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

I. Effects on Sodium-Sensitive Phosphorylation and Potassium-Sensitive Dephosphorylation

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SUMMARY

The hydrolysis of ATP by (Na^+ + K^+)-ATPase (EC 3.6.1.3) involves Na^+-dependent phosphorylation of the microsomal protein, and its breakdown is accelerated by K^+. *N*-Ethylmaleimide, a sulfhydryl reagent, inhibited (Na^+ + K^+)-ATPase by affecting either Na^+-dependent phosphorylation or K^+-sensitive dephosphorylation, depending upon the concentration of the inhibitor used and/or the presence of physiological ligands. In a ligand-free medium, lower concentrations of *N*-ethylmaleimide preferentially inhibited K^+-sensitive dephosphorylation. To decrease Na^+-dependent phosphorylation, a higher concentration of the inhibitor was required, but the rates of inhibition of ATP-hydrolyzing activity and K^+-sensitive dephosphorylation were always similar.

Physiological ligands influenced the sensitivity of the inhibition of either phosphorylation or dephosphorylation to *N*-ethylamleimide. The rates of inhibition of ATP-hydrolyzing activity and dephosphorylation were increased by Na^+ and decreased by K^+. These effects of monovalent cations could be reversed by nucleoside di- and triphosphates. In the presence of Mg^{++} alone or with either inorganic phosphate, or of Na^+ plus ATP, the ability of *N*-ethylmaleimide to inhibit phosphorylation was markedly increased, with a concomitant loss of its effect on the dephosphorylation step.

The results suggest that *N*-ethylmaleimide may differentiate between the two major conformational states of (Na^+ + K^+)-ATPase.

INTRODUCTION

The Na^+- and K^+-dependent adenosine triphosphate (EC 3.6.1.3) participates in the transport of Na^+ and K^+ across cell membranes (1–4). The pump seems to be stoichiometric. In human erythrocytes, 3 atoms of Na^+ are transported outward for 2 of K^+ inward, with the cleavage of one terminal phosphate group of intracellular ATP in the presence of intracellular Mg^{++} (5–7).

Details of the intermediate steps in the hydrolysis of ATP are needed to gain an understanding of the mechanism of the sodium pump. Studies with [^{32}P]ATP indicate that in the presence of Na^+ and Mg^{++} ^{32}P is incorporated into the microsomal protein, and K^+ facilitates the breakdown of the phosphoprotein (8–11). Although the requirement of Na^+ for phosphorylation is

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highly specific, K^+ may be replaced by other monovalent cations (8, 11). Mg^{++} may also be replaced by other divalent cations, such as Mn^{++}, but Ca^{++} produces marked inhibition (8, 11). The initial step of phosphorylation is reversible, and in the presence of a low concentration of Mg^{++} an Na^+-dependent ADP-ATP exchange is observed (12, 13).

Elucidation of the mechanism of cation transport is hampered by the difficulty of purifying the $(Na^+ + K^+)$-ATPase (14–16). As an alternative, further insight into the mechanism of the Na^+ pump may be obtained by employing irreversible inhibitors. The transport enzyme is inhibited by diisopropyl fluorophosphate, hydroxylamine, and sulfhydryl reagents (17–23). DFP[2] and hydroxylamine in the presence of Ca^{++} inhibit $(Na^+ + K^+)$-ATPase by the inhibition of Na^+-dependent phosphorylation (19, 24). On the other hand, NEM, a sulfhydryl reagent, does not affect the phosphorylation step but prevents K^+-dependent dephosphorylation (11), resulting in the stimulation of ADP-ATP exchange (25). The sensitivity of microsomal $(Na^+ + K^+)$-ATPase to irreversible inhibitors is altered by the presence of different ligands. For example, while Na^+ increases and K^+ decreases the initial rate of inhibition by NEM, it is K^+ which potentiates the inhibition of $(Na^+ + K^+)$-ATPase by DFP (17, 18, 21).

There appear to be differences between the mechanisms and sites of action of the same or different sulfhydryl inhibitors (19, 26). Information regarding the mechanism of inhibition of $(Na^+ + K^+)$-ATPase by NEM is fragmentary. In this paper and the following one (27), the effects of NEM, and their modifications imposed by ligands, on different steps in the reaction sequence of the transport enzyme are examined in greater detail. In this paper NEM is shown to react at two different sites on the microsomal $(Na^+ + K^+)$-ATPase. The reactivity of NEM at each site depends on the conformational state of $(Na^+ + K^+)$-ATPase imposed by added ligands.

[2] The abbreviations used are: DFP, diisopropyl fluorophosphate; NEM, *N*-ethylmaleimide.

