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Renal transport of cationic drugs

Author
Hsyu, Poe-Hirr,

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Renal Transport of Cationic Drugs

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Poe-Hirr Hsyu, B.S. (Pharm). National Taiwan University

DISSERTATION

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To

My wife shu-pin

My mother Tseng-Wei

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Abstract

Renal excretion is a major route for drug elimination from the body.and biotransformation. However, few studies have focused on understanding the cellular and molecular mechanisms involved in the renal excretion of drugs. A transport system in the proximal tubule of the kidney is responsible for the active secretion of many basic drugs as well as endogenous compounds. Although recent studies have greatly enhanced our knowledge about organic cation transport in the renal proximal tubule, many questions remain unanswered. The overall goals of the studies described in this dissertation were to further characterize the molecular and cellular mechanisms involved in the transport of organic cations in renal brush border membrane. In addition, we carried out clinical studies to determine whether the renal clearance of basic drugs may be stereoselective. We demonstrated that tyrosine residues are essential for organic cation transport across the brush border membrane; that the transport of organic cations in the brush border membrane (exchange with protons or cotransport with hydroxyl ions) involves a true energetic activation mechanism; and that organic anions interact with the transporter for organic cations in the brush border membrane. In addition, we demonstrated that, similar to hepatic metabolism, renal excretion of drugs may be stereoselective. However, the transport of basic drugs in the brush border membrane (either secretion by the organic cation transport system or reabsorption by the basic amino acid transport system) does not involve stereoselective mechanisms suggesting that transport in the basolateral membrane or biotransformation in the kidney may be responsible for the stereoselective excretion of basic drugs by the kidney.

Chapter 1
Introduction

The kidney is a major organ for the elimination of chemicals from the body. Renal excretion of chemicals may involve several processes including glomerular filtration, active secretion, passive reabsorption and active reabsorption. Depending on the physico-chemical properties of the compounds, one of these processes may be dominant. If a compound is predominantly excreted by glomerular filtration, renal clearance of the compound should approximate the glomerular filtration rate (GFR) which is generally estimated from the renal clearance of creatinine or inulin. On the other hand, excretion by active secretion should result in renal clearance greater than GFR and may approach the renal plasma flow rate which is usually estimated from the renal clearance of p-aminohippurate at low concentrations. Extensive reabsorption of compounds may produce insignificant renal excretion. Reabsorption can be active as exemplified by the reabsorption of endogenous compounds such as glucose, amino acids, sodium chloride, sodium bicarbonate, and ascorbic acid. It is not clear at the present time whether exogenous compounds are actively reabsorbed. If a compound exhibits adequate lipophilicity, it may be reabsorbed by passive diffusion. A compound such as urea may also be reabsorbed by facilitated diffusion. Unionized species of a compound diffuses through the biomembrane more easily. Thus, the renal reabsorption of weak acids and bases may be dependent on the pH of the urine since ionization is a function of pH. For example, methamphetamine, a weak base, is mostly unionized at alkaline pH's and mostly ionized at acidic pH's. Consequently, renal reabsorption of methamphetamine is greater at high urine pH's than at low urine pH's (Beckett

and Rowland, 1965).

History

A transport system for the active secretion of organic cations (primary, secondary, tertiary, and quaternary amines which are positively charged at physiological pH's) in the kidney was first reported in 1947 (Rennick et al.; Sperber). Rennick et al. demonstrated that the renal excretion of intravenously administered tetraethylammonium (TEA) in the dog was 2-2.5 times that of simultaneously injected creatinine, a compound which is predominantly excreted by glomerular filtration. Thus, the data indicated that TEA was actively secreted in the canine kidneys. Sperber (1947, 1948) demonstrated the renal secretion of guanidine, methylguanidine, peperidine and N^1-methylnicotinamide (NMN) in the hen. Since then, renal secretion of numerous organic cations including endogenous and exogenous compounds has been demonstrated in humans, dog, rabbit, rat and chicken (Table 1).

Techniques Used for the Study of Renal Organic Cation Transport

A number of techniques have been used to study the renal excretion of organic cations. Among these, renal clearance, sliced renal cortex, perfused and nonperfused isolated proximal tubules, stop flow, micropuncture, and isolated brush border and basolateral membrane have been the most frequently used.

Renal Clearance

Renal clearance is defined as the ratio of renal excretion rate to plasma

concentration and can be determined by measuring the plasma and urine concentrations of a compound. The method is simple and straightforward. No surgery is required. Net excretory functions of the kidney as a whole can be determined. When a marker for GFR is included in the study, the net secretion or reabsorption of a compound can be calculated. However, reabsorption and secretion cannot be quantitatively differentiated. The use of the technique does not provide information on the location and mechanisms of drug transport.

A variation of the renal clearance method was developed by Sperber (1947) who took advantage of the renal-portal circulatory system of avians which partially supplies blood to peritubular capillaries of the kidney without passing through the glomeruli. A compound injected into one ipsilateral leg vein of a chicken will be secreted by the kidney before entering the systemic circulation. By comparing the amount of the compound excreted in the urine by the ipsilateral kidney to the amount excreted by the contralateral kidney, an apparent tubular extraction fraction can be obtained as the difference in the amount excreted by the two kidneys divided by the infusion rate of the drug. Because a substantial fraction of the compound may be excreted by the kidney before entering the systemic circulation, toxicologic or pharmacologic effects which might interfere with the measurement of renal transport are minimal. This is particularly advantageous when studying the transport of organic cations many of which are pharmacologically potent. The major disadvantage of this technique is that it can be only used in avian species. Findings from avian kidneys may not be applicable to mammalian kidneys.

Renal Cortical Slices

The cortex of the kidney can be cut into thin slices and then incubated in a

physiological medium with radiolabelled substrates. Organic cations accumulate in the slices. Potential inhibitors of organic cation uptake can be studied. The effects of metabolic intermediates and inhibitors of cellular metabolism on the transport of organic cations can be determined. However, cellular binding and transport can not be easily separated. To do so, one must use specific metabolic inhibitors which may interfere with binding processes. The information obtained from studies in renal cortical slices is presumed to reflect the function of the basolateral membrane since the lumen collapses in these preparations. The accumulation of organic cations in the slices is slow and may not relate to the intact kidney. Thus, this technique, although often employed in earlier studies, is now seldom used.

Stop Flow

Stop flow is a relatively simple procedure to locate the transport site of a compound. The ureter of an animal (usually a dog) is catheterized and ligated. An intravenous infusion of an organic compound in mannitol solution is started while the ureteral catheter is clamped shut so that the GFR in that kidney is very close to zero. The clamp is then released and the rapid outflowing urine is collected in serial samples. The first samples represent urine trapped in the most distal portions of the tubule whereas the later samples represent urine trapped in more proximal portions of the tubule. Secreted organic cations are present in increased concentrations in certain samples. The times of collection of these samples infers the location of the secretion site. For example, increased concentrations of a compound in samples which also show increased concentrations of p-aminohippurate, a reference compound secreted at the proximal tubule, may suggest the secretion site is in the proximal areas. Because water is reabsorbed in both proximal and distal parts of the nephron,

inulin is usually co-administered to trace the water movement. This method is excellent for locating the transport site of compounds. However, artifacts from the clamped ureters must be considered. The method is also less accurate for compounds transported in the proximal tubules since the samples must travel over a relatively long distance to reach the catheters. Thus, lateral diffusion and passive reabsorption may obscure the results.

Micropuncture

In micropuncture experiments, a single cortical surface nephron in the exposed kidney is punctured with a micropipet under a microscope. Fluids containing compounds of interest are perfused and collected by separate micropipets located at various distances. A great deal of information can be obtained by this method. However, the technique is tedious and requires skill. The nephrons used are surface nephrons of proximal cortical tubules which may not be representative of all the nephrons. Therefore care must be taken in interpretation of the results.

Isolated Proximal Tubules

A segment of renal tubule can be isolated and simply incubated with radiolabelled substrates (nonperfused) or perfused with a buffer of suitable composition. In the nonperfused tubule, the lumen is collapsed and the data obtained presumably reflect the functions of the basolateral membrane. In the perfused tubule, both brush border (luminal) membrane and basolateral membrane (antiluminal) are functional. Both secretion and reabsorption of compounds can be determined. However, the transport properties of brush border membrane and basolateral membrane can not be studied separately. As with micropuncture, considerable technical skills are required to obtain good

data.

Isolated Brush Border Membrane Vesicles and Basolateral Membrane Vesicles

Isolated renal membrane vesicles have been used in recent studies of organic cation transport. Because of the biochemical differences between brush border and basolateral membranes, these two membranes can be isolated separately. The isolated membranes vesiculate spontaneously and transport of organic cations across the membranes can be studied. The technique is particularly advantageous for studying the transport of drugs because the membrane vesicles are virtually free of enzymes which may metabolize the drugs. The intravesicular and extravesicular media can be manipulated, allowing analysis of driving forces such as pH gradients, potential differences and inorganic ion gradients. The brush border and basolateral membrane can be studied separately. The disadvantages of this method include that contamination by other components of cells cannot be totally eliminated and that the membranes are derived from different cell types which may have different transport properties. The general difficulty in the extrapolation of in vitro data to in vivo situation has to be considered.

All of these techniques have advantages and disadvantages. Collectively, studies using these methods have revealed much of the characteristics of the organic cation transport system (Torretti and Weiner, 1976; Rennick, 1981). The pertinent findings from these studies are summarized in the subsequent paragraphs. Several reviews have been published on the transport of organic cations in the kidney (Peters, 1960; Torretti and Weiner, 1976,; Rennick, 1981; Ross and Holohan, 1983).

Substrates of the Organic Cation Transport System

A list of organic cations which are transported in the kidney along with the technique used in each study is presented in Table 1. Organic cations which are transported in the kidney include compounds from diverse chemical groups. Many of these compounds are important drugs which are frequently prescribed clinically. The endogenous compounds dopamine, epinephrine and norepinephrine and the exogenous compounds cimetidine, morphine, pindolol, procainamide and quinine are known to be transported by the organic cation transport system.

Among these substrates, NMN and TEA (see Fig. 1 for structures) are the two most frequently used compounds in studies of the organic cation transport system. NMN, an endogenous compound, is a metabolite of nicotinamide. TEA is a synthetic molecule. NMN and TEA were the compounds used in the first studies demonstrating that organic cations like organic anions were actively secreted in the kidney (Rennick et al., 1947; Sperber, 1947). These two compounds have been used in recent studies delineating the subcellular transport mechanisms involving the renal transport of organic cations (Sokol et al., 1985; Takano et al., 1984; Tarloff and Brand, 1986). Both compounds are quaternary ammonium compounds and as such exist as cations. Thus, passive diffusion is a minor component in these transport studies. Both compounds are actively secreted in the kidney. Radiolabelled NMN and TEA of high specific activities are available commercially. Thus, NMN and TEA have been extensively used in studies of the organic cation trnasport system. In contrast, three compounds with a high affinity for the organic cation transporter have

Table 1. Compounds Transported by the Renal Organic Cation Transport System

Compound	Technique	Animal	Reference
		Endogenous	
catecholamines	Sperber	chicken	Quebbemann & Rennick, 1969
	clearance	dog	Rennick, 1976
	isolated kidney	rat	Silva et al., 1979
choline	Sperber	chicken	Rennick, 1958
	micropuncture	dog	Acara et al., 1979
histamine	Sperber	chicken	Lindahl & Sperber,1956
NMN	Sperber	chicken	Sperber, 1947
serotonin	Sperber	chicken	Sanner & Wortman, 1962
	Sperber	chicken	Hakin & Fujimoto, 1969
thiamine	Sperber	chicken	Rennick, 1958
		Exogenous	
amiloride	clearance	human	Weiss et al., 1969
	clearance	dog	Baer et al., 1969
amprolium	clearance	dog	Baer et al., 1961
	clearance	dog	Beyer & Gerlarden, 1975
cimetidine	clearance	human	Somogyi & Gugler,1983
	Isolated tubule	rabbit	McKinney et al., 1981

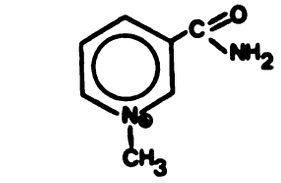
guanidine	Sperber	chicken	Sperber, 1948
hexamethonium	Sperber	chicken	Volle et al., 1960
mecamylamine	clearance	dog	Baer et al., 1956
	stop flow	dog	Pilkinton & Keyl, 1963
mepiperphenidol	Sperber	chicken	Sperber, 1948
	stop flow	dog	Pilkinton & Keyl, 1963
morphine	Sperber	chicken	Watrous et al., 1970
neostigmine	Sperber	chicken	Roberts et al., 1965
	clearance	rat	Roberts et al., 1965
pindolol	clearance	human	Hsyu & Giacomini, 1985
piperidine	Sperber	chicken	Sperber, 1948
priscoline	clearance	dog	Orloff et al., 1953
procaine	clearance	dog, rabbit	Terp, 1951
procainamide	BBMV	rabbit	McKinney & Kunnemann, 1985
quinine	clearance	dog	Torreti et al., 1962
TEA	clearance	dog	Rennick et al., 1947
	cortex slice	dog	Farah & Rennick, 1956
tolazoline	clearance	dog	Orloff et al., 1953
	Sperber	chicken	Kandel et al., 1958

been widely used as inhibitors of this system. These compounds are quinine, cyanine 863 and mepiperphenidol (Fig. 1).

Saturable transport of organic cations has been demonstrated both in vivo and in vitro. Renal clearance of TEA (at low plasma concentrations) in the dog is very close to that of p-aminohippuric acid (PAH) which can be used to estimate the renal plasma flow (Beyer et al., 1950). Rennick et al. (1954) demonstrated that in the anesthetized dog the transport rate of TEA increased with increasing TEA concentration until a maximal transport rate was reached. Sperber (1954) observed a maximal rate of transport (5 μmol/min) of NMN in the chicken. Studies using isolated perfused proximal tubules and isolated membranes also demonstrated a saturable transport mechanism for organic cations (Rennick, 1981; Ross and Holohan, 1983).

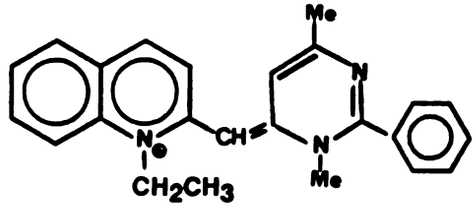
In vivo and In vitro studies have demonstrated competition for transport among the substrates. Renal clearance of NMN was rapid and reversibly decreased by mepiperphenidol in the dog (Kandel and Peters, 1957). Renal clearance of procainamide in humans is decreased by coadministration of cimetidine (Somogyi et al., 1983). Accumulation of organic cations in kidney slices is also inhibited by other organic cations (Peters, 1960). Transport of TEA and NMN in the isolated brush border membrane vesicles and basolateral membrane vesicles is inhibited by other organic cations (McKinney and Speeg, 1982; Wright,1985).

Transport of Organic Cations in Basolateral and Brush Border Membranes

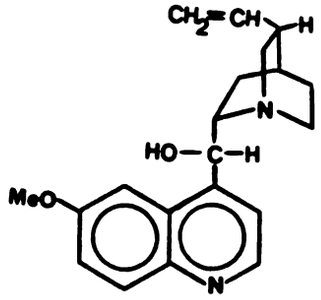


N^1-Methylnicotinamide

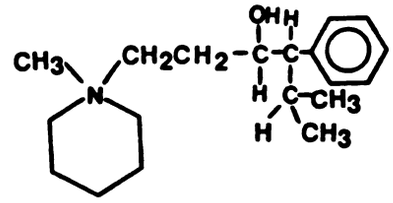
Tetraethylammonium



Cyanine 863



Quinine



Mepiperphenidol

Fig. 1. Structures of N^1-methylnicotinamide (NMN), tetraethylammonium (TEA), cyanine 863, quinine and mepiperphenidol.

For a compound to be secreted in the kidney, the compound has to be transported from the blood across the basolateral membrane of the proximal tubule into the cell and subsequently transported from the cell across the brush border membrane into the tubule lumen. Studies in isolated brush border and basolateral membrane vesicles have greatly enhanced our knowledge of mechanisms involved in the transport of the organic cations in the kidney. Saturable and specific transport of organic cations has been observed in both basolateral and brush border membrane vesicles. However, the kinetics and mechanisms of organic cation transport in these membranes differ from each other. For example, the transporter in the basolateral membrane appears to be a simple substrate system. Manipulation with various inorganic ion gradients does not affect the transport of organic cations in the basolateral membrane (Holohan and Ross, 1981). Thus, transport of organic cations in the basolateral membrane does not appear to be coupled to other ions such as Na^+ or protons. On the other hand, a number of studies have suggested that the transport of organic cations in the brush border membrane involves an organic cation-H^+ exchange (or organic cation-OH^- cotransport) system (Holohan and Ross, 1981,Takano et al.; 1983; Wright, 1985). When an outwardly directed proton gradient is created in brush border membrane vesicles, transport of organic cations is markedly enhanced. An overshoot phenomenon in which the uptake of organic cations temporarily exceeds the equilibrium value has been observed. Although the studies provide some evidence that an organic cation-proton exchange mechanism may be present, studies of proton movements in the presence of organic cation gradients are needed to decide whether a true exchange mechanism is present. In addition, the stoichiometry of this purported organic cation-proton exchange mechanism has to be addressed. Conflicting data in the literature suggest that the organic cation-proton exchanger is

electroneutral (Sokol et. al., 1985) or electrogenic with one organic cation molecule being transported in exchange for two protons (Wright, 1985).

The apparent transport constants Km and Vmax of the transporters in the brush border and basolateral membrane are also different. The Km of NMN in the brush border membrane prepared from the renal cortex of dog is lower than the Km of NMN in the basolateral membrane, whereas the Vmax in the brush border membrane is greater than that in the basolateral membrane (Kinsella et al., 1979). The Km of TEA in the brush border membranes prepared from rat is also lower than that in basolateral membrane, although the Vmax's are similar (Takano et al., 1984). The activation energies for transport of organic cations in these two membranes are also different (Kinsella et al., 1979; Takano et al., 1984).

A model for the transport of organic cations in the proximal tubule is presented in the Fig. 2. Organic cations are transported by a simple facilitated system from blood to proximal cell across the basolateral membrane in accordance with the favorable electrochemical gradient. Organic cations are then transported from the cell to the lumen via an organic cation-H^+ exchange or an organic cation-OH^- cotransport system in the brush border membrane. This process in the brush border membrane is dependent upon the pH gradient which exists across the luminal membrane of the proximal tubule. This pH gradient is created by the Na^+-H^+ transporter located in the brush border membrane or alternatively by the reabsorption of sodium bicarbonate.

Although the studies in vesicles have been generally in good accordance, studies in isolated perfused and nonperfused tubules do not support the

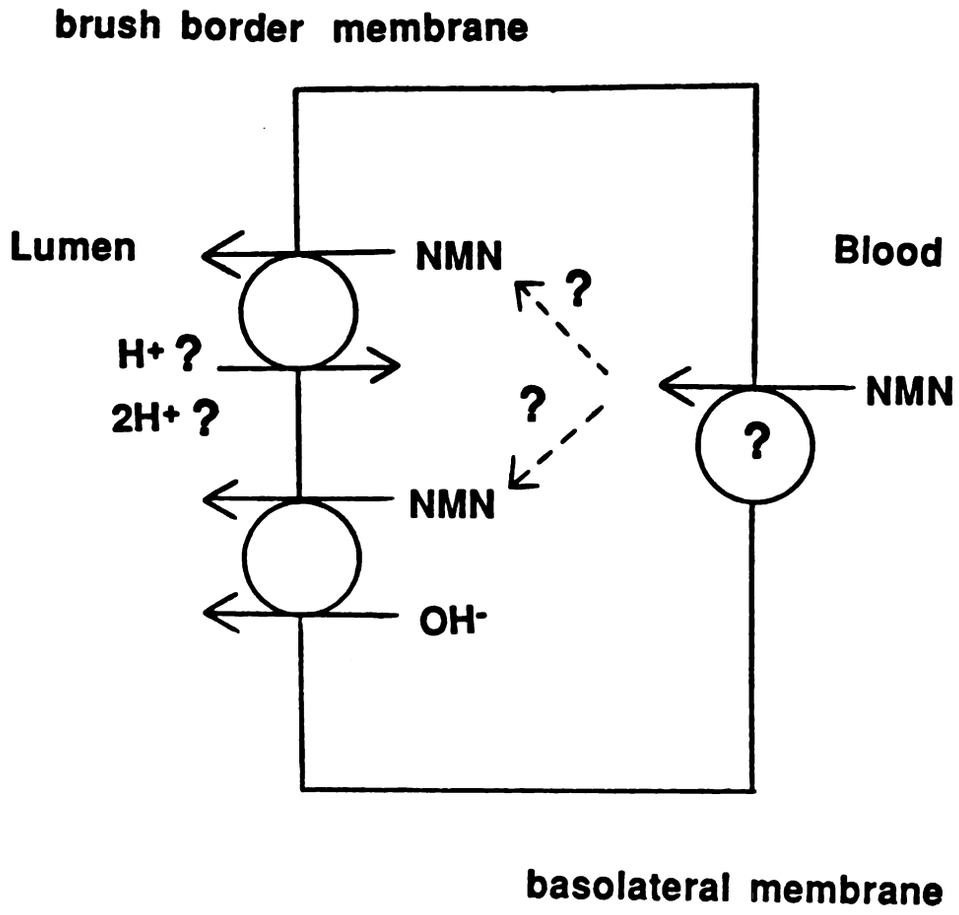


Fig. 2. A model for the transport of organic cations in the epithelial cells of the renal proximal tubule. Organic cations such as NMN are transported from blood to cell across the basolateral membrane in accordance with the favorable electrochemical gradient. NMN is then actively transported out of cell via an organic cation-H+ exchange or an organic cation-OH- cotransport. Some questions remain unanswered. Studies in isolated tubules suggest that organic cations are actively transported across the basolateral membrane. Stoichiometry of the purported exchanger (or cotransporter) in the brush border membrane is still controversial. Both one-to-one and one-to-two (organic cation-to-proton) mechanisms have been proposed.

assumption that transport of organic cations across the basolateral membrane is down an electrochemical gradient. In the isolated nonperfused tubule from rabbit, calculated concentrations of TEA in intracellular water were in excess of 100 times the concentrations of TEA in the bath (Schali et al., 1983; Tarloff and Brand, 1986). Based upon electrical potential differences the cellular concentration of TEA should be approximately 16 times the concentration in the bath. Since binding of TEA appeared to be low (Berndt, 1981; Schali et al, 1983), the data suggest that the step at the basolateral membrane requires energy.

Structure Activity Relationship

The only structural requirements for the substrates of the organic cation transport system appear to be a positive charge and a lipophilic group. Primary, secondary, tertiary and quaternary amines of endogenous and exogenous origins are transported by the system (Table 1). However, a positively charged nitrogen is not specifically required since several onium compounds including positively charged arsenic, antimony and sulfur compounds have been observed to inhibit the renal transport of NMN and TEA (Smith et al., 1967).

When a homologous series of tetraalkylammonium compounds were tested for their potency as inhibitors of NMN transport in the Sperber chicken, the potency of these compounds increased progressively as the length of the alkyl chain was increased from methyl to octyl (Green et al., 1959). For example, TEA was fourfold more potent than tetramethylammonium in inhibiting NMN transport. Similar results were also demonstrated in renal brush border membrane vesicles studies (Wright, 1985). Among dialkylamino compounds,

the compounds with longer alkyl chains also exhibited stronger inhibitory effects on the transport of NMN (Volle et al., 1959). When a hydroxyl group replaced a hydrogen in the alkyl chains of the dialkylamino compounds resuling in decreased lipophilicity, the potency of these compounds was significantly decreased. Increasing length of the alkyl chain (R) in a series of bisquaternary compounds, $^{+}R_3N\text{-}(CH_2)n\text{-}NR_3^{+}$, correlated well with the inhibitory potency of the compounds (Volle et al., 1960). Increasing length of the linking alkyl chain also appeared to increase the potency (Volle et al., 1960). These studies have tested the structural requirements of inhibition of transport and may not relate to the potency of these compounds as substrates.

Collectively, these results may suggest that the transporter for organic cations contains two domains which consists of a negatively charged site and a hydrophobic domain. The negatively charged site binds to the positively charged nitrogen of organic cations and the hydrophobic domain binds to the alkyl chain. Thus, the transporter is specific for cationic compounds and manifests a stronger affinity toward more hydrophobic compounds of a homologous series. No studies regarding the stereoselectivity of organic cation transport have been carried out. Furthermore, the structural requirements for transporters in the two membranes may be different. Studies directed at the structural requirements for transport in each membrane are needed.

Location of the Organic Cation Transporter

Using the stop flow technique, Rennick and Moe (1960) first demonstrated that tetraethylammonium was excreted in the proximal protion of the nephron. Edwards et al. (1961) in a more detailed study showed that the active secretion

of a tertiary amine, mecamylamine, took place in the proximal tubule in dogs. A back diffusion of mecamylamine within the collecting ducts was also detected when the urine was rendered alkaline by infusion of sodium bicarbonate. The transport capacity for organic cations along the proximal tubule is not homogeneous. Schali et al. (1983) using isolated proximal tubule of rabbit kidneys found that the secretion rate of tetraethylammonium was greater in the S1 segment, followed by S2 and S3. These segments are divided according to the morphological differences along the proximal tubule. S1 is the segment attached to the glomerulus and S3 is the segment connected with the loop of Henle.

Effects of Metabolic Intermediates and Inhibitors on the Renal Transport of Organic Cations

Several studies have been carried out in renal cortical slices to test the effects of metabolic intermediates and inhibitors on the renal transport of organic cations (Farah and Rennick, 1956; Farah et al, 1959; LeSher and Shideman, 1956). Basically, acetate, pyruvate and lactate stimulated the uptake of organic cations. Succinate, α-ketoglutarate and octanoate did not affect the uptake of TEA and NMN. Most metabolic inhibitors such as cyanide, malonate, fluoroacetate, iodoacetate and 2,4-dinitrophenol depressed the uptake of TEA or mepiperphenidol. Clearance studies in the dogs (Rennick and Farah, 1956), however, showed that acetate and lactate did not affect the maximal transport rate (Tm) of TEA. 2,4-Dinitrophenol decreased the Tm of TEA, but fluoroacetate and malonate were without significant effect. These findings are difficult to interpret regarding the metabolic requirements of the renal transport of organic cations in intact animals. For example, the stimulating

effect of lactate and acetate on organic cation uptake in cortical slices may be due to increased metabolism of the slices which may not occur in vivo. 2,4-Dinitrophenol, an oxidative phosphorilation decoupler, consistently deceased the maximal transport rate of NMN in the kidney in both in vivo and in vitro studies which may indicate an important role of oxidative phosphorylation in the transport of organic cations in the kidneys.

Molecular Characteristics of the Organic Cation Transporter

Although much information on the transport system in the proximal tubule of the kidney has been obtained, the transport system has not been isolated and purified. Experiments have been carried out in which ^{14}C-dibenamine, an irreversible and specific inhibitor of the renal transport of NMN, was used to label the transporter in renal cortical slices. The labeled dibenamine was found to bind to a specific protein fraction and the fraction was purified by polyacrylamide gel electrophoresis. However, this protein fraction bound to both organic cations and organic anions (Holohan et al., 1976). When the protein fraction was reconstituted to artificial phopholipid membrane vesicles, the transport of both NMN and PAH was enhanced by 45-fold (Holohan et al., 1979). Thus, the protein fraction may represent a copurification of two transporters: the organic cation transporter and the organic anion transporter.

Recently, Sokol et al. (1986) using sulfhydryl side chain modifiers have demonstrated that the transporter for organic cations in the brush border membrane contains essential sulfhydryl and disulfide groups. This work represents one of the first studies of molecular features of the transporter.

Objectives of the Thesis

Although recent studies have greatly increased our knowledge about organic cation transport in the renal proximal tubule, it is clear that many questions remain unanswered. The overall goals of the studies proposed in this dissertation were to further characterize the molecular and cellular mechanisms involved in the transport of organic cations in the renal brush border membrane. In addition we carried out clinical studies to determine whether renal transport of organic cations is stereoselective. Specifically, the following questions were addressed:

(1). Are there essential tyrosine groups present on the organic cation transporter in the brush border membrane?

(2). Does the purported organic cation-proton exchanger represent a true exchange mechanism?

(3). Do organic anions interact with organic cations in the brush border membrane?

(4). Is the renal clearance of basic drugs stereoselective in humans?

(5). Does the organic cation transporter in the brush border membrane exhibit stereoselectivity?

(6). Do basic drugs interact with the basic amino acid transport in the brush border membrane? Is this interaction stereoselective?

Below I will summarize the studies which are presented in this dissertation.

1. Identification of an Essential Amino Acid in the Transporter for Organic Cations in the Brush Border Membrane

Little is known about the molecular features of the organic cation transport

system in the brush border membrane. Although the precise structure of the transporter can only be delineated following its isolation and purification, substantial information may be obtained by more indirect methods such as the modification of essential functional groups with specific modifying agents. Covalent modification has been used extensively to investigate the specific functional groups of proteins (Means and Feeney, 1971). When an essential amino acid residue binds to a modifying agent, the capacity of the amino acid to bind to the substrate is sharply reduced in many cases and the activity of the protein is diminished accordingly. Knowledge of the specific functional groups to which the modifying agent interacts together with careful control of the experimental conditions can provide important molecular information about the transporter. Specific modifying agents have been used to identify histidine residues on the Na^+-H^+ antiporter (Grillo and Aronson, 1986) and tyrosine residues on the Na^+-glucose cotransporter (Lin et al., 1982) in brush border membrane vesicles (BBMV). Essential disulfide and sulfhydryl groups have recently been demonstrated for organic cation transport in renal BBMV (Sokol et al., 1986). In this study the tyrosine selective modifying agents, NBD-Cl and N-acetylimidazole were used to determine whether tyrosine groups may be essential for organic cation transport in the renal brush border membrane. The results suggest that tyrosine residues are indeed essential for organic cation transport and that these tyrosine groups may be located at the binding site of the transporter.

2. Organic Cation Transport in Renal Brush Border Membrane: Studies with Acridine Orange

Although trace uptake studies have demonstrated that the transport of

organic cations in brush border membranes can be stimulated by the presence of a pH gradient and suggest the existence of an organic cation-proton exchanger, several artifacts may explain the proton driven transport of organic cations. For example, creation of a temporary inside negative potential difference by the efflux of protons could have temporarily driven the transport of the positively charged organic cations. Alternatively, a catalytic effect of pH on transport could have produced an overshoot phenomenon. If the organic cation-proton exchange does exist, both proton driven transport of organic cations and organic cation driven proton transport should be demonstrable. A number of studies have demonstrated the proton driven organic cation transport (Holohan and Ross, 1981; Sokol et al., 1985; Wright, 1985). However, no one has yet demonstrated that organic cations may drive the transport of protons. In this study a pH sensitive fluorescent dye, acridine orange, was used to monitor the proton flux in the BBMV in the presence of various gradients of TEA to test whether organic cations could stimulate the transport of protons in BBMV. Our results demonstrated that organic cations can drive the transport of protons in the brush border membrane and support previous studies proposing that protons may exchange with organic cations in the brush border membrane.

3. Interactions of Organic Anions with the Organic Cation Transporter in Renal Brush Border Membrane Vesicles

It should be noted that in the above studies, no distinction has been made regarding whether an organic cation-H^+ exchange or an organic cation-OH^- cotransport is present. In this study we demonstrated that the transport of NMN in BBMV can be inhibited by probenecid and furosemide, two organic anions, and can be enhanced by L-lactate. These results may offer indirect evidence

for the presence of the organic cation-OH^- cotransporter. These organic anions may exert their effects by interacting with the OH^- (anionic) site.

4. Stereoselective Renal Clearance of Pindolol in Humans

One of the important characteristics of a transport system is its stereoselectivity towards substrates. Many transport systems in the proximal tubule are stereoselective. For example, the transport systems for glucose and amino acids are highly selective to endogenous D-glucose (Kinne et al., 1975) and L-amino acids (Silbernagl, 1975). There is little information on the stereoselectivity of the organic cation transport system. In this study, the renal clearance of the enantiomers of a model compound, pindolol, was measured to determine whether organic cations may be stereoselectively eliminated by the kidney in humans. Pindolol is administered clinically as a racemic mixture. Renal clearance of pindolol in humans exceeds the value of glomerular filtration rate indicating that it is secreted (Golightly, 1982). Thus, pindolol is an ideal compound for the clinical studies. The studies involved two steps. First I developed an HPLC assay for the determination of the concentrations of the enantiomers of pindolol in plasma and urine. Then I carried out a clinical study in humans in which the renal clearance of (+)- and (-)-pindolol was determined in normal volunteers taking a dose of 20 mg of racemic pindolol. The results demonstrated that pindolol was indeed stereoselectively cleared by the kidney in humans. The results represent the first study of stereoselective renal clearance of a basic drug and led to further studies examining the underlying mechanisms which may be stereoselective.

5. Stereoselective Studies on the Transport of Organic Cations in Renal Brush Border Membrane Vesicles

In vitro studies using isolated renal brush border membranes prepared from rabbit renal cortex were undertaken to determine the stereoselectivity of the transport of organic cations. Three pairs of optical isomers (enantiomers), mecamylamine, pindolol, and disopyramide, and two clinically used diastereomers quinine and quinidine were studied to determine whether stereoselective interactions may occur. The results demonstrated that all of these compounds inhibit the transport of NMN in the brush border membrane vesicles. However, no stereoselective inhibition was observed.

6. Effects of Pindolol on the Transport of L-Lysine in Renal Brush Border Membrane Vesicles

Studies in the literature and our previous study (Chapter 6) suggest that pindolol may be actively reabsorbed in the kidney. We postulated that the active reabsorption of pindolol may be carried out by the basic amino acid transport system in the renal proximal tubule. The interactions of organic cations with the basic amino acid transport system in brush border membranes were examined. Effects of pindolol on the transport of L-lysine in brush border membrane vesicles were studied. At high concentrations, pindolol significantly inhibited the transport of L-lysine. However, no stereoselective inhibition was observed.

Chapter 2

Essential Tyrosine Residues in the Transport of Organic Cations in Renal Brush Border Membrane Vesicles

Organic cations are transported across the brush border and basolateral membrane of epithelial cells in the proximal tubule by specific, saturable processes (Kinsella et al., 1979; Rennick, 1981; Ross and Holohan, 1983; Takano et al., 1984). Data from studies in membrane vesicles have suggested that the systems involved in the transport of the organic cations, NMN and TEA, across the brush border and basolateral membrane differ in their kinetic properties and driving forces (Kinsella et al., 1979; Takano et al., 1984). The system characterized in the brush border membrane appears to be a proton-organic cation antiport or a hydroxyl-organic cation cotransport (Holohan and Ross, 1981; Takano et al. 1984; McKinney and Kunnemann, 1985; Sokol et al., 1985; Wright, 1985). Studies in brush border membrane vesicles (BBMV) prepared from the kidney of rat (Takano et al. 1984), dog (Holohan and Ross, 1981; Sokol et al., 1985) and rabbit (McKinney and Kunnemann, 1985; Wright, 1985) have demonstrated that in the presence of an outwardly directed proton gradient, organic cations accumulate intravesicularly against a concentration gradient.

Although some information is now known about the cellular features of the organic cation transport system (Holohan and Ross, 1981; Takano et al. 1984; McKinney and Kunnemann, 1985; Sokol et al., 1985; Wright, 1985), little is known about the molecular features of the system. Using ^{14}C-dibenamine and polyacrylamide gel electrophoresis, an attempt was made to label and isolate the transporter for organic cations (Holohan et al., 1976). However, the isolated

carrier-like protein fraction bound not only to organic cations but also to organic anions (Holohan et al., 1976). Following reconstitution of the protein fraction into artificial phospholipid membrane vesicles, transport of both NMN and the organic anion, p-aminohippurate, into the vesicles was greatly enhanced (Holohan et al., 1979). These results suggest a partial purification of the organic cation transporter with the co-purification of the organic anion transporter.

The precise structure of the transporter can only be delineated following its isolation and purification. However, substantial information may be obtained by more indirect methods such as the modification of essential functional groups with specific modifying agents. Covalent modification has been used extensively to investigate the specific functional groups of proteins (Means and Feeney, 1971). When an essential amino acid residue binds to a modifying agent, the capacity of the amino acid to bind to the substrate is sharply reduced in many cases and the activity of the protein is diminished accordingly. Knowledge of the specific functional groups to which the modifying agent interacts together with careful control of the experimental conditions can provide important molecular information about the transporter. Specific modifying agents have been used to identify histidine residues on the Na^+-H^+ antiporter (Grillo and Aronson, 1986) and tyrosine residues on the Na^+-glucose cotransporter (Lin et al., 1982) in BBMV. Essential disulfide and sulfhydryl groups have recently been demonstrated for organic cation transport in the renal BBMV (Sokol et al, 1986).

In this study, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), a tyrosine modifying agent (Fig. 3; Cantley et al., 1978; Lin et al., 1982), was used to

characterize the transporter for organic cations in BBMV prepared from the cortex of rabbit kidneys. N-acetylimidazole, another tyrosine modifying agent (Fig. 3; Lin et al., 1982; Means and Feeney, 1971), was also used in some experiments to confirm the results obtained from the experiments with NBD-Cl. Our data demonstrate that treatment with NBD-Cl as well as with N-acetylimidazole inhibits the transport of organic cations in BBMV. Data are presented suggesting that the inhibition occurs as a result of the interaction with tyrosine residues and that these residues are essential for organic cation transport.

