BBA 75811

FUNCTIONS OF THE *E*–ATP AND *E*–*P* COMPLEXES IN THE MEMBRANE ATPase REACTION

ADIL E. SHAMOO* AND WILLIAM A. BRODSKY

Division of Biophysical Chemistry and Department of Physiology, Mount Sinai School of Medicine of the City University of New York, N.Y. 10010 (U.S.A.)

(Received September 27th, 1971)

SUMMARY

1. In turtle bladder microsomes incubated with 0.01 mM ATP at 0°, the Mg-dependent ATPase is 50% greater than the Ca-dependent ATPase. The addition of $Na^+ + K^+$ induces a 20% increase of Mg-ATPase, but has no effect on Ca-ATPase. The addition of Ca^{2+} to the $(Mg^{2+} + Na^+ + K^+)$-containing system eliminates the $(Na^+ + K^+)$ increment of its ATPase activity.

2. The *E*–ATP formed after incubation of microsomes with [U-^{14}C]ATP (0.01 mM ATP at 0°) amounts to an average value of 13 pmoles/mg in the presence of Ca^{2+} alone or of Mg^{2+} alone. The addition of either Na^+ or K^+ does not affect the underlying *E*–ATP formation in the presence of Mg^{2+} and/or Ca^{2+}. However, the addition of Na^+ and K^+ together induces a 32% increase in the Ca-dependent formation of *E*–ATP, but no change in the Mg-dependent formation. The addition of Ca^{2+} to the $(Mg^{2+} + Na^+ + K^+)$-containing system causes little or no change in ATP binding.

3. The phospho-protein (*E*–*P*) formed after incubation of microsomes with [γ-^{32}P]ATP (0.01 mM ATP at 0°) amounts to an average value of 19.2 pmoles/mg in the presence of Ca^{2+} alone, and 76 in the presence of Mg^{2+} alone. The addition of Na^+ causes a 3-fold increase in the amount of Ca-dependent formation of *E*–*P*, and a 2-fold increase in the Mg-dependent formation. The addition of K^+ to the $(Ca^{2+} + Na^+)$-containing mixture reduces the level of *E*–*P* to the underlying Ca-dependent level; while the same K addition to the $(Mg^{2+} + Na^+)$-system reduces the level of *E*–*P* formation to less than half (31 pmoles) of the underlying Mg-dependent level. The addition of Ca^{2+} to either the $(Mg^{2+} + Na^+)$ or the $(Mg^{2+} + Na^+ + K^+)$ system produces no change in phospho-protein formation.

4. Present data provide support for the hypothesis holding that the presence of Mg^{2+}, Na^+ and K^+ together is required in the first intermediary reaction step of the $(Na^+ + K^+)$-ATPase sequence. Although Ca can substitute for Mg in the first step of the $(Na^+ + K^+)$-ATPase sequence, it apparently forms a stable $(Ca^{2+} + Na^+ + K^+)$-*E*–ATP complex which does not break down into the final hydrolytic products. It is inferred that the Na-induced increment of phosphoprotein formation occurs in a reaction path which is in parallel to that of $(Na^+ + K^+)$-ATPase. Possible roles of *E*–ATP and *E*–*P* in sodium transport are adduced.

* Mailing address: Mount Sinai School of Medicine of the City Univ. of New York, 220 East 23rd Street, New York, N.Y. 10010, U.S.A.

INTRODUCTION

Interactions between the Ca-dependent and the Mg-dependent, Na^+, K^+-stimulatable ATPase have been reported in rabbit kidney cortex and membranes of human erythrocytes[1]; in sarcoplasmic reticulum extracts of amphibian and mammalian skeletal muscle and in other tissues[2–8]; and in the acid-labile ^{14}C binding by erythrocyte ghost membranes previously incubated with [8-^{14}C]ATP[9]. The latter study, showing a dependency of the acid-labile ^{14}C binding on Ca as well as on Mg, suggested to us that our data on the acid-stable binding of [^{14}C]ATP to microsomes of turtle bladder epithelial cells[10] could be similarly non-specific and consequently not necessarily related to ($Na^+ + K^+$)-ATPase.

Our previously reported data on the binding of ^{14}C to microsomes established the existence of an *E*–ATP complex, described some of its chemical properties, and showed that this binding remains unchanged after additions of Na^+ and/or K^+, but is decreased by ouabain only in the presence of Mg^{2+}, Na^+, and K^+ (ref. 11).

The purposes of the present paper were: (a) to correlate changes in the hydrolytic activity with concomitantly occurring changes in the levels of the *E*–ATP and *E*–*P* complexes in the microsomes; and (b) to determine the nature of the interactions of Na^+ and K^+ with the Ca and with the Mg form of the enzyme.

