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Organic cation transport in avian renal brush-border membrane vesicles

Villalobos, Alice Renee Avila, Ph.D.
The University of Arizona, 1993
ORGANIC CATION TRANSPORT IN AVIAN RENAL
BRUSH-BORDER MEMBRANE VESICLES

by
Alice Renee Avila Villalobos

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PHYSIOLOGY
In partial fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY
WITH A MAJOR IN PHYSIOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1993
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Alice Renee Avila Villalobos entitled Organic Cation Transport in Avian Renal Brush-Border Membrane Vesicles and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Dr. Eldon J. Braun
Date 8/30/93

Dr. William H. Dantzler
Date 8/30/93

Dr. Patricia B. Hoye
Date 8/30/93

Dr. And T. Major
Date 8/30/93

Dr. Stephen H. Wright
Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Date
STATEMENT BY AUTHOR

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SIGNED: [Signature]
Acknowledgements

I formally thank the members of my dissertation committee - Eldon J. Braun, William H. Dantzler, Patricia B. Hoyer, Ana M. Pajor, Michael A. Wells, Stephen H. Wright, and J.Larry Renfro, for their time, patience, and guidance. I also thank the Department of Physiology and the Physiological Sciences Program for their continual academic expertise and administrative support.

Many people supported me - academically, technically, and morally in completing my doctoral research and writing my dissertation. However, I would like to especially thank the following individuals:

- Dr. Eldon J. Braun, for being such a dedicated advisor;
- Myra Pacelli, for being a good listener and friend;
- Patricia Goggans, for being helping me focus on my goals;
- Stephani Boykin, for being so empathetic and good-hearted;
- and, Carrie Grove, for being such a faithful friend.

For their love and encouragement, I thank my parents -

- Armida Avila, for being a good mother and for making my world so large;
- and Thomas Villalobos - my father, for being the hardest working man I have ever met.

I thank my brothers -

- Thomas and Andrew, for always looking out for me and for never allowing me to use my being a girl as an excuse not to succeed.

Finally, I thank God, the one who has given me life and a reason to live.
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Abstract

The objective of this study was to characterize the transport of organic cations (OCs) at the luminal brush-border membrane of the avian renal proximal tubule. Using membrane vesicles isolated from renal tissue of chickens (*Gallus domesticus*), transport of the exogenous OC [14C]tetraethylammonium (TEA) was measured by rapid filtration under various ionic conditions. A trans proton gradient stimulated concentrative uptake of TEA. Tetraethylammonium/proton exchange was a saturable, qualitatively symmetrical process that was indirectly coupled to Na+/H+ antiport. Proton-driven uptake of TEA was not electrogenic; however, it was sensitive to changes in transmembrane potential. Proton-driven TEA uptake was apparently selectively inhibited by OCs. To evaluate substrate specificity of OC/H+ exchanger, a battery of endogenous and exogenous OCs were tested for cis inhibition and trans stimulation of [14C]TEA transport. Although amiloride, cimetidine, meperphenidol, procainamide, quinidine, quinine, ranitidine and thiamine were potent cis inhibitors of TEA transport, they were poorly transported by the exchanger. Conversely, acetylcholine, choline, epinephrine, guanidine, isoproterenol, \( N^1 \)methylnicotinamide, serotonin, and unlabeled TEA, modestly inhibited TEA transport, but trans stimulated transport. Inhibitor-induced changes in the kinetic parameters of TEA/H+ exchange suggested serotonin and thiamine competed with TEA for the substrate site on the exchanger, whereas inhibition by amiloride, procainamide, and quinidine apparently involved binding to allosteric sites on the carrier. These data suggest luminal transport of OCs in the avian proximal tubule is qualitatively similar to that in mammals. Moreover, the OC/H+ exchanger of avian BBMV has greater affinity for exogenous compounds than for endogenous compounds; however, it has a greater capacity to transport endogenous OCs than exogenous OCs.
Chapter 1
Introduction

Statement of Purpose

Secretion of organic cations (OCs) by the vertebrate renal tubule was first demonstrated by Sperber in 1947 in the chicken. Sperber showed that a compound infused into the avian renal portal vein via the iliac vein was secreted by the ipsilateral kidney prior to entering the systemic vasculature and being filtered at the glomerulus (Sperber 1947). Since its inception, the "Sperber chicken" preparation has been used extensively as an in vivo model for the study of renal transport of a wide variety of inert and toxic OCs. However, the subcellular and molecular mechanisms involved in the transport of OCs have been studied in the greatest detail in mammalian systems. Although the chicken is used as an in vivo model for the study of the transport of OCs, the subcellular mechanisms of transport in the bird are only assumed to be similar to those in the mammal. The objective of the research presented in this doctoral thesis was to probe the mechanism by which OCs are transported across the luminal brush-border membrane of the avian renal proximal tubule. Transport of the exogenous OC [\textsuperscript{14}C]tetraethylammonium was examined under various ionic conditions in luminal membrane vesicles isolated from the renal cortex of the domestic chicken (\textit{Gallus domesticus}).

I. General aspects of organic cation excretion

The magnitude and direction of specific ion gradients across the plasma membrane must be maintained for individual cells to function efficiently. Ion pumps and ion channels located within the plasma membrane selectively transport compounds in and out of cells, and thereby regulate intracellular ionic content and volume. However, because the plasma
membrane is semi-permeable, the composition of the intracellular compartment is sensitive to changes in the ionic composition of the interstitial fluid which bathes the cells and is functionally continuous with the plasma. Thus, the concerted workings of individual cells as a complete unit (i.e., an organ) demand tight regulation of the total osmolality, ion content, and volume of the plasma. The vertebrate kidney functions to regulate plasma composition by selectively manipulating concentrations of inorganic and organic ions. The mechanisms by which the kidney regulates the plasma concentrations of organic cations will be discussed in this dissertation.

Renal secretion of OCs by the vertebrate kidney was first demonstrated in 1947. In a standard renal clearance study in humans and dogs, Rennick and colleagues (1947) showed that the exogenous OC tetraethylammonium (TEA) underwent net tubular secretion. That same year, Sperber demonstrated net tubular secretion of the endogenous OC N\(^{1}\)-methylnicotinamide (NMN) in the chicken (1947). Renal transport of OCs has since been examined in monkeys, pigs, rats, snakes and fish. By similar in vivo clearance studies, other investigators have demonstrated renal tubular secretion of several other OCs (Table 1.1). These compounds vary in their derivation, function, and structure. However, each of these compounds is a primary, secondary, tertiary or quaternary amine. Secondly, because the pK\(_a\) of the nitrogen group is 7.1 or greater, these compounds should be positively charged at physiologic pH 7.4.

At normal concentrations, endogenous OCs such as acetylcholine and histamine play significant roles in mediating body function, whereas exogenous OCs such as amiloride and methadone facilitate recovery from, or management of, pathological conditions. However, at excessive or insufficient concentrations these OCs can be fatal. Thus, the kidney’s role in regulating plasma concentrations of OCs is of vital importance.
Table 1.1. Representative organic cations secreted by the mammalian and avian kidney. References are given in parentheses.

<table>
<thead>
<tr>
<th>Mammals</th>
<th>Birds</th>
</tr>
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<tbody>
<tr>
<td><strong>Endogenous Organic Cations</strong></td>
<td><strong>Endogenous Organic Cations</strong></td>
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<td>Acetylcholine (Acara and Rennick 1972)</td>
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<td>Dopamine (Rennick 1968)</td>
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<tr>
<td>Cinchonine (Jailer et al. 1947)</td>
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<td>Isoproterenol (Lifschitz et al. 1973)</td>
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<td>Mepiperphenidol (Torretti et al. 1962)</td>
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<td>Methadone (Baselt and Casarett 1972)</td>
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<td>Procaine (Terp 1951)</td>
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<td>Procainamide (Christian et al. 1984)</td>
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<tr>
<td>Tolazoline (Torretti et al. 1962)</td>
<td>TEA (Rennick et al. 1954)</td>
</tr>
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</table>
a. Direction of transepithelial flux of organic cations

Regulation of the plasma concentration of OCs as well as other compounds and the subsequent production of urine by the kidney involves integration of three processes: glomerular filtration, tubular reabsorption, and tubular secretion. Some OCs transported by the renal tubule are freely filtered at the glomerulus (e.g., NMN; Beyer et al. 1950). However, binding of OCs to plasma proteins may vary. In dogs, for example, approximately 70% of quinine in the plasma is protein bound (Torretti et al. 1962); whereas approximately 50% of dopamine is bound (Rennick 1968).

In whole animal clearance studies, clearance ratios (i.e., the ratio of the renal clearance of the test compound to the clearance of inulin or creatinine) are calculated to determine the net transepithelial flux of a test OC. Clearance ratios greater than 1 indicate that a test OC undergoes net tubular secretion; whereas ratios less than one indicate net tubular reabsorption of the compound. By this method investigators have determined that several endogenous OC undergo net tubular secretion at experimentally elevated plasma concentrations. Few studies have examined renal transport of endogenous OCs at physiological concentrations. The exceptions are choline and NMN. N¹-methylnicotinamide is a normal constituent of urine (Huff and Perlzweig 1943; Johnson et al. 1945). However, the presence of a given compound in the urine can be attributed to glomerular filtration alone; therefore, excretion of an endogenous OC does not necessarily indicate it is secreted. Clearance studies in dogs (Farah and Frazer 1961) and rabbits (Besseghir et al. 1981) which demonstrated net tubular secretion of NMN also demonstrated simultaneous reabsorption of NMN. Moreover, net tubular reabsorption of NMN has also been demonstrated in the isolated perfused tubule preparation of the snake (Dantzler and Brokl 1986) in which the lumen-to-bath flux of NMN exceeded the bath-to-lumen flux over NMN concentrations of 0-200 μM. Bidirectional transport of the endogenous OC choline has also been documented. As shown in the chicken (Acara 1975;
Acara and Rennick 1973; Rennick 1958), dog (Acara and Rennick 1973), rabbit (Besseghir et al. 1981) and rat (Acara et al. 1979; Tremble et al. 1974), the net flux of choline is dependent on the plasma concentration. At physiological levels (< 20 μM), choline undergoes net tubular reabsorption. However, when the plasma concentration is approximately doubled in chickens, rabbits and dogs or approximately tripled in rats net tubular secretion of choline is observed.

Although the exogenous OCs examined to date have been shown to undergo net tubular secretion, stop-flow studies in the dog (Pilkington and Keyl 1963; Rennick and Moe 1960) and isolated tubule perfusion studies in snakes (Hawk and Dantzler 1984) and rabbits (McKinney et al. 1981; McKinney and Speeg 1982) indicate that renal excretion of exogenous OCs such as TEA and procainamide can involve a reabsorptive component.

b. Site of transport

Stop-flow studies in dogs demonstrated that secretion of TEA (Rennick and Moe 1960), mepiperphenidol and mecamylamine (Pilkington and Keyl 1963) is localized to the proximal renal tubule. In the rat, micropuncture studies showed that NMN secretion is also restricted to the proximal tubule (Ross et al. 1975). Perfusion studies on isolated proximal tubules of snakes and rabbits have demonstrated net secretion of TEA, cimetidine and procainamide (Hawk and Dantzler 1984; McKinney and Speeg 1982; McKinney et al. 1981; Schäli et al. 1983). On the other hand, in these tubules, NMN can undergo net tubule reabsorption at high concentrations (Besseghir et al. 1990; Dantzler and Brokl 1986).

Stop-flow studies in the dog demonstrated that choline is bidirectionally transported by the proximal tubule (Acara and Rennick 1973). Micropuncture studies in the rat indicated that choline is secreted and reabsorbed at the proximal convoluted tubule and could be further reabsorbed at the pars recta and loop of Henle (Acara et al. 1979). Although many exogenous OCs undergo net tubular secretion, a single stop-flow study in dogs
demonstrated that under conditions of alkaluria reabsorption of mepiperphenidol and mecamylamine (Pilkington and Key 1963) is localized to the distal tubule. Collectively, these findings indicate that secretion of OCs is localized to the proximal segment of the renal tubule.

c. Carrier-mediated transport of organic cations

Secretion of OCs involves carrier-mediated transport as indicated by in vivo and in vitro studies that have directly demonstrated saturation and inhibition of secretion. Saturation of the excretion of OCs has been demonstrated by renal clearance studies in several mammalian species (Besseghir et al. 1981; Beyer et al. 1950; Ross et al. 1975; Rennick and Farah 1956; Rennick et al. 1947; Schäli et al. 1983; Weber et al. 1991), as well as in the chicken (Rennick et al. 1977; Springate et al. 1987). Moreover, studies in isolated perfused snake renal tubules have demonstrated saturation of the secretory flux of TEA (Hawk and Dantzler 1984) and NMN (Dantzler and Brokl 1986). The competitive inhibition of the excretion of OCs also indicates the presence of a carrier-mediated secretory pathway for OCs in the kidney. For example, Rennick and co-workers have demonstrated in the chicken that TEA and choline competitively inhibit excretion of one another (Rennick et al. 1977); a similar observation was made for cimetidine and thiamine (Rennick et al. 1984). Furthermore, these investigators also demonstrated that excretion of cimetidine in the chicken was competitively inhibited by the OCs ranitidine, thiamine, procainamide, guanidine, and choline (Rennick et al. 1984). This indicated that OCs can share a common secretory pathway in vivo.

The weak inhibitory interactions of OCs and organic anions has indicated the renal secretory pathways for these compounds are functionally separate. Inhibitors of organic anion transport such as probenecid, phenol red, acetate, penicillin and bromcresol green fail to inhibit OC transport (Lifschitz et al. 1973; Besseghir and Rennick 1981; Beyer et al.
1953; Farah et al. 1959; Sanner 1963; Rennick and Yoss 1962; Rennick and Farah 1956). Conversely, inhibitors of OC transport such as mepiperphenidol, cyanine 863, quinine, quinidine, ranitidine, amiloride and procainamide are poor inhibitors of organic anion excretion (see reviews by Rennick 1981 and Roch-Ramel et al. 1992). Further evidence for distinct transport pathways for OCs and organic anions can be found in the failure of PAH, in concentrations sufficient to obtain tubular secretory transport maximum (Tm), to depress secretion of the OCs. As first demonstrated by Sperber (1948) in the chicken, NMN, guanidine, methylguanidine and piperidine each failed to inhibit excretion of PAH; conversely, PAH failed to inhibit OC excretion. In studies on dogs, PAH infused intravenously in doses sufficient to obtain a Tm, failed to inhibit excretion of TEA (Rennick and Farah 1956), NMN (Beyer et al. 1950), and mepiperphenidol (Beyer et al. 1953). Conversely, at plasma levels sufficient to attain Tm for excretion, TEA, NMN and mepiperphenidol failed to decrease PAH excretion. Thus, OCs are excreted via a carrier-mediated transport pathway that appears to be functionally separate from that for organic anions.

II. Transepithelial secretion of organic cations in mammals

Renal tubular secretion refers to the movement of substances out of the peritubular space (functionally continuous with the plasma), across the renal cell, and into the tubule lumen. Accordingly, secretion of OCs by the proximal tubule occurs in three steps: transport across the basolateral membrane into the cell; migration across the intracellular compartment; and finally, transport across the luminal or brush-border membrane into the lumen. In vitro perfusion of rabbit and snake renal tubules indicates that OCs are secreted across the epithelium against a concentration gradient, as the reported lumen-to-bath concentration ratio for TEA is 2 (Schäli et al. 1983), for procainamide 6 (McKinney 1984), and for cimetidine as high as 26 (McKinney et al. 1981). Being positively charged at
physiological pH, OCs are secreted across the negative intracellular compartment against an electrical gradient. The net secretion of OCs against an electrochemical gradient is an energy-consuming, active process.

Based on studies of OC transport in dog renal basolateral membrane (BLMV) and brush-border membrane vesicles (BBMV; Holohan and Ross 1981; Holohan and Ross 1980; Kinsella et al. 1979) and in mouse renal cortical slices (Holm 1978; 1977a; 1977b; 1972), Holohan and co-workers were first to propose a model for OC secretion in the mammalian proximal tubule (Figure 1.1). According to this model, mediated transport of OCs into the cell from the peritubular space involves potential-driven facilitated diffusion and exchange for intracellular OCs. Efflux of OCs at the luminal membrane involves mediated exchange for protons, this being a secondary active transport process, driven by the cell-to-lumen proton gradient. The proton gradient at the brush-border membrane is in part generated by sodium-driven Na⁺/H⁺ antiport. Thus, OC secretion is indirectly, but energetically linked to the cell-to-lumen sodium gradient established by primary active extrusion of sodium by Na⁺,K⁺-ATPase at the basolateral membrane. Additionally, the intracellular negative potential that drives uptake of OCs at the basolateral membrane is ultimately sustained by Na⁺,K⁺-ATPase.

If OCs were accumulated by passive diffusion alone, the predicted tissue-to-medium concentration ratios (T/M ratios) as calculated by the Nernst equation would be 10, assuming on an average intracellular negative potential of 60 mV. However, in isolated rabbit tubules T/M ratios for OCs such as TEA and procainamide have been reported to be as high as 100 (McKinney 1983; McKinney et al. 1984; Schäli and Roch-Ramel 1982; Schäli et al. 1983; Tarloff and Brand 1986). For example, in non-perfused rabbit tubules, Tarloff and Brand (1986) assumed an intracellular negative potential of 57 mV and calculated a T/M ratio for TEA of 7.6; however, the measured value was approximately 104. Moreover, in examining TEA secretion in perfused snake tubules
Figure 1.1 A model for transepithelial secretion of organic cations in the mammalian proximal tubule. The Na\(^+\),K\(^+\)-ATPase pump (1) at the peritubular or basolateral membrane generates an inwardly-directed sodium gradient at the basolateral and luminal membranes. Cellular uptake of organic cations (OC\(^+\)) involves facilitated diffusion driven by the intracellular negative potential (2), as well OC/OC exchange (3). Luminal efflux of OCs involves OC/H\(^+\) exchange (4) driven by the cell-to-lumen proton gradient generated in part by Na\(^+\)/H\(^+\) antiport (5). Reproduced from Holohan and Ross (1981).
Hawk and Dantzler (1984) found the T/M ratio was twice the predicted value even when 2.5 mM NaCN was added to the bathing medium. Values this large could not be accounted for by passive potential-driven facilitated diffusion.

A transport mechanism which could account for these discrepancies has yet to be characterized (Pritchard and Miller 1991). However, intracellular sequestration may contribute to overestimate of the apparent intracellular concentrations of OCs. For instance, as shown by Berndt (1981), NMN and TEA bind extensively to cytosolic proteins in renal cortical slices of the rat and rabbit, and McIsaac (1969) found substantial binding of cyanine in renal slices of the chicken. More recently, Pritchard et al. (1993) demonstrated proton-driven TEA accumulation within endosomal vesicles of the rat renal cortex. This suggests that sequestration of OCs within endosomal vesicles in vivo may serve to reduce free cytosolic concentrations. If such intracellular binding or compartmentalization is not accounted for, the measured intracellular concentration would be an overestimate of the actual intracellular activity of the OC. Therefore, the actual cell-to-bath chemical gradient may be of a lesser magnitude than assumed, and the negative intracellular potential alone may be sufficient to support passive accumulation of OCs across the basolateral membrane.

In this same regard, the cell-to-lumen chemical gradient may be less than assumed, and the cell-to-lumen electrical gradient (cell negative relative to lumen) may exceed the chemical gradient. Thus, OCs may be transported against an electrochemical gradient. Not knowing the activity of OCs in the cytosol, the energetics of mediated transport at each pole of the epithelial cell cannot be completely described. Regardless, transport of OCs at the luminal membrane by an OC/H+ exchange mechanism, although not necessarily rate-limiting, is a secondary active transport process.
a. Luminal organic cation/proton exchange in mammals

i. mechanism of organic cation/proton exchange

Using radiolabeled NMN, Holohan and Ross (1981) first demonstrated countertransport of OCs and protons using dog renal BBMV. A reproduction of the model for OC/proton antiport or exchange (OC/H\(^+\) exchange) initially proposed by Holohan and Ross (1981) is presented in Figure 1.2. In this model, the unloaded exchanger is negatively charged, and the substrate site on the exchanger alternately appears at the cytoplasmic and extracellular or luminal face of the brush-border membrane. As a proton binds to the substrate site at the luminal face, the exchanger reorients to the cytoplasmic side of the membrane where the proton dissociates. An intracellular OC then binds to the substrate site and the exchanger reorients to the luminal face where the cation dissociates. By this mechanism an OC is transported against its concentration gradient coupled to the chemical energy stored in an opposing or trans proton gradient. The reorientation of an unloaded carrier is a rare event and occurs at a very slow rate. Binding of a proton to the substrate site catalyzes the reorientation of the exchanger as indicated by studies in rabbit BBMV in which a trans proton gradient increased the \(J_{\text{max}}\) for TEA uptake without altering the affinity of the carrier for TEA (i.e., \(K_m\) was not altered; Wright and Wunz 1987). However, studies in dog BBMV in which a trans proton gradient induced a decrease in the \(K_m\), as well as an increase in \(J_{\text{max}}\) for NMN suggest a trans proton gradient also induces an increase in the affinity as well as the transport capacity of the carrier for OCs (Sokol et al. 1988).

Proton-driven uptake of several OCs has been demonstrated in BBMV isolated from renal tissue of the dog, rabbit, rat, and human (Table 1.2). Generally, in studying the mechanism of luminal OC/H\(^+\) exchange the translocation of a radiolabeled OC has been monitored. However, using the pH-sensitive fluorescent dye acridine orange, it has been shown in renal BBMV that protons are indeed physically exchanged for OCs and are
Figure 1.2. A model for carrier-mediated organic cation/proton exchange at the luminal membrane of the proximal tubule. Theoretically, the unloaded carrier (C⁻) is negatively charged, and reorients at a very slow rate. Binding of a proton to the carrier catalyzes reorientation of the carrier from the lumen to the intracellular face of the membrane where the proton dissociates. An intracellular substrate (S⁺) binds to the substrate site on the carrier, and the carrier reorients to the luminal compartment where the substrate dissociates. The lumen-to-cell proton gradient drives reorientation or turnover the carrier, and thus drives luminal efflux of cationic substrates against an electrochemical gradient. Reproduced from Holohan and Ross (1981).
Table 1.2. Organic cations countertransported for protons in vertebrate renal BBMV.

<table>
<thead>
<tr>
<th>Organic Cation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>amiloride*</td>
<td>Wright and Wunz 1989</td>
</tr>
<tr>
<td>cephalaxin</td>
<td>Inui et al. 1985</td>
</tr>
<tr>
<td>gentamicin</td>
<td>Sokol et al. 1989</td>
</tr>
<tr>
<td>guanidine*</td>
<td>Miyamoto et al. 1989</td>
</tr>
<tr>
<td>mepiperphenidol*</td>
<td>Rafizadeh et al. 1986</td>
</tr>
<tr>
<td>MPP+</td>
<td>Lazaruk and Wright 1990</td>
</tr>
<tr>
<td>MPTP</td>
<td>Sokol et al. 1987</td>
</tr>
<tr>
<td>morphine*</td>
<td>Rafizadeh et al. 1986</td>
</tr>
<tr>
<td>procainamide*</td>
<td>McKinney and Kunneman 1985</td>
</tr>
<tr>
<td>verapamil</td>
<td>Sokol et al. 1989</td>
</tr>
</tbody>
</table>

*undergoes net secretion *in vivo.*
transported against a concentration gradient. In such studies, vesicles are preloaded with dye, the fluorescence of which decreases as the proton concentration increases (i.e., pH decreases). As shown by Giacomini and co-workers, an inwardly-directed TEA gradient increased the rate of fluorescence recovery in rabbit and human renal BBMV, indicating trans stimulation of efflux of protons by TEA (Hsyu and Giacomini 1987; Ott et al. 1991). Specifically, in rabbit BBMV, an outwardly-directed TEA gradient decreased the fluorescence, indicating that TEA also trans stimulated proton influx. In a similar manner, Sokol et al. (1988) found an outwardly-directed NMN gradient stimulated influx of protons under voltage clamped conditions in dog BBMV.

Countertransport or exchange of one OC for a second (i.e., OC/OC exchange) has also been demonstrated in preparations of renal BBMV from the dog, rabbit, rat, and human. Concentrative uptake of radiolabeled NMN in renal BBMV is known to be trans stimulated by unlabeled NMN, as well as mepiperphenidol, choline, TEA, tetramethylammonium (TMA), gentamicin, and verapamil (Holohan and Ross 1980; Sokol et al. 1989; Wright 1985). Similarly, transport of radiolabeled TEA against a concentration gradient in BBMV is supported by trans gradients of unlabeled TEA, NMN, morphine, MPP+ (Griffiths et al. 1992; Lazaruk and Wright 1990; Rafizadeh et al. 1987; Rafizadeh et al. 1986; Wright and Wunz 1988). Countertransport of amiloride and TEA has been observed in rabbit BBMV (Wright and Wunz 1989).

Based on kinetic studies of TEA transport in rabbit BBMV, Wright and Wunz (1988) determined that OC/H+ exchange and OC/OC exchange represent different operational modes of a common carrier. In those studies, unlabeled TEA trans stimulated concentrative uptake of radiolabeled TEA in a dose-dependent manner and induced an increase in the maximal rate of uptake ($U_{\text{max}}$) without altering the Michaelis constant ($K_m$). Furthermore, the trans stimulatory effects of protons and unlabeled TEA on the uptake of radiolabeled TEA were not additive. These results indicate protons and TEA compete for a
common substrate site or set of mutually exclusive substrate sites on the cytoplasmic (intravesicular) face of the exchanger. An increase in the external proton concentration increased the apparent $K_m$ without changing $J_{max}$, indicating that protons and TEA also competed for a common substrate site or set of mutually exclusive substrate sites on the luminal (or extravesicular) face of the exchanger. Competitive inhibition of TEA uptake by protons has been observed in other rabbit renal BBMV preparations (Jung et al. 1989), as well as in cultured opposum kidney cell monolayers (Yuan et al. 1991).

As originally noted in dog BBMV by Holohan and Ross (1981), protons, as well as the OCs choline, mepiperphenidol, TEA, TMA, and unlabeled NMN trans stimulated concentrative uptake, as well as efflux of NMN; whereas, Wright and co-workers demonstrated trans stimulation of uptake and efflux of TEA in rabbit renal BBMV by protons and TEA (Dantzler et al. 1989; Lazuruk and Wright 1990; Wright and Wunz 1987). Thus, the operation of the carrier to mediate OC/H\(^+\) exchange and OC/OC exchange is qualitatively symmetrical in each mode.

ii. effects of electrical and chemical gradients on mediated OC/H\(^+\) exchange

It has been demonstrated that an OC/H\(^+\) exchange mechanism mediates transport of TEA and procainamide at the apical membrane of the continuous renal epithelial cell lines, LLC-PK\(_1\) (Saito et al. 1992; Takano et al. 1992) and OK (Yuan et al. 1991). Similarly, studies in isolated perfused proximal tubules of the rabbit indicate that luminal transport of OCs involves OC/H\(^+\) exchange. In these tubules, the transepithelial secretion of procainamide (McKinney 1984) and efflux of TEA from tubule cell at the luminal membrane (Dantzler et al. 1989) are stimulated by acidification of the perfusate within the tubule lumen. Luminal secretion of OCs via OC/OC exchange in the intact proximal tubule has also been observed in isolated perfused tubules of the rabbit in which efflux of
intracellular TEA was stimulated in the presence of increasing concentrations of substrate in the lumen (Dantzler et al. 1989). However, in speculating on the physiological function of luminal membrane OC/H⁺ exchanger in the renal regulation of plasma concentrations of various OCs, the mechanistic properties of the antiporter as observed in vitro must be considered in light of our knowledge of the physical and biochemical properties of the renal proximal tubule in vivo.

The affinity of the carrier for protons is very high; the reported values for the apparent $K_{H^+}$ for TEA/H⁺ exchange in rabbit renal BBMV range from 30 nM to 166 nM (Jung et al. 1989; Wright and Wunz 1988; Wright and Wunz 1987). The corresponding pH values range from 6.78 to 7.52. As measured in vivo, the luminal proton concentration in the rat renal proximal tubule is 200 nM (pH 6.70); whereas the intracellular proton concentration is approximately 63 nM (pH 7.2) (Yoshimoto and Frömter 1984). These data would suggest the luminal proton concentration is sufficient to catalyze reorientation or turnover of the exchanger. Thus, at physiologic pH the carrier should readily reorient.

Holohan and Ross (1981), originally proposed that secretion of OCs via OC/H⁺ exchange was driven by the lumen-to-cell proton gradient. Although the 3.17 fold lumen-to-cell proton gradient could drive OC/H⁺ exchange, it is exceeded by the cell-to-lumen gradient of OCs. For example, in isolated perfused rabbit renal tubules, the cell-to-lumen TEA gradient was approximately 50-fold (Schäli et al. 1983). However, the actual cell-to-lumen gradients of OCs in the proximal tubule have yet to be measured in vivo.

Nevertheless, these data suggest secretory OC/H⁺ exchange may be driven by the lumen-directed OC gradient rather than a cell-directed proton gradient. Still, as seen in isolated perfused renal tubules of the rabbit, secretion of OCs are stimulated by acidification of the perfusate (McKinney 1984; Dantzler et al. 1989).
The transepithelial secretion of OCs cannot be completely understood or appreciated without considering mechanisms by which other compounds are transported by the renal tubule. Based on the finding that a trans sodium gradient stimulated uptake of NMN in dog renal BBMV, Holohan and Ross proposed that secretory OC/H+ exchange at the luminal membrane was indirectly, but energetically coupled to the large lumen-to-cell sodium gradient generated by Na+,K+-ATPase at the basolateral membrane. As seen in renal BBMV of the rat and rabbit, sodium trans stimulates transport of amiloride (Wright and Wunz 1989), procainamide (McKinney and Kunneman 1985), NMN (Wright 1985), and TEA (Rafizadeh et al. 1987; Takano et al. 1984). Moreover, sodium-dependency of tubule secretion of OCs has been demonstrated by in vitro perfusion of isolated rabbit tubules in which removal of sodium from the perfusate decreased transepithelial secretion of procainamide (McKinney 1984). In a similar study, addition of ouabain to the bathing medium substantially inhibited secretion of procainamide and cimetidine in rabbit tubules (McKinney and Speeg 1982), suggesting an indirect role of Na+,K+-ATPase in transepithelial secretion of OCs. Furthermore, in vivo perfusion of the renal portal system with ouabain attenuates NMN excretion in the Sperber chicken preparation (Nechay and Pardee 1965).

