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Structural specificity of organic cation transport in rabbit renal brush border membrane vesicles

Ayer, Katherine Dorothy, M.S.

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U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

STRUCTURAL SPECIFICITY OF ORGANIC CATION TRANSPORT IN RABBIT RENAL BRUSH BORDER MEMBRANE VESICLES

by

Katherine Dorothy Ayer

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STATEMENT BY AUTHOR

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SIGNED: Katherine D. Ayer

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Stephen H. Wright
Associate Professor
of Physiology

7/22/88
Date

David L. Nelson
Associate Professor
Pharmacology and Toxicology

7/22/88
Date

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TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABSTRACT

Organic cations (OC's) are actively secreted by the renal proximal tubules in a number of species. The transepithelial transport of OC's involves a secondary active OC/H^+ exchange process at the brush border (luminal) membrane. This study employed rabbit renal brush border membrane vesicles (BBMV) to investigate the structural requirements associated with substrate recognition at the OC transporter. A number of compounds (an N-alkylammonium series, an N^1-alkylpyridinium series and some clinically important organic bases) were tested for their ability to competitively block the uptake of radioactively labelled tetraethylammonium (TEA) into BBMV. The inhibitory effectiveness of these compounds was correlated to the degree of hydrophobicity surrounding the positively-charged nitrogenous nucleus common to all the inhibitors. Preloading BBMV with N^1-substituted pyridines trans-stimulated the uptake of TEA, suggesting that these compounds are translocated substrates for the OC transporter. The activity of the OC transport inhibitor and neurotoxin 1-methyl-4-phenylpyridinium was of special interest, and thus its transport characteristics were fully evaluated.

CHAPTER 1

INTRODUCTION

The kidney is the major site for the elimination of endogenous waste products as well as xenobiotics and their metabolites. There are two major pathways for these elimination processes: 1) filtration at the glomerulus, and 2) secretion directly from the blood into the lumen of the nephron. Secretion of a compound directly from the blood to the tubular filtrate augments the clearance of a compound which may also be filtered at the glomerulus. Secretion may occur either by the passive movement of a compound down its electrochemical gradient from the blood into the cell, and subsequently into the lumen of the nephron, or by a carrier-mediated process that may or may not involve an input of energy.

The renal secretion of organic anions and the transport of essential biomolecules such as glucose and amino acids have been extensively studied and will not be reviewed here. In contrast, the mechanism of the transepithelial secretion of organic cations and bases (OC's) has not been well characterized and is the focus of this literature review.

Renal tubular secretion of an organic cation was first described by Sperber (1947) for N^1-methylnicotinamide (NMN) in chickens and by Rennick et al. (1947) for tetraethylammonium (TEA) in dogs. Many studies since then have shown that a number of organic cations are secreted by the kidney (for example: choline, dopamine, amiloride, and morphine (Rennick, 1981)) through a pathway (or set of pathways) that is functionally separate from that for the organic anions (typically para-aminohippuric acid) (see Beyer et al., 1950; Farah and Rennick, 1956). Using the stop-flow technique Rennick and Moe (1960) showed that TEA secretion was localized to the proximal tubule of the nephron. Until the late 1970's OC secretion studies were based primarily on the use of clearance or kidney slice techniques with a variety of animal models, including the chicken, dog and rat (see Rennick, 1981). Although these studies were important in demonstrating the presence of OC secretory processes and in localizing them to the proximal tubule, they were not able to separate the transport activity at the basolateral (or peritubular) membrane from that at the brush-border (or luminal) membrane; i.e. to delineate the processes at each membrane which were responsible for the transepithelial movement of OC's.

Separation of basolateral from brush-border membranes.

Kinsella et al. (1979a) reported a technique for isolating brush-border and basolateral membranes from dog renal cortex, which is composed primarily of proximal and distal nephron segments. This technique involved homogenization of the renal cortex, followed by differential centrifugation to separate cell organelles from the plasma membrane. The resulting plasma membrane-enriched fraction was then treated with a solution of divalent cations (Ca^{2+}, Mg^{2+}, and Mn^{2+}) which caused the aggregation of basolateral and any intracellular membranes which were subsequently pelleted by a low-speed centrifugation step. The brush-border membranes were left suspended in the supernatant and were pelleted-out separately. A divalent cation precipitation method had been previously reported by Booth and Kenny (1974) for isolation of rabbit renal microvillus membranes. The technique used by Kinsella et al. (1979a) was the first to isolate relatively pure fractions of both brush-border and basolateral membranes by combining the divalent cation precipitation method for isolation of brush-border membranes with the subsequent separation of basolateral membranes on a sucrose density gradient. Enzyme markers were used to evaluate the purity of each of the membrane fractions, with alkaline phosphatase, maltase, sucrase, trehalase, and gamma-glutamyltransferase serving as markers for the brush-border membranes; and Na+-K+-ATPase as the marker for the basolateral membrane. Intracellular membranes were identified using the following markers: glucose-6-phosphatase as a marker for endoplasmic reticular membranes; succinate dehydrogenase as a marker for mitochondria; and acid phosphatase as a marker for lysosomal membranes. The brush-border membrane fraction was found to be enriched approximately 7-fold with luminal enzyme markers compared to the basolateral membrane fraction, and conversely, the basolateral membrane fraction was found to be enriched approximately 22-fold with basolateral enzyme markers compared to the brush-border preparation. The membranes were shown to form small vesicles whose orientation was 76.5% and 86% right-side out for basolateral membrane vesicles (BLMV) and brush-border membrane vesicles (BBMV), respectively. This degree of "purity" was considered adequate to support the use of these membranes in studies of organic cation transport, and represents the norm with respect to the degree of membrane enrichment typically used in studies with isolated epithelial plasma membranes (Murer and Kinne, 1980). It should be emphasized that the purity of a membrane preparation needed to implement a transport study varies depending on the type of question that is being addressed. If a transport system known to be present only in the plasma membrane is being studied, then the membrane preparation may be relatively crude since other membrane fractions will not interfere with the interpretation of the data. However, if one is studying a system whose cellular distribution is unknown or which may have subtle differences between different membrane types, then a highly purified system is necessary (Sachs et al., 1980).

Carrier-mediated transport of OC's by BBMV

The presence of a specific carrier-mediated transport system for a particular substrate or class of substrates in isolated membrane vesicles is typically recognized after the following criteria have been met: 1) Saturability - the transport must reach a maximum velocity, indicating that all carriers are transporting substrate maximally; 2) Specificity - the transport system should only be inhibited by structural analogs of the substrate; and 3) Trans-stimulation (or countertransport) of tracer substrate flux by the presence of the same, non-radioactively labelled, substrate or an analog of the substrate at the opposite membrane surface (Murer and Kinne, 1980). [Note: Trans-stimulation experiments may be difficult to interpret in that a negative result does not always imply that a compound is not a substrate for the transporter. The kinds of effects seen in trans-stimulation experiments are model-dependent, and thus may give different results depending on the experimental protocol employed.] The presence of an active transport system is verified only by the demonstration of net flux of a substrate against its electrochemical gradient (Christensen, 1975).

Kinsella et al. (1979b) were the first to report specific carrier-mediated transport of an organic cation, NMN, in renal BBMV and BLMV. The transport systems for NMN and para-aminohippuric acid (PAH; an organic anion) were mutually exclusive, and NMN transport was inhibited by other organic cations to varying degrees. The transport of NMN in the BLMV was kinetically distinct from that in BBMV, in that the apparent Michaelis constants for transport (Kt; the concentration of substrate that half-saturates the transport process) and the Jmax (maximal rate of transport) values for NMN transport were different between the two membranes. This group was also the first to report that a concentration gradient of non-radiolabelled NMN on the trans side (the side of the membrane opposite to the side where the radiolabelled substrate is) could accelerate the influx and efflux of radiolabelled NMN in BBMV. Significantly, they saw no trans effects in BLMV. This led to the hypothesis that the active step in transepithelial NMN transport occurs in the brush-border membrane, whereas transport across the basolateral membrane is a passive process. Subsequent studies (Holohan and Ross, 1980) showed that a wide variety of organic cations could drive the concentrative transport of NMN into and out of BBMV, but not BLMV, indicating that these OC's (tetramethylammonium (TMA); TEA; tetrabutylammonium (TBA); mepiperphenidol; and choline) were also substrates for the OC transporter in BBMV. The lack of concentrative uptake in BLMV provided further evidence that OC transport in BLMV was a facilitated diffusion process. A model for transepithelial transport in the proximal tubule was proposed which suggested that OC's enter the proximal tubular cell down their electrochemical gradient by a facilitated diffusion mechanism, and are actively transported across the brush-border membrane into the tubular lumen, although the source of energy for active secretion of OC's was not identified (Kinsella et al., 1979b).

Driving force for OC transport in BBMV.

Early efforts to determine the energy source for OC secretion in kidney slice preparations focused primarily on the effects of metabolic inhibitors. Farah and Rennick (1956) and Farah et al. (1959) demonstrated that metabolic inhibitors including sodium cyanide, sodium azide, and 2,4-dinitrophenol inhibited the uptake of NMN and TEA into dog renal slices. Sung and Johnstone (1965) observed that replacement of sodium in the bathing medium with sucrose inhibited the uptake of choline into rat kidney cortical slices. Further investigation by Holm (1978) on the effects of inorganic ions revealed that the removal of calcium from the bathing medium, or the replacement of sodium with lithium, decreased the uptake of TEA into mouse renal cortical slices.

All of these early studies were difficult to interpret since disruption of cellular integrity could have been producing the effects observed. The energy source for the concentrative transport of NMN into BBMV was thought to be a gradient for some endogenous ion since many OC's had been shown to stimulate NMN transport. Sodium was considered a likely candidate given its role as a co-substrate for glucose and other organic solutes (Hopfer et al., 1973; Samarzija and Fromter, 1976). However, Holohan and Ross (1981) demonstrated that a sodium gradient was ineffective at driving uphill transport of NMN into dog BBMV. Significantly, they demonstrated that a pH gradient (inside lower than outside) could drive the uphill or concentrative transport of radiolabelled NMN into BBMV, suggesting the presence of an OC/H^+ exchange process. The proton gradient effect was not seen in BLMV. The stimulation of transport in BBMV was not due to a catalytic effect of protons on the movement of NMN since the only energy available in the vesicle system was that present in the proton gradient; catalysis alone cannot account for the transient concentrative accumulation of NMN seen in BBMV in the presence of the proton gradient. It should be noted that OC uptake into right-side-out BBMV represents a reabsorptive (rather then secretory) process in the intact proximal tubule. However, Holohan and Ross (1981) and Wright and Wunz (1987b) have shown that OC influx into, and efflux from, BBMV are qualitatively similar processes, and thus uptake of OC's into BBMV is representative of movement of OC's from the cell to the lumen of intact proximal tubules.

Two other observations were noted regarding NMN transport in dog BBMV and helped to clarify the developing model of OC transport across the luminal membrane. First, the establishment of a sodium gradient from inside to outside was also found to stimulate the uptake of NMN into vesicles, but only in the presence of a low buffer concentration. This led to the hypothesis that a Na^+ gradient from inside to outside could generate a proton gradient from inside to outside via the 1:1 exchange of Na^+ and protons through the Na^+/H^+ antiport system found in renal BBMV (Aronson, 1985), and the proton gradient thereby established could in turn stimulate the uptake of NMN. This effect was masked at high buffer capacity since the proton gradient would not be established under those conditions. The physiological implication of this secondary active transport system for OC's is that the energy in the large concentration gradient for sodium reabsorption by the proximal tubule would provide the energy for OC transport by the luminal membrane. Takano et al. (1984) and Rafizadeh et al., (1987) have since gone on to demonstrate that concentrative transport of TEA into rat and rabbit BBMV, respectively, is driven by an outwardly-directed Na^+ gradient, and this uptake was due to the transient generation of an outwardly-directed proton gradient for H^+/TEA exchange.