METHODS

Materials

The mucosal epithelial cells of the urinary bladder of *Pseudemys scripta* (fresh water turtles) were isolated by incubation of the freshly excised bladders in EDTA-containing Ringers by the method previously described[12]. Mucosal cells so harvested were then subjected to homogenization and differential ultracentrifugation in order to separate the microsomal fraction from the rest of the cell fractions. The microsomal pellets (re-suspended at concentrations of about 2 mg/ml of protein) were used immediately after centrifugation or were stored at –30° for 1–3 weeks prior to use in an experiment. Precise details on the techniques of the homogenization and differential centrifugation have been reported previously[12].

The Tris salt of non-radioactive ATP was obtained from Sigma Chemical Company St. Louis, Mo. [γ-^{32}P]ATP as the sodium salt (specific activity 2 C/mmole) and [U-^{14}C]ATP as the ammonium salt (specific activity 568 mC/mmole) were obtained from Amersham/Searle Company, Arlington Heights, Ill.

Assay of initial rates of hydrolysis

For the assay of hydrolysis, the composition of the incubation mixture, in terms of final concentrations was as follows: 0.01 mM [γ-^{32}P]ATP (specific activity 2 C/mmole) or 1.0 mM ATP; 85 mM NaCl; 15 mM KCl; 3.0 mM $MgCl_2$ and/or 1.0 mM $CaCl_2$; 40 mM Tris–HCL (pH 7.3); 0.1 mM Tris–EDTA; and 4 μg of microsomal protein in a final volume of 100 μl.

The assay media (less ATP) were allowed to pre-incubate in tubes (5 mm × 50 mm) for at least 10 min at 0° before initiating the hydrolysis by the addition of [γ-^{32}P]ATP. The resulting mixture was incubated at 0° for 2–10 min prior to termination of the reaction by addition of 25 μl of 25 % (w/v) of $HClO_4$. The rest of the procedure for

determining the P_i released was carried out as has been previously described[10]. The amount of microsomal protein was determined by the method of LOWRY *et al.*[13].

Assay of ^{14}C labelling of microsomal proteins

An aliquot of microsomal proteins, 0.04–0.20 mg, was incubated for 50 sec at 0° (unless otherwise indicated) in a medium containing 0.01 mM of [U-^{14}C]ATP (specific activity 568 mC/mmole), 3 mM $MgCl_2$ or 1 mM $CaCl_2$, 40 mM Tris–HCl (pH, 7.3) as buffer, 0.1 mM EDTA–Tris, 85 mM NaCl, 15 mM KCl in a final volume of 100 μl. Reactions were terminated by addition of 100 μl of 25% (w/v) $HClO_4$ after which the mixture was centrifuged at 15000 $\times$ *g* for 15 min at 0°.

The $HClO_4$ precipitate was washed and centrifuged 5 times or more with 200-μl aliquots of a cold (0°) solution containing 10% $HClO_4$, 0.015 M disodium ATP, and 0.05 M NaH_2PO_4. After each centrifugation the supernatant was checked for radioactivity. The repeated washings and centrifugations were stopped only after little or no radioactivity was detected in the supernatant. The precipitates were then removed into a scintillation vial containing 10 ml of 90% formic acid. The precipitate together with the formic acid in the vial was left for 1/2 h. Then 10 ml of toluene scintillation counting solution containing 50% absolute alcohol were added, and the radioactivity measured in the Beckman scintillation counter. The amount of protein labelling was estimated from counts of ^{14}C in the known amount of protein together with the specific activity of [U-^{14}C]ATP.

Assay of ^{32}P labelling of protein

Microsomal pellets, 0.04–0.20 mg were incubated in the same medium as described above, except that 0.01 mM [γ-^{32}P]ATP (specific activity 2 C/mmole), instead of [U-^{14}C]ATP, was used.

Definitions

The terms, Ca-ATPase or Ca-dependent ATPase, as used in this report, mean the ATPase activity of the microsomes in the presence of calcium alone. The terms, Mg-ATPase or Mg-dependent ATPase, mean the ATPase activity of the microsomes in the presence of magnesium alone. Correspondingly, the term, $(Na^+ + K^+)$-ATPase, means the increment of microsomal ATPase induced by the addition of Na^+ and K^+ together to the microsomes incubated with calcium or magnesium or with both calcium and magnesium.

RESULTS

Hydrolysis

Most of the studies on microsomal binding of ATP reported previously by us[11,14,15] were performed with the microsomes in the presence of ATP at final concentrations of 0.01 mM at 0°. However, our work on microsomal ATPase[10,16] was usually performed with microsomes in the presence of ATP at final concentrations of 1.0–3.0 mM at 38°. To compare the microsomal studies on binding with those on ATPase activity, experiments on ATPase were performed on microsomes in the presence of 0.01 mM ATP at 0°.

