Biochimica et Biophysica Acta, 483 (1977) 181–192

BBA 68181

STUDIES ON (Na^++K^+) ACTIVATED ATPase. XLI

EFFECTS OF *N*-ETHYLMALEIMIDE ON OVERALL AND PARTIAL REACTIONS

B.M. SCHOOT, A.F.M. SCHOOTS, J.J.H.H.M. DE PONT, F.M.A.H. SCHUURMANS STEKHOVEN and S.L. BONTING

Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)

(Received January 17th, 1977)

Summary

1. Preincubation with N-ethylmaleimide inhibits the overall activity of highly purified (Na^++K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) preparations of rabbit kidney outer medulla.

2. This inhibition is decreased by addition of ATP or 4-nitrophenylphosphate under non-phosphorylating conditions, and also by addition of ADP or adenylylimidodiphosphate.

3. *N*-ethylmaleimide treatment leads to inhibition of K^+-stimulated 4-nitrophenylphosphatase activity, Na^+-stimulated ATPase activity, and phosphorylation by ATP as well as by inorganic phosphate. These inhibitions strictly parallel that of the overal (Na^++K^+)-ATPase reaction.

4. *N*-ethylmaleimide lowers the number of sites which are phosphorylated by inorganic phosphate, without affecting the dissociation constant of the enzyme-phosphate complex.

5. *N*-ethylmaleimide does not affect the relative stimulation by ATP of the K^+-stimulated 4-nitrophenylphosphatase activity.

6. These effects of *N*-ethylmaleimide can be explained as a complete loss of active enzyme, either by reaction of *N*-ethylmaleimide inside the active center, or by alterations in the quaternary structure through reactions outside the active center.

Introduction

The enzyme (Na^++K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) is involved in the active transport of sodium and potassium ions across the plasma

Abbreviations: (Na^++K^+)-ATPase, Sodium plus potassium activated adenosinetriphosphatase; CDTA, *trans*-1,2-diamino cyclohexane-*N,N,N'N'*,-tetraacetic acid.

membranes of animal cells [1—5]. The molecular structure and reaction mechanism of this system have so far been elucidated only to a minor degree. One way of obtaining more insight is by comparing the effects of group-specific modification on the overall reaction and the partial reactions of the system.

In the present study we have used *N*-ethylmaleimide, which is generally thought to react with sulfhydryl groups at neutral pH. It has previously been shown that after reaction with *N*-ethylmaleimide (Na^++K^+)-ATPase activity is inhibited [6—9] and that the degree of inhibition depends on the kind of ligands (Na^+, K^+, Mg^{2+}, ATP) present during reaction [8].

From a comparison of the effects of *N*-ethylmaleimide on the overall reaction and the Na^+-stimulated ADP-ATP phosphate exchange reaction, Fahn et al. [7] have concluded that *N*-ethylmaleimide inhibition of the overall reaction occurs through blocking of the transition of an ADP-sensitive phosphorylated intermediate to a K^+-sensitive form. There is, however, conflicting evidence on this point. Some effects of *N*-ethylmaleimide on the partial reactions of the system appear to agree with this model [7—15]. On the other hand, there are also arguments against this explanation of the *N*-ethylmaleimide inhibition mechanism. Klodos and Skou [16] recently raised some doubts about the concept of two phosphenzymes as intermediates in the hydrolysis of ATP by (Na^++K^+)-ATPase. Although Fujita et al. [15] reported parallel inhibition of the K^+-dependent 4-nitrophenylphosphatase reaction and the overall reaction by *N*-ethylmaleimide, Robinson [17] found no inhibition of the former activity. The stimulation of the Na^+-dependent ADP-ATP phosphate exchange activity after inhibition of the (Na^++K^+)-ATPase activity by *N*-ethylmaleimide has been found in electric eel electroplax microsomes, but not in rat brain microsomes [7]. In contrast to the finding of Siegel et al. [14], Hegyvary [13] reported that *N*-ethylmaleimide does not completely inhibit the ouabain-dependent phosphorylation by P_i. Furthermore, *N*-ethylmaleimide decreases the number of ATP binding sites without any effect on the affinity of ATP for the residual sites [18].

In view of this conflicting evidence, obtained with crude enzyme preparations, we have decided to investigate the effect of *N*-ethylmaleimide on a purified preparation. We find a parallel inhibition of all partial reactions and the overall reaction, which appears to rule out the specific blocking of the transition of an ADP-sensitive phosphorylated intermediate to a K^+-sensitive form.

Materials and Methods

Enzyme preparation

Purified (Na^++K^+)-ATPase was obtained from rabbit kidney outer medulla microsomes by extraction with sodium dodecyl sulfate and continuous sucrose density gradient centrifugation as described by Jørgensen [19].

The highly active (Na^++K^+)-ATPase fractions thus obtained were incubated for 30 min at 37°C in a medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 25 mM imidazole · HCl (pH 7.4) to remove ATP, and were then centrifuged for 10 min at 300 000 × *g*. The resulting pellets were washed twice by resuspension and centrifugation in 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5). The preparations were stored at −20°C in a buffer containing 250 mM su-

crose, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5). Their specific (Na^++K^+)-ATPase activity amounted to 1000—2000 μmol ATP split · mg protein^{-1} · h^{-1}, free of ouabain-insensitive ATPase activity, and the specific K^+-stimulated, ouabain-inhibited 4-nitrophenylphosphatase activity was 200—400 μmol 4-nitrophenylphosphate split · mg protein^{-1} · h^{-1}.

