Kinetic Studies on a Brain Microsomal Adenosine Triphosphatase. II. Potassium-Dependent Phosphatase Activity*

Joseph D. Robinson

ABSTRACT: A ($Na^+ + K^+$)-dependent adenosine triphosphatase preparation from rat brain catalyzed a K^+-dependent hydrolysis of *p*-nitrophenylphosphate. The response of the *p*-nitrophenylphosphate to K^+ did not follow Michaelis–Menten kinetics, but gave a Lineweaver–Burk plot that curved upward at low K^+ concentrations, consistent with a positive cooperative allosteric activation. Na^+ inhibited by raising the $K_{0.5}$ for K^+ without affecting V_{max}. However, in the presence of increasing concentrations of Na^+ the response of the enzyme to K^+ gave a Lineweaver–Burk plot that curved downward at low K^+ concentrations, consistent with Na^+ converting the K^+ activation from a positive into a negative cooperative response. This Na^+-induced change in the response to K^+ suggested that the Na^+ inhibition was not due to simple competition with K^+. Furthermore, the inhibition by Na^+ similarly followed cooperative kinetics which in turn were modified by the K^+ concentration. At a constant molar ratio of Na^+ to K^+ only V_{max} was affected. Cytosine triphosphate in the absence of Na^+ inhibited the *p*-nitrophenylphosphate, but with Na^+ it stimulated activity with an abolition of the Na^+-induced negative cooperative response to K^+ and a 70-fold reduction in the $K_{0.5}$ for K^+. ATP with Na^+ also reduced the $K_{0.5}$ and converted the cooperative response into a positive one. Furthermore, Na^+ in the presence of low concentrations of K^+ or of cytosine triphosphate (without K^+) stimulated *p*-nitrophenylphosphate activity. These data suggest that in addition to possible competition with K^+ at K^+ sites, Na^+ could modify *p*-nitrophenylphosphate activity through interactions at its own sites, and that the *p*-nitrophenylphosphate has coexisting regulatory sites for K^+, Na^+, and nucleotides. These properties of the *p*-nitrophenylphosphate may reflect similar features of the related ($Na^+ + K^+$)-dependent adenosine triphosphatase, such that interdependence between distinct Na^+ and K^+ activating sites may modify the enzymatic reaction and the regulation of ion movements.

The active transport of Na^+ and K^+ has been related convincingly to a ($Na^+ + K^+$)-dependent ATPase present in the cell membranes of a large number of tissues (Skou, 1965). However, a detailed description of this process has proved elusive, due largely to the inability to purify the membrane-bound enzyme and to the complexity of the reaction, which appears to involve a Na^+-dependent phosphorylation of the enzyme and a K^+-dependent hydrolysis (Albers, 1967). Recent attention has focused on these partial reactions, and a strong argument has developed for considering a K^+-dependent phosphatase described in several partially purified ATPase preparations as representing the terminal hydrolytic step (Askari and Koyal, 1968).

This study concerns certain kinetic properties of the K^+-dependent phosphatase present in a partially purified ATPase preparation from rat brain. It was begun for comparison with the kinetic properties of the ($Na^+ + K^+$)-dependent ATPase (Robinson, 1967) in an effort to describe those variables associated with the terminal step and to examine the relationship between the activators of the two steps. In addition, since previous kinetic studies (Squires, 1965; Robinson, 1967) had been questioned on the basis of possible competition between activating monovalent cations (Albers *et al.*, 1968; Priestland and Whittam, 1968; Robinson, 1968a), it seemed of interest to examine a process requiring only one monovalent cation where such competition could be avoided. The data appear pertinent to considerations of the number and kinds of sites for monovalent cations, and their relationships with each other and with activating nucleotides. These results are presented in support of the previous description of certain allosteric interactions in the ($Na^+ + K^+$)-dependent ATPase that may be relevant both to the mechanism of ion transport (Jardetzky, 1966; Robinson, 1967) and to the control of ion movements and cell volume (Robinson, 1968b).

Methods

The ($Na^+ + K^+$)-dependent ATPase preparation was obtained from a rat brain microsomal preparation by treatment with deoxycholate and then NaI, as previously described (Robinson, 1967), except that final suspension was in 0.01 M Tris-HCl (pH 7.8).

The standard incubation medium contained 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM NPP[1] as the Tris salt, 10 mM KCl, and the enzyme preparation (0.2 mg of protein/ml). Incubation was for 10–20 min at 30° unless otherwise stated; under the conditions used the activity was linear with time during these periods, allowing initial velocities to be estimated. The reaction was stopped by adding cold trichloroacetic acid to a final concentration of 5%, and the mixture was centrifuged. A 0.2-ml portion of the supernatant fluid was added to 1.0 ml of 1 M Tris and the absorbance was measured at 420 mμ.

* From the Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, New York 13210. *Received January 22, 1969.* This work was supported by U. S. Public Health Service Grant NB-05430.

[1] Abbreviations used are: NPP, *p*-nitrophenylphosphate; NPPase, *p*-nitrophenylphosphatase.

