Title
Mechanisms of cimetidine renal transport in isolated membrane vesicles and renal clearance studies in humans

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Mechanisms of Cimetidine Renal Transport In Isolated Membrane Vesicles and Renal Clearance Studies In Humans.

by

Lee G. Gisclon

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Dedication

I dedicate this thesis to my parents who provided the opportunity for my education, and to Marty who provided the encouragement to complete it.

Acknowledgments

I wish to acknowledge the patience and guidance provided throughout this thesis work by Doctors Kathleen Giacomini, Leslie Benet, David Warnock, and Betty Hoener. Ms. Fee Mi Wong deserves special credit for many long hours of laboratory work.

Abstract

Mechanisms of Cimetidine Renal Transport In Isolated Membrane Vesicles And Renal Clearance Studies In Humans.

Lee G. Gisclon

The mechanisms of renal transport of the histamine H_2-receptor antagonist cimetidine were studied in vitro using isolated luminal membrane vesicles prepared from rabbit kidney.[1] Cimetidine was found to accumulate in the vesicles with time. This uptake was responsive to changes in the extravesicular osmolarity, indicating transport into intravesicular space. Both saturable and nonsaturable processes were required to characterize the uptake of cimetidine. Michaelis-Menten parameters were obtained for the saturable component of cimetidine uptake. An initial outwardly-directed proton gradient was found to stimulate cimetidine uptake and this enhanced uptake was inhibited by other organic bases and cations as well as by the organic anion probenecid. The effect of probenecid on cimetidine transport was not due to effects on membrane binding, vesicle volume, or membrane potential, and the data suggest that the inhibition specifically involved the transport system for cimetidine in the luminal membrane. Subsequent studies have confirmed that probenecid competitively inhibits the transport of other organic cations across the luminal membrane of the renal proximal tubule.

The effect of creatinine on the transport of

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cimetidine across the luminal membrane of the proximal tubule was evaluated directly using isolated membrane vesicles.[2] Creatinine was found to produce concentration dependent inhibition of the proton gradient stimulated transport of cimetidine in luminal membrane vesicles prepared from rabbit kidney. These data are consistent with the observation that cimetidine inhibits the renal secretion of creatinine in vivo and suggest that cimetidine and creatinine may share a common transport system in the luminal membrane. Creatinine concentrations required to inhibit cimetidine transport were high (i.e. 0.01 to 0.5 M), suggesting that the affinity of creatinine for the transport system is low compared to that of cimetidine.

Because of our finding that probenecid inhibited cimetidine transport in luminal membrane vesicles, we sought to determine the relevance of this inhibition to drug therapy. Accordingly, we examined the interaction between probenecid and cimetidine in humans.[3] In a randomized crossover study of six healthy male subjects, probenecid was found to transiently decrease the renal clearance of cimetidine. In the first hour after cimetidine administration, probenecid significantly decreased both the glomerular filtration rate (GFR) and the net renal secretory clearance of cimetidine without increasing its nonrenal clearance. The transient nature of this inhibition resulted in no significant changes in the

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overall elimination kinetics for cimetidine. These data are consistent with our findings from in vitro studies, and suggest that interactions between other concurrently administered anion-cation drug combinations may occur.

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Introduction

Cimetidine is a histamine H_2-receptor antagonist widely used in the treatment of gastric acid hypersecretory disease. The drug is eliminated from the body by both biotransformation and renal excretion. Although there have been a number of studies elucidating the mechanisms involved in the biotransformation of cimetidine and its effects on the biotransformation of other compounds, there have been only a few studies on the mechanism(s) of the renal excretion of this drug.

Overall Thesis Objectives and Scope

The overall objectives of this thesis are to determine the cellular mechanisms involved in the renal transport of cimetidine and to ascertain the ramifications of these mechanisms on drug therapy.

Specific Objectives

The specific objectives of this thesis are:

1. To characterize the mechanisms involved in the transport of cimetidine across the luminal membrane of the renal proximal tubule.

2. To determine the effect of creatinine on cimetidine transport in luminal membrane vesicles prepared from rabbit renal cortex.

3. To determine the effect of probenecid on the renal clearance of cimetidine in humans.

Chapter I

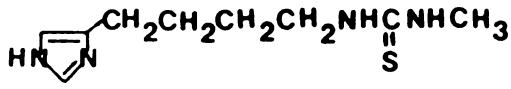
Cimetidine Background Information

A) Development

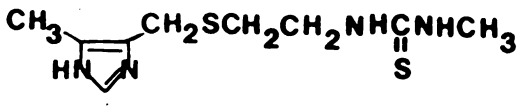
Folkow et al.[4] (1948) first suggested the possibility of more than one histamine receptor based on studies in which available antihistamines were not found to be effective in blocking histamine stimulated increases in gastric acid secretion or heart rate. Black et al.[5] (1972) were the first to define and specifically antagonize the histamine H_2-receptor, as determined by inhibition of histamine-stimulated gastric acid secretion by burimamide. However, this compound, which was the 200th synthesized, was not marketed because it was not active when administered orally. Metiamide was subsequently found to have oral potency and was used in human clinical trials, but its use was stopped because of fatal granulocytopenias thought to be due to the thiourea moiety of the compound.[6] After synthesis and testing of another 500 compounds, cimetidine (Smith Kline and French) was developed and approved for human use in England in 1976 and in the U.S. in 1977 and is now one of the most widely prescribed drugs in the world. Currently, ranitidine (Glaxo) and famotidine (Merck Sharp & Dohme) have also been approved for use in the U.S. Figure I-1 contains the structures of several H_2-receptor antagonists. Burimamide, metiamide, and tiotidine, are no

Figure I-1. Structures of histamine H_2-receptor antagonists.

BURIMAMIDE

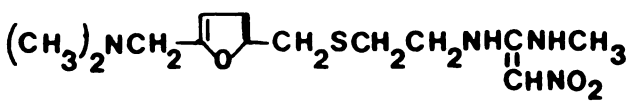


METIAMIDE

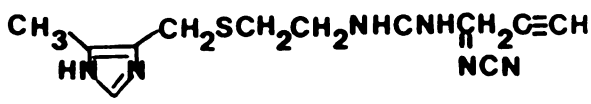


CIMETIDINE

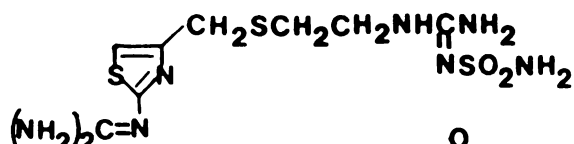
RANITIDINE



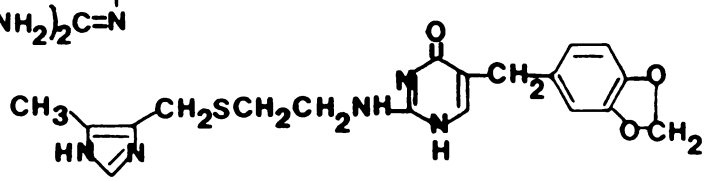
ETINTIDINE



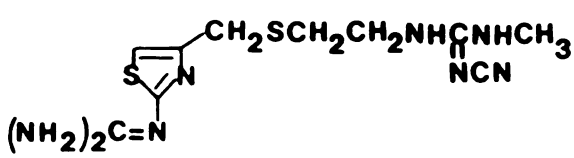
TIOTIDINE



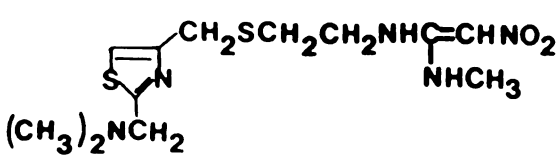
OXMETIDINE



FAMOTIDINE



NIZATIDINE



longer used in humans; etintidine, oxmetidine, and nizatidine are undergoing clinical trials.

B) Clinical Use.

Cimetidine is approved for use in the U.S. by the Food and Drug Administration for treatment of duodenal ulcers, gastric ulcers, and pathological hypersecretory disease (i.e. Zollinger-Ellison syndrome, systemic mastocytosis, multiple endocrine adenoma).[7] In addition, it is widely used to prevent stress induced ulceration and bleeding (i.e. from trauma, surgery, infection, or renal failure), to alleviate symptoms of gastroesophageal reflux, as an adjunct therapy to enzyme supplementation in pancreatic insufficiency, to neutralize stomach acid in order to prevent complications of gastric aspiration during anesthesia, and for various allergic and urticarial conditions.[7] Doses of cimetidine range from 1200 mg per day for treating uncomplicated duodenal ulcer, to as high as 3600 mg per day for treating pathological hypersecretory disease.[7]

C) Adverse Effects.

Among the adverse reactions reported to be associated with cimetidine use are central nervous system (CNS) toxicities, cardiovascular effects, endocrine effects, and hematological toxicities. Comprehensive evaluation of the incidence of these reports has revealed that adverse effects

resulting from cimetidine are extremely rare.[8,9,10] CNS toxicity has been observed in 7.3 in 100,000 patients, mental confusion in 1.1 in 100,000, and hematologic toxicities in 2.3 in 100,000. Recent evidence has been presented to show that the cardiovascular effects associated with rapidly administered intravenous doses of cimetidine may be due to a reduction in total peripheral resistance with no effect on cardiac performance.[11] Patients with compromised ability to eliminate cimetidine, i.e. the elderly, those with multiple organ failure, and shock and trauma patients appear to be most susceptible to the drug's adverse effects and must be closely monitored.[12]

D) Cimetidine Pharmacokinetics.

1. Absorption Site.

Studies in the rat have shown that cimetidine is poorly absorbed in the stomach and is differentially absorbed in the intestine according to the following order, ileum > duodenum > jejunum > colon.[13] Active membrane transport for cimetidine has been demonstrated in the rat using small intestinal membrane preparations.[14] In humans, the importance of the ileal and duodenal absorption sites has been shown in studies in which absorption was not affected by partial gastrectomy,[15] or by jejunostomy.[16] In addition to the oral tablet and the intravenous dosage forms, cimetidine is also marketed in intramuscular (IM) and oral

liquid dosage forms. The IM dosage form is rapidly and completely absorbed.[17,18] The oral liquid dosage form is equivalent in absorption to the oral tablet.[18]

2) Dual Plasma Peak.

A dual peak is often present in the plasma cimetidine concentration vs time profiles of fasted subjects dosed orally with cimetidine.[18-24] The dual peak is reported not to be present when subjects are fed.[19,21] In subjects taking metoclopramide, a stimulant of upper GI motility, the dual peak is not observed but bioavailability declines.[25,26]

Although the precise mechanism for this observation is not known, several theories have been proposed to explain the dual peaks. The theories include; a) discontinuous or site specific absorption of cimetidine,[13] b) storage of unchanged cimetidine in hepatic parenchymal tissue or gall bladder with spontaneous or food stimulated release,[24,27] and c) enterohepatic recycling in which the sulfoxide metabolite is reduced back to cimetidine by fecal bacteria and reabsorbed as such.[20]

3) Bioavailability.

The bioavailability of orally administered cimetidine has been studied in healthy subjects,[18,20] in patients with ulcer disease,[19] in patients with cirrhosis,[28,29] and in patients with renal disease.[30,31] Bioavailability has generally been reported to range from 70 to 90%,[12] except in

some individuals in whom unspecified disease or intrinsic factors cause a substantial reduction (i.e. 25-30% bioavailability).[29,32]

Food does not affect the extent of absorption but does cause delayed absorption of cimetidine.[21,33,34] Antacids of high neutralizing capacity (26-41 mmol/10ml) have been reported to decrease the AUC of cimetidine by 20-35%.[25,35,36]

4) Distribution.

Cimetidine is a weak base (pKa 6.8) and a very polar molecule with a high degree of water solubility (11.4 gm/L) and a relatively low octanol/water partition coefficient (2.5).[37] These physicochemical characteristics would favor partitioning of cimetidine into aqueous physiologic space. Data to support this argument comes from a limited number of tissue distribution studies performed in animals and in postmortem samples obtained from humans.[38-40] In these studies, 70% of the total amount of cimetidine in the body was determined to be in skeletal muscle, which, because of its large contribution to total body weight, was the primary tissue storage site. The liver, kidney, and lung were found to have tissue/plasma concentration ratios for cimetidine greater than 1.0. Ratios for the kidney and gallbladder were from 10 to 15, for liver and lung from 2 to 5, and for skeletal muscle from 1 to 2.

The accumulation of cimetidine in fat was found to be

negligible, reflecting the hydrophilic nature of the molecule. However, cimetidine does penetrate the blood-brain barrier, resulting in mean CSF/serum concentration ratios ranging from 0.03 to 0.24 in patients without hepatic or renal disease.[38-42] Certain disease states may enhance the penetration of cimetidine into, or decrease the elimination of cimetidine from the CNS. For example, patients with liver disease have been reported to have higher CSF/serum concentration ratios.[38] Cimetidine distributes into red blood cells attaining concentrations equal to those in plasma.[19] Cimetidine is secreted in the breast milk of humans, achieving concentrations 5 to 12 times higher than those in plasma.[43]

Cimetidine is approximately 10 to 20% bound to plasma proteins (albumin) over the concentration range of 0.05 to 50 ug/ml.[19,44] Binding of cimetidine to alpha-1-acid glycoproteins has not been thoroughly studied.[12] The steady state volume of distribution of cimetidine is 1.0 L/Kg, which is approximately equal to total body water, and apparently remains constant in a variety of disease states including renal failure and hepatic disease.[12,45] Somogyi et al.[19] have reported that the Vd_{ss} declines with age. The decrease in Vd_{ss} with age may be related to the relative increase in body fat and decrease in muscle mass which occurs with aging.

5) Metabolic Pathways.

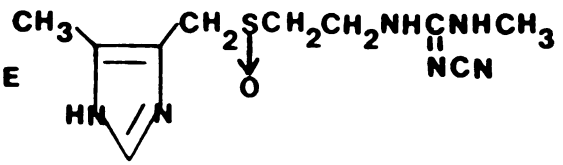
Detailed studies of cimetidine metabolism in humans have been limited, and the metabolic fate of the compound has not yet been precisely determined. Studies by Taylor et al.[44] and Mitchell et al.,[46] have positively identified three metabolites of cimetidine in man (Figure I-2). The primary metabolite is cimetidine sulfoxide, which accounts for 10 to 15% of the dose. The 5-hydroxymethyl derivative is a minor metabolite, accounting for 4% of the dose. Guanylurea cimetidine is also a minor metabolite in normal individuals (1-2%), however, its formation has been reported to be increased in burn patients.[47] In the studies by both Taylor et al.[44] and Mitchell et al.,[46] an additional polar metabolite was found which accounted for as much as 24% of the cimetidine dose. The latter group tentatively identified the metabolite as the N′-glucuronide of cimetidine. If confirmed, this would be the major metabolite of cimetidine in man.

6) Elimination.

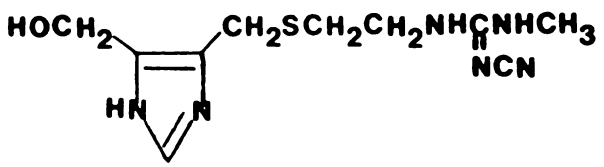
When cimetidine is administered intravenously to healthy subjects, about 70% (range, 50-80%)[45] of the dose is recovered as unchanged drug in the urine, another 15% is recovered in urine as the three metabolites, while 15% remains unaccounted for but may be the, as yet unconfirmed, glucuronide metabolite.[12] Oral administration results in less unchanged drug being excreted in the urine,[45] with the

Figure I-2. Metabolites of cimetidine formed in humans.

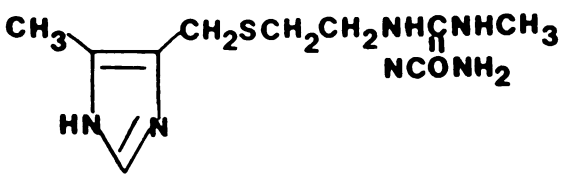
CIMETIDINE SULFOXIDE



HYDROXYMETHYL CIMETIDINE



GUANYLUREA CIMETIDINE



amount of the unidentified metabolite rising to as high as 40% of the dose.[12] Only about 2% of a dose is excreted in the bile.[45]

It is thus apparent that renal excretion is the primary route of elimination for both cimetidine and its metabolites and that renal function will determine the rate at which these compounds are removed from the body. Total body clearance (CL_s) of cimetidine in healthy young adults was reported to be 500 to 600 ml/min in a review of the literature by Somogyi et al.[45] This high systemic clearance is accounted for primarily by high renal clearances (CL_r) which were reported to range from 400 to 600 ml/min in the reviews by Ziemniak et al.,[12] Somogyi et al.,[45] and Abate et al.[48] Recent studies are included in a summary of the clearance data (mean +/- SD) for cimetidine obtained from humans (Table 1). When the data for healthy young adults is averaged, the mean +/- SEM for systemic clearance is 595 +/- 20 ml/min and for renal clearance is 409 +/- 21 ml/min from 24 and 20 separate studies respectively (Table 1). Renal clearances three to four times greater than GFR indicate that cimetidine is extensively secreted by the renal tubules in addition to being filtered by the glomerulus.

It should be noted that a great deal of variability has been reported in the systemic and renal clearances of cimetidine and that studies are difficult to compare unless patients are carefully matched for age and renal function. In addition, evidence has recently been presented to show

TABLE I-1

Investigator	Subjects	N	Age	Dose/Route	CL_{CR}	CL_S	CL_R	CL_{NR}	$T_{1/2}$
Griffiths[13]	Healthy	4		100 mg IV		444 +/-51			1.8
Ma[30]	Healthy	12	23-32	300 mg IV	'Normal'				1.5
	Renal	5	34-66	300 mg IV	49-87				2.6
	Failure	5	34-66	300 mg IV	19-34				2.9
		6	34-66	300 mg IV	0-9				3.7
Walkenstein[18]	Normals	12		300 mg IV		536 +/-126			1.8
Grahnen[20]	Healthy	10	22-25	100 mg IV		808 +/-302	600 +/-212		2.1
Larsson[22]	Renal	6	26-76	200 mg po	0-2				5.5
	Disease	10	"	"	0-5				5.2
	"	4	"	"	2-5				4.8
	"	15	"	"	5-15				3.2
	"	7	"	"	15-30				3.2
	"	12	"	"	15-52				3.3
	"	5	"	"	30-52				3.3
	"	46	"	"	'normal'				2.1
Krichman[49]	Healthy	3		300 mg IV		678 +/-177	465 +/-191		1.3
	Hemodial.	5		300 mg IV		224 +/-93	4.3 +/-4.3		3.5
Burgess[50]	Healthy	13		300 mg IV		541 +/-149	381 +/-156		1.8
	Hemodial.	7		300 mg IV		183 +/-95	3.1 +/-4		4.5
Somogyi[19]	Ulcer	6	38-61	200 mg IV	98 +/-36	475 +/-149	282 +/-126	193	2.1
	Ulcer	6	28-64	200 mg IV	103 +/-27	515 +/-238	307 +/-221	208	2.0
Randolph[51]	Renal Failure			Data For Cimetidine Sulfoxide Half Life					
	Mild		300 mg IV						4.9
	Moderate								10.5
	Severe								26.9

Investigator	Subjects	N	Age	Dose/Route	CL_{CR}	CL_S	CL_R	CL_{NR}	$T_{1/2}$
Schentag[38]	Healthy	12	26+/-3	300 mg IV	108+/-22	583+/-140	451+/-123	132	1.9
	Normal	6	46+/-21	300 mg IV	89+/-18	511+/-93	305+/-123	206	2
	Renal	5	63+/-13	300 mg IV	6+/-25	193+/-24	30+/-11	163	4.8
	Hepatic	12	56+/-14	300 mg IV	73+/-27	463+/-145	327+/-119	136	2.9
	Renal+ Hepatic	8	73+/-12	300 mg IV	15+/-10	182+/-110	68+/-57	114	6.7
Larsson[52]	Renal	1	29-77	200 mg po	12		49		4.5
	Disease	3	41-64	200 mg po	15-30		99+/-27		4.8
		5	43-66	200 mg po	30-50		163+/-15		3.5
		5	31-63	200 mg po	50-75		168+/-15		3.1
Sonne[28]	Cirrhotic	9	45-76	200 mg IV	73+/-8	606+/-64			2.0
	Ulcer	11	21-71	200 mg IV	95+/-10	556+/-44			2.3
Bodemar[53]	Normal	10	19-71	200 mg IV	113+/-12	655+/-55	375+/-33		1.8
Lebert[54]	Normal	8	21-35	300 mg IV		642+/-58			1.5
Larsson[31]	Ulcer	9	27-66	200 mg IV	79-110	718+/-80	326+/-40	123+/-102	2.0
	Hemodial.	10	44-78	200 mg IV	< 5	214+/-19	2.5+/-0.8	210+/-19	3.9
Gugler[29]	Ulcer	12	28-64	200 mg IV	94+/-138	495+/-190	293+/-166	191+/-46	2.0
	Cirrhotic	14	33-69	200 mg IV	24+/-168	487+/-249	364+/-185	123+/-102	2.2
Pancorbo[55]	Trauma	9	19-50	300 mg IV	109+/-28	631+/-74			2.3
Guay[56]	Normal	8	30	300 mg IV	91+/-11	710+/-189	371+/-174	368+/-159	2.0
	Renal Disease	8	36	300 mg IV	1.6+/-1.1	147+/-39	2.5+/-2.3	139+/-43	12.8
Bianchi[57]	Healthy	8	49	200 mg po	91	792+/-88			2.1
	cirrhotic	16	49	200 mg po	90	797+/-42			2.0
Bjaeldager[58]	Hemodial.	7	25-66	200 mg IV	< 3	272+/-55			2.9

Investigator	Subjects	N	Age	Dose/Route	CL_{CR}	CL_S	CL_R	CL_{NR}	$T_{1/2}$
Ziemniak[59]	Healthy	12		300 m3cg IV		535 +/-126			1.9
	Hepatic	8	58 +/-15	300 mg IV	50 +/-23	353 +/-69			2.3
	Cirrhotic	8	52 +/-15	300 mg IV	79 +/-35	690 +/-238			2.3
	Normal	4		300 mg IV		613 +/-329			1.7
Cello[60]	Cirrhosis	11	27-57	300 mg po	50-146	575 +/-67	303 +/-38	272 +/-37	2.9
Ziemniak[61]	Normal	12	26 +/-3	300 mg IV	105 +/-28	539 +/-126	413 +/-122	119 +/-35	1.9
	Ulcer	12	49 +/-12	200 mg IV	105 +/-35	490 +/-175	280 +/-140	182 +/-56	2.0
Ziemniak[47]	Normal	12		300mg IV	108 +/-22	539 +/-126	413 +/-112	119 +/-35	1.9
	Burn	11	32 +/-12	300 mg IV	126 +/-146	434 +/-119	231 +/-112	196 +/-56	2.5
	Burn	6	32 +/-12	300 mg IV	155 +/-157	455 +/-126	364 +/-77	84 +/-42	2.2
Abernethy[62]	Normal	16	23-66	300 mg IV		579 +/-39			2,1
	Obese	13	22-64	300 mg IV		616 +/-34			2.2
Bauer[63]	Normal	6	28 +/-4	600 mg IV	121 +/-11	637 +/-114	318 +/-38		1.9
	Obese	6	28 +/-4	600 mg IV	158 +/-12	1147 +/-266	808 +/-205		1.2
Martyn[64]	Healthy	9	23-40	300mg IV		574 +/-42			2.2
	Burn	8	18-42	300 mg IV		931 +/-84			1.5
LLoyd[65]	Critical Ill Peds.	30	4-15	26mg/kg Per 24hr		728 +/-280			1.4
VanCrugten[66]	Healthy	6	19-23	400 mg po	90 +/-12	599 +/-188			2.0
Christian[67]	Normal	6		300 mg IV			450 +/-19		

Investigator	Subjects	N	Age	Dose/Route	CL_{CR}	CL_S	CL_R	CL_{NR}	$T_{1/2}$
Muirhead[68]	Healthy	6	21-29	400 mg po			505 +/-102		2.0
Glaclon[3]	Healthy	6	26 +/-3	300 mg IV	116 +/-8	495 +/-25	417 +/-109	102 +/-48	2.3

N = number of subjects.
Age = years.
Clearances were normalized, where possible, to 70 kg body weight
CL_{cr} = ml per minute.
CL_s = total clearance (ml/min).
CL_r = renal clearance (ml/min).
CL_{nr} = nonrenal clearance (ml/min).
t 1/2 = half-life in hours.

that obese subjects who are otherwise healthy and who have normal serum creatinine levels exhibit much higher systemic and renal clearances for cimetidine.[63] Thus, the additional variable of lean body weight should be considered when comparing clearances for cimetidine obtained from different studies.

