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THE $(Na^+ + K^+)$-DEPENDENT ATPase IN THE ISOLATED MUCOSAL CELLS OF TURTLE BLADDER

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SUMMARY

1. In the microsomal fraction of isolated mucosal cells of turtle bladders, addition of Na^+ and K^+ to Mg^{2+}-containing mixtures increased ATPase activity by 100–150%; and the $(Na^+ + K^+)$-dependent activity was completely inhibited by ouabain.

2. Apparent K_m values, with respect to ATP were: 0.22 mM for the $(Na^+ + K^+)$-dependent, and 0.18 mM for the Mg^{2+}-dependent activity. The apparent K_m for Na^+ was 4.5 mM; and that for K^+, 0.36 and 2.9 mM.

3. No effects on activity were found after addition of amiloride, furosemide, and acetazolamide. In contrast, 0.1 mM *N*-ethylmaleimide inhibited the $(Na^+ + K^+)$-dependent activity by 45% without affecting the Mg^{2+}-dependent activity.

4. For Mg^{2+}-dependent ATPase, the order of nucleotide preference was: ATP > GTP > ITP > CTP > UTP > ADP; and for $(Na^+ + K^+)$-dependent ATPase, the order was: ATP > ITP > CTP > GTP, while the activity with UTP or ADP was negligible.

5. The pH optimum of the activity with $Na^+ + K^+ + Mg^{2+}$ was 7.3–7.5; while that with Mg^{2+} alone was a weak function of the pH.

6. Activity of ATPase increased as a function of temperature (15–45°). The calculated activation energy was 13.0 kcal/mole for the Mg^{2+}-dependent and 27.5 kcal/mole for the $(Na^+ + K^+)$-dependent activity.

INTRODUCTION

The isolated bladder of the fresh water turtle, *Pseudemys scripta*, possesses mechanisms for the active transport of Na^+, Cl^- and HCO_3^- under conditions of open-circuit[1] and under conditions of short-circuiting[2,3]. Recently reported work has demonstrated that ouabain added to the serosal bathing fluid (final concn. 0.1 mM) of the short-circuited bladder suppressed the net transport of Na^+ completely and irreversibly without affecting that of Cl^- or HCO_3^- (ref. 4). In crude homogenates and in

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microsomal fractions of the epithelial cells, there was demonstrated a $(Na^+ + K^+)$-stimulatable, ouabain-inhibitable ATPase activity[4].

The purposes of the present paper were to investigate further the distribution of the $(Na^+ + K^+)$-dependent ATPase activity in various fractions of the mucosal cells; to determine the effects of pH and temperature; to evaluate the kinetic parameters such as K_m and v_{max} of the ATPase activity with respect to ATP and with respect to Na^+ and/or K^+ as the substrate; to determine the effect of various inhibitors of Na^+ transport; and to determine the substrate specificity for the nucleotides — ATP, ITP, CTP, GTP, UTP, and ADP.

METHODS

Isolation of mucosal cells

The necks of the ten bladders, in the form of closed sacs, were tied to the outlet of a Luer lock syringe, filled with and immersed in Ca^{2+}-free Ringer solution[4] containing 17 mM HCO_3^-, and 2 mM EDTA, and incubated for 30–40 min at 25°. After incubation, walls of the sacs were rubbed gently against one another for 2–3 min, as recommended by LIPMAN *et al.*[5] (a maneuver which released the epithelial cells into the mucosal fluid). The cell-containing mucosal fluid was removed from the sacs and subjected to a series of homogenizations and centrifugations.

Isolation of microsomal fractions

During the procedure for obtaining the various centrifugal fractions from isolated mucosal cells, low temperatures (0–2°) were maintained throughout. Isolated cells, obtained by the EDTA treatment of ten bladders and suspended in a total volume of about 250 ml of Ringer solution, were carried through the previously described[4] procedures of homogenization and differential ultracentrifugation in order to obtain the microsomal fractions.

A microsomal pellet obtained in the last centrifugation step (65000 × *g* for 60 min) was suspended in 15–20 ml of 1 mM EDTA, stored at −10°, and used as an enzyme source for all the experiments reported here. In some cases, the pellets obtained at 10000 and 20000 × *g*, as well as all of the supernatants, were examined for ATPase activity. Protein concentration (of the enzyme suspension used in the incubation procedure), determined by the method of LOWRY *et al.*[6], was used as the normalizing parameter for enzyme activity.