TABLE I: Comparison of Relative Specific Activities of Successive NPPase Preparations.[a]

Preparation	Rel NPPase Sp Act. (units/min mg of protein)			
	No KCl, A	With 10 mM KCl, B	B − A, C	Ratio of C/A
Microsomal	0.14	0.40	0.26	1.9
Deoxycholate treated	0.12	0.98	0.86	7.2
NaI treated	0.042	1.04	1.00	24

[a] The relative specific activities (micromoles of NPP hydrolyzed per minute per milligram of protein) of successive NPPase preparations, obtained as previously described (Robinson, 1967), were compared. Initial velocities were measured during incubations in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, and 3 mM NPP (column A), or in media containing in addition 10 mM KCl (column B). Column C, the difference in activities due to KCl, is taken to represent the K^+-dependent NPPase activity.

TABLE II: Variations in NPPase Activity with $MgCl_2$ and NPP Concentrations.[a]

$MgCl_2$ Concn (mM)	Rel Act. of the K^+-Dependent NPPase (units/min mg of protein)			
	With 1 mM NPP	With 3 mM NPP	With 6 mM NPP	With 10 mM NPP
0.3		0.28		
0.6	0.36	0.50		
1.0	0.51	0.68		
1.5		0.89		
3.0	0.49	1.00	1.41	1.57
6.0		0.98	1.44	1.65
10.0	0.46	0.95	1.43	1.65

[a] The relative activities of the K^+-dependent NPPase were compared in media containing 50 mM Tris-HCl (pH 7.8), 10 mM KCl, and the concentrations of $MgCl_2$ and NPP shown.

Values were corrected for the *p*-nitrophenol content of zero-time incubations.

In measuring ATPase and CTPase activity, the nucleotide as the Tris salt was substituted for NPP, plus the amounts of NaCl and KCl stated in the text; optimal concentrations for measuring ATPase activity were 90 mM NaCl plus 10 mM KCl. Activity was measured in terms of the production of P_i as previously described (Robinson, 1967). Protein was measured by the biuret method using bovine serum albumin as a standard.

Stability constants for the complex between NPP and Mg^{2+} were estimated by the method of Walaas (1958). Dowex 1 resin in the chloride form (Bio-Rad Laboratories AG-1 X-8, 100–200 mesh) was washed and air dried, and then 25 mg of resin was added per ml of test media. The media contained 50 mM Tris-HCl (pH 7.8), 0.1 mM NPP (Tris salt), either 10 mM KCl or 10 mM KCl plus 10 mM NaCl, and $MgCl_2$ ranging from 30 to 0.3 mM. After being shaken with the resin for 2 hr at 30° the mixtures were centrifuged and the amount of NPP in the supernatant material was measured by its absorbance at 310 mμ. Controls containing no resin, no NPP, and no $MgCl_2$ were run concurrently.

NPP, ATP, and CTP were purchased from Sigma Chemical Co. as the sodium salts and converted into the Tris salts (Ulrich, 1963). All solutions were made in water that had been redistilled from an all-glass still.

The experimental points presented are the average of five or more experiments in duplicate, and are accompanied where appropriate by the standard error of the mean. Statistical significance was calculated by the t test; the criterion for significance was $P < 0.05$.

Results

Characteristics of the NPPase Activity. The three successive preparations hydrolyzed NPP to *p*-nitrophenol, but with sequential treatments the K^+-independent activity decreased markedly relative to the K^+-dependent NPPase (Table I), similar to the relative activities of ($Na^+ + K^+$)-independent and -dependent ATPase activities of these fractions (Robinson, 1967). All subsequent experiments were performed with the NaI-treated fraction. The fresh preparation had a K^+-dependent activity (Table I, C) of 0.048 μmole of NPP hydrolyzed/min mg of protein; this was 8% of the rate of ATP hydrolysis under optimal conditions (90 mM NaCl and 10 mM KCl). Activity in the absence of added KCl (Table I, A) was presumed to represent residual nonspecific phosphatase activity; "total NPPase activity" refers to the K^+-independent and K^+-dependent activity (Table I, B).

Because of variations in the absolute activity of different NaI preparations and a slow decline in activity during storage, enzyme velocities are expressed hereafter relative to the K^+-dependent activity of concurrent control incubations in the standard medium.

The pH optimum with 10 mM KCl was near 7.8, with activity declining only slightly at higher pH values.

The optimal concentrations of $MgCl_2$ were broad, but centered about a 1:1 molar ratio with NPP (Table II). With 3 mM NPP and 10 mM KCl, the K_m for $MgCl_2$ was 1.8 mM. In the absence of $MgCl_2$ there was essentially no activity.

Stability of the Mg–NPP Complex. Because of the apparent 1:1 optimal ratio between $MgCl_2$ and NPP concentrations it was of interest to know if this ratio reflected a tight complex between Mg^{2+} and NPP. If the substrate for the reaction were the Mg–NPP complex, then for a given NPP concentration increasing the $MgCl_2$ concentration would increase the concentration of the complex, and hence should increase the reaction velocity, until essentially all the NPP was complexed. The stability constant for the complex, measured as described under Methods, was 170 M^{-1} in the presence of 10 mM KCl; adding 10 mM NaCl to the media did not change the stability constant measurably. These data indicate that in the standard medium only one-fifth of the NPP existed as the Mg complex, and hence the NPP:Mg ratio may reflect the optima of the enzymatic binding sites.

