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Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase by Ethacrynic Acid: Two Modes of Action

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SUMMARY

Ethacrynic acid inhibited sodium- and potassium-dependent adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3) by affecting two different steps of enzyme turnover. First, ethacrynic acid blocked phosphorylation of (Na^+ + K^+)-ATPase with insignificant effect upon the dephosphorylation step when the Na^+:K^+ ratio was 10. It also prevented the ADP-ATP exchange reaction. The reduction in specific activity of the enzyme by ethacrynic acid was closely correlated with the degree of inhibition of phosphorylation and ADP-ATP exchange. Second, ethacrynic acid stabilized the spontaneous disappearance of the phosphorylated intermediate and slightly decreased the apparent affinity for K^+. The rate of decay of the phosphorylated intermediate in the presence of ADP was not significantly affected by ethacrynic acid treatment. The decrease in apparent affinity for K^+ could not be observed in an assay medium with an Na^+:K^+ ratio of 4. When the concentrations of Na^+ and K^+ were changed to those found in extracellular fluid, ethacrynic acid-treated enzyme showed a 20–30% decrease in specific activity as compared to the usual assay system. The inhibition of phosphorylation by ethacrynic acid appears to be of significance for inhibition of enzyme activity *in vitro*, while the stabilization of the phosphorylated intermediate may be involved in drug-induced diuresis.

INTRODUCTION

There is extensive evidence that sodium- and potassium-dependent adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3) plays a major role in the transport of monovalent cations across cell membranes (1–4). Although the transport enzyme is found in the microsomal fraction of kidney homogenates (1, 5), its involvement in the active reabsorption of sodium in the renal tubule has not been firmly established. There is, however, some circumstantial evidence which implicates (Na^+ + K^+)-ATPase in the conservation of salt and water. (a) Cardenolides, which are specific inhibitors of (Na^+ + K^+)-ATPase, reduce the renal reabsorption of sodium (6–10). (b) Both the enzymatic inhibition and natriuretic effect of cardiac glycosides can be reversed by potassium (11–13). (c) Perfusion of amphibian kidneys with potassium-free solutions greatly reduces sodium absorption while increasing the excretion of sodium in the urine (14). (d) When renal reabsorption of sodium is chronically increased or diminished, the renal (Na^+ + K^+)-ATPase changes in an adaptive way (15). (e) A positive correlation exists between natriuresis and inhibition of renal (Na^+ + K^+)-ATPase by ouabain (16). (f) (Na^+ + K^+)-ATPase in the distal tubule is 4–5 times more active than in the proximal segment (17). This cor-

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relates well with the possible site of action of ethacrynic acid and mercurials at the ascending limb of Henle's loop (18–20).

The possible function of $(Na^+ + K^+)$-ATPase in sodium reabsorption by the renal tubule may be elucidated by exploring its role in drug-induced diuresis. Inhibition of $(Na^+ + K^+)$-ATPase *in vitro* by thiol-reactive natriuretics such as mercurials (21–23) and ethacrynic acid (24) has been demonstrated. Although diuretic as well as nondiuretic mercurials inhibited rat renal $(Na^+ + K^+)$-ATPase *in vitro*, only the natriuretic mercurials effectively inhibited the enzyme when injected into the whole animal (25). Furthermore, the maximal diuresis produced by ethacrynic acid or mercurial diuretics could not be enhanced by ouabain, and natriuresis induced by injection of ouabain was not further increased by thiol-reactive diuretics (26). Moreover, prior administration of nondiuretic mercurials did not affect ouabain-induced natriuresis (26). These facts suggest that the receptors for thiol-reactive diuretics and ouabain may be the same.

There is some experimental evidence which indicates that diuresis induced by ethacrynic acid and mercurials may not be mediated by inhibition of $(Na^+ + K^+)$-ATPase. Diuretic and nondiuretic sulfhydryl-reactive agents inhibit dog kidney $(Na^+ + K^+)$-ATPase *in vitro*, but none of these drugs affects the enzyme activity when administered *in vivo*. Conversely, although administration of ethacrynic acid *in vivo* inhibits the rat kidney microsomal $(Na^+ + K^+)$-ATPase (27), it does not cause natriuresis in rats (27–30). The binding of ethacrynic acid to the membrane fraction of dog kidney is 1000–2000 times less than the binding required for 50% inhibition of the enzyme activity (24, 26).

