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OCCURRENCE AND CHARACTERISTICS OF ^{18}O-EXCHANGE REACTIONS CATALYZED BY SODIUM- AND POTASSIUM-DEPENDENT ADENOSINE TRIPHOSPHATASES*

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Running Title: ^{18}O EXCHANGE REACTIONS OF (Na^+, K^+)-ATPases

SUMMARY

Microsomal preparations of the (Na^+, K^+)-ATPase from porcine outer medulla and electroplax catalyzed a rapid Mg^{2+}- and K^+-dependent exchange of water oxygens with inorganic phosphate in the absence of ATP or ADP. Exchange activity was unaffected by uncouplers and inhibitors of oxidative phosphorylation but was inhibited by Na^+, ouabain, N,N'-dicyclohexylcarbodiimide, or p-mercuribenzoate. No nucleotide requirement for the exchange could be demonstrated.

Addition of ATP to a Na^+-inhibited system resulted in an exchange of oxygens of medium P_i concomitant with ATP hydrolysis. This ATP-induced exchange amounted to 1.5 oxygen atoms per P_i released. Neither ADP nor adenylyl methylene diphosphonate would serve to activate the exchange.

Treatment of the preparations with deoxycholate apparently uncoupled the medium $P_i \rightleftarrows HOH$ exchange but was without affect on the ATP-induced exchange.

The data suggest that the exchange arises by partial reversal of a late step in ATP hydrolysis by the (Na^+, K^+)-ATPase.

INTRODUCTION

A sodium- and potassium-dependent adenosine triphosphatase, intimately associated with cell membranes, is believed to be responsible for the coupled active transport of sodium and potassium and of certain organic solutes across eucaryotic cell membranes (1,2). The mechanism by which energy from ATP is coupled to transport by this specialized ATPase is not understood (3).

Several ATP-associated energy-transducing systems in which the hydrolytic cleavage of ATP is coupled to an energy-requiring process, as in contractile systems, or in which ATP is formed by elimination of water from P_i and ADP, as in oxidative and photosynthetic phosphorylation, have been found to catalyze oxygen exchanges between substrates and H_2O. Recognition that energy-coupling mechanisms within energy-transducing systems may have similarities of molecular design provides impetus to search for mechanistic analogies among specialized ATPases, oxidative and photosynthetic phosphorylation, and myosin-actomyosin contractile systems. Measurement of oxygen exchange reactions and the fate of phosphate oxygens with these systems has given insights into their interactions with ATP, P_i and H_2O (4-8).

The studies reported here serve to document the occurrence and properties of rapid oxygen-18 exchange reactions catalyzed by the membrane-bound (Na^+,K^+)-ATPase isolated from two diverse sources, the outer medulla of porcine kidney and the electroplax of the electric eel. Preliminary presentations of some of this data have appeared (9).

I. EXPERIMENTAL PROCEDURES

Materials

Electrophorus electricus was obtained from World Wide Aquarium Traders, Marina del Rey, Los Angeles, California. $^{32}P_i$ from various commercial sources was purified before use as described by DeLuca et al. (10). Normalized water of approximately 1.4 atom % excess ^{18}O was obtained from Yeda Research and Development Company, Rehovoth, Israel. Oligomycin was purchased from the Wisconsin Alumni Research Foundation, Madison, Wisconsin. 5-Chloro-3-t-butyl,2'-chloro-4'-nitrosalicylanilide (S-13) was the gift of Monsanto

Chemicals. Carbonyl cyanide m-chlorophenylhydrazone (m-CCCP) was obtained from DuPont Laboratories. Ouabain, L-Histidine, sodium deoxycholate, and Tris-base were purchased from Sigma Chemicals. Imidazole was purchased from Eastman and was recrystallized from acetone. Disodium adenosine triphosphate was obtained from Boehringer and was converted to the Tris form before use. N,N'-Dicyclohexylcarbodiimide was purchased from Aldrich Chemical Company and was dissolved in absolute ethanol to give a concentration of 10 mM. Adenylyl methylene diphosphonate was the gift of Dr. Roberts A. Smith.

Methods

Enzyme isolation and assay. (Na^+,K^+)-dependent ATPase from porcine kidney was prepared by modification of the method of Jørgensen and Skou (11). All procedures were carried out at 4°C unless otherwise noted. Tissue from the dark red outer medulla was obtained by dissection on a chilled glass plate of longitudinal bisections of fresh porcine kidneys. The tissue was suspended in a 10-fold volume of 0.03 M histidine, 0.25 M sucrose (pH 7.2, 20°C) and homogenized in a Waring blender for one minute. The homogenate was filtered through gauze and centrifuged at 7,000 x g for 15 minutes. The resultant pellet was homogenized in one-third the original volume of buffer and was recentrifuged. The two supernatants were combined and spun at 42,000 x g for 30 minutes. The supernatant was decanted. In order to lower the endogenous K^+,Na^+ and P_i concentrations, the remaining pellet was resuspended in a 5-fold volume of fresh buffer and was recentrifuged at 42,000 x g for 30 minutes. The pellet was suspended in buffer to a concentration of 20-30 mg per ml and was frozen in isopropanol-dry ice and stored at -20°C. The ATPase preparation was stable for several months under these

conditions. Approximately 85 mg of microsomes were obtained per kidney. The specific activity of the (Na^+,K^+)-ATPase was 30-100 units per mg protein; ouabain-insensitive ATPase was routinely 5-8 fold lower.

(Na^+,K^+)-ATPase from electroplax was isolated from freshly killed eels by the method of Albers et al. (12), with the following exceptions. The supernatants resulting from the 9,000 x g centrifugation were spun at 42,000 x g for one hour. The 42,000 x g pellet was suspended in 0.05 M Tris (pH 7.5, 20°C) and was spun again at 42,000 x g to lower endogenous K^+,Na^+ and P_i. The specific activity of the enzyme was 250-450 units per mg of protein; ouabain insensitive ATPase was routinely 10-20 fold lower.

Protein was determined by the method of Lowry et al. (13) after precipitation and wash with 5% TCA at 0-4°C.

For total ATPase activity, the enzyme assay contained 140 mM NaCl, 20 mM KCl, 3 mM Tris-ATP, 5 mM $MgCl_2$, 30 mM histidine (pH 7.5, 37°C), and an appropriate amount of the microsomal fraction in a total volume of 1.00 ml. The reaction was commenced by addition of enzyme to the assay components equilibrated at 37°C. After 10-20% hydrolysis of ATP, the reaction was quenched by the addition of 1.0 ml 1.5% ammonium molybdate in 1.0 N H_2SO_4. Inorganic phosphate was extracted into isobutyl alcohol-benzene and was determined by the method of Martin and Doty (14). Hydrolysis of ATP in the acid molybdate was negligible in the time of assay at 0°C. In some experiments release of ^{32}P from ATP-γ-^{32}P was employed for determination of ATPase activity. In these instances aliquots of the isobutyl alcohol-benzene phase were transferred to planchets, evaporated and the ^{32}P counted. ATP-γ-^{32}P was prepared essentially as described by Post and Sen (15) with the exception that it was purified by the DEAE-bicarbonate chromatographic

procedure of Ramaley et al. (16).

To correct for Mg^{2+}-dependent adenosine triphosphatase activity, samples were assayed in the presence of 1 mM ouabain by adding 100 µl of a 10 mM solution to selected tubes. ATPase specific activities were expressed as micromoles of P_i released per milligram of membrane protein per hour, and (Na^+, K^+)-ATPase activity was expressed as the difference between total and Mg^{2+}-ATPase activities.