TABLE III: Summary of Kinetic Parameters for Potassium.[a]

NPP Concn (mM)	$MgCl_2$ Concn (mM)	Additions	Kinetic Parameters		
			$K_{0.5}$ (mM)	$\|n\|$	V_{max}[b]
10	3.0	None	3.66	1.46	1.92
	6.0		4.04	1.48	2.09
	10.0		4.98	1.44	2.27
3	1.5	None	1.71	1.51	0.95
	3.0		1.92	1.41	1.10
	6.0		2.82	1.43	1.14
3	1.5	NaCl, 10 mM	3.95	0.99	1.07
	3.0		3.64	0.95	1.10
	6.0		4.58	1.02	1.16
3	3.0	NaCl, 20 mM	7.10	0.73	1.10
3	3.0	NaCl (3:1 ratio to KCl)	1.91	1.45	0.58
3	1.5	CTP, 0.3 mM, plus NaCl, 10 mM	0.054	1.02	0.62
	3.0		0.056	1.00	0.84
	6.0		0.079	1.00	0.87
3	3.0	CTP, 0.3 mM, plus NaCl, 20 mM	0.10	1.03	0.87
3	3.0	ATP, 0.3 mM, plus NaCl, 20 mM	0.074	1.39	0.21

[a] This table summarizes the values of the kinetic parameters for K^+ found in Figures 2, 3, 4, and 8. V_{max} was estimated graphically from Lineweaver–Burk plots, and $K_{0.5}$ and $|n|$ were calculated from Hill plots by the method of least squares. [b] Relative to the activity in the standard medium defined as 1.00.

TABLE IV: Sodium, Potassium, and CTP Interactions.[a]

NaCl Concn (mM)	Relative NPPase Activity		
	No K^+ or CTP	With 0.5 mM KCl	With 0.3 mM CTP
0		0.140 ± 0.005	0.016 ± 0.005
3	0.012 ± 0.011		
10	0.042 ± 0.006	0.200 ± 0.008[b]	0.172 ± 0.009[b]
20	0.043 ± 0.004	0.238 ± 0.008[b]	0.084 ± 0.007[b]
30	0.037 ± 0.007	0.241 ± 0.009[b]	

[a] The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM NPP, and NaCl, KCl, and CTP as indicated. The initial velocities listed represent increments over the NPPase activity in the absence of KCl, NaCl, and CTP (presumed to represent traces of nonspecific cation-dependent phosphatase activity contaminating the preparation), which was measured concurrently. The data are expressed relative to that of concurrent controls in the standard medium, defined as 1.00. [b] These velocities are significantly greater than the sum of the velocities with the separate additives measured at the same time.

Effects of NPP. As the concentration of NPP was increased the initial velocity was increased, but with an indication of substrate inhibition at higher concentrations. The data are presented in Figure 1 for experiments with 10 mM KCl and with 1 mM KCl, in terms of the total NPPase activity. From concurrent measurements of the substrate–velocity relationship in the absence of KCl it was possible to estimate the kinetic parameters for the K^+-dependent activity, using the relationship for two enzymes acting on one substrate, as previously described (Robinson, 1967). From such calculations the relative V_{max} was 2.2 and K_m was 3.4 with 10 mM KCl, whereas with 1 mM KCl both V_{max} and K_m decreased to 0.34 and 0.24 mM. In these experiments the $MgCl_2$ concentration was varied with the NPP to maintain a 1:1 molar ratio, in light of the above experiments indicating that such a ratio appeared optimal. However, the K_m for NPP in the presence of 10 mM KCl was not appreciably changed at a constant $MgCl_2$ concentration of 6 or 3 mM.

Effects of Potassium. In the following studies with varying KCl and NaCl concentrations initial experiments were performed in which choline chloride was added so that the ionic strength was always constant; however, the inclusion of choline chloride had no effect on the measured kinetic parameters and it was thereafter omitted for simplicity.

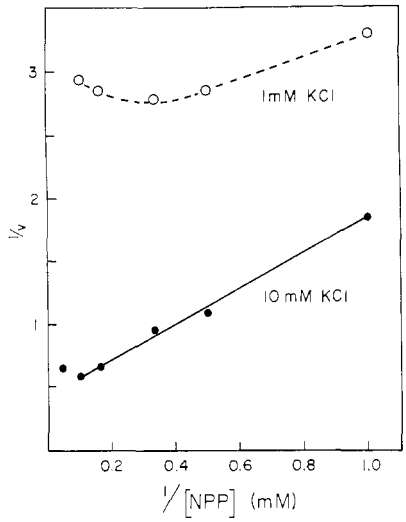

FIGURE 1: The effects of NPP on the total NPPase activity. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), either 10 mM KCl (●—●) or 1 mM KCl (O---O), and equimolar concentrations of $MgCl_2$ and NPP. Initial velocities are expressed relative to the K^+-dependent NPPase activity in the standard medium (10 mM KCl, 3 mM $MgCl_2$, and 3 mM NPP) and the data are presented in the form of a Lineweaver–Burk double-reciprocal plot.

