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Presence of an Extramitochondrial Anion-Stimulated ATPase in the Rabbit Kidney: Localization Along the Nephron and Effect of Corticosteroids

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Summary. To determine whether kidney membrane fractions contain an extramitochondrial anion-stimulated ATPase, we compared the pharmacological and kinetic properties of HCO_3-ATPase activities in mitochondrial and microsomal fractions prepared from rabbit kidney cortex and outer medulla. The results indicated that this activity differed markedly in each type of fraction. Microsomal HCO_3-ATPase was less sensitive than mitochondrial ATPase to azide, oligomycin, DCCD and thiocyanate, but was more sensitive to filipin and displayed different dependency towards ATP, magnesium and pH. Microsomal ATPase activity was stimulated by sulfite much more strongly than by bicarbonate, whereas mitochondrial activity was stimulated by both these anions to a similar extent. These results demonstrate the presence of an extramitochondrial HCO_3-ATPase in kidney membrane fractions. HCO_3-ATPase was also measured in single microdissected segments of the rabbit nephron using a radiochemical microassay previously developed for tubular Na, K-ATPase activity. An enzyme with the pharmacological and kinetic properties of the microsomal enzyme was detected in both proximal tubule, distal convoluted tubule and collecting duct, but the thick ascending limb was devoid of any detectable activity. Long-term DOCA administration markedly increased HCO_3-ATPase activity in the distal convoluted and collecting tubule. The insensitivity of microsomal HCO_3-ATPase to vanadate indicates that it belongs to the F_0-F_1 class of ATPases, and might therefore be involved in proton transport. This hypothesis is also supported by the localization of tubular HCO_3-ATPase activity at the sites of urinary acidification.

Key Words mitochondrial ATPase inhibitors · proton ATPase · isolated nephron segments · urine acidification · proton transport

Introduction

Various kinds of anion-stimulated ATPase activity have been reported in a number of tissues and organs [12, 14, 45, 48], including the kidney [8, 22, 26, 31]. In epithelial cells, these ATPases are thought to be involved in transepithelial transport of chloride and/or bicarbonate. However, to fulfill such a function, these ATPases would have to be located in the cell's plasma membrane. This raises the problem of determining the exact location of anion-stimulated ATPase activity inside the cell. Although HCO_3-ATPase activity was shown to be present in both mitochondrial and microsomal fractions prepared from cell homogenates [11, 26, 47], several authors [11, 28, 43, 47] have proposed that the presence of ATPase activity in microsomal fractions was due to mitochondrial contamination.

Many attempts have been made to establish the subcellular origin of HCO_3-ATPase activity. Most of the experiments concerned are based on the separation of mitochondrial and microsomal membranes by differential and density gradient centrifugation, the purity of the two types of membranes being ascertained from their relative enrichment in various specific enzymatic markers. This approach involves two major pitfalls: firstly, the changes in the activity of an enzymatic marker observed at various steps of membrane purification may reflect its enrichment or disappearance, but also its unmasking or denaturation during the procedure. Secondly, membrane fusion may occur during purification, thus leading to the co-purification of markers originally present in different membranes.

We therefore used another experimental approach in our attempt to characterize the HCO_3-ATPase activity of extramitochondrial origin in kidney microsomes; the aim of this approach was to distinguish between HCO_3-ATPase activity of mitochondrial and microsomal origin, on the basis of pharmacological and kinetic parameters. For this purpose, we prepared mitochondrial and microsomal fractions from rabbit kidney homogenates, and characterized the pharmacological and kinetic properties of the HCO_3-ATPase activity present in each fraction. Results obtained by this approach demonstrated the existence of an extramitochondrial anion-stimulated ATPase activity in rabbit kidney membrane fractions. From its pharmacological properties, it was suggested that this enzyme was a

proton pump. We therefore attempted to search whether this enzyme might be implicated in urinary acidification. For this purpose, we developed a microassay allowing the measurement of this enzyme activity in single nephron segments in order to determine whether it was localized at the sites of urine acidification. Furthermore, we analyzed the effect of chronic-DOCA administration on the enzyme since this procedure is known to enhance distal urine acidification. Although this approach is indirect, results of the present study suggest that extramitochondrial HCO_3-ATPase might be implicated in the process of tubular proton secretion.

Materials and Methods

Animals

Experiments were performed on three groups of male New Zealand rabbits weighing 1 to 1.5 kg and fed the usual laboratory diet. Animals in group I were not treated and were used for preparation of kidney homogenates and for setting the ATPase microassay on single nephron segments. For 11 to 18 days before the experiments, animals in group III were subcutaneously injected daily with 5 mg deoxycorticosterone acetate (DOCA) dissolved in olive oil. Control animals of group II received the same volume of solvent during this period.

Studies on Kidney Homogenates

Preparation of Mitochondrial and Microsomal Fraction

Animals were anesthetized with pentobarbital and bled from the carotid. Their kidneys were quickly removed and immersed in cold homogenization solution containing 250 mM saccharose, 5 mM EDTA, and 30 mM histidine at pH 7.4. The cortical and outer medullary portions of each kidney were separately dissected in the cold. Pieces of tissue were blotted on filter paper, weighed and homogenized in 10 volumes (wt/vol) of the above homogenization medium in the presence of 0.2% (wt/vol) deoxycholic acid, employing 6 to 10 strokes at 400 rpm in a loose-fitting Teflon®-glass homogenizer. The mitochondrial and microsomal fractions were obtained by differential centrifugation in homogenization medium as follows: The crude homogenate was centrifuged twice for 15 min at 600 × *g* and the pellet (P_2) was discarded. The supernatant (S_2) was then centrifuged for 15 min at 10,000 × *g* and the pellet (P_3) and supernatant (S_3) thus formed were respectively used to prepare the mitochondrial and microsomal fractions. S_3 was centrifuged for 90 min at 100,000 × *g* and the formed pellet (P_4) was resuspended (0.5 to 1 μg protein/μl) and constituted the microsomal fraction. P_3 was resuspended and centrifuged for 15 min at 10,000 × *g* and the pellet formed was resuspended (0.1 to 0.5 μg protein/μl) and constituted the mitochondrial fraction. All steps were carried out at 0 to 4°C. Aliquot fractions of 100 μl were stored at −20°C for several weeks without altering the anion-stimulated ATPase activity.

Determination of ATPase Activity

ATPase activity was measured by a radiochemical assay using 10 μl of tissue fraction in a total volume of 100 μl. After prewarming the reaction mixture (80 μl of incubation medium plus 10 μl of tissue fraction) for 5 min at 37°C, the reaction was started by adding 10 μl of 25 mM ATP. Unless otherwise indicated, incubation lasted for 10 min for mitochondrial fractions and 20 min for microsomal fractions. They were carried out at 37°C in a shaking water bath, and terminated by the addition of 0.5 ml of 5% (wt/vol) ice-cold trichloroacetic acid solution. Samples were then rapidly cooled and 1.5 ml of a 10% (wt/vol) suspension of activated charcoal was added. After 10-min centrifugation at 1,500 × *g*, the radioactivity of 1 ml of the supernatant was measured by liquid scintillation counting.

Unless otherwise indicated, ATPase activity was measured in a medium containing 40 mM glycylglycine, 2 mM ouabain (to block Na,K-ATPase activity), 1.5 mM $MgSO_4$, 2.5 mM Tris-ATP, and ≃1 nCi/μl γ^{32}P-ATP (New England Nuclear, Boston, 2 to 10 Ci · $mmol^{-1}$), in the presence of 30 mM $NaHCO_3$ for total ATPase activity measurement, and in the absence of $NaHCO_3$ for basal Mg-ATPase activity measurement. For the determination of extramitochondrial ATPase activity in microsomal fractions, 2.5 mM sodium azide was added. The pH of all solutions was 7.5.

When calculating ATPase activity, corrections were made for the spontaneous nonenzymatic breakdown of ATP, measured as the inorganic phosphate released in the absence of enzyme, and for the volume of supernatant on which the radioactivity was counted. The protein content of the different fractions was determined by the method of Lowry et al. [33] using crystalline bovine serum albumin as standard. ATPase activity was expressed as micromoles of inorganic phosphate released per milligram of protein per hour. Bicarbonate-dependent ATPase activity was taken as the difference between the activities measured in the presence and absence of bicarbonate.