The conflict regarding the role of $(Na^+ + K^+)$-ATPase in ethacrynic acid- and mercurial-induced natriuresis (31) results from limited knowledge of the mechanism of inhibition of cation transport by sulfhydryl-reactive agents at the molecular level. Cation transport is believed to involve at least two steps (32). The initial step consists of the Na^+-induced phosphorylation of the enzyme by ATP in the presence of Mg^{++}, followed by a K^+-induced dephosphorylation. If the concentration of Mg^{++} is reduced sufficiently below the levels that are optimal for the over-all reaction, it is possible to demonstrate an Na^+-dependent ADP-ATP transphosphorylation (33, 34). In this investigation ethacrynic acid has been used as a tool representative of thiol-reactive diuretics to study the differences in the modes of action of various sulfhydryl-reactive agents on the inhibition of $(Na^+ + K^+)$-ATPase and to distinguish between the actions of diuretic and nondiuretic compounds. The effects of ethacrynic acid on the formation, breakdown, and stability of the phosphorylated intermediate of $(Na^+ + K^+)$-ATPase have been studied. The significance of these effects for a possible inhibitory role of ethacrynic acid against $(Na^+ + K^+)$-ATPase in the pharmacological action of the drug is discussed.

MATERIALS AND METHODS

Materials. $(Na^+ + K^+)$-ATPase from guinea pig kidney and $(\gamma\text{-}^{32}P)$-ATP were prepared as described by Post and Sen (35, 36). The enzyme preparation was stored at 4° in a solution containing 10 mM imidazole and 0.1 mM H_4EDTA, with the pH adjusted to 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 cleaves 1 μmole of ATP per minute at 37°. Inorganic ^{32}P was obtained from Tracerlab, Waltham, Mass. Universally labeled ^{14}C-ADP was purchased from Schwarz BioResearch as the trilithium salt; specific activity was about 25 mCi/mmole. Ouabain and the disodium salts of ATP and ADP were obtained from Sigma. The sodium salts of the nucleotides were converted to Tris salts by ion exchange chromatography. Ethacrynic acid was a gift from Dr. John E. Baer, Merck Institute for Therapeutic Research, West Point, Pa. The stock solution of ethacrynic acid was prepared as follows: 100 mM ethacrynic acid solution was made by adding 303 mg of ethacrynic acid and 121.2 mg of Tris to 10 ml of deionized water. The resulting solution

had a pH of 7 ± 0.1 and was used within 15 min of its preparation.

Unless otherwise indicated, ethacrynic acid treatment was carried out as follows. Enzyme with a protein concentration of 1.2–2.5 mg/ml was treated with a particular concentration of ethacrynic acid, with or without added ligands, for different intervals of time at 37°. The enzyme was then washed three times with 10 mM imidazole–glycylglycine buffer, pH 7.4 ± 0.1, to remove any bound drug or added ligands. It was resuspended in the same buffer solution, and the specific activity, phosphorylation activity, and transphosphorylation activity were determined.

Phosphorylation. The reaction mixture contained 0.4 mM $MgCl_2$, 16 mM NaCl, and 0.04 mM (γ-^{32}P)-ATP besides 10 mM imidazole–glycylglycine buffer, pH 7.4 ± 0.1. Each tube contained 1.0 ml of reaction mixture with enzyme containing 0.4–0.8 mg of protein. The reaction was started with (γ-^{32}P)-ATP and was terminated with 5% trichloracetic acid at 10 sec. The amount of phosphorylated intermediate formed was determined by previously described methods (32, 37). Total phosphate was estimated by a modification of the method of Bartlett (38), and protein by the method of Lowry *et al.* (39).

To estimate dephosphorylation, the procedure was the same as described for phosphorylation except that 1.6 mM KCl was added 10 sec after the (γ-^{32}P)-ATP and the reaction was stopped with acid 10 sec later.

The specific activity of the enzyme was determined by the method described previously (35).

Transphosphorylation. The method was a slight modification of the one described by Fahn *et al.* (33). The ^{14}C-nucleoside diphosphate solution was dried at room temperature under nitrogen, and carrier nucleotide in the form of the Tris salt was added to obtain a stock solution of about 12 mCi/mmole. The incubation medium consisted of 4.0 mM Tris-ATP, 1 mM Tris-^{14}C-ADP, 0.4 mM $MgCl_2$ ± 32 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°. The reaction was stopped by placing the tubes in a boiling water bath for 2 min, and 0.1 ml of the reaction mixture was removed for protein determination. Then the tubes were centrifuged at 33,000 × *g* for 20 min and placed in an ice bath, where they remained until the samples were chromatographed. Aliquots of 50 µl of each supernatant were applied on No. 3MM Whatman chromatographic paper, along with nucleotide standards. Separation was achieved by descending chromatography overnight (about 16 hr) in a solvent system consisting of isobutyric acid–ammonia (concentrated)–water in a ratio of 66:1:33 by volume. On the next day the papers were dried in air and the nucleotides were identified by short-wavelength ultraviolet light. Paper strips were cut and counted in 10 ml of scintillation medium (300 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 5 g of 2,5-diphenyloxazole in a total of 1 liter of toluene) in a Packard Tri-Carb liquid scintillation spectrometer. The microsomal enzyme fraction obtained from guinea pig kidney contained adenylate kinase. Therefore, for each exchange experiment with or without Na^+, adenylate kinase activity was determined by adopting the same procedure as described above except that 4.0 mM Tris-ATP was omitted from the incubation medium. The Na^+-stimulated net exchange was calculated by subtracting that found in the absence of Na^+ and Tris-ATP from that obtained in the presence of Na^+ and ATP. The Na^+-stimulated exchange was about 75% of the total ^{14}C-ADP–ATP exchange.

