

# Kinetics of Na-ATPase Activity by the Na,K Pump

## *Interactions of the Phosphorylated Intermediates with Na<sup>+</sup>, Tris<sup>+</sup>, and K<sup>+</sup>*

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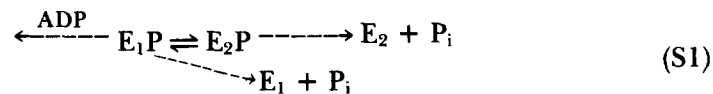
**ABSTRACT** To determine the biochemical events of Na<sup>+</sup> transport, we studied the interactions of Na<sup>+</sup>, Tris<sup>+</sup>, and K<sup>+</sup> with the phosphorylated intermediates of Na,K-ATPase from ox brain. The enzyme was phosphorylated by incubation at 0°C with 1 mM Mg<sup>2+</sup>, 25 μM [<sup>32</sup>P]ATP, and 20–600 mM Na<sup>+</sup> with or without Tris<sup>+</sup>, and the dephosphorylation kinetics of [<sup>32</sup>P]EP were studied after addition of (1) 1 mM ATP, (2) 2.5 mM ADP, (3) 1 mM ATP plus 20 mM K<sup>+</sup>, and (4) 2.5 mM ADP plus Na<sup>+</sup> up to 600 mM. In dephosphorylation types 2–4, the curves were bi- or multiphasic. “ADP-sensitive EP” and “K<sup>+</sup>-sensitive EP” were determined by extrapolation of the slow phase of the curves to the ordinate and their sum was always larger than E<sub>total</sub>. These results required a minimal model consisting of three consecutive EP pools, A, B, and C, where A was ADP sensitive and both B and C were K<sup>+</sup> sensitive. At high [Na<sup>+</sup>], B was converted rapidly to A (type 4 experiment). The seven rate coefficients were dependent on [Na<sup>+</sup>], [Tris<sup>+</sup>], and [K<sup>+</sup>], and to explain this we developed a comprehensive model for cation interaction with EP. The model has the following features: A, B, and C are equilibrium mixtures of EP forms; EP in A has two to three Na ions bound at high-affinity (internal) sites, pool B has three, and pool C has two to three low-affinity (external) sites. The putative high-affinity outside Na<sup>+</sup> site may be on E<sub>2</sub>P in pool C. The A → B conversion is blocked by K<sup>+</sup> (and Tris<sup>+</sup>). We conclude that pool A can be an intermediate only in the Na-ATPase reaction and not in the normal operation of the Na,K pump.

### INTRODUCTION

In 1957 Skou reported the discovery of Na,K-ATPase and suggested that this enzyme represented the physiological machinery for active transport of Na<sup>+</sup> and K<sup>+</sup>. Although the identity of the Na,K-ATPase and the active transport system, also called the sodium pump, is considered firmly established, there is still no unambiguous scheme correlating the enzymatic mechanism and the transport kinetics.

The Post-Albers scheme for Na,K-ATPase (e.g., Albers, 1967; Post et al., 1972) is almost universally used as a frame of reference in describing the kinetics of the enzyme. Recently, expanded versions of the original scheme have been proposed with the purpose of defining the biochemical events associated with ion movements during different transport modes of the pump (Karlsh et al., 1978; Robinson and Flashner, 1979). In these reaction models the phosphorylated enzyme intermediates,  $E_1P$  and  $E_2P$ , occupy a central position (for references to earlier work in this field see Klodos et al., 1981). However, it has from time to time been disputed whether the same phosphoenzymes are intermediates in both the Na-ATPase and the Na,K-ATPase activity (Skou, 1965, 1975; Whittam and Chipperfield, 1975; Garrahan and Garay, 1976; Klodos and Nørby, 1979), and several problems regarding the interaction of  $E_1P$  and  $E_2P$  with the transported cations remain.

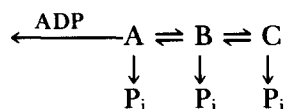
In a recent paper from this laboratory (Klodos et al., 1981), we demonstrated that the dephosphorylation kinetics of the acid-stable phosphointermediates of Na,K-ATPase under certain conditions— $[Na^+] = 150$  mM,  $[K^+] = 0$ , and various ADP concentrations—could be described by a two-compartment model, scheme S1, involving the consecutive formation of  $E_1P$  (ADP-sensitive) and  $E_2P$ :



Together with the conclusions from a parallel study on the interaction of Na,K-ATPase with Mg, ATP, and  $K^+$  (Plesner and Plesner, 1981*a, b*; Plesner et al., 1981), the kinetic characterization of the phosphoenzymes led us to propose a new, minimal model for the activities of Na,K-ATPase (Plesner et al., 1981). This model is different from the Post-Albers scheme in that  $E_1P$  is an intermediate in ATP hydrolysis in the absence of  $K^+$  but not in its presence. However, it was clear that the two-component phosphoenzyme description (S1) was inadequate to explain the known effects of variation in  $[Na^+]$  on, for instance, the  $E_1P/E_2P$  ratio, ATP:ADP exchange, Na-ATPase activity, and Na:Na exchange.

With the aim of characterizing these interactions of the cations with the phosphoenzymes of Na,K-ATPase, we have investigated the effect of  $Na^+$  (from 20 to 600 mM),  $K^+$  (20 mM), and ADP (2.5 mM) on the dephosphorylation time course of preformed, radioactive phosphointermediates,  $[^{32}P]EP$ . In one series of experiments, Tris (tris-[hydroxymethyl]-aminomethane) was added to keep the ionic strength constant.

The study somewhat surprisingly disclosed that the sum of ADP-sensitive and K-sensitive EP was  $>100\%$ , which clearly indicates the insufficiency of the two-pool model in S1. The results could, however, be accurately fitted to a three-pool model:



where A is ADP-sensitive EP, B is Na-sensitive in the sense that it is converted to A by  $\text{Na}^+$  acting at low-affinity sites, and C, as well as B, is dephosphorylated rapidly by  $\text{K}^+$ . Computer simulation allowed estimation of each of the rate coefficients involved.

To explain the dependence of the rate coefficients on  $\text{Na}^+$ ,  $\text{Tris}^+$ , and  $\text{K}^+$ , we propose that this three-pool model is a condensation of the detailed reaction scheme shown in Fig. 8. Besides being able to provide a quantitative analysis of the data, this scheme assigns component unitary processes (Hearon et al., 1959) to the composite three-pool model (allowing only one event to take place at each reaction step) and furthermore encompasses the sidedness of the transport system. Our analysis calls for a loosening of the strict classic concepts of ADP-sensitive and K-sensitive phosphoenzymes and it gives further support to the proposal that ATP hydrolysis in the presence of  $\text{Na}^+$  alone (Na-ATPase) proceeds via intermediates, some (if not all) of which are different from those of the Na,K-ATPase cycle.

A preliminary account of some of the experiments and of the comprehensive model has been given by Klodos et al. (1983).

#### MATERIALS AND METHODS

##### *Enzyme Preparation*

The Na,K-ATPase preparations were from ox brain (Klodos et al., 1975) and had the same properties as described earlier (Klodos et al., 1981; Plesner et al., 1981): specific activity,  $\sim 3 \text{ U} \cdot (\text{mg protein})^{-1}$ , maximum phosphorylation sites,  $\sim 300\text{--}360 \text{ pmol} \cdot (\text{mg protein})^{-1}$ .

##### *Phosphorylation and Dephosphorylation Experiments*

The experimental conditions (see also *Experimental Plan* and Fig. 1), including the determination of acid-stable  $^{32}\text{P}$ EP specific for Na,K-ATPase, were as described in detail by Klodos et al. (1981) unless otherwise indicated. It should be emphasized that the phosphorylation was always performed without added  $\text{K}^+$  and with  $[\text{Na}^+] \geq 20 \text{ mM}$ , and that under these conditions, almost 100% of the enzyme was phosphorylated in steady state. In one series of experiments, Tris buffer (pH 7.4 at  $0^\circ\text{C}$ ) was added to the medium so that  $[\text{Na}^+] + [\text{Tris}^+]$  was constantly 300 mM. The anion was always chloride.

Na-ATPase activity was measured at  $0.5\text{--}1^\circ\text{C}$  either under the conditions of the phosphorylation experiment with  $25 \mu\text{M}$   $^{32}\text{P}$ ATP (Fig. 1) or with 1 mM ATP (which contributes  $\sim 7 \text{ mM}$   $\text{Tris}^+$  to the assay; see below) as the substrate. The enzyme concentration was  $\sim 50 \text{ nM}$ . The  $^{32}\text{P}$  released was determined by the method of Lindberg and Ernster (1956) with the modification that the organic phase, which was isobutanol, and the water phase were pre-equilibrated with each other before use. Inorganic phosphate, when 1 mM ATP was the substrate, was determined as described by Ottolenghi (1975) with the modification (suggested by P. Ottolenghi) that 5% sodium dodecyl sulfate be added to the ascorbic acid/ammonium heptamolybdate reagent to abolish an opacity caused by the high protein concentration. The ouabain-independent activity was measured at  $0^\circ\text{C}$  after preincubation of the enzyme at  $37^\circ\text{C}$  for 30 min with  $10^{-4} \text{ M}$  ouabain (without ATP).

##### *Reagents*

ATP and ADP were obtained as sodium salts from Boehringer Mannheim Biochem-

icals, Federal Republic of Germany, and [ $\gamma^{32}\text{P}$ ]ATP was obtained from New England Nuclear, Boston, MA. ATP was converted to its Tris<sup>+</sup> salt by chromatography on a Dowex I column (Sigma Chemical Co., St. Louis, MO), and [ $\gamma^{32}\text{P}$ ]ATP was purified on DEAE-Sephadex G-25 (Nørby and Jensen, 1971). Purified ATP contained <0.5 mol% ADP. Since the K<sup>+</sup> contamination in commercial ADP batches can be rather high (corresponding to  $\sim 100 \mu\text{M}$  K<sup>+</sup> in a 2.5-mM ADP solution), ADP was purified by chromatography on a Dowex 50 WH<sup>+</sup> column. The acid eluate was neutralized immediately by propanediol. All other reagents were reagent grade.

### *Experimental Plan*

The underlying hypothesis of the experiments to be described is that the reaction sequence of Na-ATPase leading from the ADP-sensitive EP form to the unphosphorylated form(s) (presumably, but not exclusively, via the K<sup>+</sup>-sensitive form(s)) consists of several steps and therefore several different EP intermediates. One or more of these steps must involve a reaction with Na<sup>+</sup> and/or K<sup>+</sup>.

The experiments were therefore designed with the purpose of (a) localizing the reactions involving Na<sup>+</sup> and K<sup>+</sup> and (b) achieving a qualitative and quantitative description of the reaction sequence involving the acid-stable phosphorylated intermediates of Na-ATPase.

The experiments can be divided into two series. In the first, the enzyme was phosphorylated (see also Klodos et al., 1981) with  $25 \mu\text{M}$  [ $\gamma^{32}\text{P}$ ]ATP, 1 mM Mg<sup>2+</sup>, and [Na<sup>+</sup>] = 20–600 mM. In the second series, Tris buffer (pH 7.4 at 0°C) was added so that during phosphorylation and dephosphorylation [Na<sup>+</sup>] + [Tris<sup>+</sup>] was 300 mM.

In each of these series the dephosphorylation of the preformed radioactive [ $^{32}\text{P}$ ]EP intermediates (in their steady state proportions) was followed for up to 10 s under four different conditions. Under all conditions, further phosphorylation by radioactive ATP was immediately reduced to a very low rate (Klodos et al., 1981), either by the addition of unlabeled ATP to reduce the specific activity, or by the addition of ADP, which reduces the concentration of E by forming EADP. To illustrate the general experimental layout, examples of experiment types 1, 2, and 3 (below) are given in Fig. 1.

(1) **AFTER THE ADDITION OF 1 MM ATP** This experiment measures the sum of the rates of dephosphorylation of all [ $^{32}\text{P}$ ]EP species involved. Note that the addition of ATP contributes  $\sim 7$  mM Tris<sup>+</sup> to the reaction mixture.

(2) **AFTER THE ADDITION OF 2.5 MM ADP** The dephosphorylation curves are biphasic (Klodos et al., 1981). The ADP-sensitive [ $^{32}\text{P}$ ]EP and species converted rapidly to this intermediate are removed at a high rate. The slow phase may therefore serve to characterize the dephosphorylation, as well as the conversion to ADP-sensitive intermediates, of all the other [ $^{32}\text{P}$ ]EP species.

(3) **AFTER THE ADDITION OF 1 MM ATP AND 20 MM K<sup>+</sup>** In analogy to experiment type 2, this experiment allows the determination of the proportion of K-sensitive EP's and species rapidly converted thereto. Similarly, the slow phase [and the curves are indeed biphasic with a substantial proportion ([Na<sup>+</sup>]-dependent) of EP turning over slowly] serves to characterize the turnover of a K<sup>+</sup>-insensitive EP pool.

(4) **AFTER THE ADDITION OF 2.5 MM ADP AND NA<sup>+</sup> UP TO 600 MM** This illustrates the importance of Na<sup>+</sup> in the conversion of ADP-insensitive to ADP-sensitive EP and is vital for our attempts to localize the decisive step(s).

### EXPERIMENTAL RESULTS

In the following section we first report the results of the different types of experiment. We then outline the strategy for computer simulation of the

data according to the three-pool model (scheme S2) and give the results of this simulation in terms of steady state values for the pool sizes and values for the rate coefficients. Finally, we present an evaluation of the dependence of these apparent rate constants on  $[\text{Na}^+]$ ,  $[\text{K}^+]$ , and  $[\text{Tris}^+]$  in the light of the detailed reaction scheme (Fig. 8).

