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Mechanistic Analysis of the (Na^+,K^+)ATPase Using New Pseudosubstrates†

Christian Gache, Bernard Rossi, and Michel Lazdunski*

ABSTRACT: 2,4-Dinitrophenyl phosphate and β-(2-furyl)-acryloyl phosphate are shown to be excellent substrates for the (Na^+,K^+)ATPase of axonal membranes. With K^+ and Na^+ both present, the maximal activity of the enzyme on dinitrophenyl phosphate and furylacryloyl phosphate is, respectively, 1.7 and 3.9 times greater than that observed with ATP. In the presence of K^+ only, the advantage of using the synthetic substrates rather than ATP is even greater, since 2,4-dinitrophenyl phosphate and furylacryloyl phosphate are hydrolyzed by the ATPase at maximal rates that are 100 and 200 times greater, respectively, than that of ATP. In the presence of K^+ only, the two substrates are hydrolyzed 6 and 13 times faster than *p*-nitrophenyl phosphate, the more usual pseudosubstrate. The dependence of substrate concentration, pH, and Na^+ and K^+ concentration on the ATPase activity is reported for these two substrates. Detailed results concerning the Na^+ and K^+ dependence of the rates of hydrolysis of 2,4-dinitrophenyl phosphate and β-(2-furyl)acryloyl phosphate are used to propose a mechanism of interaction between Na^+ and K^+ sites essential for enzyme activity. A good fit to experimental data was obtained with a model involving four Na^+ and two K^+ sites per mole of ATPase. Na^+ binding to its sites in this model is characterized by positive and negative cooperativity. The proposed model suggests a mechanism by which Na^+ modulates the cooperativity for K^+ binding.

Most of the kinetic data which have lead to the present knowledge of the mechanism of the (Na^+,K^+)ATPase[1] of eucaryotic cells have been obtained with ATP itself or with ATP analogues (Schwartz et al., 1972; Dahl and Hokin, 1974; Askari, 1974; Skou, 1975; Whittam and Chipperfield, 1975; Glynn and Karlish, 1975; Karlish et al., 1976). It has been shown that the (Na^+,K^+)ATPase hydrolyzes ATP in a stepwise fashion involving a Na^+-dependent phosphorylation of the enzyme followed by a K^+-dependent hydrolysis of the phosphoenzyme intermediate. It is also well known that (Na^+,K^+)ATPase preparations invariably exhibit an ouabain-inhibitable K^+-dependent phosphatase activity (Judah et al., 1962; Yoshida et al., 1969; Koyal et al., 1971). *p*-Nitrophenyl phosphate and acetyl phosphate which have free energies of hydrolysis higher than typical phosphate esters will also serve as substrates for the ATPase. The (Na^+,K^+)ATPase activity on these substrates occurring in the presence of K^+ only has often been compared to the final step in the reaction sequence of ATP hydrolysis, i.e., the K^+-dependent hydrolysis of the phosphoenzyme intermediate.

The purpose of this paper is to provide detailed information concerning the steady-state kinetic properties of the (Na^+,K^+)ATPase on two new synthetic substrates, 2,4-dinitrophenyl phosphate and β-(2-furyl)acryloyl phosphate. These substrates which have high free energies of hydrolysis are much better substrates than *p*-nitrophenyl phosphate and even ATP itself. The work presented in this paper is carried out with a pure membrane preparation (Balerna et al., 1975) containing

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[1] Abbreviations used are: (Na^+,K^+)ATPase, sodium, potassium activated adenosine triphosphatase (EC 3.6.1.3); Tris, tris(hydroxymethyl)-aminomethane; NADH, reduced nicotinamide adenine dinucleotide.

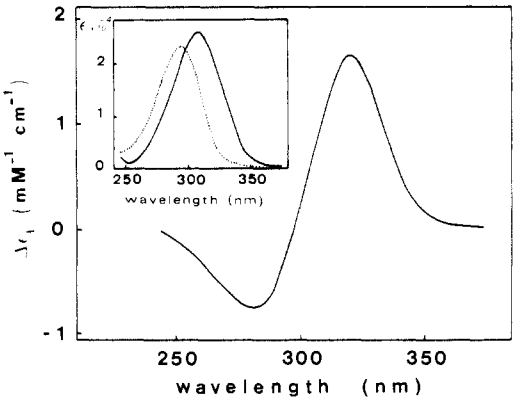

FIGURE 1: Difference spectrum between furylacryloyl phosphate and furylacrylic acid. The difference between the molar absorption coefficients (ϵ) of the two compounds was plotted as a function of wavelength. Reference beam, furylacrylic acid; signal beam, furylacryloyl phosphate. Furylacryloyl phosphate was hydrolyzed by alkaline phosphatase (or by our ATPase) to furylacrylate. Inset shows spectra for the two compounds: (—) furylacryloyl phosphate; (···) furylacrylate. Spectra were taken in a 50 mM triethanolamine buffer, pH 7.5, 20 mM K^+, 20 mM Mg^{2+}.

the membrane (Na^+,K^+)ATPase of crab nerves, a biological system that has played an important role in the development of present concepts concerning the active transport of Na^+ and K^+ (Skou, 1957). Several possible and useful models of the (Na^+,K^+)ATPase action have been recently proposed (Skou, 1971; Stein et al., 1973; Schön et al., 1974; Grisham and Mildvan, 1974; Lindenmayer et al., 1974; Albers et al., 1974; Robinson, 1970, 1976). However, in spite of considerable experimental work in the last 20 years, too many data are still lacking to present a detailed mechanism of the sodium–potassium pump which can be universally accepted. One of the most important and fundamental unresolved questions concerns the number of Na^+ and K^+ sites per mole of enzyme and the precise understanding of the mechanism by which the sites interact to generate catalytic activity. As will be seen in this work, the use of the new pseudosubstrates provides useful, although indirect, information about the mechanism of interaction between Na^+ and K^+ sites.

Materials and Methods

Materials

Phosphoenolpyruvate, NADH, lactate dehydrogenase, and pyruvate kinase were obtained from Boehringer. The dicyclohexylammonium salt of *p*-nitrophenyl phosphate was obtained from Calbiochem and its di-Tris salt from Sigma. 2,4-Dinitrophenyl phosphate in the form of its lutidinium salt was prepared according to Kirby and Varvoglis (1966). The barium salt of β-(2-furyl)acryloyl phosphate, prepared according to Malhotra and Bernhard (1968), was kindly supplied by Drs. Bernhard, Seydoux, and Rossi. The dibarium salt of ATP was converted to the free-acid form on a Bio-Rad AG 50 W-X 8 ion exchanger and then neutralized with triethanolamine. Crystalline suspensions of lactate dehydrogenase and pyruvate kinase were centrifuged and passed through a Sephadex G-25 column before use. $MgCl_2$, KCl, NaCl, and all other reagents were of the highest grade commercially available. All experiments were carried out in distilled and deionized water.