Assay of ATPase activity

In the assay for total ATPase activity (that in the presence of $Na^+ + K^+ + Mg^{2+}$), the composition of reaction mixture, expressed in terms of final millimolar concentration in the incubation flask was: ATP (Tris or disodium salt), 2.0; $MgCl_2$, 3.0; EDTA, 0.2; imidazole, 40; histidine, 40; NaCl, 84; and KCl, 15. To this was added the enzyme-containing aliquot of 0.5–1.0 ml of the raw cell homogenate or of any one of the centrifugal pellets containing 50–100 μg protein. The final pH was 7.3; and the final volume in the flask was 5 ml.

In the assay for Mg^{2+}-dependent ATPase activity the composition of the incubation mixture was the same as that for $(Na^+$-K^+-$Mg^{2+})$-dependent activity (total ATPase) except that choline chloride (100 mM) was substituted for NaCl and KCl

In many cases the Mg^{2+}-dependent ATPase activity was estimated from that measured in the presence of Na^+, K^+, Mg^{2+} and ouabain (0.1 mM).

After 5 min of preincubation, the reaction was started by addition of ATP, allowed to proceed for 10 min at 38°, and stopped by addition of 5.0 ml of cold $HClO_4$, 6%. Control tubes, carried through all incubations, were of two types (those without ATP and those without the enzyme). Aliquots of the final mixture were analyzed for P_i by the method of BERENBLUM AND CHAIN[7].

According to the current convention[8,9], the ATPase activities were defined operationally as: (a) that which catalyzes the hydrolysis of ATP in the presence of Mg^{2+} (Mg^{2+}-dependent ATPase) and, (b) that which catalyzes the hydrolysis of ATP in the presence of Na^+, K^+, and Mg^{2+} (total ATPase). Total ATPase *minus* Mg^{2+}-dependent ATPase was called $(Na^+ + K^+)$-dependent ATPase.

The initial reaction velocity was determined, as previously described[4], from the P_i released during the first 10 min of incubation. The initial concentrations of ATP varied from 0 to 2.0 mM; and no more than 20% of the ATP was degraded during the course of the incubation.

Sources of material

Ouabain (G-strophanthidin), Tris·ATP, disodium ATP, disodium ITP, disodium GTP, disodium CTP, disodium UDP, disodium UTP, *N*-ethylmaleimide, L-histidine, imidazole grade I, Tris, and EDTA were obtained from Sigma Chemical Co., St. Louis, Mo. Furosemide was obtained from Hoechst Pharmaceutical Co., Cincinnati, Ohio, and amiloride was obtained from Dr. J. E. Baer at Merck, Sharpe and Dohme, West Point, P.

RESULTS

Fractional distribution and stability

Distribution. Table I presents data on the distribution and partial purification of $(Na^+$-K^+-$Mg^{2+})$-dependent ATPase activity in a representative experiment on a pool of epithelial cells from ten turtle bladders. In this instance, the enzyme activities and protein contents were determined for each and every pellet and supernatant. No enzymatic activity was detectable in any of the supernatant fractions.

The three columns on the right side of Table I present values for specific activity. In the crude homogenates of mucosal cells, the $(Na^+$-K^+-$Mg^{2+})$-dependent or total ATPase activity (A) and the ouabain-sensitive moiety of this activity (A − B) were 8.7 and 2.5 μmoles per mg protein per h, respectively. The activity increased progressively during the centrifugal separation of the different cell fractions. For example, the $(Na^+ + K^+)$-dependent moiety of the activity (A − B) increased from 2.5 in the crude homogenate to 64.5 μmoles P_i per mg protein per h in the microsomal fraction (65000 × *g*·1 h) − a 26-fold increase; while the Mg^{2+}-dependent moiety of activity increased from 6.2 in the homogenate to 42.5 in the microsomal fraction − a 7-fold increase. Similar increases in specific activity were found in the centrifugal fractions derived from ten other pools of epithelial cells.

The four columns on the left of Table I present values for volume; for activity of $(Mg^{2+}$-Na^+-$K^+)$-dependent ATPase; and for amount of protein in each of the designated fractions.

TABLE I

DISTRIBUTION AND RECOVERY OF ATPase ACTIVITY AND OF TOTAL PROTEIN IN THE DESIGNATED CENTRIFUGAL FRACTIONS ORIGINATED FROM A POOL OF ISOLATED MUCOSAL CELLS FROM TEN TURTLE BLADDERS

Fractions were obtained by technique described in METHODS sections. Exact composition of each incubation mixture and definition of Mg^{2+}- and $(Na^+ + K^+)$-dependent ATPase can be found under *Assay of ATPase activity* of METHODS section. The unit of activity is defined as the amount of P_i in μmoles released into the incubation fluid per h.

