MECHANISMS FOR MODULATION OF RENAL UPTAKE OF ORGANIC CATIONS

BY

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Modulation of the mechanism for tubule uptake of the achiral organic cation amantadine was investigated. Rat renal cortical proximal and distal tubules were purified using a Percoll density gradient. Replacement of bicarbonate with other anions in the incubating medium resulted in a decrease in affinity and maximal transport capacity for uptake of amantadine by proximal and distal tubules. Replacement of bicarbonate with racemic lactate resulted in a decrease in affinity but not capacity. However, in the presence of bicarbonate, lactate inhibited amantadine tubule uptake. Quinine exhibited a higher potency of inhibition than quinidine for proximal tubule amantadine uptake in bicarbonate buffer. Quinine and quinidine were equipotent in their inhibition of amantadine distal tubule uptake. However, the inhibitory potency of quinine, but not quinidine, decreased in the absence of bicarbonate thereby causing the stereoselective inhibition of amantadine proximal tubule uptake to be reversed. However, stereoselectivity of inhibition returned to control levels upon addition of bicarbonate. Absence of bicarbonate decreased the inhibitory potency of quinidine but not quinine for distal tubule amantadine uptake. A decrement in extracellular sodium did not alter amantadine tubule uptake. A decrease in extracellular chloride resulted in a decrease in affinity for uptake of amantadine by distal tubules only. Neither high nor low extracellular potassium altered amantadine tubule uptake in the presence of bicarbonate. High extracellular calcium (5 *m*M) inhibited amantadine tubule uptake in the presence and absence of bicarbonate. Except for ouabain, the cardiac glycosides tested did not affect amantadine tubule uptake in the bicarbonate buffer. The inhibitory effect of ouabain on amantadine uptake was bicarbonate-dependent and did not involve entry of ouabain into the cell. Moreover, under hypokalemic conditions, ouabain enhanced proximal tubule amantadine uptake. A similar enhancement was observed for the distal tubules under hyponatremic conditions. Altogether, the present results suggest that there exist bicarbonate-dependent and bicarbonate-independent amantadine uptake sites. The former

possess high affinity and capacity and the latter possess low affinity and capacity. The bicarbonate-dependent sites are quinine-sensitive and the bicarbonate-independent uptake sites are quinidine-sensitive in the proximal tubules. The opposite appears to be true for distal tubule uptake sites. In the absence of bicarbonate, the bicarbonate-dependent amantadine uptake sites in both tubules may be modulated by lactate in a less efficient manner. However, in the presence of bicarbonate, lactate competes for modulation of the bicarbonate-dependent sites and this competition is reflected as inhibition of uptake. Activity of the Na^+/K^+-ATPase and the basolateral membrane electrical potential are not rate-limiting for influx of amantadine into the renal tubule cell.

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I will be forever indebted to Ms. Paula Macedo for her friendship and her academic and moral support throughout my graduate student years. It made swimming against the current a lot easier. Lastly, but not *leastly*, I wish to thank all my friends, past and present, for tolerating my everchanging, overstressed and overstressing personality throughout my training to become a "Doctor". I imagine it was not easy. Very special thanks to my dear friend Gregory Mathew Smith, for his ubiquitous and never-ending support.

I wish to dedicate this thesis to my mother, Ms. Ana Escobar who, without knowing it, has taught me the meaning of perseverance and stubbornness, two qualities that are indispensable to a person and researcher. Without them, I would have given up a long, long time ago. Literally, I would not have made it this far if it had not been for her.

If you can dream - and not make dreams your master, ...

If you can fill the unforgiving minute

With sixty seconds' worth of distance run,

Yours is the Earth and everything that's in it,...

(Kipling, *If*)

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ABBREVIATIONS

ACZ	Acetazolamide (Diamox®)
BSA	Bovine Serum Albumin
CA	Carbonic anhydrase
DMSO	Dimethylsulfoxide
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
Kd	Dissociation constant
KHS	Krebs Henseleit Solution
Ki	Constant of inhibition.
Km	Apparent affinity constant for uptake
NMN	N^1-methylnicotinamide
PAH	para-aminohippuric acid
Q	quinine
QD	quinidine
TEA	tetraethylammonium
Tris	Tris[hydroxymethyl]aminomethane
Vmax	Apparent maximal transport capacity for uptake

INTRODUCTION

PREAMBLE.

The present dissertation focuses on the mechanism for uptake of organic cations by the renal tubules, a system that has been relatively poorly researched.

All the present studies were performed using *in vitro* preparations. The advantage of such a preparation is that conditions of study may be closely monitored and controlled. As well, undesirable systemic effects that the drugs being tested would have with an *in vivo* situation are obviated. However, it is important to stress the fact that because these studies are done *ex vivo*, the extent to which these observations can be extrapolated to the *in vivo* situation remains to be determined. Moreover, inter-species extrapolation needs to be confirmed.

Some data that have come from our laboratory have already been confirmed using human tissue and clinical studies (Wong 1992*a* & 1992*b*; Gaudry *et al*, 1993). Therefore, we are confident that the *in vitro* preparation, as well as the fact that rat tissue is used, may be largely valid.