MATERIALS AND METHODS

The microsomal $(Na^+ + K^+)$-ATPase from guinea pig kidney and [^{32}P]ATP were prepared as described by Post and Sen (28, 29). [^{3}H]-Ouabain was a product of New England Nuclear Corporation, and [^{32}P]inorganic phosphate, which was used for labeling ATP, was obtained from Tracerlab. *p*-Nitrophenyl phosphate, ouabain, the disodium salts of ATP and ADP, and other nucleotides were purchased from Sigma Chemical Company. The sodium salts of the nucleotides were converted to Tris salts by ion-exchange chromatography with Dowex 50–Tris.

Prior incubation with NEM was carried out as follows. Microsomal preparations with protein concentrations between 1.2 and 2.5 mg/ml were incubated with a particular concentration of NEM in 10 mM imidazole–glycylglycine buffer, pH 7.0 ± 0.1, for the desired periods of time at 37°, with or without added ligands. The drug-enzyme interaction was stopped by centrifugation at 45,900 × *g* for 5 min at 0°. The supernatant solution was discarded. Following treatment with ouabain, the microsomes were resuspended in 10 ml of the imidazole–glycylglycine buffer and incubated at 37° for 10 min to dissociate the enzyme-oubain complex (30). The suspension was then centrifuged at 45,900 × *g* for 10 min, the supernatant fraction was discarded, and the enzyme pellet was again resuspended in 10 ml of the same buffer. The washing procedure was repeated twice; each time the microsomal suspension was incubated at 37° for 10 min before centrifugation. When ouabain was not present in the prior incubation medium, the microsomes were washed three times with buffer as described above, but the 10-min incubation at 37° after each washing was omitted. The washed microsomes were finally resuspended in imidazole–glycylglycine buffer (pH 6.9 ± 0.1) and used to determine ATP-hydrolyzing activity (28), K^+-sensitive phosphatase activity, [^{3}H]ouabain binding, and the incorporation of ^{32}P into the microsomal protein from [^{32}P]ATP. A similar preparation of kidney microsomes, treated with fluorescein mercuric acetate, was washed three times as described above,

and the final pellet was resuspended in buffer and dialyzed for 12 hr. No unbound mercuric acetate could be detected in the washed preparation, indicating that our procedure removes all unbound drug.

Experiments to measure phosphorylation and dephosphorylation by the treated and untreated microsomes were carried out in an ice bath. One milliliter of reaction mixture contained 0.6–1 mg of microsomal protein, 0.4 mM Mg^{++}, 16 mM Na^+, and 0.04 mM [^{32}P]ATP with or without 1.6 mM K^+. The reaction was started by the addition of [^{32}P]ATP (specific activity, 25–75 × 10^6 cpm/μmole) was stopped 10 sec later with 5% trichloracetic acid containing 0.6 mM disodium ATP and 0.8 mM H_3PO_4. To estimate dephosphorylation, K^+ was added 10 sec after the reaction had been started with [^{32}P]ATP. The reaction was terminated with trichloracetic acid 20 sec after the addition of [^{32}P]ATP. The amount of ^{32}P incorporated into the microsomal protein was determined as previously described (8, 31). Total phosphate was estimated by a modification of the method of Bartlett (32), and protein, by the method of Lowry *et al.* (33). [^{3}H]Ouabain binding was measured by the method of Matsui and Schwartz (34), and has been described previously (30).

All experiments described in the present paper and the following one were performed at least twice, and the results shown are averages.

RESULTS

Effect of NEM on Na^+-dependent phosphorylation and K^+-dependent dephosphorylation. Ethacrynic acid, a sulfhydryl reagent, caused inhibition of phosphorylation and ADP-ATP exchange activity in the kidney microsomes that correlated well with inhibition of enzyme activity (22, 23, 26). On the other hand, NEM (1 mM) inhibited K^+-sensitive dephosphorylation in electroplax microsomes and stimulated ADP-ATP exchange activity with little effect on phosphorylation (11, 25). We therefore tested the effects of higher concentrations of NEM on the Na^+-dependent phosphorylation step of kidney microsomes to ascertain whether NEM would react with the probable site of action of ethacrynic acid. Even when the concentration of NEM was raised to 5 mM there was no significant effect on Na^+-dependent phosphorylation. Na^+ has been

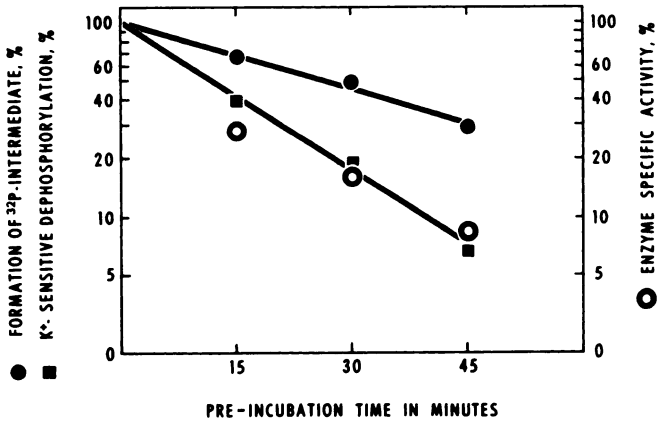

FIG. 1. *Effect of NEM on Na^+-dependent phosphorylation and K^+-dependent dephosphorylation of (Na^+ + K^+)-ATPase*