Enzyme assays

(Na^++K^+)-ATPase activity. This was determined as the difference in P_i production at 37° C in a medium containing 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2ATP, 30 mM imidazole · HCl (pH 7.4) and the same medium without 10 mM KCl, but with addition of 0.1 mM ouabain.

The ATPase reaction was stopped by adding 1.5 ml 8.6% trichloroacetic acid to 400 μl of reaction mixture. The amount of inorganic phosphate formed was determined by addition of 1.5 ml 0.66 M H_2SO_4, 1.15% ammonium heptamolybdate, 9.6% $FeSO_4$ · 6 H_2O, and reading the absorbance of the solutions at 700 nm after 30 min at room temperature (ref. 1, p. 261). Solutions with standard phosphate concentrations were used as reference.

In a second procedure the (Na^++K^+)-ATPase activity was determined by adding 1 μM γ^{32}P ATP (in addition to unlabeled ATP) to the reaction mixture. After stopping the reaction and staining for the P_i formed, the reduced phosphomolybdate complex is extracted into 3 ml isobutanol (20). To 1-ml aliquots of the isobutanol layer 10 ml Aquasol were added, and the mixture was counted for ^{32}P in a liquid scintillation analyser.

Na^+-stimulated ATPase activity. This was measured as the difference in P_i production at 37° C in a medium, containing 100 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 μM γ^{32}P ATP, 30 mM imidazole · HCl (pH 7.4), and a medium of the same composition containing in addition 0.1 mM ouabain.

The P_i production was measured by stopping the reaction at a given time by addition of 1.5 ml of 8.6% trichloroacetic acid in 0.1 mM H_3PO_4 to 400 μl reaction mixture, followed by staining and extraction of the $^{32}P_i$ into isobutanol as described in the preceding section. In some experiments, in which the inhibition of the Na^+-ATPase activity by potassium ions was determined, 10 mM KCl was added to the medium without ouabain.

K^+-stimulated 4-nitrophenylphosphatase activity. This was determined as the difference in 4-nitrophenol production at 37° C in a medium containing 10 mM KCl, 6 mM $MgCl_2$, 5 mM 4-nitrophenylphosphate, 1 mM CDTA, 30 mM imidazole · HCl (pH 7.4), and a medium of the same composition but without KCl, and with 0.1 mM ouabain. The 4-nitrophenol concentration was determined by measuring the absorbance at 410 nm after stopping the reaction by addition of 2 ml 0.5 M NaOH to 400 μl reaction mixture.

Phosphorylation by ATP. This was carried out in a medium containing 100 mM NaCl, 5 mM $MgCl_2$, 20 μM γ^{32}P ATP, 30 mM imidazole · HCl (pH 7.4). Phosphorylation was started by adding the enzyme (50—250 μg protein · ml^{-1}) to the medium. After 3 s at 37° C or 15 s at 0° C the reaction was stopped by addition of 2 ml of a solution of 5% trichloroacetic acid in 0.1 M H_3PO_4 to 100 μl of reaction mixture. The denatured protein was removed by filtration over a Selectron AE 95 filter (1.2 μm pore size, Schleicher & Schüll, Dassel, G.F.R.). After washing the filters 3 times with 5 ml stopping solution, the fil-

ters were dissolved in 10 ml Aquasol, and counted for ^{32}P in a liquid scintillation analyser. Blanks were prepared by adding the stopping solution to the $\gamma^{32}P$ ATP-containing medium before addition of the enzyme. The maximal amount of protein on the filter never exceeded 25 μg.

Phosphorylation by P_i. This was carried out in a medium containing: imidazole phosphate, at the stated concentration, purified $^{32}P_i$ ($6 \cdot 10^5$ dpm per 100 μl medium), 4 mM $MgCl_2$, 50 mM imidazole · HCl (pH 7.0), 250 μg protein · ml^{-1}.

After 4 min at 0° C the protein was denatured by adding 2 ml trichloroacetic acid in 0.1 M H_3PO_4, and the phosphorylated protein was filtered off and treated as described in the preceding section.

Blanks are prepared by adding the denaturing solution to the medium before the enzyme protein. Preliminary experiments gave high blank values for phosphorylation, which did not decrease with increasing non-radioactive P_i concentration. These high blank values were probably due to a radioisotope contamination of the carrier-free $^{32}P_i$ solution [21]. This contamination was removed by mixing the $^{32}P_i$ stock solution with boiled rabbit kidney outer medulla microsomes (500 μg · ml^{-1}), followed by centrifugation. The supernatant was used as purified carrier-free $^{32}P_i$, and contained little or no radioisotope contamination.

Treatment with ouabain. This was carried out as described by Schuurmans Stekhoven et al. [22], after reaction of the enzyme with *N*-ethylmaleimide. The *N*-ethylmaleimide-treated enzyme was incubated with 0.1 mM ouabain in 5 mM $MgCl_2$, 50 mM imidazole · HCl (pH 7.0), 5 mM dithioerythritol during 30 min at room temperature.

