

OUABAIN BINDING TO RENAL TUBULES OF THE RABBIT

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ABSTRACT

It is well known that ouabain, a specific inhibitor of Na-K ATPase-dependent transport, interferes with renal tubular salt reabsorption. In this study, we employed radiochemical methods to measure the kinetics of [³H]ouabain binding to slices of rabbit renal medulla and high resolution quantitative autoradiography to determine the location and number of cellular binding sites. The kinetics obeyed a simple bimolecular reaction with an association constant of $2.86 \pm 0.63 \text{ SD} \times 10^3 \text{ M}^{-1} \text{ min}^{-1}$ and a dissociation constant of $1.46 \times 10^{-3} \text{ min}^{-1}$, yielding an equilibrium binding constant of $0.51 \times 10^{-6} \text{ M}$. Binding was highly dependent upon temperature. At a concentration of 10^{-6} M , the rate of accumulation between 25°C and 35°C exhibited a Q_{10} of 1.8. At 0°C the rate of ouabain dissociation was negligible. The specificity of binding was demonstrated with increasing potassium concentrations. At a concentration of 1 μM , 6 mM, and 50 mM K^+ produced a 2.5- and 7-fold decrease, respectively, in the rate of ouabain accumulation observed at zero K^+ . Binding was completely inhibited by 1 mM strophanthin K. The major site of ouabain binding was the thick ascending limb; little or no binding was observed in thin limbs and collecting ducts. Moreover, binding was confined to the basolateral membranes. From autoradiographic grain density measurements, it was estimated that each cell contains over 4×10^6 ouabain binding sites or Na-K ATPase molecules. These results taken together with physiological and biochemical observations suggest that Na-K ATPase plays a key role in salt reabsorption by this segment.

KEY WORDS Na-K ATPase · renal medulla · [³H]ouabain · autoradiography · rabbit

It is generally accepted that the counter-current configuration of Henle's loop plays a critical role in the renal excretion and conservation of water by the mammalian kidney (5). However, there is less than general agreement on the specific transport mechanisms of the various segments of these loops. Micropuncture studies have clearly established that tubular fluid becomes concentrated as it flows down the descending limb (27) and that during the return passage through the ascending

limb there is a relatively greater loss of solute than water, such that fluid entering the distal tubule is more dilute than cortical plasma. Interestingly, the ascending limb has two morphologically distinct regions, one with thin cells having few mitochondria and the other with thicker cells possessing more metabolic organelles (50). It is still unclear whether or not tubular fluid dilution occurs by active salt transport in the thin ascending limb (47, 33, 39, 72) as is apparently the case of the thick ascending limb (9, 59). Thus, an alternative approach to studying this problem is warranted.

A large body of evidence has accumulated indicating that the Na-K ATPase isolatable from plasma membranes is responsible for Na⁺ movement across cell membranes (24, 54). A key observation from these investigations is that ouabain is a specific inhibitor of both enzyme activity in microsomes and ion transport in intact cells. Moreover, it exhibits quantitatively similar dose-response and binding relationships for both processes (24, 30, 32, 64).

Further study has also indicated that there is a strong correlation between the levels of Na-K ATPase activity and salt-transporting capacities in a variety of tissues (7, 16, 36). In recent years, autoradiography of [³H]ouabain has been used to localize Na-K ATPase-type transport sites in a variety of epithelial tissues (21, 36, 43, 44, 56, 57, 65). The present study examines the binding characteristics of ouabain to whole slices of renal medulla and, as well, measures autoradiographically the binding of ouabain to cell membranes of the several tubular segments in these slices.

MATERIALS AND METHODS

Kidney tissue was obtained from female New Zealand white rabbits, weighing 2–3 kg. They were anesthetized with sodium pentobarbital, a ventral abdominal incision was made, and the kidney(s) were removed. A kidney half with the outer capsule peeled off was placed in a Stadie Riggs-type microtome (Arthur H. Thomas Co., Philadelphia, Pa.) and sliced sagittally, 0.5–1.0 mm thick. The slice was trimmed to isolate either a red band of medulla or a strip of papilla. Tissue strips were preincubated in beakers of balanced salt solution buffered with bicarbonate at pH 7.3 and gassed with 95% O₂, 5% CO₂ for 20 min to allow metabolic stabilization. The temperature was maintained at 25°C (unless otherwise specified) by a constant temperature water bath. This is a commonly used temperature for incubating tissue slices since Mudge (48) found that the steady-state accumulation of K⁺ in kidney cortex slices was more favorable at 25°C than 38°C, and since Whittam and Willis (71) showed that ouabain had the same effect on ion transport at this lower temperature as at body temperature.

Radiochemical Measurements

For radiochemical measurement of ouabain binding, tissue was placed in test solutions of tritiated ouabain (0.2 μCi/ml) and [¹⁴C]inulin (to estimate extracellular space). Reversibility of binding was studied by washing in buffer before counting. At the end of incubation, tissue slices were blotted on filter paper, weighed, placed in 1 ml of 0.1 N nitric acid for 24 h to promote extraction of radioactivity, neutralized with 0.1 N sodium hydroxide, and counted after addition of Bray's

solution (8). These and samples of incubation solutions were counted in a liquid scintillation counter (Packard Instrument Co., Inc., Model 2002, Downers Grove, Ill.).

Autoradiography

Tissue strips of medulla or papilla were incubated in test solutions of tritiated ouabain (25 μCi/ml). After incubation, they were rinsed in iced saline for 20 min to eliminate unbound ouabain from extracellular space, cut into small blocks, and frozen in liquid propane (–175°C) cooled with liquid nitrogen. Frozen tissue was either stored in liquid nitrogen until freeze-dried or freeze-dried immediately. The details of this procedure have been described elsewhere (67). In summary, the dried tissue was placed in a desiccator and fixed with osmium vapor for 24 h. It was infiltrated under vacuum in Spurr's low viscosity embedding media (Polysciences, Inc., Warrington, Pa.) to which had been added 1% Dow Corning silicone oil 200 (Dow Corning Corp., Midland, Mich.). The tissue blocks were then oriented in flat embedding molds, each chamber of which was filled with epoxy resin. After the plastic was cured in an oven for 24 h, 1–2 μm sections were cut over water on a Porter-Blum ultramicrotome, Model MT-2 (Dupont, Sorvall Operations, Newtown, Conn.). Sections picked up with a wire loop were placed on glass slides and coated with nuclear track emulsion (Kodak NTB2) by dipping. The slides, after drying, were placed in sealed light-tight boxes, exposed for varying lengths of time, developed (Kodak D-19), fixed, stained with Richardson's stain (58), cover slipped, viewed under oil-immersion bright-field illumination, and photographed. Appropriate controls were carried out to insure that positive tissue chemography and latent image fading (60) were not present.