Determination of $P_i \rightleftarrows HOH$ Exchange. Reaction mixtures were prepared essentially as outlined in Table I. The incubation medium was brought to 37°C, unless otherwise stated, and the reaction was initiated by the addition of the microsomal protein. The reaction was quenched by the addition of cold perchloric acid to a final concentration of 0.4 M. Denatured protein was removed by a ten minute centrifugation at 2,000 x g. The resulting supernatant was extracted with an equal volume of isobutanol-benzene (1:1) and was centrifuged. The organic phase was discarded, and the aqueous layer was made 1 N in H^+ with 12 N HCl and 0.012 M in ammonium molybdate. Phosphate was extracted into isobutanol-benzene and was further purified as described by Chaney and Boyer (17). ^{18}O was determined by the guanidine-HCl procedure as described by Boyer and Bryan (18).

Calculation of the P_i Oxygen Exchange. In the absence of adenosine triphosphate hydrolysis, the methods described by Boyer and Bryan (18) were employed.

In the presence of adenosine triphosphate hydrolysis, calculations were made based on the following equations as described by Dempsey et al. (5) where P = mM P_i released from ATP; P_o = initial mM P_i; $^{18}O_1$ and $^{18}O_2$ = initial and final atom % excess ^{18}O in the P_i; $^{18}O_w$ = atom % excess ^{18}O in H_2O.

When $P_i{}^{18}O$ and $H_2{}^{16}O$ are present, the following approximates total exchange from average values of excess ^{18}O in the P_i:

$$\frac{P_o{}^{18}O_1}{P + P_o} - {}^{18}O_2 = \text{atom \% excess } {}^{18}O \text{ lost by exchange} \quad (1)$$

$$\frac{\text{atom \% excess } {}^{18}O \text{ lost by exchange}}{{}^{18}O_1} = \text{fraction of exchange } (F_{ex}) \quad (2)$$

$$4 \text{ (average } P_i \text{ concentration) } \ln (1-F_{ex}) = \text{mM } P_i \rightleftarrows \text{HOH} \quad (3)$$

$$(\text{mM } P_i \rightleftarrows \text{HOH exchange}) \text{ (ml volume)} = \mu\text{atoms O exchange} \quad (4)$$

When $P_i{}^{16}O$ and $H_2{}^{18}O$ are present, the atom % excess ^{18}O of the final P_i introduced by exchange is given by

$${}^{18}O_2 - P\,\frac{{}^{18}O_W}{4(P_o + P)} = \text{atom \% excess introduced by exchange} \quad (5)$$

$$\frac{\text{atom \% excess introduced by exchange}}{{}^{18}O_W} = \text{fraction exchange } (F_{ex}) \quad (6)$$

Millimolarity of $P_i \rightleftarrows$ HOH exchange and μatoms total exchange may be calculated according to equations 3 and 4.

Determination of the $P_i \rightleftarrows$ ATP and ATP $\rightleftarrows$ HOH Exchanges. The $P_i \rightleftarrows$ ATP exchange was determined by use of ^{32}P with separation of P_i from adenine nucleotides by extraction of the phosphomolybdate complex (19). ^{18}O in the ATP was determined by adsorption of nucleotides on charcoal followed by acid hydrolysis to liberate the β- and γ-phosphoryl groups and measurement of the ^{18}O in the P_i formed (20).

II. RESULTS

Effects of Na^+ and K^+ on the $P_i \rightleftarrows$ HOH Exchange. In the absence of added ADP or ATP and in the presence of K^+ and Mg^{2+}, the membrane-bound

(Na^+,K^+)-ATPase will catalyze the rapid exchange of oxygen between water and inorganic phosphate (Table I). The $P_i \rightleftarrows HOH$ exchange rates were about 20 fold the rate of ouabain-sensitive ATPase activity. The exchange was reduced to less than 4% of the original level when analyzed in the presence of 140 mM Na^+. A small amount of exchange was present in the absence of added K^+, which might be accounted for by endogenous K^+ (21,22) or a low level of inorganic pyrophosphatase (23) in the medullar preparation. When both Na^+ and K^+ were present under conditions as for Table I, the exchanges were reduced to 2% and 16% for the electroplax and medullar preparations, respectively.

Mg^{2+} Requirement. Exchange of phosphate oxygens with water required the presence of Mg^{2+} (Table II). EGTA, which does not chelate appreciable Mg^{2+} under the experimental conditions, slightly activated the exchange indicating probable removal of inhibitory heavy metal ions. KF at 0.3 mM produced only slight inhibition.

A Mg^{2+} requirement for the $P_i \rightleftarrows HOH$ exchange has been established in submitochondrial particles by Mitchell et al. (24), in myosin and actomyosin by Dempsey and Boyer (25) and in heavy meromyosin by Yount and Koshland (26).

Effect of Ouabain. Ouabain, a cardiotonic steroid which produces a specific and stoichiometric inhibition of the Na^+,K^+-pump and the (Na^+,K^+)-ATPase (2), produced 92-97% inhibition of the exchange (Table II and III). Inhibition as high as 99% has been obtained in some cases, suggesting that the exchange reflects a catalytic capacity of the (Na^+,K^+)-ATPase.

Effects of Sulfhydryl Reagents. Data presented in Table II indicate that reagents which attack sulfhydryl groups produce varying effects on the $P_i \rightleftarrows HOH$ exchange. Millimolar concentrations of the haloacetates appear to

activate whereas 26 μM p-mercuribenzoate inactivates the exchange, suggesting the possible involvement of more than one type of -SH group in the exchange process. Differential responses towards sulfhydryl reagents have been noted in the contractile ATPases; p-mercuribenzoate at micromolar concentrations was found to activate myosin, actomyosin and heavy meromyosin ATPase activity whereas higher concentrations produced inactivation (27-31). Reaction of the contractile ATPases as well as the (Na^+,K^+)-ATPase with N-ethylmaleimide has given similar responses (31,32). p-Mercuribenzoate is also known to inhibit oxidative phosphorylation and to decrease the prominent $P_i \rightleftarrows HOH$ exchange (28).

Effect of Hydroxylamine. Interestingly, hydroxylamine activated the exchange (Table II). As will be emphasized later, the ATPase can be phosphorylated by inorganic phosphate to form an acyl phosphate intermediate, and it is probably through this intermediate that the $P_i \rightleftarrows HOH$ exchange proceeds. The native acyl phosphate derived from ATP or P_i is not susceptible to hydroxylaminolysis whereas the trichloroacetic acid-denatured protein-bound acyl phosphate is susceptible (33,34). The lack of inhibition of hydroxylamine on the $P_i \rightleftarrows HOH$ exchange agrees well with these previous results obtained on ATPase activity. Activation of the exchange may be due to the fact that hydroxylamine serves as a partial substitute for K^+ in the (Na^+,K^+)-ATPase (35).

Effects of Uncouplers and Inhibitors of Oxidative Phosphorylation. Data in Table III demonstrate that these compounds do not inhibit the microsomal exchange reaction under conditions where the mitochondrial exchange would be nearly fully inhibited. Subtle differential effects of activation and inactivation are produced by 2,4-dinitrophenol and oligomycin

on the medullar and electroplax enzymes, respectively, and are similar to results obtained by Dempsey (36) on 2,4-dinitrophenol inhibition and activation of actomyosin and myosin, respectively; these results may reflect differences in protein conformation or accessibility to catalytic or regulatory sites.

The inhibitory effects of oligomycin on the (Na^+, K^+)-ATPase are reversed on addition of purified phospholipids (37), and thus the differential responses between the two preparations may reflect a qualitative or quantitative difference in phospholipid content. 2,4-Dinitrophenol has been reported to have no affect on a detergent-NaI treated preparation of the (Na^+, K^+)-ATPase (38) or the electroplax enzyme (39) whereas oligomycin is a potent inhibitor of the enzyme (40).

Concentration and Time Dependency of the Exchange Reaction. The oxygen exchange was found to be linear with time and its rate a function of pH, with a broad optimum from pH 6.3 to 7.3. The apparent K_m for P_i was estimated to be 6 mM using the medullar preparation, and 1 mM using the electroplax preparation. The apparent K_m's for K^+ and Mg^{2+} were likewise estimated to be 0.89 and 0.69 mM using the electroplax preparation.