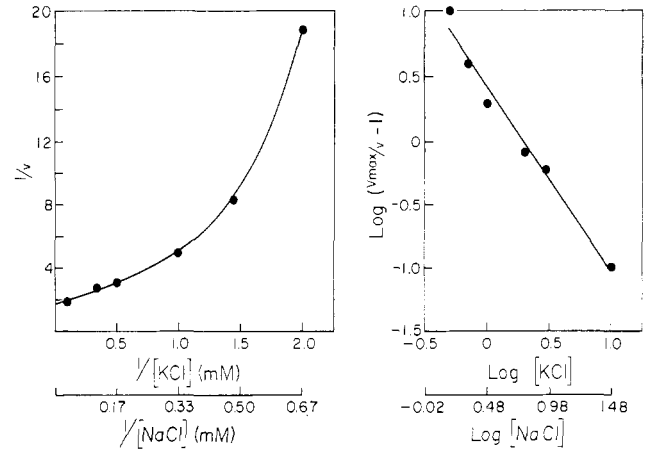

FIGURE 3: The effect of varying both Na^+ and K^+ on the K^+-dependent NPPase activity. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM NPP, and the concentrations of NaCl and KCl indicated, at a 3:1 molar ratio. Initial velocities are expressed relative to that in the standard medium. In the left-hand panel the data are presented as a Lineweaver–Burk plot, and in the right-hand panel as a Hill plot.

The relationship between the initial rate of the K^+-dependent NPPase activity and the concentration of KCl did not follow Michaelis–Menten kinetics, but gave a sigmoidal effector–velocity curve, a Lineweaver–Burk plot that curved upward at low KCl concentrations, and a Hill plot with a slope $|n| > 1.0$ (Figure 2). At a higher NPP concentration (Figure 2; Table III) the degree of sigmoidicity as indicated by $|n|$ did not change, whereas both V_{max} and the $K_{0.5}$ for K^+ increased ($K_{0.5}$ is the concentration of effector for half-maximal velocity (Koshland *et al.*, 1966) and may be derived from the Hill equation (Robinson, 1967)).

Addition of NaCl to the incubation medium inhibited NPPase activity. When NaCl and KCl were varied together at a constant molar ratio of 3:1 (Figure 3) V_{max} was decreased, but both the $K_{0.5}$ for K^+ and $|n|$ were unchanged (Table III). On the other hand, with a constant level of NaCl the kinetic parameters for K^+ changed differently: there was no effect on V_{max} but an increase in the $K_{0.5}$ for K^+ (Figure 4; Table III) as might be expected of a competitive inhibitor. However, Na^+ also markedly decreased $|n|$ for K^+ such that with 20 mM NaCl $|n| < 1.0$ (Figure 4 and Table III).

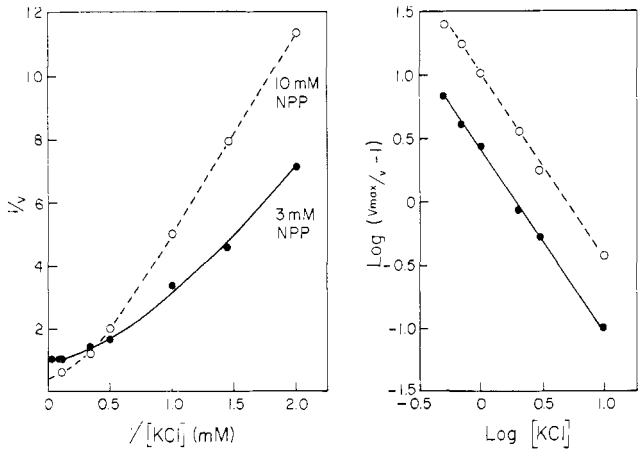

FIGURE 2: The effects of KCl on the K^+-dependent NPPase activity. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), the concentration of KCl indicated, and either 10 mM NPP and $MgCl_2$ (O-----O) or 3 mM NPP and $MgCl_2$ (●——●). Initial velocities are expressed relative to that in the standard medium (3 mM $MgCl_2$ and NPP and 10 mM KCl). In the left-hand panel the data are presented as a Lineweaver–Burk plot, and in the right-hand panel as a Hill plot with straight lines drawn by the method of least squares.

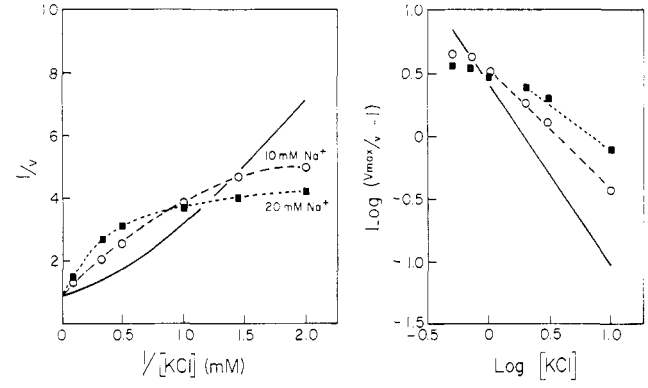

FIGURE 4: The effects of NaCl on KCl and the K^+-dependent NPPase activity. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM NPP, the concentration of KCl indicated, and either 10 mM NaCl (O-----O) or 20 mM NaCl (■-----■). Initial velocities are expressed relative to that in the standard medium (no NaCl). In the left-hand panel the data are presented as a Lineweaver–Burk plot, and in the right-hand panel as a Hill plot; for the latter, two points with 10 mM Na^+ and three points with 20 mM Na^+ were omitted in calculating the least-squares lines, as indicated by the length of the line. In each panel the solid line represents the curve from Figure 2 with 3 mM NPP, redrawn for comparison.