Fixative Perfusion

It is known that mammalian renal tubules collapse upon interruption of their blood supply. Thus, to ensure that ouabain reached the luminal membranes, we explored two methods of maintaining the tubules open. Attempts to gain access by infusing labeled ouabain into the renal artery directly were unsuccessful due to renal circulatory collapse, apparently caused by the substitution of dextran-electrolyte solutions for blood. In a variation of this method, we perfused the kidney with formaldehyde fixative before removal from the rabbit; the rapid action of this fixative maintained the tubules open for the subsequent *in vitro* incubation. Radiochemical measurements revealed that binding in fixed tissue was more than one half that in unfixated tissue slices. With the animal lying on its right side, a retroperitoneal approach was made to the left kidney, a no. 20 needle was inserted into the renal artery, and 4% formaldehyde buffered with 0.156 M phosphate (53) was infused with a constant volume pump (Harvard Apparatus Co., Inc.,

Millis, Mass.) at 5.6 ml/min for 5 min. The kidney was removed, sliced in cold fixative, and incubated in ^3H -ouabain mixed in buffered fixative. The tissue was then rinsed in iced buffer and frozen for autoradiography.

Grain Density Counting

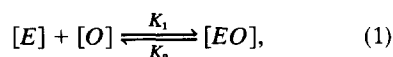
For quantitative autoradiography, tissue sections were mounted on slides along with sections of freeze-dried media containing 5% albumin. Sections were photographed on 35 mm film and enlarged to a total magnification of $\times 4,200$. Each print was mounted on the table of a graphic digitizer (Science Associates, Inc., Princeton, N. J.) and grains, tubular interstitial, medium and background boundaries were marked with a digitizer pen over the surface of the photograph. The digitized x, y coordinates were transmitted to a computer, (6) which calculated from these raw data the medium and tubular grain densities.

RESULTS

Kinetics

In order to obtain some insight into the characteristics of ouabain inhibition and determine the conditions of maximum specific binding, we measured the dependence of ^3H -ouabain accumulation in kidney whole slices on concentration and time of incubation. As shown in Fig. 1, binding to medulla was substantial even at low drug concentration, e.g., $1 \mu\text{M}$, and the initial rate of accumulation increased with concentration. At each concentration, uptake tended toward a plateau with longer incubation times. This behavior agrees with studies of binding to isolated microsomes containing $\text{Na}^+\text{-K}^+$ ATPase activity (1, 2, 30, 70) and to a variety of whole cells and tissues (3, 4). In contrast to medulla, the papilla accumulated very little ^3H -ouabain under identical conditions (not shown).

From these measurements, we estimated the total number of binding sites for a given mass of medulla and the extent to which they were filled at varying incubation times and concentration of ouabain. To do this, we formulated a model which would enable extrapolation to equilibrium conditions, since equilibrium is obviously not achieved for most of the concentrations in Fig. 1. The simplest model to assume in quantitatively describing these kinetics is a bimolecular reaction of the type:



where $[E]$ is free enzyme concentration, $[O]$,

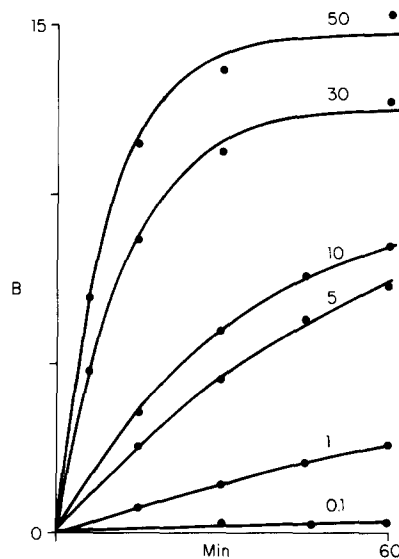


FIGURE 1 Dependence of ^3H -ouabain binding ($B, \times 10^{-8}$ mols/kg tissue) to medullary slices on time at several concentrations; numbers above curves indicate concentration in 10^{-6} M U. The curves represent the best fits to the function $EO = \alpha[1 - \exp - (\beta t)]$. Each point represents the measured mean of at least eight slices from four animals. SE bars which are of the same magnitude as Figs. 4 and 5 were omitted for clarity.

ouabain concentration, $[EO]$, ouabain-enzyme complex concentration, and K_1, K_2 , the association and dissociation rate constants, respectively. As can be shown by elementary analysis of chemical kinetics (46), the relationship between enzyme-ouabain complex concentration and time is given by:

$$EO = \alpha [1 - \exp - (\beta t)], \quad (2)$$

where

$$\alpha = E_T[O]/[O] + (K_2/K_1)$$

and

$$\beta = K_1[O] + K_2.$$

The curves in Fig. 1 are plots of Eq. 2 for each of the concentrations indicated. The values of α and β giving the best fits (Table I) were calculated using a nonlinear method (11) in which the best fit was considered to be those values yielding a minimum in the error function S^2 defined by:

$$S^2 = \left[\sum_{i=1}^N (T_i - M_i)^2 \right] / N - f, \quad (3)$$

where T is theoretical binding, M is measured

binding, f is the number of function parameters, in this case two and N is the total number of measurements.

With prolonged incubation times, equilibrium conditions are approached and the concentration of enzyme-ouabain complex becomes equal to α . Assuming this condition, Eq. 2 can be rearranged to give the familiar Michaelian relationship:

$$\alpha = [EO]_{\infty} = E_T \frac{[O]}{[O] + K_m}, \quad (4)$$

where the subscript ∞ denotes concentration at infinite time and K_m is given by K_2/K_1 . The values of $[EO]_{\infty}$ taken from Table I are plotted as a function of concentration in Fig. 2. The theoretical curve is a plot of Eq. 4. The best fitting value of E_T (13.53×10^{-6} mol/kg) and K_m (0.51×10^{-6} M) were determined by nonlinear methods as described above. The apparent dissociation constant (K_m) of 0.51×10^{-6} M is in good agreement with the value, 3.3×10^{-6} M, reported for rabbit cortex slices by Almendares and Kleinzeller (3) and is one to two orders of magnitude higher than that (1.4×10^{-7} M) cited for HeLa cells (4) or beef kidney (0.47×10^{-8} M) and guinea pig kidney (1.6×10^{-7} M) (15).

