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Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase by *N*-Ethylmaleimide

II. Effects on Sodium-Activated Transphosphorylation

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SUMMARY

An Na^+-stimulated ADP-ATP exchange reaction could be demonstrated in the microsomes obtained from guinea pig kidney. In agreement with previous results, *N*-ethylmaleimide was found to affect the Na^+-stimulated exchange reaction in two different ways, probably by reacting at two distinct sites on the (Na^+ + K^+)-dependent ATPase (EC 3.6.1.3). The reactivity of these two sites to *N*-ethylmaleimide depended on the conformational state of the transport enzyme system.

The ability of *N*-ethylmaleimide to distinguish between the inward-facing (E_1) and outward-facing (E_2) conformations of (Na^+ + K^+)-ATPase was used to determine the conformational state of the transport enzyme system in the presence of different physiological ligands, either individually or in combination. All physiological ligands except Mg^{++} (i.e., Na^+, K^+, and ATP) stabilized the E_1 conformation of (Na^+ + K^+)-ATPase. The E_2 conformation of the enzyme could be obtained either by treatment with Mg^{++} alone or by phosphorylation of (Na^+ + K^+)-ATPase to E_2-P.

ATP by itself did not appear to change the E_1 conformation of the enzyme to E_2 by occupancy of the "modifier" site, in either the presence or absence of *p*-nitrophenyl phosphate. Although both the E_2-P and E_2 forms of the enzyme have a high apparent affinity for ouabain, the glycoside binds to Na^+-E_1-ATP and Mg^{++}-E_1-ATP or E_1-P to a significant extent.

The present findings support the hypothesis that Na^+-dependent phosphorylation is part of the ATP-hydrolyzing activity of the (Na^+ + K^+)-ATPase and that in the presence of Na^+ the E_2 conformation of the transport enzyme system may be obtained only by phosphorylation of the (Na^+ + K^+)-ATPase.

INTRODUCTION

In order to understand the mechanism of microsomal (Na^+ + K^+)-ATPase (EC 3.6.1.3)-mediated transport of monovalent cations across cell membranes, it is necessary to know the intermediate steps in the hydrolysis of ATP by the enzyme system. The observations that incorporation of phosphate from [^{32}P]ATP into the microsomes is stimulated by Na^+ and reduced by K^+ suggest that Na^+-dependent phosphorylation and subsequent K^+-dependent dephosphorylation are two possible intermediate steps in the hydrolysis of ATP (1–6).

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Recently some objections have been raised to the hypothesis stated above, in which the Na^+-dependent phosphorylation is considered to be a manifestation of $(Na^+ + K^+)$-ATPase. It has been suggested that phosphorylation in the presence of Na^+, Mg^{++}, and ATP may be due to the occupancy by Na^+ of a "K^+- binding site," and that the effect of K^+ is not to increase the rate of dephosphorylation but to decrease phosphorylation (7, 8).

Active transport implies conformational change in the pumping mechanism. This conformational change in $(Na^+ + K^+)$-ATPase is believed to be induced by the formation of a phosphorylated intermediate (1). Alternatively, ATP has been suggested to produce alterations in the conformations of $(Na^+ + K^+)$-ATPase which lead to Na^+ translocation without the formation of a phosphorylated intermediate (7, 8). Ouabain, which is an effective inhibitor of $(Na^+ + K^+)$-ATPase only when present extracellularly (9, 10), can bind to the microsomes in the presence of nucleotides which ordinarily do not phosphorylate the enzyme (11–13). This has been considered to support the hypothesis that it is ATP, and not Na^+-dependent phosphorylation, which changes the conformation of the transport enzyme system (14). Furthermore, the activating effect of ATP on K^+-dependent phosphatase in the presence of Na^+ is offered as further evendence for the proposal that Na^+-dependent ATP binding to the enzyme complex, rather than phosphorylation of the enzyme by ATP, may be the primary cause of certain conformational changes in the membrane and perhaps may play a role in the process of Na^+ translocation through the membrane (15, 16).

In this paper the effect of NEM[2] on the ADP-ATP exchange activity of kidney microsomes has been studied. In agreement with the findings of previous workers, a distinct Na^+-dependent transphosphorylation was observed in the presence of low Mg^{++} concentrations, which was markedly stimulated by NEM (17–22). The present results confirm our previous finding that NEM probably reacts at two different sites of $(Na^+ + K^+)$-ATPase and that its reactivity to these sites is altered, depending on the conformational state of the enzyme (23). The ability of NEM to distinguish qualitatively between two major conformations of $(Na^+ + K^+)$-ATPase has been used to determine the possible conformational state of $(Na^+ + K^+)$-ATPase as induced by ligands, either individually or in combination. Finally, the present findings support the concept that the hydrolysis of ATP by microsomal $(Na^+ + K^+)$-ATPase is mediated by the formation of a phosphorylated intermediate and that this incorporation of phosphate into the enzyme protein produces a conformational change in $(Na^+ + K^+)$-ATPase which probably leads to Na^+ translocation in the intact membrane. A preliminary report of this work has been published (24).

[2] The abbreviation used is: NEM, *N*-ethylmaleimide.