The relation of P_i concentration to the $P_i \rightleftarrows HOH$ exchange in other systems has received only limited attention. Dempsey et al. have noted a high apparent K_m of 50 mM for the medium exchange catalyzed by actomyosin. A precise K_m value for the medium exchange value catalyzed by heavy meromyosin has not been determined but an upper limit of 10^{-4} M has been assigned (30).

Lack of Nucleotide Requirement for the $P_i \rightleftarrows HOH$ Exchange. The requirement of the $P_i \rightleftarrows HOH$ exchange for nucleotides has been shown previously for several energy-transducing systems. An apparent absolute requirement of

ADP for the $P_i \rightleftarrows HOH$ exchange of mitochondrial oxidative phosphorylation has been established by Jones and Boyer (41). The pronounced stimulation of the $P_i \rightleftarrows HOH$ exchange during net photophosphorylation by adenine nucleotides, as noted by Shavit et al. (20), has been interpreted to indicate that such a requirement exists. In addition, a nucleotide requirement for the medium $P_i \rightleftarrows HOH$ of myosin, natural and reconstituted actomyosin and heavy meromyosin has been reported by several workers (5,30).

Results of the measurement of effects of added ADP on the $P_i \rightleftarrows HOH$ exchange catalyzed by a (Na^+,K^+)-ATPase preparation, which has been previously depleted by endogenous nucleotides by an ion-exchange procedure, are given in Table IV. The expected decrease in the extent of exchange due to Mg^{2+} chelation by ADP is also included. The data show that added ADP does not activate the exchange and that there is no apparent requirement of ADP for the exchange. Slight inhibition by 5 mM ADP was noted with the native preparation whereas prior treatment with the ion-exchange resin apparently sensitized the enzyme to ADP thereby producing 50% inhibition of exchange. This may be interpreted as indicating that ion-exchange treatment removed a non-inhibitory, firmly-bound anion at the ADP site thus allowing ADP entry, binding and inhibition.

Lack of $P_i \rightleftarrows ATP$ and $ATP \rightleftarrows HOH$ Exchanges. The data in Table V show the relative rates of the $P_i \rightleftarrows ATP$ and the $ATP \rightleftarrows HOH$ exchanges occurring during ATP hydrolysis compared to the rate of the $P_i \rightleftarrows HOH$ in the absence of added ATP. The (Na^+,K^+)-ATPase does not catalyze a $P_i \rightleftarrows ATP$ exchange under the conditions employed for ATP cleavage, in agreement with previous reports (42,43). In addition, the enzyme does not catalyze an $ATP \rightleftarrows HOH$ exchange. The enzyme will, however, catalyze an $ADP \rightleftarrows ATP$ exchange (2,3,43).

$P_i \rightleftarrows$ HOH Exchange during ATP Cleavage. Hydrolytic cleavage of ATP to form ADP and P_i must result in the incorporation of at least one oxygen atom into the ADP or P_i; all incorporation appears to be in the P_i. Data shown in Fig. 1 indicate that there is an appreciable, relatively constant incorporation of ^{18}O from $H^{18}OH$ into P_i in excess of that expected for ATP hydrolysis. Figure 2 shows that this extra incorporation is linear with ATP hydrolysis and shows no lag period.

It was previously noted in Table I that Na^+, at a concentration optimal for (Na^+,K^+)-ATPase activity and in the absence of added ATP, severely inhibited the medullar $P_i \rightleftarrows$ HOH exchange. Table VI demonstrates that the exchange is inhibited by Na^+ and ouabain and that an extra incorporation of ^{18}O occurs during the course of ATP hydrolysis. This extra incorporation represents either apparent reversal of Na^+ inhibition by ATP with restoral of the medium exchange, or it represents a new exchange which arises solely during ATP cleavage.

The extra exchange of H_2O oxygen into phosphate during ATP cleavage by myosin and actomyosin has been previously recognized to be of two types--that occurring preferentially with P_i formed from cleavage of ATP, designated as "intermediate exchange," and that occurring with P_i of the reaction medium, designated as "medium exchange" (4). Results from experiments designed to assess whether the ATP-dependent exchange was medium or intermediate exchange are presented in Table VI. Measurements were made of the comparative amount of exchange as determined by the loss of ^{18}O from ^{18}O-labeled P_i and the gain of ^{18}O into P_i from ^{18}O-labeled water. Exchange occurring with a phosphoryl group, with P_i of the medium, or with P_i released from ATP would result in incorporation of ^{18}O from water into P_i. In contrast,

exchange as measured by loss of ^{18}O from ^{18}O-labeled P_i would reflect only exchange with P_i of the medium. Line 4 of Table VI indicates within the limits of experimental error, that the gain of exygen from water balances the loss of oxygen from P_i and that the observed exchange may be accounted for as "medium exchange"-- hereafter referred to as the ATP-dependent medium exchange. As noted in Table VI the ATP-dependent medium exchange is also inhibited by ouabain.

Further results presented in Table VII indicate that neither adenylyl methylene diphosphonate nor ADP can induce the exchange and suggest that ATP cleavage may be necessary for restoration of the exchange.

Effect of Deoxycholate on the Medium and ATP-dependent Medium Exchange. An apparent uncoupling of the medium exchange reaction from the (Na^+, K^+)-ATPase activity was observed following prior treatment of the enzyme with varying amounts of deoxycholate. As can be seen in Fig. 3a, the exchange capacity fell precipitously with 50% inhibition produced at 0.021% deoxycholate whereas ATPase activity was activated at even higher deoxycholate levels. Characteristically, brain and kidney ATPases are activated by low concentrations of deoxycholate (1).

However, in marked contrast to the ability of the detergent to inhibit the exchange at a very low Na^+ concentration, the ATP-dependent medium exchange, depicted in Fig. 3b, which was measured at a high Na^+ concentration optimal for ATPase activity, was relatively insensitive to deoxycholate and appeared to parallel the response shown by ATPase activity.

DCCD Inhibition of the Exchange Reaction and the ATPase. Carbodiimides are known to act as potent inhibiters of membrane ATPase systems presumably involved in energy transduction processes (44-49). Carbodiimides also

inactivate the (Na^+,K^+)-ATPase as well as the K^+-dependent p-nitrophenyl phosphatase, a reaction believed to represent the terminal dephosphorylation stage of ATP hydrolysis by the (Na^+,K^+)-ATPase (3). Incubation of the electroplax (Na^+,K^+)-ATPase with 0.5 mM DCCD (0.53 μmoles/mg protein) resulted in an initial stimulation of (Na^+,K^+)-ATPase activity followed by a linear progressive inactivation with a $t_{1/2}$ of about 25 minutes (Fig. 4). A stimulatory effect of DCCD towards (Na^+,K^+)-ATPase activity of erythrocyte membranes has been noted previously by Godin and Schrier (48). Data in Fig. 4 also show that the $P_i \rightleftarrows HOH$ exchange exhibited by the enzyme preparation was likewise susceptible to DCCD inhibition (0.45 mM, 0.33 μmoles/mg protein). In contrast to the ATPase activity, however, the exchange reaction exhibited an apparent biphasic response to DCCD. There is rapid 22% inhibition of the exchange capacity ($t_{1/2} > 1$ min) followed by a second slow phase of inactivation ($t_{1/2}$ about 60 min). Schoner and Schmidt (45) in previous studies on DCCD inhibition of a cerebral (Na^+,K^+)-ATPase have noted multiple temperature-dependent, reacting components. These responses may reflect subtle differences in the conformational state of the membrane fragments or accessibility to reactive membrane components. Conformational changes in the region 14-20° have been previously invoked to explain biphasic Arrhenius plots of (Na^+,K^+)-ATPase activity (50,51).