Studies on Isolated Nephron Segments

Tubule Microdissection

Animals were anesthetized and heparinized and their left kidney was excised and perfused with a chilled collagenase solution containing (mM): 137 NaCl, 5 KCl, 0.8 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 1 $MgCl_2$, 10 Tris-HCl, 1 $CaCl_2$, 0.1% (wt/vol) collagenase (Flow Laboratories, France) and 0.2% bovine serum albumin, pH 7.4. Thin pyramids cut along the corticopapillary axis were incubated in the same solution at 35°C and then rinsed and stored in the cold until use. Tubules were dissected freehand under a stereomicroscope in cold microdissection medium similar to the collagenase solution except that collagenase and albumin were omitted and that $CaCl_2$ was 0.25 mM. Pieces of nephron were individually transferred to the concavity of a sunken bacteriological slide, and photographed. The slides were then tightly covered and stored on ice until assay. Nephron segments were characterized according to topographical and morphological criteria as follows: Segments of proximal convoluted tubules (PCT) were dissected in the superficial kidney cortex next to their attachment to the glomeruli, and therefore consisted of the S_1 portion. Proximal straight tubules, or pars recta (PR), were dissected from the outer stripe of the outer medulla and were characterized by their attachment to the thin limb of Henle's loop. Medullary and cortical portions of the thick ascending limb

of Henle's loop (MAL and CAL) were obtained from the inner stripe of the outer medulla and from the cortex, next to the macula densa, respectively. The initial, bright (DCT_b) and late granular (DCT_g) portions of the distal convoluted tubule were dissected separately. Collecting tubules were dissected from either the cortex (cortical collecting tubule: CCT) below the last branching or from the outer medulla (medullary collecting tubule: MCT). In addition to these topographical criteria, the different nephron segments were also distinguished from each other according to their appearance and relative diameter.

Tubule Permeabilization

Without pretreatment to increase their permeability, tubules exhibited a low and variable HCO_3-ATPase activity, probably due to limited access of ATP to the enzyme's catalytic site. We have, therefore, evaluated the effect of deoxycholate (DOC) solubilization, a procedure aimed at increasing cell permeability and ATPase activity. For this purpose individual tubules were transferred with a fine polyethylene catheter onto a dried droplet of BSA in the center of a small piece of aluminum foil placed beforehand in each of the 21 cylindrical wells of a chilled aluminum plaque. The isotonic solution was replaced by 5 μl of cold distilled water, and after 15 min at 0 to 4°C the droplet surrounding the tubule was again aspirated and replaced by 0.2 μl of a solution of sodium deoxycholate. The plaques were then brought into contact with dry ice for rapid freezing and ATPase activity was determined as described below. Figure 1 depicts the changes in ATPase activity in response to various doses of deoxycholate in PR. Magnesium-dependent ATPase activity decreased progressively with increasing concentrations of DOC and became nul in the presence of 0.5% DOC, whereas HCO_3-ATPase activity, almost undetectable in the absence of DOC, increased with rising concentrations of DOC up to 0.3% and decreased thereafter. This biphasic pattern of HCO_3-ATPase activity might be explained by the superimposition of two phenomena: First, the unmasking of functional sites probably in response to tubular permeabilization in the presence of low concentrations of DOC, and secondly, the enzyme inactivation which is preponderant with DOC concentrations over 0.3%. Morphologically, an almost complete dissociation of tubular architecture was observed after the freezing-thawing step in the presence of 0.3% DOC. In further studies, tubules were pretreated with 0.3% DOC before incubation.

Incubation

The frozen plaque was placed on ice and its content allowed to thaw gradually. One microliter of incubation medium (*see* composition below) was added to each sample and the reaction was started by immersing the plaques in a water bath at 37°C and continued without stirring for 15 min. Incubation was stopped by replacing the plaques on crushed ice and by adding 15 μl of 10% (wt/vol) activated charcoal suspension in 5% trichloroacetic acid to each tubule. Activated charcoal was used to bind unhydrolyzed ATP, and the labeled inorganic phosphate was separated by filtration under vacuum through a Millipore cellulose filter (HAWP 01300, Millipore Corp., Molsheim, France). The sample was rinsed with 0.7 ml of a cold phosphate buffer solution directly into counting vials, and the radioactivity determined by liquid scintillation counting.

The incubation medium for determination of total ATPase activity contained (mM): 30 $NaHCO_3$, 1 $MgSO_4$, 2 ouabain, 2.5 sodium azide, 40 glycylglycine, 2.5 Tris ATP, and tracer amounts (=5 nCi/μl) of γ^{32}P-ATP (10 Ci/mmol). For determination of basal, bicarbonate-insensitive (Mg-dependent) ATPase activity, $NaHCO_3$ was omitted. The pH of both solutions was 7.4.

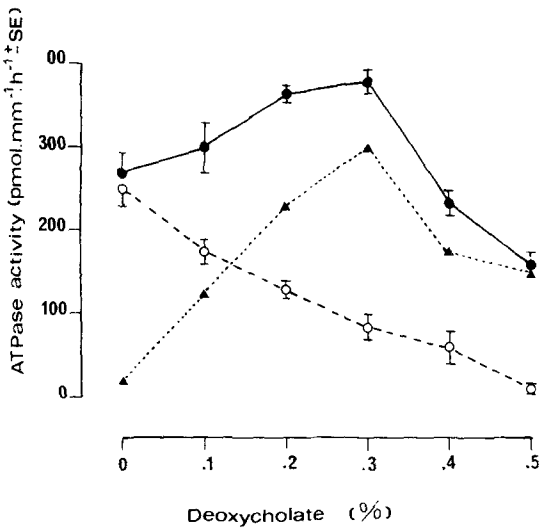

Fig. 1. Effect of sodium deoxycholate on total (–●–), Mg-dependent (–○–) and HCO_3-ATPase (–▲–) activity in rabbit pars recta. Total and Mg-dependent ATPase activity were determined on four to five samples with each deoxycholate concentration, and HCO_3-ATPase activity is the difference between the mean values

The concentrations of ATP and bicarbonate were chosen after checking that they allowed to measure ATPase activity under V_{max} conditions. The effect of increasing ATP concentration between 0.5 and 5 mM on ATPase activity was evaluated on PR dissected from the same kidney (*not shown*). Total and Mg-ATPase activity reached a plateau at ATP concentration of 2.5 mM, and, accordingly, this concentration of ATP was chosen. In separate experiments the concentration of bicarbonate was altered while keeping ionic strength constant (*not shown*). In both PR and DCT_g, ATPase activity was dependent on bicarbonate concentration in a sigmoidal fashion, suggesting a positive cooperativity. This was confirmed by the Hill's plot which indicated n_{Hill} of 1.85 and 2.17 in the PR and DCT_g, respectively. Concentration of 30 mM bicarbonate was on the plateau of maximal activity, and was chosen for subsequent assays.

Using these substrate concentrations we evaluated total and Mg-ATPase activity in PR as a function of the incubation time and of the tubular length. The increment in enzyme activity as a function of time was linear over the initial 30 min for both total ATPase and Mg-ATPase (*not shown*). Incubation for 15 min thus ensures that the two activities are measured under V_{max} conditions, and this duration was subsequently used in all assays. Total and Mg-dependent-ATPase activity were also linearly dependent (r = 0.932, n = 22 and r = 0.969, n = 21, respectively) on tubule length within a wide range from 0.25 to 1.70 mm (*not shown*).

Calculations

To examine total and Mg-dependent ATPase for each segment of

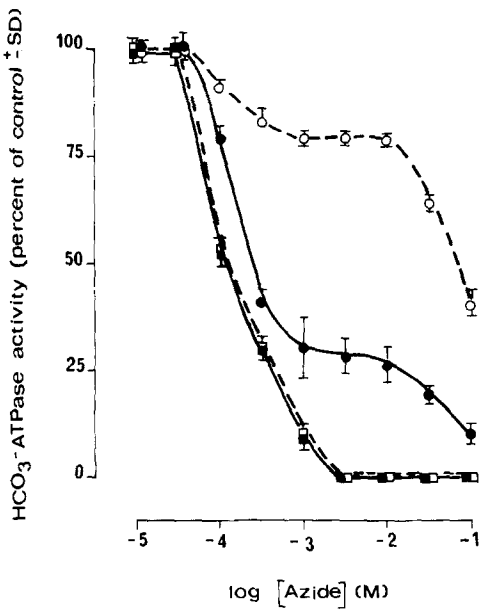

Fig. 2. Inhibition by sodium azide of HCO_3-ATPase activity in mitochondrial (□, ■) and microsomal (○, ●) fractions prepared from rabbit kidney cortex (■, ●) and outer medulla (□, ○). Enzyme activity is expressed as the percent of activity measured in the absence of azide. Each point is the mean ± SD of three separate experiments. Absolute values measured in the absence of azide were (in μmol/hr/mg protein ± SD): Mitochondria, cortex: 98.9 ± 9.9; medulla: 206.8 ± 24.8; Microsomes, cortex: 10.2 ± 2.7; medulla: 22.6 ± 1.6. In the presence of 2.5 mM azide, HCO_3-ATPase activity was completely inhibited in mitochondrial fractions but only partially inhibited in microsomal fractions