The approach to this comparison was to determine first the ATPase activity

in the presence of 1.0 mM ATP at 38° – the usual conditions (Table I); secondly, the activity in the presence of 1.0 mM ATP at 0° – to determine the effect of temperature (Table II); and finally, the activity in the presence of 0.01 mM ATP at 0° – to determine the effect of substrate concentration at 0° (Table III).

Table I presents data on the ATPase activity of microsomes incubated in the presence of 1.0 mM ATP at 38°. Group A includes the mean values for the activity of the Mg^{2+}-containing incubation mixtures; group B, those of the Ca^{2+}-containing incubation mixtures; and group C, those of the $(Ca^{2+} + Mg^{2+})$-containing mixtures.

The mean level of Ca-dependent ATPase, 13.5 μmoles/mg per h was not significantly different from that of Mg-dependent ATPase. Whereas, the addition of Na^+ and K^+ together more than doubled the level of Mg-dependent ATPase, the same addition of Na^+ and K^+ had no effect on the level of Ca-dependent ATPase.

Of special interest was the fact that the addition of Ca^{2+} to the $(Mg^{2+} + Na^+$

TABLE I

ATPase ACTIVITY AT 38° IN MICROSOMES INCUBATED FOR 10 min WITH [γ-^{32}P]ATP (specific activity 10^5 counts/min per μmole)

The composition of the incubation fluid in terms of final millimolar concentration was: Tris–ATP, 1.0; $MgCl_2$, 3.0; NaCl, 85.0; KCl, 15; $CaCl_2$, 1.0; Tris–HCl, 40; final pH, 7.3; final volume, 100 μl; total amount of microsomal protein, 3–5 μg. The procedures for adding the materials, starting and stopping the incubation, and for final determinations are described in the methods section. The values are the means ± S.E. in six experiments performed on three batches of microsomes.

Ionic conditions	*ATPase activity (μmoles/mg protein per h)*
A. Mg^{2+}	14.0 ± 0.8
$Mg^{2+} + Na^+ + K^+$	30.2 ± 1.2
B. Ca^{2+}	13.5 ± 1.0
$Ca^{2+} + Na^+ + K^+$	12.0 ± 0.6
C. $Mg^{2+} + Na^+ + K^+ + Ca^{2+}$	14.1 ± 0.3

TABLE II

ATPase ACTIVITY AT 0° IN MICROSOMES INCUBATED FOR 10 min WITH 1.0 mM OF [γ-^{32}P]ATP

The values are the means ± S.E. in six experiments performed on three batches of microsomes. The rest of the conditions are similar to those described for Table I.

Ionic conditions	*ATPase activity (nmoles/mg protein per h)*
A. Mg^{2+}	2700 ± 24
$Mg^{2+} + Na^+ + K^+$	2750 ± 20
B. Ca^{2+}	1600 ± 12
$Ca^{2+} + Na^+ + K^+$	1600 ± 14
C. $Mg^{2+} + Na^+ + K^+ + Ca^{2+}$	1100 ± 8

$+ K^+$)-containing microsomes completely eliminated the $(Na^+ + K^+)$ increment of Mg-ATPase, an effect of calcium resembling that of ouabain on $(Na^+ + K^+)$-ATPase.

Table II, cast in the same format as that of Table I, presents data on the ATPase activity of microsomes incubated in the presence of 1.0 mM ATP at 0°.

Of interest was the fact that the $(Na^+ + K^+)$ increment of Mg^{2+}-dependent activity (group A) practically vanished at 0°. Either the $(Na^+ + K^+)$-ATPase is inactivated at 0°, or the requisite concentrations of substrate and ions at 0° are different from those at 38°.

The ATPase activity in the presence of Mg^{2+} alone (or $Mg^{2+} + Na^+ + K^+$) 2700 nmoles/mg per h (group A) was 60 % greater than that in the presence of Ca^{2+} alone (or $Ca^{2+} + Na^+ + K^+$, see group B.).

The addition of Ca^{2+} to the $(Mg^{2+} + Na^+ + K^+)$-containing incubation fluid (see group C) caused an inhibition of a 60 % ($P < 0.001$) of this ATPase activity; and even reduced the level of ATPase activity to less than those of both of the underlying Mg-dependent and the underlying Ca-dependent level.

TABLE III

ATPase ACTIVITY AT 0° IN MICROSOMES INCUBATED FOR 10 min WITH 0.01 [γ-^{32}P]ATP

The values are the means ± S.E. in 6 experiments performed on 3 batches of microsomes. The rest of the conditions are similar to those described for Table I.