AMANTADINE.

Amantadine is a pharmacological agent. It is used as prophylaxis against viral influenza A and for symptomatic relief of Parkinson's disease (Schwab *et al*, 1969; Parkes, 1974; Oxford and Galbraith, 1980; Aoki and Sitar, 1988). It is an achiral, weak base (pKa 10.1), that is almost completely ionized at physiological pH. It is largely non-metabolized in the body and its excretion from the body occurs mainly via the kidneys (Koppel and Tenczer, 1985; Cedarbaum, 1987; Aoki and Sitar, 1988).

Previous reports from this laboratory have shown that amantadine is actively accumulated by renal human and rat tissue (Wong *et al*, 1990, 1991, 1992*a* & 1992*b*, 1993). Amantadine is actively transported by the renal tubules and it is dependent on tubule metabolic activity. As well, our laboratory has shown that exogenous organic

cations inhibit the renal tubule uptake of amantadine (Wong 1990, 1991, 1992*a* & 1992*b*, 1993).

PART I. GENERAL PRINCIPLES OF TRANSPORT.

Since the present dissertation deals with transport system(s), it is important that basic principles of transport be reviewed prior to the development of the research findings.

I.1 SIMPLE DIFFUSION.

Thermodynamically, molecules move across an interphase with a rate represented by the following equation:

$$V = K_d (S_1 - S_2) \qquad [1]$$

where V is the transport rate of the molecule, S_1 and S_2 are the concentrations of the substrate at points A and B, and K_d is the diffusion constant (Christensen, 1962).

It is obvious from formula [1] that for uncharged molecules (without any influence of electrochemical interactions) the rate of migration is linearly dependent on the concentration gradient, i.e. $S_1 - S_2$. Further, the diffusion rate also depends on the material traversed, represented in formula [1] as K_d, the diffusion constant. K_d depends on temperature, among other factors. Therefore, kinetics of transport processes will depend on the temperature at which the assays are performed. Kinetic data obtained should be compared only with data obtained from assays performed at the same temperature.

Biological membranes provide a hydrophobic barrier to any polar and charged solutes. However, mediated transport has evolved so that such compounds are assimilated by the cell selectively.

I.2 FACILITATED DIFFUSION.

This type of transport is important for accelerating the movement of non-lipid-soluble molecules across the membrane. The membrane component that aids in the transport is termed the carrier. Carriers are merely catalysts and hence are recycled. Moreover, since their quantity is finite, this type of transport exhibits saturation, i.e. at high substrate concentrations all the carriers are used and transport does not increase.

Facilitated transport is not to be confused with other systems such as protein pores which are non-enzymic transport systems. Although similar kinetics are involved under non-saturating substrate concentrations, protein pores only allow for the passage of ions and/or ionized molecules, and this type of flux is completely dependent on diffusion.

If facilitation of transport occurs by combination of the substrate S, with a mediating structure C, to form a complex CS, the following scheme can be written,

$$S_{out} + C_{out} \Rightarrow CS \Rightarrow S_{in} + C_{in}$$

Then, the rate of passage through the membrane is given by:

$$V = Kd\,(CS_1 - CS_2) \qquad [2]$$

where Kd is the diffusion coefficient, CS_1 and CS_2 are the concentrations of the carrier-substrate complex on either side of the membrane.

For net transport in a system not able to transport the substrate against its concentration gradient, i.e. an equilibrative system, the net transfer across the membrane is given by the rate of diffusion of the carrier-substrate complex (Widdas, 1953; Rosenberg and Wilbrandt, 1955):

$$V = D'C_t ((S_1 - S_2) Km / (S_1+Km) (S_2+Km)) \quad [3]$$

$$= Vmax ((S_1 - S_2) Km / (S_1+Km) (S_2+Km)) \quad [4]$$

where D' is the permeability constant of the carrier-substrate complex, S_1 and S_2 are the substrate concentrations on either side of the membrane, Km is the dissociation constant for the carrier-substrate complex, and Vmax is the maximal transport capacity of the carrier. However, under conditions where no substrate exists on the opposite side of the membrane ($S_2 = 0$), formula [4] simplifies, and the rate of the unidirectional flux of a substrate across the membrane using a carrier of limited capacity is given by the following relationship, proposed by Michaelis and Menten (1913):

$$V = Vmax \ (S / (S + Km)) \quad [5]$$

where Vmax is the maximal transport capacity of the system, S is the substrate concentration and Km is the concentration of substrate that will produce half the maximal rate (i.e. ½ Vmax). This relationship assumes that the rate-limiting parameter is the traversal of the complex across the membrane and not the formation and dissociation of the carrier-substrate complex. The dissociation constants must be the same for C and CS. To obtain a unidirectional flux, the initial rate must be measured, or at least the concentration of the substrate on the opposite side of the membrane, i.e. the side to which the substrate is being transported, must be relatively low compared to the Km for transport from that side. Further explanation of these parameters is provided below.

I.3 ACTIVE TRANSPORT.

