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Urinary acidification in turtle bladder is due to a reversible proton-translocating ATPase

(epithelial transport/bioenergetics/proton transport)

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ABSTRACT **Adverse proton electrochemical gradients ($\Delta\tilde{\mu}_H$) applied across the turtle urinary bladder decrease active H^+ transport in this epithelium. A $\Delta\tilde{\mu}_H$ of 180 mV abolishes both transport and its tightly coupled metabolic reaction. Larger gradients should, in theory, reverse the direction of H^+ transport and the metabolic reaction leading to synthesis of ATP if the pump is an ATPase, or cause an increase in the oxidized state of a redox pair if it is a redox pump. To distinguish between these two possibilities, we measured ATP levels in epithelial cells that were poisoned to inhibit cellular mechanisms of ATP synthesis. At $\Delta\tilde{\mu}_H$ of 120 mV or less no ATP synthesis was found. At $\Delta\tilde{\mu}_H$ of greater than 120 mV there was a linear increase in ATP synthesis. Dinitrophenol, a H^+ carrier, prevented synthesis at $\Delta\tilde{\mu}_H$ of 310 mV. Dicyclohexylcarbodiimide, an inhibitor of H^+ transport that works at the cell surface, prevented ATP synthesis at $\Delta\tilde{\mu}_H$ of 310 mV. These results demonstrate that a reversible proton-translocating ATPase in the mucosal border of the bladder is the H^+ pump responsible for urinary acidification.**

Although many details of acid excretion by the kidney have been studied, the nature of the pump responsible for urinary acidification remained unknown (1, 2). Many ion pumps are ATPases; however, the role of redox pumps in epithelial transport continues to intrigue physiologists. Proton-translocating ATPases and redox mechanisms are widely distributed in nature, especially in mitochondria, bacteria, and chloroplasts (3). It is likely that the proton pump in urinary epithelia is either an ATPase or a redox pump. To distinguish between them, previous investigators have relied on studies testing the preferential utilization of various substrates in metabolic pathways that are coupled to H^+ transport (4, 5). Because many intermediates are shared by several pathways and because the redox potential appears to be in equilibrium with the free energy of ATP hydrolysis (6), it is evident that these studies can be misleading.

Another approach had been the measurement of the stoichiometry between ion transport and O_2 consumption (or CO_2 production). Investigators had assumed that for redox systems the limiting stoichiometry would be 2 H^+ per $\frac{1}{2}$ O_2 (7). When higher values were found, it was thought that a redox mechanism was excluded. It is now evident that the stoichiometry between H^+ transport and O_2 consumption could be as high as 4 H^+ per $\frac{1}{2}$ O_2 (8). Furthermore, even finding this value does not distinguish between the two types of pumps, nor does finding a higher value tell us that the pump is an ATPase. In this paper we use a direct approach that relies on identification of the specific high-energy intermediate used by the pump.

The H^+ pump of the turtle urinary bladder offers an opportunity to study this problem. This epithelium, an excellent model of renal acid excretion, has an active proton pump that is tightly linked to a metabolic reaction. Application of an increasingly adverse electrochemical gradient across this membrane leads to a linear decline in the rate of transport and the rate of coupled reaction (4). At a gradient of ≈180 mV the pump stops (1, 2). Larger gradients should, in theory at least, reverse the direction of flow of protons and of the metabolic reaction, leading to the synthesis of ATP if the pump is an ATPase or to an increase in the oxidized state of the redox pair if the pump is a redox pump. Because it is not clear which redox pair would be directly involved in the proton translocation, we decided to measure ATP levels. However, using ATP levels to distinguish between redox pumps and ATPases is subject to some restrictions. Because of the aforementioned inevitable presence of common metabolic intermediates and the near equilibrium between the redox potential and the free energy of ATP hydrolysis, an increase in ATP levels could be produced even if the pump is a redox mechanism. Furthermore, an increase in ATP levels in normal cells could easily be buffered by all the ATP consuming and generating reactions in the cell. For these reasons we decided to poison all ATP generating reactions. In this poisoned state any new ATP synthesis in response to a reversed proton flow through the pump is prima-facie evidence for the presence of a reversible ATPase. We found that, indeed, ATP was synthesized in response to reversed proton flow. Based on this and other evidence we conclude that the H^+ pump of the turtle bladder is a reversible proton-translocating ATPase.