METHODS

$(Na^+ + K^+)$-ATPase from guinea pig kidney and [γ-^{32}P]ATP were prepared as described by Post and Sen (25, 26). The enzyme preparation was stored at 4° in a solution containing 10 mM imidazole and 0.1 mM tetrahydro-EDTA adjusted to pH 6.9 ± 0.1 with HCl. The specific activity of the $(Na^+ + K^+)$-ATPase was 2.3–4.5 units/mg of protein. One unit of enzyme activity represents 1 μmole of ATP cleaved per minute at 37°. ^{32}P was obtained from Tracerlab. Universally labeled [^{14}C]ADP was purchased from Schwarz BioResearch as the trilithium salt; specific activity was about 25 mCi/mmole. [^{3}H]Ouabain was a product of New England Nuclear Corporation. Ouabain, NEM, and the disodium salts of ATP and ADP were obtained from Sigma Chemical Company. The sodium salts of the nucleotides were converted to Tris salts by ion-exchange chromatography.

Preliminary treatment with NEM and phosphorylation were carried out as described in the accompanying paper (23). Total phosphate was estimated by a modification of the method of Bartlett (27), and protein, by the method of Lowry *et al.* (28). Binding of [^{3}H]ouabain was determined by

the method of Matsui and Schwartz (11) as described previously (29). The specific activity of the [^{3}H]ouabain was 100 Ci/mole. The Na^+-dependent ADP-ATP exchange activity was estimated by the method described previously (30), except that adenylate kinase activity was determined by measuring the amount of [^{14}C]AMP formed as described by Blostein (22). Thus Na^+-stimulated net exchange was calculated by subtracting the amount of [^{14}C]AMP formed form the total [^{14}C]ATP synthesized in each experimental tube. The usual incubation medium consisted of 4.0 mM Tris-ATP, 1 mM Tris-[^{14}C]ADP, 0.1 mM $MgCl_2$ ± 1.5 mM NaCl, 10 mM imidazole–glycylglycine (pH 7.4 ± 0.1), and about 0.10–0.15 mg of protein in a total volume of 0.5 ml. The samples, in 10-ml centrifuge tubes, were incubated for 15 min at 25°.

RESULTS

Optimal Na^+ and Mg^{++} concentrations for transphosphorylation. Fahn *et al.* (17, 18) have reported that there was considerable disparity in the conditions required to produce the maximal exchange rate between the native and NEM-treated microsomes of the eel electric organ. By employing a lower concentration of Mg^{++} it was possible to make the two conditions more comparable and to improve the exchange rate slightly. A maximal exchange rate for the native microsomes was obtained when the concentrations of Mg^{++} and Na^+ were 0.1 mM and 1.5 mM, respectively (Fig. 1). Following NEM treatment, the maximal exchange rate was obtained when 0.4 mM Mg^{++} and 3 mM Na^+ were employed (Fig. 1). For all subsequent experiments, the concentrations of Mg^{++} and Na^+ for both native and NEM-treated microsomes were maintained at 0.1 mM and 1.5 mM, respectively. In agreement with the findings of Fahn *et al.* (18), at a constant ATP concentration of 4 mM the exchange rate was maximal when the ADP:ATP ratio was 1:4 (not shown). Since the optimal pH for both ATP hydrolysis and ADP-ATP ex-

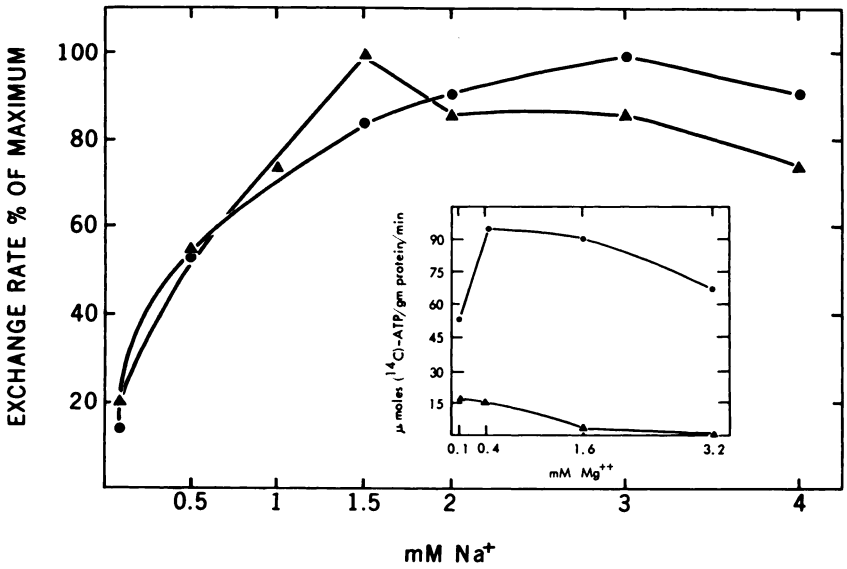

FIG. 1. *Activation of ADP-ATP exchange reaction by different concentrations of Na^+ or Mg^{++} in native (▲) and NEM-treated (●) microsomes*

The treated microsomal preparation was first incubated with 2.5 mM NEM, 16 mM Na^+, 3 mM Tris-ATP, and 0.25 mM ouabain at 37° for 30 min. Treated microsomes were washed free of all added ligands as described previously (23). Incubation conditions for exchange reactions are described in the text, but various concentrations of either Na^+ or Mg^{++} were employed. A constant concentration of Mg^{++} (0.1 mM) was employed for each experimental point, and in the inset 1.5 mM Na^+ was used along with the indicated concentrations of Mg^{++}. The same microsomal preparation was used for all experimental points.