Fraction	*Volume* (*ml*)	*Activity* $Mg^{2+} + K^+ + Na^+$		*Protein concn.* (*mg/ml*)	*Specific activity* (*units/mg*)		
		Units/ml	*Total units*		*A* $Mg^{2+} + Na^+ + K^+$ (*no ouabain*)	*B* $Mg^{2+} + Na^+ + K^+$ + *ouabain*	*A* − *B* $Na^+ + K^+$
Mucosal cells homogenate	90	16.2	1457	1.87	8.7	6.2	2.5
10000 × *g* pellet	20	19.2	389	0.98	19.6	16.2	3.4
20000 × *g* pellet	21	17.0	358	0.50	34.1	19.8	14.3
65000 × *g* pellet	21	53.7	1128	0.50	107	42.5	64.5

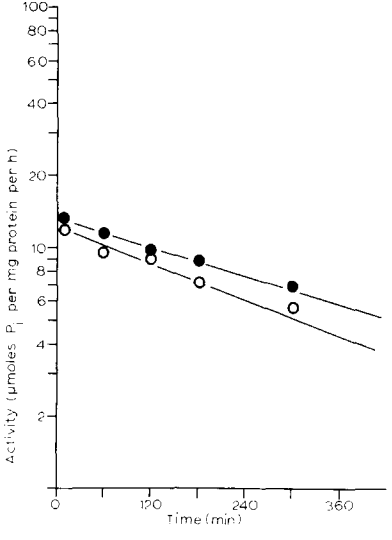


Fig. 1. Semi-log plot of Mg^{2+}-dependent ATPase activity (○—○), and $(Na^+ + K^+)$-dependent ATPase activity (●—●) *vs.* time. $(Na^+ + K^+)$-dependent activity is defined as that measured in the presence of Mg^{2+}, Na^+ and K^+ less that in the presence of Mg^{2+}, Na^+, K^+ and ouabain.

Of the total protein in the cell homogenate, 85 % was recovered from all of the supernatants and pellet fractions. The microsomal pellet (65000 × *g*) contained 6 % of the original total protein.

Whereas the total protein in all of the pellets and supernatants amounted to 85 % of that in the original homogenate, the total (Na^+-K^+-Mg^{2+})-dependent activity amounted to 128 %, and the Mg^{2+}-dependent activity (derivable from values under Column B) to 93 % of its activity in the original homogenate. From these data, it was estimated that the recovery of $(Na^+ + K^+)$-dependent ATPase activity was 213 % of what it was in the original homogenate.

The 213 % yield of $(Na^+ + K^+)$-dependent ATPase recovered in all centrifugal fractions was interpreted as the consequence of an inhibition of this activity in the original homogenate. Such an inhibition could have been due to the existence of naturally occurring inhibitors such as histone-like substances released from the nuclei during homogenization of the cells[10]; or to certain unspecified effects of high concentration of other proteins in the cell homogenate.

Stability. Having achieved the partial isolation and partial purification of a comparatively high specific activity of ATPase in the microsomal fraction, the next step was to determine the stability of the activity during storage at −10°, and during the course of incubation at 38°.

Fig. 1 is a semi-log plot of values of activity of the Mg^{2+}-dependent and of the $(Na^+ + K^+)$-dependent enzyme as a function of time of incubation at 38°. The linear pattern of the plot indicates that both decay rates were exponential functions, from which the half-life of the enzyme activities could be calculated. The half-times of decay of both enzyme activities ($(Na^+ + K^+)$-dependent and Mg^{2+}-dependent) were 300 and 240 min, respectively. This means that less than 3 % of the enzyme activity would be lost during a 10–15-min interval at 38° (which was the maximal time of incubation in any or all of the present experiments).

Not shown in the figure are data obtained on the corresponding activities after storage at −10°. Every 4–5 days, aliquots of the microsomal pellet, stored at −10°, were brought to 38° and tested (during a 10–15-min period) for the Mg^{2+}-dependent and for the $(Na^+ + K^+)$-dependent ATPase activity. Both activities yielded logarithmic patterns which were qualitatively, but not quantitatively similar to those in Fig. 1. The estimated half-life of either activity at −10° was 35–36 days.

Enzymatic properties

In the microsomal pellets isolated from the mucosal epithelial cells of the turtle bladder, the ATPase activity has an absolute requirement for Mg^{2+} as well as a cation-sensitivity with respect to Na^+ and K^+ together[4].

