Kinetic Studies on a Brain Microsomal Adenosine Triphosphatase. Evidence Suggesting Conformational Changes*

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ABSTRACT: Adenosine triphosphatase (ATPase) activity of three sequential preparations from rat brain microsomes was studied. The final preparation, obtained after NaI treatment, displayed an absolute requirement for Na^+ plus K^+; this finding together with kinetic studies indicated that two distinct Mg^{2+}-dependent ATPase activities existed in the microsomes, one independent of monovalent cations and the other having an absolute requirement for both Na^+ and K^+. Kinetic studies on the (Na^+ + K^+)-dependent ATPase revealed several allosteric effects. (1) Variation of either Na^+ or K^+ concentration altered the reaction velocity in a manner indicating a cooperative homotropic effect; (2) changes in the concentration of Na^+ and K^+ altered both the K_m for ATP and the V_{max} indicating a heterotropic effect of the salts; and (3) variations in ATP concentration did not demonstrate cooperative homotropic effects, but did alter both the degree of the cooperative effects and the effective half-maximal concentrations of Na^+ and K^+, indicating a heterotropic effect. Estimates of the energy of activation suggested that increases in salt concentration caused increases in the entropy of activation of the ATPase. These data support proposals that conformational changes, which have been implicated in allosteric processes and in entropy changes, occur in the (Na^+ + K^+)-dependent ATPase during activity, possibly in relation to membrane transport of cations.

Although the (Na^+ + K^+)-stimulated ATPase[1] has been related convincingly to membrane ion transport (Skou, 1965), the mechanism of this transport remains uncertain. Plausible hypotheses involve conformational changes in the enzyme molecule causing concomitant changes in transmembrane channels or actual transport of enzyme-bound ions (Whittam and Ager, 1965; Opit and Charnock, 1965; Jardetzky, 1966). Evidence supporting these hypotheses is meager, in part due to the difficulty in detecting any such change within the membrane fragments, for attempts to solubilize or purify the enzyme to a major extent have failed. Recently, kinetic evidence of allosteric effects, which imply conformational changes, have been reported with an enzyme preparation containing both salt-stimulated and salt-independent ATPase activity (Squires, 1965), and structural alterations in membrane fragments, as indicated by light-scattering and permeability changes, were shown to be caused specifically by ATP (Robinson, 1967).

It seemed of interest to examine the kinetic properties of membrane ATPase preparations not only in terms of allosteric effects, but also as an expansion of the scanty and conflicting kinetic data available and as an indication of whether the two activities (salt dependent and salt independent) represent two different enzymes or one. Furthermore, by using a preparation displaying only the salt-dependent activity certain kinetic artifacts might be avoided.

Data are presented supporting the contention that the two activities represent two enzymes, rather than merely a stimulation above a salt-independent level or that the salt-independent activity is in fact salt dependent, but fully stimulated *in situ* (Järnefelt, 1964). No evidence was found indicating that one enzyme was related to the other either as a degradation product or as a subunit, although such a formulation appears attractive. Certain allosteric properties were demonstrable with the salt-dependent enzyme. Both Na^+ and K^+ exhibited cooperative effects and also altered the K_m for ATP; ATP, although not showing cooperative effects, did alter the effective half-maximal concentration for the salts; and measurements of energies of activation suggested changes in entropy of activation and corresponding conformational changes during ATP hydrolysis.

Methods

The microsomal preparation was obtained from rat brains homogenized in nine volumes of 0.25 M sucrose–0.001 M EDTA (pH 7.0) by first centrifuging the homogenate for 12 min at 18,000*g* and next centrifuging the supernatant material for 25 min at 30,000*g* (Robinson, 1965). For studies with the crude microsomal preparation this pellet was then washed with 0.25 M sucrose and finally suspended in 0.25 M sucrose (about 8 ml/g of brain) and was used within 3 hr of preparation.

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[1] Abbreviations used: ATPase, adenosine triphosphatase; DOC, sodium deoxycholate.

TABLE I: Activity of the ATPase Preparations.[a]

	Preparation		
	Microsomal	DOC Treated	NaI Treated
Enzymatic activity (μmoles of P_i/mg of protein per min)			
A. In Mg^{2+} medium	0.44	0.36	
B. In (Mg^{2+} + Na^+ + K^+) medium	0.67	0.91	0.60
C. Difference B − A	0.23	0.55	0.60
Ratio of activities C:A	0.52	1.5	>50
Recovery (per cent)			
Of activity C	(100)	89	42
Of protein	(100)	37	16
Relative specific activity of C	(1)	2.4	2.6

[a] The three preparations (described under Methods) were incubated at 30° in both the Mg^{2+} medium, containing 50 mM Tris-HCl (pH 7.3), 3 mM $MgCl_2$, and 3 mM Tris-ATP, or in the Mg^{2+} + Na^+ + K^+ medium, containing in addition 90 mM NaCl plus 10 mM KCl. The initial velocity of P_i production was measured, and the difference in rate between incubations in the two media is listed as activity C. Recoveries of activity and protein and relative specific activity are based on the corresponding values with the microsomal preparation.