Data for the nonrenal clearance of cimetidine are also variable. In literature reviews, Ziemniak et al.[12] and Somogyi et al.,[45] report mean values of 144 and 200 ml/min respectively. The mean +/- SEM for nonrenal clearance of cimetidine after intravenous administration in normal or healthy subjects from 11 studies in Table 1 was 209 +/- 29 ml/min.

The elimination half-life for cimetidine reported in the reviews by Ziemniak et al.[12] and Somogyi et al.,[45] is 2 hours. A half-life of 1.94 +/- .05 hr (mean +/- SEM) was calculated from data compiled for healthy subjects in 20 studies (Table 1).

E) Factors Affecting Elimination.

1) Age.

There is little data on the pharmacokinetics and pharmacodynamics of cimetidine in neonates and children. Although cimetidine might be expected to exhibit low renal clearances in these populations because of depressed tubular development,[45] a few studies in neonates[69,70] have shown

that these patients have an enhanced ability to eliminate cimetidine and may require higher doses. Studies in children[61,65,71,72] have also demonstrated that they have ability to eliminate cimetidine more rapidly (10 to 14 ml/min/kg CL_s) than adults (7 to 8 ml/min/kg CL_s).[12,45]

Both total systemic clearance[19,73] and renal clearance[74,75] have been reported to be inversely correlated with age and are decreased in the elderly. The changes in these parameters have been attributed to declining renal function (GFR) with age.[76] In the elderly, the elimination half-life is also correlated with age, however, the magnitude of change is not as great as would be expected, in part because of a proportional decline in both Vd_{ss} and CL_s.[45]

2) Renal Disease.

As would be expected for a compound which is cleared primarily by the kidney, the CL_s of cimetidine is drastically reduced in renal failure. The CL_s was reported to be reduced to 20 to 50% of normal in the review by Somogyi et al.,[45] depending on the degree of renal failure. In severe renal failure, the CL_r of cimetidine may be reduced to less than 10% of normal (Table 1), and it is the drastic reduction in this clearance, often to less than 5 ml/min, which primarily affects CL_s. Substantial increases in the half-life are observed in patients with severe renal failure or in patients with both renal and hepatic

disease.[12] The literature is conflicting as to whether metabolic clearance remains unchanged,[38,45,50] or is decreased by renal disease.[31,56]

Unlike the parent compound, the sulfoxide and hydroxymethyl metabolites of cimetidine are cleared from the body almost exclusively by the kidney.[12,46] With normal renal function, these metabolites have elimination half lives similar to cimetidine,[45] while in severe renal failure the half life of the sulfoxide may rise to as much as 27 hours.[51] (Table 1).

3) Hepatic Disease.

The effect of hepatic diseases on elimination of cimetidine has not yet been precisely defined.[12,45] (Table 1). An approximately equal number of studies argue for and against hepatic diseases decreasing total systemic and nonrenal clearance of cimetidine.[12] It is likely that difficulties in classification of hepatic impairment contribute to differences in interpretation of the effects of these diseases on cimetidine disposition. It is clear however that patients with both hepatic and renal failure have greatly diminished ability to eliminate the drug and often are subject to accumulation and attendant adverse effects.[38]

F) Drug Interactions.

The information presented on drug interactions in this section was collected from the reviews by Somogyi et al.,[77] Bauman et al.,[78] Greene et al.,[79] Sorkin et al.,[80] Powell et al.,[81] and Gerber et al.,[82] as well as a review of the most recent literature.[66-68] For an extensive listing of all drug interactions reported with cimetidine, the reader is referred to the review by Ziemniak et al.[12]

Cimetidine drug interactions in theory can occur by four mechanisms:[82] 1) by altering gastric pH, cimetidine may affect absorption of acid labile drugs, or drugs which have pH dependent solubility, 2) cimetidine may inhibit the cytochrome p-450 or p-448 mediated metabolism of other drugs, 3) by decreasing hepatic blood flow, cimetidine may decrease the hepatic extraction of drugs which exhibit flow dependent clearance, and 4) compounds which are secreted by the renal proximal tubules may compete with cimetidine for membrane mediated transport processes.

Among the extensive list of drug interactions reported, the following are considered to be most clinically significant.[77,80]

1. The absorption of ketoconazole, a drug which exhibits increasingly poor water solubility with increasing pH, is decreased when administered concurrently with cimetidine.

2. Cimetidine induced inhibition of metabolism or decreased hepatic blood flow results in altered elimination

kinetics of the following drugs which are cleared by hepatic metabolism: 1) warfarin, 2) benzodiazepines including diazepam, desmethyldiazepam, and chlordiazepoxide, 3) phenytoin, 4) B-blockers including propranolol, metoprolol, and labetalol, and 5) theophylline.

3. The renal clearance of the bases procainamide and N-acetylprocainamide are decreased by cimetidine due to competition for renal proximal tubular transport. Cimetidine also inhibits the renal clearance of creatinine without affecting the GFR.[50,83]

Drugs which have been shown to alter the pharmacokinetics of cimetidine include antacids of high neutralizing capacity,[25,35,36] anticholinergics,[26] and metoclopramide,[25,26] all of which decrease the bioavailability of cimetidine. Phenobarbital pretreatment has been shown to cause enhanced enzymatic biotransformation of cimetidine in humans, resulting in an increased nonrenal clearance of cimetidine.[84]

G) Cimetidine Renal Transport.

Evidence for net renal secretion of cimetidine in humans has been obtained from in vivo studies in which the renal clearance of cimetidine in healthy adults has been shown to exceed GFR by a factor of three or four fold.[45] These findings suggest that cimetidine is actively secreted from plasma into urine against a concentration gradient. Based on a limited number of in vivo studies in which

cimetidine has been shown to inhibit the renal clearance of organic bases: ranitidine,[66] triamterene,[68] procainamide,[85,86] and N-acetylprocainamide,[85,86] and one study in which cimetidine renal clearance was inhibited by the organic base ketoconazole,[67] cimetidine is thought to be secreted by the organic cation transport system in man.

More definitive studies of the renal tubular transport of cimetidine have been conducted in animals. These studies are discussed in detail below.

1. In Vivo Studies In The Rat.

Weiner et al.[87] studied the renal clearance of intravenously administered cimetidine in anesthetized rats. Renal clearances were determined under steady state conditions following administration of loading doses and sustaining infusions of cimetidine. The ratio of cimetidine renal clearance to GFR (determined by inulin clearance) decreased from 2.6 to 1.3 with plasma concentrations increasing from 2 to 200 ug/ml, indicating that the renal secretion of cimetidine was saturable. The renal clearance of cimetidine was decreased by 30% with concurrent infusion of bicarbonate, suggesting that cimetidine may be reabsorbed by nonionic diffusion in the kidney. There was no evidence for urine flow dependent renal clearance.

The effect of cimetidine on the renal clearances of radiolabeled forms of the organic anion para-aminohippurate (PAH) and the quaternary cation tetraethylammonium (TEA)

were determined at steady state. Cimetidine did not affect the renal clearance ratio of PAH, but completely eliminated net secretion of TEA (renal clearance ratio TEA/GFR < 1). These experiments suggest that the renal transport of organic cations but not organic anions is inhibitable by cimetidine, and imply that cimetidine is transported by the renal organic cation system and is not transported by the PAH transport system.

This study was important because saturability of cimetidine renal secretion was demonstrated, providing evidence for carrier mediated renal transport. However, it should be noted that the clearance ratios for renal elimination were determined at cimetidine plasma concentrations of approximately 2, 50, 90, and 200 ug/ml. Because therapeutic concnetrations of cimetidine are at or below 2 ug/ml, it would be of interest to more precisely define the saturable renal elimination process.

2. In Vitro Studies With Perfused Proximal Tubules From Rabbit Kidney.

McKinney et al.[88,89] studied the renal transport of cimetidine in isolated perfused superficial proximal tubules from rabbit kidney. The individual tubules were perfused while in a bath containing radiolabeled cimetidine. Fluid from the perfused tubule was collected and the concentration of labeled cimetidine in the tubular fluid determined. Cimetidine concentrations were found to be 15 to 26 times

higher in the tubular fluid than in the bath, indicating that active secretion had occurred. Temperature and concentration dependent cimetidine secretion was also demonstrated. The rate of cimetidine transport from lumen to bath, determined by adding labeled cimetidine to the perfusate, was 10-20% of the bath to lumen transport rate and this transport was only slightly temperature dependent and was not inhibitable by ouabain. These findings suggest that the reabsorption was passive in nature.

The organic bases quinine, quinidine, tolazoline, procainamide, N-acetylprocainamide, and cimetidine sulfoxide, at bath concentrations ranging from 10^{-5} to 10^{-3} M, produced concentration dependent inhibition of cimetidine transport from bath to lumen. Similarly, the organic anions, probenecid and PAH, produced concentration dependent inhibition of the bath to lumen transport of cimetidine. Creatinine also was found to inhibit cimetidine transport, although to a lesser extent than the other compounds tested. Ouabain, at 10^{-5} M, when added to the highest concentration of each inhibitor tested, was found to produce further inhibition of cimetidine transport for all inhibitors except quinine. Quinine had, by itself, virtually eliminated cimetidine transport.

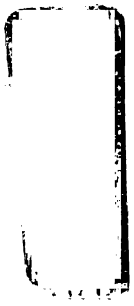
These studies are important for understanding the mechanisms of cimetidine renal transport because they were the first studies in isolated renal tissue. The observed effect of quinine on cimetidine transport is particularly

relevant because quinine is known to be a potent and specific inhibitor of renal organic cation transport.[90] The results with the other basic compounds confirm that cimetidine transport is inhibited by organic cations and suggest that cimetidine is transported by the organic cation system.

The effects of probenecid and PAH on cimetidine secretion are more difficult to explain because the organic anion and organic cation transport systems are thought to be separate and distinct.[90] The authors speculated that cimetidine could be transported by either the organic cation or the organic anion transport systems, but was preferentially transported by the cation system. However, this conclusion was based on data obtained from transport across intact cells involving two functionally distinct membranes (discussion in Chapter II), and is premature because no effort was made to investigate the mechanism of the interaction and thus substantiate the conclusion.

3. In Vitro Studies With Canine Renal Cortical Slices.

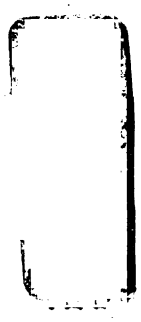
Cacini et al.[91] conducted experiments in which the uptake of radiolabeled cimetidine (at 10^{-6} M) was studied in thin cortical slices prepared from canine kidney. Cimetidine accumulated in the slices with time, achieving a slice to medium concentration ratio of three at equilibrium, thus demonstrating concentrative uptake. No evidence was found for metabolism when tissue extracts were analyzed by



thin layer chromatography. To distinguish transport from membrane binding, the uptake studies were also performed in the presence of nitrogen and sodium cyanide, inhibitors of active transport processes. These inhibitors reduced the uptake of cimetidine by 60 and 70% of control respectively, indicating that the majority of uptake of cimetidine was dependent on metabolic energy.

The specific inhibitors of organic cation transport, quinine and cyanine 863,[90] at 10^{-3} and 10^{-4} M respectively, produced 60-70% inhibition of the equilibrium uptake of cimetidine. In similar experiments probenecid (10^{-5} M) inhibited equilibrium cimetidine uptake by 20%, but produced only minimal additional inhibition at higher concentrations (to 10^{-3} M). PAH (10^{-3} M) did not inhibit cimetidine uptake at either initial times or at equilibrium. Inhibition of the equilibrium uptake of radiolabeled PAH (10^{-6} M) by cimetidine (10^{-4} M) was shown, however, no inhibition was observed for cimetidine at 10^{-5} M. Inhibition of uptake of radiolabeled TEA (10^{-6} M) by probenecid was also shown, although concentrations higher than those which produced inhibition of cimetidine uptake were required (i.e. 5×10^{-4} M).

Conclusions reached from this study were: 1) that cimetidine uptake was saturable and energy dependent, 2) that the uptake of cimetidine was mediated by the organic cation transport system, 3) that cimetidine transport was sensitive to probenecid but not PAH and that the inhibition



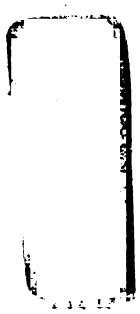
by probenecid was not due to a transport system shared with PAH, and 4) that the basolateral membrane was responsible for cimetidine transport.

This study confirms the previous findings for cimetidine transport relative to its carrier mediated nature and the effects of organic cations and anions. In theory, the study identifies a role for the basolateral membrane in cimetidine transport because in renal cortical slices, the lumen is thought to be occluded. The study was not rigorously conducted in terms of the concentration dependent effects of the inhibitors and the conclusions about the absence of inhibition by PAH are not supported by experimental evidence.

4. In Vivo Studies In The Sperber Chicken.

Rennick and coworkers[92] studied tubular secretion of cimetidine using the Sperber chicken model, in which all of the venous return from one leg is routed to the peritubular perfusion of one kidney before reaching the systemic circulation. By measuring urinary excretion of a substance infused into one leg and separately measuring urinary excretion in each kidney, the tubular excretory function of the infused kidney can be investigated.

Renal transport of radiolabeled cimetidine was found to be saturable and was shown to occur at a rate 88% of that of PAH, a compound which is completely secreted in one pass through the kidney. The organic cations, cimetidine,



ranitidine, thiamine, procainamide, guanidine, and choline, produced concentration dependent inhibition of cimetidine transport in order of decreasing potency. Surprisingly, the organic cation, quinine, was ineffective as an inhibitor of cimetidine transport. Cimetidine in turn produced concentration dependent inhibition of transport of the radiolabeled cations, thiamine, triamterene, and TEA. Cimetidine was found to be metabolized by the kidney to the sulfoxide and hydroxymethyl derivatives, and the amounts of these metabolites in urine were highest from the infused kidney.

These were well designed studies which clearly demonstrated the in vivo inhibition of cimetidine transport by endogenous cations (i.e. thiamine, guanidine, and choline) known to be transported by the renal organic cation transport system.[90] The lack of effect of quinine on cimetidine secretion is unexplained. The studies were also the first to demonstrate that cimetidine could be metabolized by the kidney. The hydroxymethyl and sulfoxide metabolites were produced at 2.5 and 9% of the rate at which cimetidine reached the kidney, rates which are in agreement with the percentages of these metabolites formed in humans after cimetidine administration.[45] The very high extraction efficiency of the kidney for eliminating cimetidine (88% of PAH excretion) confirms the high renal clearance observed in other species and directly demonstrates that cimetidine is extensively secreted by the renal tubules. It would have

been interesting if the investigators had tested the specificity of cimetidine transport by examining the effect of the anions, probenecid and PAH.

5. Studies In Basolateral and Luminal Membrane Vesicles From Rat Renal Cortex.

Takano et al.,[93] studied cimetidine transport in membrane vesicles prepared from rat kidney. Cimetidine (10^{-2} M) was shown to inhibit the uptake of labeled TEA (5 X 10^{-4} M) into both basolateral and luminal membrane vesicles. Transport of TEA was at a higher rate in the luminal membrane and inhibition by cimetidine was greater in the luminal membrane than in the basolateral membrane. Cimetidine (10^{-4} to 10^{-3} M) and TEA (10^{-4} to 10^{-3} M) were reported to produce concentration dependent inhibition of the initial proton gradient-stimulated uptake of labeled TEA (10^{-4} M). In the reverse experiment, TEA (6 X 10^{-4} M) inhibited the proton gradient-stimulated uptake of labeled cimetidine (2.5 X 10^{-5} M) in the luminal membrane.

These studies examined transport systems for organic cations in both the luminal and basolateral membranes. Previously, Holohan and Ross[94] identified transport systems for organic cations in luminal and basolateral membranes and demonstrated proton gradient-stimulated transport of the quaternary cation, N^1-methylnicotinamide. These investigators also advanced the theory that organic cation transport in the luminal membrane is a secondarily active

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system driven by a proton gradient oriented from lumen to cell and maintained by the Na^+/H^+ antiport system of the luminal membrane.[94]

Unfortunately, the studies by Takano et al.[93] are made less valid by lack of adequate controls, i.e. no effort was made to demonstrate saturability, or intravesicular uptake, and the studies purporting to demonstrate concentration dependent inhibition of proton gradient driven TEA transport in the luminal membrane only show that inhibition was maximal at the lowest concentration (10^{-4} M) of inhibitor (cimetidine) tested.

6. Conclusions.

The studies presented in this section as well as the work in humans have clearly demonstrated that cimetidine is secreted in the proximal tubule via a saturable transport system. There is evidence for mediated transport for cimetidine in luminal membranes as well as in antiluminal membranes. The inhibitor studies with organic cations have shown that the renal organic cation transport system is involved in cimetidine renal secretion. The effects of the organic anions, probenecid and PAH, are unusual and were not explained in these studies. Major questions remaining to be answered are: 1) What are the mechanisms of cimetidine transport in the luminal membrane, the theoretical site of active transport for organic cations;[94] 2) Are the effects of probenecid and PAH due specifically to direct effects on

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the organic cation transport system; 3) Can the inhibition of organic cation transport by organic anions be demonstrated in vivo in humans; and 4) What are the clinical implications of this interaction. These questions form the basis for the majority of this thesis project.

Chapter II

Renal Transport Studies Using Isolated Membrane Vesicles

A) Background.

An understanding of the important role of the renal proximal tubule in the conservation and elimination of electrolytes, endogenous substances, and exogenously administered compounds has brought about an awareness of the need to understand the cellular mechanisms by which these transport processes occur. Historically, non-invasive renal clearance and isolated perfused kidney techniques have provided an overall analysis of tubular transport functions.[95] With development of in vivo micropuncture and microperfusion methods, transport functions of individual nephrons accessible from the kidney surface could be studied, and with simultaneous perfusion of the tubular lumen and the peritubular capillaries, driving forces for transport could be analyzed.[95] In vitro microperfusion methods have allowed transport studies in nephron segments which are not accessible at the kidney surface.[95] Non-invasive techniques including ion and gas selective microprobes, optical spectroscopy, and nuclear magnetic resonance are now also being used to study renal transport.[96] With development of monoclonal cell lines, it is possible to study renal transport in homogeneous cell types isolated from various renal tissues.[97]

The renal proximal tubule is comprised of a conical monolayer of epithelial cells which forms the tubular lumen. The transporting membrane on the plasma surface of the tubular cell is the basolateral membrane and on the luminal surface is the brush border (luminal) membrane. The directional transport of solutes from plasma to lumen and vice versa requires that these membranes be functionally polar.[98] With development of methods to fractionate and separate these functionally distinct membranes into isolated membrane vesicles, it has become possible to study the mechanisms of solute transport in the membrane of interest via radiotracer techniques.

B) Advantages and Disadvantages of Using Membrane Vesicles For Transport Studies.[99]

1. **Advantages.**

a) Transport in the membrane of interest can be studied separately from the effects of other transporting membranes of the cell.

b) Because cytoplasmic components are eliminated in membrane isolation steps, substrate transport can be studied without interference from substrate metabolism. Also, the energy sources available to the intact cell are removed and conditions can be controlled by the the investigator in order to determine the driving forces for solute transport.

c) The intravesicular and extravesicular environments of membrane vesicles can be precisely controlled by the

investigator, thus the effect of changing a single variable can be determined for one membrane without influence from the other membranes and cellular organelles.

d) The kinetic properties of the transport system can be evaluated because the concentrations of substrate and cosubstrate on both sides of the membrane are known at time zero. These properties cannot be determined in intact cells because essentially all substrates are either metabolized, sequestered in the membrane or the cell, or transported by different transport systems in different membranes.[98]

2. **Disadvantages.**

a) Due to the traumatic and prolonged physical methods for preparing membrane vesicles and the in vitro fusion of the membrane to form the vesicle, alterations of permeability and transport properties are possible.

b) Inactivation of transport systems can occur in the membrane vesicle preparation. Therefore, the absence of a particular transport phenomenon in membrane vesicle studies does not absolutely exclude its in vivo presence. On the other hand, the presence of a transport phenomenon in vesicle studies usually is convincing evidence for its presence in the intact tissue of origin.

c) Membrane vesicle isolation and transport studies are generally carried out in artificial solutions which may quantitatively and qualitatively affect the functioning of transport systems as compared to the in vivo situation.

C) Methods of Membrane Isolation.

The basolateral and luminal membranes of the proximal tubule differ with respect to properties other than their functional polarity. These membranes are known to have different buoyant densities and negative surface charge densities, properties which allow for their separation by differential precipitation, and/or density gradient centrifugation.[98]

1. Luminal Membrane Isolation.

Because luminal membranes have a high density of negative surface charge relative to basolateral and other membranes, they can neutralize the positive charges of divalent cations without aggregating.[100] Cytoplasmic and basolateral membranes cannot compensate for the two positive charges and cross-linking occurs, allowing separation by centrifugation. The differential precipitation-centrifugation scheme for isolation of luminal membrane vesicles shown in Figure II-1 is a modification of the method of Booth and Kenny,[100] and was used to prepare luminal membrane vesicles from rabbit renal cortex for the cimetidine transport studies described in Chapters III and IV of this thesis. EGTA (Ethyleneglycol-bis-(p-aminoethyl ether)-NNN$'$N$'$-tetraacetic acid) was added to remove calcium, the presence of which causes the vesicles to be leaky due to calcium dependent hydrolytic enzymes released during homogenization.[101] Magnesium was added to provide divalent cation. The sealed luminal membrane vesicles shown in

Figure II-1. Schematic diagram of the differential precipitation/centrifugation method for isolation of luminal membrane vesicles from rabbit renal cortex.