Methods

Membrane Preparation. Axonal plasma membranes were isolated from walking leg nerves of the crab *Cancer pagurus* as described by Balerna et al. (1975). Before use, membranes of fraction II were centrifuged for 60 min at 100 000*g* and resuspended in 50 mM triethanolamine chloride buffer (pH 7.5). The final specific activity of the (Na^+,K^+)ATPase was about 1 μmol min^{-1} (mg of protein)$^{-1}$ at 25 °C and pH 7.5 under optimum ionic concentrations (Gache et al., 1976). Ouabain (1 mM) produced more than 99% inhibition. The membrane preparation was completely devoid of acid or alkaline phosphatase activities (Balerna et al., 1975). Protein concentrations were measured according to Hartree (1972) after suspending membrane samples in a 15 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer at pH 7.5.

Determination of (Na^+,K^+)ATPase Activity. The ATPase activity was routinely assayed at 25 °C by the pyruvate kinase–lactate dehydrogenase linked system under conditions which have been published elsewhere (Gache et al., 1976).

The Activity of the (Na^+,K^+)ATPase on Synthetic Organic Phosphates. Nitrophenyl phosphatase activities were assayed by following spectrophotometrically the liberation of *p*-nitrophenol or 2,4-dinitrophenol at 410 nm (ϵ_{410} for the *p*-nitrophenolate anion and 2,4-dinitrophenolate anion are 1.7×10^4 and 0.95×10^4 M^{-1} cm^{-1}, respectively) with spectrophotometers (Beckman Acta M VI, Perkin-Elmer 356 or Varian Techtron 635) equipped with thermostated cell holders.

The spectral characteristics of β-(2-furyl)acryloyl phosphate have been published by Malhotra and Bernhard (1968). Upon hydrolysis of this substrate to furylacryloic acid, the spectrum is shifted towards shorter wavelengths (Figure 1, inset). The difference spectrum between substrate and product is given in Figure 1. It shows that furylacryloyl phosphate hydrolysis can be followed in the near-ultraviolet range. Maximum sensitivity is obtained near 320 nm. However, because of the high absorption of the substrate, this wavelength cannot be used at all substrate concentrations. For this reason, kinetic results at high substrate concentrations were obtained at longer wavelength (between 345 and 355 nm).

Inhibition by Ouabain. Axonal membranes were incubated for 15 h at 1 °C in the presence of different concentrations of ouabain in a 50 mM triethanolamine chloride buffer, pH 7.5, with 5 mM Mg^{2+} and 5 mM inorganic phosphate. Activities on synthetic substrates were measured as previously described.

Computing Procedure. Kinetic data were fitted to the Hill equation (Hill, 1910)

$$v = \frac{V_m[S]^{n_H}}{K_{0.5}{}^{n_H} + [S]^{n_H}}$$

where V_m, $K_{0.5}$, and n_H stand for maximum velocity, half maximum effect, and Hill coefficient, respectively. All computations were carried out with a Wang 2200 calculator according to Atkins (1973). The program described by this author allows the simultaneous determination of the three parameters V_m, $K_{0.5}$, and n_H which give the best fit to the experimental data. In each figure of this paper, the curve presented is the theoretical curve giving the best fit with the experimental data. V_m, $K_{0.5}$, and n_H values which appear in the tables are computed values.

Results

Ouabain and ATP Inhibition of Pseudosubstrates Hydrolysis. It has been shown previously (Gache et al., 1976), on the membrane preparation used in this work, that the hydrolytic activities of the (Na^+,K^+)ATPase on the natural

TABLE I: Kinetic Parameters for Substrates under Different Ionic Conditions.[a]

Substrate	(Na^+,K^+)ATPase conditions V_m (nm min^{-1} mg^{-1})	K_M (mM)	n_H	K^+ phosphatases conditions V_m (nm min^{-1} mg^{-1})	K_M (mM)	n_H
ATP	1000	0.12	1	~20	0.2	0.7
p-Nitrophenyl phosphate	95	3.2	1	300	2.2	1
2,4-Dinitrophenyl phosphate	1650	1.2	1	1900	0.6	1
β-(2-Furyl)acryloyl phosphate	3900	0.45	1	4100	0.2	1

[a] (Na^+,K^+)ATPase conditions: 100 mM Na^+, 10 mM K^+. K^+ phosphatases conditions: no Na^+, 20 mM K^+. Mg^{2+} concentrations are 5 mM with ATP as substrate, 20 mM with nitrophenyl phosphates as substrates and 50 mM with furylacryloyl phosphate. 25 °C, triethanolamine chloride, 50 mM, pH 7.5.

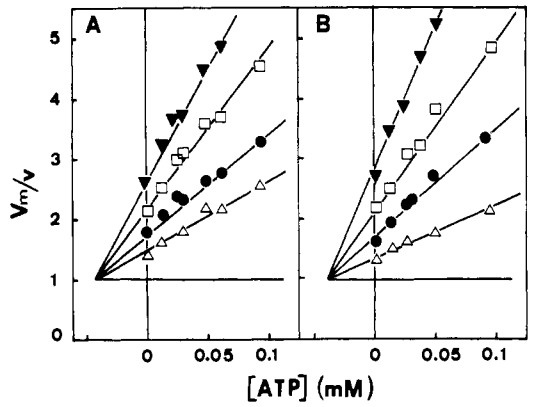

FIGURE 2: ATP inhibitions of the K^+ phosphatases activities. Dixon plots of inhibition by ATP of the ATPase catalyzed hydrolyses of dinitrophenyl phosphate (A) and furylacryloyl phosphate (B). Concentrations of dinitrophenyl phosphate: (▼) 0.5 mM; (□) 0.75 mM; (●) 1 mM; (△) 2.5 mM. Concentrations of furylacryloyl phosphate: (▼) 0.12 mM; (□) 0.16 mM; (●) 0.32 mM; (△) 0.48 mM. Other experimental conditions are: 25 °C, pH 7.5, no Na^+, 20 mM K^+. Mg^{2+} concentrations were 20 and 50 mM in experiments with dinitrophenyl phosphate and furylacryloyl phosphate, respectively.