RESULTS

Inhibition of (Na^+ + K^+)-ATPase by ethacrynic acid. Duggan and Noll (24) were the first to report inhibition of (Na^+ + K^+)-ATPase of guinea pig kidney cortex by ethacrynic acid. They found that the K_i for ethacrynic acid was 0.2 mM. However, Nechay *et al.* (26) reported that at concentrations "approaching the limits of solubility" of ethacrynic acid, i.e., 0.5–1.0 mM, only 50% of the (Na^+ + K^+)-dependent portion of enzyme activity was inhibited. Since Nechay *et al.* (26) did not study the

effects of prior incubation with ethacrynic acid upon enzyme activity, we decided to plot the time curve for ethacrynic acid inhibition of (Na^+ + K^+)-ATPase. Results are shown in Fig. 1. The enzyme activity could be completely inhibited by 1.1 mM ethacrynic acid when it was incubated with the drug for 80 min.

The protein concentration in the above experiment was low (12.5 μg/ml) and unsuitable for studying the effect of ethacrynic acid on the phosphorylation and dephosphorylation reactions. When the protein concentration was raised to between 1.2 and 2.5 mg/ml in the enzyme suspension, 1.1 mM ethacrynic acid was unable to inhibit the enzyme activity following incubation for 90 min. It was of interest, therefore, to study the effect of protein concentration upon ethacrynic acid-induced inhibition of (Na^+ + K^+)-ATPase. Results are shown in Fig. 2. As the ratio of concentration of the drug to protein was increased by lowering the protein concentration, there was a more rapid inhibition of enzyme activity. Under these conditions, 50% inhibition of the enzymatic activity was obtained with a protein concentration of 0.118 mg/ml. This seems to indicate that nonspecific binding of ethacrynic acid is high.

Effect of ethacrynic acid on phosphorylation and dephosphorylation steps of (Na^+ + K^+)-ATPase. Since transport of Na^+ and K^+ involves Na^+-induced phosphorylation and K^+-induced dephosphorylation (32), the effects of ethacrynic acid on these two steps were examined. Figure 3 shows the results of a simultaneous study of enzymes treated with ethacrynic acid for different intervals of time, with respect to specific activity of ATP hydrolysis and Na^+-induced phosphorylation. There was good correlation between these two parameters, indicating that inhibition of (Na^+ + K^+)-ATPase by ethacrynic acid may be mediated by blocking the phosphorylation step.

N-Ethylmaleimide, a sulfhydryl inhibitor like ethacrynic acid, has been shown to block dephosphorylation without any effect on phosphorylation (40). Therefore, we studied the phosphorylation, dephosphoryla-

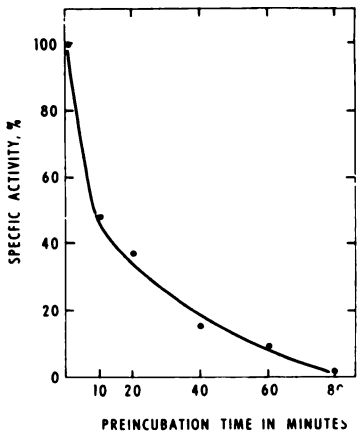


FIG. 1. *Time curve for inhibition of enzyme turnover by ethacrynic acid*

The reaction mixture contained 1.1 mM ethacrynic acid and 12.5 μg of protein. The incubation was carried out at 37°. At the times indicated, the enzyme activity was tested by transferring 0.1 ml of the treated enzyme suspension to 1.0 ml of solution containing 3.3 mM $MgCl_2$ and 3.8 mM disodium ATP, together with either 83.3 mM NaCl and 20.8 mM KCl or 0.25 mM ouabain. Enzymatic assay was carried out by the method of Post and Sen (35).