The points in the figures concerning the dephosphorylation experiments are data points and the curves are those obtained with the rate coefficients determined by simulation of the three-pool model. The headings of the sections below refer to the type of experiment described in the preceding section.

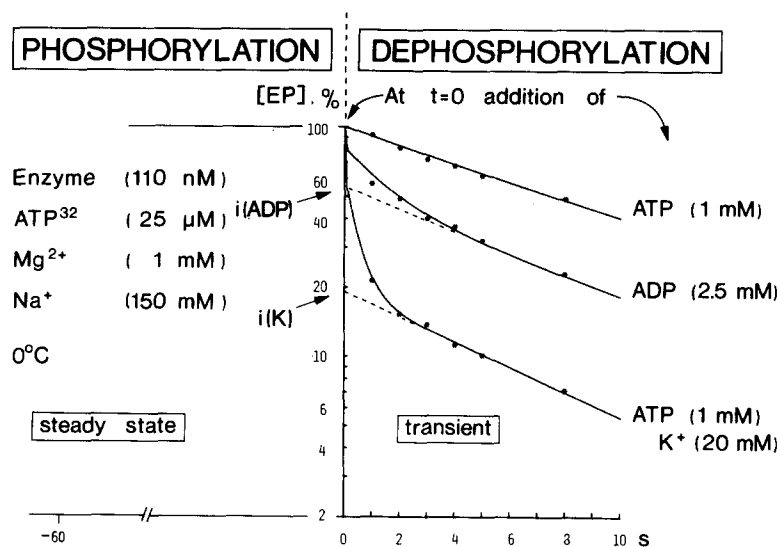


FIGURE 1. A typical experiment, here with  $[\text{Na}^+] = 150 \text{ mM}$ , illustrating the conditions for phosphorylation and three (types 1, 2, and 3; see text) of the four types of dephosphorylation experiments performed in the present paper. The curves are computer simulations of the three-pool model in scheme S2 and the intercepts  $i\text{ADP}$  and  $i\text{K}$  are obtained by linear extrapolation (by eye) using the data points. These intercepts are used in the computer simulation as described in the text. Note that  $100\% - i\text{ADP} + 100\% - i\text{K} = 45\% + 81\% = 126\%$  is  $>100\%$  (see Table I).

#### (1) Dephosphorylation with 1 mM ATP

The ATP curves of Figs. 2 and 3 clearly illustrate that the rate of dephosphorylation of the EP pools as a whole increases with  $[\text{Na}^+]$ . The effect levels off at 300 mM, and at 600 mM the rate is again lower. Comparison of Figs. 2 and 3 reveals that  $\text{Tris}^+$ , at any given  $\text{Na}^+$  concentration, inhibited dephosphorylation.

Although the curves appear monoexponential and have often been assumed to be so, close inspection of these and previously published curves reveals that this is not necessarily so. The curves through the points of the type 1 experiments in Figs. 2 and 3 are actually calculated from the sum of three exponentials related to the three-pool model (see below).

## (2) Dephosphorylation with 2.5 mM ADP

Two principal features of these experiments are apparent from Figs. 2 and 3. First,  $\text{Na}^+$  increases the proportion of EP that is rapidly dephosphorylated

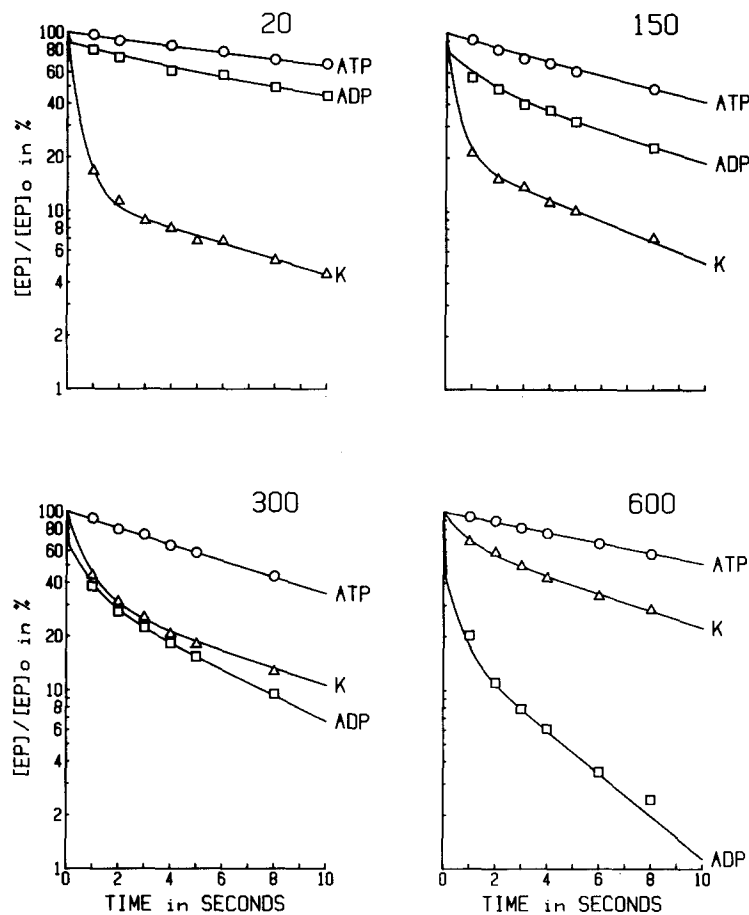


FIGURE 2. Dephosphorylation curves at  $[\text{Na}^+] = 20, 150, 300,$  and  $600 \text{ mM}$  after addition of  $1 \text{ mM ATP}, 2.5 \text{ mM ADP},$  or  $1 \text{ mM ATP} + 20 \text{ mM K}^+.$   $[\text{Na}^+]$  was the same during phosphorylation and dephosphorylation. A similar experiment with  $50 \text{ mM Na}^+$  is not shown. The experiments were performed at  $0^\circ\text{C}$  as described in Materials and Methods; see also Fig. 1. The points are data points expressed as  $100\% \cdot [\text{EP}]/[\text{EP}]_0,$  which is calculated as  $(\text{EP}'_t - \text{EP}'_\infty)/(\text{EP}'_0 - \text{EP}'_\infty)$  (see Klodos et al., 1981), where  $\text{EP}'_0$  was  $300\text{--}360$  and  $\text{EP}'_\infty$  (EP dephosphorylated for 5 min) was  $\sim 10 \text{ pmol P} \cdot \text{mg protein}^{-1}.$  Every point is the mean value of at least three experiments. The SEM was on the average  $\pm 3 \text{ pmol P} \cdot \text{mg protein}^{-1},$  which corresponds to  $\pm 1\%.$  The curves are computer simulations of the three-pool model (S2) as described in the text.

by ADP, called the ADP-sensitive EP, here estimated (see Fig. 1) as  $100\% - i\text{ADP}$  (Table I). Second, the rate of dephosphorylation of the remaining EP, ADP-insensitive EP, is higher with a higher  $\text{Na}^+$  concentration. This part

of the dephosphorylation seems to follow a monoexponential course at least at high  $[\text{Na}^+]$  (see also Figs. 1 and 4 in Klodos et al. [1981]).

Again, the results with added  $\text{Tris}^+$  are somewhat different.  $\text{Tris}^+$ , at a given  $[\text{Na}^+]$  up to 150 mM, increased the proportion of ADP-sensitive EP and slowed the decay of the remaining EP species.

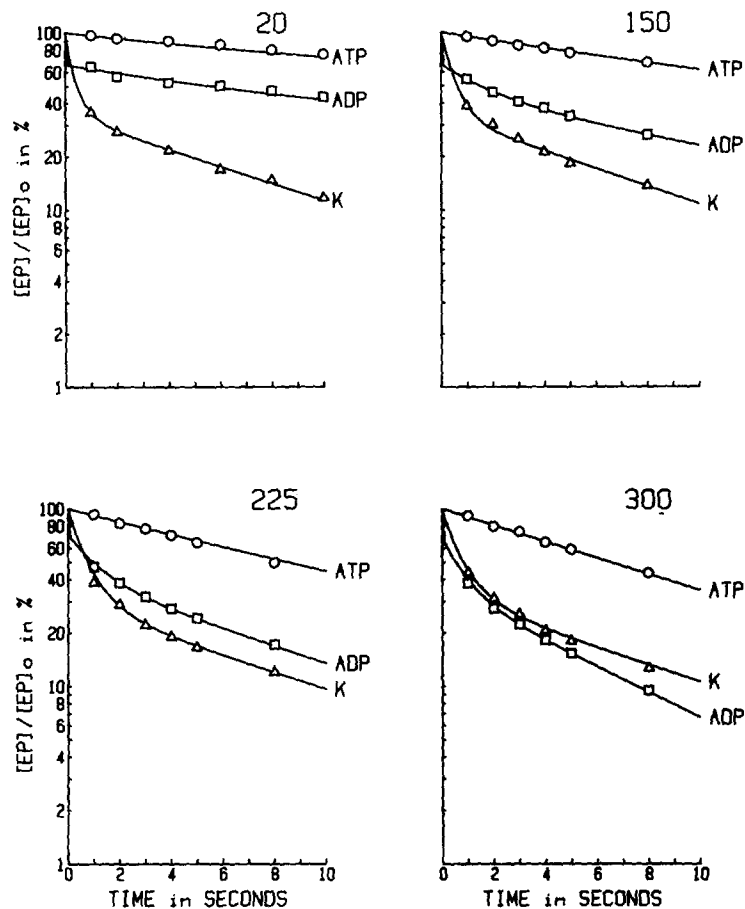


FIGURE 3. Dephosphorylation curves (as in Fig. 2) at  $[\text{Na}^+] = 20, 150, 225,$  or  $300$  mM in Tris buffer. The experimental conditions were such that  $[\text{Na}^+] + [\text{Tris}^+]$  was held constant at  $300$  mM both during phosphorylation and dephosphorylation. The  $[\text{Na}^+] = 300$  mM experiment is from Fig. 2 for comparison. An experiment with  $50$  mM  $\text{Na}^+ + 250$  mM  $\text{Tris}^+$  was also performed (not shown). See Figs. 1 and 2 for further details.

### (3) Dephosphorylation with $1$ mM ATP and $20$ mM $\text{K}^+$

The proportion of  $^{32}\text{P}$ EP dephosphorylated rapidly by addition of ATP +  $\text{K}^+$  is estimated as  $100\% - iK$  (see Figs. 1–3 and Table I). This fraction, which can be called  $\text{K}^+$ -sensitive EP, decreases with a rise in  $[\text{Na}^+]$ , and at a given  $[\text{Na}^+]$ ,  $\text{Tris}^+$  decreases the proportion of this EP form. There is an

indication that the slope of the initial fast decay (ATP + K curves in Figs. 2 and 3) is  $\text{Na}^+$  dependent. The slope of slow phase of these curves, however, is affected by neither  $[\text{Na}^+]$  nor  $[\text{Tris}^+]$ . The corresponding rate coefficient is approximately equal to  $0.1 \text{ s}^{-1}$ . The EP component represented by this slow phase is ADP-sensitive EP because it disappears very rapidly if ADP is added together with ATP and  $\text{K}^+$  (not shown).

(4) *Dephosphorylation with 2.5 mM ADP +  $\text{Na}^+$  Up to 600 mM*

As shown in the foregoing experiments of types 2 and 3, the steady state composition of the EP pool with respect to ADP-sensitive and K-sensitive EP is determined by the composition of the phosphorylation medium, notably the  $\text{Na}^+$  and the  $\text{Tris}^+$  concentrations. In Fig. 4 we demonstrate that for a given steady state situation, addition of  $\text{Na}^+$  together with ADP at the start of the dephosphorylation experiment changes the course of  $[\text{}^{32}\text{P}]\text{EP}$  decay.

TABLE I  
Proportion (%) of ADP-sensitive  $E_1\text{P}$  and  $\text{K}^+$ -sensitive  $E_2\text{P}$  as a Function of  $[\text{Na}^+]$  and  $[\text{Tris}^+]$

$[\text{Na}^+]$	$[\text{Tris}^+]$	$E_1\text{P}$ (100% - iADP)	$E_2\text{P}$ (100% - iK)	$E_1\text{P} + E_2\text{P}$
<i>mM</i>	<i>mM</i>			
20	0	15	88	103
50	0	27	87	114
150	0	45	81	126
300	0	67	68	135
600	0	83	38	121
20	280	35	68	103
50	250	38	66	104
150	150	48	65	113
225	75	57	70	127

The ordinate intercepts iADP and iK were determined as described in Fig. 1 and the text.

It is obvious that the slope of the slow phase is increased by  $\text{Na}^+$ , as is expected from the results of the type 2 experiments. More interesting is the fact that the proportion of ADP-sensitive EP, measured as 100% - iADP, is increased by the addition of  $\text{Na}^+$  to the dephosphorylation medium (see Fig. 4; e.g., enzyme phosphorylated with 20 mM  $\text{Na}^+$ : with 20 mM  $\text{Na}^+$  in the dephosphorylation medium, 100% - iADP is ~20%; with 600 mM it is ~60%). This apparently indicates that  $\text{Na}^+$ , in a concentration-dependent way, can convert an ADP-insensitive  $[\text{}^{32}\text{P}]\text{EP}$  pool (or part of it) during the dephosphorylation period to ADP-sensitive EP. We have previously (Klodos et al., 1983) called this pool  $\text{Na}^+$ -sensitive EP.

Before we describe the curve-fitting method by which the experiments are evaluated, we want to single out two features of the results obtained.