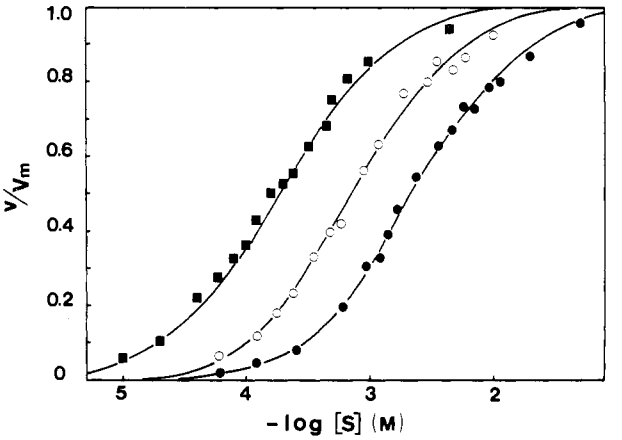

FIGURE 3: Substrate concentration dependence of the rate of hydrolysis of nitrophenyl phosphates and furylacryloyl phosphate. Measurements were carried out at 25 °C, pH 7.5, 20 mM Mg^{2+}, 20 mM K^+, as described under the experimental section. (○) Dinitrophenyl phosphate; (■) furylacryloyl phosphate; (●) *p*-nitrophenyl phosphate. Curves are normalized to v/V_m. True maximum velocities are given in Table I.

substrate ATP and on the synthetic substrate *p*-nitrophenyl phosphate are inhibited by ouabain in a similar way. As it will be seen in detail later in the text, 2,4-dinitrophenyl phosphate and furylacryloyl phosphate are excellent pseudosubstrates of the (Na^+,K^+)ATPase. The characteristics of ouabain inhibition of the phosphatase activity of the (Na^+,K^+)ATPase were found to be identical with *p*-nitrophenyl phosphate, 2,4-dinitrophenyl phosphate, and β-(2-furyl)acryloyl phosphate.

Dixon plots for the inhibition of the ATPase hydrolysis of dinitrophenyl phosphate and furylacryloyl phosphate by ATP are presented in Figure 2A,B. As expected, ATP is a competitive inhibitor of the hydrolysis of the pseudosubstrates. The apparent inhibition constants for ATP measured from dinitrophenyl phosphate and furylacryloyl phosphate hydrolyses are 45 and 40 μM, respectively.

A parallel series of determination using the classical pseudosubstrate *p*-nitrophenyl phosphate gave a value of K_I = 45 μM.

Comparative Kinetic Parameters for the Hydrolysis of ATP and Synthetic Substrates by the (Na^+,K^+)ATPase. It is well known that maximum rates of ATP hydrolysis by the enzyme are observed when both Na^+ and K^+ are present (Skou, 1957; Dahl and Hokin, 1974; Askari, 1974). Conversely, the catalyzed hydrolysis of *p*-nitrophenyl phosphate is faster in the absence of Na^+. Kinetic parameters related to the hydrolysis of dinitrophenyl phosphate and furylacryloyl phosphate have been determined in K^+ alone and in the presence of both Na^+ and K^+. The substrate concentration dependence of the rate of hydrolysis follows a simple Michaelis–Menten law with a Hill coefficient of 1.0 in the presence and in the absence of Na^+. Typical results are presented in Figure 3. The data obtained with the new pseudosubstrates are compared in Table I with those which can be obtained using *p*-nitrophenyl phosphate and ATP under the same experimental conditions.

Whereas a Michaelis–Menten behavior is always observed with pseudosubstrates, in the absence or in the presence of Na^+, the situation is different with the natural substrate. ATPase-catalyzed ATP hydrolysis follows Michaelis–Menten kinetics when sodium is present but exhibits negative cooperativity in its absence (see also Gache et al., 1976).

In terms of K_M values *p*-nitrophenyl phosphate is the worst substrate (higher K_M) in the absence or presence of Na^+, the best substrates being ATP and furylacryloyl phosphate. The most interesting observation is that dinitrophenyl phosphate and furylacryloyl phosphate give a maximum velocity which is even higher than that observed for ATP, even in the presence of Na^+. The difference in maximum velocities is dramatically increased if Na^+ is absent.

2,4-Dinitrophenyl phosphate is a very convenient substrate for the analysis of the influence of pH upon kinetic parameters, the pK of the chromophoric 2,4-dinitrophenolate being near 4.1 at 25 °C. The comparative pH dependence of the AT-

TABLE II: Potassium Stimulations of the (Na^+,K^+)ATPase Activities on Dinitrophenyl Phosphate and Furylacryloyl Phosphate in the Presence of Increasing Sodium Concentrations.[a]

[Na^+] (mM)	Act. on dinitrophenyl phosphate			Act. on Furylacryloyl phosphate		
	V_m	$K_{0.5}$ (mM)	n_H	V_m	$K_{0.5}$ (mM)	n_H
0	100	5.2	1.88	100	1.6	1.72
0.05				100	2	1.72
0.5				99	2.25	1.13
1	102	4.8	1.9	98	1.07	0.77
2	98	4.67	1.65			
3	98	4.24	1.2			
5	99	3.4	0.9	100	0.48	0.94
10	98	1.3	0.94			
20	100	0.9	1.43			
100	95	1.7	1.6	97	1.05	1.5
200	75	2.3	1.86	80	1.61	1.7
400	40	3.58	2.0			

[a] 25 °C, 50 mM triethanolamine chloride, pH 7.5, 5 mM dinitrophenyl phosphate and 20 mM Mg^{2+}, or 2 mM furylacryloyl phosphate and 50 mM Mg^{2+}. Maximum velocity in the absence of sodium is taken as 100.

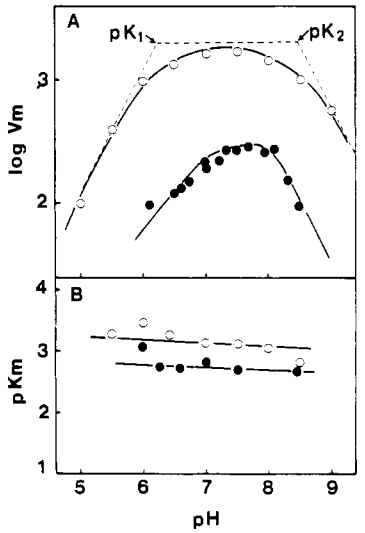

FIGURE 4: pH dependence of kinetic parameters for the hydrolysis of nitrophenyl phosphates. (A) pH dependence of log V_m. (B) pH dependence of pK_m. (○) Dinitrophenyl phosphate hydrolysis; (●) *p*-nitrophenyl phosphate hydrolysis. Measurements were done in 100 mM buffers adjusted to the desired pH values at 25 °C. These buffers were: acetate at pH 4.5, 5.0, and 5.5; 2-(*N*-morpholino)ethanesulfonic acid at pH 5.5, 6, 6.5, and 7; tris(hydroxymethyl)aminomethane at pH 8, 8.5, and 9.