TABLE 1

Effect of NEM on ADP-ATP exchange and adenylate kinase activity of kidney microsomes

Prior incubation with NEM was conducted as described previously (23). The exchange reaction was carried out as described in the text.

Prior treatment	Total activity		Adenylate kinase activity		Net exchange		Net Na^+-stimulated exchange ($A - B$)	ATP-hydrolyzing activity
	$Na^+ + Mg^{++}$	Mg^{++}	$Na^+ + Mg^{++}$	Mg^{++}	$Na^+ + Mg^{++}$ (A)	Mg^{++} (B)		
	μmoles [^{14}C]ATP/g protein/min		*μmoles [^{14}C]ATP/g protein/min*		*μmoles [^{14}C]ATP/g protein/min*			*μmoles P_i/mg protein/hr*
Control	40	18	7	9	33	9	24	207
2.5 mM NEM	73	10	7	7	66	3	63	127
2.5 mM NEM + 2 mM K^+	59	11	8	7	51	4	47	150
2.5 mM NEM + 2 mM K^+ + 3 mM ATP	99	21	10	9	89	12	77	92

change is about 7.5 (18), all exchange reactions were carried out at pH 7.4 ± 0.1 (20).

Effect of adenylate kinase on kidney microsomes. Electroplax microsomes do not exhibit any adenylate kinase activity (12). However, such activity was present in the kidney microsomes, and results of a typical experiment are shown in Table 1. The maximum contribution of adenylate kinase in the presence of Na^+ was between 15 and 20% of the total [^{14}C]ATP formed. Although NEM inhibited Na^+-insensitive, Mg^{++}-stimulated exchange, as found by Fahn *et al.* (18), it did not decrease adenylate kinase activity. However, the addition of ATP to the incubation medium protected against the inhibition of Mg^{++}-stimulated exchange by NEM. Furthermore, in agreement with our finding on the effects of NEM on phosphorylation and dephosphorylation (23), K^+ retarded while ATP and K^+ facilitated the stimulation of Na^+-dependent transphosphorylation by NEM.

The Na^+-dependent ADP-ATP exchange activity was found to be linear for more than 40 min in guinea pig kidney microsomes under the present experimental conditions (Fig. 2). However, the Na^+-independent exchange activity was linear for only 20 min. NEM inhibited the $(Na^+ + K^+)$-ATPase, decreasing either Na^+-dependent phosphorylation or K^+-sensitive dephosphorylation (23). Kidney microsomes treated with NEM to inhibit the $(Na^+ + K^+)$-ATPase partially by either of the two pathways exhibited Na^+-dependent exchange activity which was linear for at least 15 min (Fig. 2). Therefore, in all experiments described in the present paper, the exchange activity was measured for 15 min.

Next the optimal concentration of NEM for stimulation of Na^+-dependent transphosphorylation was determined. When K^+ and ATP were present in the incubation medium, maximal stimulation was obtained with 5 mM NEM. Higher concentrations of NEM were not tried, because at 10 mM NEM both the phosphorylation and dephosphorylation steps are affected, indicating loss of selectivity of NEM for its preferential site of action (23).

Changes in reactivity of NEM to $(Na^+ + K^+)$-ATPase, depending on conformational state of enzyme system. $(Na^+ + K^+)$-ATPase is believed to exist in two major conformations, the inward-facing (E_1) and the outward-facing (E_2) (31). The inward-facing form may be obtained either in a ligand-free medium or in the presence of Na^+ (12, 32, 33). Under both these conditions there was stimulation of Na^+-dependent exchange, indicating that when the enzyme is in the E_1 conformation NEM reacts at one site to stimulate Na^+-dependent transphosphorylation (Table 2) and inhibit K^+-sensitive dephosphorylation (23). Consistent with the finding of the effect of NEM on phosphorylation, there was some inhibition of the ex-