The microsomes were incubated with 10 mM NEM at 37° for the periods indicated. Each sample was then washed three times with buffer as described in the text. The ^{32}P incorporated by the treated and untreated microsomes was determined by the procedure outlined in the text. The untreated enzyme had a specific activity of 141.8 μmoles of P_i per milligram of protein per hour and incorporated 227.5 pmoles of ^{32}P per milligram of protein in the presence of Na^+. The amount of the ^{32}P labeled intermediate formed in the NEM-treated microsomes is expressed as a percentage of ^{32}P incorporated by the control microsomes, which had not been exposed to NEM.

shown to facilitate the over-all inhibition of enzyme activity by NEM (21). However, the effect of 5 mM NEM on the phosphorylation step of $(Na^+ + K^+)$-ATPase was the same whether or not Na^+ was present in the prior incubation medium (results not shown). When the concentration of NEM was raised to 10 mM, there was definite inhibition of phosphorylation (Fig. 1), indicating that NEM, like ethacrynic acid, probably reacts at a site to prevent the Na^+-dependent incorporation of ^{32}P from [^{32}P]ATP into kidney microsomes.

The inhibition of phosphorylation by 10 mM NEM (Fig. 1) and the previously reported effects of this sulfhydryl reagent on K^+-sensitive dephosphorylation (11) raise the obvious question as to which of these two steps is more intimately related to the inhibition of ATP-hydrolyzing activity. Therefore the effects of 10 mM NEM on three parameters—phosphorylation, dephosphorylation, and ATP-hydrolytic activity—were determined. The rates of inhibition of K^+-sensitive dephosphorylation and ATP-hydrolytic activity were similar, while the rate of inhibition of phosphorylation followed an independent course (Fig. 1).

Effects of Na^+, K^+, and ATP on inhibition of phosphorylation and dephosphorylation of $(Na^+ + K^+)$-ATPase by NEM. The initial rate of inhibition of $(Na^+ + K^+)$-ATPase activity by NEM is increased by Na^+ and decreased by K^+ (21, 35). Addition of ATP along with Na^+ or K^+ to the prior incubation medium reverses the effects of these two monovalent cations (21, 35). When ouabain is present in the prior incubation medium together with ATP, it is Na^+ and not K^+ which more markedly decreases the activity (21, 35). These changes in the rates of inhibition produced by different combinations of ligands may be due to alterations in the reactivity of NEM toward its two different sites of action as defined in the preceding section. This possibility was examined by measuring the Na^+-dependent phosphorylation and K^+-dependent dephosphorylation of NEM-treated microsomes in the presence of different ligands (Table 1). Incubation of kidney microsomes and NEM with Na^+ plus ATP or with Na^+ plus ATP and ouabain gave almost the same degree of Na^+-dependent phosphorylation. Although NEM produced only a small decrease in K^+-sensitive dephosphorylation in the presence of Na^+ and

TABLE 1

Modifications of effects of NEM on inhibition of phosphorylation and dephosphorylation by monovalent cations in the presence and absence of ouabain or ATP

The microsomes were incubated with or without 5 mM NEM at 37° for 30 min. The Na^+-dependent incorporation of ^{32}P and its reduction by K^+ were determined as described in the text. ^{32}P incorporated is expressed as picomoles per micromole of enzyme phosphate in experiment 1, and as picomoles per milligram of protein in experiments 2 and 3.

Incubation conditions	Phosphorylation: ^{32}P incorporated in absence of K^+ (A)	Dephosphorylation	
		Microsomal protein-bound ^{32}P present following addition of K^+ (B)	K^+-dependent dephosphorylation ($A - B$)
Experiment 1			
No NEM	401	92	309
40 mM Na^+ + 3 mM ATP + 5 mM NEM	376	128	248
40 mM Na^+ + 3 mM ATP + 0.25 mM ouabain + 5 mM NEM	374	320	54
Experiment 2			
No NEM	186	49	137
2 mM K^+ + 5 mM NEM	180	76	104
Experiment 3			
No NEM	216	46	170
2 mM K^+ + 3 mM ATP + 5 mM NEM	217	200	17

ATP, there was substantial protection of the phosphorylated intermediate against K^+-sensitive breakdown when Na^+, ATP and ouabain were added. Similarly, with K^+ in the prior incubation medium, there was little inhibition of K^+-sensitive dephosphorylation by NEM (Table 1). However, this effect was markedly enhanced when ATP was also added to the prior incubation medium together with K^+. There was no significant effect on Na^+-dependent phosphorylation by NEM in the presence of either K^+ or K^+ plus ATP.