The second observation was that the electrical potential difference (PD) across BBMV membranes was found not to be a force which supported active transport of OC transport in BBMV (Takano et al., 1984; Sokol et al., 1985; Rafizadeh et al., 1987; Wright and Wunz, 1987b). Thus, proton diffusion potentials generated by pH gradients could be dismissed as a cause for the uphill transport of OC's seen in the presence of an outwardly-directed proton gradients. Furthermore, these observations also suggested that the stoichiometry of OC/H^+ exchange is 1:1, a conclusion that has been supported on both kinetic (Takano et al., 1984; Rafizadeh et al., 1987; Wright and Wunz, 1987b) and thermodynamic (Sokol et al., 1985; Wright and Wunz, 1987b) grounds as well.

Peritubular (or basolateral) OC transport.

Kinsella et al. (1979b) noted a difference in the transport processes between BLMV and BBMV in their initial studies of organic cations in isolated vesicles. Since that time many other groups have reported that OC transport in renal BLMV is a carrier-mediated process, but cannot drive the concentrative accumulation of OC's, i.e., it is not an active transport process (Takano et al., 1984, 1985; Wright and Wunz, 1987b). However, studies employing isolated intact segments of renal proximal tubules have suggested that the transport of OC's across the basolateral membrane is an active process.

In initial studies employing intact proximal tubules, McKinney at al. (1981, 1982) described the active secretion of the organic bases cimetidine and procainamide by isolated perfused proximal tubules. The concentrations of both cimetidine and procainamide in the luminal fluid were 7- to 10-fold higher than the concentrations in the bathing solution, indicating active secretion from bath-to-lumen. Both cimetidine and procainamide secretion were blocked by the presence of the non-radioactively labelled OC's cimetidine, procainamide, quinidine, and N-acetylprocainamide in the bathing medium, as well as by ouabain (an inhibitor of Na^+-K^+-ATPase) and hypothermia (25 °C lower than 37.5 °C). Ouabain inhibition of secretion provided further evidence that the transport of organic bases was linked, at least indirectly, to the electrochemical gradient for Na, as first suggested by Holohan and Ross (1981). Subsequent studies have demonstrated active secretion of TEA in isolated perfused rabbit proximal tubule (Schali et al., 1983) and snake (*Thamnophis* spp.) proximal tubule (Hawk and Dantzler, 1984). Interestingly, the transport of NMN in snake proximal tubules has been shown to differ from that for TEA as it seems to follow a reabsorptive rather than secretory pathway which is separate from that for TEA (Dantzler and Brokl, 1986, 1987).

Uptake studies in isolated, non-perfused proximal tubules in both snake and rabbit indicate that TEA uptake across the basolateral membrane is an active carrier-mediated process, i.e., uptake occurs against an electrochemical gradient for TEA (Schali et al., 1983; Hawk and Dantzler, 1984; Tarloff and Brand, 1986). This observation has so far not been supported by studies using isolated BLMV, and thus the energy requirements of the OC transport step in the basolateral membrane are still unresolved.

Heterogeneity of OC transport along the length of the proximal tubule.

Rennick and Moe (1960) provided evidence that OC transport was localized in the proximal tubule. However, the proximal tubule is composed of three distinct regions: the S1 (early convoluted proximal tubule), S2 (early straight proximal tubule) and S3 (distal straight proximal tubule) regions. Using isolated, perfused segments of proximal tubules McKinney (1982) demonstrated that procainamide secretory rates in rabbit were ranked S1 > S2 > S3 in cortical nephrons, and S1 = S2 > S3 in juxtamedullary nephrons, thereby demonstrating an axial heterogeneity of OC transport along the proximal tubule. The same qualitative results were obtained by Schali et al. (1983) for TEA secretion in isolated perfused proximal tubules, but the rates of uptake in the 3 segments were similar in nonperfused tubules (i.e., a bath-to-cell flux). This implied that the difference in secretory rates was due to differences in the rate of transport at the brush-border membrane. Montrose-Rafizadeh et al. (1987) found that BBMV derived from tissue rich in S1 and S2 segments had a higher Jmax for TEA uptake than BBMV rich in S3, although the Kt values were similar in both BBMV preparations. They interpreted these results as indicating that the differences in transport rates were due to a higher density of OC transporters for TEA in the S1 and S2 segments, since the two preparations had similar total available transport volumes. This may be of importance when comparing kinetic values from membrane preparations isolated by different methods since each method may isolate slightly different segments of the proximal tubule.

It is now generally accepted that OC transport across the brush-border membrane is a carrier-mediated process that is saturable, specific for organic cations, and can be driven by the 1:1 exchange of protons for OC's via a secondary active transport process. The transport process at the basolateral membrane is not so clear, in that data from isolated

BLMV suggest it is a facilitated diffusion process, whereas data from intact tubule studies support an active transport model.

Functional groups on the OC transporter necessary for OC transport.

An early attempt by Holohan and coworkers (1976, 1979) to isolate the OC transporter met with technical difficulty. A purified protein fraction isolated by this group bound both organic cations and anions, and reconstitution of this protein fraction into liposomes resulted in the transport of both PAH and NMN, suggesting that it contained both the anion and cation transporters. Since these experiments no further work on the isolation and purification of the OC transporter has been published. However, some information as to the structure of the transporter has been gained using more indirect methods.

The structural specificity of a transport system for substrate often depends on the types of functional groups at or close to the binding site(s) and translocation pathways for the substrate. Specific modifying agents have been used to gain information as to what types of residues comprise the essential functional groups of the OC transporter. Inui et al. (1984) found that TEA transport in BBMV isolated from cells of the kidney epithelial line LLC-PK1 could be inhibited by $HgCl_2$, a compound known to interfere with sulfhydryl groups on proteins. Sokol et al. (1986) and Hori et al. (1987) demonstrated that OC transport was inhibited by the presence of sulfhydryl-modifying reagents in dog and rat BBMV, respectively. In the rat, this effect was reversed by subsequent treatment with free thiols such as dithiothreitol, glutathione, and cysteine, and was prevented by co-incubation with a large concentration of TEA, which is consistent with the presence of a sulfhydryl group at the binding site for TEA (Hori et al. 1987). However, Sokol et al. (1986) showed that the disulfide reducing agent dithiothreitol inhibited the uptake of NMN in dog BBMV (and this effect was prevented by excess NMN), suggesting the presence of a disulfide moiety at the NMN binding site. These differences may reflect differences in the OC transporter from dog and rat. The presence of an essential tyrosine moiety at the binding site for NMN transport in rabbit BBMV was identified by Hsyu and Giacomini (1987) using the tyrosine-modifying reagents N-acetylimidazole and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl). NBD-Cl also reacts with sulfhydryl groups, and subsequent treatment with 2-mercaptoethanol (a disulfide reducing agent) did not affect the inhibition of NMN transport by NBD-Cl, indicating that a sulfhydryl moiety is probably not present at the NMN binding site in the rabbit. The presence of a carboxylate moiety at the proton binding site in dog BBMV was suggested by Sokol et al. (1987a). The rate of inactivation of NMN transport by N,N'-dicyclohexylcarbodiimide (DCCD; a hydrophobic carboxylate modifying reagent) was not affected by the presence of NMN, leading these authors to suggest that the proton and NMN binding sites were topographically distinct. However, these data are also consistent with the presence of an essential carboxylate moiety elsewhere than the binding site for protons on the transporter since an excess of protons did not protect against inhibition of transport.

Data gathered from the use of "group-specific" modifying agents has to be interpreted with caution, because the observation that a modifying reagent inhibits transport may be merely the result of a nonspecific alteration of the transporter by the reagent, and not a direct effect on an "essential" functional group.

Kinetics of interaction of OC's with the transporter in BBMV.

There are four distinct processes that occur in the carrier-mediated transport of a substrate across a lipid membrane. These are: 1) binding of the substrate to the carrier at one face of the membrane, 2) translocation of the substrate from one face of the membrane to the other through the lipid bilayer, 3) release of the substrate on the opposite face of the membrane, and 4) reorientation of the carrier to the original face of the membrane with or without a new substrate bound. Two of these processes, the binding of substrate to the carrier and the rate of translocation, can be quantitated kinetically. The affinity of the carrier for a particular substrate influences how well that substrate will be transported; if the affinity is high, substrate will bind more readily, and therefore, at a given concentration, have a higher probability of occupying the binding site of the carrier. The apparent affinity of the carrier for a particular substrate is measured by calculating the Michaelis-constant (Kt) for transport of substrate by the carrier. The Kt is the concentration of substrate that results in half-maximal transport. A relative measure of the translocation rate of the carrier through the membrane is given by the Jmax for transport, i.e. the maximal rate of transport. This may differ depending on the substrate that is bound to the carrier. A higher Jmax for one substrate over a second substrate would indicate that the carrier undergoes the conformational changes associated with translocation more efficiently depending on the characteristics of the substrate bound to it. Thus, the structure of the molecule binding to and being translocated by the carrier is important to the effective function of the carrier.

The transport of substrate by a carrier is inhibited by molecules that compete with the substrate for binding (competitive inhibitors). The apparent affinity of the carrier in BBMV for a competitive inhibitor can be estimated by measuring uptake of a radioactively labelled substrate in the presence of increasing concentrations of inhibitor. An estimate of the Ki (inhibitor constant) is given by the concentration of inhibitor that decreases the rate of uptake of a low concentration (<<Kt) of substrate to one-half its maximal value. The equation used to generate a value for Ki for an inhibitor is $Ka = Kt\ (1 + i/Ki)$, where Ka is the apparent Michaelis constant for transport in the presence of some concentration (i)

of inhibitor, Kt is the Michaelis constant for transport in the absence of inhibitor, and Ki is the inhibitor constant.

A number of researchers have determined kinetic constants (Kt, Jmax, or Ki values) for substrates that interact with the OC transporter in BBMV. Kinsella et al. (1979b) reported a Kt for NMN transport in dog BBMV, under pH equilibrium conditions, of 1.6 mM with a Jmax of 20 nmol/mg protein-min, although, more recently, this group (Sokol et al., 1988) reported that the Kt for NMN was 94 μM and the Jmax was 0.45 nmol/mg protein-min in the same preparation. The reason for this discrepancy in the kinetic parameters is unclear and is an issue that should be addressed. In rabbit BBMV, Wright (1985) reported a Kt and Jmax of 0.6 mM and 5.0 nmol/mg protein-min, respectively, for NMN transport under pH gradient conditions. Hsyu and Giacomini (1987) reported Kt and Jmax values of 1.5 mM and 2.0 nmol/mg protein-min., respectively, under pH equilibrium conditions in rabbit BBMV.

The kinetics of TEA transport have been studied in both rabbit and rat BBMV. Both Wright and Wunz (1987b) and Rafizadeh et al. (1987) reported Kt values in the range of 0.12 - 0.33 mM and Jmax values in the range of 5.7 - 8.4 nmol/mg protein-min for TEA uptake into rabbit BBMV under pH gradient conditions (pH 6.0in : 7.5out). Takano et al. (1984) reported Kt and Jmax values of 0.8 mM and 7.4 nmol/mg protein-min, respectively for TEA uptake into rat BBMV under pH equilibrium conditions.

The kinetic basis for the cis and trans-effects of protons has been addressed by Wright and Wunz (1987b) and Sokol et al. (1988). Sokol et al. (1988) reported that the Kt and Jmax for NMN influx into dog BBMV decrease and increase, respectively, in the presence of an outwardly-directed proton gradient. This would indicate that the presence of protons on the inside of the vesicles increases the affinity of the transporter for the binding of NMN on the outside face of the membrane, presumably by producing some sort of conformational change in the binding site for NMN on the opposite face. The inrease in Jmax in the presence of a proton gradient was also observed by Wright and Wunz (1987b) for TEA influx into rabbit BBMV, although there was no effect of pH on the Kt for TEA. This suggests that the carrier reorients to the outside more quickly when a proton is bound (there is a higher chance of a proton being bound when the pH inside is decreased) than when the carrier is empty. The presence of a large cis concentration of protons inhibits the uptake of both NMN (Wright, 1985) and TEA (Wright and Wunz, 1987b) into rabbit BBMV, suggesting that TEA and protons may compete for a common binding site or mutually exclusive set of binding sites, whereas, Sokol et al. (1987a) suggest that the proton and NMN binding sites are topographically distinct in dog BBMV. These differences in the effects of protons on the kinetics of OC transport may reflect differences in the characteristics of NMN and TEA transport as reported by Dantzler and Brokl (1986, 1987) in the snake, or more likely, differences between the transporters in dog and rabbit. It is clear that very little comparative kinetic data has been generated for the OC transport system in renal BBMV, and thus, the functional characteristics of the transporter are still unclear.