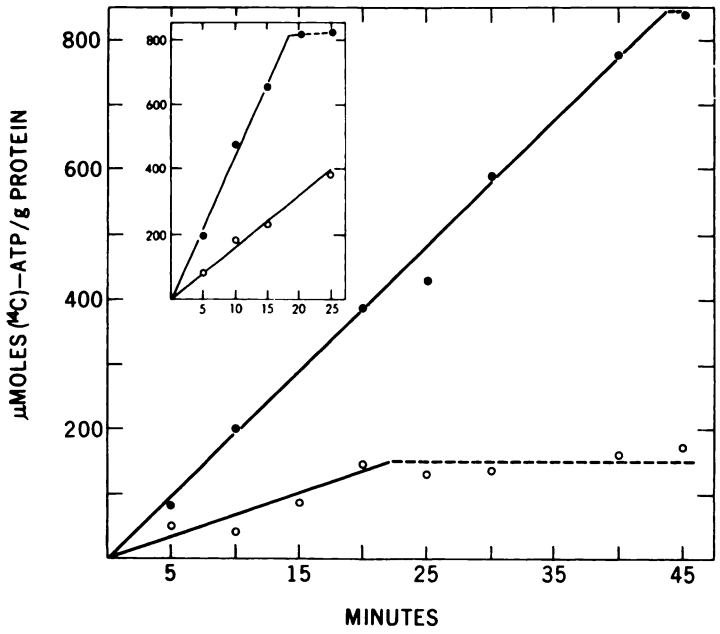

FIG. 2. *Time course of ADP-ATP exchange reaction for native and NEM-treated microsomes*

The incubation conditions for the exchange reaction are described in the text, and the Na^+-dependent (●) and Na^+-independent (○) ADP-ATP exchange activities shown were obtained after subtracting those due to adenylate kinase. The inset shows the time course of ADP-ATP exchange for NEM-treated microsomes. The enzyme was first incubated at 37° with 2.5 mM NEM either with 16 mM Na^+, 3 mM Tris-ATP, and 0.25 mM ouabain for 30 min (●) or with 4 mM Mg^{++} for 15 min (○). The same microsomal preparation was used for all experimental points. The lines were fitted by the method of least squares.

TABLE 2

Effect of NEM on E_1 conformation of $(Na^+ + K^+)$ − ATPase

Prior treatment with NEM was conducted as described previously (23). The exchange reaction was carried out as described in the text.

Prior treatment	Preliminary incubation at 37°	Net Na^+-stimulated exchange
	min	*μmoles [^{14}C]ATP/g protein/min*
Control	30	16
5 mM NEM	30	33
5 mM NEM + 16 mM Na^+	15	86
5 mM NEM + 16 mM Na^+	45	64

change rate when the sulfhydryl reagent was incubated in the presence of Na^+ for 45 min (23). The stimulation of the exchange rate by NEM when the enzyme is in the E_1 conformation seems to be preferential.

The E_2 conformation of $(Na^+ + K^+)$-ATPase may be obtained by phosphorylating the enzyme in the presence of Na^+, Mg^{++}, and ATP (12, 31–33). Under such conditions NEM probably reacts at another site to inhibit the exchange (Fig. 3). In nine experiments in which NEM was first incubated with kidney microsomes in the presence of Na^+, Mg^{++}, and ATP, exchange was inhibited in four, no significant effect was seen in two, and in the remaining three an unexpected stimulation of Na^+-dependent transphosphorylation was observed. These observations may be due to slow turnover of the enzyme even in the presence of Na^+, Mg^{++}, and ATP, which permitted NEM to react with both the E_1 and E_2 forms (32, 34). Variability in these results may be due to the differences in the rates of turnover of different microsomal preparations, depending on their purity. This turnover is blocked or markedly inhibited by ouabain (33), and after prior incubation of NEM with the

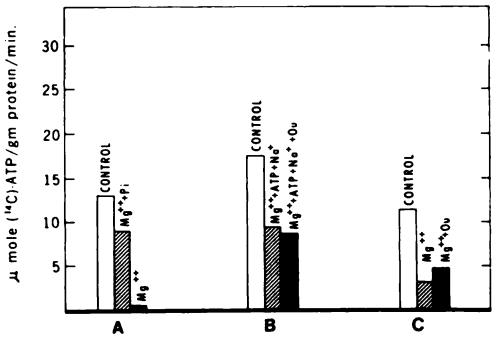

FIG. 3. *Effect of NEM on E_2 conformation of $(Na^+ + K^+)$-ATPase*

Microsomes were first incubated with 5 mM NEM under the following conditions: *A*, either with 4 mM Mg^{++} or with 4 mM Mg^{++} plus 1 mM inorganic phosphate for 30 min; *B*, either with 4 mM Mg^{++} plus 3 mM ATP and 100 mM Na^+ or with 4mM Mg^{++}, 3 mM ATP, 100 mM Na^+, and 0.25 mM ouabain (Ou) for 60 min; *C*, either with 4 mM Mg^{++} or with 4 mM Mg^{++} plus 0.25 mM ouabain for 45 min. The treated microsomes then were washed free of all ligands as described previously (23), and exchange reactions for the treated and native enzymes were carried out as described in the text. Three different enzyme preparations were used in *A*, *B*, and *C*.

kidney microsomes in the presence of ouabain as well as Na^+, Mg^{++}, and ATP there was consistent inhibition of exchange in five experiments (Fig. 3).

Since extracellular ouabain inhibits the $(Na^+ + K^+)$-ATPase of squid giant axon and red blood cells (9, 10), the enzyme is believed to have a higher apparent affinity for ouabain when it is in the E_2 conformation (31–33). Several workers have shown that [³H]ouabain binding is facilitated by either Mg^{++} alone or Mg^{++} plus inorganic phosphate (12–14, 29). We tested the effect of NEM under these two conditions and always found inhibition of the exchange (Fig. 3), indicating that when the enzyme was in the E_2 conformation NEM reacted preferentially with another site to inhibit both Na^+-dependent exchange and ^{32}P incorporation into microsomal protein (23).