Ionic conditions	*ATPase activity (nmoles/mg protein per h)*	*Probability*
A. Mg^{2+}	208 ± 8	
$Mg^{2+} + Na^+$	200 ± 7	
$Mg^{2+} + K^+$	190 ± 6	
$Mg^{2+} + Na^+ + K^+$	251 ± 9	
$\Delta (Na^+ + K^+)$	43 ± 6	$P < 0.001$
B. Ca^{2+}	141 ± 11	
$Ca^{2+} + Na^+$	135 ± 8	
$Ca^{2+} + K^+$	130 ± 10	
$Ca^{2+} + Na^+ + K^+$	141 ± 9	
$\Delta (Na^+ + K^+)$	0.0 ± 1.0	$P > 0.9$
C. $(Mg^{2+} + Ca^{2+})$	240 ± 12	
$(Mg^{2+} + Ca^{2+}) + Na^+$	226 ± 9	
$(Mg^{2+} + Ca^{2+}) + K^+$	195 ± 10	
$(Mg^{2+} + Ca^{2+}) + Na^+ + K^+$	200 ± 9	

Table III, cast in the same format as those of the previous tables, shows the data on ionic requirements of microsomal ATPase activity at 0° with 0.01 mM ATP, the same conditions under which the binding studies to be reported herein were performed. Of interest is the fact that the $(Na^+ + K^+)$ increment of the Mg-dependent ATPase activity, 43 nmoles/mg/per h, was readily detectable at 0° when the ATP concentration was 0.01 instead of 1.0 mM (compare with group A of previous table).

The level of Mg-dependent ATPase, 208 nmoles/mg per h, (group A) was almost 50 % greater than that of Ca-dependent ATPase (group B), but was 20 % less than the activity in the presence of $Mg^{2+} + Ca^{2+}$ (group C). Evidently the two cations act together on ATPase in some unspecified, partially additive manner.

The addition of either Na^+ alone or of K^+ alone to either the Mg-containing or to the Ca-containing enzyme produced little, if any change in the underlying level of ATPase activity (groups A and B). However, the addition of Na^+ alone to the $(Mg^{2+} + C^{2+})$-containing mixture (group C) caused significant decreases (8 and 20 % respectively) in the underlying level of ATPase activity.

The addition of Na^+ and K^+ together in the presence of Ca^{2+} did not accelerate the Ca-dependent ATPase activity (group B); which contrasts with the 20 % acceleration in the presence of Mg^{2+} (group A). The absence of a $(Na^+ + K^+)$-induced increment of ATPase in the presence of $Ca^{2+} + Mg^{2+}$, means that the addition of Ca^{2+} to the $(Mg^{2+} + Na^+ + K^+)$-containing system inhibited the $(Na^+ + K^+)$-stimulated moiety of the Mg-dependent ATPase. Alternatively, the addition of Na^+ and K^+ together inhibited 20 % of the activity of the $(Mg^{2+} + Ca^{2+})$-containing enzyme.

These findings on turtle bladder microsomes are in harmony with findings of others working with different tissues. For example, BOEGMAN *et al.*[2] found that the Ca-dependent ATPase of sarcoplasmic reticulum of frog skeletal muscle was not changed by additions of $Na^+ + K^+$. In addition, a Ca-induced inhibition of $(Na^+ + K^+)$-ATPase has been reported in muscle and erythrocytes[2,3,5,17].

The conditions for the hydrolytic activities obtained and shown in Table III, 0° and 0.01 mM ATP, were maintained in all subsequent experiments on binding of ^{32}P or ^{14}C to microsomes incubated with either [^{32}P]ATP or [U-^{14}C]ATP respectively. This permitted a valid comparison of data on enzymatic hydrolysis (ATPase activities) with those on the binding of ATP and/or *P* to the enzyme.

ATP binding

Table IV shows the results of experiments on ATP binding to Mg-containing, to Ca-containing or to $(Mg^{2+} + Ca^{2+})$-containing microsomal mixtures incubated with [U-^{14}]ATP. The amount of ^{14}C-binding under any of the ionic conditions reported herein, has been corrected for the amount of binding to the $HClO_4$, "killed" microsomes which amounted to no more than 15 % of that bound to enzymatically intact microsomes.

TABLE IV

BINDING OF ATP TO MICROSOMES INCUBATED AT 0° FOR 50 sec WITH [U-^{14}C]ATP

Specific activity of [U-^{14}C]ATP was about 10^9 counts/min per μmole, ATP, 0.01 mM. The values are the means ± S.E. in six experiments performed on three batches of microsomes. The rest of conditions are similar to those described in METHODS.