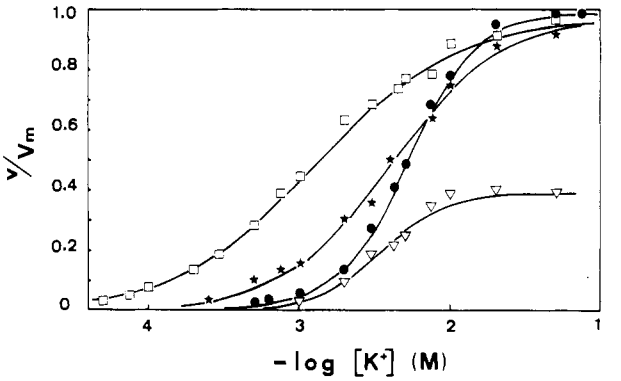

FIGURE 5: The K^+ concentration dependence of the catalyzed transformation of dinitrophenyl phosphate at different Na^+ concentrations. Sodium concentrations were: (●) no sodium; (★) 3 mM; (□), 10 mM; (▽), 400 mM. 5 mM dinitrophenyl phosphate, 20 mM Mg^{2+}, 25 °C, pH 7.5, 50 mM triethanolamine. The same results were obtained when experiments were carried out in 500 mM triethanolamine buffer showing that there are no ionic strength effects.

Pase-catalyzed hydrolysis of *p*-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate is presented in Figure 4.

V_m values are drastically affected by pH changes, whereas K_M values hardly vary. The V_m-pH profile obtained with 2,4-dinitrophenyl phosphate was analyzed according to Dixon and Webb (1964). It indicates the essentiality of 2 ionizable groups with pK values of 6.2 and 8.5.

Potassium Stimulation of Synthetic Substrates Hydrolysis in the Absence of Sodium. Figure 5 shows that K^+ stimulation of the enzymatic activity in the absence of Na^+ is cooperative for dinitrophenyl phosphate ($n_{H(K^+)}$ = 1.9). The same situation was observed for the furylacryloyl phosphate ($n_{H(K^+)}$ = 1.7). Half-maximum stimulations ($K_{0.5(K^+)}$) are 5 and 1 mM for dinitrophenyl phosphate and furylacryloyl phosphate, respectively, as compared to 5.8 mM for *p*-nitrophenyl phosphate (Gache et al., 1976).

Potassium Stimulation of Synthetic Substrate Hydrolysis in the Presence of Sodium. Potassium stimulations of the hydrolytic activity using dinitrophenyl phosphate and furylacryloyl phosphate have also been analyzed at different sodium concentrations between 0 and 400 mM Na^+. Typical curves of stimulation obtained with dinitrophenyl phosphate are presented in Figure 5. Detailed kinetic data for the two substrates are listed in Table II.

The Na^+ concentration controls both Hill coefficients and $K_{0.5}$ values for K^+. In the absence of Na^+, K^+ stimulation is cooperative, as we have already seen. As Na^+ concentration is increased, the Hill coefficient decreases to a value of less than 1, indicating negative cooperativity. It then increases to a maximal value of 2. $K_{0.5(K^+)}$ values follow a similar behavior. They first decrease at low concentrations of Na^+ and as the concentration of Na^+ is increased they return to the original higher value obtained in the absence of Na^+.

Variations of $K_{0.5(K^+)}$ and $n_{H(K^+)}$ with Na^+ concentrations are presented in Figure 6A for the catalyzed hydrolysis of dinitrophenyl phosphate. The relationship between $n_{H(K^+)}$ and $K_{0.5(K^+)}$ at different Na^+ concentrations is presented in Figure 6B for dinitrophenyl phosphate and furylacryloyl phosphate.

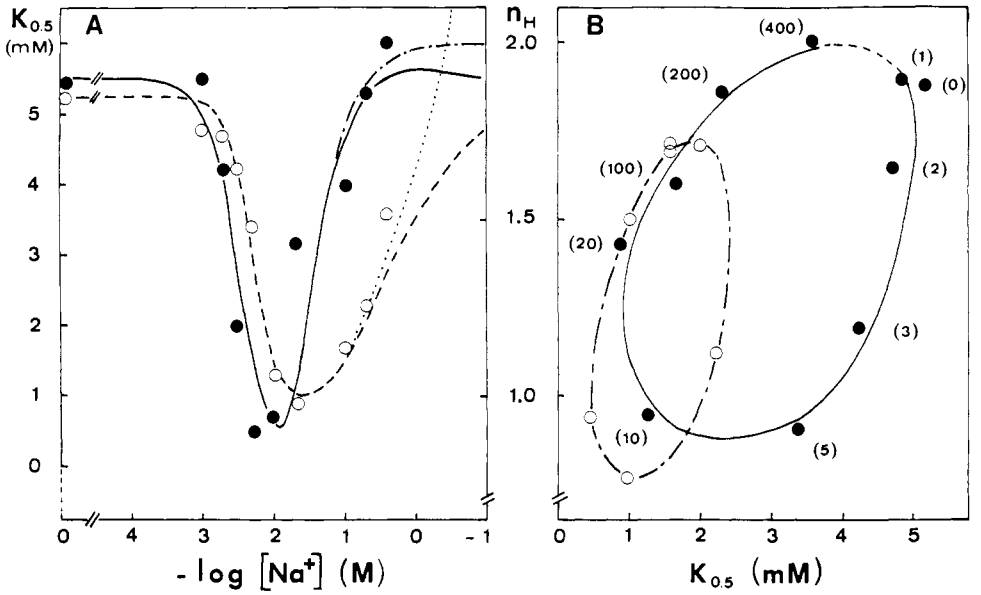

FIGURE 6: Heterotropic interactions between K^+ and Na^+ sites. (A) The data presented are those listed in Table II for dinitrophenyl phosphate. Sodium concentration dependence of $K_{0.5(K^+)}$ (○, - - -, and • • •) and $n_{H(K^+)}$ (●, —, and - - -). Solid and dotted curves are theoretical curves calculated from the model presented in Figure 8 with the equations developed in the Appendix. The values of association constants for Na^+ and K^+ which give a reasonably good fit of the experimental data are given in the legend of Figure 8. (B) The relationship between $n_{H(K^+)}$ and $K_{0.5(K^+)}$ at different Na^+ concentrations for furylacryloyl phosphate (○) and dinitrophenyl phosphate (●) hydrolyses. Values between brackets give the Na^+ concentrations (in mM) for the dinitrophenyl phosphate hydrolysis.