The systems discussed so far lead to equilibrium, not accumulation. However, many substrates attain intracellular concentrations that are much higher than those

predicted by simple diffusion or by carrier-mediated diffusion. According to equation [4], at equilibrium $S_1 = S_2$ and no net transfer of substrate is observed. Kinetically, the requirements for uphill transport may be outlined as follows (Rosenberg and Wilbrandt, 1955):

$$V = K_d (CS_1 - CS_2) = D' ((C_1S_1 / Km) - (C_2S_2 / Km)) \qquad [6]$$

with the factors as previously described. Since this type of transport leads to accumulation, at $S_1 = S_2$, $V \neq 0$. This condition can be produced if the ratio C_1:C_2 is changed or if the affinities of the carrier for the substrate on either side of the membrane are different. The ratio of the two values of Km is therefore the maximal distribution ratio that the transport can produce.

Obviously, if the affinity of the carrier is to be increased in such a way that uphill transport is produced, energy must be available from an energy-yielding reaction. Part of this energy is presumed to become available to decrease affinity of the carrier for the modified carrier-substrate complex in order to permit its dissociation thereby generating elevated levels of the substrate. This type of active transport in which energy is directly coupled to the transport process is known as primary active transport.

However, not all transport systems are of the primary active type. Secondary active transport is that in which a substrate is transported against its concentration gradient coupled to the transport of another substrate down its concentration gradient. In this way, one uphill transport would be driven indirectly by the energy used directly for the transport of another substrate The direction of transport of the two substrates may be either in the same (cotransporters) or in the opposite (antiporters) direction.

Tertiary active transport is an extension of secondary active transport in that the driving force created by the movement of the second substrate in the secondary active transport is used to drive the exchange or co-transport of a third substrate. An example of

this type of transport is that of organic cation and anion transport by renal tubule cells (*vide infra*).

Kinetically,

$$S' = S / Km \qquad [7]$$

where S' is the relative substrate concentration, and S and Km as previously described. Then, the rate of transport of a substrate R, in the presence of a concentration gradient of a second substrate S, is given by the following equation (Wilbrandt and Rosenberg, 1961):

$$V_R = Vmax (R' (S'_2 - S'_1) / ((S'_1 + R' + 1) (S'_2 + R' + 1))) \qquad [8]$$

where R' = R / Km. It is apparent from equation [8] that in order for transport of R to stop, $S_1' = S_2'$. In other words, R will be transported as long as there exists a concentration gradient for S. Moreover, transport of R will occur uphill and it will continue until the following relation is satisfied:

$$R_1/R_2 = S_1' + 1 / S_2' + 1 \qquad [9]$$

the above ratio defines the maximal accumulation that the system can attain.

PART II: TRANSPORT KINETICS.

As shown before, the movement of a substrate across the membrane using a carrier of limited capacity is given by equation [5], proposed by Michaelis and Menten (1913). The derivation of this relationship can be found in most biochemistry textbooks (Zubay, 1988):

$$V = (Vmax \cdot S) / (Km + S)$$

This relationship describes a square parabola, the Langmuir isotherm, that exhibits saturation (Fig. I-1). As described above, Vmax is the maximal transport capacity of the system. It provides an indication of carrier density (number of carriers) active during the transport process. It is important to emphasize that no direct correlation exists between Vmax and the number of transporters present in a system. The latter value may be determined by Scatchard plots (Scatchard, 1949). Km is the concentration of substrate that will produce half the maximal rate (i.e. ½ Vmax). The value of Km gives an indication of the dissociation constant (Ks) for the carrier-substrate complex. Generally, 1/Km is used as a measure of the affinity of the carrier for the substrate so that a large Km is indicative of low affinity binding. Km is dependent on several factors such as pH and temperature of the environment, so that great care must be taken when comparisons of measured Km values are made among laboratories.

In practice, before the advent of computers, it was difficult to determine the parameters Km and Vmax from the Langmuir isotherm because of the difficulty of approximating the asymptote for Vmax. Hence the error in the estimation of Vmax would lead to an error in the calculation of Km (substrate concentration at ½ Vmax).

Lineweaver and Burk (1934) proposed the following equation obtained by a simple mathematical manipulation of the Michaelis-Menten relationship:

$$1/v = (Km/Vmax)\,(1/S) + 1/Vmax \qquad [10]$$

Therefore, a plot of 1/v *vs* 1/S will yield a straight line whose y-intercept will equal 1/Vmax and whose x-intercept will equal -1/Km. A major disadvantage of this approach is that because it is a double reciprocal plot, the low values of the substrate concentration will have a larger role in determining the position of the fitted line whereas