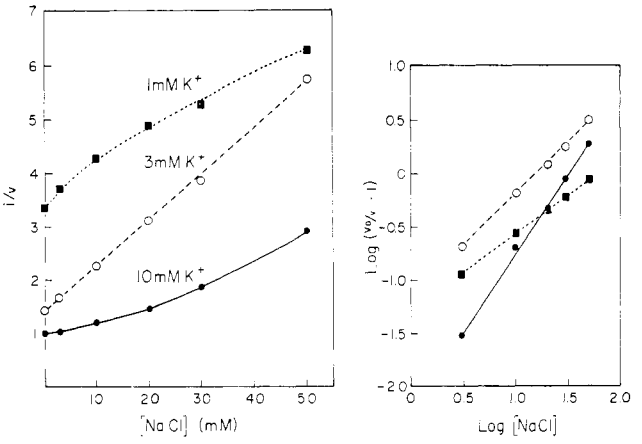

FIGURE 5: The effects of NaCl on the K^+-dependent NPPase activity. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM NPP, the amount of NaCl indicated, and either 10 mM KCl (●——●), 3 mM KCl (O- - - -O), or 1 mM KCl (■-----■). Initial velocities are expressed relative to that in the standard medium. In the left-hand panel the data are presented as a Dixon plot, and in the right-hand panel as a Hill plot in terms of V_0, the velocity in the absence of NaCl.

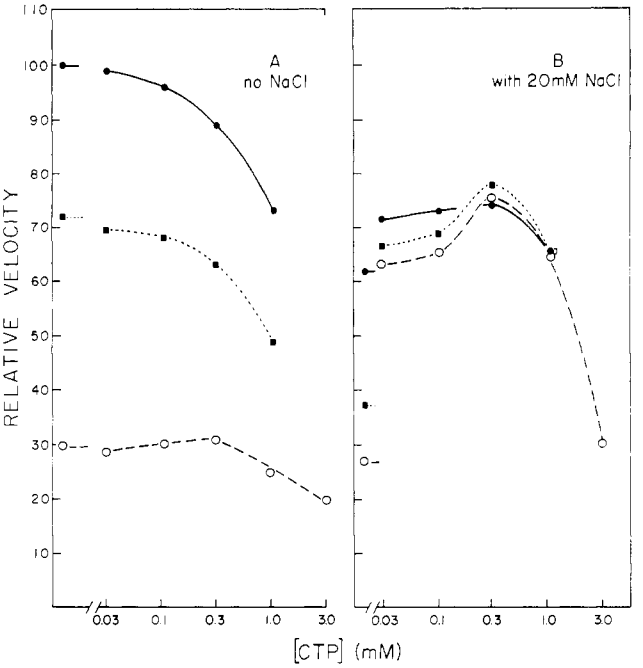

FIGURE 6: The effects of CTP on K^+-dependent NPPase activity. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3mM NPP, the amount of CTP indicated, 10 mM (●——●), 3 mM (■------■), or 1 mM (O------O) KCl, and either no NaCl (left panel) or 20 mM NaCl (right panel). Initial velocities are expressed relative to that in the standard medium.

Some reservations must be stressed about the latter experiments, for it can be seen that at KCl concentrations below 1 mM the points on the Hill plot do not lie on a straight line, and this deviation is greater at the higher NaCl concentration. The discrepancy is in the direction of a greater velocity than described by the Hill equation for the line, and as shown in Table IV the presence of high (10–20 mM) NaCl with low (0.5 mM) KCl significantly increased NNPase activity to more than the sum of the two alone.

Effects of Sodium. In the absence of KCl, NaCl stimulated NPPase activity very slightly (Table IV). In the presence of KCl the relationship of the NaCl concentration to activity depended upon the KCl concentration. Thus with 10 mM KCl the Dixon plot (Dixon and Webb, 1964) curved upward at high NaCl concentrations; the Hill plot showed $|n| > 1.0$, and the $K_{-0.5}$ for Na^+ (the concentration to produce half the uninhibited velocity, V_0) was 32 mM (Figure 5 and Table V). As the KCl concentration was reduced, $|n|$ decreased to less than 1.0, whereas the value for $K_{-0.5}$ first fell and then rose at lower KCl concentrations. The apparent increase in the $K_{-0.5}$ with 1.0 mM KCl may reflect the synergism cited above (Table IV) between high NaCl and low KCl.

The K_i for Na^+ inhibition was estimated to be about 6 mM from the extrapolation of the Dixon plots to their common point of intersection with the horizontal line at a height $1/V_{max}$. Such an intersection is consistent with competitive inhibition (Dixon and Webb, 1964).

Effects of Nucleotides. Adding CTP to the standard incubation medium inhibited NPPase activity, but the addition of NaCl with CTP stimulated NPPase at lower KCl concentrations (Figure 6). The optimal CTP concentration was about 0.3 mM, and with either 1 or 3 mM KCl the concentration of NaCl for half-maximal stimulation was about 2.5 mM (Figure 7).

In the presence of 0.3 mM CTP and either 10 or 20 mM NaCl the $K_{0.5}$ for K^+ was markedly decreased to about $^1/_{70}$ that with NaCl alone (Figure 8 and Table III). Furthermore, $|n|$ was 1.0 at both NaCl concentrations.

Although 0.3 mM CTP had practically no effect on NPPase activity in the absence of NaCl and KCl, in the presence of 10 mM NaCl, NPPase activity was significantly greater than the sum of each alone (Table IV), similar to the effect of low KCl with NaCl.