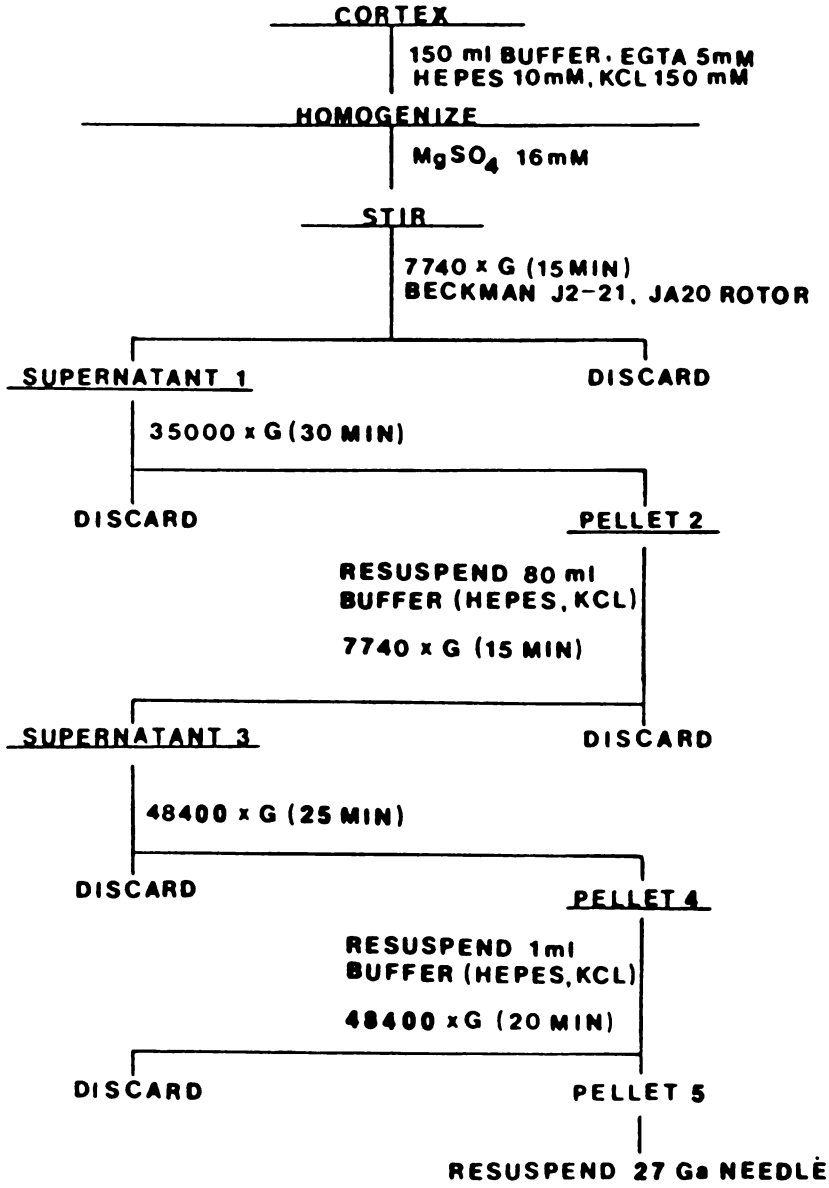


Figure II-2 are an electron microscope photograph (magnified 25,000X) of vesicles prepared by the divalent cation method. For a more detailed report of various methods used to prepare luminal membrane vesicles, the reader may consult the review by Murer and Gmaj.[98]

2. Basolateral Membrane Isolation.

The basolateral membrane does not have the rigid cytoskeletal structure that the luminal membrane does. Therefore, isolation techniques using a more gentle homogenization procedure are required.[98] In contrast to the luminal membrane, no selective method is available for isolation of basolateral membranes and the membrane vesicles will represent a mixture of plasma membranes from all the cells present in the original tissue.[98] However, enzyme methods are available by which to determine the tissue source of the majority of the basolateral membranes.[98] Methods utilizing centrifugation in self-orienting Percoll gradients are currently favored for isolation of the basolateral membrane.[98] Additional methods for isolation of basolateral membrane vesicles are presented in the review by Murer and Gmaj.[98]

D) Enzyme Markers For Membrane Identification.

Other differences between the luminal and basolateral membranes include the enzymes known to be more or less selectively associated with each membrane. By monitoring enzyme activities, the purity and/or cross-contamination of

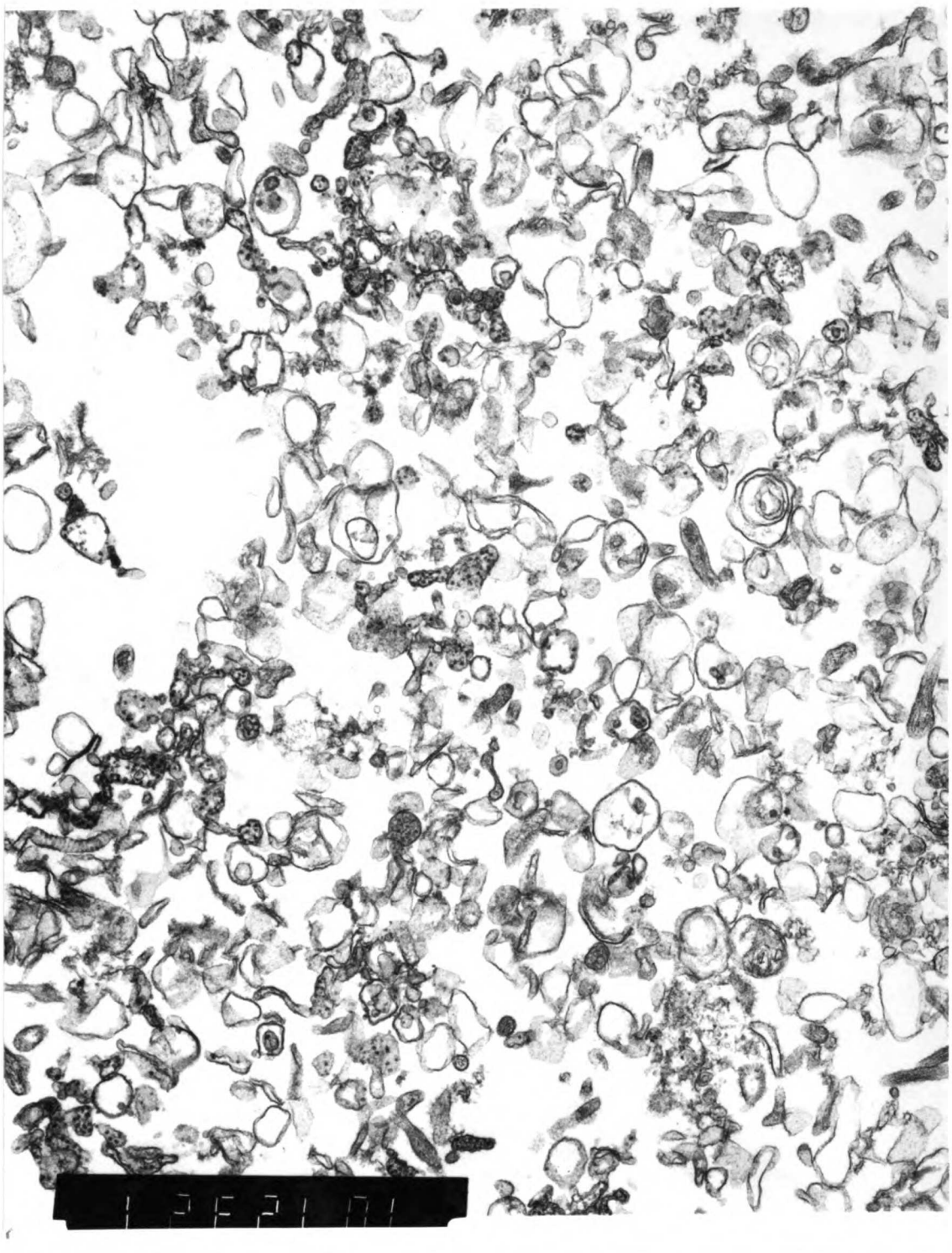


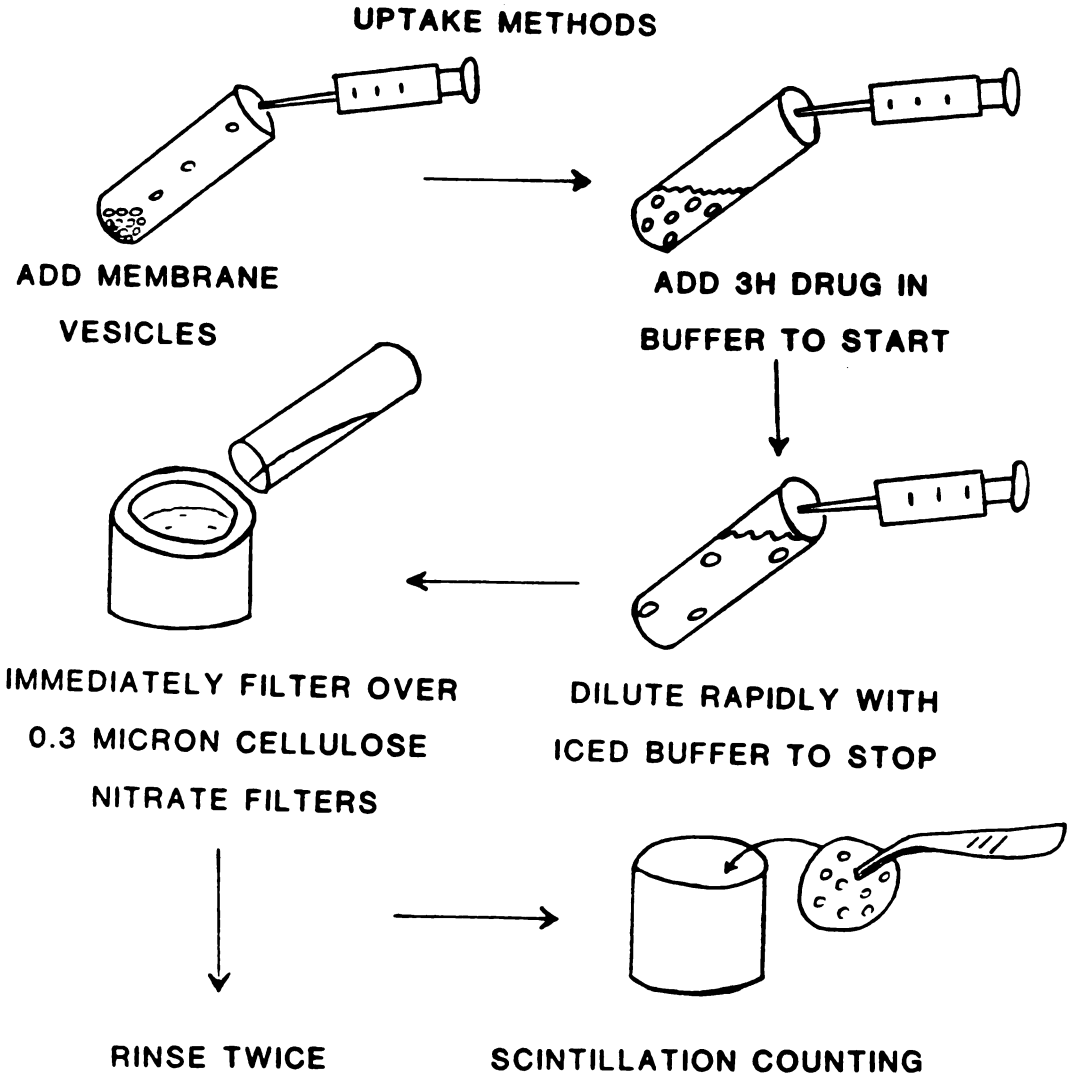
Figure II-2. Electron microscope photograph (magnified X 25,000) of sealed luminal membrane vesicles prepared from rabbit renal cortex by the divalent cation precipitation-differential centrifugation method.

the desired membrane can be determined. Enzymes used to identify the presence of the luminal membrane include: maltase, trehalase, gamma-glutamyltranspeptidase, aminopeptidase M, and alkaline phosphatase.[98] Enzymes associated with the basolateral membrane include: Na/K ATPase, Ca-ATPase, and hormone-stimulated adenylate cyclase.[98] Enrichment of activities of maltase and Na/K-ATPase from the original homogenate to the final fraction were used to monitor purity of separation of the luminal membranes for the studies described in Chapters III and IV. Detailed methods for the enzyme assays are presented in the appendices to this thesis.

E) Methods Used to Measure Solute Transport.

Uptake of radiolabeled tracer into membrane vesicles followed by rapid filtration is the most commonly used method to study solute transport. With practice and experience it is possible to measure uptakes with precision at times as low as 5 seconds. Uptakes can be accurately measured at 2 seconds if two investigators participate. With automatic mixing and diluting devices, uptakes at times of less than 1 second have been reported.[102] The uptake reaction is stopped without loss of intravesicular solute by rapid dilution with a large volume of iced stop mix, generally consisting of the same buffer in which the vesicles were suspended. The schematic diagram in Figure II-3 shows the general method of performing uptake

Figure II-3. Schematic diagram of the physical methods used in conducting radiotracer uptake studies with membrane vesicles.



measurements.

Intravesicular solute uptake can be distinguished from membrane binding by osmotically shrinking the vesicles with varying concentrations of an impermeable solute such as sucrose, then comparing uptakes at equilibrium.[1] In theory, at infinite extravesicular osmolarity the vesicles will shrink until no intravesicular volume exists, and any tracer remaining associated at equilibrium is due to membrane binding. Osmotically induced lysing of vesicles may also be used to determine intravesicular solute uptake. In the lysis method, the uptake reaction is stopped with iced deionized distilled water (DDW) in place of buffer. The DDW creates an osmotic gradient causing the vesicles to swell and burst, thus releasing intravesicular contents into the extravesicular medium where they are greatly diluted. Radioactivity remaining associated with the vesicles after lysis may be presumed to be bound to membranes. By comparing the uptakes stopped with buffer to those stopped with DDW, the degree of membrane binding can be determined.

Optical methods are also available for studying specific transport systems. Examples include the study of electrogenic transport systems using hydrophobic dyes, the study of proton transport with the fluorescent dye, acridine orange, and the study of osmotic flow of solutes using light scattering techniques.[98]

F) Criteria Used To Demonstrate the Existence of a Transport System.

A number of criteria are usually used to confirm the presence of a transport system in membrane vesicles. These criteria include:

1. Saturability.

Carrier mediated membrane transport systems exhibit saturability, as opposed to simple diffusion systems which are not saturable.[98] Thus, nonlinearity in saturation experiments supports the presence of a saturable transport system.

2. Specificity.

Concentration dependent inhibition of uptake of labeled substrate by an unlabeled form of a chemically similar compound on the same side of the membrane (cis-inhibition) provides evidence that the two compounds are transported simultaneously by the same transport system.[98] Lack of inhibition by chemically dissimilar compounds provides additional evidence that the membrane carrier is selective for substrates of a particular chemical class.

3. Countertransport.

A property of a carrier mediated transport system is that it exhibits the phenomenon of countertransport or trans-stimulation.[98] This can be demonstrated by preloading vesicles with a high concentration of unlabeled substrate and then measuring uptake of a tracer of the same or a chemically similar substrate. An overshoot of the

equilibrium value of uptake attained in the absence of unlabeled substrate demonstrates countertransport.

4. Temperature Dependence.

Carrier mediated transport systems exhibit a greater dependence on temperature than simple diffusion systems and their activities are nonlinear with respect to temperature.[98] An Arrhenius plot of the natural logarithm of uptake rate vs 1/T in theory should exhibit two slopes for a carrier mediated transport system, the slopes changing at the phase transition of membrane lipids.

Summary.

Despite the disadvantages listed for studies conducted with membrane vesicles, they have proven invaluable as a means of establishing the mechanisms of solute transport in renal as well as intestinal and other organ systems. The mechanisms for glucose and amino acid transport are now understood because of the use of isolated membrane vesicles. In this thesis, membrane vesicles were used to elucidate the characteristics of cimetidine transport in luminal membranes according to many of the criteria listed above.

Chapter III

Cimetidine Transport Studies In Luminal Membrane Vesicles Prepared From Rabbit Renal Cortex

A) Background

As described in Chapter I, prior to this work cimetidine renal transport had been studied in cortical slices from canine kidney,[91] in the Sperber chicken preparation,[92] in vivo in the rat,[87] in luminal membrane vesicles from rat cortex,[93] and in isolated perfused proximal tubules from the rabbit.[88,89] With the exception of the work conducted with rat cortical vesicles, which was of limited scope, these studies were carried out with preparations using intact tissue. As discussed in Chapter II, the transport of cimetidine observed in such preparations is a composite of the transport events occurring at both the basolateral and luminal membranes as well as the transport and/or metabolic events occurring at the intracellular organelles. Because the currently accepted theory for organic cation transport in the proximal tubule predicts that the active transport step occurs in the luminal membrane,[94] we chose to study the mechanisms involved in cimetidine transport across the luminal membrane of the renal proximal tubule.[1]

Since cimetidine is a basic compound (pKa 6.8) and exists in part as the monocation at physiologic pH, the organic cation transport system of the renal proximal tubule

might be expected to be involved in its secretion. Several studies have demonstrated that, as expected, cimetidine transport can be inhibited by organic bases and cations and is thought to occur by the organic cation transport system.[87-89,91-93]

However, investigators have also demonstrated that cimetidine transport in renal cortical slices prepared from dog kidney,[91] and in isolated perfused proximal tubules of rabbit kidney[88] can be inhibited by the organic anion, probenecid, and to a lesser extent by PAH. Phloridzin, a compound which inhibits organic anion but not organic cation transport, has also been observed to inhibit cimetidine secretion in the rabbit proximal tubule.[88] Since cimetidine does not exist in an anionic form in the physiologic pH range, the inhibition of secretion caused by the organic anions, and phloridzin is unexplained. The organic anion and cation transport systems of the renal proximal tubule appear to be distinct, and cross-inhibition between systems is not thought to occur.[90] Collectively, these findings suggest that a heterogeneous group of compounds including bases, quaternary cations, and some organic acids can affect the renal secretory transport of cimetidine.

B) Objectives.

The specific objectives of this study were to:

1. Characterize the transport of cimetidine in luminal membrane vesicles isolated from rabbit renal cortex.

2. Determine the effect of an initial, oppositely-directed proton gradient on cimetidine transport.

3. Determine the effects of the organic cations, N^1-methylnicotinamide (NMN), ranitidine, and cimetidine sulfoxide, as well as the effect of the organic anion, probenecid, on cimetidine luminal membrane transport.

C) Methods.

1. Isolation of Luminal Membrane Vesicles.

Luminal membrane vesicles were prepared by the method of Booth and Kenny,[100] as modified previously,[103-105] and adapted as noted here. A male New Zealand white rabbit, weighing 2 to 3 Kg, was sacrificed by concussion followed by decapitation and each kidney was flushed <u>in situ</u> with 40 ml of ice-cold homogenizing buffer consisting of 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 150 mM KCL, and 5 mM EGTA, adjusted to pH 7.4 with KOH. The buffer used for all other steps of the procedure was 10 mM HEPES with 150 mM KCL, pH 7.4, (called HK buffer). The membranes and buffers were kept ice cold during the entire procedure. The cortex was removed, coarsely minced, and homogenized for 4 minutes in 150 ml homogenizing buffer with a Sorvall Omni-Mixer Model 17105 set at high speed. Magnesium sulfate was added to the homogenate to achieve a final concentration of 16 mM, and the mixture was rapidly stirred for 20 minutes. The luminal membranes were separated according to the differential

precipitation/centrifugation scheme outlined in Figure II-1, using a Beckman refrigerated centrifuge Model J2-21 with a JA-20 rotor. Vesicles were prepared the day before use and stored at 4^0 C overnight. For transport studies, the membranes were diluted to a protein concentration of 20 mg/ml.

2. Protein Determination.

Protein concentrations were determined by a modification of the method of Lowry et al.,[106] which is presented in detail in the appendices.

3. Enzyme Determination.

The enzyme methods for maltase[107,108,109] and Na/K ATPase[110] used to determine the purity or contamination of the luminal membrane preparation are presented in detail in the appendices. Briefly, for maltase, an enzyme marker for the luminal membrane, the activity was assessed by a coupled enzyme assay system in which the rate of reduction of NADP to NADPH was monitored spectrophotometrically. Na/K ATPase, also determined by a coupled enzyme assay, was used to assess contamination of luminal membrane vesicles by basolateral membranes.

4) Transport Methods.

The general physical method of uptake studies was presented in Figure II-3 and is outlined briefly below.

a) For cimetidine uptake studies in the absence of an initial proton gradient, the reaction was initiated by adding 20 ul of reaction mix to 5 ul of luminal membrane

vesicles (protein concentration 20 mg/ml) with immediate vortexing. The reaction mix consisted of 50 uM cimetidine (as 0.225 uCi ^{3}H-cimetidine, specific activity 15-30 Ci/mmol, plus unlabeled cimetidine) in HK buffer at pH 7.4, and inhibitor if used. The uptakes were stopped by rapid dilution with 4 ml iced buffer and were immediately filtered by vacuum using a Hoefer filter manifold, Model FH 225V, with 0.3 um cellulose nitrate filters , type PHWP, Millipore/Waters Corporation. Two additional 4 ml rinses of iced buffer were filtered before removing the filters for scintillation counting. Blanks were prepared by carrying out the above procedures using the same reaction mix used in the methods discussed in this section. The radioactivity associated with the blank filters was averaged and subtracted from the radioactivity associated with the filters containing vesicles. The filters were placed in 10 ml Amersham ACS^{R} scintillation fluid and the radioactivity was determined with a Beckman Scintillation Counter, Model LS 7800. The counting efficiency ranged from 36 to 40%.

b) For cimetidine studies with an initial proton gradient, vesicles were suspended in 20 ml of 10 mM MES (2-[N-morpholino]ethanesulfonic acid), 150 mM KCL buffer, pH 6.0 (MK buffer). After 1 hr equilibration at 4^{0} C, the vesicles were centrifuged for 20 min at 20,000 RPM. The supernatant was removed and the vesicles were resuspended in MK buffer and adjusted to a protein concentration of 20 mg/ml. To start the uptake reaction,

20 ul of HK buffer, pH 7.4, containing labeled and unlabeled cimetidine as before, and inhibitors if used, were added to 5 ul of luminal membrane suspension. The uptake was stopped with iced HK buffer as before.

c) Cimetidine countertransport studies were conducted both in the presence and in the absence of an initial proton gradient. The studies were performed by incubating the vesicles with 2.4 X 10^{-4} M unlabeled cimetidine in HK buffer, pH 7.4, or in MK buffer, pH 6.0, for 1 hr at 4^{0} C. The uptakes were initiated by adding 30 ul of HK buffer containing 1.4 X 10^{-5} M unlabeled cimetidine and the same amount of labeled cimetidine as previously used. These studies were conducted both in the presence and in the absence of an initial proton gradient.

d) For glucose uptakes, 35 ul of vesicles (20 mg/ml protein) were added to 140 ul of reaction mix. The mix consisted of HK buffer with 1.44 X 10^{-4} M D-[^{3}H]-glucose (as 9.5 uCi ^{3}H-glucose plus unlabeled D-glucose), pH 7.4. Reaction mix for Na^{+}-stimulated glucose uptakes was identical except that 150 mM NaCl was used in place of 150 mM KCl. After combining vesicles with the reaction mix, samples of 25 ul were removed periodically and quenched as described for cimetidine uptakes.