Ionic conditions	*ATP-binding (pmoles/mg protein)*	*Ionic conditions*	*ATP-binding (pmoles/mg protein)*
A. Mg^{2+}	13.4 ± 1.0	B. Ca^{2+}	13.1 ± 1.2
$Mg^{2+} + Na^+$	13.8 ± 1.1	$Ca^{2+} + Na^+$	13.8 ± 1.6
$Mg^{2+} + K^+$	13.0 ± 1.2	$Ca^{2+} + K^+$	13.5 ± 1.0
$Mg^{2+} + (Na^+ + K^+)$	14.0 ± 1.2	$Ca^{2+} + (Na^+ + K^+)$	18.2 ± 1.5
C. $Mg^{2+} + Ca^{2+}$	13.4 ± 1.3	$\Delta(Na^+ + K^+)_{Ca^{2+}}$	5.1 ± 1.2
$Mg^{2+} + Ca^{2+} + Na^+$	11.0 ± 1.2	$P < 0.01$	
$Mg^{2+} + Ca^{2+} + K^+$	12.8 ± 1.1		
$Mg^{2+} + Ca^{2+} + Na^+ + K^+$	15.0 ± 1.6		

The acid-stable binding of ^{14}C to the microsomes in the presence of Mg^{2+} alone (group A) was the same as that in the presence of Ca^{2+} alone (group B) or that in the presence of $Mg^{2+} + Ca^{2+}$ (group C).

The addition of either Na^+ alone of K^+ alone to the Mg-containing incubation mixture, to the Ca-containing mixture, or to the ($Mg^{2+} + Ca^{2+}$)-containing mixture caused no significant change in the amount of binding of ATP to the microsomes.

The addition of Na^+ and K^+ together produced little or no change in ATP binding in the Mg-containing mixture (group A). However, the same addition increased the ATP binding in the Ca-containing mixture by 38 % (from 13.0 to 18.2 pmoles/mg, see group B). The addition of $Na^+ + K^+$ produced a small, but not statistically significant increase (12 %) of the ATP binding in the ($Mg^{2+} + Ca^{2+}$)-containing mixture (group C). These data suggest that Ca addition blocks the conversion of *E*–ATP to products in the ($Ca^{2+} + Na^+ + K^+$)-containing mixture and/or in the ($Mg^{2+} + Na + K^+$)-containing mixture.

Not shown are: (a) data from one set of paired control experiments showing a small amount of binding of ATP to microsomes (2–3 pmoles/mg incubated with [^{14}C]-ATP in the absence of either Mg^{2+} or Ca^{2+}, *i.e.* with the buffer alone or with the buffer *plus* Na^+ and/or K^+ (ref. 11); and (b) data from another set of paired controls showing that there was no detectable ^{14}C binding to microsomes incubated with [8-^{14}C]ADP (60 mC/mmole) under any of the aforementioned ionic conditions. The blank values subtracted from both of the aforementioned control experiments were those due to the $HClO_4$ "killed" blanks.

P binding

Table V presents data from experiments on ^{32}P binding to microsomes incubated with [γ-^{32}P]ATP (2 C/mmole) in the presence of: (A) Mg-containing incubation mixtures; (B) Ca-containing mixtures; or (C) ($Mg^{2+} + Ca^{2+}$)-containing mixtures.

Parenthetically, the amount of phosphoprotein formed under any or all of the present conditions was 3–12 times that of *E*–ATP formed concomitantly under the same conditions. Thus the *E*–*P* formation in the presence of $Mg^{2+} + Na^+$, (161

TABLE V

BINDING OF *P* TO MICROSOMES INCUBATED AT 0° FOR 50 sec WITH [γ-^{32}P]ATP

Specific activity of [γ-^{32}P]ATP, 2 C/mmole, ATP, 0.01 mM. The values are the mean ± S.E. in six experiments performed on three batches of microsomes. The rest of conditions are similar to those described in METHODS.

Ionic conditions	*P-binding (pmoles/mg protein)*	*Ionic conditions*	*P-binding (pmoles/mg protein)*
A. Mg^{2+}	76.0 ± 3.0	B. Ca^{2+}	19.2 ± 2.0
$Mg^{2+} + Na^+$	161 ± 3.0	$Ca^{2+} + Na^+$	57.9 ± 1.5
$Mg^{2+} + K^+$	75.0 ± 4.0	$Ca^{2+} + K^+$	19.0 ± 1.5
$Mg^{2+} + Na^+ + K^+$	31.0 ± 0.5	$Ca^{2+} + Na^+ + K^+$	20.0 ± 1.0
$\Delta(Na^+)$	85.2 ± 4.0	$\Delta(Na^+)$	36.5 ± 2.0
C. ($Mg^{2+} + Ca^{2+}$)	44.2 ± 3.0		
($Mg^{2+} + Ca^{2+}$) + Na^+	165 ± 3.0		
($Mg^{2+} + Ca$) + K^+	43.0 ± 2.5		
($Mg^{2+} + Ca^{2+}$) + $Na^+ + K^+$	30.0 ± 0.4		

pmoles/mg) was nearly 13 times the *E*–ATP formation in the presence of $Mg^{2+} + Na^+$. However, these data cannot provide the stoichiometric relationship between *E*–ATP and *E*–*P* in the ATPase sequence because of the marked differences in the kinetics of formation of the two complexes. The amounts compared were each measured after 50 sec of incubation at 0°. But the formation of *E*–ATP reaches its maximal level within 2 sec of incubation[11], and that of *E*–*P* after 20–25 sec (ref. 10).