(a) Traditionally, EP in steady state is considered to consist of ADP-sensitive EP and  $\text{K}^+$ -sensitive EP, sometimes called  $E_1\text{P}$  and  $E_2\text{P}$ , respectively, so that



$EP = E_1P + E_2P$ . It is customary (e.g., Kuriki and Racker, 1976; Hara and Nakao, 1981) to determine  $E_1P$  as  $100\% - iADP$  and  $E_2P$  as  $100\% - iK$  (see Fig. 1). Using this procedure, we find that when  $[Na^+] = 20$  mM during

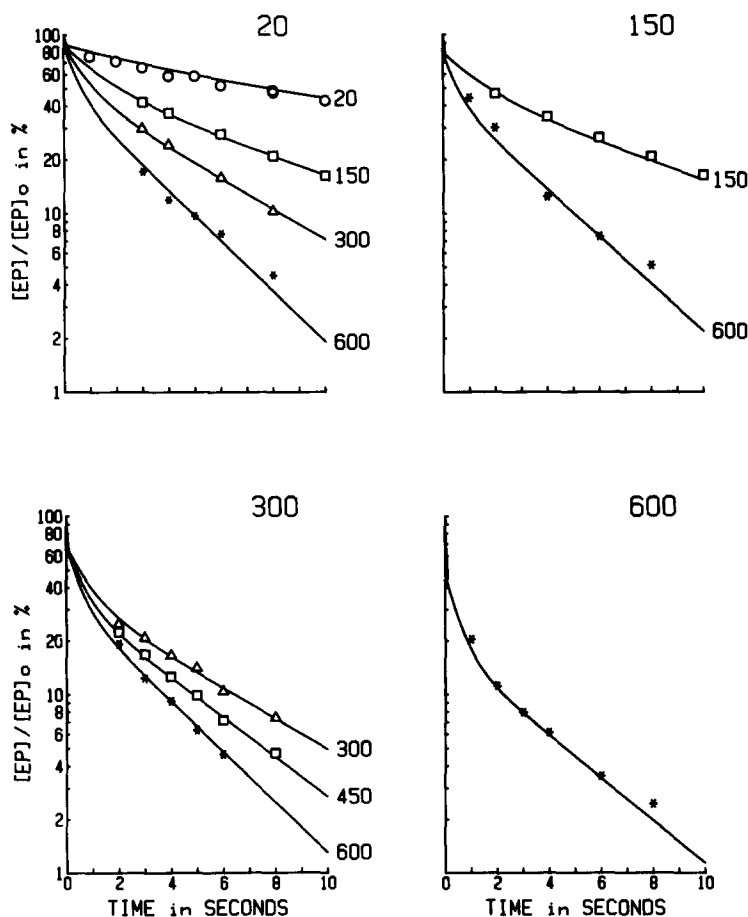


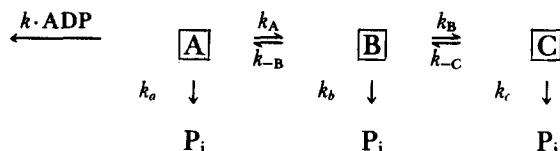
FIGURE 4. Dephosphorylation curves with ADP +  $Na^+$  corresponding to experiment type 4 (see text). The enzyme was phosphorylated as described in the presence of 20, 50 (not shown), 150, 300, or 600 mM  $Na^+$ , as indicated at the top of each panel. Dephosphorylation was accomplished by addition of 2.5 mM ADP and  $Na^+$  to give the final  $Na^+$  concentration shown for each curve. The 600-mM experiment in the lower right panel is the same as that shown in Fig. 2. See Figs. 1 and 2 for further experimental details. The curves are calculated using the values for  $A_0$ ,  $B_0$ , and  $C_0$  characteristic for  $[Na^+]$  during phosphorylation as initial values at  $t = 0$  and the rate coefficients of the three-pool model (S2) corresponding to  $[Na^+]$  during dephosphorylation.

phosphorylation,  $E_1P + E_2P$  is close to the expected 100%, but with  $[Na^+] > 50$  mM,  $E_1P + E_2P$  becomes  $>100\%$  (Table I).

(b) With a given steady state composition of EP, the value for  $100\% -$

iADP = ADP-sensitive EP can clearly be increased by increasing  $[Na^+]$  during dephosphorylation. Thus,  $Na^+$  increased both the rate of dephosphorylation and the apparent proportion of ADP-sensitive EP.<sup>1</sup>

Both these features are unexplainable by a traditional two-pool model and we were forced to try the three-pool model shown in scheme S2 as the minimum requirement for a successful curve fitting.



SCHEME S2. Three-pool model for the interconversion and hydrolysis of the phosphoenzymes of Na-ATPase. A, B, and C are different phosphoenzyme pools. For the experiments in which  $[Tris^+] + [Na^+]$  was 300 mM, the coefficients have a subscript T, e.g.,  $k_{AT}$ ,  $k_{-BT}$ , etc.

This scheme implies that A is ADP sensitive and  $E_{ADP}^{\sim P}$  (which of course cannot react with ADP) is thus not included among the acid-stable EP forms in this scheme. This seems justified for the following reasons. Let us assume that  $E_{ADP}^{\sim P}$  was acid stable and constituted a significant (measurable) proportion of the EP forms at steady state (a hypothetical pool X, not shown in S2, but placed to the left of A in scheme S2:  $\xleftarrow{k_X} X \xleftarrow{k \cdot ADP} A$ ). Since the addition of ADP, which stops phosphorylation and converts A to X, leads to a rapid disappearance of a portion of the acid-stable EP,  $k_X$  must be large. But if  $E_{ADP}^{\sim P}$  is acid stable and  $k_X$  is large, the addition of unlabeled ATP (see experiment type 1;  $k \cdot ADP \cong 0$ ) would also lead to a significant drop in the  $[^{32}P]EP$  concentration, a drop corresponding to  $[X]$ , and this is not observed (Figs. 1-3). We therefore conclude that if  $E_{ADP}^{\sim P}$  is acid stable, it is present in insignificant amounts.

#### KINETIC ANALYSIS OF DATA

As mentioned in the Introduction, all results under different conditions could be accurately fitted to the three-pool model shown in scheme S2. The mathematical formulation of that model is shown in Fig. 5. The three-pool model does not permit an analytical solution as regards the dephosphorylation equations, and the curves (which are a sum of three exponentials) must therefore be generated by numerical methods. This necessitates a primary estimation (see below) of reasonable starting values for the parameters in these equations. The strategy used in and the logic behind the estimation of the necessary starting values for the curve fitting were as follows.

*Assumptions and Preliminary Estimates of  $[A]_0$ ,  $[B]_0$ , and  $[C]_0$*

(i) Dephosphorylation of pool C (and perhaps B), but not A, is fast in the presence of  $K^+$  (the ATP +  $K^+$  curves). Therefore, the ordinate intercept of

<sup>1</sup> If this effect of  $Na^+$  were due to an increased rate of conversion of  $E_2P$  to  $E_1P$  in a two-pool system,  $Na^+$  would not change the intercept but only the slope of the ADP-insensitive decay (see Klodos et al., 1981, for an analysis of the two-pool model).

the slow phase,  $iK$  (see Figs. 1–3), is equal to  $[A]_o + a[B]_o$ , where  $0 \leq a < 1$ . As a first approximation, we assume that the monoexponential behavior of the slow phase reflects that both C and B are dephosphorylated rapidly so that  $a \cong 0$ , which makes  $iK$  a reliable starting value for  $[A]_o$ .

(ii) Pool A is assumed to disappear very rapidly after addition of 2.5 mM ADP (the ADP curves, Figs. 1–4). Thereafter, the curve follows the curve corresponding to a two-pool model described by Klodos et al. (1981) and the extrapolated ordinate intercept of the “final” monoexponential phase,  $iADP$ , is thus equal to  $[C]_o + b[B]_o$ , where  $0 \leq b < 1$ . Note that  $iK + iADP = [A]_o + [C]_o + (a + b)[B]_o$  is always  $< 100\%$ . This means that  $(a + b) < 1$  and thus  $b < 1$ .

#### STEADY STATE

$$\frac{[A]_o}{[B]_o} = \frac{1}{k_A} (k_{-B} + k_b + k_c \cdot \frac{k_B}{k_{-C} + k_c}) \quad \frac{[C]_o}{[B]_o} = \frac{k_B}{k_{-C} + k_c}$$

$$[A]_o + [B]_o + [C]_o = 100$$

#### DEPHOSPHORYLATION

$$[A]_{t,dt} = [A]_t - ([A]_t \cdot (k_A + k_a) - [B]_t \cdot k_{-B}) \cdot dt$$

$$[B]_{t,dt} = [B]_t - ([B]_t \cdot (k_B + k_b + k_{-B}) - [A]_t \cdot k_A - [C]_t \cdot k_{-C}) \cdot dt$$

$$[C]_{t,dt} = [C]_t - ([C]_t \cdot (k_C + k_{-C}) - [B]_t \cdot k_B) \cdot dt$$

FIGURE 5. Mathematical model for the three-pool model shown in scheme S2. It should be noted that in the computer simulation, as described in the text, the rate constants are only allowed to vary with the concentration of  $Na^+$ ,  $Tris^+$ , and  $K^+$ . In experiments of types 3 (where  $K^+$  is added) and 4 (where  $Na^+$  is added to the dephosphorylation medium), one can thus have one set of rate constants during steady state and another set during dephosphorylation. Dephosphorylation with 2.5 mM ADP is assumed to result in  $[A]_t = 0$  just after the addition of ADP (see Klodos et al., 1981).  $[A]_o$ , of course, is not zero. See text for further details.

(iii) With a constant steady state composition, determined by  $[Na^+]$  during phosphorylation, the addition of  $Na^+$  to ADP decreases  $iADP$ , as shown in Fig. 4. This is taken as evidence for an increased rate of conversion of B to A, and therefore  $iADP$  will approach  $[C]_o$  with increasing  $[Na^+]$ .

In summary, an estimate of  $[A]_o$  and  $[C]_o$  is obtained from points  $i$  and  $iii$ , respectively, and the estimate of  $[B]_o$  is then calculated as  $100\% - [A]_o - [C]_o$ .

#### Preliminary Estimates of Rate Coefficients

The general assumption is that the rate coefficients are dependent upon  $[Na^+]$ ,  $[K^+]$ , and  $[Tris^+]$  only, as noted also in the legend to Fig. 5.

(iv) The slope of the dephosphorylation curves after the addition of cold ATP (the ATP curves, Figs. 1–3) is a weighted (with respect to  $[A]$ ,  $[B]$ , and  $[C]$ ) average of  $k_a$ ,  $k_b$ , and  $k_c$ .

(v) As described under point *ii*, the ADP curves follow a two-pool model after  $\sim 1$  s and  $[A]_i$  is accordingly set to 0. The early curvature (Figs. 1–4) is primarily dependent on  $(k_{-B} + k_b)$ , whereas the slope of the monoexponential phase is determined mostly by  $k_r$  (and  $k_{-C}$ ) (cf. Klodos et al., 1981). The slope and the extrapolated ordinate intercepts of the curve will depend on  $[A]_o$ ,  $[B]_o$ , and  $[C]_o$ , and on  $k_{-B} + k_b$ .

(vi) Estimates of  $k_A$  and  $k_B$  can now be obtained from the steady state equations. Note that  $k_B/(k_{-C} + k_r)$  appears in both steady state expressions.

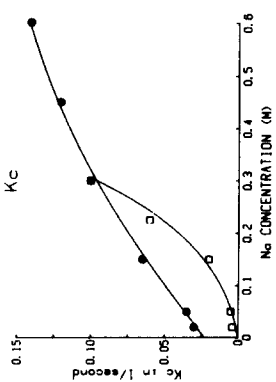
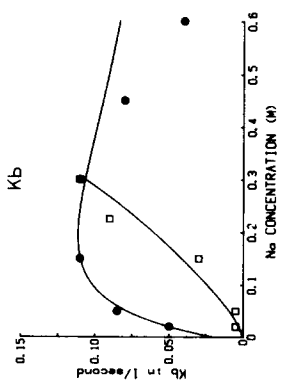
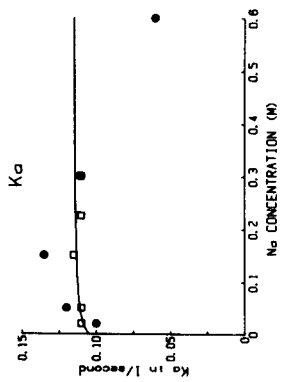
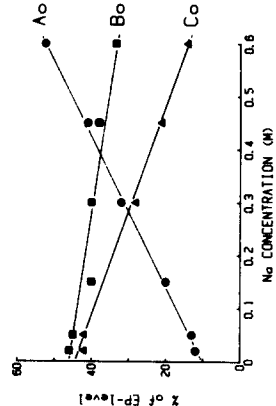
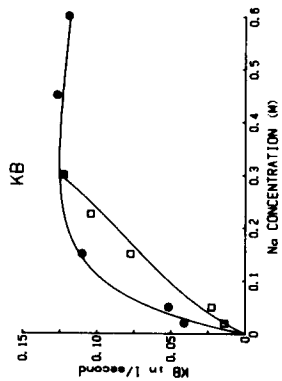
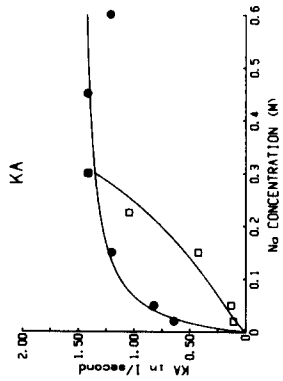
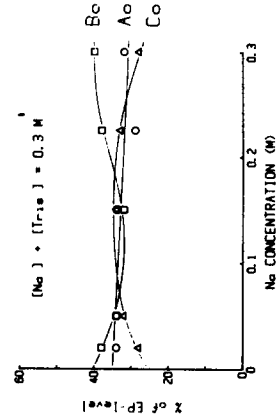
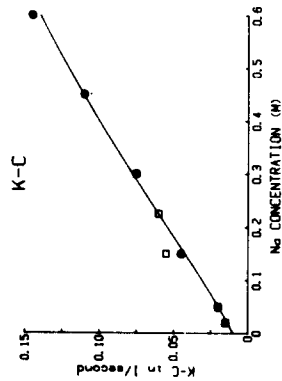
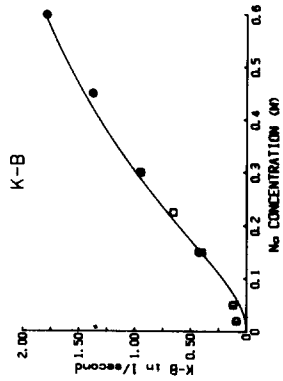
#### *Curve Fitting Using the Three-Pool Model*