Structural specificity of OC transport

Cis-inhibition of uptake of substrate by competitive inhibitors of the transport process has been shown to provide useful information about the substrate specificity of transport systems (e.g. Wright et al., 1980; Stevens and Wright, 1985). Measuring the uptake of a single substrate, and ranking the inhibitory effectiveness of a variety of competitive inhibitors, can provide information as to what types of molecules the carrier has the highest affinity for. A competitive inhibitor is a compound whose inhibitory effects can be overcome by increased concentrations of substrate, in accordance with the theory of mass action. Although a number of compounds have been shown to inhibit the transport of OC's such as NMN and TEA in BBMV, the relative affinity of the transporter for these compounds has not been systematically investigated. Most studies have shown the effects of one concentration of a number of different inhibitors on the uptake of either NMN or TEA into BBMV. This has provided a qualitative estimate of the inhibitory effectivenenss of these OC's, but not a quantitative ranking of how well the transporter binds these compounds. For example, Wright and Wunz (1987b) reported that 1 mM amiloride and 1 mM cimetidine were more effective inhibitors of radioactively labelled TEA transport into BBMV than 1 mM concentrations of either non-radioactively labelled TEA or NMN, suggesting that the OC transporter has a higher affinity for the former two compounds than the two latter compounds. Takano et al. (1985) reported that 10 mM cimetidine inhibited TEA uptake into rat BBMV more effectively than 10 mM TEA, although use of such high concentrations of inhibitors made it difficult to draw quantitative conclusions about the inhibitory effectiveness of these compounds. Determination of apparent Ki values for a series of inhibitors allows a more accurate ranking of inhibitory effectiveness, as well as providing the opportunity to establish quantitative criteria to describe substrate-transporter interaction. For example, Wright (1985) showed that the apparent inhibitory constants (Ki's) for tetramethylammonium (TMA), TEA, and tetrapropylammonium (TPA) on NMN transport were 0.95, 0.19, and 0.02 mM, respectively. This suggested that increasing the length of the alkyl chain around the ammonium nitrogen increased the affinity of the transporter for the inhibitor. This was the first real attempt to probe the structural specificity of the OC transporter in BBMV, i.e., to determine what types of molecules the OC transporter will interact with.

It is important to note that inhibitors of transport need not serve as transported substrates themselves. There are several examples of transport inhibitors that merely bind to a transport receptor (for example: phlorizin inhibition of sodium-coupled glucose transport; or amiloride inhibition of the Na^+/H^+ exchanger). Thus, the question of whether an inhibitor serves as a translocated substrate is a separate, but important, issue with respect to the structure-activity interaction of a compound at a transporter. The most common experiments used to determine whether a compound is translocated are countertransport studies, because the test compound does not have to be radioactively labelled. Trans-stimulation (or countertransport) of the flux of a radioactively labelled substrate by a concentration gradient for a test compound oriented in the opposite direction is consistent with transport of the two compounds by the same carrier. A number of groups have demonstrated countertransport of OC's in both BBMV and BLMV (Holohan and Ross, 1980; Wright, 1985; Wright and Wunz, 1987b; Rafizadeh et al., 1986,1987). The positive stimulation of uptake under these conditions was interpreted as evidence that the carrier is able to bind both substrates at either face of the membrane, although McKinney and Kunnemann (1985, 1987) offered an alternative hypothesis. They suggested that stimulation of 3H-procainamide and 3H-cimetidine uptake produced by a trans concentration of unlabelled procainamide involved the development of a secondary proton gradient via procainamide/H^+ exchange, which then drove the H^+/OC exchange to drive the labelled OC in the BBMV. In other words, they suggested that the transporter is asymmetrical in its ability to bind substrate, i.e., can bind only protons on one face and substrate on the opposite face. However, Wright and Wunz (1987b) demonstrated that protons and OC's compete for binding at the external face of the exchanger in rabbit BBMV, implying that protons and OC's are able to bind to the same site, or at least on the same side of the exchanger.

There are several key assumptions implicit in the use of countertransport experiments as a means of demonstrating that two substrates share a common transport pathway. First, there is the assumption that a fully-loaded carrier translocates through the membrane at a faster rate than an unloaded carrier. However, if the rate constants for translocation of an unloaded carrier are very large, then the countertransport will not be observed, i.e. the trans concentration of test compound will not stimulate the translocation rate of the carrier over the rate in the absence of the trans substrate (Stein, 1986). Thus, a conclusion as to whether the test compound is transported can only be made if the experiments yield a positive result, because the rate constants for translocation of an unloaded carrier are not known. A negative result, on the other hand, only indicates that the question is still unanswered. Second, a problem in the design of some countertransport experiments measuring influx of tracer substrate (i.e. pre-equilibrating BBMV with the test compound) is the carry-over of test compound into the transport buffer when the BBMV suspension and transport buffer are mixed (see METHODS for details of transport studies). If the concentration of the test compound in the vesicle suspension is high the carry-over concentration may also be large, and could produce cis-inhibition of tracer uptake. Thus the stimulatory effects of the trans test compound may be balanced or overcome by the inhibitory effects of the carry-over. Control experiments should be carried out in the presence of the same cis concentration of test substrate that will be carried over in the preload experiments. This has not always been done and may have led to erroneous interpretations of the data.

In the work comprising my thesis research I have addressed two issues. First, I have investigated the structure-activity relationships of a variety of inhibitors of TEA transport in rabbit renal BBMV. Two homologous series of molecules, a tetraalkylammonium series, and an N^1-alkylpyridinium series, were used to systematically test the effects of increasing hydrophobicity around the positively-charged nitrogenous nucleus of these inhibitor molecules on the uptake of TEA into BBMV. This provided quantitative information on the affinity of the transporter for a number of organic cations. Second, once it was established that a compound was an inhibitor of OC transport, it was of interest to determine whether that compound was actually translocated by the transporter. Translocation was demonstrated for a few of the OC inhibitors studied.

CHAPTER TWO

METHODS AND MATERIALS

Brush Border Membrane Vesicle Preparation

This renal brush border membrane vesicle (BBMV) preparation is a modification of that first described by Kinsella et al. (1979a), and adapted by Wright and Wunz (1987a). New Zealand White rabbits were killed with an intravenous overdose of sodium pentobarbital ($\simeq$ 125 mg/kg). The kidneys were cleared of blood by perfusion in situ with $\simeq$ 30 ml cold sucrose-HEPES (HS-2) buffer (250 mM sucrose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffered to pH 7.0 with tris(hydroxymethyl)aminomethane (TRIS)). The kidneys were dissected free and the outer cortical membrane was removed. The cortex was dissected free from the medulla and papilla, chopped with a razor blade, and this cortical starting material was weighed and added to a fixed volume of ice-cold HS-2 buffer (150 ml per 2 kidneys). This suspension was homogenized, while sitting on ice, using an OCI Instruments Model 17105 Omni-Mixer set at maximum speed for 4 minutes. The homogenate was poured into centrifuge tubes and centrifuged in a Beckman Model J2-21 centrifuge with a JA-20 rotor at 1090 x g for 10 minutes (all centrifugation steps were performed at 4 ^{o}C). The supernatant, containing plasma and some intracellular membranes plus soluble cell constituents, was collected and centrifuged at 9800 x g for 10 minutes. The supernatant and the top loose pellet were collected and centrifuged at 48400 x g for 20 minutes. The supernatant, which is enriched in both brush border and basolateral membranes, was then poured off and the pellet resuspended in a divalent cation buffer (5 mM $CaCl_2$, 5 mM $MgCl_2$, 100 mM mannitol, 25 mM HEPES-TRIS, pH 7.0), using a Dounce homogenizer and type A pestle, with the volume of buffer calculated as 5 ml of divalent cation buffer per gram of renal cortical starting material.

This incubation with divalent cations results in a selective aggregation of basolateral and intracellular membranes, leaving brush border membranes in free suspension (Schmitz et al., 1973). The divalent cation resuspension was incubated in an ice bath for 45 minutes, after which the suspension was centrifuged at 1480 x g for 12 minutes to pellet out the aggregated basolateral and intracellular membranes. The resulting supernatant was collected and recentrifuged at 48400 x g for 20 minutes, providing the brush border membrane-enriched pellet. The supernatant (containing soluble proteins) was discarded and the pellet was resuspended in the desired buffer by homogenizing the pellet with a large volume ($\simeq$ 40 ml) of the desired buffer using a CSA "Caframo" Stirrer Type RZR1 glass-Teflon homogenizer at a setting of 4 for 10 strokes. This resuspension was centrifuged at 48400 x g for 20 minutes, yielding the final brush border membrane pellet. The pellet was then resuspended in a small volume of the desired buffer, using the glass-Teflon homogenizer at a setting of 4 for 10 strokes, (the exact compositions of the resuspension buffers for each experiment are outlined in the figure legends) to yield the final membrane preparation. The membrane preparation was left on ice overnight for use in transport studies the following day. The amount of membrane protein in a vesicle preparation was determined using the Bio-Rad Protein Assay with bovine gamma globulin as a standard. The concentration of protein was consistently 15 - 40 mg/ml.

The purity of the BBMV preparation was assessed by Wright and Wunz (1987b) using enzymatic markers. Alkaline phoshatase and trehalase are enzymes commonly used as markers for the brush border membrane of the renal proximal tubule; Na^+-K^+-ATPase and K^+-dependent p-nitrophenylphosphatase (K-PNPPase) are marker enzymes for the basolateral membrane; and succinate dehydrogenase (SDH) is an enzyme used to assess mitochondrial contamination. The BBMV preparation was enriched 11.4 ± 0.77 and 14.2 ± 1.76-fold with alkaline phosphatase and trehalase activities, respectively, over the activities present in the initial homogenate. The preparation was enriched 0.8 ± 0.29 and 0.4 ± 0.31-fold with Na^+-K^+-ATPase and K-PNPPase activities, respectively, and SDH activity was increased 0.7 ± 0.18-fold. These data indicate that the vesicle preparation is highly enriched with brush border membranes and has very little contamination by basolateral membranes or mitochondria.

Measurement of Brush Border Membrane Transport

Influx Studies

The uptake of radiolabelled substrate into brush border membrane vesicles (BBMV) was measured using a rapid filtration technique with a single manifold filter apparatus, as described previously (Wright et al., 1983). Ninety microliters (μl) of a transport buffer containing the radiolabelled substrate and the desired extravesicular salt and buffer composition (the composition of the transport buffers is outlined in the figure legends) was added to the bottom of a 10 x 75 mm plastic test-tube. Ten μl of vesicle suspension was added to the side of the tube, above the level of the transport buffer, and at "time zero" the tube was vortexed to mix the vesicle suspension with the transport buffer. The transport reaction was stopped at the appropriate time point by adding one millilitre (ml) of ice-cold iso-osmotic stop solution (150 mM KCl, 5 mM HEPES at pH 7.5) to the reaction tube and vortexing. One ml of the stopped solution was then rapidly filtered under vacuum through a Millipore 0.45 μM HAWP nitrocellulose filter, and the filter was rinsed once with four ml of ice-cold stop solution. The filter was transferred to a scintillation vial to which was added six ml of scintillation cocktail. The radioactive substrate on the filter was determined by a Beckman LS 3801 liquid scintillation counter using an external standard to correct for quench. Nonspecific binding of substrate to the vesicles and the filter was determined by adding 1 ml of ice-cold stop solution to the transport buffer before adding the vesicle suspension and filtering this solution directly after mixing. Any radioactivity associated with the filter would thus be due to reactions other than the uptake of radiolabel into BBMV. Diffusion of substrate into the vesicles was determined by measuring the uptake of substrate in the presence of 20 mM tetraethylammonium (TEA; a compound known to be a substrate for the organic cation transporter, with a half-saturation constant (Kt) of approximately 150 μM), because this saturating concentration of TEA would effectively block any transport-mediated uptake of substrate into the BBMV (Wright and Wunz, 1987b). Uptake of substrate into vesicles was calculated as moles of substrate taken up per milligram of protein. All transport measurements were performed at room temperature (22-25 ^{0}C).