The deoxycholate-treated microsomes (DOC preparation) were obtained by suspending the crude microsomal preparation in 0.25 M sucrose and adding an equal volume of 1.0 M KCl containing 1.5 mg of sodium deoxycholate/ml (Järnefelt, 1964). The suspension was mixed in a TenBroek homogenizer and then centrifuged for 30 min at 30,000*g*. For studies with the DOC preparation this pellet was washed with 20 volumes of 0.25 M sucrose and finally suspended in 0.25 M sucrose (about 6 ml/g of brain) and was used within 3 hr of preparation.

The NaI-treated microsomes (NaI preparation) were obtained by suspending the initial pellet after DOC treatment in a mixture of 2.0 M NaI, 2.5 mM EDTA, 5.0 mM cysteine, and 2.0 mM NaATP (pH 8.0) (Nakao *et al.*, 1965). This suspension was mixed in a TenBroek homogenizer and allowed to stand at 0° for 30 min; it was then diluted with water to 0.8 M NaI and centrifuged for 30 min at 100,000*g*. The resultant pellet was washed twice with 0.01 M Tris-HCl (pH 7.3) and finally suspended in the same medium (about 1.5 ml/g of brain). Aliquots of this preparation were stored at −20° and were used within 1 week.

Two standard incubation media were used. (i) The Mg^{2+} medium contained 0.1 ml of enzyme preparation/ml and 50 mM Tris-HCl (pH 7.3), 3 mM $MgCl_2$, and 3 mM Tris-ATP; and (ii) the (Mg^{2+} + Na^+ + K^+) medium contained in addition 90 mM NaCl plus 10 mM KCl. When the ATP concentration was changed the $MgCl_2$ concentration was changed correspondingly, to maintain an equimolar ratio. Incubation was for 4 min at 30° unless otherwise indicated.

ATPase activity was measured in terms of the production of P_i (Lowry and López, 1946) corrected for zero-time incubations. Protein was measured by the biuret method using bovine serum albumin as a standard.

ATP was purchased from Sigma Chemical Co. as the sodium salt and converted to the Tris salt (Ulrich, 1963).

Results

Purification Procedures. The three ATPase preparations varied in the ratio of activity with Mg^{2+} to activity with Mg^{2+} + Na^+ + K^+ (Table I). Thus the properties of these two activities could be compared at different degrees of relative purity and of membrane modification.

Treatment with NaI caused the major change in relative activity, decreasing activity in the Mg^{2+} medium to less than 2 per cent of that with Mg^{2+} + Na^+ + K^+, and affording a preparation containing essentially only the (Na^+ + K^+)-dependent activity (Table I). However, the specific activity of the (Na^+ + K^+)-dependent activity increased only 2.6-fold through the preparation procedures and only slightly over the DOC treatment alone.

The pellet obtained after NaI treatment consisted of a fluffy layer above a dense layer; the specific activity of the dense layer was greater than that of the fluffy layer (to a relative specific activity of 5.6), but since both were free of (Na^+ + K^+)-independent activity both layers were routinely used in order to increase the yield.

Since the specific activities of the NaI preparations varied, the rates of P_i production were expressed, for kinetic analysis, relative to that at 30° in the standard Na^+ + K^+ medium with 3 mM ATP, which was given a value of 1.0. Such controls were run with each incubation. The NaI preparation required Mg^{2+} and both Na^+ and K^+; it was inactive with Ca^{2+} and was almost totally blocked by 0.1 mM ouabain.

Attempts to isolate the (Na^+ + K^+)-independent

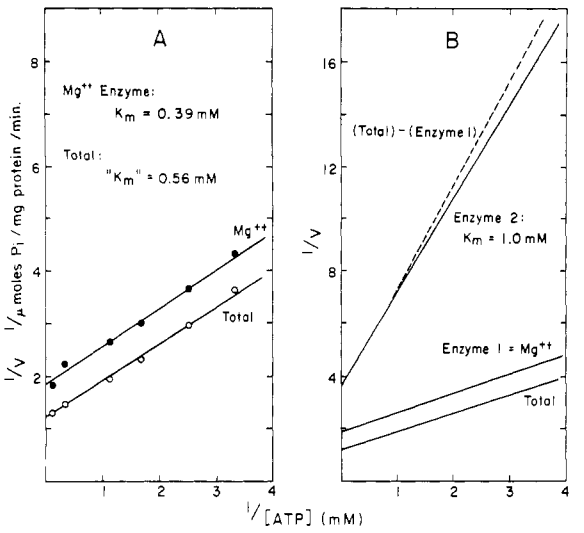

FIGURE 1: ATPase activity of the microsomal preparation. In panel A the initial rate of P_i production is plotted against ATP concentration in the Lineweaver–Burk form. Incubations were at 30° (a) in the Mg^{2+} medium (●——●) containing 0.1 ml of the microsomal preparation/ml, 50 mM Tris-HCl (pH 7.3), and equimolar concentrations of $MgCl_2$ and Tris-ATP; and (b) in the same medium containing in addition 90 mM NaCl plus 10 mM KCl (○——○). Each point is the average of 8 or more determinations, and the lines were drawn by the method of least squares. In panel B the two Lineweaver–Burk lines are redrawn and labeled, respectively, "enzyme 1" and "total activity;" the line representing enzyme 2 was calculated from these, as described in the text, using the expression for "two enzymes acting on one substrate." The upward curving line (- - - -), labeled "(total) − (enzyme 1)" results from subtracting, for each substrate concentration, the velocity of enzyme 1 from the total velocity, and plotting this difference in the Lineweaver–Burk form.