The pattern of microsomal ATPase activity *versus* simultaneous concentrations of Na^+ and K^+ in the incubation mixture was similar to that first reported by SKOU[11] in crab nerve microsomes. The maximal activity in this microsomal preparation was attained in the presence of Na^+ (60–90 mM) together with K^+ (10–20 mM).

Cationic concentrations (mM) for measuring (Mg^{2+}-Na^+-K^+)-dependent activity in the present work was: Mg^{2+}, 3; Na^+, 85; K^+, 15; while the pH of the incubation mixture was fixed at 7.3.

Accordingly, a series of experiments were performed on microsomal pellets in

TABLE II

MEAN VALUES FOR THE ATPase ACTIVITY OF MICROSOMAL PELLETS INCUBATED UNDER THE FIVE CATIONIC CONDITIONS DESIGNATED

In all cases, the composition of the reaction mixture, expressed in terms of final millimolar concentration in the 5 ml of incubation fluid was: Tris·ATP, 2.0; $MgCl_2$, 3.0; EDTA, 0.2; imidazole, 40; histidine, 40; and final pH 7.3. The cations were added to each reaction mixture as designated at the top of each column, and the final millimolar concentrations of the salts were: NaCl, 85; and KCl, 15; while that of ouabain was 0.1 mM. The conditions and concentrations specified for the last two columns on the right were employed throughout the remainder of the present work, except where otherwise specified.

Statistical parameters	*Specific activity (μmoles P_i per mg protein/h)*				
	Mg^{2+}	$Mg^{2+} + Na^+$	$Mg^{2+} + K^+$	$Mg^{2+} + K^+ + Na^+$	$Mg^{2+} + K^+ + Na^+$ + *ouabain*
Mean ± S.E.	32.0 ± 2.16	31.9 ± 1.20	31.7 ± 1.87	73.2 ± 6.49	32.2 ± 1.96
P (Mg^{2+})	—	$P > 0.8$	$P > 0.8$	$P < 0.001$	$P > 0.8$
n	9	5	5	9	9

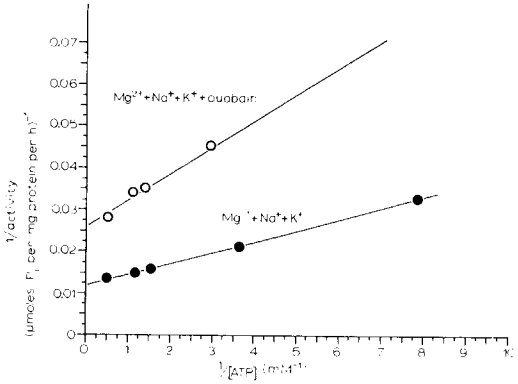


Fig. 2. Lineweaver–Burk plot of ($Na^+ + K^+$)-dependent ATPase activity (●—●), and Mg^{2+}-dependent ATPase activity (○—○) *vs.* molar concentration of ATP. Concentration of non-ATP constituents were the same as those described for Fig. 1 in METHODS.

the presence of: Mg^{2+}; $Mg^{2+} + Na^+$; $Mg^{2+} + K^+$; $Mg^{2+} + Na^+ + K^+$; and $Mg^{2+} + Na^+ + K^+ +$ ouabain.

Table II presents mean values for ATPase activity under the aforementioned conditions. As expected, the mean ATPase activity in the presence of $Mg^{2+} + Na^+ + K^+$ (73.2 μmoles per mg per h) was greater than that in the presence of Mg^{2+}; $Mg^{2+} + Na^+$; or $Mg^{2+} + K^+$ ($P < 0.001$). Moreover the baseline (Mg^{2+}-dependent) rate was not perceptibly altered after addition of either Na^+ or K^+ alone ($P > 0.8$ in both cases).

The table also shows that the activity of a ouabain-treated microsomal preparation in the presence of $Na^+ + K^+ + Mg^{2+}$ was the same as that of the preparation in the presence of Mg^{2+} alone ($P > 0.8$). This suggested that the ouabain-treated preparation in the presence of all three cations could be taken as equivalent to the preparation in the presence of Mg^{2+} alone.

NaI treatment. Exposure of microsomal pellets to NaI as recommended by NAKAO *et al.*[12] and others[13] presumably reduces the Mg^{2+}-dependent, but not the $(Na^+ + K^+)$-dependent ATPase activity. In two experiments on turtle bladder microsomes, NaI decreased the activity of both the Mg^{2+}-dependent and $(Na^+ + K^+)$-dependent ATPase by 10–20 % (an effect much less than that found in other tissues[12,13]).