Efflux Studies

BBMV were preloaded with radiolabelled TEA by adding the radiolabelled substrate to the final vesicle resuspension solution, and allowing this suspension to incubate for at least 1 hour at room temperature (25 ^{0}C). Studies of the time course of TEA uptake have shown that these conditions result in an equilibrium distribution of TEA across the vesicle membrane (i.e., equal TEA concentrations inside and outside the BBMV). 400 μl aliquots of the vesicle suspension were added to the bottom of plastic test-tubes, and 5 μl of a solution containing 2 - 202.5 mM of a test compound was added as a drop to the side of the test-tube. When mixed with the vesicle suspension, the result was the establishment of an inwardly-directed gradient of the test substrate, with no effective change (< 2%) in the equilibrium distribution of the radiolabelled TEA. At "time zero" the tube was vortexed, and at appropriate time points after mixing a 50 μl aliquot of the reaction mixture was removed and pipetted into 2 ml of an ice-cold iso-osmotic stop solution in borosilicate glass test-tubes. The stopped reaction mixture was vortexed and rapidly filtered under vacuum through 0.45 μM HAWP nitrocellulose filters, which were then rinsed once with 4 ml of the ice-cold stop solution. The filters were transferred to scintillation vials to which was added 6 ml of scintillation cocktail, and the radioactivity measured as described above.

Chemical Synthesis

The N^1-alkylpyridinium compounds were synthesized using the method of Kosower and Skorcz (1960). Pyridine was dried over calcium hydride overnight and distilled. 0.15 moles of the dried pyridine was mixed with 0.10 moles of the appropriate iodoalkane and 25 ml ethanol in a round-bottom flask, and refluxed for 4 to 8 hours using a heating mantle and reflux condensor. Recrystallization of N^1-methylpyridinium iodide (NMP) and N^1-ethylpyridinium iodide (NEP) was carried out in 100% ethanol, and recrystallization of N^1-isopropylpyridinium was carried out by dissolving the crude solid in a small volume of ethanol and adding an excess of cold isopropanol until recrystallization occurred. Purity of the compounds was determined by melting point. The values obtained corresponded to those found in the literature.

Chemicals

^{3}H-TEA-bromide (110 mCi/mmole) and ^{3}H-MPP-acetate (88.4 Ci/mmole) were purchased from New England Nuclear (DuPont). ^{14}C-TEA-bromide (56 mCi/mmole) was purchased from Wizard Laboratories. All other chemicals were purchased from common sources and were the highest quality available.

Statistics

The statistical significance of observed differences between two experimental situations was determined using a Student's t test. Differences at the 0.05 level were considered significant. A non-linear regression algorithm was used for all kinetic analyses of transport.

CHAPTER THREE

STRUCTURAL SPECIFICITY OF OC TRANSPORT

RESULTS AND DISCUSSION

Effect of pH on the time course of TEA uptake into BBMV.

The uptake of ^{3}H-TEA into BBMV under pH equilibrium conditions (pH 7.5in : 7.5out) was comparatively slow and within 15 to 60 seconds reached an equilibrium condition that was constant for at least 1 hour (Figure 1). An outwardly-directed proton gradient (pH 6.0in : 7.5out) stimulated the initial (5 sec) rate of TEA uptake by 4-fold (128.5 ± 7.5 vs. 31.2 ± 2.3 pmol/mg). Furthermore, in contrast to the results seen under pH equilibrium conditions, TEA accumulation at 5, 15, and 60 sec, exceeded that measured at 1 hour by which time the pH gradient was presumed to have dissipated, indicating that TEA was actively concentrated inside the BBMV against its electrochemical gradient. This transient "overshoot" phenomenon indicated that some energy source was driving the uphill transport of TEA into the BBMV. The only source of energy provided in this system was that present in the outwardly-directed proton gradient. One hour uptakes were the same for both the pH gradient and pH equilibrium conditions, indicating that the increased uptakes at the 5, 15, and 60 sec. time points under pH gradient conditions were not due to changes in intravesicular volume. The observations provide good evidence for the presence of a TEA/H^+ exchange system (or a TEA/OH^- cotransport system) in the rabbit renal BBMV, as has been reported previously (Rafizadeh et al., 1986; Wright and Wunz, 1987b). The 1 hour equilibrium uptake of ^{3}H-TEA in the presence of 600 mM sucrose was approximately 30% of the uptake in the absence of sucrose, idicating that approximately 30% of radiolabel associated with the vesicles was due to binding of ^{3}H-TEA.

Inhibition of TEA uptake by other organic cations and bases.

Figure 2a shows the effects increasing concentrations of two non-radioactively labelled OC's (tetrapropylammonium, TPA; and N^1-ethylpyridinium, NEP) in the extravesicular buffer on the uptake of 25 μM ^{3}H-TEA into BBMV. Increasing the concentration of either TPA or NEP in the transport buffer decreased the 3 sec. uptake of TEA. The curve describing the effect of TPA on ^{3}H-TEA transport was shifted left of the curve for NEP, indicating that TPA was a more potent inhibitor than NEP. Figure 2b presents the same data as in Figure 2a in the form of a modified Dixon plot. Due to the fact that the ^{3}H-TEA concentration used in this study ($\simeq$ 20 μM) was much less than the Kt for TEA transport ($\simeq$ 150 μM; Wright and Wunz, 1987b) the x-intercepts of the least-squares regression lines in this plot provide an estimate of the apparent Ki value for that inhibitor (x-intercept = -Ki). The Ki, or inhibitor constant, is the concentration of inhibitor that reduced the uptake of ^{3}H-TEA to one-half its maximal value. The Ki values for TPA and NEP from one representative experiment were 15.0 and 234 μM, respectively. Assuming that the inhibitory effect of these compounds on TEA transport were competitive in nature, these Ki values represent a relative measure of the apparent affinity of the transporter for these inhibitors. Table 1 lists the Ki values for a large number of OC's and bases which were of interest according to their structure and/or their previous use in studies of renal OC transport (see APPENDIX I for chemical structures). All of the apparent Ki values listed in Table 1 were estimated by the method described in figures 2a and 2b.

Table 2a lists the Ki values for a series of inhibitors which are structurally similar to TEA - the n-alkylammonium bromides. The apparent affinity of the transporter for these compounds increased as the length of their hydrophobic side chains increased. Indeed, the affinity of the transporter for TPeA was approximately 10,000 times greater than its affinity for TMA. Thus it was hypothesized that increasing the hydrophobicity surrounding the positively-charged nitrogenous nucleus increases the affinity of the transporter for a compound, as was previously suggested by Wright (1985). This hypothesis was tested by examining the effects of a series of N^1-alkylpyridinium iodides on ^{3}H-TEA transport. A series of these substituted pyridinium molecules were synthesized for this purpose. Table 2b shows that as the length or bulkiness of the alkyl group bonded to the positively-charged nitrogen increased, the apparent affinity of the transporter for that compound also increased. The data presented in Tables 2a and 2b provide evidence that the OC transporter preferentially binds compounds which have a highly lipophilic environment surrounding a positively charged nitrogenous nucleus.

Countertransport of TEA with identified inhibitors.

The inhibitory effects described above provide information concerning the nature of interaction of two series of ligands (substrates) with the substrate binding site on the OC transporter. They do not, however, provide any information as to whether the various inhibitors examined here are themselves substrates for transport. In other words, once bound, which, if any, of the inhibitor-transporter complexes undergo a translocation reaction. This question was addressed for several of the inhibitors used in this study by testing whether gradients of these compounds could stimulate the countertransport of ^{3}H-TEA in a manner analogous to that seen with protons. Pre-equilibrating BBMV in a buffer containing 500 μM TEA, NEP, or NIPP stimulated the initial (5 sec.) rate of uptake of ^{3}H-TEA by 243 ± 16.8, 211 ± 5.9, and 263 ± 29.7 %, respectively, over their respective control values under pH equilibrium conditions (Figure 3). These experiments provide evidence that the energy established in a concentration gradient for each of these substrates can drive the uphill transport of ^{3}H-TEA, similar to the effect of preloading the vesicles with an excess of protons (see Figure 1). Therefore, these data suggest that an antiport system is present which exchanges the preloaded OC with extravesicular ^{3}H-TEA. NMN and NMP were also shown to be substrates for this transporter using countertransport experiments (Figure 3). The nominal (1 hour) equilibrium uptakes for all conditions was similar in both the preloaded and control vesicles, indicating that the intravesicular volumes were the same.

Structure-activity relationships between inhibitors.

The structural similarity between the N-alkylammonium and N^1-alkylpyridinium series' of inhibitors allows a relatively straightforward comparison of their structure-activity relationships with the binding site on the OC transporter. However, the other molecules listed in Table 1 have more complicated structures and are, therefore, more difficult to categorize. 1-methyl-4-phenylpyridinium (MPP) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are structural analogs of the N^1-alkylpyridinium series of molecules, and have Ki values lower than any of the N^1-alkylpyridinium molecules studied here. This suggests that the transporter may prefer to bind a molecule which has a lipophilic substitution on the para position of the pyridinium ring. However, it is not clear whether a 6-membered ring substitution creates a better substrate, or if any large hydrophobic group would confer the increased affinity for binding. MPP has a slightly lower Ki than MPTP, indicating that the conformation of the aromatic pyridine ring (planar) may be preferential to that of the pyrimidine ring containing a double bond which constrains the ring into a partial chair conformation. Paraquat is also stucturally similar to the N^1-alkylpyridinium series. In fact, it is actually two N^1-methylpyridinium molecules linked at their para positions. The Ki value for paraquat (97 μM) is 10-fold lower than that for N^1-methylpyridinium (1.1 mM), suggesting that the paraquat molecule, with its two positively-charged nitrogens, may have a higher chance of binding to a negatively-charged substrate binding site, or that the transporter has greater affinity for compounds with two positively-charged centers. However, NIPP had a lower Ki than paraquat, indicating that the double positive charge is probably not very important. It is likely that the effect of the two positive charges gives the transporter a higher apparent affinity for paraquat than N^1-methylpyridinium due to a higher probability for binding, and that this effect is augmented by an increased hydrophobicity of the molecule. It would be very interesting to measure the relative Ki value of an N^1-ethyl- or N^1-isopropyl-substituted paraquat-type molecule to confirm or disprove this theory. The structure of MPP also closely resembles that of paraquat, yet MPP is a more potent inhibitor of OC transport by approximately 10 times. MPP lacks the second positively-charged nitrogen present on the paraquat molecule, and this provides further evidence that the hydrophobicity of a molecule is what primarily dictates how well the transporter will bind that molecule.

Quinidine and mepiperphenidol (Darstine) have been recognized for many years as inhibitors of organic cation secretion in intact animals and in kidney slice preparations, and of transport in isolated vesicle studies (Rennick, 1981). However, the mechanism of inhibition of these compounds has not been studied in detail. Quinidine is an organic base with a complicated ring structure that has two titratable nitrogens ($pK_1 = 5.4$; $pK_2 = 10$). At pH 7.5 (the extravesicular buffer pH in all inhibitor kinetics experiments) one of the ring nitrogens will be protonated and thus carry a positive charge. The quinidine molecule is relatively nonpolar, with only two small polar functional groups in a large hydrophobic structure. The very small Ki value for quinidine makes it a valuable tool for probing structural specificity requirements of related molecules, such as 4-substituted quinolines.