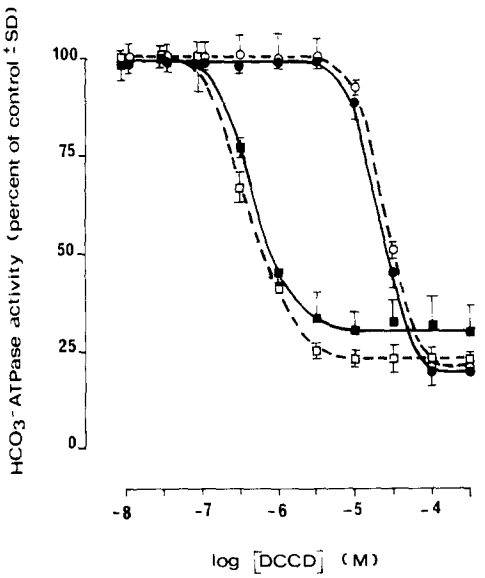

Fig. 3. Inhibition by N,N'-dicyclohexylcarbodiimide (DCCD) of HCO_3-ATPase activity in mitochondrial (□, ■) and microsomal (○, ●) fractions prepared from rabbit kidney cortex (■, ●) and outer medulla (□, ○). Each point is the mean ± SD of three separate experiments. In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. Enzyme activity is expressed as the percent of activity measured in the absence of DCCD

the nephron, 10 samples of each of its morphologically distinct regions were distributed randomly into two groups, one for measuring total, and the other Mg-dependent ATPase. HCO_3-dependent ATPase was taken as the difference between the means of each group. In addition, five blanks (incubated without tubules) were used in each assay to determine the nonenzymatic hydrolysis of ATP. ATPase activity was expressed as picomoles inorganic phosphate liberated per millimeter of tubule length per hour.

Results are expressed as means ± SE. Statistical significance was assessed by Student's *t*-test for unpaired data, *P* values less than 0.05 being considered significant.

Results

Pharmacological Properties of HCO_3-ATPase in Mitochondrial and Microsomal Rabbit Kidney Fractions

Sodium Azide

Figure 2 shows the mean variations in HCO_3-ATPase activity in mitochondrial and microsomal fractions of cortical and medullary origin in response to various concentrations of sodium azide. Results are expressed as the percent of activity measured in the absence of azide. HCO_3-ATPase activity in cortical and medullary mitochondrial fractions was inhibited in a dose-dependent fashion for sodium azide concentrations between 2.5×10^{-5} and 2.5×10^{-3} M ($K_i = 10^{-4}$ M). In the presence of 2.5 mM azide, mitochondrial HCO_3-ATPase activity was completely inhibited. In the microsomal fractions, HCO_3-ATPase activity was inhibited according to a biphasic pattern: Within the concentration range which completely inhibited HCO_3-ATPase activity in mitochondrial fractions, sodium azide reduced by only 20 to 75% this activity in microsomal fractions. For sodium azide concentrations exceeding 10^{-2} M, a second inhibitory phase was observed. These results suggest that microsomal HCO_3-ATPase activity originates in two different enzymes, one of which displays the same sensitivity to azide as the mitochondrial HCO_3-ATPase and is probably present owing to mitochondrial contamination, and another which is insensitive to azide and therefore cannot be of mitochondrial origin. Mitochondrial contamination accounted for 20 and 75% of the microsomal HCO_3-ATPase activity in the medulla and cortex, respectively. In the microsomal fraction,

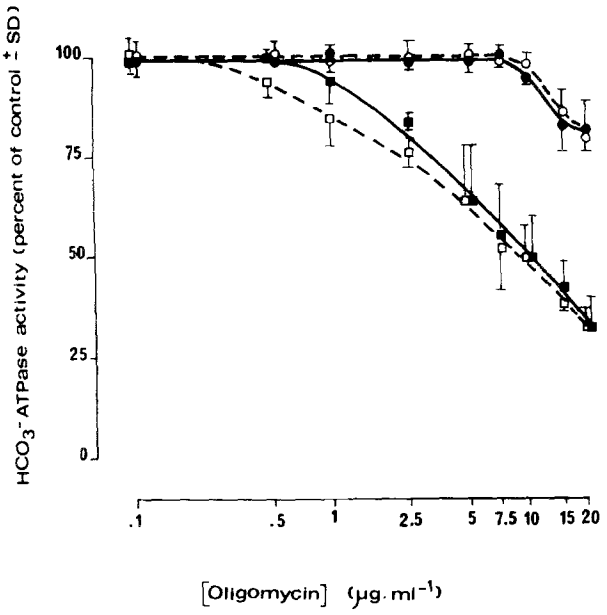

Fig. 4. Inhibition by oligomycin of HCO_3-ATPase activity in mitochondrial (□, ■) and microsomal (○, ●) fractions prepared from rabbit kidney cortex (■, ●) and outer medulla (□, ○). Each point is the mean ± SD of three separate experiments. In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. Enzyme activity is expressed as the percent of activity measured in the absence of oligomycin

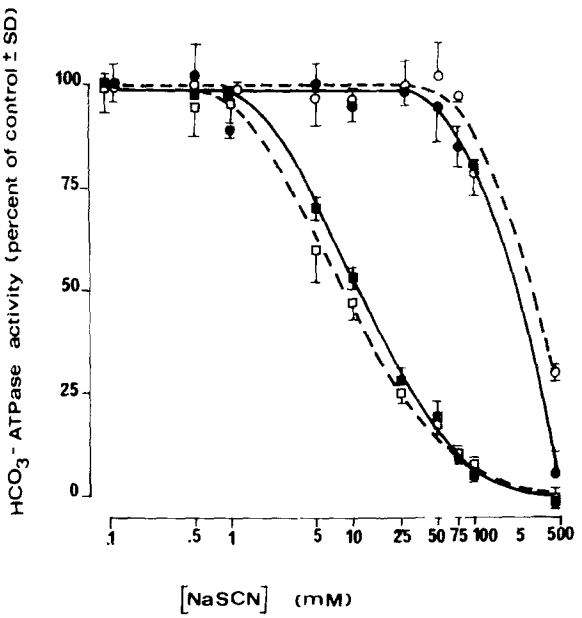

Fig. 5. Inhibition by thiocyanate of HCO_3-ATPase activity in mitochondrial (□, ■) and microsomal (○, ●) fractions prepared from rabbit kidney cortex (■, ●) and outer medulla (□, ○). Each point is the mean ± SD of three separate experiments. In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. Enzyme activity is expressed as the percent of activity measured in the absence of thiocyanate

the mean azide insensitive (extra mitochondrial) HCO_3-ATPase activity (in μmol/mg protein/hr ± SD) was 2.9 ± 0.9, $n = 3$ and 18.0 ± 1.3, $n = 3$, in the cortex and outer medulla, respectively. In further studies, we used 2.5 mM azide in the incubation medium to determine HCO_3-ATPase activity in microsomal fractions, which enabled us to measure the extramitochondrial moiety of this activity.

DCCD

Another mitochondrial inhibitor, DCCD (N, N'-dicyclohexylcarbodiimide), was also tested for its capacity to alter HCO_3-ATPase activity in mitochondrial and microsomal rabbit kidney fractions (Fig. 3). DCCD inhibited this activity by 70 to 80% in both types of fractions but the mitochondrial ATPase was almost 100 times more sensitive to DCCD than the microsomal one (mitochondria, $K_i = 5 \times 10^{-7}$ M; microsomes, $K_i = 2 \times 10^{-5}$ M).

Oligomycin

Figure 4 shows the action of a third mitochondrial inhibitor, oligomycin, on HCO_3-ATPase activity in mitochondrial and microsomal kidney fractions. Here again, mitochondrial HCO_3-ATPase was more sensitive to oligomycin than the microsomal enzyme. Thus, mitochondrial HCO_3-ATPase activity decreased by 70% in the presence of 20 μg/ml oligomycin (the threshold being observed for 1 μg/ml), whereas in microsomes, this activity only decreased by about 20%, even with high doses of oligomycin (>10 μg/ml).

Thiocyanate

The responses of mitochondrial and microsomal HCO_3-ATPase to various doses of thiocyanate resemble their responses to oligomycin (Fig. 5). The doses of thiocyanate that produced the threshold of inhibition of microsomal HCO_3-ATPase (>50 mM) were higher than those that induced half-maximal inhibition of mitochondrial ATPase (10 mM).

Vanadate

HCO_3-ATPase activity in both mitochondrial and microsomal fractions was not very sensitive to vanadate since concentrations of over 10^{-5} M only induced mild inhibition (*not shown*).