Finally, luminal efflux of OCs via proton exchange would involve removal of positively charged substrates from the negative intracellular compartment of the renal tubule. As suggested by Pritchard and Miller (1991), if luminal OC/H+ exchange were not an electroneutral process, exit of OCs from the cell via such a mechanism would ultimately require an increased production and expenditure of ATP to maintain the transmembrane potential, as well as the sodium and potassium gradients at each pole of the tubule cell. However, studies examining the effects of membrane potential on OC/H+ exchange indicate that this is an electroneutral antiport mechanism. As seen in BBMV of the rabbit (Rafizadeh et al. 1987; Wright and Wunz 1987) and rat (Takano et al. 1984), TEA
transport is not electrogenic, as membrane potential is insufficient to support uphill transport of TEA. Proton-driven transport of NMN is also seen to be insensitive to changes in membrane potential in BBMV of the dog (Sokol et al. 1985) and human (Ott et al. 1991); however, in rabbit BBMV, NMN transport is evidently electrogenic (Wright 1985). The stoichiometric studies of proton-driven exchange of NMN and TEA conducted in BBMV of the dog (Sokol et al. 1985) and rabbit (Wright and Wunz 1987), verified transport was electroneutral; in each case the coupling ratio was 1:1. Interestingly, although proton-driven TEA uptake in rabbit renal BBMV is electroneutral, Wright and Wunz (1987) found uptake was stimulated by an intravesicular-positive potential. These investigators suggested the lumen-positive membrane potential in the intact proximal tubule (Barrat et al. 1974) may serve to facilitate secretory OC/H+ exchange.

b. luminal transport of organic cations in non-mammalian vertebrates

The in vitro studies carried out in mammalian renal systems indicate that an OC/H+ exchange mechanism could function in vivo to secrete OCs across the brush-border membrane of the proximal tubule. The current data on the subcellular mechanisms of OC transport in mammals, although not complete, are rather extensive relative to that for non-mammalian vertebrates. Although data on transport of OCs in non-mammalian vertebrates are limited, studies in isolated perfused renal proximal tubules and isolated renal brush-border membranes of snake have contributed to our understanding of the mechanisms involved in the transport of OCs in non-mammalian species.

Using radiolabeled TEA and NMN, Dantzler and colleagues have examined the mechanisms of luminal transport of OCs in the snake proximal tubule; these are qualitatively similar to those described for the rabbit. As is seen in perfused tubules of the rabbit (Schäli et al. 1983), a net secretory flux of TEA was measured in snake tubules (Hawk and Dantzler 1984). Luminal efflux of labeled TEA is stimulated by decreases in
luminal pH, as well as by increases in luminal concentrations of unlabeled TEA and choline (Dantzler and Brokl 1988). Similarly, in isolated snake BBMV, uphill transport of labeled TEA was supported by a trans proton gradient. Uptake was also trans stimulated by unlabeled TEA and choline (Dantzler et al. 1991). Thus, an OC/H+ exchange mechanism must mediate TEA transport at the luminal membrane of intact snake proximal tubule as is seen in intact rabbit tubules (Dantzler et al. 1989). Transport of TEA in BBMV is also trans stimulated by sodium. Sodium stimulation of TEA uptake was most probably secondary to generation of trans proton gradient as an amiloride-sensitive Na+/H+ antiport is present in snake BBMV. However, secretion of TEA in perfused tubules was not altered by removal of sodium from the perfusate (Hawk and Dantzler 1984). Finally, although not confirmed in the intact tubule, TEA transport in snake BBMV is electroneutral (Dantzler et al. 1991).

Although a net secretory flux of TEA is measured in perfused snake renal tubules, a net reabsorptive flux of NMN is measured in these tubules (Dantzler and Brokl 1986), as is seen in rabbit tubules (Besseghir et al. 1990). Transepithelial reabsorptive and secretory fluxes of NMN were sodium-dependent (Dantzler and Brokl 1986). TEA in concentrations as high as 20 mM in either the perfusate or bathing medium, or both, had no effect on either the secretory or reabsorptive fluxes of NMN in the snake tubule. These data suggest the transepithelial pathways for these two compounds are separate (Dantzler and Brokl 1986). Furthermore, neither the secretory flux nor the luminal efflux of NMN was influenced by acidification of the perfusate or the addition of choline to the perfusate (Dantzler and Brokl 1987). Luminal uptake of NMN was blocked by NMN analogues N¹-hexylnicotinamide (NHN) and N¹-ethylnicotinamide (NEN), but not by TEA or mepiperphenidol, suggesting that a quaternary ammonium configuration is not the sole requisite for substrate compatibility with the luminal transporter for NMN (Dantzler and
Brokl 1986). Thus, as seen in the rabbit (Besseghir et al. 1990; Schäli et al. 1983), NMN is a poor substrate for the OC/H+ exchange mechanism in the snake proximal tubule.

These detailed studies in snake renal proximal tubules indicate that luminal transport of TEA is qualitatively similar to that in mammals. However, there are no data to suggest that TEA or any other OC is transported via an OC/H+ exchange mechanism in other non-mammalian vertebrates. Although Sperber found no correlation between urinary excretion of OCs (e.g., guanidine, NMN, piperidine) and changes in urine pH in the chicken (Sperber 1948), urate precipitates in avian urine may serve to buffer changes in urine pH (Long 1982; Long and Skadhauge 1983; Wolbach 1955). Thus, we cannot eliminate the possibility that OCs are transported across the avian luminal membrane via OC/H+ exchange based on Sperber's negative findings. Brokl and Dantzler (1993) have recently begun studying OC transport in isolated non-perfused proximal tubules of the chicken and have demonstrated concentrative accumulation of TEA across the basolateral membrane. However, to date there are no reported data on the luminal transport of OCs in the avian proximal tubule. A more complete understanding of the mechanisms, as well as the physiological relevance of OC transport in the vertebrate kidney requires consideration of similarities, as well as the differences among mammalian and non-mammalian species.

c. substrate specificity of luminal brush-border OC/H+ exchange

The luminal OC/H+ exchanger is specific for OCs as indicated by the weak inhibitory effects of organic anions on transport in renal BBMV. Organic anions such as p-aminohippurate (PAH), o-iodohippurate, and probenecid are poor inhibitors of OC transport; whereas cationic compounds such as mepiperphenidol, quinine, tetrabutylammonium, and cimetidine are potent inhibitors of OC transport (e.g., Kinsella et al. 1979; Ott et al. 1991; Wright 1985; Wright and Wunz 1989). However, a limited number of OCs are known to be transported by the luminal OC/H+ exchanger. Of the
more than thirty OCs known to undergo net tubular secretion by the renal tubule in vivo (Table 1.1), only two endogenous OCs (i.e., NMN and guanidine) and six exogenous OCs (i.e., amiloride, cimetidine, mepiperphenidol, morphine, procainamide, and TEA) are known to be transported by the luminal brush-border OC/H⁺ exchanger (Table 1.2). In addition, other exogenous OCs cephalaxin, gentamicin, 1-methyl-4-phenylpyridinium, (MPP⁺), and verapamil which are not excreted by the intact kidney are also transported by the exchanger (Table 1.2).

In vitro studies on isolated perfused tubules of the rabbit and snake indicate the luminal OC/H⁺ exchanger plays a more significant physiological role in the secretion of exogenous OCs than endogenous OCs. Secretion of the exogenous OC procainamide in perfused rabbit tubules (McKinney and Speeg 1982), as well as the luminal efflux of the exogenous OC TEA from perfused rabbit (Dantzler et al. 1989) and snake tubules (Dantzler and Brokhl 1988) is stimulated by an increase in the trans (i.e., luminal) proton concentration. On the other hand, secretion of the endogenous OC NMN is not altered by changes in luminal pH in perfused tubules of the rabbit (Besseghir et al. 1990) or snake (Dantzler and Brokhl 1987). Secretion of other endogenous OCs has not been directly studied in vitro; however, the endogenous cation choline is known to trans stimulate luminal efflux of TEA in perfused snake tubules (Dantzler and Brokhl 1988). This would suggest that choline like TEA is transported by the luminal OC/H⁺ exchanger in the intact proximal tubule. However, as shown by Wright et al. (1992) in rabbit renal BBMV, the Jₘₐₓ for proton-driven uptake of choline is approximately 40 nmol·mg⁻¹·min⁻¹, and the affinity of the OC/H⁺ exchanger for choline is very low (Kₘ for choline is ~10 mM). Instead choline is transported via a separate high affinity, potential driven, facilitated pathway. In addition, Miyamoto et al. (1989) showed concentrative uptake of the endogenous cation guanidine in the presence of a trans proton gradient in rabbit renal
BBMV. Although reciprocal inhibition of proton-driven uptake by guanidine and TEA indicated these two compounds are transported by a common OC/H⁺ antiport mechanism, kinetic studies suggested that guanidine was transported by multiple carriers. Thus, the available data indicate that the luminal brush-border membrane OC/H⁺ exchanger plays a more significant physiological role in the renal regulation of plasma levels of exogenous cations than that of endogenous OCs. However, data on luminal transport of endogenous OCs in the renal proximal tubules is limited to choline, guanidine, and NMN. Dopamine and serotonin were shown to inhibit NMN/H⁺ exchange in the dog renal BBMV (Sokol et al. 1987). No other endogenous OCs have been tested for possible transport by the renal luminal OC/H⁺ exchanger.
III. Objectives

The Sperber chicken preparation is used extensively as an *in vivo* model for the study of OC secretion by the vertebrate kidney. Such studies have assumed that the subcellular mechanisms mediating transport of OCs in the avian proximal tubule are similar to those described in mammalian systems. The first objective of my study was to determine if transport of OCs at the luminal membrane of the avian renal proximal tubule involves mediated exchange for protons as shown for mammals. Using $[^{14}\text{C}]$tetraethylammonium ($[^{14}\text{C}]$TEA; Figure 1.3) as a model substrate, I examined OC transport in BBMV isolated from chicken renal tissue in the presence of various transmembrane gradients.

Data on the transport of various exogenous OCs across the renal brush-border membranes of mammals and snake suggest the luminal OC/H$^+$ exchange mechanism functions *in vivo* to regulate plasma concentrations of exogenous OCs. However, the available data on the transport of endogenous OCs indicate that the physiological role of the luminal OC/H$^+$ exchanger in regulation of these OCs is questionable. Thus, the second objective of my study was to determine whether OCs other than TEA were transported by the OC/H$^+$ exchanger in avian renal BBMV. To evaluate substrate specificity of the exchanger, I tested the ability of several endogenous and exogenous OCs to *cis* inhibit proton-driven $[^{14}\text{C}]$TEA uptake and *trans* stimulate $[^{14}\text{C}]$TEA efflux.
Figure 1.3. The structural formula of the exogenous organic cation tetraethylammonium.
Chapter 2

Characterization of Organic Cation Transport by Avian Renal Brush-Border Membrane Vesicles

I. Introduction

The secretion of organic cations (OCs) by the vertebrate kidney was first demonstrated in 1947. Ivar Sperber showed net tubular secretion of the endogenous OC \( N^1 \)-methylnicotinamide (NMN) in chickens (Sperber 1947), while Barbara Rennick and colleagues (Rennick et al. 1947) demonstrated net tubular secretion of the exogenous OC tetaethylammonium (TEA) in humans and dogs. In his studies, Sperber infused NMN directly into the renal portal system, having previously determined that the OC would be transported by renal tubules of the ipsilateral kidney prior to entering the systemic vasculature and being filtered at the glomeruli (Sperber 1946). This perfusion of the avian renal portal system, known as the Sperber chicken technique, has been used extensively as an \textit{in vivo} model to study transport processes in the vertebrate kidney. A considerable understanding of OC secretion in birds has been derived by this method. However, because techniques, such as micropuncture and microperfusion of avian renal tubules have proven to be very difficult, our understanding of the cellular mechanisms of OC secretion in birds is limited. In contrast, OC secretion in the mammalian kidney has been well studied in the intact organ, individual tubule and at the subcellular level by a variety of \textit{in vivo} and \textit{in vitro} techniques. Although the Sperber chicken has been used for studying OC secretion \textit{in vivo}, the subcellular mechanisms of OC secretion in avian kidney are only assumed to be similar to those in mammals. Therefore, the purpose of these studies was to probe the cellular mechanisms of OC secretion in the avian kidney.

Organic cations are actively secreted against an electrochemical gradient at the proximal segment of the renal tubule as demonstrated in stop-flow studies in dogs.
(Pilkington and Keyl 1963; Rennick and Moe 1960) and in isolated perfused tubules of rabbits (Dantzler et al. 1989; McKinney 1984; McKinney et al. 1981; McKinney and Speeg 1982; Schäli et al. 1983) and snakes (Hawk and Dantzler 1984). The current model for transepithelial secretion of OCs is based on data obtained predominantly from studies in mammals (for review see Ref. Pritchard and Miller 1991, Ross and Holohan 1983). Briefly, OCs are initially transported from the peritubular space across the basolateral membrane by either OC/OC exchange or electrogenic facilitated diffusion (Holohan and Ross 1980). Transport of OCs across the cell interior to the luminal brush-border membrane (BBM) may involve simple diffusion and possibly, as recent data suggest, endosomal vesicular transport (Pritchard et al. 1993). At the BBM, OCs are transported by OC/H+ exchange or antiport (Holohan and Ross 1981).

Transport of OCs at the BBM from a negative intracellular compartment into the less negative lumen is an active process, as positively charged compounds are transported against an electrochemical gradient. Based on studies in isolated BBM vesicles (BBMV), Holohan and Ross (1981) hypothesized that this transport step involved an OC/H+ exchange or antiport mechanism that was indirectly coupled to Na+/H+ exchange. Numerous studies have shown that OCs, such as NMN, TEA, procainamide, morphine, guanidine, and cimetidine are exchanged for H+ or OCs in BBMV of several mammalian species: the dog (Holohan and Ross 1981; Holohan and Ross 1980; Sokol et al. 1987), rat (Griffiths et al. 1992; Inui et al. 1985; Katsura et al. 1991; Takano et al. 1985; Takano et al. 1984), rabbit (Gisclon et al. 1987; Lazaruk and Wright 1990; McKinney and Kunneman 1987; Miyamoto et al. 1989; Rafizadeh et al. 1987; Rafizadeh et al. 1986; Wright 1985; Wright and Wunz 1989; Wright and Wunz 1988; Wright and Wunz 1987), and human (Ott et al. 1991b). These findings are in agreement with observations made for isolated perfused renal tubules of rabbits, in which the secretion of radiolabeled TEA across the
luminal membrane is stimulated by a decrease in luminal pH and by an increase in the luminal concentration of TEA (Dantzler et al. 1989).

In comparison, data on luminal OC secretion in the nonmammalian kidney are limited. Dantzler and colleagues have demonstrated that luminal transport of TEA is coupled to the counter flux of H⁺ and OCs in isolated perfused tubules (Dantzler and Brokl 1988; Hawk and Dantzler 1984) and in BBMV of snakes (Dantzler et al. 1991). Similarly, in the crab (Cancer borealis) urinary bladder TEA secretion increased in response to the acidification of the luminal compartment (Miller et al. 1989). There has been no direct demonstration of OC/H⁺ exchange, however, in the renal BBM of other nonmammalian vertebrates. Due to the technical difficulties associated with isolating viable avian proximal tubules, I elected to examine OC transport in BBMV isolated from chicken kidneys. By this method, I was able to examine OC transport as it occurs independently of intracellular transport mechanisms. Under various ionic conditions, I measured transport of [¹⁴C]tetraethylammonium (TEA), an exogenous OC and model substrate for the OC/H⁺ exchanger. The data indicated that the mechanisms of OC transport in avian renal BBMV are qualitatively similar to those in mammalian BBMV. The transport of TEA is physically coupled to the trans flux of H⁺. Proton-driven TEA uptake is a mediated, saturable process that is indirectly coupled to Na⁺ transport. Finally, TEA/H⁺ exchange is specifically inhibited by cationic transport inhibitors, such as mepiperphenidol and quinine, but not organic anions such as p-aminohippurate (PAH).
II. Materials and Methods

a. Animals and harvesting of tissue

White Leghorn (*Gallus domesticus*) hens age 6-8 mo weighing 1-1.5 kg were used in this study. Prior to their use, animals were housed at the University farm with free access to commercial lay mash and water. Unanesthetized animals were decapitated using poultry shears (J.A. Henckels® Germany) and exsanguinated. The body cavity was immediately opened and the viscera were removed to expose the kidneys.

The kidneys were manually perfused *in situ*. The external iliac and ischiatic arteries and veins were clamped with hemostats to insure forced perfusion of the kidneys. A cannula (polyethylene tubing-50) was inserted into the dorsal aorta and secured with "00" silk suture. The kidneys were perfused with approximately 80 ml of ice-chilled "homogenization buffer" (in mM: mannitol 50, Tris-HEPES 1, pH 7.4); the "perfusate" (a mixture of blood and homogenization buffer) exited the kidney via the caudal vena cava. This perfusion served to clear the organ of excess blood to minimize contamination of the final BBMV suspension with erythrocyte membranes. Such contamination may alter experimental results, as the erythrocyte membrane contains a variety of transport proteins. Once the kidneys were well blanched they were extracted and momentarily placed in cold homogenization buffer. On a paper towel, all extra-renal tissue (excess fat, the renal capsule, fragments of blood vessels, nerves and ureteral branches) was dissected from each kidney. In a disposable petri dish, the kidneys were minced with a steel razor and weighed to the nearest 0.1 g. Tissue from a single animal, 8 - 20 g, was used in each BBMV preparation.

b. Brush-border membrane preparation

Brush-border membrane vesicles are isolated from avian renal proximal tubules by means of Ca⁺² precipitation and differential centrifugation following a protocol developed
by Renfro and Clark (1984). In this procedure all solutions are refrigerated (4°C) or ice-chilled immediately prior to usage. All homogenization vessels are retained in an ice bath during homogenization of tissue or resuspension of vesicles. Finally, all centrifugations are carried out at 4°C.

The minced kidney is diluted with homogenization buffer (10 ml per gram wet weight of tissue) and homogenized using an OCI® Omni-Mixer set at speed 10 for two 2-min periods separated by a 2-min reprieve. The initial homogenate is diluted 2-fold with homogenization buffer, and 100 mM CaCl₂ is added to a final concentration of 10 mM. The "Ca⁺²-homogenate" solution is rapidly stirred in an ice bath for 20 min. Calcium binds to membrane vesicles of all origins; however, binding causes the density of vesicles derived from mitochondrial, endoplasmic reticular and basolateral membranes to exceed that of BBMV. Brush-border vesicles are then easily isolated from non-brush-border membrane vesicles by a series of differential centrifugations. The Ca⁺²-homogenate solution is centrifuged using a SS-34 rotor (Sorvall® RC-5B centrifuge) at 1500 × g for 10 min. As a result, non-brushed border vesicles are pelleted and subsequently discarded. The supernatant containing BBMV and residual non-brush-border vesicles, is retained and centrifuged at 3000 × g for 10 min. A second pellet of non-brush-border vesicles is formed and also discarded. The remaining supernatant, containing BBMV, is centrifuged at 26,000 × g for 30 min to pellet BBMV. The resulting supernatant is decanted and discarded. The centrifuge tubes are inverted at room temperature for approximately 5 min to allow excess supernatant to drain from the BBMV pellets. Pellets are gently removed from the bottom of the centrifuge tubes with the aid of a rubber policeman and a minimal volume of intravesicular or "vesicle" buffer. In a glass Potter-Elvehjem (5 ml) vessel this pellet is suspended in vesicle buffer with a Teflon® pestle (400 rpm, 10 strokes). The suspension is transferred to a centrifuge tube and sufficient vesicle buffer is added to prevent implosion of the centrifuge tube during the final centrifugation at 26,000 × g for 30
min. This final centrifugation serves to "preload" the vesicles with medium appropriate for the specified enzyme or transport assay. The final BBMV pellet is resuspended in vesicle buffer using a 1 or 2 ml glass Potter-Elvehjem vessel and a Teflon® pestle (400 rpm, 10 strokes). The volume of the final BBMV suspension varied (0.75 - 1.5 ml) as determined by the experimental protocol. For measurement of enzyme activities and for measurement of [14C]methyl-D-glucose uptake, vesicles were suspended in a mannitol Tris-HEPES buffer. For measurement of [14C]tetraethylammonium bromide uptake, vesicles were suspended in a KCl-mannitol buffer titrated with HEPES-KOH. The specific composition of the vesicle buffers and incubation or transport buffers for each individual experiment is described in the figure legends.

c. Analytical methods

Protein concentration was measured using a Bio Rad® protein assay. A standard curve was generated using Bio Rad protein Standard I-bovine plasma γ-globulin. The specific activities of alkaline phosphatase and Na⁺,K⁺-ATPase were determined by the methods outlined by Mircheff and Wright (1976) and that of succinate dehydorogenase by the method of Pennington (1961).

d. Transport studies

Uptake of radiolabeled isotope was measured using the rapid filtration technique (Hopfer et al. 1973). In a polystyrene tube (Falcon®, 12 x 75 mm), 5 or 10 μl of vesicles were diluted with 195 or 90 μl, respectively, of incubation or "transport" medium containing trace amounts of [14C]-labeled substrate and simultaneously vortexed. Vesicles were incubated at room temperature (25°C). The transport reaction was terminated by adding 1 ml "ice cold" isosmotic KCl "stop" buffer (containing CaCl₂ 2 mM, HgCl₂ 0.1 mM, and Tris-HEPES 20 mM, pH 7.8) while simultaneously vortexing the mixture. One
milliliter of this mixture was immediately collected on a prewetted HAWP 024 Millipore filter (pore size 0.45 μm) under vacuum and washed with an additional 4 ml of cold stop buffer. The filter was then placed in a scintillation vial with 10 ml of scintillation fluid (EcoLite™, ICN Biomedicals, Inc.), and the radioactivity was measured using Beckman LS 5801 spectrometer. Correction was made for nonspecific binding of isotope to the filter at each time point. Uptake was expressed in picomoles [14C]-labeled substrate per milligram vesicle protein. At each time point, unless otherwise stated, uptake was measured in triplicate in at least three separate vesicle preparations.

In a preliminary experiment, to determine whether [14C]TEA was transported into an osmotically active space, I examined the effects of extravesicular osmolality on TEA uptake. The 2 hr uptake of 50 μM [14C]TEA was measured in BBMV incubated in the presence of increasing concentrations of sucrose (0 - 600 mM) (Fig 2.1). Uptake was measured in triplicate and plotted against the inverse of the extravesicular osmolality (1/Osm). The uptake of [14C]TEA decreased with increasing extravesicular osmolality; the relationship between these two parameters was expressed by linear regression. These data indicated that the intravesicular volume decreased in response to the increasing extravesicular osmolality. Theoretically, as the vesicles "shrank", the proportion of the total [14C]TEA bound to BBMV would increase. Because the exact reflection coefficients of the solutes present in the system (i.e., mannitol, K⁺, Cl⁻, HEPES, Tris) are not known, the exact fraction of [14C]TEA bound to the vesicle membrane could not be calculated. However, extrapolation of the line to infinite osmolality, 1/Osm = 0, (i.e., calculation of the y-intercept) gives an estimate of the percent of isotope bound to BBMV. In this preparation, membrane-bound isotope represented approximately 25% of the total measured [14C]TEA.
Figure 2.1. Effect of increasing extravesicular osmolality on the equilibrium uptake of [14C]tetraethylammonium. Two hour uptake of [14C]TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in medium containing (in mM) 100 KCl, 200 mannitol and 5 HEPES-KOH (pH 6.0). At 25°C, 10 μl BBMV were diluted with 90 μl transport medium containing ~60 μM [14C]TEA-Br, 100 mM KCl, 200 mM mannitol, 5 mM HEPES-KOH (pH 7.5) plus 0, 50, 100, 200, 300, 400, 500 or 600 mM sucrose. 

(n = 1; triplicate measurements; mean ± S.D.)
e. Chemicals

$[^{14}\text{C}]$methyl-D-glucose (286.90 mCi/mmol) was obtained from New England Nuclear. $[^{14}\text{C}]$tetraethylammonium bromide (56 mCi/mmol) was purchased from Wizard Labs (West Sacramento, CA). All other chemicals were obtained from standard sources.

f. Statistical analysis

Data are expressed as the mean ± S.E. Data were compared using Student’s t-test for paired observations. Differences at the 0.05 level were considered significant.
III. Results

a. Biochemical analysis

The functional integrity of the final BBMY suspension was evaluated on the basis of protein yield, the specific activity of selected enzymes, and the transport characteristics of the isolated membranes. The mean protein yield was 0.35% (Table 2.1). The protein concentration of the final BBMV suspension was 4 - 13 mg/ml. The mean concentration for each uptake experiment is reported in the figure legends.

The biochemical purity of the final BBMY suspension was ascertained by measuring the specific activities of "marker" enzymes in the vesicle suspension and the initial kidney homogenate. In the kidney, alkaline phosphatase is a marker enzyme for the luminal or brush border membrane, Na⁺-K⁺, ATPase for the basolateral membrane, and succinate dehydrogenase for the inner mitochondrial membrane. The greater the proportion of a defined membrane fraction in the BBMV suspension, the greater the "enrichment ratio" of the corresponding marker enzyme. The enrichment ratio is the quotient of the specific activity of a marker enzyme in the BBMV suspension divided by specific activity in the initial kidney homogenate. Ideally, a BBMV suspension is not contaminated with basolateral or mitochondrial membranes. Thus, the enrichment ratio of alkaline phosphatase in the BBMV suspension should be high (≥10) while the enrichment ratios of other marker enzymes should not exceed one. Accordingly, in my BBMV suspension, alkaline phosphatase was enriched 18-fold over that of the initial homogenate, while Na⁺-K⁺, ATPase and succinate dehydrogenase were not enriched (Table 2.1). Thus, this BBMV suspension was relatively pure.

b. Transport properties of avian renal BBMV.

To determine if avian BBMV maintained transport properties characteristic of the native BBM, the uptake of 100 μM [¹⁴C]methyl-D-glucose was measured in the presence
Table 2.1. Protein yield and enzyme enrichment ratios in the final suspension of avian brush-border membrane vesicles.

<table>
<thead>
<tr>
<th>Protein Content (mg protein)</th>
<th>Enzyme Specific Activities (μmol·mg⁻¹·hr⁻¹)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkaline Phosphatase</td>
<td>Na⁺-K⁺-ATPase</td>
</tr>
<tr>
<td>IH: 1750.35 ± 113.27</td>
<td>0.31 ± 0.045</td>
<td>3.68 ± 0.90</td>
</tr>
<tr>
<td>BBMV: 6.52 ± 1.16</td>
<td>5.78 ± 0.85</td>
<td>1.22 ± 0.50</td>
</tr>
</tbody>
</table>

ER: 18.46 (± 0.37)

Percent: 0.35% (± 0.047%)

Data are expressed as the mean ± standard error.

ER: enzyme enrichment ratio = \frac{\text{specific activity BBMV}}{\text{specific activity IH}}

IH: initial kidney homogenate

BBMV: final brush-border membrane vesicle suspension

percent protein yield = \frac{\text{total protein IH}}{\text{total protein BBMV}}
and absence of an inwardly directed or cis sodium gradient at various time intervals over 2 hr (Fig 2.2). In the presence of a cis sodium gradient (100 mM NaCl), there was a transient accumulation of glucose above equilibrium values. This phenomenon is referred to as an "overshoot" and is indicative of the concentrative uptake of substrate, in this case, glucose (Aronson 1981). In contrast, a cis K⁺ gradient (100 mM KCl) did not generate an overshoot. The vesicle content of glucose at equilibrium (2 hr) was the same in the presence and absence of sodium. Therefore, the stimulation of glucose transport by sodium was not due to changes in vesicle volume. Thus, in these renal BBMV, glucose uptake was physically coupled to sodium influx. These data also confirmed that a transmembrane gradient of Na⁺ could be maintained in avian BBMV.