Reaction with N-ethylmaleimide. This was performed, unless otherwise stated, for 30 min at 37° C in a medium containing 25 mM imidazole · HCl (pH 7.5), 2 mM CDTA, and up to 50 μg protein · ml^{-1}, *N*-ethylmaleimide at the stated concentration, and other additives as stated.

The reaction with *N*-ethylmaleimide was ended by addition of at least a 5-fold molar excess dithioerythritol. Blanks were prepared by adding dithioerythritol to the *N*-ethylmaleimide solution 10 min prior to addition of the enzyme. After pre-incubation with *N*-ethylmaleimide the reaction mixture was kept on ice, and aliquots were taken for testing enzymatic activities.

In some experiments additives added during the *N*-ethylmaleimide reaction (e.g. KCl, 4-nitrophenylphosphate, ATP) may interfere with the subsequent enzymatic assay. In those cases the preparation was first subjected to column chromatography. The *N*-ethylmaleimide reaction mixture (100 μl) was placed on a Sephadex G 25 coarse column (100 × 5 mm), equilibrated in 25 mM imidazole · HCl (pH 7.5), 2 mM CDTA. The enzyme was eluted in a 1 ml fraction after 0.9 ml elution with the equilibration buffer.

Although the *N*-ethylmaleimide solutions were always freshly prepared, there was some variation in the degree of inhibition, apparently due to slow decomposition of *N*-ethylmaleimide in the solid state. The inhibition curve in Fig. 4 was, therefore, determined with a fresh batch of *N*-ethylmaleimide.

Protein determinations. These were carried out after a trichloroacetic acid precipitation, as described by Jørgensen [19], with bovine serum albumin as standard.

Materials. ATP (disodium salt), ADP (free acid) and adenylylimidodiphosphate (Li_4^+ salt) were supplied by Boehringer (Mannheim, G.F.R.). 4-Nitrophenylphosphate (disodium salt) was obtained from Merck (Darmstadt, G.F.R.), and converted to the imidazole salt by ion exchange chromatography over a Dowex 50 column (H^+ form) and subsequent neutralization with imidazole. $\gamma^{32}P$ ATP (3.0 Ci · $mmol^{-1}$ initial specific radioactivity) was obtained from the Radiochemical Centre, Amersham, England. Carrier free $^{32}P_i$, in aqueous solution, and Aquasol were purchased from NEN Chemicals (Frankfurt am Main, G.F.R.). All other chemicals were of reagent grade.

Results

Effects of N-ethylmaleimide on the (Na^++K^+)-ATPase activity

Preincubation of purified (Na^++K^+)-ATPase with *N*-ethylmaleimide causes inactivation of the ATPase activity. Fig. 1 shows that the inhibition rate follows pseudo first-order kinetics, both in the presence and absence of Mg^{2+}. In the presence of Mg^{2+} the inhibition rate is higher. In all further experiments the reaction with *N*-ethylmaleimide was performed in the absence of Mg^{2+} and in the presence of CDTA.

Addition of ATP, ADP or adenylylimidodiphosphate to the *N*-ethylmaleimide

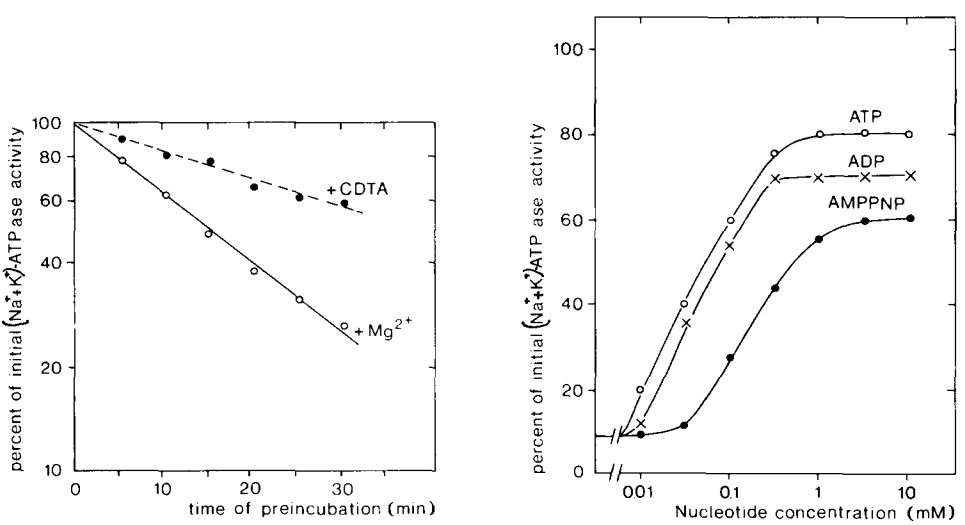

Fig. 1. Inhibition of (Na^++K^+)-ATPase activity by *N*-ethylmaleimide. The reaction mixture for preincubation with *N*-ethylmaleimide (NEM) contained: 7.5 μg protein · ml^{-1}, 2 mM CDTA (●) or 5 mM $MgCl_2$ (○), 0.7 mM *N*-ethylmaleimide, 25 mM imidazole · HCl (pH 7.5). After varying times of preincubation at 37°C, aliquots were removed for assay of (Na^++K^+)-ATPase activity as described in Materials and Methods.