The amount of *P*-binding in the presence of Mg^{2+}, (76 pmoles/mg) was nearly four times greater than that in the presence of Ca^{2+}; and almost twice that in the presence of $Mg^{2+} + Ca^{2+}$. Apparently the addition of Ca^{2+} inhibited the formation of phosphoprotein in the presence of Mg^{2+} (see group C). The exact nature of this interaction is not readily evident without a complete kinetic pattern of this activity with respect to Ca^{2+} and Mg^{2+} alone and/or together.

The addition of Na^+ increased the phosphoprotein formation under all 3 conditions. For example, Na^+ addition more than doubled the *P*-binding in the presence of Mg, trebled the binding in the presence of calcium, and quadrupled the binding in the presence of $Mg^{2+} + Ca^{2+}$.

The addition of K^+, by itself had no detectable effect on the binding under the conditions set for groups A, B, and C.

The addition of Na^+ and K^+ together halved the phosphoprotein formed in the presence of Mg^{2+}, but had no effect on that formed in the presence of Ca^{2+}, or on that formed in the presence of $Mg^{2+} + Ca^{2+}$. Assuming that the sodium-induced increment of phosphoprotein formed in the presence of Ca^{2+} is the same substance as that formed in the presence of Mg^{2+}, it follows that the Na-induced increment of phosphorylation is not uniquely dependent upon the presence of Mg^{2+}. Nevertheless, the value of the sodium increment of Ca-dependent *P* binding was 43 % of that of the sodium increment of Mg-dependent *P* binding.

It is of interest to regard the $(Na^+ + K^+)$ additions in terms of the addition of K^+ to: (a) the $(Mg^{2+} + Na^+)$-containing mixture; (b) the $(Ca^{2+} + Na^+)$-containing mixture; and to (c) the $(Mg^{2+} + Ca^{2+} + Na^+)$-containing mixture.

The addition of K^+ to the $(Mg^{2+} + Na^+)$-containing incubation mixture decreased the level of phosphoprotein to half of the underlying level formed in the presence of Mg^{2+} alone. The addition of K^+ to the $(Ca^{2+} + Na^+)$-containing, as well as to the $(Mg^{2+} + Ca^{2+} + Na^+)$-containing mixture decreased the level of phosphoprotein to the same level as that formed in the presence of Ca^+ and to that formed in the presence of $Mg^{2+} + Ca^{2+}$.

SKOU[18,19] has reported that the addition of K^+ to a $(Mg^{2+} + Na^+)$-containing microsomal mixture will reduce the phosphoprotein formation to levels below those found in the presence of Mg^{2+} alone. The data in Table V, as well as those of SKOU, suggest that part of the phosphoprotein formed in the presence of Mg^{2+} is the same as or is somehow related to the phosphoprotein formed in the presence of $Mg^{2+} + Na^+$. The K-induced effect has been interpreted as a K-dependent dephosphorylation step in the $(Na^+ + K^+)$-ATPase reaction, because it is associated with the increment of hydrolysis observed on addition of K^+ to the $(Mg^{2+} + Na^+)$-containing mixture of microsomes Mg^{2+}. However, SKOU's data on ITP show that addition of K^+ reduces the level of phosphorylation in the presence of $Mg^{2+} + Na^+$, and, at the same time, actually decreases the overall rate of hydrolysis[18,19]. The presently reported results in Table V on *P*-binding in the presence of $Ca^{2+} + Na^+ + K^+$ and $Ca^{2+} + Na^+$ also

demonstrate that addition of K^+ decreases the level of phosphoprotein formation; and that this event occurs concomitantly with no change in the overall rate of ATP hydrolysis (see Table III). Therefore, the K-induced decrease of phosphorylation of microsomes in the presence of $Mg^{2+} + Na^+$ as well as that in the presence of $Ca^{2+} + Na^+$ must be due to a blocking action of the K^+ ion on the phosphorylation of the protein rather than to an accelerating action of the K^+ ion on the dephosphorylation of the protein. The lack of an effect of calcium on the phosphorylation of the ($Mg^{2+} + Na^+ + K^+$)-containing microsomes must be considered together with its concomitant effects under the same conditions, *e.g.* a small increase in the formation of *E*–ATP together with complete inhibition of ($Na^+ + K^+$)-ATPase. In this connection, assume that the ($Na^+ + K^+$) ATPase sequence is as follows.