To determine if Na⁺/glucose cotransport was electrogenic, 5 sec uptake of [¹⁴C]methyl-D-glucose was measured in the presence of a cis Na⁺ gradient at various transmembrane potentials manipulated by used of KCl and the potassium ionophore, valinomycin. This protocol was first use by Murer and Hopfer (1974) to demonstrate electrogenic Na⁺/glucose cotransport in intestinal BBMV of rats. In my study, an inside negative potential was imposed in BBMV by pre-equilibration with 100 mM KCl and valinomycin (20 µg/mg BBM protein) and subsequent dilution (10X) with K⁺-free transport medium. To generate an inside positive potential, BBMV were pre-equilibrated with K⁺-free buffer and valinomycin and diluted (10X) with transport medium containing 111.12 mM KCl. Finally, to short circuit or "voltage clamp" the membrane potential, BBMV were pre-equilibrated with 100 mM KCl and valinomycin and diluted (10X) with transport medium of similar K⁺ concentration. The results show that an inside negative potential significantly stimulated glucose uptake above control (voltage clamped condition 44.9 ± 0.83 to 87.0 ± 5.05 pmol·mg protein⁻¹·S⁻¹), whereas an inside positive potential significantly inhibited uptake (44.9 ± 0.83 to 19.2 ± 3.19 pmol·mg protein⁻¹·S⁻¹). In either case, imposition of a transmembrane potential did not significantly alter vesicle
Figure 2.2. Time course of [14C]methyl-D-glucose uptake in the presence and absence of an inwardly-directed Na+ gradient. Uptake of [14C]methyl-D-glucose was measured in BBMV prepared in vesicle buffer containing (in mM) 300 mannitol, 3 NaN3, 20 Tris-HEPES (pH 7.4). At 25°C, 10 μl BBMV were diluted with 90 μl isosmotic mannitol transport medium containing 100 μM [14C]methyl-D-glucose, 20 mM Tris-HEPES (pH 7.4) and either 111.11 mM KCl (○) or 111.11 mM NaCl (●). (n = 3; mean ± S.E.; *P < 0.05)
content of glucose at equilibrium (2 hr). These data confirmed the presence of electrogenic
Na+/glucose cotransport in avian renal BBMV.

c. Effects of a trans H+ gradient on [14C]TEA transport

Organic cation/H+ exchange has been demonstrated repeatedly in BBMV isolated
from the kidneys of dogs, rabbits, rats, humans, and snakes. To determine if OC/H+
exchange was present in avian renal BBMV, uptake of 50 µM [14C]tetraethylammonium
bromide (TEA) was measured in the presence and absence of a outwardly-directed or trans
H+ gradient (pH_{in} 6.0:pH_{out} 7.5) (Fig 2.3a). A trans H+ gradient significantly stimulated
the initial uptake of TEA and supported a 4-fold overshoot. The 2 hr uptake of TEA was
not significantly altered by changes in transmembrane gradient of H+, indicating H+
stimulation of uptake did not involve changes in vesicle volume or membrane permeability.
In one experiment, I also examined the stimulatory effects of protons on the efflux of TEA
from BBMV (Fig 2.3b). The efflux of 150 µM [14C]TEA from BBMV was stimulated
only in presence of a trans H+ gradient (pH_{in} 7.5:pH_{out} 6.0). Stimulation of TEA efflux
was not due to changes in vesicle volume, as marked differences in the equilibrium content
of [14C]TEA were not observed.

The translocation of the positively charged TEA may have been driven by a negative
diffusion potential generated by the trans flux of H+, rather than by the carrier-mediated
exchange of TEA and H+. To test this, I measured proton-driven uptake of 50 µM 14C-
TEA under voltage clamped conditions (K+ equilibrium, plus valinomycin) in the presence
and absence of the H+-specific ionophore, carbonyl cyanide p-(trifluoromethoxy)
phenylhydrazone (FCCP) (Fig 2.4). This result indicated that concentrative uptake of
TEA persisted in the presence of a trans H+ gradient under voltage clamped conditions.
The addition of 30 µM FCCP, which effectively served to collapse the trans H+ gradient,
prevented generation of an overshoot. The effect of FCCP was specific, as 5 sec
Figure 2.3. Time course of $[^{14}C]$tetraethylammonium transport in the presence and absence of a counter-directed $H^+$ gradient. Uptake of $[^{14}C]$TEA (Fig 2.3a) was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in media containing (in mM) 200 mannitol, 100 KCl, 10 HEPES-KOH, pH 6.0 or pH 7.5. At 25°C, 10 μl of BBMV were diluted with 90 μl of isosmotic mannitol transport media containing ~50 μM $[^{14}C]$TEA-Br, 100 mM KCl and 10 mM HEPES-KOH, pH 6.0 or pH 7.5. (n = 3, mean ± S.E., * P < 0.05). In a similar manner, efflux of $[^{14}C]$TEA from BBMV was monitored (Fig 2.3b). BBMV were prepared and pre-equilibrated (1 hr, 25°C) in media containing 200 mM mannitol, 100 mM KCl, 10 mM HEPES-KOH, pH 6.0 or pH 7.5 and ~250 μM $[^{14}C]$TEA-Br. At 25°C, 10 μl of BBMV were diluted with 90 μl of isosmotic mannitol transport media containing 100 mM KCl and 10 mM HEPES-KOH, pH 6.0 or pH 7.5. (n = 1; triplicate measurements, mean ± S.E.) In both figures: ■ pH Equilibrium 6.0; □ pH in 6.0:pH out 7.5; ● pH Equilibrium 7.5; ○ pH in 7.5:pH out 6.0.
[^14C]TEA uptake was further inhibited by the addition of 20 mM unlabeled TEA (Fig 2.4). These data suggest that TEA transport was physically coupled to the trans flux of H\(^+\) and that an OC/H\(^+\) exchange or antiport mechanism is expressed in avian renal BBMV.

d. Kinetic parameters of proton-driven [^14C]TEA uptake

The kinetic parameters of H\(^+\)-driven [^14C]TEA uptake were measured by the "tracer-displacement" protocol outlined by Malo and Berteloot (1991). In a preliminary study, the time course of 100 µM [^14C]TEA uptake via H\(^+\) exchange was measured; uptake was linear through 3 sec (Fig. 2.5). In these kinetic studies, the 3 sec uptake of 100 µM 14C-TEA was measured in the presence of increasing cis concentrations of unlabeled (cold) TEA (0 - 20 mM) in 5 separate BBMV preparations. Representative data from one experiment are displayed in Figure 2.6. In using the tracer-displacement method to analyze the kinetics of TEA uptake, I assumed that unlabeled TEA competitively inhibited transport of [^14C]TEA. Mathematically, the initial rate of [^14C]TEA uptake, \( J \), was expressed as a function of the concentration of cold TEA, [TEA]. This relationship can be described by the Henri-Michaelis-Menten velocity equation for enzyme kinetics in the presence of a competitive inhibitor (Segal 1971):

\[
J = \frac{J_{\text{max}} [^14\text{C-TEA}]}{K_t + [^14\text{C-TEA}] + [\text{TEA}]} + C.
\]

This equation includes the following terms: \( J_{\text{max}} \), the maximal rate of uptake; [^14C-TEA], the concentration of [^14C]TEA; \( K_t \), the Michaelis constant or the TEA concentration that yields \( \frac{1}{2} J_{\text{max}} \); and [TEA], the concentration of unlabeled TEA. The constant \( C \) represents the fraction of [^14C]TEA that is either transported by non-saturable process(es) or non-specifically bound to the BBMV or both. However, in the absence of a non-saturable pathway for TEA accumulation, \( C \) equals zero. The initial rate of [^14C]TEA
Figure 2.4. Time course of $[^{14}\text{C}]$tetraethylammonium uptake in the presence of *trans* $\text{H}^+$ gradient and a $\text{H}^+$ ionophore under voltage clamped conditions. In these studies, the $\text{H}^+$ ionophore carbamyl cyanide ($p$-trifluoromethoxy)phenylhydrazone (FCCP) was used. Uptake of $[^{14}\text{C}]$TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in medium containing (in mM) 200 mannitol, 100 KCl, 20 μM valinomycin, 5 HEPES-KOH (pH 6.0). At 25°C, 10 μl of BBMV were diluted with 90 μl of isomotic mannitol transport media containing ~50 μM $[^{14}\text{C}]$TEA-Br, 20 μM valinomycin, 100 mM KCl and 5 HEPES-KOH (pH 7.5). Transport media contained no ionophore, ●; 30 μM FCCP, ○; or 30 μM FCCP and 22.22 mM unlabeled TEA-Br . ■.

(n = 3; mean ± S.E.; * P<0.05)
Figure 2.5. Time course of 100 μM $[^{14}\text{C}]$tetraethylammonium uptake in the presence of a trans H$^+$ gradient. One to five second uptake of $[^{14}\text{C}]$TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in medium containing 100 mM KCl, 200 mM mannitol and 5 mM HEPES-KOH (pH 6.0). At 25°C, 10 μl of BBMV were incubated with 90 μl of isosmotic mannitol transport media containing ~100 μM $[^{14}\text{C}]$TEA-Br, 100 mM KCl, 5 mM HEPES-KOH (pH 7.5). (n = 1; triplicate measurements; mean ± S.D.)
Figure 2.6. The initial rate of $[^{14}C]$tetraethylammonium uptake in the presence of increasing cis concentrations of unlabeled tetraethylammonium. Three second uptake of $[^{14}C]$TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in medium containing 100 mM KCl, 200 mM mannitol and 5 mM HEPES-KOH (pH 6.0). At 25°C, 10 µl of BBMV were incubated with 90 µl of isosmotic mannitol transport media containing ~100 µM $[^{14}C]$TEA-Br, 100 mM KCl, 5 mM HEPES-KOH (pH 7.5) and increasing concentrations of unlabeled TEA (0-20 mM). Although uptake was measured in 5 separate BBMV preparations, data from a representative experiment is displayed here. In this particular experiment, the $K_t$ and $J_{max}$ for TEA were 400 µM and 23 nmol·mg protein⁻¹·min⁻¹, respectively. ($n = 1$, triplicate measurements)
uptake decreased with increasing cis concentrations of cold TEA, asymptotically approaching zero. This indicated H+-driven TEA uptake is a carrier-mediated, saturable process. However, the displacement curve did not reach a zero asymptote. This implied the presence of a non-specific, non-saturable component of [14C]TEA transport that was not inhibited by cold TEA in concentrations as high as 20 mM. The calculated value of $C$ was 0.03 nmol·mg protein$^{-1}$·min$^{-1}$; the corresponding apparent diffusion coefficient was $2.61 \times 10^{-8}$ l·mg protein$^{-1}$·min$^{-1}$. Most simply, this indicated the presence of a minor non-saturable TEA transport process(es), such as simple diffusion. However, as previously noted, the measured uptake of [14C]TEA, even in the presence of 20 mM cold TEA, may include nonspecifically bound isotope. The $J_{\max}$ for TEA, an index of the carrier's capacity to transport TEA, was 24.56 nmol·mg protein$^{-1}$·min$^{-1}$ ± 5.94 nmol·mg protein$^{-1}$·min$^{-1}$. The $K_t$ for TEA, an index of the carrier's affinity for TEA, was 531 μM ± 113.14 μM.

e. Cis inhibitory effects of organic compounds on [14C]TEA/H+ exchange

A general assessment of the substrate specificity of OC/H+ exchange was conducted by examining cis inhibition of TEA/H+ exchange by various organic compounds. The 5 sec uptake of 50 μM [14C]TEA was measured in the presence of a trans H+ gradient and cis 20 mM unlabeled TEA or 1 mM cis concentrations of each inhibitor (Table 2.2). Twenty millimolar unlabeled TEA inhibited 5 sec [14C]TEA uptake by 98% and prevented generation of an overshoot (Appendix A). This again suggested TEA uptake by a diffusive pathway was minimal. In 1 mM concentrations, unlabeled TEA and NMN each profoundly inhibited initial uptake (estimated at 5 sec) and blocked the uphill transport of TEA (Appendix A). By comparison, choline modestly inhibited the initial uptake of TEA (~40%) and did not completely abolish the H+-driven overshoot (Appendix A). Each of the cationic transport inhibitors cimetidine, mepiperphenidol
Table 2.2. Cis-inhibition of five second $[^{14}C]$tetraethylammonium uptake by various compounds.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Control</th>
<th>w/Inhibitor</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEA (20 mM)</td>
<td>157.10±36.13</td>
<td>4.03*±1.60</td>
<td>97%</td>
</tr>
<tr>
<td>TEA (1 mM)</td>
<td>164.4±36.02</td>
<td>45.00*±8.98</td>
<td>74%</td>
</tr>
<tr>
<td>NMN (1 mM)</td>
<td>190.46±19.14</td>
<td>55.35*±5.76</td>
<td>71%</td>
</tr>
<tr>
<td>Choline (1 mM)</td>
<td>165.53±25.05</td>
<td>99.87*±2.43</td>
<td>40%</td>
</tr>
<tr>
<td>Darstine (1 mM)</td>
<td>169.52±37.49</td>
<td>10.97*±2.43</td>
<td>94%</td>
</tr>
<tr>
<td>Cimetidine (1 mM)</td>
<td>169.52±37.49</td>
<td>6.20*±3.11</td>
<td>96%</td>
</tr>
<tr>
<td>Quinidine (1 mM)</td>
<td>164.40±36.02</td>
<td>4.07*±0.33</td>
<td>98%</td>
</tr>
<tr>
<td>Quinine (1 mM)</td>
<td>197.76±10.73</td>
<td>2.96*±0.71</td>
<td>99%</td>
</tr>
<tr>
<td>Ranitidine (1 mM)</td>
<td>197.76±10.73</td>
<td>4.07*±1.03</td>
<td>98%</td>
</tr>
<tr>
<td>PAH (1 mM)</td>
<td>190.46±19.14</td>
<td>168.66±33.25</td>
<td>11%</td>
</tr>
<tr>
<td>Probenecid (1 mM)</td>
<td>190.46±19.14</td>
<td>125.89*±10.95</td>
<td>34%</td>
</tr>
</tbody>
</table>

Five second uptake of $[^{14}C]$TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in a mannitol medium containing: 100 mM KCl and 5 mM HEPES-KOH (pH 6.0). At 25°C, 10 μl BBMV were diluted with 90 μl isosmotic mannitol transport medium containing ~50 μM $[^{14}C]$TEA-Br, 100 mM KCl, 5 mM HEPES-KOH (pH 7.5), and no inhibitor, 22.22 mM unlabeled TEA-Br, or 1.11 mM of the respective test compound. (n = 3; mean ± S.E.; *P < 0.05)
(Darstine), quinidine, quinine, and ranitidine inhibited uptake by at least 93%. In contrast, the organic anions p-aminohippurate (PAH) and probenecid were poor inhibitors of TEA uptake. Although probenecid inhibited 5 sec uptake by ~30%, neither anion markedly diminished the H+-generated overshoot of TEA uptake (Appendix A). However, probenecid modestly, though significantly inhibited the initial uptake of TEA (~30%).

Uptake at equilibrium (2 hr) was not significantly altered in the presence of any of the tested compounds (Appendix A). Thus, when observed, inhibition by the organic cation or anion was not due to a nonspecific perturbation of the transporter or vesicle membrane lipid bilayer.

**f. effects of Na+ on [14C]TEA transport**

To define the potential role of Na+ and Na+/H+ exchange in the luminal transport of OCs in the avian proximal tubule, I examined the effects of Na+ on the uptake of 50 μM [14C]TEA in these BBMV (Fig 2.7). In the absence of a H+ gradient (pH<sub>in</sub> = pH<sub>out</sub> 7.5), I measured TEA uptake in the presence of trans and cis Na gradients (100 mM NaCl), and at Na+ equilibrium (100 mM NaCl). A significant stimulation of the initial rate of TEA uptake (estimated at 5 sec) and a transient accumulation of TEA above equilibrium values were observed only in the presence of a trans Na+ gradient. There were no significant differences between equilibrium values measured in the presence and absence Na+. Thus, the effects of Na+ did not involve changes in vesicle volume. However, the observed trans stimulation of [14C]TEA uptake by Na+ may involve one or more mechanisms: generation of an intravesicular negative diffusion potential by the efflux of Na+, carrier-mediated Na+/TEA exchange, or generation of an outwardly-directed H+ by Na+/H+ exchange.

To define the mechanisms of Na+ trans stimulation, uptake of 50 μM [14C]TEA was measured at pH equilibrium (pH<sub>in</sub> = pH<sub>out</sub> 7.5) under voltage clamped conditions in the presence of a trans Na+ gradient and 30 μM FCCP (Fig 2.8). Presumably, generation
Figure 2.7. Time course of $[^{14}C]$tetraethylammonium uptake in the presence of a *trans* Na$^+$ gradient at pH equilibrium. Uptake of TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in medium containing 5 mM HEPES-KOH (pH 7.5), 100 mM KCl and 300 mM mannitol or 100 mM mannitol and 100 mM NaCl. At 25°C, 10μl of BBMV were diluted with 90 μl of isosmotic mannitol transport media containing ~50 μM $[^{14}C]$TEA-Br, 5 mM HEPES-KOH (pH 7.5), 100 mM KCl and no NaCl, 111.11 mM NaCl, or 100 mM NaCl. Legend: 〇 Control (No NaCl); △ 100 mM *trans* NaCl (Na$^+_{\text{in}}$ $>$ Na$^+_{\text{out}}$); ● 100 mM *cis* NaCl (Na$^+_{\text{out}}$ $>$ Na$^+_{\text{in}}$); ▲ Na$^+$ Equilibrium (100 mM NaCl). (n = 3; mean ± S.E.; * P<0.05)
Figure 2.8. Time course of [14C]tetraethylammonium uptake under voltage clamped conditions in the presence of a trans Na⁺ gradient and a H⁺ ionophore. Uptake of TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in medium containing 20 μM valinomycin, 5 mM HEPES-KOH (pH 7.5), 100 mM KCl, 100 mM NaCl plus 100 mM mannitol and no FCCP, 100 mM mannitol and 30 μM FCCP, or 58 mM mannitol, 22.22 mM unlabeled TEA and 30 μM FCCP. At 25°C, 10 μl BBMV were diluted with 90 μl of isosmotic mannitol buffer containing ~50 μM [14C]TEA-Br, 5 mM HEPES-KOH (pH 7.5), 100 mM KCl, 20 μM valinomycin and no FCCP (○), 30 μM FCCP (●), or 22.22 mM unlabeled TEA and 30 μM FCCP (■). (n = 3; mean ± S.E.; * P<0.05)
of both a trans H⁺ gradient via Na⁺/H⁺ exchange, as well as a Na⁺ diffusion potential were precluded under such conditions. Short-circuiting the transmembrane potential did not block uphill transport of TEA in the presence of a trans Na⁺ gradient, suggesting the trans stimulatory effects of Na⁺ were not a result of a negative diffusion potential alone. In the presence of FCCP, although the initial rate of uptake (i.e., 5 sec) was not altered, a trans Na⁺ gradient failed to support concentrative uptake of TEA. These effects of FCCP were specific, as the addition of 20 mM unlabeled TEA further reduced the initial rate of uptake. The inhibitory effect of FCCP indicated Na⁺ trans-stimulation of TEA uptake was not a result of Na⁺/TEA exchange, but a secondary result of the generation of a trans H⁺ gradient. Equilibrium values of TEA uptake were significantly increased in the presence of FCCP. This may have been a result of a time-dependent, non-specific effect of FCCP.

g. trans effects of organic cations on tetraethylammonium uptake.

Finally, to determine whether TEA could be transported in exchange for organic cations in these avian BBMV, I monitored the time course of [¹⁴C]TEA uptake in BBMV pre-equilibrated with 1 mM concentrations of unlabeled TEA, NMN, and choline (Fig 2.9). (Note in these particular experiments, BBMV were diluted 40-fold, rather than 10-fold, with transport medium. At this dilution, the cis "carry-over" concentration of the test OCs was 25 µM.) In the absence of a H⁺ gradient (pH<sub>in</sub> = pH<sub>out</sub> 7.5), uptake of 50 µM [¹⁴C]TEA was measured in the absence and presence of trans 1 mM of each OC, and in the presence of cis 25 µM of each OC. Trans gradients of unlabeled TEA and NMN produced 3.5- and 2.5 fold increases in the initial rate of TEA (estimated at 5 sec; Fig 2.8a, b), respectively. Furthermore, in each case, accumulation of TEA was significantly stimulated above equilibrium. Choline did not trans-stimulate TEA accumulation at any time point. The OCs tested significantly altered uptake at equilibrium. The observed trans-stimulation
Figure 2.9. Time course of [14C]tetraethylammonium uptake at pH equilibrium in the presence trans gradients of organic cations. Uptake of TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in medium containing 5 mM HEPES-KOH (pH 7.5), 100 mM KCl and either 202 mM mannitol or 200 mM mannitol and 1 mM of unlabeled TEA (Fig 2.9a), NMN (Fig 2.9b), and choline (Fig 2.9c). At 25°C, 5 μl BBMV were diluted with 195 μl isosmotic mannitol transport buffer containing ~50 μM [14C]TEA-Br, 5 mM HEPES-KOH (pH 7.5), 100 mM KCl and no cation or 27.8 μM of the respective organic cation. Legend: □, control (1 mM trans mannitol; ■, 1 mM trans OC+ (OC+in >OC+out); △, 25 μM cis OC+ (OC+out >OC+in). (n = 3; mean ± S.E.; * P<0.05)
by OCs might have been secondary to the generation of either a trans gradient of H⁺ by OC/H⁺ exchange or a negative diffusion potential generated by the efflux of cations or H⁺. Under voltage-clamped conditions (potassium equilibrium plus valinomycin), 30 μM FCCP did not impede trans-stimulation of [¹⁴C]TEA uptake by TEA or NMN (Appendix B). Thus, these data indicated the uptake of [¹⁴C]TEA was physically coupled to the efflux of the OCs TEA and NMN, as well as H⁺.
IV. Discussion

In this study, I examined the mechanisms of organic cation transport across the apical membrane of the avian renal proximal tubule using BBMV. I isolated BBMV from the proximal tubules of hens by means of Ca\(^{2+}\) precipitation and differential centrifugation. As judged by the enzyme enrichment ratios and transport properties of the final BBMV suspension, these vesicles maintained biochemical and functional integrity. The BBM marker enzyme alkaline phosphatase was enriched 18-fold (Table 2.1). In other renal BBMV preparations of the chick and snake, reported enrichment ratios for alkaline phosphatase were 15 and 14, respectively (Benyajati and Dantzler 1987, Renfro and Clark 1984). In the present study, glucose was transported by a Na\(^{+}\)-dependent electrogenic process as first shown in renal BBMV of the rabbit (Aronson and Sacktor 1974; Beck and Sacktor 1975). In addition, these results are similar to those seen in other chicken renal BBMV preparations (Renfro and Clark 1984; Kou and Austic 1987). Thus, the enzymatic and transport characteristics of the chicken renal BBMV in the present study are comparable to those of renal BBMV of other vertebrates. Moreover, these BBMV maintained biochemical and functional properties characteristic of the intact BBM and were suitable for the study of the transport of TEA.

Tetraethylammonium (TEA) is one of many organic cations known to undergo net tubular secretion in the intact chicken kidney (for review, see Rennick 1981, Wideman 1988). However, our understanding of the cellular mechanisms involved in transepithelial secretion in birds as well as other nonmammalian vertebrates is limited. According to the current model for the transepithelial secretion of OCs, transport across the BBM of the proximal tubule is the rate-limiting step. Thus, our first objective was to describe the mechanisms of luminal secretion of OCs in birds using BBMV isolated from chicken renal proximal tubules. Second, I sought to compare the mechanisms of OC transport in avian BBMV to those previously described in mammalian BBMV. I monitored changes in the
uptake of the exogenous OC \([^{14}\text{C}]\)TEA in response to alterations in the transmembrane gradients of H+, Na+ and various organic ions.

In chicken renal BBMV, a \textit{trans} H+ gradient stimulated TEA uptake producing a 5-fold transient accumulation above equilibrium (Fig 2.3a). Proton \textit{trans}-stimulation of TEA persisted under voltage clamped conditions (potassium equilibrium plus valinomycin) (Fig 2.4). However, addition of the H+ ionophore FCCP prevented generation of the proton-driven overshoot (Fig 2.4), indicating that TEA transport was physically coupled to the \textit{trans} flux of H+. Thus, OC/H+ exchange or antiport was present in these avian renal BBMV. (Note that OC/H+ exchange is thermodynamically equivalent to OC/OH\textsuperscript{-} cotransport (Murer, Hopfer and Kinne 1976)). Similarly, TEA is transported in exchange for H+ in BBMV isolated from the kidney of the rabbit (Dantzler et al. 1989; Miyamoto et al. 1989; Rafizadeh et al. 1987; Rafizadeh et al. 1986, Wright and Wunz 1988, Wright and Wunz 1987), rat (Hori et al. 1985, Katsura et al. 1991, Takano et al. 1984), human (Ott et al. 1991), and snake (Dantzler et al. 1991). Studies in perfused tubules of both the rabbit (Dantzler et al. 1989) and snake (Dantzler and Brok 1987) in which decreasing luminal pH stimulated efflux of TEA across the intact BBM confirm the presence of a luminal OC/H+ exchanger. To my knowledge, there has been no previous report of an OC/H+ exchange mechanism in the BBM of the avian renal proximal tubule.

Clearance studies in chickens have shown that renal excretion of TEA is a saturable process (Milton and Odlind 1985; Springate et al. 1987; Rennick et al. 1977; Rennick et al. 1956; Rennick et al. 1954). Likewise, in the present study as determined by analysis of the kinetic parameters of the initial rate of TEA uptake (Fig 2.6), accumulation of TEA in the presence of a \textit{trans} H+ gradient was a carrier mediated, saturable process; however, a minor non-saturable component was also present. The Michaelis constant or \(K_t\) for TEA was \(\sim 530 \mu\text{M}\), and the maximal rate of TEA uptake or \(J_{\text{max}}\) was \(\sim 25 \text{ nmol}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}\). These kinetic values for TEA/H+ exchange are similar to those reported for snake
and rabbit renal BBMV. In snake BBMV, the $K_t$ and $J_{max}$ for TEA were \(~475\ \mu M\) and \(~2\ \text{nmol·mg protein}^{-1}·\text{min}^{-1}\), respectively. For rabbit BBMV, Wright and colleagues reported $K_t$ and $J_{max}$ of \(~100\ \mu M\) and \(10\ \text{nmol·mg protein}^{-1}·\text{min}^{-1}\), respectively (Lazaruk and Wright 1990; Wright and Wunz 1988), and Rafizadeh and co-workers reported corresponding values of \(~330\ \mu M\) and \(~9\ \text{nmol·mg protein}^{-1}·\text{min}^{-1}\) (Rafizadeh et al. 1987). The relatively narrow range of the reported values of $K_t$ for TEA suggests similar affinity of the OC/H$^+$ exchanger for TEA among these species. In a stricter sense, however, as the $K_t$ for TEA is greatest in chicken BBMV, the affinity of the OC/H$^+$ exchanger for TEA is apparently lowest in this animal. An analogous comparison of values of $J_{max}$ among species suggest the capacity of the OC/H$^+$ exchanger to transport TEA is greatest in the chicken, being twice that in rabbits and 12 times that in snakes. This variation may be due to species differences or differences in experimental conditions. Such differences have yet to be examined. Nonetheless, the kinetic analysis of the of TEA uptake indicated that the OC/H$^+$ exchanger expressed in avian renal BBMV has an affinity and transport capacity for TEA comparable to that in mammals.

A general assessment of the substrate specificity of OC/H$^+$ exchange was conducted by examining cis-inhibition of $[^{14}\text{C}]$TEA/H$^+$ exchange by various organic compounds (Table 2.2). The initial uptake of $[^{14}\text{C}]$TEA (5 sec) was significantly inhibited by each OC tested (unlabeled TEA, NMN, choline, cimetidine, mepiperphenidol, quinidine, quinine and ranitidine). Each of these cationic compounds inhibits OC/H$^+$ exchange in renal BBMV of mammals (Gisclon et al 1987; Ott et al. 1991; Miyamoto et al. 1989; Rafizadeh et al. 1987; Wright and Wunz 1987). Moreover, every OC tested except quinidine is known to inhibit excretion of OCs in chickens (Milton and Odlind 1985; Springate et al. 1987; Rennick et al. 1984; Rennick et al. 1977; Green et al. 1959; Rennick et al. 1984; Bessighir and Rennick 1981). Choline, the weakest of the cationic inhibitors, was the only OC that did not completely prevent generation of a H$^+$-driven overshoot in
TEA uptake. The implications of the weak inhibitory effects of choline will be more completely addressed later in this chapter in conjunction with the data on the trans effects of choline on transport. As suggested by my data, inhibition of OC excretion may be due in part to an inhibition of OC transport across the apical membrane of the proximal tubule cell.

Unlike organic cations, the organic anions probenecid and PAH poorly inhibited TEA transport. Neither anion markedly altered the magnitude of the TEA overshoot. Similarly, PAH fails to inhibit OC/H\(^+\) exchange in mammalian BBMV (Rafizadeh et al. 1987; Griffiths et al. 1992; Kinsella et al. 1979; Ott et al. 1991; Wright and Wunz 1991). Although probenecid is considered a prototypic inhibitor of organic anion transport, it significantly decreased the initial uptake of TEA in avian BBMV (Table 2.2) and is known to inhibit proton-driven uptake of cimetidine (McKinney and Kunneman 1987; Gisclon et al. 1987). Moreover, Hsyu et al. (1988) demonstrated that probenecid competitively inhibited proton-driven uptake of NMN in rabbit renal BBMV. In avian BBMV, as well as those of the rabbit, the equilibrium uptake of the OC was not altered by probenecid, indicating its inhibitory interaction with OC transport was specific.

Overall, the inhibitory effects of organic cations and anions indicate that the OC/H\(^+\) exchanger in avian BBMV is specific for organic cations. These data are consistent with renal clearance studies in chickens (Besseghir and Rennick 1981; Sperber 1948; Rennick 1960; Rennick 1958; Rennick et al. 1956; Sanner 1963) and dogs (Beyer and Gelarden 1975; Beyer et al. 1953; Lifschitz et al. 1973; Orloff et al. 1953; Rennick and Moe 1960; Rennick et al. 1947). At plasma concentrations sufficient to attain tubular transport maxima, organic anions (e.g., PAH, phenol red, probenecid) do not inhibit the excretion of OCs. Conversely, at similar plasma concentrations, OCs (e.g., TEA, NMN, isoproterenol) do not inhibit the excretion of organic anions. These findings indicate the transport pathways for the renal excretion of OCs and organic anions are distinct.
Although I did not examine the orientation of the avian BBMV, previous investigators, by means of electron-microscopic and immunological methods, determined that intestinal BBMV are predominantly (~95%) oriented right-side out (Haase et al. 1978). Therefore, assuming avian renal BBMV behave similarly, uptake is analogous to a reabsorptive flux across the luminal membrane of the proximal tubule, and efflux is analogous to a secretory flux. Both the uptake and efflux of TEA are trans-stimulated by $H^+$, indicating TEA/$H^+$ exchange was qualitatively symmetrical (Fig 2.3a, b). Thus, although I specifically characterized TEA uptake, the mechanisms of TEA efflux are likely to be qualitatively similar. It is not unreasonable to assume that my conclusions regarding the mechanisms of TEA uptake may be applied in describing the transport of OCs across the BBM of the intact avian proximal tubule. However, the presence of an OC/$H^+$ exchanger in these isolated renal BBMV does not necessitate a physiological role for this transporter in secretion of OC in the intact avian kidney. The possible role of a luminal OC/$H^+$ exchanger in the tubular secretion of OCs must be considered in light of our current knowledge of the physiology of avian kidney.