Methods

Preparation of Brush Border Membrane Vesicles

Brush border membrane vesicles were prepared by a method involving addition of divalent cations and differential centrifugation (Booth and Kenny, 1974; Ives et al., 1983). For each preparation one male New Zealand white rabbit weighing 2-3 kg was sacrificed. The kidneys were perfused with 30 ml ice-cold HK buffer (10 mM HEPES and 150 mM KCl, pH adjusted with KOH to 7.4) containing 5 mM EGTA. Cortex was then trimmed from the medulla and homogenized in 200 ml ice-cold HK-EGTA buffer at full speed for four minutes with a tissue homogenizer (Sorvall^{TM} Omni-Mixer, Dupont Co., Newtown, CT). Magnesium sulfate was added to the homogenate to a concentration of 16 mM. The mixture was stirred for 20 minutes at 0^0C and poured into four centrifuge tubes. Brush border membrane vesicles were obtained following a series of centrifugations at 4^0C. All centrifugations were performed in a JA 20 rotor of a Beckman JA-21 centrifuge (Beckman Instruments Inc., San Jose, CA). The first

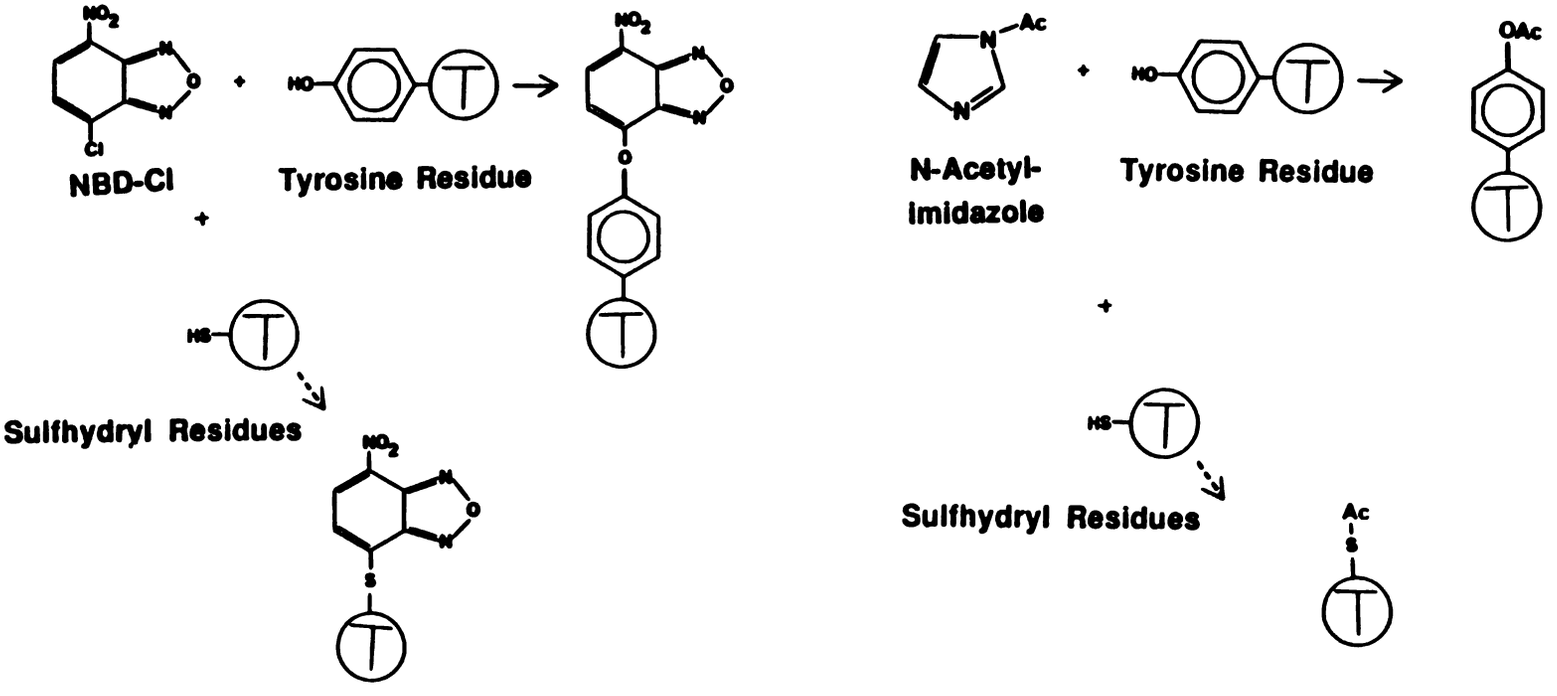


Fig. 3. The interactions of NBD-Cl with tyrosine residues and sulfhydryl residues (left panel). NBD-Cl reacts with the phenolic group of tyrosine residues in the transporter (T) to form ether derivative. NBD-Cl also reacts with sulfhydryl residues in the transporter to form thioether derivative. The interactions of N-acetylimidazole with tyrosine residues and sulfhydryl residues (right panel) are also illustrated.

centrifugation was at 8,000 rpm for 15 minutes. The pellets (p1) were discarded and supernatants (s1) were centrifuged at 17,000 rpm for 30 minutes. The supernatants (s2) were aspirated off. The pellets (p2) were resuspended in HK buffer (20 ml per tube) by forcing the mixture through a 22 gauge needle with a 35 ml syringe. The resuspended mixture was recentrifuged at 8,000 rpm for 15 minutes. The pellets (p3) were discarded and the supernatants (s3) were saved and centrifuged at 20,000 rpm for 25 minutes. The resulting pellets (p4) were resuspended with HK buffer (0.5 ml per tube) through a 27 gauge needle and 1 ml syringe. The suspension was centrifuged at 20,000 rpm for 20 minutes. Pellets (p5) were resuspended with a 27 gauge needle and a 1 ml syringe. The protein concentration of p5, determined by the method of Lowry (Lowry et al., 1951), was adjusted to 20 mg/ml.

Characterization of the Brush Border Membrane

The activities of two marker enzymes: Na^+-K^+-ATPase for the basolateral membranes and maltase for the brush border membranes, were measured in the tissue homogenate and p5 to determine the enrichment of brush border membranes and contamination of basolateral membranes in the preparation. Maltase activity was determined by the rate of glucose release from maltose (Ives et al., 1983; Turner and Moran, 1982) with a commercial kit for the analysis of glucose (Glucose assay vials, No. 15-UV, Sigma Chemical Co., St. Louis, MO). Glucose was converted to glucose-6-phosphate (G-6-P) by ATP in the presence of hexokinase. G-6-P was subsequently oxidized to 6-phosphogluconic acid (6-PG), coupled with the reduction of NADP to NADPH. As NADPH has a high optical absorbance at 340 nm and NADP has no absorbance at this wavelength, the activity of maltase can be monitored by

measuring the rate of absorbance increase at 340 nm. The scheme of the reactions is as follows:

$$\text{Maltose} \xrightarrow{\text{Maltase}} 2\ \text{Glucose}$$

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NADP} \xrightarrow{\text{G-6-P Dehydrogenase}} \text{6-PG} + \text{NADPH}$$

One volume of tissue homogenate was diluted with one volume of HK buffer and one volume of p5 was diluted with 24 volumes of HK buffer. Contents of one Glucose assay vial which included all the ingredients except maltose and maltase were reconstituted with 31 ml of ice-cold distilled water. Maltose was added to 10 ml of the reconstituted assay solution to a concentration of 1mg/ml. Twenty-five µl of the diluted tissue homogenates or the diluted p5 were mixed with one ml of assay solution with maltose, The mixture was taken into a spectrophotometer (Ultrospec 4050, LKB Instruments Inc., Gaithersburg, MD) with an automatic sipper and temperature controller (Model 4070, LKB Instruments Inc.). The optical absorbance at 340 nm was monitored for 10 minutes at 25^0C. Rate of increase of the optical absorbance was calculated as the slope of a plot of absorbance versus time. Control experiments were carried out as described above except that no maltose was added to the assay solution. The rate of absorbance increase of the control was subtracted. The rate was then converted to specific activity of maltase. The enhancement of maltase activity in p5 compared to the homogenate ranged between 8- to 14-fold.

Na^+-K^+-ATPase activity was determined by the method of Schoner et al. (1967). In this procedure, ATP is dissociated to ADP and phosphate (Pi). ADP

then reacts with phosphoenolpyruvate (PEP) to form pyruvate and ATP in the presence of pyruvate kinase. Pyruvate is reduced to lactate which is coupled with the oxidation of NADH to NAD^+ by lactate dehydrogenase. NADH has strong optical absorbance at 340 nm, whereas NAD^+ does not absorb light of this wavelength. Thus, the activity of Na^+-K^+-ATPase can be determined by monitoring the decrease of optical absorbance at 340 nm. The scheme of the reactions is as follows:

$$\text{ATP} \xrightarrow{\text{Na}^+\text{-K}^+\text{-ATPase}} \text{ADP} + \text{Pi}$$

$$\text{PEP} + \text{ADP} \xrightarrow{\text{Pyruvate kinase}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{Lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+$$

A buffer containing 62.5 mM imidazole, 62.5 mM NH_4Cl, 3.125 mM $MgCl_2$ and 1.25 mM EGTA, pH adjusted to 7.3 with HCl (buffer A) was prepared. Reaction mix was prepared by mixing 18.75 ml of buffer A with 2.813 ml of 1 M Na Cl. Cofactor mix containing 3.6 mM NADH, 15.15 mM ATP, 2.06 mM PEP, 100 units/ml pyruvate kinase and 110 units/ml lactate dehdrogenase in ice-cold buffer A was prepared daily. Eight hundred µl reaction mix, 100 µl cofactor mix and 100 µl 50 mM imidazole solution (pH 7.3) were mixed together. Five to one hundred µl of the tissue homogenates or p5 were added to the mixture. The absorbance change at 340 nm of the mixture was monitored for 1 minute. Because ATP is spontaneously dissociated to ADP and Pi even in the absence of Na^+-K^+-ATPase, it was necessary to determine this rate of spontaneous dissociation. This was determined by carry out the experiments as described as above except that ouabain (1 mM) was also present which completely inhibited the activity of Na^+-K^+-ATPase. The rate measured in the presence of ouabain was then subtracted from the rate obtained in the absence of ouabain. The

enhancement of Na^+-K^+-ATPase activity range between 0- to 1.5-fold.

Na^+-glucose cotransport was also monitored to ascertain the presence of functional brush border membranes. Glucose is cotransported with Na^+ in the renal brush border membrane but not in the basolateral membrane. In the presence of an inwardly directed Na^+ gradient, the uptake of glucose in the brush border membrane vesicles is stimulated and glucose temporarily accumulates against a concentration gradient. Five µl of vesicles (20 mg/ml) loaded with HK buffer was incubated with 20 µl of pH 7.4 buffer (150 mM NaCl and 10 mM HEPES, pH adjusted with NaOH) containing 0.175 mM D-glucose and 1.4 µCi D-^{3}H-glucose. Incubations were carried out for 0.5, 1, 2.5, 5, and 60 minutes. The radioactivity associated with the vesicles was measured as described in the section on NMN Transport Studies. The typical overshoot phenomenon (Kinne et al., 1975) was observed in each experiment (Fig. 4).

Modification with NBD-Cl and N-Acetylimidazole

A volume of BBMV (20 mg/ml) was added to an equal volume of HK buffer containing various concentrations of NBD-Cl or 10 mM N-acetylimidazole and incubated at 25^{0}C for one hour. The final concentrations of NBD-Cl were 0, 0.02, 0.1, 0.2, 0.3, 0.5, 1.0, 1.5, and 2.0 mM. In some experiments, 2-mercaptoethanol was added to a final concentration of 2 mM together with the NBD-Cl or N-acetylimidazole solution to prevent the interaction of sulfhydryl residues with the modifying agents (Cantley et al., 1978). The chemicals were then removed by dilution and centrifugation as follows: The incubation solution was diluted twentyfold with ice-cold HK buffer. The mixture was centrifuged at 20,000 rpm for twenty minutes at 4^{0}C. The supernatants were

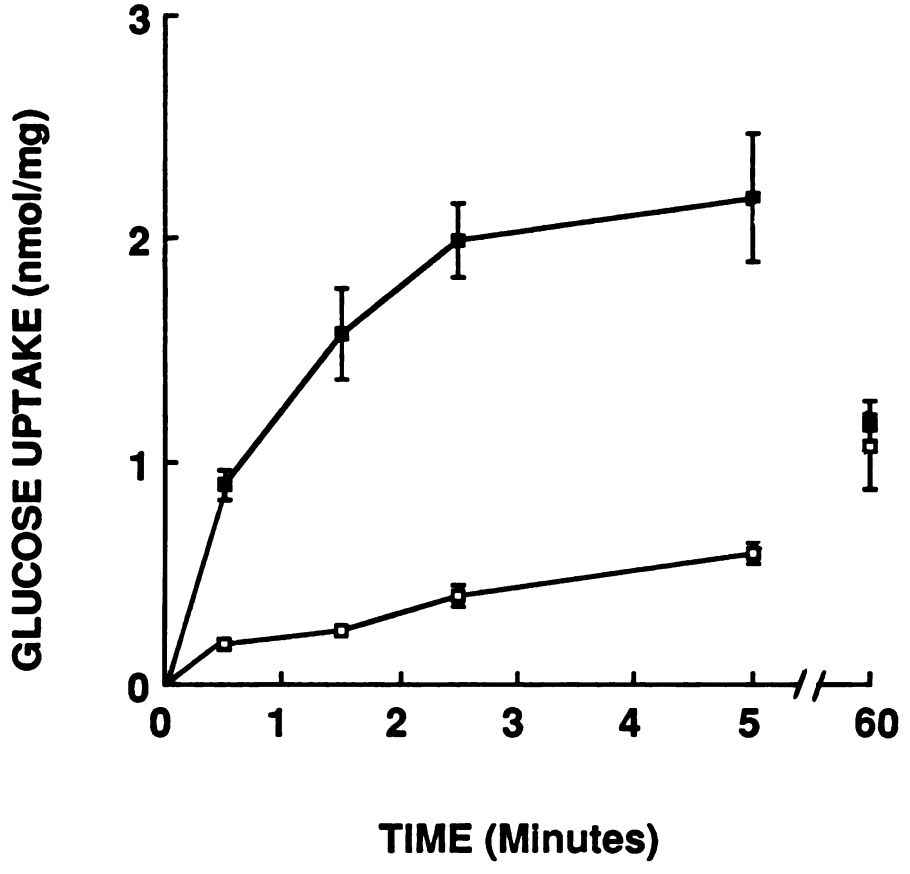


Fig. 4. Glucose uptake in the presence of a Na^+ gradient (■) and in the absence of a Na^+ gradient (□). Vesicles loaded with pH 7.4 HK buffer were incubated with a solution with 0.175 mM D-glucose and 1.4 μCi D-^{3}H-glucose in either pH 7.4 HK buffer or in pH 7.4 HNa buffer (150 mM NaCl and 10 mM HEPES). Each data point represents mean±SE of three experiments carried out in three membrane preparations.

discarded and the pellets resuspended in the same volume of ice-cold buffer and recentrifuged again at 20,000 rpm for 20 minutes. The pellets were collected and resuspended with HK buffer to a final concentration of approximately 20 mg/ml.

For the time dependent interaction of NBD-Cl and BBMV, BBMV were incubated with 0.15 mM NBD-Cl and 2 mM 2-mercaptoethanol for 0, 0.5, 1, 2, 5, 10, 30, and 60 minutes. The chemicals were then removed by dilution and centrifugation as described above. In all the other studies, incubation with NBD-Cl (0.15 mM) or N-acetylimidazole (5 mM) was carried out for one hour at 25^0C and the chemicals removed by dilution and centrifugation as described above.

In the TEA protection experiments, TEA was incubated with BBMV for 10 minutes. An equal volume of NBD-Cl or N-acetylimidazole solution was then added to the vesicles and incubated for one hour. The final concentrations of the chemicals were: TEA, 100 mM; NBD-Cl, 0.15 mM or N-acetylimidazole, 5 mM. The chemicals were removed at the end of the incubation.

Optical Absorbance Change of the Reaction of BBMV with NBD-Cl

A UV-visible spectral scan was carried out in the control and NBD-Cl treated vesicles. A wavelength scan showed that the maximal absorbance change occurred at 450 nm. Therefore, the optical absorbance of the vesicles reacting with NBD-Cl was monitored at 450 nm for one hour. One hundred µl of BBMV was mixed with 400 µl of HK buffer and 500 µl of NBD-Cl (0.3 mM) in HK buffer. The absorbance at 450 nm was recorded for one hour at 25^0C in a

spectrophotometer equipped with a temperature controller.

Uptake Studies

Ten µl of vesicles or HK buffer (as filter blanks) was mixed with 20 µl of HK buffer containing approximately 1.6 µM ^{3}H-N^{1}-methylnicotinamide (NMN) and incubated for various times at 25^{0}C. At the end of the incubations, 3 ml of ice cold HK buffer was added to stop the transport process. The mixture was immediately filtered under vacuum through a membrane filter (0.3 µm, PH type, Millipore Co., Bedford, MA) in a ten place filtration manifold (Hoefer Scientific Instruments, San Francisco, CA). The filter was washed twice with 3 ml ice cold buffer and placed in 10 ml scintillant (Scinti Verse™II, Fisher Scientific Co., Fair Lawn, NJ). Radioactivity associated with the filter was determined in a Beckman LS 7800 liquid scintillation counter (Beckman Instruments Inc., San Jose, CA). The radioactivity associated with the filter blank was determined as above except that 10 µl of buffer was used instead of 10 µl of vesicles. This value was subtracted from the total radioactivity to obtain the radioactivity associated with the vesicles. Counting efficiency ranged between 38 and 40%.

The initial uptake of NMN in the control membranes and the NBD-Cl treated membranes as a function of concentration of NMN was also studied. Unlabelled NMN was added to ^{3}H-NMN (approximately 1.6 µM) and vesicles to obtain final concentrations ranging between 10^{-2} and 10^{-6} M. The uptake was carried out as previously described.

Studies of L-lysine transport in the control and the NBD-Cl treated membrane vesicles were carried out as follows. Ten µl of the control or NBD-Cl

treated membrane vesicles was incubated with 50 µl L-lysine solution (1.2 * 10^{-5} M unlabelled L-lysine and approximately 50 nM ^{3}H-L-lysine in HK buffer, pH 7.4) for 8 seconds and one hour and the uptake of L-lysine in the vesicles was determined.

NMN Uptake in BBMV in the Presence of an Outwardly Directed Proton Gradient

BBMV were incubated in 0.15 mM NBD-Cl or HK buffer for one hour at 25^{0}C, pH 7.4. The vesicles were diluted and centrifuged twice as previously described except that the diluting buffer was pH 6.0 HK buffer. In this way, the pH of the intravesicular space was decreased to 6.0. The final protein concentration of the resuspended BBMV was adjusted to 10 mg/ml. Twenty µl of BBMV was added to 80 µl of pH 7.5 HK buffer containing approximately 1.2 µM of ^{3}H-NMN. The reaction mixture was incubated for 8 and 30 seconds, 1, 2, 5,10, and 60 minutes. Uptake procedures were carried out as described in the Uptake Studies Section.

Studies with Acridine Orange

Acridine orange was used as an "on line" indicator of proton movement (Warnock et al., 1982). Acridine orange is a weak base which binds to the vesicles when an outwardly directed proton gradient is present (Lee and Forte, 1978). The compound fluoresces and its fluorescence is quenched when it is bound to the vesicles. Thus by measuring the fluorescence of acridine orange the movement of protons in the media can be monitored.

Vesicles were incubated with NBD-Cl or HK buffer (as a control) as previously described. The mixture was diluted and centrifuged twice with pH 6.0 HK buffer. The final protein concentration of the preparation was adjusted to 20 mg/ml with pH 6.0 HK buffer. Twenty-five μl of the vesicles were added to 2.5 ml of pH 7.5 HK buffer containing 6 μM acridine orange in a cuvette. The fluorescence of the solution was adjusted to 100% at an excitation wavelength of 492 nm and an emission wavelength of 530 nm. The fluorescence was monitored for 10 minute at room temperature. The maximal change of fluorescence (equilibrium value - lowest value) and the initial rate of fluorescence recovery, determined from the tangents of the linear segments of the curves during the first six seconds after addition of the vesicles, for the control and NBD-Cl treated BBMV were calculated and compared. Fluorescence monitoring was carried out in a fluorescence spectrophotometer (MPF-2A, Perkin Elmer, Norwalk, CN) equipped with a QPD-33 recorder (Perkin-Elmer). Vesicles loaded with pH 7.5 HK buffer were also added to the acridine orange solution as a control.

Determination of Free Sulfhydryl Residues in BBMV

Because NBD-Cl also reacts with sulfhydryl residues and sulfhydryl residues have been recently demonstrated to be essential for the transport of NMN in BBMV (Sokol et al., 1986), it was important to determine whether the inhibitory effect of NBD-Cl on NMN transport was caused by the interaction of NBD-Cl with sulfhydryl groups. We measured the free sulfhydryl residues in untreated BBMV and in BBMV that had been treated with 0.15 mM NBD-Cl. We also measured the free sulfhydryl residues in the BBMV that had been treated with both NBD-Cl and 2-mercaptoethanol (2 mM). Ellman's reagent, DNTB,

was used to measure the free sulfhydryl groups in the vesicles (Tse et al., 1983). Twenty to fifty µl of vesicles containing less than 0.8 mg of protein were mixed with pH 7.4 buffer in a total volume of 400 µl. The mixture was then vortexed with 800 µl of 50 mM Tris-HCl buffer, pH 8.8, and 20 µl of 10 mM DNTB in methanol. After a 60 minutes incubation period at 25^0C, the mixture was centrifuged at 20,000 rpm for 20 minutes to spin down the vesicles. The absorbance of the supernatant was determined at 412 nm. The concentration of sulfhydryl residues was calculated from a standard curve obtained from known concentrations of reduced glutathione ranging from 0 to 60 nM.

Data Analysis

The data describing transport rate versus concentration of NMN were fit to a Michaelis-Menten model (see Appendix A) as follows:

$$\text{Rate} = \text{Tmax} * C / (\text{Km} + C)$$

where Tmax represents the maximal transport rate; Km represents the concentration needed to reach half of the Tmax and C represents the concentration of NMN in the extravesicular solution. The fitting procedure used was the FIT FUNCTION Procedure on the PROPHET computer system which is a nonlinear least squares regression based on iterations (PROPHET Statistics, National Institutes of Health). The data were also fit to the following model:

$$\text{Rate} = \text{Tmax} * C / (\text{Km} + C) + F * C$$

where F is the coefficient for the linear, nonsaturable transport of NMN across BBMV. However, because F was not significantly different from zero in all of the fits, and Tmax and Km were not significantly different from those obtained from the simple model, the first model was used.

Unless otherwise specified, all data points were determined in three replicates and three experiments were performed in three separate membrane preparations for each experiment. Data are presented either as mean±SD of a representative experiment of three qualitatively similar experiments or as mean±SD of three replicate experiments. Details of each experiment are described in the figure legends.

Statistical differences were determined by Student's t-test. When more than two experiments were compared, analysis of variance was used to detect statistical differences among the groups. If statistical differences were observed, the Newman-Keuls multiple range test was used to test if any two groups were statistically different. Probability (p) at the 0.05 level was considered significant.

Chemicals

^{3}H-N^{1}-methylnicotinamide (2.8 Ci/mmole) was purchased from ICN (Irvine, CA). ^{3}H-D-Glucose (30 Ci/mmole) and ^{3}H-L-lysine (97 Ci/mmole) were obtained from New England Nuclear (Boston, MA). All the other chemicals were purchased either from Aldrich or Sigma.

Results

The uptake of ^{3}H-NMN in BBMV as a function of time is depicted in Fig. 5. NMN accumulated in the vesicles with time. Equilibrium was reached in one hour. Also shown is the uptake of NMN in BBMV in which ice-cold distilled water was substituted for the buffer "stop-mix". The amount of ^{3}H-NMN

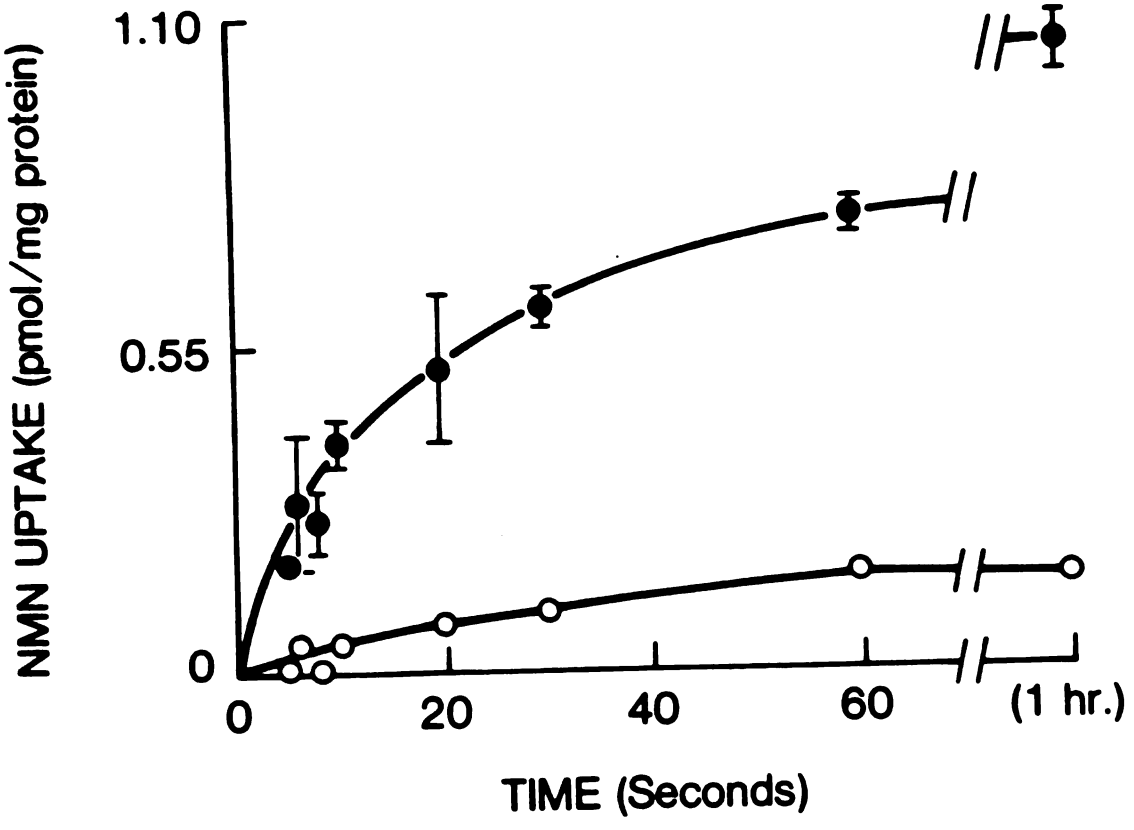


Fig. 5. The time course of NMN uptake in control (●) and lysed (○) BBMV at pH 7.4, 25^{0}C. Data are from a representative experiment. Each data point represents the mean of triplicate measurements. Vertical lines represent ± 1SD. Where the vertical line is absent, the SD is encompassed within the point.

associated with these lysed vesicles presumably represents bound ^{3}H-NMN. The percentage bound at any time can be calculated as the uptake in the lysed vesicles divided by the uptake of ^{3}H-NMN in unlysed vesicles at that time. The percentage bound to BBMV averaged 15% and was not time dependent. This percentage is consistent with the value obtained by Wright (1985) who estimated the binding of NMN to BBMV prepared from rabbit kidney by determining the equilibrium uptake of NMN in increasing osmolarities. Also, Kinsella et al. (1979) reported that binding of NMN was less than 15% in BBMV prepared from dog renal cortex. The 15% was considered minimal and no correction was made for the binding.

The decrease in NMN transport by treatment with 0.15 mM NBD-Cl as a function of incubation time is presented in Fig 6. The transport rate of NMN decreased rapidly and reached the maximal inhibition within one hour. The time to reach half of the maximal inhibition ($t_{1/2}$) was 4.3±3.2 minutes. The change in absorbance at 450 nm of BBMV following incubation with NBD-Cl is also presented in Fig. 6. Initially, the absorbance increased rapidly and approached a plateau at approximately 40 minutes. The maximal change in absorbance at one hour was 0.214±0.007 absorbance unit. The time to reach half of the maximal value was 6.7±1.1 minutes. These two half-lives were reasonably close. BBMV or NBD-Cl incubated with HK buffer for one hour did not show an absorbance change. One hour was selected as an appropriate incubation time for the subsequent experiments.

The Initial transport rate of NMN at 8 second in BBMV as a function of NBD-Cl concentration is shown in Fig. 7. NBD-Cl reduced the transport rate of NMN in a concentration dependent fashion. The highest concentration of NBD-

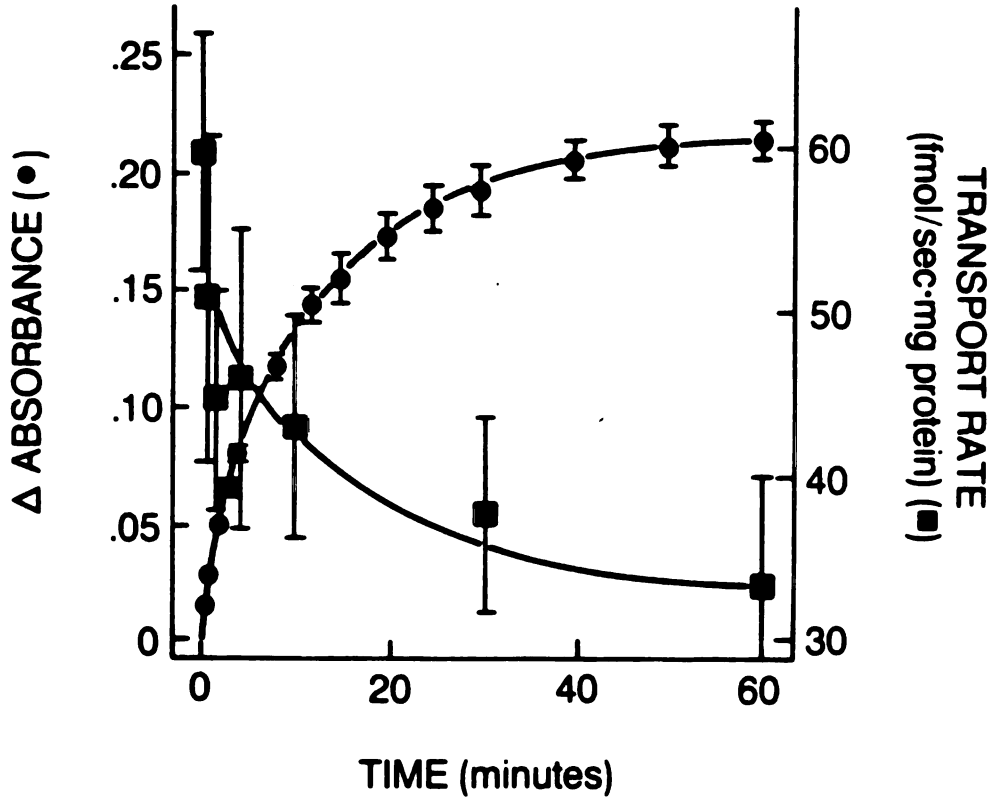


Fig. 6. The time course of optical absorbance change (●) of BBMV following incubation with NBD-Cl at 25^{0}C. The incubation medium was HK buffer (pH 7.4) containing 0.15 mM NBD-Cl. The time course of the inactivation of NMN transport (■) is also presented in the same figure. The initial rate of NMN transport was measured at 8 seconds, 25^{0}C. ^{3}H-NMN concentration in the incubation media was approximately 3.0 μM.

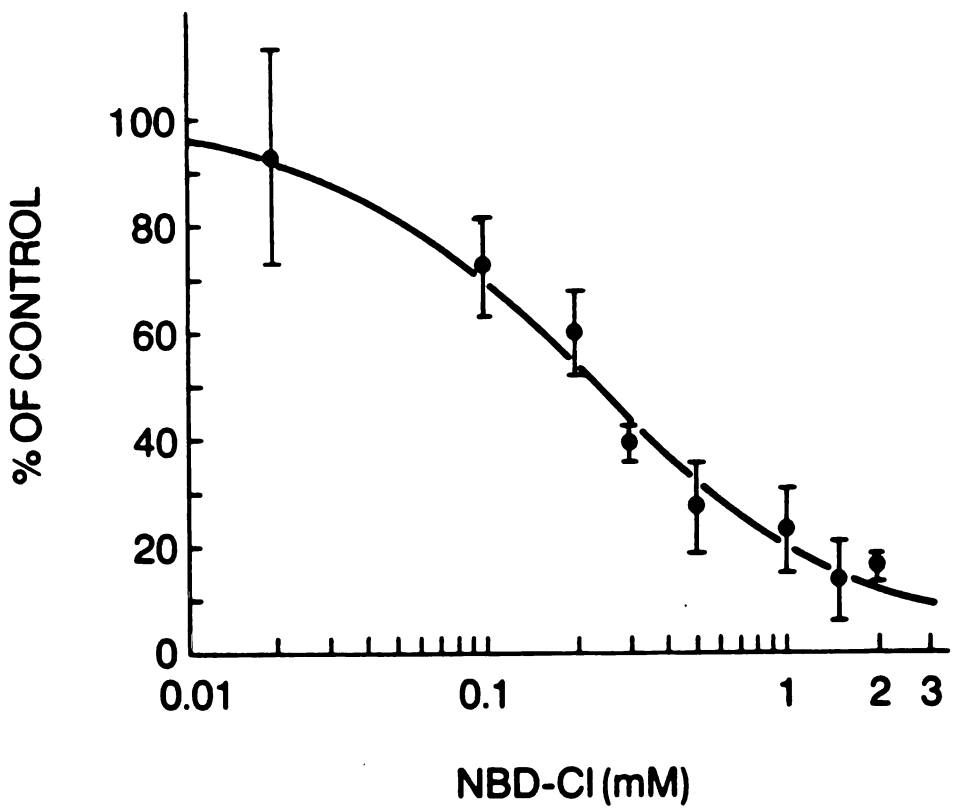


Fig. 7. The effect of various concentrations of NBD-Cl on NMN transport in BBMV. Initial NMN transport rate was measured at 8 seconds. ^{3}H-NMN concentration was approximately 3.0 μM. Each symbol represents mean of three experiments. Vertical lines represent ± 1SE.

Cl (2 mM) produced approximately an 85% reduction in the transport rate. The IC_{50} of NBD-Cl was estimated to be 0.22 mM. However, the equilibrium uptake of NMN was decreased in vesicles incubated with concentrations of NBD-Cl greater than 0.2 mM suggesting that at higher concentrations NBD-Cl might disrupt the integrity of the vesicles or alter the vesicle size. At concentrations less than or equal to 0.2 mM, NBD-Cl did not significantly affect the equilibrium uptake. In the remaining experiments 0.15 mM NBD-Cl was used. Treatment of the vesicles with 2-mercaptoethanol did not alter the effect of NBD-Cl (0.2 and 2.0 mM) on NMN uptake at initial times or at equilibrium.

The initial transport rates of NMN in the control BBMV and in BBMV treated with NBD-Cl (0.15 mM) or N-acetylimidazole (5 mM) under various conditions are summarized in Table 2. Treatment of BBMV with NBD-Cl or N-acetylimidazole reduced the initial uptake of NMN to 50% and 60% respectively. The uptake of NMN at one hour was not significantly different among control (1.19±0.05 pmol/mg protein), NBD-Cl treated (1.15±0.01 pmol/mg protein) and N-acetylimidazole treated (1.09±0.10 pmol/mg protein) BBMV indicating that neither vesicle size nor integrity was changed by the side chain modifiers. The addition of 2-mercaptoethanol did not significantly change the transport rate of NMN in the control or treated vesicles (Table 2). The uptake of ^{3}H-NMN at one hour was not significantly different among these groups (1.31±0.15 pmol/mg protein for control, 1.29±0.36 pmol/mg protein for NBD-Cl and 2-mercaptoethanol treated BBMV and 1.20±0.20 pmol/mg protein for N-acetylimidazole and 2-mercaptoethanol treated BBMV). In contrast to NMN transport, the transport rate of L-lysine in the control membranes at 8 second was 239±49 fmol/s·mg protein (fmoles per second per mg of protein) and was not significantly different from that in the NBD-Cl treated membranes

Table 2. Transport Rate of NMN in BBMV under Various Conditions

Membrane	NMN Transport Rate[a] (fmol/s·mg protein)		
	-[b]	2-mercaptoethanol[c] (2mM)	TEA[d] (100 mM)
Control	35.0±8.0	35.5±11.2	43.5±8.9
NBD-Cl treated	16.8±7.0†	18.0±9.5†	44.2±4.4
N-acetylimidazole treated	20.8±6.2†	18.0±10.1†	47.8±1.9

[a] Transport rate of NMN in control and treated BBMV was measured at 8 second at pH 7.4, 25^{0}C.

[b] BBMV were incubated for one hour in either pH 7.4 HK buffer (control), pH 7.4 HK buffer containing 0.15 mM NBD-Cl (NBD-Cl treated) or pH 7.4 HK buffer containing 5 mM N-acetylimidazole (N-acetylimidazole treated).

[c] 2-mercaptoethanol (2 mM) was also present in the incubation media.

[d] BBMV were preincubated with 100 mM of TEA for 10 minutes.

† Statistically different from control ($p < 0.05$).

(230±60 fmol/s·mg protein). Equilibrium uptake of L-lysine was not changed by the treatment of the vesicles with NBD-Cl.

The reduction of NMN transport could be prevented by preincubation with 100 mM TEA (Table 2). The initial rate of transport of ^{3}H-NMN in the control BBMV incubated with TEA or BBMV incubated with NBD-Cl and TEA or N-acetylimidazole and TEA were not statistically different. The uptake at one hour was also not significantly different among these experiments (1.03±0.09 pmol/mg protein, 1.00±0.10 pmol/mg protein, and 0.96±0.14 pmol/mg protein, respectively).

Data obtained from a representative Michaelis-Menten experiment of NMN transport in control and NBD-Cl (0.15 mM) treated BBMV are presented in Fig. 8. In three experiments the maximal transport rate (Tmax) of NMN in control BBMV was 32.6±8.4 pmol/s·mg protein and the Km was 1.47±0.23 mM; whereas the Tmax in the NBD-Cl treated BBMV was 18.4±4.3 pmol/s·mg protein and Km was 1.72±0.55 mM. Treatment with NBD-Cl reduced the Tmax values significantly ($p < 0.05$), but did not affect the Km values significantly.

As shown in Fig. 9, the transport of NMN in the BBMV was accelerated by the presence of an outwardly directed proton gradient. The NMN uptake was transiently threefold (3.38 pmol/mg protein versus 1.05 pmol/mg protein) greater than the equilibrium values at 30 second (overshoot phenomenon). This overshoot was reduced to 2.05 pmol/mg protein in the NBD-Cl treated BBMV, whereas the equilibrium value was unchanged. The initial rate of NMN transport when the pH gradient was present was fourfold greater than the initial rate when there was no pH gradient (141±54 fmol/s·mg protein versus 35±8

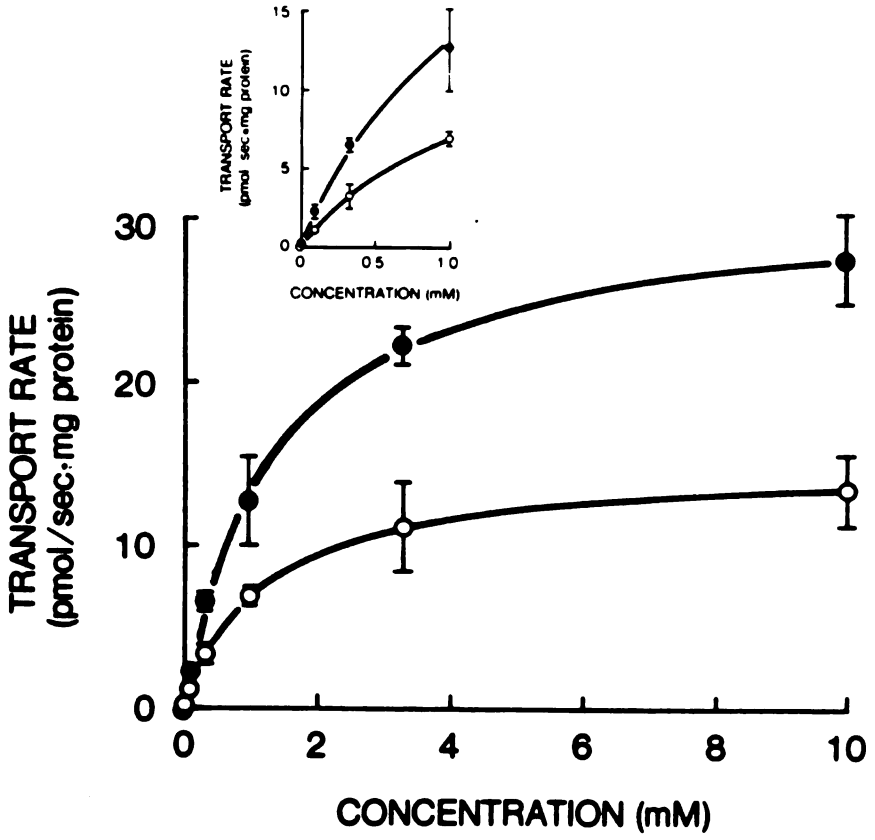


Fig. 8. The initial transport rate of NMN at 8 second in control (●) and NBD-Cl treated (○) BBMV at pH 7.4, 25^{0}C as a function of concentration of NMN. The data are from a representative experiment. The curves represent the computer generated fit to the equation: Rate = Tmax * C/ (Km + C). The insert shows the transport rate of NMN at lower concentrations together with the computer generated curves. The concentrations used in this experiment were (in M): $2.0*10^{-6}$ (not visible in the graph), $1.2*10^{-5}$, $1.0*10^{-4}$, $3.3*10^{-4}$, $1.0*10^{-3}$, $3.3*10^{-3}$, and $1.0*10^{-2}$. In this experiment, the estimated Tmax for the control was 31.3 pmol/s.mg protein and for NBD-Cl treated BBMV was 15.0 pmol/s.mg protein. The estimated Km was 1.38 mM for the control and 1.18 for the NBD-Cl treated BBMV.