The starting values obtained were then fed into an HP 85 desk computer (Hewlett-Packard Co., Palo Alto, CA), which was programmed with the equations in Fig. 5. The time step,  $dt$ , could be varied between 1 and 50 ms without any consequence for the shape and position of the simulated curves, and a step of 10 ms was finally chosen. The rate coefficients and  $[A]_o$ ,  $[B]_o$ , and  $[C]_o$  were then varied by trial and error until a satisfactory fit to the data points was obtained. The fact that there are, for each steady state condition, no less than four different dephosphorylation experiments places considerable constraints on the variability of the results of this curve fitting (see Fig. 6). The restrictions imposed by the steady state expressions were of course obeyed and so were the conditions described in the legend to Fig. 5. One further note regarding the simulation of type 3 experiments (dephosphorylation with ATP + K<sup>+</sup>): for these dephosphorylation conditions,  $k_{-B}$  was initially set to 0 as a starting value, which is equivalent to saying that B and C are dephosphorylated so rapidly that the transfer of B to A is negligible. As will be discussed later, this turned out not to be true at high Na<sup>+</sup>.

The results of the curve fitting are given first in Figs. 1–4, where the drawn curves illustrate the efficacy of fit between the data and the three-pool model. Second, Fig. 6 shows the individual rate coefficients by which these curves were simulated, as well as the values for  $[A]_o$ ,  $[B]_o$ , and  $[C]_o$  as a function of Na<sup>+</sup> concentration, with and without added Tris<sup>+</sup>. Note that the rate coefficients without Tris<sup>+</sup> (closed symbols in Fig. 6) may be influenced

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FIGURE 6. (*opposite*) The rate coefficients (panels 1–7) and steady state pool sizes (panels 8 and 9) of the three-pool model (S2) as a function of  $[Na^+]$ . Closed symbols are for experiments without Tris<sup>+</sup>; open symbols are for  $[Na^+] + [Tris^+] = 300$  mM. The points represent the values obtained by curve fitting of the three-pool model to the data in Figs. 2–4, as described in the text. The range of variation in the rate coefficients, which will still give satisfactory curves through the data points in Figs. 1–4, is not more than  $\pm 0.01$  s<sup>-1</sup> for  $k_a$ ,  $k_b$ ,  $k_B$ ,  $k_{-C}$ , and  $k_r$ , and  $\pm 0.1$  s<sup>-1</sup> for  $k_A$  and  $k_{-B}$ . Greater variations clearly resulted in systematic differences between the computed model curves and the data. The curves in this figure are calculated from the affinity constants and the rate constants (Tables II and III) corresponding to the comprehensive model in Fig. 8.



to a certain, slight extent by the  $\sim 7$  mM of  $\text{Tris}^+$  added with the ATP in experiments of types 1 and 4 (see Materials and Methods).

It is obvious that the rate coefficients of the three-pool model, with the possible exception of  $k_a$ , are very much dependent on  $\text{Na}^+$  concentration. Interestingly enough, the forward reaction constants  $k_A$  and  $k_B$ , as well as the hydrolysis rate coefficients  $k_b$  and  $k_c$ , are lower with  $\text{Tris}^+$  than without, whereas those leading in the direction of pool A, namely  $k_{-B}$  and  $k_{-C}$ , are unaffected.  $k_A$  is  $\sim 10$  times higher and  $k_{-B}$  at  $\text{Na}^+ \sim 150$  mM is  $\sim 5$ – $10$  times higher than the other rate coefficients. The steady state consequence of these relationships is illustrated by the variations in the pool sizes  $[A]_o$ ,  $[B]_o$ , and  $[C]_o$  with the  $\text{Na}^+$  and  $\text{Tris}^+$  concentrations shown in Fig. 6.

#### *Comparison of Directly Measured and Calculated Na-ATPase Activity*

From the hydrolysis rate coefficients and the pool sizes given in Fig. 6 one may calculate the expected overall rate of  $\text{P}_i$  production as a function of  $[\text{Na}^+]$  (with or without  $\text{Tris}^+$ ) as

$$v = k_a[A]_o + k_b[B]_o + k_c[C]_o.$$

Comparison between  $v$  (calculated) and  $v$  (measured) shows that with  $[\text{Na}^+] + [\text{Tris}^+] = 300$  mM the agreement is excellent (Fig. 7A).

As regards the experiments without  $\text{Tris}^+$ , the situation is not so clear. First of all, the measured activity with  $25 \mu\text{M}$   $[\text{}^{32}\text{P}]\text{ATP}$  is 10–30% higher than that with 1 mM ATP (Fig. 7B) with  $\text{Na}^+$  up to 225 mM. This may be explained by the fact that our unlabeled ATP (as mentioned several times above) contains  $\text{Tris}^+$  and contributes  $\sim 7$  mM  $\text{Tris}^+$  to the assay since  $\text{Tris}^+$  in this range of  $\text{Na}^+$  concentrations inhibits the hydrolysis (Fig. 7A and the hydrolysis rate constants  $k_b$  and  $k_c$  in Fig. 6). Similarly, J. C. Skou (personal communication), using  $\text{Tris-ATP}$  as substrate, has observed an inhibition above  $100 \mu\text{M}$  ATP of Na-ATPase at  $37^\circ\text{C}$  with Na up to 150 mM, whereas L. Plesner and I. W. Plesner (personal communication) did not observe such an inhibition, probably because they used  $\text{Na}_3\text{ATP}$  instead of  $\text{Tris-ATP}$ .

Second, for  $\text{Na}^+ \geq 300$  mM, the activity with  $25 \mu\text{M}$  ATP is 10–15% lower than that with 1 mM ATP. The reason for this is not clear, but it might reflect an ionic strength effect on the  $K_{0.5}$  for ATP for the Na-ATPase reaction. It is obvious from Fig. 7B, however, that also in the experiments without  $\text{Tris}^+$  there is agreement between the calculated hydrolysis rate and that measured under conditions corresponding to those under which the hydrolysis rate constants were determined. This agreement might be taken as support for the validity of the computer simulation in describing the system.

#### *A Model for Interpreting the Cation Dependence of Rate Constants*

To explain and extract further information from the relationships shown in Fig. 6 between the parameters of the three-pool model and  $[\text{Na}^+]$  and  $[\text{Tris}^+]$ , it is necessary to expand the three-pool model to a more detailed reaction scheme. Before we undertake this task, we should point out that the form of the curves through the data points in Fig. 6 immediately excludes

the possibility that the interconversions in the three-pool model are simply additions (binding) of  $\text{Na}^+$  to or dissociation of  $\text{Na}^+$  from A, B, and C. In the case of binding reactions, the rate coefficients would not "saturate" with  $\text{Na}^+$

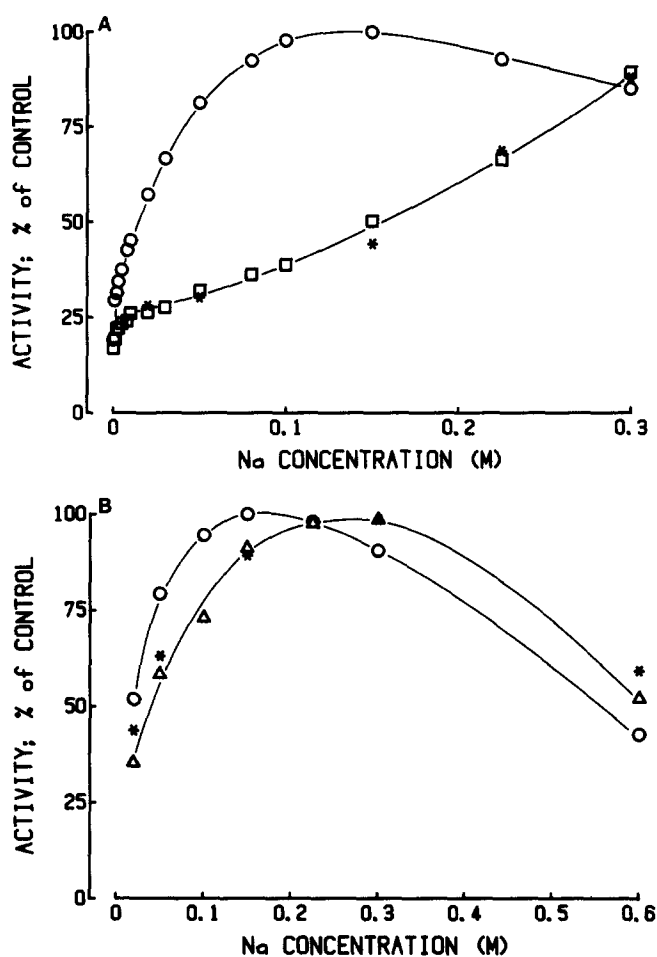


FIGURE 7. (A) Na-ATPase activity at  $0^{\circ}\text{C}$  with  $25\ \mu\text{M}$  [ $^{32}\text{P}$ ]ATP as the substrate (see Materials and Methods) as a function of  $\text{Na}^+$  concentration in the absence of  $\text{Tris}^+$  (O) and with  $[\text{Na}^+] + [\text{Tris}^+] = 300\ \text{mM}$  (□). The values represented by a star are calculated as described in the text from the steady state pool sizes of the three-pool model (S2) and the hydrolysis rate coefficients for the  $\text{Na}^+ + \text{Tris}^+$  experiment; see Fig. 6, open symbols. The activity corresponding to 100% was measured with  $150\ \text{mM}$   $\text{Na}^+$ ,  $1\ \text{mM}$   $\text{Mg}^{2+}$ , and  $30\ \text{mM}$  imidazole (no  $\text{Tris}$ ). It was  $2.7\ \text{nmol}\cdot(\text{mg protein})^{-1}\cdot\text{min}^{-1}$ . (B) Na-ATPase activity at  $0^{\circ}\text{C}$  in the absence of  $\text{Tris}$ , with  $25\ \mu\text{M}$  [ $^{32}\text{P}$ ]ATP (O) or with  $1\ \text{mM}$  ATP (which contributes  $\sim 7\ \text{mM}$   $\text{Tris}^+$  to the assay) ( $\Delta$ ). Control activity measured as above was  $1.9\ \text{nmol}\cdot(\text{mg protein})^{-1}\cdot\text{min}^{-1}$ . The values symbolized by a star are calculated as described in the text (see also above) using values for pool sizes and hydrolysis rate coefficients from Fig. 6 (no  $\text{Tris}^+$ , closed symbols).

as do  $k_A$  and  $k_B$ , and for dissociation reactions, the coefficients are independent of  $\text{Na}^+$ . Similarly, the observed dependence of the hydrolysis rate coefficients  $k_b$  and  $k_c$  on cation concentration is incompatible with such a simple reaction scheme.

One obvious way of explaining the data in Fig. 6 is to assume that the EP pools in the three-pool model, A, B, and C, all consist of an equilibrium mixture of EP species capable of binding  $\text{Na}^+$ . Within a given pool of phosphoenzymes, e.g., pool B, the EP species then would differ in the degree of occupancy of their  $\text{Na}^+$  sites and therefore possibly also in their properties

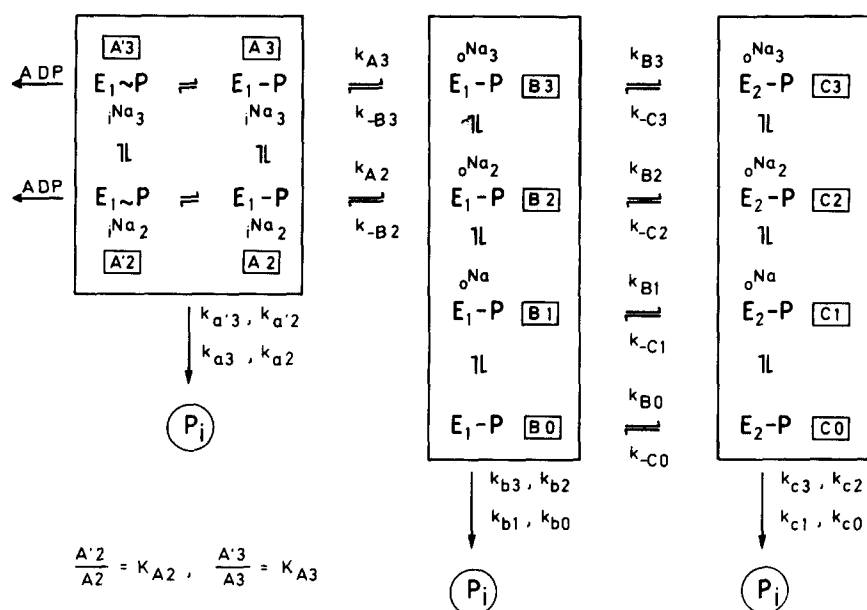


FIGURE 8. Comprehensive model for the interconversion of the acid-stable EP forms of Na-ATPase. The letters *i* and *o* before bound Na signify that Na is bound to internally oriented (cytoplasmic) sites and external sites, respectively. The site binding constants (not shown in the figure) are denoted  $\alpha$ ,  $\beta$ , and  $\gamma$  for  $\text{Na}^+$  binding in pools A, B, and C, respectively. Within the three pools, the EP species are supposed to be in equilibrium. The model is described in further detail in the text and so is the reaction of  $\text{Tris}^+$  and  $\text{K}^+$  with the different EP forms.

in regard to hydrolysis and conversion to the corresponding species in pools A and C. The model shown in Fig. 8 is an example of the application of these principles. It further illustrates how we have broken the overall reaction scheme, the three-pool model, down into unitary processes applying the criterion that in chemical and enzymatic reactions only one event (e.g., binding or release of a ligand, conformational change in the enzyme protein, hydrolysis [or other changes] of a chemical bond) takes place at each step (Hearon et al., 1959).