The avian kidney is composed of a heterogeneous population of nephrons (Braun and Dantzler 1972). Approximately 85% of these nephrons are "reptilian-type" nephrons that have no loops of Henle and are located in the cortex. The remaining 15% are "mammalian-type" nephrons which have loops of Henle is located in the medulla. Mammalian-type nephrons also have higher single nephron glomerular filtration rates, a greater capacity to reabsorb Na$^+$ and water and can concentrate the urine. How the two types of nephrons differ in their abilities to secrete OCs is not known. Presumably, the BBM of the proximal tubules of the two types of nephrons were proportionally represented in the final BBMV suspension. However, I cannot predict to what extent, if at all, the BBM OC/$H^+$ exchanger is preferentially expressed or functions in each nephron population.
Holohan and Ross (1981) proposed luminal efflux of OCs from the proximal tubule cell via OC/H⁺ exchange was driven by the lumen-to-cell H⁺ gradient. However, micropuncture studies in European starlings demonstrated that there was no transepithelial H⁺ gradient across the proximal tubule of the most superficial reptilian-type nephrons; the peritubular blood pH and luminal pH were 7.58 (Laverty and Alberici 1987). The intracellular pH of the avian proximal tubule cell has not been measured. However, if I assume the intracellular pH is 7.4, or 7.2 as measured in the rats (Yoshitomi and Frömter 1984), there would be a cell-to-lumen or cis-directed H⁺ gradient relative to the secretory flux of OC. Yet, in avian BBMV, a cis H⁺ gradient inhibited TEA transport (Fig 2.3a,b). Thus, physiologically, the orientation of the H⁺ gradient across the BBM of the avian proximal tubule is apparently not conducive to secretory OC/H⁺ exchange. However, as first hypothesized by Holohan and Ross (1981), secretion of OCs is indirectly driven by potential energy stored in the large lumen-to-cell Na⁺ gradient across the BBM. Organic cations are secreted in exchange for H⁺ initially secreted into the lumen by Na⁺/H⁺ antiport at the BBM (Murer et al. 1976; Kinsella and Aronson 1980). The indirect coupling of Na⁺ transport to the transport of several OCs including TEA has been demonstrated in renal BBMV of mammals (Holohan and Ross 1981; McKinney and Kunneman 1985; Rafizadeh et al. 1987; Takano et al. 1984; Wright 1985; Wright and Wunz 1989) and snakes (Dantzler et al. 1991). Sodium-dependent OC secretion has been observed in the intact proximal tubule as well. In perfused snake renal tubules, the lumen-to-bath flux of TEA is significantly decreased by the removal of Na⁺ from the tubular lumen (Hawk and Dantzler 1984).

In avian BBMV TEA transport was also indirectly linked to Na⁺ transport presumably through Na⁺/H⁺ exchange (Fig. 2.7, Fig 2.8). Admittedly, I only indirectly demonstrated the presence an Na⁺/H⁺ exchanger in these BBMV. Ideally, this hypothesis would be tested using the Na⁺/H⁺ exchange inhibitor amiloride (Kinsella and Aronson
However, amiloride is actively secreted by OC transport system in the chicken (Besseghir and Rennick 1981; Rennick et al. 1984) and competitively inhibits TEA/H+ exchange in rabbit renal BBMV (Wright and Wunz 1989). Furthermore, I did not verify the presence of Na+/H+ exchange by measuring H+-dependent transport of Na+ as demonstrated in renal BBMV of the snake (Dantzler et al. 1991). However, the present data indicate a Na+/H+ exchange process functions in avian BBMV. Specifically, the inhibition of TEA uptake by the H+ ionophore FCCP indicates Na+ trans stimulation of TEA uptake was secondary to the generation of an outwardly directed H+ gradient presumably by Na+/H+ exchange (Fig. 2.8). If Na+ trans stimulation represented direct Na+/TEA exchange, FCCP would have been ineffective. Moreover, these data suggested that, despite the absence of an appropriate H+ gradient across the intact avian BBM, the inwardly-directed Na+ gradient may indirectly drive the secretory OC/H+ exchange at the luminal membrane of the avian proximal tubule. However, because a transepithelial potential exists across the avian proximal tubule (lumen negative, -2.24 mV; Laverty and Alberici 1983), an analysis of the electrogenicity and stoichiometry of OC/H+ exchange would further our understanding of the physiological role of the organic cation exchanger.

An OC/OC antiport mechanism was also present in these BBMV, as indicated by the concentrative uptake of [14C]TEA in the presence of outwardly-directed gradients of unlabeled TEA and NMN (Fig 2.9a,b). Trans stimulation of radiolabeled TEA by unlabeled TEA was previously observed in renal BBMV of mammals (Dantzler et al. 1989; Griffiths et al. 1992; Katsura et al. 1991; Lazaruk and Wright 1990; Ott et al. 1991; Rafizadeh et al. 1986; Takano et al. 1984; Wright and Wunz 1988; Wright et al. 1992) and snakes (Dantzler et al. 1991). Similarly, in perfused renal tubules of rabbits (Dantzler et al. 1989) and snakes, the efflux of intracellular TEA across the intact BBM is trans stimulated
by unlabeled TEA (Dantzler and Brokl 1987). In avian BBMV, NMN both *trans*
stimulated uphill transport of TEA (Fig 2.9a, b) and *cis*-inhibited TEA/H⁺ exchange
(Table 2.2), indicating that TEA and NMN are each transported across the BBM by the
OC/H⁺ exchanger. Previous studies in chickens demonstrated dose-dependent reciprocal
inhibition of the excretion by NMN and TEA, indicating the two OCs also compete for a
carrier-mediated transport process in the intact kidney (Green et al. 1959). The present
findings suggest that the mutual inhibitory effects of TEA and NMN may involve
competition for a common transporter at the BBM of the proximal tubule.

My data conflict with previous reports of the *trans*-stimulatory effects of NMN on
TEA transport (Fig 2.9b). In human renal BBMV, NMN *trans* stimulated the initial uptake
of TEA, but failed to generate an overshoot (Ott et al. 1991). In contrast, Rafizadeh and
co-workers found NMN *cis* inhibited TEA/H⁺ exchange, but did not *trans*-stimulate TEA
transport in rabbit renal BBMV (Rafizadeh et al. 1986). In that same study, TEA both
*trans* stimulated and *cis* inhibited NMN transport. However, NMN was not transported in
exchange for H⁺. These investigators concluded that NMN and TEA shared a common
transporter that has a greater affinity for TEA, than for NMN. In a separate preparation of
rabbit renal BBMV, Wright demonstrated that NMN transport was coupled to the counter
flux of H⁺ and unlabeled NMN (Wright 1985). However, studies in perfused proximal
tubules of the rabbit (Besseghir et al. 1990) and the snake (Dantzler and Brokl 1987)
suggest that luminal OC exchange may not play a major role in the secretion of NMN. In
each species, neither a decrease in luminal pH, nor the addition of unlabeled NMN to the
lumen stimulated NMN efflux from the tubule cell.

Unlike TEA and NMN, choline failed to *trans* stimulate TEA transport in avian
BBMV (Fig 2.9c). In contrast, choline *trans* stimulates TEA uptake in renal BBMV of
snakes (Dantzler et al. 1991) and rabbits (Wright and Wunz 1992). Likewise, choline *trans*
stimulates TEA efflux from the cells of perfused proximal tubules (Dantzler and Brokl
Lazaruk and Wright (1990) demonstrated that minimizing the competition of protons and OCs for the binding site on the exchanger enhanced the capacity of OCs to trans stimulate TEA transport in rabbit renal BBMV. This was done by decreasing the intra- and extravesicular H⁺ concentration from 30 nM to 3 nM (i.e., increasing the pH from 7.5 to 8.5). I did not examine the trans effects of choline at a pH greater than 7.5. These experimental conditions did, however, accommodate trans-stimulation of [14C]TEA transport by NMN and unlabeled TEA. Although the kinetics of choline/H⁺ exchange were not directly examined, the low inhibitory potency of choline (Table 2.2) and its inability to trans stimulate TEA transport (Fig 2.9c) suggest the OC exchanger's affinity for choline is lower than for TEA. My data are consistent with those of previous renal clearance studies in chickens, dogs, rabbits, and rats. At supraphysiological concentrations, choline undergoes net tubular secretion and may competitively inhibit TEA excretion. However, although choline inhibits excretion of [14C]TEA in the chicken, 4 times as much choline as unlabeled TEA is required to produce an equal degree of inhibition (Rennick et al. 1977). These findings suggested a common secretory pathway for the two OCs (Rennick et al. 1977). At physiological levels (< 100 µM), however, choline is reabsorbed by the renal tubule (Acara and Rennick 1973, Acara et al. 1979; Besseghir et al. 1981). Recently, in rabbit renal BBMV, Wright et al. (1992) demonstrated the OC/H⁺ exchanger's affinity for choline was extremely low; the $K_t$ for choline was 10 mM. Moreover, unlike that for TEA, the primary mechanism for choline transport was electrogenic facilitated diffusion with a $K_t$ for choline of ~100 µM (Wright et al. 1992).

In Figure 2.10 is presented a schematic representation of OC transport across the BBM of the avian proximal tubule cell based on the present data. Briefly, the mechanisms of OC transport in avian renal BBMV were qualitatively similar to those in mammalian BBMV. Proton-driven transport of tetraethylammonium is a carrier-mediated, saturable process. Tetraethylammonium/proton exchange was specific for organic cations and
indirectly coupled to Na\(^+\) transport, presumably through Na\(^+\)/H\(^+\) exchange. Although there is no lumen-to-cell proton gradient in the proximal tubule of the most superficial nephrons of the avian kidney, the large inwardly-directed Na\(^+\) gradient across the BBM may indirectly drive secretory OC/H\(^+\) exchange. The endogenous organic cation NMN was also transported by the OC exchanger, and choline was apparently a poor substrate for the OC exchanger. A more stringent evaluation of the substrate specificity of the antiporter is required to understand its role in regulating plasma concentrations of endogenous and exogenous OCs. Finally, these data validate the continued application of the avian kidney as a model for organic cation secretion by the vertebrate kidney.
Figure 2.10. Model for luminal transport of organic cations in the avian renal proximal tubule.
Chapter 3

Effects of transmembrane potential on organic cation transport in avian renal brush border membranes

I. Introduction

Active secretion of organic cations by the vertebrate renal proximal tubule has been demonstrated by stop-flow studies in dogs (Pilkington and Keyl 1963; Rennick and Moe 1960) as well as in isolated perfused renal tubules of the rabbit (Dantzler et al. 1989) and snake (Dantzler and Brokl 1986; Hawk and Dantzler 1984). Based on studies of canine renal BBMV, Holohan and Ross first proposed that organic cations were secreted across the luminal or brush-border membrane of the mammalian renal proximal tubule by a proton exchange mechanism (OC/H⁺ exchange; Holohan and Ross 1981). These investigators further hypothesized that luminal transport of organic cations was driven by the lumen-to-cell proton gradient generated in part by secretion of protons via Na⁺/H⁺ antiport. Sodium/proton antiport is driven by the inwardly-directed sodium gradient ultimately established by the Na⁺,K⁺-ATPase exchange pump at the peritubular membrane (Holohan and Ross 1981). The energetic coupling of OC/H⁺ exchange to sodium transport has been demonstrated in BBMV of the rabbit (McKinney and Kunneman 1985; Rennick et al. 1987; Wright 1985; Wright and Wunz 1989), snake (Dantzler et al. 1991) and rat (Takano et al. 1984).

In the preceding chapter, I presented data on transport of the organic cation tetraethylammonium in BBMV isolated from renal proximal tubules of domestic chickens. Tetraethylammonium (TEA) was transported against a concentration gradient by carrier-mediated exchange for H⁺ (Fig 2.3a). Additionally, concentrative uptake of TEA in BBMV was indirectly stimulated by a trans sodium gradient via Na⁺/H⁺ antiport (Fig 2.7, Fig
2.8), suggesting that the secretory OC/H⁺ exchange at the BBM in vivo may be indirectly, but energetically linked to sodium transport, as previously proposed for mammals (Holohan and Ross 1981). However, unlike the mammalian proximal renal tubule, a lumen to cell proton gradient apparently does not exist across the BBM of the intact avian renal tubule, as indicated by direct measurements of luminal pH (~7.60) in superficial nephrons of the avian kidney (Laverty and Alberici 1987). These investigators also measured a transepithelial potential difference, lumen-negative (~2.25 mV). In the absence of an appropriate proton gradient across the luminal membrane, carrier-mediated exchange of H⁺ and TEA would be more susceptible to changes in the transmembrane electrochemical gradients. Furthermore, as OC/H⁺ exchange involves countertransport of two charged substrates, if such a mechanism were not electroneutral, it would require a greater expenditure of metabolic energy to drive luminal transport of organic cations.

Thus, to further investigate the mechanism of OC/H⁺ exchange and understand the function of a luminal OC/H⁺ exchange mechanism in the secretion of organic cations in the bird, I examined the effect of transmembrane electrical potential difference (PD) on [¹⁴C]TEA transport in isolated renal brush-border membrane vesicles. Using the potassium-specific ionophore valinomycin to increase membrane conductance to potassium, transmembrane potassium gradients were manipulated to create a potassium diffusion potential and thereby generate a transient transmembrane PD (Murer and Hopfer 1973). The effects of transmembrane potential on influx and efflux of [¹⁴C]TEA, were examined in the presence and absence of trans H⁺ gradient. Although TEA transport was not electrogenic, transport of the organic cation was sensitive to changes in PD. Proton-driven uptake of TEA was stimulated when the vesicular lumen was made transiently negative, whereas efflux of TEA was inhibited when the extravesicular compartment was made positive. To verify that TEA/H⁺ exchange was electroneutral, I attempted to determine the stoichiometry of the antiport mechanism using the static head method.
(Kinsella and Aronson 1982; Turner and Moran 1982). Based on a conservative analysis of the data, I concluded the coupling ratio of TEA/H+ exchange is slightly greater than 1 (H+:TEA). However, based on the present data and previous data on TEA transport, I hold that TEA/H+ exchange involves no net transfer of charge.
II. Material and Methods

a. Animals.

White Leghorn hens (*Gallus domesticus*) age 6 - 8 mos weighing 1.5 - 2 kg were used in this study. Prior to their use, birds were housed at the University farm and given free access to water and commercial mash. Unanesthetized animals were decapitated using poultry sheers (J.A. Henkels®, Germany) and exsanguinated. Immediately following exsanguination, the peritoneal cavity was opened, and the viscera were removed to expose the kidneys.

b. Harvesting of tissue and brush-border membrane preparation

Brush-border membrane vesicles (BBMV) were isolated from avian renal proximal tubules by means of Ca^{2+} precipitation and differential centrifugation following a protocol developed by Renfro and Clark (1984). The procedure for harvesting tissue and isolating BBMV has been previously described in detail (Chapter 2). The biochemical purity of the final BBMV suspension was evaluated on the basis of protein yield and the enrichment of membrane-specific enzymes as specified in Chapter 2 (Table 2.1). The mean protein yield was approximately 0.35%. The activity of the brush-border enzyme alkaline phosphatase in the final BBMV suspension was enriched approximately 18-fold over that in the initial homogenate. In contrast, the specific activities of the basolateral and mitochondrial enzymes were not enriched. Thus, the basolateral and mitochondrial membrane contamination of the final BBMV suspension was minimal.

c. Transport assay

The transport of radiolabeled substrate by avian BBMV was measured by rapid filtration technique in the manner described in detail in Chapter 2.
d. Statistical analysis

In those experiments examining sodium-dependent uptake of $[^{14}\text{C}]$methyl-D-glucose and the effects of transmembrane electrical potential difference on $[^{14}\text{C}]$TEA uptake, unless otherwise stated, uptake was measured at each prescribed time point in triplicate in at least three separate BBMV preparations (i.e., $n = 3$). Data were expressed as mean ± S.E. and analyzed by Student paired t-test. In experiments testing the effects of transmembrane electrical potential difference on the efflux of $[^{14}\text{C}]$TEA from BBMV, vesicle content of $[^{14}\text{C}]$TEA was measured at 5, 10 and 15 sec and 2 hr in triplicate in three separate BBMV preparations. These data were expressed as a percent of the initial content in the presence of a potassium gradient, and were analyzed by three-way analysis of variance (ANOVA). Probability at the 0.05 level was considered significant.

e. Chemicals

$[^{14}\text{C}]$methyl-D-glucose (286.90 mCi/mmol) was purchased from New England Nuclear, and $[^{14}\text{C}]$ tetraethylammonium bromide (56 mCi/mmol) from Wizard Labs (West Sacramento, CA). All other chemicals were obtained from standard sources and were of the purest grade.
III. Results

a. Effects of membrane PD on sodium-dependent glucose transport

Prior to testing the effect of transmembrane potential difference (PD) on TEA transport, I first determined if a PD could be established in avian BBMV. As first demonstrated in BBMV of rat intestine by Murer and Hopfer (1974) and later in BBMV of rabbit cortex by Beck and Sacktor (1975), Na+/glucose cotransport is electrogenic. In each case, sodium-dependent glucose uptake was stimulated in the presence of a negative intravesicular PD.

The PD may be manipulated by using potassium gradients and valinomycin. However, valinomycin is known to inhibit transport of organic cations including TEA in renal BBMV of the rabbit (Miyamoto et al. 1989; Rafizadeh et al. 1987; Wright 1985) and dog (Sokol et al. 1985). Alternatively, a transmembrane gradients of a permeant anions such as iodide or thiocyanate have been successfully used to create membrane diffusion potentials in BBMV isolated from rabbit intestine (Gunther et al. 1984) and renal cortex (Beck and Sacktor 1975; Berteloot 1986; Wright and Wunz 1987), as well snake renal BBMV (Dantzler et al. 1991), to demonstrate electrogenic Na+/glucose transport. Brush-border membrane vesicles isolated from rabbit intestine (Gunther et al. 1984) and renal cortex (Berteloot 1986) and snake renal cortex (Dantzler et al. 1991) were shown to be more permeable to iodide and isethionate than to either potassium or chloride. Thiocyanate is a more permeant anion than thiocyanate in rabbit renal BBMV (Beck and Sacktor 1975).

In a preliminary experiment, I indirectly tested the permeability of avian renal BBM to various anions by assessing the stimulatory effects of inwardly-directed gradients of various anions on sodium-dependent glucose uptake (Fig 3.1). Uptake of 110 μM [14C]methyl-D-glucose was measured in the presence and absence of inwardly-directed gradients of sodium salts (100 mM) of chloride, gluconate, iodide, isethionate, and thiocyanate (n = 1; triplicate measurements). Irrespective of the accompanying anion, the
Figure 3.1. The effects of various anions on sodium-dependent glucose uptake. Uptake of $[^{14}C]$methyl-D-glucose was measured in BBMV prepared in vesicle buffer containing (in mM) 300 mannitol, 3 NaN$_3$, 20 Tris-HEPES (pH 7.4). At 25°C, 10 µl BBMV were diluted with 90 µl isosmotic mannitol transport medium containing 100 µM $[^{14}C]$methyl-D-glucose, 20 mM Tris-HEPES (pH 7.4) and either 111.11 mM mannitol (○) or 111.11 mM sodium salts of various anions (see legend). (n = 1, triplicate measurements; mean ± S.E.; *P < 0.05)
inwardly-directed sodium gradient supported an overshoot in glucose uptake that peaked at 15 sec. The stimulatory effect of anions on the initial uptake of glucose (estimated at 5 sec) increased in the following order: gluconate, isethionate, iodide, thiocynate, chloride. In the same order of effectiveness, these anions induced increases in the peak of the sodium-induced overshoot. However, vesicle content of glucose at 2 hr was not markedly different for the various anions. These data indicated that the avian renal BBM was most permeable to chloride, in addition to indicating that Na⁺/glucose cotransport was electrogenic. However, these data conflict with the findings of Gunther et al. (1984) and others (Berteloot 1986) for BBMV of other species which indicated that iodide and thiocyanate were more permeant anions than chloride. Thus, I elected not to use anions to create diffusion potentials and manipulate the membrane PD. Instead, I opted to use potassium gradients and potassium-specific ionophore valinomycin.

By manipulating the transmembrane gradient of potassium (KCl) in the presence of valinomycin (20 µg/mg BBM protein) a potassium diffusion potential was generated which transiently established a PD across the vesicle membrane (Murer and Hopfer 1974; Beck and Sacktor 1975). In this manner, I determined whether changes in PD influenced the 5 sec uptake of [14C]methyl-D-glucose in the presence of an inwardly-directed sodium gradient. Theoretically, generation of PD was prevented or clamped at 0 mV in the presence of valinomycin and equal intra- and extravascular concentrations of KCl (100 mM); in this state, the intravesicular content of [14C]methyl-D-glucose was 44.88 ± 0.83 pmol·mg protein⁻¹·5 s⁻¹. When the intravesicular compartment was made transiently negative in the presence of an outwardly-directed potassium gradient (100 mM KCl in vs 10 mM KCl out) and valinomycin, uptake increased significantly (87.03 ± 5.05 pmol·mg protein⁻¹·5 s⁻¹). Conversely, rendering the intravesicular compartment positive, in the presence of an inwardly-directed potassium gradient (0 mM KCl in vs. 100 mM out), uptake significantly decreased (19.15 ± 3.19 pmol·mg protein⁻¹·5 s⁻¹). However, the
vesicular content of glucose at equilibrium (2 hr) was not markedly affected by the ionic conditions imposed to manipulate PD. These data confirmed that Na⁺/glucose cotransport was electrogenic in avian renal BBMV and verified the efficacy of the method to generate a transmembrane electrical potential difference.

b. Effects of transmembrane potential on influx of [¹⁴C]TEA

Altering the membrane PD using potassium gradients and valinomycin, I examined the effects of PD on uptake of 50 µM [¹⁴C]TEA in BBMV in the presence and absence of an proton gradient (Fig 3.2). In the absence of a proton gradient (pH_in = pH_out 7.5), generation of an intravesicular-negative or intravesicular-positive membrane PD did not support concentrative uptake of [¹⁴C]TEA (i.e. no overshoot was produced; Fig 3-2a). Valinomycin did not influence TEA uptake in the absence of a H⁺ gradient. In the absence of a potassium gradient ([KCl]out = [KCl]in), uptake of TEA at 5 and 15 sec as well as at 2hr was not significantly altered by valinomycin. Although the uptake of TEA was stimulated in the presence of an inside-negative PD at 5 sec (28.19 ± 4.34 to 43.98 ± 9.27 pmol·mg protein⁻¹·s⁻¹) and 15 sec (41.79 ± 6.83 to 70.81 ± 15.84 pmol·mg protein⁻¹·s⁻¹), these increases were not significant (P < 0.15). Uptake at 5 and 15 sec was not altered in by a inside-positive PD. The ionic conditions imposed to generate an inside-negative PD ([KCl]_in > [K⁺]out, plus valinomycin) induced an increase, though not significant, in the vesicle content of TEA at equilibrium (108.27 ± 33.95 to 71.21 ± 13.8 pmol·mg protein⁻¹·2 h⁻¹), whereas those imposed to generate an inside-positive potential ([KCl]out > [KCl]in, plus valinomycin) induced an significant increase in equilibrium content (54.76 ± 13.01 to 71.21 ± 13.80 pmol·mg protein⁻¹·2 h⁻¹).

In contrast, a transient overshoot in TEA uptake was generated in the presence of a H⁺ gradient (pH_in 6.0:pH_out 7.5), regardless of the ionic conditions and subsequent changes in membrane PD (Fig 3-2b). However, proton-driven TEA influx was
Figure 3.2 Effects of membrane potential on $^{14}$C-tetraethylammonium uptake in the presence and absence of an inwardly-directed (trans) proton gradient. Uptake of TEA was measured in BBMV prepared and prequillibrated (1 hr; 25°C) in a mannitol media, pH 7.5 (Fig 3.2a) or pH 6.0 (Fig 3.2b; 10 mM HEPES-KOH) with or without valinomycin (Val). Valinomycin-free vesicle medium contained 100 mM KCl, whereas valinomycin media contained 20 μg valinomycin/mg protein and 100 mM KCl or no KCl. At 25°C, 10 μl BBMV were diluted with 90 μl isomotic mannitol transport media containing ~50 μM $^{14}$C-TEA-Br, 5 mM HEPES (pH 7.5) and 111.11, 100 mM or 0 mM KCl. □, no valinomycin at KCl equilibrium; O, PD clamped at 0mV ($K^{+}_{in} = K^{+}_{out}$ 100 mM, plus VAL); △, inside-negative PD ($K^{+}_{in} > K^{+}_{out}$, plus Val); Δ, inside-positive PD ($K^{+}_{out} > K^{+}_{in}$, plus Val). (n = 3; mean ± S.E.; * P<0.05).
significantly inhibited in the presence of valinomycin. In the absence of a potassium gradient ([KCl]_{out} = [KCl]_{in}), valinomycin (20 μg/mg protein) reduced the initial uptake of TEA (5 sec) (77.5 ± S.D. 1.85%), as well as significantly decreasing uptake at the peak of the overshoot (15 sec) 67.2 ± S.D. 8.65%. In spite of the inhibitory effects of valinomycin, generation of a transient intravesicular-negative PD significantly stimulated TEA/H\(^+\) exchange, increasing 5 sec uptake of TEA (60.02 ± 0.644 to 97.37 ± 5.85 pmol·mg protein\(^{-1}\)·5 s\(^{-1}\)) as well as the height of the overshoot (15 sec) by approximately 50%. Proton-driven TEA uptake in the presence of an inside-negative PD was nonetheless markedly less than that observed in the absence of valinomycin. An inside-negative PD stimulated uptake, an inside-positive PD did not alter the initial uptake (5 sec) of TEA; however, uptake was significantly reduced at 15 sec (144.35 ± 2.81 to 136.44 ± 6.84 pmol·mg protein\(^{-1}\)·15 s\(^{-1}\)). In the presence of valinomycin, the 2 hr vesicle content of TEA at potassium equilibrium was 60.19 ± 3.77 pmol·mg protein\(^{-1}\)·2 h\(^{-1}\). The ionic conditions imposed to create a transient inside-negative PD significantly increased equilibrium TEA content (92.57 ± 3.72 pmol·mg protein\(^{-1}\)·2 h\(^{-1}\)); conversely, conditions imposed to generate an inside-positive PD significantly decreased TEA content (45.28 ± 0.83 pmol·mg protein\(^{-1}\)·2 h\(^{-1}\)). However, in the absence of valinomycin at potassium equilibrium, 2 hr content of TEA was 100.76 ± 14.23 pmol·mg protein\(^{-1}\)·2 h\(^{-1}\), suggesting that valinomycin and potassium had long-term additive effects on the membrane binding of isotope and possibly altered membrane permeability.

The inhibitory effects of valinomycin on TEA transport particularly in the presence of a trans H\(^+\) gradient may have been attributed to the effects of ethanol on the membrane lipid bilayer, as the valinomycin-BBMV suspension contained approximately 1% ethanol. In a pilot experiment, I measured uptake of 65 μM \(^{14}\)C]TEA in BBMV suspended in 1% ethanol in the presence of H\(^+\) gradient (pH\(_{in}\) 6.0:pH\(_{out}\) 7.5) under potassium equilibrium (100 mM KCl) (n = 1, triplicate measurements; Fig 3-3). Although countertransport of
Figure 3.3. The effects of ethanol on the time course of proton-driven [14C]tetraethylammonium transport. Uptake of TEA was measured in BBMV prepared and pre-equilibrated (1 hr, 25°C) in media containing 200 mM mannitol, 100 mM KCl, 10 mM HEPES-KOH, pH 6.0 and no ethanol (●) or 1% absolute ethanol (○). At 25°C, 10 µl of BBMV were diluted with 90 µl of isosmotic mannitol transport media containing ~65 µM [14C]TEA-Br, 100 mM KCl and 5 mM HEPES-KOH, pH 7.5. (n = 1, triplicate measurements; mean ± S.D).
TEA and protons persisted in the presence of 1% ethanol, the initial uptake (5 sec) was decreased approximately 15%. At later time points (15 - 60 sec) uptake was inhibited by approximately 15 - 20% as well. However, 2 hr equilibrium values of vesicular content of [14C]TEA were virtually identical (EtOH 46.97 ± SD 5.74 vs control: 47.00 ± SD 2.11 pmol·mg protein⁻¹·2 h⁻¹).

c. Effects of transmembrane potential on efflux of [14C]TEA

Haase et al. (1978) demonstrated that BBMV from rabbit intestine were oriented right-side-out (i.e., the extracellular face of the intact brush-border membrane is exposed to the extravesicular compartment in vitro). Thus, influx, or uptake of substrate into vesicles is functionally analogous to reabsorption across the BBM in vivo, whereas efflux of substrate from BBMV is functionally equivalent to secretion of substrate across the BBM. Therefore, to further evaluate the effects of membrane PD on TEA transport, I examined efflux of 250 μM [14C]TEA from BBMV in the presence and absence of a trans H⁺ gradient (Fig 3-4). The efflux of [14C]TEA was monitored by measuring vesicle content of [14C]TEA at 5, 10 and 15 sec and 2 hr in triplicate in three separate BBMV preparations. The effects of an inside-negative or positive membrane PD on the time course of TEA efflux were assessed by comparing the percent of [14C]TEA retained in the presence of the PD to corresponding values measured under conditions of a clamped PD (0 mV, theoretically). By similar statistical analysis, the effects of valinomycin (20 μg/mg BBM protein) on TEA efflux under conditions of potassium equilibrium (100 mm KCl) were evaluated by comparing vesicle content of [14C]TEA in the absence of valinomycin to data recorded in the presence of valinomycin.