Unlike CTP, ATP over the concentration range 0.001–0.3 mM did not increase NPPase activity in the presence of 20 mM NaCl and 1 mM KCl. The rate with 20 mM NaCl plus 0.3 mM ATP was, however, greater than without NaCl. Furthermore, ATP in the absence of NaCl increased the $K_{0.5}$ for K^+ to 4.7 mM, but in the presence of 20 mM NaCl the $K_{0.5}$ for K^+ was strikingly reduced to 0.074 mM with $|n| = 1.4$ (Figure 8 and Table III).

TABLE V: Summary of Kinetic Parameters for Sodium.[a]

KCl Concn (mM)	$MgCl_2$ Concn (mM)	Kinetic Parameters	
		$K_{-0.5}$ (mM)	$\|n\|$
10	1.5	26	1.39
	3.0	32	1.46
	6.0	33	1.35
3	3.0	16	0.96
1	3.0	59	0.73

[a] This table summarizes the values of the kinetic parameters for Na^+ found in Figure 5. $K_{-0.5}$ and $|n|$ were calculated from Hill plots by the method of least squares.

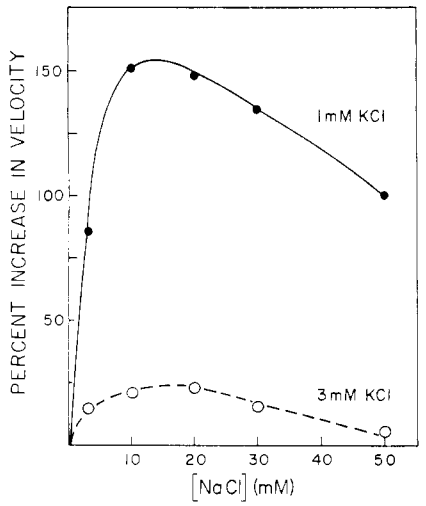

FIGURE 7: The effect of NaCl on NPPase activity in the presence of CTP. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM NPP, 0.3 mM CTP, the amount of NaCl indicated, and either 1 mM (●——●) or 3 mM KCl (○-----○). Data are expressed as the percentage increase in initial velocity of the K^+-dependent NPPase activity compared with concurrent controls in the absence of NaCl.

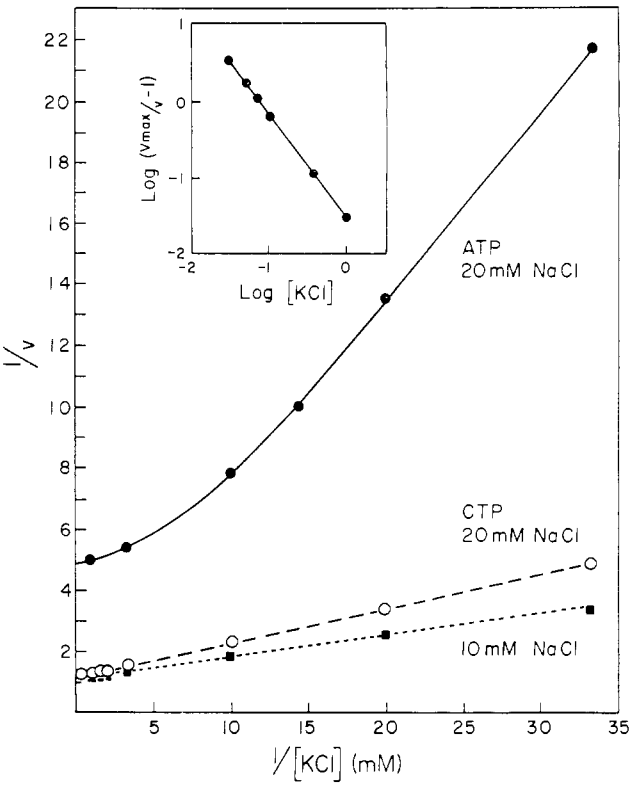

FIGURE 8: The effect of nucleotides plus Na^+ on the response of the NPPase to K^+. The enzyme preparation was incubated in media containing 50 mM Tris-HCl (pH 7.8), 3 mM $MgCl_2$, 3 mM NPP, the concentration of KCl indicated, and either 10 mM NaCl and 0.3 mM CTP (■-----■), 20 mM NaCl and CTP (○-----○), or 20 mM NaCl and 0.3 mM ATP (●——●). Initial velocities of the K^+-dependent NPPase activity are expressed relative to that in the standard medium, and are presented as a Lineweaver–Burk plot; the data for ATP are replotted in the insert as a Hill plot.

Effects of $MgCl_2$ on the Kinetic Parameters for K^+ and Na^+. Since the effects of the monovalent cations might represent, at least in part, interaction with the Mg^{2+} activation, experiments were conducted at $MgCl_2$ concentrations above and below those optimal with 10 mM KCl. Values of the kinetic parameters for K^+ and Na^+ at various $MgCl_2$ concentrations are included in Tables III and V for representative cation effects. These data show that $MgCl_2$ influenced the $K_{0.5}$ for the monovalent cations somewhat, but, unlike the effects of NaCl on K^+ activation, had little effect on the pattern of the response, as indicated by $|n|$. Conversely, at low KCl concentrations the optimal concentrations of $MgCl_2$ were slightly lower.

CTPase Activity. In a medium containing 50 mM Tris-HCl (pH 7.8), 0.3 mM CTP as the Tris salt, 0.3 mM $MgCl_2$, 20 mM NaCl, and 1 mM KCl, the enzyme preparation liberated P_i at a rate of 0.05 μmole/min mg of protein; this was about one-fourth the rate with ATP under these conditions.