III. DISCUSSION

The results presented in this study clearly demonstrate that (Na^+,K^+)-ATPase preparations are capable of catalyzing a rapid, K^+-dependent $P_i \rightleftarrows HOH$ exchange. The nearly complete inhibition of the exchange by ouabain, a specific inhibitor of the (Na^+,K^+)-ATPase demonstrates that the exchange

is a catalytic capacity of the (Na^+,K^+)-ATPase. The lack of inhibition by uncouplers and inhibitors of oxidative phosphorylation and the particularly significant effects of K^+ and Na^+ on the exchange further serve to demonstrate the origin of the exchange.

Of particular interest is the fact that there was no demonstrable requirement of the $P_i \rightleftarrows HOH$ for ADP. Characteristics of the ADP requirement for the $P_i \rightleftarrows HOH$ of oxidative phosphorylation indicate that the exchange occurs as a result of reversal of overall formation of ATP and HOH from ADP and P_i (4). Although an ADP requirement for the $P_i \rightleftarrows HOH$ exchange of contractile ATPases has been firmly established, their apparent lack of $P_i \rightleftarrows ATP$ and $ATP \rightleftarrows HOH$ exchanges make it unlikely that the $P_i \rightleftarrows HOH$ exchange involves reversal of ATP binding and cleavage. Reversal of cleavage of firmly bound ATP remains possible. Our results on lack of ADP requirement and lack of $P_i \rightleftarrows ATP$ and $ATP \rightleftarrows HOH$ exchanges make it extremely unlikely that the $P_i \rightleftarrows HOH$ exchange of the (Na^+,K^+)-ATPase involves total reversal of ATP cleavage. The lack of an ADP requirement could be interpreted to indicate the presence of a firmly bound ADP at the catalytic site; however, there is no present evidence for a firmly bound ADP. Also, the lack of ADP stimulation of the exchange after treatment of the membrane preparation with an anion exchange resin argues against such a proposal. The ADP inhibition of the exchange after resin treatment may signify competitive inhibition or inhibition due to interaction at some regulatory site.

Ouabain apparently acts to stabilize a phosphoenzyme formed from P_i. The phosphoenzyme has been shown to be an acyl phosphate (52,53) and to be identical to that formed from ATP, p-nitrophenyl phosphate or acetyl

phosphate (54,55). The enzyme can also be phosphorylated by P_i in the absence of ouabain (9). Toda et al. (56) have also documented this latter observation and have found high levels of phosphoenzyme in the presence of K^+ and Mg^{++}, the conditions under which the $P_i \rightleftarrows HOH$ exchange is manifest. It is also noteworthy that phosphorylation of the native enzyme by P_i was inhibited by Na^+ and by added nucleotide, conditions under which the $P_i \rightleftarrows HOH$ exchange is inactivated (Tables I and VI).

Rapid mixing and quenching experiments of Kanazawa et al. (57) have established the kinetic competency of the phosphoryl enzyme as an intermediate in the hydrolysis of ATP by the (Na^+,K^+)-ATPase. Although kinetic evaluations are currently lacking, it appears likely that a covalent phosphoenzyme, an acyl phosphate, is a catalytic intermediate in the $P_i \rightleftarrows HOH$ exchange reaction. Discussion on the mechanistic origin of the exchange will be given in a future publication concerning ^{18}O incorporation into the enzyme acyl phosphate intermediate. Briefly, the mechanism is consistent with reversible elimination of water from P_i involving displacement by a carboxylate anion.

Inactivation of the $P_i \rightleftarrows HOH$ exchange by DCCD occurred to about the same extent as inactivation of the (Na^+,K^+)-ATPase activity, providing additional support for the localization of the exchange. Interestingly, the Mg^{2+}-ATPase activity associated with the electroplax and the medullar preparations is more susceptible to DCCD than the hydrolytic and exchange reactions of the (Na^+,K^+)-ATPase. Similar observations were made with the erythrocyte enzyme by Godin and Schrier (48). The apparent susceptibility of these membrane systems to carbodiimides and the resulting similar biochemical consequences possibly reflect a common structural or functional organization essential for the energy transduction properties of these membranes.

The similar mode of action of DCCD and oligomycin in mitochondria has been established by Beechy and coworkers (58). Oligomycin is a potent inhibitor of the (Na^+,K^+)-ATPase and blocks the proposed $E_1P \rightarrow E_2$-P transition (3) and is without affect on the K^+-activated acyl phosphatase (59). Since the $P_i \rightleftarrows HOH$ exchange and the K^+-activated acyl phosphatase activity were unaffected by oligomycin but were inhibited by carbodiimides, the sites of action of these two inhibitors may not be identical in the (Na^+,K^+)-ATPase. There is some evidence that oligomycin may be able to interact with the phosphatase site and, at low concentrations, partially overcome Na^+ inhibition and, at higher concentrations, block ATP reversal of Na^+ inhibition of the K^+-activated phosphatase activity (59).

Significantly, it was found in this study that Na^+ could drastically reduce the rate of the $P_i \rightleftarrows HOH$ exchange and that the exchange may be restored by the simultaneous presence of Na^+ and ATP. These observations correlate well with previous results obtained with the K^+-activated phosphatase. Na^+ or nucleotides are known to inhibit the K^+-activated phosphatase (59-62), whereas phosphatase activity is restored by the simultaneous presence of Na^+ and nucleoside triphosphates (60-63). It is not known whether phosphorylation by the nucleoside triphosphate is necessary for this effect. Phosphorylation may not be a prerequisite since reversal of Na^+ inhibition of the K^+-activated phosphatase could also be produced by nucleoside triphosphates which are cleaved slowly or not at all by the (Na^+,K^+)-ATPase. However, oligomycin can block the ATP reversal of Na^+ inhibition, suggesting perhaps that phosphorylation is necessary (59). It is possible that ATP binds at an effector site producing a conformational change restoring the catalytic component of the phosphatase site. This

proposal has been alluded to by others (3,50,61,62,64). In this vein, Robinson (65,66) has suggested that the phosphorylated enzyme may function as a more efficient phosphatase. It is also possible that binding or cleavage of ATP changes the binding affinity for Na^+. Such a process may be intimately involved with the energy-coupling reaction of transport.

Recently, tests in this laboratory of the $P_i \rightleftarrows HOH$ exchange capacity of other membrane preparations have indicated that the Ca^{2+}-activated ATPase from rabbit muscle sarcoplasmic reticulum is capable of catalyzing a similar exchange (67). The effects of Ca^{2+} and Mg^{2+} on the $P_i \rightleftarrows HOH$ exchange reaction of this ATPase are qualitatively similar to the effects of Na^+ and K^+, respectively, on the exchange reaction of the (Na^+,K^+)-ATPase. Experiments also show a nearly reciprocal relationship between Ca^{2+} inhibition of the $P_i \rightleftarrows HOH$ exchange and the Ca^{2+} stimulation of the ATPase. These and other results indicate also that reversal of a late step in ATP hydrolysis may be responsible for the exchange reaction. It has likewise been demonstrated that ATP can reverse Ca^{2+} inhibition of the exchange in a manner similar to the ATP reversal of Na^+ inhibition in the (Na^+,K^+)-ATPase. Apparent energy-dependent changes in binding affinities of the primary ions deserves closer scrutiny.

The ATP-dependent medium exchange could have arisen through a different mechanism than the medium exchange, which occurred in the presence of Mg^{2+} and K^+ alone. As noted in Table VI, analysis of the loss of ^{18}O from ^{18}O-labeled phosphate was found to balance the gain of ^{18}O from ^{18}O-labeled water into phosphate during ATP cleavage indicating that the ATP dependent exchange was also medium exchange. Consequently, under the conditions of ATP cleavage and in the presence of suboptimal concentrations

of $MgHPO_4^-$, or free Mg^{2+}, a relatively large amount of back reaction occurred. Thus the phosphoenzyme intermediate appears to be formed simultaneously from ATP as well as P_i under the conditions for maximal ATP cleavage. The lack of $P_i \rightleftarrows$ ATP and ATP $\rightleftarrows$ HOH exchanges emphasize the apparent irreversibility of the overall reaction *in vitro*.