ATPase from brain microsomes were unsuccessful. The procedure described for erythrocyte Mg^{2+}-dependent ATPase (Nakao *et al.*, 1963) produced no material. Procedures for extracting contractile proteins from muscle (Mihalyi and Rowe, 1966) and from liver mitochondria (Vignais *et al.*, 1963) produced only traces of material that did not form contractile fibers nor display superprecipitation with ATP, and were presumably nonspecific remnants of the microsomal material.

Rate and Substrate Concentration. Initial velocities were estimated by selecting brief incubation periods over which P_i production was linear with time. Since preliminary experiments showed that maximal activity was obtained with a Mg^{2+}:ATP concentration ratio of 1, both at low (0.6 mM) and control (3.0 mM) concentrations of ATP, this ratio was maintained in all experiments.

With the microsomal preparation the K_m for P_i production from ATP in the Mg^{2+} medium was 0.39 mM (Figure 1). The Lineweaver–Burk plot of the total rate of P_i production in the $Mg^{2+} + Na^+ + K^+$ medium gave a straight line with an apparent K_m of 0.56 mM. However, on the assumption that P_i production in this medium represented the output of two distinct enzymes (a Mg^{2+}-dependent ATPase and a ($Mg^{2+} + Na^+ + K^+$)-dependent ATPase), this plot of total activity represents really the line described for "two enzymes acting on one substrate" (Dixon and Webb, 1964).

$$v_t = \frac{V_{max_1}}{1 + \frac{K_{m_1}}{S}} + \frac{V_{max_2}}{1 + \frac{K_{m_2}}{S}}$$

where v_t = velocity of total activity; V_{max_1}, V_{max_2} = maximal velocities for enzymes 1 and 2; K_{m_1}, K_{m_2} = Michaelis constants for enzymes 1 and 2; and S = substrate concentration. A plot of $1/v_t$ against $1/S$ gives an hyperbola, but appears to be a straight line, only bending downward at very high S (Dixon and Webb, 1964).

From this equation the V_{max} and K_m for the ($Na^+ + K^+$)-dependent enzyme can be calculated, knowing V_{max} and K_m for the Mg^{2+}-dependent enzyme (Figure 1A) and v_t as a function of S (Figure 1A). The V_{max} was 0.27 μmole of P_i/mg of protein per min with a K_m of 1.0 mM.

Furthermore, the line calculated for the ($Na^+ + K^+$)-dependent enzyme is different from that obtained by merely subtracting, for each substrate concentration, the Mg^{2+}-dependent rate of P_i production from the total rate (Figure 1B). The spurious line, plotted from the subtracted points, while lying close to the calculated line at high substrate concentrations, curves upward on the Lineweaver–Burk plot, falsely suggesting a deviation from Michaelis–Menten kinetics for the ($Na^+ + K^+$)-dependent enzyme.

With the DOC preparation the K_m for the Mg^{2+}-dependent enzyme increased to 0.46 mM, whereas the K_m for the ($Na^+ + K^+$)-dependent enzyme, calculated from the total activity as described above, decreased to 0.68 mM.

With the NaI preparation activity with Mg^{2+} alone was too small to estimate K_m; for the ($Na^+ + K^+$)-dependent enzyme the K_m had further decreased to 0.56 mM. As the concentration of Na^+ and K^+ was decreased (maintaining the constant molar ratio of 9:1) the K_m for ATP decreased along with V_{max} (Figure 2A).

The Lineweaver–Burk plots fitted straight lines well (Figures 1 and 2). As a further indication of agreement with Michaelis–Menten kinetics the data were replotted in terms of the Hill equation (Monod *et al.*, 1963), $\log (V_{max} - v) - 1 = \log K_h - n \log S$, where K_h = Hill constant; for Michaelis–Menten kinetics $K_h = K_m$; and n = slope of the Hill plot, and is 1.0 for Michaelis-Menten kinetics. For all three salt concentrations the Hill plot had a slope of 1 (Figure 2B)