Carrying the analysis further, the association rate constant is obtained from α and β :

$$K_1 = \alpha \cdot \beta / E_T [O]. \quad (5)$$

Substituting paired values of α and β from Table I into Eq. 5 yields an average value for K_1 of 2.86 ± 0.63 SD $\times 10^3$ /M min. The corresponding, average dissociation rate constant (K_2) calculated from K_m and K_1 is 1.46×10^{-3} /min.

Temperature Dependence

A question of particular importance to the

TABLE I
Values of α and β Used to Determine the
Theoretical Curves of Figure 1.*

Ouabain $\times 10^{-6}$ M	α $\times 10^{-6}$ mol Kg $^{-1}$	β $\times 10^{-2}$ min $^{-1}$	S
0.1	2.71	0.15	0.06
1	9.35	0.58	0.05
5	11.48	1.76	0.11
10	10.44	2.91	0.05
30	12.71	8.16	0.46
50	14.87	11.05	0.63

* Symbols are defined in the text.

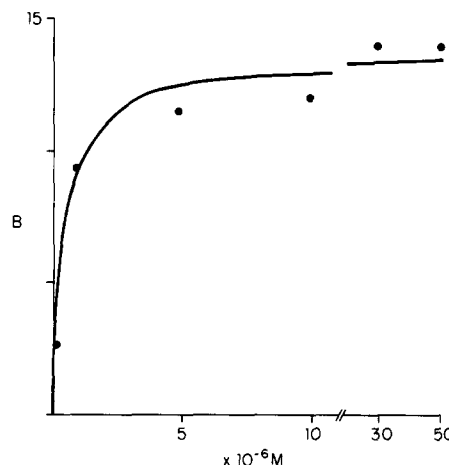


FIGURE 2 Equilibrium binding: the points are the values of α in Table I and the curve is a plot of Eq. 4. The values of K_m (0.51×10^{-6} M) and E_T (13.53×10^{-6} mol/kg tissue) were determined by nonlinear methods.

autoradiographic studies was whether or not unbound ouabain could be washed out at low temperatures without appreciably disturbing the bound ouabain. Clearly, the absence of extracellular ouabain would improve the signal-to-noise ratio of the autoradiograph. This question was examined by incubating slices in 10^{-6} M [3 H]ouabain (0.1μ Ci/ml) for 30 min and then transferring them to a runout solution containing 10^{-3} M unlabeled ouabain. During the first 75 min, the temperature was maintained at 0° C and thereafter at 25° C. The runout solution was changed periodically and its [3 H]ouabain content measured. Similar experiments were conducted with [3 H]inulin (0.1 mg/ml and 0.1μ Ci/ml). The high concentration of unlabeled ouabain prevented rebinding once [3 H]ouabain dissociated. As shown in Fig. 3, there was a rapid loss of extracellular [3 H]ouabain at 0° C which was less than 20% of the total present. Upon warming to 25° C, there was an additional loss of [3 H]ouabain. The time course followed a simple exponential function as predicted by Eq. 1. This behavior is very much in contrast to that of [3 H]inulin whose runout is more than 90% complete after 25 min. Moreover, the runout rate of inulin increases only slightly when the temperature is increased to 25° C (not shown). The runout of inulin does not follow a simple exponential, since the rate of runout from a particular tissue region depends on the diffusion distance. This varies from zero at the surface to about 0.25 mm at the center.

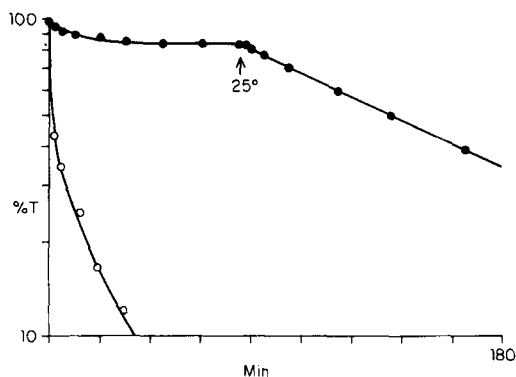


FIGURE 3 Kinetics of [^3H]ouabain dissociation and its dependence on temperature. The experimental protocol is described in the text. %T indicates the fraction of labeled compound remaining in the tissue (note log scale); each division on the abscissa is 20 min. Closed circles represent [^3H]ouabain, and open circles represent [^3H]inulin. Each point is the average from three pieces of tissue. Curves were drawn by eye. The average inulin space before washout was 0.3 ml/g tissue.

The dissociation constant at 25°C, determined from the slope of the [^3H]ouabain runout curve (Fig. 3), is $7.9 \times 10^{-3} \text{ min}^{-1}$. This agrees reasonably well with the above value of $1.2 \times 10^{-3} \text{ min}^{-1}$ computed indirectly from K_1 and K_m . It is somewhat larger because the association constant (K_1) measured from ouabain uptake is undoubtedly too small due to diffusion delays in reaching the sites of reaction. Diffusion does not interfere in the determination of the dissociation rate in the runout experiments because dissociation is clearly the rate-limiting step in these kinetics.

The dependence of binding on temperature was examined further by measuring the time-course of accumulation at several temperatures in $1 \mu\text{M}$ tritiated ouabain. As shown in Fig. 4, uptake at 4°C was negligible. Almendares and Kleinzeller (3) also found a negligible ouabain uptake in slices of rabbit kidney cortex at this temperature. Uptake at 25°C (Fig. 4) was quite substantial and was nearly doubled by increasing the temperature to 35°C. The Q_{10} of the uptake rate measured over the linear part of the curve was 1.8. Over the same temperature range, Erdman and Schoner (17) reported a similar increase in ouabain binding to microsomes while Baker and Willis (4) found a fourfold increase in ouabain binding to intact HeLa cells.