The $P_i \rightleftarrows$ HOH exchange occurring during ATP cleavage was linear with time and showed no lag period, similar to results obtained with myosin and heavy meromyosin by Swanson, Yount and Hermann (7,30). Dempsey and Boyer (5,25) and Sartorelli *et al.* (28) have observed a lag period, however, suggesting induction of conformational changes prior to appearance of the exchange. It is of interest that neither the soluble nor the membrane-bound Mg^{2+}-ATPase of *Streptococcus faecalis* catalyzes a medium or ATP-induced medium exchange (68), perhaps reflecting the totally uncoupled state of the enzyme.

Under proper conditions the incubation of microsomal fractions from brain and kidney with deoxycholate gives rise to increases in specific activity of the (Na^+, K^+)-ATPase whereas the basal Mg^{2+}-ATPase remains unchanged or decreases (1). Skou (69) has interpreted activation of the (Na^+, K^+)-ATPase as being due to exposure of latent sites in the membrane since the molecular activity remains constant. Similar marked increases in activity were observed with the porcine outer medullar enzyme employed in this study, whereas no activation of ATPase activity was observed upon similar treatment of the electroplax enzyme.

In distinct contrast to the effect of deoxycholate on the rate of ATP cleavage, however, the medium exchange was surprisingly sensitive to the detergent. These findings may be related to the observations of

Martonosi et al. (70) on the apparent detergent-induced uncoupling of Ca^{2+} transport from Ca^{2+}-ATPase activity in sarcoplasmic reticulum vesicles. They noted that ATP-dependent transport of Ca^{2+} was drastically inhibited under conditions in which Ca^{2+}-dependent ATPase activity was activated. Herman has also noted similar differential effects of sulfhydryl group modifiers on heavy meromyosin medium exchange relative to Mn^{2+}-ATPase activity (30). Apparently low amounts of detergents or sulfhydryl modifier induce subtle structural perturbations of the protein conformation resulting in a disengagement of components necessary to effect functional aspects of the ATPases. Equally surprising in this regard was that the apparent uncoupling of the $P_i \rightleftarrows HOH$ exchange could be prevented by ATP and Na^+ as was evidenced by the relatively constant amount of ATP-induced medium exchange. Structural perturbation effects by nucleotides in the presence of Na^+ have been alluded to previously.

There are an increasing number of instances where functional aspects of ATPase systems are apparently more susceptible to disruption than ATPase activity. An example is the elimination of the characteristic oxygen and nucleotide exchange reactions of the mitochondrial and chloroplast ATP synthetases while retaining ATPase activity. Perhaps, restoration of oxygen exchange capacities in the (Na^+,K^+)-ATPase may provide pertinent insights for future attempts at functional reconstitution or may, at least, provide a useful criterion for establishing such a reconstitution.

There is additional evidence that the oxygen exchanges of energy-transducing ATPases are more closely related to the function of the system rather than the rate of ATP

hydrolysis, that is, there is a general lack of correlation between oxygen exchange capacities and hydrolysis rates whereas there is a direct correlation between exchange capacities and function. With myosin, Levy et al. (6) have made important studies on the effects of nucleotides, divalent cations, and temperature and have reached the conclusion that the extent of oxygen exchange bears no simple relationship to hydrolysis rate. With heavy meromyosin Yount and Koshland (26) have also demonstrated the lack of correlation between nucleoside triphosphate hydrolysis and ^{18}O exchanges and, in addition, showed the direct correlation between the divalent cation and nucleoside triphosphate requirements for ^{18}O exchange and contraction. More direct evidence for such a correlation comes from the observation of Dempsey et al. (5) that the extent of exchange catalyzed by glycerated muscle fibers increases with increase in tension on the fiber.

Comments should be made about the investigations of Yount and coworkers who have investigated the ability of red blood cell ghosts and a brain microsomal preparation to catalyze $P_i \rightleftarrows HOH$ exchanges (30). They concluded that exchanging intermediates similar to those in muscle contraction and oxidative phosphorylation probably are not formed in the (Na^+,K^+)-ATPase. Very low level exchanges, often zero exchange capacity and frequent ambiguous results were obtained. Their findings may find explanation in the inhibitory effects of Na^+ on the exchange. Also, the brain microsomal enzyme used in their studies was treated with 0.2% deoxycholate to elicit high ATPase activity. Results also presented in this study show that 50 and 99% inhibition of the medium $P_i \rightleftarrows HOH$ exchange was obtained upon pre-treatment with 0.02 and 0.06% deoxycholate, respectively.

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FOOTNOTES

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FIGURE 1

WATER OXYGEN INCORPORATION INTO P_i RELEASED FROM ATP
AS A FUNCTION OF ATP HYDROLYSIS

Concentrations of the reaction components were glycylglycine-imidazole (50 mM, pH 7.20), 20 mM KCl, 140 mM NaCl, 40 mM ATP, 40 mM $MgCl_2$, $H^{18}OH$ (1.022 atom percent excess), and 0.38 mg medullar (Na^+,K^+)-ATPase in a total volume of 20 ml. Each succeeding point represents values of oxygen exchange and ATP hydrolysis at increasing time after start of incubation: from left to right, points represent 4, 10, 15, 17, and 20 min.

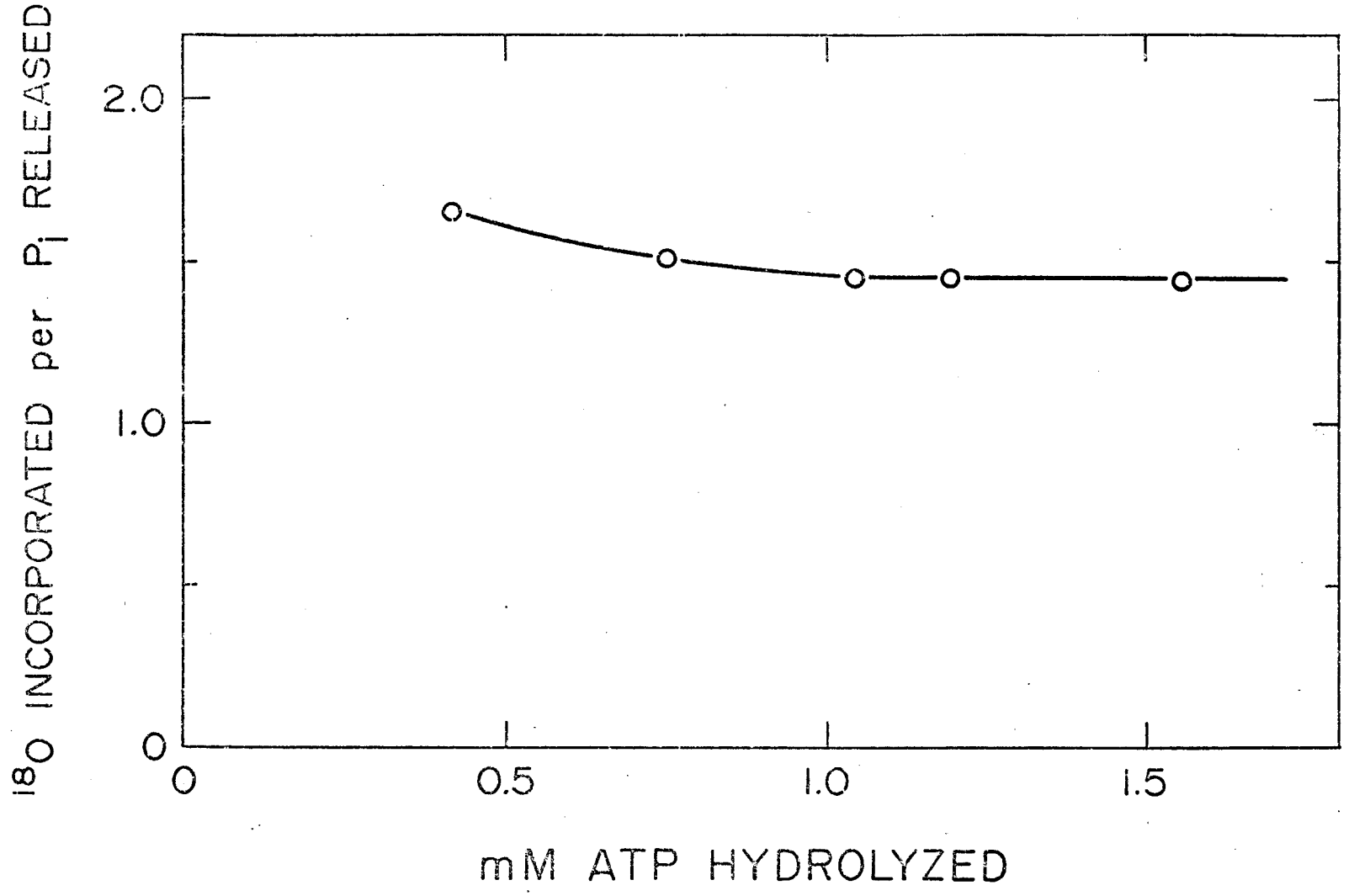
^{18}O INCORPORATED per P_i RELEASED
2.0
1.0
0
0
0.5
1.0
1.5
mM ATP HYDROLYZED