$$\underset{}{E} + \underset{}{\text{ATP}} \overset{(1)}{\leftrightharpoons} \underset{}{E\text{–ATP}} \overset{(2)}{\leftrightharpoons} \underset{}{\text{ADP}} + \underset{}{E\sim P} \overset{(3)}{\longrightarrow} \underset{}{E\text{–}P} \overset{(4)}{\longrightarrow} \underset{}{E} + \underset{}{P_i}$$

If the calcium blockade were located only at reaction 2, this would be sufficient to block completely the P_i release as was actually observed. But this type of blockade alone would result in a decrease in the formation of $E\sim P + E\text{–}P$, which clashes with the observations in phosphoprotein in Table V.

There are two choices which can be rendered consistent with the three sets of data: (a) the calcium blockade may be located at two reaction sites, 2 and 4, in the series reaction sequence postulated above; or (b) the calcium blockade is localized to one reaction site, but the ($Na^+ + K^+$)-ATPase system has a branch with a parallel set of reactions, *e.g.*

$$\begin{array}{lcl} & \overset{(1)}{} & \overset{(2)}{} \\ E + \text{ATP} & \leftrightharpoons & E\text{–ATP} \longrightarrow E + P_i + \text{ADP} \\ \Uparrow (3) & \overset{(4)}{} & \Uparrow (5) \\ E' + \text{ATP} & \leftrightharpoons & E'\sim P + \text{ADP} \end{array}$$

One path, denoted *E*, is the energy-releasing or hydrolytic path; and the other denoted *E'*, is the energy-conserving or exchange reaction path. If the calcium blockade occurs only at site 2, the ($Na^+ + K^+$)-ATPase (P_i release) would be blocked, the phosphoprotein formation would be unaffected, and the *E*–ATP formation would increase or remain constant—and all 3 predictions match the experimental data in this report. This indicates that the reaction model selected is consistent with (but not uniquely required for) a single site of calcium action. Alternatively designed models of parallel reaction paths can also be rendered consistent with the calcium effects or lack of effects on the 3 parameters (ATPase, *P*-binding, ATP-binding) of the microsomal ATPase system.

DISCUSSION

An *E*–ATP complex, the existence of which has been demonstrated recently by us[11,15] has to be the first intermediary product in direct series with the hydrolytic sequence catalyzed by ($Na^+ + K^+$)-ATPase. The simultaneous presence of Mg^{2+}, Na^+, and K^+ is required to endow the enzyme protein with its property of ouabain sensitivity not only with respect to the overall ($Na^+ + K^+$) hydrolytic increment but also with respect to the primary complexation of the substrate (ATP) to the protein[11].

Thus, ouabain reduces the formation of the E–ATP by 50 % while completely inhibiting $(Na^+ + K^+)$-ATPase. This means that Mg^{2+}, Na^+ and K^+ together are required co-factors in the very first intermediary reaction step (*i.e.* $E + ATP \rightleftharpoons E \sim ATP$) of the $(Na^+ + K^+)$-ATPase; but this does not exclude additional interactions among the 3 cations, ouabain, and the enzyme at later stages in the $(Na^+ + K^+)$-ATPase sequence.

Present data show that calcium can substitute, at least in part, for Mg^{2+} in the formation of E–ATP. For example, the $(Na^+ + K^+)$-increment of ATPase is not elicited in the calcium form of the enzyme system (*i.e.* $Ca^{2+} + Na^+ + K^+$), although a $(Na^+ + K^+)$-increment in the level of E–ATP formation does occur.

This set of data can be explained by assuming that the affinity of the $(Na^+ + K^+)$ form of the enzyme for calcium is greater than its affinity for magnesium. If this were so, the complex between the $(Na^+ + K^+)$-enzyme and calcium once formed, would not dissociate readily. Consequently the rate of P_i release due to addition of Na^+ and K^+ would be negligible while the amount of E–ATP formed would be increased as was actually observed under these conditions. The increment of E–ATP in the calcium form of the enzyme system occurs only in the simultaneous presence of all 3 cations, Ca^{2+}, Na^+, and K^+. This suggests that part of the calcium form of the $(Na^+ + K^+)$-E–ATP complex, like part of the magnesium form of the $(Na^+ + K^+)$-E–ATP complex, is actually the only form of E–ATP related directly to the $(Na^+ + K^+)$-ATPase molecule.