4) Data Analysis.

For each experiment five replicate determinations were made to generate each data point. Unless otherwise specified, the results are presented as the means +/- SE of

three separate experiments. Statistical significance of differences for the proton gradient inhibition studies of NMN and probenecid (Figure III-6a,6b) was analyzed using the Student's unpaired t-test. In the countertransport studies, the data were paired and were analyzed by the Student's paired t-test. In the inhibitor studies where multiple comparisons were made, the data, representing absolute uptake values, were analyzed by analysis of variance to determine whether differences were present. A Newman-Kuels multiple range test was then used to determine significant differences between pairs of groups. Differences were considered significant at the $P < .05$ level.

5) Materials.

All chemicals, except those noted below, were obtained from Sigma Chemical Co. (St. Louis, MO). Ranitidine was provided by Glaxo Research (Ware, Hertfordshire, UK). Cimetidine sulfoxide was provided by Smith Kline and French (Philadelphia, PA). ^{3}H-cimetidine (15-30 Ci/mmol) was purchased from Amersham (Arlington Hts., IL). ^{3}H-D-glucose (15 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

D) Results.

1) Vesicle Preparation.

The enhancement of maltase activity (mean +/- SD) in the luminal membrane vesicles, determined in 15 preparations, was 8.9 +/- 2.7. Na/K ATPase activity was not

enhanced, indicating minimal contamination with basolateral membranes. In the presence of an inwardly-directed sodium gradient, a characteristic overshoot phenomenon for the uptake of D-glucose was evident, documenting the presence of functional and sealed luminal membrane vesicles.

2) Transport Studies.

The uptake of cimetidine into the luminal membrane vesicles is shown in Figure III-1. Uptake occurred at a rapid rate for approximately 1 min and then more slowly over 4 hr. Because of the rapid initial rate of uptake, all inhibition and kinetic studies were carried out at times of 6 sec or less. Samples for equilibrium determinations were taken at 4 hr.

The initial rate of uptake of cimetidine as a function of its concentration in a representative experiment is presented in Figure III-2. The uptake appeared to be comprised of a saturable component and a nonsaturable component. Assuming that the saturable component could be described by simple Michaelis-Menten kinetics, the data were fit by computer[111] to the equation:

$$\text{Rate of uptake} = \frac{(V_{max} \times C)}{(K_m + C)} + (k_n \times C)$$

where V_{max} and K_m are the Michaelis-Menten constants, k_n is a first-order rate constant for nonsaturable processes, and C is the concentration of cimetidine in the reaction mix. The computer generated curve of the saturable process is also shown in the figure. For four separate experiments,

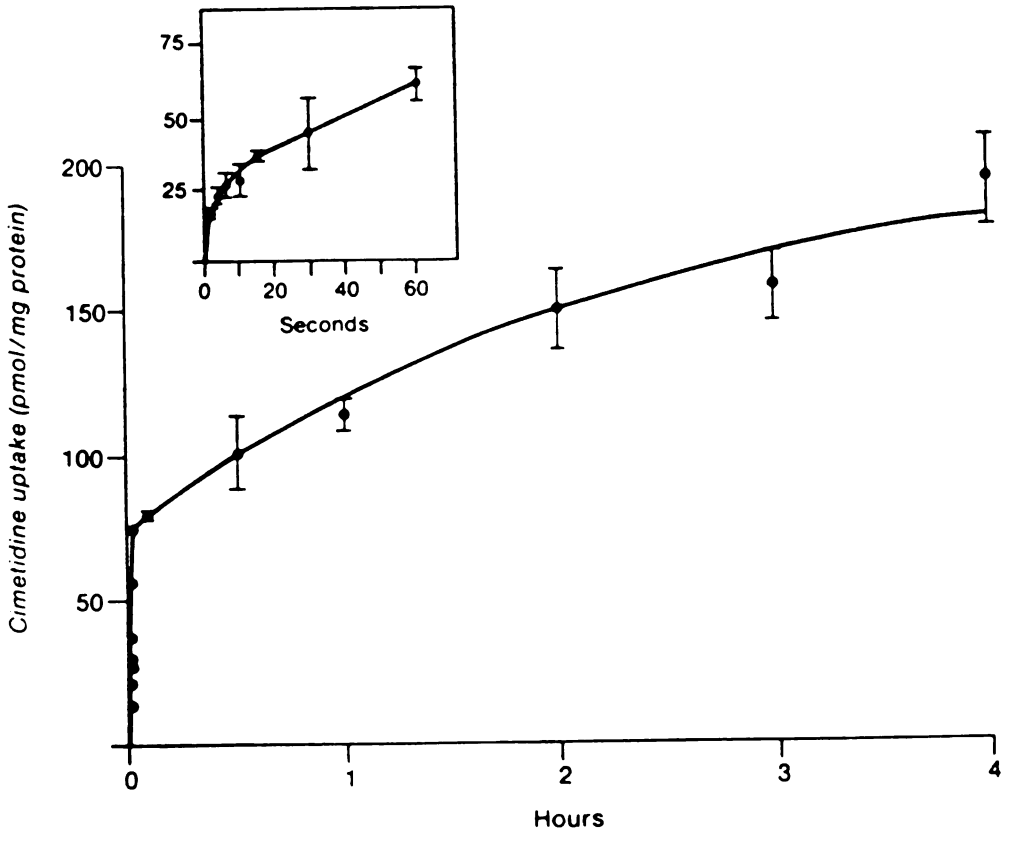


Figure III-1. Cimetidine uptake in luminal membrane vesicles as a function of time. Cimetidine concentration was 50 uM (as 0.225 uCi ^{3}H-cimetidine plus unlabeled cimetidine) in HK buffer, pH 7.4. Each point is the mean +/- SE of data obtained from 3 separate membrane preparations.

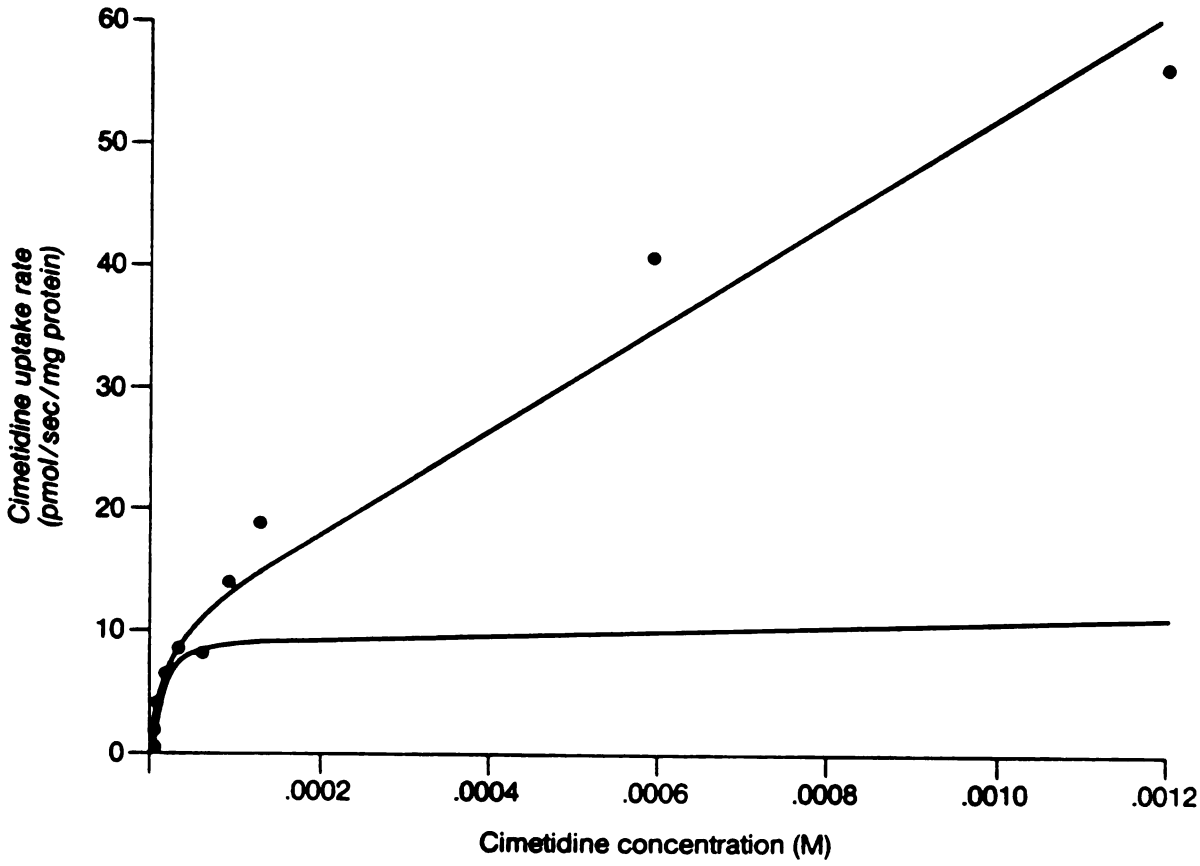


Figure III-2. Rate of uptake of cimetidine in luminal membrane vesicles as a function of cimetidine concentration. Data are from a representative experiment. Uptake was determined at 2 sec. Each data point is the mean of 5 determinations at each concentration. Curves were generated by computer as described in Results. The ascending curve is the computer fit to total uptake. The horizontal curve is the saturable component determined by subtracting nonsaturable uptake from total uptake.

the K_m (mean +/- SD) was 4.6 +/- 4.0 uM and the V_{max} (mean +/- SD) was 6.9 +/- 2.3 pmol/sec/mg protein. It should be noted that both the saturable and nonsaturable components may represent either binding or transport processes.

3) Osmolarity Studies.

To determine whether the uptake of cimetidine was due to intravesicular accumulation or binding to membranes, uptake of cimetidine was determined at 4 hr in the presence of varying concentrations of the impermeable solute, sucrose. Theoretically, at infinite sucrose osmolarity, intravesicular volume is negligible and cimetidine associated with the vesicles represents membrane bound compound. Thus the intercept of a linear plot of cimetidine uptake vs inverse sucrose osmolarity, divided by cimetidine uptake in the absence of sucrose, represents the fraction of cimetidine bound to the membranes. Cimetidine uptake was sensitive to media osmolarity as shown in Figure III-3, and was 25% membrane bound at equilibrium.

4) Inhibition Studies.

The inhibitory effects of 3 organic cations on cimetidine uptake are shown in Figure III-4. In order to differentiate inhibition of transport from inhibition of binding or effects on intravesicular volume, uptakes were carried out at initial times and at equilibrium. At initial times, statistically significant ($P < .05$) inhibition was shown for cimetidine sulfoxide at 10^{-2} and 10^{-3} M, while ranitidine and NMN inhibitions were significant at 10^{-2} M.

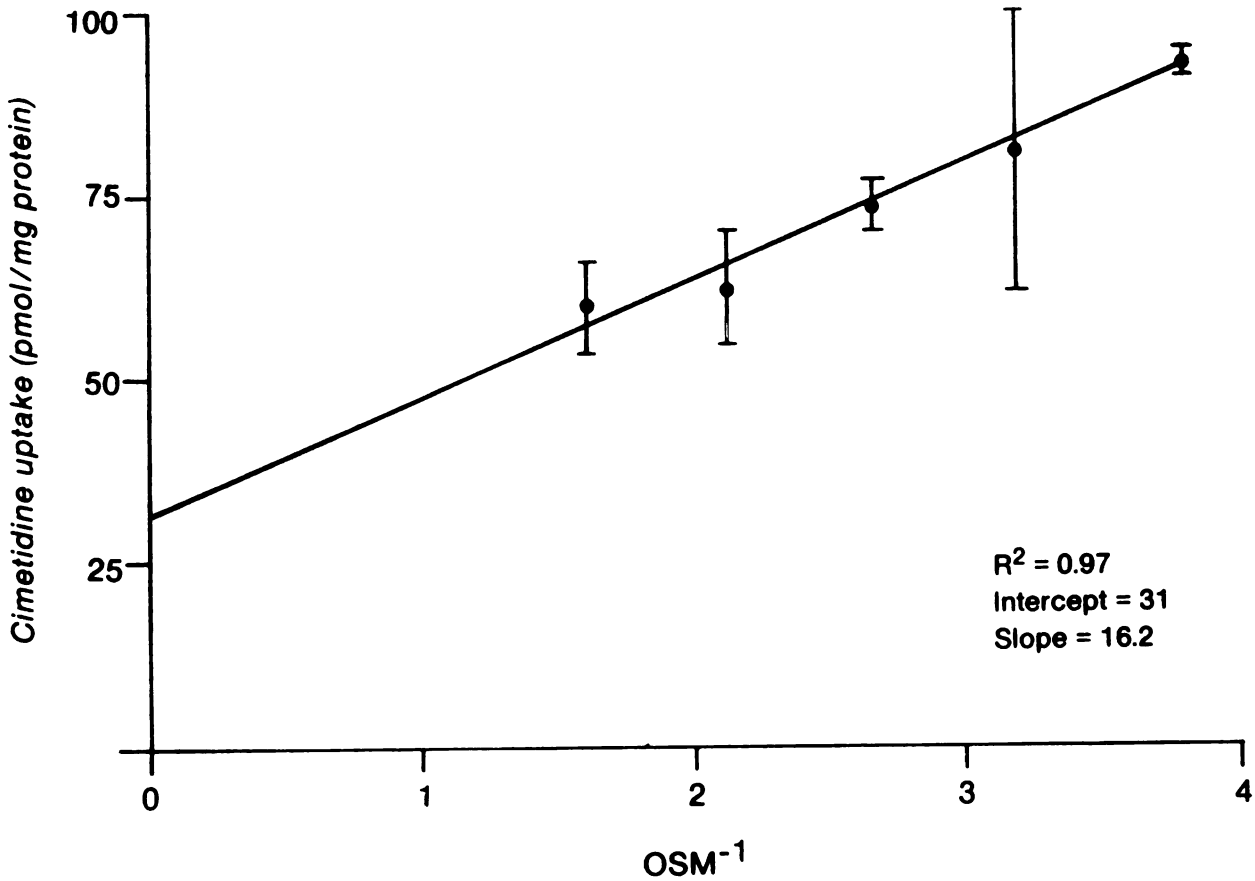


Figure III-3. Equilibrium uptake of cimetidine as a function of inverse osmolarity of sucrose in the extravesicular media. Uptake of 50 uM cimetidine was determined in luminal membrane vesicles in HK buffer containing sucrose at varying osmolarities. Each point is the mean +/- SE of data obtained from 3 separate membrane preparations. Membrane binding was determined to be 25%, as calculated by (intercept X 100)/(uptake in absence of sucrose).

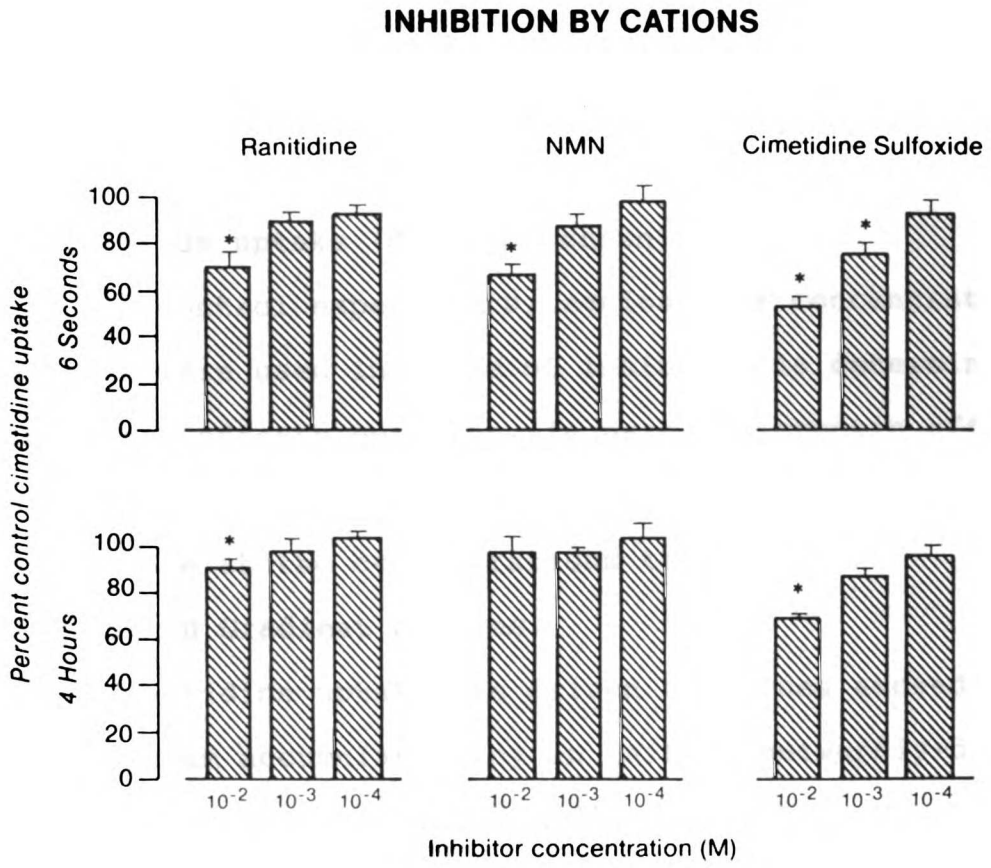


Figure III-4. Inhibition of cimetidine uptake in luminal membrane vesicles by organic cations. Uptake of 50 uM cimetidine was determined at 6 sec and at 4 hr. Effects on cimetidine uptake of inhibitors are expressed as a percent of uptake in the absence of inhibitor (mean +/- SE). Data represent at least 3 preparations of 5 determinations each. Significant differences are noted by asterisks.

None of the compounds produced significant inhibition at 10^{-4} M.

At equilibrium, cimetidine sulfoxide and ranitidine produced significant inhibitions at 10^{-2} M, although less than that observed at initial times in both cases. The inhibition of equilibrium uptake by these compounds suggests that the membrane bound cimetidine was displaced by compounds of close structural similarity. Cimetidine itself at 10^{-2} M produced 70% inhibition at initial times and, like its sulfoxide metabolite, produced 25% inhibition of equilibrium uptake (data not shown).

In control experiments, equiosmolar concentrations of sucrose were used in place of inhibitor to determine if inhibitor effects were due, in part, to osmotic effects. No decrease of cimetidine uptake was observed in the presence of sucrose at the concentrations tested.

5) pH Gradient Studies.

Cimetidine uptake was driven to values exceeding its equilibrium accumulation by an initial outwardly-directed proton gradient (or inwardly-directed hydroxide ion gradient) (Figure III-5). The proton gradient-stimulated uptake of cimetidine was inhibited significantly by 10^{-2} M NMN at times ranging from 2 through 60 seconds, but not at later times (Figure III-6a).

Similar results were obtained in 3 separate experiments with ranitidine and cimetidine sulfoxide, in which the initial uptake of cimetidine, 82.4 +/- 13.0 pmol/mg protein,

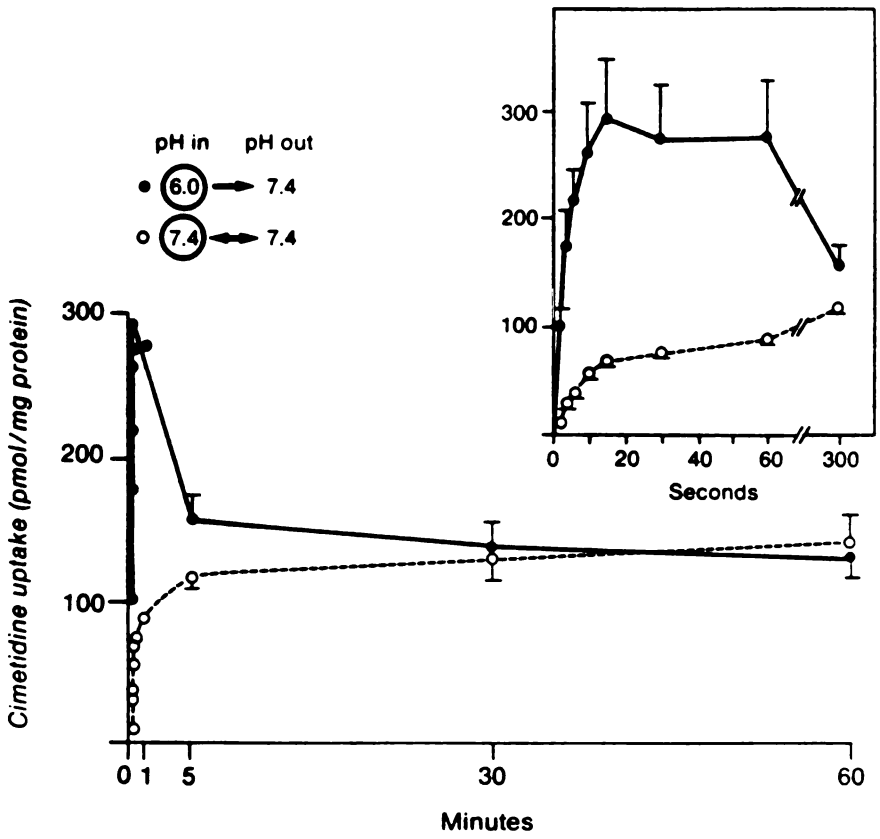


Figure III-5. Uptake of 50 uM cimetidine in luminal membrane vesicles in the presence (filled circles) and absence (open circles) of an initial outwardly-directed proton gradient. Each point represents the mean +/- SE of data obtained from 5 separate membrane preparations.

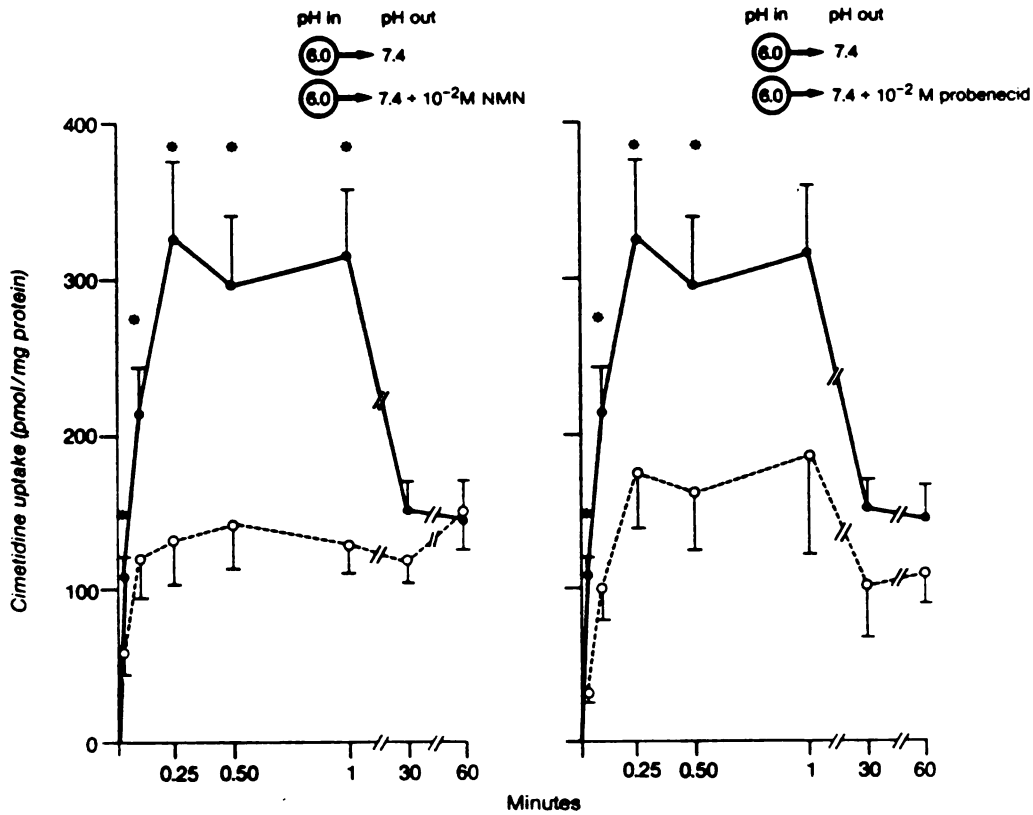


Figure III-6. (a). Proton gradient stimulated uptake of 50 uM cimetidine in the presence (open circles) and absence (filled circles) of 10^{-2} M NMN. (b). Proton gradient-stimulated uptake of 50 uM cimetidine in the presence (open circles) and absence (filled circles) of 10^{-2} M probenecid. Each point is the mean +/- SE of data from 5 separate membrane preparations. Significant differences noted by asterisks.

was reduced to 23.5 +/- 3.9 ($P < .05$) and 30.7 +/- 6.6 ($P < .05$) pmol/mg protein respectively (Figure III-7). The uptake of cimetidine at equilibrium was not significantly reduced by either of these compounds.