The design of this model (Fig. 8) is based primarily on the observations in this paper as condensed in Fig. 6. It is, for instance, necessary to have three  $\text{Na}^+$  binding steps in pool B to explain the Na dependence of  $k_{-B}$ ,  $k_B$ , and  $k_b$ , whereas one binding reaction for  $\text{Na}^+$  in pool A is sufficient to explain  $k_a$  and  $k_A$ . For pool C, two or three  $\text{Na}^+$  binding sites are necessary. It is obvious, however, that some of the details of the model, e.g., the assignment of  $\text{Na}^+$ -binding sites to external (o) or internal (i) aspects of the enzymes, cannot be derived solely from our data since we are working with a broken membrane preparation where the cytoplasmic and the external aspects of the pump are exposed to the same medium (the same  $\text{Na}^+$ ,  $\text{Tris}^+$ , and  $\text{K}^+$  concentration).

The model in Fig. 8 must therefore be considered the result of a synthesis based not only upon the results reported here but also on the many studies of Na interactions with the Na,K-ATPase performed under more physiological conditions. The relationship between our model in Fig. 8 and the results of its application in the analysis of the data in Fig. 6, and the more physiological aspects of the Na,K-ATPase, will be discussed later. It is necessary to mention here, however, that in designing this model we have correlated our analysis with the published information about Na,K-ATPase, its properties as disclosed by experiments with cells or vesicles, and its transport functions, in the following manner.

Phosphorylation by ATP in Na,K-ATPase is promoted by cytoplasmic (iNa) sodium at high-affinity sites (Blostein, 1979) and ADP-sensitive  $E_1 \sim P$  is formed first (Post et al., 1975; Fukushima and Nakao, 1981). Since A is the ADP-sensitive pool, we have assigned the internal binding sites to this pool. One of the characteristics of the Na pump is that it can operate in different modes. Of particular relevance to our model is the so-called uncoupled Na efflux, extrusion of  $\text{Na}^+$  into a medium lacking  $\text{Na}^+$  and  $\text{K}^+$  and their congeners, and Na:Na exchange between the intra- and the extracellular medium. Since the stoichiometry of uncoupled Na efflux to ATP hydrolysis is between 2 and 3 (Glynn and Karlsh, 1976) and only one  $\text{Na}^+$ -binding reaction is necessary in pool A (see above), we suggest that the  $E_1P$ 's of pool A have bound two or three Na ions. Regarding Na:Na exchange, it is relevant to our model that it seems to be ADP dependent (Glynn et al., 1971; Glynn and Hoffman, 1971; Cavieres and Glynn, 1979), that the stoichiometry of efflux to influx is 1 (Garrahan and Glynn, 1967*a*; De Weer et al., 1979), and that both efflux and influx are stimulated by extracellular sodium (oNa) acting at low-affinity sites (Garrahan and Glynn, 1967*a*; Garay and Garrahan, 1973, 1979; Beaugé and Campillo, 1976). Also relevant to this issue is the finding that  $\text{Na}^+$ , acting with rather low affinity, increases the ratio between ADP-sensitive and K-sensitive EP (Kuriki and Racker, 1976; Jørgensen and Karlsh, 1980; Hara and Nakao, 1981; this paper). ADP:ATP exchange, which clearly involves the ADP-sensitive  $E_1 \sim P$  in pool A, is also stimulated by Na acting at low-affinity sites (Tobin et al., 1973; Wildes et al., 1973; Beaugé and Glynn, 1979), and these sites are located on the external aspects of the pump (Kaplan and Hollis, 1980; Kaplan, 1982). In our analysis (see the last paragraph in this section and Table II), the binding affinities for  $\text{Na}^+$

binding to the species in pools B and C are found to be low, and the external  $\text{Na}^+$ -binding sites are therefore assigned to these two pools. This makes the  $A \rightarrow B$  reaction equivalent to the ion translocation process.

It is also important for the design of the model in Fig. 8 that the ADP sensitivity of EP is most probably lost at a step preceding the Na:Na exchange reaction since the latter is inhibited by oligomycin, whereas ADP:ATP exchange is stimulated (Garrahan and Glynn, 1967c; Blostein, 1970; Wildes et al., 1973), and that the concentration of the ADP-sensitive  $E_1 \sim P$  is greatly increased by oligomycin (as shown by Hegyvary, 1976, and I. Klodos, unpublished data). Finally, one may ask why we have proposed that the translocation (Na:Na exchange,  $A \rightleftharpoons B$ ) occurs before the "major" conformational change  $E_1P \rightarrow E_2P$  ( $B \rightarrow C$ ). An important argument is that the  $\text{Na}^+$

TABLE II  
Site Affinity Constants for  $\text{Na}^+$  and  $\text{Tris}^+$  Corresponding to the Model in Fig. 8

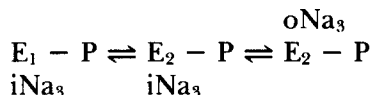
EP pool	$\text{Na}^+$ on $\text{Na}^+$ sites	Site affinity constant ( $M^{-1}$ ) for	
		$\text{Tris}^+$ on	$\text{Na}^+$ on
		Tris <sup>+</sup> site*	
A	$\alpha' = \underline{30}$ (13-30) <sup>‡</sup>	$\epsilon_A > 35$	$\rho_A > 20$
B	$\beta = \underline{5}$ (3-6)	$\epsilon_B > 35$	$\rho_B > 20$
C	$\gamma = \underline{1}$ (0.5-5)	$\epsilon_C > 35$	$\rho_C > 20$

The constants were obtained as described in the text and the Appendix. Values used in the calculation of the curves in Fig. 6 are underlined, whereas the range of values giving a satisfactory fit is shown in parentheses.

\* For values above those shown the simulated curves do not change significantly as long as  $\epsilon/\rho = 1.75$  (see Appendix).

<sup>‡</sup>  $\alpha'$  is an apparent affinity constant; see text and Appendix.

in pool B reacts at low-affinity sites [see Fig. 6 ( $k_{-B}$  as a function of  $[\text{Na}^+]$ ), and Table III], probably outside-facing sites, and that translocation therefore most likely has occurred when we go from A to B. But it should also be understood that the alternative pathway from A to C with the "major" conformational change occurring before the translocation



would open up the possibility of a quite pronounced ADP-independent Na:Na exchange, which generally does not seem to take place to any significant extent (Glynn et al., 1971; Glynn and Hoffman, 1971; Cavieres and Glynn, 1979).

In the Appendix, the expressions relating the rate coefficients of the three-pool model (S2) to the comprehensive model in Fig. 8 are presented. These expressions were fitted to the data of Fig. 6 by trial and error, and although there is a certain flexibility in this computer fitting procedure, the fact that<sup>2</sup>

<sup>2</sup> To simplify the notation, we use the general terminology  $k_j$  and  $k_{j0}$ ,  $k_{j1}$ , etc., where j can stand for A, a, -B, etc., as is also shown in Table III.

$k_j = k_{j0}$  at  $[\text{Na}^+] = 0$ ,  $k_j = k_{j\infty}$  at  $[\text{Na}^+] \rightarrow \infty$ , and that the site affinity constants ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\epsilon_A$ ,  $\rho_A$ , etc.) are common for the rate coefficient expressions for a given pool places considerable constraints on the system. The result of the curve fitting is shown in Fig. 6 and Tables II and III and gives the range of values of the affinity constants and rate constants that allow satisfactory fits of the expressions in the Appendix (Eqs. 2A, 5A–9A, etc.) to the data.

## DISCUSSION

*The Necessity of the Three-Pool Model in Scheme S2*

In the remarks introducing the three-pool model (S2) in the Results, we made it clear that since ADP-sensitive EP + K-sensitive EP was >100% (Table I)

TABLE III  
Rate Constants Corresponding to the Model in Fig. 8

j	A	a	-B	b	B	-C	c*
$k_{j3}$ ( $s^{-1}$ )	1.5 <sup>‡</sup> (1.5–1.7)	0.12 <sup>‡</sup>	4.5 (4.5–3.5)	0	0.06 (0–0.07)	0.4 (2–0.33)	0/0.24 (0–0.24)
$k_{j2}$	0 <sup>‡</sup> (0–0.3)	0.11 <sup>‡</sup>	0.3 (1–0)	0.15 (0.08–0.17)	0.18	0.3 (0.4–0)	0.33/0.07 (0.4–0.07)
$k_{j1}$			0	0.14 (0.2–0.13)	0.12 (0.15–0.1)	0.07 (0.15–0.03)	0.11/0.04 (0.2–0.04)
$k_{j0}$			0	0.02 (0–0.02)	0 (0.02–0)	0.01 (0–0.02)	0.02
$f_j$	0–0.1	1	1	0	0.3–0.4	1	0

The constants were obtained as described in the text and the Appendix. The values used in the calculation of the curves in Fig. 6 are shown separately, whereas the range of values giving a satisfactory fit is given in parentheses. Also shown is the Tris-factor,  $f$  (see Appendix).

The values for the rate constants are connected to the values for the site affinity constants given in Table II.

\* The two combinations of rate constants—0; 0.33; 0.11; 0.02 and 0.24; 0.07; 0.04; 0.02—fit the data equally well.

‡ Apparent rate constants (see Appendix). The “true” rate constants are equal to or higher than the apparent.

and since  $\text{Na}^+$  added during dephosphorylation increased ADP-sensitive EP, a three-pool model is the minimum model that can explain these results. There are four other published studies from which one can extract information regarding the sum of ADP-sensitive EP and K-sensitive EP. Kuriki and Racker (1976) have published ATP, ADP, and ATP +  $\text{K}^+$  dephosphorylation curves at 120 mM  $\text{Na}^+$  (Fig. 3 in their paper) using Na,K-ATPase from the electric organ of *Electrophorus electricus*. Although the authors do not comment on this particular problem, it is obvious that ADP-sensitive EP is ~75% and  $\text{K}^+$ -sensitive EP is ~82%, the sum being 157%. Similar results were obtained with enzymes from the same source by Yoda and Yoda (1982), who showed that the deviation from 100% of the sum of the two types of

phosphoenzyme was dependent on  $[\text{Na}^+]$ . Fukushima and Nakao (1980, 1981), studying a Ca phosphoenzyme formed with Na,K-ATPase from kidney or brain (pig or rat), also consistently observed that  $E_1P + E_2P > 100\%$ . The two types of EP were determined by extrapolation of the (apparently) monoexponential, slow phases of dephosphorylation curves with ADP and  $\text{K}^+$ , respectively. To explain this observation, they suggested that both  $E_1P$  and  $E_2P$  were overestimated because the slow phases were multi- rather than monoexponential. However, if  $E_1P$  and  $E_2P$  are measured as  $100\% - i\text{ADP}$  and  $100\% - i\text{K}$ , respectively, they will always be underestimated if the slow phase of dephosphorylation follows a multiexponential course. Furthermore, a multiexponential course of the slow phase means that the EP remaining after the very rapid disappearance of ADP- or  $\text{K}^+$ -sensitive EP must consist of at least two pools (cf. Klodos et al., 1981, for an analytical solution of this problem), which means that there must have been at least three pools initially. Regarding this point, it is of considerable interest that  $E_2P$  increased more rapidly than  $E_1P$  decreased when these components were measured as a function of time after initiation of phosphorylation (Fig. 6 in Fukushima and Nakao, 1981). This is exactly what is to be expected if A (cf. S2), formed initially, is converted relatively rapidly to B (which is both ADP and  $\text{K}^+$  sensitive at high  $\text{Na}^+$ ), and if C, being ADP-insensitive, is formed from B at a lower rate, as proposed here. Although these studies thus confirm our observation, Hara and Nakao (1981) reported for pig kidney Na,K-ATPase that "The sum of these fractions was nearly 1 in the range of 50 to 1,200 mM NaCl." These authors also do not concur with our observation that there is a decrease in  $i\text{ADP}$  when  $\text{Na}^+$  is added to the dephosphorylation medium (Fig. 6 of their paper compared with Fig. 4 in this paper). Whether this discrepancy reflects species or organ differences, or whether it is due to differences in experimental conditions like the time scale of the experiments and the technique for obtaining the ordinate intercept, is not easy to determine. We should point out, however, that with 2 M  $\text{Na}^+$  and 40 mM  $\text{K}^+$  (Fig. 2, Hara and Nakao, 1981) in the  $\text{K}^+$  dephosphorylation experiments, a significant proportion of pool B may be converted to A ( $k_{-B}$  becomes large), escaping dephosphorylation with  $\text{K}^+$  (see below in section 5).