Specificity of Binding

The antagonistic effect of potassium on ouabain

binding in both isolated enzyme preparations and intact cells is well established (25, 63). This effect is often used as a test of ouabain-specific binding. Hence, dependence of rate of binding on extracellular K was investigated in medullary slices incubated for varying times in $1 \mu\text{M}$ [^3H]ouabain. The measurements are shown in Fig. 5. Increasing the potassium concentration from 0 to 6 mM to 50 mM K produced a 2.5- and 7-fold decrease, respectively, in the rate of ouabain accumulation as measured over the first 30 min of uptake. However, at a ouabain concentration of $50 \mu\text{M}$, suppression of binding by 50 mM K was undetectable (not shown). No attempt was made to sys-

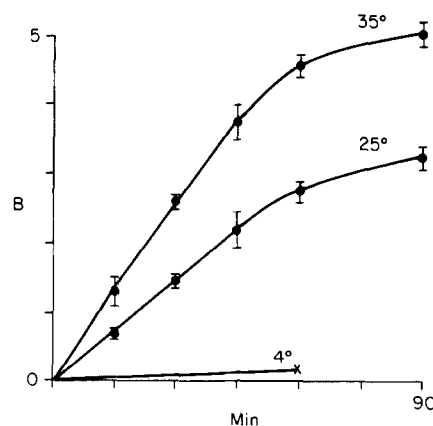


FIGURE 4 Effect of incubation temperature on the rate of [^3H]ouabain (10^{-6} M) binding (B , $\times 10^{-6} \text{ mol/kg tissue}$) to medulla slices. Each point represents the means ($\pm \text{SE}$) from four animals; x represents the mean of six tissue slices from three animals.

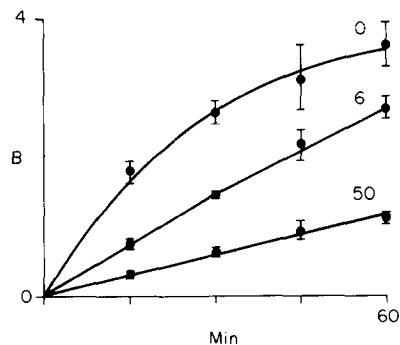


FIGURE 5 Effect of extracellular K^+ on rate of [^3H]ouabain (10^{-6} M) binding (B , $\times 10^{-6} \text{ mol/kg tissue}$) to medullary slices. Curves represent best fits to same function as Fig. 1. Incubation solutions contained the indicated concentrations of K^+ ; ionic strengths were kept constant by varying $[\text{Na}^+]$. Points represent mean values ($\pm \text{SE}$) from four animals.

tematically measure the kinetics of potassium inhibition of ouabain binding.

As a further check of the specificity of ouabain binding, medullary slices were incubated in 1 μM [^3H]ouabain containing 100 μM of strophanthin K. Strophanthin K is a closely related glycoside which has been shown to compete with ouabain for membrane binding sites (13, 25). After 30 min of incubation, the ouabain content of the tissue did not differ experimentally from that expected of extracellular space filling.

Autoradiography

Having established the basic characteristics of [^3H]ouabain binding, we were now in a position to examine its segmental and cellular distribution. Typical autoradiographs prepared from medullary slices incubated in high specific activity 1 μM [^3H]ouabain for 60 min at 25°C are shown in Fig. 6. As can be seen, there is a heavy accumulation of ouabain, i.e., high density of silver grains, associated with the darker-staining, thick ascending limb and negligible or low level binding to the lighter-staining collecting ducts. This medullary pattern of binding is very much in contrast with that of papillary slices incubated under similar conditions (Fig. 7). The level of bound ouabain is so low that it is indistinguishable from residual extracellular ouabain.

Although the precise cellular structure giving rise to the grains cannot be resolved at the light microscope level, there is ample reason to suggest that ouabain binds to basolateral membranes. Ouabain, being a large water-soluble molecule, penetrates cell membranes slowly. The basolateral membranes are known to undergo extensive interdigitation with neighboring cells, thereby producing complex intercellular spaces (42) which extend over the basal two-thirds of the cell. These facts together with the observation of a near absence of grains over the nuclear and apical regions in contrast to a dense accumulation of grains over the basilar portion of each cell argue that ouabain binds to basolateral membranes. This is particularly evident in autoradiographs of longer exposure (see Fig. 9).

Although the luminal membranes appear not to bind ouabain, the fact that tubules collapse upon interruption of their blood supply, thereby partially or totally occluding the lumen, raises a question of whether or not the [^3H]ouabain had access during incubation to cryptic binding sites.

To insure that this was not the case, we prepared autoradiographs from formaldehyde-perfused tissue. The autoradiograph of Fig. 8 is representative of fixed tissue incubated in 1 μM [^3H]ouabain for 60 min and rinsed in cold saline for 20 min. Although the tubules clearly remained open, the relative grain density distribution did not change. Corresponding radiochemical measurements of whole slices indicated that the absolute binding had been reduced to 60% of that in unfixed tissue. Thus, we cannot exclude the possibility that apical binding sites have been preferentially modified. The fact that formaldehyde-fixed tissue binds ouabain is not surprising in view of Ernst and Philpott observations (22). Some binding to apical membranes cannot be completely excluded. However, the absence of an apical grain density above background in autoradiographs of long exposure, eg., Fig. 9a, 44 days, suggest a negligible level of binding. A basal grain density distinctly above background is noticeable within 5 days of exposure (autoradiographs not shown). It should be noted that the greater membrane density in the basal region of the tubule could contribute to this difference.

We also examined the specificity of autoradiographic binding, using a reduction of binding in the presence of high K^+ as a criterion. The autoradiographs of Fig. 9 show a substantial decrease in thick ascending limb binding when [K^+] is increased from 6 to 50 mM. Quantitative analysis of similar autoradiographs, but of lower grain densities (Table II) indicate that tubular binding has been reduced by 44%, a value that compares rather well with radiochemical measurements (42%, Fig. 5).

The radiochemical kinetic analysis indicated the presence of a limited number of binding sites. This phenomenon was also observed autoradiographically. When the concentration of ouabain was increased from 1 to 500 μM while the amount of radioactive ouabain was kept constant (25 $\mu\text{Ci/ml}$), most of the binding sites were filled with unlabeled ouabain, thus producing an absence of grains (autoradiographs not shown).