The requirement of phosphorylation to produce the E_2 conformation in the presence of Na^+ is made clear in studies of the effects of Mn^{++} and Ca^{++}. Mn^{++} is known to support phosphorylation, while Ca^{++} inhibits it (1–6). Prior treatment with NEM in the

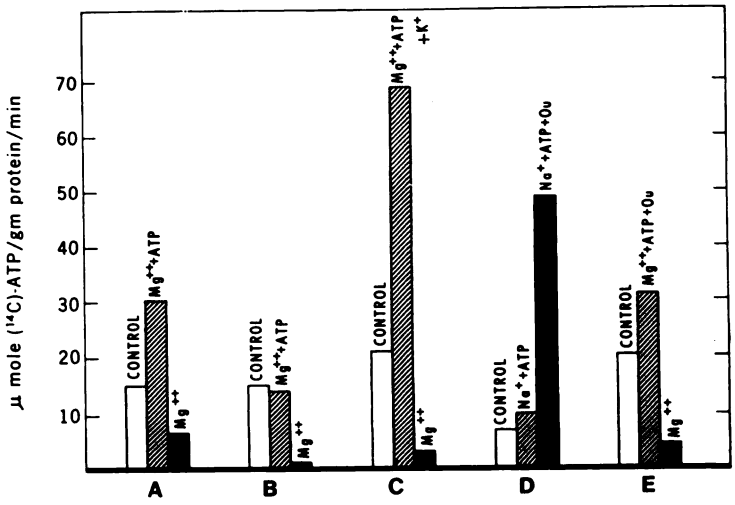

FIG. 4. *Effect of NEM on Na^+-dependent transphosphorylation in the presence of ATP or ouabain*

The microsomes were first incubated with 5 mM NEM at 37° in the presence of 1 mM Mg^{++} or of 1 mM Mg^{++} plus 3 mM Tris-ATP for 60 min (*A*), 4 mM Mg^{++} or of 4 mM Mg^{++} plus 3 mM Tris-ATP for 60 min (*B*), 1 mM Mg^{++} or of 1 mM Mg^{++}, 3 mM Tris-ATP, and 2 mM K^+ for 45 min (*C*), 16 mM Na^+ plus 3 mM Tris-ATP or of 16 mM Na^+, 3 mM Tris-ATP, and 0.25 mM ouabain (Ou) for 30 min (*D*), or 1 mM Mg^{++} or of 1 mM Mg^{++}, 3 mM Tris-ATP, and 0.25 mM ouabain for 45 min (*E*). The treated microsomes then were washed free of all added ligands (23), and exchange reaction rates were determined as described in the text.

TABLE 3

Reversal of Mg^{++} effect by Na^+ and K^+

The microsomes were first incubated with 5 mM NEM in the presence of the indicated ligands for 15 min in experiment 1 and 30 min in experiment 2. Na^+-stimulated ADP-ATP exchange was determined for washed and treated microsomes as described in the text.

Prior treatment	Net Na^+-stimulated exchange
	μmoles [^{14}C]ATP/g protein/min
Experiment 1	
Control	13
5 mM NEM + 4 mM Mg^{++}	6
5 mM NEM + 4 mM Mg^{++} + 16 mM Na^+	11
5 mM NEM + 4 mM Mg^{++} + 4 mM K^+	9
Experiment 2	
Control	13
5 mM NEM + 4 mM Mg^{++}	5
5 mM NEM + 4 mM Mg^{++} + 16 mM Na^+	21
5 mM NEM + 4 mM Mg^{++} + 4 mM K^+	14

presence of Na^+, ATP, and Mn^{++} inhibited exchange, but replacement of Mn^{++} by Ca^{++} in the prior incubation medium markedly facilitated ADP-ATP exchange (results not shown). Thus, in the presence of Na^+, occupancy of the Mg^{++}-binding site by a divalent ion is not itself sufficient to cause a conformational change to the E_2 form of (Na^+ + K^+)-ATPase. The effect depends on the ability of the divalent ion to support Na^+-dependent phosphorylation of the transport enzyme system.

ATP and E_1 conformation of (Na^+ + K^+)-ATPase. Prior incubation of NEM in the presence of Mg^{++} resulted in inhibition of exchange (Fig. 4). However, when ATP was also added to the prior incubation medium, the treated and washed enzyme showed stimulation of exchange, indicating that although Mg^{++} produced the E_2 form of (Na^+ + K^+)-ATPase, ATP restored the conformation to E_1. The ability of ATP to induce the E_1 conformation is further illustrated by our finding that addition of K^+ plus Mg^{++} and ATP to the prior incubation medium resulted in still greater stimulation of exchange (Fig. 4). This is in sharp contrast to the effect of Na^+, Mg^{++}, and ATP, which inhibited exchange as a result of phosphorylation (see Fig. 3).