K_m values. The kinetic parameters for $(Na^+ + K^+)$-dependent and for Mg^{2+}-dependent ATPase activity were determined with respect to: ATP concentration as substrate; Na^+ concentration as substrate; and K^+ concentration as substrate. In all of the kinetic calculations, the Mg^{2+}-dependent moiety of ATPase activity was measured as the residual ATPase activity remaining after addition of ouabain (0.1 mM) to a microsomal pellet incubated in the presence of Na^+, K^+ and Mg^{2+}.

Fig. 2 is a plot of values of reciprocal ATPase activity *versus* reciprocal ATP concentration for $(Na^+ + K^+)$-dependent and for Mg^{2+}-dependent activities in a representative experiment on the microsomal fraction isolated from a pool of ten turtle bladders. Estimating graphically from the Lineweaver–Burk plot of Fig. 2, the K_m for $(Na^+ + K^+)$-dependent ATPase was 0.23 mM, while that for Mg^{2+}-dependent ATPase was 0.24 mM.

Figs. 3 and 4 show Lineweaver–Burk plots derived from data on the ouabain-sensitive moiety of ATPase activity (*i.e.*, $(Na^+ + K^+)$-stimulated ATPase). The form of the plots is similar to that of Fig. 2, except that the reciprocal of the ATPase activity was plotted as a function of the reciprocal of the Na^+ concentration in the presence of constant levels of ATP, K^+ and Mg^{2+} (Fig. 3); and as a function of the reciprocal of the K^+ concentration in the presence of constant levels of ATP, Na^+ and Mg^{2+} (Fig. 4).

Whereas the apparent K_m with respect to Na^+ was clearly 4.0 mM (see Fig. 3), the K_m with respect to K^+ appeared to have two values, 0.20 and 2.0 mM (see Fig. 4). This double valued K_m was derived graphically by making two straight lines approximate the curvilinear pattern of reciprocal ATPase activity *versus* reciprocal K^+ concentration in Fig. 4. Interestingly, the single valued K_m's (with respect to K^+) reported for other tissues[9,11,13] as well as for the turtle bladder[14] ranged from 1.0 to 3.0 mM (a range which fell between the two limits of the double valued K_m shown in Fig. 4).

Over and above the single experiments shown in Figs. 2, 3 and 4, the K_m values

were determined in three experiments performed under each one of the three conditions; *i.e.*, with ATP as substrate, with Na^+ as substrate, and with K^+ as substrate. The mean values for K_m were as follows: 0.22 mM (ATP); 4.5 mM (Na^+); and 0.36 and 2.9 mM (K^+).

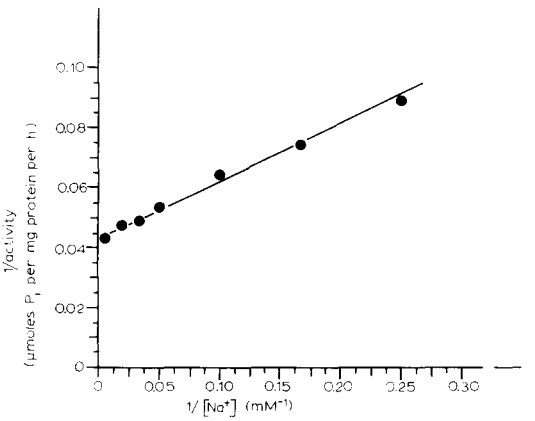


Fig. 3. Lineweaver–Burk plot of ($Na^+ + K^+$)-dependent ATPase activity *vs.* molar concentration of Na^+. Concentration of all non-Na^+ constituents as listed in METHODS.

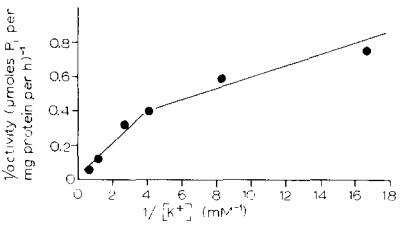


Fig. 4. Lineweaver–Burk plot of ($Na^+ + K^+$)-dependent ATPase activity *vs.* molar concentration of K^+. Concentration of all non-K^+ constituents as listed in METHODS.