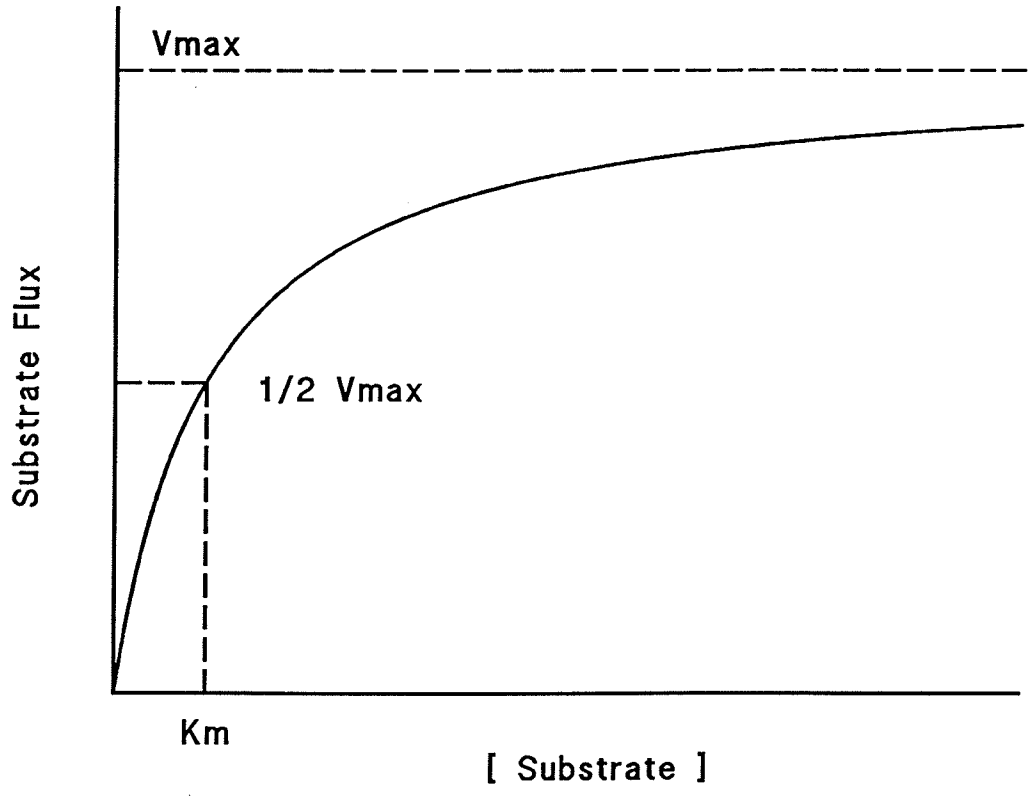


Figure I-1. Representative plot of the Langmuir isotherm obtained with the Michaelis-Menten relationship for carrier-mediated transport. Km is the concentration of substrate at ½ Vmax (the maximal transport capacity). The Km value gives an indication of the carrier affinity for the substrate.

it is these same values which are the ones likely to possess the larger proportional errors.

Further mathematical manipulation of the Lineweaver-Burk plot was proposed by Eadie (1942) to yield the following:

$$v = Vmax - (v / S) Km \qquad [11]$$

A plot of v *vs* v/S will yield a straight line whose slope is equal to -Km and the y-intercept is equal to Vmax. This transformation is known as the Eadie-Hofstee plot and it has the advantage that both intercepts have finite values, whereas the Lineweaver-Burk plot approaches infinity as the substrate concentration approaches zero. Further, the Eadie-Hofstee plot permits a more accurate examination of any interactions between substrate and transporter due to the fact that deviation from the normal Michaelis-Menten kinetics is more obvious than with the Lineweaver-Burk (Zivin and Waud, 1986).

A novel approach for determining kinetic parameters was proposed by Eisenthal and Cornish-Bowden (1974). The main concept of this approach is to treat the values of Km and Vmax as variables and v and S as constants. That is, for every v at certain S there will be an infinite number of Km and Vmax values that will satisfy such a coordinate. The relationship upon which this proposal was based is the following:

$$Vmax = v + ((v / S) Km) \qquad [12]$$

Therefore, a plot of Vmax *vs* Km with Vmax = measured v and Km = -S will yield several lines representing all the Km and Vmax values that will satisfy each measurement. However, only one value for Km and Vmax will satisfy all the coordinates, and those will be given by the coordinates at the intersection of the lines. An advantage of this plot is that it allows for the determination of the kinetic parameters without manipulation of the

data. One disadvantage with such a plot is that the lines will intersect at several points, and hence the variability of the measured parameters will be inherently high.

With the advent of computer programs, it has become easier to determine kinetic parameters without the need for manipulation of the data. Non-linear regression programs have become increasingly available to researchers. The way the program works is that it takes a first "guess" of the kinetic parameters. Using this guess, the program fits an equation to the data performing repeated iterations with new guesses obtained from the previous iteration until the residual sum of squares is minimized (Wilkinson, 1961; Cleland, 1963).

In fact, the proper way of calculating the kinetic parameters is to plot the data using one of the linear plots previously presented and to use the determined values for Km and Vmax as the first guess for the non-linear regression program. One drawback of non-linear regression fitting is that a larger amount of data than with the linear plots is needed in order to obtain reliable determinations. Altogether, it seems that both the linear and non-linear regression approaches to calculation of kinetic parameters are complementary; neither one should be taken as best.

PART III: KINETICS OF INHIBITION OF TRANSPORT.

Inhibition of transport may occur due to different combinations of interactions between inhibitor, substrate and carrier. Based on the fact that many transporters show selectivity for one conformation over another, different substrates with similar conformations will interact with the transporter. Depending on the type of interaction, inhibition of a reaction can be classified as competitive, non-competitive, or uncompetitive. Each inhibition type possesses its own characteristics.