Mepiperphenidol is composed of a piperidinium ring with a very long hydrophobic substituent. Kinsella et al. (1979b) reported a Ki value for mepiperphenidol of 80 μM on

NMN uptake in dog BBMV in contrast to their reported Kt for NMN of 1.6 mM. Similarly, the mepiperphenidol Ki value of 11 μM reported here is much lower than the Ki value for NMN (1.4 mM), and is also lower than the Kt for NMN of 0.73 mM reported previously in rabbit BBMV (Wright, 1985). Clearly, the OC transporter has a higher affinity for the complex ring structure of mepiperphenidol than for the relatively simple ring structure of NMN. The hydrophobicity of long alkyl substituent may be what makes mepiperphenidol such a potent inhibitor. The effects of the polar hydroxyl group are very likely masked by this large hydrophobic moiety. Propranolol also had a very low Ki value, and its structure is somewhat similar to that of mepiperphenidol in that it has a very hydrophobic structure except for the presence of a single hydroxyl group close to the nitrogen (pK = 9.5). This suggests that a hydrophilic or polar moiety may be an essential part of the substrate binding site, and the transporter may have higher affinity for molecules that contain an hydroxyl group close to (2-4 carbons away from) the positively-charged nitrogen, but which is embedded in a large hydrophobic environment. Procainamide may also fall into this scheme, but it is a smaller molecule with less hydrophobicity which may be the reason that it has a higher Ki value (50 μM). Choline also has an hydroxyl group close to the positively-charged nitrogen, but lacks the large hydrophobic environment of propranolol or mepiperphenidol, and this may be the reason that choline was such a poor inhibitor (Ki = 1.7 mM) compared to the others. Like procainamide and choline, neostigmine and serotonin are smaller molecules with polar substituents, and are more difficult to classify. In the case of these molecules, as with the two series examined in the present set of experiments, a series of very closely-related molecules with progressive and systematic changes in structure need to be studied to get a better understanding of the structure-activity relationship between the inhibitors and the binding site of the OC transporter.

The present results support the hypothesis that the OC transporter has a higher affinity for molecules containing a positively-charged nitrogen surrounded by a highly lipophilic environment than for more hydrophilic molecules. OC's with complex hydrophobic ring structures are very potent inhibitors of the transport of TEA by the OC transporter. A more detailed picture as to what types of molecular structures the OC transporter has greatest affinity for can only be developed by testing a much larger library of compounds for their ability to inhibit OC transport. It is not clear whether molecules containing other positively-charged atoms such as phophorous or sulfur atoms interact with the OC transporter, and this is an issue that should be investigated.

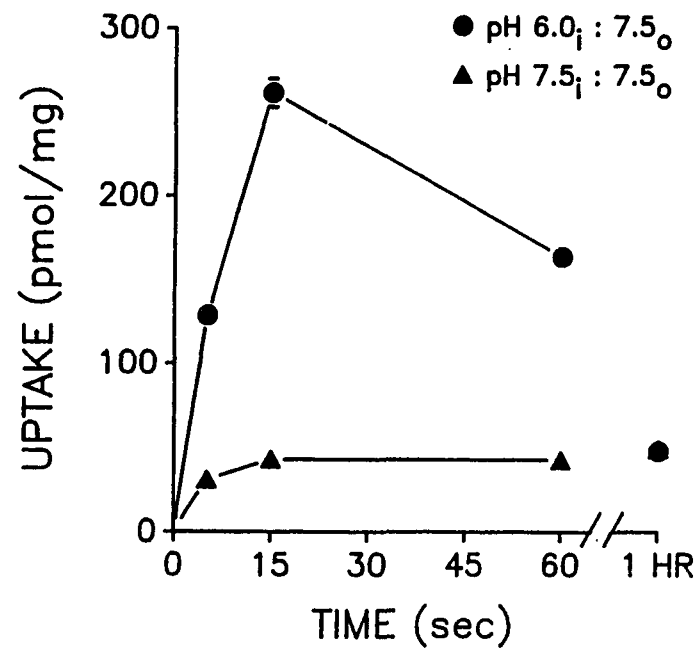


Figure 1. Time course of TEA uptake into BBMV. Vesicles were pre-equilibrated in a solution containing 100 mM KCl, 300 mM mannitol and 5 mM HEPES-KOH at pH 6.0 (●) or pH 7.5 (▲). Uptakes were measured in a transport buffer containing 100 mM KCl, 300 mM mannitol, 5 mM HEPES-KOH and 22 μM ^{3}H-TEA bromide at pH 7.5. Each point is the mean ± SEM of triplicate determinations from a representative membrane preparation.

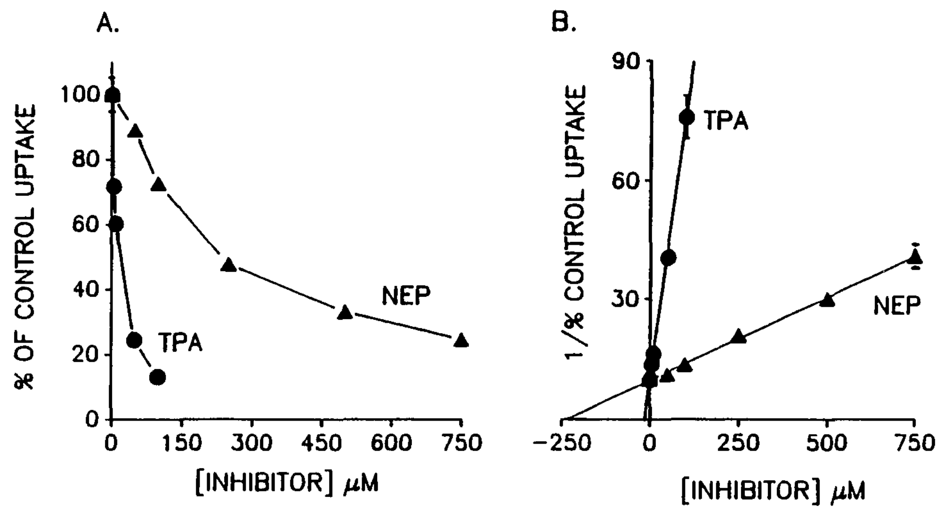


Figure 2. Inhibition of ^{3}H-TEA uptake by other organic cations. a) Vesicles were preequilibrated in a transport buffer containing 150 mM KCl and 5 mM HEPES-KOH at pH 6.0. 3 second uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, 25 μM ^{3}H-TEA bromide and increasing concentrations of either tetrapropylammonium (TPA) bromide (0-100 μM) or N^{1}-ethylpyridinium (NEP) bromide (0-750 μM), at pH 7.5. Each point is the mean ± SEM of triplicate uptakes from a representative membrane preparation. b) Same data as in figure 2a transformed on a modified Dixon plot. The x-intercepts of the least squares regression lines provide an estimate of the negative apparent Ki value for that inhibitor. Apparent Ki's for TPA and NEP from this experiment were 15.0 and 234 μM, respectively.

Table 1. Inhibitor constant (Ki) values for OC inhibitors of ^{3}H-TEA uptake in BBMV. Ki values were calculated by the same method outlined in figures 2a and 2b. Each value represents the mean ± SEM from at least three separate membrane preparations, except where indicated by numbers in parentheses.

Inhibitor	*Inhibitor Constant (Ki) (μM)*
tetrapentylammonium (TPeA)	0.57 ± 0.077
quinidine	1.40 ± 0.31
cimetidine	4.20 ± 0.85
propranolol	7.24 ± 0.87
tetrabutylammonium (TBA)	7.40 ± 1.73
mepiperphenidol (Darstine)	11.07 ± 0.20
1-methyl-4-phenylpyridinium (MPP)	11.45 ± 0.06 (2)
1-methyl-4-phenyltetrahydro-pyridine (MPTP)	20.18 ± 2.22
tetrapropylammonium (TPA)	23.91 ± 5.31
procainamide	50.44 ± 4.63
N^1-isopropylpyridinium (NIPP)	79.78 ± 7.10
paraquat (methyl viologen)	96.78 ± 18.31
tetraethylammonium (TEA)	170.16 ± 15.97
N^1-ethylpyridinium (NEP)	279.43 ± 23.22
serotonin (5-HT)	325.79 (1)
neostigmine	316.96 ± 4.90 (2)
N^1-methylpyridinium (NMP)	1141 ± 147
N^1-methylnicotinamide (NMN)	1443 ± 412
choline	1710 (1)
tetramethylammonium (TMA)	4995 ± 912

Table 2a. Ki values for the N-alkylammonium series of OC inhibitors shown in Table 1. These compound differ only in the alkyl chain length surrounding the positively-charged nitrogen.

Table 2b. Ki values for the N[1]-alkyl-substituted pyridinium series of inhibitors shown in Table 1.

Inhibitor	*Inhibitor Constant (Ki) (μM)*
A.	
TMA	4995 ± 912
TEA	170.16 ± 15.97
TPA	23.91 ± 5.31
TBA	7.40 ± 1.73
TPeA	0.57 ± 0.077
B.	
NMP	1141 ± 147
NEP	279.43 ± 23.22
NIPP	79.78 ± 7.10
NMN	1443 ± 412

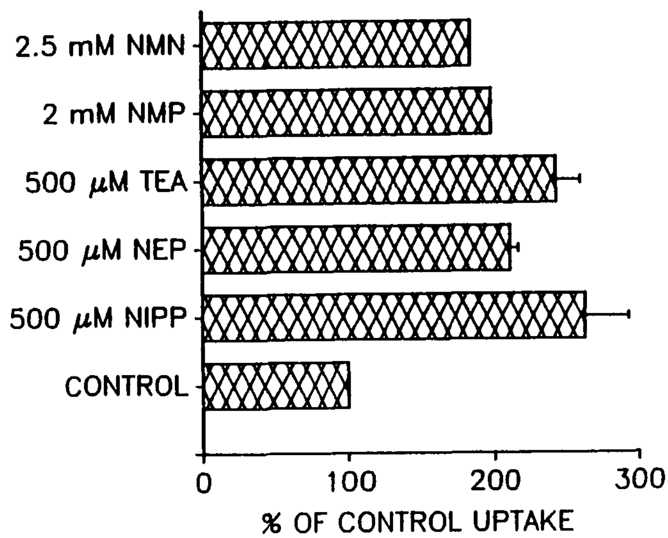


Figure 3. Countertransport of TEA by trans concentrations of some OC's. Vesicles were pre-equilibrated in a buffer containing 150 mM KCl and 5 mM HEPES-KOH (control), plus one of the following: 1) 500 μM TEA bromide; 2) 500 μM NEP iodide; 3) 500 μM NIPP iodide; 4) 2 mM NMP iodide; or 5) 2.5 mM NMN chloride. Control uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, 20 μM ^{3}H-TEA, and one-tenth the concentration of the test compound used to preload the vesicles. This cis concentration of the test compound represented that concentration established by carry-over after dilution of 10 μl of preloaded vesicle suspension with 90 μl of transport buffer in the preload experiments. Each experiment is presented as percent of its own control uptake. Data presented are the mean ± SEM from 3 separate membrane preparations (TEA and NEP); the mean ± one-half the range from two separate membrane preparations (NIPP); or data from a representative experiment (NMP and NMN).

CHAPTER FOUR

TRANSPORT OF 1-METHYL-4-PHENYL-PYRIDINIUM (MPP)

RESULTS AND DISCUSSION

Results

Effect of pH on the time course of MPP uptake.