Inhibitors. The ouabain-induced inhibition of Na^+ transport and of ($Na^+ + K^+$)-dependent ATPase raised the question of the effect of other inhibitors of Na^+ transport on the ($Na^+ + K^+$)-dependent ATPase—a question which provided the basis for the next set of experiments.

As indicated previously, ouabain (0.1 mM) inhibited all of the ($Na^+ + K^+$)-dependent moiety but none of the Mg^{2+}-dependent moiety of the microsomal ATPase. As a matter of fact, ouabain at a final concentration of 0.3 μM resulted in the inhibition of 50 % of the ($Na^+ + K^+$)-dependent ATPase activity. This degree of sensitivity to ouabain is greater (by two orders of magnitude) than that found in microsomes from other tissues[18–20].

Amiloride (0.1 mM), furosemide (0.1 mM) and acetazolamide (0.1 mM) had no inhibitory effects on ($Na^+ + K^+$)-dependent or on Mg^{2+}-dependent ATPase.

The lack of inhibition by amiloride is in accord with results of BAER *et al.*[16] on ATPase activity. However, amiloride has been shown to block Na^+ transport in the toad bladders[17] and turtle bladders (D. E. GENTILE AND W. A. BRODSKY, unpublished data).

The lack of inhibition of microsomal ATPase activity by furosemide (0.1 mM) is consistent with parallel findings in our laboratory, on the lack of effect of furosemide on Na^+ transport and short-circuiting current across the intact turtle bladders.

The sulfhydryl blocker, *N*-ethylmaleimide (0.1 mM) reduced the short-circuiting current of two turtle bladders to zero 30 min after its addition to the bathing media. However, *N*-ethylmaleimide (0.1 mM) inhibited the ($Na^+ + K^+$)-dependent moiety of microsomal ATPase by 45 % (without affecting the Mg^{2+}-dependent moiety) after it had been incubated with the microsomal pellet for 30 min.

Substrate specificity. In order to determine the specificity of the substrate required for the nucleotidase activity in the microsomal pellets, comparative experiments on the rate of hydrolysis were performed in the presence of equimolar concentrations of ITP, CTP, GTP, ADP or UTP, instead of ATP. Ionic conditions in all reaction vessels were the same as those described above (*i.e.*, $Na^+ + K^+ + Mg^{2+}$ in one reaction vessel, and $Na^+ + K^+ + Mg^{2+}$ + ouabain in the paired vessel containing another aliquot of the same microsomal pellet).

Table III presents values of specific enzymatic activity of a selected microsomal pellet with respect to its ability to catalyze the rate of P_i release from six different nucleotides.

TABLE III

NUCLEOTIDE PREFERENCE WITH RESPECT TO HYDROLYTIC ACTIVITY OF THE MICROSOMAL PELLET

All hydrolytic activities were compared to that obtained in the presence of Tris·ATP, by substitution of equimolar amounts of the Na^+ salts of the five other nucleotides. Conditions were otherwise identical to those described under METHODS and for previous tables. Substrate concn., 2.0 mM.

Substrate	*Specific activity* (*μmoles P_i per mg protein/h*)			*Activity ratio*
	A $Mg^{2+} + Na^+ + K^+ + ouabain$	*B* $Mg^{2+} + Na^+ + K^+$	*B − A*	*(B − A)/A*
ATP	27.5	49.1	21.6	0.79
ITP	16.3	29.3	13.0	0.80
CTP	9.6	16.5	6.9	0.72
GTP	25.0	27.5	2.5	0.10
ADP	4.5	4.8	0.3	0.07
UTP	8.0	8.1	0.1	—

Considering the Mg^{2+}-dependent hydrolytic activity with respect to ATP as 100%, the order of nucleotide preference was as follows: GTP, 91%; ITP, 60%; CTP, 35%; UTP, 29%; ADP, 16%.

Considering the $(Na^+ + K^+)$-dependent activity (see column designated B − A), with respect to ATP as 100%, the order of nucleotide preference was: ITP, 60%; CTP, 32%; GTP, 10%; ADP, 1.4%; UTP, 0%.

The degree of stimulation of hydrolysis by addition of $Na^+ + K^+$ (activity ratio) was about 0.80 regardless of which nucleotide was used.

These data on absolute levels of activity and on degree of stimulation by $Na^+ + K^+$ were similar to those of SKOU[21] on the comparative hydrolytic activities of ITP and ATP in crab nerve, but differed from those of others working with beef kidney microsomes[22], calf heart microsomes[13], and bovine brain microsomes[23].

pH dependency. Fig. 5 is a plot of values of $(Na^+ + K^+)$-dependent and of Mg^{2+}-dependent ATPase activity *versus* those of pH of the incubation mixture in a series of experiments on a single pool of microsomal pellets. Each point on the graph was taken from the average of at least three separate experiments on enzymatic activity.