The Influence of Na^+ Concentration upon the Catalyzed Hydrolysis of Pseudosubstrates in the Presence of K^+. As already shown in Figure 5 for dinitrophenyl phosphate, Na^+ increases the phosphatase activity of the (Na^+,K^+)ATPase at low concentrations of K^+. Figure 7 shows the characteristics of Na^+ stimulation of dinitrophenyl phosphate and furylacryloyl phosphate hydrolyses in 0.5 mM K^+. In both cases, $K_{0.5(Na^+)}$ is in the range of 3 mM and the maximum stimulation factor is close to 6. In both cases, the factor of stimulation varies cooperatively with the Na^+ concentration, $n_{H(Na^+)}$ being 2.2 and 2 for dinitrophenyl phosphate and furylacryloyl phosphate, respectively.

At high concentrations of K^+, as shown in Figure 5 for dinitrophenyl phosphate, Na^+ inhibits the enzyme activity instead of increasing it. Complete inhibition can be obtained if high enough concentrations of Na^+ are used (Figure 7, insets). For example, at 50 mM K^+, $K_{0.5}$ corresponding to the half-maximal inhibition is 350 mM for dinitrophenyl phosphate and 450 mM for furylacryloyl phosphate. In both cases, the Hill coefficient for this inhibition is 1.7. The inhibition by a high concentration of Na^+ is apparently due to competition between Na^+ and K^+ for the K^+ sites necessary for enzyme activity.

Absence of ATP Stimulation of Dinitrophenyl Phosphate and Furylacryloyl Phosphate Catalyzed Hydrolyses. Low concentrations of ATP and other nucleotide triphosphates stimulate the hydrolysis of *p*-nitrophenyl phosphate catalyzed by the (Na^+,K^+)ATPase at low K^+ and high Na^+ concentrations (Yoshida et al., 1969; Koyal et al., 1971; Swann and Albers, 1975; Robinson, 1975; Gache et al., 1976). We have therefore investigated in this work whether such a stimulation also exists for dinitrophenyl phosphate and furylacryloyl phosphate. Experimental results are reported in Figure 7. At 0.5 mM K^+, in the presence of different Na^+ concentrations, ATP produces little if any effect on the catalyzed hydrolysis of these two substrates. Under identical conditions of Na^+ and K^+ concentrations, ATP strongly stimulates the hydrolysis of *p*-nitrophenyl phosphate (Gache et al., 1976). Other concen-

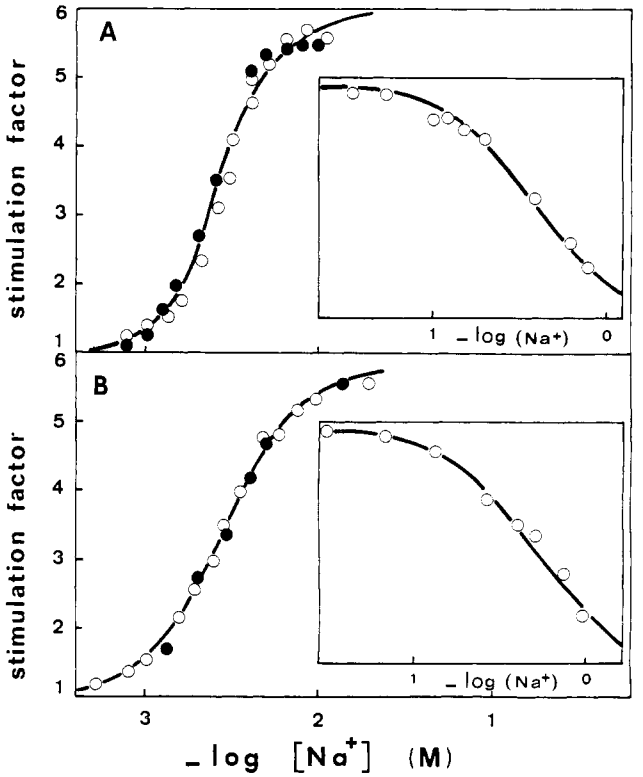

FIGURE 7: Sodium effects on K^+ phosphatase activities. (A) Activation of dinitrophenyl phosphate hydrolysis at low concentration of K^+ (0.5 mM) in the absence (○) or in the presence (●) of 20 μM ATP. (B) Activation of furylacryloyl phosphate hydrolysis at 0.5 mM K^+ in the absence (○) or in the presence (●) of 20 μM ATP. The insets in A and B represent the inhibitions of phosphatase activities toward dinitrophenyl phosphate and furylacryloyl phosphate, respectively, at the high K^+ concentration of 50 mM. Substrate concentrations were 5 mM for dinitrophenyl phosphate and 2 mM for furylacryloyl phosphate. The stimulation factor in this figure is defined as v/v_0, where v_0 is the rate measured in 0.5 mM K^+ and in the absence of Na^+, and v is the rate measured at the same K^+ concentration and in the presence of Na^+ at the indicated concentration.

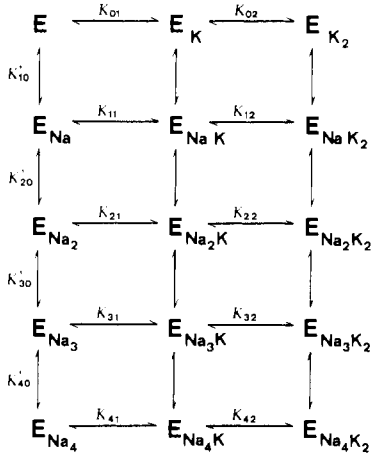

FIGURE 8: A model with four Na^+ sites and two K^+ sites to explain the experimental data of Figure 6. The constants introduced in this scheme are the microscopic constants of association of Na^+ and K^+ to the enzyme E. The values for the constants (in M^{-1}) which give a satisfactory fit of the experimental results in Figure 6 are: $K'_{10} = 1.5$, $K'_{20} = 400$, $K'_{30} = 9$, $K'_{40} = 2400$, $K_{01} = K_{11} = K_{41} = 10$, $K_{02} = K_{12} = K_{42} = 3850$, $K_{21} = 3100$, $K_{22} = 13$, $K_{31} = 250$, $K_{32} = 10^5$ for the first fit (— and - - -). The second fit (- - - and • • •) is obtained with the same values, except for $K_{41} = 1$ and $K_{42} = 9950$. This does not change the main conclusions. The values for K_{01} and K_{02} are strictly imposed. They are calculated from the stimulation parameters $K_{0.5(K^+)}$ and $n_{H(K^+)}$ in the absence of Na^+ using equations (Levitzki and Koshland, 1976) $K_{01} = (2 - n_H)/K_{0.5}n_H$ and $K_{02} = n_H/K_{0.5}(2 - n_H)$. The values for the other association constants have been retained after successive trials and assays to obtain the best fit to the experimental data.

trations of Na^+ and K^+ (K^+ concentration between 0.5 and 5 mM) were tried; in none of them was any stimulating effect by ATP observed.