Effects of ADP and other nucleotides on inhibition of (Na^+ + K^+)-ATPase by NEM in the presence of either Na^+ or K^+. The (Na^+ + K^+)-ATPase shows pronounced specificity for ATP (1), and the reversal of inhibition of enzyme activity by NEM in the presence of either Na^+ or K^+ by ATP has been attributed to substrate-induced conformational changes in the transport enzyme system (21, 35). This effect of ATP has been suggested to be involved in the translocation of Na^+ (21, 35). However, the effects of other nucleotides on the rates of inhibition of ATP-hydrolyzing activity by NEM in the presence of either Na^+ or K^+ have not been examined. ADP exhibited an effect qualitatively similar to that of ATP in reversing the effects of Na^+ and K^+ on the rate of inhibition of (Na^+ + K^+)-ATPase by NEM (Fig. 2). UTP had a slightly different influence. In the presence of K^+ plus UTP the rate of inhibition of (Na^+ + K^+)-ATP was increased as compared to that produced by K^+ only, but, in contrast to the results with ATP (21, 35) and ADP, the rate was lower with K^+ plus UTP than with Na^+ plus UTP (Fig. 2). A low concentration of ATP (10 μM) reversed the effect of Na^+ but was unable to alter the response in the presence of K^+ (Fig. 3). The effect of ITP and CTP were intermediate between those of low and high concentrations of ATP. Recently two nucleotide sites on (Na^+ + K^+)-ATPase have been recognized (36–38). It appears that occupancy of only one of these two nucleotide sites is enough to decrease the effect of Na^+ on the rate of inhibition by NEM.

Effect of NEM on ouabain-sensitive conformation of (Na^+ + K^+)-ATPase. The binding of ouabain to (Na^+ + K^+)-ATPase

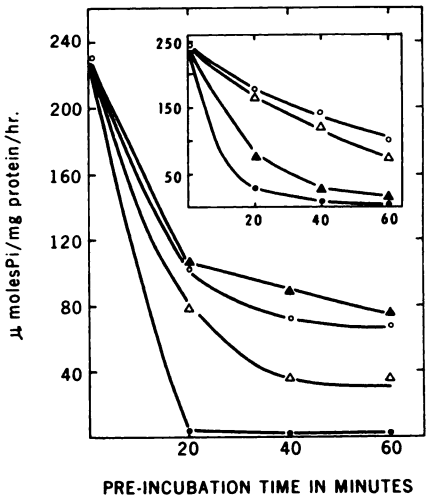

FIG. 2. *Effect of ADP and UTP on inhibition of (Na^+ + K^+)-ATPase activity by NEM in the pres- of Na^+ or K^+*

The microsomes and 5 mM were incubated with 16 mM Na^+ (●), 16 mM Na^+ plus 3 mM ADP (▲), 2 mM K^+ plus 3 mM ADP (△), or 2 mM K^+ (○) for the indicated periods. the microsomes were then washed and ATP-hydrolyzing activities determined as described in the text. In the inset, except for the replacement of 3 mM ADP by 3 mM UTP, all other conditions were the same.

is facilitated by Mg^{++} (30, 31, 34, 39, 40), and is enhanced by the addition of either inorganic phosphate or Na^+ and ATP to the incubation medium along with Mg^{++} (30,31, 34, 39–41). Since the effect of NEM on the ouabain-sensitive conformation of (Na^+ + K^+)-ATPase had not been examined, we determined the rates of inhibition of (Na^+ + K^+)-ATPase activity by prior incubation of the microsomes with NEM in the presence of Mg^{++}, of Mg^{++} plus inorganic phosphate, and of Mg^{++} plus Na^+ and ATP, with or without ouabain. There was a marked increase in the rate of inhibition when Mg^{++} either alone or with inorganic phosphate, was present in the prior incubation medium as compared to NEM only (Fig. 4). The rate of inhibition was slower in the presence of Mg^{++} plus inorganic phosphate than with Mg^{++} alone, indicating that inorganic phosphate probably gave some degree of protection against inhibition by NEM. The results further show that Mg^{++}, like Na^+, facilitated the inhibition by NEM, and that this effect

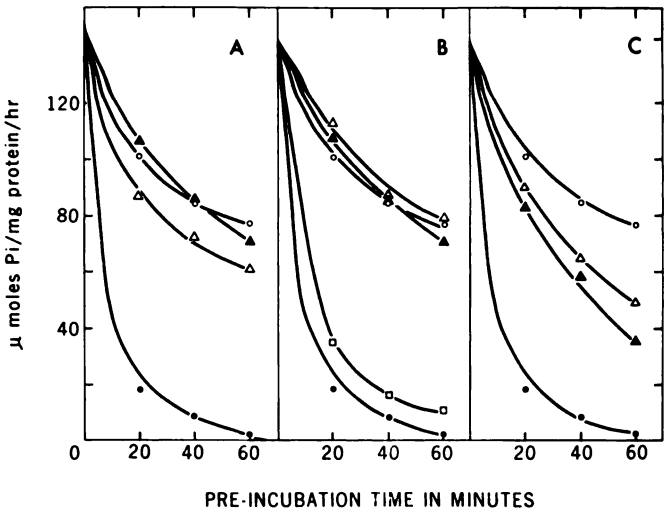

FIG. 3. *Effects of three nucleoside triphosphates on inhibition of (Na^+ + K^+)-ATPase activity by NEM in the presence of Na^+ or K^+*