Table 1. Kinetic characteristics of mitochondrial and microsomal HCO_3-ATPase[a]

		Mitochondrial HCO_3-ATPase		Microsomal HCO_3-ATPase	
		Cortex	Medulla	Cortex	Medulla
Bicarbonate	apparent K_m	10.7 ± 0.6 (3)	10.1 ± 1.1 (3)	8.6 ± 1.0 (5)[b]	7.2 ± 1.6 (5)[b]
	n_{Hill}	1.53 ± 0.08 (3)	1.65 ± 0.08 (3)	1.33 ± 0.08 (5)[b]	1.36 ± 0.10 (5)[c]
	V_{max}	108.4 ± 11.1 (3)	183.9 ± 26.3 (3)	2.5 ± 1.2 (5)[d]	19.3 ± 2.9 (5)[d]
ATP	apparent K_m	0.92 ± 0.08 (3)	1.17 ± 0.15 (3)	0.48 ± 0.07 (3)[d]	0.63 ± 0.10 (3)[d]
	V_{max}	111.1 ± 12.7 (3)	203.3 ± 18.4 (3)	2.9 ± 0.4 (3)[d]	18.2 ± 2.4 (3)[d]

[a] Values are mean ± SD, number of experiments in parentheses. Apparent K_m are expressed in mmol/liter and V_{max} in μmol/mg protein/hr.
Statistically different from mitochondrial fractions according to Student's *t*-test:
[b] $P < 0.025$;
[c] $P < 0.005$;
[d] $P < 0.001$.

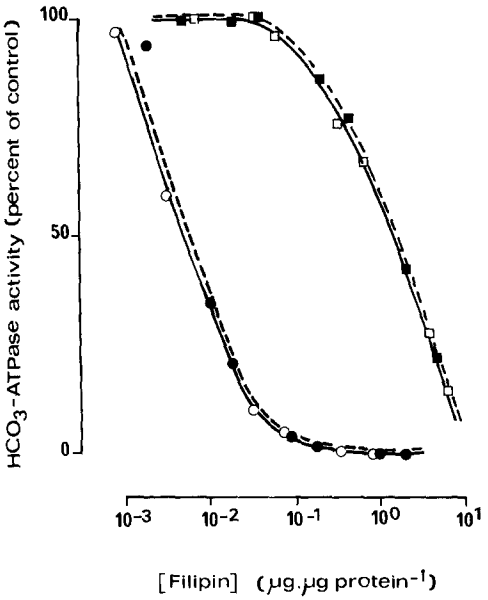

Fig. 6. Inhibition by filipin of HCO_3-ATPase activity in mitochondrial (□, ■) and microsomal (○, ●) fractions prepared from rabbit kidney cortex (■, ●) and outer medulla (□, ○). In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. Enzyme activity is expressed as the percent of activity measured in the absence of filipin

N-ethylmaleimide

HCO_3-ATPase activity was not sensitive to N-ethylmaleimide, even at high concentrations (up to 10^{-2} M), in either mitochondrial or microsomal fractions (*not shown*).

Filipin

Filipin is a polyene antibiotic which complexes membrane cholesterol [9] and therefore alters the lipid environment of proteins present in cholesterol-rich membranes. Since microsomal plasma membranes are much richer in cholesterol than mitochondrial membranes, we also tested the action of filipin on the HCO_3-ATPase activity present in microsomal and mitochondrial fractions (Fig. 6). Filipin was a potent HCO_3-ATPase inhibitor since it completely abolished the enzyme activity in both types of fractions. However, microsomal fractions were over 100-fold more sensitive than mitochondrial ones to inhibition by filipin (Microsomes: $K_i = 5 \times 10^{-3}$ μg/μg protein; Mitochondria: $K_i = 2$ μg/μg protein).

Kinetic Characteristics of Mitochondrial and Microsomal HCO_3-ATPase Activity

The second approach used to distinguish between HCO_3-ATPase activity of mitochondrial and microsomal origin was to explore their specific kinetic properties. For this study, again we used 2.5 mM sodium azide in the microsomal ATPase assay in order to inhibit the mitochondrial contaminant present in the microsomal fractions.

Bicarbonate Dependence

Stimulation of HCO_3-ATPase activity in mitochondrial and microsomal fractions by increasing bicarbonate concentrations produced similar activation patterns in cortical and medullary preparations but there were slight differences between these patterns in mitochondrial and microsomal fractions. In both fractions maximal activity was observed with bicarbonate concentrations of about 30 mM, the concentration chosen in most experiments. Higher concentrations of bicarbonate (>40 to 50 mM) slightly inhibited HCO_3-ATPase activity in microsomal but

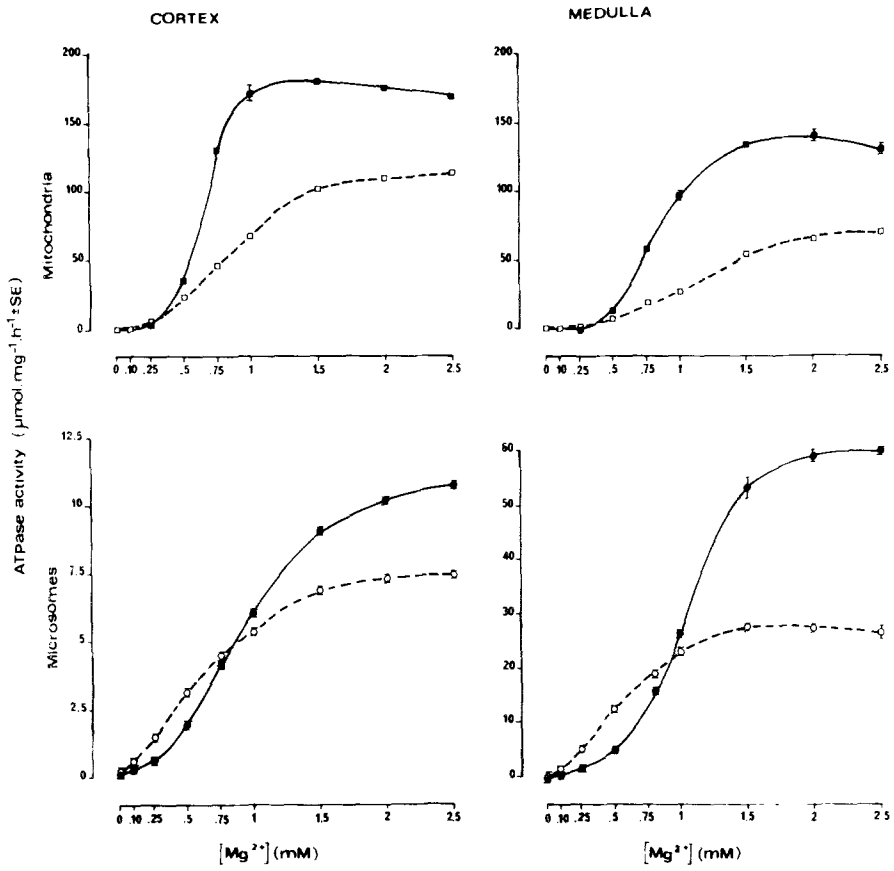

Fig. 7. Effect of the magnesium concentration on ATPase activity measured in the presence (full symbols) or absence (open symbols) of 30 mM bicarbonate, in mitochondrial fractions (upper panel) and microsomal fractions (lower panel) prepared from rabbit kidney cortex (left) and outer medulla (right). In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. Each point represents the mean ± SE of three to four determinations

not in mitochondrial fractions. In the presence of low concentrations, activation curves were sigmoidal, suggesting positive cooperativity which was confirmed by the Hill plot. Microsomal HCO_3-ATPase differed from the mitochondrial enzyme by its lower apparent K_m and n_{Hill} for bicarbonate (Table 1).

ATP Dependence

When ATP concentration was raised, HCO_3-ATPase activity increased in both microsomal and mitochondrial fractions. For both cortex and medulla, the apparent K_m for ATP was higher in mitochondrial than microsomal fractions (Table 1), again suggesting that the origin of HCO_3-ATPase activity was different in each type of fraction.