In the absence of a pH gradient (pH_in = pH_out 7.5; Fig 3-4a), the percent TEA retained in the presence of an outside-positive PD ([KCl]_out < [KCl]_in, plus valinomycin) was significantly greater than control, an indication that TEA efflux was inhibited by
Figure 3.4. The effects of transmembrane electrical potential difference on the efflux of [14C]tetraethylammonium in the presence and absence of an outwardly-directed (trans) proton gradient. Efflux of [14C]TEA from BBMV prepared and pre-equilibrated (1 hr, 25°C) in mannitol media containing ~250 μM [14C]TEA and 10 mM HEPES-KOH, pH 7.5 with or without 20 μg valinomycin/mg protein. Valinomycin-free vesicle medium contained no KCl, whereas valinomycin media contained 100 mM KCl or no KCl. At 25°C, 10 μl BBMV were diluted with 90 μl isosmotic mannitol transport media containing 10 mM HEPES, pH 7.5 (a) or pH 6.0 (b) and 111.11, 100 mM or 0 mM KCl. ●, no valinomycin (VAL) at KCl equilibrium; ◼, PD clamped at 0mV (Kᵢ = Kᵢ out 100 mM, plus VAL); □, outside-positive PD (Kᵢ > Kᵢ out, plus VAL); ▣, outside-negative PD (Kᵢ out > Kᵢ, plus VAL). (n = 3; mean ± S.E.; * P<0.05).
an outside-positive PD. However, an outside-negative PD ([KCl]_{out} > [KCl]_{in}, plus valinomycin) did not alter TEA efflux (P < 0.59). The effects of changes in PD on TEA transport may have been due to indiscriminate changes in membrane permeability or changes in intravesicular volume. The retention of [^{14}C]TEA at 2 hr at potassium equilibrium (35.95 ± SD 6.22%) was not markedly altered in the presence of valinomycin (36.51 ± SD 5.9%). The ionic conditions imposed to generate an extravesicular-positive PD slightly increased the percent TEA retained relative to control (43.61 ± SD 4.54%), whereas ionic conditions imposed to generate an outside-negative PD decreased TEA retention (26.77 ± SD 5.24%). Valinomycin inhibited efflux; as indicated by the significant increase in the percent TEA retained in the absence of a potassium gradient ([KCl]_{out} = [KCl]_{in} 100 mM).

Efflux of [^{14}C]TEA was stimulated in the presence of H^+ gradient (pH_{in} 7.5:pH_{out} 6.0; Fig 3.4b). In the absence of potassium gradient, the retention of TEA was significantly greater in the presence of valinomycin, indicating proton-stimulated efflux was inhibited by the ionophore. However, at 2 hr, TEA retention was not markedly altered in the presence of valinomycin (34.77 ± SD 3.95% vs 35.83 ± SD 5.34%). Generation of an extravesicular-positive PD, induced a slight, but significant inhibition of efflux of TEA. The ionic conditions imposed to generate an outside-positive PD slightly increased equilibrium content (34.77 ± 3.95% to 37.38 ± 5.34%). Similarly imposing a transient outside-negative PD resulted in an increased retention of TEA; however, these changes were not statistically significant (P < 0.07). The ionic conditions imposed to generate a transient outside-negative PD markedly decreased the percent of TEA retained at equilibrium (34.77 ± 3.95% to 21.81 ± SD 3.30%).
d. Determination of the coupling ratio for [$^{14}$C]TEA/H$^+$ exchange by the static head method

Failure of a PD to support concentrative uptake of TEA suggested that TEA/H$^+$ exchange was not electrogenic (Fig 3.2a). However, transport of TEA was sensitive to changes in PD. The non-specific inhibitory effects of valinomycin complicate interpretation of these data. In the presence of valinomycin, any possible inhibitory effects of PD may have been accentuated, whereas any stimulatory effects may have attenuated. Despite the electrosensitivity of TEA transport, TEA/H$^+$ exchange may be electroneutral (i.e., involve no net transfer of charge). Stimulation of proton-driven TEA uptake by an inside-negative membrane PD (Fig 3.2b) and inhibition of proton-driven efflux of TEA by an outside-positive membrane PD (Fig 3.4b), suggested that more than one TEA molecule was exchanged for a proton. Attempts were made to determine the coupling ratio of TEA/H$^+$ exchange by the static head method (Fig 3.5, 3.6, and 3.7).

The design of these experiments is based on equilibrium thermodynamics of carrier mediated transport (Aronson 1981; Kinsella and Aronson 1982; Turner and Moran 1982). Mathematically, equilibrium thermodynamics is described by the following equation:

$$\frac{[\text{TEA}]_{\text{out}}}{[\text{TEA}]_{\text{in}}} = \left(\frac{[\text{H}^+]_{\text{out}}}{[\text{H}^+]_{\text{in}}}\right)^n \exp\left(\frac{F}{RT} \Delta \Psi (n - 1)\right)$$

Equation (2-1)

Where in and out denote intra- and extravesicular concentrations of TEA and H$^+$, $n$ represents the moles of protons transported per mole TEA, $F$ is Faraday's constant, $R$ is the gas constant, $T$ is absolute temperature in degrees Kelvin, and $\Delta \Psi$ is the transmembrane electrical potential difference. If $n$ is 1.0, equation 2-1 reduces to

$$\frac{[\text{TEA}]_{\text{out}}}{[\text{TEA}]_{\text{in}}} = \left(\frac{[\text{H}^+]_{\text{out}}}{[\text{H}^+]_{\text{in}}}\right)^n$$

Equation (2-2)
and thus, not influenced by $\Delta \Psi$. Assuming that TEA/H$^+$ exchange is obligatory, regardless of the coupling ratio, translocation of TEA is physically coupled to the oppositely directed translocation of a proton. At sufficient magnitude, an outwardly-directed H$^+$ gradient would drive influx of TEA. On the other hand, an outwardly-directed TEA gradient of appropriate proportion would drive efflux of TEA. A state in which no net flux of either TEA or H$^+$ via the exchanger is generated when these two forces are equal but opposed. Such a state can be established experimentally by manipulating the transmembrane gradients of each substrate and measuring the net flux of one or the other substrate. The coupling ratio, $n$ can be calculated from the known intra- and extravesicular concentrations of TEA and H$^+$ (pH).

In these experiments, the stoichiometry of TEA/H$^+$ exchange was examined by monitoring changes in the intravesicular content of TEA under conditions in which the in to out TEA gradient is held constant at 10:1 and the in to out H$^+$ gradient was varied from 1:1 to 1:100 by varying the extravesicular pH (i.e., the pH of the transport medium). The membrane potential was not clamped at 0 mV by valinomycin in these experiments due to the non-specific inhibitory effect of the ionophore. However, Wright and Wunz (1987) in using the static head method to determine the coupling ratio of TEA/H$^+$ exchange in rabbit renal BBMV, successfully conducted experiments under conditions of KCl equilibrium (150 mM KCl in and out) citing previous work by Ives et al. (1986) which indicated the KCl conductance in rabbit renal BBMV was sufficient to short circuit the generation of a PD. Furthermore, as demonstrated in earlier experiments, the permeability of the avian renal BBM to chloride exceeded that of iodide and thiocyanate. Thus, in these experiments I measured net flux of $[^{14}\text{C}]$TEA in the presence of equal intra- and extravesicular KCl concentrations (100 mM KCl). Brush-border membrane vesicles were prepared and pre-equilibrated (1 hr, ~25°C) in vesicle medium containing 500 $\mu$M
[14C]TEA and titrated to pH 6.0 ([H+] = 1000 nM; 10 mM HEPES-KOH) and diluted 10-fold in isosmotic transport media titrated to pH 6.0 to 8.0 (10 mM HEPES-KOH). The intravesicular content of [14C]TEA was expressed as a percent of the initial content of isotope. In the first of these experiments, BBMV were incubated in transport medium with a pH of 6.0 ([H+] = 1000 nM), 7.0 ([H+] = 100 nM), or 8.0 ([H+] = 10 nM), thereby establishing in to out H+ gradients of 1:1, 10:1, and 100:1 (Fig 3.5). Thus, a 10:1 (500 μM:50 μM) in to out concentration ratio of [14C]TEA was established, which should drive efflux of [14C]TEA. Theoretically, if the stoichiometry of TEA/H+ was 1:1, the 10:1 in to out TEA gradient should have been counterbalanced by a simultaneously imposed 10:1 in to out H+ gradient (pH_{in} 6.0:pH_{out} 7.0). Thus, under these conditions, there should have been no net flux of TEA (i.e., the content of [14C]TEA should have remained constant). However, at an extravesicular pH 7.0 a net increase in [14C]TEA was measured at 3 and 5 sec. At 10 and 15 sec the vesicle content of TEA approximated the initial content. An an extravesicular pH of 8.0, a net increase in vesicle content of [14C]TEA was measured through 5 -15 sec. Unexpectedly, in the absence of a H+ gradient (pH_{in} 6.0:pH_{out} 6.0) the vesicle content of TEA remained relatively constant through 10 sec, indicating the driving force for TEA influx apparently counterbalanced that of the 10:1 in to out TEA gradient. Based on these data, the calculated coupling ratio (H+:TEA) of TEA/H+ exchange according to equation 2-2, was ∞!

Because the main variable in the preceding experiments was the magnitude of the outwardly-directed gradient across the BBM, I questioned the effects of the pH of the stop solution used to terminate the transport reaction. In instances in which the transport medium was titrated to 6.0 or 7.0, initiation of the transport reaction transiently established a 1:1 or 10:1 in to out H+ gradients, respectively. However, the stop solution pH was 7.8 ([H+] = 16 nM). Thus, during the termination of transport, the in to out H+ gradient was
Figure 3.5. Effects of external pH on the efflux of [14C]tetraethylammonium from BBMV in the presence of an outwardly-directed [14C]tetraethylammonium gradient. Efflux of [14C]TEA was measured from BBMV prepared and pre-equilibrated (1 hr, 25°C) in mannitol media containing ~500 μM [14C]TEA-Br, 100 mM KCl, and 10 mM HEPES-KOH, pH 6.0. At 25°C, 10 μl BBMV were diluted in 90 μl isosmotic mannitol transport media containing 100 mM KCl and 10 mM HEPES at pH 8.0 ( ), 7.0 ( ), or 6.0 ( ). Transport was terminated using an isosmotic stop buffer containing 10 mM HEPES-KOH, pH 7.8. (n = 1, mean of triplicate measurements).
momentarily 63:1 ([H\(^{+}\)]\(_{\text{in}}\) = 1000 nM : [H\(^{+}\)]\(_{\text{out}}\) = 16 nM) which would exceed that of 
\[^{14}\text{C}\]TEA (11:1; [TEA]\(_{\text{in}}\) = 500 μM:[TEA]\(_{\text{out}}\) = 45.45 μM) and drive net influx of \[^{14}\text{C}\]TEA. Such "post-termination" influx could explain the unexpected net increase in the vesicle content of \[^{14}\text{C}\]TEA measured in the presence of supposed 1:1 and 10:1 in to out H\(^{+}\) gradients (Fig 3.5a). Thus, the experiment was repeated using stop solutions each titrated to pHs equaling that of the corresponding transport medium (Fig 3.6). In addition, changes in vesicular content of \[^{14}\text{C}\]TEA were measured in BBMV preloaded with 500 μM \[^{14}\text{C}\]TEA and 20 mM unlabeled TEA (pH 6.0) and incubated in transport medium pH 6.0. Presumably, 20 mM cold TEA would block the carrier-mediated pathway for radiolabeled TEA. Thus, in the event of no net flux of isotope the vesicle content of \[^{14}\text{C}\]TEA would be better approximated. Indeed, under these conditions, the TEA content was virtually unchanged. However, at pH equilibrium, there was a net efflux of TEA, whereas a 100-fold in to out H\(^{+}\) gradient (pH\(_{\text{in}}\) 6.0:pH\(_{\text{out}}\) 8.0) induced a net influx of TEA. A 10:1 in to out H\(^{+}\) gradient also generated a net influx of TEA. These data suggested that the coupling ratio was not 1:1, but according to equation 2-2 more than one proton was transported per TEA molecule.

In the final attempt to determine the stoichiometry of TEA/H\(^{+}\) exchange by the static head method, BBMV were pre-equilibrated with ~500 μM \[^{14}\text{C}\]TEA at pH 6.0 and diluted 10-fold in isosmotic transport media of pH 6.0, 6.3, 6.5, 6.7, 7.0 and 8.0 (Fig 3-7). The pH of the stop solutions were titrated accordingly. In varying the pH, I attempted to more accurately approximate the magnitude of the in to out H\(^{+}\) gradient required to counterbalance the driving force of the 10:1 in to out \[^{14}\text{C}\]TEA gradient. As described for the preceding experiment, a net efflux of TEA was induced by a 1:1 out to in H\(^{+}\) gradient (pH\(_{\text{in}}\) 6.0:pH\(_{\text{out}}\) 6.0), whereas a net influx was induced by in to out H\(^{+}\) gradients of 10:1 (pH\(_{\text{in}}\) 6.0:pH\(_{\text{out}}\) 7.0) and 100:1 (pH\(_{\text{in}}\) 6.0:pH\(_{\text{out}}\) 8.0). However, no net flux of \[^{14}\text{C}\]TEA
Figure 3.6. Effects of external pH on the efflux of $^{14}\text{C}\text{]}$tetraethylammonium from BBMV in the presence of an outwardly-directed $^{14}\text{C}\text{]}$tetraethylammonium gradient: Effects of stop solution pH. Efflux of $^{14}\text{C}\text{]}$TEA was measured from BBMV prepared and pre-equilibrated (1 hr, 25°C) in mannitol media containing 100 mM KCl, and 10 mM HEPES-KOH, pH 6.0 and ~500 μM $^{14}\text{C}\text{]}$TEA-Br with and without 20 mM unlabeled TEA-Br ( ). At 25°C, 10 μl BBMV were diluted in 90 μl isosmotic mannitol transport media containing 100 mM KCl and 10 mM HEPES at pH 8.0 (□), 7.0 (○), or 6.0 (●, ■). Transport was terminated using an isosmotic stop buffer (10 mM HEPES-KOH) at pH equal to that of the corresponding transport medium. (n = 1, mean of triplicate measurements).
Refined manipulation of external pH. Efflux of [14C]TEA was measured from BBMV prepared and pre-equilibrated (1 hr, 25°C) in mannitol media containing 100 mM KCl, and 10 mM HEPES-KOH, pH 6.0 and ~500 μM [14C]TEA-Br with and without 20 mM unlabeled TEA-Br ( ). At 25°C, 10 μl BBMV were diluted in 90 μl isosmotic mannitol transport media containing 100 mM KCl and 10 mM HEPES at pH 6.0, 6.3, 6.5, 6.7, 7.0 or 8.0. Transport was terminated using an isosmotic stop buffer (10 mM HEPES-KOH) at pH equal to that of the corresponding transport medium. (n = 1, mean of triplicate measurements).
was measured in the presence of a 5:1 outwardly directed H\(^+\) gradient (pH\(_{in}\) 6.0:pH\(_{out}\) 6.7). Thus, according to equation 2-2, the coupling ratio of TEA/H\(^+\) exchange is 1.43 (H\(^+\):TEA). If this were actually the case, a net efflux of TEA should have been measured in the presence of extravesicular pH of 6.5 and 6.3 which corresponded to in to out H\(^+\) gradients of approximately 2:1 and 3:1, respectively. On the contrary, a net influx of TEA was also measured in each case.
IV. Discussion

The present data on the effects of transmembrane PD on TEA transport in avian renal BBMV indicated that carrier-mediated transport of this organic cation for H\(^+\) was not electrogenic, for in the absence of a pH gradient, a transmembrane PD did not support concentrative uptake of TEA (Fig 3.2a). The ineffectiveness of membrane PD to alter transport was not due to an inability to establish a PD. Using potassium gradients and valinomycin, I demonstrated that Na\(^+\)/glucose cotransport was electrogenic, being stimulated by a inside-negative PD and inhibited by a inside-positive PD. Although not electrogenic, carrier-mediated TEA transport was sensitive to changes in the membrane PD. Proton-driven uptake of TEA was stimulated by inside-negative PD, and slightly inhibited when the vesicular lumen was made positive (Fig 3.2b). Efflux of TEA was slightly inhibited by both the outside-negative and outside-positive PD in the absence of a pH gradient; whereas proton-driven efflux of the organic cation was inhibited only by an outside-positive PD (Fig 3.4b).

The effects of membrane PD on organic cation transport have been examined in renal BBMV of other species. The data indicate that luminal OC/H\(^+\) exchange is electroneutral. In rabbit renal BBMV, as in avian renal BBMV, Wright and Wunz (1987) demonstrated concentrative uptake of TEA could not be driven by a PD, indicating TEA transport was not electrogenic. However, uptake was sensitive to changes in PD, being stimulated by inside-positive PD and inhibited by an inside-negative PD. These investigators confirmed TEA/H\(^+\) exchange was in fact electroneutral by determining a coupling ratio of 1:1 by the static head method. Thus, the antiporter itself produced no net transfer of charge. Studies by other investigators in renal BBMV of the rabbit (Jung et al. 1989; Rafizadeh et al. 1987), rat (Takano et al. 1984), and snake (Dantzler et. al. 1991) have also indicated that transport of TEA was electroneutral.
Data on the effects of transmembrane PD on NMN transport in renal BBMV are not consistent among the species studied. As demonstrated by Sokol et al. (1985) proton-driven efflux of NMN was insensitive to alterations in membrane PD. Additionally, by the static head method, these investigators determined the stoichiometry of NMN/H⁺ exchange was 1:1, verifying the antiport mechanism was electroneutral. Similarly, Ott et al. (1991) concluded that proton-stimulated NMN uptake in human renal BBMV was also electroneutral, having observed no significant changes in uptake in response to changes in transmembrane PD. In contrast, Wright (1985) demonstrated that transport of NMN in rabbit renal BBMV was apparently electrogenic. Concentrative uptake of NMN was induced in the presence of an inside-positive PD, whereas an inside-negative PD inhibited NMN uptake. Although, no stoichiometric experiments were conducted, stimulation of NMN uptake by an inside-positive PD, suggested the coupling ratio of NMN/H⁺ exchange was 1:2, H⁺ :NMN (Wright 1985). The available data on the effects of PD on transport of procainamide and guanidine in rabbit renal BBMV, indicate transport of each organic cation via OC/H⁺ exchange is not electrogenic (McKinney and Kunneman 1985; Miyamoto et al. 1989).

In testing the effects of membrane PD on TEA transport in avian renal BBMV, the membrane PD was altered by manipulating the transmembrane potassium gradients in the presence of the ionophore valinomycin. However, valinomycin substantially inhibited TEA transport (Fig 3.2a, b; Fig 3.4a, b). The concentration of valinomycin used in this study, 20 μg/mg vesicle protein, was relatively high. However, at concentrations as low as 5 μg/mg protein the ionophore has been shown to non-specifically inhibit transport of TEA, as well as that of the NMN and guanidine in renal BBMV of other species (Miyamoto et al. 1989; Rafizadeh et al. 1987; Sokol et al. 1985; Wright 1985). The inhibitory effects of valinomycin may be concentration-dependent. In using potassium gradients and valinomycin to alter membrane PD in dog renal BBMV, Sokol et al. (1985), found at ~1.5
μg/mg protein valinomycin was sufficient to create a potassium diffusion potential, yet had no effect on proton-driven NMN efflux. However, increasing the concentration to 10 μg/mg protein produced marked inhibition of NMN transport, yet had no effect on Na+/glucose transport. In rat renal BBMV, when valinomycin was used in a similar manner for modifying membrane PD at concentrations of approximately 9 μg/mg protein, it did not inhibit TEA transport (Takano et al. 1984). In my studies, I did not titrate to the minimal concentration of valinomycin sufficient to manipulate PD. The ethanol (1%) used to solubilize the ionophore only modestly inhibited proton-driven TEA uptake (Fig 3.3).

In addition to valinomycin, the proton-specific ionophores CCCP, nigericin, and gramicidin D are also known to inhibit NMN transport in renal BBMV in the absence of a proton gradient (Wright 1985; Sokol et al. 1988). Valinomycin increases membrane permeability to potassium. The ionophore first forms a complex with a potassium ion, surrounding it with a cage of hydrophobic residues. The valinomycin-potassium complex easily crosses the membrane, generating a flux of positive charges (Stein 1990). Based on studies of transport in dog renal BBMV, Sokol and colleagues (Sokol et al. 1988; Sokol et al. 1985) postulated that at higher concentrations intercalation of the ionophore into the lipid bilayer distorts the lipid region approximate to the OC transporter, thereby prohibiting normal carrier function. Such perturbations of the membrane bilayer may explain the significant differences in equilibrium content of [14C]TEA measured in the presence of valinomycin in avian BBMV (Fig 3.2; Fig 3.4). Although TEA efflux in avian renal BBMV was inhibited by valinomycin in the presence and absence of pH gradient (Fig 3.4), uptake of TEA was inhibited by valinomycin only in the presence of a trans proton gradient (Fig 3.2a,b). In using valinomycin and potassium to generate a PD in rabbit renal BBM, Miyamoto et al. (1989) noted guanidine transport was inhibited by valinomycin only in the presence of a pH gradient. These investigators suggested that in combination, valinomycin and potassium accelerated dissipation of the H⁺ gradient and thereby inhibited proton-
driven organic cation transport. They cited the work by Ives (1985) on rabbit renal BBMV which demonstrated that valinomycin at concentration of 6 µg/mg protein plus potassium at concentrations as low as 8 mM increased proton permeability. However, Rafizadeh et al. (1987) noted inhibition of TEA uptake by valinomycin in absence of potassium in rabbit renal BBMV. In addition, in thin lipid membranes prepared from sheep erythrocytes, Andreoli and colleagues (1967) demonstrated that the order of cation permselectivity for valinomycin was H⁺ > Rb⁺ > K⁺ > Cs⁺ > Na⁺. Thus, valinomycin alone may enhance the dissipation of H⁺ gradients in renal BBMV, and thereby inhibit OC/H⁺ transport by reducing the driving force.

In interpreting the data on the effects of membrane PD on TEA transport in avian renal BBMV, I cannot disregard the inhibitory effects of valinomycin. The nonspecific effects of valinomycin may have attenuated or augmented any stimulatory or inhibitory effects of PD on TEA transport. However, under conditions of pH equilibrium in which valinomycin had no effect, an inside-negative or inside-positive membrane PD each failed to support an overshoot in TEA uptake (Fig 3-2a), thus, suggesting that TEA/H⁺ exchange was not electrogenic. Despite inhibition by valinomycin, proton-driven concentrative uptake of TEA persisted irrespective of modifications in membrane PD (Fig 3-2b). Furthermore, despite valinomycin inhibition, an inside-negative PD stimulated proton-driven TEA uptake (Fig 3-2b). I, therefore, maintain TEA/H⁺ exchange was not electrogenic, but was nonetheless sensitive to changes in membrane PD. As discussed by Turner (1981), although carrier-mediated transport of substrates may be electroneutral, the function of the carrier may be susceptible to changes in membrane potential if the unloaded, or free carrier, possesses an electrical charge.

The sensitivity of the exchange mechanism to PD may have suggested that the coupling ratio of TEA/H⁺ exchange was not 1:1. However, earlier studies demonstrated that uptake of TEA via H⁺ exchange was described by classic Michaelis-Menten kinetics
(Fig 2.6), suggesting that TEA/H\(^{+}\) exchange probably involved the exchange of one TEA molecule for one proton. I attempted to determine the coupling ratio of TEA/H\(^{+}\) exchange by the static head method to determine whether TEA/H\(^{+}\) exchange was in fact electroneutral and did not involve net transfer of charge. According to the present data, the coupling ratio of TEA/H\(^{+}\) exchange was not 1:1 or an equivalent ratio thereof (e.g., 2:2, 3:3, etc.), but was 1.43 (H\(^{+}\):TEA\(^{+}\)). Theoretically, this suggested that an average of 1.43 protons were exchanged for one TEA molecule per transport event (Fig 3.7). However, the notion that more than one proton is transported per TEA molecule is not consistent with the observed effects of PD on TEA transport. The stimulation of proton-driven TEA uptake by an inside-negative potential and inhibition of an outside-positive PD on the efflux of TEA would indicate that more than one TEA molecule was transported per proton.

Inconsistencies in the data may be attributed to a variety of technical discrepancies in the experimental design as well as less than ideal biological parameters inherent to the BBM. As discussed earlier, the transport reaction may not have actually been terminated at the prescribed time point, thus allowing for continued influx or efflux of \([^{14}\text{C}]\text{TEA}\) depending on the in to out proton gradient (Fig. 3.5). Titrating the stop solution pH to match that of the corresponding transport medium partially rectified this discrepancy (Fig 3.6). Measurements of changes in vesicle content of isotope may have been refined by other modifications of the technical protocol. The concentration of \([^{14}\text{C}]\text{TEA}\) used was 500 \(\mu\text{M}\) which nearly approximates the Michaelis constant for TEA in avian renal BBMV (Fig 2.6). At a lower concentration of \([^{14}\text{C}]\text{TEA}\) a steady state condition may have been better approximated. In addition, the absolute amount of isotope non-specifically bound to the vesicle membrane would have also been decreased. Finally, as the affinity of the exchanger for protons is very high, at an intravesicular pH of 6, the proton concentration (~1000 nM) may have been sufficient to inhibit efflux of \([^{14}\text{C}]\text{TEA}\).
Beyond the technical factors associated with using the static head method to determine the stoichiometry of TEA/H⁺ exchange, are the biological properties of the membrane which are contradictory to the some of the theoretical bases of thermodynamic equilibrium. Thus, the occurrence of *slippage* and *leakage* should be considered. Slippage refers to movement of substrate across the membrane by the exchanger that is not physically coupled to the counterflow of a second substrate. For example, this may occur when an unloaded carrier reorient from one face of the membrane to the other without initially binding and subsequently transporting a proton. Subsequent binding of a TEA molecule to the carrier results in the translocation of the TEA molecule which is not physically coupled to countertransport of a proton. Reorientation of an unloaded carrier is incompatible with the thermodynamics of the classical models of antiport transport mechanism (Stein 1990; Holohan and Ross 1981). However, such is the confrontation of idealism and reality. Leakage refers to movement of one or both substrates of the antiporter across the membrane by means other than the carrier system being tested. This may involve transport by either an alternate carrier system or a passive diffusive pathway (Stein 1990). Leakage may be minimized if alternate transport pathways have been identified and inhibited by compounds which do not alter function of the test carrier. Kinsella and Aronson (1982) have pointed out that significant leak pathways for protons exist in rabbit renal BBMV which may complicate the effective use of the static head method.

In summary, these data indicated TEA/H⁺ exchange in avian renal BBMV is not electrogenic but is electrosensitive. Stimulation of uptake of TEA by carrier mediated H⁺ exchange by an intravesicular-negative potential and the inhibition of efflux of TEA by an extravesicular-positive potential suggest that luminal secretion of TEA may be influenced by the transmembrane potential. Using double-barrel electrodes, Laverty and Alberici (1987) measured a small lumen-negative transepithelial potential difference (~2.25 mV) in the superficial proximal tubules of the avian kidney. These data suggest that the lumen-
negative potential may facilitate transport of OCs via OC/H+ exchange. In finding that TEA uptake in rabbit renal BBMV was stimulated by an intravesicular-positive PD, Wright and Wunz (1987) suggested the lumen-positive potential measured in the later segments of the mammalian proximal tubule (Barratt et al. 1974) may serve to regulate binding of OCs to the exchanger or the turnover rate of the carrier thereby facilitating the transepithelial secretion of these compounds. The lumen-negative potential in the intact avian proximal tubule may have a similar catalytic effect on TEA/H+ exchange. As indicated by my previous studies in avian renal BBMV, despite the absence of a proton gradient across the luminal membrane of the avian proximal tubule (Laverty and Alberici 1987), the cell-directed sodium gradient may energetically drive luminal secretion of organic cations by OC/H+ exchange (Fig 2.7; Fig 2.8). In addition, studies in rabbit renal BBMV suggest that affinity of the OC/H+ exchanger for H+ is very high; the apparent $K_{H^+}$ is $\sim30$ nM (Wright and Wunz 1988). This H+ concentration corresponds to an approximate pH of 7.5, the approximate pH measured in the avian renal tubule lumen (Laverty and Alberici, 1987). Thus, the luminal concentration of protons may be sufficient to catalyze turnover of the exchanger. The present data suggest the lumen-negative potential in the intact avian renal proximal tubule could facilitate secretion of OCs. Whether TEA/H+ exchange was truly electroneutral could not be determined from the present data on the stoichiometry of TEA/H+ exchange. Although further testing is necessary to resolve this issue, the kinetic parameters suggest that the exchange mechanism likely involves 1:1 exchange of TEA and protons. Collectively, the data on TEA transport in avian renal BBMV, indicate that the energetics of OC/H+ exchange are compatible with known physiological parameters such that an OC/H+ exchange could function to secrete organic cations across luminal membrane of the avian proximal tubule.
Chapter 4

Substrate specificity of organic cation/proton exchange in avian renal brush-border membranes

I. Introduction

While there are numerous studies describing transepithelial secretion of organic cations by the renal proximal tubule some details are not well understood. Of particular interest is the transport of organic cations (OCs) across the luminal brush-border membrane (BBM), the apparent rate-limiting step in tubular secretion of these compounds (Holohan and Ross 1981). Carrier-mediated $H^+$ antiport or exchange is thought to be the primary mechanism by which OCs are transported across the BBM. Such an exchange mechanism has been demonstrated in membrane vesicles isolated from renal cortical tissue of the dog (Holohan and Ross 1981), rat (Takano et al. 1985), rabbit (Wright 1985), human (Ott et al. 1991), and snake (Dantzler et al. 1991). Secretory exchange of OCs for $H^+$ has also been demonstrated in isolated perfused proximal tubules of the rabbit and snake (Schäli et al. 1983). Although the existence of the luminal or BBM OC exchanger is well documented, the physiological role of this transporter is not completely defined. In the present study, I sought to further elucidate the role of the BBM OC exchanger in the renal transport of OCs.