In a separate set of experiments, increases in osmolality or ionic strength of 5–10% (such as occasioned by addition of HCl or of NaOH) were found to have no effect on ATPase activity.

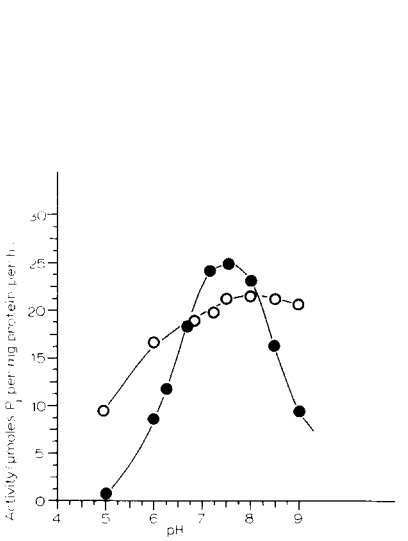
The $(Na^+ + K^+)$-dependent activity reached a maximal value at pH levels of 7.3–7.5; and the Mg^{2+}-dependent activity increased monotonically as a function of pH. The figure also shows that the activity ratio, $(Na^+ + K^+)/Mg^{2+}$, varied as a function of pH; and was 1.1 at pH 7.3, the pH used routinely in the present studies.

Temperature effects. The Mg^{2+}-dependent and $(Na^+ + K^+)$-dependent ATPase activities were measured as functions of temperature between 15 and 45°. Under the conditions of the present experiments, the initial reaction velocity (rate of P_i release) was assumed to be directly proportional to the specific rate constant, K_r, and may be substituted for it, as is commonly done in order to estimate the activation energy from the Arrhenius equation,

$$\ln K_r = -E/RT + C$$

where E is the activation energy; R, the gas constant; T, the absolute temperature; and C, the constant of integration.

Fig. 6, a plot of values of log (rate of P_i release) *versus* those of reciprocal absolute temperature, shows the two functions ($(Na^+ + K^+)$-dependent activity and Mg^{2+}-dependent activity). From the slope of the Arrhenius plot, $-E/2.3\,R$, one can estimate the activation energy for each of the two reactions.



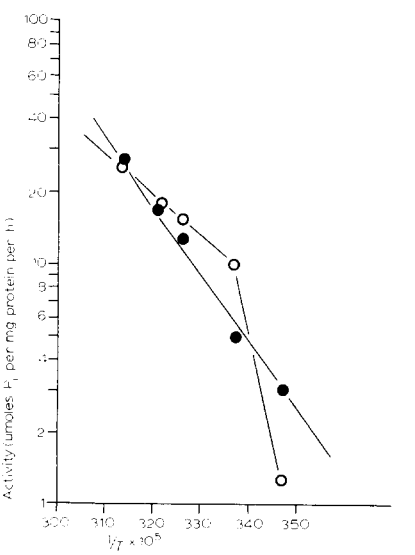


Fig. 5. ATPase activity *vs.* pH. $(Na^+ + K^+)$-dependent activity calculated as described for Fig. 2. The desired pH was obtained by addition of either HCl or NaOH to the reaction mixture, the composition of which has been described under Fig. 1 and in METHODS. ●—●, $(Na^+ + K^+)$-dependent ATPase; ○—○, Mg^{2+}-dependent ATPase.

Fig. 6. Semi-logarithmic plot of $(Na^+ + K^+)$-dependent (●—●) and of Mg^{2+}-dependent (○—○) ATPase activity *vs.* reciprocal absolute temperature.

The activation energy for the $(Na^+ + K^+)$-dependent reaction was 27.5 kcal/mole over the entire temperature range studied; while that for the Mg^{2+}-dependent reaction was 13.2 kcal/mole over the range 23–45°, and 56.2 kcal/mole over the range 15–23°. The Q_{10} values estimated between 30 and 40° were: 2.2 for the $(Na^+ + K^+)$-dependent

function; and 1.8 for the Mg^{2+}-dependent function. These values were similar in magnitude to those reported by BONTING AND CARRAVAGIO[15] for ATPase from several different tissues.