As a final test of binding specificity, autoradiographs were prepared from medullary slices incubated in [^3H]inulin 60 min and then frozen without rinsing. As the results of Fig. 10 show, there is little or no activity over the tubules but rather the activity is confined to the extracellular space. There was virtually no activity left in similarly treated tissues which had been rinsed for 20

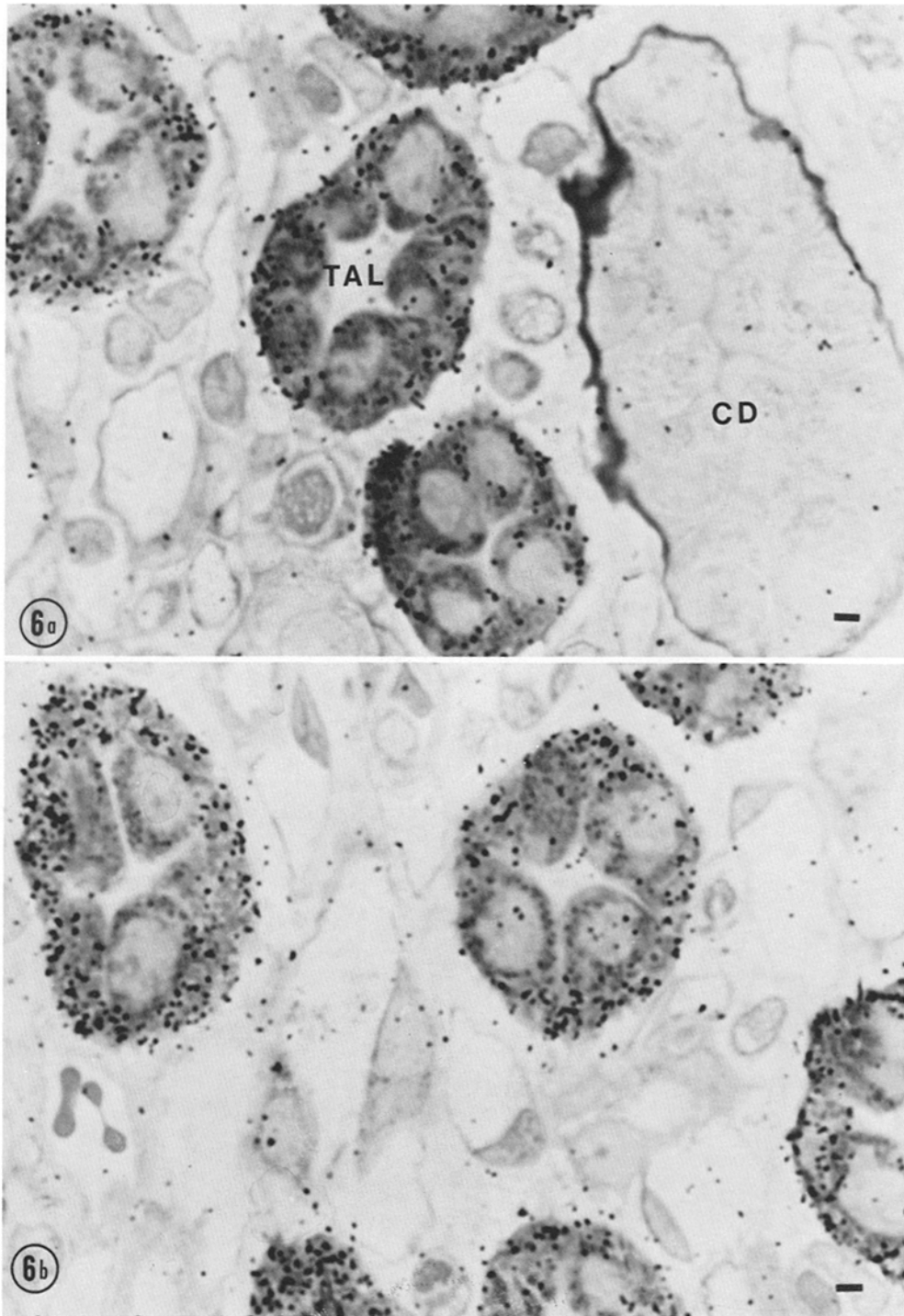


FIGURE 6 (*a* and *b*) Autoradiographs prepared from rabbit kidney medulla incubated for 60 min in 1 μ M [3 H]joubain (25 μ Ci/ml), followed by a 20-min rinse in cold buffer. The darker staining tubule (*TAL*) is thick ascending limb, and the larger-diameter, lighter staining tubule (*CD*) is collecting duct. Bar, 2 μ m, 14-day exposure.

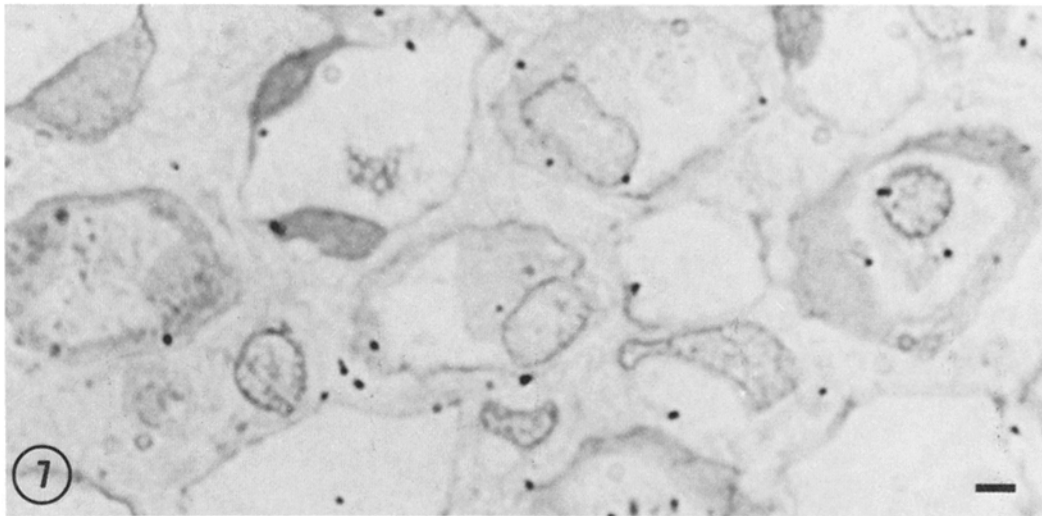


FIGURE 7 Autoradiograph of papillary slice incubated for 60 min in 1 μM [^3H]ouabain followed by a 20-min rinse in cold buffer. Bar, 2 μm , 14-day exposure.

min in inulin-free medium before freezing (not shown).