III.1 COMPETITIVE INHIBITION.

As presented in the scheme below, competitive inhibition results from an interaction of the inhibitor (I) at the same binding site on the carrier (C) as the substrate (S) being studied.

$$\begin{array}{ccccccccc} C & + & S & \Rightarrow & CS & \Rightarrow & C & + & P \\ + & & & & & & & & \\ I & & & & & & & & \\ \Updownarrow K_i & & & & & & & & \\ CI & & & & & & & & \end{array}$$

The binding of the inhibitor is a reversible reaction. Therefore, inhibition will eventually be overcome as the concentration of the substrate becomes high. The maximal rate of the reaction (Vmax) will not be affected, but as the inhibitor interferes with the binding of the substrate, it will appear that affinity for the binding site (Km) will have been altered.

In kinetic terms, the rate of the reaction in the presence of a competitive inhibitor is given by:

$$v = Vmax * S / (Km (1 + i / K_i) + S) \qquad [13]$$

where K_i is the dissociation constant of the carrier-inhibitor complex, *i* is the inhibitor concentration and the other terms as previously described. Such changes in kinetic parameters would be reflected by the Lineweaver-Burk plot as a change in the Km but not Vmax in the presence of the inhibitor.

Dixon (1953) proposed a plot of 1/v vs *i* at more than one substrate concentration as a means to determine the value of K_i (Fig. I-2) This type of plot gives lines with

different slopes which if extrapolated, intersect at a value on the abscissa equal to $-K_i$. Because it is a reciprocal transformation, the Dixon plot has many of the problems associated with the Lineweaver-Burk plot, and the determined K_i may possess large errors.

An additional problem is that the Dixon plot yields similar lines for both competitive and mixed inhibition. Therefore it is not possible to determine with certainty the type of inhibition present (*vide infra*). To resolve the problem, Cornish-Bowden proposed a complementary plot described by the following relationship (1974):

$$S / v = (Km / Vmax (1 + i / K_i)) + (S / Vmax) \qquad [14]$$

A plot of s/v vs *i* will yield straight lines at different substrate concentrations. This type of plot is used to determine whether the inhibitor interacts with the carrier-substrate complex as well, a fact that will distinguish competitive from mixed inhibition (*vide infra*). Since by definition competitive inhibition does not include interaction of the inhibitor with the complex, the Cornish-Bowden plot will yield parallel lines.

III.2 NON-COMPETITIVE INHIBITION.

The term non-competitive has been coined to define the type of inhibition in which the inhibitor interacts with the carrier and the carrier-substrate complex.

$$\begin{array}{ccccccccc} CI & + & S & \Leftrightarrow & CSI & \Rightarrow & C & + & P \\ \Updownarrow K_i & & & & \Updownarrow K_i' & & & & \\ I & & & & I & & & & \\ + & & & & + & & & & \\ C & + & S & \Leftrightarrow & CS & \Rightarrow & C & + & P \end{array}$$

where K_i' is the dissociation constant for the CSI complex, and the other terms as previously described.

This type of inhibition does not change the affinity of the substrate for the binding site. However, the maximal transport capacity will be altered, as a proportion of the carrier-substrate complexes does not result in product formation.

The rate of transport is given by the following equation:

$$v = (Vmax * S) / ((S + Km) (1 + i / K_i)) \qquad [15]$$

Such a type of inhibition is reflected by the Lineweaver-Burk plot as a change in Vmax but not Km. The Dixon plot will yield a similar graph in which the extrapolated lines will meet on the x-axis at a value equal to $-K_i$ (Dixon, 1953). However, the value of K_i' cannot be determined from the Dixon plot. The Cornish-Bowden approach will yield a plot similar to the two above in which the extrapolated lines intersect on the x-axis at a value equal to $-K_i'$ (Cornish-Bowden, 1974).

III.3 UNCOMPETITIVE INHIBITION.

This type of inhibition includes interaction of the inhibitor with the carrier-substrate complex but not with the carrier alone.

$$\begin{array}{ccccccccc} C & + & S & \Rightarrow & CS & \Rightarrow & C & + & P \\ & & & & + & & & & \\ & & & & I & & & & \\ & & & & \Updownarrow K_i' & & & & \\ & & & & CSI & & & & \end{array}$$

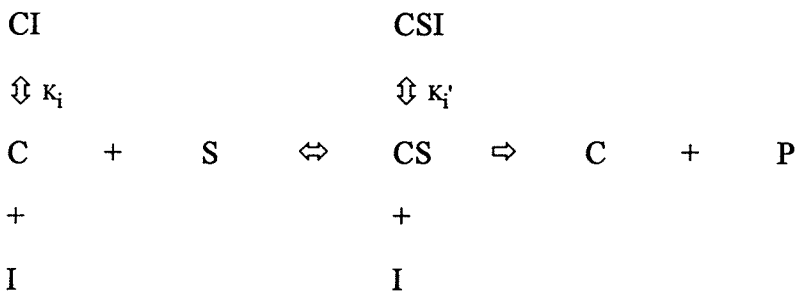
Such an inhibitory interaction results in the change of Vmax but not Km. The following formula describes the rate:

$$v = Vmax * S / ((Km + S (1 + i / K_i')) \qquad [16]$$

As there is no interaction between the inhibitor and the free carrier, the Dixon plots will yield parallel lines (Dixon, 1953). The Dixon plot represents a problem because the parameter K_i' cannot be determined. Similarly, the Lineweaver-Burk plot will yield parallel lines which will be misleading as they suggest a change in Km when theoretically there should be none. Determination of K_i' is done with the Cornish-Bowden plot which will yield lines with different slopes and whose intercept will lie above the x-axis. The extrapolated value to the abscissa will be equal to $-K_i'$ (Cornish-Bowden, 1974).