Absence of Stimulation of p-Nitrophenyl Phosphate Hydrolysis by Furylacryloyl Phosphate. Since furylacryloyl phosphate is a better substrate than ATP itself (at least in terms of maximal rate) and since ATP at low concentrations is known to stimulate the catalyzed hydrolysis of *p*-nitrophenyl phosphate, the effects of furylacryloyl phosphate on *p*-nitrophenyl phosphate hydrolysis were analyzed. Under conditions of Na^+ and K^+ concentrations in which ATP stimulates *p*-nitrophenyl phosphate hydrolysis, no stimulation was found by replacing ATP with furylacryloyl phosphate. The expected competitive inhibition of the *p*-nitrophenyl phosphatase activity at high enough concentrations of furylacryloyl phosphate was however observed.

Discussion

Two substrates have played an important role in the development of the present knowledge of the (Na^+,K^+)ATPase mechanism, the physiological substrate ATP itself and *p*-nitrophenyl phosphate. ATP is by far a better substrate than *p*-nitrophenyl phosphate in the presence of Na^+ and K^+. However, the synthetic substrate has two interesting properties which are not shown by the natural substrate. In the first place, its hydrolysis can be catalyzed with a good efficiency by the ATPase in the presence of K^+ only; secondly, the kinetic analysis of the hydrolysis is easily carried out.

The present work has shown that furylacryloyl phosphate and dinitrophenyl phosphate will probably be excellent substrates for future mechanistic work with the (Na^+,K^+)ATPase. In the presence of both Na^+ and K^+, the maximal activity of the enzyme on dinitrophenyl phosphate and furylacryloyl phosphate is, respectively, 1.7 and 3.9 times higher than that observed with ATP (Table I). In studies using K^+ only, the advantage of using the synthetic substrates rather than ATP is even greater, since 2,4-dinitrophenyl phosphate and furylacryloyl phosphate are hydrolyzed by the ATPase at maximal rates that are 100 and 200 times greater, respectively, than that of ATP. The use of the synthetic substrates provides a convenient assay for the enzyme activity. The kinetics of their hydrolysis can be followed by a direct and continuous spectrophotometric technique, whereas analysis of ATP hydrolysis necessitates either the use of a discontinuous assay to follow inorganic phosphate release (Chen et al., 1956; Lowry and Passonneau, 1972) or the continuous but indirect assay which makes use of the pyruvate kinase–lactate dehydrogenase linked system (Bucher and Pfleiderer, 1955). Dinitrophenyl phosphate and furylacryloyl phosphate will also be good substitutes of *p*-nitrophenyl phosphate, since they are hydrolyzed much faster by the ATPase molecule (Table I).

A complete analysis of the (Na^+,K^+)ATPase mechanism would necessitate detailed studies of presteady-state kinetics. These studies can now be considered with the two above substrates and since techniques have recently become available to prepare the enzyme in a purified form and in reasonable quantities from different origins (Jorgensen et al., 1971; Uesugi et al., 1971; Kyte, 1972; Lane et al., 1973; Hokin et al., 1973). The two substrates will also probably be useful in stopped-flow analyses of transient kinetics similar to those which have been already carried out with another enzyme catalyzing the hydrolysis of the same substrates, the alkaline phosphatase (Halford et al., 1972; Chappelet-Tordo et al., 1974a,b; Cathala et al., 1975).

The steady-state kinetic analysis presented in this work already permits a discussion of the interactions which occur between Na^+ and K^+ sites which modulate the enzyme activity. The most important observations are the following. (1) In the presence of Na^+ alone, pseudosubstrates are not hydrolyzed by the ATPase. However, if Na^+ by itself does not stimulate hydrolysis of pseudosubstrates, it controls the value of both $n_{H(K^+)}$ and $K_{0.5(K^+)}$. Therefore, Na^+ operates as an allosteric effector of K^+ stimulation. (2) Na^+ stimulation in the presence of K^+ is cooperative with a Hill coefficient near 2, indicating a multiplicity of Na^+ sites. K^+ stimulations in the presence of Na^+ can display pronounced positive cooperativity ($n_{H(K^+)} \simeq 1.9$), moderate positive cooperativity ($1 < n_{H(K^+)} < 1.9$), or even slight negative cooperativity ($n_{H(K^+)} = 0.9$, 0.7). (3) $n_{H(K^+)}$ and $K_{0.5(K^+)}$ values at high sodium concentrations are nearly identical with values observed in the total absence of sodium (Table II).

A model can be proposed in an attempt to explain these observations. It has the following basic properties. (1) Na^+ sites are silent with respect to catalytic activity. In consequence v/V_m is proportional to the degree of saturation of the K^+ sites ($\bar{y}_{(K^+)}$). (2) The enzyme molecule contains two specific K^+ sites and four specific Na^+ sites.

The model is presented in Figure 8. It does not take into account the possible conformational states of the enzyme molecule; only binding states have been considered. The use of the equations developed in the Appendix and an adequate choice of the microscopic binding constants give a theoretical description of the variation of $K_{0.5(K^+)}$ and $n_{H(K^+)}$ which fits reasonably well with the experimental results presented in Figure 6A. The model presented in Figure 8 and the dissociation constants given in its legend permit a calculation of the proportions of each of the major molecular forms of the enzyme as a function of Na^+ and K^+ concentrations (Figure 9).

The agreement between experimental data and their theo-

retical description in Figure 6A leads to a number of conclusions.

(1) The presence of four Na^+ sites and two K^+ sites in the model is sufficient to explain the experimental observations. It is therefore not necessary to introduce a greater number of monovalent cation binding stites. On the other hand, a smaller number of K^+ and Na^+ sites does not give a satisfactory fit to the experimental data. A minimum number of two K^+ sites is necessary to account for values of $n_{H(K^+)}$ in the neighborhood of 2. However, using the equations developed in the Appendix for a model similar to that presented in Figure 8 but with only two Na^+ sites and two K^+ sites, it has not been possible to obtain a good fit of the data of Figure 6. Because of frequent reports to the effect that the (Na^+,K^+)ATPase catalyzes the exchange of three Na^+ for two K^+ (Sen and Post, 1964; Whittam and Ager, 1965; Garrahan and Glynn, 1967) a model with three Na^+ and two K^+ sites was analyzed as above. Such a model did not give a good fit of the data presented in Figure 6.