The role of the "cation or hydrogen exchange mechanism" in the renal excretion of exogenous OCs was first proposed by Baer and colleagues (Baer et al. 1956). This hypothesis was based on studies in humans and dogs by these and other early investigators. In these studies, inducing an aciduria resulted in an increase in the excretion of the exogenous OCs mecamylamine, nicotine, quinine and quinidine (Andrews and Cornatzer 1944; Baer et al. 1956; Beyer et al. 1975; Gerhardt et al. 1969; Haag and Larson 1942; Haag et al. 1943; Jailer et al. 1947; Pilkington and Keyl 1963; Torretti et al. 1962).
Transport of these compounds by OC/H\(^+\) exchange has yet to be reported. However, several other exogenous OCs that have been shown to be secreted \textit{in vivo} are known to be countertransported for H\(^+\) in renal BBMV: amiloride (Wright and Wunz 1989), cimetidine (Gisclon et al. 1987; McKinney and Kunnemann 1987; Takano et al. 1985), mepiperphenidol (Rafizadeh et al. 1986), morphine (Rafizadeh et al. 1986), procainamide (Mckinney and Kunnemann 1985), and TEA (Dantzler et al. 1991; Dantzler et al. 1989; Hori et al. 1985; Inui et al. 1985; Katsura et al. 1991; Miyamoto et al. 1989; Rafizadeh et al. 1987; Rafizadeh et al. 1986; Takano et al. 1984; Wright and Wunz 1987). Other substrates for the OC exchanger expressed in renal BBMV are the nephrotoxins gentamicin and cephalexin, the neurotoxins 1-methyl-4-phenylpyridinium (MPTP) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP\(^+\)), and the calcium channel blocker verapamil (Inui et al. 1985b; Lazaruk and Wright 1990; Sokol et al. 1989; Sokol et al. 1987).

Involvement of the BBM OC/H\(^+\) exchange in the tubular secretion of the exogenous OCs has been demonstrated in isolated perfused proximal tubules. The transepithelial flux of procainamide in isolated perfused rabbit proximal tubules (McKinney 1984), as well as the cell-to-lumen flux of TEA in perfused tubules of the rabbit and snake (Dantzler and Brok 1988, Dantzler et al. 1989) were, in each case, stimulated by acidification of the tubule lumen. These findings suggest that secretory exchange of OCs for H\(^+\) at the BBM of the renal proximal tubule occurs \textit{in vivo}.

In contrast, the physiological role of the BBM OC exchanger in the transport of endogenous OCs is less certain. Current data indicate OC/H\(^+\) exchange is not the sole means by which these compounds are transported across the BBM. As discussed earlier, Wright and co-workers (1992) demonstrated that unlike the exogenous OC TEA, choline was poorly transported by the OC/H\(^+\) exchange mechanism in rabbit renal BBM; choline was instead transported by electrogenic facilitated diffusion. The role of the BBM OC exchange in secretion of NMN is also questionable. Countertransport of NMN for H\(^+\) and
OCs has been demonstrated in BBMV of the several mammalian species, including the dog (e.g., Holohan and Ross 1981; Holohan and Ross 1980), rat (e.g., Griffiths et al. 1992), man (Ott et al. 1991b), and rabbit (e.g., Wright 1985; Hsyu et al. 1988). However, as indicated by studies in isolated perfused renal proximal tubules of rabbits and snakes, in which secretion of NMN was unaltered by changes in luminal concentrations of H\(^+\) or OCs (Besseghir et al. 1990; Dantzler and Brokl 1987), the BBM OC exchanger may not be a major component in the secretion of NMN. Based on reciprocal inhibition studies in rabbit BBMV, guanidine may share a mediated H\(^+\) exchange pathway with TEA. However, measurements of the kinetic parameters of proton-driven uptake of each OC indicated that guanidine may be transported by multiple carriers within the BBM (Miyamoto et al. 1989). B\(\_\)O\(\_\)ther than choline, NMN, and guanidine few endogenous organic cations known to undergo net tubular secretion \textit{in vivo} have been tested for potential transport by the BBM OC exchanger. Dopamine and serotonin were each shown to inhibit proton-driven uptake of NMN in dog BBMV (Sokol et al. 1987), and creatinine was shown to inhibit proton-driven uptake of TEA in rabbit BBMV (Miyamoto et al. 1989). However, whether these or other endogenous OCs are transported by the BBM OC exchanger remains to be determined.

The significance of the renal BBM OC exchanger in the regulation of plasma levels of OCs, endogenous OCs in particular, is not fully understood. Having demonstrated the presence of an OC/H\(^+\) exchange mechanism in chicken renal BBMV (Chapter 2), my objective was to further elucidate the physiological relevancy of the exchanger. Using \(\text{[}^{14}\text{C}]\)tetraethylammonium (TEA) as a model substrate, I evaluated the substrate specificity of the avian OC exchanger for a battery of endogenous and exogenous OCs (Table 4.1). The criteria for classifying an OC as a transported substrate were: 1) \textit{cis} inhibition and 2) \textit{trans}
Table 4.1. The organic cations tested for substrate specificity in avian renal BBMV. Names, abbreviations or common name, and dissociation constants (pK<sub>a</sub>) are listed.

<table>
<thead>
<tr>
<th></th>
<th>pK&lt;sub&gt;a&lt;/sub&gt;</th>
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<tbody>
<tr>
<td><strong>Endogenous Compounds</strong></td>
<td></td>
</tr>
<tr>
<td>Acetylcholine (ACh)</td>
<td>&gt;13</td>
</tr>
<tr>
<td>Choline</td>
<td>13.9</td>
</tr>
<tr>
<td>Epinephrine</td>
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</tr>
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<td></td>
<td>8.66</td>
</tr>
<tr>
<td>Guanidine</td>
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</tr>
<tr>
<td>N&lt;sup&gt;1&lt;/sup&gt;-methyl nicotinamide (NMN)</td>
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</tr>
<tr>
<td>5-Hydroxytryptamine (Serotonin)</td>
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</tr>
<tr>
<td></td>
<td>8.9</td>
</tr>
<tr>
<td>Thiamine</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>4.8</td>
</tr>
<tr>
<td><strong>Exogenous Compounds</strong></td>
<td></td>
</tr>
<tr>
<td>Amiloride</td>
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</tr>
<tr>
<td>Cimetidine</td>
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</tr>
<tr>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>10.35</td>
</tr>
<tr>
<td></td>
<td>9.1</td>
</tr>
<tr>
<td>Procainamide</td>
<td>9.2</td>
</tr>
<tr>
<td>Mepiperphenidol (Darstine)</td>
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</tr>
<tr>
<td>Quinidine</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>Quinine</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>4.1</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>2.3</td>
</tr>
<tr>
<td>Tetraethylammonium (TEA)</td>
<td>13</td>
</tr>
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</table>
stimulation of carrier-mediated transport of the model substrate by the test OC (Holohan and Ross 1981; Wilbrandt and Rosenberg 1961). I, therefore, tested each OC for its ability to cis inhibit [$^{14}$C]TEA/H$^+$ exchange and trans stimulate [$^{14}$C]TEA efflux. Finally, to describe the general mechanisms by which several test OCs inhibited TEA transport, I also evaluated their effects on the kinetic parameters of [$^{14}$C]TEA/H$^+$ exchange.

Previous investigators have demonstrated quantitative relationships of the molecular structure of an OC and its inhibitory capacity (Reynard 1968; Ullrich et al. 1992; Ullrich et al. 1991) as well as its transport efficiency in vivo (Green et al. 1959; Quebbemann and Rennick 1969; Volle et al. 1960a, 1960b; Volle et al. 1959). However, the primary objective of this study was to determine which of several OCs previously demonstrated to be excreted by the chicken and other vertebrates were also transported by the BBM OC exchanger in chicken BBMV. Thus, the OCs tested were not selected on the basis of their chemical or structural properties. The endogenous OCs tested were acetylcholine (ACh), choline, epinephrine, guanidine, N$^1$-methylnicotinamide (NMN), serotonin and thiamine; each has been shown to undergo net tubular secretion in the Sperber chicken preparation (Table 1.1). These endogenous OCs as a group were weak inhibitors of [$^{14}$C]TEA transport; yet, with the exception of thiamine, each trans stimulated the efflux of [$^{14}$C]TEA. The exogenous compounds amiloride, isoproterenol, cimetidine, mepiperphenidol (Darstine), and TEA have been demonstrated to undergo net tubular secretion in the chicken (Table 1.1). Procainamide, quinidine, quinine, and ranitidine are transported by renal tubules of mammals and inhibit excretion of OCs in the chicken (Table 1-1). In chicken renal BBMV, the exogenous OCs were more effective cis inhibitors of [$^{14}$C]TEA transport than were endogenous OCs. However, with the exception of isoproterenol and unlabeled TEA, exogenous compounds poorly trans stimulated [$^{14}$C]TEA efflux. The inhibitor-induced changes in the kinetic parameters of proton-driven TEA uptake indicated the endogenous OCs serotonin and thiamine competed with TEA for
the substrate site on the exchanger, whereas inhibition of TEA transport by exogenous OCs amiloride, quinidine, and quinine involved binding of the OC to an allosteric site. These results indicated that although the affinity of the BBM OC exchanger was greater for exogenous OCs than for endogenous OCs, the transport capacity of the exchanger was greater for endogenous OCs.
II. Materials and Methods

a. Animals

White Leghorn roosters (*Gallus domesticus*) age 6-8 mo weighing 1.5 - 2 kg were used in this study. Prior to their use, animals were housed at the University farm and given free access to commercial mash and water. Unanesthetized animals were decapitated using poultry shears (J.A. Henckels®, Germany) and exsanguinated. The body cavity was immediately opened and the viscera were removed to expose the kidneys.

b. Harvesting of tissue and brush-border membrane preparation

Brush-border membrane vesicles (BBMV) were isolated from avian renal proximal tubules by means of Ca\(^{2+}\) precipitation and differential centrifugation following a protocol developed by Renfro and Clark (1984). The procedure for harvesting tissue and isolating BBMV has been previously described in detail (Chapter 2).

c. Analytical methods

Protein concentration was measured using a Bio Rad® protein assay and Bio Rad® protein I-bovine plasma γ-globulin as a standard. The biochemical purity of the final BBMV suspension was evaluated on the basis of enzyme enrichment (Table 2.1). The specific activity of the brush-border enzyme alkaline phosphatase in the BBMV suspension was enriched 18-fold compared to that in the initial homogenate. In contrast, the specific activities of the basolateral and mitochondrial enzymes, Na\(^{+}\)-K\(^{+}\), ATPase and succinate dehydrogenase were not enriched over those in the initial homogenate. Thus, basolateral and mitochondrial membrane contamination within the final BBMV suspension was minimal.
d. Transport studies

Transport of radiolabeled substrate was assayed at room temperature using a rapid filtration technique (Hopfer et al., '73; Wright et al '83) as described in Chapter 2. Uptake of radiolabeled substrate was expressed in picomoles $[^{14}\text{C}]$-labeled substrate per milligram vesicle protein. At each prescribed time point, unless otherwise stated, uptake was measured in triplicate in at least three separate vesicle preparations. The efflux of $[^{14}\text{C}]$TEA from BBMV was also measured by the rapid filtration technique. However, for those experiments in which efflux of $[^{14}\text{C}]$TEA was monitored, the vesicle content of $[^{14}\text{C}]$TEA was expressed as a percent of the total isotope contained within BBMV prior to the initiation of the transport reaction. The initial isotope content was measured by simultaneously vortexing and diluting 10 $\mu$l BBMV with a mixture of 1 ml of cold stop solution and 90 $\mu$l transport medium. One milliliter of this mixture was immediately collected on a prewetted filter under vacuum. The filter was washed with additional cold stop solution (4ml). As previously described, the activity of $[^{14}\text{C}]$TEA retained within the vesicles was measured by a liquid scintillation system.

As described in Chapter 2, it was verified that $[^{14}\text{C}]$TEA was transported into an osmotically active space, and the apparent fraction of membrane-bound isotope was estimated to be approximately 25% of the total measured $[^{14}\text{C}]$TEA.

e. Chemicals.

$[^{14}\text{C}]$methyl-D-glucose (286.90 mCi/mmol) was purchased from New England Nuclear. $[^{14}\text{C}]$tetraethylammonium bromide (56 mCi/mmol) was purchased from Wizard Labs (West Sacramento, CA). Mepiperphenidol (Darstine) was contributed by Merck Sharp and Dome Laboratories (Rahway, NJ.). All other chemicals were obtained from standard sources and were of the highest purity.
g. Statistical analysis

In those experiments in which Na⁺-dependent uptake of [14C]methyl-D-glucose and the proton-driven uptake of [14C]TEA were measured, the amount of substrate that accumulated within BBMV was expressed as pmol [14C]-labeled substrate per mg vesicle protein. These data were expressed as mean ± S.E. and analyzed by Student's t-test. In experiments in which cis inhibition or trans stimulation of [14C]TEA transport by organic cations were examined, data were analyzed using an analysis of variance. In those experiments in which cis inhibition of TEA uptake by organic cations was examined, uptake was expressed as the absolute value of vesicle content of isotope (pmol [14C]TEA/mg vesicle protein) and data were analyzed by analysis of variance. In examining the trans effects of organic cations on [14C]TEA efflux, I found that the absolute values of initial vesicular content of [14C]TEA measured in the presence of trans gradients of each test organic cation were consistently greater than control values (Table 4.2). Therefore, experimental and control values of vesicular content of [14C]TEA were converted and expressed as a percent of the initial content of [14C]TEA as measured in the presence and absence of the organic cation. In this form, the data were analyzed by ANOVA. Probability of the 0.05 level was considered significant.
Table 4.2. Variations in the measured initial content of [¹⁴C]tetraethylammonium in BBMV in the presence 1 mM *trans* concentrations of endogenous and exogenous organic cations. Vesicle content was measured in triplicate in each of the three experiments testing the *trans* stimulatory effects of organic cations on TEA transport. The data are expressed as the mean initial content measured in the absence (i.e., control) and presence of each organic cation for each BBMV preparation (i.e., I, II, or III). See Material and Methods for method of measurement and Figure 4.2 for experimental design.

<table>
<thead>
<tr>
<th>Endogenous Organic Cations</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>102.58</td>
<td>110.10</td>
<td>133.47</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>96.91</td>
<td>129.69</td>
<td>111.74</td>
</tr>
<tr>
<td>Control</td>
<td>143.12</td>
<td>102.58</td>
<td>110.10</td>
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<tr>
<td>Choline</td>
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<td>107.72</td>
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<tr>
<td>Control</td>
<td>106.69</td>
<td>102.58</td>
<td>110.10</td>
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<tr>
<td>Epinephrine</td>
<td>117.07</td>
<td>113.38</td>
<td>128.31</td>
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<tr>
<td>Control</td>
<td>143.12</td>
<td>102.58</td>
<td>110.10</td>
</tr>
<tr>
<td>NMN</td>
<td>140.25</td>
<td>103.94</td>
<td>125.17</td>
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<tr>
<td>Control</td>
<td>120.00</td>
<td>102.58</td>
<td>110.10</td>
</tr>
<tr>
<td>Serotonin</td>
<td>124.23</td>
<td>120.26</td>
<td>134.92</td>
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<tr>
<td>Control</td>
<td>106.69</td>
<td>102.58</td>
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<tr>
<td>Thiamine</td>
<td>108.78</td>
<td>125.81</td>
<td>137.82</td>
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<tr>
<td>Control</td>
<td>106.69</td>
<td>102.58</td>
<td>89.81</td>
</tr>
<tr>
<td>Guanidine</td>
<td>100.58</td>
<td>119.65</td>
<td>101.36</td>
</tr>
</tbody>
</table>
Table 4.2 con't. Variations in the measured initial content of $[^{14}\text{C}]$tetraethylammonium in avian renal BBMV in the presence 1 mM *trans* concentrations of endogenous and exogenous organic cations.

<table>
<thead>
<tr>
<th>Exogenous Organic Cations</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>102.58</td>
<td>110.10</td>
<td>133.47</td>
</tr>
<tr>
<td>Amiloride</td>
<td>139.09</td>
<td>163.66</td>
<td>142.47</td>
</tr>
<tr>
<td>Control</td>
<td>120.00</td>
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<td>89.81</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>152.53</td>
<td>116.05</td>
<td>138.10</td>
</tr>
<tr>
<td>Control</td>
<td>143.12</td>
<td>104.16</td>
<td>89.81</td>
</tr>
<tr>
<td>Mepiperphenidol</td>
<td>148.86</td>
<td>98.86</td>
<td>103.30</td>
</tr>
<tr>
<td>Control</td>
<td>143.12</td>
<td>104.16</td>
<td>89.81</td>
</tr>
<tr>
<td>Quinidine</td>
<td>173.81</td>
<td>152.64</td>
<td>145.68</td>
</tr>
<tr>
<td>Control</td>
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<td>104.16</td>
<td>89.81</td>
</tr>
<tr>
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<td>186.14</td>
<td>127.92</td>
<td>130.08</td>
</tr>
<tr>
<td>Control</td>
<td>120.00</td>
<td>104.16</td>
<td>89.81</td>
</tr>
<tr>
<td>Procainamide</td>
<td>129.51</td>
<td>84.77</td>
<td>102.50</td>
</tr>
<tr>
<td>Control</td>
<td>120.00</td>
<td>104.16</td>
<td>89.81</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>132.34</td>
<td>108.75</td>
<td>96.55</td>
</tr>
<tr>
<td>Control</td>
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<td>110.10</td>
<td>133.47</td>
</tr>
<tr>
<td>Isoproteronol</td>
<td>135.99</td>
<td>117.60</td>
<td>124.50</td>
</tr>
<tr>
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<td>110.10</td>
<td>104.16</td>
<td>89.81</td>
</tr>
<tr>
<td>TEA</td>
<td>137.15</td>
<td>98.76</td>
<td>93.96</td>
</tr>
</tbody>
</table>
III. Results

a. *Cis* inhibition of $[^{14}\text{C}]\text{TEA}/H^+$ exchange by organic cations

Theoretically, if $[^{14}\text{C}]\text{TEA}$ and a test OC were transported by a common carrier, the flux of the model substrate $[^{14}\text{C}]\text{TEA}$ should decrease in the presence of the test OC when the transmembrane gradients of both compounds are directed toward the same vesicular compartment. "Cis inhibition" refers to the impedence of the transport of one substrate by a second substrate when the compounds are at the same face of the vesicle membrane. Thus, I evaluated the specificity of the OC/H$^+$ exchanger by testing the abilities of several endogenous and exogenous organic cations to *cis* inhibit TEA/H$^+$ exchange. As described earlier, at concentrations of 1 mM the exogenous OCs cimetidine, mepiperphenidol, quinidine, quinine, and ranitidine each decreased uptake of $[^{14}\text{C}]\text{TEA}$ by approximately 90% or more. Thus, to refine the assessment of the affinity of the OC exchanger, I examined the inhibitory effects of these and other OCs at concentrations of 100 μM. Five second uptake of 65 μM $[^{14}\text{C}]\text{TEA}$ was measured in the presence of a H$^+$ gradient (pH$_{\text{in}}$ 6.0: pH$_{\text{out}}$ 7.5) and 100 μM of each organic cation (Fig 4.1a, b).

The endogenous cations acetylcholine (ACh), choline, and guanidine failed to significantly decrease TEA uptake (p < 0.08; Fig 4.1a). However, thiamine and serotonin moderately inhibited TEA transport, decreasing uptake by approximately 60% and 45%, respectively. By comparison, epinephrine and NMN were less effective; each inhibited uptake by approximately 20%. As described earlier, 1 mM choline modestly inhibited uptake of $[^{14}\text{C}]\text{TEA}$ (table from chapter 1); however, the inhibitory effectiveness of ACh or guanidine at concentrations greater than 100 μM was not examined. In contrast, the exogenous cations were relatively potent *cis* inhibitors of TEA/H$^+$ exchange (Fig 4.1b). For example, amiloride and quinidine each inhibited uptake by at least 90%. Raniditine, quinine, and cimetidine were nearly as effective, decreasing uptake by approximately 85%.
Figure 4.1. *Cis* effects of organic cations on proton-driven \([^{14}\text{C}]\)tetraethylammonium uptake. Vesicles were pre-equilibrated (1 hr, 25°C) in medium (pH 6.0). At 25°C, 5 sec uptake of \([^{14}\text{C}]\)TEA was measured by diluting 10 µl BBMV with 90 µl isosmotic transport medium (pH 7.5) containing ~65 µM \([^{14}\text{C}]\)TEA-Br and 111.11 µM of each test OC. Vesicle and transport media contained (in mM) 100 KCl, 200 mannitol and 10 HEPES-KOH (pH 7.5). Each organic cation was tested in three separate BBMV preparations (n = 3). Uptake is expressed as a percent of uptake in the absence of inhibitor (mean ± S.D); however, absolute values of uptake were statistically analyzed by ANOVA. (* p < 0.05).
However, the inhibitory capacities of some exogenous cations were comparable to those of endogenous cations. For instance, decreases in TEA uptake measured in the presence of procainamide (\(=55\%\)) and mepiperphenidol (Darstine, \(=65\%\)) were comparable to that measured in the presence of thiamine (\(=60\%\)). Unlabeled TEA inhibited uptake by 20\% as did the endogenous cations NMN and epinephrine. Isoproterenol, a slightly more effective inhibitor than TEA, decreased uptake of \(^{14}\text{C}\)TEA by approximately 30\%. Excluding the endogenous organic cations ACh, choline, and guanidine, the \textit{cis} inhibitory potency of those organic cations tested at 100 \(\mu\text{M}\) decreased in the order: quinidine > amiloride > quinine > cimetidine \(\approx\) ranitidine > Darstine > thiamine > procainamide > serotonin > isoproterenol > TEA = epinephrine = NMN. Using the inhibitory potency as an index of the OC/H\(^+\) exchanger's affinity, these results suggested the overall affinity of the OC exchanger is greater for exogenous organic cations than for endogenous organic cations.

\textbf{b. \textit{Trans} stimulation of \(^{14}\text{C}\)TEA efflux by organic cations}

The ability of organic cations such as quinidine and amiloride to profoundly \textit{cis} inhibit TEA/H\(^+\) exchange suggested that the OC exchanger has a high affinity for these exogenous organic cations. However, \textit{cis} inhibition of TEA/H\(^+\) exchange by an organic cation is not a necessary indication the organic cation itself is a transported substrate of the OC exchanger. Transport of ions by an exchange mechanism involves physical coupling of the flux of one substrate to the counter flux of a second. Thus, if a test compound was transported by the OC exchanger, the carrier-mediated flux of \(^{14}\text{C}\)TEA should be stimulated in the presence of a counter-directed transmembrane gradient of the test compound. Augmentation of mediated transport of a model substrate by second substrate present in the vesicular compartment into which the first is transported is referred to as "\textit{trans} stimulation". Therefore, I examined the capacity of the OC exchanger to transport cations other than TEA by testing \textit{trans} stimulation of TEA transport. At pH equilibrium
(pH 7.5), the efflux of 150 μM [14C]TEA was measured at 5 and 15 sec in the absence and presence of 1 mM trans concentrations of endogenous (Fig 4.2a) and exogenous (Fig 4.2b) organic cations. As previously mentioned in Material and Methods, the experimental and control values of the vesicular content of [14C]TEA were expressed as percents of the initial vesicular content of isotope as measured in the presence or absence of an organic cation. As [14C]TEA effluxed from BBMV, the percent of the initial [14C]TEA retained within BBMV would decrease. Efflux was considered to be trans stimulated by an organic cation if the percent of [14C]TEA retained in BBMV was significantly (p < 0.05) less than control. Trans stimulatory effects of each organic cation were statistically analyzed by comparing the percent of [14C]TEA retained at 5 sec and 15 sec in the presence of the test compound to the corresponding set of 5 sec and 15 sec control values by an analysis of variance.

As a group, the endogenous organic cations trans stimulated the efflux of [14C]TEA to a greater extent than did exogenous organic cations (Fig 4.2). However, thiamine, the most potent endogenous cis inhibitor of TEA uptake, failed to significantly (p < 0.07) trans stimulate TEA efflux; the percent of [14C]TEA retained in the BBMV in the absence and presence of a trans thiamine gradient were not significantly different. Conversely, ACh, choline, and guanidine, compounds that did not cis inhibit TEA uptake, each significantly trans stimulated the efflux of TEA. Choline markedly stimulated TEA efflux at 5 and 15 sec by an average of 17% and 10% respectively. Although ACh and guanidine each trans stimulated TEA efflux at 5 sec by approximately 10%, stimulation by these cations was less marked at 15 sec. Epinephrine, NMN, and serotonin were the only endogenous organic cations to both cis inhibit and trans stimulate TEA transport; each organic cation trans stimulated TEA efflux by approximately 15% at 5 sec and by approximately 10% at 15 sec.
Figure 4.2. *Trans* effects of organic cations on $^{14}$C]tetraethylammonium efflux from BBMV. Efflux of $^{14}$C]TEA from BBMV was measured at 5 and 15 sec in the presence and absence of *trans* gradients of endogenous (a) and exogenous (b) OCs. Following pre-equilibration (1h, 25°C) in vesicle medium (pH 7.5) containing ~150 µM $^{14}$C]TEA, 10 µl BBMV were diluted (10X) in transport medium (pH 7.5) containing 1.11 mM of an OC (●) or 1.11 mM mannitol (■). Vesicle and transport media contained (in mM) 100 KCl, 200 mannitol and 10 HEPES-KOH (pH 7.5). Retention of $^{14}$C]TEA within BBMV at 5 and 15 sec was expressed as a percent of the initial $^{14}$C]TEA content. *Trans* effects of each OC were tested in three separate BBMV preparations (n = 3), and efflux was measured in triplicate in each preparation. (*, p < 0.05)
Figure 4.2 con't. *Trans* effects of organic cations on [14C]tetraethylammonium efflux from avian renal BBMV.
In contrast, the exogenous organic cations were, generally, ineffective \textit{trans} stimulators of TEA efflux (Fig 4.2b). Unlabeled TEA and isoproterenol, modest \textit{cis} inhibitors of [\textsuperscript{14}C]TEA uptake, were the only exogenous compounds to significantly \textit{trans} stimulate [\textsuperscript{14}C]TEA efflux. Unlabeled TEA stimulated 5 sec efflux of [\textsuperscript{14}C]TEA an average of 22\% and 15 sec efflux an average of 10\%. Isoproterenol \textit{trans} stimulated 5 sec and 15 sec efflux by approximately 13\% and 7\%, respectively. In the presence of the remaining exogenous organic cations, efflux of [\textsuperscript{14}C]TEA was \textit{trans} inhibited, rather than \textit{trans} stimulated; the percent of [\textsuperscript{14}C]TEA retained within the vesicles at 5 and 15 sec was significantly greater than in their absence. In the presence of \textit{trans} gradients of amiloride, mepiperphenidol and procainamide, efflux of [\textsuperscript{14}C]TEA over 15 sec was impeded such that the percent of isotope retained within BBMV exceeded control values by as much as 50\%. In the presence of \textit{trans} gradients of cimetidine, quinidine, quinine, and raniditine the percent of [\textsuperscript{14}C]TEA retained within the BBMV was greater than control, and moreover, exceeded the initial content of isotope by as much as 18\% in some instances. Thus, although certain exogenous OCs (excluding TEA and isoproterenol) were potent \textit{cis} inhibitors of [\textsuperscript{14}C]TEA, these compounds poorly \textit{trans} stimulated transport of [\textsuperscript{14}C]TEA. The observed \textit{trans} inhibition of efflux of [\textsuperscript{14}C]TEA by these cations suggested they were either not translocated by the OC exchanger or are translocated at very low rates. Collectively, these results suggested that endogenous organic cations were more effective \textit{trans} stimulators of TEA transport than were exogenous organic cations.

A preliminary experiment was conducted to determine whether the observed \textit{trans} stimulation and inhibition of TEA efflux by OCs was the result of substrate-carrier interaction rather than indiscriminate changes in vesicle volume, membrane permeability, or binding of [\textsuperscript{14}C]TEA to the vesicle membrane. Brush-border membrane vesicles of a single preparation were pre-equilibrated with 190 \mu M [\textsuperscript{14}C]TEA and the vesicular content of [\textsuperscript{14}C]TEA was measured prior to initiation of transport and following a 2hr incubation.
in the absence and presence of *trans* 1 mM concentrations of each organic cation (triplicate measurements, n = 1; Table 4.3). All endogenous organic cations (including thiamine) as well as unlabeled TEA and isoproterenol increased the initial isotope content by an average of 9%. By comparison, in the presence of the remaining exogenous cations, which both profoundly *cis* and *trans* inhibited TEA transport, the initial content of [14C]TEA was markedly greater than control values. Ranitidine was the exception, increasing [14C]TEA content by only 6%. However, mepiperphenidol and procainamide, moderate inhibitors of TEA transport, increased initial content by approximately 15%. Whereas, in the presence of *trans* gradients of the more effective *cis* inhibitors amiloride, cimetidine and quinine, the corresponding initial vesicular content of [14C]TEA exceeded control values by 23%, 31%, and 40%. In the presence of quinidine, the most potent inhibitor of both uptake and efflux of TEA, the initial vesicular content of isotope was 55% greater than control. Similar increases were observed in experiments examining the *trans* effects of organic cations on the 5 and 15 sec efflux of [14C]TEA (Table 4.2). Overall, the initial vesicular content of [14C]TEA was greater in the presence of *trans* gradients of exogenous organic cations than endogenous cations. More specifically, the initial content of [14C]TEA were greatest in the presence of organic cations which most effectively *cis* and *trans* inhibited TEA transport. Conversely, the initial isotope content was only slightly greater than control when measured in the presence of those organic cations which moderately *cis* inhibited and *trans* stimulated TEA transport.