The uptake of ^{3}H-MPP into BBMV under pH equilibrium conditions (pH 7.5in : 7.5out) was comparatively slow and never exceeded the uptake observed at 1 hour (the nominal equilibrium time point, at which all solute and energy gradients have dissipated) (Figure 4). As was observed for TEA uptake (Figure 1), the presence of an outwardly directed proton gradient (pH 6.0in : 7.5out) stimulated the uptake of ^{3}H-MPP into BBMV (Figure 4). Data from three separate experiments show that the initial (5 sec) rate of MPP uptake in the presence of a proton gradient was stimulated 9.6 ± 2.48-fold over that in the absence of a proton gradient. MPP was accumulated inside the vesicles against its concentration gradient, as indicated by the greater uptake at 5, 15, and 60 seconds than at the 1 hour nominal equilibrium. This transient "overshoot" was not observed in the absence of a proton gradient. In these experiments the only source of energy available to drive uphill transport was the energy in the proton electrochemical gradient, and this represents good evidence for the presence of a H^+/MPP exchange process, or an OH^-/MPP co-transport process, within the BBMV. Addition of 20 mM TEA to the transport buffer under pH gradient conditions completely abolished the proton-stimulated uptake of MPP into BBMV, although the nominal equilibrium uptake was not different from that observed in the absence of 20 mM TEA. These results indicate that MPP uptake can be blocked by a competing substrate. Evidence showing that TEA is a competitive inhibitor of MPP transport is described in a later section.

Kinetics of MPP uptake

The rate of uptake of MPP into BBMV was measured as a function of the concentration of MPP in the transport buffer under pH gradient conditions (pH 6.0in : 7.5out). The uptake consisted of both a saturable and a non-saturable component, and was best described by the equation:

$$J = \frac{Jmax\,[S]}{Kt + [S]} + D[S]$$

where J is the rate of uptake of MPP at a given MPP concentration ([S]) in the transport buffer, Jmax is the maximal rate of MPP uptake, Kt is the Michaelis-Menton constant for MPP in this system (i.e. the concentration of MPP that produces one-half maximal transport) and D is a coefficient that takes into account non-saturable components of uptake (i.e. diffusion and binding). The non-saturable component was corrected for by measuring the uptake of MPP in the presence of 20 mM TEA (approximately 150 times the Kt for TEA transport), which has been shown to saturate the carrier-mediated transport of organic cations via the OC transporter (Wright and Wunz, 1987b). Uptake measured under these conditions is attributed to diffusion and non-specific membrane binding, and was used to correct for non-saturable uptake of ^{3}H-MPP at all other MPP concentrations. This reduced the above equation to a simple Michaelis-Menton relationship for carrier-mediated transport into BBMV (Figure 5). The Michaelis-Menton equation was fit to the data using a nonlinear regression algorithm. The values for Jmax and Kt for MPP transport from three separate experiments were calculated as 3.3 ± 0.22 nmol/mg-min and 7.8 ± 1.59 μM, respectively.

Effect of MPP on proton-driven TEA transport.

The proton-driven antiport of MPP resembled the system identified in rabbit renal BBMV for the uptake of other OC's such as N^1-methylnicotinamide (NMN) (Holohan and Ross, 1981; Rafizadeh et al., 1986) and TEA (Takano et al., 1984; Rafizadeh et al., 1987; Wright and Wunz, 1987b). Furthermore, a large concentration of TEA (20 mM) blocked the uptake of ^{3}H-MPP (Figure 4). Therefore, the effect of MPP as an inhibitor of TEA uptake was examined to determine whether these two substrates shared a common transport pathway. Figure 6 shows the effects of increasing concentrations of unlabelled MPP on the uptake of ^{3}H-TEA. As the concentration of MPP in the transport buffer increased, the uptake of 20 μM ^{3}H-TEA was reduced, indicating that MPP was interacting with the TEA transporter in some manner. Figure 6b presents the same data transformed into a modified Dixon plot. The x-intercept of the regression line in this plot provides an estimate of the apparent negative Ki value for MPP (concentration of MPP that reduced the rate of TEA uptake to one-half maximum). The average Ki value for MPP was 11.5 μM (n=2). As a corollary, increasing concentrations of TEA in the transport buffer inhibited ^{3}H-MPP uptake into BBMV, with an apparent Ki value of 180 μM (n=1).

Interactions between MPP and TEA transport

The observations presented above suggested that TEA and MPP interact at a common transport site. A quantitative test of this hypothesis was made through the measurement of the kinetics of reciprocal inhibition of these two compounds. The rate of TEA uptake was measured as a function of [TEA] in the presence of 25 μM MPP (approximately 3 times the Kt for MPP); and the rate of MPP uptake was measured as a function of the [MPP] in the presence of 150 μM TEA (approximately the Kt value). Figure 7 presents Augustinsson-Woolf-Hofstee plots (J vs. J/S) of the data from these experiments.

The presence of 25 μM MPP in the transport buffer decreased the rate of TEA uptake through an increase in the apparent Kt (461 ± 70 vs. 99.2 ± 2.90 μM), but had no effect on the Jmax (14.3 ± 2.81 vs. 12.4 ± 0.75 nmol/mg-min) of the transport process. Similarly, the presence of 150 μM TEA in the transport buffer decreased the rate of MPP uptake through an effect on the Kt (18.8 ± 2.1 vs. 7.8 ± 1.59 μM) with no effect on the Jmax (3.8 ± 0.27 vs. 3.3 ± 0.22 nmol/mg-min). The Ki value for MPP on the uptake of TEA was calculated using the equation: $Ka = Kt\ (1+i/Ki)$, where Ka is the apparent Michaelis constant for TEA uptake in the presence of MPP and Kt is the Michaelis constant for TEA determined in the absence of a competing organic cation. The Ki value for MPP on the uptake of TEA was 7.68 ± 1.95 μM, which is similar to the value of Kt for MPP (7.8 μM), suggesting that MPP is indeed a competitive inhibitor of TEA uptake. The Ki value for TEA on the uptake of MPP was determined to be 113 ± 35.2 μM, which compared reasonably well with the Kt for TEA (99.2 μM), and with the Kt of 150 μM previously reported (Wright and Wunz, 1987b).

Countertransport of MPP and TEA

A common test of whether two substrates share a common transport pathway is to measure the ability of one substrate to trans-stimulate the transport of the second compound. Therefore, experiments were performed to test the mutual trans effects of MPP and TEA on the uptake of these two OC's. When BBMV were preloaded with 1 mM TEA the initial (5 sec) rate of uptake of both ^{3}H-TEA and ^{3}H-MPP were stimulated over that seen under non-preloaded conditions (Figure 8). However, preloading BBMV with 50 μM MPP did not stimulate the initial rate of uptake of ^{3}H-TEA over control conditions, although it did slightly stimulate the initial rate of uptake of ^{3}H-MPP. The failure of intravesicular MPP to stimulate TEA uptake could have been due to problems inherent with the design of these countertransport experiments. If the turnover rate for the transporter with MPP bound is slow compared to the turnover rate which occurs when the transporter is occupied by either TEA or a proton, then preloading the BBMV with MPP could have no stimulatory effect or, indeed, could trans-inhibit uptake of TEA. Thus, the protocol used in the experiments shown in Figure 8 is limited in its predictive ability because although a positive result (i.e. trans-stimulation) supports the hypothesis for a common transport pathway, a negative result could have several possible explanations. These include: 1) that the turnover rate for the countertransported substrate may be comparatively slow, 2) that the substrate may merely bind to the transporter to block translocation, or 3) that the compound may not be a substrate for the transporter.

An alternative experimental protocol was used to try to provide more definitive evidence as to whether MPP could drive countertransport of TEA. BBMV were pre-equilibrated with ^{3}H-TEA after which a small volume of a concentrated solution of the countertransport test compound was added to an aliquot of BBMV suspension (creating a concentration gradient for the test compound from outside to inside the BBMV). Addition of the test compound resulted in a less than 2% change in the extravesicular concentration of ^{3}H-TEA at time zero, i.e. ^{3}H-TEA was still effectively at an equilibrium distribution. The time course of the content of ^{3}H-TEA inside the vesicles was then monitored after addition of the test compound. If the test compound was a substrate for the same transporter as TEA, then the concentration gradient of the test compound from outside to inside the BBMV would be predicted to provide the energy to drive TEA out of its equilibrium condition via a test compound/TEA exchange process. On the other hand, if the test compound were to merely bind to the transporter and block the transport of TEA, then no change should occur in vesicle TEA content. The loss of TEA from vesicles due to changes in osmolarity after the addition of the test compound (which was always less than 2.5 mOSM) was ruled out by adding a small volume of concentrated KCl to the control vesicle suspension. Figure 9 presents the results obtained from experiments using MPP and NMN as countertransport test compounds. NMN is an OC that has been studied extensively as a substrate for the OC transporter (Kinsella et al., 1979b; Holohan and Ross, 1980, 1981; Wright, 1985), and was used as a positive control. NMN decreased the content of ^{3}H-TEA by as much as 35% (at 30 sec) over control. MPP also decreased the content of intravesicular TEA by up to 19% (at 15 sec), thereby supporting the conclusion that MPP is a substrate for a common OC transporter with TEA.

Discussion

1-methyl-4-phenylpyridinium (MPP) is an organic cation implicated in the onset of a premature Parkinson's-like syndrome in some illicit drug users (Langston et al., 1983). MPP proved interesting in studies of OC transport in rabbit BBMV because of its chemical structure (see Chapter 1). Furthermore, it has been shown to be a potent inhibitor of OC transport (Sokol et al., 1987b). Of particular interest was the suggestion by Sokol et al. that MPP is a non-transported inhibitor of OC transport. Based on its structural similarity to the N^1-substituted pyridinium molecules, which were shown to be transported (Chapter 1), this suggestion was surprising. Thus, independent experiments were performed to determine the behaviour of MPP at the OC transporter.

The present results indicate that MPP is transported by the OC transporter in BBMV via a H^+/MPP exchange mechanism (Fig. 4), similar to the mechanism previously described for other organic cations such as: NMN (Holohan and Ross, 1981; Wright, 1985), TEA (Wright and Wunz, 1987b; Rafizadeh et al., 1986;), amiloride (Wright and Wunz, unpublished observations), procainamide (McKinney and Kunnemann, 1985), and cimetidine (McKinney and Kunnemann, 1987; Takano et al., 1985). These results contradict those reported by Sokol et al. (1987b), and this is probably attributable to the fact that the present experiments measured the uptake of ^{3}H-MPP directly into BBMV, whereas the previous work had been done using non-radioactively labelled MPP in countertransport studies, which may lead to erroneous conclusions (see below). Sokol et al. (1987b) did show that the effects of MPP are specific for the OC transporter.

On kinetic grounds, TEA and MPP appeared to share a common transport pathway (Figures 6 and 7). First, the two substrates displayed the characteristics of mutual inhibition. Increasing concentrations of TEA in the extravesicular buffer reduced the 3 sec. uptake of ^{3}H-MPP into BBMV, and likewise, increasing concentrations of extravesicular MPP reduced the uptake of ^{3}H-TEA. Second, the presence of MPP increased the Kt for TEA transport but had no effect on the Jmax. Similarly, the presence of TEA increased the Kt for MPP transport without changing the Jmax. The observation that the Kt for transport of a substrate is increased in the presence of an inhibitor, without influencing the Jmax is strong kinetic evidence that the two substrates are acting at the same site. Third, proof that two substrates share the same carrier lies in the fact that the Ki and Kt for a substrate should be similar if the substrate can also serve as an inhibitor for the same transporter (Christensen, 1975). The Ki for MPP on ^{3}H-TEA uptake (7.68 μM) was similar to the Kt for MPP (7.8 μM), and the Ki for TEA on ^{3}H-MPP uptake (113 μM) was close to the Kt for TEA (99.2 μM) (Figure 7). Data from independent experiments showed that the Ki for MPP was 11.5 μM (see Table 1) and the Kt for MPP was 7.8 μM (Figure 5). Conversely, the Ki for TEA on MPP uptake was 180 μM, again similar to the Kt for TEA reported above. The Jmax for MPP was approximately 4-fold lower than the Jmax for TEA. Given the evidence that these substrates share a common transport pathway, the difference in Jmax suggests that the turnover rate of the carrier is lower when MPP is bound than when TEA is bound.