Fig. 2. Effect of nucleotides on inhibition of (Na^++K^+)-ATPase activity by *N*-ethylmaleimide. The reaction with *N*-ethylmaleimide (NEM) was carried out at 37°C for 30 min in media containing 7.5 μg protein · ml^{-1}, 10 mM *N*-ethylmaleimide, 25 mM imidazole · HCl (pH 7.5), 2 mM CDTA (5 mM when ATP is present) and ATP (Tris salt) (○) or ADP (imidazole salt) (X) or adenylylimidodiphosphate (tetralithium salt; AMPPNP) (●) in the concentrations indicated. After reaction with *N*-ethylmaleimide (Na^++K^+)-ATPase activity was assayed as described in Materials and Methods. Corrections are made for the effects of added nucleotides on (Na^++K^+)-ATPase activity.

reaction mixture (10 mM *N*-ethylmaleimide, 30 min preincubation) lowers the inhibition of the $(Na^+ + K^+)$-ATPase activity. Their half-maximal concentrations were 35, 39 and 160 μM, respectively (Fig. 2). Protection by ATP only occurs when it is bound to the enzyme without phosphorylation taking place. Addition of $MgCl_2$ during the reaction with *N*-ethylmaleimide under these conditions abolished the protective effect of ATP (Table I). Partial protection (up to 25%) against inhibition of the $(Na^+ + K^+)$-ATPase activity by *N*-ethylmaleimide was given by 4-nitrophenylphosphate. This effect also seems to depend on binding of 4-nitrophenylphosphate to the enzyme, since it disappears when phosphorylation of the enzyme by this substance [23] can occur (presence of Mg^{2+} with and without K^+ ions, Table I).

The inhibitory effect of *N*-ethylmaleimide was pH-dependent, increasing with increasing pH (Fig. 3). The persistence of inhibition at pH values below 7.0 indicates reaction with sulfhydryl groups [24]. In all further experiments the reaction with *N*-ethylmaleimide took place at pH 7.5.

Inhibition as a function of the *N*-ethylmaleimide concentration during preincubation at pH 7.5 is shown in Fig. 4. Half-maximal inhibition is obtained at 0.7 mM ($pI_{50} = 3.2$).

Effects of N-ethylmaleimide on the K^+-stimulated 4-nitrophenylphosphatase activity

The K^+-stimulated 4-nitrophenylphosphatase reaction, which is inhibited by ouabain, is thought to represent the dephosphorylation step of the ATPase system. Preincubation of $(Na^+ + K^+)$-ATPase preparations with *N*-ethylmaleimide led to parallel reduction of the $(Na^+ + K^+)$-ATPase- and K^+-stimulated 4-nitrophenylphosphatase activities, independent of the pH of the reaction mixture (Fig. 3), and of the concentration of *N*-ethylmaleimide (Fig. 4).

The degree of inhibition of the K^+-stimulated 4-nitrophenylphosphatase activity by preincubation with various concentrations of *N*-ethylmaleimide is in-

TABLE I

EFFECTS OF VARIOUS LIGANDS ON INHIBITION BY *N*-ETHYLMALEIMIDE

Treatment with *N*-ethylmaleimide was carried out in a medium containing: 10 mM *N*-ethylmaleimide (except for the control), 25 mM imidazole · HCl (pH 7.5), 100 μg protein · ml^{-1} and ligands as indicated in the table, for 30 min at 37°C. After reaction with *N*-ethylmaleimide the ligands were removed by gel filtration on a Sephadex G25 column, and assays for K^+-stimulated 4-nitrophenyl phosphatase (K-pNPPase) and $(Na^+ + K^+)$-ATPase activity were performed as described in Materials and Methods. Results from 3 experiments carried out in duplicate are presented as means with standard error of the mean.

Ligands added during *N*-ethylmaleimide reaction	Remaining activity	
	K-pNPPase (%)	$(Na^+ + K^+)$-ATPase (%)
Control (no *N*-ethylmaleimide)	100	100
4 mM CDTA	7.6 ± 0.6	6.2 ± 0.4
10 mM ATP + 4 mM CDTA	56.8 ± 2	69.5 ± 7
10 mM ATP + 100 mM NaCl + 5 mM $MgCl_2$	14.4 ± 3.5	7.6 ± 1.8
10 mM 4-nitrophenylphosphate + 4 mM CDTA	26.1 ± 1.8	21.1 ± 2
10 mM 4-nitrophenylphosphate + 5 mM $MgCl_2$	13.7 ± 0.9	5.5 ± 0.1
10 mM 4-nitrophenylphosphate + 5 mM $MgCl_2$ + 5 mM KCl	6.8 ± 1.2	4.6 ± 1.3