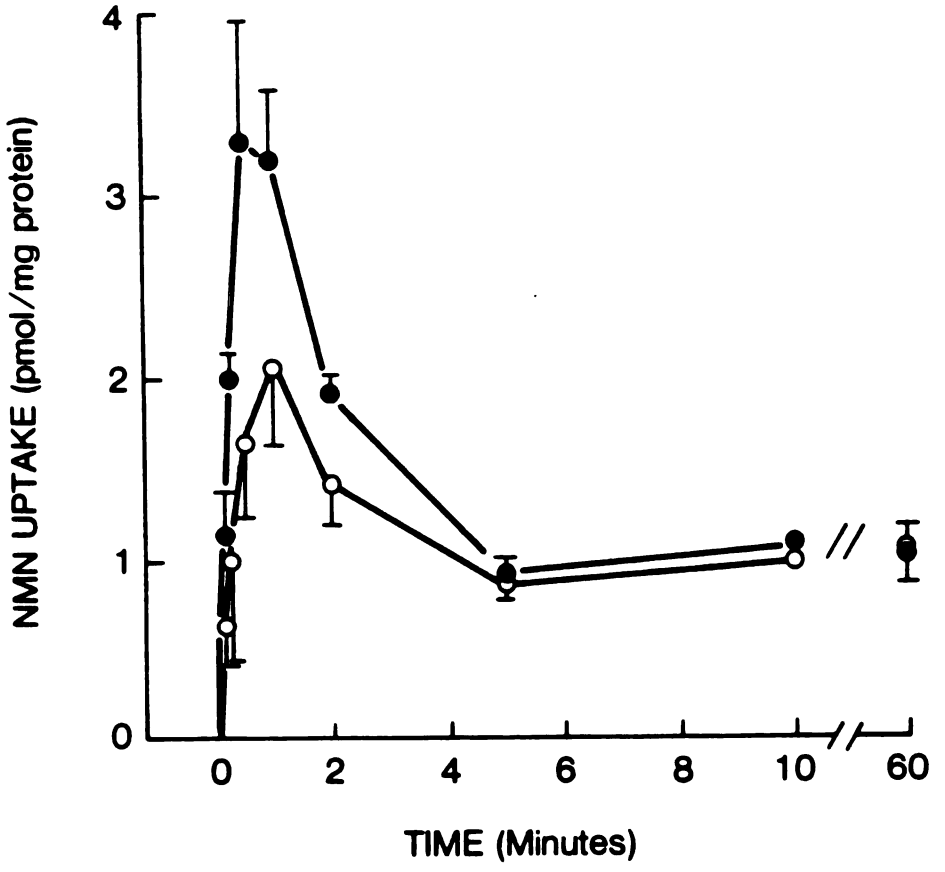


Fig. 9. Proton driven NMN uptake versus time in control (●) and in NBD-Cl treated (○) BBMV. Vesicles were loaded with pH 6.0 HK buffer and incubated with pH 7.5 HK buffer containing approximately 1.6 μM ^{3}H-NMN. Each data point represents mean±SE of three experiments carried out in three membrane preparations.

fmol/s·mg protein). The initial rate of NMN transport in the presence of an outwardly directed proton gradient was reduced to half (77±42 fmol/s·mg protein) in the NBD-Cl treated BBMV.

Since organic cation transport in BBMV is accelerated by a pH gradient, the attenuation of the initial uptake and overshoot in the NBD-Cl treated BBMV could have been caused by an increase in the proton permeability in the NBD-Cl treated BBMV. The permeability of the vesicles to protons in the control and NBD-Cl treated vesicles was tested. Figure 10 shows the fluorescence change observed in control and NBD-Cl treated BBMV loaded with pH 6.0 buffer and incubated in pH 7.5 buffer containing acridine orange. Acridine orange bound instantly to the vesicles in the presence of the pH gradient. Consequently, the fluorescence of the system rapidly decreased. As the pH gradient gradually dissipated, acridine orange moved outward and the fluorescence rose to the equilibrium value. The equilibrium value was lower than 100% because of the turbidity produced by the BBMV. The maximal fluorescence change observed in the control BBMV was 15.6±1.0 fluorescence units which was not significantly different from that of the treated BBMV (15.1±1.1 fluorescence units). The initial rate of return of fluorescence was 0.162±0.018 fluorescence unit/s for control and was not significantly different from 0.159±0.020 fluorescence unit/s obtained in the NBD-Cl treated BBMV. These results suggest that treatment of the vesicles with NBD-Cl did not alter the permeability of the BBMV to protons.

To assess the possibility that NBD-Cl was interacting with sulfhydryl groups (Fig. 3) we determined the amount of free sulfhydryl groups in the untreated BBMV and NBD-Cl treated BBMV. In addition, we determined whether 2-mercaptoethanol restored the free sulfhydryl residues which had

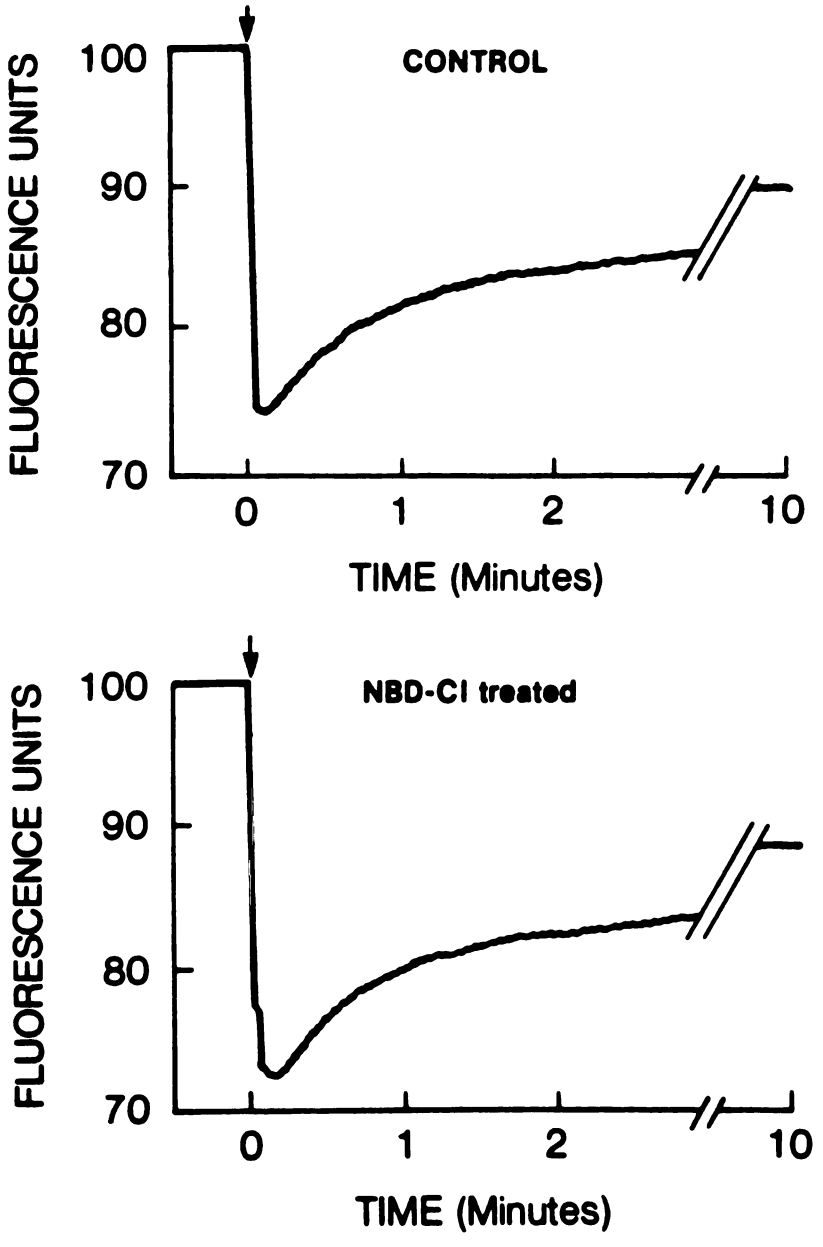


Fig. 10. Data from a representative experiment showing the change of fluorescence of acridine orange following addition of untreated BBMV (upper panel) and BBMV that had been treated with NBD-Cl (lower panel). Both control and NBD-Cl treated vesicles were loaded with pH 6.0 HK buffer and incubated with pH 7.5 HK buffer containing 6 μM acridine orange. The arrows indicate the time at which the vesicles were added to the acridine orange solution.

reacted with NBD-Cl. The amount of free sulfhydryl groups in the untreated BBMV was 37.4±9.4 nmol/mg protein (n = 4) and was reduced to 31.9±10.8 nmol/mg protein in the NBD-Cl treated BBMV (n = 4, $p < 0.05$). In the BBMV treated with both NBD-Cl and 2-mercaptoethanol, the amount of free sulfhydryl residues was 36.7±11.5 nmol/mg protein (n = 4) which was not significantly different from the the amount of sulfhydryl groups measured in the untreated BBMV. In vesicles that had been solubilized with tergitol (NP40, 0.9%), similar trends were also observed, i.e., NBD-Cl produced a 15% decrease in sulfhydryl groups, and 2-mercaptoethanol could prevent this decrease. These results indicate that NBD-Cl bound to approximately 15% of the total sulfhydryl residues in the BBMV. 2-Mercaptoethanol restored the free sulfhydryl residues that had reacted with NBD-Cl.

Discussion

In this study we demonstrated that the side chain modifying agents, NBD-Cl and N-acetylimidazole, reduced the initial uptake of NMN in BBMV. This reduction was not due to a change in the integrity of the vesicles, or a decrease in the intravesicular volume since the uptake at equilibrium was not different between the control and the treated BBMV.

Although NBD-Cl and N-acetylimidazole are more specifically reactive with tyrosine, these agents also react with other functional groups. However, by carefully choosing the pH and reaction time, the side reactions can be minimized. For example, NBD-Cl also reacts with primary amines, but the reactivity of amino groups toward NBD-Cl is suppressed below pH 8.0 (Aboderin et al., 1973). The reaction of NBD-Cl with tyrosine and lysine in

aqueous solution was monitored at 25^{0}C at pH 7.4. Tyrosine interacted substantially with NBD-Cl in 12 hours, whereas lysine did not show any interaction with NBD-Cl in the same period of time. Under the experimental conditions of this study, the major functional groups reacting with the two reagents are sulfhydryl groups and the phenolic group of tyrosine (Fig. 3). However, the thioether bond and the thioester bond formed by the interaction of sulfhydryl groups with NBD-Cl and N-acetylimidazole can be dissociated with 2-mercaptoethanol (Cantley et al., 1978; Lin et al., 1982). We measured the free sulfhydryl residues in the NBD-Cl treated and untreated BBMV. Under the conditions of our experiments, NBD-Cl bound to approximately 15% of the total sulfhydryl groups. Treatment with 2-mercaptoethanol (2 mM) completely restored the free sulfhydryl residues that had reacted with NBD-Cl indicating that the thioether groups had been dissociated by 2-mercaptoethanol.

The studies demonstrated that treatment of the vesicles with NBD-Cl reduced the initial rate of NMN transport and that the reduction was dependent upon the NBD-Cl concentration (Fig. 7). When a concentration of 0.15 mM NBD-Cl was used, the initial rate of NMN transport was reduced to 50%. The addition of 2-mercaptoethanol (2 mM) did not significantly affect the transport of NMN in the control or treated BBMV (Table 2). Since the data demonstrated that 2-mercaptoethanol dissociated the thioether bond formed from the reaction of NBD-Cl and sulfhydryl residues, these results suggest that the effect of NBD-Cl on NMN transport was not due to its reaction with sulfhydryl residues; rather tyrosine residues appear to be the functional groups to which NBD-Cl was reacting to reduce the transport of organic cations. The results do not contradict the findings of Sokol et al. (1986) who demonstrated that sulfhydryl residues are essential functional groups for organic cation transport in BBMV.

Sokol et al. demonstrated that a disulfide-reducing agent, dithiothreitol, decreased the activity of NMN transporter in renal BBMV. Thus, one would expect 2-mercaptoethanol, also a disulfide-reducing agent, to reduce the transport of NMN in the vesicles in our experiments. However, Sokol et al. (1986) also showed that the interaction between dithiothreitol and the transporter was reversed by washing with buffer probably due to protein reoxidation when the reducing agent was removed. In our study, the 2-mercaptoethanol-treated vesicles were washed twice with HK buffer before the NMN transport in the vesicles was determined. It is quite conceivable that the interaction between 2-mercaptoethanol and the disulfide residues in the vesicles was also reversed by the washing procedures. As a result the transport of NMN was not affected by the treatment of vesicles with 2-mercaptoethanol.

Because membrane lipids have many functional groups which may potentially interact with NBD-Cl, it is possible that the inhibitory effects of NBD-Cl on NMN transport resulted from non-specific modifications of membrane lipids. If this were the case, one would expect that other transport systems in the BBMV might be similarly affected. The observation that L-lysine transport and proton permeability were unaltered by NBD-Cl treatment suggests that treatment of the BBMV with NBD-Cl (0.15 mM) does not generally affect brush border membrane transporters.

To determine the nature of the inhibitory effects of NBD-Cl on NMN uptake into BBMV, we measured the initial rate of uptake of NMN as a function of concentration in control BBMV and in vesicles that had been treated with NBD-Cl (0.15 mM). The Tmax was decreased in the NBD-Cl treated BBMV, whereas

the apparent Km was not changed. These results are consistent with a reversible noncompetitive mechanism or an irreversible inactivation (covalent bonding). In all experiments the BBMV treated with NBD-Cl were diluted twice with twentyfold volume of ice-cold buffer. If the interaction of NBD-Cl with BBMV were readily reversible, NBD-Cl should have been washed out during the washing procedures and its inhibitory effects on NMN transport nullified. Aboderin et al. (1973) have demonstrated that NBD-Cl forms covalent bonds with tyrosine in solution which might suggest that the same reaction is occurring in the interaction between NBD-Cl and tyrosine residues in BBMV. The apparent Km (1.47 mM) of NMN obtained in the untreated vesicles was similar to the 1.58 mM value obtained by Kinsella et al. in BBMV from dog kidney (1979) and the 0.63 mM value obtained by Wright in BBMV from rabbit (1985). The apparent Tmax (32.6 pmol/s·mg) obtained in the untreated vesicles is reasonably similar to the value of 85 pmol/s·mg reported by Wright (1985), but not to the value of 332 pmol/s·mg reported by Kinsella el al. (1979). Species differences may explain the greater Tmax obtained by Kinsella et al. (1979).

To determine whether the tyrosine residues may be located at the binding site for organic cations, we carried out studies in which TEA was used to protect tyrosine residues from both NBD-Cl and N-acetylimidazole. If the tyrosine residues are at the binding site for organic cations, TEA, present at higher concentrations should bind to these tyrosine residues and thus prevent these residues from reacting with NBD-Cl and N-acetylimidazole. On the other hand if the tyrosine residues are not at the binding site, the addition of TEA might not have protective effects. We observed that in the presence of 100 mM TEA neither side chain modifier inhibited NMN transport suggesting that the tyrosine residues may be located at the binding site of the organic cation transporter. In

contrast, in HK buffer without BBMV 100 mM TEA did not inhibit the interaction of tyrosine (0.15 mM) and NBD-Cl (0.15 mM) suggesting that TEA does not simply interact with tyrosine residues and implying that a "binding site" is involved. Although these results suggest that the modified tyrosine residues are at the binding site for NMN transport, the data do not exclude the possibility that the presence of a high concentration of TEA might change the conformation of the transporter and consequently prevent the interaction of tyrosine and NBD-Cl.

Several investigators have demonstrated that the transport of organic cations in BBMV can be accelerated in the presence of an outwardly directed proton gradient (Holohan and Ross, 1981; Takano et al., 1984; McKinney and Kunnemann, 1985; Sokol et al., 1985; Wright, 1985). In this study we confirmed that in the presence of a proton gradient there was an overshoot of NMN uptake. This overshoot and initial uptake of NMN was reduced to approximately half in the NBD-Cl treated BBMV (Fig. 9). Since the permeability of protons in BBMV was unaffected by the NBD-Cl treatment as demonstrated in the acridine orange studies (Fig. 10), the reduction in the overshoot and initial transport rate of NMN could not have been caused by a faster dissipation of the pH gradient in the NBD-Cl treated BBMV. These decreases probably resulted from a reduced activity of the organic cation-proton antiporter in the NBD-Cl treated BBMV.

In summary, we have demonstrated that treatment with the side chain modifiers, NBD-Cl and N-acetylimidazole, results in an inhibition of organic cation transport in the vesicles. The interaction between NBD-Cl and the transporter is both time and concentration dependent. Results have been presented suggesting that the functional groups being modified by NBD-Cl are

tyrosine residues. The tyrosine residues are essential for the transport of organic cations in BBMV.

Chapter 3

Organic Cation Transport in the Renal Brush Border Membrane: Studies with Acridine Orange

Many endogenous and exogenous organic cations are actively secreted in the proximal tubule of the kidney via a specific transport system (Torretti et al., 1962; Rennick, 1981; Ross et al., 1975; Kinsella et al., 1979; Holohan and Ross, 1981). Studies in isolated cortical membrane vesicles have demonstrated that the transporters of organic cations in the brush border and basolateral membrane differ in their kinetic properties and driving forces (Kinsella et al., 1979; Takano et al., 1984). In the basolateral membrane, organic cations seem to be transported by a simple substrate-transporter system, although there is some controversy about whether the system is facilitated (Holohan and Ross, 1981; Kinsella et al., 1979) or active (Schali et al., 1983; Tarloff and Brand, 1986). In contrast, the organic cations, tetraethylammonium (TEA) (Takano et al., 1984), N^1-methylnicotinamide (NMN) (Holohan and Ross, 1981; Sokol et al., 1985; Wright, 1985), and procainamide (McKinney and Kunnemann, 1985) accumulate in isolated brush border membrane vesicles (BBMV) against a concentration gradient (overshoot phenomenon) in the presence of an outwardly directed proton gradient. Based on these studies, organic cation-proton exchange (or organic cation-hydroxyl cotransport since the two mechanisms cannot be easily distinguished) has been proposed as the mechanism for organic cation transport across the renal brush border membrane (For the reason of simplicity, the mechanism will be referred to as organic cation-proton exchange here). Although the studies are consistent with an organic cation-proton exchange mechanism, alternative mechanisms are also compatible with the data. For example, creation of a

temporary inside negative potential difference by the efflux of protons could temporarily drive the transport of the positively charged organic cations. Alternatively, a catalytic effect of pH on transport could produce an overshoot phenomenon (See Appendix B).

As reviewed by Turner (1983), a cotransporter or antiporter must be capable of energy transduction. As such it must be able to transport the activator, i.e., the transport of a substrate must be coupled with the cotransport or the exchange of the activator. In the case of the organic cation-proton antiporter, this implies that a gradient of organic cations must be able to drive the counter movement of protons. In the previous studies (Kinsella et al., 1979; Holohan and Ross, 1981; Takano et al., 1984), organic cation transport was specifically monitored in the presence of a pH gradient. To date there has been no study in which organic cation driven proton transport has been specifically investigated. The purpose of this study was to monitor the movement of protons in the presence of favorable organic cation gradients to determine whether a true exchange mechanism is present. Using the pH sensitive dye, acridine orange (Fig. 11), we demonstrated that in isolated BBMV the efflux of protons was accelerated in the presence of an inwardly directed gradient of the organic cation, TEA. Increasing the TEA gradient produced an increasing rate of efflux of protons although the system was saturable. These data demonstrate that TEA can drive the transport of protons and offer more direct evidence to support previous data suggesting that organic cations are transported across the renal brush border membrane by a proton exchange or a hydroxyl cotransport mechanism.

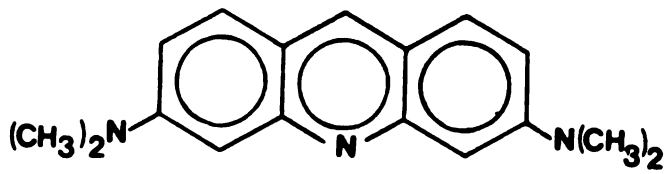


Fig. 11. Structure of acridine orange.

Methods

Preparation of BBMV

Brush border membrane vesicles were prepared by a method involving magnesium aggregation and differential centrifugation developed by Booth and Kenny (1974) and modified in our laboratory (Hsyu and Giacomini, 1987; Chapter 2). Vesicles were resuspended to a concentration of 20 mg/ml.

Studies of ^{3}H-NMN Uptake

For the pH gradient studies, 10 µl of pH 6.0 HK10 buffer (10 mM HEPES, 150 mM KCl; as blanks) or 10 µl of vesicles loaded with a pH 6.0 HK10 buffer was mixed with 40 µl of pH 7.4 HK10 buffer containing approximately 1.6 µM ^{3}H-NMN and incubated for 8, 15, 30 seconds, 1, 2.5, 5 and 60 minutes at room temperature. For studies in the absence of a pH gradient, 10 µl of vesicles loaded with the pH 7.4 HK10 buffer were incubated with 20 µl of the pH 7.4 HK10 buffer containing ^{3}H-NMN for 8, 30 seconds and 1, 2.5, 5, and 60 minutes. In some studies uptake of NMN in the presence of 6 µM acridine orange (AO) at 8 seconds and 60 minutes was determined. Uptake of NMN associated with the vesicles was determined by the rapid filtration method.

pH Jump Studies

Vesicles were loaded with pH 6.0 HK.1 buffer (0.1 mM HEPES, 155 mM KCl). A 6 µM solution of AO in pH 7.5 HK.1 buffer was prepared and 2.5 ml was pipetted into a disposable acrylic cuvette (Super-VU, Fisher Scientific Co.). The

fluorescence of the solution was adjusted to 100% at an excitation wavelength of 492 nm and an emission wavelength of 530 nm. Fifteen µl of vesicles was placed in a small ladle[1] and was added to the cuvette through a small port that had been drilled in the top of the fluorometer. Aliquots of 2 M TEA solution were added 6 seconds later to achieve final concentrations ranging between 10 mM and 50 mM. The fluorescence was monitored for 1 minute at room temperature. The initial rate of fluorescence recovery following the addition of TEA was determined from the tangents of the linear segments of the curves during the first three seconds (to be shown subsequently in Fig. 13). Fluorescence monitoring was carried out in a fluorescence spectrophotometer (MPF-2A, Perkin Elmer, Norwalk, CN) equipped with a QPD-33 recorder (Perkin-Elmer). Vesicles loaded with pH 7.5 HK.1 buffer were also added to AO solution as a control.

Studies in Vesicles Loaded with TEA or Na^+

Vesicles were loaded with either 155 mM TEA-Cl, 0.1 mM HEPES (HTEA.1 buffer), pH 7.5 or 155 mM NaCl, 0.1 mM HEPES, pH 7.5 (HNa.1 buffer). Fifteen µl of vesicles was added to the cuvette containing 2.5 ml AO solution, pH 7.5. The fluorescence of the mixture was monitored for 6 minutes. When a steady-state fluorescence quenching was achieved, aliquots of TEA-Cl or NaCl solution were added to the cuvette. Vesicles were also loaded with pH

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[1] The ladle was made from a plastic four-ribbed plunger of a one-ml syringe. One end of a rib was cut off. The end disk was softened on a hot plate covered with aluminum foil and molded to a ladle with two sides attached to two uncut ribbs. I would like to thank Dr. Harlan Ives for demonstrating how to make the ladle.

6.0 HTEA.1 buffer and added to either pH 7.5 AO solution or pH 7.5 HTEA.1 buffer containing 6 μM AO.

Data Analysis

AO fluorescence, although related to the pH gradient, is difficult to accurately calibrate in the presence of the small pH gradients present in this study. Thus, data obtained in this study are expressed as rate of fluorescence recovery rather than as flux of protons. The data of rate of fluorescence recovery versus external concentration of TEA were transformed to rate/conc versus conc according to Hanes (1932). Linear least squares regression was performed to obtained slope and intercept. Vmax was calculated as 1/slope and Km was calculated as intercept/slope.

All experiments were determined in triplicate and three experiments were performed in three separate membrane preparations for each study. Statistical differences between two groups were determined by Student's t-test. Probability (p) at the 0.05 level was considered significant.

Chemicals

^{3}H-N^{1}-Methynicotinamide (2.8 Ci/mmole) was purchased from ICN (Irvine, CA). All other chemicals were purchased either from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) and were the highest grade available.

Results

Preliminary studies

Before we began the studies, we demonstrated that a pH gradient driven overshoot phenomenon for organic cation transport could be observed in our membrane preparations. Figure 12 depicts the uptake of NMN in the presence and absence of an outwardly directed proton gradient. In the absence of the pH gradient, NMN uptake increased with time and approached 90% of the equilibrium value within 5 minutes. The uptake of NMN in the presence of the outwardly directed proton gradient was enhanced as expected and an overshoot 3-fold greater than the equilibrium uptake was observed.

Next, it was important to determine whether AO affected the transport of organic cations. In the absence of a pH gradient, the rate of NMN transport in the BBMV at 8 sec was 44.7±8.8 fmol/s·mg in the absence of AO and was 41.6±0.5 fmol/s·mg in the presence of AO (6 μM). These rates were not significantly different suggesting that AO (6 μM) did not affect the transport of NMN in the BBMV. The equilibrium uptake of NMN at one hour was also not affected by AO (758±166 fmol/mg versus 795±96 fmol/mg) suggesting that AO did not affect the size or integrity of the vesicles. However, in the presence of a pH gradient, a decrease in the initial rate of transport of NMN was observed in the presence of AO (6 μM). This effect was not observed when concentration of NMN were above 10 mM.

Because NMN quenched the fluorescence of AO, and TEA at concentrations as high as 50 mM did not alter the fluorescence of AO, TEA was

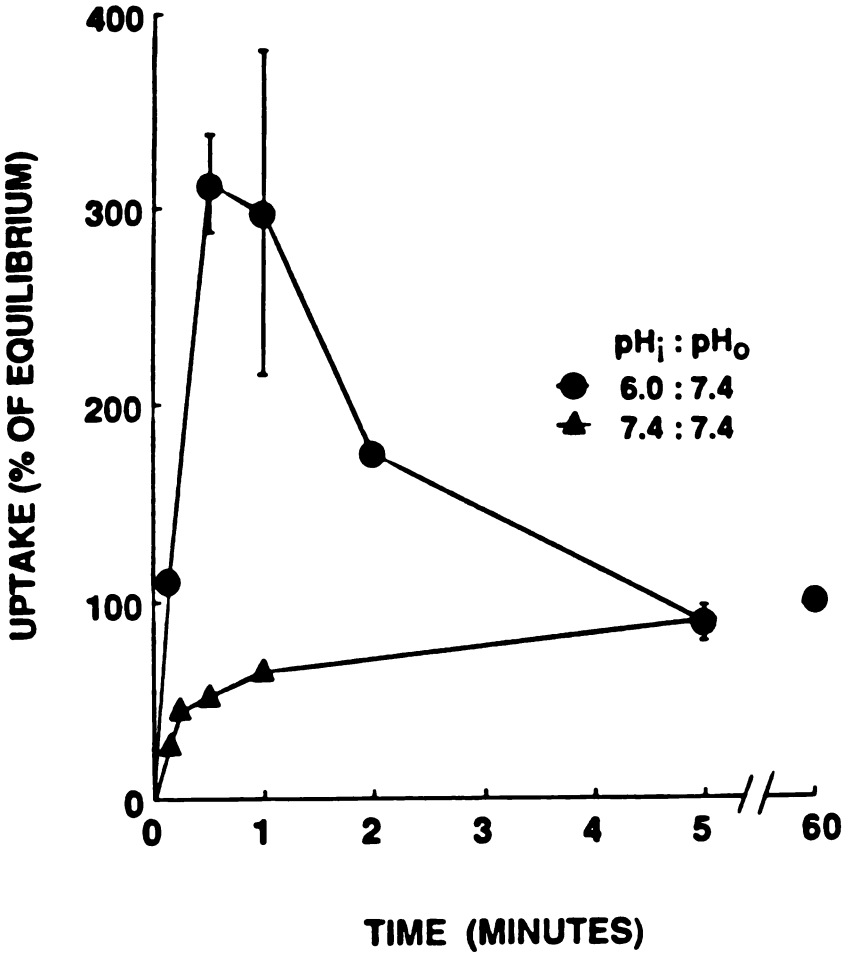


Fig. 12. Time course of NMN uptake in renal brush border membrane vesicles in the presence of a pH gradient (represented by closed squares) and absence of a pH gradient (represented by closed triangles). Data are presented as % of equilibrium uptake. Each data point represents mean±SD from three experiments. pH_i represents the intravesicular pH and pH_o represents the extravesicular pH.

used in the AO studies. It should be noted that TEA and NMN share the same transport system in BBMV and have similar kinetics (Wright, 1985; Takano et al., 1984; Holohan and Ross, 1981).

AO Studies

As shown in Fig. 13, the fluorescence of the AO solution decreased rapidly following the addition of pH 6.0 vesicles. The decrease in fluorescence occurs because there is an increased binding of AO to the vesicles in the presence of a pH gradient which results in a quenching of the fluorescence (Dell'Antone, 1972; Lee et al., 1976). The fluorescence reached a maximal change in less than 5 seconds and remained constant (steady-state) for approximately 10 seconds. Afterwards the fluorescence started to increase as the pH gradient dissipated and AO dissociated from the vesicles (not depicted in Fig. 13). The maximal fluorescence change was 17.2±1.2 fluorescence units in three experiments.

The rate of fluorescence recovery was faster in the presence of extra-vesicular TEA and was dependent upon the extravesicular TEA concentration. The rates of fluorescence recovery in the presence of TEA were as follows: [TEA]=0.16 mM,10.3±2.0 fluorescence units/min; [TEA]=0.64 mM, 12.3±1.3 fluorescence units/min; [TEA]=3.2 mM, 14.8±2.2 fluorescence units/min; [TEA]=12.7 mM, 23.5±3.2 fluorescence units/min; [TEA]=25.2 mM, 28.0±2.6 fluorescence units/min; [TEA]=40.1 mM, 37.8±4.0 fluorescence units/min and [TEA]= 50.2 mM, 39.8±4.6 fluorescence units/min. A linear Hanes transformation of the data from a typical experiment is presented in Fig. 14. In three experiments using three different membrane preparations, Km was

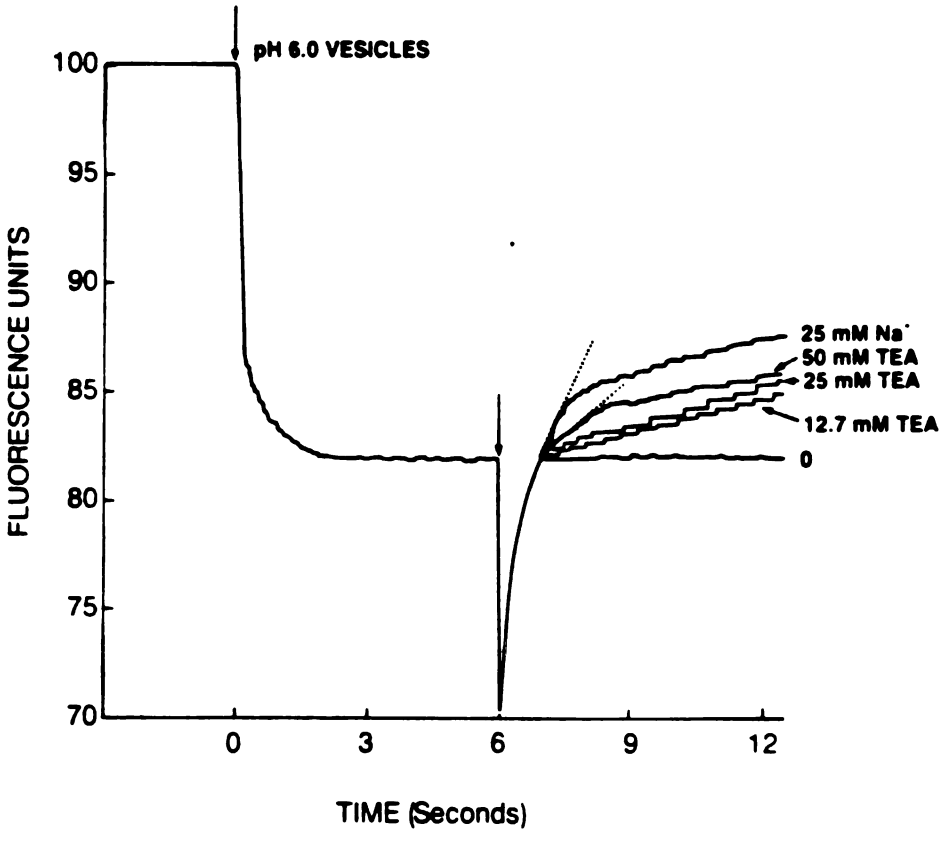


Fig. 13. Fluorescence change when renal brush border membrane vesicles loaded with pH 6.0 HK.1 buffer (155 mM KCl and 0.1 mM HEPES) were added to pH 7.5 AO solution (6 μM AO, 155 mM KCl, and 0.1 mM HEPES). The first arrow indicates the time at which the vesicles were added to the AO solution. The second arrow indicates the time at which aliquots of TEA or Na^+ solution were added to the AO solution to produce the concentrations indicated in the figure.

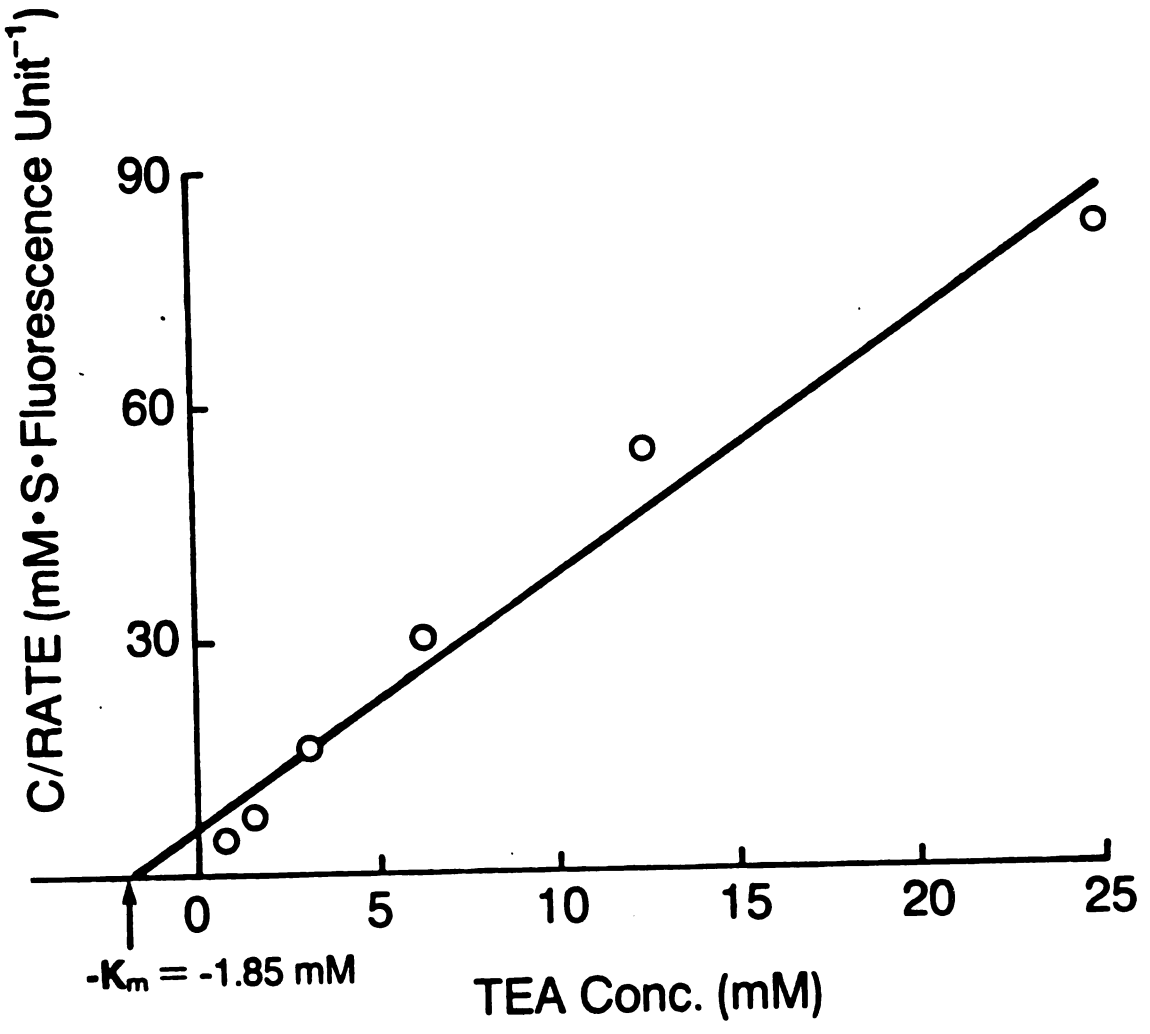


Fig. 14. Plot of data of extravesicular concentration of TEA/rate of fluorescence change versus extravesicular concentration of TEA (Hanes plot) from a representative experiment. $r = 0.986$, $p < 0.001$.

2.57±1.24 mM and Vmax was 39.8±12.6 fluorescence units/min. Control studies in which vesicles loaded with pH 7.5 HK buffer were added to AO solution produced a small increase of fluorescence (1.73±0.43 fluorescence units). In contrast, KCl (50 mM) added extravesicularly did not significantly change the fluorescence recovery rate. When vesicles loaded with pH 7.5 HK.1 buffer were added to the AO solution, a small increase in the fluorescence (1.73±0.43 fluorescence units) was observed (n = 3).

Figure 15 depicts the fluorescence change of the AO solution following the addition of vesicles loaded with either 155 mM TEA-Cl or 155 mM NaCl. Vesicles loaded with TEA produced a small, although significant, decrease in fluorescence (4.1±0.6 fluorescence units, n = 3). In contrast, vesicles loaded with Na^+ produced a much greater decrease in fluorescence. The decrease was somewhat variable, ranging from 20 to 35 fluorescence units in three experiments. Steady-state fluorescence quenching developed in approximately 3 min. This fluorescence quenching was reversed by adding Na^+ externally. External Na^+ concentrations as low as 1.6 mM produced a significantly faster rate of fluorescence recovery. In contrast, TEA (50 mM) added to the external buffer did not significantly alter the rate of fluorescence recovery.