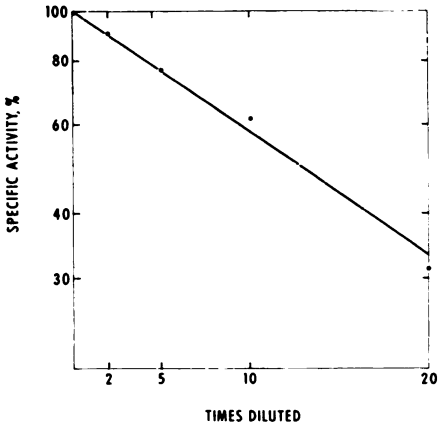


FIG. 2. *Effect of protein concentration upon ethacrynic acid-induced inhibition of (Na^+ + K^+)-ATPase*

The protein concentration of the original enzyme was 1.5 mg/ml. This was diluted as indicated, and each dilution was treated with 10 mM ethacrynic acid. The enzyme was incubated for 1 hr and then washed three times with 10 mM imidazole-glycylglycine buffer in order to remove unbound drug. The enzyme was resuspended in 10 mM imidazole–glycylglycine buffer, pH 7.4 ± 0.1, and its specific activity was determined.

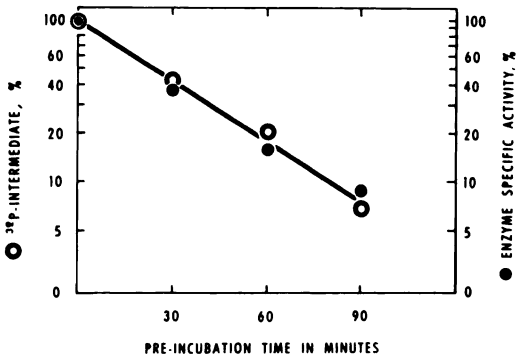


FIG. 3. *Correlation between ethacrynic acid inhibition of enzyme turnover and of phosphorylation*

The enzyme was incubated with 15 mM ethacrynic acid for the time intervals indicated and then washed free of unbound ethacrynic acid and resuspended as described in MATERIALS AND METHODS. Phosphorylation was carried out at 2° by the method described in the text.

tion, and specific activity of the enzyme treated with ethacrynic acid. Table 1 shows that although there is a good correlation between inhibition of specific activity and phosphorylation, there is no significant effect of ethacrynic acid treatment on K^+-induced dephosphorylation of the enzyme. Ethacrynic acid, therefore, reacts with the enzyme to block the phosphorylation step, with probably little effect on the dephosphorylation step.

Effect of ethacrynic acid on ^{14}C-ADP–ATP exchange. The initial step of Na^+ and K^+ transport appears to be reversible and, in the presence of Na^+, Mg^{++}, ATP, and ^{14}C-ADP, it is possible to obtain synthesis of ^{14}C-ATP, as has been shown with electroplax enzyme (33, 34) and briefly reported for the guinea pig kidney microsomal preparation (41). To confirm the effect of ethacrynic acid upon the phosphorylation step, its effect on the ADP–ATP exchange reaction was examined. The results (Fig. 4) show good correlation between inhibition of ADP–ATP exchange and specific activity.

Effect of ethacrynic acid on stability of phosphorylated intermediate. The results described thus far seem to indicate that ethacrynic acid specifically inhibits the phosphorylation step without affecting the K^+-induced dephosphorylation. However, Nechay *et al.* (26) have reported that ethacrynic acid-induced inhibition of (Na^+ + K^+)-ATPase could be reduced by increasing the concentration of K^+. Furthermore, *N*-ethylmaleimide has been shown to stabilize a phospho-enzyme form with reduced sensitivity to dephosphorylation by K^+ (40). It was therefore of interest to investigate the stability of the phosphorylated intermediate of (Na^+ + K^+)-ATPase treated with ethacrynic acid. Results are shown in Fig. 5. After phosphorylation was blocked by addition of 10 mM EDTA, the ^{32}P-intermediate of the untreated enzyme decayed rapidly. In contrast, marked stability was exhibited by the ethacrynic acid-treated enzyme.

Although the spontaneous disappearance of the ^{32}P-intermediate of the ethacrynic acid-treated enzyme was greatly reduced, rapid dephosphorylation could be produced by addition of 10 mM EDTA and 1.6 mM KCl (Fig. 6).

TABLE 1

Effect of ethacrynic acid on phosphorylation, dephosphorylation, and specific activity of (Na^+ + K^+)-ATPase

Incubation time with 15 mM ethacrynic acid	Specific activity	Inhibition of specific activity	^{32}P incorporated in absence of K^+	Inhibition of ^{32}P incorporation	^{31}P present following addition of K^+	Block of K^+-induced dephosphorylation
min	*μmoles P_i/mg protein/hr*	%	*pmoles/mg protein*	%	*pmoles/mg protein*	%
Control	126		239		43	
30	102	18	223	7	62	10
60	82	35	134	44	43	0
90	46	63	85	65	35	0

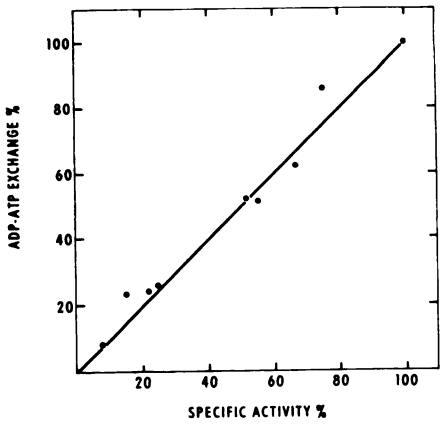


FIG. 4. *Comparison of sodium-stimulated ADP-ATP exchange and specific activity of ethacrynic acid-treated enzymes*