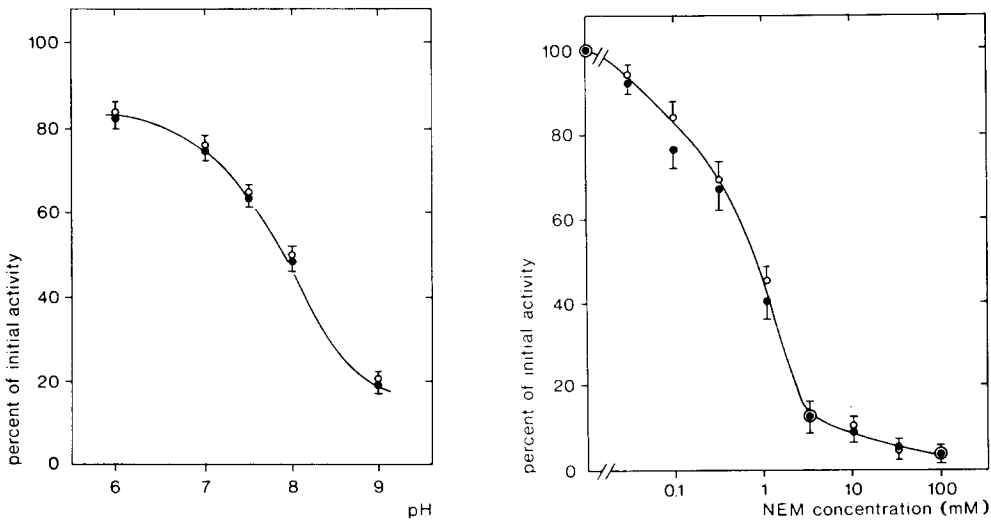

Fig. 3. Effect of pH on *N*-ethylmaleimide inhibition. (Na^++K^+) (•) and K^+-stimulated 4-nitrophenylphosphatase (○) activities were assayed in aliquots of reaction mixtures containing: 10 μg protein · ml^{-1}, 0.7 mM *N*-ethylmaleimide (NEM), 2 mM CDTA and 50 mM Tris/maleate buffer at the indicated pH values. After preincubation for 30 min at 37°C the *N*-ethylmaleimide reaction is stopped by adding dithio-erythritol to a final concentration of 10 mM. Corrections are made for the spontaneous inactivation of the enzyme at the given pH.

Fig. 4. *N*-ethylmaleimide inhibition of (Na^++K^+)-ATPase and K^+ stimulated 4-nitrophenylphosphatase activities. The *N*-ethylmaleimide reaction mixture contained: 7.5 μg protein · ml^{-1}, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5) and *N*-ethylmaleimide (NEM) at stated concentrations. After preincubation for 30 min at 37°C the *N*-ethylmaleimide reaction was ended by adding dithioerythritol (5-fold molar excess), and aliquots of the mixture were assayed for (Na^++K^+)-ATPase activity (•) and K^+ stimulated 4-nitrophenylphosphatase (K-pNPPase) activity (○) as described in Materials and Methods. Fresh batches of *N*-ethylmaleimide were used in these experiments.

dependent of the kind and concentration of ligands present during assay (Fig. 5). The stimulation of this activity, in the presence of 0.5 mM K^+, by 20 mM Na^+ (20% stimulation) or by 20 mM Na^+ + 0.1 mM ATP (150% stimulation) is not changed by reaction with *N*-ethylmaleimide.

Effects of N-ethylmaleimide on the Na^+-stimulated ATPase activity

In the presence of micromolar ATP concentrations the ATPase activity is stimulated by Na^+ ions alone, whereas simultaneous addition of 100 mM Na^+ and 10 mM K^+ ions stimulates less than with Na^+ alone [20]. Post et al. [25] suggest that only the E_1 conformation of the enzyme is involved in this Na^+-stimulated ATPase activity. If *N*-ethylmaleimide only inhibited the conformational change of a phosphorylated intermediate from an E_1 (ADP-sensitive) to an E_2 (K^+-sensitive) form, no inhibitory effect of *N*-ethylmaleimide on this Na^+-stimulated ATPase activity would be expected. As shown in Table II, the latter activity is inhibited to the same extent as the (Na^++K^+)-ATPase activity. Moreover, the inhibition of the Na^+-stimulated ATPase activity by K^+ ions is not changed by *N*-ethylmaleimide treatment, suggesting that the affinity of K^+ ions for the inhibitory site is not changed.