III.4 MIXED INHIBITION.

Mixed inhibition may be described as a combination of competitive and uncompetitive inhibition (Cornish-Bowden, 1974). That is, the inhibitor interacts both with the carrier and the carrier-substrate complex but the substrate cannot bind to the carrier-inhibitor complex. The following scheme describes the interactions:



Two inhibition constants need to be measured, Ki for the carrier-inhibitor complex and K_i' for the CSI complex. As described above, the Dixon plot will yield the K_i (Dixon, 1953) and Cornish-Bowden plot will yield K_i' (Cornish-Bowden, 1974).

A summary of the different plots obtained for the different types of inhibition using the Dixon and Cornish-Bowden plots is presented in Figure I-2.

III.5 CHENG-PRUSOFF ANALYSIS.

In 1973, Cheng and Prusoff proposed a semilogarithmic plot of v *vs* [i] in which multiple sites of action can be detected. However, the disadvantage of this plot is that the obtained value for IC_{50} (the concentration of the inhibitor that will cause the rate of the reaction to decrease by 50% of that in the absence of the inhibitor) depends on the concentration of the substrate used for the assay. To correct for this factor, the following formula was proposed:

$$IC_{50} = K_i (1+ S / Km) \qquad [17]$$

The terms in this relationship change slightly with the type of inhibition encountered. Concerns have been raised that at the present time many researchers incorrectly use the Cheng-Prusoff equation (Craig, 1993; Leff and Dougall, 1993). Therefore, it is important that the type of inhibition is determined, using the Dixon and Cornish-Bowden plots, prior to the calculation of K_i from IC_{50} values. It is imperative that great care is taken to ensure that the proper equation is used when calculating the K_i values from the Cheng-Prusoff plot.

PART IV: RENAL EPITHELIAL UPTAKE.

Prior to the explanation of the renal transport of charged organic molecules, it is important to consider contributions made by renal physiology and anatomy.