It was of particular interest to determine the effect of probenecid on proton gradient-stimulated cimetidine uptake. As shown in Figure III-6b, probenecid significantly inhibited the stimulated uptake of cimetidine from 2 through 30 seconds. Preliminary experiments have also demonstrated that the anion, furosemide, at 10^{-2} M, inhibited the proton gradient-stimulated uptake of cimetidine. The effects of varying probenecid concentrations over a 40 fold range (2.5×10^{-4} to 1×10^{-2} M) on proton gradient-stimulated cimetidine uptake are presented in Figure III-8. Concentration dependent inhibition of cimetidine uptake in the presence of probenecid is evident and statistically significant ($P < .05$) for the three highest concentrations of probenecid.

6) Countertransport Studies.

Experiments were conducted to determine if loading the vesicles with a higher concentration of unlabeled cimetidine could increase the initial rate of uptake of cimetidine. Proton gradient-stimulated uptake of cimetidine was enhanced by preloading the vesicles with a 5 fold higher intravesicular concentration of cimetidine. The uptake was significantly greater at 15 and 30 sec in the preloaded as compared to unloaded vesicles (Figure III-9). Similar

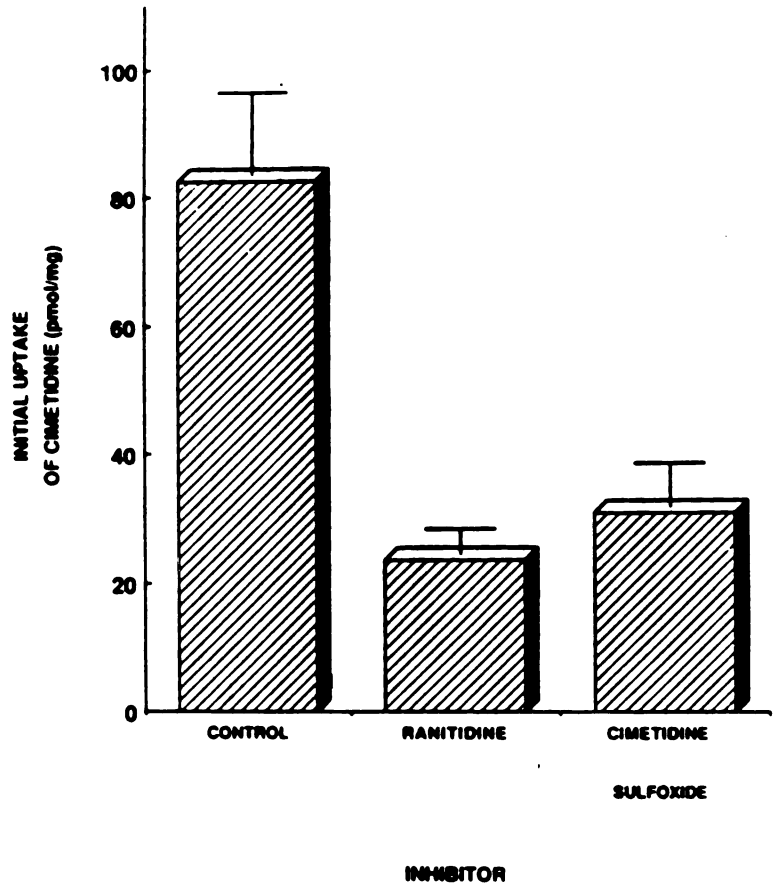


Figure III-7. Inhibition by ranitidine and cimetidine sulfoxide of proton gradient driven cimetidine uptake in luminal membrane vesicles. Data are the mean +/- SE from 3 separate preparations of 5 determinations each.

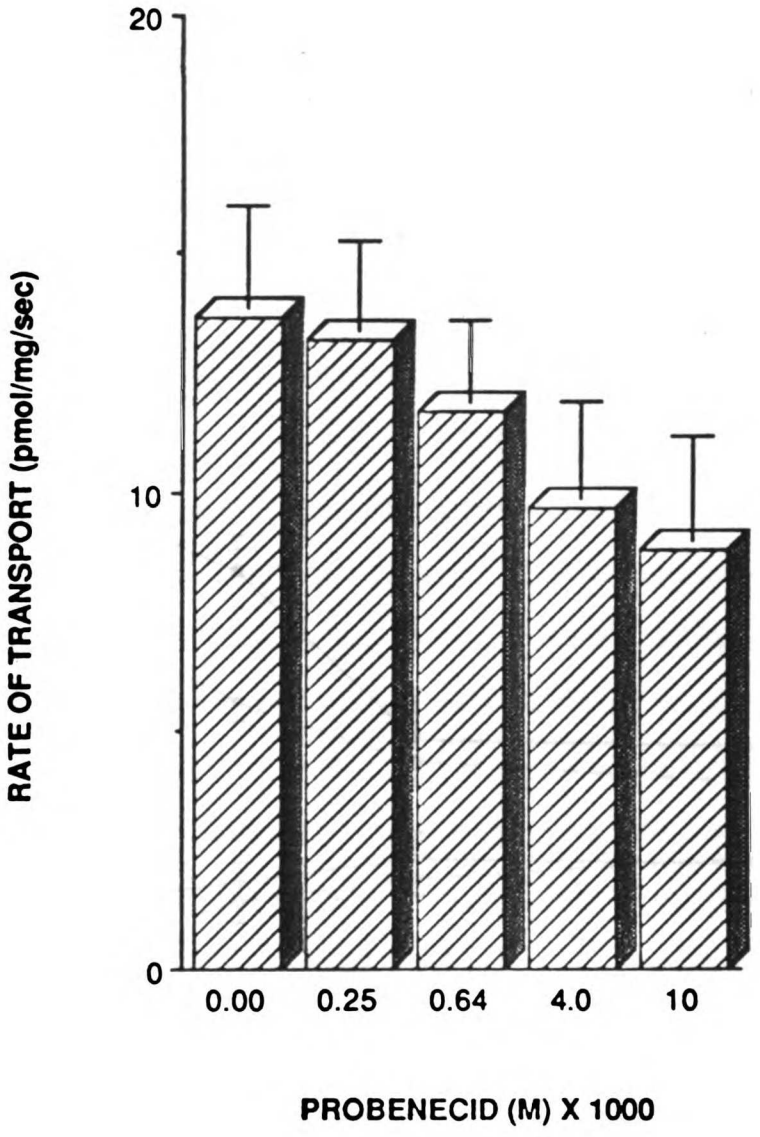


Figure III-8. Inhibition of the proton gradient-stimulated uptake of cimetidine by probenecid. Uptake of cimetidine in the absence of probenecid is shown in the first column, followed by uptake in the presence of increasing probenecid concentrations. Data represent the mean +/- SE of 3 separate membrane preparations.

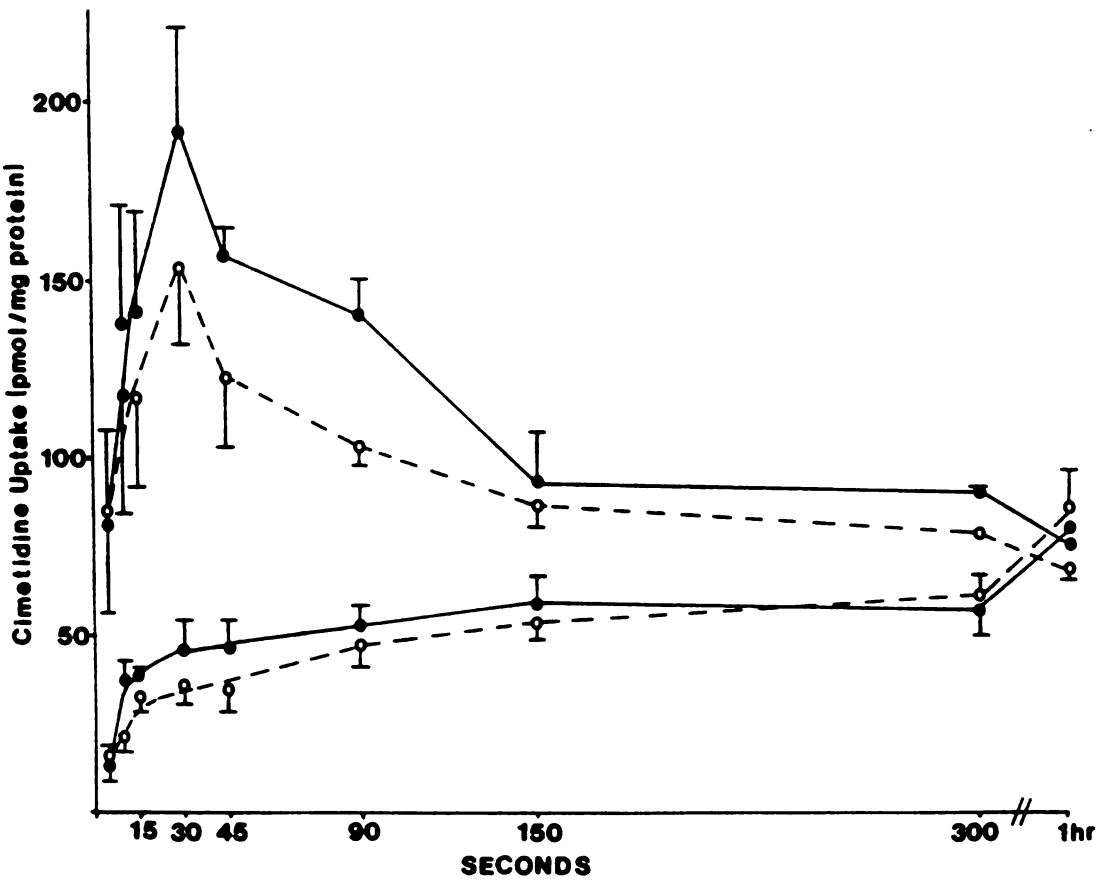


Figure III-9. Uptake of cimetidine in luminal membrane vesicles (open circles) and in luminal membrane vesicles preloaded with cimetidine (filled circles). Uptake in the presence of a proton gradient (top 2 curves) and in the absence of a proton gradient (bottom 2 curves). Each point is the mean +/- SE of data from 3 separate preparations. Significant differences were obtained at 15 and 30 sec in the presence of a proton gradient.

studies were conducted in the absence of an initial proton gradient. Although uptakes tended to be enhanced for preloaded vesicles in the absence of a proton gradient, the differences were not statistically significant (Figure III-9).

F) Discussion.

The renal transport of the organic base, cimetidine, has been studied in various whole animal and intact renal cell preparations from the rat, rabbit, dog, chicken, and man.[85-89,91-93] Several of these studies have shown that cimetidine transport in the kidney can be inhibited in a concentration dependent manner by compounds different in chemical nature from the organic bases or quaternary cations.[88,91] These non-cationic inhibitors have included diverse chemical groups such as the organic anions PAH and probenecid, the neutral compound creatinine, and phloridzin, an inhibitor of D-glucose and PAH renal transport.

In whole cell preparations, it is difficult to determine whether inhibition of transport occurs as a result of the effect of an inhibitor on the transporter per se, or as a result of indirect effects on cellular function. Furthermore, the effects of specific driving forces for transport such as ion gradients are not easily interpreted in such preparations. Accordingly, we have studied the mechanisms of cimetidine transport in isolated luminal membrane vesicles. Studies of time dependent cimetidine

uptake, saturation experiments, membrane binding experiments, and studies of inhibitors were conducted in the absence of an initial proton gradient to determine whether cimetidine was transported intravesicularly and to elucidate the characteristics of this transport in the absence of a secondary driving force.

The uptake of cimetidine as a function of time was also studied in vesicles in which an initial outwardly-directed proton gradient was present. Because the transport of the organic cations NMN and TEA is known to be driven by an initial outwardly-directed proton gradient in renal luminal membrane vesicles,[93,94,112] we performed these studies with cimetidine. We also carried out inhibitor studies and studies of countertransport in vesicles in which an outwardly-directed proton gradient was present. Presumably, in the presence of the gradient, a greater proportion of cimetidine is transported via the exchange pathway. In the absence of this driving force, passive or nonionic diffusion might play a greater role in the intravesicular accumulation of cimetidine.

There have been two previous studies of cimetidine transport in isolated luminal membrane vesicles. The first, by Takano et al.,[93] was carried out in luminal and antiluminal membrane vesicles from rat kidney. These investigators demonstrated an overshoot phenomenon for cimetidine uptake in the presence of an outwardly-directed proton gradient in luminal membrane vesicles. Cimetidine

was reported to inhibit the luminal membrane transport of TEA, but not to affect D-glucose or PAH transport, suggesting that cimetidine and TEA share a common transport system in the luminal membrane. Neither countertransport nor the degree of membrane binding of cimetidine was reported.

In the second study, published in abstract form,[113] cimetidine transport was examined in luminal membrane vesicles prepared from rabbit kidney. An outwardly-directed proton gradient was reported to drive cimetidine uptake above its equilibrium accumulation, and the bases, cimetidine, procainamide, and quinidine were found to inhibit the proton gradient-stimulated uptake. Cimetidine uptake was reported to be enhanced by preloading the vesicles with procainamide. Saturability studies defining Michaelis-Menten parameters were not performed in either of the previous investigations. Furthermore, the effect of the organic anion probenecid on cimetidine transport was not investigated in the previous studies.

In the present study we observed that cimetidine accumulated in luminal membrane vesicles as a function of time (Figure III-1). The slow accumulation of cimetidine from 30 min to 4 hr, as seen in Figure III-1, may imply a slow passive diffusion or binding process. Of the total cimetidine uptake, 75% could be accounted for by intravesicular accumulation, and 25% was due to membrane binding (Figure III-3).

The rate of uptake of cimetidine as a function of concentration could be described by an equation with both saturable and nonsaturable components (Figure III-2). This suggests that cimetidine may be transported into the vesicles by both saturable transport and by passive diffusion. With a pKa of 6.8, cimetidine would exist primarily (80%) as the uncharged species at pH 7.4, and thus nonionic diffusion down its concentration gradient into the vesicles could account for a portion of the initial uptake. This interpretation is consistent with the data in Figure III-2 and may also explain the lack of complete inhibition of the initial uptake by high concentrations of inhibitors (Figure III-4).

The finding of an enhanced uptake of cimetidine into vesicles preloaded with a higher concentration of cimetidine strongly suggests the presence of a facilitated transport system (Figure III-9). The enhanced uptake of [^{3}H]-cimetidine may have been due to a direct exchange of unlabeled cimetidine for [^{3}H]-cimetidine, or the unlabeled cimetidine may have exchanged with a proton, thus creating a proton gradient that in turn drove [^{3}H]-cimetidine uptake. The latter mechanism was proposed previously by McKinney et al.[114] when studying transport of the organic base procainamide in isolated luminal membrane vesicles. Regardless of the mechanism, the enhanced uptake observed in this study suggests that a facilitated transport mechanism is present.

In this study we found that the luminal membrane transport of cimetidine in the absence of an initial proton gradient could be inhibited by the organic bases ranitidine and cimetidine sulfoxide, and the quaternary cation NMN (Figure III-4). The observation that none of the inhibitors, even at 10^{-2} M, could inhibit more than 45% of the initial cimetidine uptake is consistent with a passive diffusion component as previously discussed. Our experiments, in which cimetidine itself, at 10^{-2} M, could not inhibit more that 70% of its initial uptake support this possibility, i.e. that 30% of the initial uptake may not be inhibitable. All of the inhibitors studied exhibited a higher degree of inhibition when cimetidine transport was driven by an outwardly-directed proton gradient, suggesting that under these conditions, a larger fraction of cimetidine may be transported by a saturable or inhibitable process. The fact that unlabeled cimetidine at 10^{-2} M could inhibit 70% of its initial uptake, whereas NMN and ranitidine could maximally inhibit 35% suggests that a component of cimetidine uptake (about 35%) may not be inhibitable by cations other than cimetidine itself. Support for this argument is provided from the inhibition data in Figure III-4, in which cimetidine sulfoxide, the structurally similar major metabolite of cimetidine, was capable of inhibiting 45% of the initial cimetidine uptake. Alternatively, the organic cations and bases may simply have a lower affinity for the transporter than cimetidine. The K_m for NMN

transport in isolated luminal membrane vesicles prepared from rabbit kidney[112] is 100-fold greater than the K_m that we observed for cimetidine in this study.

Organic cation transport from the proximal tubule cell across the luminal membrane and into the luminal fluid has been described by Holohan and Ross[94] as an active process requiring a driving force. It is postulated that the proton gradient from luminal fluid to tubule cell provides the driving force, with an organic cation exchanging for a proton.[94] In the presence of an initial outwardly-directed proton gradient, accumulation of the quaternary cations NMN[94,112] and TEA,[93] and the bases procainamide[114] and cimetidine,[93,113] have been reported to temporarily exceed equilibrium accumulation in luminal membrane vesicles. The proton gradient driven overshoot phenomenon was also observed for cimetidine in this study (Figure III-5). The overshoot presumably was caused by protons inside the vesicles exchanging for external cimetidine, as mediated by the proton/organic cation transport mechanism. An alternative explanation for the overshoot could be that increased ionization of a base would occur at the lower intravesicular pH, resulting in trapping of the ionized species and producing a temporary overshoot. Because cimetidine is a base, either mechanism may have produced the observed overshoot phenomenon. However, the proton/organic cation exchange mechanism is increasingly well documented for quaternary cations[94,112,114] which can not be subject to

pH gradient trapping.

In this study, the proton gradient-stimulated uptake of cimetidine was inhibited by the quaternary cation NMN, by the bases cimetidine sulfoxide and ranitidine, and by the anion probenecid (Figures III-6a,6b,7,8). Proton gradient-stimulated uptake of the cations NMN, TEA and procainamide have been reported to be inhibited by various other cations and bases in agreement with the proton/organic cation exchange mechanism.[93,94,112-114]

As discussed before, several investigators have observed inhibition of cimetidine transport by probenecid in whole tissue preparations.[88,91] Because probenecid is an anion, it might not be expected to inhibit the transport of the base cimetidine via competition for the same carrier. McKinney et al.[88] demonstrated that the observed inhibition of cimetidine transport in isolated perfused tubules was not caused by nonspecific toxic effects of probenecid. In the present study, probenecid did not significantly affect the sodium gradient driven uptake of D-glucose (Figure III-10). These findings suggest that the inhibition of cimetidine uptake by probenecid was not caused by nonspecific effects on membranes or membrane transporters. Furthermore, because sodium gradient driven transport of D-glucose is sensitive to potential differences, the lack of effect by probenecid would indicate that probenecid does not generate potential differences and therefore could not affect cimetidine transport by this mechanism. Equilibrium uptake of

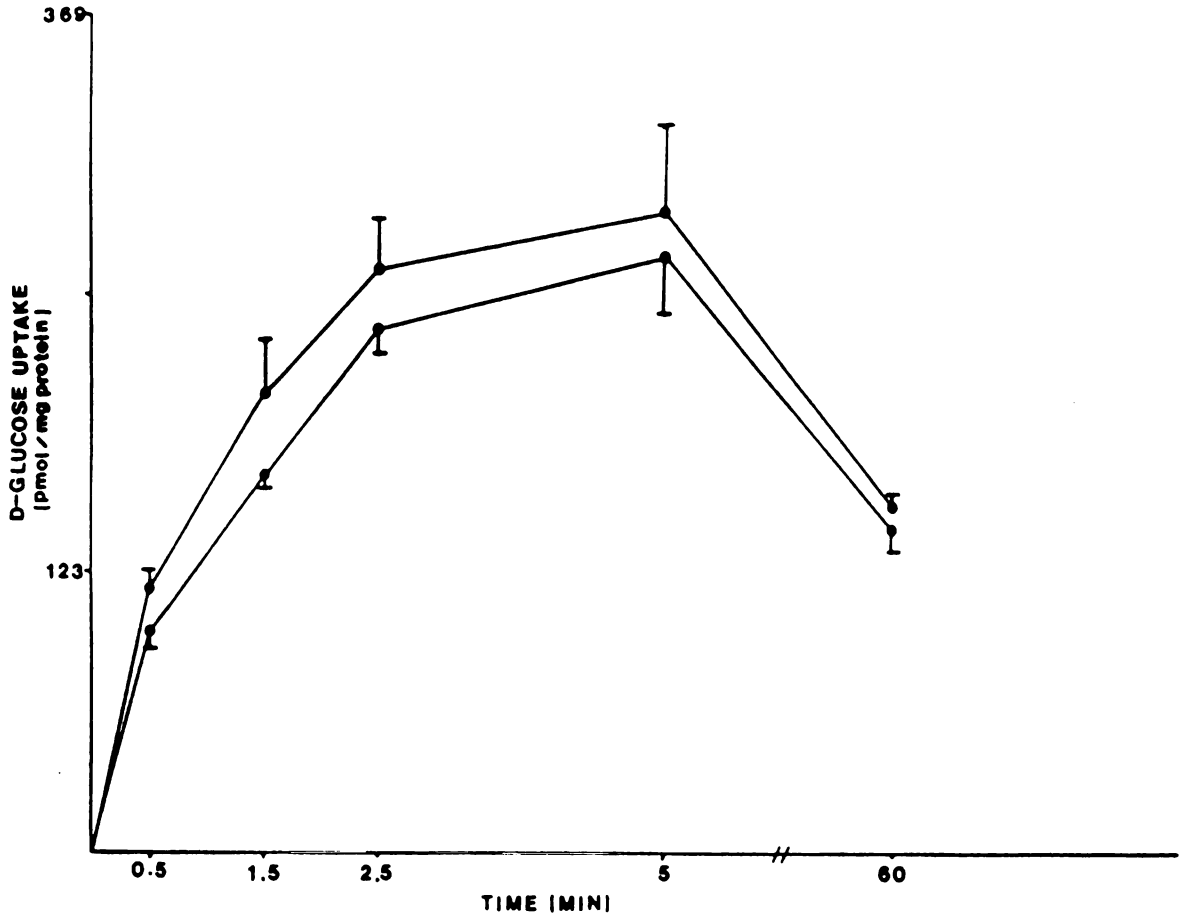


Figure III-10. Time course of the Na^+ gradient stimulated uptake of D-glucose in the absence (upper curve) and in the presence (lower curve) of 10^{-2} M probenecid. Probenecid slightly but not significantly decreased D-glucose uptake.

cimetidine was unchanged in the presence of probenecid, indicating that vesicle volume or membrane binding of cimetidine was not changed by probenecid. Thus it can be concluded that probenecid has a direct effect on the proton gradient-stimulated uptake of cimetidine at the luminal membrane.