Reversal of effect of Mg^{++} by Na^+ and K^+. The binding of ouabain in the presence of Mg^{++} may be reversed by Na^+, and this has been interpreted in terms of conformational change produced by Na^+ (29, 33). Therefore the effects of Na^+ and K^+ on the inhibition of Na^+-dependent transphosphorylation produced by NEM in the presence of Mg^{++} were examined. Table 3 shows that although Na^+ could convert the inhibitory effect of NEM in the presence of Mg^{++} into a stimulatory effect on ADP-ATP exchange, K^+ prevented the inhibition. Thus each ligand but Mg^{++} (i.e., Na^+, ATP, and K^+) appears to stabilize the E_1 conformation. However, the E_2 conformation may also

TABLE 4

Binding of [^3H]ouabain to kidney microsomes

Native microsomes (0.2 mg) in the presence of the indicated ligands were exposed to 0.25 μmole of [^3H]ouabain for 100 sec, either at 37° (A) or at 0° (B). Ouabain binding was measured as described in the text.

Incubation medium	[^3H]Ouabain bound	Maximal ouabain bound
	pmoles/mg protein	%
Series A (37°)		
2 mM Tris-ATP	0	0
100 mM Na^+ + 2 mM Tris-ATP	64.5	72
2 mM Mg^{++} + 2 mM Tris-ATP	53.0	59
2 mM Mg^{++} + 2 mM Tris-ATP + 100 mM Na^+	89.4	100
2 mM Mg^{++}	41.1	45.8
2 mM Mg^{++} + 100 mM Na^+	23.4	26
Series B (0°)		
100 mM Na^+ + 2 mM Mg^{++} + 2 mM Tris-ATP	80.3	100
2 mM Mg^{++} + 2 mM Tris-ATP	60.1	74.5

be obtained by phosphorylating the enzyme in the presence of Na^+, Mg^{++}, and ATP.

Ouabain and E_1 conformation of $(Na^+ + K^+)$-ATPase. The requirement of phosphorylation for ouabain binding in the presence of Na^+ is controversial (31). [^{3}H]Ouabain binding to $(Na^+ + K^+)$-ATPase has been demonstrated in the presence of Na^+ plus ATP, Mg^{++} plus ATP, or Na^+ plus Mg^{++} and ADP (11–13, 35). Although no phosphorylation should take place under these conditions, [^{3}H]ouabain binding is believed to occur because of the presence of residual Mg^{++}, Na^+, and adenylate kinase activity in the microsomal preparation, which may give rise to a small amount of phosphorylation, enough to support ouabain binding (29, 33). However, no attempts were made in previous studies to test this possibility by determining the amount of phosphorylation and ouabain binding to the same microsomal preparation under different conditions. Table 4 shows that a considerable amount of ouabain was bound in the presence of Na^+ and ATP or of Mg^{++} and ATP. The amount of ouabain bound to the kidney microsomes as a percentage of the maximal binding obtained in the presence Na^+, Mg^{++}, and ATP compares well with the percentage of [^{3}H]ouabain bound to heart microsomes (35) or with percentage inhibition of the ATP-hydrolyzing activity of electroplax $(Na^+ + K^+)$-ATPase (12) under these two conditions. In spite of ouabain binding in the presence of Na^+ plus ATP or of Mg^{++} plus ATP, little or no ^{32}P was incorporated into microsomal protein under either of these two conditions (Table 5). Furthermore, the difference between these two effects cannot be attributed to differences in the temperatures at which the [^{3}H]ouabain-binding and phosphorylation studies were carried out, because [^{3}H]ouabain binding in the presence of Mg^{++} and ATP was not reduced even at 0° (Table 4). Under identical conditions, with or without ouabain, the amounts of [^{32}P]inorganic phosphate released were similar (Table 5).

Therefore, in the presence of Mg^{++} plus ATP or of Na^+ plus ATP, ouabain probably binds in the absence of phosphoryltion to the E_1 conformation of $(Na^+ + K^+)$-ATPase. Once ouabain binds to the microsomes, however, the enzyme may change its conformation to E_2. This was tested by

TABLE 5

Phosphorylation of kidney microsomes with [^{32}P]ATP

The reaction mixture, containing 0.6 mg of microsomal protein in imidazole–glycylglycine buffer, pH 7.4 ± 1, and other additions as indicated, was first incubated for 5 min on ice. The phosphorylation reaction was started by the addition of 0.04 mM [^{32}P]ATP and stopped with 39 ml of 5% trichloracetic acid 100 sec later. When ouabain was present, it was added 5 sec before the [^{32}P]ATP. The incorporation of ^{32}P into microsomal protein was determined as described previously (23). The supernatant fraction obtained after the first addition of trichloracetic acid to each sample was saved, and 1 ml of each supernatant was counted for ^{32}P before and after shaking with 0.5 g of charcoal to estimate the amount of free [^{32}P]inorganic phosphate. The specific activity of [^{32}P]ATP was 66.5 × 10^6 cpm/μmole.