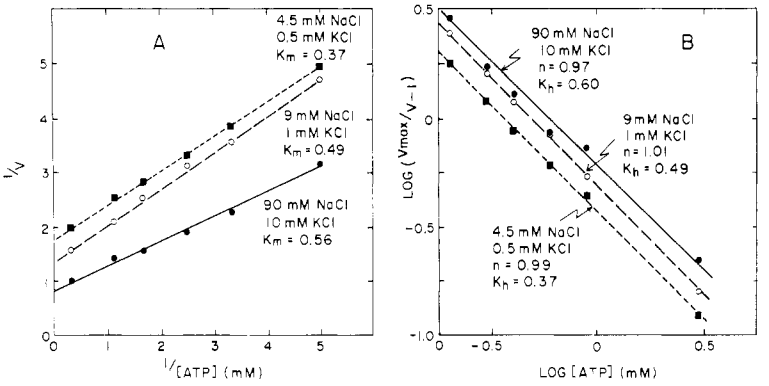

FIGURE 2: Effects of salt and ATP concentrations on the ATPase activity of the NaI preparation. In panel A the rate of P_i production, in arbitrary units, is plotted against the concentration of ATP in the Lineweaver–Burk form. Incubations were at 30° in media containing 0.1 ml of the NaI preparation/ml, 50 mM Tris-HCl (pH 7.3), equimolar concentrations of $MgCl_2$ and Tris-ATP, and NaCl plus KCl at one of the concentrations indicated. In panel B the same data are presented in the form of a Hill plot. Each point represents the average of six or more determinations, and lines for both plots were drawn by the method of least squares.

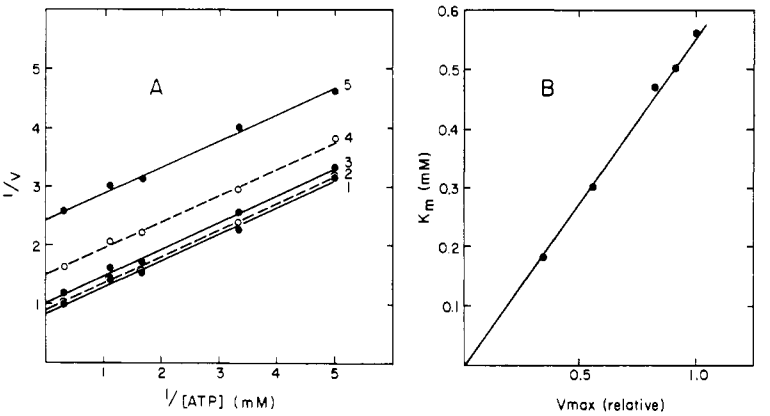

FIGURE 3: Effects of KCl on the substrate–velocity relationships. In panel A the rate of P_i production, in arbitrary units, is plotted against the concentration of ATP in the Lineweaver–Burk form at different concentrations of KCl. Incubations were at 30° in media containing 0.1 ml of the NaI preparation/ml, 50 mM Tris-HCl (pH 7.3), equimolar concentrations of $MgCl_2$ and Tris-ATP, 90 mM NaCl, and varying concentrations of KCl: (1) 10, (2) 4, (3) 2, (4) 1, and (5) 0.5 mM. In panel B the data are replotted in terms of the K_m for ATP at each KCl concentration against the corresponding V_{max}. Each point is the average of five or more determinations.

(Least-squares lines were drawn for both the Lineweaver–Burk and the Hill plots.)

The K_m for ATP, measured at 90 mM NaCl but with varying concentrations of KCl, increased with increasing KCl (Figure 3A). The plot of each K_m against the corresponding V_{max} gave a straight line passing through the origin (Figure 3B).

At 20° the K_m for ATP in the $Na^+ + K^+$ medium was only 0.39 mM with the NaI preparation.

Rate and Salt Concentrations. Only data with the NaI preparation are reported.

In the presence of 90 mM NaCl the rate of P_i production increased as the K^+ concentration was increased from 0.25 to 10 mM. Lineweaver–Burk plots in terms of KCl concentration were not linear but curved upward at low concentrations of KCl (Figure 4A); this was more marked at the higher ATP concentration. Hill plots of these data (Figure 4B) gave straight lines with a slope $n > 1$, further indicating a deviation from Michaelis–Menten kinetics.

From the Hill equation the concentration of reactant at half-maximal velocity, $K_{0.5}$ (Koshland *et al.*, 1966), is given by $K_{0.5} = K_h^{(1/n)}$. The $K_{0.5}$ for KCl was 0.41 mM at the lower concentration of ATP (0.6 mM). but increased to 0.73 mM at the control concentration of ATP (3.0 mM). Correspondingly, n was greater at the higher concentration of ATP.

To assess the significance of the difference in n at the two ATP concentrations n was calculated for each experiment by the method of least squares, and these