*The Comprehensive Model (Fig. 8) for  $\text{Na}^+$  and  $\text{Tris}^+$  Effects on EP*

Although the three-pool model was necessary and sufficient to accurately describe all our data (Figs. 1–4), it must be expanded so that the dependence of its rate coefficients on  $[\text{Na}^+]$ ,  $[\text{Tris}^+]$ , and  $[\text{K}^+]$  can be explained. The excellent fit of the model to the data on the one hand and the dependence of the coefficients on ionic conditions on the other hand suggests that the three pools of A, B, and C must be composed of a number of EP species that are in ion-dependent equilibrium with one another. A further incentive for presenting a rather detailed model is the large amount of additional published information on the enzymological and transport properties of Na,K-ATPase in the presence of  $\text{Na}^+$  (and absence of  $\text{K}^+$ ). Some of this information is summarized in *A Model for Interpreting the Cation Dependence of Rate Constants* (see above).

Together with our results and our application of the repeatedly stated maxim that an enzymatic reaction can be divided into steps each of which represents only one event (so-called unitary processes), we have developed the model in Fig. 8 as a comprehensive but nevertheless minimal model. A simplified version of this model is discussed by Nørby and Klodos (1982) and Nørby (1983).

It should be noted that we do not operate with simultaneous  $\text{Na}^+$  occupancy of inner and outer sites in our model since this is not required to explain our results. In fact, simultaneous occupancy would make A3 and B3 (see Fig. 8) indistinguishable, i.e., both would equal  $E_1(i\text{Na}_3, o\text{Na}_3)\text{P}$ . Garrahan and co-workers (Garay and Garrahan, 1973; Garrahan and Garay, 1974; Garrahan et al., 1979) found that although simultaneous occupancy is not a requirement, it gives the simplest mathematical model for their Na-ATPase and Na:Na exchange results. Their Na-ATPase studies will be discussed below.

In the model in Fig. 8, we use the notation  $E_1$  in pools A and B. This does not mean that we consider the enzyme protein to have exactly the same conformation in these two pools (evidently the EP's have different properties in our model). However, we do suggest that the major conformational change takes place after the translocation of  $\text{Na}^+$ , as was suggested by Post et al. (1972) a decade ago. For a review of the conformations of Na,K-ATPase, see Jørgensen (1982). We want to indicate also that although our data can be fitted well by the expanded scheme in Fig. 8, which assumes the equivalency of the  $\text{Na}^+$  sites in pool B and the separate equivalency of the  $\text{Na}^+$  sites in pool C, this fit cannot be taken as proof of the equivalency of these sites. Rather, it reflects our limited perspective of the present pools as examined in these dephosphorylation measurements. For a separate discussion of the high affinity external  $\text{Na}^+$ -site, see *High-Affinity External  $\text{Na}^+$  Site* below.

#### *The Effect of $\text{Na}^+$*

It is our belief that the efficacy of fit of our model to the data presented here, together with the reasoning behind its formulation, warrants its use as a frame of reference (a biochemical and enzymological correlate) for the several properties, partial reactions, and transport modes, which, in the absence of  $\text{K}^+$ , are influenced by the  $\text{Na}^+$  concentration. The reader is referred to recent reviews by Garrahan and Garay (1976), Robinson and Flashner (1979), and Cantley (1981), which cover these subjects. In this section we shall limit the discussion to the effect of  $\text{Na}^+$ . The effect of  $\text{Tris}^+$ , which may influence the partial reactions and transport, as it has the phosphoenzymes and Na-ATPase, is discussed below (*The Effect of  $\text{Tris}^+$* ). We shall also assume that the substantial increase in ionic strength when  $\text{Na}^+$  is increased from 20 to 600 mM is responsible for the deviation between the model and the data as regards  $k_a$  and  $k_b$  (Fig. 6). Skou (1974, 1979) has reported a similar inhibitory effect of high ionic strength on both pNPPase and Na,K-ATPase activity.

Before we proceed with the discussion of the  $\text{Na}^+$  effect, we should point out that the dephosphorylation constants obtained in the present study are generally of the same magnitude as those calculated by other authors from

the "linear" parts of ATP, ADP, or ATP + K<sup>+</sup> dephosphorylation curves (Post et al., 1972; Kuriki and Racker, 1976; Hegyvary, 1976; Fukushima and Nakao, 1980, 1981; Klodos et al., 1981; Hara and Nakao, 1981).

*Internal Na<sup>+</sup> and the Properties of Pool A*

The lowest Na<sup>+</sup> concentration in the experiments reported in this paper is 20 mM. With 25 μM ATP and 1 mM Mg<sup>2+</sup>, this gives maximum phosphorylation, in accordance with the  $K_{0.5}$  for Na<sup>+</sup> for phosphorylation being much lower than 20 mM, since Na<sup>+</sup> is acting on high-affinity cytoplasmic sites (Blostein, 1979). Similarly, when uncoupled Na<sup>+</sup> efflux (Sachs, 1970; Karlsh and Glynn, 1974; Blostein, 1979), Na:Na exchange (Garrahan and Glynn, 1967*a*; Sachs, 1970; Garay and Garrahan, 1973), ATP:ADP exchange (Wildes et al., 1973; Beaugé and Glynn, 1979; Kaplan and Hollis, 1980; Kaplan, 1982), and Na-ATPase activity (Post et al., 1972; Beaugé and Glynn, 1979; Garrahan et al., 1979; Blostein, 1979) are studied, the internal Na<sup>+</sup> sites have a high affinity for Na<sup>+</sup> and in most cases they seem to be saturated at relatively low Na<sup>+</sup> concentrations. It was therefore not expected that the efflux rate constant,  $k_A$ , in S2 and Fig. 8 would increase with Na<sup>+</sup> with an apparent affinity constant as low as ~30 M<sup>-1</sup>, which corresponds to a  $K_{\text{diss}}$  of ~30 mM. In this connection it must be remembered that the number of internally bound Na ions needed for phosphorylation is unknown (one might be sufficient, as suggested by Mårdh and Post [1977]). For ATP:ADP exchange, two might be necessary (Beaugé and Glynn, 1979), whereas Na efflux is best described by assuming that three (Garay and Garrahan, 1973) or two to three (Karlsh and Pick, 1981) Na ions must be bound for maximal efflux. Our suggestion that the composition of pool A changes with Na and that  $k_{A2} < k_{A3}$  (Fig. 8, Table III) agrees with these ideas.

Comparing quantitative aspects such as apparent  $K_m$  or  $K_{0.5}$  values requires that species differences, temperature, etc., be taken into consideration. However, although a  $K_{0.5}$  of ~30 mM ( $1/\alpha'$ , Table II) suggests a rather low-affinity site to be in operation, it must be noted (see Appendix and Fig. 8) that the "true" affinity constant,  $\alpha$ , is  $\alpha'(1 + K_{A2})/(1 + K_{A3})$ , so that the "true"  $K_{\text{diss}}$  for Na<sup>+</sup> may be considerably lower than 30 mM.

Our model in Fig. 8 sets some constraints on the mechanism by which oligomycin can simultaneously inhibit Na:Na exchange (Garrahan and Glynn, 1967*c*; Sachs, 1980) and stimulate ATP:ADP exchange (Blostein, 1970; Wildes et al., 1973) and E<sub>1</sub>~P formation (Hegyvary, 1976; I. Klodos, unpublished data) in a reversible manner. The simplest possibility is that oligomycin binds to  $i\text{Na}_2\text{E}_1\sim\text{P}$  and/or  $i\text{Na}_3\text{E}_1\sim\text{P}$  in such a manner that the ADP sensitivity of these forms is stabilized (conversion to  $i\text{Na}_3\text{E}_1\text{P}$  is inhibited). Oligomycin may also bind to all the species in pool A and inhibit translocation of Na<sup>+</sup> ( $k_A \rightarrow 0$ , as with Tris<sup>+</sup> or K<sup>+</sup>; see below) without changing drastically the equilibria within the A pool.

*External Na<sup>+</sup> and the Properties of Pools B and C*

Na<sup>+</sup> in concentrations higher than that needed to saturate the high-affinity internal sites is important for a number of properties and partial reactions of

the Na,K-ATPase. We propose that these effects of  $\text{Na}^+$ , which are elicited on rather low-affinity sites, may be due to interactions of  $\text{Na}^+$  with pools B and C. First, our model provides a detailed mechanistic explanation of the role of  $\text{Na}^+$  in increasing the  $E_1P/E_2P$  ratio. Here it must be borne in mind that not only does pool A increase with  $[\text{Na}^+]$  at the expense of pool C (Fig. 6), but also that more and more of pool B becomes ADP-sensitive because of the increasing ratio of  $k_{-B}/k_B$  at higher  $\text{Na}^+$ . This effect of  $\text{Na}^+$  is clearly a low-affinity phenomenon (and Tris dependent [Fig. 6]; see also Kuriki and Racker, 1976; Jørgensen and Karlsh, 1980; Hara and Nakao, 1981; Yoda and Yoda, 1982), and it is parallel to a similar low-affinity effect of  $\text{Na}^+$  on ATP:ADP exchange (Wildes et al., 1973; Beaugé and Glynn, 1979; Kaplan and Hollis, 1980). Presumably, it is  $\text{Na}^+$  bound to external sites that promotes this phenomenon (Kaplan and Hollis, 1980; Kaplan, 1982), which shows little or no tendency to saturate in the region of 20 to 140–200 mM  $\text{Na}^+$ , as with  $k_{-B}$  (or  $k_{-C}$ ).

Our model also indicates part of the route that must be followed when ATP is synthesized from inorganic phosphate and ADP in what is called the backwards-running or reversal of the pump. In pump reversal (Glynn et al., 1970),  $\text{K}^+$  efflux and the simultaneous ATP synthesis by red blood cells (Glynn and Lew, 1970) increases linearly with external  $[\text{Na}^+]$ . Similarly, when  $E_2P$  is formed from  $E + P_i$ , synthesis of ATP can be invoked by adding ADP and high concentrations of  $\text{Na}^+$ , the apparent  $K_{0.5}$  for  $\text{Na}^+$  being 0.3–1 M (Post et al., 1974; Taniguchi and Post, 1975). This is what one would expect from Fig. 8 and the affinities and rate constants in Tables II and III: driving  $E_2P$  toward  $i\text{Na}_3E_1\sim P$  is possible only with very high  $\text{Na}^+$  concentrations. A similar relation has been observed between Na:Na exchange and external  $[\text{Na}^+]$  by several laboratories, e.g., Garrahan and Glynn (1967a), Garay and Garrahan (1973), and there is ample evidence that two to three sites must be occupied before  $\text{Na}^+$  influx can take place (Garay and Garrahan, 1973). This is equivalent to our observation that  $k_{-B0}$  and  $k_{-B1}$  are both zero (Fig. 8, Table III).

As regards the Na-ATPase activity, the effect of  $\text{Na}^+$  on the redistribution of the three pools and on the dephosphorylation constants does not provide a simple kinetic formula for the relationship between Na-ATPase activity,  $v$ , and  $[\text{Na}^+]$  observed by us and by Glynn and Karlsh (1976), Beaugé and Glynn (1979), and Garrahan et al. (1979). The versatility of our model in accounting also for this relationship is apparent from the agreement between the calculated and the directly observed  $v$ 's in Fig. 7. Note that here the low-affinity sites of pools B and C play a role. Two further points regarding Na-ATPase activity need to be emphasized. First, our data are best simulated if it is assumed that  $k_{b3}$  (and perhaps  $k_{r3}$ ) is zero, which would suggest inhibition (lower rate constants) at very high  $[\text{Na}^+]$ . This is in line with the measurements shown in Fig. 7B and the findings of Hara and Nakao (1981) and Forgac and Chin (1982), but it was not observed by Mårdh and Post (1977) (or by P. Ottolenghi, personal communication), probably because the latter authors used  $\text{Tris}^+$  to keep  $[\text{Tris}^+] + [\text{Na}^+]$  constant (see also Fig. 7A and *The Effect*

of  $\text{Tris}^+$  below). Second, the suggestion by Glynn and Karlish (1976) and Lee and Blostein (1980) that on the low-affinity sites  $\text{Na}^+$  acts like  $\text{K}^+$  in promoting dephosphorylation is also consistent with the result that only occupation of one or two sites is necessary for instituting hydrolysis. This is the type of  $\text{Na}^+$  interaction that may result in  $\text{Na}:\text{Na}$  exchange in the absence of ADP, and, accompanied by ATP hydrolysis, the  $\text{Na}^+$  influx proceeding via  $\text{E}_2\text{P}(\text{Na})_o \rightarrow \text{E}_2(\text{Na})_o \rightarrow \text{E}_1(\text{Na})_i$ . It is clear, however, that at saturating external  $\text{Na}^+$  and with ADP in the cells,  $\text{Na}:\text{Na}$  exchange in our model will be accompanied by little hydrolysis because (a)  $k_{b3}$  and  $k_{r3}$  are 0, and (b)  $k_A$  and  $k_{-B}$  will both be at least one order of magnitude higher than  $k_a$ , as shown in Fig. 6.

#### *High-Affinity External $\text{Na}^+$ Site*

One feature of the interaction between  $\text{Na},\text{K}\text{-ATPase}$  and  $\text{Na}^+$  is the inhibition by low extracellular  $[\text{Na}^+]$  ( $<5$  mM) of (a) uncoupled  $\text{Na}^+$  efflux (Garrahan and Glynn, 1967a; Sachs, 1970; Lew et al., 1973; Karlish and Glynn, 1974; Beaugé and Campillo, 1976), (b) the ATP hydrolysis associated herewith (Karlish and Glynn, 1974; Glynn and Karlish, 1976), and (c) ATP:ADP exchange (Beaugé and Glynn, 1979; Kaplan and Hollis, 1980; Kaplan, 1982). Although our experiments are performed at  $[\text{Na}^+] > 20$  mM, and although the inhibition by  $\text{Na}^+$  at the high-affinity extracellular site is reversed at higher  $[\text{Na}^+]$  concentrations, this phenomenon may nevertheless be important for the interpretation of our model at 0–20 mM  $\text{Na}^+$ .