Incubation conditions	^{32}P-Labeled intermediate	^{32}P-incorporated	$^{32}P_i$ in supernatant	[^{32}P]ATP hydrolyzed
	pmoles/mg protein	*% maximum*	*pmoles/ml*	*pmoles*
1. [^{32}P]ATP mixture			27.6	
2. 0.04 mM ATP			26.6	0
3. 0.04 mM ATP + 100 mM Na^+	21.8	9.9	27.2	21
4. 0.04 mM ATP + 100 mM Na^+ + 2 mM Mg^{++}	220	100	300.6	493
5. 0.04 mM ATP + 2 mM Mg^{++}	10.7	4.9	268.5	252
6. 0.04 mM ATP + 10 mM K^+ + 2 mM Mg^{++}			223.9	196
7. 0.04 mM ATP + 100 mM Na^+ + 0.25 mM ouabain	25.2	11.4	23.6	21
8. 0.04 mM ATP + 2 mM Mg^{++} + 0.25 mM ouabain	24.8	11.2	264.0	261

TABLE 6

Conformational state of (Na^+ + K^+)-ATPase in the presence of different ligands as predicted by effects of NEM and ouabain

Ligands	Effect of NEM					Effect of ouabain	
	Phosphorylation	Dephosphorylation	Exchange rate	Other observations	Conformation predicted	[^{3}H]-Ouabain binding	Conformation predicted
Na^+	None	Rapid ↓[a]	Rapid ↑		E_1	Low	E_1
K^+	None	Slow	Slow ↑	Reverses Mg^{++} effect	E_1	Low	E_1[b]
ATP	None	Slow ↓	Slow ↑	Reverses Mg^{++} effect	E_1	Low	E_1
Mg^{++}	Rapid ↓	None	Rapid ↓		E_2	High	E_2
Na^+ + ATP	None	Slow ↓	Slow ↑		E_1	High	E_2[b]
K^+ + ATP	None	Rapid ↓	Rapid ↑		E_1	None	None
Mg^{++} + ATP	None	Slow ↓	Slow ↑		E_1	High	E_2[b]
Mg^{++} + ATP + K^+	None	Rapid ↓	Rapid ↑		E_1	None	None
Mg^{++} + ATP + Na^+	Slow ↓	None	Slow ↓ (variable)		E_2	High	E_2
Mg^{++} + P_i	Rapid ↓	None	Rapid ↓		E_2	High	E_2

[a] Downward arrows denote inhibition; upward arrows, stimulation.

[b] Other interpretations are possible.

incubating a microsomal preparation with NEM in the presence of Na^+ and ATP or of Mg^{++} and ATP, with or without ouabain. In the presence of Na^+ plus ATP, NEM stimulated the exchange rate, and this stimulation was enhanced by adding ouabain (Fig. 4). In the presence of Mg^{++} plus ATP and ouabain, NEM still stimulated the exchange rate, but the result was not significantly different from the stimulation produced in the presence of Mg^{++} and ATP only. This confirms that in the presence of Na^+ and ATP or of Mg^{++} and ATP ouabain binds to (Na^+ + K^+)-ATPase even in the E_1 conformation.

Reaction of NEM with (Na^+ + K^+)-ATPase in the presence of Na^+, K^+, Mg^{++}, and ATP. NEM appears to distinguish between the two conformations of (Na^+ + K^+)-ATPase by reacting at two different sites, depending on the conformational state of the enzyme. How will the reactivity of NEM to the two sites be affected if the (Na^+ + K^+)-ATPase turns over rapidly? When NEM was incubated with Na^+, K^+, Mg^{++}, ATP, and kidney microsomes, the treated and washed enzyme exhibited stimulation of exchange from a control value of 18 to 49 μmoles of [^{14}C]ATP per gram of protein per minute. This result helps to explain the apparent discrepancy between the effects of NEM on phosphorylation and on exchange in the presence of Na^+, Mg^{++}, and ATP. Phosphorylation was consistently more inhibited than dephosphorylation (23) when the microsomes were incubated with NEM in the presence of Na^+, Mg^{++}, and ATP; in contrast, the results observed in the exchange reaction (Fig. 3) were quite variable.

Different ligands, individually or in combination, favor different conformations of (Na^+ + K^+)-ATPase. The conformational states predicted from the effects of NEM and ouabain in the presence of different ligands are shown in Table 6.

DISCUSSION

Na^+-dependent ADP-ATP exchange was first demonstrated by Fahn *et al.* (17, 18) in microsomes obtained from the electric organ of *Electrophorus electricus*. Subsequently Stahl reported two types of exchange

reaction with rat brain microsomes (19, 20). With high Mg^{++} concentrations the exchange reaction was insensitive to Na^+ and exhibited broad nucleotide specificity, whereas with low Mg^{++} it showed an absolute requirement for ATP and was stimulated by Na^+ only (19, 20). Preliminary reports on Na^+-dependent transphosphorylation in kidney microsomes have been published (24, 36). Two types of exchange activity were also observed in the kidney microsomes.[3] With low Mg^{++} concentrations ADP-ATP exchange rate was maximal with 1.5 mM Na^+ and 0.1 mM Mg^{++} for the native enzyme (Fig. 1). The optimal conditions for the NEM-treated enzyme were 3 mM Na^+ and 0.4 mM Mg^{++}. Similar differences between the native and NEM-treated enzymes have been reported for electroplax microsomes (17, 18).