Magnesium Dependence

Both basal ATPase activity (Mg-ATPase) and total activity (Mg-ATPase + HCO_3-ATPase) in the various membrane fractions increased in a sigmoidal fashion with rising concentrations of magnesium (Fig. 7). Magnesium dependence was similar in cortical and medullary fractions but markedly different in mitochondrial and microsomal preparations. In the microsomal fractions, 30 mM bicarbonate significantly inhibited the basal Mg-ATPase activity induced by magnesium concentrations below 0.9 mM, whereas at higher magnesium concentrations, bicarbonate obviously acted as a stimulant. Although this inhibition also occurred in mitochondrial fractions, it was quantitatively much smaller and was observed for a narrower range of magnesium concentrations (<0.20 mM). In four separate experiments, the mean magnesium concentrations ± SD at which total ATPase activity was equal to Mg-ATPase activity were as follows: for microsomes: cortex, 0.78 ± 0.08 mM; medulla, 0.74 ± 0.17 mM; and for mitochondria: cortex, 0.18 ± 0.09; medulla, 0.18 ± 0.09. To measure HCO_3-ATPase activity, we decided to use a magnesium concentration of 1.5 mM, which although not optimal, constitutes a good compromise between the optimal concentrations respectively required for microsomal and mitochondrial fractions.

pH Dependence

The changes occurring in HCO_3-ATPase activity when the pH in the incubation medium was raised

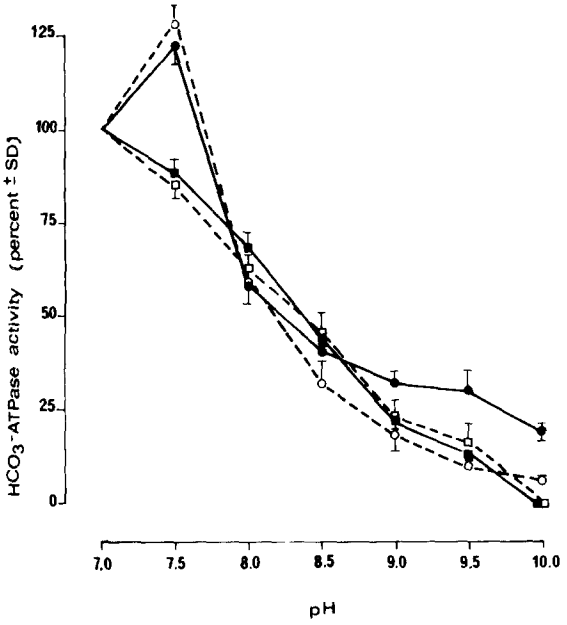

Fig. 8. Effect on pH on HCO_3-ATPase activity in mitochondrial fractions (□, ■) and microsomal fractions (○, ●) prepared from rabbit kidney cortex (■, ●) and outer medulla (□, ○). In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. Each point is the mean ± SD of three separate experiments

to levels between 7.0 and 10.0 (Fig. 8) showed that in mitochondrial fractions, this activity was almost linearly dependent on pH and became zero around pH 10.0. In microsomal fractions, HCO_3-ATPase activity was maximum at pH 7.5 and rapidly decreased at higher pH.

Anion Dependence

ATPase activity is stimulated by several mineral ions in addition to bicarbonate, including chloride, sulfate, nitrate and sulfite, as well as by organic anions including citrate, acetate, maleate, glucuronate and oxalate. These different anions are generally held to activate the same molecular entity, whose specificity toward stimulating anions is rather poor. However, the relative stimulating power of these anions differs from tissue to tissue. We therefore investigated the ATPase activities respectively originating in kidney mitochondria and microsomes to see whether they had the same anionic specificity. For this purpose, we compared the dependence of mitochondrial and microsomal ATPase on chloride, bicarbonate, sulfate and sulfite.

In the results summarized in Fig. 9, ATPase activities are expressed as percent of basal activities (determined in the presence of 1.5 mM sulfate as the only mineral anion). Anionic specificity was similar in cortical and medullary fractions but markedly different in mitochondria and microsomes. In mitochondria, bicarbonate was the most efficient anion and stimulated basal Mg-ATPase activity three- to fourfold. Sulfite was also a potent stimulator, although it became slightly inhibitory at concentrations above 50 mM. Conversely, chloride did not activate mitochondrial ATPase activity and even inhibited basal Mg-ATPase activity. Sulfate slightly stimulated ATPase activity at concentrations below 20 mM and inhibited basal ATPase activity at higher concentrations. In the microsomes, sulfite was by far the most potent stimulator of ATPase activity, followed by bicarbonate and chloride. Sulfate had no significant effect on basal activity.

Two conclusions can be drawn from these results: First, the presence of 1.5 mM sulfate in the basal assay medium ($MgSO_4$) does not significantly alter ATPase activity and therefore does not lead to underestimation of anion-stimulated activity. Second, anion-stimulated ATPase activity clearly has different anionic specificity in mitochondria and microsomes, which is another argument for the extramitochondrial origin of this activity in microsomes.

In view of these findings, one may wonder whether, in the same membrane preparation, stimulation of these ATPase activities by several anions involves the same molecular entity, or it reflects the presence of several enzymes, each one stimulated by a single anion. If there were several distinct enzymes, the different anion specificities found for the microsomal and mitochondrial fractions could not be taken as an argument indicating that microsomal and mitochondrial HCO_3-ATPase are different. These results would rather indicate the existence of several enzymes, some being present in the mitochondria, e.g. HCO_3-ATPase and SO_3-ATPase, and others in the microsomes, e.g. HCO_3-ATPase, SO_3-ATPase and Cl-ATPase. In that case, the difference in the stimulating efficiency of the various anions in these two fractions would represent the relative proportions of these enzymes present in each type of fraction.

To ascertain whether the different anions stimulated one or several enzymes, we tested whether or not their stimulatory actions were additive. If each anion stimulates a different enzyme, the ATPase activity determined in the simultaneous presence of maximal concentrations of two anions should be the

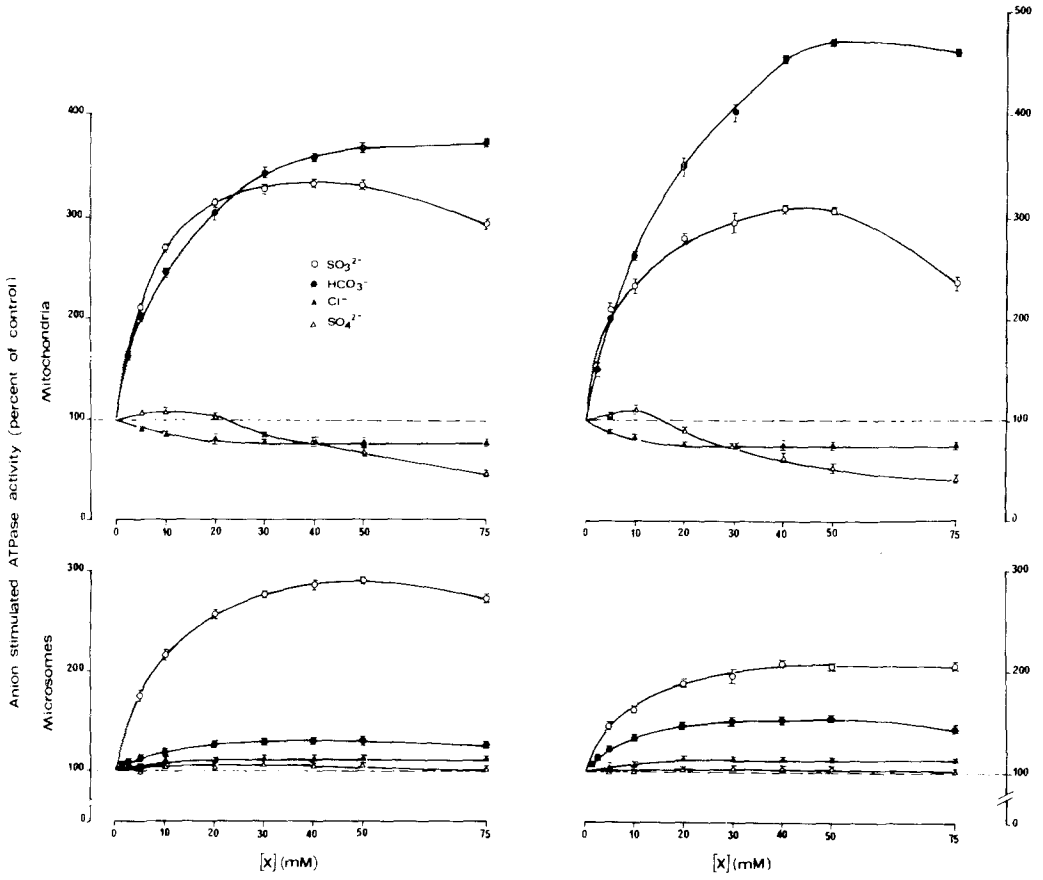

Fig. 9. Effect of bicarbonate (●), sulfite (○), chloride (▲) and sulfate (△) concentrations on the ATPase activity measured in mitochondrial fractions (upper panel) and microsomal fractions (lower panel) prepared from rabbit kidney cortex (left) and outer medulla (right). In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. ATPase activity is expressed as the percent of activity determined in the absence of anion (Mg-ATPase). Each point represents the means ± SE of three to four determinations

sum of the activities observed in the presence of each anion. Conversely, if each anion stimulates the same enzyme, the ATPase activities should not be additive, and the stimulation observed in the presence of two anions should not exceed the maximal stimulation induced by the most efficient anion. The results depicted in Fig. 10 indicate that in mitochondrial fractions, the responses to bicarbonate and sulfite were not additive. Similarly, in the microsomes, the response to bicarbonate was not additive to that of either chloride or sulfite. In the presence of bicarbonate and sulfite, ATPase activity was even lower than in the presence of sulfite alone, probably because high concentrations of anion are mildly inhibitory, and/or because of competition for the occupancy of the stimulatory site.