Chapter IV

Inhibition of Cimetidine Transport By Creatinine In Luminal Membrane Vesicles Prepared From Rabbit Kidney

A) Background

Creatinine, an endogenous imidazole compound, is the end product of creatine metabolism in muscle tissue.[115] The rate of creatinine production is considered to be constant in most individuals and is closely related to body weight, age, and sex.[116,117] Creatinine, pKa of 4.83,[118] exists in the uncharged state at physiologic pH although it is often referred to as an organic cation.

Creatinine is eliminated from the body by the kidney, primarily by glomerular filtration.[117] It generally fits the requirements of a marker for GFR, which are: 1) that it is freely filtered at the glomerulus; 2) that it is biologically inert and not metabolized; 3) that it is not protein bound; and 4) that it is neither secreted nor reabsorbed by the tubules. Thus creatinine clearance, or its clearance as estimated from serum concentrations, is extensively used in clinical practice to estimate the GFR,[117] which is considered to be the best single parameter available for determining renal function.[119]

Creatinine deviates from being an ideal marker for GFR because it is secreted to a limited extent by the proximal tubules.[117] The extent to which GFR is overestimated because of creatinine secretion is the subject of two

thorough studies recently published.[120,121] Creatinine has also been reported to be reabsorbed by the renal tubules,[122,123] and to be eliminated by metabolism,[123,124] although the precise extent to which these pathways of elimination affect creatinine clearance is not clear.[117]

The renal secretory transport of creatinine has been reported to occur by both the organic anion and the organic cation transport systems of the proximal tubule in various species.[125,126] As discussed in Chapters I and III, cimetidine also contains an imidazole ring, and has been reported to be secreted by the organic cation transport system. In the rabbit, cimetidine transport is inhibited in a concentration dependent manner by creatinine.[88] In humans, cimetidine at therapeutic concentrations decreases creatinine clearance.[83] A striking example of the extent to which creatinine secretion is inhibited by cimetidine was given in the study by Shemesh et al.,[121] in which secretion of creatinine in humans with renal disease was virtually eliminated by one 300 mg intravenous dose of cimetidine.

These reports, as well as reports of inhibition of creatinine clearance by other cationic drugs,[127] suggest that creatinine may share the same transport system with organic cations in the proximal tubule. These interactions are important from a clinical perspective, and, because creatinine is not charged at physiologic pH, the interaction is interesting from a transport perspective. We studied the

nature of this interaction at the luminal membrane, a site of active transport for organic cations.

B) Objective.

The objective of this study was to determine whether creatinine directly inhibits cimetidine transport across the luminal membrane of the renal proximal tubule.

C) Methods.

Luminal membrane vesicles were prepared from rabbit renal cortex by a modification of the method of Booth and Kenny,[100] as reported in detail previously.(Chapter III) The transport of cimetidine in luminal membrane vesicles was studied under conditions in which cimetidine transport was driven by an initial outwardly-directed proton gradient.[1] Briefly, luminal membrane vesicles were equilibrated and resuspended in buffer containing 10 mM MES, 150 mM KCL, at pH 6.0, and adjusted to a protein concentration of 20 mg/ml. To initiate the transport reaction, 20 ul of reaction mix containing 10 mM HEPES, 150 mM KCL, 50 uM cimetidine as unlabeled cimetidine with 0.225 uCi ^{3}H-cimetidine (specific activity 15-30 Ci/mmol), pH 7.4, were added to 5 ul of membrane vesicles. Inhibition studies were conducted with varying concentrations of creatinine in the reaction mix. The remainder of the procedures for conducting transport studies are described in detail in Chapter III of this thesis.

D) Materials.

^{3}H-cimetidine (15-30 Ci/mmol) was obtained from Amersham (Arlington Hts., IL). All other chemicals were obtained from Sigma Chemical Co, (St. Louis, MO).

E) Results and Discussion.

The time course of cimetidine uptake in the absence of creatinine and in the presence of 0.05 M creatinine is shown in Figure IV-1. Each point represents the mean +/- SEM of data from 3 separate experiments. Uptake of cimetidine into the vesicles was decreased significantly in the presence of creatinine ($P < 0.05$, Student's paired t-test) at all times except 5 min and 60 min. These data demonstrate that the observed inhibition of cimetidine uptake produced by creatinine was not due to alterations of cimetidine membrane binding or vesicle volume and strongly suggest that inhibition of cimetidine transport was the mechanism involved.

Concentration dependent inhibition experiments were conducted by adding creatinine in varying amounts to the reaction mix. The initial rate of cimetidine uptake, assessed at 6 sec, was measured in the presence of creatinine ranging in concentration from 0.0125 to 0.5 M. The results of these studies, presented as a Dixon plot in Figure IV-2, demonstrate concentration dependent inhibition of the initial rate of cimetidine transport by creatinine.

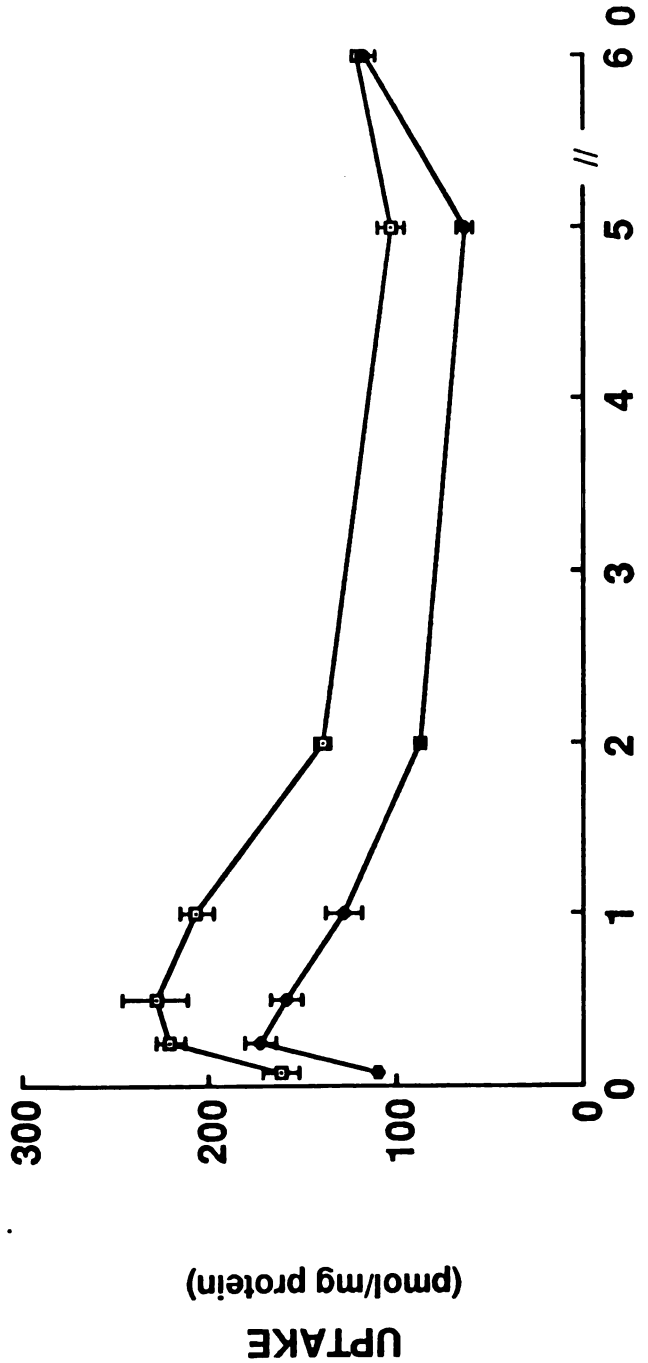


Figure IV-1. The time course of cimetidine uptake in the absence of creatinine (□), and in the presence (●) of 5 X 10^{-2} M creatinine. Each point is the mean +/- SE of data from 3 separate experiments.

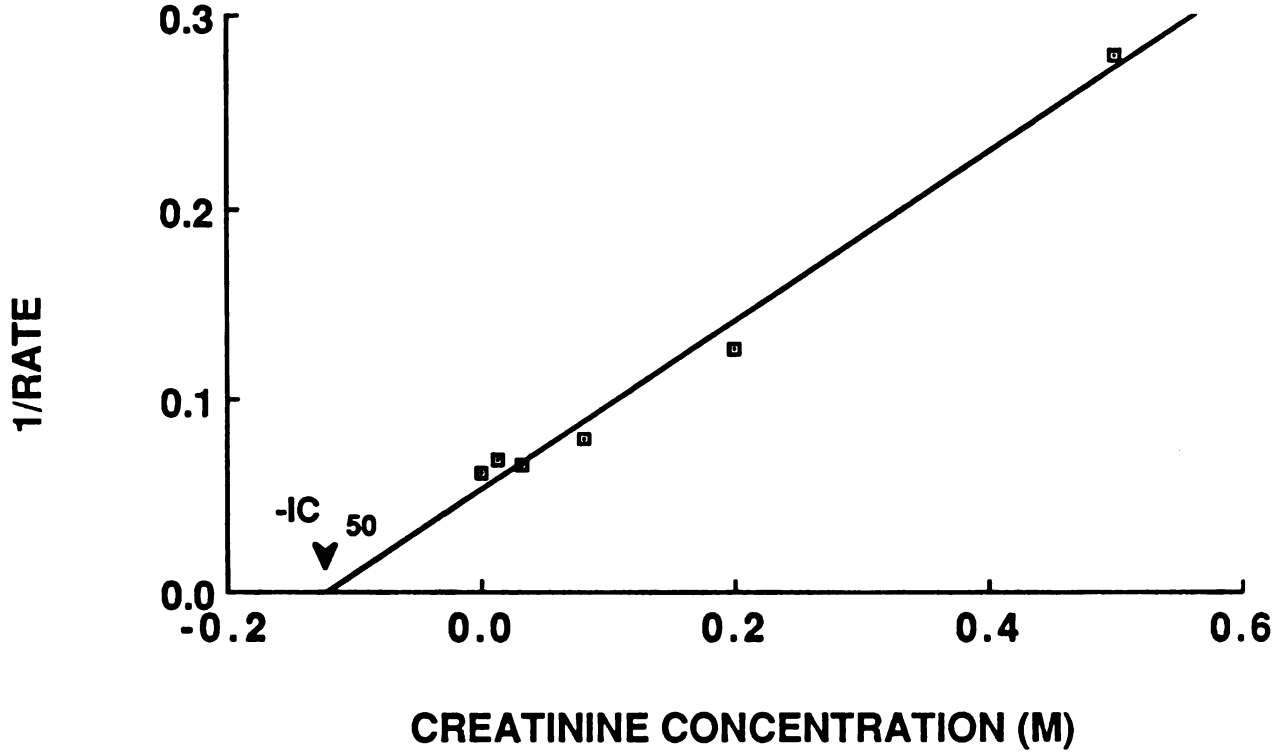


Figure IV-2. Dixon plot of the effect of varying concentrations of creatinine on the initial rate of cimetidine uptake. Each point represents the mean of data obtained from 3 separate experiments. The concentration of creatinine required to produce 50% inhibition (IC_{50}) of the initial rate of cimetidine uptake is shown as the X axis intercept. For these experiments, IC_{50} = 0.123 M.

Each data point represents the mean of data from 3 separate experiments. Extrapolation of the regression line to the X axis gives a value of 0.123 M, which represents the negative of the creatinine concentration required to produce 50% inhibition of the initial rate of cimetidine transport.

From these data, we conclude that creatinine inhibits the transport of cimetidine in luminal membrane vesicles and that the inhibition occurs in a concentration dependent manner. It is difficult to explain mechanistically why an uncharged molecule such as creatinine would inhibit the transport of cimetidine. Both molecules contain an imidazole ring, and it is possible that the interaction may involve displacement of the imidazole ring from a binding site on the transport carrier.

Creatinine was not a potent inhibitor of cimetidine transport (IC_{50} approximately 0.12 M) in comparison to N^1-methylnicotinamide, ranitidine, or cimetidine sulfoxide.(Chapter III). These organic cations, at concentrations of 0.01 M, inhibited at least 50% of the proton gradient-stimulated uptake of cimetidine in luminal membrane vesicles.(Chapter III). The high concentrations of creatinine (much higher than those present physiologically) that were required to inhibit the transport of cimetidine in this study suggest that cimetidine has a much higher affinity for the carrier than creatinine. This higher affinity may explain, in part, the effective inhibition of creatinine secretion produced by cimetidine in vivo.

Chapter V

Inhibition of Cimetidine Renal Elimination By Probenecid In Humans

A) Background.

Considerable progress has been made in understanding the physiologic and pharmacokinetic mechanisms involved in drug-drug interactions. For drugs that are transported in the renal tubules, important drug interactions may occur when one drug inhibits the renal secretion of another drug. These interactions, such as the interaction between probenecid and the penicillins, may be exploited for therapeutic purposes. More commonly, the interactions may result in adverse drug effects. It is generally assumed that the system involved in the transport of organic cations in the proximal tubule is distinct from the system(s) involved in the transport of organic anions and that organic anions do not inhibit the transport of organic cations and vice versa.[90] This assumption is based upon a number of studies, performed in vivo as well as in vitro, using renal cortical slices and isolated luminal and antiluminal membrane vesicles, demonstrating a lack of cross-inhibition between substrates of the organic cation and anion systems.[90] Accordingly, the drug-drug interactions in the kidney that have been observed clinically have been anion-anion or cation-cation interactions.

In apparent conflict with these observations, a few studies in the literature suggest that, in fact, there may be interactions between organic cations and organic anions in the proximal tubule. For example, McKinney and co-workers have demonstrated that the organic anion probenecid, a classical inhibitor of organic anion transport, inhibits the secretion of the organic cation cimetidine in isolated perfused tubules from rabbit kidney.[88] Probenecid has also been observed to inhibit the uptake of cimetidine in cortical slices prepared from canine kidney.[91] Recently, we have observed that this interaction occurs, at least in part, at the luminal membrane (Chapter III). Collectively, these studies suggest that organic anions may inhibit renal tubular transport of organic cations in vitro. Major questions that have not been addressed are whether organic anion-cation interactions occur in vivo, whether these interactions may be clinically relevant to rational drug use, and the nature of the mechanism which produces the interaction between these compounds.

B) Objectives.

The purpose of this study was to determine whether organic anions may inhibit the renal excretion of organic cations in humans. For this purpose, we used the organic anion, probenecid and the organic cation, cimetidine. These compounds were selected as model compounds primarily because

of the in vitro evidence suggesting that the two compounds may interact in the kidney.

C) Methods.

1. Experimental Procedures.

This study was approved by the Committee on Human Research, University of California, San Francisco. Six male subjects ranging in age from 22 to 29 years gave informed consent and participated in the study. They were of normal weight for height and age according to the weight tables of the Metropolitan Life Insurance Company, 1983.[128] The subjects had normal medical histories and had no evidence of disease as determined by physical examination, ECG, urinalysis, and blood chemistry (SMA-25). Serum creatinine, and the ratio of blood urea nitrogen to serum creatinine were within normal limits for all subjects.

The subjects were instructed not to take any drug, other than those used in the study, for a period from one week prior to the first study day and extending until the last study day was completed. None of the subjects used tobacco. No caffeine or alcohol containing beverages were allowed on study days. Food, including dextrose containing beverages, was withheld from midnight prior to study day until two hours after cimetidine dosing. Food was then allowed ad lib.

The study involved a two-period (A and B) randomized crossover design with seven days separating the first study

day from the second. On each study day, an intravenous catheter was placed in the cephalic vein of each forearm. One catheter was used for drug infusion and the other was used for blood sampling. In period A, an oral fluid load of 1000 ml water was given 2.5 hours before administration of cimetidine in order to maintain urine flow and to prevent crystallization of inulin in the renal tubules. Thirty minutes later, 500 ml of water was administered orally, followed by 500 ml/hr for the next three hours. After this, fluids were allowed ad lib. Two hours prior to cimetidine dosing, a bolus dose of 2 gm inulin in normal saline was given intravenously, and an intravenous infusion of inulin in normal saline (9.5 mg/ml) was started by infusion pump and continued at a rate of 9.5 mg/min for four hours. Cimetidine, 300 mg in 45 ml normal saline, was administered intravenously at a constant rate over 15 minutes via an infusion pump. In period B, subjects were dosed to steady state with probenecid by administering a 500 mg oral dose every six hours for 13 doses prior to cimetidine administration. The last dose of probenecid was given 3 hours before cimetidine administration. The rest of the protocol for period B was identical to period A.

Blood samples were collected in 10 ml heparinized Venoject^{R} vacuum tubes, Terumo Medical (Elkton, MD). Plasma was harvested by centrifugation and stored at -20^{o} C until analyzed. Blood samples were collected at 0 hour just prior to cimetidine dosing, and at 5, 10, 15, 30, and 45 minutes,

and at 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 7, and 9 hours after the start of the cimetidine infusion.

Urine was collected at the following intervals; 0-.5, .5-1, 1-1.5, 1.5-2, 2-4, 4-6, 6-8, and 8-10 hours after the start of the cimetidine infusion. After each collection, volume and pH were recorded and a 10 ml aliquot was frozen at -20^{o}C until analyzed.

2. Chemical Analysis, Cimetidine.

Plasma and urine were analyzed for cimetidine by a modification of the method of Guay et al.[129] Twenty ul of the internal standard, ranitidine, in methanol (0.05 ug/ul), 80 ul methanol, 100 ul 1 N NaOH, and 5 ml methylene chloride were added to 500 ul plasma in a 15 ml centrifuge tube. The tube was tightly sealed and rotated for 20 minutes on a rotary mixer. The mixture was centrifuged for 5 min at 2000 rpm and the upper layer was removed by vacuum suction. A 4.5 ml aliquot of the organic phase was transferred to a new test tube and evaporated to dryness under nitrogen. Samples were reconstituted in 100 ul mobile phase just before injection onto the chromatograph.

A similar procedure was used for urine except that urine sample volumes were 20 ul, which were diluted with 480 ul deionized distilled water. Twenty ul ranitidine in methanol (0.075 ug/ul) was added as the internal standard. The remainder of the extraction procedure was identical to that for plasma.

Samples were chromatographed using a Waters Microbondapak C_{18} reversed phase column, 3.9 mm X 30 cm, Millipore Waters Corp (Bedford, MA), a Waters variable wavelength UV detector Model# 481 set at 228 nm, and a Waters pump Model # M-45. The mobile phase was 93% 10 mM K_2HPO_4, pH 4.8, and 7%, by volume, acetonitrile. A flow rate of 2 ml/min was used. In this system, cimetidine eluted at 7.5 min and ranitidine eluted at 10.5 min in regions of the chromatograph free from interference from endogenous compounds. Cimetidine sulfoxide, the primary metabolite of cimetidine,[45] eluted near the solvent front. In addition, this metabolite did not extract well in methylene chloride and therefore did not interfere with the assay of cimetidine. No interference from probenecid or from inulin was detected. Standard curves were prepared daily from blood bank plasma and from blank (0 hour) urine from each subject. Standard curves prepared from plasma were linear over the range of cimetidine concentrations from 0.1 to 20 ug/ml and were linear for urine over the range of concentrations from 20 to 200 ug/ml.

The limit of assay sensitivity, defined as a peak height five times baseline noise, was approximately 100 ng/ml from 500 ul plasma. This assay method produced inter- and intra-day coefficients of variation of less than 10%.

3. Chemical Analysis, Inulin.

Inulin was assayed in urine and plasma by the

spectrophotometric method of Heyrovsky.[130] The method is presented in detail in the appendices.

D) Materials.

Ranitidine HCl was obtained from Glaxo LTD (Ware, Hertfordshire, England), cimetidine for analytical use was purchased from Sigma Chemical Co (St Louis, MO), probenecid tablets (lot # J2702) were manufactured by Merck Sharp and Dohme (West Point, PA), and cimetidine for injection (lot # 245T17) was manufactured by Smith Kline and French Laboratories (Carolina, PR). Sodium Chloride Injection 0.9% USP (lot # 7C016N7) was manufactured by Travenol Labs Inc. (Deerfield, IL), and Inulin and Sodium Chloride Injection USP (lot # 30012) was purchased from American Critical Care (McGaw Park, IL). Methylene chloride, acetonitrile, and methanol were all of HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals and reagents were obtained from Sigma Chemical Co.

E) Pharmacokinetic Analysis

The pharmacokinetic parameters for cimetidine were determined as follows: area under the plasma concentration vs time curve (AUC) by the linear trapezoidal rule with extrapolation to infinity; total systemic clearance (CL_s) as Dose/$AUC_{0-\infty}$; the amount of unchanged cimetidine excreted in the urine in a collection interval (Ae_{0-t}) as urine cimetidine conc X urine vol; renal clearance (CL_r), unless

noted otherwise, as the slope of a plot of the renal excretion rate vs midpoint plasma concentration forced through the origin; nonrenal clearance (CL_{nr}) as CL_s minus Ae/AUC; and net clearance by renal secretion (CL_{rs}) estimated as CL_r minus fu X glomerular filtration rate (GFR), where fu is the fraction unbound and is equal to 0.8,[45] and GFR as inulin clearance (urine inulin conc X urine vol)/(plasma inulin conc X interval time(min)) measured in the first urine collection interval after cimetidine administration. The plasma concentration vs time data of cimetidine were fit by computer[131] to a two compartment model with a zero order infusion of 15 minutes duration. The steady-state volume of distribution (Vd_{ss}) was calculated by a model independent method using the area under the first moment of the plasma concentration vs time curve, correcting for the 15 minute infusion time.[132,133]

F) Statistical Analysis.

Unless otherwise specified, results are presented as the mean +/- SD of the data obtained from six subjects. Statistical significance of differences was determined by the Student's paired t-test and was considered significant at the .05 level.

G) Results.