It is possible that the reason that vesicles loaded with TEA produced a relatively small change in the fluorescence units in comparison to the vesicles loaded with Na^+ was that TEA prevented the binding of AO to the vesicles. We determined the change in fluorescence following the addition of vesicles loaded with pH 6.0 HTEA.1 buffer to pH 7.5 HTEA.1 buffer or HK.1 buffer containing 6 μM AO. The maximal fluorescence change (16.9±1.8 fluorescence units)

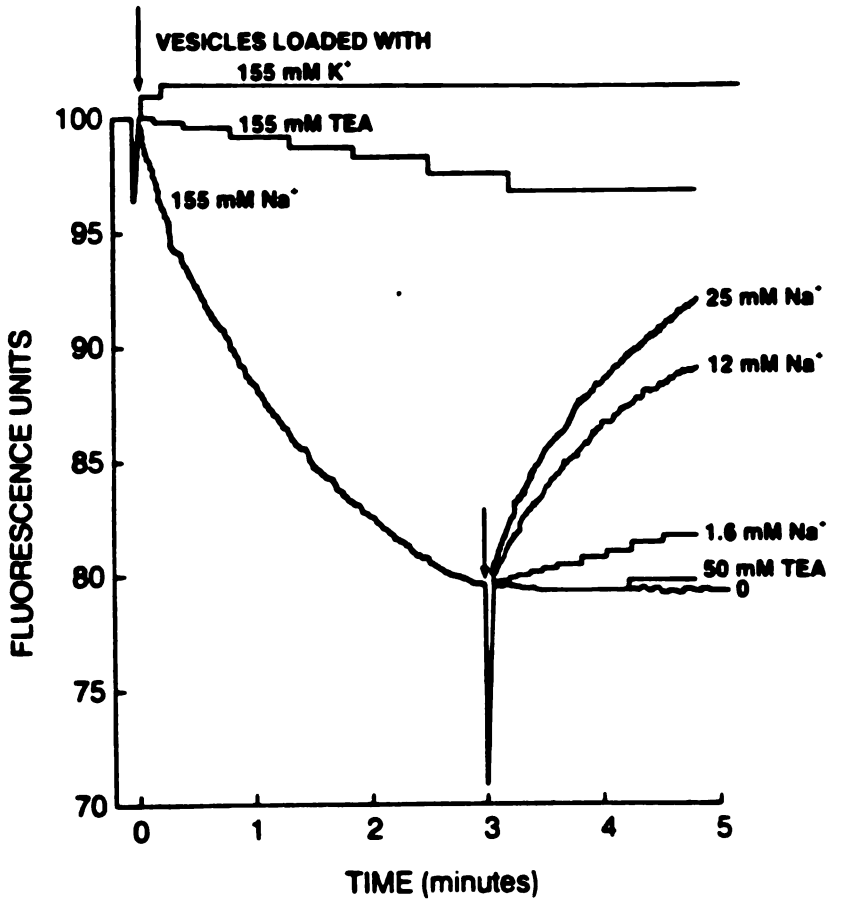


Fig. 15. Fluorescence change when vesicles loaded with either pH 7.5 HNa.1 buffer (155 mM NaCl and 0.1 mM HEPES), pH 7.5 HTEA.1 buffer (155 mM TEA.Cl and 0.1 mM HEPES), or pH 7.5 HK.1 buffer (155 mM KCl and 0.1 mM HEPES) were incubated in pH 7.5 AO solution (6 μM AO, 155 mM KCl, and 0.1 mM HEPES). The first arrow indicates the time at which the vesicles were added to the AO solution. The second arrow indicates the time at which aliquots of TEA or Na+ solution were added to the AO solution to produce the concentrations indicated in the figure.

obtained in the above experiments was not different from that (17.2±1.2 fluorescence units) obtained in experiments in which vesicles loaded with pH 6.0 HK.1 buffer were added to pH 7.5 AO solution. These results suggest that TEA did not affect the binding of AO to the vesicles.

Discussion

Several research groups have demonstrated that organic cations accumulate in BBMV in the presence of an outwardly directed proton gradient (Kinsella et al., 1979; Ross and Holohan, 1983; Takano et al., 1984). Based upon the demonstration of an apparent "overshoot phenomenon", it has been concluded that organic cations are transported out of proximal tubule cells by a proton exchange mechanism. Although an organic cation-proton exchange mechanism is consistent with the data obtained in the previous studies (Holohan and Ross, 1981; McKinney and Kunnemann, 1985; Takano et al., 1984; Wright, 1985), the apparent "overshoot phenomenon" could have been produced by other mechanisms. First, because organic cations are positively charged, a temporary negative potential difference created by the rapid efflux of protons could have driven organic cation transport against a concentration gradient in the BBMV. Conflicting data in the literature suggest that the organic cation-proton exchanger is electroneutral (Sokol et al., 1985) or electrogenic with one organic cation molecule being transported in exchange for two protons (Wright, 1985). In either case, an inside negative potential difference would not be expected to drive organic cation transport.

It is also possible that the apparent "overshoot phenomenon" of organic cations in the presence of a pH gradient could have been due to a catalytic

effect of pH on organic cation transport (Appendix B). A catalytic effect implies that the activator (in this case the protons) binds to the transporter and changes the substrate binding affinity or translocation rate. But the activator need not be transported. In fact, the rate of organic cation transport in BBMV appears to be a function of pH (Holohan and Ross, 1981; Takano et al., 1984; Wright, 1985). At lower pH's, the rate of transport of organic cations is lower and increases with increasing pH. Thus, in the presence of a lower intravesicular pH, organic cations may be transported at a slower rate outwardly. As the pH gradient dissipates, the outward transport rate will progressively increase. This catalytic effect of pH on transport could have produced the observed "overshoot phenomenon" in the previous studies (Holohan and Ross, 1981; McKinney and Kunnemann, 1985; Takano et al., 1984; Wright, 1985).

An overshoot phenomenon may also have been caused by enhanced nonspecific binding of the organic cations to the vesicles in the presence of an outwardly directed proton gradient. Such pH gradient-dependent binding to biomembranes has been observed for several basic dyes (Lee et al., 1976, Lee and Forte, 1978).

It is clear that if a true organic cation-proton antiporter exists, then a favorable gradient of organic cations should drive the movement of protons. The movement of both substrate and cosubstrate has been demonstrated for the Na^+-glucose cotransporter (Kinne et al., 1975; Beck and Sacktor, 1978) and the Na^+-H^+ exchanger (Kinsella and Aronson, 1981; Warnock et al., 1982). In this study we investigated the organic cation-proton exchanger by specifically monitoring proton movement with acridine orange. This compound has been used to monitor the pH gradient generated by K^+-H^+-ATPase in gastric

membrane vesicles (Lee and Forte, 1978), and to investigate the kinetics of the Na^+-H^+ exchanger in renal BBMV (Warnock et al., 1982). The mechanism of fluorescence change of acridine orange as a function of a pH gradient was initially thought to result from a pH dependent distribution of acridine orange according to the Henderson-Hasselbalch equation. Later studies, however, demonstrated that binding of acridine orange to vesicles in the presence of a pH gradient was a more likely mechanism (Lee et al., 1976; Rabon et al., 1978). In our study, a pH gradient of 1.5 units across the vesicles produced a fluorescence change of 17.2 units which corresponded to the fluorescence of an acridine orange solution with a concentration of 4.1 μM. Since 6 μM of acridine orange was initially present, presumably the decrease of concentration of acridine orange was caused by the diffusion of acridine orange into the vesicles. Assuming an intravesicular volume of 1 μl/mg (Ives, 1985), the intravesicular concentration of acridine orange would be 13 mM. Using the Henderson-Hasselbalch equation, a concentration of only 190 μM would be predicted. Thus, pH distribution of acridine orange alone can not explain the dramatic fluorescence change and a pH dependent binding of acridine orange is likely.

In this study we observed that when vesicles loaded with pH 6.0 HK.1 buffer were added to the pH 7.5 AO solution, the fluorescence of the AO solution decreased as expected. An inwardly directed TEA gradient accelerated the rate of fluorescence recovery suggesting that TEA had accelerated the efflux of protons. However, several artifacts may have caused this faster fluorescence recovery. First, TEA may have inhibited the binding of AO to the vesicles resulting in an increased concentration of AO in the solution and an increased fluorescence. Secondly, TEA may have nonspecifically increased the

permeability of the membrane to protons resulting in a rapid dissipation of the pH gradient. These mechanisms were excluded in the pH jump experiments in which HTEA.1 buffer was used as the intra- and extravesicular medium. High concentrations of TEA (155 mM) did not affect the maximal fluorescence change or rate of fluorescence recovery. Therefore the data indicate that TEA accelerates the efflux of protons and are compatible with an organic cation-proton exchange mechanism.

The data obtained in this study are consistent with a saturable exchange mechanism. The initial rate of efflux of protons (determined as the initial rate of recovery of fluorescence) increased with increasing concentrations of TEA until a maximal rate of efflux occurred at approximately 40 mM of TEA, i.e., increasing the concentrations of TEA could not further accelerate the efflux of protons. It is also possible that at high concentrations of TEA, the rate of dissociation of AO from the vesicles became the rate limiting step. Evidence against this possibility was obtained in the studies shown in Fig. 13. Sodium added externally produced a much faster rate of recovery of the fluorescence of AO than did TEA at the highest extravesicular concentration. Further evidence demonstrating that AO dissociation is not the rate limiting step was obtained by Warnock et al. (1982). Using nigericin, a K^+-H^+ ionophore, these investigators demonstrated that in BBMV the rate of binding of AO to the vesicles was faster than proton movement accelerated by nigericin and was not the rate limiting step.

The Vmax obtained in this study (39.8 fluorescence units) can be roughly converted to flux of protons. The relationship between fluorescence change and pH was 0.17±0.07 pH unit/fluorescence unit when pH change was smaller

than 0.4 unit. This relationship was obtained by calibrating fluorescence change in acridine orange solution incubated with vesicles loaded with buffers of various pH's. Intravesicular buffer capacity was determined by titrating lysed BBMV in 20 ml of 0.9% tergitol (NP 40) solution with 0.05 N KOH (Ives, 1985). The buffer capacity of BBMV was determined to be 250 pmol/mg protein/pH unit (Appendix C). Using this information, the Vmax was converted to 28.3 pmol/s·mg for TEA driven proton transport. This value is very similar to the Vmax, 32.6±8.4 pmol/s.mg, obtained for NMN transport (Hsyu and Giacomini, 1987). The results appear to agree with a one to one exchange of organic cations and protons (Sokol et al., 1985) since the Vmax of organic cation driven proton transport is similar to the Vmax of organic cation transport. However, the Vmax obtained in this study has a large standard deviation (45% of the mean) due to the difficulty in correlating fluorescence change to pH unit. Thus, the possibility of two protons in exchange with one organic cation as proposed by Wright (1985) can not be excluded.

Because our preliminary studies indicated that AO (6 μM) may interfere with the pH gradient dependent transport of organic cations at low concentrations, the apparent Michaelis-Menten constant (Km) for proton transport calculated from Hanes plot (Fig. 14) of 2.57±1.24 mM must be interpreted with some caution. This constant represents the concentration of TEA which produced a rate of proton flux which was one half of the maximal rate of proton flux.

When vesicles were loaded with 155 mM TEA, the maximal fluorescence decrease was 4.1±0.6 fluorescence units, roughly corresponding to a pH gradient between 0.5 and 1.0 pH unit (Fig. 15). It is important to point out that

the rate of proton efflux in the presence of an inwardly directed TEA gradient (Fig. 13) should not be directly compared to the rate of proton influx in the presence of an outwardly directed TEA gradient (Fig. 15). The experiments were carried out at two different intravesicular pH's. The buffer capacity of HEPES (pKa 7.5) is much greater at pH 7.4 than at pH 6.0 and would be expected to diminish the intravesicular pH changes and thereby result in a diminished fluorescence change. In addition, the transport of organic cations is slower in the absence of a pH gradient (Fig. 12, Takano 1984). Thus one might anticipate a slower exchange rate under these conditions (Fig. 15).

In comparison to TEA loaded vesicles, Na^+ loaded vesicles produced a substantially greater maximal fluorescence change (20-35 fluorescence units) indicating that the intravesicular pH was lower than 6.0. These results may suggest that the TEA-proton exchanger operates at a slower rate than that of the Na^+-H^+ exchanger. This hypothesis is consistent with the fact that the Vmax of Na^+-H^+ exchange is 113 nmol/min.mg protein in BBMV prepared from rabbit kidney (Kinsella and Aronson, 1981) which is 23-fold greater than the Vmax of 5 nmol/min.mg protein obtained for organic cation-proton exchange in rabbit renal BBMV (Wright, 1985). If the rate of exchange of protons with TEA were sufficiently slow, then one would expect that at a higher buffer capacity the exchange mechanism would be less detectable. The capacity of the buffer would not be exceeded and the pH would not change. We increased the buffer capacity of the intra- and extravesicular solutions by increasing the HEPES concentration to 10 mM. When vesicles loaded with HTEA10 buffer (pH 7.5) were placed in pH 7.5 AO solution, no decrease in fluorescence was observed. At the higher buffer capacity inwardly directed gradients of TEA were unable to increase the rate of fluorescence recovery. In contrast, when Na^+ was added

externally (25 mM) the rate of fluorescence recovery was accelerated (98 fluorescence units/min), albeit not as accelerated as in similar studies in a buffer with a lower capacity (172 fluorescence units/min). The Na^+ loaded vesicles also produced smaller maximal fluorescence changes (15-20%) in the presence of 10 mM HEPES. These results suggest that the amount of protons transported in exchange for TEA in BBMV was small so that 10 mM HEPES was able to neutralize the protons and to maintain the original pH. On the other hand, the proton gradient generated by Na^+-H^+ exchange in BBMV exceeded the buffer capacity of 10 mM HEPES and changed the intravesicular pH significantly.

The fluorescence quenching of the Na^+ loaded vesicles was reversed by adding Na^+ externally but not by adding TEA. Because Na^+ created an outwardly directed proton gradient, one would expect that the TEA exchange mechanism would increase the dissipation rate of the proton gradient. Thus, adding TEA externally should increase the rate of fluorescence recovery. However, TEA was added under steady-state conditions when a considerable amount of Na^+ had already been transported extravesicularly. Thus the rate of proton efflux produced by the TEA gradient may have been negligible in comparison to the combined rate of proton efflux produced by the sodium present in the external medium and the passive diffusion rate of protons which may be great at the low intravesicular pH. Alternatively, the external Na^+ may compete with TEA for exchange with protons. This would indicate that the Na^+-H^+ exchanger has a higher affinity for protons than the exchanger of TEA and protons and consequently protons were unavailable to the TEA transporter.

In summary, we have demonstrated that the efflux of protons in BBMV

was accelerated in the presence of a concentration gradient of TEA. Vesicles loaded with TEA also generated an intravesicular pH gradient. These results are consistent with the presence of an antiport of TEA and protons or a cotransport of TEA and hydroxyl ions in the renal brush border membrane. Although alternative mechanisms such as a catalytic effect of pH on organic cation transport may also be present, this study provides direct evidence for an exchanger of organic cations and protons or a cotransporter of organic cations and hydroxyl ions in the renal brush border membrane.

Chapter 4

Interactions of Organic Anions with the Organic Cation Transporter in Renal Brush Border Membrane Vesicles

Distinct driving forces and mechanisms have been identified for organic cation transport in the brush border and basolateral membrane of the proximal tubule (Kinsella et al., 1979; Rennick, 1981; Holohan and Ross, 1981). For example, the transport of organic cations across the basolateral membrane appears to be via a simple substrate transport system, whereas organic cation transport across the brush border membrane appears to be driven by a proton exchange (or hydroxyl cotransport) mechanism. Several studies (Kinsella et al., 1979; Rennick, 1981; Wright, 1985) have demonstrated that the transport of organic cations can be inhibited by other organic cations, but not by organic anions such as probenecid and p-aminohippurate (PAH). Therefore, it is generally assumed that there is a specific transporter for organic cations which is not affected by organic anions.

However, there are also data in the literature suggesting that there may be interactions between organic anions and organic cations in the renal proximal tubule. Sewing and Kaplowitz (1979) noted that coadministration of probenecid prolonged the terminal half-life of cimetidine in the rat. Since cimetidine is predominately eliminated by renal secretion in the rat, the data suggest that probenecid may inhibit the renal secretion of cimetidine. McKinney et al. (1981) demonstrated that the renal secretion of cimetidine in isolated perfused rabbit proximal tubules is decreased by probenecid in a concentration dependent fashion. Accumulation of cimetidine in canine renal cortical slices was also inhibited by probenecid (Cacini et al., 1982). Preliminary data from

our laboratory demonstrate that probenecid inhibits the proton gradient driven uptake of cimetidine in brush border membrane vesicles prepared from rabbit renal cortex (Gisclon et al., in press). Collectively, these studies have led us to speculate: (I). that the interaction may not be specific for cimetidine and probenecid, but may involve a general interaction between organic cations and organic anions in the proximal tubule; (II). that the interaction may occur at the brush border membrane ; and (III). that the purported exchange mechanism between organic cations and H^+ ions in the brush border membrane may actually be a cotransport system of organic cation and OH^- ions.

To test these hypotheses we investigated the effects of several organic anions including probenecid, furosemide and L-lactate on the transport of N^1-methylnicotinamide (NMN), the classical substrate of the organic cation transporter, in brush border membrane vesicles prepared from rabbit renal cortex. Our data demonstrate that in the presence of an outwardly directed proton gradient the transport of NMN in BBMV is significantly inhibited by both probenecid and furosemide. The inhibitory effect of probenecid is concentration dependent. NMN transport in the absence of a pH gradient is also inhibited by probenecid (10 mM). In contrast to probenecid and furosemide, L-lactate produced a small but significant overshoot of NMN.

Methods

NMN Transport Studies

Brush border membrane vesicles were prepared by a method involving magnesium-EGTA aggregation and differential centrifugation as developed by

Booth and Kenny (1974) and modified in our laboratory (Hsyu and Giacomini, 1987; Chapter 2). Ten µl of vesicles (20 mg/ml) loaded with pH 6.0 HK buffer (HEPES 10 mM and KCl 150 mM, pH with KOH) were incubated with 40 µl pH 7.4 HK buffer containing ^{3}H-NMN (2.0 µM) alone or ^{3}H-NMN (2.0 µM) and either 10 mM of probenecid or furosemide for 8, 15, and 30 seconds, and 1, 2.5, 5, 60 and 120 minutes. Control experiments were carried out in which sucrose (20 mM) was added to the incubation media to ascertain the effects of an osmotic gradient on the transport of NMN. Incubations were stopped by rapid addition of 3 ml of ice cold HK buffer. Radioactivity associated with the vesicles was determined by the rapid filtration method (Chapter 2). The concentration dependent effect of probenecid (ranging from 0.1 to 10 mM) on NMN transport was determined as described above at 8 seconds (initial time) and at 2 hours (equilibrium). When transport studies in the absence of a pH gradient were carried out, 10 µl of vesicles loaded with pH 7.4 HK buffer were incubated with 20 µl of pH 7.4 buffer containing ^{3}H-NMN and 0, 1 or 10 mM of probenecid for 8, 30 seconds and 1, 2.5 and 120 minutes.

The effects of several anions on the transport of NMN in the absence of a pH gradient were studied as described above except that the concentration of the anions was 50 mM and that the buffer used contained 10 mM HEPES, 50 mM KCl and 200 mM mannitol. The salts used were L-lactic acid, PAH, KCl, potassium phosphate, potassium isethionate, and glutamic acid.

Acridine Orange Studies

Acridine orange, a fluorescent probe for pH gradient (Warnock et al., 1982; Chapter 3), was used to ascertain the effect of probenecid on the pH

gradient in the membrane vesicles. Fifteen µl of vesicles (20 mg/ml) loaded with pH 6.0 HK buffer were added to 2.5 ml of pH 7.4 HK buffer containing 6 µM acridine orange and 0 or 10 mM of probenecid. The fluorescence of the mixture was monitored for 1 minute using a fluorescence spectrophotometer (MPF-2A, Perkin-Elmer, Norwalk, CN) equipped with a QPD-33 recorder (Perkin-Elmer). Excitation wavelength was set at 492 nm and emission wavelength was 530 nm. The fluorescence of the acridine orange solution was adjusted to 100%. Probenecid (10 mM) itself quenched 15-20% of the fluorescence of acridine orange. However, the initial fluorescence of acridine orange was readjusted to 100% when probenecid was present. After the addition of the vesicles, the maximal change of fluorescence and initial rate of fluorescence recovery, measured at 10 seconds after the vesicles were added, were determined.

Na^+-glucose transport

Five µl of vesicles (20 mg/ml) loaded with HK buffer were incubated with 20 µl of pH 7.4 buffer (150 mM NaCl and 10 mM HEPES) containing 0.175 mM D-glucose and 1.4 µCi ^{3}H-D-glucose. Incubations were carried out for 0.5, 1, 2.5, 5, and 60 minutes. The experiments were stopped and the radioactivity associated with the vesicles was measured as described previously in the section on NMN Transport Studies.

Data analysis

Unless otherwise specified, each data point in each experiment was determined as the mean of three replicates. For each study three experiments were performed in three separate membrane preparations. Data are presented

as mean±SD. Statistical differences were determined using Student's t-test.

In the studies carried out at various concentrations of probenecid, the rates of NMN transport in the BBMV were transformed to 1/rate and plotted against concentration of probenecid (Dixon plot). Linear least squares regression was performed for each experiment. The intercept at the abscissa represents $-IC_{50}$.

Chemicals

^{3}H-N^{1}-methylnicotinamide (2.8 Ci/mmole) was purchased from ICN (Irvine, CA). ^{3}H-D-Glucose (30 Ci/mmole) was obtained from New England Nuclear (Boston, MA). All the other chemicals were purchased either from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) and were the highest grade available.

Results

As depicted in the left panel of Fig. 16, the uptake of NMN in the presence of a pH gradient in the BBMV exhibited a typical overshoot phenomenon. The uptake was significantly reduced by probenecid (10 mM) and furosemide (10 mM) as demonstrated by the elimination of the overshoot phenomenon. Probenecid was the more potent inhibitor of NMN transport reducing the initial uptake of NMN to 14.8% of the control value (0.97±0.22 pmol/mg), whereas furosemide reduced it to 30.6 %. The uptake at equilibrium was not changed by probenecid (2.04±0.27 pmol/mg vs. 2.02±0.41 pmol/mg for

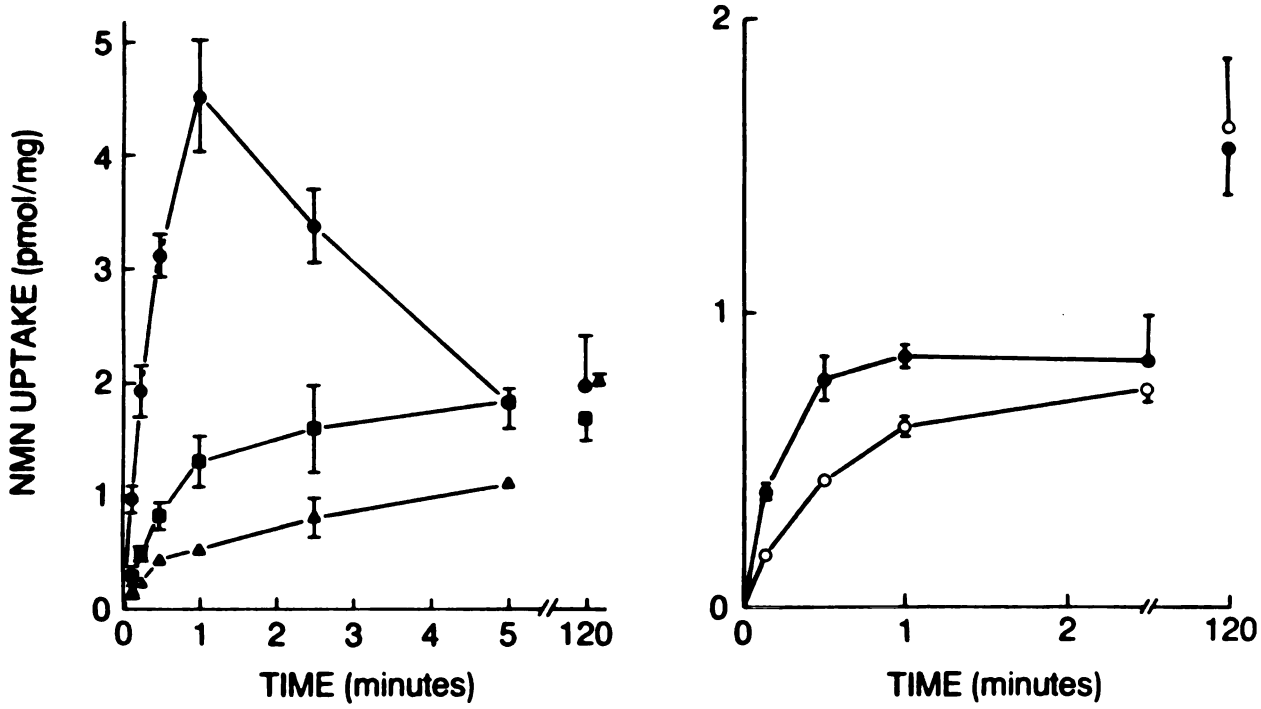


Fig.16. The time course of proton gradient stimulated NMN uptake (pH 6.0-pH 7.4, inside acidic) in renal brush border membrane vesicles in the absence of (●), or in the presence of 10 mM probenecid (▲), or in the presence of 10 mM furosemide (■) is depicted in the left panel. Each data point represents the mean±SE obtained from six (control) or three experiments (probenecid and furosemide). The time course of NMN uptake (pH 7.4-pH 7.4,) in the absence (●) or presence of 10 mM probenecid (○) in renal brush border membrane vesicles is depicted in the right panel. Each data point represents the mean±SD obtained from three experiments. In both panels, NMN uptake was significantly decreased at earlier times, but not at equilibrium by the organic anions.

control) or furosemide Indicating that the integrity and the size of the vesicles were not affected. Sucrose did not significantly decrease the uptake of NMN (Fig. 17). However, there was a small (< 15%), although not significant, decrease in NMN uptake at all time points which probably reflected a slight reduction in vesicle size caused by the osmotic effects of sucrose.

The inhibition of NMN uptake by probenecid (10 mM) in the absence of a pH gradient is depicted in Fig. 16 (right panel). The uptake of NMN at 8, 30 and 60 seconds, but not at 2 hours was significantly decreased. The initial uptake at 8 second was reduced to 44.7% of the control value indicating that in the absence of a pH gradient probenecid produced a significant inhibition in NMN uptake. The effects of probenecid on NMN uptake, although significant, were smaller than the effects obtained in the presence of a pH gradient. Probenecid at a concentration of 1 mM did not significantly inhibit the uptake of NMN in the absence of a pH gradient.

A Dixon plot of the data obtained from a representative experiment is shown in Fig. 18. A linear relationship between 1/rate of NMN transport and concentration of probenecid is illustrated by the straight regression line. Correlation coefficients (r) in three experiment were all greater than 0.99. IC_{50} obtained from the intercept on the abscissa was 2.31±1.18 mM (n = 3).

It was possible that probenecid may have caused an inhibition in the pH dependent uptake of NMN by accelerating the dissipation of the proton gradient. Thus, we carried out studies in which acridine orange was used to monitor the proton gradient. A representative acridine orange experiment is presented in Fig. 19. In the absence of probenecid, the fluorescence of acridine

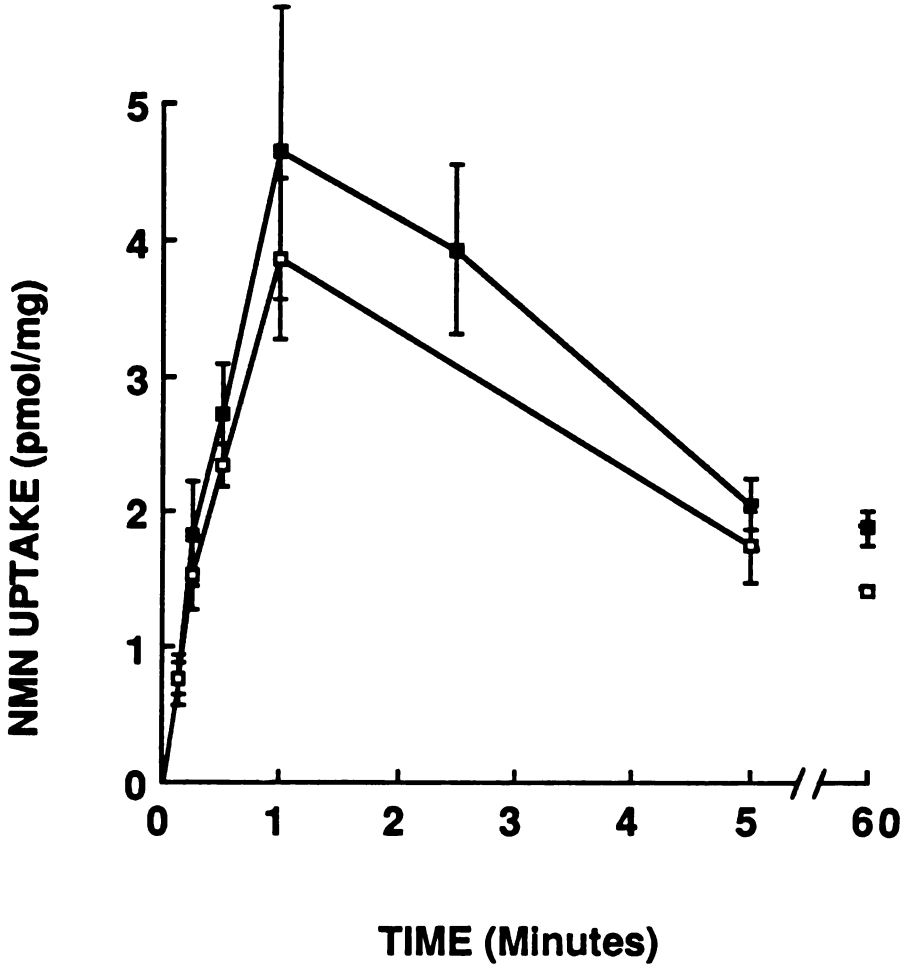


Fig. 17. The time course of proton gradient stimulated NMN uptake (pH 6.0-pH 7.4, inside acidic) in renal brush border membrane vesicles in the absence of (■), or in the presence (□) of 20 mM sucrose. Each data point represents mean±SE from three experiments.

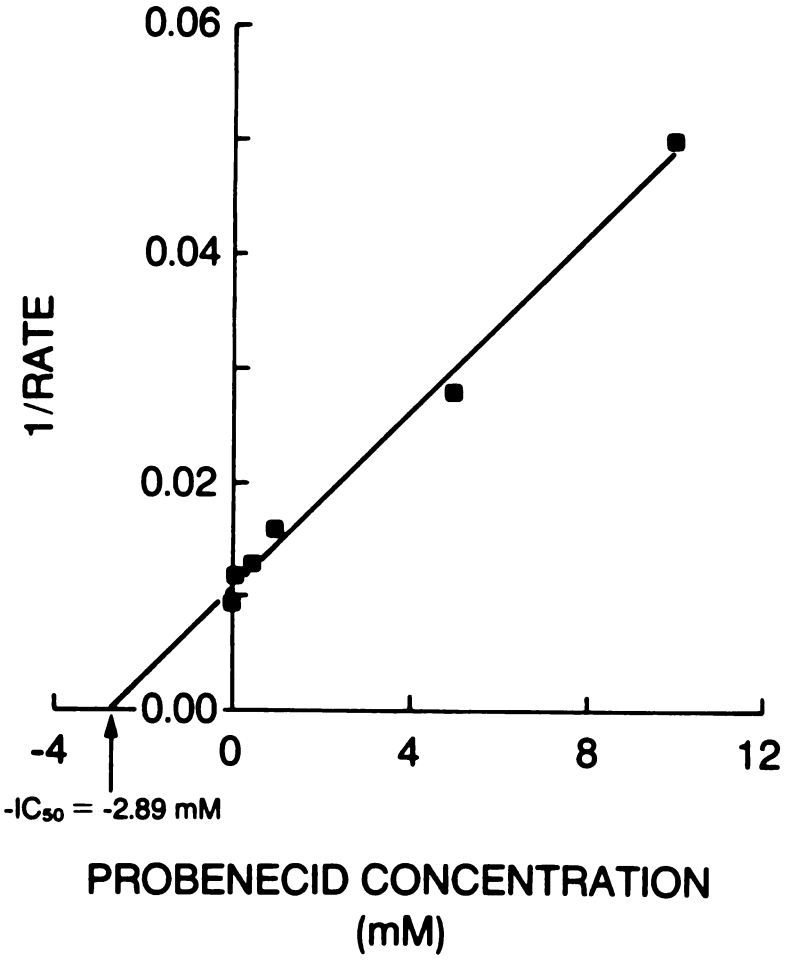


Fig. 18. Dixon plot showing a representative concentration dependent inhibition experiment. The inverse of the rate of NMN transport in BBMV is plotted against the concentration of probenecid. The IC_{50} is obtained from the intercept of abscissa.

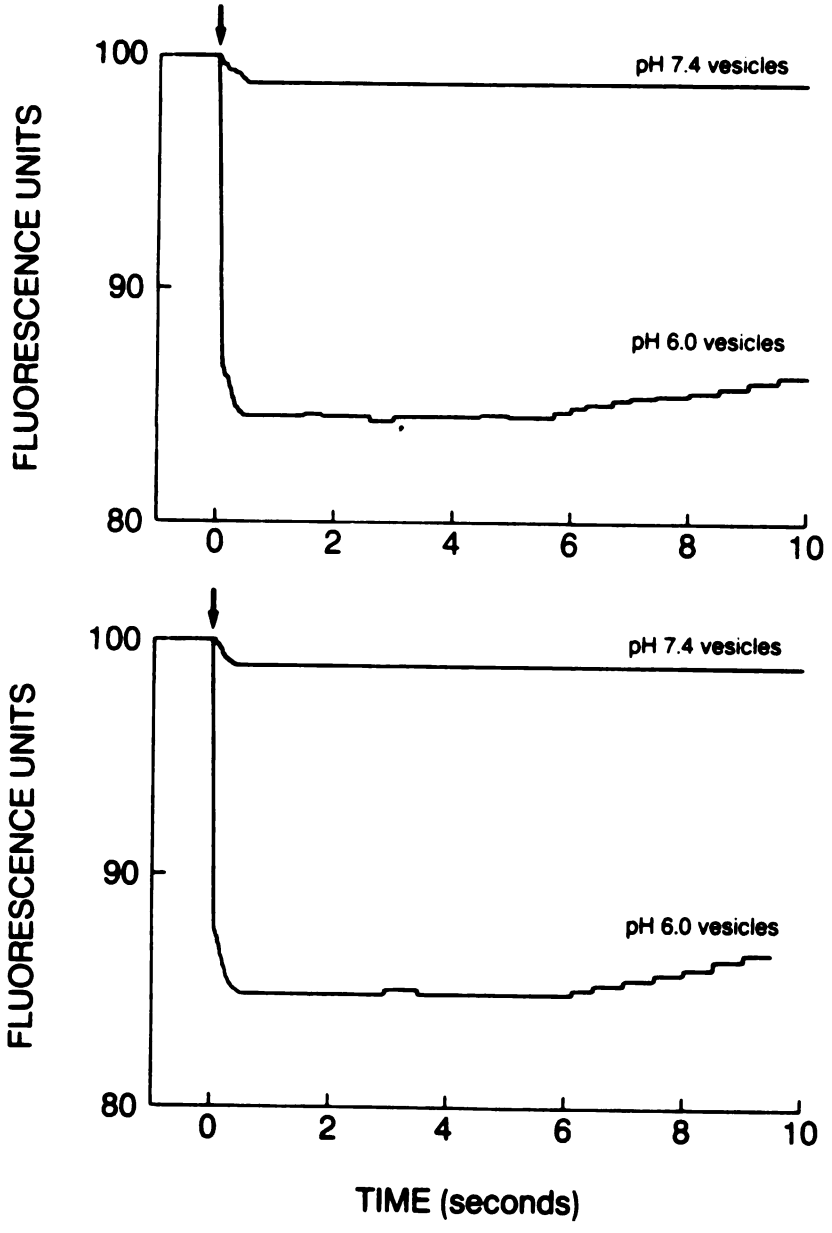


Fig. 19. Data from a representative experiment showing the change of fluorescence of acridine orange following the addition of BBMV loaded with pH 6.0 HK to a solution of 6 μM acridine orange in pH 7.4 HK buffer (upper panel) or to a solution of 6 μM acridine orange and 10 mM probenecid in pH 7.4 HK buffer (lower panel). The arrows indicate the time at which the vesicles were added to the acridine orange solution.

orange decreased rapidly following the addition of vesicles loaded with pH 6.0 HK buffer. The decrease in fluorescence is due to pH gradient dependent binding of AO to the vesicles which results in a quenching of the fluorescence. A steady state was reached and maintained for a few seconds followed by a dissipation in the proton gradient as indicated by the AO fluorescence returning to baseline values. The presence of 10 mM probenecid did not significantly affect the maximal change in fluorescence (16.1±0.08 fluorescence unit versus 15.6±1.0 fluorescence unit for the control). The initial rate of fluorescence recovery in the presence of 10 mM probenecid (0.168±0.018 fluorescence unit/s) was also not different from that of the control 0.160±0.017 fluorescence unit/s). These results suggest that probenecid does not affect the proton gradient or the permeability of the protons in the BBMV.

L-Lactate (50 mM) significantly increased the uptake of NMN at earlier times (less than or equal to one minute). A small overshoot was observed at one minute which was significantly greater than the equilibrium uptake (Fig. 20). These results suggest that lactate can drive the uptake of NMN in the absence of a pH gradient. None of the other anions tested including PAH, chloride, phosphate, isethionate, and glutamic acid were able to increase the transport of NMN (Table 3).

Glucose transport in the BBMV exhibited a typical overshoot phenomenon (Fig. 21) when an inwardly directed Na^+ gradient was present. The Na^+ driven glucose uptake was slightly (< 10%), although not significantly, inhibited by 10 mM probenecid. These results suggest that probenecid did not inhibit NMN transport by producing nonspecific effects on transport systems in general, or by altering membrane potential since the Na^+-glucose

Table 3. The Effects of Anions on the Transport of NMN in BBMV

Compound	Uptake (8 s)	Uptake (1 hr)	n
% control	% control		
control	100	100	5
chloride	95±25	84±9	4
glutamate	90±6	76±1*	3
isethionate	62±4*	73±2*	2
L-lactate	136±23*	93±8	5
PAH	102±34	92±13	5
phosphate	92±13	54±5*	3

The values are mean±SE ($n \geq 3$) or mean±range ($n = 2$) as percentage of the uptake for the control experiment. The mean uptake for control at 8 seconds is 0.36±0.02 pmol/mg and the mean uptake for control at 1 hour is 1.25±0.06 pmol/mg.

* values are significantly different from the control ($p < 0.05$).

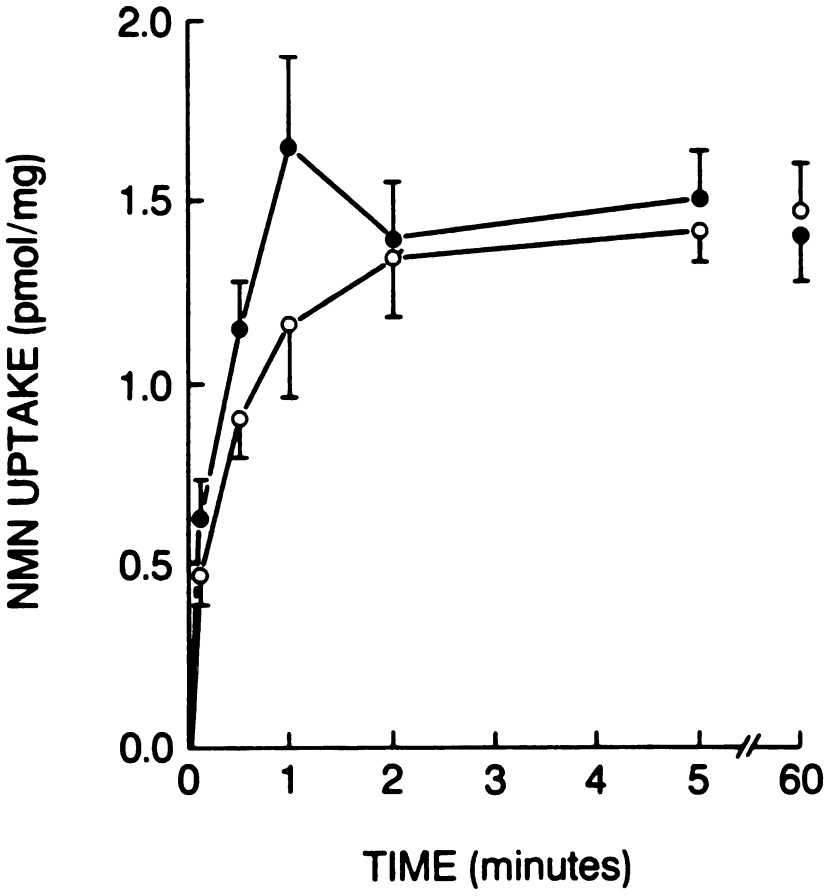


Fig. 20. The time course of NMN uptake (pH 7.4-pH 7.4,) in the absence (○) or presence of 50 mM lactate (●) in renal brush border membrane vesicles. Each data point represents mean±SE from five experiments. Values are significantly different from the control up to one minute .