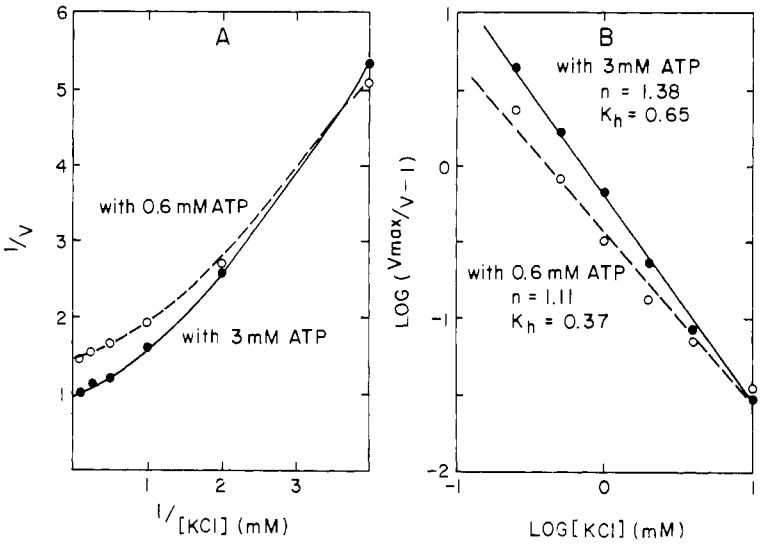

FIGURE 4: The effect of KCl concentration on ATPase activity. In panel A the initial rate of P_i production, in arbitrary units, is plotted against the concentration of KCl in the Lineweaver–Burk form. Incubations were at 30° in media containing 0.1 ml of the NaI preparation/ml, 50 mM Tris-HCl (pH 7.3), 90 mM NaCl, and varying concentrations of KCl with: (a) 3 mM Tris-ATP and 3 mM $MgCl_2$ (●——●) or (b) 0.6 mM Tris-ATP and 0.6 mM $MgCl_2$ (○- - -○). In panel B these data are replotted in the Hill form. Each point is the average of six or more determinations, and the straight lines were drawn by the method of least squares.

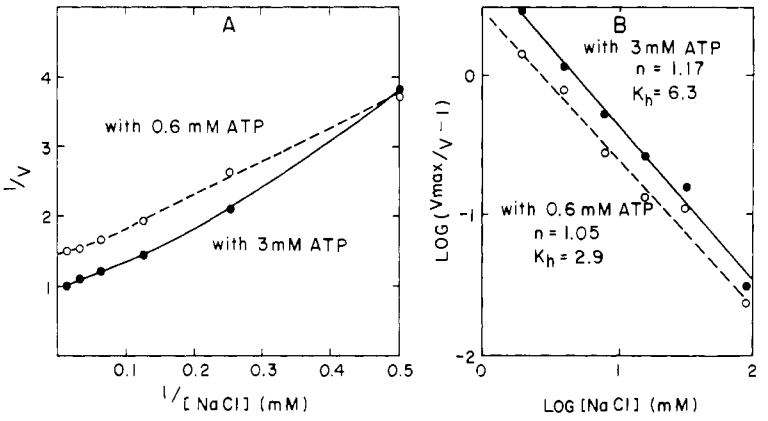

FIGURE 5: The effect of NaCl concentration on ATPase activity. The experiments were performed and data were plotted as in Figure 4 except that the incubation medium contained, in all cases, 10 mM KCl and the NaCl concentration was varied.

values for n at each ATP concentration were then compared using the student Fisher t test. The values of n at the two ATP concentrations were significantly different at the $p < 0.01$ level.

In the presence of 10 mM KCl the rate of P_i production increased as the NaCl concentration was increased from 2 to 90 mM. The Lineweaver–Burk plots again curved upward at low salt concentrations and Hill plots gave straight lines with $n > 1$ (Figure 5). As with KCl, at the standard concentration of ATP both the $K_{0.5}$ for NaCl (4.8 mM) and n, the slope, of the Hill plot (1.17), were greater than that at the lower concentration of ATP ($K_{0.5}$ = 2.8 mM; n = 1.05). The difference for n was significant at the $p < 0.01$ level.

At 20° with 3 mM ATP the values were lower than at 30°. For KCl, n was 1.31 and $K_{0.5}$ was 0.54 mM; for NaCl, n was 1.19 and $K_{0.5}$ was 3.8 mM.

When the concentrations of both NaCl and KCl were varied together, maintaining the molar ratio of 9:1, the Lineweaver–Burk plot had a downward bend at high salt concentrations (Figure 6A), whereas the Hill plot gave a straight line with $n < 1.0$ (Figure 6B). From the Hill plot the $K_{0.5}$ for KCl was 0.67 mM,

slightly lower than that found at a constant 90 mM NaCl concentration, whereas the $K_{0.5}$ for NaCl was 5.8 mM, higher than that found at a constant 10 mM KCl concentration.

At 20° these experiments gave somewhat lower values for both; the $K_{0.5}$ for KCl was 0.50 mM and for NaCl was 5.4 mM; n was 0.77. As at 30° the $K_{0.5}$ for KCl was less than that at a constant high NaCl concentration, whereas the $K_{0.5}$ for NaCl was greater than that at a constant high KCl concentration.

Since varying the Na^+ and K^+ concentrations altered the osmolarity and the ionic strength of the medium, parallel experiments were performed to investigate these variables. For all experiments in which NaCl and KCl were varied corresponding incubations were performed in which sufficient choline chloride was added so that the sum of the concentrations of the three salts was maintained at 100 mM. No discernable difference was observed between experiments without choline chloride and those with choline chloride added to maintain a constant total salt concentration.