Semi-logarithmic plasma cimetidine concentration vs time plots following administration of cimetidine alone and

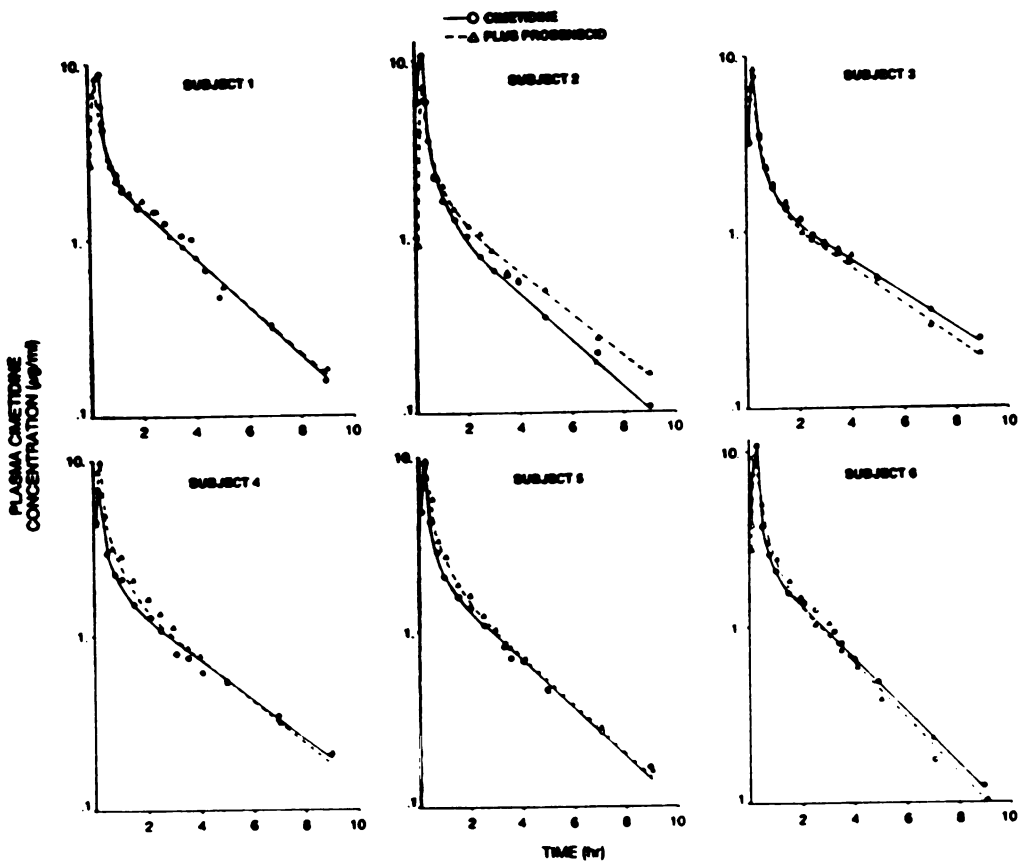


Figure V-1. Semilogarithmic plasma cimetidine concentration vs time plots for 6 subjects who received 300 mg cimetidine intravenously over 15 minutes (○), or 300 mg cimetidine intravenously following steady-state administration of probenecid (500 mg po q6h) (△). Curves were generated by computer fit of the data to a two compartment model.

with probenecid are shown in Figure V-1 for each subject. The actual data points as well as the computer generated curves are shown. The data for each individual were highly reproducible between study days and there was no apparent change in the overall elimination kinetics of cimetidine between treatments. The plasma concentration curves declined bi-exponentially in each subject. A slight hump was apparent after 2 hours in the curves of subjects 1 and 6, which coincided with the time the subjects were fed. The mean +/- SD of the pharmacokinetic parameters for cimetidine including Vd_{ss}, AUC, $t_{1/2}$, Ae_{0-10hr}, CL_s, Cl_{nr}, CL_r, GFR, and CL_{rs} are presented for the two treatments in Table V-1. There were statistically significant decreases in GFR, CL_r, and CL_{rs} of cimetidine when probenecid was administered. Data for each individual are presented in tabular form in the appendices.

Renal clearances of cimetidine were determined from the slope of the renal excretion rate vs midpoint plasma concentration plots forced through the origin with no weighting of data (Figure V-2). The renal clearances (mean +/- SD) for treatments of cimetidine and cimetidine with probenecid were 417 +/- 109 and 324 +/- 103 ml/min, respectively ($p < .005$). Renal clearances (mean +/- SD) obtained from the renal excretion rate plots with weighting of data (1/Y) were 377 +/- 74 vs 319 +/- 86 ml/min ($p < .01$) for cimetidine and for cimetidine with probenecid respectively. The greatest differences in renal clearances

Table V-1

Parameter	Cimetidine	Cimetidine plus probenecid	P
Vd_{ss} (L/Kg)	1.00 +/- .2	0.96+/-.2	NS
AUC (ug-hr/ml)	10.1 +/- .5	10.6 +/- 1.1	NS
$t_{1/2}$ (hr)	2.3 +/- .4	2.3 +/- .4	NS
Ae_{0-10hr} (%dose)	76 +/- 8	72 +/- 10	NS
CL_s (ml/min)	495 +/- 25	475 +/- 52	NS
GFR (ml/min)	116 +/- 8	83 +/- 20	<.01
CL_r* (ml/min)	417 +/- 109	324 +/- 103	<.005
CL_r** (ml/min)	377 +/- 74	319 +/- 86	<.01
CL_r*** (ml/min)	393 +/- 53	350 +/- 60	NS
CL_{nr} (ml/min)	102 +/- 48	125 +/- 52	NS
Cl_{rs} (ml/min)	310 +/- 117	248 +/- 106	<.05

*Calculated from the slope of the renal excretion rate vs Cp mid plot with no weighting of data.

**Calculated from the slope of the renal excretion rate vs Cp mid plot with data weighted 1/Y.

***Calculated as AE/AUC.

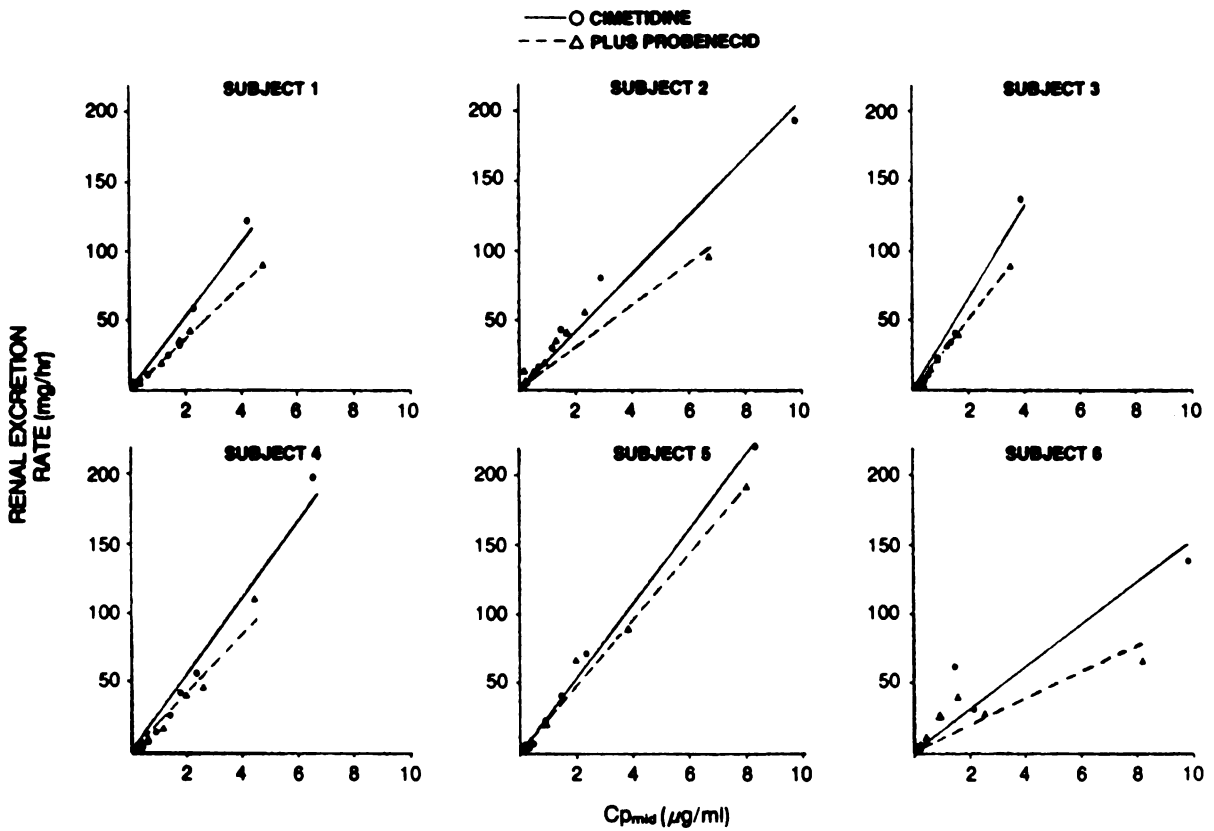


Figure V-2. Renal excretion rate vs midpoint plasma cimetidine concentration plots for 6 subjects. Data represent treatment of cimetidine alone (O), or cimetidine with probenecid (Δ). Renal clearance was calculated by obtaining the slope of the regression line forced through the origin.

were associated with the first or second urine collection intervals (one-half to one hour after cimetidine dosing). During the first urine collection interval, both the renal excretion rate (167 +/- 38 vs 107 +/- 44 mg/hr, $P < .01$) and the renal clearance (508 +/- 125 vs 364 +/- 106 ml/min, $P < .02$) of cimetidine were markedly reduced by probenecid. Renal clearances, calculated by averaging the fractional clearances of all collection intervals (Ae_{0-t}/AUC_{0-t}), were also significantly reduced by probenecid (376 +/- 47 vs 330 +/- 45 ml/min, $p < .05$).

Nonrenal clearances were not significantly different between treatments (102 +/- 48 vs 125 +/- 52 ml/min) for cimetidine and for cimetidine with probenecid. We chose to calculate nonrenal clearance by subtracting renal clearance, determined as AE/AUC, from total clearance because we believed this method would minimize the bias apparent in the renal excretion rate plots.

The GFR, determined by inulin clearance in the first urine collection interval after cimetidine administration, for the two treatments in five subjects is shown in Figure V-3. Inulin clearance could not be determined for one of the subjects due to analytical problems. A significantly lower GFR was observed when probenecid was administered with cimetidine as compared to cimetidine alone (83 +/- 20 vs 116 +/- 8 ml/min , $P < .01$). The renal clearance, and the estimated net clearance by tubular secretion for cimetidine in the individual subjects for both treatments are shown in

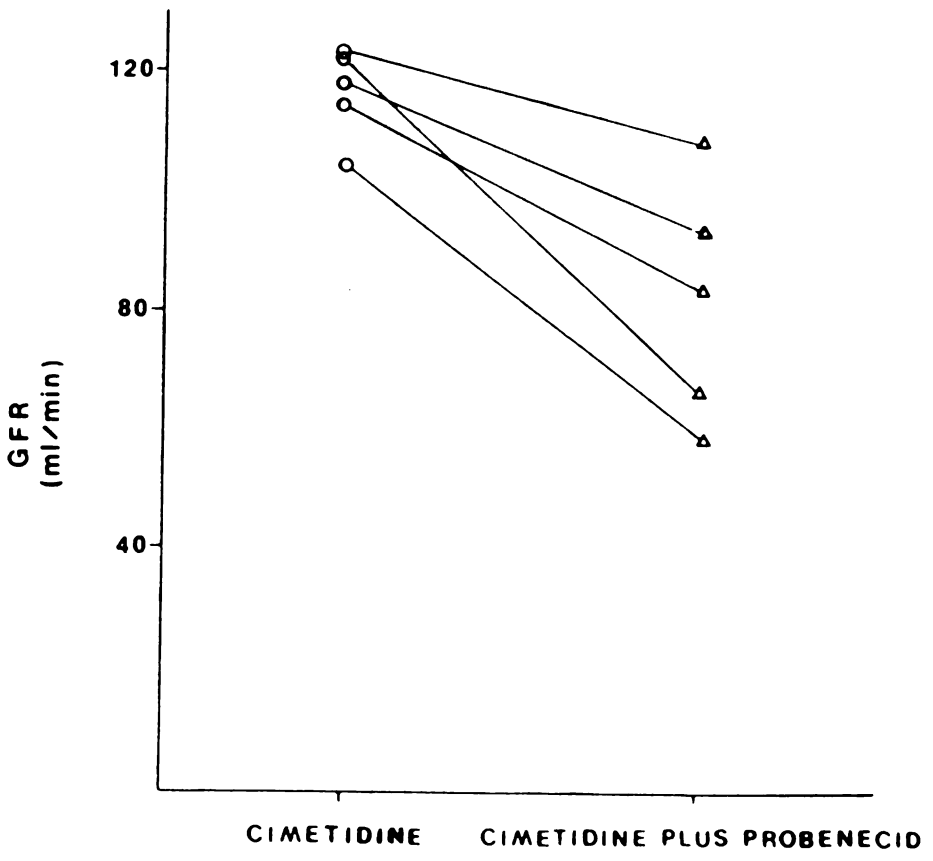


Figure V-3. Glomerular filtration rate as estimated by inulin clearance during the first urine collection interval, following intravenous administration of 300 mg cimetidine (O) or 300 mg cimetidine with probenecid (Δ) to 5 subjects.

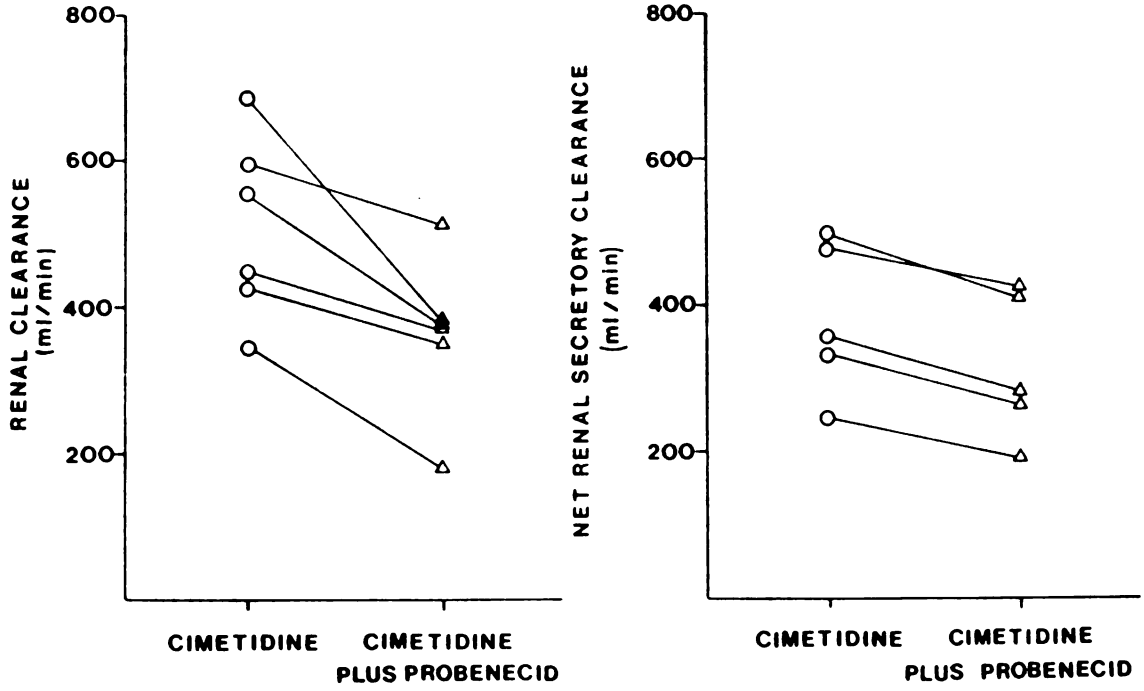


Figure V-4. The renal clearance and the net clearance by renal secretion of cimetidine following administration of 300 mg cimetidine alone (O) or 300 mg cimetidine with probenecid (Δ). Data were obtained from the first urine collection interval.

Figure V-4. These data were obtained in the first urine collection interval at the same time GFR was determined. In addition to decreasing GFR, probenecid significantly reduced the net secretory component of renal clearance (380 +/- 104 vs 314 +/- 100 ml/min, $P < .001$) in the first collection period. A statistically significant reduction in the secretory clearance was also observed when data from all collection intervals were averaged (310 +/- 117 vs 248 +/- 106 ml/min, $p < .05$). It should be noted that GFR was determined only in the first urine collection interval and this analysis assumes that GFR remains constant.

G) Discussion.

The pharmacokinetics of cimetidine have been studied frequently in humans, and several comprehensive reviews of this literature have been published in recent years.[12,45,48] The pharmacokinetic parameters obtained for cimetidine in this study and those reported in recent literature reviews[12,45] are presented for comparison in Table V-2. It can be seen that the data obtained in the present study are in agreement with those obtained from healthy adults in many other studies compiled from the literature.

In healthy young adults, the total systemic clearance of cimetidine may be as high as 500 to 600 ml/min,[45] although considerable variation is known to occur.[45] The high systemic clearance for cimetidine is largely accounted for by its renal clearance which may be 3 or 4 times greater

Table V-2

Reference	Vd_{ss} (L/Kg)	CL_s (ml/min)	CL_r (ml/min)	CL_{nr} (ml/min)	$t_{1/2}$ (hr)	fe (% dose)
Somogyi[45]	.8-1.2	500-600	400-600	200	2.0	50-80
Ziemniak[12]	1.0	500-600	400-600	144	2.0	70
Present Study	$1\pm.2$	495 ± 25	417 ± 109	102 ± 48	$2.3\pm.4$	76 ± 8

than GFR.[45] Therefore, cimetidine is secreted by the renal tubules in addition to being cleared by filtration in the glomerulus. Cimetidine, a weak base of pKa 6.8 is 20% ionized at pH 7.4 and would be expected to be transported in the renal tubules by the organic cation transport system.[90] The renal proximal tubular transport of cimetidine has been studied in vitro and in vivo in a variety of species including the rat,[87,93] rabbit,[1,88,89] dog,[91] and chicken.[92] Data from these studies, in which cimetidine transport was inhibited by other organic bases and quaternary cations, have suggested that the renal tubular transport of cimetidine is mediated by the organic cation transport system. Studies in humans, demonstrating that cimetidine inhibits the renal clearance of the organic bases procainamide,[85,86] ranitidine,[66] and triamterene,[68] and in which the renal clearance of cimetidine was inhibited by the base ketoconazole,[67] have suggested that, in the human kidney, cimetidine is also secreted by the organic cation transport system.

Probenecid, pKa 3.4, is an organic acid which is widely used for treating chronic gout.[134] It is also used to inhibit secretion of penicillin by the renal tubules.[134] Probenecid is a classic inhibitor, and a substrate, of the renal organic anion transport system(s).[135] The compound is both secreted and reabsorbed in the proximal tubules,[135] however, only 5 to 11% of an orally administered dose is actually eliminated unchanged in the urine.[134] Probenecid

metabolism, 70% oxidation of alkyl side chains and 20% glucuronide conjugation, accounts for 90% of its total systemic clearance.[134]

An early report, published as an abstract, presented data showing that probenecid increased the elimination half-life of cimetidine in vivo in rats.[136] Because cimetidine is known to be eliminated primarily by the kidney, it can be postulated that the increased half-life may have been due to probenecid or its metabolite(s) causing a decrease in the renal clearance of cimetidine, although renal clearances were not actually reported in the study. Probenecid had previously been reported to inhibit the renal secretion of a series of basic catecholamine derivatives in the chicken.[137-139] Several in vitro studies[1,88,91] have since demonstrated that probenecid can inhibit the renal proximal tubular transport of cimetidine, and that the inhibition occurs in part at the luminal membrane of the proximal tubule (Chapter III).

The inhibition of renal tubular transport of an organic base by an organic acid is unusual and contradicts our current understanding of organic anion and organic cation transport mechanisms.[90] The question of whether this interaction is demonstrable in vivo is important from the perspective of ascertaining the biological relevance of this interaction to the whole animal and determining the clinical importance of the interaction to drug therapy.

In the present study, probenecid did not alter the elimination of cimetidine sufficiently to cause clinically significant differences in the plasma concentration vs time profiles (Figure V-1). However, probenecid did cause a significant reduction in the renal clearance of cimetidine. This effect cannot be unequivocally assigned to probenecid because it is possible that one or more of the probenecid metabolites may have been involved. However, the inhibition of cimetidine transport by probenecid in renal luminal membrane vesicles,[1] which are virtually devoid of metabolic capability, would support the argument that probenecid itself can produce inhibition.

The reduction in the renal clearance of cimetidine in this study was particularly evident in the first hour after cimetidine administration. Inspection of the renal excretion rate plots of Figure V-2 reveals that the data for the two treatments are in relative agreement for the later time periods in which plasma cimetidine concentrations were below 2 ug/ml. Probenecid levels would also be expected to be low during this time. The most notable differences occurred during the earliest urine collection intervals. In this study, probenecid was administered 3 hours before cimetidine. According to the data of Selen et al.,[140] the time to peak probenecid concentrations for orally administered doses of 500 mg is 3 hours. Therefore, probenecid concentrations in plasma would have been at or near maximum during the time when the renal clearance of

cimetidine was maximally decreased. Probenecid is accumulated in the kidney,[134,135] and it is probable that renal concentrations of probenecid were also high during the time of maximum inhibition of cimetidine renal clearance. The expected half-life of probenecid at these doses is 4 hours.[134,140] Therefore, the probenecid concentrations would have been declining in parallel with the diminishing effect on cimetidine renal clearance and may explain the transitory nature of the inhibition observed.

The short-lived nature of the inhibition of renal clearance provides an explanation for the observation that plasma cimetidine concentrations did not change significantly between treatments. The similarity in the amounts of cimetidine excreted unchanged in the urine in each study period can be explained by the transient effect of probenecid on the renal clearance of cimetidine.

Consistent with other researchers who have reported decreases in GFR of 27%[141] and 22%[142] in humans after probenecid administration, we observed a 28% reduction in GFR with probenecid. The mechanism for this effect is not known. Despite the contribution of the reduced GFR in decreasing the renal clearance, the net clearance by renal secretion of cimetidine was significantly decreased by probenecid treatment (Figure V-4).

A decrease in the net renal clearance by secretion of cimetidine in the presence of probenecid and/or its metabolites could be explained by an increased plasma

protein binding, an enhanced biotransformation in the kidney, an increase in tubular reabsorption, or by a diminished tubular secretion of cimetidine.

Cimetidine is approximately 20% bound to plasma proteins over the range of concentrations from .05 to 50 ug/ml.[45] To attribute the observed changes in renal clearance to an effect of probenecid on the plasma protein binding of cimetidine, one would have to postulate that probenecid increased the binding of cimetidine to plasma proteins. We feel this is an extremely unlikely mechanism since protein binding interactions usually occur by displacement as opposed to enhancement of binding.

Renal metabolism of cimetidine has been demonstrated in the chicken,[92] however, the renal biotransformation of cimetidine has never been studied in man. Although the effects of probenecid on the metabolic fate of other compounds are complex,[134] we could find no evidence in the literature to indicate that probenecid has ever been observed to alter the metabolism of cimetidine. For these reasons, and because there was no change in the nonrenal clearance of cimetidine between treatments in the present study, we feel that this mechanism for the decreased renal clearance of cimetidine is unlikely.

Probenecid could theoretically have increased the rate of reabsorption of cimetidine by increasing urine pH or decreasing urine flow. However, the greatest effect of probenecid on the renal clearance of cimetidine occurred

within one hour after cimetidine administration. During this interval, as well as during other intervals, there was no significant effect of probenecid on either urine pH or urine flow. Therefore, the most plausible explanation, consistent with the in vitro findings discussed previously, is that probenecid caused inhibition of the renal secretion of cimetidine.