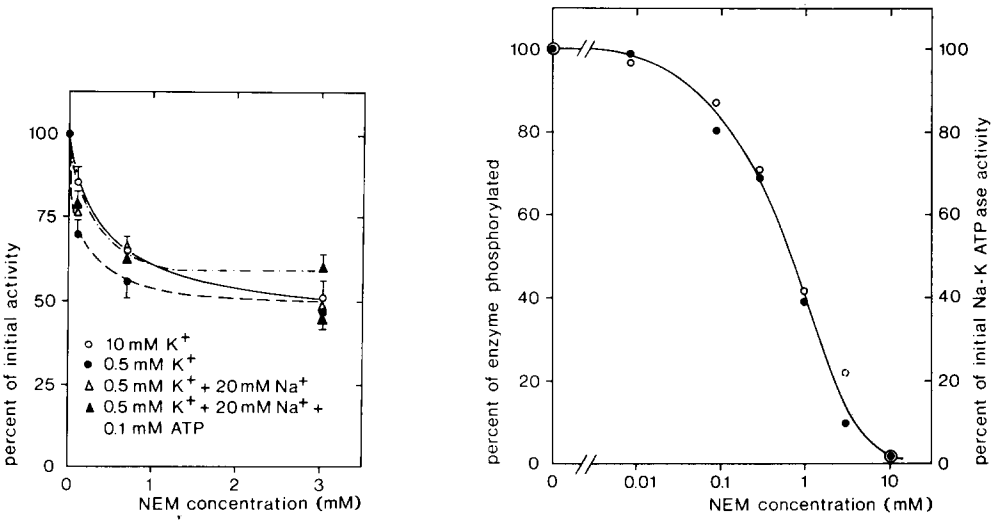

Fig. 5. Influence of *N*-ethylmaleimide on variously determined K^+ stimulated 4-nitrophenylphosphatase activity. Preincubation with *N*-ethylmaleimide (NEM) was carried out for 30 min at 37°C in media containing 10 μg protein · ml^{-1}, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5) and *N*-ethylmaleimide at the indicated concentrations. The reaction was ended by adding a 5-fold molar excess of dithiothreitol. K^+-stimulated 4-nitrophenylphosphatase (K-pNPPase) activity was determined as described in Materials and Methods, in the presence of 10 mM KCl (○) or 0.5 mM KCl (●) or 0.5 mM KCl plus 20 mM NaCl (△) or 0.5 mM KCl plus 20 mM NaCl plus 0.1 mM ATP (▲). The 4-nitrophenylphosphatase activities are presented as percent of initial activity.

Fig. 6. *N*-ethylmaleimide inhibition of phosphorylation by ATP and of $(Na^+ + K^+)$-ATPase activity. Preincubation with *N*-ethylmaleimide (NEM) was carried out for 30 min at 37°C in a medium containing 200 μg protein . ml^{-1}, 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5) and *N*-ethylmaleimide at indicated concentrations. After stopping the reaction, the amount of enzyme phosphorylated by ATP at 0°C (○) and $(Na^+ + K^+)$-ATPase activity (●) were determined as described in Materials and Methods.

Effects of N-ethylmaleimide on phosphorylation

$(Na^+ + K^+)$-ATPase preparations can be phosphorylated by ATP in the presence of Mg^{2+} and Na^+ ions. As Fig. 6 shows, the degree of phosphorylation and the $(Na^+ + K^+)$-ATPase activity are decreased to the same extent after preincu-

TABLE II

EFFECT OF *N*-ETHYLMALEIMIDE ON Na^+-STIMULATED ATPase ACTIVITY

The preincubation of the enzyme was carried out in medium containing: 2 mM CDTA, 25 mM imidazole · HCl (pH 7.5), 10 μg protein · ml^{-1} and *N*-ethylmaleimide at a concentration as indicated, for 30 min at 37°C. After stopping the preincubation by addition of dithioerythritol to a final concentration of 10 mM, aliquots were assayed for Na^+-stimulated ATPase and $(Na^+ + K^+)$-ATPase activity (radioactive procedure), as described in Materials and Methods. Results from 2 experiments carried out in duplicate are presented as means with standard error of the mean.

Concentration of *N*-ethylmaleimide during preincubation (mM)	Percent of initial activity		
	Na^+ stimulated ATPase		$(Na^+ + K^+)$-ATPase
	— KCl	+ 10 mM KCl	
0	≡100	55 ± 1	≡100
2	32 ± 2	13 ± 4	33 ± 3

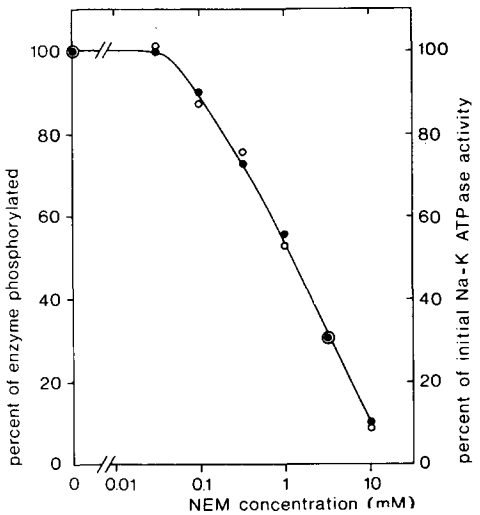

Fig. 7. *N*-ethylmaleimide inhibition of phosphorylation by P_i in the preseence of ouabain and of $(Na^+ + K^+)$-ATPase activity. Preincubation with *N*-ethylmaleimide (NEM) was carried out as described in the legend of Fig. 6. Thereafter aliquots were taken for ouabain treatment and subsequent phosphorylation by 50 μM P_i (○) and for $(Na^+ + K^+)$-ATPase assay, as described in Materials and Methods.

bation with various concentrations of *N*-ethylmaleimide. This is true, whether the phosphorylation is performed at 0°C (Fig. 6) or at 37°C (results not shown).

The enzyme can also be phosphorylated by P_i. In previous studies with crude enzyme preparations, preincubation with ouabain and Mg^{2+} was necessary to obtain appreciable phosphorylation. In our highly purified preparations phosphorylation by P_i in the absence of ouabain was not much less than that in its presence. This phosphorylation reaction is also inhibited by prior reaction with *N*-ethylmaleimide, and again to about the same extent as the $(Na^+ + K^+)$-ATPase

TABLE III

EFFECT OF *N*-ETHYLMALEIMIDE TREATMENT ON PHOSPHORYLATION BY INORGANIC PHOSPHATE

Treatment with *N*-ethylmaleimide (NEM) was carried out in a medium containing 0.7 mM *N*-ethylmaleimide, 25 mM imidazole · HCl (pH 7.5), 200 μg protein · ml^{-1}, 2 mM CDTA, for 30 min at 37°C. The *N*-ethylmaleimide reaction was ended by addition of excess dithioerythritol. Ouabain treatment was carried out as described in Materials and Methods. After treatment with *N*-ethylmaleimide $(Na^+ + K^+)$-ATPase activity and phosphorylation capacity at varying P_i concentrations (1—100 μM) were determined as described in Materials and Methods.