These results demonstrate that mitochondrial and microsomal fractions each contain a single anion-dependent ATPase activity which in the mitochondria is stimulated by bicarbonate or sulfite, and in the microsomes by sulfite and to a lesser extent by bicarbonate and chloride.

Properties of the Tubular HCO_3-ATPase

Extramitochondrial Origin of Tubular HCO_3-ATPase Activity

In view of the pharmacological and kinetic differences observed between mitochondrial and microsomal HCO_3-ATPase activities, we decided to discriminate these two components on tubular segments on the basis of their sensitivity to sodium azide and of their anionic specificity.

Figure 11 shows the dependency of HCO_3-ATPase activity on sodium azide in the PR. The inhibitory action of sodium azide was biphasic: HCO_3-ATPase activity decreased markedly for az-

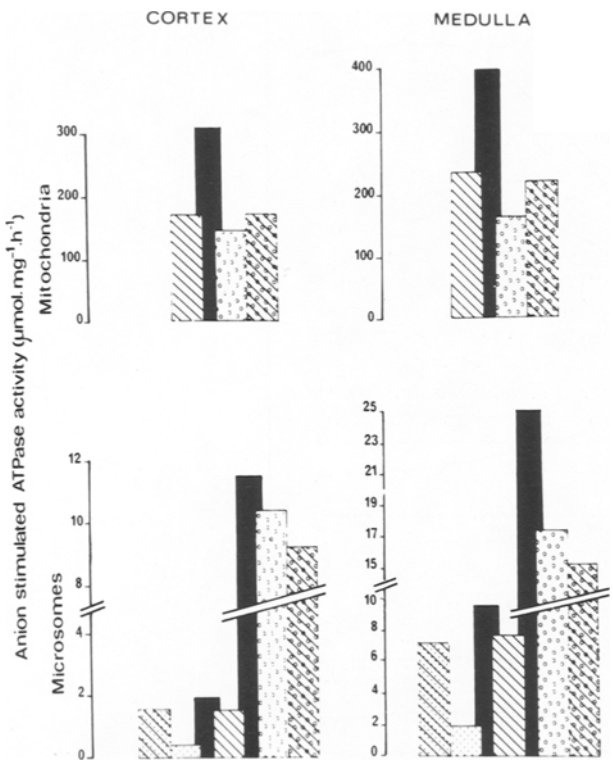

Fig. 10. Increase in ATPase activity after stimulation by 50 mM of each of the following: bicarbonate (▨), chloride (▩), sulfite (▩), bicarbonate + chloride (▨) or bicarbonate + sulfite (▨) in mitochondrial fractions (upper panel) and microsomal fractions (lower panel) prepared from rabbit kidney cortex (left) and outer medulla (right). In the microsomal fractions, ATPase activity was determined in the presence of 2.5 mM sodium azide. Black bars between the columns for two anions indicate the theoretical activity which would be expected if these anions had additive effects

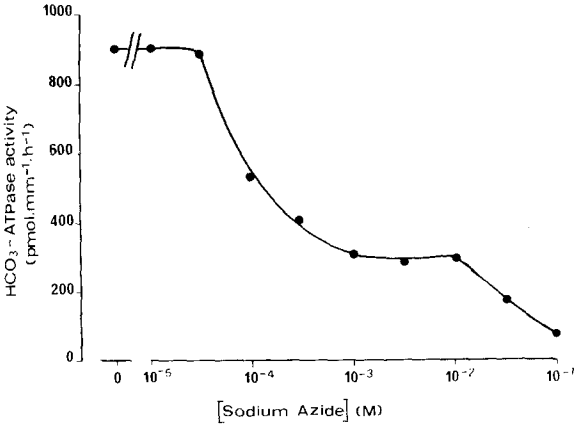

Fig. 11. Inhibition of HCO_3-ATPase activity from rabbit pars recta as a function of sodium azide concentration. Each point is the difference between the means of two groups of four to five samples in which total and Mg-ATPase activity were determined

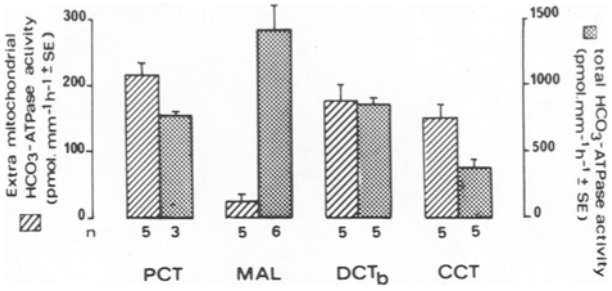

Fig. 12. HCO_3-ATPase activity measured in the presence (extramitochondrial HCO_3-ATPase) or in the absence (total HCO_3-ATPase) of 2.5 mM sodium azide in the proximal convoluted tubule (PCT), medullary thick ascending limb of Henle's loop (MAL), bright portion of the distal convoluted tubule (DCT_b) and cortical tubule (CCT). *n* indicates the number of animals

ide concentrations comprised between 3×10^{-5} and 10^{-3} M, reached a plateau until 10^{-2} M and further decreased thereafter. These results, which are similar to those described on microsomal fractions (Fig. 2) suggest that in the presence of 2.5 mM sodium azide, HCO_3-ATPase activity from mitochondrial origin was completely inhibited whereas the extramitochondrial one was not altered. Therefore, this concentration of sodium azide was adopted to determined the extramitochondrial moiety of HCO_3-ATPase activity in isolated nephron segments.

Furthermore, HCO_3-ATPase activities determined either in the presence or the absence of sodium azide were compared in several nephron segments. Results in Fig. 12 indicate that not only ATPase activities were much lower in the presence of sodium azide, but also the profile of the activity along the nephron was different in the presence and the absence of the mitochondrial inhibitor. In the absence of sodium azide, HCO_3-ATPase activity was highest in the medullary thick ascending limb of Henle's loop, intermediate in the proximal and distal convoluted tubule and lowest in the cortical collecting tubule, whereas the extramitochondrial HCO_3-ATPase activity (measured in the presence of 2.5 mM sodium azide) was similar in the proximal and distal convoluted tubule and collecting tubule, and almost undetectable in the medullary thick ascending limb.

Finally, we compared the stimulatory efficiency of bicarbonate and sulfite on tubular extramitochondrial ATPase since it was shown that these two anions had similar effects on the mitochondrial enzyme, whereas on microsomal fractions sulfite was more potent than bicarbonate to stimulate ATPase

Table 2. Stimulatory action of bicarbonate and sulfite on extramitochondrial anion-dependent ATPase activity along the rabbit nephron[a]

	Base	$+HCO_3^-$	Δ HCO_3	$+SO_3^{2-}$	Δ SO_3^{2-}
PCT	552 ± 28 (5)	712 ± 64 (5)	160	1777 ± 230 (4)	1225
PR	481 ± 72 (4)	727 ± 46 (5)	246	1566 ± 218 (5)	1082
MAL	84 ± 56 (5)	98 ± 46 (5)	14	174 ± 58 (5)	90
CAL	128 ± 27 (5)	128 ± 40 (4)	0	99 ± 36 (5)	−29
DCT_b	503 ± 53 (5)	684 ± 30 (5)	181	3167 ± 361 (5)	2664
DCT_g	290 ± 74 (5)	524 ± 107 (5)	234	4220 ± 233 (5)	3930
CCT	646 ± 66 (5)	808 ± 44 (4)	162	1646 ± 86 (4)	1000
MCT	269 ± 55 (5)	495 ± 100 (5)	226	2930 ± 182 (5)	2661

[a] ATPase activity in the different rabbit nephron segments in the absence of added anion (Base) or in the presence of either 25 mM bicarbonate ($+HCO_3^-$) or 25 mM sulfite ($+SO_3^{2-}$). Activities are in pmol · mm^{-1} · hr^{-1} ± SD; number of segments in parentheses.

Table 3. Effect of different pharmacological agents on tubular HCO_3-ATPase activity

	PCT	PR	DCT_b	CCT
Oligomycin 10 μg ml^{-1}	98.3, 97.4	98.2, 100.2	NT	101.6, 96.7
N-ethylmaleimide 1 mM	96.4, 101.6	NT	96.5, 102.6	NT
Vanadate 10^{-5} M	100.6, 97.4	99.0, 102.0	88.4, 98.3	87.7, 109.6
DCCD 3 μM	98.3, 99.2	107.6, 100.8	NT	97.5, 90.8
30 μM	44.0, 39.6	64.8, 51.7	NT	52.4, 39.6
Thiocyanate 25 mM	117.5, 98.6	91.4, 98.3	101.1, 97.2	85.8, 106.7

[a] Results are individual values of two different experiments and are expressed as percent of the control HCO_3-ATPase activity determined on the same animals. NT, not tested.