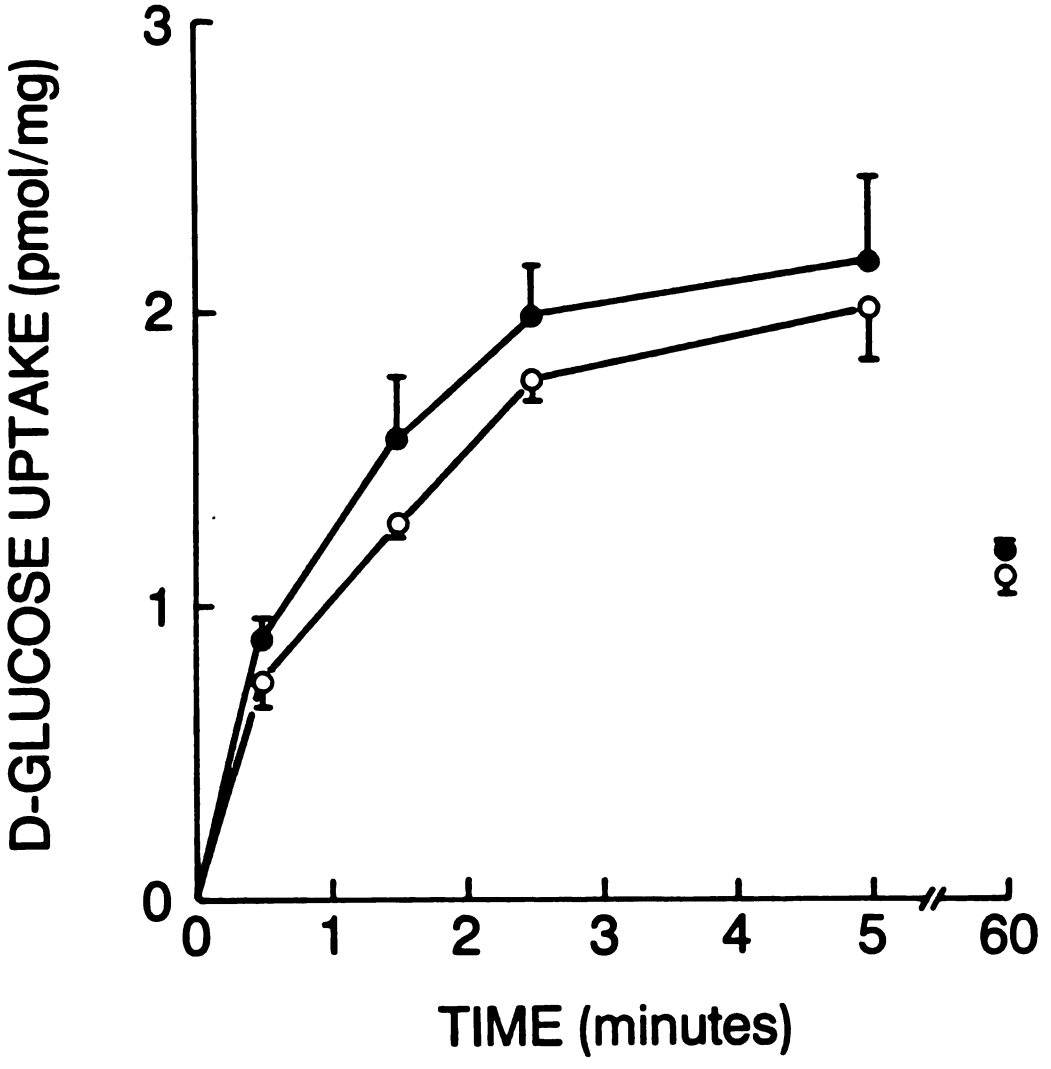


Fig. 21. The time course of Na^+-glucose uptake (pH 7.4-pH 7.4,) in the absence (●) or presence of 10 mM probenecid (○) in renal brush border membrane vesicles. Each data point represents mean±SE from three experiments.

cotransporter is sensitive to differences in potential (Kinne et al., 1975).

Discussion

A number of studies have indicated that the systems involved in the secretion of organic anions and organic cations are distinct (Kinsella et al., 1979; Rennick, 1981; Ross and Holohan, 1983; Wright, 1985). Competitive inhibitors of one system usually do not inhibit the transport of substrates of the other system. However, several studies, both in vivo and in vitro, indicate that probenecid, the classical inhibitor of the organic anion transport system in the proximal tubule, also inhibits the renal transport of cimetidine. A recent study in this laboratory demonstrated that probenecid decreases the transport of cimetidine in renal brush border membrane vesicles (Gisclon et al., in press). A major question is whether the observed interaction between probenecid and cimetidine is limited to these two compounds or whether organic anions may generally inhibit the transport of organic cations. To address this question, we studied the effects of probenecid and furosemide, two compounds known to inhibit organic anion transport, on the transport of NMN, a classical substrate of the organic cation transport system, in renal brush border membrane vesicles.

The results of this study demonstrated that the transport of NMN in the BBMV in the presence of an outwardly directed proton gradient was significantly reduced by both probenecid and furosemide (Fig. 16). Concentrations of probenecid as low as 0.1 mM were able to reduce the transport of NMN significantly. The IC_{50} of probenecid (2.31 mM) is somewhat greater than the Km value for NMN of 0.6 mM (6) in rabbit BBMV. This value is very similar to the Ki of probenecid (2.3 mM) for the PAH transport system in the renal brush

border membrane of rat (Kinsella et al., 1979), but is substantially higher than the Ki of probenecid (0.30 mM) for the PAH transport system in the renal brush border membrane of dog (Kinsella et al., 1979). However, the mechanism of inhibition (competitive, noncompetitive or uncompetitive) was not investigated. Therefore, the IC_{50} can not be appropriately converted to a Ki.

Four possible mechanisms may explain the inhibitory effects of these organic anions on the transport of NMN. First, the organic anions may have nonspecific membrane effects on the brush border membrane and thus inhibit transporters in general by reducing their capacities for transport. However, Na^+-glucose transport in the BBMV was not significantly affected by 10 mM probenecid (Fig. 21) indicating that at least one transporter is normally functional. Furthermore, neither probenecid nor furosemide altered the uptake of NMN in BBMV at equilibrium suggesting that these compounds did not affect the integrity or size of the vesicles. These results suggest that nonspecific effects on transporters in general is an unlikely mechanism.

Secondly, organic anions may affect the permeability of BBMV to protons or otherwise cause the dissipation of the proton gradient which in turn would reduce the rate of transport of NMN. The studies of acridine orange suggest that the permeability of BBMV to protons was not changed by probenecid (Fig. 19). The studies in which probenecid (10 mM) inhibited the uptake of NMN in the absence of a pH gradient also support the hypothesis that the inhibitory effects of probenecid on NMN uptake were not due to a dissipation of the pH gradient. Probenecid appears to be a less potent inhibitor of NMN transport in the absence of a pH gradient. This may explain why other investigators who have studied the effects of probenecid on NMN transport in isolated membrane

vesicles have failed to observe the inhibition.

Another possible mechanism that may explain the effects of probenecid and furosemide on NMN transport is that these organic anions may change the potential difference across the membrane and indirectly inhibit the transport of NMN. This would imply that the transport of NMN in the brush border membrane is electrogenic and thus can be affected by alterations in potential difference. The rheogenicity of organic cation transport in BBMV is controversial. An electroneutral organic cation proton exchange has been proposed by Sokol et al. (1985), whereas a rheogenic mechanism involving an NMN-$2H^+$ exchanger has been proposed by Wright (1985). If the transport is electroneutral as proposed by Sokol et al., then changes in potential difference would not affect the the transport of NMN. On the other hand if Wright's model is correct and assuming an inwardly directed concentration gradient of organic anions creates an intravesicularly negative potential difference, then transport of NMN would be reduced. However, if the presence of probenecid in the extravesicular medium produces a negative potential intravesicularly, Na^+-glucose transport which is sensitive to potential difference in the vesicles should be enhanced in the presence of the same concentration of probenecid. In our studies, the Na^+-glucose transport was slightly reduced by 10 mM probenecid.

The final mechanism involves the direct interaction of organic anions with the organic cation transporter. Several groups (Holohan and Ross, 1981; Takano et al., 1984; Wright, 1985, Sokol et al., 1985; McKinney and Kunnemann, 1985) have proposed that the transporter of organic cations is an antiport of organic cations and protons, although it can not be distinguished

from a cotransport of organic cations and hydroxyl ions (Holohan and Ross, 1981). It is possible, albeit unlikely, that organic anions compete directly with NMN or protons for binding to the transporter and thereby inhibit the transport of NMN in the BBMV. An intriguing possibility is that the organic cation transporter is actually a cotransporter of organic cations and hydroxyl ions and organic anions such as probenecid and furosemide act at the OH^- site. Furosemide has been shown to inhibit the Cl^--Cl^- exchange (Brazy and Gunn, 1976) and Na^+-K^+-$2CL^-$ (Greger et al., 1983) cotransport, presumably by acting at the binding site for inorganic anions. It is conceivable that in our study probenecid and furosemide inhibited the transport of NMN by affecting the OH^- site of an NMN-OH^- cotransporter. Alternatively, probenecid and furosemide may interact with a third site which modulates the activity of the organic cation transporter. Further studies are needed to delineate the exact mechanism of the observed interaction between NMN and organic anions.

If an organic cation-hydroxyl cotransport exists, some anions should be able to replace hydroxyl as substrates. These anions should stimulate the transport of NMN. We examined the effects of several anions including chloride, phosphate, PAH, L-lactate, isethionate, and glutamate representing substrates of several transport systems in brush border membrane. Among these, L-lactate was able to create an overshoot of NMN. L-lactate can be transported by the chloride transport system in the erythrocyte (Halestrap., 1976). This suggests that L-lactate, a small molecule, can replace inorganic anions such as chloride, as a substrate. It is conceivable that lactate also acts as a substrate in the binding site of OH^- in an organic cation-OH^- transporter. The overshoot of NMN transport created by the L-lactate gradient was small compared to the overshoot created by the pH gradient. This small overshoot

may be due to weak affinity of lactate to the transporter and/or due to a competing transport system which specifically transports lactate (Murer et al., 1981). The results indicate that there is a binding site for anions in the organic cation transporter which interacts with inhibitors and substrates. This binding site appears to exhibit some specificity toward anions. For example, L-lactate, probenecid, and furosemide interacted with this site, whereas PAH and chloride did not (Table 3). A model for the NMN-OH^- cotransport in the renal brush border membrane is depicted in Fig. 22. There are two binding sites: one for organic cations such as NMN, the other for the anions where OH^- and L-lactate are substrates and probenecid and furosemide are inhibitors.

Our results have implications not only to our understanding of the mechanism involving organic cation transport in the proximal tubule, but possibly also to potential drug-drug interactions which may occur between organic cations and organic anions clinically. One can estimate the expected intracellular concentration of probenecid in the proximal tubule following therapeutic doses. In a clinical study, a 2 gm oral dose of probenecid produced a mean plasma concentration of 150 μg/ml (0.53 mM) in humans (Selen et al., 1982). Assuming a plasma protein binding of 85% (Selen et al., 1982) and a fifteenfold accumulation of probenecid in the proximal tubule cell (Sheikh and Stahl, 1977), one can obtain an expected cellular concentration of probenecid of 1 mM. In this study, 1 mM probenecid resulted in a 44% decrease in the initial NMN uptake in the BBMV. Certainly, one cannot directly extrapolate data obtained in vitro in animal tissue to the clinical situation. However, it is clear that the concentrations of probenecid that inhibited the transport of NMN in BBMV are of the same order of magnitude as those which may be present in the cells of proximal tubule after therapeutic doses. Thus, coadministration of

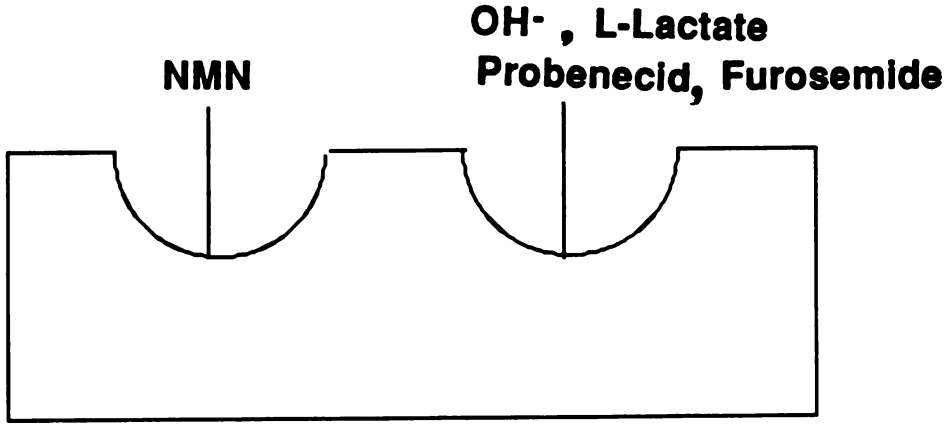


Fig. 22. A model for the organic cation-OH^- cotransporter in the renal brush border membrane. There are two binding sites: one for organic cations as represented by NMN, the other for anions where OH and L-lactate are substrates and probenecid and furosemide are inhibitors.

probenecid with organic cations might produce a decrease in the renal secretion of organic cations. It will be of interest to conduct clinical studies to determine whether such interactions actually occur.

In summary, we have demonstrated that the transport of organic cations in BBMV can be inhibited by probenecid and furosemide or accelerated by L-lactate. The results may suggest the presence of an organic cation-OH^- cotransport in BBMV. The organic anions may exert their effects by interacting with the OH^- (anionic) site.

Chapter 5

High Performance Liquid Chromatographic Determination of the Enantiomers of Pindolol in Biological Fluids

Pindolol [1-(indole-4-yloxy)-3-isopropylamino-2-propanol], a non-selective β-adrenergic blocking agent with intrinsic sympathomimetic activity, is used clinically in the treatment of hypertension (Persson and Ulrich, 1973) and angina pectoris (Frithz and Nordgren, 1975). As with most β-adrenergic blocking agents, pindolol (Visken) is marketed as a racemic mixture of two optical isomers. (-)-Pindolol, the more potent enantiomer, is responsible for much of the therapeutic action of pindolol (Jeppsson et al., 1984). Because enantiomers often differ in their pharmacokinetic and pharmacodynamic properties, it is desirable to measure each enantiomer individually in biological fluids after administration of a racemic mixture.

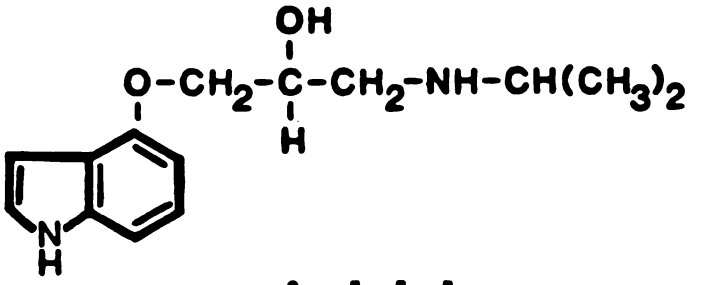
Several derivatizing agents have been used for the stereospecific HPLC assay of β–adrenoceptor blocking agents. Among these, N-trifluoroacetyl-(-)-prolyl chloride (Silber and Riegelman, 1980) is susceptible to racemization during storage and a low temperature must be achieved for the reaction of this reagent and propranolol. *Tert*-butoxycarbonyl-L-leucine anhydride (Hermansson and Bahr, 1982) is not commercially available and the reaction with β–adrenoceptor blocking agents involves several steps. R-(+)-Phenylethyl isocyanate (Thompson et al., 1982; Wilson and Walle, 1984) has never been successfully used for the derivatization of β–adrenoceptor blocking agents in plasma. None of these agents has been specifically used for the determination of concentrations of pindolol enantiomers in the plasma or urine.

This Chapter describes the first stereospecific high performance liquid chromatographic method for the measurement of the enantiomers of pindolol in human plasma and urine. The procedure involves a three-step solvent extraction of pindolol from plasma and urine, followed by derivatization at room temperature with S-(-)-α–methylbenzyl isocyanate (Fig. 23). The diastereomers formed are then separated by HPLC and detected by fluorescence detection. The procedure is sufficiently sensitive to allow quantitation of the individual enantiomers after therapeutic doses of racemic pindolol. Although the method has been developed to determine (+)- and (-)-pindolol in biological fluids, data are presented demonstrating the adaptability of the procedure to other β–adrenoceptor blocking agents.

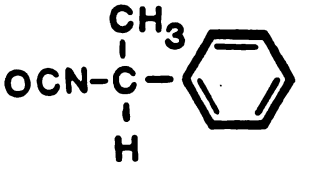
Methods

Sample Preparation

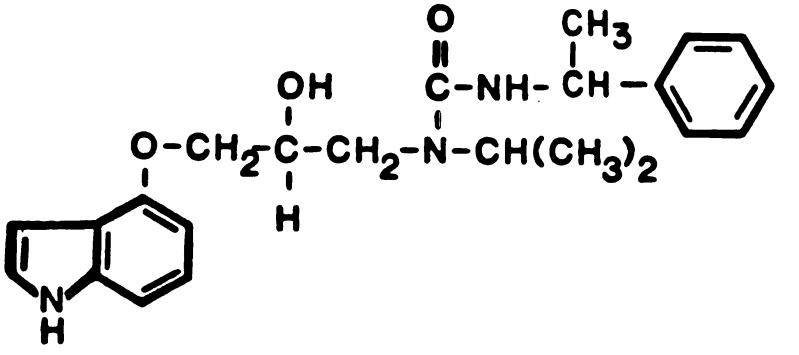
One ml of plasma and an aliquot of 15-180 µl of a racemic pindolol standard solution (1.2 ng/µl) were mixed in a 20 X 150 mm disposable borosilicate culture tube. One-half ml of 2 M NaOH was added and mixed with the sample. Five ml of ether was added and the resulting mixture vortexed for one minute and then centrifuged at 1000 g for 5 minutes. The aqueous and organic phase were separated by inserting the test tube into a dry ice-acetone bath and decanting the ether layer into another culture tube after the water layer had frozen. One-half ml of 1 M HCl was added to the decanted ether layer which was then vortexed for 1 minute and centrifuged at 1000 g for 5 minutes. The aqueous layer was retained by discarding the ether layer after immersion of the tube into a dry ice-acetone bath. One-half ml of 2 M NaOH and 5 ml of ether were then added to the thawed aqueous phase and the mixture vortexed for 1



pindolol



S-(-)-α-methylbenzyl isocyanate



urea derivative

Fig. 23. Structures of pindolol, S-(-)-α-methylbenzyl isocyanate and their urea derivative.

minute. The ether layer was separated from the aqueous layer as described above.

Five ml of urine containing approximately 25 μg of racemic pindolol was diluted with various volumes of distilled water to obtain suitable concentrations of pindolol. One-half ml of the appropriately diluted solution was used for the assay. The extraction was identical to that described for plasma except that 0.3 ml of 2 M NaOH solution and 0.25 ml of 1 M HCl solution were used instead.

The ether layer obtained from the procedures described in the preceding paragraphs was evaporated under a nitrogen gas stream at room temperature. Fifty μl of derivatizing solution of S-(-)-α–methylbenzyl isocyanate was then added and the sample was vortexed for 30 seconds. The solution was evaporated under a nitrogen stream and reconstituted with 100 μl of mobile phase (see below). Eighty μl was injected onto the column for plasma samples. Forty μl was injected for urine samples.

Chromatography

The HPLC system used in this study included a pump (Beckman 110A, Beckman Instruments, Inc., Fullerton, CA), an injector (Waters U6K, Water Associates, Milford, MA) and a variable wavelength fluorescence detector (Kratos FS 970 fluorometer and Kratos GM 970 monochromator, Kratos Analytical Instruments, Ramsey, NJ). The column was a reversed-phased C18 column (Altex ultrasphere ODS 5 μm, 4.6 X 150 mm, Beckman Instruments Inc., Berkeley, CA). The recorder was OmniScribe D-5000 Strip Chart Recorder (Houston Instruments, Austin, TX). The mobile phase was methanol:water

(62:38, v/v). The flow rate was 1.2 ml/min with a column inlet pressure of approximately 1900 psi. The excitation wavelength of fluorescence was set at 209 nm with a filter cutoff at 320 nm. The sensitivity was adjusted to 0.05 μA for plasma samples and to 0.1 μA for urine samples.

Identification of Peaks

To ascertain which peaks corresponded to (+)- and (-)-pindolol, 1 μg of racemic pindolol was treated with 100 μl of the derivatizing solution of S-(-)-α–methylbenzyl isocyanate either with or without the addition of 0.5 μg (+)-pindolol. Five μl of each solution was injected onto the column. Chromatograms of these two solutions were compared to differentiate peaks of (+)- and (-)-pindolol derivatives. Each enantiomer (0.5 μg) was also derivatized and chromatographed. The retention time of each diastereomer was compared with those obtained following derivatization of racemic pindolol.

Characteristics of the Assay

Calibration

The assay was calibrated by analyzing 1.0 ml aliquots of blank plasma to which approximately 15-220 ng of racemic pindolol had been added or 0.5 ml blank urine to which approximately 100-2500 ng of racemic pindolol had been added. Three or four replicates were measured for each concentration. Six concentrations were used for each calibration curve. In each sample, the absolute heights of the (+)- and (-)-pindolol derivative peaks were measured and plotted against the concentration of each enantiomer. Linear least squares

regression was applied to determine the slopes and intercepts. From these, the concentrations of (+)- and (-)-pindolol in each unknown sample were obtained.

Reproducibility and Precision

Absolute peak heights were measured and the coefficient of variation (CV) was calculated at each concentration. Reproducibility measurements were based upon a minimum of three replicates of peak heights obtained on a single day. Slopes of the calibration curves for plasma and urine samples were determined daily. The variation of the slopes sobtained on three different days was used as a measure of day-to-day variance. Using the calibration curve the calculated concentration of each enantiomer was obtained from the peak height and differences between the calculated concentration and the initial concentration present (precision) were determined.

Derivatization Efficiency

The efficiency of derivatization was determined by treating 24 µg racemic pindolol with 24 µg of the derivatizing agent in 100 µl of chloroform. Ten µl of the solution was applied to a TLC plate and the mixture was developed with chloroform : ethyl acetate (30 :70). unreacted pindolol was used as an indicator of the derivatizing efficiency. Pindolol and its derivatives were detected at 254 nm.

Recovery from Extraction

One ml aliquots of water, plasma and urine were spiked with known

quantities of racemic pindolol (17.8 and 214 ng for plasma and water; 238 and 4750 ng for urine). After the samples were extracted, derivatized and chromatographed as previously described, the peak heights for the (+)-pindolol and (-)-pindolol derivatives were compared with peak heights obtained when the same amount of each enantiomer in chloroform was treated with S-(-)-α–methylbenzyl isocyanate and injected directly onto the column. Four replicate determinations at each concentration were performed.

Identification of Derivatives

To differentiate between carbamate and urea diastereomers, 30 mg of racemic pindolol was treated with S-(-)-α–methylbenzyl isocyanate in 30 ml of chloroform for 10 minutes at room temperature. The solution was then extracted twice with 20 ml of 0.1 M HCl. Ten µl of the organic phase and the combined aqueous phase was applied to TLC plates and developed as previously described. The developed plates were analyzed for unreacted pindolol and pindolol derivatives. The chloroform layer was evaporated under reduced pressure and was reconstituted with 2 ml of absolute alcohol. The alcohol solution was submitted for chemical ionization mass spectral analysis.

Interference by Other Compounds

Because of the nature of the extraction, acidic and neutral drugs are not expected to interfere with this assay. Several basic drugs with native fluorescence were similarly derivatized with S-(-)-α–methylbenzyl isocyanate and chromatographed to detect possible interferences.

Search for an Internal standard

Several β–adrenoceptor blocking agents (acebutotol, atenolol and *tert*-butylpindolol) and indole compounds (α–ethyltryptamine, indole-acetone, indole acetonitrile, 7-methyltryptamine, N-ω–methyltryptamine, tryptophol, tryptamine, indole-3-carbinol) were also tested as internal standards. These compounds were similarly derivatized with S-(-)-α-methylbenzyl isocyanate and chromatographed to determine whether the retention times were appropriate.

Application of the Assay to Other β–Adrenoceptor Blocking Agents

Acebutolol HCl and atenolol HCl (approximately 1 mg each) were dissolved in one ml of distilled water. NaOH (1 M, 50 µl) and 3 ml of ethyl acetate were added and the samples vortexed to extract the free base. The ethyl acetate solution (2.5 ml) was evaporated under a nitrogen stream. The derivatizing agent in chloroform (500 µl) was then added. The chloroform solution was evaporated under a nitrogen stream and reconstituted with 500 µl of mobile phase. Aliquots of the atenolol (5 µl) and the acebutolol (2 µl) were injected onto the HPLC system. The mobile phase was methanol : water (50 : 50) for the atenolol sample and methanol : water (55 : 45) for the acebutolol sample. The flow rate was 1.2 ml/min for the atenolol and 2.0 ml/min for the acebutolol derivatives. The excitation wavelength was adjusted to 220 nm for both samples.

Chemicals

S-(-)-α–methylbenzyl isocyanate (Aldrich Chemical Co., Milwaukee, WI), (±)-pindolol (Sigma Chemical Company, St. Louis, MO), (+)- and (-)-pindolol (Sandoz Inc., East Hanover, NJ) were used without further purification. Anhydrous ether (Mallinckrodt Inc., Paris, KY) was redistilled from sodium aluminum hydride before use. Chloroform ((Mallinckrodt Inc.) was dried with calcium chloride. Methanol (J.T. Baker Chemical Co., Phillipsburg, NJ) was HPLC grade. TLC plates (MC/B Manufacturing Chemist Inc., Cincinnati, OH), were precoated silica gel 60 TLC sheets with a fluorescent indicator (F254), 20 X 20 cm.

Racemic pindolol (~ 60 mg) was dissolved in 100 ml of methanol as a stock solution and stored at -20^{0}C. Standard solutions were prepared by dilution with distilled water to the appropriate concentration immediately before use. Daily, 2 µl of S-(-)-α–methylbenzyl isocyanate was dissolved in 10 ml chloroform as the derivatizing solution.

Results

Chromatography and Identification of Peaks

Chromatograms of typical plasma and urine samples are shown in Fig. 24. Baseline resolution of the diastereomers in both plasma and urine sample was obtained with retention times of 13.8 and 15.6 minutes. In the sample spiked with the (+)-pindolol, the peak height at 13.8 minutes was doubled indicating that this peak corresponded to the (+)-pindolol derivative. When individual enantiomers were used instead of the racemate, the (+)-pindolol derivative showed only one peak with a retention time of 13.7 minute and the (-)-pindolol

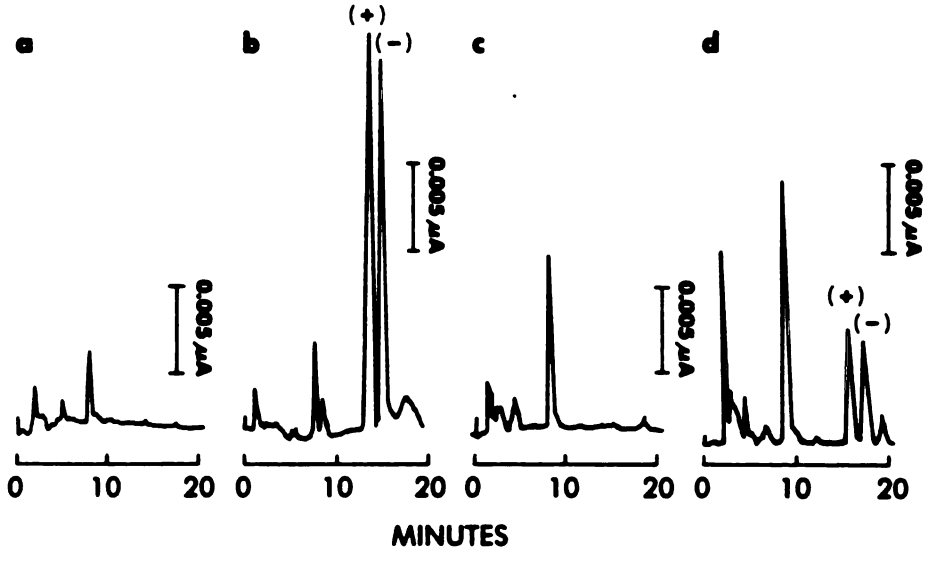


Fig. 24. HPLC chromatograms obtained after extraction of racemic pindolol followed by derivatization with S-(-)-α-methylbenzyl isocyanate. Key: (a) blank plasma sample, (b) plasma sample initially containing 100 ng/ml racemic pindolol, (c) blank urine sample, (d) urine sample initially containing 100 ng/ml racemic pindolol.

derivative, 15.6 minutes.

Characteristics of the Assay

Calibration

Peak heights of the (+)- and (-)-pindolol derivatives correlated linearly with concentrations of both enantiomers in the range of concentrations tested. Correlation coefficients of the lines were > 0.99 (Table 4). The limit of detection, defined as four times the baseline noise, was 2 ng/ml for each enantiomer in plasma. There was little variation in the observed peak heights when plasma samples from different individuals were used to construct the calibration curves.

Reproducibility and Precision

Data showing the reproducibility of the assays of plasma and urine samples are summarized in Table 5. The CV, used as an indicator of reproducibility, was less than 12% at each concentration of the individual enantiomers. The day-to-day CV was approximately 12% for plasma samples and approximately 10% for urine samples (Table 4) for both enantiomers. The calculated concentrations of enantiomers were within 10% of the initial concentrations present. When 1-ml plasma samples containing 101 ng of (+)-pindolol and 24.8 ng of (-)-pindolol were assayed, the calculated amounts were 98.5 ng (+)-pindolol (CV = 7.0%, n = 4) and 24.9 ng (-)-pindolol (CV = 6.3%, n = 4). The calculated amounts for plasma samples containing 24.8 ng (+)-pindolol and 100 ng (-)-pindolol were 23.6 ng (+)-pindolol (CV = 3.7%, n = 4) and 103 ng (-)-pindolol (CV = 8.0%, n = 4).

Table 4. Calibration Curves for the Pindolol Enantiomers

Sample	Slope[a]	Intercept[b]	Correlation Coefficient (r)
(+)-Pindolol[c]	0.256 ± 0.030	0.074 ± 0.081	0.999
(-)-Pindolol[c]	0.240 ± 0.028	-0.058 ± 0.077	0.999
(+)-Pindolol[d]	0.0305 ± 0.0030	-0.0171 ± 0.0387	0.994
(-)-Pindolol[d]	0.0279 ± 0.0028	-0.0211 ± 0.0325	0.996

[a] Values of the slopes are the mean±SD from three different days.

[b] Not significantly different from zero.

[c] Plasma samples.

[d] Urine samples.

Table 5. Reproducibility and Precision of the Assay for (+)- and (-)-Pindolol in Plasma and Urine

Expected Conc. ng/ml	solvent	Calculated Conc. ng/ml (+)-pin[a,b]	Calculated Conc. ng/ml (-)-pinl[a,b]
8.92	plasma	8.47 (9.30)	8.89 (7.48)
17.8	plasma	17.0 (5.47)	17.0 (5.48)
35.6	plasma	35.9 (3.29)	33.8 (6.16)
53.5[c]	plasma	55.1 (3.19)	56.6 (2.08)
77.2	plasma	78.0 (7.07)	78.4 (7.91)
107	plasma	106 (11.6)	105 (11.9)
119	urine	117 (8.94)	118 (10.6)
238	urine	218 (5.48)	219 (2.89)
475	urine	505 (4.69)	505 (2.42)
950	urine	990 (3.83)	974 (5.80)
1910	urine	1840 (6.43)	1860 (6.70)
2380	urine	2330 (4.02)	2450 (4.65)

[a] Mean concentrations of four measurements of each pindolol enantiomer calculated from peak height.

[b] Value in the parentheses represneteds coefficient of variation calculated from four measurements at each concentration.

[c] Only three measurements were carried out.

Derivatization Efficiency

The R_f values of the (+)-pindolol derivative, (-)-pindolol derivative and unreacted pindolol in the TLC system were 0.72, 0.67 and 0, respectively. When a 10-µl aliquot of the derivative (prepared from 2400 ng of pindolol) was developed on the TLC system, no trace of unreacted pindolol could be found. Experiments demonstrated that pindolol would be undetectable only when its concentration was ≤ 20 ng. Thus, it is concluded that the derivatization efficiency was > 99%.

Recovery from Extraction

Recoveries of (+)- and (-)-pindolol from plasma, urine and water are tabulated in Table 6. Plasma and water had similar recoveries, whereas urine samples had lower, but consistent, recoveries.

Interference by Other Compounds

None of the retention times of the drugs tested overlapped with those of the pindolol diastereomers. The retention times were either < 5 minutes (morphine, naloxone, and prazosin) or > 45 minutes (desipramine, imipramine, (+)-metoprolol, practolol, propanthelol, quinine, and verapamil).

Search for an Internal standard

The criteria used for selection of an internal standard were that the compound should react with S-(-)-α–methylbenzyl isocyanate and fluoresce, be

Table 6. Total Recovery from the Extraction of (+)-Pindolol and (-)-Pindolol

Conc. ng/ml	solvent	enantiomer	%Recovery	CV[a]
8.91	plasma	(+)-pin	75.0	10.9
8.91	plasma	(-)-pin	73.6	7.12
107	plasma	(+)-pin	80.0	6.00
107	plasma	(-)-pin	76.9	5.48
238	urine	(+)-pin	35.2	8.92
238	urine	(-)-pin	33.1	10.6
4750	urine	(+)-pin	35.0	6.43
4750	urine	(-)-pin	33.1	6.72
107	water	(+)-pin	80.3	4.76
107	water	(-)-pin	75.0	5.10

[a] Coefficient of variation obtained from four replicates at each concentration.

extracted and chromatograph similarly to pindolol. Two compounds, 1-(indole-4-yloxy)-3-(*tert*-butylamino)-2-propanol (*tert*-butylpindolol) and N-ω–methyl-tryptamine, fulfilled the criteria (Table 7). However, we were unable to obtain either enantiomer of *tert*-butylpindolol. Racemic *tert*-butylpindolol resulted in two incompletely resolved peaks at 22.4 and 23.6 minutes in this procedure. N-ω–methyltryptamine after reacting with S-(-)-α–methylbenzyl isocyanate had a retention time of 12 minutes. When N-ω–methyltryptamine was added to the plasma and urine samples containing racemic pindolol, the coefficient of variation of the peak height ratio of pindolol versus N-ω-methyltryptamine was somewhat higher (approximately 10-20%) than those using peak heights of pindolol alone. The higher variation may have been produced by the pH's used in the extraction which may have caused the partial degradation of N-ω–methyltryptamine. In this study, because the total recovery of the pindolol diastereomers was constant (Table 6) and the assay was both reproducible and linear when using absolute peak heights (Tables 4 and 5), an internal standard was not included. However, these two compounds can be used as internal standards.

Identification of Derivatives

There are two functional groups on the pindolol molecule that could react with S-(-)-α–methylbenzyl isocyanate: the chiral secondary alcohol to form carbamate derivatives, and the secondary amine to form urea derivatives (Fig. 23). Evidence was obtained in this study suggesting that the urea derivatives were formed. First, the derivatives were not extracted with 0.1 N HCl from chloroform which suggests the presence of urea derivatives. Since the carbamate derivatives would contain the readily protonated nitrogen atom

Table 7. Retention Times of Compounds Tested

compound	retention time (min)
acebutolol (racemic)	13.5 and 15.0
atenolol (racemic)	5.2 and 5.4*
tert-butylpindolol (racemic)	22.4 and 23.6*
α–ethyltryptamine	14.2
indole-3-acetone	2
indole acetonitrile	-
7-methyltryptamine	13.5
N-ω–methyltryptamine	12
tryptophol	2 and 16
tryptamine	7.9
indole-3-carbinol	2.2

* Two peaks were partially resolved.

found in pindolol, these derivatives should be soluble in acid. Secondly, the mass spectrum was consistent with urea derivatives (Fig. 25). A molecular ion (MH^+) was observed at m/z 396, suggesting that only one molecule of S-(-)-α–methylbenzyl isocyanate reacted with one molecule of pindolol. The ion at m/z 378 resulting from the loss of H_2O suggests the existence of a free hydroxyl group in the molecule.

Application of the Assay to Other β–Adrenoceptor Blocking Agents

Baseline separation of the diastereomers of atenolol and acebutolol was achieved (Fig. 26). The retention times were 18.4 and 19.5 minutes for the diastereomers of atenolol and 26.3 and 28.6 minutes for the diastereomers of acebutolol.

Discussion

β–Adrenoceptor blocking agents are drugs frequently used in the treatment of hypertension (Persson and Ulrich, 1973) and angina pectoris (Frithz and Nordgren, 1975). Most of them are clinically administered as a racemic mixture, although the (-)-enantiomer is usually approximately one hundredfold more potent than the (+)-enantiomer in β–adrenoceptor blocking activity (Jeppsson et al., 1984; Barrett and Cullum, 1968). Enantiomers often exhibit different pharmacokinetic characteristics. For example, S-(-)-propranolol is cleared more slowly than R-(+)-propranolol following oral doses of racemic propranolol in humans (Silber et al., 1982). It is therefore important to be able to quantitate both enantiomers in biological fluids in the pharmacokinetic,

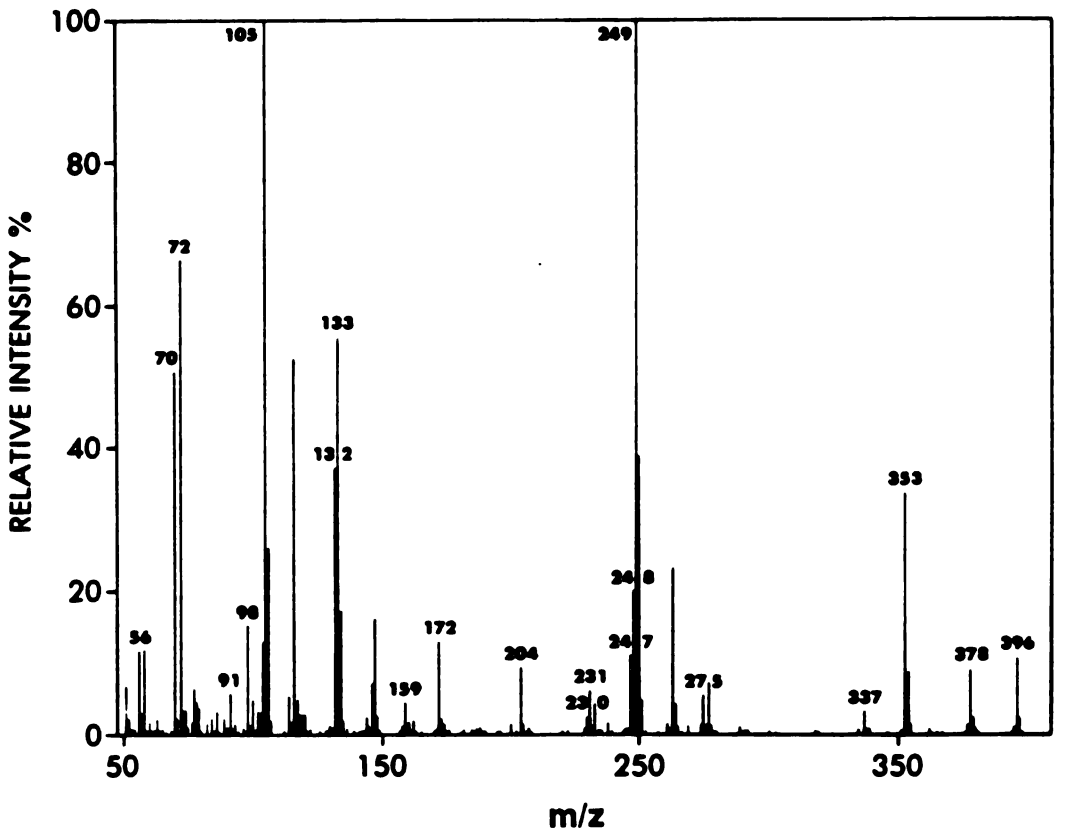


Fig. 25. Chemical ionization mass spectra of the compounds resulting from the reaction of pindolol with S-(-)-α-methylbenzyl isocyanate.