Discussion

This discussion is presented in terms of allosteric processes (Monod *et al.*, 1965; Koshland *et al.*, 1966; Atkinson, 1966) as described for the ATPase (Squires, 1965; Robinson, 1967). The assumption is made that, aside from contaminating traces of cation-independent phosphatases found in crude preparations, the K^+-dependent phosphatase is a single enzymatic entity, albeit in various states of activity. The interpretation of kinetic data is rarely unambiguous, and alternative explanations must be kept in mind; with only the impure enzyme available more direct measurements (*e.g.*, physical studies, ion binding affinities, and capacities) were not possible.

In this framework K^+ may be classed as a cooperative homotropic allosteric activator of the NPPase, as indicated by the Hill plot with $|n| > 1.0$ (Figure 2); replotting the data of Fujita *et al.* (1966) will also give a Hill plot for K^+ with $|n| > 1.0$. K^+ is also a heterotropic allosteric modifier toward the substrate, affecting the K_m for NPP (Figure 1), and NPP is a heterotropic modifier toward K^+, affecting the $K_{0.5}$ but not altering $|n|$ (Figure 2).

On the other hand, Na^+ inhibited the NPPase in a manner suggesting competition with K^+. V_{max} was unaffected while the $K_{0.5}$ for K^+ was increased (Figure 4). However, this inhibition is not simple competition since Na^+ also affected the degree of cooperativity toward K^+, changing K^+ from a positive cooperative activator with $|n| > 1.0$, to a negative cooperative activator (Conway and Koshland, 1968), with $|n| < 1.0$ (Figure 4). Moreover, Na^+ itself engaged in cooperative allosteric interactions, with K^+ as a heterotropic modifier changing the Na^+ interactions from positive to negative (Figure 5).

Models for cooperative effects propose a set of interacting sites for the homotropic effector; conceivably a fraction of the K^+ sites could be competitively usurped by Na^+ and through such occupancy subvert the K^+ interactions at the remaining sites from a positive to a negative cooperative interaction. But these inhibitory Na^+ sites need not be identical with the K^+ sites to mediate the response, and several lines of inquiry suggest that other sites were available to Na^+. Thus with a constant molar ratio of Na^+ to K^+ V_{max} was reduced, indicating that Na^+ affected the system, but neither $|n|$ nor the

$K_{0.5}$ for K^+ was altered (Figure 3), as would be expected if Na^+ occupied K^+ sites. Furthermore, the slight activation of NPPase by Na^+ was increased by K^+ (Table IV); such synergism is difficult to reconcile with a competition between the ions for the same sites.

A further indication of a distinct role for Na^+ was apparent in experiments with nucleotides. CTP in the absence of Na^+ inhibited the NPPase, but with Na^+ it stimulated (Figure 6). Such a response to nucleotides was first described by Fujita *et al.* (1966) who interpreted it as a decreased sensitivity of the enzyme to Na^+, but Nagai and Yoshida (1966) found that both CTP and ATP increased activity over that in the absence of Na^+, and proposed instead an allosteric process of some sort. As shown in Figure 8, the stimulation by nucleotides plus Na^+ may be interpreted as a heterotropic modification of the NPPase toward K^+, with an abolition of the Na^+-induced negative cooperative effect and a remarkable decrease in $K_{0.5}$. Similar data on a reduced $K_{0.5}$ for K^+ in the presence of nucleotide and Na^+ has recently appeared (Yoshida *et al.*, 1969). Furthermore, Na^+ activated the NPPase in the presence of CTP but the absence of K^+ (Table IV), reminiscent of the synergism between Na^+ and low concentrations of K^+. Perhaps either with low K^+ concentrations or with nucleotides an activating site for the NPPase becomes available to Na^+.

The concentration of $MgCl_2$ also affected the kinetic values for Na^+ and K^+, and it cannot be dismissed as an independent variable. However, for a given NPP concentration, increases in the $MgCl_2$ concentration chiefly raised the $K_{0.5}$ for K^+ and had little effect on V_{max}. Unlike added NaCl or KCl, varying the $MgCl_2$ over a fourfold range had essentially no effect on $|n|$ (Tables III and V). Since the same patterns of responses to monovalent cations were obtained over this range of $MgCl_2$ contrations, it seems unlikely that the effects of the monovalent cations result largely from changes in the Mg^{2+} activation or that the Mg^{2+} alters markedly the form of the response to monovalent cations. Nevertheless, interpretation of these results with an enzyme subject simultaneously to at least five activating ions (*viz.*, NPP, CTP, Mg^{2+}, Na^+, and K^+) must be tempered with caution.