Preloading BBMV with 1 mM TEA stimulated the uptakes of both ^{3}H-MPP and 14C-TEA, providing still more evidence that the two compounds share the same carrier. However, preloading with 50 μM MPP did not stimulate the uptake of 14C-TEA, and only slightly stimulated the uptake of ^{3}H-MPP. Sokol et al. (1987b) also found that preloading dog renal BBMV with MPP failed to stimulate uptake of NMN, although MPTP did trans-stimulate NMN transport. Based on this observation they concluded that MPP bound to the OC transporter but was not translocated, whereas MPTP was a translocated substrate. The present results suggest that this conclusion may have been premature. As indicated in the RESULTS section of this chapter it is difficult to interpret a negative result from the protocol used in the experiments presented in Figure 8. Furthermore, in the same experiments it was shown that TEA preloading could drive the countertransport of MPP into rabbit BBMV, and the modified countertransport protocol did show that MPP could drive the transport of TEA against its concentration gradient.

The lack of stimulation of TEA uptake by preloading BBMV with MPP could not be immediately regarded as proof that MPP was not translocated since we had independently shown that in fact MPP was translocated. The problem seems to lie in an inherent defect in the design of these countertransport experiments. If, for example, the turnover rate of the carrier loaded with MPP was much slower that the turnover of the TEA-loaded carrier, then orientation of the carrier from the inside to the outside of the vesicle would be slowed when BBMV were preloaded with MPP. This in turn would result in fewer radiolabelled substrate molecules being allowed entry into the vesicles per unit time via translocation from the outside to the inside of the vesicles, since fewer carriers would be oriented towards the outside. In fact, a very slow turnover rate may actually result in an inhibition of uptake compared to control values if the turnover rate for the transporter with substrate bound was lower than the turnover rate of a proton-loaded carrier. This result was seen for some substrates used in these experiments (ex. paraquat, unpublished observations). The lack of stimulation by MPP was not attributable to carry-over of the trans concentration of the substrate, which could then produce cis-inhibition, because the control experiments were run in a transport buffer containing the same concentration of the inhibitor on the cis side (see Figure 8 legend). The lower Jmax for MPP compared to TEA, and the observation that preloading with MPP did not stimulate the uptake of TEA gives support to the hypothesis that a slow turnover rate could mask a countertransport effect. An alternative explanation could be that the test substrate may be transported through a different carrier, and the concentration gradient for the test substrate may set up a transient proton gradient which then drives the efflux of TEA through the TEA/H^+ exchanger. However, the kinetic evidence presented above indicates that TEA and MPP are transported by the same carrier and this favors the hypothesis that MPP and TEA are counter-exchanged one-for-the-other.

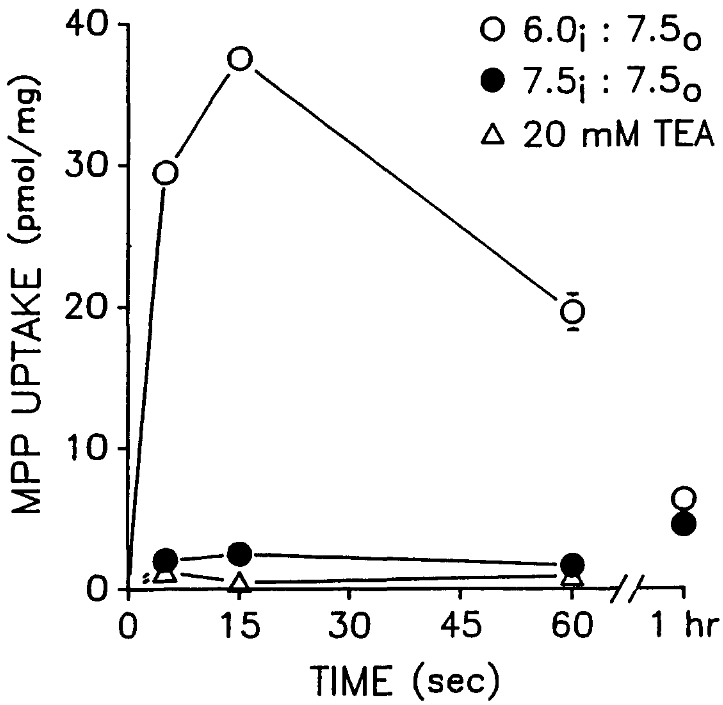


Figure 4. Time course of uptake of MPP into BBMV. Vesicles were pre-equilibrated in a solution containing 150 mM KCl and 5 mM HEPES-KOH at pH 6.0 (o,Δ) or 7.5 (•). Uptakes were measured in transport buffers containing 150 mM KCl, 5 mM HEPES-KOH, 9.0 nM ^{3}H-MPP acetate plus 1 uM unlabelled MPP iodide at pH 7.5 with (Δ) or without 20 mM TEA bromide (o,•). Each point is the mean ± SEM of triplicate determinations of uptake from a representative experiment.

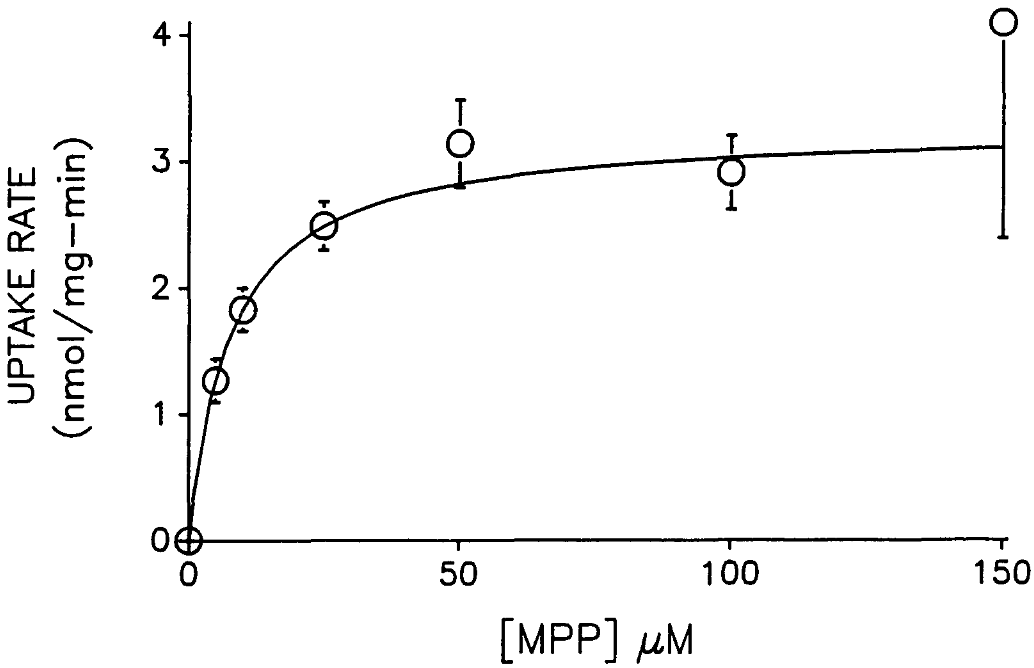


Figure 5. Effect of increasing extravesicular MPP concentration on the rate of MPP uptake into BBMV. Vesicles were pre-equilibrated in a solution containing 150 mM KCl and 5 mM HEPES-KOH at pH 6.0. 3 sec. uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, 9.0 nM ^{3}H-MPP acetate and increasing concentrations of unlabelled MPP iodide (0-150 μM). The non-saturable component of ^{3}H-MPP uptake was determined by measuring uptake in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH 9.0 nM 3H-MPP acetate and 20 mM TEA bromide, and was subtracted from all other uptake values. Each point represents the mean ± SEM of uptakes calculated from three separate membrane preparations.

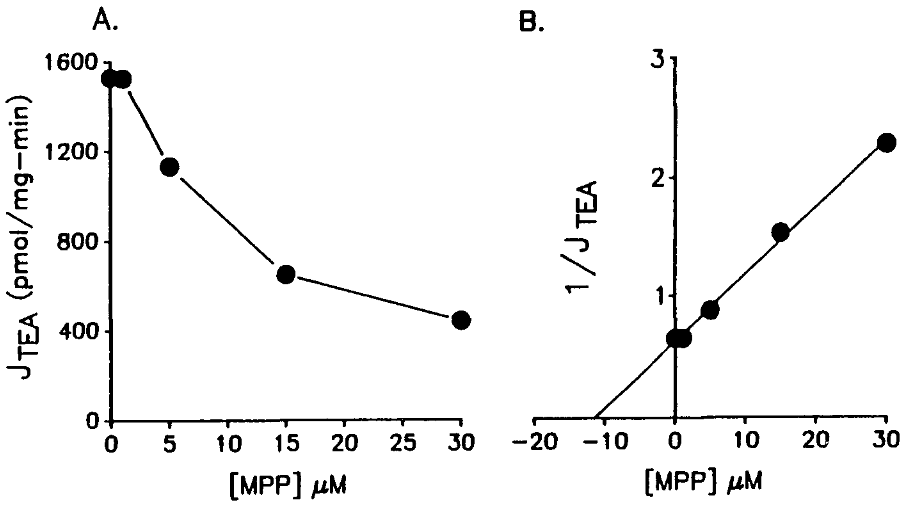


Figure 6. A) Inhibition of uptake of 20 μM ^{3}H-TEA by increasing extravesicular concentrations of MPP. Vesicles were pre-equilibrated in a buffer containing 150 mM KCl and 5 mM HEPES-KOH at pH 6.0. 3 sec. uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, 20 μM ^{3}H-TEA, and increasing concentrations of MPP iodide (1-30 μM). Only carrier-mediated uptake is expressed (total uptake minus that determined in the presence of 20 mM TEA bromide). Each point is the mean ± SEM of triplicate uptake determinations from a representative experiment. B) Modified Dixon plot of data presented in Figure 6A. The x-intercept of the least-squares regression line is 11.4 μM, and provides an estimate of the apparent inhibitor constant (Ki) value of MPP against TEA transport.

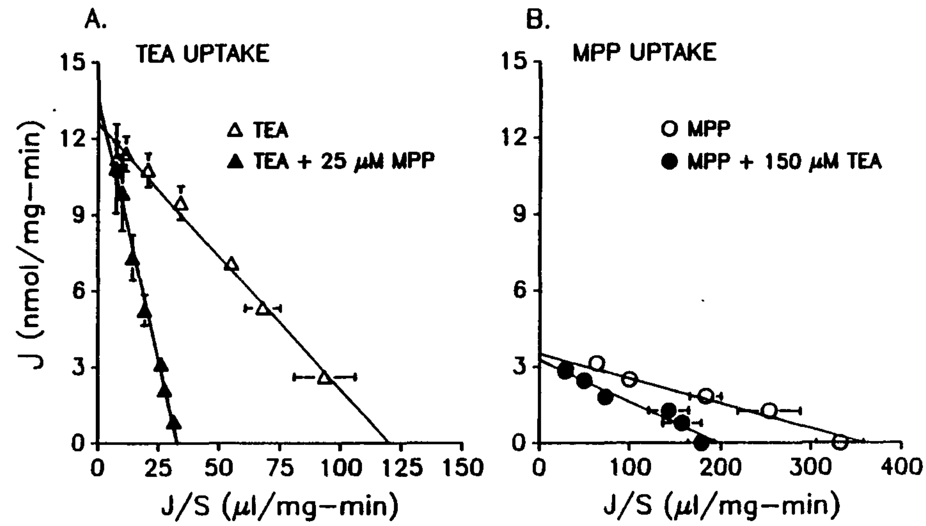


Figure 7. A) Effect of extravesicular MPP on the kinetics of TEA uptake. Vesicles were preequilibrated in a solution containing 150 mM KCl and 5 mM HEPES-KOH at pH 6.0. 3 sec. uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, 25 μM MPP iodide, 25-36 μM 14C-TEA, and increasing concentrations of non-radioactively labelled TEA bromide (0-1500 μM). Only carrier-mediated uptake is presented (total uptake minus uptake determined in the presence of 20 mM TEA bromide). The y-intercept provides and estimate of the Jmax, and the negative slope of the least-squares regression line provides an estimate of the Kt for transport under each condition. Each point is the mean ± SEM from 3 separate experiments. B) Effect of extravesicular TEA on the kinetics of MPP uptake. Vesicles were preequilibrated in same solution as in 7A. 3 sec. uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, 150 μM TEA bromide, 9.0 nM ^{3}H-MPP acetate, and increasing concentrations of non-radioactively labelled MPP iodide (5-150 μM). Only carrier-mediated uptake is presented. Each point is the mean ± SEM from three separate experiments.