The microsomes and 5 mM NEM were incubated with 16 mM Na^+ (●), 16 mM Na^+ plus nucleotide (▲), 2 mM K^+ (○), or 2 mM K^+ plus nucleotide (△, □) for the indicated periods. The microsomes were then washed and ATP-hydrolyzing activities determined as described in the text. Nucleotides: *A*, 3 mM CTP; *B*, 10 μM ATP (△, ▲) or 3 mM ATP (□); *C*, 3 mM ITP.

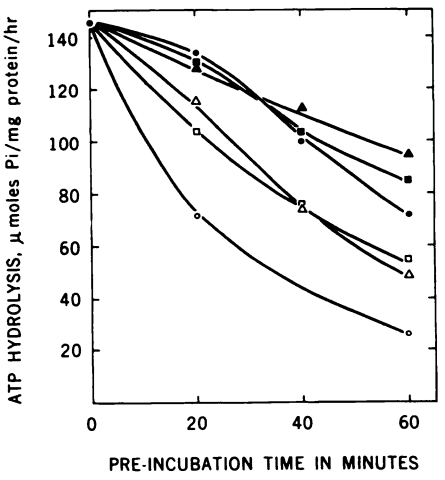

FIG. 4. *Reactivity of NEM to ouabain-sensitive conformation of (Na^+ + K^+)-ATPase*

The microsomes and 5 mM NEM were incubated with 4 mM Mg^{++} (○), 4 mM Mg^{++} plus 1 mM inorganic phosphate with (□) or without (△) 0.25 mM ouabain, or 4 mM Mg^{++} plus 100 mM Na^+ and 3 mM ATP (▲), or with 4 mM Mg^{++}, 100 mM Na^+, 3 mM ATP, and 0.25 mM ouabain (■), or no added ligand (●), for the indicated periods. The microsomes were then washed and ATP-hydrolyzing activities determined as described in the text.

of Mg^{++}, even in the presence of Na^+, was antagonized by ATP.

Next we tested the phosphorylation and dephosphorylation steps of the (Na^+ + K^+)-ATPase reaction after the microsomes had been allowed to react with NEM in the presence of Na^+, Mg^{++}, and ATP or with Mg^{++} plus inorganic phosphate, with or without ouabain. Since ouabain inhibits both dephosphorylation and rephosphorylation (8, 31), it was important to use an enzyme preparation to which ouabain binding is reversible. Table 2 shows that microsomes treated with ouabain in the presence of Mg^{++} plus inorganic phosphate underwent phosphorylation and dephosphorylation to almost the same degree as untreated kidney microsomes after three washings with imidazole–glycylglycine buffer as described in MATERIALS AND METHODS. When NEM had been treated with (Na^+ + K^+)-ATPase in the presence of Mg^{++} plus inorganic phosphate, with or without ouabain, the treated and washed microsomes exhibited no inhibition of K^+-sensitive dephosphorylation, but there was marked inhibition of Na^+-dependent incorporation of ^{32}P into kidney microsomes (Table 2). Similar results were ob-

TABLE 2

Effect of ouabain-sensitive conformation on inhibition of phosphorylation and dephosphorylation of $(Na^+ + K^+)$-ATPase by NEM

The method is described in the text.

Incubation conditions	Prior incubation time	Phosphorylation		Dephosphorylation		
		^{32}P incorporated in absence of K^+ (*A*)	Inhibition of phosphorylation	Microsomal protein-bound ^{32}P following addition of K^+ (*B*)	K^+-dependent dephosphorylation (*A* − *B*)	Inhibition of K^+-dependent dephosphorylation
	min	*pmoles/mg protein*	%	*pmoles/mg protein*	*pmoles/mg protein*	%
Experiment 1						
No addition	45	227		64	163	
5 mM NEM	45	204	10	178	26	78
Experiment 2						
4 mM Mg^{++} + 1 mM P_i	45	161		27	134	
4 mM Mg^{++} + 1 mM P_i + 0.25 mM ouabain	45	155	4	27	128	0
4 mM Mg^{++} + 1 mM P_i + 5 mM NEM	45	79	49	19	60	9
4 mM Mg^{++} + 1 mM P_i + 5 mM NEM + 0.25 mM ouabain	45	81	52	21	60	10
Experiment 3						
100 mM Na^+ + 3 mM Mg^{++} + 4 mM ATP	45	166		45	121	
100 mM Na^+ + 3 mM Mg^{++} + 4 mM ATP + 5 mM NEM	15	152	10	57	95	14
100 mM Na^+ + 3 mM Mg^{++} + 4 mM ATP + 5 mM NEM	30	134	19	45	89	9
100 mM Na^+ + 3 mM Mg^{++} + 4 mM ATP + 5 mM NEM	45	127	33	42	85	8

tained when microsomes were incubated with NEM in the presence of Na^+, Mg^{++}, and ATP (Table 2). There was progressive inhibition of phosphorylation with increasing incubation time, but little effect on dephosphorylation.