DISCUSSION

Coupling between ATPase and transport

Ouabain abolishes the net transport of Na^+ (ref. 4) and reduces the rate of O_2 consumption in the intact turtle bladder; and inhibits the $(Na^+ + K^+)$-dependent ATPase activity in the isolated microsomal pellet of this tissue. In other tissues, the binding of ouabain to microsomal protein is increased by Na^+ and decreased by K^+ (refs. 24 and 25). This suggests that the free energy of the $(Na^+ + K^+)$-dependent ATP hydrolysis is coupled to the Na^+ transport mechanism. Thus, ouabain may inhibit the reaction providing free energy for transport; or the mechanism coupling ATP hydrolysis to transport; or the pump-carrier operation in the membrane.

Estimates of the stoichiometry between Na^+ transport and ATP hydrolysis[15,26], or between Na^+ transport and O_2 consumption[26] have been made without exact knowledge of the mechanism which couples Na^+ transport to the ATPase activity. Nevertheless, the Na^+ dependency of transport in the intact system is similar to the Na^+ dependency of the microsomal ATPase in red cells[27].

Despite the limitations of such kinetic similarities, we estimated the ratio of Na^+ transported to ATP hydrolyzed, assuming that the estimated cell concentration of ATP, 0.25–0.30 mM (ref. 28) is near that corresponding to the K_m of the $(Na^+ + K^+)$-dependent ATPase reaction.

The mean value for net transport of Na^+ is 7.5 μequiv per mg of microsomal protein per h (ref. 3); and present data show that the $(Na^+ + K^+)$-dependent ATPase-catalyzed rate of release of P_i is 10–20 μmoles per mg of microsomal protein per h at the assumed ATP concentration of 0.25 mM. Therefore, 3.5–7.0 equivalents of Na^+ are transported for each mole of P_i released (a range similar to that found (0.7–15) in a wide variety of Na^+ transporting tissues[26]).

By an analogous procedure, it was estimated that 15 equivalents of Na^+ are transported for each mole of the Na^+-dependent consumption of O_2 (a value slightly less than Na^+/O_2 ratio of 16–20 estimated for frog skin[29] and toad bladder[30]).

Na^+-dependent O_2 consumption is defined as the measured decrement in O_2 consumption after replacing the ambient Na^+ with choline or after adding ouabain (0.1 mM) to the incubation mixture of whole respiring bladder wall or mucosal layer (M. E. LEFEVRE AND W. A. BRODSKY, unpublished data). The estimated ratio of the $(Na^+ + K^+)$-dependent P_i release to Na^+-dependent O_2 consumed was 2.0 to 4.3.

Even if ATP were the sole source of transport energy, the stoichiometry cannot provide data showing the nature of coupling between a spatially oriented chemical reaction in the membrane phase and a directionally oriented transport process.

Properties of bladder ATPase

The presently reported values of the pH optimum, of the Q_{10}, and of the K_m's for ATP and Na^+, but not those of K_m for K^+ were similar to corresponding values reported elsewhere[11,13,14].

The double valued K_m for K^+ (shown in Fig. 4 of this report) is consistent with

the existence of two K^+ binding sites on the microsomal protein. One site has a high affinity and low capacity for K^+, while the other has a low affinity and a high capacity for K^+ binding. Although not the unique explanation, this picture is similar to a previous hypothesis of SKOU[31] based on a large mass of kinetic data.

The broad spectrum of the Mg^{2+}-dependent nucleotide hydrolyses is in accord with similar data obtained from several microsomal sources[11,13,22,23] (although the degree of hydrolysis of each nucleotide was not exactly the same in comparing any one tissue with another).

The spectrum of the $(Na^+ + K^+)$-dependent nucleotide hydrolyses was narrower than that of the Mg^{2+}-dependent hydrolyses. Whereas our data on ITP and ATP in the turtle bladder resembled those of SKOU[21] in the crab nerve, they differed from those of others in beef kidney[22], calf[13], and bovine brain[23].

The discontinuity in the Arrhenius plot of Mg^{2+}-dependent activity *versus* reciprocal absolute temperature in the neighborhood corresponding to 23° is consistent with several possibilities suggested for such phenomena by DIXON AND WEBB[32] (*e.g.*, with two parallel or two successive reactions between enzyme and the same substrate; or with two forms of the enzyme, or with a reversible inactivation of the enzyme at certain temperatures). Moreover the discontinuity in the Arrhenius plot of the Mg^{2+}-dependent ATPase of the turtle bladder is similar to that in the plot of myosin ATPase (also a Mg^{2+}-dependent ATPase) at 16° with ATP or ITP as substrate[33].

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