These findings provide an interesting contrast to the conclusions reached in a recent study by van Crugten and co-workers.[66] These investigators found that, in normal subjects, the organic anion cephalothin had no effect on the renal clearance of cimetidine and vice versa. Based on these observations, and the fact that cephalothin is an anion and is secreted by the renal organic anion transporter, the authors concluded that organic anions and organic cations do not interact in the human kidney. Our data are in apparent conflict with these conclusions, however, one must consider the relative inhibitory potencies of probenecid and cephalothin and their concentrations in relation to potency. It is possible that the concentrations of cephalothin in their study were insufficient to inhibit cimetidine transport.

Previous work with water and electrolyte[88] and glucose[1] transport has provided evidence that probenecid does not produce its inhibitory effect on tubular transport by toxic mechanism(s). Rather, the inhibition of cimetidine renal secretion by probenecid suggests an interaction with the

carrier mediated mechanism(s) of membrane transport for cimetidine. Further studies from this laboratory have suggested that the interaction between the organic anion probenecid and the organic cation cimetidine is representative of a general interaction between organic anions and organic cations at the luminal membrane.[143] The actual mechanism by which the organic anions produce inhibition of membrane transport of organic cations is now being explored.

In summary, the results of this study have demonstrated that probenecid inhibits the renal elimination of cimetidine in humans. The inhibition was mediated by a reduction in GFR as well as by an apparent inhibition of the tubular secretory component of cimetidine renal excretion. This interaction is of importance from a mechanistic standpoint and it is the first demonstration of a renal interaction between organic anions and organic cations in humans.

Chapter VI

Conclusions

A) Cimetidine Transport In Luminal Membrane Vesicles.

Luminal membrane vesicles were isolated from rabbit renal cortex by a differential precipitation-centrifugation method. The functional integrity of transport systems in the vesicles was demonstrated by Na^+-gradient stimulated uptake of D-glucose. These studies also have demonstrated that the vesicles were tightly sealed and were osmotically reactive. Purity of the membrane preparation was demonstrated by monitoring the enzyme markers, maltase and Na^+/K^+ ATPase.

Transport of cimetidine in the vesicles was characterized by studying the time course of uptake of cimetidine into the vesicles, quantitating the degree of membrane binding of cimetidine, ascertaining the effect of concentration on the initial rate of uptake, and determining the effect of an initial outwardly-directed proton gradient on cimetidine uptake. The effects of inhibitors on the uptake of cimetidine in the absence and in the presence of the proton gradient were determined. The data indicate that both saturable and nonsaturable processes were involved in the uptake of cimetidine in the vesicles. The inhibition of cimetidine transport by organic cations and the fact that a proton gradient could stimulate the transport of cimetidine

suggests that cimetidine transport is mediated by the organic cation transport system in the luminal membrane.[94]

The finding that probenecid inhibited the proton gradient-stimulated transport of cimetidine in luminal membrane vesicles agrees with the data obtained in isolated perfused tubules[88] and in renal cortical slices.[91] The studies in this thesis have demonstrated that this interaction occurs at the luminal membrane of the proximal tubule and appears to be a result of a direct effect of probenecid on the cimetidine transporter and not related to general inhibition of membrane transport or membrane toxicity. These findings suggest that the transport of organic cations may be affected by organic anions and are contradictory to the theory that substrates of one system do not affect transport of substrates of the other system.[90] Subsequent studies in this laboratory have confirmed that the organic anions, probenecid and furosemide, inhibit transport of organic cations other than cimetidine.[143]

Inhibition studies with the endogenous neutral compound creatinine have demonstrated that it inhibits proton gradient-stimulated cimetidine transport in luminal membrane vesicles in a concentration dependent manner. These data suggest that cimetidine and creatinine may share a common transport system in the luminal membrane of the proximal tubule. This may be the mechanism for the inhibition of creatinine secretion by cimetidine observed clinically in humans.[83] The high concentrations of creatinine required to

produce inhibition indicate that cimetidine, in comparison to creatinine, has a much higher affinity for the transport system.

B) Inhibition of Cimetidine Renal Clearance By Probenecid In Humans.

The data for the cimetidine renal clearance studies of Chapter V are in agreement with human clearance data reported in recent literature reviews.[12,45] Cimetidine was found to be eliminated primarily by renal routes in healthy normal volunteers. Renal clearances approximately 3.5 times GFR indicate that cimetidine is extensively secreted by the renal tubules. Probenecid administered orally three hours before cimetidine was found to significantly decrease the renal clearance of cimetidine for up to one hour after cimetidine administration. The decreased renal clearance of cimetidine was determined to occur by inhibition of tubular secretion as well as reduction of GFR.

These findings demonstrate that the effects of probenecid on cimetidine transport are biologically relevant and suggest that interactions between organic anions and organic cations in the renal tubules may occur in vivo. Probenecid did not alter the overall elimination kinetics of cimetidine and the interaction is therefore not of clinical significance for this combination of drugs. However, the interaction between other concurrently administered anion-

cation drug combinations could be clinically relevant and has not been studied.

The findings of these studies are of great interest from the standpoint of the mechanisms of renal transport of organic bases and organic acids. The relevance of this research applies not only to elimination of exogenously administered compounds but also to elimination of endogenous organic bases and acids.

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Appendices

1. Protein Assay Procedure.

A) Reagents.

1) 2% Na_2CO_3 in 1 N NaOH, 50 ml.

2) 1%Na/K tartrate in deionized distilled H_2O (DDW), 0.5 ml.

3) 1% $CuSO_4$ in DDW, 0.5 ml.

4) One-half strength Folin-phenol reagent, in DDW, 300 ul.

B) Dilutions For Luminal Membrane Vesicle Assay.

1) First homogenate, dilute 1/2 with DDW, use 15 ul and 30 ul sample volumes, and dilute 1/4 with DDW, use 30 ul and 60 ul sample volumes. Samples are further diluted to 600 ul with DDW before adding assay reagents.

2) Final pellet (luminal membrane vesicles), dilute 1/25 with DDW, use 15 ul and 30 ul sample volumes, and dilute 1/50 with DDW, use 30 ul and 60 ul sample volumes. Samples are further diluted to 600 ul with DDW.

c) Assay Procedure.

Reagents may be prepared separately and stored tightly sealed for several months. Just before performing the assay, 50 ml $NaOH/Na_2CO_3$ solution is combined with 0.5 ml Na/K tartrate and 0.5 ml $CuSO_4$. If the samples are cloudy after addition of $CuSO_4$, a poor standard curve results and this can be corrected by preparing and rerunning with fresh $NaOH/Na_2CO_3$. The diluted samples are placed in a 5 ml test

tube and 3.0 ml of the combined reagents are added, vortexed and allowed to stand for at least 15 min to solubilize the proteins. While vortexing, 300 ul Folin-phenol reagent are rapidly added to the combined sample/reagent mix and allowed to stand at least 30 minutes. Absorbance is read at 650 nm with a spectrophotometer, using disposable plastic cuvettes or an automatic sipper. Blanks are prepared by adding buffer in the same dilutions used for membrane samples and their absorbance is subtracted from samples and standards.

D) Standard Curve.

Bovine serum albumin in DDW (1mg/ml) is used as a protein standard. Volumes of 5, 10, 15, 30, and 50 ul are prepared for the standard curve as described above for the samples. Protein concentrations are determined from the linear regression curve of the standards.

2. Enzymatic Determination of Purity/Contamination of Isolated Luminal Membrane Vesicles.

A) Maltase Enzyme Assay.

Activity of the enzyme maltase is used as a marker for determining the relative concentration of luminal membranes in the final centrifuge fraction, compared to other cortical membranes which are present in the first homogenate. Quantitation of maltase activity is performed using a coupled enzyme assay system[107-109] as follows:

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

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

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

The activity of maltase is monitored by determining the rate of reduction of NADP (no absorbance at 340 nm) to NADPH (high absorbance at 340 nm).

1) Reagents.

All reagents except maltase (present in the luminal membrane fraction) and maltose are contained in the Sigma glucose assay kit,[107] which is reconstituted just before the assay by adding 31 ml DDW. An aliquot of this is saved for blanks. With the remainder, a 1.0 mg/ml solution of maltose is prepared.

2) Sample Dilutions.

Samples are prepared as:

25 ul first homogenate (undiluted) + 1.0 ml assay mix, 25 ul final luminal membrane diluted 1/10 with DDW + 1.0 ml assay mix, and 25 ul luminal membrane diluted 1/20 + 1.0 ml assay mix. Blanks are prepared with buffer + assay mix containing maltose.

3) Assay Procedure.

The spectrophotometer is set at 340 nm, with the temperature control set either at 25^0 C or at 37^0 C. Regardless of which temperature is used, the assay mix must remain refrigerated until just prior to use. Use the autosipper to aspirate the samples. The reaction is allowed to proceed for 10 minutes, and the reaction rate is determined from a linear portion of the curve as change in absorbance/min for the sample minus change in absorbance/min of the blank.

4) Calculations.

Enzyme activity was calculated as:

$$\frac{(\text{Change A/min})(\text{ml reaction mixture})(1000\ \text{ul/ml})(\text{dilution})}{(\text{ul sample volume})(6.2)}$$

where Change A/min is the slope of the rate plot with blank subtracted, dilution is the dilution factor, and 6.2 is the molar extinction coefficient of NADPH.

Enrichment of maltase activity is calculated as:

$$\frac{(\text{Activity of luminal membrane fraction/ mg protein})}{(\text{Activity of first homogenate/mg protein})}$$

The mean +/- SD of enrichment of maltase activity for 15 separate luminal membrane preparations prepared in this laboratory was 8.9 +/- 2.7.

B) Na/K ATPase Assay.

Contamination of the luminal membrane vesicles with basolateral membrane vesicles was monitored by a modification of the optical method for quantifying Na/K ATPase activity of Schoner et al.[110] This assay is also a coupled enzyme system by which the activity of ATPase can be determined by monitoring the rate of production of NAD from NADH as follows:

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

$$PEP + ADP \xrightarrow{Pyruvate\ Kinase} Pyruvate + ATP$$

$$Pyruvate + NADH \xrightarrow{Lactate\ Dehydrogenase} Lactate + NAD^+.$$

1) Reagents (final molar concentrations required).

Buffer A.

62.5 mM Imidazole HCl, pH 7.3

62.5 mM NH_4Cl

3.125 mM $MgCl_2$

1.25 mM EGTA

pH to 7.5 with HCl, keep at room temperature.

Buffer B.

50.0 mM Imidazole HCl, pH 7.3

Reaction Mix.

18.75 ml Buffer A

2.813 ml 1 M NaCl

QS to 30 ml with DDW, divide into equal volumes, add sufficient ouabain to deliver a concentration of 1.25 mM to one portion.

Cofactor Mix.

NADH .36 mM

ATP 1.51 mM

PEP .21 mM

PK 10.0 mM

LDH 11.0 mM

Dissolve in Buffer A, QS to 3.33 ml, keep iced.

2) Assay Procedure.

Pipette 800 ul reaction mix with and without ouabain into separate test tubes and add 100 ul cofactor mix. Determine the sample volume required (usually 5 to 100 ul) and add sufficient quantity of Buffer B to the mixture above to give a total 1.0 ml after addition of the sample. Add 5 to 100 ul sample, vortex well and use the autosipper to take up the sample.

The spectrophotometer is set at 340 nm and the reaction is run at 37^0 C. The reaction is allowed to run 5 to 10 min.

3) Calculations.

The rate is determined from the slope of a linear portion of the curve as Change A/min sample minus Change A/min for reaction mix with ouabain. The remainder of the calculations are identical to the assay for maltase.

3. Inulin Assay Procedure.

A) Reagents.

1. Indole-3-acetic acid 0.5% in 95% ethanol. Keep refrigerated.

2. Concentrated HCl (37% w/w).

3. Trichloroacetic acid 10% in DDW.

4. Inulin standard (0.05 ug/ul) in DDW. Add inulin to boiling DDW to dissolve, cool and bring to volume. Prepare fresh daily.

B) Sample dilutions.

Plasma samples may be assayed undiluted. Urine samples are diluted 1/100 with DDW.

C) Assay procedure.

To 250 ul of plasma or diluted urine add 1.0 ml TCA, vortex and allow to stand for 10 minutes. Centrifuge at 5000 RPM for 5 minutes. To 250 ul of the supernatant add 50 ul indole-3-acetic acid and 2.0 ml HCl. Vortex and place samples in a shaking water bath for 75 minutes at 37^0 C. Cool the samples to room temperature. Absorbance of the samples is read at 520 nm on the spectrophotometer.

D) Standard curves.

Plasma or diluted urine are spiked with 5, 10, 20, 30, and 50 ul of inulin standard solution. Blanks are prepared by adding DDW to plasma or urine, instead of the inulin standard, and are subtracted from standards and samples. Inulin concentrations are determined from the linear regression curve of the standards.

E) Calculations.

Inulin clearance is calculated by the following formula: $CL_{in} = \frac{\text{urine conc. X urine vol}}{\text{plasma conc. X time of interval}}$

F) Precautions.

Concentrated HCl produces caustic and corrosive fumes. Samples should be handled with gloves in a fume hood and cuvettes should be tightly capped to prevent damage to the spectrophotometer.

CLINICAL DATA FOR CIMETIDINE-PROBENECID STUDY

Subject 1, Day 1. Treatment: cimetidine

Time (Hr) From Infusion Start	Cp (ug/ml)
0.420	8.98
0.500	5.81
0.583	4.23
0.833	2.66
1.08	2.18
1.33	1.91
1.83	1.55
2.47	1.47
2.83	1.26
3.48	1.06
3.83	1.02
4.33	0.678
4.92	0.489
6.83	0.328
8.83	0.155

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpoint**	Cp Mid (ug/ml)	Urine Flow (ml/min)	Urine pH
0.833	101.7	0.583	4.23	11.0	7.0
1.25	24.52	1.04	2.25	10.4	7.0
1.83	19.13	1.54	1.75	7.6	7.0
3.83	31.07	2.33	1.46	4.1	6.0
5.83	21.34	4.83	0.62	1.2	5.3
7.83	7.022	6.83	0.31	0.64	5.1
9.83	6.670	8.83	0.16	1.2	5.0

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 1, Day 2. Treatment: cimetidine. + probenecid.

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	2.66
0.167	6.63
0.250	8.19
0.517	4.73
0.750	2.91
1.03	2.41
1.50	1.89
2.00	1.70
2.48	1.49
3.03	1.05
3.50	0.923
4.00	0.802
5.03	0.541
7.00	0.307
9.00	0.181

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpoint**	Cp Mid (ug/ml)	Urine Flow (ml/min)	Urine pH
1.03	93.67	0.515	4.75	9.4	6.1
1.53	21.11	1.28	2.12	4.0	5.3
2.03	17.69	1.78	1.75	6.2	5.9
3.95	37.73	2.99	1.14	3.3	5.1
6.03	17.17	4.99	0.602	4.7	6.0
8.03	9.176	7.03	0.313	1.8	5.7
10.0	5.748	9.03	0.165	3.9	6.0

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 2, Day 1. Treatment: cimetidine + probenecid

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	0.890
0.167	4.02
0.250	7.11
0.533	3.59
0.767	2.26
1.02	1.99
1.50	1.45
2.03	1.15
2.50	1.05
2.98	0.821
3.48	0.635
3.98	0.565
5.02	0.506
6.98	0.263
9.00	0.163

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min).....	pH
0.483	45.90	0.242	6.73	13.1	7.0
1.07	33.56	0.775	2.25	7.7	6.9
1.57	20.57	1.32	1.673	8.7	7.0
2.07	17.59	1.82	1.26	8.2	6.9
3.93	36.74	3.00	0.830	5.1	6.7
5.98	20.90	4.96	0.470	1.6	6.6
8.00	11.94	6.99	0.27	1.3	5.5
9.95	9.35	8.98	0.155	3.9	6.8

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 2, Day 2. Treatment: cimetidine.

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	5.85
0.167	9.30
0.250	10.92
0.45	5.90
0.750	2.21
1.02	1.61
1.48	1.24
2.00	1.00
2.53	0.770
3.03	0.640
3.53	0.600
3.97	0.550
5.02	0.342
7.05	0.215
9.10	0.108

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.383	74.12	0.192	9.76	18.9	6.7
0.966	47.36	0.675	2.82	10.2	6.8
1.45	20.46	1.21	1.45	10.4	6.7
1.92	14.18	1.68	1.14	10.9	6.7
4.02	33.60	2.97	0.640	6.8	6.7
6.00	18.64	5.00	0.342	4.4	6.0
8.03	8.12	7.02	0.215	2.3	5.7
9.93	3.39	8.98	0.112	2.5	6.1

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 3, Day 1. Treatment: cimetidine

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	3,20
0.167	6.51
0.250	7.64
0.500	3.56
0.750	2.35
1.00	1.79
1.53	1.39
2.02	1.19
2.52	0.953
3.00	0.836
3.52	0.757
3.98	0.664
5.00	0.532
7.00	0.351
8.92	0.246

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.950	131.6	0.475	3.84	10.1	6.3
1.50	22.80	1.23	1.50	4.0	5.9
2.00	17.89	1.75	1.28	6.6	6.0
4.00	44.61	3.00	0.836	6.4	6.2
6.00	22.02	5.00	0.532	1.9	5.9
8.00	12.34	7.00	0.351	0.87	5.0
10.0	7.12	9.00	0.246	3.4	6.4

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 3, Day 2. Treatment: cimetidine + probenecid

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	3.26
0.167	5.78
0.283	8.29
0.500	3.47
0.800	2.29
1.00	1.89
1.50	1.49
2.12	0.980
2.55	0.900
3.00	0.860
3.50	0.800
4.00	0.730
5.00	0.540
7.00	0.290
8.92	0.200

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
1.00	89.48	0.500	3.47	9.0	5.9
1.50	20.03	1.25	1.65	2.9	5.2
2.00	15.95	1.75	1.22	6.9	5.6
4.00	47.70	3.00	0.850	8.3	6.2
6.00	26.23	5.00	0.540	5.1	7.1
8.00	5.311	7.00	0.320	0.79	6.8
10.0	6.809	9.00	0.190	2.1	7.0

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subjec 4, Day 1. Treatment: cimetidine

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	4.51
0.167	6.83
0.250	6.48
0.533	2.91
0.800	2.21
1.02	2.13
1.55	1.50
2.08	1.27
2.50	1.06
3.03	0.781
3.55	0.748
4/03	0.615
4.95	0.544
7.03	0.346
9.07	0.203

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.483	96.41	0.24	6.52	11.6	7.0
1.07	33.20	0.77	2.28	12.0	6.9
1.48	18.31	1.28	1.72	16.4	6.8
2.18	18.65	1.83	1.37	3.6	7.0
3.85	23.12	3.02	0.844	5.0	6.8
5.90	13.50	4.88	0.546	9.3	6.7
7.83	5.954	6.87	0.344	2.1	6.8
9.86	5.005	8.85	0.216	6.0	6.9

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 4, Day 2. Treatment: cimetidine + probenecid.

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	4.48
0.167	8.68
0.250	9.83
0.417	4.90
0.733	3.15
1.02	2.84
1.52	2.10
2.03	1.62
2.48	1.33
2.98	1.13
3.48	0.858
3.98	0.770
5.00	0.555
7.02	0.312
9.07	0.213

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.967	107.6	0.484	4.46	9.3	6.9
1.45	21.82	1.21	2.53	15.8	6.9
1.92	19.42	1.68	1.94	16.1	6.9
3.94	32.64	2.93	1.15	8.4	6.8
6.01	17.50	4.98	0.574	2.8	6.6
8.04	10.75	7.03	0.339	1.4	5.7
9.98	5.33	8.97	0.206	2.7	6.1

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 5, Day 1. Treatment: cimetidine + probenecid.

Time (Hr) From Infusion Start	Cp (ug/ml)
0.167	6.84
0.250	9.46
0.500	5.87
0.750	3.38
1.02	2.67
1.50	1.89
2.00	1.62
2.47	1.25
2.97	1.03
3.52	0.810
4.12	0.700
5.00	0.470
7.02	0.280
9.03	0.160

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.417	79.78	0.208	8.03	19.4	6.9
0.967	49.14	0.692	3.84	8.9	6.8
1.93	46.79	1.45	1.96	11.4	6.8
1.96	46.22	3.05	0.960	3.6	6.3
7.93	32.64	6.05	0.380	0.73	5.3
9.91	6.891	8.92	0.155	0.99	5.6

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 5, Day 2. Treatment: cimetidine.

Time (Hr) From Start Infusion	Cp (ug/ml)
0.083	4.96
0.167	7.45
0.250	9.29
0.479	4.22
0.733	2.83
1.00	2.09
1.52	1.59
2.00	1.37
2.50	1.09
3.27	0.806
3.55	0.700
4.00	0.675
4.95	0.464
7.03	0.264
8.93	0.168

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.417	87.91	0.208	8.31	13.0	6.7
1.417	71.70	0.917	2.30	9.9	6.5
1.917	20.98	1.67	1.46	13.0	6.6
3.950	45.81	2.93	0.90	5.0	6.4
6.970	19.68	5.46	0.42	1.4	6.2
8.040	3.99	7.51	0.235	0.78	5.6
9.970	11.59	9.01	0.150	5.4	6.1

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 6, Day 1. Treatment: cimetidine + probenecid.

Time (Hr) From Infusion Start	Cp (ug/ml)
0.083	2.78
0.167	7.45
0.250	8.90
0.524	5.00
0.750	3.00
1.05	2.45
1.54	1.83
1.99	1.47
2.52	1/01
3.03	1.04
3.48	0.723
4.17	0.585
5.03	0.389
7.08	0.232
9.17	0.097

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.417	26.70	0.208	8.13	17.8	7.4
1.43	28.03	0.920	2.60	5.3	7.1
2.13	27.72	1.78	1.63	11.1	6.9
4.12	51.90	3.13	0.860	9.0	6.5
6.00	15.49	5.06	0.420	5.1	6.4
8,08	8.288	7.04	0.200	2.6	5.6
9.93	9.650	9.00	0.100	4.5	6.6

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.

Subject 6, Day 2. Treatment: cimetidine.

Time (Hr) From Infusion Start	Cp (ug/ml)
0.086	3.79
0.183	9.32
0.256	10.9
0.519	3.78
0.770	2.58
1.00	2.09
1.52	1.55
2.05	1.39
2.54	1.23
3.22	0.945
3.48	0.810
4.03	0.653
4.97	0.489
7.08	0.173
8.98	0.122

Time (Hr) Interval*	Ae Interval (mg)	Time (Hr) Midpt**	Cp Mid (ug/ml)	Urine Flow (ml/min)	pH
0.417	58.02	0.208	9.85	19.6	7.0
1.55	34.19	0.980	2.10	4.8	6.6
2.18	39.33	1.87	1.40	14.2	6.6
3.82	46.54	3.09	0.900	7.7	6.5
5.83	16.90	4.92	0.480	2.8	7.1
8.03	5.01	6.93	0.225	1.2	6.9
9.93	3.77	8.98	0.105	2.5	6.0

*Time elapsed from start cimetidine infusion to end collection interval.

**Time elapsed from start cimetidine infusion to midpoint collection interval.