Enzyme preparations were treated with 15 mM ethacrynic acid for different intervals of time (up to 45 min) at 37° to obtain 50% or less inhibition of activity. Since Na^+ enhances inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid (42), enzyme preparations inhibited more than 50% were obtained by prior treatment with 10 mM ethacrynic acid and 100 mM Na^+ for different intervals of time (up to 30 min). The Na^+-stimulated net exchange reaction in control and treated and washed enzymes was determined as described in the text. The rate of Na^+-stimulated exchange of the control enzyme was 20.0 μmoles of ^{14}C-ATP synthesized per gram of protein per minute.

Inhibition of phosphorylation and the stabilization of the ^{32}P-intermediate appeared to be two different effects of ethacrynic acid. To confirm this, an enzyme preparation was treated with 10 mM ethacrynic acid at 37° for 30 min. This washed enzyme incorporated about the same number of micromoles of ^{32}P per kilogram of protein as the untreated enzyme, and had the same specific ATPase activity. However, the treated but uninhibited enzyme exhibited marked stabilization of the ^{32}P-intermediate, which was not significantly different from that shown by an enzyme that was 50% inhibited by ethacrynic acid (Fig. 6). This indicates that ethacrynic acid initially stabilized the phospho-enzyme complex, following which inhibition of phosphorylation resulted in blockade of the specific activity.

Effect of ADP on phosphorylated intermediate of $(Na^+ + K^+)$-ATPase treated with ethacrynic acid. $(Na^+ + K^+)$-ATPase treated with *N*-ethylmaleimide produces a phosphorylated intermediate with decreased sensitivity to K^+ and markedly increased susceptibility to dephosphorylation by ADP (34, 40, 41). We therefore tested the effect of ADP upon the ^{32}P-intermediate of the ethacrynic acid-treated enzyme. Results are shown in Fig. 7. ADP did not significantly alter the rate of dephosphorylation of the ethacrynic acid-treated enzyme. This indicates that ethacrynic acid stabilizes a potassium-sensitive phosphorylated intermediate.

Sensitivity of ^{32}P-intermediate of ethacrynic acid-treated enzyme to low concentrations of K^+. Close examination of Fig. 6 shows that although K^+ dephosphorylated the ^{32}P-intermediate of $(Na^+ + K^+)$-ATPase treated with ethacrynic acid, the rate of decay was slower than that of an untreated enzyme preparation (41). This suggested

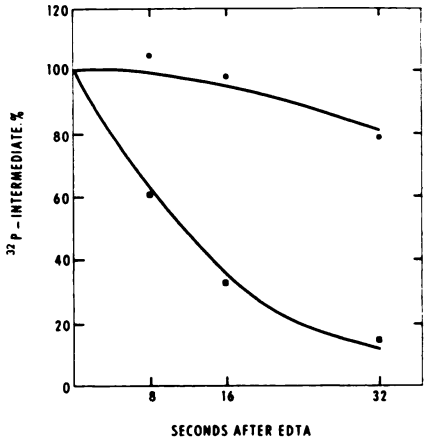


FIG. 5. *Stabilization of phosphorylated intermediate by ethacrynic acid*

The reaction mixture for the control enzyme (■——■) or ethacrynic acid-treated enzyme (●——●) contained 16 mM NaCl and 0.4 mM $MgCl_2$. The reaction was started 10 sec before zero time with 0.04 mM ^{32}P-ATP. At zero time, 10 mM $(Tris)_4$ EDTA was added to chelate the free Mg^{++} and block further phosphorylation. The reaction was stopped with acid at times indicated. The control enzyme had a specific activity of 276 μmoles of P_i per milligram of protein per hour and incorporated 320 μmoles of ^{32}P per kilogram of protein. The ethacrynic acid-treated enzyme was 50% inhibited.