FIGURE 2

TIME COURSE OF TOTAL OXYGEN EXCHANGE AND ATP HYDROLYSIS

Conditions were as described in Fig. 1

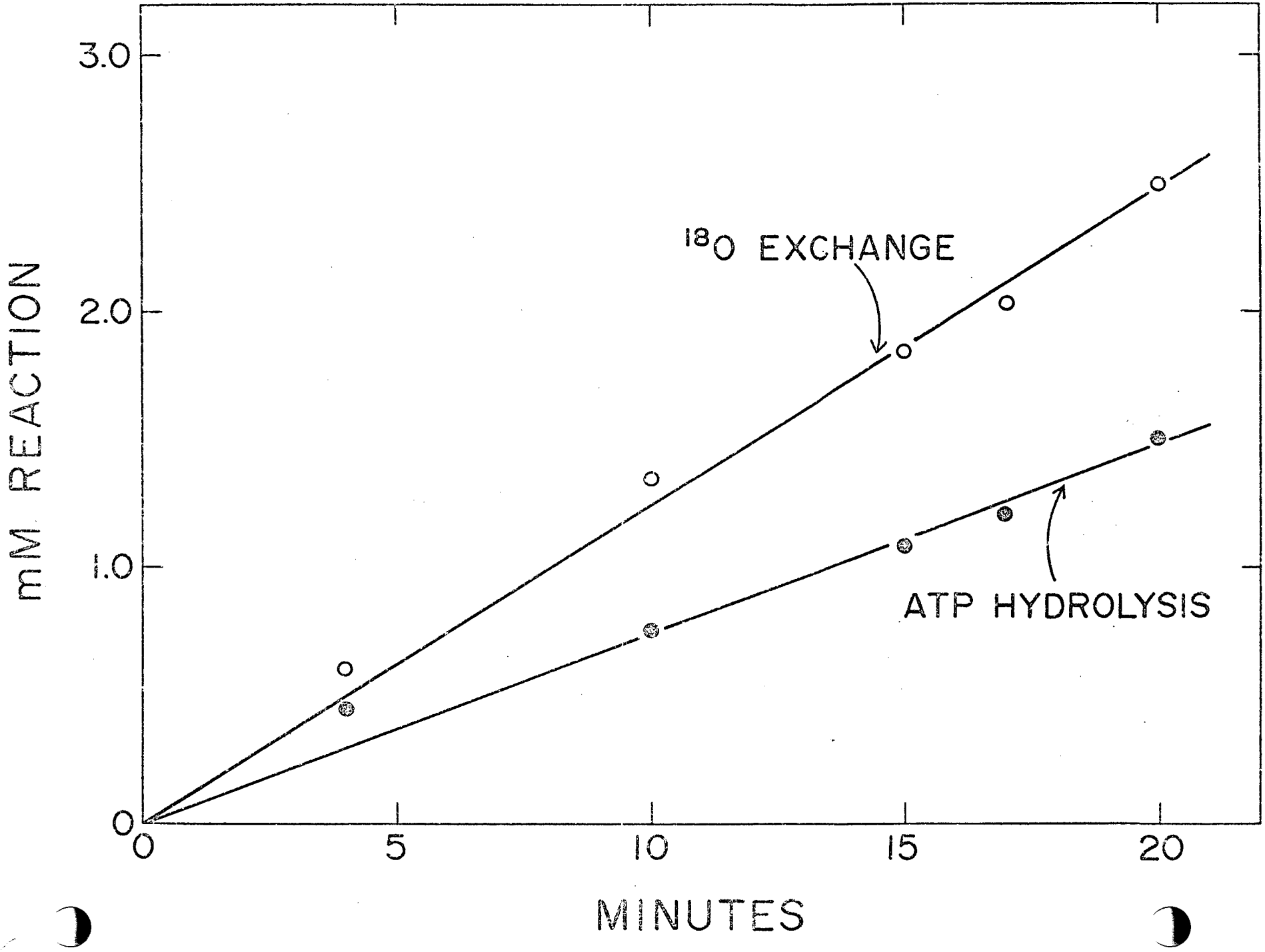
3.0
2.0
1.0
0
mM REACTION
^{18}O EXCHANGE
ATP HYDROLYSIS
0
5
10
15
20
MINUTES

FIGURE 3

THE EFFECT OF PRETREATMENT OF THE MEDULLAR (Na^+, K^+)-ATPase WITH VARYING AMOUNTS OF SODIUM DEOXYCHOLATE

A, effect on $P_i \rightleftarrows HOH$ exchange and (Na^+, K^+)-ATPase. B, effect on ATP-induced oxygen exchange. Enzyme was incubated with deoxycholate as described by Skou (11).