Reversal by Na^+ of NEM-induced inhibition of phosphorylation in the presence of Mg^{++}. The binding of ouabain to $(Na^+ + K^+)$-ATPase in the presence of Mg^{++} may be reversed by the addition of Na^+ (30, 31). If the site at which NEM reacts to cause inhibition of phosphorylation acquires an apparent higher affinity for the inhibitor, as a result of conformational change of the transport enzyme system in the presence of Mg^{++}, this should be reversed, at least partially, by Na^+. The results in Table 3 were obtained in an experiment designed to test this possibility. In the presence of Mg^{++} only, NEM markedly inhibited the Na^+-dependent phosphorylation, with no apparent effect on K^+-sensitive dephosphorylation. When Na^+ was also added to the prior incubation medium, there was an increase in ^{32}P incorporation into kidney microsomes and significant inhibition of K^+-sensitive dephosphorylation. The results indicate a reversal by Na^+ of the Mg^{++} effect.

Seigel *et al.* (42) have found that the inhibition of dephosphorylation by NEM does not prevent [^{3}H]ouabain binding to electroplax microsomes by the Na^+-stimulated pathway (30). The results obtained with guinea pig kidney microsomes were little different. There was some inhibition of ouabain binding by NEM after prior incubation of kidney microsomes, even for shorter periods. However, [^{3}H]ouabain binding was always higher either with Mg^{++} plus P_i or

TABLE 3

Partial reversal by Na^+ of Mg^{++}- and NEM-induced inhibition of phosphorylation of $(Na^+ + K^+)$-ATPase

The method is described in the text.

Incubation conditions	Prior incubation time	Phosphorylation		Dephosphorylation		
		^{32}P incorporated in absence of K^+ (*A*)	Inhibition of phosphorylation	Microsomal protein-bound ^{32}P following addition of K^+ (*B*)	K^+-dependent dephosphorylation (*A* − *B*)	Inhibition of dephosphorylation
	min	*pmoles/mg protein*	%	*pmoles/mg protein*	*pmoles/mg protein*	%
Experiment 1						
No NEM	15	136		18	118	
4 mM Mg^+ + 5 mM NEM	5	99	23	15	84	2
4 mM Mg^{++} + 100 mM Na^+ + 5 mM NEM	5	124	9	27	97	11
4 mM Mg^{++} + 5 mM NEM	15	65	53	15	49	23
4 mM Mg^{++} + 100 mM Na^+ + 5 mM NEM	15	94	31	34	60	37
Experiment 2						
No NEM	60	290		53	237	
4 mM Mg^{++} + 5 mM NEM	60	80	72	31	49	25
4mM Mg^{++} + 100 mM Na^+ + 5mM NEM	60	136	51	97	39	63

with Mg^{++}, ATP, and Na^+ than in the presence of Mg^{++} only. This indicates that NEM treatment did not significantly affect [^3H]-ouabain binding.

DISCUSSION

The hydrolysis of ATP by $(Na^+ + K^+)$-ATPase consists of at least two steps: Na^+-dependent phosphorylation of the microsomes and K^+-dependent hydrolysis of the resulting acyl phosphate (8–11). NEM can reduce the enzyme turnover by affecting either step, depending on the concentration of the inhibitor used and the presence of different ligands in the prior incubation medium. In a ligand-free medium, lower concentrations of NEM almost selectively inhibited K^+-sensitive dephosphorylation, with little or no effect on the formation of the phosphorylated intermediate (Fig. 1). Even when a higher concentration of NEM was used to inhibit the Na^+-dependent phosphorylation, the rates of inhibition of ATP hydrolysis and K^+-sensitive dephosphorylation were similar (Fig. 1). This suggests that although NEM reacts at two different sites of $(Na^+ + K^+)$-ATPase, the site which affects K^+-sensitive dephosphorylation has a much higher apparent affinity for the sulfhydryl reagent.

However, in the presence of Mg^{++} (Table 3), Mg^{++} plus inorganic phosphate (Table 2), and Na^+ plus Mg^{++} with ATP (Table 2), the reactivity of NEM was altered and the site involved in the inhibition of Na^+-dependent phosphorylation had an apparently higher affinity for the inhibitor. There are two possible explanations of this alteration in apparent affinity of $(Na^+ + K^+)$-ATPase for NEM. First, alteration of the reactivity with NEM was found only when Mg^{++} was present in the prior incubation medium. It is possible that the primary site of action for NEM, i.e., that involved in the inhibition of K^+-sensitive dephosphorylation, is at or near the Mg^{++}-binding site. Therefore, whenever Mg^{++} was present in the prior incubation medium, it protected against inhibition of dephosphorylation, resulting in the reaction of NEM at another site to prevent the formation of the phosphorylated intermediate. Second, the effect of Mg^{++} in the pres-

ence and absence of other ligands on the apparent affinity of $(Na^+ + K^+)$-ATPase for NEM may have been due to the conformational changes produced in the transport enzyme system.