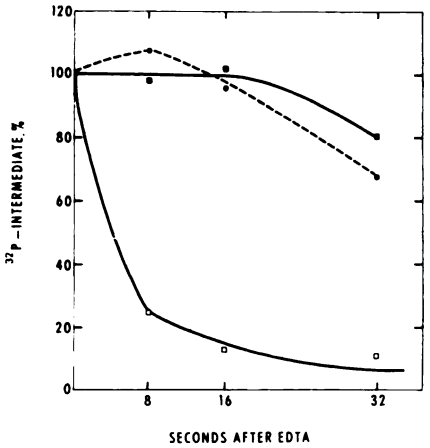


FIG. 6. *Effect of K^+ on stability of phosphorylated intermediate of ethacrynic acid-treated enzyme*

The enzyme was treated with either 15 mM (■——■, □——□) or 10 mM (●- - -●) ethacrynic acid at 37° for ½ hr (as described for Fig. 2). The reaction mixture was the same as for Fig. 5. At zero time 10 mM (Tris)$_4$ EDTA (■——■, ●- - -●) or 10 mM (Tris)$_4$ EDTA plus 1.6 mM KCl (□——□) was added. The reaction was stopped with acid at the times indicated. The specific activities of the untreated enzyme and of enzymes treated with 10 mM and 15 mM ethacrynic acid were 147, 141.5, and 73.2 μmoles of P_i per milligram of protein per hour, respectively.

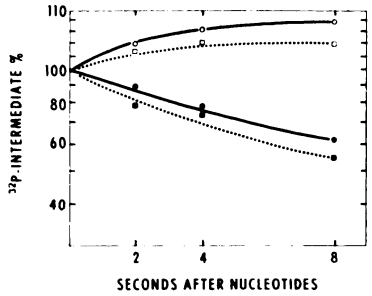


FIG. 7. *Sensitivity of phosphorylated intermediate of ethacrynic acid-treated enzyme to ADP*

The reaction mixture for the control enzyme (●——●, ■-----■) and ethacrynic acid-treated enzyme (○——○, □-----□) contained 16 mM NaCl and 0.4 mM $MgCl_2$. The reaction was started 10 sec before zero time with 0.04 mM ^{32}P-ATP. At zero time the radioactive ATP was diluted with a 50-fold excess of unlabeled ATP either with (□-----□, ■-----■) or without (○——○, ●——●) 5 mM ADP. The reaction was stopped with acid at the times indicated. The ethacrynic acid-treated enzyme was uninhibited.

that ethacrynic acid probably reduces the apparent affinity for K^+. The effect of low concentrations of K^+ on the phosphorylated intermediate of the ethacrynic acid-treated enzyme is shown in Fig. 8. While 0.04 mM K^+ slowly dephosphorylated an untreated enzyme, ethacrynic acid treatment completely prevented dephosphorylation for at least 8 sec. Even in the presence of 0.1 mM K^+, slightly slower disappearance of the ^{32}P-labeled intermediate occurred with the ethacrynic acid-treated enzyme. These findings indicate a shift in the apparent affinity of the phosphorylated intermediate for K^+.

The pulse-labeling technique of Post *et al.* (41) offered a convenient method of demonstrating simultaneously the lack of effect of low K^+ concentration on the phosphorylation step and the decreased sensitivity of ethacrynic acid-treated enzyme to dephosphorylation. Results are shown in Fig. 9. The rate of dephosphorylation in the presence of 0.04 mM K^+ was markedly slower with ethacrynic acid-treated en-

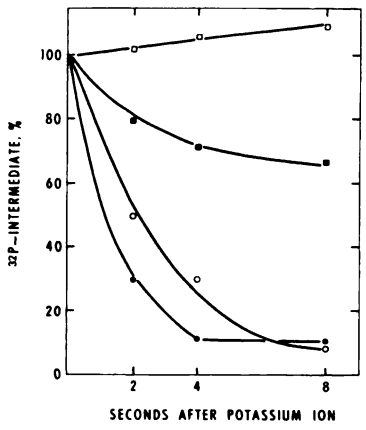


FIG. 8. *Sensitivity of phosphorylated intermediate of ethacrynic acid-treated enzyme to various concentrations of K^+*

The experimental procedure was similar to that described in Fig. 7 for both control enzyme (■——■, ●——●) and ethacrynic acid-treated enzyme (□——□, ○——○), except that for unlabeled ATP, either 0.04 mM (■——■, □——□) or 0.1 mM (●——●, ○——○) KCl was added. The reaction was then stopped at the indicated times with acid. The ethacrynic acid-treated enzymes were uninhibited.

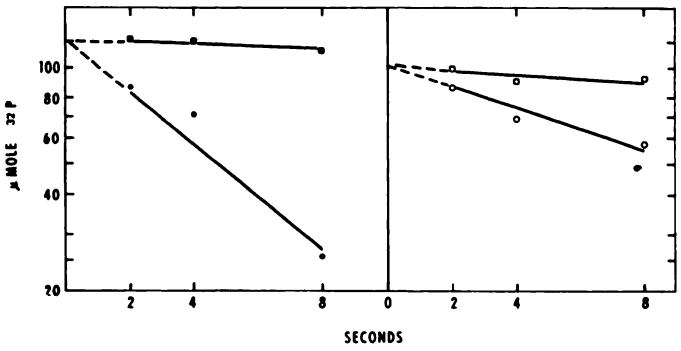


FIG. 9. *Effect of K+ on formation and breakdown of phosphorylated intermediate*

The initial reaction mixture for both control enzyme (■——■, ●——●) and ethacrynic acid-treated enzyme (□——□, ○——○) contained 1.0 mM (Tris)$_4$ EDTA to trap traces of Mg^{++} in the enzyme preparation, as well as 16 mM NaCl and 0.04 mM ^{32}P-ATP. The reaction was started by a solution containing 2 mM unlabeled (Tris)$_4$ ATP and 1.6 mM $MgCl_2$ and was stopped at the indicated times with acid. When present, 0.04 mM K^+ (●——●, ○——○) was included in the initial reaction mixture. The data points shown by squares were obtained in the absence of potassium.