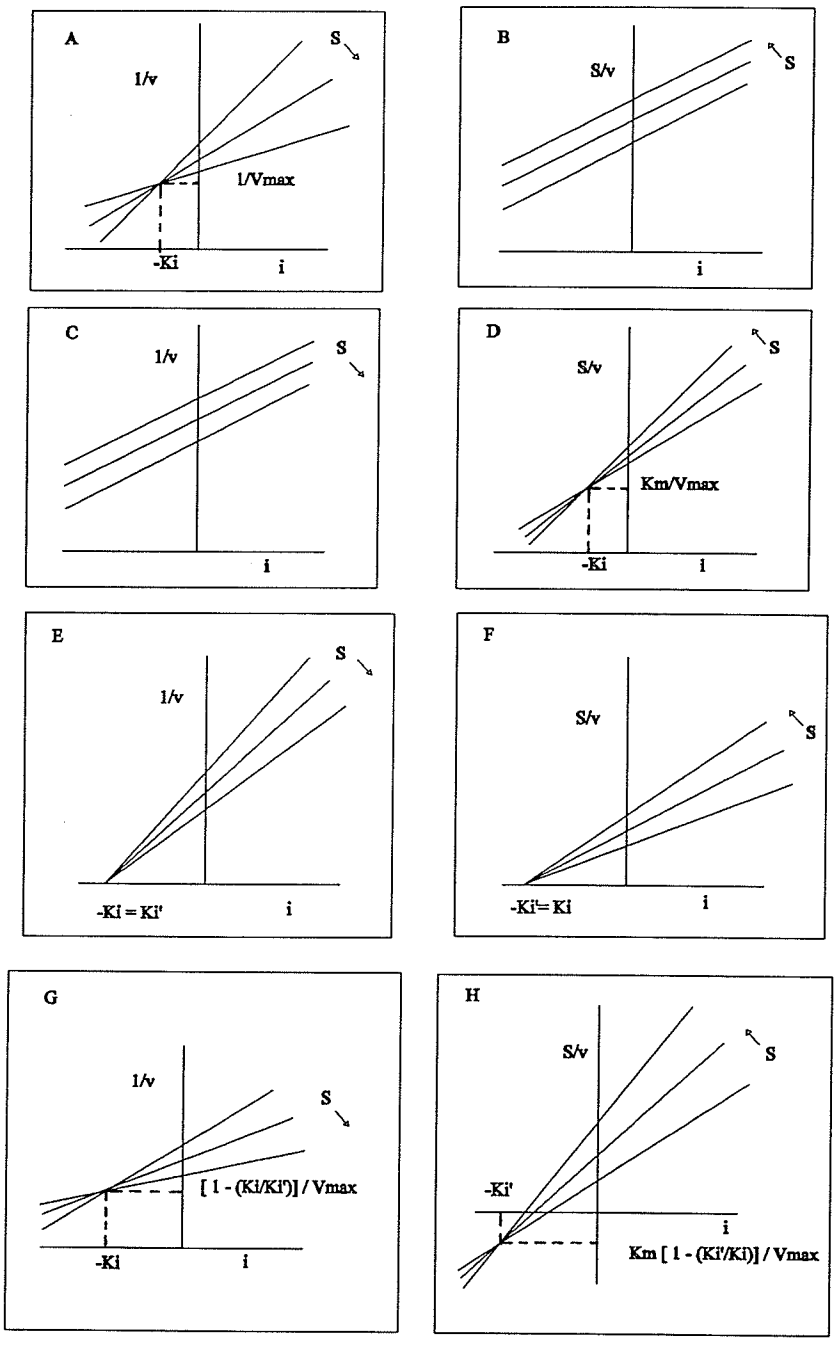


Figure I-2. Summary of the plots obtained with the Dixon (A, C, E, G) and Cornish-Bowden (B, D, F, H) approaches to determining inhibitory constants Ki and Ki' for competitive (A, B) , uncompetitive (C, D), non-competitive (E, F), and mixed inhibition (G, H)(Cornish-Bowden, 1974).

IV.1 RENAL PHYSIOLOGY

The functional unit of the kidney is the nephron. There exist approximately one million nephrons in the human kidney. There are three types of nephrons: superficial, midcortical and juxtamedullary nephrons. They are functionally and morphologically different (Beeuwkes, 1980; Bulger and Dobyan, 1982). However, anatomically they are all divided grossly into similar segments (Kriz and Bankir, 1988). All nephrons are composed of a filtering component (the glomerulus) and a connecting tubular part. Blood arrives at the glomerulus which serves as a filter for small molecules present in the plasma. This process is known as ultrafiltration. The protein-free ultrafiltrate flows into the tubular part connected to the glomerulus by the Bowman's capsule. Throughout its course, the tubular component is formed of a monolayer of epithelial cells resting on a basement membrane. There is considerable heterogeneity among the different parts of the tubule. For the purpose of reference, the tubule is grossly divided into four sections. The proximal tubule receives the ultrafiltrate, and it is this part of the nephron in which most of the reabsorptive processes for the filtrate components are present. Further flow of the ultrafiltrate will move it into the loop of Henle. The loop is divided in turn into descending (descends into the medulla), and ascending loops (which returns into the cortex). At the end of the ascending loop of Henle, the distal tubule begins, whence the connecting and collecting ducts follow (Vander, 1991).

As well, within these gross divisions, there are further anatomical divisions of the proximal tubule. The first segment of the proximal tubule is convoluted, *pars convoluta*, and it is known as the S1 segment. The S2 segment follows and it lies between the *pars convoluta* and the S3 segment or *pars recta*, the part that connects the proximal tubule with the loop of Henle. There is extensive functional heterogeneity among these segments as well.

The tubule epithelia possess the ability to remove compounds from the ultrafiltrate and to return them to the circulation. This process is known as reabsorption. The

opposite process is present also, whereby substrates present in the blood which did not filter through the glomerulus are actively transported into the lumen. This process is known as secretion. Whether the substrate appears in the urine will depend on the extent to which it is filtered, secreted, and reabsorbed.

Both foreign and endogenous compounds are metabolized to charged organic molecules within the body (Pritchard and Miller, 1993). Most of these compounds are toxic and must be eliminated from the body as promptly as possible. The kidney is responsible for most of the removal of these organic anions and cations, and specific transporters are present in the renal tubules to perform this task. However, these systems are not specific for the kidney. Similar mechanisms are present in the liver (Despopoulos, 1966; Barany, 1973), choroid plexus (Barany, 1972), the ciliary body (Becker and Forbes, 1961; Barany, 1972), and the thyroid (Hamilton, 1953). Both organic anion and cation secretory systems have been reported to be separate from each other, although several qualitative similarities have also been reported (Pritchard and Miller, 1993).

IV.2 ORGANIC ANION RENAL TRANSPORT.

The tubule epithelia actively secrete a large number of different organic anions, both foreign and endogenous (Table I-1). Many of these compounds gain access to the luminal space *via* filtration through the glomerulus, so that the secretory pathway adds to their excretion *via* the urine.

Historically, the first reports of tubule secretion of organic anions were made by Marshall and associates who showed that the dye, phenol red, stained the tubules of the dog (Marshall and Vicker, 1923; Marshall and Grafflin, 1928; Marshall, 1931), rat, and frog (Edwards and Marshall, 1924) renal cortex without the presence of glomerular filtration. Shannon (1938) showed similar results with the marine teleost, *Lophius americanus*, whose kidney consists almost exclusively of proximal tubules. Later reports, using other species (Chambers and Kempton, 1933), stop-flow (Malvin *et al*, 1958) and

Table I-1. List of representative compounds secreted by the organic anion renal transport system.