Energy of Activation. Preliminary experiments showed that, for a given set of experimental conditions, the Arrhenius plots were linear from 30 to 20°. Thus the energy of activation (E) could be estimated by measuring the rate of P_i production, under those conditions, at 30 and 20°. Thus the energy of activation (E) could be estimated by measuring the rate of P_i production, under those conditions, at 30 and 20°, employing the common assumption that initial velocity is directly proportional to k_r, the specific rate constant, and may be substituted for it in the Arrhenius equation, $\ln k_r = (-E/RT) + C$, where R = gas constant, T = absolute temperature, and C = constant.

With the microsomal preparation, E for P_i production in the Mg^{2+} medium was 8.0 kcal (Table II). For the $(Na^+ + K^+)$-dependent enzyme, E could be estimated by subtracting, for each temperature, the Mg^{2+}-dependent activity from the total activity with $Mg^{2+} + Na^+ + K^+$ (this was valid since at high substrate concentrations the activity of each enzyme alone is essentially additive); such calculation gave E = 19.5 kcal (Table II).

With the DOC preparation, E for the Mg^{2+}-dependent activity increased to 15 kcal, whereas E for the $(Na^+ + K^+)$-dependent enzyme, calculated as above, remained constant at 19.7 kcal (Table II).

With the NaI preparation, E for the Mg^{2+}-dependent activity could not be determined because of negligible activity. E for the $(Na^+ + K^+)$-dependent enzyme remained at 19.9 kcal (Table II).

As the concentration of salts was increased and the rate of P_i production increased, E increased (Table III). This occurred whether each salt was carried separately or both together.

Attempts to Interconvert the Enzymes. Attempts to convert the $(Na^+ + K^+)$-dependent ATPase into a $(Na^+ + K^+)$-independent enzyme were unsuccessful. Methods tried on the NaI preparation included: incubation or preincubation with divalent cations, with sulfhydryl reagents, and with detergents; autolysis; aging at pH 6.0 or 8.0; and sonication in dilute buffer, detergents, and in 10% butanol. No marked increase in the $(Na^+ + K^+)$-independent activity could be shown, although many procedures reduced the $(Na^+ + K^+)$-dependent activity. With the microsomal and DOC preparations some treatments decreased the $(Na^+ + K^+)$-dependent activity more than the independent, but none caused a net increase in $(Na^+ + K^+)$-independent activity.

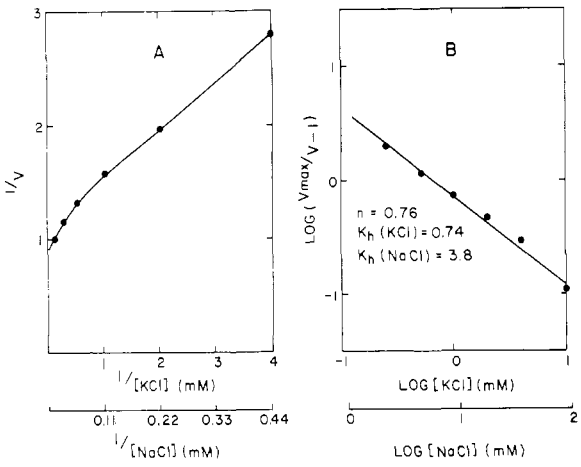

FIGURE 6: The effect of varying both NaCl and KCl on ATPase activity. The experiments were performed and data were plotted as in Figure 4, except that the incubation medium contained, in all cases, 3 mM $MgCl_2$ and 3 mM Tris-ATP and the concentrations of both NaCl and KCl were varied together, maintaining a molar ratio of 9:1.

TABLE II: Energies of Activation ATPase Preparations.[a]

Preparation	Energy of Activation of P_i Production (kcal)	
	$Na^+ + K^+$ Independent	$Na^+ + K^+$ Dependent
Microsomal	8.0	19.5
DOC treated	15.0	19.7
NaI treated		19.9

[a] Energies of activation were determined from measurements of the initial rate of P_i production in both the Mg^{2+} medium and the $Mg^{2+} + Na^+ + K^+$ medium, as described for Table I, both at 30 and 20°. The $(Na^+ + K^+)$-dependent activity refers to the total activity in the $Mg^{2+} + Na^+ + K^+$ medium less that in the Mg^{2+} medium.

Discussion

The results support best the contention that two

TABLE III: Changes in Energy of Activation with Changes in the Salt Content of the Medium.[a]

A. Variation in KCl Concn		B. Variation in NaCl Concn		C. Variation in Both NaCl and KCl Concn		
90 mM NaCl + KCl of (mM)	Energy of Activation (kcal)	10 mM KCl + NaCl (mM)	Energy of Activation (kcal)	NaCl Concn (mM)	KCl Concn (mM)	Energy of Activation (kcal)
0.25	11.8	2	17.6	2.25	0.25	17.6
0.50	14.6	4	18.5	4.5	0.5	18.4
1.0	17.0	8	18.8	9.0	1.0	19.0
2.0	19.6	16	18.2	18	2	19.5
4.0	19.8	32	19.3	36	4	19.6
10	19.9	90	19.9	90	10	19.9

