brain $(Na^+ + K^+)$-ATPase to sodium. This study was carried out in the presence of low sodium and low potassium concentrations. A three-site model rather than a two-site model more closely predicts responses to sodium for beef brain $(Na^+ + K^+)$-

ATPase preparations. While it is possible that this discrepancy reflects species differences, a two-site model fits our data obtained at low potassium (0·5 mM) considerably better than the data at higher potassium (Fig. 7*a*).

Sodium and potassium activation of $(Na^+ + K^+)$-ATPase is consistent with a model that requires three sodium ions and two potassium ions to interact with their respective activation sites to effect one turnover cycle of the enzyme (eqn. (10); $n = 3$; $K_a = 1{\cdot}39$ mM; $K_i = 11{\cdot}7$ mM). If these ions are also transported and one ATP is hydrolysed per turnover cycle, the activation model is in accord with the apparent stoichiometry of the sodium pump (Sen & Post, 1964; Gardos, 1964; Whittam & Ager, 1965; Garrahan & Glynn, 1967*d*).

The models for sodium and potassium activation of $(Na^+ + K^+)$-ATPase have several physical implications for the interaction of cations with the pump-enzyme system. (*a*) Cation interactions with the activation sites of the models are random; thus, cation binding to one site does not induce formation of the second site for potassium activation or second and third sites for sodium activation. Similarly, the activation sites are independent in the autosteric or allosteric sense (Koshland & Neets, 1968). These restrictions do not preclude cation-induced fitting as the cation interacts with the groups which compose an individual site. (*b*) The relative differences in affinities for potassium and sodium between the two potassium-activation sites indicate that electrostatic interactions *per se* do not determine affinities for the cations; otherwise, the difference in dissociation constants for potassium between the sites (i.e. 0·213 *vs.* 0·091) should be reflected by similar (though not necessarily proportional) or no differences in the dissociation constants for sodium. The dissociation constants for sodium, however, change in the opposite direction (i.e. 13·7 *vs.* 74·1), suggesting that the physical dimensions of the sites contribute to the affinities for sodium and potassium. Size-restrictive sites, for example, might cause these differences if the site with high affinity for potassium and low affinity for sodium was slightly larger than the other site, since dehydrated sodium is smaller than dehydrated potassium.

The argument for a role in size restriction becomes even stronger if the sodium-activation sites are converted to potassium-activation sites within the enzyme (pump) turnover cycle (Shaw, 1955; Caldwell, Hodgkin, Keynes & Shaw, 1960) and considerable though indirect (i.e. derived from studies on fragmented membrane preparations) evidence is consistent with a sequential (or consecutive) transport of sodium and potassium (Post, Sen & Rosenthal, 1965; Albers, Koval & Siegel, 1968). *As an alternative to the model analysed in this paper*, therefore, we propose a scheme (Fig. 10) that depicts a possible physical mechanism for consecutive

transport. This scheme explains the stoichiometry of the sodium pump (i.e. three sodiums pumped out/two potassiums pumped in/ATP hydrolysed) and a mechanism for cardiac glycoside interaction with the pump. It is based on the requirements that an intermediate in the reaction be an enzyme-acylphosphate (Post *et al.* 1965; Nagano, Kanazawa, Mizuno, Tashima, Nakao & Nakao, 1965; Hokin, Sastry, Galsworthy & Yoda, 1965); that the interior of the pump protein(s) be dehydrated; and that sodium efflux precedes potassium influx.