	With ouabain treatment	Without ouabain treatment
$(Na^+ + K^+)$-ATPase activity, remaining after NEM treatment	45%	72%
Maximum amount of P_i binding sites remaining after NEM treatment	60%	73%
K_{diss} of the enzyme — P_i complex,		
a. without NEM treatment	21 μM	36 μM
b. after NEM treatment	18 μM	31 μM

activity. Inhibition of the two activities as a function of the *N*-ethylmaleimide concentration is shown in Fig. 7. In other experiments the phosphorylation has been determined as a function of the P_i concentration. From these data the dissociation constant of the phosphate-enzyme complex was calculated by means of a Scatchard plot. It appears that the dissocation constant is not changed by prior treatment with *N*-ethylmaleimide, but that the number of binding sites is reduced (Table III).

Discussion

The experiments reported in this paper clearly indicate that the purified $(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla is inhibited by pretreatment with *N*-ethylmaleimide, and that this inhibition is antagonized by nucleotides and 4-nitrophenylphosphate under conditions excluding phosphorylation. Our study further shows that the partial reactions are also inhibited, and that their inhibition runs entirely parallel with that of the overall activity.

The latter finding is in contrast with several previous reports. Our results confirm the parallel inhibition of $(Na^+ + K^+)$-ATPase and K^+-stimulated 4-nitrophenylphosphatase activity observed by Fujita et al. [15]. The main discrepancy between our results and those previously reported concerns the parallelism of inhibition by *N*-ethylmaleimide of $(Na^+ + K^+)$-ATPase activity and enzyme phosphorylation by ATP [7,10,11] and P_i [13,14]. Two possible explanations for this discrepancy occur to us. First, in nearly all previous studies rather crude enzyme preparations have been used. This means that phosphorylation experiments may have given appreciable errors due to contamination with other proteins which can be phosphorylated by ATP. Secondly, the purified preparation may differ from the crude ones with respect to accessibility of groups reacting with *N*-ethylmaleimide. There are suggestions [8] that more than one group may react with *N*-ethylmaleimide with parallel loss of enzyme activity, so different groups may have been reacting in the various studies. This may explain why Patzelt-Wenczler et al. [11] find less inhibition of phosphorylation by ATP than of $(Na^+ + K^+)$-ATPase activity after treatment with *N*-ethylmaleimide, but equal inhibition of both parameters after treatment with another sulfhydryl reagent, viz. 5,5′ dithiobis-(2-nitrobenzoic acid).

Another problem is the uncertainty about the nature of the group which reacts with *N*-ethylmaleimide. Previous authors, reporting inhibition of $(Na^+ + K^+)$-ATPase activity by treatment with *N*-ethylmaleimide, have simply assumed that reaction with a sulfhydryl group is responsible. Our finding that inhibition of $(Na^+ + K^+)$-ATPase activity also occurs at pH values below 7 may indicate that *N*-ethylmaleimide reacts with sulfhydryl groups, since reaction with amino groups will mainly take place at pH values above 8.0 [24]. However, reaction of *N*-ethylmaleimide with amino acid or protein amino groups can occur at a pH as low as 7.0 [26,27,28]. Since in most studies *N*-ethylmaleimide treatment has been carried out in the pH range 7.0—7.5, there is no absolute certainty about the nature of the functional group(s) reacting with *N*-ethylmaleimide.

The conclusion of Fahn et al. [7] that the main effect of *N*-ethylmaleimide would be the inhibition of the conversion of an ADP-sensitive (E_1-P) to a K^+-sensitive phosphorylated intermediate (E_2-P) is not supported by our results. Their conclusion conflicts with the parallel inhibition of the overall $(Na^+ + K^+)$-

ATPase reaction and of the binding of ATP [18], of the phosphorylation by ATP and of the sodium-activated ATPase activity, which are all reactions preceding the formation of an E_1-P intermediate.

Our results lead to a different explanation for the effects of *N*-ethylmaleimide. We propose that this substance reacts progressively with one or more vital functional groups of the enzyme, thereby completely abolishing the activity of an increasing number of enzyme molecules. Support for this assumption is derived from (1) the strong parallelism in inhibition of the partial reactions and the overall reaction of the enzyme system, (2) the lack of change in the parameters of the remaining enzyme activity (K_{diss} for the enzyme · ATP complex [18] and for the enzyme · P_i complex, stimulation by 20 mM Na^+ with or without 0.1 mM ATP of the K^+-stimulated 4-nitrophenylphosphatase activity at 0.5 mM K^+).

This vital functional group could be located either inside or outside the catalytic center of the enzyme system. The first possibility is favored by the protective effects of ATP and its analogs and of 4-nitrophenylphosphate, and also by the finding that phosphorylation by ATP increases the number of mol *N*-ethylmaleimide bound per mol (Na^++K^+)-ATPase [29]. On the other hand, substances which appear to react with a group inside the catalytic center (sulfhydryl reactive ATP analogues, [11]; 2,3-butanedione, [30]) inhibit K^+-stimulated 4-nitrophenylphosphatase activity less than the (Na^++K^+)-ATPase activity, whereas *N*-ethylmaleimide inhibits these two activities in parallel.