zyme as compared to control (Na^+ + K^+)-ATPase. This confirms the decreased apparent affinity of ethacrynic acid-treated enzyme for K^+.

Specific activity of ethacrynic acid-treated enzyme at physiological concentrations of Na^+ and K^+. The ratio of the concentrations of Na^+ and K^+ in the assay medium for (Na^+ + K^+)-ATPase varies from 4 to 10 in the system *in vitro* (24, 25, 43). However, in the extracellular fluid, the Na^+:K^+ ratio is about 36 (44). Since ethacrynic acid reduced the sensitivity of the enzyme to K^+, it may be a more effective inhibitor of (Na^+ + K^+)-ATPase in a physiological ionic milieu than in the routinely employed assay medium *in vitro*, where the K^+ concentration is high. Specific activities of treated and untreated enzyme in the presence of two different concentration ratios of Na^+ and K^+ are shown in Table 2. For the control enzyme there was no significant difference at the two ionic concentrations, in agreement with the findings of Kinsolving *et al.* (45). However, with ethacrynic acid-treated enzyme there was a significant increase in the inhibition of (Na^+ + K^+)-ATPase activity when the Na^+:K^+ concentration ratio was raised from 4 to 36.5. This finding suggests that ethacrynic acid may inhibit enzyme activity in the whole animal even though this is not observed in the artificial assay system *in vitro* usually employed.

DISCUSSION

A variety of thiol-reactive natriuretic agents have been shown to inhibit the renal microsomal (Na^+ + K^+)-ATPase *in vitro* (21–24). The data suggest that inhibition of this enzyme could be the mechanism whereby these drugs inhibit renal sodium transport. Establishment of (Na^+ + K^+)-ATPase as the renal receptor for natriuretic agents has been hampered, first, because nondiuretic sulfhydryl inhibitors have been shown to react with (Na^+ + K^+)-ATPase, and, second, because no correlation exists between diuresis and inhibition of (Na^+ + K^+)-ATPase when a natriuretic agent is administered *in vivo*.

Although much information is available regarding the effects of various sulfhydryl inhibitors on (Na^+ + K^+)-ATPase, few studies have been directed toward determining the precise mechanism of such inhibition of enzyme activity. Studies with (γ-^{32}P)-ATP indicate that in the presence of Na^+ and Mg^{++} radioactivity is incorporated into the enzyme protein (32). If K^+ is added after the formation of the phospho-protein, a rapid breakdown of the protein complex takes place, liberating free inorganic phosphate. These observa-

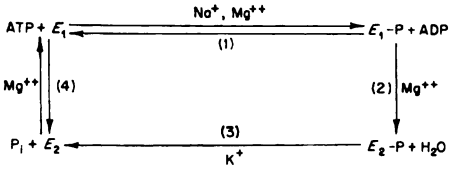
TABLE 2

Effect of Na^+:K^+ concentration ratio on ethacrynic acid-induced inhibition

Enzymes were treated at 37° with 10 mM ethacrynic acid for 30 min in experiment 1, with 15 mM ethacrynic acid for 30 min in experiment 2, or with 10 mM ethacrynic acid plus 100 mM Na^+ for 15 min in experiment 3. The treated enzymes were washed free of drug and Na^+, and specific activities of treated and untreated microsomes were determined as described in the text.

Experiment	Specific activity		
	Na^+:K^+ = 4 (A)	Na^+:K^+ = 36.25 (B)	(A − B)/A × 100
	μmoles P_i/mg protein/hr		%
Experiment 1			
Control	139	151	
Ethacrynic acid-treated	121	98	19
Experiment 2			
Control	126	125	0
Ethacrynic acid-treated	82	55	33
Experiment 3			
Control	108	116	
Ethacrynic acid-treated	39	29	26

tions led Post and Sen (46) to propose a two-step reaction scheme for the breakdown of ATP. If the magnesium ion concentration is reduced sufficiently below the levels optimal for the over-all reaction, it is possible to demonstrate a sodium-dependent ADP-ATP transphosphorylation (33, 34). Furthermore, treatment of the enzyme with *N*-ethylmaleimide, which does not block sodium-induced phosphorylation, greatly stimulates ADP-ATP exchange (34, 40). These observations led Albers and his associates (33, 47) to propose the following multistage reaction scheme (see also ref. 41). Ouabain, the well-known inhibitor of (Na^+ + K^+)-ATPase, probably acts at step 3 (37, 48, 49), while *N*-ethylmaleimide and oligomycin prevent transformation of E_1-P to E_2-P (40, 50, 51). Very little is known about the site of action of other inhibitors of (Na^+ + K^+)-ATPase, including diuretics.