Grain Density Measurements

In order to quantitatively relate these autoradiographic observations to the radiochemical measurements on whole tissue and determine the density of binding sites in thick ascending limbs, we carried out a grain counting analysis of the autoradiographs. Comparing these results, presented in Table II, with the radiochemical measurements of Fig. 1, it can be seen that the thick ascending limb binds two to three times more ouabain than whole tissue. This is not unexpected, since a large fraction of a tissue slice consists of extracellular space and cells of low level binding. Especially noteworthy in both measurements is that at 1 μM ouabain the relative changes in binding due to alterations in medium $[\text{K}^+]$ are the same (see Fig. 5). At 50 μM ouabain, 50 mM K^+ did not reduce the level of binding.

DISCUSSION

The radiochemical measurements of this study clearly demonstrate the presence of a large number of ouabain binding sites in the medulla and very few sites in the papilla of the rabbit kidney. Moreover, the low value of the equilibrium binding constant ($K_m = 0.51 \times 10^{-6}$ M) determined from the kinetic measurements as well as the sensitivity of the binding to $[\text{K}^+]$ indicates that the ouabain binds to the Na-K ATPase of these

tissues. These findings are consistent with Na-K ATPase measurements in mammalian kidney microsomes in which activity is reported to be two to four times higher in the medulla than in the cortex of dog, cat, and rabbit (7, 31, 35). Similarly, Nechay et al. (49) reported a high medullary, intermediate cortical, and low papillary enzyme activity in human kidney.

Segmental Distribution

Our autoradiographic experiments, which go far beyond the resolution of tissue slice chemical analyses, further show that the medullary Na-K ATPase is located almost exclusively in the thick ascending limb. This central observation is in agreement with the measurements of Schmidt and Dubach (61) on rat nephron. Using a combination of tubular microdissection and microchemical analysis, these investigators found thick ascending limb (inner stripe) Na-K ATPase activity to be seven or more times as great as that of the proximal tubule, twice that of the distal tubule (cortex), and twice that of the collecting duct. We observed a much lower ouabain binding to medullary collecting ducts relative to the thick ascending limb. This apparent discrepancy is probably due to the fact that their measurements included cortical as well as medullary collecting duct. The absence or low level of binding to thin limbs and collecting ducts in the medulla and papilla (Figs. 6, 7, 8, and 10) imply that Na-K ATPase-dependent transport is either absent or quite small in

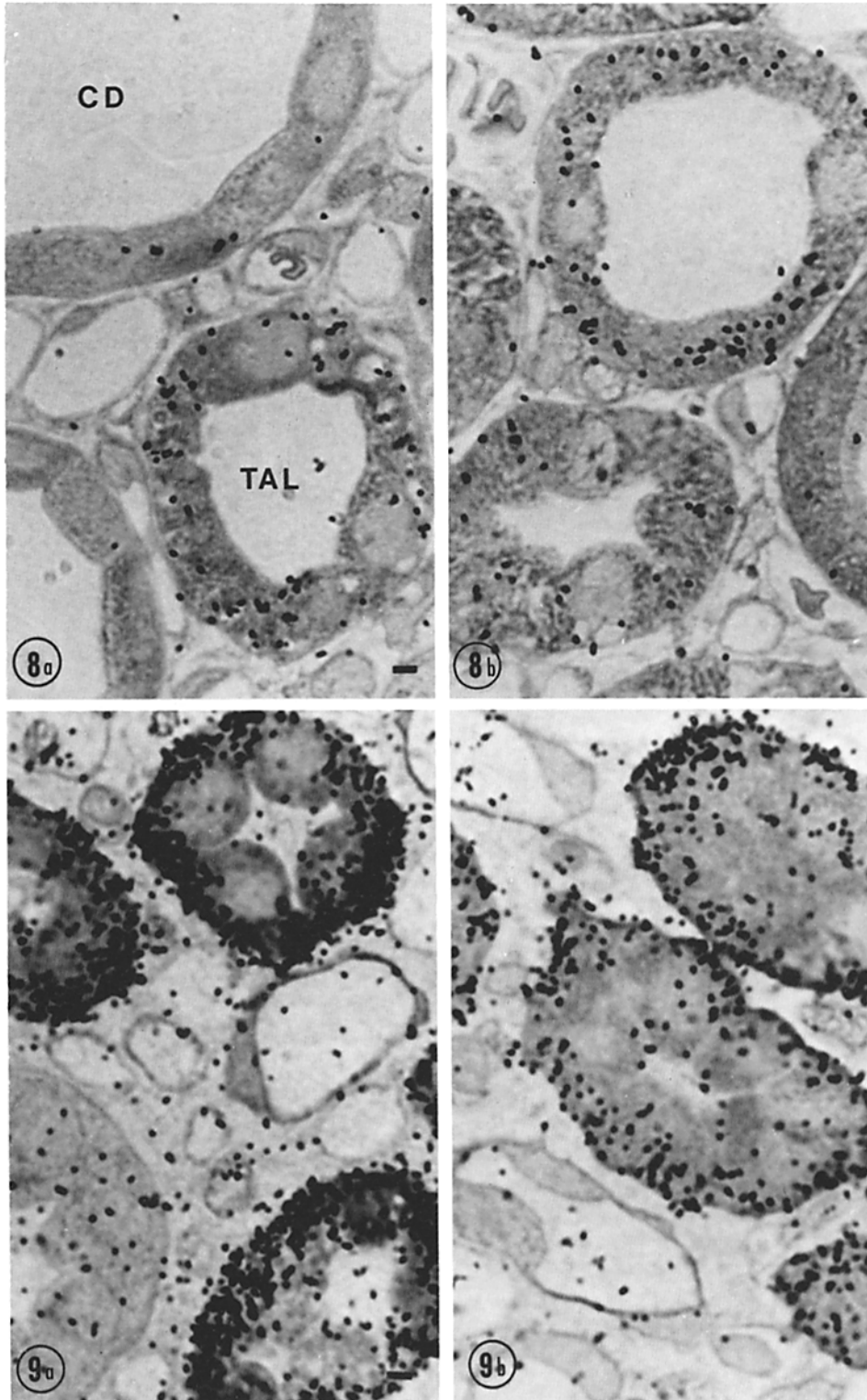


FIGURE 8 (*a* and *b*) Autoradiographs of rabbit kidney medulla arterially perfused *in situ* with 4% formaldehyde 5 min. Kidneys were then removed, sliced, and incubated in $1 \mu\text{M}$ $[^3\text{H}]$ ouabain for 60 min, and rinsed in cold buffer for 20 min. Thick ascending limb (TAL) and collecting duct (CD) are indicated. Bar, $2 \mu\text{m}$, 16-day exposure.