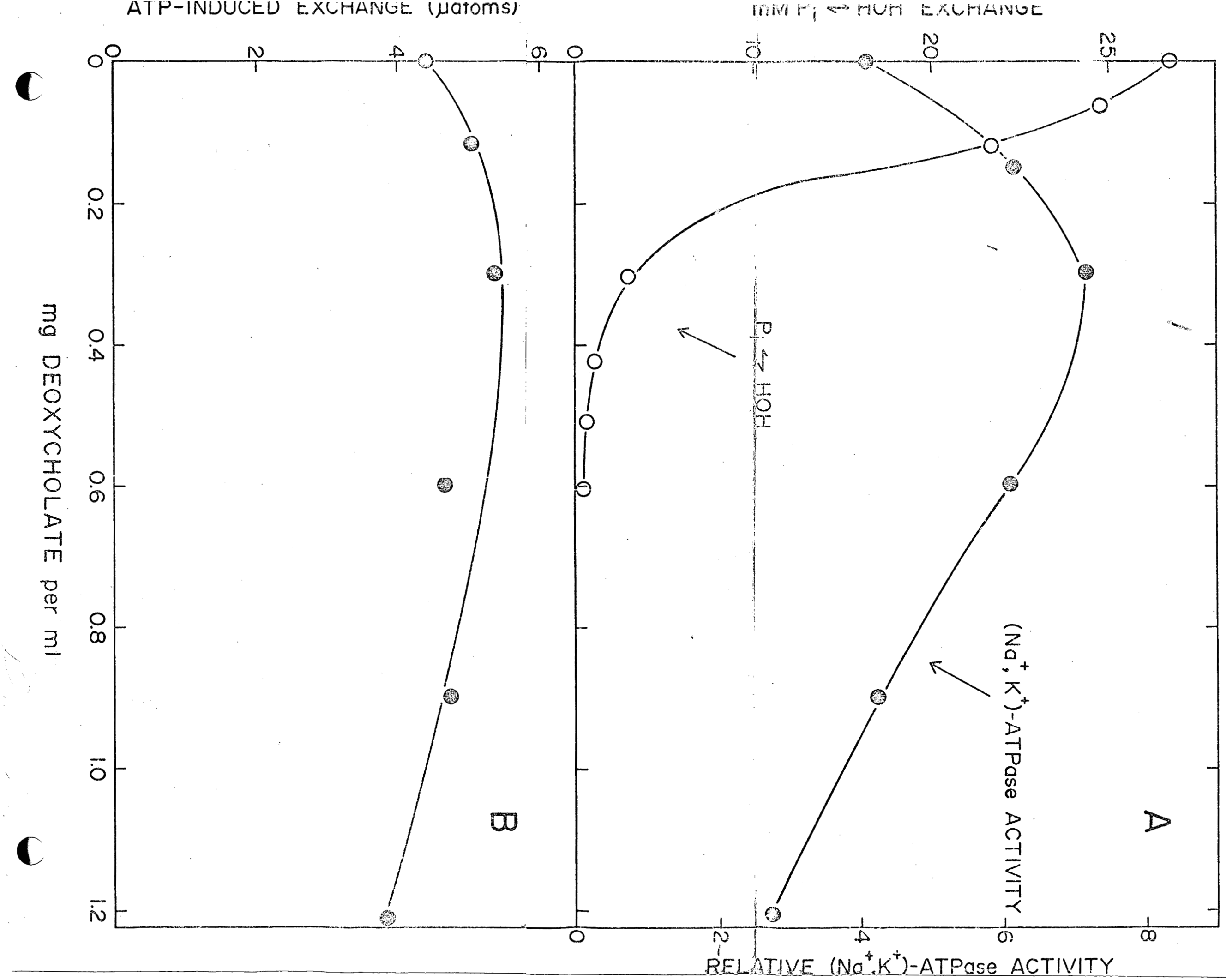
A
B
RELATIVE (Na^+,K^+)-ATPase ACTIVITY
(Na^+,K^+)-ATPase ACTIVITY
$P_i \rightleftharpoons HOH$
mM $P_i \rightleftharpoons$ HOH EXCHANGE
ATP-INDUCED EXCHANGE (μatoms)
mg DEOXYCHOLATE per ml

FIGURE 4

EFFECT OF TIME OF INCUBATION WITH N,N'-DICYCLOHEXYLCARBODIIMIDE (DCCD) ON $P_i \rightleftarrows HOH$ EXCHANGE AND (Na^+, K^+)-ATPase ACTIVITY

Conditions of incubation were: $P_i \rightleftarrows HOH$ exchange, 329 nmoles DCCD per mg protein (0.45 mM DCCD), 50 mM imidazole pH 7.2; (Na^+, K^+)-ATPase activity, 531 nmoles DCCD per mg protein (0.5 mM DCCD), 50 mM imidazole pH 7.2. Samples were incubated for the indicated time and then diluted 20:1 for assay. Conditions for assay were: $P_i \rightleftarrows HOH$ exchange, 5 mM $MgCl_2$, 40 mM KH_2PO_4, 50 mM imidazole glycylglycine (pH 7.2) and 0.84 atom percent excess $H^{18}OH$, 25°C, 120 minutes; (Na^+, K^+)-ATPase activity, as described in Methods. All measurements were corrected for controls containing no DCCD.

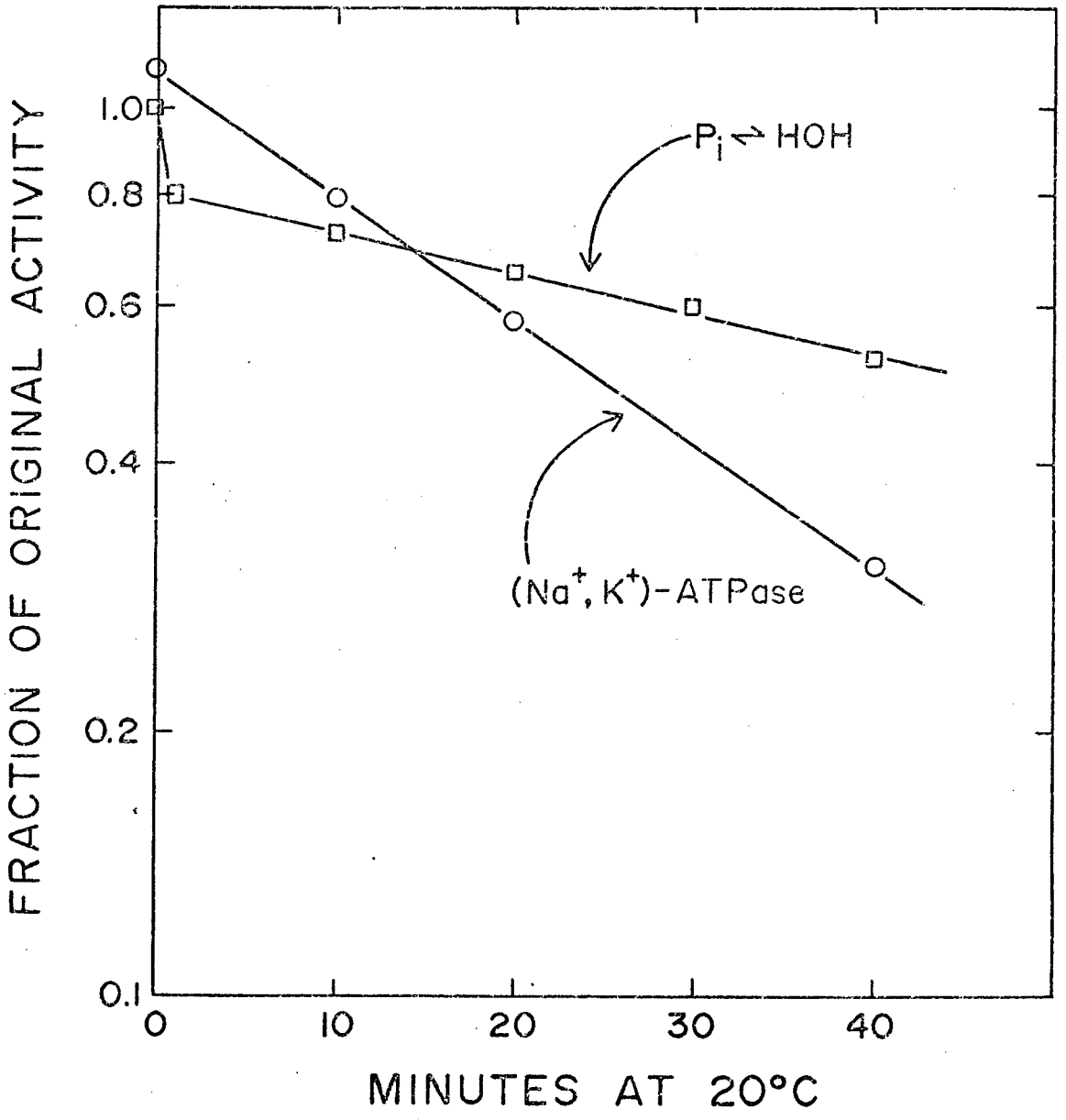
FRACTION OF ORIGINAL ACTIVITY
1.0
0.8
0.6
0.4
0.2
0.1
0
10
20
30
40
MINUTES AT 20°C
$P_i \rightleftharpoons HOH$
(Na^+, K^+)-ATPase

TABLE I

K^+-ACTIVATION AND Na^+-INHIBITION OF A $P_i \rightleftarrows HOH$ EXCHANGE REACTION

Medullar reaction mixtures contained 37 mM Tris-phosphate and 50 mM imidazole-glycylglycine (pH 7.20), 5.0 mM $MgCl_2$, 0.3 mg kidney (Na^+,K^+)-ATPase per ml, $H^{18}OH$ (1.099 atom percent excess), ± 140 mM NaCl, and ± 20 mM KCl and were incubated for 60 minutes at 25°C. In addition to the above, the electroplax reaction mixtures contained 0.98 mM KH_2PO_4, 0.63 mg electroplax (Na^+,K^+)-ATPase, and $H^{18}OH$ (1.14 atom percent excess) and were incubated for 10 minutes at 37°C. Analyses and calculations were made as described under "Experimental Procedure."