Besides inhibiting (Na^+ + K^+)-ATPase *in vivo* (27), ethacrynic acid has been shown to inhibit Na^+ transport across several non-renal epithelial tissues, such as small intestine of hamster (52), rat (53), and rabbit (54) and isolated toad urinary bladder (55), and to inhibit Na^+ efflux from human erythrocytes (56). Furthermore, Chez *et al.* (54) have recently demonstrated inhibition of linked sodium and potassium transport in rabbit ileal mucosa. In the present work, ethacrynic acid was found to block sodium-stimulated phosphorylation to a degree which correlated well with the inhibition of specific activity (Fig. 3). When the Na^+:K^+ concentration ratio was 10, ethacrynic acid did not block potassium-induced dephosphorlyation (Table 1). This indicates that ethacrynic acid acts at step 1 in the above scheme. This is further supported by the observed inhibition of the ADP-ATP exchange reaction in ethacrynic acid-treated enzyme, which paralleled the inhibition of specific activity (Fig. 4).

Although both *N*-ethylmaleimide and ethacrynic acid are sulfhydryl inhibitors, their primary sites of action appear to be different. Whereas ethacrynic acid inhibits formation of the phosphorylated intermediate, *N*-ethylmaleimide permits sodium-induced phosphorylation but prevents the conversion of a potassium-insensitive intermediate to a potassium-sensitive one. However, *N*-ethylmaleimide seems to react at an additional site, and under certain conditions it is possible to demonstrate inhibition of phosphorylation and exchange by *N*-ethylmaleimide (42, 57).

The site of action of ethacrynic acid at step 1 appears to be different from that of ouabain. This is further substantiated by studying the binding of ^{3}H-ouabain to ethacrynic acid-treated enzyme (58). Enzyme preparations which were inhibited 50% or more by ethacrynic acid bound ^{3}H-

ouabain as well as untreated $(Na^+ + K^+)$-ATPase.

So far we have discussed one mode of inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid. This seems to be of importance in the inhibition of enzyme activity only *in vitro*. The concentration of ethacrynic acid employed to inhibit $(Na^+ + K^+)$-ATPase is much higher than doses required to induce natriuresis (24, 26), and there appears to be a considerable amount of nonspecific binding (Fig. 2). Therefore, unless the kidney has some special mechanism for transporting or concentrating ethacrynic acid at the receptor site, inhibition of phosphorylation is an unlikely biochemical mechanism of production of diuresis by this drug.

Ethacrynic acid has been found to stabilize the phosphorylated intermediate by markedly slowing down the spontaneous breakdown of E_2-P (Fig. 5) and by decreasing its susceptibility to low concentrations of K^+ (Figs. 8 and 9). Since ADP did not dephosphorylate the ethacrynic acid-treated enzyme significantly faster than untreated enzyme (Fig. 7), the stabilized form must have been E_2-P rather than E_1-P. Therefore, the second mode by which ethacrynic acid inhibits $(Na^+ + K^+)$-ATPase appears to be by slowing down dephosphorylation by K^+ (step 3). Not only is this site of action of ethacrynic acid identical with that of ouabain, but there are other common features. Both these inhibitors stabilize E_2-P (37), and their degree of inhibition of dephosphorylation is affected by the concentration of K^+ (59). There are, however, some differences between these two drugs. While the stabilization of E_2-P by ethacrynic acid is a little more marked than by ouabain, its sensitivity to K^+ is less affected. This latter effect probably makes ethacrynic acid a much weaker inhibitor of $(Na^+ + K^+)$-ATPase as compared to ouabain.

Of the two modes of inhibition of $(Na^+ + K^+)$-ATPase by ethacrynic acid, viz. stabilization of the phosphorylated intermediate and inhibition of its formation, the former precedes the latter (Fig. 6). Stabilization of E_2-P seems to us of immense pharmacological significance. If this type of inhibition were achieved in the whole animal, no decrease in $(Na^+ + K^+)$-ATPase activity would be observed *in vitro* at $Na^+:K^+$ concentration ratios of 4–10, which have been employed by most previous workers. However, the enzyme may turn over more slowly *in vivo*, since the $Na^+:K^+$ ratio in the extracellular fluid is 36, and under these conditions inhibition by ethacrynic acid may be more readily observed (Table 2). This point is currently under investigation.

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