Differences in values of [14C]TEA retained within BBMV following 2 hr incubation in the presence of exogenous and endogenous cations were less pronounced (Table 4.3). With few exceptions, absolute values of equilibrium [14C]TEA content in the presence of organic cations were no more than 10% greater than control. In the presence of unlabeled TEA and ACh, 2 hr [14C]TEA content was approximately 10% less than control, and in the presence of serotonin, content was 17% greater than control. To compare the
Table 4.3. *Trans* effects of organic cations on the initial and two hour content of [\(^{14}\)C]tetraethylammonium.

<table>
<thead>
<tr>
<th>Content [(^{14})C]TEA (pmol/mg protein)</th>
<th>% retained 2hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>110.37</td>
</tr>
<tr>
<td><strong>Endogenous organic cations</strong></td>
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</tr>
<tr>
<td>Serotonin</td>
<td>119.98</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>122.97</td>
</tr>
<tr>
<td>Thiamine</td>
<td>117.99</td>
</tr>
<tr>
<td>Guanidine</td>
<td>118.24</td>
</tr>
<tr>
<td>Ach</td>
<td>123.16</td>
</tr>
<tr>
<td>Choline</td>
<td>120.60</td>
</tr>
<tr>
<td>NMN</td>
<td>119.30</td>
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<tr>
<td><strong>Exogenous organic cations</strong></td>
<td></td>
</tr>
<tr>
<td>TEA</td>
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</tr>
<tr>
<td>Procainamide</td>
<td>130.67</td>
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<tr>
<td>Isoproterenol</td>
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</tr>
<tr>
<td>Quinidine</td>
<td>170.74</td>
</tr>
<tr>
<td>Darstine</td>
<td>127.20</td>
</tr>
<tr>
<td>Quinine</td>
<td>154.26</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>144.35</td>
</tr>
</tbody>
</table>

Vesicles were preequilibrated with ~190 μM [\(^{14}\)C]TEA-Br (1 hr, 25°C), and diluted 10X in transport medium containing 1 mM of each organic cation. Vesicle content was measured in triplicate prior to initiation of transport and following two hour incubation in BBMV of a single vesicle preparation (n = 1). See Figure 4.2 for experimental design.
trans effects of organic cations on 2 hr efflux of [\textsuperscript{14}C]TEA, each absolute value of equilibrium [\textsuperscript{14}C]TEA content was converted to a percent of the initial vesicular [\textsuperscript{14}C]TEA content measured in the presence of the organic cation. In the control state, the mean percent of [\textsuperscript{14}C]TEA retained at equilibrium was 33.3% (± S.D. 0.43%). The percent of [\textsuperscript{14}C]TEA retained in the presence of organic cations, excluding quinidine, varied (25.9% - 35.2%); however, the mean percent was 31.1% (± S.D. 2.7%). The absolute values of equilibrium content of isotope were greatest when measured in the presence of quinine and quinidine; these organic cations increased isotope content by 41% and 217%, respectively. The greatest percent retention of [\textsuperscript{14}C]TEA, nearly 47%, was also observed in the presence of quinidine.

c. Effects of organic cations on kinetic parameters of TEA/H\textsuperscript{+} exchange

The ability of an organic cation to cis inhibit and trans stimulate transport of TEA may be a direct consequence of the substrate-carrier binding interaction. The inability of potent cis inhibitors of TEA/H\textsuperscript{+} exchange (e.g., amiloride, mepiperphenidol, ranitidine) to trans stimulate TEA transport suggests these compounds may not necessarily compete with TEA for the substrate site on the exchanger. To describe the general mode of inhibition by endogenous and exogenous cations, I evaluated changes in the kinetic parameters of the initial rates of proton-driven TEA uptake induced by amiloride (15 μM), procainamide (75 μM), quinidine (5 μM), serotonin (150 μM), and thiamine (100 μM). Using a "tracer-displacement" protocol (Malo and Berteloot '91), I measured 3 sec uptake of 100 μM [\textsuperscript{14}C]TEA in the presence of a H\textsuperscript{+} gradient (pH\textsubscript{in} 6.0:pH\textsubscript{out} 7.5) and increasing concentrations of unlabeled TEA (0 - 10 mM) in the presence and absence of each organic cation. (Earlier, I determined that uptake of 100 μM [\textsuperscript{14}C]TEA was linear through 3 sec (Fig 2.5).) Each OC was tested in two separate BBMV preparations.
The relationship of the rate of $^{14}$C-TEA uptake ($J$) and concentration of unlabeled TEA ([TEA]) is described by the equation:

$$J = \frac{J_{\text{max}} \cdot [^{14}\text{C-TEA}]}{K_t + [^{14}\text{C-TEA}] + [\text{TEA}]} + C,$$

Equation 4-1

where $J_{\text{max}}$ is the maximal rate of carrier-mediated $^{14}$C-TEA uptake; $K_t$ is the Michaelis constant; $[^{14}\text{C-TEA}]$, the concentration of $^{14}$C-TEA; and $C$ represents the non-saturable transport or membrane binding of $^{14}$C-TEA. The principles of this method are explained in greater detail in chapter I. Briefly, this method is based on the assumption that unlabeled (cold) TEA competitively inhibits binding of $^{14}$C-TEA to the substrate site on the exchanger, such that the uptake of $^{14}$C-TEA will decrease in the presence of increasing concentrations of unlabeled TEA. Indeed, in each experiment, the $^{14}$C-TEA uptake decreased with increasing concentrations of cold TEA in the presence and absence of inhibitor. The control and experimental values of $J_{\text{max}}$ and $K_t$ for $^{14}$C-TEA calculated for each inhibitor are listed in Table 4.4. Following the general principles of enzyme inhibition outlined by Segal (1976), the inhibitor-induced changes in $J_{\text{max}}$ and $K_t$ for $^{14}$C-TEA were evaluated to describe the interaction of each organic cation with the OC/H$^+$ exchanger.

In these kinetic experiments, the mean $K_t$ and $J_{\text{max}}$ for TEA in the absence of inhibitor was 323.08 μM ± S.E. 40.20 μM and 10.58 nmol·mg protein$^{-1}$·sec$^{-1}$ ± S.E. 1.01 nmol·mg protein$^{-1}$·sec$^{-1}$, respectively ($n = 7$, Table 4.4). The endogenous cations serotonin and thiamine induced increases in the apparent $K_t$ ($K_{\text{tap}}$) for TEA, but had no effect on the $J_{\text{max}}$, thereby probably acted as competitive inhibitors of TEA/H$^+$ exchange. In the presence of 150 μM serotonin, the $K_{\text{tap}}$ for TEA increased (e.g., 260 μM vs. 545 μM); however, $J_{\text{max}}$ remained relatively constant (e.g., 8.30 nmol·mg protein$^{-1}$·sec$^{-1}$ vs.
Table 4.4. Inhibitor-induced changes in the kinetic parameters of proton-driven $[^{14}\text{C}]$TEA uptake. See Figure 4.3 for description of experiment.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Trial</th>
<th>$K_t^a$ (µM)</th>
<th>$K_{tapp}^b$ (µM)</th>
<th>$J_{max}^c$ (nmol·mg$^{-1}$·min$^{-1}$)</th>
<th>$J_{max}^d$ (nmol·mg$^{-1}$·min$^{-1}$)</th>
<th>$K_i^e$ (µM)</th>
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</thead>
<tbody>
<tr>
<td>Amiloride 15 µM</td>
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<td>212.92</td>
<td>320.81</td>
<td>11.83</td>
<td>4.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>285.17</td>
<td>221.93</td>
<td>11.55</td>
<td>4.04</td>
<td></td>
</tr>
<tr>
<td>Procainamide 75 µM</td>
<td>1</td>
<td>212.92</td>
<td>264.67</td>
<td>11.83</td>
<td>3.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>285.17</td>
<td>678.95</td>
<td>11.55</td>
<td>11.98</td>
<td></td>
</tr>
<tr>
<td>Quinidine 5 µM</td>
<td>1</td>
<td>356.70</td>
<td>192.00</td>
<td>14.96</td>
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<tr>
<td></td>
<td>2</td>
<td>230.90</td>
<td>134.30</td>
<td>7.37</td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>Serotonin 150 µM</td>
<td>1</td>
<td>260.23</td>
<td>545.29</td>
<td>8.30</td>
<td>8.13</td>
<td>136.93</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>331.90</td>
<td>7.37</td>
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<td>1377.13</td>
<td>8.45</td>
<td>15.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>504.75</td>
<td>973.28</td>
<td>11.59</td>
<td>11.75</td>
<td>107.73</td>
</tr>
</tbody>
</table>

a: $K_t$, Michaelis constant, no inhibitor  
b: $K_{tapp}$, apparent Michaelis constant  
c: $J_{max}$, maximal rate of uptake control  
d: $J_{max}$, maximal rate of uptake in the presence of inhibitor  
e: $K_i$, inhibitory constant.
8.13 nmol·mg protein⁻¹·sec⁻¹; Table 4.4). The inhibitor constant \( (K_i) \) for serotonin was calculated by the following equation (Segal 1976):

\[
\frac{J}{J_{\text{max}}} = \frac{[^{14}\text{C}]\text{TEA}}{K_t} \frac{1}{1 + \frac{[^{14}\text{C}]\text{TEA}}{K_t} + \frac{[I]}{K_t}},
\]

where \([I]\) is the concentration of the inhibitor in \( \mu\text{M} \). The average calculated inhibitor constant \( (K_i) \) for serotonin was approximately 245 \( \mu\text{M} \). These data indicated serotonin was a competitive inhibitor of TEA/H⁺ exchange, and as such, serotonin cis inhibited TEA uptake by competing for the substrate site on the exchanger. These results were in agreement with the observation of significant trans stimulation of TEA efflux by serotonin which indicated serotonin was itself transported by the OC exchanger.

The changes in kinetic parameters of TEA transport induced by 100 \( \mu\text{M} \) thiamine indicated this endogenous OC acted as a competitive inhibitor of TEA/H⁺ exchange (Table 4.4). In the first of two experimental trials, thiamine induced an increase in the \( K_{tapp} \) for TEA (505 \( \mu\text{M} \) vs 973 \( \mu\text{M} \)) without altering \( J_{\text{max}} \) (11.59 nmol·mg protein⁻¹·sec⁻¹ vs. 11.75 nmol·mg protein⁻¹·sec⁻¹). Thus, thiamine probably acted as a competitive inhibitor of TEA/H⁺ exchange, having an approximate \( K_i \) of 108 \( \mu\text{M} \). In the second trial, thiamine also induced an increase in the \( K_{tapp} \) for TEA (411 \( \mu\text{M} \) vs. 1377 \( \mu\text{M} \)); however, the \( J_{\text{max}} \) for TEA also increased (8.45 nmol·mg protein⁻¹·sec⁻¹ vs. 15.51 nmol·mg protein⁻¹·sec⁻¹). The increase in the \( K_{tapp} \) for TEA is a characteristic effect of a competitive inhibitor. However, the increase in \( J_{\text{max}} \) for TEA was not anticipated as 100 \( \mu\text{M} \) thiamine decreased the 5 sec uptake of TEA by approximately 60% (Fig 4.1a). Generally, these data indicated serotonin and thiamine were competitive inhibitors of OC/H⁺ exchange. The \( K_i \)'s for serotonin and thiamine are indicative of the OC exchanger's affinity for these organic
cations (Segal 1976); the lower the $K_i$, the greater the affinity of the exchanger for the organic cation. The $K_i$ for thiamine was less than that for serotonin (~110 μM vs. ~245 μM). Thus, as also indicated by the thiamine's greater capacity to cis inhibit $[^{14}C]$TEA/H$^+$ exchange (Fig 4.1a), the OC exchanger's affinity for thiamine is greater than for serotonin.

In contrast, changes in the kinetic parameters of TEA/H$^+$ exchange induced by the exogenous OCs amiloride, procainamide, and quinidine were not characteristic of competitive inhibitors (Table 4.4). In the presence of 15 μM amiloride, the $J_{\text{max}}$ for TEA was decreased (e.g., 12 nmol·mg protein$^{-1}$·sec$^{-1}$ vs. 4 nmol·mg protein$^{-1}$·sec$^{-1}$), whereas $K_{tapp}$ for TEA remained relatively constant (e.g., 285 μM vs. 223 μM). Such changes are characteristic of a non-competitive inhibitor, indicating cis inhibition of TEA/H$^+$ exchange by amiloride was in part the result of the inhibitor binding to an allosteric site on the exchanger. Procainamide (75 μM) did not induce consistent changes in the kinetic parameters of TEA/H$^+$ exchange. In one case, procainamide acted as a non-competitive inhibitor, failing to markedly alter the $K_{tapp}$ for TEA (213 vs. 265 μM) while decreasing the $J_{\text{max}}$ (12 nmol·mg protein$^{-1}$·sec$^{-1}$ to 3.5 nmol·mg protein$^{-1}$·sec$^{-1}$). However, in a second trial procainamide acted as a competitive inhibitor, increasing the $K_{tapp}$ from 285 μM to 679 μM and failing to alter $J_{\text{max}}$ (11.55 nmol·mg protein$^{-1}$·sec$^{-1}$ vs. 11.98 nmol·mg protein$^{-1}$·sec$^{-1}$) with an approximate $K_i$ of 50 μM. These results suggest that procainamide inhibited TEA transport by binding to the substrate site and possibly an allosteric site on the OC exchanger. At 5 μM, quinidine acted as an uncompetitive inhibitor of TEA/H$^+$ exchange, inducing decreases in both $K_{tapp}$ and $J_{\text{max}}$ for TEA. For example, in one case, the $K_{tapp}$ for TEA decreased from 357 μM to 192 μM and the $J_{\text{max}}$ decreased from 15 nmol·mg protein$^{-1}$·sec$^{-1}$ to 3 nmol·mg protein$^{-1}$·sec$^{-1}$. Theoretically, interaction of the uncompetitive inhibitor involves an obligate order of binding of substrate and inhibitor; the inhibitor binds to an allosteric site on the carrier only after the substrate binds to the substrate site. Because these cations possibly bind to both the active and allosteric
sites, calculation of $K_i$'s for these compounds was not valid. Having only tested the inhibitory effects of a single concentration of each exogenous cation, I could not accurately calculate the IC$_{50}$ of these exogenous cations (i.e., concentration of inhibitor which decreases uptake of TEA by 50%; Segal 1976).
IV. Discussion

In an earlier chapter, I presented data that demonstrated that the exogenous OC $[^{14}\text{C}]\text{TEA}$ was transported via carrier-mediated exchange for $\text{H}^+$ as well as the OCs NMN and TEA in avian renal BBMV (Fig 2.3; Fig 2.9). TEA/$\text{H}^+$ exchange was saturable and indirectly coupled to Na$^+$ transport (Fig 2.7; Fig 2.8). Those data suggested that luminal transport of OCs in the avian kidney was qualitatively comparable to that in mammals (see review by Ross and Holohan 1983) and reptiles (Dantzler et al. 1991). According to the current models of the vertebrate kidney, mediated exchange of OCs for $\text{H}^+$ is the primary mechanism by which organic cations are secreted at the luminal membrane of the proximal tubule (Holohan and Ross 1981). Nearly all exogenous OCs transported by the OC exchanger in vitro are known to undergo net tubular secretion in vivo. Such findings suggest a physiological role of the BBM OC exchanger in the renal transport of exogenous OCs. Although many endogenous OCs are secreted by the renal tubules in vivo including vital compounds such as the neurotransmitters 5-hydroxytryptamine (serotonin) and ACh and the vitamins thiamine and riboflavin (Table 1.1), few endogenous OCs have been tested as transported substrates of the renal BBM OC exchanger. Consequently, the potential role of the OC exchanger in the renal regulation of plasma levels of endogenous OCs is not fully understood. Thus, to further elucidate the physiological significance of the OC exchanger, I assessed the substrate specificity of the luminal OC exchanger by examining the cis inhibitory and trans stimulatory effects of several endogenous and exogenous OCs on the exchange of $[^{14}\text{C}]\text{TEA}$ in chicken renal BBMV.

To facilitate the discussion of these data on the cis and trans effects of OCs on the transport of TEA in chicken renal BBMV, a model of the BBM OC exchanger patterned after that previously presented by Holohan and Ross (1981) will be used (Fig 1.2). In this
model, the substrate site on the exchanger alternately appears at the intravesicular and extravesicular face of the vesicle membrane.

In chicken renal BBMV, uptake and efflux of $[^{14}\text{C}]\text{TEA}$ was stimulated in the presence of \textit{trans} gradients of $\text{H}^+$ and OCs (Fig 2.3, 2.9. Therefore, two assumptions are apparently valid. First, the exchanger translocates OCs and protons bidirectionally. Second, the substrate sites for OCs and $\text{H}^+$, whether one in the same or mutually exclusive, may appear at either face of the membrane (Wright and Wunz 1988). Transport of the model substrate $[^{14}\text{C}]\text{TEA}$ is physically coupled to the counter directed flux of either $\text{H}^+$ or OCs across the membrane by the following scheme: 1) A proton or an OC binds to the substrate site on the exchanger at the intravesicular face of the membrane. 2) The substrate-exchanger complex subsequently reorients to the extravesicular face where the substrate dissociates. 3) $[^{14}\text{C}]\text{TEA}$ binds to the substrate site at the extravesicular face. 4) The $[^{14}\text{C}]\text{TEA}$-exchanger complex reorients to the intravesicular membrane face at which $[^{14}\text{C}]\text{TEA}$ debinds. In this manner, transport of an OC (e.g. $[^{14}\text{C}]\text{TEA}$) against its concentration gradient can be "driven" by high \textit{trans} concentrations of either $\text{H}^+$ or OCs. By this mechanism the secretory exchange of OC across the brush-border or luminal membrane of the renal proximal tubule is directly driven by the lumen-to-cell $\text{H}^+$ gradient generated by luminal $\text{Na}^+$/\text{H}^+ antiport. Finally, reorientation of an unloaded carrier is a rare event. Although the carrier mediates translocation of substrates across the membrane bilayer, it is the binding of the substrate to the substrate site which catalyses reorientation of the carrier (Holohan and Ross 1981).

Mediated transport of $[^{14}\text{C}]\text{TEA}$ by the BBM OC exchanger requires that the cation binding to the substrate site on the carrier. Thus, binding of $[^{14}\text{C}]\text{TEA}$ to the exchanger and its subsequent translocation would be inhibited by a second substrate present in the same face of the membrane (Holohan and Ross 1981; Wilbrandt and Rosenberg 1961).
Based on this principle, the affinity of the BBM exchanger in chicken BBMV was assessed by examining the *cis* inhibitory effects of OCs on mediated exchange of $[^{14}\text{C}]\text{TEA}$ for $\text{H}^+$. The exogenous OCs were more effective *cis* inhibitors of $[^{14}\text{C}]\text{TEA}/\text{H}^+$ exchanger, than were endogenous compounds (Fig 4.1a, b); there were, however, exceptions to this generalization. Although the inhibitory potencies of the exogenous OCs quinidine, amiloride, quinine, cimetidine and ranitidine markedly exceeded those of any endogenous OC, the same could not be said of the other exogenous OCs tested. The capacities of mepiperphenidol and procainamide to *cis* inhibit $[^{14}\text{C}]\text{TEA}$ uptake were comparable to that of thiamine. The inhibitory potencies of isoproterenol and unlabeled TEA were matched and, in some cases, exceeded by those of epinephrine, NMN, and serotonin. On the other hand, the endogenous cations ACh, choline and guanidine each failed to *cis* inhibit TEA transport. Several OCs tested in chicken BBMV were previously shown by Rennick and co-workers (1984) to inhibit renal excretion of $[^{14}\text{C}]\text{cimetidine}$ in chickens in decreasing order of potency: cimetidine, raniditine, thiamine, procainamide, guanidine, choline. The effectiveness of these same OCs to *cis* inhibit $[^{14}\text{C}]\text{TEA}/\text{H}^+$ exchange in isolated avian BBMV decreased in similar order (Fig 4.1a,b). These data on *cis* inhibition of proton-driven TEA uptake by different OCs indicated the affinity of the luminal OC exchanger in chicken BBMV was greater for the exogenous organic cations than for endogenous cations.

Although the relative affinity of the luminal OC exchanger for various OCs may be gauged by their capacities to *cis* inhibit $[^{14}\text{C}]\text{TEA}/\text{H}^+$ exchange, the capacity of the exchanger to transport these same compounds can not necessarily be assessed by the same criterion. The essence of an exchange mechanism is the physical coupling of the transmembrane flux of one substrate to the opposing flux of a second (Fig 1.2; Holohan and Ross 1981; Wilbrandt and Rosengerg 1961). In theory, an OC transported by the BBM OC exchanger should *trans* stimulate transport of the model substrate $[^{14}\text{C}]\text{TEA}$. 
For the specific set of OCs tested in this study, the ability to trans stimulate $[^{14}\text{C}]$TEA transport was apparently inversely related to the cis inhibitory potency of an OC. Those compounds which cis inhibited proton-driven uptake of $[^{14}\text{C}]$TEA by 40% or less (i.e., Ach, choline, epinephrine, isoproterenol, guanidine, NMN, serotonin, and unlabeled TEA) significantly trans stimulated efflux of $[^{14}\text{C}]$TEA. In contrast, OCs which decreased TEA uptake by 55% or more either failed to significantly trans stimulate efflux of $[^{14}\text{C}]$TEA (i.e., thiamine), or, moreover, trans inhibited efflux (i.e., amiloride, cimetidine, mepiperphenidol, procainamide, quinidine, quinine, and ranitidine) (Figure 4.2a,b). Thus, although the affinity of the BBM OC exchanger was generally greater for exogenous OCs than endogenous OCs, the latter were apparently transported with greater efficiency by the avian BBM OC/H$^+$ exchanger.

Stimulation of $[^{14}\text{C}]$TEA efflux in the presence of inwardly-directed gradients of OCs may have been secondary to the generation of either a trans negative diffusion potential by influx of cations or an inwardly directed H$^+$ gradient via OC/H$^+$ exchange. I did not directly examine the trans effects of OCs on $[^{14}\text{C}]$TEA efflux under voltage-clamped conditions or in the presence of a proton ionophore (e.g., FCCP). However, in separate experiments, I demonstrated that a trans negative membrane potential did not stimulate the efflux of $[^{14}\text{C}]$TEA (Fig 3.4) or the initial uptake of $[^{14}\text{C}]$TEA in the absence of a trans H$^+$ gradient (Fig 3.2a). Moreover, because of their size and polarity these OCs are not likely to diffuse across the lipid bilayer at a rate sufficient to generate a trans membrane potential for the 15 sec duration of the transport reaction.

The most pertinent objection to acceptance of the observed trans stimulation of $[^{14}\text{C}]$TEA efflux by test OCs as conclusive proof that these compounds are transported by the BBM OC exchanger is based on the fact I did not demonstrate equilibrium displacement of the vesicular content of $[^{14}\text{C}]$TEA (i.e. an overshoot or an undershoot) in the presence of a trans gradient of a given OC (Aronson 1981). The apparent trans stimulation of
[14C]TEA from BBMV may have been the result of either a chemically induced increase in membrane permeability to [14C]TEA or a decrease in the binding of isotope to the vesicle membrane, rather than a direct exchange of [14C]TEA for the test OC. Conversely, increased retention of [14C]TEA within BBMV may have been attributed to decreases in either membrane permeability or membrane binding of isotope, rather than to specific trans inhibition of carrier-mediated efflux of [14C]TEA. Preliminary experiments indicated indiscriminate changes in vesicle volume, membrane permeability, or nonspecific binding of [14C]TEA were not major factors in the observed trans effects of OCs on [14C]TEA transport. Values of initial content of [14C]TEA within BBMV measured in the presence of each OC were consistently greater than control values (Table 4.2; Table 4.3). The exception was unlabeled TEA, which possibly decreased binding of isotope by displacing isotope bound to the filter and BBMV. Although the increase the initial vesicular content of [14C]TEA in the presence of the various organic cations varied (6% - 40%), the qualitative effect was consistent. This indicated that in the control state (i.e. in the absence of organic cations), [14C]TEA continued to efflux from BBMV after the transport reaction was supposedly terminated. Although BBMV were pre-equilibrated with [14C]TEA (~190 μM) prior to measurements of the initial isotope content, they remained in a dynamic, rather than a static state. Thus, dilution of the BBMV with transport medium and stop solution established a transient gradient of [14C]TEA (190 μM_in:1.7 μM_out) that would thermodynamically favor net efflux of [14C]TEA. The Hg^{2+} (HgCl₂, 100 μM) and Ca^{2+} (CaCl₂, 2 mM) in the stop solution should have prevented or at least minimized the residual efflux of TEA. Moreover, at concentrations of approximately 100 μM, HgCl is known to completely block concentrative proton-driven uptake of cimetidine in rabbit renal BBMV (Inui et al. 1985a, 1985b) and that of TEA in rat renal renal BBMV (Takano et al. 1985). These data suggested Hg^{2+} and Ca^{2+} did not adequately serve their intended purpose. However, I did not titrate the concentration of HgCl₂ and CaCl₂ at which "residual" efflux
was blocked. Because residual efflux was trans inhibited by nearly all OCs, this was most likely a carrier-mediated process. Moreover, with the exception of ranitidine, the same compounds seen to trans inhibit 5 and 15 sec efflux of $[^{14}\text{C}]\text{TEA}$ (i.e. amiloride, cimetidine, mepiperphenidol, procainamide, quinidine, and quinine) and most effectively cis inhibit $[^{14}\text{C}]\text{TEA}$ uptake, were also the most potent trans inhibitors of the residual efflux of isotope. The converse was also true; those compounds seen to modestly cis inhibit TEA transport and trans stimulate TEA efflux only modestly inhibited residual efflux.

In contrast, the absolute, as well as the converted values of the vesicular content of $[^{14}\text{C}]\text{TEA}$ measured at equilibrium in the presence of OCs were not markedly different from control values (Table 4.3). This indicated these alterations, if any, in either membrane permeability or membrane binding of $[^{14}\text{C}]\text{TEA}$ were relatively uniform in the presence of the OCs tested. Both the absolute and converted equilibrium content of $[^{14}\text{C}]\text{TEA}$ were, however, substantially greater than control when measured in the presence of quinine and quinidine, suggesting that the apparent trans inhibitory effects of these OCs were sustained over time. Increases in equilibrium values of such magnitude may represent either increases in binding of $[^{14}\text{C}]\text{TEA}$ to the vesicle membrane or actual increases in the intravesicular concentration of $[^{14}\text{C}]\text{TEA}$ or both. Being very lipophyllic compounds (Ott et al. 1991a), quinine and quinidine may have partially intercalated into the lipid bilayer during the 2 hr incubation, thereby altering the biophysical properties of the membrane (e.g., membrane permeability, membrane fluidity, surface charges). Subsequently, binding of $[^{14}\text{C}]\text{TEA}$ to the intra- and extravescicluar face of the vesicle membrane may have been enhanced. Alternatively, these and other potent inhibitors of OC/H$^+$ exchange may bind to the exchanger with great affinity, but are either not translocated or are exchanged at rates too low to produce measurable changes in $[^{14}\text{C}]\text{TEA}$ transport. Slow rates of dissociation of the inhibitor-exchanger complex or translocation of
a tightly bound inhibitor would effectively immobilize the OC exchanger, and thereby prohibit carrier-mediated efflux of TEA from the vesicle. Moreover, if an additional long-term effect of these compounds was a decrease in membrane permeability, 2 hours may not have been sufficient time for $[^{14}\text{C}]{\text{TEA}}$ to equilibrate between the intravesicular and extravesicular compartments.

The relatively uniform increases in equilibrium content of $[^{14}\text{C}]{\text{TEA}}$ induced by OCs and the general correlation of the increment of increase in the initial content of $[^{14}\text{C}]{\text{TEA}}$ with the inhibitory potency of a given OC suggested the apparent trans inhibition and trans stimulation of $[^{14}\text{C}]{\text{TEA}}$ efflux were most likely the result of a specific physical interaction of OCs with the OC exchanger. If indiscriminate changes in the biochemical properties of the vesicle membrane had been the cause for the trans stimulation or inhibition of efflux, a correlation, albeit qualitative, would not have been observed. I, therefore, concluded that stimulation of $[^{14}\text{C}]{\text{TEA}}$ efflux in the presence of trans gradients of OCs such as serotonin and epinephrine represented mediated countertransport of substrate via the BBM OC exchanger, whereas trans inhibition of efflux by OCs such as ranitidine and cimetidine was indicative of the exchanger's low transport capacity for these OCs.