[a] Energies of activation were determined from measurements of the initial rate of P_i production at 20 and 30°. Incubation was in the Mg^{2+} medium, described in Table I, with the following additions: in column A, with 90 mM NaCl plus KCl as indicated; in column B, with 10 mM KCl plus NaCl as indicated; and in column C with NaCl and KCl as indicated.

distinct enzymes are present in the microsomal preparation: a Mg^{2+}-dependent ATPase and a (Mg^{2+} + Na^+ + K^+)-dependent ATPase. It is unlikely that the (Na^+ + K^+)-independent ATPase is really salt dependent but fully activated *in situ* (Järnefelt, 1964) since its K_m and energy of activation differed greatly from the salt-dependent enzyme (Figure 1, Table I). And preparation of a salt-dependent ATPase free of salt-independent activity (as well as differences in K_m and E values) argues against a salt-independent level of activity on which is superimposed Na^+ + K^+ activation. The following discussion will deal with that ATPase having an absolute requirement for both Na^+ and K^+.

Since the kinetics of the salt-dependent ATPase did not follow the classical Michaelis–Menten expressions (Figure 4–6) it seemed of interest to consider these processes in terms of allosteric enzyme effects, as suggested by Squires (1965).

Allosteric effects (Monod *et al.*, 1963, 1965; Koshland *et al.*, 1966; Atkinson, 1966) are concerned with interactions between topographically distinct sites on an enzyme by which effector substances at the allosteric site(s), by inducing conformational changes in the protein, alter the enzymatic properties at the active site. Although subunits with distinct effector and enzymatic sites have been demonstrated for some enzymes (*e.g.*, Gerhart and Schachman, 1965) and direct evidence of conformational changes resulting from association with an allosteric effector has been reported (Dratz and Calvin, 1966), the identification of allosteric properties has usually been through kinetic analyses. Deviation from Michaelis–Menten kinetics, however, need not imply allosteric processes; for example, Gawron *et al.* (1966) in a detailed kinetic study of succinic dehydrogenase explained nonlinear Lineweaver–Burk plots in terms of a multistage reaction sequence. Furthermore, kinetic data alone are generally inadequate for distinguishing between alternative models of subunit interaction (Koshland *et al.*, 1966).

Despite these reservations it may be pertinent to consider this enzyme in terms of allosteric models since the (Na^+ + K^+)-dependent ATPase exhibited both major allosteric processes described by Monod *et al.* (1965): (1) heterotropic effects, and (2) homotropic or cooperative effects.

Heterotropic effects refer to processes in which the allosteric modifier alters the V_{max} and/or the K_m for the substrate (or for another allosteric modifier). The concentration of Na^+ and K^+ altered both the V_{max} and the K_m for ATP (Figures 2 and 3) and thus the salts fulfill the criteria for heterotropic allosteric modifiers.

Homotropic effects refer to processes in which low concentrations of the allosteric modifier increase the efficacy of additional quantities of that same substance. Such cooperative effects result in sigmoidal velocity plots, Lineweaver–Burk plots that curve upward at low substrate concentrations, and Hill plots in which the slope (n) is greater than 1. In the Hill equation, which is a generalized kinetic expression (Atkinson, 1966), n has been interpreted as a measure of both the number of sites for the reactant and the strength of interaction between them (Atkinson *et al.*, 1965).

Both Na^+ and K^+, when each was varied independently (Figures 4 and 5), exhibited cooperative homotropic effects, *i.e.*, the Lineweaver–Burk plots curved upward and in the Hill plots $n > 1$. These results are thus in accord with the report of Squires (1965). As might be expected with conformational changes, the allosteric effects were smaller at lower temperatures (*e.g.*, n decreased toward 1.0).

When both Na^+ and K^+ were varied together at a constant molar ratio (9:1) the Lineweaver–Burk plot showed a downward curve at high concentrations (Figure 6A); such a curve has been attributed to two different binding sites (Datta and Prakash, 1966).

The Hill plot gave a straight line with $n < 1$ (Figure 6B); this has been interpreted as indicating more than one type of binding site (Wyman, 1964). Analysis of both plots fits a model with each ion binding to its own specific sites. However, the $K_{0.5}$ for Na^+ was markedly smaller when both ions were kept at a constant molar ratio, suggesting competition between Na^+ and K^+ for the Na^+ site.

For hemoglobin values of n in the Hill plot have been shown to vary with the salt concentration of the medium (Rossi-Fanelli *et al.*, 1961). However, with the ATPase there was no change in the kinetic values when choline chloride was added to maintain constant salt concentrations, indicating that ionic strength and osmolarity alone were not responsible for the kinetic effects of Na^+ and K^+.