Conclusions that models with two K^+ and only two or three Na^+ sites do not describe adequately the experimental data are not surprising. Even an eye examination of the curves in Figure 6 indicates that four Na^+ sites are needed to explain the data. The decrease of $n_{H(K^+)}$ observed in the low-concentration range of Na^+ is cooperative with respect to Na^+ concentration: $n_{H(K^+)}$ passes from 1.9 to 0.9, the minimum value of $n_{H(K^+)}$, between 1 and 5–10 mM Na^+. Similarly, the increase of $n_{H(K^+)}$ from 0.9 to 1.9 in the higher range of Na^+ concentration is observed between 5–10 and 200 mM Na^+. Such a variation is also characterized by a positive cooperativity of the $n_{H(K^+)}$ variation with respect to Na^+. The existence of a cooperative behavior for the decrease of $n_{H(K^+)}$ at low Na^+ concentration obviously necessitates the introduction in the model of at least two Na^+ sites linked by a positive cooperativity. Similarly, the generation of a cooperative behavior for the increase of $n_{H(K^+)}$ at high Na^+ concentrations will necessitate the introduction of two other Na^+ sites also linked by a positive cooperativity. The qualitative analysis of the curve shown in Figure 6 will therefore predict a model having four Na^+ sites belonging to two different families.

(2) Values for the association constants used in the model of Figure 8 are given in the legend of that figure. They indicate that Na^+ binding to its sites is characterized both by positive and negative cooperativity. There are two families of Na^+ sites (K'_{10} and K'_{20}, on one hand, K'_{30} and K'_{40}, on the other hand), each containing two sites. The two families are related by negative cooperativity. Within each family of Na^+ sites there is positive cooperativity between the sites ($K'_{10} < K'_{20}$; $K'_{30} < K'_{40}$).

(3) Na^+ modulates the cooperativity for K^+ binding in the following way. At low concentration of Na^+, form E_{Na} shows a positive cooperativity; as the concentration of Na^+ is increased, form E_{Na_2} gives a negative cooperativity, while the subsequent forms E_{Na_3} and E_{Na_4} return the system to a positive cooperativity.

(4) In a medium range of concentrations ($[Na^+]$ = 10 mM and $[K^+]$ = 1 mM) the main form which accumulates in the model system to catalyze the substrate hydrolysis is the E_{Na2K} complex. This complex is characterized by a half-saturation for both cationic ligands. It has been formed by a cooperative binding of Na^+ on half of the Na^+ sites and a Michaelis–Menten type binding of K^+, also on half of the available K^+ sites.

At concentrations of Na^+ and K^+ between 10 and 100 mM, the form of the enzyme which accumulates, representing more than 70% of the total enzyme concentration, is $E_{Na_3K_2}$. This range of concentrations of Na^+ and K^+ corresponds to physiological concentrations of these cations. In crab nerve, internal Na^+ concentration is near 40 mM and external K^+ concentration near 10 mM. The observed accumulation of $E_{Na_3K_2}$ in the physiological range of concentrations of Na^+ and K^+ is of a particular interest in view of the frequent observation that the (Na^+,K^+)ATPase catalyzes the exchange of three Na^+ for two K^+.

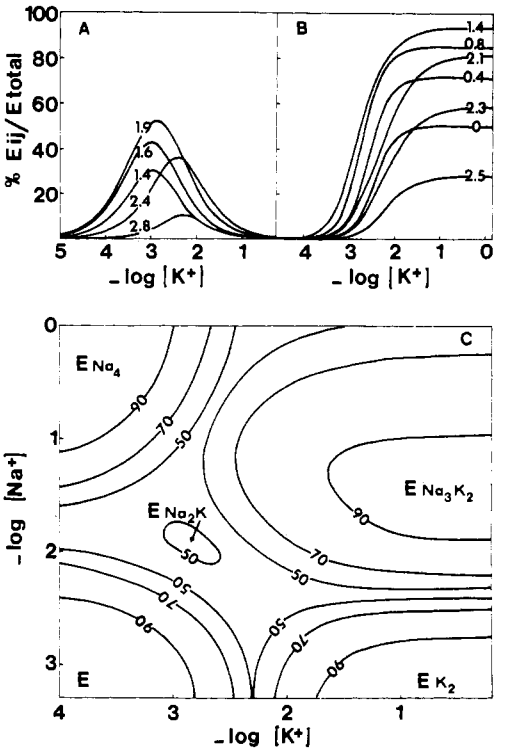

FIGURE 9: Calculated proportions of species E, E_{Na_4}, E_{Na_2K}, E_{K_2}, and $E_{Na_3K_2}$ as a function of Na^+ and K^+ concentrations. Fractional concentrations of each species, $[E_{ij}]/[E_{total}]$, were computed using equations developed in the Appendix for the model which appears in Figure 8 with the dissociation constants given in its legend. A and B are theoretical curves giving the variation of $[E_{Na_2K}]/[E_{total}]$ (A) and $[E_{Na_3K_2}]/[E_{total}]$ (B) as a function of K^+ concentrations at different Na^+ concentrations. Numbers given in the figures indicate values of $-\log [Na^+]$. C presents a diagram of isoconcentration lines (for $[E_{ij}]/[E_{total}]$ = 50, 70, and 90%) of species E, E_{K_2}, E_{Na_4}, E_{Na_2K} and $E_{Na_3K_2}$ in the plane $-\log [Na^+]/-\log [K^+]$. Species which appear in this figure are those which accumulate in reasonable proportions. Isoconcentrations lines for E_{Na_2} and $E_{Na_4K_2}$ are not presented in order to simplify the diagram. These two species are never present in proportions representing more than 25–30% of the total enzyme concentrations and this happens in very restricted zones of Na^+ and K^+ concentrations. All other species, not mentioned in this figure and which appear in the model of Figure 8, never accumulate in proportions higher than 5% of the total.

Although direct determinations of ^{22}Na binding to a (Na^+,K^+)ATPase have been attempted (Kaniike et al., 1976), at the present moment there is still a nearly total lack of knowledge of directly determined binding stoichiometries of Na^+ and K^+ to the enzyme. However, information is available on the subunit structure of the (Na^+,K^+)ATPase. (Na^+,K^+)-ATPases, including the axonal ATPase (Balerna et al., 1975), have molecular weights of about 250 000. The most recent determinations propose an $\alpha_2\beta_2$ structure for the enzyme (where α is the catalytic subunit, mol wt $\simeq$ 100 000) (Kyte, 1972; Jorgensen, 1974; Perrone et al., 1975; Kyte, 1975). Such a structure will easily accommodate two K^+ and four Na^+ sites. Furthermore, its oligomeric character will allow it to

generate all sorts of cooperative behavior, positive or negative.