The model substrate $[^{14}\text{C}]{\text{TEA}}$ is transported by the OC/H$^+$ exchange mechanism in chicken BBMV, as indicated by the concentrative uptake of $[^{14}\text{C}]{\text{TEA}}$ in the presence of trans gradient of H$^+$ and OCs (Fig 2.3a; Fig 2.9). In strict compliance with the criteria for substrate specificity of an antiport or exchange transport system, the endogenous OCs epinephrine, NMN, and serotonin and the exogenous OC isoproterenol were also transported substrates of the avian BBM OC exchanger, being the only test compounds to both cis inhibit (Fig 4.1a,b) and trans stimulate transport of $[^{14}\text{C}]{\text{TEA}}$ (Fig 4.2a, b). As it was seen in chicken BBMV to inhibit TEA/H$^+$ exchange, serotonin was previously demonstrated to cis inhibit proton-driven uptake of NMN in dog renal BBMV (Sokol et al
1987) and that of guanidine in BBMV isolated from human intestine (Miyamoto et al. 1988) and placenta (Ganapathy et al. 1988). Serotonin, as well as epinephrine and isoproterenol are each known to undergo net tubular secretion by the OC transport system of the avian kidney (Rennick and Yoss 1962; Quebbeman and Rennick 1969; Sanner and Wortman 1962; Silva et al. 1979) as well as mammals (Ball et al. 1982; Lifschitz et al 1973; Sandler and Spector 1961). Granted as much as 70% of the serotonin reaching the kidney is metabolized to 5-hydroxyindoleacetic acid (Hakim et al. 1970) and over 50% of the epinephrine to metanephrine (Ball et al. 1982; Silva et al. 1979). However, these data suggest the unmetabolized forms of these OCs and isoproterenol may be transported by the OC exchange system at the BBM membrane of the proximal tubule in vivo.

Tetraethylammonium and NMN share a common transport pathway in the intact avian kidney (Springate et al 1987). The ability of NMN to cis inhibit $[^{14}\text{C}]$TEA/H$^+$ exchange (Table 2.2; Fig 4.1a) as well as trans stimulate both the efflux (Fig 4.2a), as well as the concentrative uptake of $[^{14}\text{C}]$TEA in chicken BBMV (Fig 2.9) suggested luminal secretory OC exchange may be a common transport pathway shared by these OCs in vivo. However, as discussed previously, the apparent role of the luminal exchanger in the renal transport of NMN may vary among species. For example, in rabbit renal BBMV, NMN is transported by OC/H$^+$ exchange (Hsyu et al. 1988; Wright 1985). However, an analogous mechanism apparently contributes little to secretion of NMN in the intact proximal tubule of that species. Secretion of NMN in isolated perfused renal tubules of rabbits, as well as snakes, remained relatively constant despite changes in luminal concentrations of H$^+$ or OCs (Besseghir et al. 1990; Dantzler and Brokl 1987).

Although ACh, choline, and guanidine each trans stimulated efflux of $[^{14}\text{C}]$TEA (Fig 4.2a), each failed to cis inhibit $[^{14}\text{C}]$TEA/H$^+$ exchange (Fig 4.1a). Substrate-carrier interactions of this nature indicated these OCs were transported by the avian OC exchanger;
however, the affinity of the carrier for these compounds was relatively low. The data are consistent with previous findings on the renal transport of choline in chickens and mammals. Briefly, at physiological plasma concentrations (>100 μM) choline undergoes net tubular secretion, sharing a common secretory transepithelial pathway with TEA (Rennick et al. 1977). The affinity of the OC secretory transport pathway is significantly lower for choline than for TEA (Rennick et al. 1977). As demonstrated by Wright and co-workers (1992) in rabbit renal BBMV, the affinity of the luminal OC exchanger was much lower for choline than that for TEA (i.e., $K_t$ for choline of ~10 mM vs. ~100 μM for TEA; Lazaruk and Wright 1990). These same investigators demonstrated the presence of an electrogenic facilitated pathway having a high affinity for choline (i.e., $K_t$ for choline ~100 μM; Wright et al. 1992). This was postulated to be the primary mechanism mediating luminal reabsorption of choline as it undergoes net tubular reabsorption at physiological levels (2 - 10 μM; Wright et al. 1992). Acetylcholine and guanidine have been shown to undergo net tubular secretion in the chicken (Acara and Rennick 1972b; Sperber 1948); however, whether these endogenous OCs are secreted or reabsorbed by the renal tubule at physiological plasma concentrations is not known. Acetylcholine is actively secreted by the avian renal tubule unmetabolized (Acara and Rennick 1972b). Although choline was shown to inhibit renal excretion of ACh, reciprocal inhibition by ACh was not observed (Acara and Rennick 1976), thereby, indicating the affinity of the secretory transport pathway for ACh is lower than for choline.

My conclusion that the affinity of the OC exchanger is lower for guanidine than for other OCs was also consistent with previous findings on the renal excretion of OCs in birds. Guanidine was shown to inhibit renal excretion of cimetidine (Rennick et al. 1984), choline (Acara and Rennick 1972b), and amiloride (Besseghir and Rennick 1981) in chickens; however, it is less effective than other cationic transport inhibitors (e.g., cyanine,
mepiperphenidol, quinine). Thus, the affinity of the transepithelial OC transport pathway for guanidine is also relatively low. In contrast to my findings in chicken renal BBMV, Ott and colleagues reported that guanidine failed to trans stimulate TEA uptake in human renal BBMV (Ott et al '91). The cis inhibitory effects of guanidine on TEA transport were not examined in that study. Guanidine and TEA were each shown to be transported by Oc/H+ exchange in rabbit renal BBMV by Miyamoto et al. (1989). Kinetic analyses of proton-driven uptake of each OC suggested that the transport pathways for these cations were functionally separate. In this study, the Eadie-Hofstee plot ($J$ vs. $J/[S]$) of TEA/H+ exchange was linear, indicating operation of a single carrier system for TEA; however, the plot for guanidine/H+ exchange was curvilinear, suggesting transport of substrate by multiple carriers. The kinetic parameters of guanidine/H+ exchange were reportedly unaltered in the presence of 10 mM TEA (Miyamoto et al. 1989). In addition, although TEA/H+ exchange was apparently more susceptible to cis inhibition by any given inhibitor, unlabeled TEA was a more effective inhibitor of radiolabeled TEA than was guanidine. These data indicated guanidine shared a common BBM OC carrier with TEA and other OCs, but was possibly also transported by an additional, separate mediated pathway.

Collectively, these data indicated the OC exchanger expressed in chicken renal BBMV preferentially transported endogenous OCs, with the exception of thiamine. Rennick and co-workers had previously shown thiamine not only inhibited renal excretion of cimetidine, choline and TEA in the chicken, but that it also shared a common transepithelial secretory pathway with these compounds (Acara and Rennick 1976; Rennick 1958; Rennick et al. 1984). Though the most potent endogenous cis inhibitor of $[^{14}\text{C}]$TEA uptake in avian BBMV (Fig 4.1a), thiamine was the only endogenous OC that did not significantly trans stimulate efflux of $[^{14}\text{C}]$TEA (Fig 4.2a). As indicated by the changes in the kinetic parameters of TEA uptake induced by thiamine, this OC competed with TEA for a common substrate site on the exchanger. Despite this and the exchanger's
moderate affinity for thiamine, it was poorly transported. These data suggested that inhibition of OC transport by thiamine in vivo may involve inhibition of transport of OCs at the BBM.

In chicken renal BBMV, the specificity of the OC exchanger for exogenous OCs other than isoproterenol and TEA was very low. The affinity of the OC exchanger for amiloride, cimetidine, mepiperphenidol, quinidine, quinine, and ranitidine was very high as indicated by their cis inhibitory potencies. Although each OC has been demonstrated to undergo net tubular secretion in chickens or other vertebrates (Table 1.1), these exogenous cations were poorly transported in chicken renal BBMV under the present experimental conditions. The cis inhibitory effects of exogenous OCs on transport of TEA were consistent with findings in renal BBMV of other species. As seen here, amiloride, cimetidine, mepiperphenidol, and quinine inhibited TEA transport in rabbit BBMV (Myamoto et al. 1989; Wright and Wunz 1987; Rafizadeh et al. 1987). Quinidine, ranitidine, and procainamide are known to inhibit proton-driven uptake of OCs such as NMN, cimetidine, and procainamide in renal BBMV of humans and rabbit (McKinney and Kunnemann 1985; McKinney and Kunnemann 1987; Ott et al. 1991a, b; Gisclon et al. 1987). However, these compounds failed to trans stimulate transport of [14C]TEA, but instead trans inhibited transport under the present experimental conditions, indicating that these compounds were either not transported or are transported at very low rates by the avian renal OC exchanger. Several exogenous OCs poorly transported by the OC exchanger in chicken BBMV in the absence of a H⁺ gradient are known to be transported by carrier mediated OC exchange in renal BBMV of other species. Mepiperphenidol, shown to trans stimulate initial uptake of NMN in dog BBMV (Holohan and Ross 1980), also trans stimulated uphill transport of TEA in rabbit BBMV (Rafizadeh et al. 1987). Moreover, concentrative uptake of amiloride, procainamide, and cimetidine via by OC/H⁺
exchange has been demonstrated in renal BBMV of the rabbit (McKinney and Kunnemann 1985; McKinney and Kunnemann 1987; Wright and Wunz 1989).

The inability of potent cis inhibitors of TEA transport to trans stimulate OC transport in chicken BBMV was not a novel finding. Cimetidine, quinidine, and ranitidine to my knowledge had not been previously tested for trans stimulatory effects on BBM transport of OC. However, quinine failed to trans stimulate TEA uptake in rabbit renal BBMV (Rafizadeh et al. 1987), and this is consistent with the present data. Investigators have found that other exogenous OCs transported by OC/H+ exchange, poorly trans stimulated transport of OCs. McKinney and Kunneman demonstrated proton driven exchange of procainamide and cimetidine in rabbit renal BBMV (McKinney and Kunnemann 1985; McKinney and Kunnemann 1987). However, trans stimulation of the uptake of [3H]procainamide or [3H]cimetidine by unlabeled procainamide was secondary to generation of a trans H+ gradient via H+ exchange (In these experiments, however, the cis carry-over concentration of procainamide was 1.11 mM. This concentration of procainamide may have been sufficient to inhibit uptake of 50 μM of [3H]-substrate).

Wright and co-workers demonstrated in separate studies that amiloride and MPP+ were each transported via OC/H+ exchange in rabbit BBMV. The kinetics of the inhibitory interactions of each OC and TEA indicated amiloride and MPP+ each competed with TEA for common substrate site on the OC exchanger (Lazaruk and Wright 1990; Wright and Wunz 1987). As would be expected, TEA trans stimulated uptake of amiloride and MPP+; however, reciprocal stimulation by amiloride or MPP+ was not observed (Lazaruk and Wright 1990). Similarly, Sokol et al. 1987 found that MPP+ trans inhibited, rather than trans stimulated NMN efflux in dog BBMV.

The rate-limiting event in the mediated exchange of OCs is reorientation of the carrier. Theoretically, the rate of reorientation or turnover should be accelerated in the presence of a large trans concentration of a transported substrate, and thusly, the cis-to-
trans flux or rate of translocation of a model substrate should be stimulated. It has been postulated that failure of potent cationic inhibitors to trans stimulate OC transport might be due to either a slow turnover rate of the loaded carrier or a slow rate of dissociation of the substrate-carrier complex, or both. In the extreme case, the inability to trans stimulate indicates the OC was not translocated (Lazaruk and Wright 1990; Rafizadeh et al. 1987; Sokol et al. 1987). Lazaruk and Wright "unmasked" counter-stimulation of TEA transport by MPP⁺ for TEA in rabbit renal BBMV by decreasing the intra- and extravesicular concentrations of H⁺ from 30 nM to 3 nM. In the rabbit, the apparent $K_{H^+}$ for OC/H⁺ exchange is approximately 30 nM (Wright and Wunz 1988). Therefore, at pH 7.5, the concentration of H⁺ would be sufficient to catalyze turnover of the exchanger. Thus, if the rate of carrier turnover or substrate-carrier dissociation for MPP⁺ were slower than that for H⁺, any subsequent translocation of TEA would be not be measurable. Accordingly, in decreasing the H⁺ concentration (i.e., increasing pH to 8.5), proton-driven turnover of the exchanger was attenuated and stimulation of TEA transport in the presence of a trans gradient of MPP⁺ was then observed. However, trans MPP⁺ supported only approximately one third the cis-to-trans flux of radiolabeled TEA supported by unlabeled TEA (Lazaruk and Wright 1990). In my experiments, I examined the trans effects of OCs on [¹⁴C]TEA transport in the presence of intra- and extravesicular H⁺ concentrations of 30 nM (pH 7.5). However, I did not examine the trans effects of OCs in the presence of a decreased H⁺ concentration.

The relatively slow turnover rate of the potent inhibitors of OC transport such as quinidine and cimetidine may be a direct, but adverse consequence of the high affinity of the OC exchanger for these compounds. If such a compound binds too tightly to the substrate site on the carrier, the substrate-carrier complex cannot make the conformational changes necessary to reorient the carrier from one side of the membrane to the other at an adequate rate. The tightness with which the substrate binds to the carrier may also result in
a low dissociation rate of the OC from the exchanger (Krupka 1989). As indicated by
changes in the kinetic parameters of the TEA/H+ exchange by representative OCs in
chicken BBMV, the capacity of the OC exchanger to translocate an OC, as well as the
inhibitory potency of an OC may be a direct consequence of binding of substrate to an
allosteric site on the carrier (Table 4.4). Amiloride and procainamide were apparently
noncompetitive inhibitors of TEA/H+ exchanger, and quinidine an uncompetitive inhibitor.
Thus, these exogenous OCs, seen here to trans inhibit transport [14C]TEA, possibly
bound to allosteric sites on the exchanger. In renal BBMV of the rabbit, amiloride and
quinidine, as well as quinine were shown to inhibit OC/H+ exchange in a competitive
manner (Wright and Wunz 1987; Ott et al. 1991a), thereby indicating inhibition involved
competition for a common substrate site on the exchanger. Binding of an OC to an allosteric
site and the subsequent changes in carrier configuration may result in altered affinity of the
substrate site for TEA in addition to decreased rates of turnover and translocation of the
substrate-exchanger complex. Based on the present data, I cannot say whether these
exogenous compounds bound exclusively to allosteric sites. Indeed, if cationic inhibitors
of OC/H+ exchange bound simultaneously to the substrate site and an allosteric site, the
tightness with which an OC bound to the exchanger may be enhanced. In the extreme case,
any such substrate-carrier interactions may effectively immobilize the carrier and, thus,
result in trans inhibition of TEA transport. In contrast, serotonin, a transported substrate of
the avian OC exchanger, was shown to compete with TEA for the substrate site on carrier,
as apparently did thiamine. Although thiamine failed to trans stimulate transport of TEA, it
did not trans inhibit transport.

The inverse relationship between the cis inhibitory potency of a test OC and its
apparent transport efficiency in chicken BBMV was consistent with data on excretion of
several potent inhibitors of OC transport. The maximum tubular transport rates (Tm's)
reported for several OCs tested in the chicken are listed in Table 4.5. As demonstrated in
Table 4.5. Transport maximum values for some organic cations excreted by the chicken. References are listed in parentheses.

<table>
<thead>
<tr>
<th>Organic Cation</th>
<th>Transport Maximum (µmol·kg⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride</td>
<td>0.012    (Besseghir and Rennick 1981)</td>
</tr>
<tr>
<td>Choline</td>
<td>1.2      (Acara and Rennick 1972)</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>0.065    (Springate et al. 1987)</td>
</tr>
<tr>
<td>NMN</td>
<td>2.5      (Sperber 1948)</td>
</tr>
<tr>
<td>Quinine</td>
<td>Negligible (Volle et al. 1960)</td>
</tr>
<tr>
<td>TEA</td>
<td>0.5      (Rennick et al. 1977)</td>
</tr>
</tbody>
</table>
chicken BBMV, the potent inhibitors quinine, amiloride, and cimetidine are also poorly transported by the kidney \textit{in vivo}. By comparison, TEA, NMN, and choline, shown to modestly inhibit OC transport \textit{in vitro} (Fig 4.1a), as well as \textit{in vivo} (Springate et al. 1987), are transported by the intact avian renal tubules at much higher rates. A comparison of the reported $K_t$ s for several OCs transported by the OC/H$^+$ exchanger in rabbit renal BBMV indicates an analogous relationship between the affinity of the OC/H$^+$ exchanger for a given OC and the respective maximal turnover rate of that OC-exchanger complex ($J_{\text{max}}$; Table 4.6). For instance, amiloride, the $K_t$ for which is 7.5 $\mu$M, has a $J_{\text{max}}$ of approximately 3 nmol-mg$^{-1}$-min$^{-1}$; whereas the $K_t$ for choline is 10 mM, the $J_{\text{max}}$ is 38 nmol-mg$^{-1}$-min$^{-1}$. The exception in the reported data is procainamide. Although the OC/H$^+$ exchanger has a relatively moderate affinity for procainamide ($K_t$ for procainamide: 540 $\mu$M), the $J_{\text{max}}$ for this OC is equal to that of amiloride. Similarly, the affinity of the avian OC exchanger for procainamide was also moderate (Fig 4.1b); whereas, the apparent turnover rate of the exchanger in the presence of procainamide was low (Fig 4.2b).

The OC/H$^+$ exchanger is thought to be the primary mechanism by which OCs are secreted at the luminal membrane of the proximal tubule and, thus, a major component in the renal clearance of exogenous OCs (Holohan and Ross 1981). The present data on substrate specificity of the OC exchanger in chicken renal BBMV and previously reported data on transport of OCs both \textit{in vivo} and \textit{in vitro} indicate exogenous OCs are transported at relatively low rates. The $K_t$ or $K_{\text{tapp}}$ for several exogenous OCs closely approximate the reported plasma concentrations achieved when these compounds are taken in prescription dosages (Table 4.6). Although the affinity of the BBM OC exchanger is high enough to transport these compounds at concentrations present \textit{in vivo}, the low rates at which these exogenous OCs are transported suggest these compounds are slowly cleared from the plasma. Thus, when cationic drugs such as amiloride and procainamide are administered simultaneously, the precautions taken to avoid excessive dosages and
Table 4.6. Kinetic parameters of organic cation transport in rabbit renal brush-border membrane vesicles. References are listed in parentheses.

<table>
<thead>
<tr>
<th>Organic Cation</th>
<th>$K_t$ (μM)</th>
<th>$J_{\text{max}}$ (nmol·mg⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride</td>
<td>7.5</td>
<td>5</td>
</tr>
<tr>
<td>Choline</td>
<td>10,000</td>
<td>38</td>
</tr>
<tr>
<td>Cimetidine</td>
<td>4.6</td>
<td>0.41</td>
</tr>
<tr>
<td>Guanidine</td>
<td>280</td>
<td>1.72</td>
</tr>
<tr>
<td></td>
<td>3,400</td>
<td>11.2</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>7.8</td>
<td>3.3</td>
</tr>
<tr>
<td>NMN</td>
<td>630</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>2,610</td>
<td>7.0</td>
</tr>
<tr>
<td>Procainamide</td>
<td>540</td>
<td>2.8</td>
</tr>
<tr>
<td>Quinidine</td>
<td>2.5*</td>
<td></td>
</tr>
<tr>
<td>Quinine</td>
<td>2.4*</td>
<td></td>
</tr>
<tr>
<td>TEA</td>
<td>100</td>
<td>12.4</td>
</tr>
<tr>
<td></td>
<td>490</td>
<td>2.68</td>
</tr>
</tbody>
</table>

*apparent $K_t$
possibly, fatal competitive inhibition of renal excretion are warranted. In contrast, the $K_t$ and $K_{t_{app}}$ for endogenous OCs transported by OC/H$^+$ exchange exceed the corresponding reported physiological substrate concentrations. As seen in chicken renal BBMV, the $K_{t_{app}}$ for serotonin was approximately 250 $\mu$M (Table 4.4), yet the plasma concentrations of serotonin are approximately 0.04 $\mu$M. Perhaps, as postulated for choline (Wright et al. 1992), the OC exchanger is involved in renal transport of other endogenous OCs such as serotonin at supraphysiological, rather than physiological concentrations.

The affinity of a transport system for a given substrate may vary depending on the experimental procedure. For example, in snakes, the OC/H$^+$ exchanger expressed in isolated renal BBMV has a $K_t$ for TEA of $\sim$475 $\mu$M (Dantzler et al. 1991). By comparison, in the intact isolated perfused proximal tubule, the affinity of the OC transport pathway in the BBM was much higher; the $K_t$ for TEA was 5.9 $\mu$M (Hawk and Dantzler 1984). Thus, although the concentration of the various endogenous and exogenous OC tested in the present study exceed the physiological or pharmacological plasma concentrations, the relative cis and trans effects of these compounds should not be disregarded. In addition, tubule perfusion studies are necessary to verify the role of BBM OC/H$^+$ exchanger in the transepithelial transport of various OCs. For example, NMN is transported via OC/H$^+$ exchange in rabbit renal BBMV (Hsyu et al. 1988; Wright 1985); however, in isolated perfused rabbit renal tubules OC/H$^+$ exchange was apparently not involved in NMN transepithelial secretion (Besseghir et al. 1990).

The integrative functions of the liver and kidney in regulating the plasma levels of endogenous and exogenous OCs also should be addressed in future studies. If, for example, a poorly transported exogenous OC such as quinine is not adequately cleared by the renal tubules (Table 4.5), the compound may circulate through the liver where it is
metabolized. Thus, future studies should examine the transport of renal, as well as hepatic metabolites of endogenous and exogenous OCs.

In summary, the OCs ACh, choline, epinephrine, guanidine, isoproterenol, NMN, serotonin which were previously demonstrated to undergo net tubular secretion in the intact chicken were also transported by the OC exchanger expressed in chicken renal BBMV. Thus, the OC/H+ exchange mechanism at the luminal membrane of the proximal tubule may contribute to the net transport of these OCs by the renal tubule in vivo. With few exceptions, the overall affinity of the OC exchanger was greater for exogenous OCs than for endogenous OCs. As observed in renal BBMV of other vertebrates, those OCs for which the exchanger possessed the greatest affinity (i.e., amiloride, cimetidine, mepiperphenidol, procainamide, quinidine, quinine, ranitidine, and thiamine) were poorly transported in the absence of a trans H+ gradient. Thus, in general exogenous OCs were transported less efficiently than endogenous OCs. The exogenous OCs amiloride, procainamide and quinidine were shown to bind to allosteric site on the carrier, explaining in part their capacity to cis and trans inhibit OC transport in renal BBMV.
Summary

In summary, $[^{14}\text{C}]$tetraethylammonium is countertransported for protons, as well as OCs in avian renal brush-border membrane vesicles. Mediated OC/H$^+$ exchange is not electrogenic and is specific for OCs. Proton-driven uptake of TEA was indirectly coupled to sodium transport, presumably by indirect generation of a trans proton gradient via Na$^+/H^+$ antiport. The OC/H$^+$ exchanger of the avian brush-border membrane had a strong affinity for the exogenous OCsamiloride, cimetidine, mepiperphenidol, procainamide, quinidine, quinine and ranitidine, as well as the endogenous cation thiamine; however, these compounds were poorly transported by the exchanger in the absence of a proton gradient. Previous investigators have demonstrated that compounds such as amiloride, cimetidine and quinine are potent inhibitors of OC excretion in the bird, but are poorly transported in vivo. Kinetic studies suggested the inhibitory potency and low transport rates of exogenous compounds is in part due to binding of substrate to allosteric sites on the luminal exchanger. In contrast, acetylcholine, choline, epinephrine, guanidine, isoproterenol, N$^1$-methylnicotinamide, serotonin, and unlabeled TEA, which were previously known to be readily excreted, modestly inhibited TEA transport, but were transported by the OC exchanger in these vesicles. In conclusion, as in mammals, luminal OC transport in the avian proximal tubule involves a proton exchange mechanism that is indirectly coupled to Na$^+/H^+$ antiport. Likewise, as seen in mammals, the OC/H$^+$ exchanger of the avian luminal membranes has a greater affinity for exogenous cations, but a greater capacity to transport endogenous organic cations.
APPENDIX A

*Cis* inhibitory effects of various organic compounds on proton-driven $[^{14}\text{C}]$tetraethylammonium uptake.
Cis inhibitory effects of organic compounds on \([^{14}C]\)tetraethylammonium uptake via H+ exchange were tested in BBMV pre-equilibrated (1 hr, 25°C) in mannitol medium containing 100 mM KCl and 5 mM HEPES-KOH, pH 6.0. At 25°C, 10 μl BBMV were diluted with 90 μl isosmotic mannitol transport media containing ~50 μM \([^{14}C]\)TEA-Br, 100 mM KCl, 5 mM HEPES-KOH (pH 7.5), and either 22.22 mM unlabeled TEA-Br or 1.11 mM of inhibitor. Uptake is expressed as pmol \([^{14}C]\)TEA/mg protein. (n = 3; mean ± S.E.)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>5 sec</th>
<th>No Inhibitor</th>
<th>2 h</th>
<th>Inhibitor</th>
<th>15 sec</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM TEA</td>
<td>157.10</td>
<td>294.63</td>
<td>57.72</td>
<td>4.03</td>
<td>4.52</td>
<td>46.27</td>
</tr>
<tr>
<td>±36.13</td>
<td>±53.08</td>
<td>±2.21</td>
<td>±1.60</td>
<td>±2.39</td>
<td>±5.08</td>
<td></td>
</tr>
<tr>
<td>1 mM TEA-Br</td>
<td>164.4</td>
<td>325.70</td>
<td>56.99</td>
<td>45.00</td>
<td>68.13</td>
<td>50.58</td>
</tr>
<tr>
<td>±36.02</td>
<td>±47.50</td>
<td>±2.56</td>
<td>±8.98</td>
<td>±17.63</td>
<td>±4.60</td>
<td></td>
</tr>
<tr>
<td>1 mM NMN-Cl</td>
<td>190.46</td>
<td>335.62</td>
<td>55.53</td>
<td>55.35</td>
<td>77.79</td>
<td>66.12</td>
</tr>
<tr>
<td>±19.14</td>
<td>±46.52</td>
<td>±0.57</td>
<td>±5.76</td>
<td>±3.30</td>
<td>±2.22</td>
<td></td>
</tr>
<tr>
<td>1 mM Choline-Cl</td>
<td>165.53</td>
<td>325.7</td>
<td>57.22</td>
<td>99.87</td>
<td>124.23</td>
<td>48.1</td>
</tr>
<tr>
<td>±36.86</td>
<td>±47.50</td>
<td>±2.44</td>
<td>±25.05</td>
<td>±28.87</td>
<td>±9.35</td>
<td></td>
</tr>
<tr>
<td>1 mM Darstine</td>
<td>169.52</td>
<td>333.02</td>
<td>64.20</td>
<td>10.97</td>
<td>23.8</td>
<td>52.40</td>
</tr>
<tr>
<td>±37.49</td>
<td>±49.04</td>
<td>±2.10</td>
<td>±2.43</td>
<td>±5.23</td>
<td>±13.20</td>
<td></td>
</tr>
<tr>
<td>1 mM Cimetidine</td>
<td>169.52</td>
<td>333.02</td>
<td>64.20</td>
<td>6.20</td>
<td>8.49</td>
<td>51.70</td>
</tr>
<tr>
<td>±37.49</td>
<td>±49.04</td>
<td>±2.10</td>
<td>±3.11</td>
<td>±4.61</td>
<td>±13.70</td>
<td></td>
</tr>
<tr>
<td>1 mM Quinidine</td>
<td>164.40</td>
<td>325.70</td>
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<td>4.07</td>
<td>2.75</td>
<td>43.01</td>
</tr>
<tr>
<td>±36.02</td>
<td>±47.50</td>
<td>±3.42</td>
<td>±0.33</td>
<td>±1.73</td>
<td>±1.39</td>
<td></td>
</tr>
<tr>
<td>1 mM Quinine</td>
<td>197.76</td>
<td>366.69</td>
<td>60.45</td>
<td>2.96</td>
<td>3.72</td>
<td>63.14</td>
</tr>
<tr>
<td>±10.73</td>
<td>±18.15</td>
<td>±3.85</td>
<td>±0.71</td>
<td>±1.78</td>
<td>±6.23</td>
<td></td>
</tr>
<tr>
<td>1 mM Ranitidine</td>
<td>197.76</td>
<td>366.69</td>
<td>60.45</td>
<td>4.07</td>
<td>7.93</td>
<td>62.5</td>
</tr>
<tr>
<td>±10.73</td>
<td>±18.15</td>
<td>±3.85</td>
<td>±1.03</td>
<td>±1.24</td>
<td>±2.40</td>
<td></td>
</tr>
<tr>
<td>1 mM PAH</td>
<td>190.46</td>
<td>335.62</td>
<td>55.53</td>
<td>168.66</td>
<td>277.12</td>
<td>58.64</td>
</tr>
<tr>
<td>±19.14</td>
<td>±46.52</td>
<td>±0.57</td>
<td>±33.25</td>
<td>±39.64</td>
<td>±2.94</td>
<td></td>
</tr>
<tr>
<td>1 mM Probenecid</td>
<td>190.46</td>
<td>335.62</td>
<td>55.53</td>
<td>125.89</td>
<td>229.56</td>
<td>61.45</td>
</tr>
<tr>
<td>±19.14</td>
<td>±46.52</td>
<td>±0.57</td>
<td>±10.95</td>
<td>±30.39</td>
<td>±5.05</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX B:

Trans stimulation of [\(^{14}\text{C}\)]tetraethylammonium uptake by organic cations in the presence of a proton ionophore.
In a preliminary experiment, uptake of [14C]TEA was measured in avian renal BBMV in the presence of trans gradients of unlabeled TEA (A) or NMN (B) and the H+ ionophore FCCP. Vesicles were prepared and pre-equilibrated (1 hr, 25°C) in mannitol medium containing 5 mM HEPES-KOH (pH 7.5), 100 mM KCl, 20 μg valinomycin/mg protein and no OC, 1 mM OC, or 1 mM OC plus 30 μM FCCP. At 25°C, 5 μl BBMV were diluted with 195 μl isosmotic mannitol buffer containing ~50 μM [14C]TEA-Br, 5 mM HEPES-KOH (pH 7.5), 100 mM KCl and no cation or 27.8 μM OC. o, control (1 mM trans mannitol); Δ, 1 mM trans OC+ (OC+in >OC+out); Δ, 1 mM trans OC+ plus FCCP; o, 25 μM cis OC+ (OC+out >OC+in).

(n = 1; mean of triplicate measurements)
References