METHODS AND MATERIALS

Paired hemibladders from fresh water turtles, *Pseudemys scripta elegans*, were removed with a minimum of handling. Each hemibladder was washed with a phosphate-free Ringer's solution containing 110 mM NaCl, 1 mM $MgCl_2$, 3.5 mM KCl, 1 mM $CaCl_2$, and 2.5 mM $NaHCO_3$ per liter and then glued to a polyethylene ring using Eastman 910 glue. The ring was then mounted in an Ussing chamber. The hemibladders were bathed in the above Ringer's solution and bubbled with room air. The area of the exposed surface of the membrane was 8.3 cm^2.

The open circuit potential difference was recorded and then the bladders were short-circuited by using an automatic voltage clamp. After this, 1 mM ouabain was added to the serosal solution, and 0.1 mM amiloride was added to the mucosal solution. These additions caused a reversal of the short-circuit current (1). The reversed short-circuit current has been shown to be a H^+ current (1, 4).

As soon as the experiment was completed, the hemibladders, still on the rings, were removed from the chamber, washed with Ringer's solution, and immediately immersed in liquid nitrogen. The mucosal surface of the hemibladder was then scraped

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Abbreviations: DNP, 2,4-dinitrophenol; DCCD, *N,N'*-dicyclohexylcarbodiimide; $\Delta\tilde{\mu}_H$, transepithelial proton electrochemical gradient; $\Delta\psi$, transepithelial electric potential difference.

lightly with a scalpel that had been cooled to the temperature of liquid nitrogen. Light microscopy showed that this process yielded mucosal cells in the scrapings. The amount scraped from each hemibladder was ≈1.5 mg of protein.

The scraped cells were then placed in a tube containing 3 ml of frozen 2% perchloric acid. The tube was allowed to thaw in an ice bath for ≈30 min. The suspension was centrifuged at 18,000 rpm at 4°C for 10 min. The supernatant was made alkaline to a phenolphthalein end point with a solution containing 7.5 M KOH and 100 mM K_2HPO_4, centrifuged in a clinical centrifuge at 400 rpm for 2 min, and then removed to an ice bath. The supernatant was used to measure ATP. The pellet from the perchloric acid extraction was dissolved in 0.5 M NaOH at room temperature and used for protein determination.

ATP was measured by a modification of the firefly lantern luciferin–luciferase method reported by Kimmich *et al.* (9). Protein was measured by the Lowry method as modified by Hartree (10), using bovine serum albumin as a standard. The first step of the Hartree method, treatment with 0.5 M NaOH, Na tartrate, and Na_2CO_3 was changed to treatment with Na tartrate and Na_2CO_3 without additional NaOH.

Firefly lanterns, crystalline ATP, and phosphoenol pyruvate were obtained from Sigma; pyruvate kinase and myokinase were obtained from Calbiochem; bovine serum albumin was obtained from Armour Drugs; and Eastman 910 glue was purchased from Eastman Corporation.

RESULTS

The ATP content of epithelial cells scraped from hemibladders was 28.0 ± 1.4 nmol/mg of protein (±SEM) (n = 12). The average rate of H^+ transport in these bladders was 2.53 ± 0.46 nmol/min cm^2.

Cellular ATP-generating reactions were inhibited by using sodium cyanide (2 mM) and iodoacetate (5 mM). In paired hemibladders, the serosal surface of one bladder was exposed to these poisons at pH 5 for 30, 60, or 80 min. During the first 15 min of poisoning, the serosal solution was changed five times. The bladders were short-circuited throughout the experiment and the mucosal pH was ≈7.4. By 40 min, the average serosal pH increased to 6.4. ATP and protein were measured at the indicated times and the results are shown in Fig. 1. The ATP content of the control bladders averaged 25 nmol/mg of protein (n = 4) and declined in an exponential fashion with a half-time of ≈15 min. At 80 min, the luminal pH was decreased to 4.5, the serosal pH was kept at 6.4, and the bladder was voltage-clamped at 200 mV, mucosal side positive. This net electrochemical gradient of 310 mV was maintained for 40 min. In the paired bladder, the mucosal pH was kept at 7.4 and the bladder was clamped at 0 mV for 40 min. In both bladders the serosal solution contained 2 mM sodium cyanide and 5 mM iodoacetate. The ATP content of epithelial cells scraped from both bladders is shown in Fig. 1. The bladders clamped at a $\Delta\tilde{\mu}_H$ = −60 mV* had ATP levels of 0.4 nmol/mg of protein, whereas those clamped at $\Delta\tilde{\mu}_H$ of 310 mV had ATP contents of 10.2 nmol/mg of protein. These results demonstrate that application of a $\Delta\tilde{\mu}_H$ of 310 mV results in an increase in cellular ATP content.