FIGURE 9 (*a* and *b*) Effect of K^+ on binding to thick ascending limbs. (*a*) Autoradiograph prepared from tissue incubated as in Fig. 6, 6 mM K^+ . (*b*) Autoradiograph prepared as in Fig. 6, except that K^+ concentration was raised to 50 mM . Bar, $2 \mu\text{m}$, 44-day exposure.

TABLE II
Grain Density Measurements of Thick Ascending Limbs

[Ouabain]*	[K*]	Grains/ μm^2 tubule†	N‡	Bound ouabain
μM	mM			10^{-6} mol/liter of tubule
1	6	0.45 ± 0.11	47	3.0
1	50	0.20 ± 0.11	47	1.3
50	6	0.17 ± 0.03	16	55.0
50	50	0.18 ± 0.03	34	60.0
Medium				
			0.15 \pm 0.02	48

* 60-min incubation; 25 $\mu\text{Ci/ml}$ of [^3H]ouabain.

† Average \pm SD.

‡ No. of tubular profiles measured in several sections; each profile contained 50 or more grains. Medium areas contained a comparable number of grains.

|| Calculated as: Tubular grain density/medium grain density \times medium concentration.

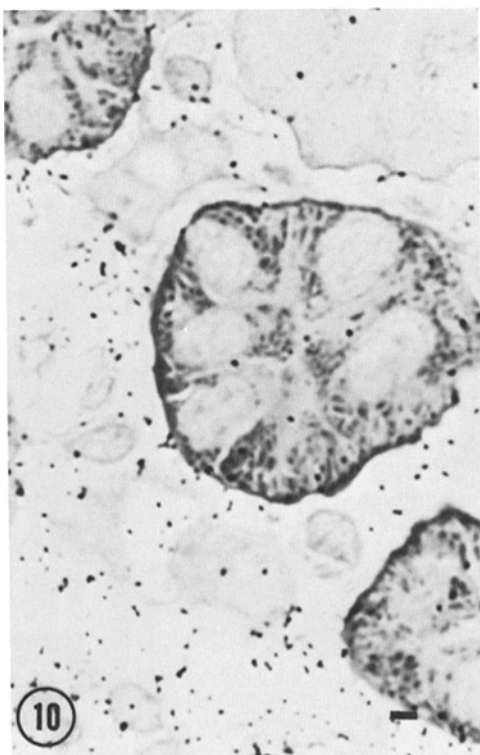


FIGURE 10 [^3H]inulin autoradiograph of medulla prepared from tissue incubated for 60 min at 1 mg/ml (25 $\mu\text{Ci/mg}$). Bar, 2 μm , 45-day exposure.

these structures. Imai and Kokko (33) found no evidence of active salt transport in isolated, perfused, thin ascending limbs of rabbits, and, simi-

larly, there is a lack of evidence for active transport in the descending limb (38). Thus, it is not surprising to find little or no ouabain binding in these segments. Although there are a number of studies which show that salt is actively transported out of cortical collecting ducts (28, 29, 68), medullary collecting ducts remain relatively unstudied. The low level binding in this study may reflect either a low capacity Na-K ATPase-dependent pump or, quite possibly, a different type of salt-transporting mechanism.

Membrane Site Density

As argued in the Results section, the thick ascending limb binding sites are located predominantly if not exclusively on the basolateral membranes. From the molecular point of view, the number of Na-K ATPase sites per unit area of membrane is of special interest. The grain density measurements of Table II provide some of the essential data for calculating this parameter. Taking maximum binding to be 55×10^{-6} mol/liter of tubule and one ouabain molecule per transport site and approximating the volume of a single cell as a cube 5 μm on edge, we calculate that there are 4.1×10^6 transport sites/cell. Assuming no magnification of membrane area and no binding to the apical membrane, we calculate that there would be 55 \AA between centers of adjacent Na-K ATPase particles. This value differs insignificantly from the estimated diameter (50 \AA) of the purified enzyme (34). Hence, without membrane magnification, nearly all of the membrane will be occupied by enzyme. Measurements of basolateral membrane area of thick ascending limb cells are not available; however, a rough estimate is that the basal invagination (50) increases the area by a factor of 5–10. This would yield a comfortable average spacing of 125–175 \AA between centers. The present estimate of 4.1×10^6 sites per cell is much less than that found for the salt-secreting chloride cell of the teleost gill (1.5×10^8 sites/cell, reference 36). On the other hand, it is significantly larger than that reported for the HeLa cell (8×10^5 sites/cell) and cultured cells of guinea pig kidney (7.5×10^5 sites per cell) reported by Baker and Willis (4), and considerably larger than the values of 200–300 and 6×10^4 sites/cell for mammalian (12, 32) and fish (10) erythrocytes. The higher number of sites for the chloride cell as compared to the ascending limb cell is probably due to the larger volume and the higher membrane magnification factor of the former. Clearly, the number of sites per unit area of

membrane is a more meaningful index of comparison; unfortunately, quantitative estimates of plasma membrane area for most cells are not available.