Addition	Oxygens exchanged per P_i present	
	medullar	electroplax
None	0.12	-
Na^+	< 0.02	< 0.06
K^+	1.21	2.83
Na^+,K^+	0.19	< 0.06

TABLE II

EFFECTS OF VARIOUS COMPOUNDS ON THE $P_i \rightleftarrows HOH$ EXCHANGE REACTION

Reaction mixtures contained 50 mM glycylglycine-imidazole (pH 7.20), 5 mM $MgCl_2$, 3.0 mg kidney (Na^+,K^+)-ATPase per ml, and 40 mM KH_2PO_4 and were incubated for 60 minutes at 37°C.

Addition	Oxygen Exchange	
	O/P_i	% of control
None	4.51	100
Minus $MgCl_2$	0.74	16
5 mM EDTA	0.06	1
5 mM EGTA	4.86	108
0.3 mM KF	3.87	85
1 mM ouabain	0.34	8
26 μM p-mercuribenzoate	0.24	5
1.9 mM bromoacetate	5.60	124
2.7 mM chloroacetate	5.06	112
5 mM Tris-acetate	4.61	102
25 mM NH_2OH	6.10	135

TABLE III

EFFECTS OF VARIOUS INHIBITORS ON THE $P_i \rightleftarrows HOH$ EXCHANGE REACTION

Reaction mixtures contained 50 mM glycylglycine-imidazole (pH 7.20), 40 mM KH_2PO_4, 5 mM $MgCl_2$, and 1.2 and 1.0 mg respectively of kidney or electroplax (Na^+,K^+)-ATPase per ml and were incubated at 37°C for 60 minutes.

Addition	Electroplax		Medullar	
	O/P_i	% of control	O/P_i	% of control
None	0.64	100	2.68	100
800 μM 2,4-dinitrophenol	8.84	91	3.28	122
1 μM 5-chloro-3-t-butyl,2'chloro-4'-nitrosalicylanilide	9.67	101	3.38	126
11 μg/ml oligomycin	8.84	91	3.03	113
36 μM m-chlorocarbonylcyanide phenylhydrazone	-	-	2.68	100
1 mM ouabain	0.29	3	0.13	5

TABLE IV

EFFECT OF ADP ON THE $P_i \rightleftarrows HOH$ EXCHANGE REACTION

Reaction mixtures contained 50 mM glycylglycine-imidazole (pH 7.20), 39.2 mM KH_2PO_4, 5 mM $MgCl_2$, 0.93 mg electroplax (Na^+,K^+)-ATPase per ml, ± NaADP, and $H^{18}OH$ (0.886 atom percent excess) and were incubated for 60 minutes at 37°C.

Dowex treated	ADP	Oxygens exchanged per P_i	Inhibition	Calculated inhibition due to chelation of Mg^{2+} by ADP	Inhibition due to ADP
			%	%	%
-	0	12.9	0	0	0
+	0	12.9	0	0	0
+	0.5 mM	11.0	15	4	11
-	5 mM	8.90	31	23	8
+	5 mM	3.61	72	23	49

TABLE V

RELATIVE RATES OF $P_i \rightleftarrows$ ATP, ATP $\rightleftarrows$ HOH AND $P_i \rightleftarrows$ HOH EXCHANGES

The porcine kidney outer medullar enzyme was used (specific activities of the (Na^+, K^+)-ATPase and Mg^{2+}-ATPase were 0.20 and 0.17 units/mg, respectively). All tubes contained the following: 50 mM imidazole-glycylglycine (pH 7.20), 20 mM KCl, and 5 mM $MgCl_2$. $P_i \rightleftarrows$ ATP: the reaction mixture also contained 10.22 mM Na_2ATP, 1.04 mM $^{32}P_i$ (1.36×10^6 cpm per ml), 1.45 mg enzyme, and 140 mM NaCl. Total volume was 5.0 ml. The reaction time was 8 minutes at 37°C resulting in 36% hydrolysis of ATP. ATP $\rightleftarrows$ HOH: the reaction tube also contained 10.22 mM Na_2ATP, 1.155 atom percent excess $H^{18}OH$, 0.29 mg enzyme, and 140 mM NaCl. Total volume was 1.0 ml. Reaction time was 30 minutes at 37°C resulting in 71.5% hydrolysis of ATP. The final atom percent ^{18}O in ATP was 0.204. $P_i \rightleftarrows$ HOH: the reaction mixture also contained 0.29 mg enzyme, 39.2 mM KH_2PO_4, and 1.016 atom percent excess $H^{18}OH$, total volume was 1.0 ml. Reaction time was 30 minutes at 37°C.

Exchange Reaction	Total Exchange (μatoms/hr/mg protein)	Per Cent of $P_i \rightleftarrows$ HOH Exchange
$P_i \rightleftarrows$ ATP	0.049 ± 0.013	0.02
ATP $\rightleftarrows$ HOH	3.3 ± 4.1	0
$P_i \rightleftarrows$ HOH	252 ± 5	100

TABLE VI

ATP-DEPENDENT RESTORATION OF MEDIUM P_i ⇄ HOH IN PRESENCE OF Na^+

Reaction mixtures contained the following: glycylglycine-imidazole (50 mM, pH 7.20), 5 mM $MgCl_2$, 0.98 mM KH_2PO_4, 20 mM KCl, 0.63 mg electroplax (Na^+,K^+)-ATPase, ± 140 mM NaCl, ± $H^{18}OH$ (1.140 atom percent excess), ± ^{18}O in the P_i (1.67 atom percent excess), ± 5 mM Na_2ATP, or ± 1 mM ouabain and were incubated for 10 minutes at 37°C.

Additions	µatoms oxygen exchanged	
	$H^{18}OH$	$P_i{}^{18}O$
None	2.83	-
Na^+	< 0.06	< 0.06
Ouabain	< 0.06	< 0.06
ATP, Na^+	2.14*	2.15*
ATP, Na^+, Ouabain	< 0.00*	< 0.06

*Corrected for oxygen incorporation due to ATP cleavage.

TABLE VII

LACK OF RESTORATION OF MEDIUM P_i ⇄ HOH EXCHANGE BY AN ATP ANALOG

Reaction mixtures contained glycylglycine-imidazole (50 mM, pH 7.20), 5 mM $MgCl_2$, 1.0 mg kidney (Na^+,K^+)-ATPase, 20 mM KCl, and 140 mM NaCl, total volume was 5 ml. The reaction was run for 60 minutes at 37°C.

Addition	mM P_i	µatoms oxygen exchanged
Minus NaCl	1.27	18.4
None	1.27	0.796
1.2 mM adenylyl methylene diphosphonate	1.27	0.438
1 mM ADP	1.27	0.820
5 mM ATP	1.43	4.17*

*Corrected for oxygen incorporation due to ATP cleavage.