Application of transepithelial electrochemical gradients on the turtle bladder results in a linear decline in the rate of H^+ transport (1, 2). Not only was the rate of net transport decreased, but so was the rate of oxidative metabolism, suggesting that the electrochemical gradient decreased *active* H^+ transport (4). At a $\Delta\tilde{\mu}_H$ of ≈180 mV, both transport and its coupled metabolic reaction were abolished. Greater $\Delta\tilde{\mu}_H$ should, in theory at least, reverse the direction of both processes. If the pump is an ATPase, ATP should be synthesized. However, ATP will be synthesized only at $\Delta\tilde{\mu}_H$ greater than the reversal potential. In normal bladders that would be ≈180 mV, whereas in poisoned bladders we expect it to be much less.†

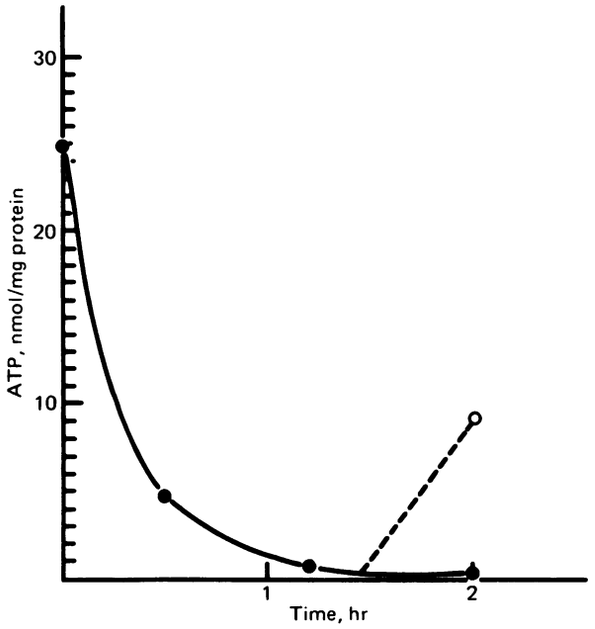

FIG. 1. Effect of addition of iodoacetate (2 mM) and sodium cyanide (2 mM) to the serosal side at t = 0. ATP levels were measured at designated times. ●, Experiments in which the transepithelial proton electrochemical gradient ($\Delta\tilde{\mu}_H$) was −60 mV. ○, $\Delta\tilde{\mu}_H$ of 310 mV was applied for 40 min. The dashed line should not be taken to imply that ATP synthesis is linear over this period of time. Indeed it appears to be highly nonlinear, with initial rates being much higher than later rates.

We tested the effect of different $\Delta\tilde{\mu}_H$ on ATP synthesis in 17 pairs of hemibladders that were poisoned for 80 min by serosal iodoacetate and cyanide in a manner identical to the experiment shown in Fig. 1. One hemibladder in each pair was exposed to a $\Delta\tilde{\mu}_H$ of 310 mV (mucosal pH 4.5, serosal pH 6.4, and $\Delta\psi$ of 200 mV), whereas the other bladder was exposed to $\Delta\tilde{\mu}_H$ of −60, 120, or 210 mV for the final 40 min. The potential difference did not exceed 200 mV nor was the mucosal pH lower than 4.5 in any experiment. However, the contribution of $\Delta\psi$ and ΔpH to the $\Delta\tilde{\mu}_H$ was varied. The ATP levels in both bladders was measured after 40 min of exposure to the $\Delta\tilde{\mu}_H$, and the results are shown in Fig. 2. No ATP was synthesized at a $\Delta\tilde{\mu}_H$ of −60 or 120 mV; ATP levels at $\Delta\tilde{\mu}_H$ = −60 mV were 0.5 ± 0.1 nmol/mg of protein (n = 6) and at $\Delta\tilde{\mu}_H$ of 120 mV they were 0.6 ± 0.2 nmol/mg of protein (n = 6). At a $\Delta\tilde{\mu}_H$ of

* $\Delta\tilde{\mu}_H$ was the sum of the transepithelial pH difference, ΔpH (serosal minus mucosal), and the transepithelial potential difference ($\Delta\psi$) (mucosal side positive to the serosal side). Previous estimates have shown that the voltage drop across the luminal membrane accounts for ≈80% of that across the whole epithelium (11).