ATP may bind before phosphorylation (not shown) to effect potassium dissociation from the carrier (Nørby & Jensen, 1971; Jensen & Nørby, 1971; Hegyvary & Post, 1971; Post, Hegyvary & Kume, 1972; also, see Siegel & Goodwin, 1972). The true substrate, however, is probably the magnesium.ATP complex and transfer of phosphate from ATP to the acyl group requires magnesium (Post *et al.* 1965). Orientation of the three-compartment conformer towards the inside *vs.* the outside is consistent with a magnesium-dependent conversion of the enzyme acylphosphate from one functional form to another (Albers *et al.* 1968). Thus, in the presence of low magnesium the acylphosphate three-compartment conformer would be oriented towards the inside such that an ATP–ADP exchange reaction (Fahn, Koval & Albers, 1966; Fahn, Hurley, Koval & Albers, 1966; Post *et al.* 1969) is possible, whereas in the presence of high magnesium the complex would be oriented towards the external surface such that the exchange reaction is not favoured. A low rate for the spontaneous break-down of the acylphosphate in the absence of potassium (Post *et al.* 1972) is accounted for if water is rate-limiting in the region of the acylphosphate. In the presence of potassium a much higher rate of break-down is explained if potassium greatly increases the availability of water for hydrolysis of the acylphosphate. While the entrance of water is shown from the external surface, it is equally possible that water enters from the internal surface. The scheme as presented also appears to be consistent with a sodium:sodium exchange (Garrahan & Glynn, 1967*a*), a potassium:potassium exchange (Glynn, Lew & Lüthi, 1970) or complete reversal of the pump such that ATP is synthesized under appropriate conditions (Garrahan & Glynn, 1967*c*; Glynn & Lew, 1970; Lant & Whittam, 1968; Priestland & Whittam, 1969; Lant, Priestland & Whittam, 1970).

The two-compartment conformer is represented by nonequivalent compartments, whereas the three-compartment conformer depicts equivalency of cation interaction sites. However, the scheme *per se* does not suffer by making the former equivalent or the latter nonequivalent. Similarly, the sites may be interdependent in an allosteric or autosteric sense (Squires, 1965; Robinson, 1967, 1968, 1970) or be independent

(Garrahan & Glynn, 1967*b*; Priestland & Whittam, 1968; Garrahan, 1969; Baker *et al.* 1969*b*).

The evidence for the sequential transport model is derived from enzymatic characterization of fragmented-membrane preparations (Post *et al.* 1965; Albers *et al.* 1968; Post *et al.* 1969) and requires that a sodium-induced chemical change precede the potassium interaction. While this is attractive, the possibility of a requirement for simultaneous sodium and potassium interaction, as suggested by the analysis herein described and the data of Baker *et al.* (1969 *b*) and of Hoffman & Tosteson (1971), must be entertained. Skou & Hilberg (1969) discussed the possibility that the sodium- and potassium-activation sites exist at the same time (consistent with simultaneous transport) and that phosphorylation by ATP (absence of potassium) is a consequence of sodium occupation of both species of activation sites. In the presence of potassium, sodium would occupy only the sodium-activation sites and potassium would interact with the potassium-activation sites to prevent phosphorylation. This, of course, does not preclude chemical changes in the state of the enzyme during transport, but may be inconsistent with a role for the phosphorylated intermediate, acylphosphate, in the turnover cycle of the pump. A major point of this paper is that the sodium- and potassium-activation data for a fragmented-membrane preparation are consistent with a model for simultaneous transport that accounts for the stoichiometry of the reaction.

A limited amount of structural information is available with respect to $(Na^+ + K^+)$-ATPase, but recent purification studies (Kyte, 1972; Lane, Copenhaver, Lindenmayer & Schwartz, 1973) suggest that the enzyme is composed of two proteins, one of which can be phosphorylated and a second which is a glycoprotein (Kyte, 1972). If the two proteins are in fact required for activity, the glycoprotein may serve as a regulator of the pump. A glycoprotein of erythrocyte membranes appeared to penetrate the membrane and have components existing in the extracellular and perhaps intracellular phases. In addition to a high number of negative charges due to the carbohydrate residues, this protein has considerable hydrophobic character (Marchesi, Tillack, Jackson, Segrest & Scott, 1972; Segrest, Jackson, Marchesi, Guyer & Terry, 1972). Thus, it is attractive to suggest that cation interaction with the glycoprotein modulates pump activity. In addition, the glycoprotein may constitute part of (or be?) the cardiac glycoside receptor.

Note added in proof. Whilst this paper was being revised, a paper by Albers & Koval (1973) appeared. They examined potassium activation of *p*-nitrophenylphosphatase activity manifested by *Electrophorus* electric organ $(Na^+ + K^+)$-ATPase. They suggest that potassium activation (in

absence of sodium) reflects two types of sites, one of which modulates substrate accessibility and a second which stimulates the turnover rate. Also, an abstract by Garrahan & Garay (1973) recently appeared that summarized some experiments on resealed erythrocyte ghost preparations. They concluded that cation movements catalysed by the pump are not consistent with a sequential transport mechanism but rather with simultaneous transport.

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