A model for the transport of monovalent cations involving the formation of a phosphorylated intermediate has been proposed (for a recent review, see ref. 31). Several objections have been raised against this model, particularly concerning the role of the phosphorylated intermediate in the transformation of enzyme conformation and the consequent translocation of cations through the membrane. An alternative hypothesis for the mechanism of cation transport is based on the assumption that it is ATP and not the phosphorylated intermediate which changes the conformation from the inward- to the outward-facing form (7, 8, 37, 38).

Askari and Rao (15, 16) suggested that although ATP hydrolysis by (Na^+ + K^+)-ATPase is mediated by phosphorylation of microsomal protein, "the primary event in producing certain conformational changes in the membrane" is the occupancy by ATP of the modifier site. This interpretation was supported by the observation that efflux of Na^+ across the red blood cell membrane in the presence of *p*-nitrophenyl phosphate was inhibited by external Na^+, and this inhibition could be reversed by nucleotides (ATP or CTP) and oligomycin (15, 16). However, preliminary treatment of the kidney microsomes with NEM in the presence of ATP plus either Na^+ or K^+ resulted in stimulation of exchange, indicating that ATP binds to the E_1 form (Table 1 and Fig. 4). Moreover, the E_2 form induced by Mg^{++} could be reversed to E_1 by ATP (Fig. 4). This suggests that it is phosphorylation and not occupancy of the modifier site by ATP that is necessary to induce the E_2 conformation.

More recently, Yoshida *et al.* (39) and Robinson (40) have shown that activation by nucleotides of K^+-sensitive phosphatase in the presence of Na^+ is due to the decrease in K_m for K^+. It therefore appears that the increased efflux of Na^+ observed in intact cells, in the presence of *p*-nitrophenyl phosphate and Na^+ at low concentrations of nucleotides, was due to an increase in the apparent affinity for K^+ rather than to some conformational changes that led to Na^+ translocation through the membrane (16).

Although the efflux of Na^+ associated with membrane-bound (Na^+ + K^+)-ATPase shows a specific requirement for ATP, [^{3}H]ouabain binding to the fragmented microsomes may be supported by different nucleoside di- or triphosphates in the presence of Na^+ and Mg^{++} (11–14). From the lack of specificity of ATP in the binding reactions, Albers *et al.* (12) and Hoffman (13) concluded that phosphorylation of (Na^+ + K^+)-ATPase is not essential for the binding of ouabain. This conclusion was consistent with an earlier report of Fahn *et al.* (6), who found that nucleoside triphosphates other than ATP can neither phosphorylate nor prevent Na^+-dependent phosphorylation with [^{32}P]ATP by electroplax microsomes. Since ouabain binds to an intact membrane only extracellularly (9, 10), Schwartz *et al.* (14) suggested that ATP and other nucleotides cause primarily an alteration in enzyme structure which somehow leads to the transport of ions without the formation of *E*-P.

The question of nucleotide specificity was re-examined by Schöner *et al.* (41) and Skou and Hilberg (7), who observed phosphorylation with [^{32}P]ITP and [^{32}P]GTP in ox brain microsomes. Although the rate of hydrolysis was much slower with either ITP or GTP than with ATP (41), the similarity in both rate and extent (42) of ouabain binding with

[3] Unpublished observations.

ITP and GTP was explained by Matsui and Schwartz (11) as being due to interaction of ouabain with the small amount of E-P formed and its subsequent removal from the equilibrium system, which stimulated further phosphorylation. If this hypothesis is correct, ^{32}P incorporation into the microsomes should be greater in the presence of ouabain than in its absence. Such an effect was not observed (Table 5). Furthermore, in the presence of either Na^+ plus ATP or Mg^{++} plus ATP, ouabain binding occurred with little or no phosphorylation (Tables 4 and 5). These results are in agreement with the earlier conclusion of Albers *et al.* (12) and Hoffman (13).

However, it may still be asked whether ATP changes the conformation of (Na^+ + K^+)-ATPase from E_1 to E_2 in the presence of either Na^+ or Mg^{++} to support ouabain binding, as suggested by Schwartz *et al.* (14). This seems unlikely, for several reasons. First, prior incubation of kidney microsomes with NEM in the presence of ATP, ouabain, and either Na^+ or Mg^{++} stimulated the exchange rate, indicating ouabain binding to the E_1 conformation (Fig. 4). Second, [^{3}H]ouabain binding to the NEM-treated enzyme has been demonstrated (23, 43). Third, Post *et al.* (32) have shown that dephosphorylation of NEM-treated microsomes by ADP was not affected by ouabain at 0°, but that when the incubation temperature was raised to 23° dephosphorylation was inhibited, indicating binding of ouabain to E_1-P at the higher temperature. Thus the present results suggest that although the E_2-P form of the enzyme has a high apparent affinity for ouabain, under some conditions ouabain may bind to E_1-P or even to Na-E_1-ATP and Mg-E_1-ATP.

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