Table 4. HCO_3-ATPase and Mg-ATPase activities in individual nephron segments of control and DOCA-treated rabbits[a]

	Control			DOCA-treated		
	n	HCO_3-ATPase	Mg-ATPase	*n*	HCO_3-ATPase	Mg-ATPase
PCT	5	198 ± 13	241 ± 53	6	235 ± 37	248 ± 30
PR	5	285 ± 13	152 ± 41	6	267 ± 22	137 ± 34
MAL	5	3 ± 3	127 ± 46	6	9 ± 4	123 ± 33
CAL	4	9 ± 7	42 ± 18	4	16 ± 9	54 ± 32
DCT_b	5	276 ± 13	152 ± 27	5	374 ± 21[b]	191 ± 64
DCT_g	4	300 ± 23	116 ± 82	6	419 ± 18[b]	139 ± 63
CCT	5	98 ± 13	130 ± 31	6	215 ± 17[c]	179 ± 67
MCT	4	96 ± 12	83 ± 56	4	177 ± 14[c]	81 ± 53

[a] Each value is the mean ± SE of *n* rabbits. ATPase activity is expressed as picomoles per millimeter per hour.
Statistical significance from Student's *t*-test:
[b] $P < 0.005$;
[c] $P < 0.001$.

activity (Fig. 9). Results presented in Table 2 indicate that in all segments, the anion-stimulated ATPase activity determined in the presence of 2.5 mM sodium azide was higher with sulfite than bicarbonate.

Pharmacological Properties of Tubular HCO_3-ATPase

In addition to its insensitivity to sodium azide and its higher response to sulfite than bicarbonate, tubu-

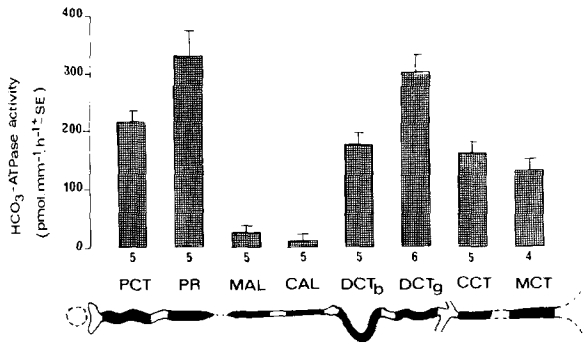

Fig. 13. HCO_3-ATPase activity along the rabbit nephron. PCT, proximal convoluted tubule; PR, pars recta; MAL and CAL, medullary and cortical thick ascending limb of Henle's loop; DCT_b and DCT_g, bright and granular portion of the distal convoluted tubule; CCT and MCT, cortical and outer medullary collecting tubule. *n* indicates the number of animals

lar HCO_3-ATPase shares several other properties with the extramitochondrial enzyme described in microsomal fractions. As shown in Table 3, HCO_3-ATPase activity of different nephron segments was insensitive to 10 μg ml^{-1} oligomycin, 1 mM N-ethylmaleimide, 10^{-5} M vanadate, 25 mM thiocyanate and 3 μM dicyclohexylcarbodiimide (DCCD). In the presence of 30 μM DCCD, the enzyme activity was reduced by approximately half, as in microsomal fractions.

Localization of HCO_3-ATPase Along the Nephron

HCO_3-ATPase Along the Nephron of Normal Rabbits

Figure 13 shows HCO_3-ATPase activity in individual nephron segments from normal animals. The highest HCO_3-ATPase activity (300 to 330 pmol · mm^{-1} · h^{-1}) was found in the straight portion of the proximal tubule and the late granular distal convoluted tubule. HCO_3-ATPase activity was lower (130 to 210 pmol · mm^{-1} · h^{-1}) in the proximal convoluted tubule, the initial bright portion of the distal convoluted tubule and the collecting tubule in its cortical and outer medullary portions. Enzyme activity in the medullary and cortical thick ascending limb of Henle's loop was not statistically different from zero.

Effects of DOCA Treatment

As similar ATPase activity was measured in DOCA-treated animals at various intervals between days 11 and 18 of treatment, the data were pooled.

Changes in HCO_3-ATPase activity were confined to the distal segments of the nephron, as this activity was not altered by DOCA treatment in any other segments examined (Table 4). Compared with controls, HCO_3-ATPase activity in DOCA-treated animals averaged 135 and 140% in the bright and granular portions of the distal convoluted tubule, respectively, and 219 and 184% in the cortical and outer medullary collecting tubules, respectively. DOCA administration had no effect on HCO_3-insensitive-ATPase in any nephron segment, as Mg-ATPase activity was similar in control and DOCA-treated animals (Table 4).

Because alterations in enzyme activity were located at the sites where structural changes were previously found after similar treatment [15, 23, 49], it appeared necessary to express the results not only in terms of tubule length but also as a function of protein content. Therefore, the results were recalculated for the various nephron segments using the protein content per unit length we reported previously [15]. The data in Table 5 clearly show that HCO_3-ATPase activity expressed per microgram of protein was similar in the various nephron segments of control and DOCA-treated animals, except in the cortical and outer medullary collecting tubule where it still increased by 62 and 42%, respectively.

Discussion

One of the purposes of this study was to determine whether HCO_3-ATPase is an intrinsic component of microsomal membranes or whether it entirely results from the mitochondrial contaminant present in such fractions. On the basis of the sensitivity to sodium azide (Fig. 2) it appears that part of the HCO_3-ATPase activity present in microsomal fractions prepared from rabbit kidney probably originates in the mitochondria. However, the azide-insensitive moiety of this microsomal HCO_3-ATPase activity has pharmacological and kinetic properties which clearly distinguish it from a mitochondrial contaminant: 1) It is less sensitive than the mitochondrial enzyme to oligomycin and DCCD (Figs. 3, 4), two compounds known to interact directly with the subunits of mitochondrial ATPase; 2) It is less sensitive to thiocyanate (Fig. 5); 3) It displays different sensitivities to magnesium, pH, and anions (Figs. 7, 8, 9); 4) Finally, it is much more sensitive to filipin than mitochondrial ATPase activity (Fig. 6), suggesting that it is embedded in a cholesterol-rich membrane. HCO_3-ATPase activity was also localized in specific segments of the rabbit nephron, i.e., the proximal tubule in its convoluted and straight portions, the distal convoluted tubule and

the cortical and medullary collecting tubule (Fig. 13). This tubular enzyme is most likely the same as that described in microsome fractions prepared from kidney homogenates since it shares most of its properties: It is insensitive to sodium azide (Fig. 11), oligomycin, N-ethylmaleimide, vanadate and thiocyanate (Table 3), has similar sensitivity to DCCD (Table 3), and is better stimulated by sulfite than bicarbonate (Table 2). The absence of any detectable HCO_3-ATPase activity in the thick ascending limb of Henle's loop, a segment rich in mitochondria [40] and in the mitochondrial enzymatic marker citrate synthase [34], is an additional evidence for its extramitochondrial origin. The demonstration of the presence of an extramitochondrial HCO_3-ATPase activity in kidney microsomes is consonant with recent findings of anion-stimulated ATPase activities in the plasma membranes of numerous tissues and species such as the gills of the goldfish [12], eel [4], rainbow trout [5] and fiddler crab [10], the insect rectum [29], the intestine of *Aplysia* [17] and of the rat [21], and of the mantel of the freshwater clam [50].