In contrast to a previous report (Squires, 1965), ATP did not show a cooperative homotropic effect, *i.e.*, the Lineweaver–Burk plots were linear and in the Hill plot $n = 1$ (Figures 1 and 2). A linear Lineweaver–Burk plot was also obtained for ATP with a purified salt-dependent ATPase from heart (Matsui and Schwartz, 1966). This discrepancy may be due to an artifact arising when two enzymes are present acting on one substrate: the activity of the second enzyme may not be calculated, for all substrate concentrations, by subtracting the velocity of the first from the total velocity. Such an erroneous calculation inherently produces an upward-bending Lineweaver–Burk plot and a Hill plot with $n > 1$ (Figure 1B). In other reports where such subtractions were made (Wheeler and Whittam, 1962; Chignell and Titus, 1966) Hill plots would show $n > 1$.

ATP, did however, exert a heterotropic effect on the $K_{0.5}$ values for the salts, increasing them as the ATP concentration was increased (Figures 4 and 5). Increased homotropic effects of the salts (increased n) with increased ATP concentration does not fit the Monod–Wyman–Changeux (1965) model in which, as the heterotropic reagent saturates the enzyme, homotropic effects disappear.

When the ATP concentration was varied the Mg^{2+} concentration also was varied to maintain a 1:1 molar ratio, since preliminary experiments, in accord with the report of Wheeler and Whittam (1962), indicated that this was the optimal ratio at high and low ATP concentrations. Furthermore, maintaining this ratio precluded variations in ATP–Mg^{2+} complexing, which could affect reaction rate. The possibility remains that enzyme–metal complexes, rather than nucleotide–metal complexes, was a significant factor (see Kuby and Noltmann, 1962).

Earlier reports (Swanson, 1966; Gruener and Avi-Dor, 1966) have also noted higher values of the energy of activation (E) for the ($Na^+ + K^+$)-dependent ATPase than for the Mg^{2+}-dependent ATPase (Table II). The increase in E with increasing salt concentration (Table III), which has not been described previously, may be of some interest in relation to the proposed allosteric processes.

In simplest form kinetic theory relates the rate constant (k_r) to the energy of activation and the entropy of activation

$$k_r = \frac{\bar{k}T}{h} e^{\Delta S^{\ddagger}/R} e^{RT - E/RT}$$

where $\bar{k}$ = Boltzmann's constant, h = Planck's constant' and $\Delta S^{\ddagger}$ = entropy of activation. Alterations in the system that tend to increase k_r, and thus the velocity of the enzymatic process, must increase $\Delta S^{\ddagger}$ and/or decrease E.

For the ATPase, increasing salt concentration, which increased reaction rate, was associated with an increase in E (Table III). Hence, if E increased while the reaction rate increased (from the addition of salts to the medium) then $\Delta S^{\ddagger}$ must have increased to override the change in E.

Although ignorance of the concentration of the enzyme and of the detailed reaction sequence prohibits the use of a less superficial application of kinetic theory, some heuristic implications may be derived from this approach. The entropy term may be interpreted as a measure of the change in the order of the system during catalysis, as reflected by changes in solvation and in inter- and intramolecular relationships. Hence these estimates of elevated values of $\Delta S^{\ddagger}$ for the ATPase suggest conformational changes, dependent on salt concentration, occurring in the enzyme–reactant complex during ATP hydrolysis, and support the preceding observations on allosteric effects of the salts on the enzyme.

Reaction mechanisms for ATP hydrolysis propose a Na^+-dependent phosphorylation of the enzyme with a K^+-specific liberation of P_i (Fahn *et al.*, 1966). With this proposal, changes in K^+ concentration should specifically vary the velocity of the terminal step in P_i production. Under circumstances in which k_{+2} may be varied independently of k_{+1} and k_{-1} it has been proposed that a plot of K_m against V_{max} should give, at $V_{max} = 0$, K_s, the enzyme–substrate dissociation constant (Slater and Bonner, 1952). Such a plot (Figure 3B) gave instead a line passing through the origin, which has been interpreted (Dixon and Webb, 1964) as indicating either that $k_{-1} = 0$ (which seems unlikely in this case because of evidence for ADP–ATP exchange (Fahn *et al.*, 1966)) or that an intermediate product is previously liberated.

It has been proposed that all enzymes displaying allosteric effects contain subunits (Monod *et al.*, 1965), and a bacterial membrane ATPase was shown (Abrams and Baron, 1967) to contain catalytic subunits and noncatalytic, possibly regulatory, subunits, on the pattern of aspartate transcarbamylase (Gerhart and Schachman, 1965). From these findings it is tempting to consider the membrane salt-dependent ATPase as being composed of heterogeneous subunits: catalytic subunits interacting with ATP, and regulatory subunits interacting with Na^+ and K^+ (and possibly acting as carriers for these ions). Then the salt-independent ATPase might represent, at least in part, catalytic

subunits dissociated from the regulatory subunits during preparation. With ATPase preparations from erythrocytes partial "uncoupling" of the salt-dependent ATPase was accomplished by sonic irradiation (Askari and Fratantoni, 1963) and detergents (Chan, 1967) so that ATPase activity could be stimulated by either Na^+ or K^+ alone. However, no support for this model could be obtained with the brain preparation; possibly with the NaI preparation dissociated subunits were unstable.

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