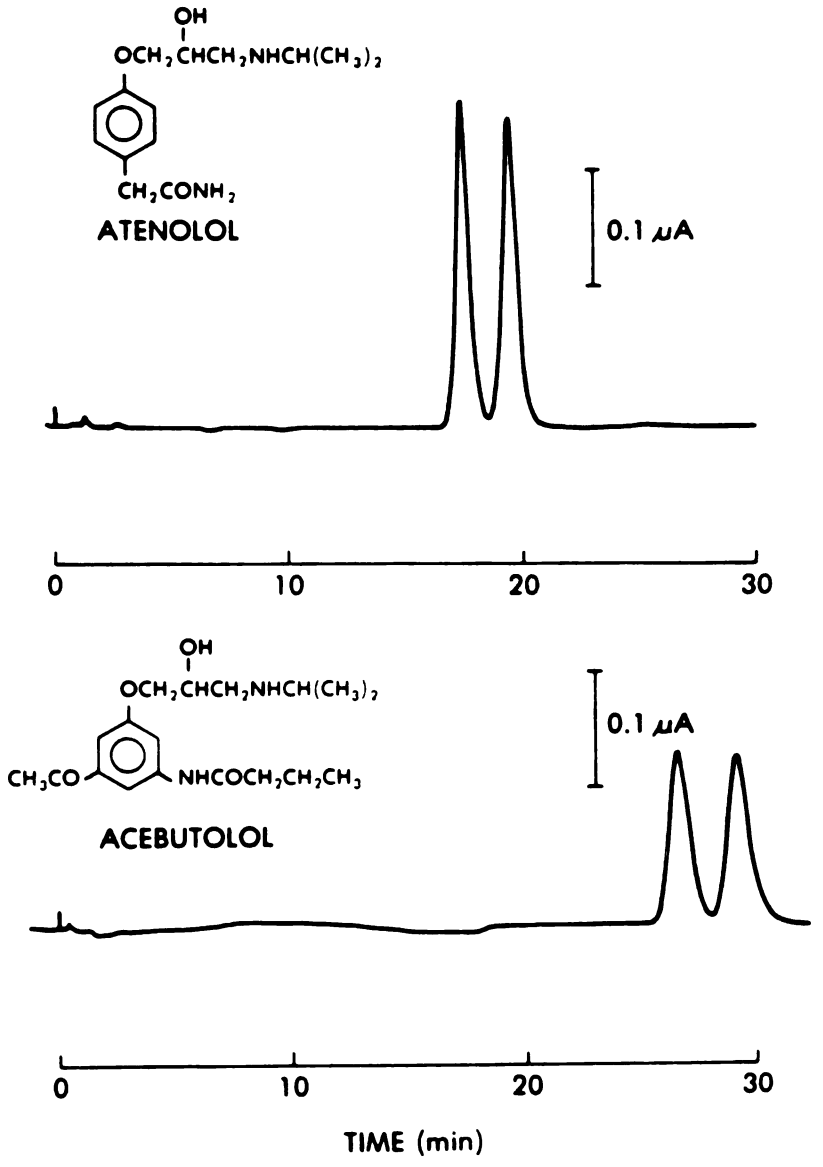


Fig. 26. HPLC chromatograms obtained after extraction of racemic atenolol and acebutolol followed by derivatization with S-(-)-α-methylbenzyl isocyanate. Structures of atenolol and acebutolol are also presented.

pharmacodynamic studies of β–adrenoceptor blocking agents.

Our study demonstrated that the reaction of S-(-)-α–methylbenzyl isocyanate with pindolol is fast and complete with no detectable side reactions. The reaction was carried out at room temperature rather than at the lower temperatures required in previously published stereospecific assays for β–adrenoceptor blocking agents (Silber and Riegelman, 1980). The diastereomers of pindolol appear to be urea compounds as opposed to carbamate derivatives as indicated in the mass spectrum (Fig. 25). When different equivalents (2-500) of S-(-)-α–methylbenzyl isocyanate were added to pindolol in chloroform, the retention times and peak heights of the two diastereomers were unchanged which indicated no carbamate was formed even in the presence of excessively high equivalents of the derivatizing agent. These results are in agreement with Thompson et al. (1982) who observed that the enantiomers of propranolol formed urea compounds with R-(+)-α–methylbenzyl isocyanate. Separation of the diastereomers of pindolol was easily accomplished by either TLC or HPLC. Using the HPLC system described in this study, we obtained baseline resolution of the two diastereomers of pindolol (Fig. 24). Interfering peaks present in plasma and urine were eliminated by the three-step extraction procedure.

The assay of both enantiomers of pindolol was linear in the concentration range tested in urine and plasma (Table 4). Reproducibility and precision were good even when one of the enantiomers predominated (approximately 4 : 1). It is important to calibrate the assay in the appropriate biological fluid because of differences in recovery of drug from urine and plasma (Table 6). Lower recoveries of pindolol from urine have been reported previously (Pacha, 1969).

A number of drugs with native fluorescence including various tricyclic antidepressants, opiates and calcium channel blocking agents were tested for interference. As none of them interfered with the assay, this assay appears to be useful in monitoring (+)- and (-)-pindolol concentrations in clinical situations where other drugs may be present. This procedure has been successfully applied in a clinical study of pindolol in humans in which the renal clearance of pindolol was demonstrated to be stereoselective (Hsyu and Giacomini, 1985; Chapter 6). No interfering peaks from metabolites of pindolol were observed in the clinical samples. All known metabolites of pindolol except isopropylamine in humans are acidic or amphoteric compounds which would be excluded by the extraction procedure. Isopropylamine is nonfluorescent and is quite different structurally from pindolol; therefore it is unlikely that this compound would interfere with the analysis of pindolol.

The baseline resolution of atenolol and acebutolol (Fig. 26) demonstrated that this assay may have broad uses for stereospecific quantitation of many β–adrenoceptor blocking agents. These agents all have a secondary amino moiety b to the OH group which can react with S-(-)-α–methylbenzyl isocyanate. The diastereomers can then be separated by standard chromatographic methods.

Chapter 6

Stereoselective Renal Clearance of Pindolol in Humans

A number of drugs are marketed as racemic mixtures. Although enantiomers have identical physical and chemical properties, the chiral macromolecules, especially proteins, in the body are very specific to the spatial arrangement of drug molecules. Consequently, stereospecific or stereo-selective interactions between macromolecules and drugs are frequently encountered. These interactions result in stereoselective pharmacokinetics and pharmacodynamics of drugs. For example, S-(-)-propranolol is a considerably more potent β–adrenoceptor blocking agent than the R-enantiomer (Barrett and Cullum, 1968). In addition, the clearance of propranolol in human beings is stereoselective, with the S-enantiomer cleared less rapidly than the R-enantiomer (Silber and Riegelman, 1980; Silber et al., 1982).

Stereoselective hepatic metabolism of drugs has been well documented both in vivo and in vitro (Testa and Jenner, 1980). The clinical implications and complexities of stereoselective metabolism are exemplified by the interaction of warfarin and phenylbutazone (Lewis et al., 1974). The clearance of S-warfarin, the more potent enantiomer, is reduced approximately to one half by coadministration of phenylbutazone, whereas the clearance of R-warfarin is almost doubled. The measured clearance of racemate , however, is unaffected. Thus, in the presence of of phenylbutazone, plasma concentrations of racemic warfarin remain unchanged, whereas the anticoagulant effect of warfarin is augmented.

In contrast to the numerous studies regarding stereoselective hepatic

metabolism of drugs, few studies have determined whether stereoselective renal excretion of drugs occurs. Renal excretion is a major route of elimination of drugs and their metabolites and is composed of three processes: glomerular filtration, tubular secretion and tubular reabsorption. Of these, tubular secretion and reabsorption, which can involve saturable carrier mediated processes, may be stereoselective. For example, the active reabsorption of endogenous compounds such as amino acids in the proximal tubule is stereoselective (Ross and Holohan, 1983). Two systems are primarily responsible for the active secretion of drugs from the body: one for organic anions and another for organic cations. At present, stereoselective renal elimination of either cationic or anionic drugs has been rarely studied.

Pindolol (Visken, Sandoz Inc., East Hanover, NJ), an organic base, is a clinically important β–adrenoceptor blocking agent. In humans, the drug is cleared approximately equally by both metabolism and renal excretion (Golightly, 1982). The renal clearance of racemic pindolol exceeds the glomerular filtration rate (GFR) which indicates that the compound is actively secreted. In this study, we examined the stereoselective pharmacokinetics of pindolol in humans. Particular emphasis was placed on measuring the renal clearance of the individual enantiomers. The stereospecific high performance liquid chromatographic assay for pindolol in biologic fluids developed in our laboratory enabled us to quantitate the concentrations of both enantiomers simultaneously in plasma and urine (Hsyu and Giacomini, 1986, Chapter 5). Thus, we were able to measure the renal clearance of each enantiomer in the presence of the other enantiomer under identical physiological conditions.

Our results indicate that the renal clearance of pindolol is stereoselective

in humans. The mechanisms that may be responsible for the observed stereo-selective renal clearance are discussed.

Methods

Clinical Protocol

Six healthy male volunteers between 20 and 30 years of age, weighing between 60 and 80 Kg, were studied. None had a history of diabetes, asthma, or other respiratory, hepatic, renal, or cardiovascular disease. Informed consent was obtained after the purpose, procedures, and risks of the experiments were explained to the volunteers. The volunteers were instructed not to take any drugs or alcoholic beverages for 2 weeks before the experiments. After an overnight fast, an indwelling polyethylene venous catheter with a Teflon obdurator (20G2 longdwel obdurator; Becton-Dickinson, Rutherford, NJ) was placed in a forearm vein of each subject. Subjects swallowed two 10-mg tablets of pindolol along with 240 ml of lukewarm water. Blood samples were collected through the catheter into rubber sealed glass test tubes containing heparin as an anticoagulant (10-ml Vacutainer heparin tube; Becton-Dickinson). No heparin was administered to the subjects. Forty ml of blood was collected before drug administration and 10 ml of blood were collected at approximately 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10,12, and 24 hours after drug administration. Blood was centrifuged immediately at 1000 g and the plasma was transferred into screw-topped glass vials. Urine was collected into plastic graduated containers at intervals of 0-0.5, 0.5-1, 1-2, 2-3, 3-4, 4-6, 6-8, 8-12, and 12-24 hours after drug administration. To facilitate urination, the subjects drank an additional 240 ml of lukewarm water at the end of each of the first

three urine collection intervals. Immediately after collection, the pH and volume of the urine were measured and recorded, and a 20-ml aliquot was saved in a screw-capped glass scintillation counting vial. Plasma and urine were stored at -20^{0}C until analysis.

The experiments were carried out in the Drug Studies Unit of the University of California, San Francisco.

Analytical

Concentrations of (+)- and (-)-pindolol in plasma and urine were determined by a stereospecific high performance liquid chromatographic procedure (Hsyu and Giacomini, 1986, Chapter 5).

Creatinine concentration in the plasma and urine was determined by a creatinine diagnostic kit (Sigma Chemical Co., St. Louis, MO), in which the colorimetric reaction between creatinine and picric acid was used to quantitate the creatinine concentration. Clearance of creatinine was calculated and used to estimate the GFR value in each subject.

Plasma Protein Binding

The binding of (+)- and (-)-pindolol to plasma proteins was determined by equilibrium dialysis. Two-ml dialysis cells (Dianorm System, Spectrum Medical, Los Angeles, CA). separated into two compartments by a cellophane membrane (Spectra por II, Spectrum Medical) with a molecular weight cutoff of 12,000-14,000, were used. The dialysis was carried out at 37^{0}C for 4 hours,

during which time the cells were gently rotated. In preliminary studies, the 4-hour dialysis time was found to be sufficiently long to achieve equilibrium (greater than five times the time required to reach 50% of equilibrium value). The effect of concentration on the binding of pindolol to plasma proteins was also examined in the preliminary studies. The results indicated that the fraction unbound (fu) of both enantiomers of pindolol was constant when the concentrations of racemic pindolol were < 1.5 μg/ml). Therefore, 800 ng of racemic pindolol was added to one ml of blank plasma from each subject and dialyzed against 1 ml of isotonic Sorensens phosphate buffer (0.13 M, pH 7.4) in triplicate. After dialysis, the concentrations of (+)- and (-)-pindolol in the plasma and in the buffer were determined by the stereospecific HPLC procedure described previously. The fu value was calculated as the concentration of each enantiomer in the buffer divided by the concentration of the same enantiomer in the plasma.

Data Analysis

Because the plasma concentrations of (+)- and (-)-pindolol at 24 hours in each subject were below the detection limit of the HPLC procedure, the area under the plasma concentration vs. time curve (AUC) of the individual enantiomers of pindolol was calculated by the linear trapezoidal rule from time 0 to 12 hour and extrapolated to time infinity. The extrapolated area under the curve from 12 hours to time infinity , AUC_{EX}, was obtained by the formula $AUC_{EX} = C_{last}/k$, where C_{last} is the last measured concentration of the enantiomers (usually at 12 hours) and k is the terminal elimination rate constant obtained from the curve fitting. In each subject, the plasma concentration-time curve of each enantiomer was fit to a one-compartment model with zero order

absorption using DRUGFUN from the PROPHET system (Holford, 1982a). Zero order rather than first order absorption was used to fit the plasma concentration-time curve because a better fit as judged by the Schwartz criterion (Holford, 1982b) was obtained. The mechanisms involved in the absorption of pindolol were not studied and should not be implied by the the fitting procedure. The terminal half-life of the individual pindolol enantiomer in each subject was obtained from the fits as 0.693/k. To obtain the renal clearance of each enantiomer, the excretion rate was plotted against the computer-fit plasma concentration at the midpoint of each urine collection interval and a linear least squares regression analysis forced through the origin was applied. The slope of this line is the renal clearance. Renal clearance was also calculated as Ae^{∞}/AUC, where Ae^{∞} is the amount of unchanged pindolol enantiomer excreted in the urine from time zero to time infinity.

To ascertain whether renal clearance was dependent upon urine pH, urine flow rate, time, or plasma concentration, fractional renal clearances were calculated for each urine collection period as the excretion rate divided by the measured plasma concentration obtained at the midpoint of the urine collection intervals. Plots of fractional renal clearance vs. urine pH, urine flow rate, time and plasma concentration were examined visually and also by linear least squares regression analysis for systematic trends.

The GFR in each subject was estimated from the creatinine clearance calculated as the excretion rate of creatinine divided by the concentration of creatinine.

A statistical analysis to determine whether there were differences between

(+)- and (-)-pindolol in the individual pharmacokinetic parameters were carried out using Student's *t* test.

Results

Plasma concentration-time profiles of both enantiomers in all subjects are presented in Fig. 27. The measured plasma concentrations for each subject are tabulated in Apeedix D. The data can be described by a one compartment model with an absorption phase. In each subject, the maximal concentrations of (+)- and (-)-pindolol occurred at the same time (between 1 and 2 hours after drug administration). In addition, the maximal concentrations of (+)- and (-)-pindolol were identical in each subject and averaged 66±13 ng/ml in the six subjects. However, the plasma concentration of (+)- and (-)-pindolol tended to diverge at later times, with concentrations of (-)-pindolol being slightly higher than those of (+)-pindolol in five of the six subjects.

Certain pharmacokinetic parameters of (+)- and (-)-pindolol are summarized in Table 8. The AUC and the terminal half-life of (-)-pindolol were greater than those of (+)-pindolol ($p < 0.05$). In all subjects, the amount of (-)-pindolol excreted unchanged in the urine during the experiment (Ae^{∞}) was greater than that of (+)-pindolol. Since the experiment was carried out for five to six half-lives of (+)- and (-)-pindolol, the amounts in Table 8 approximate the total amount of the enantiomer that would be excreted. Of the 10-mg dose of each enantiomer, the fraction of (-)-pindolol excreted in the urine in the six subjects was 0.430±0.067 and 0.361±0.074 for (+)-pindolol ($p < 0.05$). The measured urine concentrations and volumes over each time interval for the individual subjects are tabulated in Appendix E.

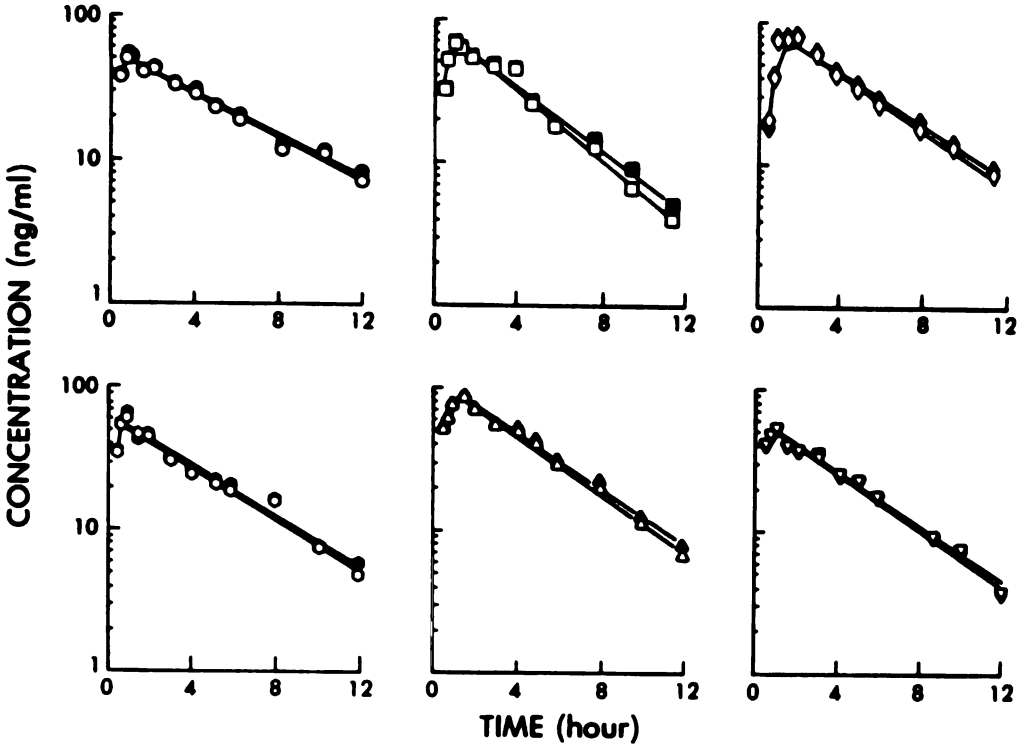


Fig. 27. Plasma concentration-time profiles of (+)-pindolol (open symbols) and (-)-pindolol (closed symbols) in each subject after administration of an oral dose of 20 mg of racemic pindolol. The curves represent the best fit to a one-compartment model with an absorption phase. Each symbol represents an individual subject as listed in Table 8.

Table 8. Pharmacokinetic Parameters of (+)-Pindolol and (-)-Pindolol in Humans

Subject	AUC ng/ml·hr		Half-life hr		Ae^{∞} mg	
	(+)	(-)	(+)	(-)	(+)	(-)
K.C. (●)[a]	310	321	4.04	4.22	3.20	3.99
F.W. (◖)	347	364	2.73	3.02	4.78	5.41
K.D. (◆)	462	477	3.37	3.57	3.64	4.15
P.E. (■)	299	304	3.38	3.50	3.57	4.19
B.D. (▲)	453	477	2.93	3.12	2.56	3.43
M.P. (▼)	283	279	3.12	3.03	3.92	4.62
Mean	359	370*	3.26	3.41*	3.61	4.30*
SD	79	87	0.46	0.46	0.74	0.67

*The difference between (+)- and (-)-pindolol is statistically significant ($p < 0.05$) by Student's paired t test.

[a]Symbols in the parentheses represent indivual subjects are consistently used in Figures 27-33.

Figure 28 depicts plots of the renal excretion rate vs plasma concentration of the individual enantiomers in each of the six subjects. In several but not all subjects, there was a tendency for the renal clearance to be greater at higher plasma concentrations. The slopes of the lines (forced through the origin) represent the renal clearance of (+)- and (-)-pindolol and are graphically presented in Fig. 29. The mean renal clearance of (-)-pindolol was 240±55 ml/min and was statistically greater than that the value of 200±51 ml/min for (+)-pindolol ($p < 0.01$). When renal clearance was calculated as Ae^{∞} /AUC, similar stereoselective differences were observed (176±55 ml/min for (+)-pindolol, 204±61 ml/min for (-)-pindolol; $p < 0.005$). The net clearance by secretion, calculated as renal clearance - fu x GFR, is also shown in Fig. 29. The net clearance by secretion of (-)-pindolol was 196±47 ml/min and was approximately 30% higher on the average than that of (+)-pindolol (157±48 ml/min; $p < 0.01$).

Plots of renal clearance vs. urine pH, time and plasma concentration in each subject demonstrated no systematic trends (Appendix F), which suggests no dependence of renal clearance on these factors. When linear regression was applied, the slopes of the plots of renal clearance vs time or plasma concentration in five of the six subjects were not significantly different from zero. With regard to urine pH, none of the slopes was significantly different from zero. However, in three of six subjects, for either (+)- or (-)-pindolol the slope of renal clearance vs. urine flow was significantly greater than zero ($p < 0.05$).

The fu values for (+)- and (-)-pindolol in each of the six subjects are shown in Table 9. There were no significant differences in the fu of (+)- and (-)-pindolol, which averaged 0.45. The renal clearances of the unbound

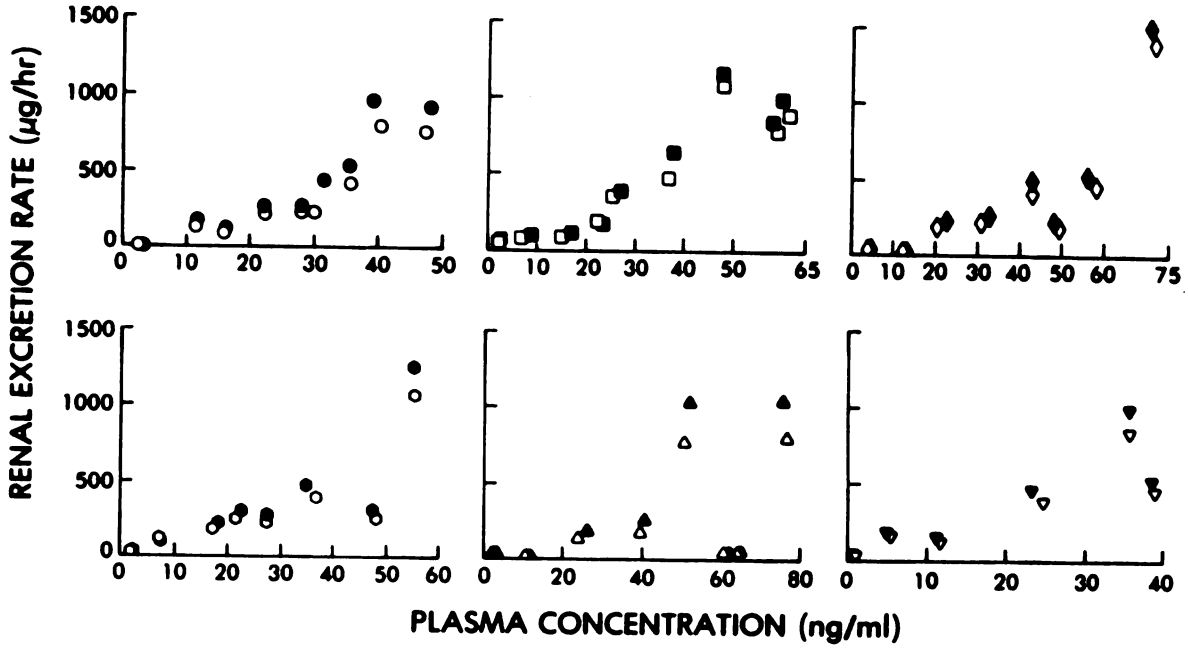


Fig. 28. Renal excretion rate vs. plasma concentration (obtained at the midpoint of the urine collection interval) of (+)-pindolol (open symbols) and (-)-pindolol (closed symbols).

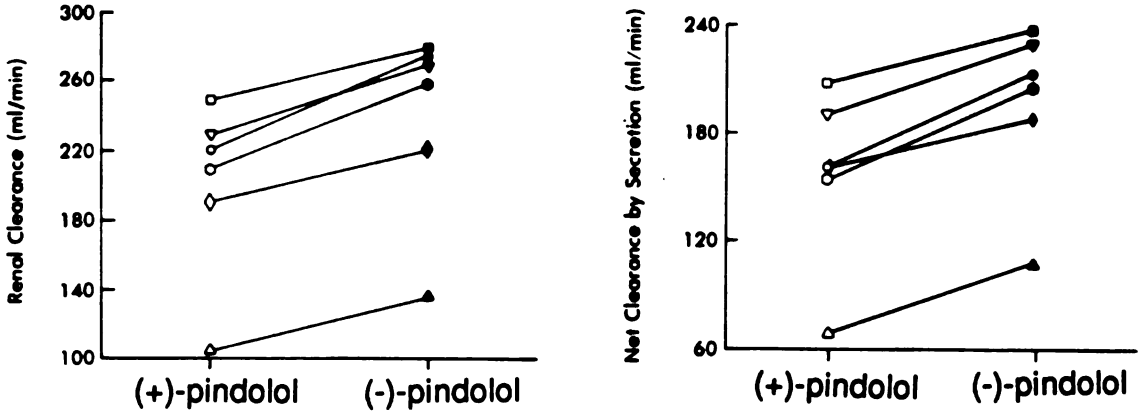


Fig. 29. Renal clearance (left) and net clearance by secretion (right) of (+)- and (-)-pindolol in each subject. Net clearance by secretion was calculated as renal clearance minus fu x GFR.

Table 9. Protein Binding and Renal Excretion of (+)-Pindolol and (-)-Pindolol

Subject	fu[a]		Unbound renal clearance[b] ml/min		GFR ml/min
	(+)	(-)	(+)	(-)	
K.C.	0.56	0.59	395	469	107
F.W.	0.46	0.45	546	618	91
K.D.	0.40	0.44	479	498	75
P.E.	0.54	0.53	386	485	102
B.D.	0.43	0.35	243	386	84
M.P.	0.36	670	670	748	114
Mean	0.45	0.45	453	534*	96
SD	0.08	0.09	147	128	15

[a] Each value represents mean of three replicates.

[b] Unbound renal clearance calculated as renal clearance divided by fu.

* Statistically different by Student's paired t test.

enantiomer, calculated as renal clearance divided by fu, are also listed in Table 9 together with GFR. The unbound renal clearance of both enantiomers was approximately five fold greater than the GFR, which demonstrates the existence of an active secretory process.

Discussion

Although much has been learned in the past decade about stereoselective metabolism of drugs, little is known about stereoselective renal excretion. In this study, we used pindolol as a model compound to study whether stereo-selective renal clearance of an organic base can be demonstrated clinically. Pindolol was ideally suited as a model compound for the following reasons. First, clinical studies with racemic pindolol (Meier, 1982) have demonstrated that the drug is actively secreted in the kidneys. Second, a stereoselective HPLC procedure developed in our laboratory enabled us to determine the concentration of each enantiomer in the presence of the other and thus to study the renal clearance of the individual enantiomers under identical physiological conditions. In addition, because racemic pindolol is the clinically used compound, determining the renal clearance of the individual enantiomers after the administration of the racemate is more relevant to therapy with pindolol.

The results of the present study indicate that pindolol is eliminated from the kidney stereoselectively (Fig. 28 and Fig. 29). Since each enantiomer was excreted from the kidney under identical physiological conditions, differences in factors such as urine pH or time cannot explain the observed stereoselective renal clearance.

There are three possible mechanisms that can account for the stereoselective renal clearance of pindolol: (I) stereoselective plasma protein binding, (II) stereoselective renal transport, and (III) stereoselective renal metabolism. Although pindolol was bound to plasma proteins with a fraction bound of 0.55, no stereoselective difference in the binding of the individual enantiomers was observed (Table 9). Thus plasma protein binding cannot explain the observed stereoselective renal clearance of pindolol.

In this study, the renal clearance of each enantiomer was measured conventionally from the rate of excretion of unchanged enantiomer and the plasma concentration rather than from the renal blood flow and the extraction ratio of the enantiomer across the kidney. Thus, the renal clearance actually reflects the net excretion of unchanged enantiomer and not the total renal elimination (excretion plus the metabolism). The renal clearance of a drug which is filtered, secreted and reabsorbed can be described by the following equations:

$CL_r = (dAe/dt)/C_p$

$= \text{filtration clearance+secretion clearance-reabsorption clearance}$

$= fu * GFR + CL_{sec} - CL_{reab}$

$CL_{sec} = fu * Tmax/(Km + fu * C_p)$

$CL_{reab} = fu * Tmax'/(Km' + fu * C_p) + CL_{diff}$

where dAe/dt represents the renal excretion rate, C_p represents the plasma concentration, CL_r represents renal clearance, CL_{sec} represents secretion clearance, CL_{reab} represents reabsorption clearance, CL_{diff} represents diffusion clearance, Tmax and Tmax' represent maximal transport rates for secretion and reabsorption respectively, Km and Km' represent concentrations needed to reach half of the maximal transport rates for secretion

and reabsorption respectively, and fu represents unbound fraction. Both secretion and reabsorption processes may involve saturable mechanisms which are carried out by specific transporters. The unionized drug may also be significantly reabsorbed by passive diffusion in the kidney.

Renal metabolism represents a pathway that competes with the excretion of unchanged drug and, if present, diminishes the renal clearance of the enantiomer as calculated by the conventional method. Therefore, stereoselective differences in the metabolism of pindolol by the kidney could have produced the observed stereoselective renal clearance of pindolol. However, several studies in the literature suggest that renal metabolism of pindolol in humans may not occur. In patients with renal disease, no change in the metabolic clearance of pindolol was observed (Ohnhaus et al., 1974; Oie and Levy, 1975). If metabolism in the kidney were an important route of elimination, one might expect that the metabolic clearance of pindolol would be impaired in the diseased kidneys. In patients with liver disease, a substantial decrease in the clearance of racemic pindolol has been observed, which suggests the metabolism of pindolol is occurring to a large extent in the liver (Ohnhaus et al., 1982a; Ohnhaus et al., 1982b). If metabolism by the kidney were an important pathway of elimination, only a small decrease in the clearance of pindolol in patients with liver disease would have been observed. Although none of these studies excludes the possibility that stereoselective renal metabolism of pindolol occurs in humans, the studies suggest that if renal metabolism does occur, it is a very minor route of elimination. However, it is possible that stereoselective renal metabolism, although a minor route, may account for the differences in the renal clearance of (+)- and (-)-pindolol. If this were the case, (+)-pindolol would be cleared faster by renal metabolism than (-)-pindolol,

resulting in a lower measured renal clearance of the (+)-pindolol. This hypothesis is consistent with other data in this study that suggest that (+)-pindolol is cleared by nonrenal metabolism more quickly than (-)-pindolol (see below).

Another mechanism that may have been responsible for the stereoselective renal clearance of pindolol is stereoselective renal transport. In agreement with other studies (Meier, 1982; Gugler et al., 1974), we observed that the renal clearance of unbound pindolol was greater than the GFR, which indicates that pindolol is actively secreted by the kidneys. The data do not exclude the possibility that reabsorption, either passive or active, may also be occurring. Since > 95% of pindolol is positively charged at physiological pH values (Lemaire and Tillement, 1982), it is possible that the organic cation transport system in the proximal tubule is involved in the secretion of pindolol from the kidney (Rennick, 1981) and that this system is stereoselectively transporting the drug. Upon perusal of the literature, we did not find any information regarding the stereoselectivity of the organic cation transport system.

In this study, the mean difference in net clearance by secretion of (+)- and (-)-pindolol was approximately 30%. Since pindolol has a relatively high therapeutic ratio (Ohnhaus et al., 1982b), the clinical significance of these differences is probably unimportant. However, the study may have clinical implications for other racemic organic bases that are renally eliminated and general implications for the organic cation transport system of the renal proximal tubule.

In general, the renal clearance of the individual enantiomers was not dependent upon plasma concentration, time, or urine pH. In three of six subjects, there was a significant correlation of renal clearance vs. urine flow, which suggests that the enantiomers are reabsorbed by passive diffusion. In addition, in two or three of the subjects, the fractional renal clearance tended to be larger when plasma concentrations were higher. Balant et al. (1981) also observed that in subjects who had received a single oral dose of pindolol, the renal clearance of racemic pindolol was greater at early times when plasma concentrations were higher. This transiently greater renal clearance may be explained by early arterial-venous differences in drug concentration or by saturable reabsorption processes in the kidney which may be stereoselective.

In addition to studying directly the renal clearance of the enantiomers of pindolol, we also examined stereoselective differences in other pharmacokinetic properties. We observed that the AUC and Ae^{∞} of (-)-pindolol were greater than those of (+)-pindolol. Because AUC and Ae^{∞} after oral administration are determined by both bioavailability and total body clearance, these data suggest that the bioavailability of (-)-pindolol is higher or that the total body clearance is lower than that of (+)-pindolol. Since the total body clearance is the sum of renal and nonrenal clearance and we observed that the the renal clearance of (-)-pindolol was greater than that of (+)-pindolol, these data suggest that (-)-pindolol may be cleared more slowly by a nonrenal route such as metabolism than the corresponding (+)-enantiomer. The longer half-life of (-)-pindolol also supports the notion that the total body clearance of (-)-pindolol is lower than that of (+)-pindolol and therefore (-)-pindolol is eliminated more slowly by metabolism. Alternatively, the longer half-life of (-)-pindolol may be a result of a larger apparent volume of distribution; however, because the

drug was orally administered, the volume of distribution could not be determined. The simplest explanation for the observed stereoselective differences in half-life, AUC, Ae^{∞}, and renal clearance is that there is both stereoselective metabolism and renal excretion, with (-)-pindolol being excreted more rapidly by the kidney and cleared more slowly by nonrenal mechanisms than the corresponding (+)-enantiomer.

In summary, this study has demonstrated that pindolol is eliminated from the kidneys in humans by stereoselective mechanisms. Since binding of pindolol to plasma proteins was not stereoselective, renal handling mechanisms are implicated. A discussion has been presented supporting the notion that either or both stereoselective renal transport or/and stereoselective renal metabolism is/are occurring. In addition to stereoselective renal elimination, the data suggest that pindolol is also eliminated by stereoselective metabolism.

Chapter 7

Relationships between Intrinsic Sympathetic Tone and the Acute Effects of Pindolol on Heart Rate and Blood Pressure

Pindolol is a nonselective β–adrenoceptor blocking agent with intrinsic sympathomimetic activity (Carruthers and Twum-Barima, 1982). Several studies have demonstrated that despite its intrinsic sympathomimetic activity, therapeutic doses of pindolol may decrease the resting heart rate (Aellig, 1977; Carruthers and Twum-Barima, 1982). However, there have been no studies demonstrating a relationship between heart rate and plasma concentration of pindolol. In fact, Carruthers and Twum-Barima (1982) administered cumulative oral doses of pindolol ranging between 2.5 and 57.5 mg to normal volunteers and observed that the effect of pindolol on the resting heart rate was independent of dose and therefore, by inference, independent of plasma concentration.

Although chronic doses of pindolol reduced the blood pressure, there is some controversy in the literature regarding the effects of acute doses of pindolol on resting blood pressure. Using mean data, several investigators have reported that acute doses of pindolol produce no effects on resting blood pressure (Svendsen el al., 1979, 1980; Silke et al., 1983). In contrast, in one study when data from individual hypertensive patients were analyzed separately, an acute dose of pindolol produced a decrease, or surprisingly, an increase in resting blood pressure in several patients (Anavekar et al., 1975). These variable and somewhat unexpected effects were unexplained.

In a recent study (Hsyu and Giacomini, 1985; Chapter 6), we reported the

pharmacokinetics of the enantiomers of pindolol in normal volunteers after a single oral dose. In this chapter we present the effects of pindolol on the resting heart rate and the resting blood pressure in these healthy volunteers. Our data demonstrated that pindolol significantly reduced the resting heart rate and the negative chronotropic effect was related to the plasma concentration of (-)-pindolol. The effects of pindolol on blood pressure were variable. Some of this variability could be related to the intrinsic sympathetic tone of each individual.

Methods

The clinical protocol has been described in Chapter 6. Blood pressure and heart rate were measured in subjects in the supine position immediately before the collection of each blood sample. Blood pressure measurements were determined by a sphygmomanometer and expressed as mean arterial pressure. The heart rate was obtained from an electrocardiogram (1511B electrocardiograph, four-lead; Hewlett-Packard Co., Palo Alto, CA)

The pharmacodynamic analysis was carried out as follows. Mean arterial pressure was calculated as diastolic pressure + 1/3 * (systolic pressure - diastolic pressure). The heart rate and mean arterial pressure were plotted against the plasma concentration of (-)-pindolol, the active isomer (Jeppsson et al., 1984), in each subject. Linear regression was applied to each data set and the slope of each line examined to determine if is was significantly different from zero. The slope of heart rate vs. plasma concentration (normalized negative chronotropic effect of pindolol) obtained in each of the subjects was related to the initial heart of each subject by linear regression. The slope of mean arterial pressure vs. plasma concentration (normalized tensive effect of pindolol) was

related to initial mean arterial pressure, Initial heart rate and normalized negative chronotropic effect of pindolol. Student's *t* test was used to determine whether the slope of each regression line was significantly different from zero.

Results

Heart rate and mean arterial pressure in each subject during the study period are listed in Appendix D. Figure 30 depicts the plots of heart rate vs. plasma concentration of (-)-pindolol in each subject. A negative slope was obtained in all six subjects indicating that the heart rate was significantly reduced after a single oral dose of pindolol. The slope was significantly different from zero ($p < 0.05$) in four of the six subjects. Figure 31 is a plot of the individual normalized negative chronotropic effect of pindolol vs. the initial heart rate. The correlation coefficient (r) was -0.917 ($p < 0.01$). The data indicate that the greater the initial heart rate, the more prominent the negative chronotropic effect of pindolol.

Pindolol exerted different effects on mean arterial pressure in each of the subjects. In some subjects mean arterial pressure increased, in others it decreased and in others it was unchanged. The slope of mean arterial pressure versus plasma concentration of (-)-pindolol ranged from 0.12 to 0.22. Among those one was significantly greater than zero ($p < 0.05$), two were significantly smaller than zero, and three were not significantly different from zero. Figure 32. depicts mean arterial pressure plotted against the plasma concentration of (-)-pindolol in two of the six subjects. In these two subjects pindolol significantly affected mean arterial pressure. However, in one subject, mean arterial pressure was significantly increased and in the other, mean

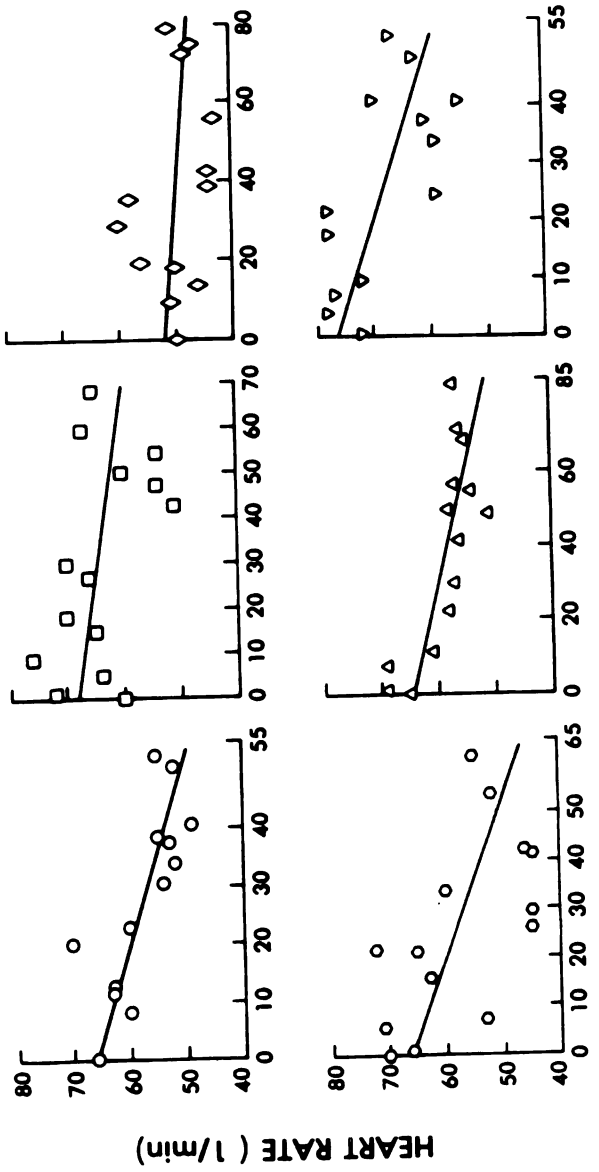


Fig. 30. Heart rate vs. plasma concentration of (-)-pindolol in each subject. The lines were obtained by linear least squares regression analysis. The slopes were significantly different from zero in subjects represented by the circles, hexagons, triangles, and inverted triangles.