If the origin of microsomal HCO_3-ATPase is extramitochondrial, what then is its intracellular location? Renal microsomal fractions include membranes from various origins: endoplasmic reticulum, lysosomes, plasma membranes, Golgi apparatus, endosomes, etc. Therefore, it is not known in which subcellular compartment the microsomal HCO_3-ATPase described in this study is located. In this respect, it is worth noting that determination of HCO_3-ATPase activity in isolated nephron segments required partial solubilization of the system with deoxycholate, a procedure which is not required to determine ATPase activity located in tubular cell plasma membrane, such as Na,K-ATPase [13]. This difference probably reflects a different intracellular location, HCO_3-ATPase being less accessible to exogenous substrates than ATPase of the basolateral membrane as expected if it were located in the luminal membrane or in intracellular vesicles. Several authors reported the presence of an HCO_3-ATPase activity displaying some characteristics of the present kidney microsomal enzyme in brush-border membranes prepared from rat kidney cortex [8, 26] suggesting that the HCO_3-ATPase in kidney microsomes might be located in the luminal membrane of tubular cells. However, it is noteworthy that in the study by Kinne-Saffran and Kinne [26], alkaline phosphatase activity (a marker of the proximal tubule brush border) was enriched 10.7-fold in the brush-border membranes, whereas HCO_3-ATPase was only enriched 3.4-fold, suggesting that alkaline phosphatase and HCO_3-ATPase had different locations within the luminal brush border. In agreement with the luminal location of the anion-stimulated ATPase, Iyengar and colleagues [22] reported in dog renal medulla a 26-fold enrichment of SO_3-ATPase activity in a membrane fraction they tentatively identified as apical membranes of the collecting duct. In contrast, Knauf and colleagues recently reported that the presence of an HCO_3-ATPase in brush-border membranes of rat proximal tubules was due to mitochondrial contamination [28]. Our results, however, clearly demonstrate the existence of an extramitochondrial HCO_3-ATPase activity in kidney membrane fractions. If anion-stimulated ATPase is indeed associated with the luminal membrane of proximal and collecting tubules, it might not have the same location as alkaline phosphatase in this membrane which is known to be heterogeneous as regards its protein composition [24]. One possibility would be that HCO_3-ATPase activity is located in the coated pits or on the endocytic vesicles just below the luminal membrane whereas alkaline phosphatase is located more distally on the microvilli. This might explain the differences in enrichment observed by Kinne-Saffran and Kinne [26] for alkaline phosphatase and HCO_3-ATPase, and also the absence of extramitochondrial HCO_3-ATPase reported by Knauf et al. [28] if we assume that their membrane preparations were partially or totally devoid of the most basal portion of the brush-border membranes.

The last problem to be discussed concerns the possible function of this enzyme. Ion-translocating ATPases may be classified into at least two categories, E_1-E_2 and F_0-F_1, according to their properties. ATPases of the E_1-E_2 class, such as Na,K-ATPase, Ca,Mg-ATPase or gastric H,K-ATPase, are characterized in part by the presence of a phosphorylated intermediate in their catalytic cycle which renders them sensitive to vanadate. In contrast, F_0-F_1 type ATPases have no phosphorylated intermediate, and

Table 5. HCO_3-ATPase activity per protein content in individual nephron segments of control and DOCA-treated rabbits[a]

	Control	DOCA-treated
PCT	627 ± 41 (5)	695 ± 109 (6)
PR	983 ± 45 (5)	937 ± 77 (6)
MAL	13 ± 13 (5)	40 ± 18 (6)
CAL	80 ± 63 (4)	152 ± 86 (4)
DCT_b	1160 ± 55 (5)	1100 ± 62 (5)
DCT_g	1277 ± 98 (4)	1111 ± 48 (6)
CCT	508 ± 67 (5)	824 ± 65 (6)[b]
MCT	410 ± 51 (4)	582 ± 46 (4)[b]

[a] HCO_3-ATPase activity is expressed as pmoles per microgram protein per hour ± SE; number of animals is given in parentheses. Calculations were done using the relationship between protein content and tubular length according to El Mernissi et al. [15].
[b] $P < 0.05$ according to Students *t*-test.

thus are insensitive to vanadate. The latter class includes ATPases present in the mitochondria, lysosomes, clathrin-coated vesicles, and endosomes, all of which are known to transport protons. Although microsomal kidney HCO_3-ATPase is different from mitochondrial ATPase, it belongs to the F_0-F_1 class because it is insensitive to vanadate, and it is therefore likely to transport protons. In that case it would no longer be justified to deduce that, as bicarbonate stimulates ATPase activity, ATPase primarily drives bicarbonate transport. Instead of being transport, anions might thus act as regulators of this proton pump, as already described for mitochondrial ATPase [41], a system sensitive to anions and capable of performing ATP-driven transport, in which it has been proposed that the anions regulate the ATPase activity allosterically. Physiologically, bicarbonate might thus fulfill two functions in proton secretion: stimulation of the proton pump and buffering of the protons extruded in the luminal fluid by the pump.

If one admits that the extramitochondrial HCO_3-ATPase works as a proton pump, the question arises whether it could be implicated in proton secretion and/or bicarbonate reabsorption across the tubular epithelium. Gottschalk and colleagues first demonstrated that in the rat, urine acidification occurs in the proximal tubule, the distal convoluted tubule and collecting duct [19], the nephron segments in which we localized the extramitochondrial HCO_3-ATPase activity (Fig. 13). Two mechanisms of proton secretion have been identified in the mammalian kidney: Na/H exchange and active, sodium-independent proton secretion. However, the exact contribution of the two modes of proton secretion in the different segments is still controversial. Electroneutral Na/H exchange has been demonstrated in rat and rabbit brush-border membrane vesicles [27, 38] as well as in isolated rabbit proximal tubules [2], and probably accounts for the major fraction of proton secretion in the proximal tubule. However, McKinney and Burg [35], and Burg and Green [6] suggested that part of the acidification observed on rabbit proximal tubules perfused *in vitro* might be sodium independent, and thus primarily active. Bichara and colleagues [3] also provided evidence for the presence of a primary proton pump in suspensions of rabbit proximal tubules. In the rat proximal tubule, Frömter and Gessner [16], and Chan and Giebisch [7] also suggested that part of proton/bicarbonate transport was active and sodium independent. The rabbit cortical collecting tubule is able to either reabsorb or secrete protons [36, 37] whereas the medullary collecting tubule constantly secretes protons [32, 46]. All these processes were shown to be active and the existence of an electrogenic proton pump has been postulated in these segments [1]. Thus the localization of HCO_3-ATPase in the proximal and collecting tubule found in this study is also in agreement with the sites of active proton pumping, and is compatible with a role of this enzyme in proton secretion through the nephron epithelium. The hypothesis that HCO_3-ATPase described in the present study might be located in subapical vesicles and be involved in proton transport is consonant with several observations showing that ATP-dependent proton pumps are present in membrane vesicles from kidney cortex [25, 42] and medulla [18]. These proton pumps are located in subapical endocytic vesicles in both proximal [42, 44] and collecting tubule cells [44]. However, tubular HCO_3-ATPase is insensitive to N-ethylmaleimide (Table 3) in contrast to these proton pumps. Whether this difference in sensitivity to NEM indicates two distinct enzymes, or is due to assay conditions will have be to elucidated.

The presence of a proton-ATPase was also demonstrated in kidney lysosomes [20]. Such a localization for the tubular HCO_3-ATPase is most unlikely since the lysosomal proton pump is also sensitive to NEM [20], and furthermore, the distribution along the nephron of lysosomal markers such as acid phosphatase and N-acetyl-D-glucosaminidase [30, 39] is quite different from the profile of HCO_3-ATPase.

Recently, Stone and colleagues [46] reported that, in the rabbit medullary collecting duct, adrenalectomy reduced bicarbonate reabsorptive rate by more than 90% whereas chronic DOCA administration almost doubled it. Thus, we evaluated whether chronic DOCA administration might alter HCO_3-ATPase activity in target segments for mineralocorticoids. Results in Table 4 indicate that chronic DOCA treatment markedly increased HCO_3-ATPase activity in the collecting tubule and, although to a lesser extent, in the distal convoluted tubule. It is known, however, that chronic administration of DOCA induces the hypertrophy of these distal segments of the rabbit nephron [15, 23, 49]. This raises the problem whether the observed changes in HCO_3-ATPase activity are specific corticosteroids or whether they are secondary to the overall increase in protein content and membrane surface observed after chronic DOCA treatment. Since DOCA treatment increased tubular HCO_3-ATPase activity and protein content by a similar factor in the distal convoluted tubule (Table 5), it is likely that changes in ATPase activity observed in this segment are nonspecific. However, in the collecting tubule, HCO_3-ATPase activity increased proportionally more than the protein content (Table 5), suggesting that induction of HCO_3-ATPase

might be a specific effect of corticosteroids involved in the alteration of H^+/HCO_3^- transport observed in this segment [46]. In order to further study the possible relationship between HCO_3-ATPase activity and mineralocorticoids, we shall attempt to evaluate the short-term changes in ATPase activity in response to administration of aldosterone to adrenalectomized rabbits since it was shown that *in vitro* addition of aldosterone significantly increased bicarbonate reabsorption by MCT segments harvested from adrenalectomized rabbits [46].

In conclusion, the present results demonstrate that renal microsomes display intrinsic anion-stimulated ATPase activity which cannot be ascribed to mitochondrial contamination. This enzyme is insensitive to azide, oligomycin, and thiocyanate and only slightly sensitive to DCCD and thus displays pharmacological properties of proton-ATPases of the F_0-F_1 class. Finally, it is located exclusively in the proximal tubule, the distal convoluted tubule and the collecting tubule, that is at the sites of urinary acidification and of active proton pumping, suggesting that it might be implicated in proton secretion through the luminal border of tubular cells.

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