† The gradient at which the reaction should reverse is determined by (2):

$$(\Delta\tilde{\mu})_{J_H=0} = Z\,\Delta G_{ATP},$$

in which Z is the stoichiometry and ΔG_{ATP} is the free energy of ATP hydrolysis [≈11.9 kcal/mol (unpublished results)]. If during poisoning all of the ATP was converted to ADP and P_i, then ΔG will decrease to ≈7.1 kcal/mol. Hence, the reversal potential will be ≈110 mV.

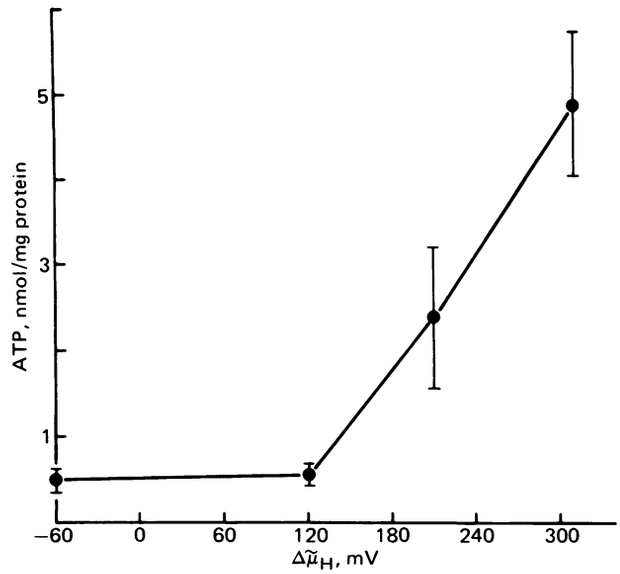

FIG. 2. Effect of application of $\Delta\tilde{\mu}_H$ on ATP levels in epithelial cells. $\Delta\tilde{\mu}_H$ was applied for 40 min and was composed of a $\Delta\psi$ and a ΔpH in different combinations.

210 mV ATP levels were 2.4 ± 0.8 nmol/mg of protein (n = 5), whereas at 310 mV they were 4.9 ± 0.9 nmol/mg of protein (n = 17). Fig. 3 shows that ATP can be synthesized in response to ΔpH or $\Delta\psi$ provided that the sum of the two exceeds 120 mV. These results demonstrate that gradients greater than 120 mV result in ATP synthesis. The response of ATP synthesis to applied gradients was apparently linear over this range. These results suggest that ATP was synthesized by the H^+ pump. In a set of four experiments we tested the "symmetry" of the response of bladders to a change in ΔpH. Both members of four pairs of poisoned hemibladders were clamped at a $\Delta\psi$ of 200 mV and the luminal pH was titrated to pH 6.0 in both, but the serosal pH was 5.5 in one and 7.5 in the other. The ATP level in those with a serosal pH of 5.5 was 3.17 nmol/mg of protein ($\Delta\tilde{\mu}_H$ = 200 mV), whereas in the bladder in which the serosal pH was 7.5 ($\Delta\tilde{\mu}_H$ = 320 mV) it was 5.72 nmol/mg of protein ($P < 0.05$). Hence, it appears that serosal alkalinization in this setting is similar to mucosal acidification.

It is possible, however, that the proton electrochemical gradient induced a flux of H^+ into the cells that in turn altered the cellular environment in such a manner as to lead to ATP synthesis by the mitochondria even in these poisoned cells. To test this possibility we increased proton entry into the cells by using dinitrophenol (DNP). This agent specifically translocates protons in artificial (12) and natural membranes, including the luminal border of the turtle bladder (4). If the ATP was synthesized by the mitochondria as a result of cellular acidification, we would expect more ATP to be synthesized in response to a given electrochemical gradient. On the other hand, if ATP was synthesized by a H^+ pump located in the luminal border in response to an electrochemical gradient, then DNP should decrease the synthesis because it will collapse the gradient across the luminal border.