The first possibility seems unlikely, for three reasons. First, replacement of Mg^{++} by Ca^{++} did not alter the apparent affinity of $(Na^+ + K^+)$-ATPase for NEM, although these two divalent ions probably compete for the same site on $(Na^+ + K^+)$-ATPase (27). Second, Mg^{++} plus ATP, with or without K^+, did not prevent the reaction of NEM with its primary site of action to stimulate ADP-ATP exchange (27). Third, the rates of inhibition of ATP hydrolysis by NEM in the presence of Mg^{++} and of Na were not very different. If alteration of the apparent affinity of $(Na^+ + K^+)$-ATPase for NEM were due to competitive protection by Mg^{++}, the reactivity with NEM resulting in inhibition of the K^+-sensitive dephosphorylation should decrease, with no significant effect on the ability of this agent to inhibit Na^+-dependent phosphorylation. However, Mg^{++} not only decreased the ability of NEM to inhibit dephosphorylation but also enhanced its capacity to inhibit phosphorylation. Therefore the effects of Mg^{++} can only be explained on the basis of conformational changes in $(Na^+ + K^+)$-ATPase.

Results described in the present paper strongly support the concept of the existence of two major conformations of $(Na^+ + K^+)$-ATPase (4). When the enzyme was in the E_1 form, NEM almost selectively reacted with a site at which it inhibited K^+-sensitive dephosphorylation (Fig. 1 and Table 1). To transform the conformation of the enzyme from E_1 to E_2, Mg^{++} seemed to be an essential requirement. In the presence of Mg^{++} (Table 3), Mg^{++} plus Na^+ and ATP (Table 2), or Mg^{++} plus inorganic phosphate (Table 2), NEM could not bind to the site involved in the inhibition of dephosphorylation and reacted readily with another site to inhibit the formation of the phosphorylated intermediate. Thus changes in the reactivity of NEM toward its two different sites are due to changes between two major conformational states of $(Na^+ + K^+)$-ATPase.

The transport enzyme $(Na^+ + K^+)$-ATPase is believed to exhibit allosteric interactions (4, 34–47). Fahn *et al.* (11), who first demonstrated inhibition of dephosphorylation by NEM, interpreted their results on the basis of stabilization of the E_1 conformation of the transport enzyme system by the sulfhydryl inhibitor. Thus, the primary effect of NEM may well be stabilization of either of the two major conformations of $(Na^+ + K^+)$-ATPase, with no direct effect on phosphorylation or dephosphorylation. Therefore, another possible interpretation for the findings described in this paper is that NEM reacts with only one site, and its ability to inhibit phosphorylation when the enzyme is in the E_2 conformation may be due to prevention of the conversion of the outward-facing form of $(Na^+ + K^+)$-ATPase to the inward-facing conformation. This possibility seems unlikely on the basis of ouabain-binding studies. Although ouabain binds more selectively with the E_2 conformation of $(Na^+ + K^+)$-ATPase (4, 30, 31, 34, 39), enzyme which had been treated with NEM to inhibit phosphorylation showed no binding of [^{3}H]ouabain in a ligand-free medium.

Since the rates of inhibition of $(Na^+ + K^+)$-ATPase by NEM in the presence of Na^+ or K^+ could be reduced by ATP, Skou and Hilberg (21, 35) suggested that ATP changes the affinities of Na^+ and K^+ for the transport enzyme system as a result of conformational change in $(Na^+ + K^+)$-ATPase, and that this effect of ATP is involved in the transport of monovalent cations. However, ADP, like ATP, decreased the rate of inhibition of $(Na^+ + K^+)$-ATPase by NEM in the presence of Na^+ and increased it when Na^+ was replaced by K^+ (Fig. 2). Moreover, CTP and, to a lesser extent, ITP exhibited qualitatively similar results (Fig. 3). Thus, despite the marked specificity of ATP for Na^+ transport, the alterations in the rates of inhibition of $(Na^+ + K^+)$-ATPase by NEM in the presence of monovalent cations can be produced by a variety of nucleotides.