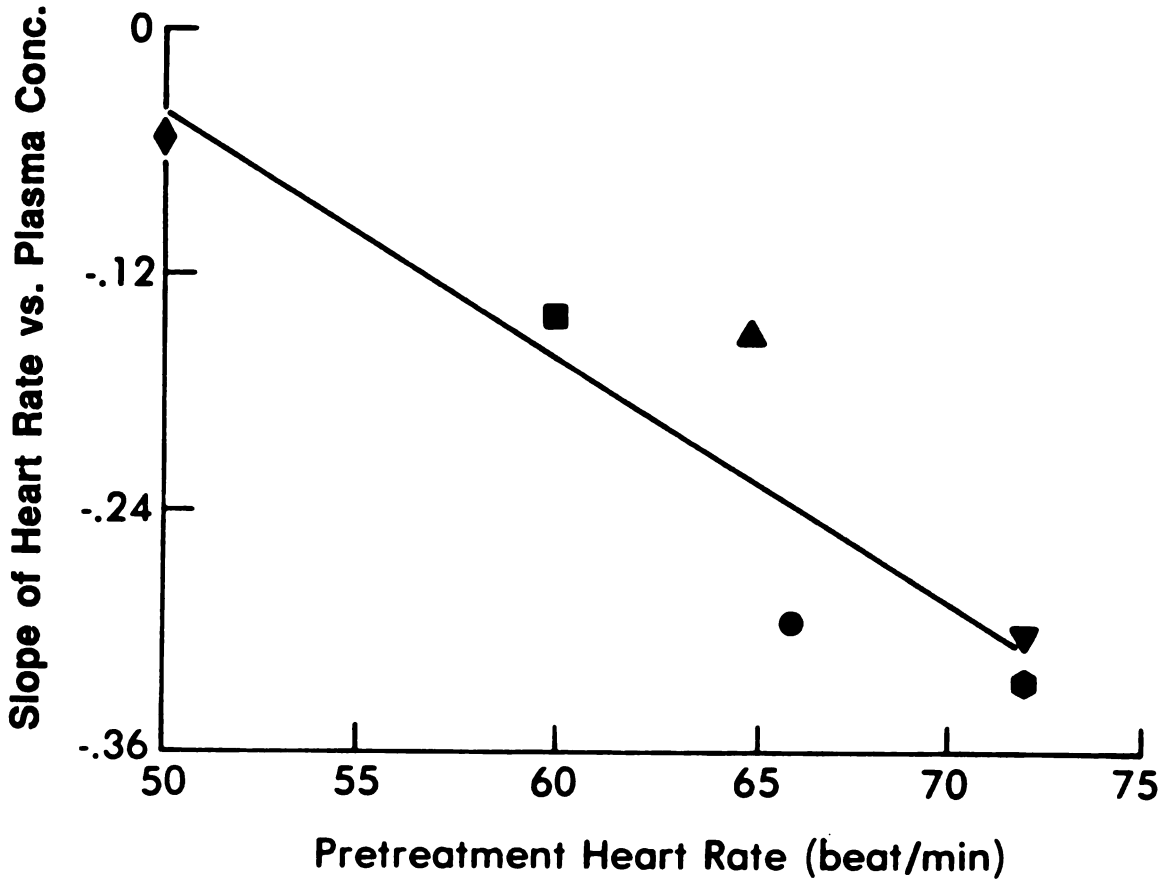


Fig. 31. Slopes of a line describing the dependence of the heart rate on plasma concentration in each individual subject plotted against the pretreatment heart rate. The line represents the best fit line with a correlation coefficient of -0.917 ($p < 0.01$).

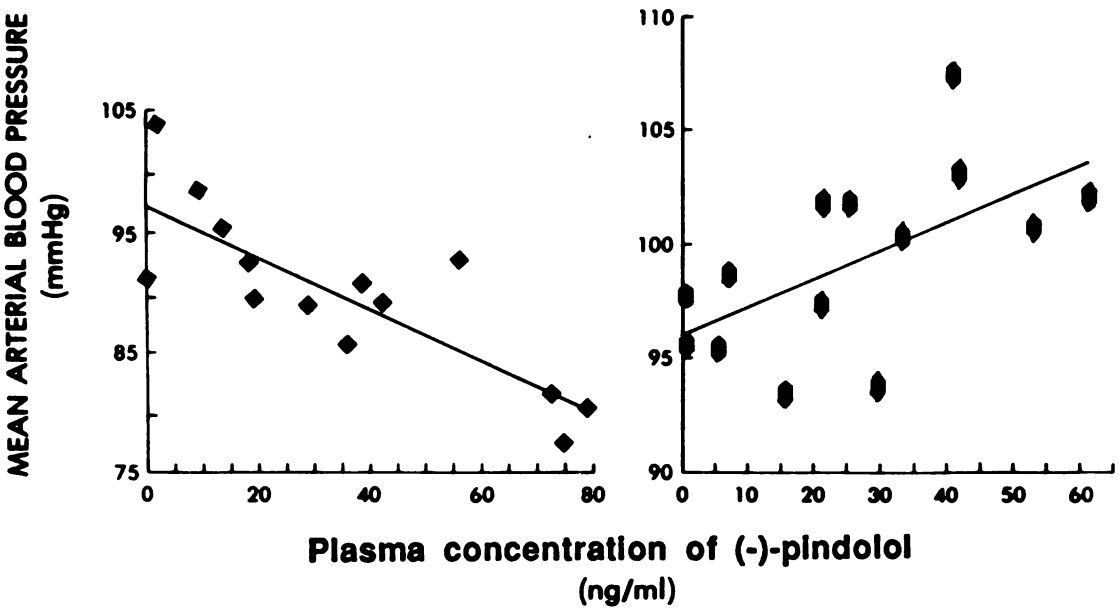


Fig. 32. Mean arterial pressure vs plasma concentration of (-)-pindolol in two subjects. Linear regression line is also presented in each graph. The slope of the regression line of the right panel is significantly greater than zero, whereas that of the left panel is significantly lower smaller than zero.

arterial pressure was significantly decreased.

Upon further examination of the data, we observed that the normalized tensive effect of pindolol was significantly related to the initial heart rate ($r = 0.76$, $p < 0.05$; Fig. 33). Furthermore, this effect was significantly related to the normalized negative chronotropic effect of pindolol ($r = -0.94$, $p < 0.01$, Fig. 33). In the subjects in which pindolol produced the smaller inhibitory effects on heart rate, significant hypotensive effects on mean arterial pressure were observed. On the other hand, pindolol increased blood pressure in one subject in whom pindolol exerted the greatest negative chronotropic effects. The normalized tensive effect was not significantly related to initial mean arterial pressure ($r = -0.06$, $p > 0.9$).

Discussion

The data presented in this study represent the first attempt to relate the plasma concentration of pindolol (in this case (-)-pindolol) to its effects on heart rate. The results demonstrate a good relationship between the resting heart rate and plasma concentration of pindolol (Fig. 31). In all volunteers, the slope of the line relating heart rate to plasma concentration was negative, and in four of the six subjects, the slope was significantly different from zero. In accordance with other investigators, we have observed that the effect of pindolol on the resting heart rate is largely determined by an individual's sympathetic tone which is closely related to the initial heart rate of the individual (Carruthers and Twum-Barima, 1982; Rosenthal et al., 1979).

In contrast to the general decrease in heart rate, mean arterial pressure in

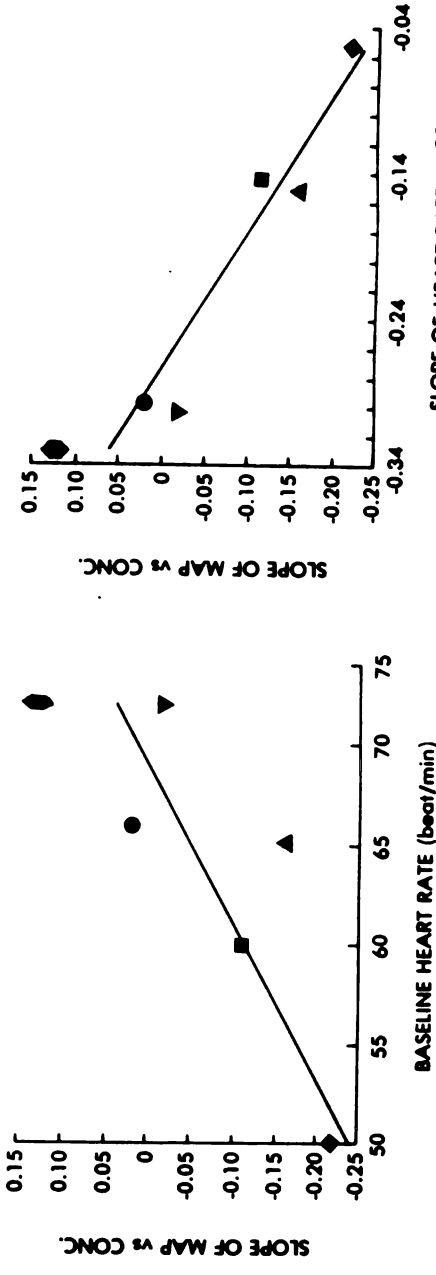


Fig. 33. Slope obtained from the plot of mean arterial vs plasma concentration of (-)-pindolol in each subject is plotted against the baseline heart rate (left panel) and against the slope obtained from the plot of heart rate vs plasma concentration of (-)-pindolol (right panel).

the healthy volunteers was variably affected by an oral dose of pindolol in this study. In one subject the mean arterial pressure increased, in others it decreased and in others it was unchanged. To explain these variable effects of pindolol on mean arterial pressure, we examined the data closely. Our data suggested that the effects of pindolol were significantly related to the initial heart rate and the normalized negative chronotropic effect (Fig. 33). Although one can never imply cause and effect relationships based upon correlations, the highly significant correlation coefficient suggest that cause and effect may exist. Physiologically, mean arterial pressure is related to the product of cardiac output and peripheral vascular resistance according to the hydraulic equation. Pindolol may affect peripheral resistance by two opposing mechanisms. As a β–adrenoceptor blocking agent, pindolol may reduce β–mediated vasodilitation resulting in an increase in peripheral vascular resistance (Hoffman, 1982). Alternatively, due to its intrinsic sympathomimetic activity, pindolol may induce peripheral vasodilitation resulting in a decrease in the peripheral vascular resistance. In the subjects with a high intrinsic sympathetic tone pindolol may have acted as a β–adrenoceptor blocking agent producing an increase in peripheral resistance which causes mean arterial pressure to rise. In contrast, in the subjects with a low intrinsic sympathetic tone, the intrinsic sympathomimetic activity of pindolol may have predominated resulting in a reduction in mean arterial pressure. Although the above mechanism is consistent with our observations, other mechanisms related to the effects of the drug on myocardial functions may also explain the data.

Irrespective of the physiological mechanism, it is apparent that there is a relationship between intrinsic sympathetic tone and the variable effects of pindolol on mean arterial pressure. The normalized tensive effect was more

significantly related to the normalized negative chronotropic effect than to the initial heart because the ability of pindolol to reduce resting heart rate is a better index of intrinsic sympathetic tone than resting heart rate itself (Tanaka et al., 1982). Because of the more complex mechanisms involved in the hypotensive effects of β–adrenoceptor blocking agents following chronic administration, these data should not be extrapolated to the chronic therapeutic situation. The sample size in this study is small and only one subject exhibited an increased blood pressure. Thus, further studies are needed to establish if pindolol will produce hypertensive effects on patients with high intrinsic sympathetic activity. Most data in the literature are mean data of all the subjects and can not be used to resolve this issue. However, in a previous study Anavekar et al. (1981) observed an increased blood pressure in several hypertensive subjects following a single oral dose of pindolol, although no explanation was given by the investigators, it is possible that these subjects had high intrinsic sympathetic tones.

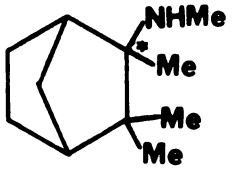
The results of this study suggest that intrinsic sympathetic tone should be considered when assessing the acute effects of β–adrenoceptor blocking agents with intrinsic sympathomimetic activity on heart rate and blood pressure. Pindolol produced opposite effects on blood pressure depending upon the subjects' intrinsic sympathetic tone. Grouping the subjects together tended to obscure the diverse effects.

Chapter 8

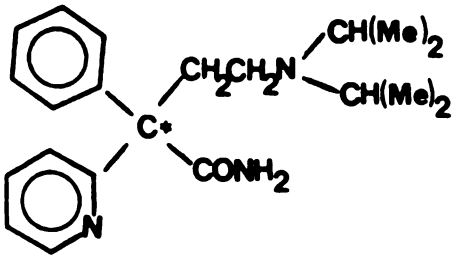
Lack of Significant Stereoselectivity of Organic Cation Transport in Brush Border Membrane Vesicles

We have recently demonstrated that the renal clearance of pindolol in humans is stereoselective (Hsyu and Giacomini, 1985, Chapter 6). Since pindolol is actively secreted, the results may suggest that pindolol is stereoselectively secreted in the proximal tubule in humans via the organic cation transport system in the kidney, although other possibilities such as stereoselective renal reabsorption or renal metabolism cannot be excluded. Stereoselective interactions with the organic cation transport system may occur at either the brush border or the basolateral membrane of the proximal tubule. To date there have been no studies examining the stereoselectivity of the organic cation transport system in either membrane.

In this study, we examined the interaction of three pairs of enantiomers, (+)- and (-)-pindolol, (+)- and (-)-disopyramide, and (+)- and (-)-mecamylamine (Fig. 34) with the organic cation transporter in the brush border membrane to determine whether the system was stereoselective. Studies were carried out in brush border membrane vesicles (BBMV) prepared from rabbit renal cortex. Two of the compounds, pindolol and mecamylamine, appear to be actively secreted in the kidney (Chapter 6, Baer et al., 1956). The clearance of disopyramide in the dog is stereoselective (Giacomini et al., 1980). The results demonstrate absence of notable stereoselective interactions of the enantiomeric pairs with the transporter.



mecamylamine



disopyramide

Fig. 34. Structures of mecamylamine and disopyramide. The chiral carbons are indicated by asterisks.

Methods

BBMV were prepared by a method employing EGTA-magnesium aggregation followed by differential centrifugation (Hsyu and Giacomini, 1987; Chapter 2). Because of the difficulty in obtaining radiolabelled enantiomers, we did not study the transport of the enantiomers in BBMV directly. Instead, the inhibitory effect of each enantiomer on the transport of NMN, a classical substrate for organic cation transport system, in BBMV was determined. Ten μl of vesicles (20 mg/ml) loaded with pH 7.4 HK buffer (150 mM KCl and 10 mM HEPES) were incubated with 20 μl pH 7.4 HK buffer containing ^{3}H-NMN (2.4 μM) alone or ^{3}H-NMN (2.4 μM) and various concentrations of (+)-pindolol, (-)-pindolol, (+)-mecamylamine, (-)-mecamylamine, (+)-disopyramide, and (-)-disopyramide for 8 seconds and 1 hour.

The IC_{50} of each enantiomer was calculated from a Dixon plot (1/rate versus inhibitor concentration) as the absolute value of the abscissa intercept.

Unless otherwise specified, each data point in each experiment was determined as the mean of three replicates. For each study three experiments were performed in three separate membrane preparations. Data are presented as the mean±SD. The paired *t* test or analysis of variance was used when applicable. A probability (p) less than 0.05 was considered statistically significant.

Results

The initial transport rate of NMN was inhibited progressively by

increasing concentration of each enantiomer (Fig 35). The IC_{50}'s (in mM) of the enantiomers were: (+)-mecamylamine = 0.75±0.33, (-)-mecamylamine = 0.5±0.3, (+)-disopyramide = 2.2±1.1, and (-)-disopyramide = 1.9±0.5. Because of the limited solubility of pindolol, the IC_{50} of either enantiomer of pindolol could not be determined. However, 1 mM of (+)-pindolol inhibited NMN transport (27.6±4.1 pmol/s·mg) to 52% (14.4±1.3 fmol/s·mg) and (-)-pindolol inhibited that to 53% (14.6±1.2 fmol/s·mg) suggesting that the IC_{50} of either pindolol enantiomer was approximately 1 mM. No significant stereoselectivity was observed for any pairs of these compounds. However, between pairs, both enantiomers of mecamylamine were significantly more potent than the enantiomers of disopyramide ($p < 0.05$). At the concentration of 1 mM, mecamylamine enantiomers were more potent than pindolol enantiomers ($p < 0.05$).

Discussion

In this study, we examined the interaction of three pairs of enantiomers with the transport system for organic cations in the brush border membrane of the renal proximal tubule to determine whether these compounds interacted stereoselectively with this system. We studied the interaction of the enantiomers with NMN, a classical substrate of the organic cation transport system. Because pindolol is actively secreted in the kidney, we postulated that the observed stereoselective renal clearance of pindolol was caused by the stereoselective secretion of pindolol. Since pindolol is positively charged at physiological pH's, it is likely that pindolol is transported by the transport system for organic cations. In addition, two other pairs of enantiomers, (+)- and (-)-mecamylamine and (+)- and (-)-disopyramide were used. These compounds

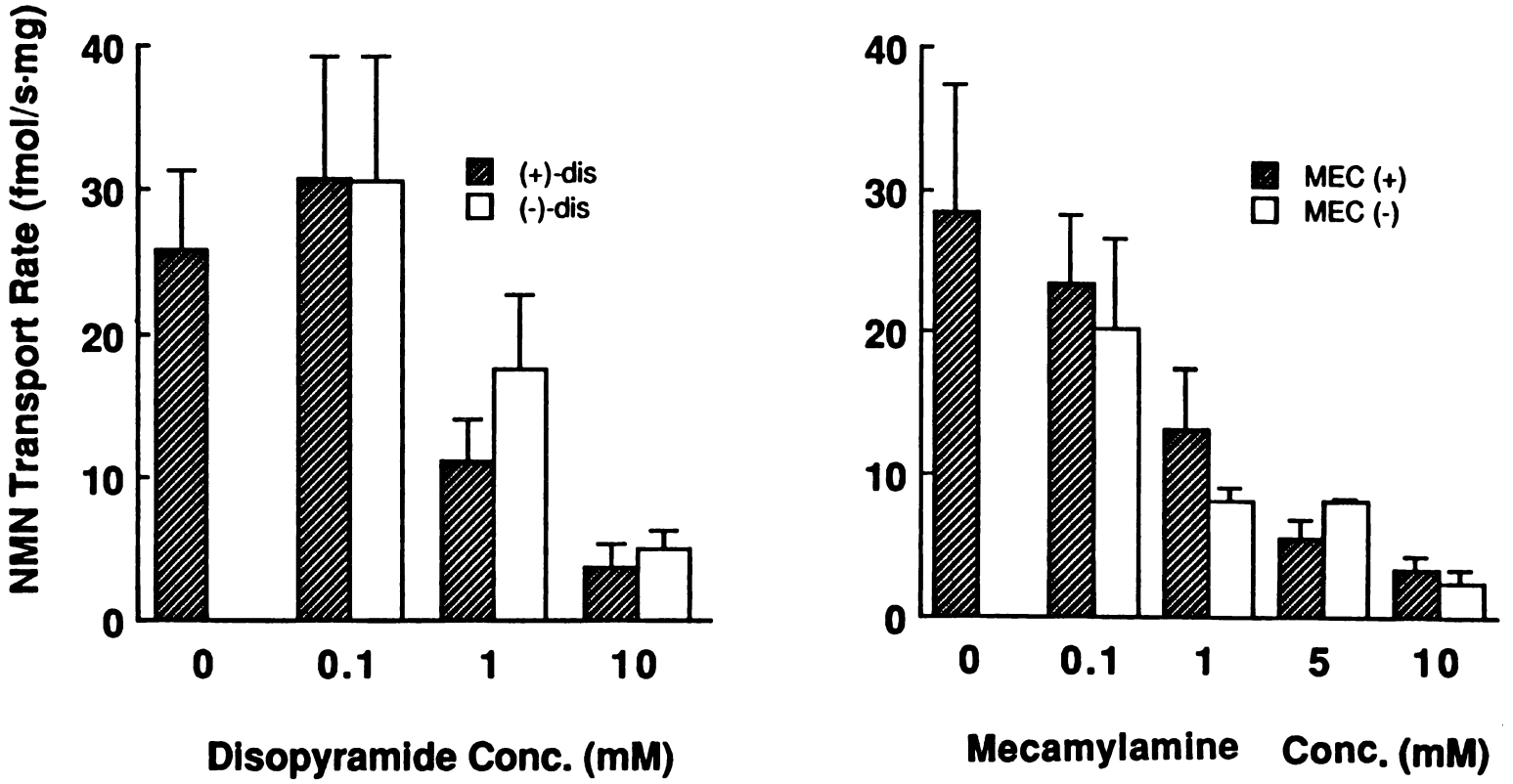


Fig. 35. Inhibition of NMN transport in BBMV by the enantiomers of disopyramide (left panel) and mecamylamine (right panel). The transport studies were carried out at 25^{0}C, pH 7.4. Data are presented as mean±SE of three experiments.

were selected because they are actively secreted by the kidney and because they represent sequential structural modifications. In mecamylamine, the asymmetric carbon atom is α to the amino group. In pindolol, the asymmetric carbon atom is β to the amino group. In disopyramide, the asymmetric carbon atom is γ to the amino group. Thus, the three compounds seemed reasonable to study the stereoselective interaction of basic drugs with the transporter for organic cations.

We chose to study the interaction in brush border membrane vesicles prepared from the renal cortex of rabbit. The brush border membrane appears to be the site where the active step of secretion of organic cations takes place (Rennick, 1981, Holohan and Ross, 1981) and consequently, may exhibit stereoselectivity. The Michaelis-Menten constant, Km, of NMN transport in the brush border membrane from different mammalian species including the dog, the rat and rabbit appear to agree well with each other (Kinsella et al., 1979, Wright, 1985, Chapter 2). Thus, data from rabbit should have implications for other species including humans.

The organic cations used in this study included compounds of diverse chemical structures, yet all inhibited the transport of NMN. Notably the interactions were not stereoselective (Fig. 35). To exhibit stereoselectivity, the binding site of the transporter needs three-point binding so that spatial orientation of the substrates and inhibitors will be important. Our data suggest that the organic cation transporter in the brush border membrane may not require three-point binding and accordingly does not exhibit stereoselectivity. These results are consistent with earlier studies suggesting that the two structural requirements for the interactions with the organic cation transport

system are a positively charged group and a lipophilic side chain (Peters, 1960).

In summary, the binding site of the transporter for organic cations in BBMV may consist of a negatively charged domain and a hydrophobic domain. The lack of stereoselective of the transport system is consistent with this deduction since only two contact points are present which can not exhibit stereoselectivity. This transporter is somewhat unusual in that it does not exhibit stereoselectivity. Transporters for endogenous hexose and amino acids in the renal proximal tubule appear to exhibit marked stereoselectivity (Kinne et al., 1975; Ross and Holohan, 1983).

In conclusion, we have observed that the transport system for NMN in renal BBMV prepared from rabbit cortex was inhibited by organic cations. This transport system does not appear to be significantly stereoselective. These results do not appear to explain the clinical data demonstrating stereoselective renal clearance of pindolol (Chapter 6). However, these discrepancies may be caused by species differences, i.e., the transport of organic cations in the brush border membrane is stereoselective in humans but not in the rabbit. This is unlikely because the transport system for organic cations is quite similar among mammalian species (Rennick 1981). Alternatively, the stereoselective transport of organic cations may be occurring in the basolateral membranes or via another transport system in either the brush border or basolateral membrane. Another possibility is that the renal transport of organic cations is not stereo-selective and the differences in renal clearance of (+)- and (-)-pindolol observed clinically were a result of stereoselective renal metabolism.

Chapter 9

The Effects of Pindolol on the Transport of L-Lysine in Renal Brush Border Membrane Vesicles

In a recent clinical study, we demonstrated that the renal clearance of pindolol is stereoselective (Hsyu and Giacomini, 1985; Chapter 6) and that pindolol is actively secreted in the kidney. Additionally, our data suggested that saturable reabsorption of pindolol in the kidney may also occur (Chapter 6). Thus, we postulated that the stereoselective renal clearance may have been due to stereoselective renal transport or stereoselective renal metabolism in the reabsorptive or secretory direction.

In this study we examined the interaction of pindolol at the system responsible for the active reabsorption of basic amino acids in the brush border membrane of the renal proximal tubule. We postulated that pindolol may be actively reabsorbed by this system because similar to basic amino acids, pindolol is positively charged at physiological pH's. In addition, there are data in the literature suggesting that organic cations and basic amino acids may interact in the proximal tubule (Rennick, 1981). Acara and Rennick (1976) demonstrated that the excretion of choline, an organic cation, is enhanced by coadministration of lysine in Sperber chickens. This enhanced excretion may have been caused by competition of choline and lysine at a reabsorption site.

Basic amino acids are actively reabsorbed at the brush border membrane in the renal proximal tubule by a specific transport system (Silbernagl et al., 1975; Schafer and Barfuss, 1980). The transport of basic amino acids is saturable and stereoselective for L-configured basic amino acids including L-

lysine, L-arginine and L-ornithine. Thus, it was possible that in our previous study (Chapter 6) pindolol was being reabsorbed by this system via a stereo-selective mechanism.

To determine whether pindolol interacts with the basic amino acid transporter, we examined the effect of (+)-pindolol, (-)-pindolol and racemic pindolol on the transport of L-lysine in brush border membrane vesicles (BBMV) prepared from rabbit renal cortex. Our results demonstrate that pindolol interacts weakly with this system and that the interaction is not stereoselective.

Methods

BBMV are prepared by a method employing EGTA-magnesium aggregation followed by differential centrifugations (Chapter 2). Transport studies were carried out as follows. Ten µl of vesicles (20 mg/ml) loaded with pH 7.4 HK buffer (150 mM KCl and 10 mM HEPES) were incubated with 50 µl pH 7.4 HK buffer containing ^{3}H-L-lysine (approximately 50 nM) and unlabeled L-lysine ($1.2 * 10^{-5}$ M) alone or ^{3}H-L-lysine, unlabelled L-lysine and racemic pindolol (1.2, 6, and 12 mM) for various times. The uptake of L-lysine in BBMV was determined by the rapid filtration method. The effects of several compounds including L-arginine, L-ornithine, L-tyrosine, NMN, TEA, quinine (1 mM), (+)- and (-)-pindolol (1, 5 , and 10 mM) on the initial transport of L-lysine were also determined at initial times (between 8 and 16 seconds) and at two hours as described above.

Binding of L-lysine to the vesicles was determined by the method of increasing sucrose osmotic gradients. Uptake of L-lysine into vesicles at two

hours was determined as described above except that various concentrations of impermeable sucrose were present extravesicularly. The uptake of L-lysine was then plotted against the inverse of the sucrose concentration in the extravesicular medium. The intercept (representing the uptake of L-lysine at infinite sucrose gradient) divided by the uptake of L-lysine in the absence of sucrose represents the fraction of L-lysine bound to the vesicles.

Michaelis-Menten kinetics of L-lysine transport in the BBMV were also studied. The transport of L-lysine (approximately 50 nM) in the presence of L-lysine and D-lysine (10^{-3} to 10^{-5} M) at 8 seconds and 2 hours were studied. The Ki's of L-lysine and D-lysine were obtained from a Dixon plot (1/rate versus inhibitor concentration) as the absolute value of the intercept at the abscissa.

Unless otherwise specified, each data point in each experiment was determined as the mean of three replicates. For each study three experiments were performed in three separate membrane preparations. Data are presented as mean±SE. Statistical differences were determined by Student's *t* test.

Results

Binding of L-lysine to BBMV was determined by measuring the equilibrium uptake of L-lysine ($1*10^{-5}$ M) in the presence of various osmotic pressures obtained by adding various amount of the impermeable solute, sucrose, extravesicularly. In the presence of increasing concentrations of sucrose, there is a progressive shrinking of the vesicles. The intercept of a plot of uptake of L-lysine versus the inverse of osmolarity should represent the amount of L-lysine bound to the vesicles, i.e., the amount of the compound associated with the

vesicles when the intravesicular space is infinitesimal. The fraction bound can be obtained by dividing the intercept by the uptake of L-lysine in the absence of sucrose. Binding of L-lysine in BBMV was 14.9±2.3% (mean±range, n=2). Data from a representative experiment are depicted in Fig. 36. This value is substantially lower than the 50% reported by Stieger et al. (1983).

Michaelis-Menten studies demonstrated that L-lysine was transported stereoselectively in the brush border membrane vesicles. Fig. 37 depicts the results from a representative experiment. For three experiments the Ki of L-lysine was 0.27±0.10 mM and the Ki of D-lysine was 1.0±0.1 mM. Consistent with previous studies, the transporter has a greater affinity for L-lysine, the endogenous enantiomer.

The initial transport of L-lysine was variably affected by different compounds (Table 10). As expected, the basic amino acid L-arginine (1 mM) significantly decreased the transport of L-lysine. The basic amino acid L-ornithine (1 mM) decreased the transport of L-lysine, although this decrease was not statistically significant. The neutral amino acid L-tyrosine (1 mM) had no inhibitory effect on the transport of L-lysine. Among non-amino acid compounds only quinine exhibited significant inhibitory effects at 1 mM. None of the compounds significantly altered the equilibrium uptake of L-lysine suggesting that the compounds were not altering the vesicle size or the binding of L-lysine to the vesicles.

Pindolol did not significantly decrease the initial transport of L-lysine at the concentrations used except at 10 mM. The time course of L-lysine uptake in the absence and presence of 10 mM of racemic pindolol is presented in Fig. 38.

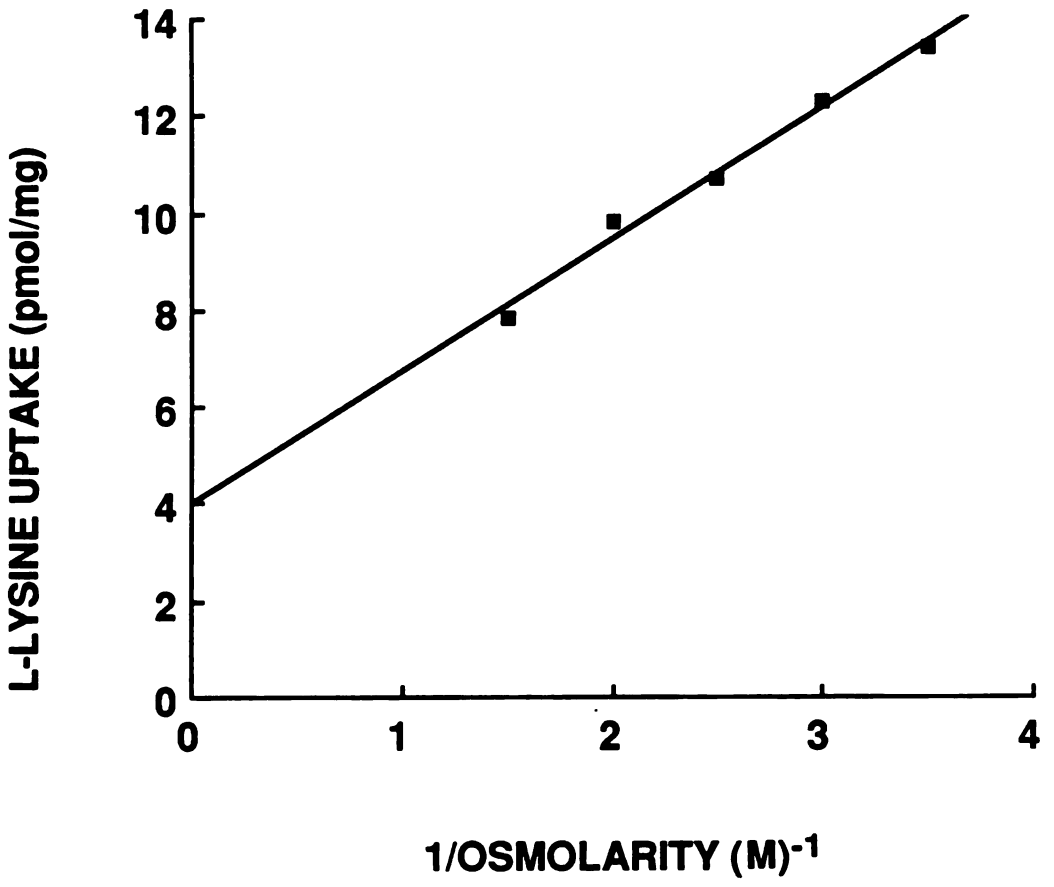


Fig. 36. Uptake of L-lysine in BBMV at equilibrium as function of extravesicular sucrose osmolarity. The intercept of the regression line represents the uptake of L-lysine in BBMV at infinite osmolarity. This value divided by the equilibrium uptake of L-lysine in the absence of sucrose represents the fraction of L-lysine bound to the membrane. Data from a typical experiment are presented.

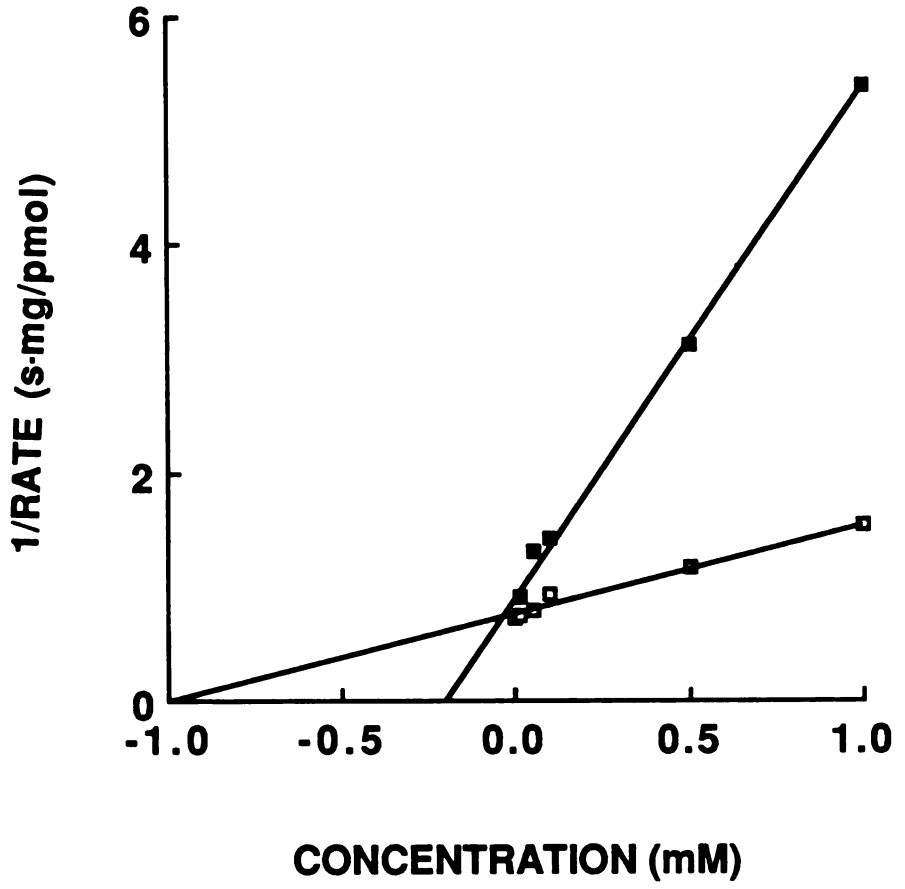


Fig. 37. Dixon plot of the inverse of the initial transport rate of L-lysine (45 nM) in the presence of various concentrations of L-lysine (■) and D-lysine (□) in BBMV. Data are from a representative experiment. In this experiment, the Ki of L-lysine was 0.20 mM and the Ki of D-lysine was 1.0 mM.

Table 10. Effects of Compounds on the Transport of L-Lysine

compounds	rate (% control)*
control	100
L-arginine	56±10†
L-ornithine	77±15
L-tyrosine	122±35
NMN	107±9
NMN (10 mM)	104±5
pindolol	104±21
pindolol (5 mM)	75±16
pindolol (10 mM)	49±5†
quinine	77±2†
TEA	99±5
TEA (10 mM)	102±6

* Data are mean±SE from three experiments and are expressed as percentage of the rate of the control. The concentration of compound, unless specified, was 1 mM. In all experiments ^{3}H-L-lysine (~ 50 nM) and unlabeled L-lysine (10 mM) were present.

† Significantly different from the control ($p < 0.05$)

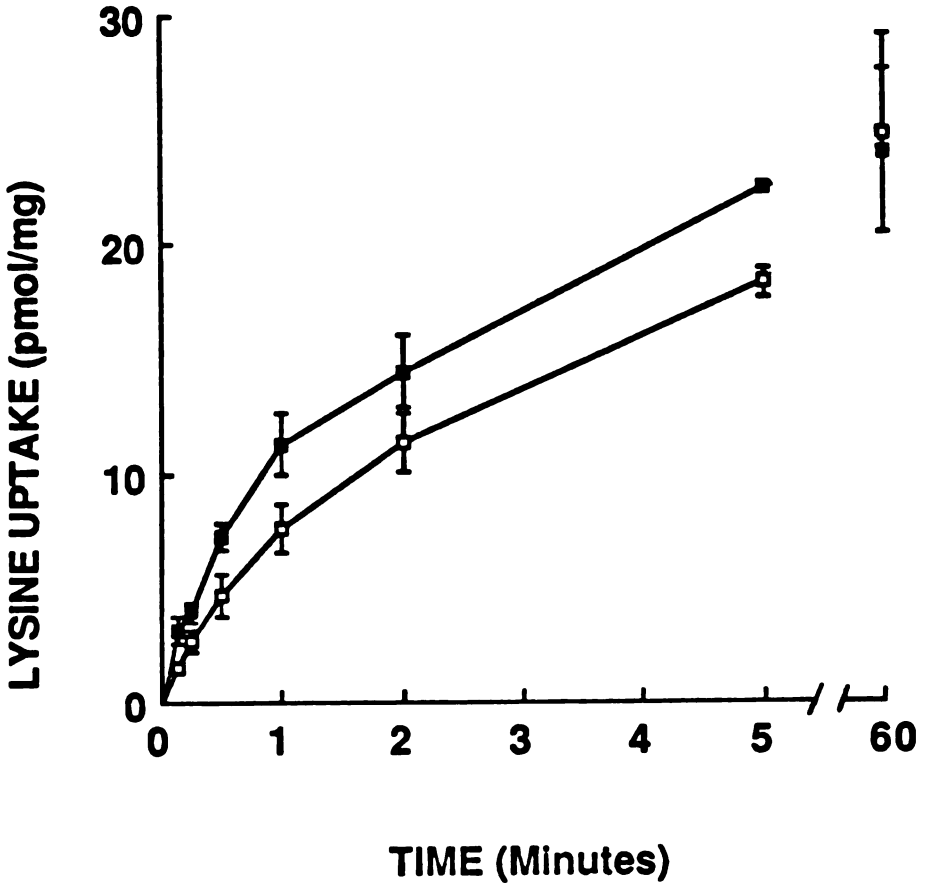


Fig. 38. Time course of L-lysine uptake in the presence (□) and absence (■) of 10 mM of racemic pindolol. Data are presented as mean±SE of three experiments.