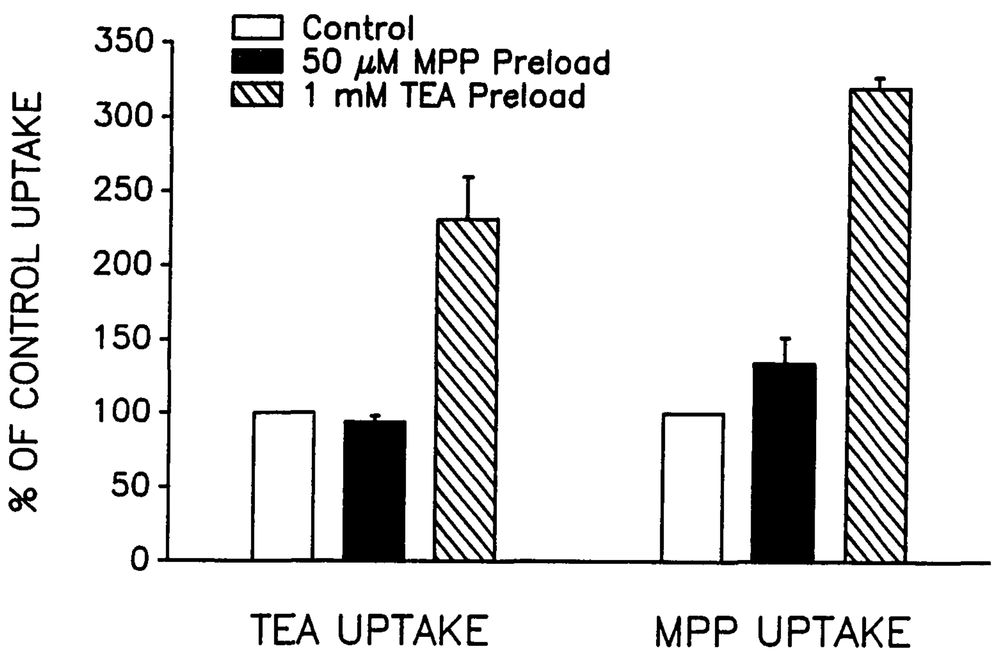


Figure 8. Countertransport with TEA and MPP. Vesicles were preequilibrated with 150 mM KCl and 5 mM HEPES-KOH at pH 7.5 (control), with 50 μM unlabelled MPP iodide (MPP preload), or 1 mM unlabelled TEA bromide (TEA preload). 5 sec. and 1 hour uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, and either 30 μM ^{14}C-TEA or 9 nM ^{3}H-MPP. Control uptakes were measured in a transport buffer containing 150 mM KCl, 5 mM HEPES-KOH, and either 5.0 μM unlabelled MPP iodide (MPP control) or 0.10 mM unlabelled TEA bromide (TEA control). Each bar represents the mean + SEM from three separate membrane preparations.

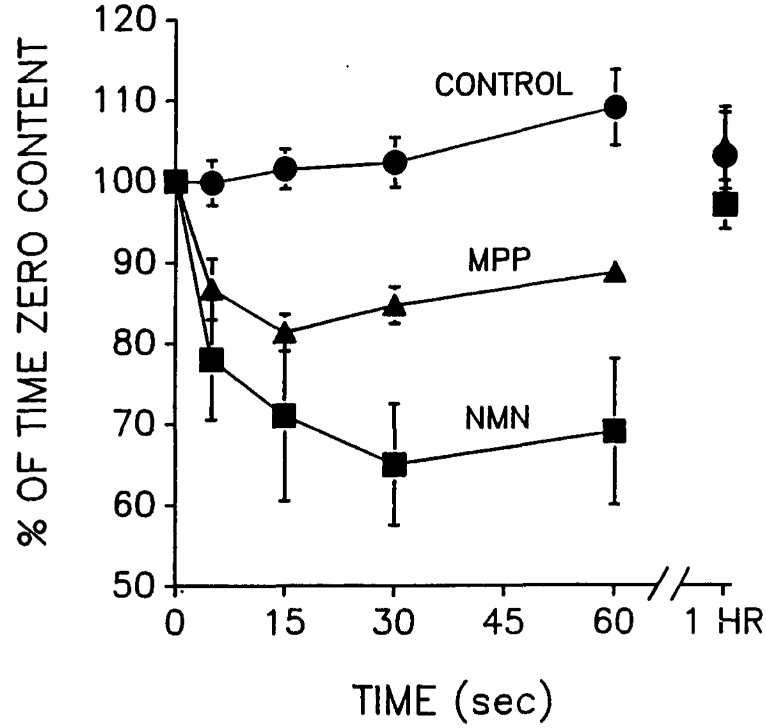


Figure 9. Net efflux of TEA from BBMV driven by trans concentrations of organic cations. Vesicles were pre-equilibrated with 150 mM KCl, 5 mM HEPES-KOH, and 25 μM ^{3}H-TEA bromide. The initial (time zero) equilibrium ^{3}H-TEA content of the vesicles was determined by adding 50 μl of the vesicle suspension to 2 ml of ice-cold iso-osmotic stop solution, which was then vortexed and filtered through a 0.45 μM Millipore filter under vacuum, after which the filter was rinsed with 4 ml of ice-cold stop solution. 5 μl of a concentrated solution of the test compound (202.5 mM KCl for controls, 8.1 mM MPP iodide plus 194.4 mM KCl for MPP, and 202.5 mM for NMN chloride), was then added to the side of a test-tube containing 400 μl of vesicle suspension, and vortexed. This resulted in extravesicular (cis) concentrations of 100 μM and 2.5 mM for MPP and NMN, respectively, which changed the cis concentration of ^{3}H-TEA by less than 2%. 50 μl samples of the vesicle suspension were removed at the indicated time points and added to 2 ml of ice-cold stop solution and filtered as above. Data are expressed as vesicle content of ^{3}H-TEA as a function of time after addition of the test compound. Each point represents the mean $\pm$ SEM from 4 (control) or 3 (MPP) membrane preparations, or the mean $\pm$ one-half the range (NMN) from 2 membrane preparations.

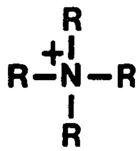
CONCLUSION

This thesis has presented the first rigorous evaluation of the structure-activity relationships at the organic cation transporter in rabbit renal BBMV. Evidence has been presented to show that many of the inhibitors of OC transport are also translocated substrates, and in particular, that contrary to a previous report, MPP is a translocated substrate for the OC/H^+ exchanger.

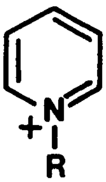
The degree of hydrophobicity surrounding the positively-charged nitrogenous nucleus of an inhibitor molecule is correlated to the affinity of the transporter for that inhibitor. In both the N-alkylammonium and N^1-alkylpyridinium series' of molecules, systematically increasing the length of the alkyl chains bonded to the positively-charged nitrogen dramatically decreased the Ki value of the inhibitor. Organic cations with complex lipophilic ring structures are potent inhibitors of the OC transporter, further supporting the argument that hydrophobicity is an important prerequisite for high affinity interaction with the transporter. However, a much larger library of compounds must be studied before a more detailed picture of the structure-activity relationship between the transporter and substrate can be mapped out.

The importance of quantitating and ranking the inhibitory effectiveness of compounds that interact with the OC transporter was observed in commonly used countertransport experiments. The magnitude of the Ki value for an inhibitor will dictate what concentrations of that compound may be used effectively in countertransport studies, and thus help investigators to interpret a negative result more effectively. This will hopefully lead investigators to understand the importance of running control experiments that take into account the carry-over of an inhibitor from the vesicle suspension into the extravesicular transport buffer.

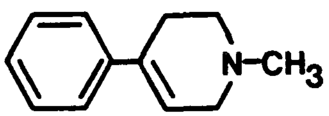
The experiments presented here showing net efflux of radioactively-labelled substrate from vesicles in the presence of an extravesicular concentration of test substrate represent the first time such experiments have been employed in OC transport studies. This experimental protocol will help investigators determine which OC's are transported substrates, and which are only able to bind to the transporter, but do not undergo a translocation step. The importance of this new experimental protocol became obvious in the characterization of MPP transport, where a previous study had suggested that MPP was not a translocated substrate. The experiments performed in the previous study were repeated and the same results obtained. However, independent evidence that MPP was translocated led to the development of the new countertransport protocol, and to the realization that a rigorous characterization of transport of a compound must be carried out before drawing conclusions as to the behaviour of that compound with the OC transporter.



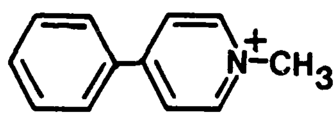
R = CH_3 (TMA)
C_2H_5 (TEA)
C_3H_7 (TPA)
C_4H_9 (TBA)
C_5H_{11} (TPeA)



R = CH_3 (NMP)
C_2H_5 (NEP)
C_3H_7 (NIPP)



1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)



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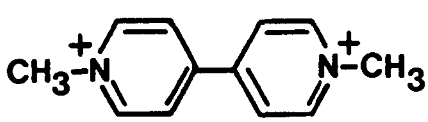

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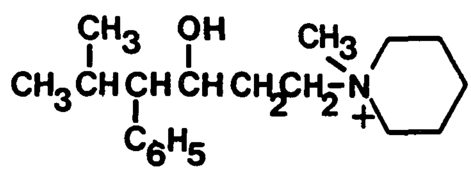
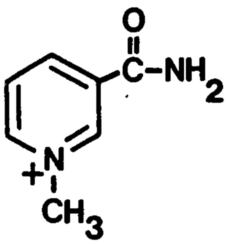


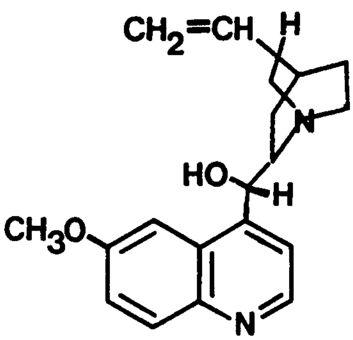
mepiperphenidol

APPENDIX I Structures of chemical inhibitors

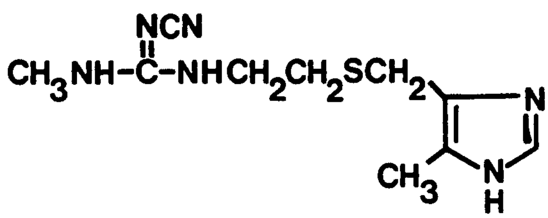


N^1-methylnicotinamide

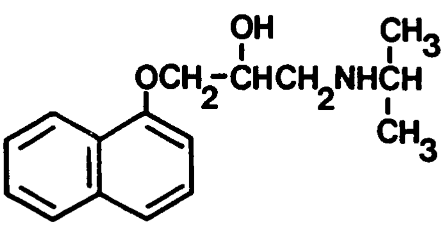
procainamide



quinidine



cimetidine



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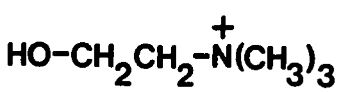
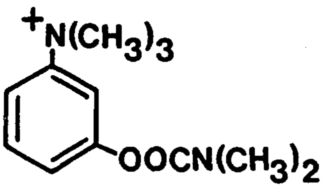
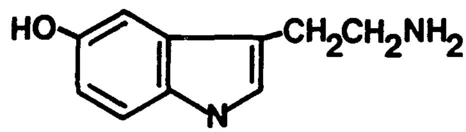


choline

APPENDIX I (cont'd)



neostigmine



serotonin

APPENDIX I (cont'd)

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