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REFERENCES

1. J. C. Skou, *Physiol. Rev.* **45**, 596 (1965).
2. R. W. Albers, *Annu. Rev. Biochem.* **36**, 727 (1967).

3. R. L. Post, *in* "Regulatory Functions of Biological Membranes" (J. Järnefelt, ed.), p. 163. Elsevier, Amsterdam, 1968.
4. R. Whittam and K. P. Wheeler, *Annu. Rev. Physiol.* **32**, 21 (1970).
5. A. K. Sen and R. L. Post, *J. Biol. Chem.* **239**, 345 (1964).
6. R. Whittam and M. E. Ager, *Biochem. J.* **97**, 214 (1965).
7. P. J. Garrahan and I. M. Glynn, *J. Physiol. (London)* **192**, 217 (1967).
8. R. L. Post, A. K. Sen and A. S. Rosenthal, *J. Biol. Chem.* **240**, 1437 (1965).
9. K. Ahmed and J. D. Judah, *Biochim. Biophys. Acta* **93**, 603 (1964).
10. L. E. Hokin, P. S. Sastry, P. R. Galsworthy and A. Yoda, *Proc. Nat. Acad. Sci. U. S. A.* **54**, 177 (1965).
11. S. Fahn, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **243**, 1993 (1968).
12. S. Fahn, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **241**, 1882 (1966).
13. W. I. Stahl, *J. Neurochem.* **15**, 511 (1968).
14. S. P. Banerjee, I. L. Dwosh, V. K. Khanna and A. K. Sen, *Biochim. Biophys. Acta* **211**, 345 (1970).
15. A. Kahlenberg, N. C. Dulak, J. F. Dixon, P. R. Galsworthy and L. E. Hokin, *Arch. Biochem. Biophys.* **131**, 253 (1969).
16. D. W. Towle and J. H. Copenhaver, Jr., *Biochim. Biophys. Acta* **203**, 124 (1970).
17. L. E. Hokin and A. Yoda, *Proc. Nat. Acad. Sci. U. S. A.* **52**, 454 (1964).
18. L. E. Hokin, A. Yoda and R. Sandhu, *Biochim. Biophys. Acta* **126**, 100 (1966).
19. H. Bader, A. B. Wilkes and D. H. Jean, *Biochim. Biophys. Acta* **198**, 583 (1970).
20. C. F. Chignell and E. Titus, *Biochim. Biophys. Acta* **159**, 345 (1968).
21. J. C. Skou and C. Hilberg, *Biochim. Biophys. Acta* **110**, 359 (1965).
22. S. P. Banerjee, V. K. Khanna and A. K. Sen, *Mol. Pharmacol.* **6**, 680 (1970).
23. S. P. Banerjee, V. K. Khanna and A. K. Sen, *Biochem. Pharmacol.* **20**, 1649 (1971).
24. G. Sachs, E. Z. Finley, T. Tusji and B. I. Hirschowitz, *Arch. Biochem. Biophys.* **134**, 497 (1969).
25. S. Fahn, M. R. Hurley, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **241**, 1890 (1966).
26. S. P. Banerjee and A. K. Sen, *Fed. Proc.* **28**, 589 (1969).
27. S. P. Banerjee, S. M. E. Wong and A. K. Sen, *Mol. Pharmacol.* **8**, 18 (1972).
28. R. L. Post and A. K. Sen, *Methods Enzymol.* **10**, 762 (1967).
29. R. L. Post and A. K. Sen, *Methods Enzymol.* **10**, 773 (1967).
30. T. Tobin and A. K. Sen, *Biochim. Biophys. Acta* **198**, 120 (1970).
31. A. K. Sen, T. Tobin and R. L. Post, *J. Biol. Chem.* **244**, 6596 (1969).
32. G. R. Bartlett, *J. Biol. Chem.* **234**, 466 (1959).
33. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
34. H. Matsui and A. Schwartz, *Biochim. Biophys. Acta* **151**, 655 (1968).
35. J. C. Skou, *Protoplasma* **63**, 303 (1967).
36. T. Kanazawa, M. Saito and Y. Tonomura, *J. Biochem. (Tokyo)* **61**, 555 (1967).
37. A. Czerwinski, H. J. Gitelman and L. G. Welt, *Amer. J. Physiol.* **213**, 786 (1967).
38. A. H. Neufeld and H. M. Levy, *J. Biol. Chem.* **244**, 6493 (1969).
39. R. W. Albers, G. J. Koval and G. J. Seigel, *Mol. Pharmacol.* **4**, 324 (1968).
40. J. F. Hoffman, *J. Gen. Physiol.* **54**, 343S (1969).
41. A. Schwartz, H. Matsui and A. Laughter, *Science* **159**, 323 (1968).
42. G. J. Seigel, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **244**, 3264 (1969).
43. R. F. Squires, *Biochem. Biophys. Res. Commun.* **19**, 27 (1965).
44. J. D. Robinson, *Biochemistry* **6**, 3250 (1967).
45. J. D. Robinson, *Biochemistry* **8**, 3348 (1969).
46. T. Tobin, S. P. Banerjee and A. K. Sen, *Nature* **225**, 745 (1970).
47. R. L. Post, S. Kume, T. Tobin, B. Orcutt and A. K. Sen, *J. Gen. Physiol.* **54**, 306S (1969).