It may be of interest now to make a rapid comparison between the ATPase-catalyzed hydrolysis of 2,4-dinitrophenyl phosphate and furylacryloyl phosphate, on the one hand, and *p*-nitrophenyl phosphate on the other hand. Although the efficiency of hydrolysis is quite different, *p*-nitrophenyl phosphate shares a number of properties displayed by the other synthetic substrates. The Hill coefficient for K^+, $n_{H(K^+)}$, determined in the absence of Na^+ is near 2 as for the other substrates. The presence of Na^+ generates a negative cooperativity of the K^+ stimulation; curves relating stimulation of ATPase activity to K^+ concentrations contain intermediary plateau regions. For example, at a Na^+ concentration of 100 mM, increasing K^+ concentration gives a stimulation of the activity up to 2 mM K^+. This is followed by a plateau of activity between 2 and 20 mM K^+, then by a new increase of the stimulation factor (Gache et al., 1976). Without going into a detailed analysis in these previously published results, it is obvious that the data obtained for *p*-nitrophenyl phosphate also indicate the presence of two classes of K^+ sites which are linked by a negative cooperativity modulated by the presence of Na^+. We have now established that at the high Na^+ concentration of 400 mM, the negative cooperativity for the response disappears; $n_{H(K^+)}$ resumes a high value of 2, a behavior similar to other synthetic substrates studied in this paper. All these results indicate that the modulation by Na^+ and K^+ of the ATPase activity towards *p*-nitrophenyl phosphate could be interpreted by the model presented in Figure 8. One of the important differences between *p*-nitrophenyl phosphate and the other organic phosphates studied in this work is observed in the presence of ATP. Simultaneous addition of ATP and Na^+ produces a large stimulation of the activity towards *p*-nitrophenyl phosphate at low levels of K^+. No such behavior has been observed with dinitrophenyl phosphate and furylacryloyl phosphate. Stimulation by ATP of pseudosubstrate hydrolysis might only arise when the pseudosubstrate is a poor substrate for the (Na^+,K^+)ATPase as compared to ATP itself. This phenomenon would not be observed when the pseudosubstrate is a better substrate than ATP itself.

It is interesting finally to compare results obtained for the ATPase-catalyzed hydrolysis of the pseudosubstrates to those obtained for the natural substrate ATP (Gache et al., 1976). With ATP, $n_{H(Na^+)}$ is near 2 in a very large range of K^+ concentrations (between 0.1 and 200 mM K^+). This value of $n_{H(Na^+)}$ is nearly identical to that which has been found with the pseudosubstrates. Also when ATP is used as substrate, $n_{H(K^+)}$ is found to be near 1 between 5 and 100 mM Na^+. This value of $n_{H(K^+)}$ is similar to that found for the pseudosubstrates used in this work at Na^+ concentration in the vicinity of 10 mM (Figure 6 and Table II). We have now checked that at a very high Na^+ concentration, $n_{H(K^+)}$ with ATP reaches a high value of 1.65. This again is in agreement with that observed with the pseudosubstrate. Although there are obviously many analogies between kinetic properties of pseudosubstrates, on the one hand, and ATP, on the other hand, there is one important difference. When ATP is used as substrate, both Na^+ and K^+ stimulate the catalytic activity (Skou, 1957; Askari, 1974). When pseudosubstrates are used instead, K^+ is the only stimulatory ion; Na^+ does not stimulate, it only modulates the K^+ response. Moreover, whereas K^+ stimulation is observed for pseudosubstrates hydrolysis in the total absence of Na^+, such a stimulation does not exist with ATP under the same conditions. In the total absence of Na^+, catalytic activity for ATP hydrolysis exists. It is inhibited by ouabain but it is not stimulated by K^+. The existence of these differences suggests that the model proposed in Figure 8 to explain the behavior of the pseudosubstrates must be adapted slightly to explain the kinetic features of hydrolysis of ATP.

The model proposed in this paper introduces the notion of negative cooperativity and even half-site reactivity for Na^+ and K^+ sites. It is evidently of interest to know if such properties have been observed for substrate binding or transformation. The answer is not known for pseudosubstrates but it is well established that negative cooperativity is a basic property of ATP binding and transformation for (Na^+,K^+)ATPases of various origins (Hegyvary and Post, 1971; Glynn and Karlish, 1976; Robinson, 1976; Gache et al., 1976).

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Appendix

This appendix gives the phenomenological description of the model presented in Figure 8.

The enzyme, E, is in equilibrium with the two ligands, Na^+ and K^+. The enzyme contains m potassium sites and n sodium sites. $[E_{ij}]$ represents the enzymatic complex with i sites occupied by Na^+ and j sites occupied by K^+. Macroscopic equilibrium constants for K^+ and Na^+ binding are defined by eq 1

$$K_{ij} = \frac{[E_{ij}]}{[E_{i(j-1)}][K]}, \qquad K_{ij}' = \frac{[E_{ij}]}{[E_{(i-1)j}][Na]} \tag{1}$$

[Na] and [K] represent concentrations of sodium and potassium ions. The binding polynomial, P (Wyman 1965), for this system can be written

$$P = \sum_{i=0}^{n} \sum_{j=0}^{m} \varphi_{i0}\psi_{ij}[Na]^i[K]^j \tag{2}$$

where $\psi_{ij} = \prod_{j=1}^{m} K_{ij}$ and $\varphi_{ij} = \prod_{i=1}^{n} K_{ij}'$ (with $\psi_{i0} = \varphi_{0j} = 1$). The saturation function for K^+ is defined (Viratelle and Seydoux, 1975) as

$$\bar{y}_{[K^+]} = \frac{1}{m}\frac{[K]}{P}\frac{dP}{d[K]} \tag{3}$$

Homotropic interactions (positive or negative cooperativity) observed for K^+ stimulations impose the existence of at least two K^+ sites.

If we take $m = 2$, the expression for $K_{0.5}$ is derived from eq 3 when $\bar{y}_{K^+} = 1/2$ and $[K] = K_{0.5}$, which gives

$$K_{0.5} = \left(\frac{\sum_{i=0}^{n} \varphi_{i0}[Na]^i}{\sum_{i=0}^{n} \varphi_{i0}\psi_{i,2}[Na]^i} \right)^{1/2} \tag{4}$$

The Hill coefficient at half saturation is calculated according to Viratelle and Seydoux (1975) with eq 5, where $\dot{P}$ and $\ddot{P}$ represent, respectively, the first and second derivative of the binding polynomial, P, with respect to [K].

$$n_H = 1 + K_{0.5}\frac{2\ddot{P}\dot{P} - \dot{P}^2}{\dot{P}(2P - K_{0.5}\dot{P})} \tag{5}$$

In the text, microscopic association constants are discussed rather than the macroscopic ones used in this Appendix.

Macroscopic and microscopic constants for the ith binding step in an n step mechanism are related by the well-known equation (Edsall and Wyman, 1958)

$$K_{i(\text{macro})} = \frac{n-i+1}{i} K_{i(\text{micro})}$$

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