It has been suggested that the inhibition results from high-affinity binding of one  $\text{Na}^+$  (Cavieres and Ellory, 1975; Garay and Garrahan, 1979) to  $\text{E}_2\text{P}$ , which inhibits the dephosphorylation of this compound (Lew et al., 1973; Cavieres and Ellory, 1975; Glynn and Karlish, 1976), thereby disrupting the cycle before the formation of  $\text{E}_1\text{P}$ , which plays a crucial role in the processes mentioned. Now, if this high-affinity site is one of the three external  $\text{Na}^+$  sites, the three sites cannot have the same affinity for  $\text{Na}^+$  as assumed in our model (Appendix). The consequence of this is that our data at  $[\text{Na}^+] = 20\text{--}600$  mM, where the high-affinity site is saturated, must be described by a two-site model. When such a model, assuming two equal, independent, low-affinity sites in each of the pools B and C ( $\beta = 2$  and  $\gamma = 0.5 \text{ M}^{-1}$ ), is fitted to our data, the results given in Fig. 9 and Table IV are obtained. Comparison of Figs. 6 and 9 will reveal that the fit to  $k_b$  (and perhaps  $k_B$ ) is best with the equal three-site model, whereas  $k_{-C}$  and  $k_c$  fit equally well. One may speculate whether this reflects the presence of a high-affinity external site on  $\text{E}_2\text{P}$  in pool C only and not on the species in pool B.

#### *The Effect of $\text{Tris}^+$*

The original purpose of adding  $\text{Tris}^+$  in the series of experiments where  $[\text{Na}^+] + [\text{Tris}^+]$  was kept constant at 300 mM was to see whether the  $\text{Na}^+$  effect at  $[\text{Na}^+] = 20\text{--}300$  mM was influenced by ionic strength. The profound difference between the hydrolysis curves (Fig. 7A) with and without  $\text{Tris}^+$  might well be thought of as an ionic strength effect, especially as the rate of hydrolysis declines when  $[\text{Na}^+]$  is increased from 300 to 600 mM. However,



on the basis of our analysis of all the data, we shall argue in favor of a specific effect of  $\text{Tris}^+$  on what we shall call a "Tris site."

It is clear from the simulation of the three-pool model (Fig. 6) that  $k_{-B}$  and  $k_{-C}$  (and probably also  $k_u$ ) are independent of the presence of  $\text{Tris}^+$ . When these coefficients are evaluated according to the comprehensive model in

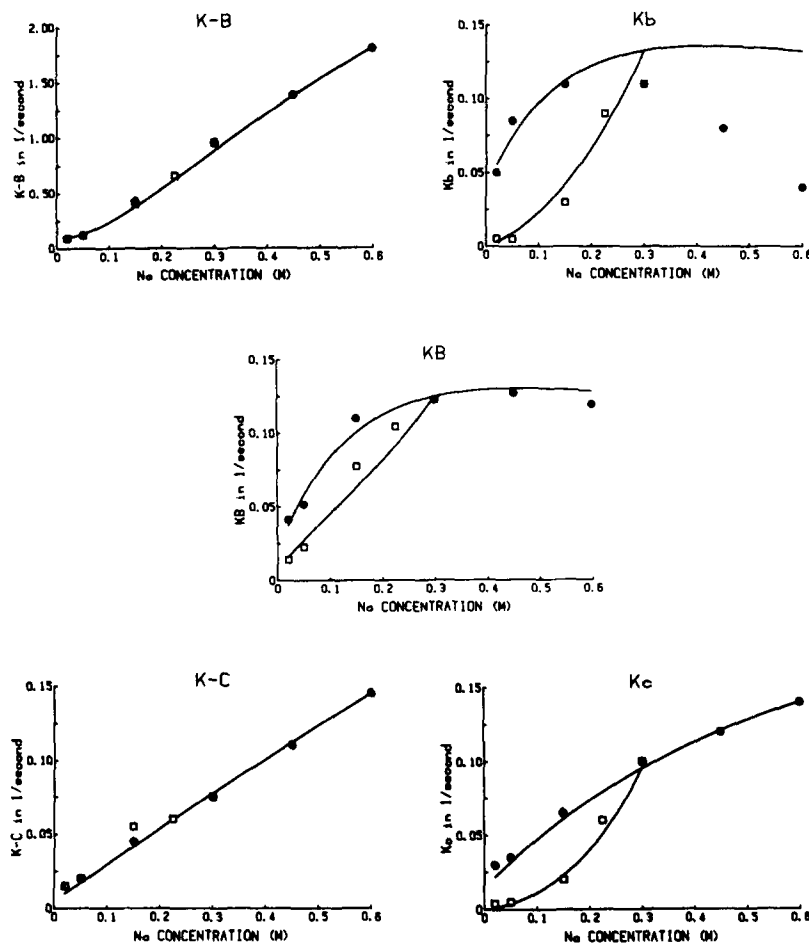


FIGURE 9. The result of fitting a model with one high-affinity external  $\text{Na}^+$  site ( $K_{\text{diss}} < 5 \text{ mM}$ ) and two equal and independent low-affinity external  $\text{Na}^+$  sites to the rate coefficients for pools B and C (see Fig. 6 for further details on the data points). The constants used in the calculation of the curves are given in Table IV and in the text (*High-Affinity External  $\text{Na}^+$  Site* in the Discussion).

Fig. 8 (see the Appendix), it appears that only those species with three  $\text{Na}^+$ , B3 and C3, are converted to A3 and B3, respectively, since  $k_{j0}$ ,  $k_{j1}$ , and  $k_{j2}$  ( $j = -B$  or  $-C$ ) are close to zero (Table III). From these premises we can conclude that  $\text{Tris}^+$  does not alter the composition of pool B or C and it

seems to be without influence on the site affinity constant for  $\text{Na}^+$ . It is thus unlikely that Tris reacts on the  $\text{Na}^+$  sites. There is no indication of a  $\text{Na}^+$  effect of  $\text{Tris}^+$ .

On the other hand,  $k_A$ ,  $k_b$ ,  $k_B$ , and  $k_c$  are all dramatically decreased by  $\text{Tris}^+$ , and this, together with the indifference of the other constants, is most easily explained as follows: within a given pool—A, B, or C— $\text{Tris}^+$  reacts with all species on a Tris site with the same affinity,  $\epsilon_A$ ,  $\epsilon_B$ , or  $\epsilon_C$ , and the EP complexes with  $\text{Tris}^+$  have in some but not all respects properties different from those without  $\text{Tris}^+$ . The close correlation between the observed and calculated hydrolysis rates (Fig. 7A) supports this analysis. Finally, with the above assumptions and the additional feature of a competition (Appendix) between  $\text{Na}^+$  and  $\text{Tris}^+$  on the Tris site, we were also able to simulate satisfactorily the rate-constant curves for the Tris experiments in Fig. 6.

According to what we have just said, the discrepancy between the Na-ATPase curves with and without  $\text{Tris}^+$  is the result of a specific  $\text{Tris}^+$  effect rather than an effect of variation in ionic strength. This should be taken into consideration in the interpretation of several published Na-ATPase curves:

TABLE IV  
Rate Constants Corresponding to a Model Like That in Fig. 8 But with One High-Affinity  $\text{Na}^+$  Site in Pools B and C

j	-B	b	B	-C	c
$k_{j3}$ ( $\text{s}^{-1}$ )	6	0	0	1	0
$k_{j2}$	0	0.25	0.25	0.25	0.37
$k_{j1}$	0.1	0.04	0.02	0	0.02
$f_j$	1	0	0.4	1	0

The rate constants are those used in the calculation of the curves in Fig. 9. See the Discussion.

Post et al. (1972) had no  $\text{Tris}^+$  in their Na-ATPase assay (but measured dephosphorylation rate constants as a function of  $[\text{Na}^+]$  in the presence of up to 720 mM  $\text{Tris}^+$ ); Mårdh and Post (1977) used 30 mM  $\text{Tris}$  and varied  $[\text{Na}^+]$ . Both Beaugé and Glynn (1979) and Garrahan et al. (1979) kept  $[\text{Na}^+] + [\text{Tris}^+]$  constant, at 180 and 150 mM, respectively. Choline has also been used to keep ionic strength constant (Glynn and Karlisch, 1976; Lee and Blostein, 1980), but we do not know whether choline has a specific effect on Na,K-ATPase.

Besides inhibiting the hydrolysis by decreasing  $k_b$  and  $k_c$ ,  $\text{Tris}^+$  also inhibits the conversion of A to B (the ion translocation step in Fig. 8), since it decreases  $k_A$  toward zero (Fig. 6 and Table III;  $f_A \sim 0$ ).  $\text{Tris}^+$ , so to say, keeps the EP on the true  $\text{E}_1\text{P}$  form with high-affinity internal sites for  $\text{Na}^+$ . This effect of  $\text{Tris}^+$  may be analogous to that observed for the dephosphoenzymes by Skou and Esmann (1980), who found that  $\text{Tris}^+$  (and certain other protonated buffers) favored the formation of a sodium-like conformation of the enzyme. Of equal interest is the report by Jensen and Ottolenghi

(1984) that Na,K-ATPase at low ionic strength occurs in a form with a high affinity for  $K^+$ , a low affinity for ATP, and presumably a low affinity for  $Na^+$ . Addition of 15–75 mM Tris<sup>+</sup> (Nørby and Jensen, 1971; Jensen et al., 1983) converts the enzyme to a form with a high affinity for ATP, and presumably a high affinity for  $Na^+$ . The affinity for Tris<sup>+</sup> in these experiments seems to be higher than  $100 M^{-1}$  (J. C. Skou, personal communication), and our value for the affinity to the phosphoenzymes,  $\epsilon > 35 M^{-1}$  (Table II), thus seems to be comparable to that for the dephosphoenzymes.

#### *The Effect of $K^+$*

The dephosphorylation experiments in the presence of 1 mM ATP and 20 mM  $K^+$  are best simulated by setting a high value for the hydrolysis rate constants of B and C in scheme S2, e.g.,  $k_b = 2 s^{-1}$  and  $k_c = 5 s^{-1}$ ; for  $[Na^+] < 300$  mM, we assume that  $k_A$  (see below) and  $k_{-B}$  are 0. For  $[Na^+] = 600$  mM, the following coefficients were used ( $s^{-1}$ ):  $k_b = 1$ ,  $k_c = 0.8$ ,  $k_A = 0.075$ , and  $k_{-B} = 0.5$ . The other coefficients were those shown in Fig. 6 for the appropriate  $Na^+$  concentration.<sup>3</sup> This is equivalent to a situation where external  $K^+$  competes with  $Na^+$  in pools B and C, and by binding to the EP species in both pools, it speeds up dephosphorylation of  $K^+$ -sensitive EP, as is generally accepted. After a short time, then, only pool A is left, and one might expect the slope of this slow dephosphorylation phase to correspond to  $k_a + k_A$  (remember [Fig. 6] that for  $[Na^+] = 20$ –300 mM,  $k_a \cong 0.12 s^{-1}$  and  $k_A = 0.7$ – $1.2 s^{-1}$ ). However, the observed slope (Figs. 2 and 3) corresponds in all cases to a rate coefficient of only  $0.1 s^{-1}$  and thus is close to  $k_a$  (and not  $k_a + k_A$ ). This means that  $k_A$  must be drastically reduced by  $K^+$ . We interpret this to show that  $K^+$  reacts with the species in pool A (the ADP-sensitive EP) and blocks the translocation of  $Na^+$  from inside sites to outside sites (i.e.,  $k_A = 0$ ) and we conclude that  $K^+$  has very much the same effect as Tris<sup>+</sup> on this step (see above). This effect of  $K^+$  on  $k_A$  was unexpected, but a survey of the literature for the relatively few  $K^+$  dephosphorylation curves revealed that this may not have been a unique observation, although it has not really been commented on before. Thus, with enzyme preparations from different sources, Kuriki and Racker (1976), Foster and Ahmed (1977), Fukushima and Nakao (1980, 1981) (with  $Ca^{2+}$  phosphoenzyme), and Hara and Nakao (1981) all demonstrated that dephosphorylation with CDTA (or ATP) +  $K^+$  is biphasic and that the slope of the second, slow phase is comparable to that of the dephosphorylation curves in the absence of  $K^+$ , as observed in the present paper (Figs. 1–3; note that  $k_a$ ,  $k_b$ , and  $k_c$  are of the same magnitude; Fig. 6). We should like further to emphasize that the rate constant for the decay of the second component of the ATP +  $K^+$  curve is independent of  $Na^+$  and Tris<sup>+</sup> (this paper), and therefore, like  $k_a$ , it is probably not influenced by  $Na^+$  on outside sites. The implications of these findings for the reaction mechanism of Na- and Na,K-ATPase are discussed below.

<sup>3</sup> Under these circumstances the curves are simulated equally well by assuming that  $k_b$  and  $k_c$ , or  $k_b$  and  $k_{-C}$ , or  $k_b$  and  $k_{-C}$  are large. In all three cases B and C will disappear rapidly.