Membrane Location and Transport Function

A basolateral location for the Na-K ATPase is in agreement with the now classical model of an absorptive epithelium proposed by Koefoed-Johnson and Ussing (37). Quite surprisingly, there seems to be little correlation between the membrane location, i.e., apical vs. basal, and direction of transport as is predicted by this model and conventionally accepted views of Na-K ATPase-type transport. Several laboratories using [³H]ouabain autoradiography and histochemical techniques specific to Na-K ATPase have reported a basolateral location in the salt-secreting epithelia of the teleost gill (36), avian (18, 21) and reptilian (15) salt glands and sweat glands (56) as well as in the reabsorptive epithelium of the rabbit ileum (65), rat distal tubule (19), frog (43) and fish (57) bladder and frog skin (44). In another microdissection and microchemical analysis study (62), Schmidt and Dubach found little or no activity in proximal tubular brush border fragments and a specific Na-K ATPase activity in basal membrane fragments seven times that of the total tissue specimen. Similar results have been reported in intestinal epithelial cells (45) using this approach. Comparable results were obtained by Ebel et al. (14) with rat cortical membranes separated by density gradient centrifugation. However, George and Kenny (23), using a membrane separation method based on free flow electrophoresis, report appreciable brush border Na-K ATPase activity. This activity might well be due to remnants of lateral membrane (51). More recently, Kyte (40, 41) reported a Na-K ATPase ferritin conjugated antibody method for electron microscope localization of this enzyme. He observed a high density of binding to basolateral membranes and a low level of binding to apical membranes of canine proximal and distal tubular cells. To what extent nonspecific binding may have occurred was not clear. The only secretory epithelium in which an apical location has been observed is the frog choroid plexus (56).

Peaker and Linzell (52) suggested that the function of basolateral membrane Na-K ATPase found in the avian salt-secreting gland is to

maintain intracellular ionic composition in the resting state. Secretion is stimulated by increasing the basal entry of NaCl, and hence the supply of Na to an ouabain-sensitive apical pump. This scheme, although theoretically attractive, is difficult to reconcile with the observations that, in both the avian salt gland (20, 21) and the teleost gill (36), basal membrane area and Na-K ATPase increase with chronic salt stress and that the apical membrane does not bind ouabain. According to the above scheme, one would expect an increase in the apical rather than the basal membrane transport capacity. Histochemical (18) and recent ouabain binding studies (21, 36) clearly indicate a comparative absence of ouabain binding to apical membrane in these tissues. The possibility that the apical membrane possesses a ouabain-insensitive secretory pump cannot be ruled out, but then this does not account for ouabain inhibition of salt gland and gill secretion. We believe that this problem still awaits a definitive explanation and that further speculation is unwarranted here.

The role of the Na⁺-K ATPase in renal tubular transport has been critically examined by Epstein and Silva (16). They propose at least one other renal salt reabsorptive mechanism independent of Na-K ATPase. This conclusion is based on the observation that a large fraction (50%) of the filtered salt in the isolated perfused rat kidney is reabsorbed in spite of a nearly complete inhibition of Na-K ATPase. The further observation that either the removal of HCO₃ from the perfusate or the inclusion of the carbonic anhydrase inhibitor acetazolamide produced an additional 20% inhibition beyond that of ouabain points to the proximal tubule as at least one site of Na-K ATPase independent transport. The negligible ouabain binding to medullary collecting ducts suggests another possible site of ouabain-insensitive Na transport. It is particularly noteworthy that, in their study of the intact canine kidney (16), renal infusion of 10⁻⁶ M ouabain reduced Na-K ATPase activity by 50-80% and almost completely inhibited the ability to produce either a concentrated or dilute urine.

These observations confirm in the intact kidney the results obtained with isolated perfused thick ascending limbs of the rabbit. In medullary thick ascending limbs, Rocha and Kokko (59) found that 10⁻⁵ M ouabain inhibited both transtubular electrical potential and net NaCl transport by half. That greater inhibition was not found is not surprising since at 10⁻⁵ M (Fig. 1) more than 40

min is required to attain half-maximal binding. Presumably, their exposure times were much shorter. Burg and Green (9) reported similar observations for perfused cortical thick ascending limbs; however, they observed nearly complete inhibition of transepithelial electrical potential and net NaCl flux. The electrical potential dropped to zero after 10 min of exposure. Quite surprisingly, the half-time for reversal of inhibition was only 15 min. We observed a 50-min half-time for dissociation (Fig. 3). It would be of interest to know whether this apparent difference in kinetics between medullary and cortical thick limb is real.

The high density of basolateral membrane Na-K ATPase, its adaptive response to salt stress (16, 31), and the sensitivity of thick ascending limb transport to ouabain clearly suggest an integral role for this enzyme in the transepithelial movement of NaCl. If it is assumed that thick limb Na-K ATPase functions in the conventional exchange fashion, e.g., 3 Na in and 2 K out as in human erythrocytes, then the presence of large amounts of Na-K ATPase on the basal cell membrane creates a paradox, for electrical measurements in isolated perfused thick limbs indicate that Cl is actively reabsorbed. The recent observations of Goldin and Tong (26) in transport by reconstituted Na-K ATPase from canine renal medulla may provide the key clue in solving this puzzle. Purified enzyme reconstituted into artificial lipid vesicles produced an efflux (1:1 stoichiometry) of Na and Cl in the presence of ATP and K. Although NaCl efflux and ATP hydrolysis required K, there was no exchange transport between Na and K. The credibility of this exciting observation was further enhanced by observations from the same laboratory (69) that purified canine brain Na-K ATPase reconstituted in the same manner counter-transported Na and K with a stoichiometry near 3:2.

In view of these results, the possibility that salt reabsorption in thick ascending limbs occurs by an active basolateral NaCl pump requires reexamination. It is germane to note that, in the transport studies of isolated thick ascending limbs (9, 59), attempts to account for net fluxes by passive mechanisms were inconclusive. Furthermore, in one of these studies (59), the transtubular Na flux ratios did not conform to simple diffusion. However, an observation in these studies which is difficult to reconcile with a coupled NaCl pump is the failure of the transepithelial voltage to disappear in the absence of Na (replaced with choline). Two explanations come to

mind: either cellular [Na] did not go to zero and the pump continued work at a reduced rate, or removal of Na uncoupled the pump generating an electrogenic Cl pump. Substitution of another cation (K, H₃O) for Na seems unlikely since the isolated enzyme requires Na. Quite clearly, more information regarding relative permeabilities of both membranes and electrochemical gradients is needed to resolve this paradox. In concluding, we should note that some of the problems highlighted here will be discussed in more detail in a forthcoming review article (66).

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