Favoring a location of the vital functional group outside the catalytic center is the following consideration. Evidence has accumulated that the (Na^++K^+)-ATPase molecule has two catalytic centers with different affinities for ATP [31,32]. The center with high affinity is thought to be responsible for the Na^+-stimulated ATPase activity, while the low-affinity center would catalyse the K^+-stimulated 4-nitrophenylphosphatase reaction, whereas the overall reaction would involve both centers [31]. This model rules out parallel inhibition of partial and overall reactions upon reaction with a vital functional group of either one of the catalytic centers. More likely, reaction with such a group outside the catalytic centers could affect both catalytic centers equally by modification of the interaction of the subunits. The parallel protective effects of nucleotides and 4-nitrophenylphosphate could be similar to that of ATP against inactivation by sodium dodecyl sulfate (Fig. 2 in ref. 19), either by stabilization of the active enzyme molecule or by an induced conformational change [33].

The strictly parallel inhibition of the partial reactions and the overall reaction of the (Na^++K^+)-ATPase system seriously limits the usefulness of *N*-ethylmaleimide for the elucidation of the reaction mechanism of this enzyme system. However, the finding that the inhibition by *N*-ethylmaleimide is influenced by the kinds and concentrations of the ligands present may be useful in studies of the various conformational states of the enzyme.

Acknowledgements

Our thanks are due to Dr. P.L. Jørgensen for his advice concerning the purification of (Na^++K^+)-ATPase and to Miss S. de Vries for her excellent technical assistance.

References

1 Bonting, S.L. (1970) in Membranes and Ion Transport (Bittar, E.E., ed.), Vol. 1, pp. 257—286, Wiley-Interscience, London
2 Dahl, J.L. and Hokin, L.E. (1974) Annu. Rev. Biochem. 43, 327—356
3 Jørgensen, P.L. (1975) Quart. Rev. Biophys. 7, 239—274
4 Schwartz, A. Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacological Rev. 27, 3—134
5 Glynn, I.M., and Karlish, S.J.D. (1975) Annu. Rev. Physiol. 37, 13—55
6 Skou, J.C. and Hilberg, C. (1965) Biochim. Biophys. Acta 110, 359—369
7 Fahn, S., Hurley, M.R., Koval, G.J., and Albers, R.W. (1966) J. Biol. Chem. 241, 1890—1895
8 Banerjee, S.P., Wong, S.M.E., Khanna, V.K. and Sen, A.K. (1972) Mol. Pharmacol. 8, 8—17
9 Banerjee, S.P., Wong, S.M.E., and Sen, A.K. (1972) Mol. Pharmacol. 8, 18—29
10 Fahn, S., Koval, G.J., and Albers, R.W. (1968) J. Biol. Chem. 243, 1993—2002
11 Patzelt-Wenczler, R., Pauls, H., Erdmann, E. and Schoner, W. (1975) Eur. J. Biochem. 53, 301—311
12 Post, R.L., Kume, S., Tobin, T., Orcutt, B. and Sen, A.K. (1965) J. Gen. Physiol. 54, 306s—326s
13 Hegyvary, C. (1976) Biochim. Biophys. Acta 422, 365—379
14 Siegel, G.J., Koval, G.J., and Albers, R.W. (1969) J. Biol. Chem. 244, 3264—3269
15 Fujita, M., Nakao, T., Tashima, Y., Mizumo, N., Nagano, K., and Nakao, M. (1966) Biochim. Biophys. Acta 117, 42—53
16 Klodos, I., and Skou, J.C. (1975) Biochim. Biophys. Acta 391, 474—485
17 Robinson, J.D. (1970) Arch. Biochem. Biophys. 139, 164—171
18 Nørby, J.G. and Jensen, J. (1974) Ann. N.Y. Acad. Sci. 242, 158—167
19 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52
20 Neufeld, A.H. and Levy, H.M. (1969) J. Biol. Chem. 244, 6493—6497
21 Skou, J.C. and Hilberg, C. (1969) Biochim. Biophys. Acta 185, 198—219
22 Schuurmans Stekhoven, F.M.A.H., van Heeswijk, M.P.E., de Pont, J.J.H.H.M. and Bonting, S.L. (1976) Biochim. Biophys. Acta 422, 210—224
23 Robinson, J.D. (1971) Biochem. Biophys. Res. Commun. 42, 880—885
24 Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, pp. 110—114, Holden-Day Inc., San Francisco
25 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530—6540
26 Smyth, D.G., Nagamatsu, A., and Fruton, J.S. (1960) J. Am. Chem. Soc. 82, 4600—4604
27 Smyth, D.G., Blumenfeld, O.O. and Konigsberg, W. (1964) Biochem. J. 91, 589—595
28 Guidotti, G. and Konigsberg, W. (1964) J. Biol. Chem. 239, 1474—1484
29 Hart, W.M. and Titus, E.O. (1973) J. Biol. Chem. 248, 4674—4681
30 de Pont, J.J.H.H.M., Schoot, B.M., van Prooyen-van Eeden, A. and Bonting, S.L. (1977) Biochim. Biophys. Acta 213—227
31 Robinson, J.D. (1976) Biochim. Biophys. Acta 429, 1006—1019
32 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. 256, 465—496
33 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 246—257