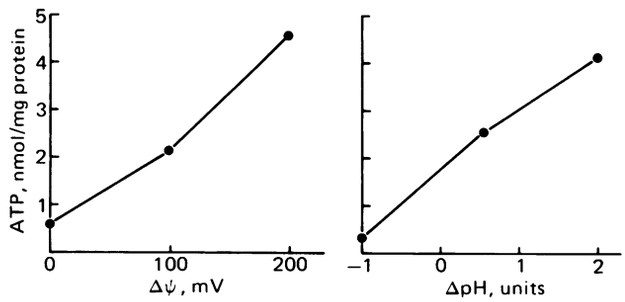

FIG. 3. Effect of a ΔpH and a $\Delta\psi$ on ATP in epithelial cells. (*Left*) The effect of increasing $\Delta\psi$ was tested in bladders clamped at a ΔpH of 2 units. (*Right*) The ΔpH was increased in bladders clamped at 180 mV. The $\Delta\tilde{\mu}_H$ was applied for 40 min.

Five paired hemibladders were mounted in Ussing chambers and poisoned for 80 min as above. To one hemibladder in each pair, DNP was added to the luminal medium in a final concentration of 2 μM. Previous experiments (4) have shown that in this concentration DNP does not enter the cell but uncouples H^+ transport at the cell surface. At this concentration the CO_2 production not related to active H^+ transport was not increased by DNP. Both hemibladders were exposed to a $\Delta\tilde{\mu}_H$ of 310 mV for the final 40 min. The ATP content of the epithelial cells from the control bladders averaged 10.7 ± 4.1 nmol/mg of protein. The DNP-treated bladders had an ATP content of 0.7 ± 0.1 nmol/mg of protein (Table 1). These results demonstrate that ATP synthesis in response to an electrochemical gradient across the luminal border is due to a specific proton channel located in that membrane. Previous studies have, in fact, suggested that the H^+ pump is located at the luminal border (1, 11).

To further evaluate the site of ATP synthesis we used the inhibitor of H^+ transport, dicyclohexylcarbodiimide (DCCD). This agent has been previously shown to decrease proton flow in the proteolipid F_0 portion of proton-translocating ATPase of bacteria and mitochondria (3, 13). Addition of 0.2 mM DCCD to the luminal surface resulted in an immediate inhibition of H^+ transport in the turtle bladder which was not reversed by washing. To insure that the effect of DCCD was on the luminal surface rather than on intracellular ATP-generating sites such as mitochondria, we measured the effect of a 15-min exposure of the luminal surface of the turtle bladder to DCCD on epithelial ATP content. In four paired hemibladders bathed with Ringer's solution at pH 7.4 and short-circuited, the initial rate of H^+ transport in the control bladder was 2.6 nmol/min cm^2 and in the bladder to receive DCCD it was 3.6 nmol/min cm^2. Exposure of the luminal surface to 0.2 mM DCCD for 15 min caused complete inhibition of H^+ transport. The resistance of the bladder increased from 3369 ± 451 ohms/cm^2 before treatment with DCCD to 3504 ± 431 ohms/cm^2 after exposure to DCCD. ATP content was measured ≈100 min later (Table 1). Control unpoisoned bladders had an ATP content of 32.6 ± 1.3 nmol/mg of protein, whereas the DCCD-treated bladders had an ATP level of 30.9 ± 2.0 nmol/mg of protein. These results indicate that the inhibition of H^+ transport by DCCD was not due to a decrease in mitochondrial ATP synthesis. Rather, it was due to an effect of DCCD on the H^+ pump itself.

Six paired hemibladders were poisoned by cyanide and iodoacetate for 80 min and clamped at a $\Delta\tilde{\mu}_H$ of 310 mV for the final 40 min. One member of each pair was treated by a 15-min exposure of DCCD to the luminal medium. The ATP content of the control bladders averaged 3.1 ± 0.6 nmol/mg of protein whereas the DCCD-treated bladders had ATP contents of 0.5 ± 0.1 nmol/mg of protein (Table 1). These results indicate that ATP synthesis in response to large electrochemical gradients was due to the H^+ pump located at the luminal border of the turtle bladder.