The uptake of L-lysine was significantly reduced at earlier times (≤ 5 minutes) but not at equilibrium. The initial rate of transport of L-lysine was 0.395±0.068 pmol/s·mg and was 0.193±0.034 pmol/s·mg in the presence of 10 mM racemic pindolol.

The effects of the enantiomers of pindolol on the transport of L-lysine were examined. (+)-Pindolol (10 mM) reduced the initial transport rate of L-lysine (0.395±0.068 pmol/s·mg) to 0.155±0.050 pmol/s·mg and (-)-pindolol (10 mM) reduced the rate to 0.155±0.022 pmol/s·mg suggesting that pindolol does not interact stereoselectively with the basic amino acid transporter in the brush border membrane vesicles. Lower concentrations of the enantiomers of pindolol (1 mM and 5 mM) did not significantly inhibit L-lysine transport. Higher concentrations of pindolol enantiomers could not be tested because of solubility limitations.

Discussion

In this study we examined the interaction of pindolol at the basic amino acid transport system in the brush border membrane of the renal proximal tubule to ascertain whether the compound interacted with this system and whether this interaction was stereoselective. Our previous clinical study suggested that pindolol was stereoselectively eliminated by the kidney. We attributed this stereoselective renal elimination to either stereoselective renal transport or stereoselective renal metabolism. Since the renal clearance of both enantiomers was greater than glomerular filtration rate, the data suggested that pindolol was actively secreted by the kidney. Interestingly, we also obtained some evidence suggesting that the compound may be reabsorbed by

a saturable mechanism (Chapter 6). In some subjects the fractional renal clearance tended to be larger when plasma concentrations of pindolol enantiomers were higher (Fig. 28). This apparent concentration dependence of renal clearance on plasma concentration was also reported by Balant et al. (1981) for racemic pindolol.

Because it appeared that pindolol was reabsorbed by a saturable mechanism, we postulated that the observed stereoselective renal clearance of pindolol may have been due to stereoselective reabsorption of the compound. There are known transport systems in the renal proximal tubule responsible for the reabsorption of many endogenous compounds including glucose, various amino acids and lactate. Of these systems , the basic amino acid transport system would be the most likely system to be involved in the reabsorption of pindolol. The rationale is that pindolol, like the basic amino acids, is positively charged at physiological pH's. Secondly, the basic amino acid transporter is known to preferentially transport L-configured basic amino acids. Thus, it is possible that pindolol may also be stereoselectively transported by the system. Finally, there is some evidence in the literature suggesting that cationic drugs may interact with basic amino acids in the kidney. Acara and Rennick (1976) demonstrated that the excretion of choline, an organic cation, is enhanced by coadministration of lysine in Sperber chickens. This enhanced excretion may have been caused by competition between choline and lysine at a reabsorption site.

We chose to examine the interaction of pindolol at the basic amino acid transporter in brush border membrane vesicles prepared from rabbit renal cortex. The brush border membrane is known to be the site of active

reabsorption of basic amino acids. Although our finding of stereoselective renal elimination of pindolol was in humans, it was necessary to carry out these studies in an animal model. We selected rabbit as the model because species differences in the transport system for basic amino acids in the kidney are minimal among mammalian species.

In this study the binding of L-lysine to BBMV was only 15% as determined by increasing osmolarity. This value is significantly lower than that (50 %) reported by Stieger et al. (1981). However, our buffer contained high concentrations of KCl, whereas Stieger et al. used non-ionizable mannitol. L-Lysine appears to bind more to membrane vesicles in the presence of non-ionizable media (Hilden and Sacktor, 1981). Different Donnan potential results from different incubation media may also contribute to this discrepancy (McNamara et al., 1986). The lower binding of L-lysine in this study is advantageous because the inhibitory effects of organic cations on L-lysine can be ascribed to inhibition of transport rather than to inhibition of binding of L-lysine to the membrane vesicles.

In this study we demonstrated that the transport of L-lysine was inhibited by basic amino acids including L-arginine and L-ornithine. The neutral amino acid, L-tyrosine, did not significantly alter the transport of L-lysine. The transport system for basic amino acids in brush border membrane is more selective for L-lysine than D-lysine as demonstrated by the lower Ki values of L-lysine (Fig. 37). The Ki of L-lysine (0.27 mM) is consistent with the reported Km values for L-lysine of 0.3-0.7 mM (Stieger et al.,1981) and 0.8 mM (Samarzija et al., 1982). This system also appears distinct from the organic cation transport system. NMN and TEA (10 mM), two classical substrates of the organic cation

transport system, did not significantly affect the transport of L-lysine. Quinine, a potent inhibitor of organic cation transport, inhibited only 22% of initial L-lysine transport at a concentration of 1 mM. The same concentration of quinine was able to completely eliminate the initial transport of NMN in BBMV (data not shown).

Pindolol (10 mM) inhibited the uptake of L-lysine at earlier times but not at equilibrium indicating that the inhibitory effects were not due to disruption of vesicles or reduction in vesicle size. The data also suggest that pindolol did not affect the binding of L-lysine significantly since uptake at equilibrium is at least partially dependent on the binding of L-lysine in BBMV. Pindolol (10 mM) decreased the initial uptake of L-lysine to 50%. Pindolol (5 mM) may have inhibited the transport of L-lysine, although the inhibition was not statistically significant. At the concentration of 1 mM pindolol did not inhibit the initial uptake of L-lysine. Because of the low potency and limited solubility of pindolol (~ 10 mM), detailed mechanistic studies of the nature and kinetics of the interaction could not be carried out. The transport system for L-lysine in BBMV exhibits low affinity toward pindolol.

Although the fact that pindolol inhibited the transport of L-lysine in the brush border membrane vesicles does not prove that pindolol is actually transported by the system, these findings together with the data from our clinical study (Chapter 6) suggest that it is possible that pindolol is reabsorbed in the proximal tubule by the L-lysine transport system. Another basic drug, quinine, also inhibited the transport of L-lysine. These data demonstrate that certain basic drugs such as pindolol and quinine can interact with basic amino acid transport which might suggest some interesting clinical interactions between

basic drugs and basic amino acids.

The transport of pindolol by the L-lysine transporter does not appear to be stereoselective since 10 mM (+)-pindolol and (-)-pindolol inhibited the initial uptake of L-lysine to the same extent. Preliminary studies also showed that (+)-pindolol and (-)-pindolol at concentrations of 1 and 5 mM had the same effects on L-lysine transport. In reference to the clinical study (Chapter 6), these data do not support the notion that the observed stereoselective renal clearance of pindolol was caused by its stereoselective reabsorption via the basic amino acid transport system at the brush border membrane in the proximal tubule. Because pindolol interacted only weakly with the transporter for basic amino acids, it is unlikely that this interaction would exhibit saturable characteristics as observed in the clinical studies (Chapter 6; Balant et al., 1981).

Chapter 10
Summary and Conclusions

The kidney and liver are the two major organs responsible for the elimination of drugs from the body. However, the mechanisms of renal excretion of drugs, especially for basic drugs are not as well understood as the mechanisms involved in the hepatic metabolism of drugs. It is generally known that drugs can be excreted in the kidney by filtration, secretion and reabsorption. But only in the last two decades with the advances in methodology such as micropuncture, isolated perfused tubules and isolated renal cell membranes have the details of drug excretion processes, especially secretion, in the kidney been examined. It has been established that there are two major systems in the proximal tubule for the renal secretion of drugs: one for organic anions and another for the organic cations. These two systems, although coexistent in the proximal tubule, have appeared to be separate and distinct systems (Rennick, 1981). There is a transporter for organic cations in the basolateral membrane which transports basic drugs from blood into the epithelial cells. A transporter in the brush border membrane transports the drugs out of the cells into the tubular lumen. Because the epithelial cells in the proximal tubule have interior negative potential differences (approximately -70 mV) under normal physiological conditions, it has been proposed that organic cations can enter and accumulate in the cell by the electrical driving force, although this mechanism remains controversial (Schali et al., 1983; Tarloff and Brand, 1986). The transport system in the basolateral membrane appears to be a simple substrate system. Transport of NMN and TEA in basolateral membrane vesicles was not stimulated by either Na^+ or H^+ gradient (Holohan and Ross, 1981; Takano et al., 1984). The transport of TEA was enhanced by a

intravesicular negative potential difference. These results are consistent with a simple substrate system. The transport of organic cations in the brush border membrane is more complicated. A number of studies have demonstrated that transport of organic cations in brush border membrane vesicles (BBMV) is stimulated by an outwardly directed proton gradient (Holohan and Ross, 1981; Takano et al., 1984; Sokol et al., 1985; Wright, 1985). An organic cation-H^+ exchange (or an organic cation-OH^- cotransport) has been proposed based on these studies. These data have enhanced our knowledge of renal excretion of organic cations. Nevertheless, many questions remain unanswered regarding the nature of the transport system for organic cations.

In the first part of the dissertation, we carried out studies to delineate the cellular and molecular mechanisms of organic cation transport in the brush border membrane. In the first study, we used tyrosine modifying agents to identify the role of tyrosine residues in the transporter for organic cations in the brush border membrane vesicles prepared from rabbit kidney cortex. The brush border membrane vesicles were treated with NBD-Cl, a tyrosine modifying agent, and transport of NMN in the NBD-Cl treated and untreated membrane was compared. Treatment with NBD-Cl decreased the transport rate of NMN in the membranes. The reduction of NMN transport increased with incubation times. NMN transport decreased with increasing concentrations of NBD-Cl. NBD-Cl also interacts with sulfhydryl residues. However, 2-mercaptoethanol was added to reverse the interaction between NBD-Cl and sulfhydryl residues. 2-Mercaptoethanol reversed the interaction of NBD-Cl and sulfhydryl residues, but did not alter the inhibitory effect of NBD-Cl on the NMN transport. These results suggest that the interaction between tyrosine residues and NBD-Cl produced the decrease in the activity of the transporter for organic cations.

Thus, tyrosine groups appear to be essential for the activity of the transporter for organic cations in the brush border membrane. The inhibitory effect of NBD-Cl was obviated when the membrane vesicles were preincubated with a high concentration of TEA which suggests that the tyrosine residues may be located at the binding site for organic cations on the transporter.

Although an organic cation-H^+ exchange (or organic OH^- cotransport) mechanism has been proposed by several research groups, most of the evidence was obtained in studies using trace uptake of organic cations in the presence of a pH gradient across the membrane in brush border membrane vesicles (Holohan and Ross, 1981; Takano et al., 1984; Sokol et al., 1985; Wright, 1985). Other mechanisms such as artifacts in potential differences and catalytic effects of the pH gradients which may also produce the observed overshoot of organic cations can not be ruled out in those studies. To establish the existence of the exchange (or cotransport) mechanism it is essential to demonstrate that not only a pH gradient can stimulate the transport of organic cations but also that a gradient of organic cations can stimulate the transport of protons (or hydroxyl ions). In Chapter 3, we used acridine orange, a fluorescent probe of proton movement, to determine the transport of protons in the presence of TEA gradients. Our results demonstrated that the transport of protons (or hydroxyl ions) was accelerated by gradients of TEA in a concentration dependent fashion. Thus, a true exchange (or cotransport) mechanism appears to be responsible for the transport of organic cations in the brush border membrane.

In previous studies organic cation transport has been presumed to be unaffected by organic anions (Kinsella et al., 1979; Rennick, 1981; Wright,

1985). In our studies (Chapter 4), we demonstrated that the transport of NMN in BBMV was affected not only by organic cations but also by organic anions. Probenecid and furosemide, two organic anions transported by the organic anion transport system in the renal proximal tubule, inhibited the transport of NMN significantly. On the other hand lactate accelerated the transport of NMN. These results suggest that organic anions also interact with the transporter for organic cations. It is unlikely that organic anions interact with the transporter at binding sites either for organic cations or protons. However, if the transporter is an organic cation-OH^- cotransport system, it is quite possible that organic anions may interact at the binding site for hydroxyl ions. Thus, our data may offer indirect evidence for an organic cation-OH^- cotransport mechanism.

In the second part of the dissertation, we investigated the question of whether drugs were stereoselectively eliminated by the kidney. For these studies, pindolol was used as a model compound. To enable us to carry out the clinical studies of pindolol, we developed a stereospecific HPLC assay for determining concentrations of pindolol in plasma and urine (Chapter 5). This method is specific, sensitive and reproducible for the enantiomers of pindolol. Preliminary experiments also demonstrated that the method can be used with minor modifications for the determination of the concentrations of the enantiomers of other β–adrenoceptor blocking agents such as atenolol and acebutolol.

In the clinical studies of pindolol in humans, we demonstrated that the renal clearance of pindolol is stereoselective (Chapter 6). The stereoselectivity between (+)-pindolol and (-)-pindolol is small (20-30%) but significant. Stereoselective renal transport or stereoselective renal metabolism may have

been the cause of the observed stereoselectivity.

We also monitored the heart rate and blood pressure of the subjects following the acute dose of pindolol (Chapter 7). Pindolol reduced the heart rate in most of the subjects, but produced variable effects on blood pressure. However, both the negative chronotropic and hypotensive effects of pindolol after a single dose seemed to be dependent upon the intrinsic sympathetic tone of the subjects.

From in vitro studies using brush border membrane vesicles, we demonstrated that enantiomers of pindolol, mecamylamine and disopyramide inhibited the transport of NMN to the same extent (Chapter 8). This implied that transport of organic cations via the transporter for organic cations in the brush border membrane was not stereoselective. Transport of L-lysine was also inhibited equally by (+)- and (-)-pindolol despite the fact that this system exhibit stronger affinity toward the L-isomer of lysine (Chapter 9). Both quinine and pindolol interacted with L-lysine transporter, but NMN and TEA did not. These results indicate that at least some organic cations interact with the transporter for basic amino acids and may be reabsorbed by this system. However, the results suggest that pindolol does not interact stereoselectively with either the organic cation transport system or the reabsorptive system for basic amino acids in the brush border membrane. Species differences may explain the discrepancy, although to date, studies of organic cation transport in different mammalian species tend to agree with each other. It is also possible that stereoselective transport of organic cations is present in the basolateral membranes rather than in the brush border membranes. Alternatively, the renal transport of pindolol and organic cations is not stereoselective. The observed

stereoselective renal clearance of pindolol may be produced by stereoselective renal metabolism.

Because of the studies carried out in this dissertation we now know that tyrosine residues are essential for organic cation transport across the brush border membrane; that the transport of organic cations in the brush border membrane (exchange with protons or cotransport with hydroxyl ions) involves a true energetic activation mechanism; and that organic anions interact with the transporter for organic cations in the brush border membrane. In addition, we know that similar to hepatic metabolism, renal excretion of drugs may be stereoselective. However, the transport of organic cations in the brush border membrane (either secretion by the organic cation transport system or reabsorption by the basic amino acid transport system) does not involve stereoselective mechanisms suggesting that transport in the basolateral membrane or renal metabolism may be responsible for the stereoselective excretion of basic drugs in the kidney.

Although many questions remain to be answered regarding the transport system for organic cations, studies in this dissertation have contributed substantially to our knowledge of the organic cation transport system, and specifically to the cellular and molecular mechanisms involved in drug transport in the kidney.

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Appendixes

A. Kinetics of Transporter-substrate Interactions

1. Simple transporter-substrate interaction

An equilibrium describing the interaction between transporter in the cell membrane and its substrate (assuming no passive diffusion) is shown below.

$$Te + Se \leftrightarrow TSe \leftrightarrow TSi \leftrightarrow Ti + Si$$

where T = Transporter, S = Substrate, TS = Transporter-substrate complex, e and i represent extra and intracellular phases, respectively. Initially, the concentration of Si is negligible. Thus, the efflux of Si is insignificant compared with the influx of Se. The equilibrium can be simplified to

$$Te + Se \underset{Ks}{\leftrightarrow} TSe \xrightarrow{\alpha} TSi \longrightarrow Ti + Si$$

where Ks = dissociation constant and α = translocation rate constant. Assuming that the translocation is the rate limiting step. Then

$v = \alpha[TSe]$ and

$Ks = [Te][Se]/[TSe]$

where v = initial transport rate of the substrate.

It is also clear that the total number of exterior transporters (Tt) is

$[Tt] = [Te] + [TSe] = Ks[TSe]/[Se] + TSe = TSe(1 + Ks/[Se])$

$[TSe] = [Tt]/(1 + Ks/[Se])$

$$\text{Thus } v = \alpha[TSe] = \frac{\alpha[Tt]}{1 + Ks/[Se]}$$

when [TSe] = [Tt], the rate should be greatest. Therefore the maximal rate

$$V_{max} = \alpha [Tt]$$

$$v = \frac{V_{max}}{1 + K_s/[Se]} = \frac{V_{max}[Se]}{[Se] + K_s}$$

Thus, the initial rate of transport of substrate in the membrane can be described by Michaelis-Menten like kinetics.

2. Competitive Inhibition

When a competitive inhibitor is present along with the substrate, the equilibria are as follows:

$$Te + Se \underset{K_s}{\leftrightarrow} TSe \xrightarrow{\alpha} TSi \rightarrow Ti + Si$$

$$+$$

$$Ie \updownarrow K_i \; Tie$$

where I = inhibitor, TIe = transporter-inhibitor complex.

$$[Tt] = [Te] + [TSe] + [TIe]$$

$$K_i = [Te][Ie]/[TIe]$$

$$v = \alpha [TSe]$$

$$v/[Tt] = \alpha [TSe]/([Te] + [TSe] + [TIe])$$

$$v = \frac{[Tt]\,\alpha\,[TSe]}{[Te] + [TSe] + [TIe]} = \frac{V_{max}\,[TSe]}{[Te] + [TSe] + [TIe]}$$

$$= \frac{V_{max}[Se]/K_s}{1 + [Se]/K_s + [Ie]/K_i}$$

$$= \frac{Vmax\,[Se]}{Ks + [Se] + Ks\,[Ie]/Ki}$$

$$= \frac{Vmax\,[Se]}{Ks\,(1 + [Ie]/Ki) + [Se]}$$

A competitive inhibitor does not alter the apparent Vmax, but alters the apparent Ks.

a. Dixon Plot

If the reciprocal is taken at both sides

$1/v = (Ks\,(1 + [Ie]/Ki) + [Se])/ Vmax\,[Se]$

$= Ks/(Vmax[Se]) * (1 + [Ie]/Ki) + 1/Vmax$

$1/v = Ks/(Vmax[Se]Ki) * [Ie] + 1/Vmax\,(1 + Ks/[Se])$

A plot of 1/v versus [I] (Dixon plot) will yield a straight line with a positive slope.

The intercept at x-axis can be solved as

$0 = Ks/(Vmax[Se]Ki) * [Ie] + 1/Vmax\,(1 + Ks/[Se])$

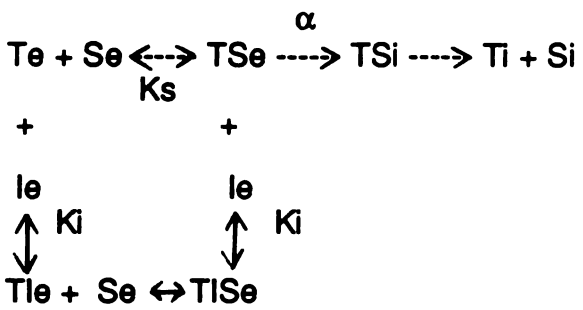
$[Ie] = -(1 + Ks/[Se])/(Ks/[Se]Ki)$

$= -Ki\,(1 + [Se]/Ks)$.

If [Se] < Ks, the intercept may represent -Ki.

3. Noncompetitive Inhibition

A noncompetitive inhibitor binds to both the transporter and the transporter-substrate complex to form transporter-inhibitor complex and transporter-substrate-inhibitor complex. These inhibitor complexes cannot be translocated directly. The equilibria in the presence of a noncompetitive inhibitor are as follows:



where I = inhibitor, TIe = transporter-inhibitor complex, TISe = transporter-substrate-inhibitor complex.

$Ks = [Te][S]/[TSe] = [TIe][S]/[TISe]$

$Ki = [Te][Ie]/[TIe] = [TSe][Ie]/[TISe]$

$[Tt] = [Te] + [TSe] + [TIe] + [TISe]$

$v = \alpha [TSe]$

$v/[Tt] = \alpha [TSe]/([Te] + [TSe] + [TIe] + [TISe])$

By the same mathematical manipulation

$$v = \frac{Vmax\,[Se]/Ks}{1 + [Se]/Ks + [Ie]/Ki + [Ie][Se]/KsKi}$$

$$= \frac{Vmax\,[Se]}{(1 + [Ie]/Ki)([Se] + Ks)}$$

$$= \frac{Vmax\,[Se](1 + [Ie]/Ki)}{Ks + [Se]}$$

The only effect of a noncompetitive inhibitor is to decrease the apparent Vmax, the Ks is not changed.

a. Dixon Plot

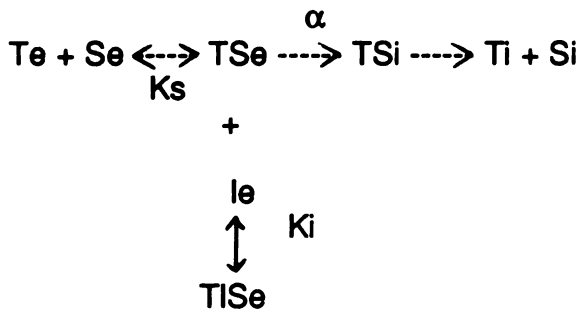
The reciprocal equation for noncompetitive inhibitor is as follows:

$1/v = (1 + Ks/[Se])/VmaxKi * [Ie] + 1/Vmax\,(1 + Ks/[Se])$

The Dixon plot will yield a straight line with the intercept of X-axis equal to Ki.

4. Uncompetitive Inhibition

An uncompetitive inhibitor binds to the transporter-substrate complex. The resulted transporter-substrate-inhibitor complex cannot be translocated directly. The equilibria in the presence of a uncompetitive inhibitor are as follows:



$Ks = [Te][S]/[TSe]$

$Ki = [TSe][Ie]/[TISe]$

$[Tt] = [Te] + [TSe] + [TISe]$

$v = \alpha [TSe]$

$v/[Tt] = \alpha [TSe]/([Te] + [TSe] + [TISe])$

By the same mathematical manipulation

$$v = \frac{Vmax\,[Se]/Ks}{1 + [Se]/Ks + [Se][Ie]/KsKi}$$

$$= \frac{Vmax\,[Se]}{Ks + [Se](1 + [Ie]/Ki)}$$

$$= \frac{Vmax\,[Se](1 + [Ie]/Ki)}{Ks(1 + [Ie]/Ki) + [Se]}$$

A uncompetitive inhibitor changes not only apparent Vmax but also apparent Ks

to the same extent.

a. Dixon Plot

The reciprocal equation for a noncompetitive inhibitor is as follows:

$1/v = 1/VmaxKi * [Ie] + 1/Vmax (1 + Ks/[Se])$

The Dixon plot will yield a straight line with the intercept of X-axis equal to -Ki(1 + Ks/[Se]). If [Se} < Ks, the intercept may be much greater than the Ki.

B. Cotransport Mechanism and Catalytic Effect

1. Cotransport mechanism

An equilibrium describing a cotransport system is shown below.

$$Te + Se + Ae \underset{Ks}{\leftrightarrow} TSAe \underset{Kt}{\leftrightarrow} TSAi \underset{Ks}{\leftrightarrow} Ti + Si + Ai$$

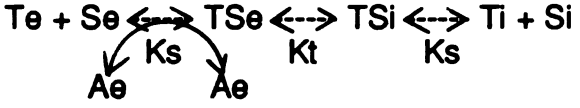
where T = Transporter, S = Substrate, A = Activator, TSA = Transporter-substrate-activator complex, Ks = Dissociation constant, Kt = Translocation constant, e and i represent extra and intracellular phases, respectively.

Ks = [Te][Se][Ae]/[TSAe] = [Ti][Si][Ai]/[TSAi]

To simplify the calculation assume that initially no activator is present and that [Se] = [Si]. At this point, if extra amount of activator is added extracellularly, then [Ae] is increased which results in the increased [TSAe]. The increase in [TSAe] in turn causes the increase in [TSAi] and eventually, increases in [Ai] and [Si]. Thus, intracellular substrate concentration is temporarily greater than the equilibrium value. Eventually, a new equilibrium is reached in which [Se] equals [Si], and [Ae] equals [Ai]. However, the equilibrium concentration of substrate is unchanged. Applying the results to vesicle studies, a temporary overshoot phenomenon will be observed.

2. Catalytic Effect

A catalytic system can be described by the following equilibrium:



Ks = [Te][Se]/[TSe] = [Ti][Si]/[TSi].

Notice that the activator Ae is not transported across the membrane. The presence of Ae, however, changes the Ks. Again assume that initially no activator is present and that [Se] equals [Si]. When activator is added extracellularly, Ks is decreased. Thus the equilibrium shifts to the increase of [TSe] which in turn increases [TSi] and [Si]. Accumulation of the substrate against a concentration gradient is also observed. However, if the membrane is impermeable to the activator, the increase in [Si] will be permanent since the activator is not transported across the membrane. However, in vesicle studies, passive diffusion of the activators such as Na^+ and H^+ does occur. Thus, the accumulation of the substrate against concentration gradients is only temporary and an apparent overshoot phenomenon is also observed in catalytic effect.

C. Titration of the Buffer Capacity of BBMV

Ten mg of BBMV was resuspended in 40 ml pH 6.0 buffer and centrifuged at 20,000rpm for 20 minutes. The supernatant was poured off and the vesicles were resuspended in 20 ml 0.9% Tergitol (NP 40) solution. The solution was titrated with 0.05 N KOH at room temperature. The pH of the solution was monitored with a pH meter. The titration curve was linear between pH 6.00-6.40. On average one µl of KOH changed the pH of the solution by 0.020 pH unit which corresponded to a buffer capacity of 250 pmol/pH unit/mg protein. The data from the two experiments are listed as follows.

volume of KOH	pH	
(µl)	sample A	sample B
0	5.99	6.02
2	6.03	6.06
4	6.07	6.10
6	6.10	6.14
8	6.15	6.18
10	6.19	6.22
12	6.23	6.25
14	6.27	6.28
16	6.31	6.33

D. Plasma Concentration-time Profiles of (+)- and (-)-Pindolol and Pulse and mean arterial pressure-time Profiles in the Subjects

1. Subject K. C.

Time (hr)	Concentration (ng/ml) (+)-pin	(-)-pin	Pulse beat/min	MAP mmHg
0.00	-	-	66	82
0.50	37.51	37.57	53	77
0.75	49.55	50.65	52	88
1.00	51.14	52.53	55	86
1.53	39.37	38.62	55	95
2.03	42.36	40.74	49	84
3.05	33.90	34.09	52	81
4.07	28.52	30.65	54	85
5.01	23.21	22.97	60	86
6.17	19.29	20.29	70	84
8.13	11.93	12.52	63	86
10.2	11.51	11.66	63	88
12.0	7.31	8.15	60	87

2. Subject F. W.

Time (hr)	Concentration (ng/ml) (+)-pin	(-)-pin	Pulse beat/min	MAP mmHg
0.00	-	-	60	90
0.50	31.47	30.06	70	89
0.75	51.08	50.10	60	89
1.02	67.79	68.03	65	76
1.52	60.76	59.36	67	86
2.02	53.03	54.39	54	79
3.05	46.39	47.24	54	77
4.22	44.05	42.77	51	79
5.08	25.69	26.86	66	85
6.25	18.12	18.27	70	82
8.30	13.04	14.82	65	80
10.2	6.72	8.84	76	82
12.3	4.10	5.05	64	86

3. Subject K. D.

Time (hr)	Concentration (ng/ml) (+)-pin	(-)-pin	Pulse beat/min	MAP mmHg
0.00	-	-	50	91
0.53	20.63	18.33	50	92
0.78	39.92	38.90	44	91
1.03	72.96	72.60	48	81
1.53	75.73	74.95	47	77
2.02	79.66	79.29	51	80
3.03	58.32	56.34	43	93
4.03	42.72	42.60	44	89
5.12	33.58	35.76	58	85
6.23	27.11	29.10	60	89
8.37	17.39	19.57	56	89
10.1	13.28	13.58	46	95
12.3	8.89	9.75	51	98

4. Subject P. E.

Time (hr)	Concentration (ng/ml) (+)-pin	(-)-pin	Pulse beat/min	MAP mmHg
0.00	-	-	72	96
0.47	34.78	33.71	60	100
0.73	54.19	53.50	52	101
0.95	60.55	61.53	55	102
1.47	47.27	41.65	45	107
1.97	44.69	42.11	46	103
2.97	30.68	29.57	45	94
3.98	24.96	26.04	45	102
5.17	20.81	21.72	72	102
5.83	19.00	21.40	65	97
7.98	15.98	15.75	63	93
10.1	7.52	7.38	53	99
11.9	4.99	5.75	71	95

5. Subject B. D.

Time (hr)	Concentration (ng/ml) (+)-pin	(-)-pin	Pulse beat/min	MAP mmHg
0.00	-	-	65	98
0.50	51.01	49.97	58	92
0.73	58.37	56.64	57	86
0.97	72.92	71.03	56	84
1.50	83.59	83.59	57	87
2.02	69.10	68.48	55	80
2.98	53.39	54.86	54	84
4.07	48.23	49.02	51	68
4.98	40.24	41.43	56	84
5.98	28.09	30.11	57	86
8.02	19.69	22.45	58	82
9.92	11.22	11.81	61	91
12.0	6.52	7.82	69	94

6. Subject M. P.

Time (hr)	Concentration (ng/ml) (+)-pin	(-)-pin	Pulse beat/min	MAP mmHg
0.00	-	-	72	95
0.50	41.76	40.89	70	86
0.75	48.36	48.52	63	87
1.00	52.81	52.12	67	88
1.57	40.61	40.75	55	91
2.08	37.74	37.59	61	84
3.05	33.83	33.89	59	83
4.08	25.99	24.58	59	88
4.95	23.22	21.65	78	87
5.88	18.40	17.54	78	82
8.58	9.70	9.59	72	85
9.95	8.05	7.05	77	84
11.9	3.99	3.73	78	88

E. Urine Volume and Concentration-time Profiles of (+)- and (-)-Pindolol in the Subjects

1. Subject K. C.

Time (hr)	Volume (ml)	Concentration (μg/ml) (+)-pin	(-)-pin
0.75	238	0.75	0.84
1.30	285	1.47	1.78
2.13	280	2.37	2.87
3.30	250	1.94	2.49
4.22	135	2.20	2.96
6.22	186	2.20	2.90
8.14	76	2.10	2.72
12.1	204	2.50	3.04
23.8	126	0.59	0.72

2. Subject F. W.

Time (hr)	Volume (ml)	Concentration (μg/ml) (+)-pin	(-)-pin
0.67	34	3.37	3.64
1.25	144	3.18	3.44
2.13	270	2.93	3.27
3.25	225	5.43	5.78
4.18	52	8.60	11.6
6.30	57	13.2	14.6
8.30	60	3.14	3.67
12.4	134	2.45	2.89
24.0	384	1.24	1.46

3. Subject K. D.

Time (hr)	Volume (ml)	Concentration (μg/ml) (+)-pin	(-)-pin
1.70	116	2.64	3.00
2.58	460	2.67	2.87
3.51	220	1.94	2.24
4.48	42	9.66	11.3
6.61	222	2.23	2.58
8.69	130	3.13	3.74
12.6	116	0.83	0.97
24.3	445	0.62	0.76

4. Subject P. E.

Time (hr)	Volume (ml)	Concentration (μg/ml) (+)-pin	(-)-pin
1.00	274	1.77	2.07
2.03	270	1.01	1.20
3.03	176	2.25	2.70
4.08	85	2.94	3.46
5.75	72	6.13	7.16
8.08	84	5.22	6.27
12.1	242	1.79	2.09
23.9	453	0.89	1.08

5. Subject B. D.

Time (hr)	Volume (ml)	Concentration (µg/ml) (+)-pin	(-)-pin
1.05	8	1.61	1.97
2.08	495	1.67	2.16
3.08	23	1.94	2.31
4.08	250	3.10	4.12
6.00	78	4.50	6.18
8.08	68	3.91	5.54
12.1	20	1.25	1.86
24.1	908	0.28	0.40

6. Subject M. P.

Time (hr)	Volume (ml)	Concentration (µg/ml) (+)-pin	(-)-pin
2.08	497	1.90	2.19
3.08	154	5.47	6.46
5.83	233	4.61	5.37
10.1	70	6.42	8.04
12.2	86	3.50	4.12
23.9	333	0.92	1.11

F. Renal Clearance, Urine pH, Time, and Plasma Concentration of (+)- and (-)-Pindolol in the Subjects

1. Subject K. C.

CL_r@ ml/min (+)-pin	CL_r@ ml/min (-)-pin	urine pH	time* hr	concentration# ng/ml (+)-pin	concentration# ng/ml (-)-pin
140	157	7.32	0.38	28	28
266	318	6.97	1.03	48	48
329	409	6.92	1.72	40	39
193	249	6.98	2.72	36	36
123	229	7.02	3.76	30	32
152	200	6.86	5.22	23	22
87	110	7.24	7.18	16	16
188	225	6.51	10.1	12	12

@ renal clearance determined at each collection period of urine samples.
* the midpoint time of the collection period of urine samples.
# the plasma concentration at the midpoint time of the collection period.

2. Subject F. W.

CL_r@ ml/min (+)-pin	CL_r@ ml/min (-)-pin	urine pH	time* hr	concentration# ng/ml (+)-pin	concentration# ng/ml (-)-pin
124	137	6.17	0.34	23	23
224	244	6.31	0.96	59	58
243	277	6.30	1.69	62	60
380	403	6.02	2.69	48	48
218	284	6.16	3.72	37	38
236	245	5.78	5.24	25	27
105	110	6.61	7.30	15	17
194	191	6.95	10.3	7	8

3. Subject K. D.

CL_r@ ml/min (+)-pin	CL_r@ ml/min (-)-pin	urine pH	time* hr	concentration# ng/ml (+)-pin	concentration# ng/ml (-)-pin
61	71	5.90	0.85	49	48
322	351	6.44	2.14	72	71
132	157	6.04	3.05	58	56
161	189	6.48	4.00	43	43
119	135	6.31	5.55	31	33
158	171	7.28	7.65	21	23
34	37	6.80	10.7	12	13

4. Subject P. E.

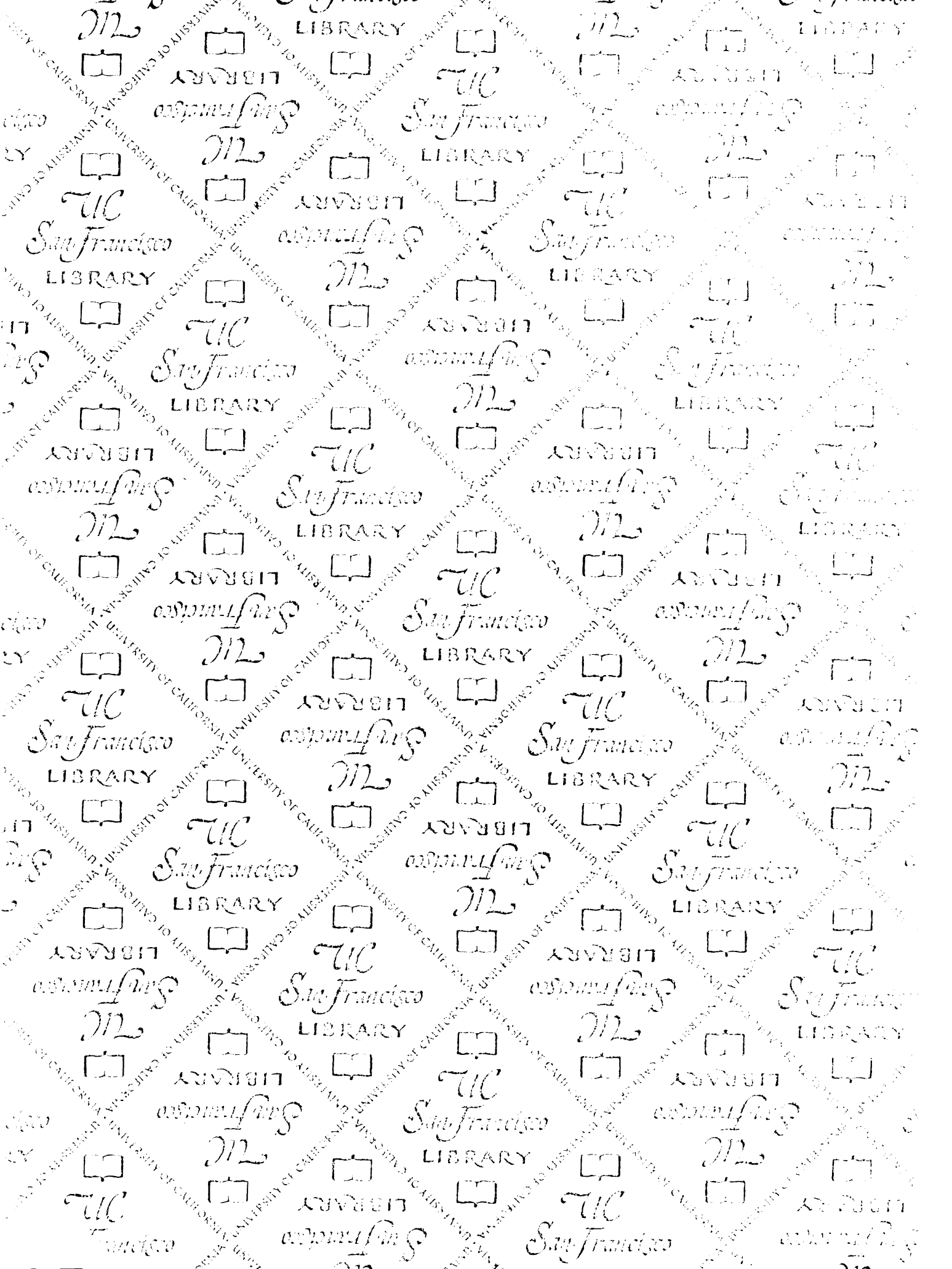
CL_r@ ml/min (+)-pin	CL_r@ ml/min (-)-pin	urine pH	time* hr	concentration# ng/ml (+)-pin	concentration# ng/ml (-)-pin
324	381	6.64	0.78	55	55
92	110	6.80	1.52	48	48
179	226	6.63	2.53	37	35
145	170	6.43	3.56	27	28
203	227	6.24	4.92	22	23
181	203	6.88	6.92	17	19
238	283	6.18	10.1	8	7

5. Subject B. D.

CL_r@ ml/min (+)-pin	CL_r@ ml/min (-)-pin	urine pH	time* hr	concentration# ng/ml (+)-pin	concentration# ng/ml (-)-pin
175	228	6.34	1.57	77	76
12	15	6.44	2.58	60	61
256	333	6.20	3.58	51	52
77	103	5.50	5.04	40	41
93	115	5.58	7.04	24	26
149	165	5.39	10.1	11	12

6. Subject M. P.

CL_r@ ml/min (+)-pin	CL_r@ ml/min (-)-pin	urine pH	time* hr	concentration# ng/ml (+)-pin	concentration# ng/ml (-)-pin
194	225	6.18	1.04	39	39
393	465	6.61	2.58	36	36
263	325	6.54	4.46	25	23
151	192	5.71	7.96	12	11
426	556	5.52	11.1	6	5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