The marked heterotropic effects of nucleotide plus Na^+ do not fit a reaction scheme with only a competition between Na^+ and K^+ for the same site, and suggest a more complex scheme with sites for Na^+, K^+, and nucleotide. For example, the data would fit a model in which K^+-dependent hydrolysis is best served by an enzyme conformation dependent upon the presence of nucleotide and Na^+ at their own sites. Such a complement of modifiers invites comparison of the NPPase with the ATPase. It is not the aim of this paper to marshall the evidence relating these two activities, but certain lines of evidence may be cited: both require Mg^{2+} and K^+ and are inhibited by ouabain, and parallelism exists in regard to treatment with NaI, thermal denaturation, tryptic digestion, and inhibition by *N*-ethylmaleimide and diisopropylphosphofluoridate (Fujita *et al.*, 1966); similar labeled peptide fragments can be isolated after incubation with either [^{32}P]acetyl phosphate or [^{32}P]ATP (Israel and Titus, 1967); and ATP and the phosphatase substrates are competitive inhibitors to each other (Bader and Sen, 1966; Israel and Titus, 1967; Sachs *et al.*, 1967; Formby and Clausen, 1968; but see Fujita *et al.*, 1966). Objections to this relationship have been based on different sensitivities to certain ions and inhibitors; however, such disparities may reflect the role of the substrate as a heterotropic modifier in the enzymatic process (Robinson, 1967) such that different substrates may induce different responses. In this vein, Askari and Koyal (1968) showed that sensitivity of the NPPase to oligomycin, an inhibitor of the terminal step in the ATPase, was dependent upon the presence of both ATP and Na^+. The present data are consistent with such a relationship.

For the ATPase these considerations suggest a concerted process with interdependent coupled activators, rather than two sequential processes each with its own activator. Many models for the ATPase (*e.g.*, Judah and Ahmed, 1964; Post *et al.*, 1965; Albers *et al.*, 1968; Lowe, 1968) describe an internal Na^+ site that is translocated to become an external K^+ site. Instead of this sequential interconversion of sites, Na^+ and K^+ sites could coexist. Such a formulation would permit separate but interdependent channels for cation movement, and the coupling between these sites would bear not only on homeostatic control but also on such varied processes as the mode of action of diphenylhydantoin in epilepsy (Festoff and Appel, 1968) and electrogenic ion pumps involved in the generation of the receptor potential in the retina (Smith *et al.*, 1968) and acetylcholine-induced postsynaptic inhibition in nerve (Pinsker and Kandel, 1969).

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Kinetics of the Resolution of Complex I (Reduced Diphosphopyridine Nucleotide–Coenzyme Q Reductase) of the Mitochondrial Electron Transport System by Chaotropic Agents*

K. A. Davis and Y. Hatefi

ABSTRACT: Chaotropic agents (NaSCN, $NaClO_4$, guanidine-HCl, and urea) resolve the enzymic assembly of complex I (reduced diphosphopyridine nucleotide–coenzyme Q reductase) resulting in the solubilization of the reduced diphosphopyridine nucleotide dehydrogenase and the iron–sulfur protein of the complex. They also cause the release of these components from the binary I–III complex and electron transport particle. The resolution of complex I is first order with respect to the appearance of soluble reduced diphosphopyridine nucleotide dehydrogenase and highly temperature dependent at low concentrations of chaotropic agents. As in the case of microsomes and other complexes of the respiratory chain, the aerobic resolution of complex I is accompanied by destruction of the iron–sulfur proteins and considerable lipid oxidation. However, under anaerobic conditions the resolution of complex I by chaotropic agents proceeds in the absence of any detectable lipid oxidation or iron–sulfur protein destruction. Reduced diphosphopyridine nucleotide, but not diphosphopyridine nucleotide or reduced triphosphopyridine nucleotide, impedes the chaotropic-induced resolution of complex I, suggesting that the reduced complex has a more stable conformation.

Recent studies in this laboratory have shown that anions such as SCN^-, ClO_4^-, and I^- (chaotropic agents) disrupt membrane structure and increase the water solubility of particulate proteins and nonelectrolytes (Hatefi and Hanstein, 1969). Physicochemical considerations have permitted the interpretation that chaotropic agents change the structure and the lipophilicity of water such that membrane hydrophobic bonds, which are mainly responsible for the stability of such structures in aqueous media, are weakened and the thermodynamic barrier for entry of nonelectrolytes and the apolar groups of proteins into the aqueous phase is lowered. These studies have further demonstrated that guanidine-HCl and urea[1] also behave like chaotropic ions in solubilization of particulate proteins and nonelectrolytes. In addition it has been shown that as a consequence of membrane resolution, chaotropic agents induce a very rapid lipid oxidation in ETP,[2] the purified complexes of the respiratory chain, and microsomes.

The resolution of complex I by chaotropic agents has yielded (a) a DPNH dehydrogenase, containing 1 mole of flavin, 4 g-atoms of iron, and 4 moles of labile sulfide per 70,000 g of protein; (b) an iron–sulfur protein; and (c) a protein fraction with solubility properties similar to the mitochondrial "structural" proteins (Hatefi and Stempel, 1967, 1969). In this process, the catalytic and inhibitor-response properties of DPNH dehydrogenase undergo vast changes as the particle-bound enzyme is rendered soluble. These changes include a tenfold increase in K_m^{DPNH}, the emergence of very high reductase activities with respect to quinones (menadione and DCIP) and ferric complexes (ferricyanide and cytochrome *c*) as electron acceptors and the appearance of an active thiol (Hatefi and Stempel, 1969).

The present study describes the kinetics of the resolution of

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[1] Hatefi and Hanstein (1969) have defined chaotropic agents as "those inorganic anions which favor the transfer of apolar groups to water." However in this paper guanidine-HCl and urea will also be referred to as chaotropic agents.

[2] Abbreviations used are: complex I, particulate, rotenone-, and piericidin A sensitive DPNH–coenzyme Q reductase; the binary I–III complex, particulate DPNH–cytochrome *c* reductase; ETP, electron transport particle; DCIP, 2,6-dichlorophenolindophenol; MDA, malondialdehyde.