There is no a priori reason to deny that the calcium form of ATPase and of its intermediates, E–ATP and E–P, may be related to the transport of calcium from the cytoplasm to the interstitial fluid of most cells including those of the turtle bladder. However, in the absence of data on intracellular levels of calcium and even on calcium flux across the bladder, such a correlation can be no more than speculative at the present time.

If all of the ionic co-factors, the ouabain effect, and the $(Na^+ + K^+)$-induced conformational change occur in the very first intermediary reaction to form E–ATP, then what is the role of the phospho-enzyme? Data of the present report suggest that the sodium-induced increment of phospho-enzyme appearing in microsomes incubated with ATP is not necessarily one of the steps in series with the intermediary reactions between ATP + E and final products in the hydrolysis of ATP catalyzed by $(Na^+ + K^+)$-ATPase. This is because the K-induced decrement of the Na-induced increment of phosphoenzyme occurs with or without an increase in the concomitant hydrolysis (see Tables III and V). For example a K-induced decrement of phospho-enzyme has been found together with a decrease in the overall hydrolysis when ITP is the substrate[18,19], or when calcium is substituted for magnesium in the reaction mixture (see Tables III and V).

Two parallel paths such as described above suggest the possibility of two functions of the $(Na^+ + K^+)$-ATPase system in the process of membrane transport. One such function, that of energy transfer from metabolism to the membrane, may reside in the E–ATP path; and the other function, sodium translocation and/or sodium potassium exchange requires the participation of the phospho-enzyme path. The present data are not sufficient to determine the exact assignment of such transport functions to intermediary enzyme paths. However, data reported elsewhere in abstract form show that the pH dependency of the formation of sodium phospho-enzyme is

superimposable on and hence a counterpart of the pH dependency of sodium transport[15,21], which is consistent with the above assignment of the functions of energy transfer and Na^+ translocation to the $(Na^+ + K^+)$-ATPase system.

ACKNOWLEDGMENTS

This study was supported, in part by the National Institute of Health Research Grant AM 13037, and Training Grant AM 0564202, and in part by the National Science Foundation Grant GB 7764.

The author thanks Miss Kathy Blaser for her technical assistance.

REFERENCES

1 F. H. EPSTEIN AND R. WHITTAM, *Biochem. J.*, 99 (1966) 232.
2 R. J. BOEGMAN, J. F. MANERY AND L. PINTERIC, *Biochim. Biophys. Acta*, 203 (1970) 506.
3 E. ROGUS, T. PRICE AND K. L. ZIERLER, *J. Physiol.*, 54 (1969) 188.
4 A. MARTONOSI, *J. Biol. Chem.*, 244 (1969) 613.
5 G. INESI, E. MARING, A. J. MURPHY AND B. H. MCFARLAND, *Arch. Biochem. Biophys.*, 138 (1970) 285.
6 J. C. SKOU, *Biochim. Biophys. Acta*, 23 (1957) 394.
7 M. SOMOGYI, *Experientia*, 20 (1964) 28.
8 R. L. POST, A. K. SEN AND A. S. ROSENTHAL, *J. Biol. Chem.*, 240 (1965) 1437.
9 E. HEINZ AND J. F. HOFFMAN, *J. Cell Comp. Physiol.*, 65 (1965) 31.
10 A. E. SHAMOO, D. E. GENTILE AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 484.
11 A. E. SHAMOO AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 241 (1971) 846.
12 R. E. SOLINGER, C. F. GONZALEZ, Y. E. SHAMOO, H. R. WYSSBROD AND W. A. BRODSKY, *Am. J. Physiol.*, 215 (1968) 249.
13 O. H. LOWRY, J. V. PASSONEAU, F. X. HASSELBERGER AND D. W. SCHULZ, *J. Biol. Chem.*, 239 (1964) 18.
14 A. E. SHAMOO AND W. A. BRODSKY, *Biophys. J.*, 11 (1971) 287a.
15 W. A. BRODSKY AND A. E. SHAMOO, *Biophys. J.*, 11 (1971) 288a.
16 A. E. SHAMOO, D. E. GENTILE AND W. A. BRODSKY, *Biochim. Biophys. Acta*, 203 (1970) 495.
17 E. T. DUNHAM AND I. M. GLYNN, *J. Physiol.*, 156 (1961) 274.
18 J. C. SKOU, *Phys. Rev.*, 45 (1965) 596.
19 J. C. SKOU AND C. HILBERG, *Biochim. Biophys. Acta*, 185 (1969) 198.
20 R. L. POST, S. KUME, T. TOBIN, B. ORCUTT AND A. K. SEN, *J. Gen. Physiol.*, 54 (1969) 306s.
21 D. E. GENTILE, A. E. SHAMOO, H. R. WYSSBROD AND W. A. BRODSKY, *Am. J. Physiol.*, 217 (1969) 652.