In one additional experiment we added DCCD to the serosal side of a pair of hemibladders that were *not* poisoned by cyanide or iodoacetate. One bladder was clamped at a $\Delta\tilde{\mu}_H$ of 370 mV, whereas the other was clamped at a $\Delta\tilde{\mu}_H$ of 0. The latter bladder had an ATP content 2 hr after the addition of DCCD of 0.2 nmol/mg of protein. This suggests that when DCCD

Table 1. ATP synthesis by mitochondria in poisoned cells

Additions	Cyanide (2 mM) + iodoacetate (5 mM) for 80 min	$\Delta\tilde{\mu}_H$ (310 mV) for 40 min	n	ATP, nmol/mg protein Control	Exp.	$\Delta \pm$ SEM
DNP (2 μM)	+	+	5	10.7	0.7	10.0 ± 4.0*
DCCD (0.2 mM)	−	−	4	32.6	30.9	1.7 ± 1.9
DCCD (0.2 mM)	+	+	6	3.1	0.5	2.6 ± 0.6*
Oligomycin (150 μg/ml)	+	+	6	5.1	0.5	4.5 ± 1.4*

Effect of various maneuvers on ATP synthesis in response to an electrochemical gradient ($\Delta\tilde{\mu}_H$). All additions were to the mucosal medium in the final concentrations shown.
* $P < 0.05$.

gains access to the interior it can inhibit ATP synthesis. The bladder treated by DCCD and exposed to a $\Delta\tilde{\mu}_H$ of 370 mV for 40 min had an ATP content of 6.5 nmol/mg of protein. These results suggest that DCCD blocks only the H^+ pump when applied from the urinary side. It also supports the evidence that the synthesis of ATP is due to the H^+ pump.

Proton-translocating ATPases from bacteria and mitochondria contain a portion (F_1) that binds oligomycin with consequent inhibition of their activity (3). To determine whether the H^+ pump of the turtle bladder shared this sensitivity, we tested the effects of oligomyclin on ATP synthesis in response to an electrochemical gradient. Six paired hemibladders were poisoned for 80 min and exposed to an electrochemical gradient of 310 mV in the last 40 min. Oligomycin dissolved in 100 μl of methanol was added to the mucosal medium to a final concentration of 150 μg/ml to one member of each pair. This caused an inhibition of H^+ transport. The control hemibladder received 100 μl of methanol. The ATP levels in the control bladders averaged 5.0 ± 1.4 nmol/mg of protein, whereas in the oligomycin-treated bladders it was 0.5 ± 0.1 nmol/mg of protein (Table 1). Although DCCD and oligomycin are by no means "specific" inhibitors of the mitochondrial ATP synthetase, the similarity between it and the turtle bladder proton pump is striking.

DISCUSSION

During application of a proton electrochemical gradient, transport of H^+ into the cell undoubtedly occurs. Cellular acidification could conceivably lead to ATP synthesis by the mitochondria. A number of experiments performed above make this possibility unlikely. Serosal alkalinization led to ATP synthesis even though we would expect the cell pH in this setting to be higher than in the other experiments. Addition of DNP in concentrations previously shown *not* to uncouple oxidative phosphorylation, abolishes ATP synthesis even though presumably it increases proton entry and cellular acidification. Addition of DCCD such that it binds only to the luminal membrane prevents ATP synthesis. Finally, addition of serosal DCCD in doses that completely abolish mitochondrial ATP synthesis does not prevent ATP synthesis in response to a proton electrochemical gradient. We conclude that the ATP synthesized in response to proton electrochemical gradients is due to a reversible ATPase located in the luminal border of the epithelium. The response of ATP synthesis to an electrical gradient indicates that this active transport pathway is "electrogenic." This strengthens the view (1) that in the turtle bladder a one for one Na/H exchange is untenable. The electromotive force of this pump is ≈180 mV and is inhibited by DCCD and oligomycin. All three of these characteristics are shared by the ATP synthetases of bacteria and mitochondria. Whether these similarities represent a fundamental identity or is fortuitous remains to be discovered.

The nature of the H^+ pump in the kidney is yet to be determined. However, it is probable that a proton-translocating ATPase is the cause of urinary acidification in the kidney. Kinne and coworkers (14, 15) found a HCO_3-stimulated ATPase in the luminal border of the proximal tubule and collecting duct, which may be the proton pump. However, no direct experiments that relate proton transport to ATP hydrolysis or synthesis are available at present.

The study of the energetics of active transport in epithelia has been hampered by the lack of methods that simultaneously measure the rate of transport and the rate of chemical reaction that is tightly coupled to it. Investigators had to rely on measurements on O_2 consumption or CO_2 production reactions that are only distantly related to the transport apparatus. The method presented in this paper is a first step towards measurements of the reactions that directly fuel the pump. With further refinements it should be possible to measure the stoichiometry between transport and ATP hydrolysis and the relationship between active transport and the ΔG of ATP hydrolysis under various physiologically interesting situations.

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