*Are the Phosphointermediates of the Na-ATPase Cycle Common to Both the Na-ATPase and the Na,K-ATPase Cycles?*

In a recent paper (Plesner et al., 1981), we addressed this question and arrived at a negative answer. The arguments were based on a kinetic analysis using the classic two-pool model, as well as a critical review of the papers by Mårdh and his colleagues (for references, see Plesner et al., 1981). In our opinion, the experiments discussed in the preceding section clearly indicate that at least the EP species of pool A (classic  $E_1P$  or ADP-sensitive EP) cannot be intermediates of the Na,K-ATPase cycle: according to what has been said above, the rate of metabolism of A in the presence of 20 mM  $K^+$  in steady

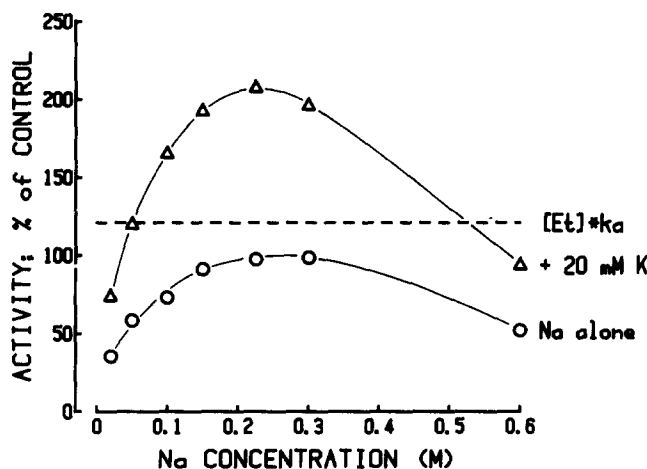


FIGURE 10. Directly measured Na-ATPase activity with 1 mM ATP (○) and Na,K-ATPase activity (△) with 1 mM ATP, 20 mM  $K^+$ , and the  $Na^+$  concentrations shown ( $t = 0-1^\circ C$ ; circumstances correspond to type 3 experiments). The dotted line represents the calculated maximal Na,K-ATPase activity under these conditions, assuming that at steady state (a) almost 100% of the enzyme is located in pool A (scheme S2, Fig. 8) and (b) that the rate constant for the metabolism of pool A is close to  $k_a$  under these conditions. See above for a discussion regarding the discrepancy between the measured (△) and the calculated (---) maximum Na,K-ATPase activity.

state is close to  $k_a \cdot [A]_o$ , and this must be equal to the rate of  $P_i$  production (ATP hydrolysis) via the intermediates of the scheme in Fig. 8. Since  $[A]_o$  cannot exceed  $[E]_{tot}$ , the calculated, maximal rate of ATP hydrolysis is  $k_a \cdot [E]_{tot}$ . When the ATPase activity is measured with or without  $K^+$  (Fig. 10), it appears that 20 mM  $K^+$  at all  $Na^+$  concentrations almost doubles the activity. However, the maximum Na,K-ATPase activity, calculated as described, is clearly lower than that measured at most  $Na^+$  concentrations. The difference between the measured and the calculated Na,K-ATPase activity becomes even more pronounced if one takes into account that the steady state value

of  $[EP]_{tot}$  in the presence of 20 mM  $K^+$ , and therefore of  $[A]_o$ , is definitely lower than  $[E]_{tot}$ , as demonstrated, for instance, by Klodos and Nørby (1979).

It is thus obvious that the hydrolysis of ATP under these conditions (with  $K^+$ ) proceeds via other intermediates than those represented in Fig. 8. This strongly argues against the commonly accepted Post-Albers scheme (Post et al., 1972; Karlsh et al., 1978), in which the intermediates of Na-ATPase are also intermediates in the presence of  $K^+$  (Na,K-ATPase activity), and it supports our earlier notion that the two types of ATP hydrolysis proceed via separate cycles (Plesner et al., 1981).

It remains to be discussed by which mechanism  $K^+$  might block the conversion of A to B. To do this,  $K^+$  must react with one or more of the EP's of pool A, but since the effect of (20 mM)  $K^+$  is completely independent of  $[Na^+]$  from 20 to almost 600 mM (Fig. 2) and of  $[Tris^+]$  (Fig. 3), it is unlikely that  $K^+$  binds to a  $Na^+$  or  $Tris^+$  site in pool A. It has been shown that low external  $K^+$  even at high external  $Na^+$  will inhibit Na:Na exchange (Garrahan and Glynn, 1967*a, b*; De Weer et al., 1979) and the ATP hydrolysis associated with uncoupled  $Na^+$  efflux (Glynn and Karlsh, 1976). This might indicate binding to external sites in pool A, but it could just as well be a result of a competition between  $Na^+$  and  $K^+$  in pool B and a subsequent accumulation of  $E_2K$  (Glynn and Karlsh, 1976).

One hypothesis, purely speculative, regarding the effect of  $K^+$ , is that  $K^+$  binds to a high-affinity, external  $K^+$  site on the  $E_1P$  species of pool A (is this the site that later becomes the high-affinity external  $Na^+$  site? see above), and by sitting there it blocks the  $A \rightarrow B$  conversion by preventing the movement of  $Na^+$  from the inside sites (through a channel?) to the outside sites.

#### *Concluding Remarks*

The model presented in scheme S2 and Fig. 8 has a necessary and sufficient number of reaction steps to explain most, if not all, the properties of Na,K-ATPase known to involve the acid-stable phosphorylated intermediates. In the light of the foregoing discussion, we want especially to emphasize the following points.

First, the phosphorylated intermediates are distributed among three major pools (and not just the classic  $E_1P$  and  $E_2P$  pools), each consisting of EP complexes which are in rapid equilibrium. The EP species of pool A have  $Na^+$  bound to two or three (probably internal) high-affinity sites, whereas the EP's of pools B and C are characterized by a binding of  $Na^+$  to three (probably external) low-affinity sites. A simultaneous effect of  $Na^+$  on internal and external sites is thus not a feature of our model. It is noteworthy that  $Na^+$  is translocated before the (presumably slow) major conformation change (from  $E_1P$  to  $E_2P$ ). Second, at 100–600 mM  $Na^+$  (in the absence of  $K^+$  and  $Tris^+$ ), the rate coefficients for the interconversion  $A \rightleftharpoons B$  are one order of magnitude higher than those for the  $B \rightleftharpoons C$  interconversion and for dephosphorylation. This might allow for a ratio of  $\sim 10:1$  or larger between the rate of Na:Na exchange and ATP hydrolysis. Third, pool A reacts rapidly with ADP (is ADP sensitive), and at relatively high  $[Na^+]$  also a part of pool

B will disappear upon addition of ADP, because of the relatively fast  $A \rightleftharpoons B$  interconversion (see above). Fourth, we suggest that the most likely explanation of the inhibitory effect of low external  $[Na^+]$  on (uncoupled)  $Na^+$  efflux and on ATP:ADP exchange is the existence of a high-affinity, external  $Na^+$  site on the  $E_2P$  of pool C. This site might be one of the three  $Na$  sites on  $E_2P$ . Fifth,  $Tris^+$  has dramatic effects: it blocks the  $A \rightarrow B$  conversion and reduces drastically the dephosphorylation rate of B and C. Finally, whereas pools B and C are both rapidly dephosphorylated after addition of  $K^+$  to the medium, the dephosphorylation of A is not affected, but its conversion to B is apparently blocked. Furthermore, the calculated rate of ATP hydrolysis in the presence of  $K^+$  is considerably smaller than the observed and we conclude that the EP species in pool A are only intermediates of the Na-ATPase but not of the Na,K-ATPase activity.

#### APPENDIX

##### *Derivation of the Relationship of the Rate Coefficients of the Three-Pool Model (S2) to the Rate Constants of the Comprehensive Model (Fig. 8), $[Na^+]$ , and $[Tris^+]$*

The derivation of these relationships is performed under the assumption (see also legend to Fig. 8) that (a) there is equilibrium within the pools and (b) the site affinity constant within a pool is independent of the degree of occupancy by sodium and independent of the presence of  $Tris^+$ . The  $Na^+$ -site affinity constants are  $\alpha$ ,  $\beta$ , and  $\gamma$  in pools A, B, and C, respectively. Furthermore,  $Tris^+$  binds to a separate  $Tris^+$  site with the same affinity to all species in a pool, the binding constants being  $\epsilon_A$ ,  $\epsilon_B$ , and  $\epsilon_C$  for the three pools. As discussed in the main text, it is necessary for obtaining a good fit of the  $Tris$  data to assume that  $Na^+$  competes with  $Tris^+$  on the  $Tris$  site, but that only the presence of  $Tris^+$  on the site has effect. The binding constants for  $Na^+$  to that site are  $\rho_A$ ,  $\rho_B$ , and  $\rho_C$ .

##### *Experiments with $Na^+$ Alone (No $Tris^+$ )*

By definition,  $[A] = [A_2] + [A'2] + [A_3] + [A'3]$ , and the rate of dephosphorylation of pool A is

$$k_a[A] = k_{a2}[A_2] + k_{a'2}[A'2] + k_{a3}[A_3] + k_{a'3}[A'3].$$

Using the equilibrium constants in Fig. 8 and the binding constant,  $\alpha$ , defined above, we get

$$k_a = \frac{(k_{a2} + k_{a'2} \cdot K_{A2}) + (k_{a3} + k_{a'3} \cdot K_{A3}) \alpha [Na^+]}{(1 + K_{A2}) + (1 + K_{A3}) \cdot \alpha \cdot [Na^+]}$$

This equation can be rearranged to

$$k_a = \frac{\frac{(k_{a2} + k_{a'2} \cdot K_{A2})}{1 + K_{A2}} + \frac{(k_{a3} + k_{a'3} \cdot K_{A3})}{1 + K_{A3}} \cdot \alpha' [Na^+]}{1 + \alpha' [Na^+]} \quad (1A)$$

$$k_a = \frac{k'_{a2} + k'_{a3} \cdot \alpha' [Na^+]}{1 + \alpha' [Na^+]} \quad (2A)$$

where  $\alpha'$  is an "apparent" affinity:

$$\alpha' = \alpha \cdot \frac{1 + K_{A3}}{1 + K_{A2}} \quad (3A)$$

and the meaning of the apparent rate constants  $k'_{a2}$  and  $k'_{a3}$  is evident from comparison of Eqs. 1A and 2A. Using the same line of reasoning,

$$k_A = \frac{k_{A2} + k_{A3} \cdot \alpha \cdot [\text{Na}^+]}{(1 + K_{A2}) + (1 + K_{A3})\alpha[\text{Na}^+]}; \quad (4A)$$

$$k_A = \frac{k'_{A2} + k'_{A3} \cdot \alpha' \cdot [\text{Na}^+]}{1 + \alpha'[\text{Na}^+]}, \quad (5A)$$

where  $\alpha'$  is defined in Eq. 3A and  $k'_{A2}$  and  $k'_{A3}$  are apparent constants equal to  $k_{A2}/(1 + K_{A2})$  and  $k_{A3}/(1 + K_{A3})$ .

By similar arguments we can write the equations for the other five rate coefficients of scheme S2:

$$k_j = \frac{k_{j0} + k_{j1} \cdot 3\beta[\text{Na}^+] + k_{j2} \cdot 3\beta^2[\text{Na}^+]^2 + k_{j3} \cdot \beta^3[\text{Na}^+]^3}{(1 + \beta[\text{Na}^+])^3}, \quad (6A)$$

where  $j = b, -B, \text{ or } B$  and, similarly,

$$k_n = \frac{k_{n0} + k_{n1} \cdot 3\gamma[\text{Na}^+] + k_{n2} \cdot 3\gamma^2[\text{Na}^+]^2 + k_{n3} \cdot \gamma^3[\text{Na}^+]^3}{(1 + \gamma[\text{Na}^+])^3}, \quad (7A)$$

where  $n = c \text{ or } -C$ .

#### *Experiments with Na<sup>+</sup> and Tris<sup>+</sup>*

The rate coefficients with Tris<sup>+</sup> bear the extra subscript T, e.g.,  $k_{BT}$ ,  $k_{B2T}$ , etc. According to the assumptions laid out in the first paragraph of this appendix, and taking pool B as an example for the method of deriving the Tris rate coefficients, we can write

$$[B] = [B0](1 + \beta\text{Na})^3(1 + \rho_B[\text{Na}^+] + \epsilon_B[\text{Tris}^+]).$$

If we also make the simple assumption that the rate constant for a particular type of reaction is changed by the same factor for all species involved, e.g.,  $k_{B3T} = f_B \cdot k_{B3}$ ,  $k_{B2T} = f_B \cdot k_{B2}$ , or  $k_{b3T} = f_b \cdot k_{b3}$  and  $k_{b2T} = f_b \cdot k_{b2}$ , it is easy to show that, for example,

$$k_{BT} = k_B \cdot \frac{1 + \rho_B[\text{Na}^+] + f_B \cdot \epsilon_B[\text{Tris}^+]}{1 + \rho_B[\text{Na}^+] + \epsilon_B[\text{Tris}^+]}, \quad (8A)$$

$$k_{bT} = k_b \cdot \frac{1 + \rho_B[\text{Na}^+] + f_b \cdot \epsilon_B[\text{Tris}^+]}{1 + \rho_B[\text{Na}^+] + \epsilon_B[\text{Tris}^+]}. \quad (9A)$$

Analogous expressions for the effect of Tris<sup>+</sup> on  $k_a$ ,  $k_A$ ,  $k_{-B}$ ,  $k_{-C}$ , and  $k_r$  have exactly the same form. An explanatory note: if  $f = 1$ , Tris<sup>+</sup> is obviously without effect. Since the Tris<sup>+</sup> coefficients of the three-pool model are always equal to or lower than those in the absence of Tris<sup>+</sup>,  $f$  is always between 1 and 0.

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