Endogenous	Exogenous
Bile salts	Acetazolamide
cAMP	Cephalothin
Fatty acids	Chlorothiazide
Hippurate	Furosemide
Hydroxybenzoates	p-Aminohippurate
Oxalate	Penicillin G
Prostaglandins	Phenol red
Urate	Probenecid
	Salicylate
	Saccharin

micropuncture (Cortney *et al*, 1965) techniques, confirmed findings by Marshall and co-workers. Subsequent studies focused on the structural requirements of the molecules transported by this system to show that both a negative charge and a large hydrophobic region are necessary (Taggart, 1958; Essig and Taggart, 1960; Knoefel *et al*, 1961; Despopoulos, 1965; Moller and Sheikh, 1983).

Although no specificity of transport was detectable, Smith *et al* (1945) showed that organic anions were capable of inhibiting transport of other organic anions, suggesting that they were all transported by a common system. Moreover, of the hippuric acid derivatives tested, p-aminohippuric acid (PAH) was the most avidly secreted by this system. Since then, PAH has been used as a prototype substrate for the organic anion transport system. Heterogeneity of PAH transport along the proximal tubules has been shown. It appears that secretion is higher in the *pars recta* (S2 and S3) than in the early convoluted (S1) segment of the tubule (Tanner and Isenberg, 1970). However, all portions of the tubule secrete PAH and differences in transport activity reflect carrier density (Vmax) rather than differences in affinity (Km).

Organic anions accumulate both intracellularly and intraluminally. The latter may be due to both intracellular and paracellular transit. Paracellular flux has been shown to be quantitatively not significant (Weeden and Jernow, 1968; Burg and Orloff, 1969; Tune *et al*, 1969).

The "classical" organic anion transport system has been extensively studied (Fig. I-3). It has been shown to be dependent on metabolic energy (Cross and Taggart, 1950). Basolateral entry of the anion is dependent on the basolateral Na^+/K^+ ATPase to create a sodium-gradient (out > in). This sodium gradient is used by an Na^+/dicarboxylate cotransporter to create a dicarboxylate gradient across the basolateral membrane (in > out). The dicarboxylate gradient is coupled to an anion/anion exchanger through which the organic anion enters the cell (Cross and Taggart, 1950; Burg and Orloff, 1969; Dantzler, 1969; Bresler and Nikiforov, 1977; Podevin *et al*, 1978; Kikuta and Hoshi,

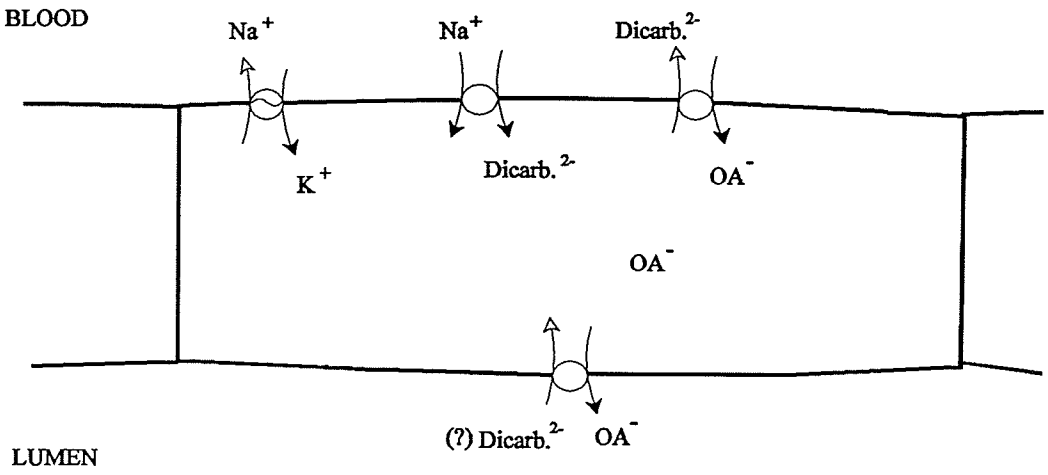


Figure I-3. Present model for the mechanism of renal transport of organic anions across the proximal tubule cell. OA^-, organic anion, $Dicarb.^{2-}$, dicarboxylate anion; (?) denotes an unknown substrate for the exchange at the brush-border membrane that may be a dicarboxylate. For explanation of the model please see text.

1979; Maxild *et al*, 1981; Sheikh and Moller, 1982; Shimada *et al*, 1987; Pritchard, 1988). Exit of the organic anion into the lumen is carried out *via* facilitated diffusion (Kinsella *et al*, 1979; Martinez *et al*, 1990).

Understanding of the organic anion renal secretion system has provided important knowledge applicable to the clinical setting. Physiologists have long used organic acids to measure renal plasma flow and functional tubule mass. Although physiologists no longer use these methods for blood flow determination, physicians still use molecules of the hippuric acid type as a qualitative means to measure kidney structure and viability. This has proven to be an invaluable tool for the assessment of viability in human transplanted kidneys (Strabb *et al*, 1973; Salvatierra *et al*, 1974).

Many xenobiotics are organic anions and the kidney is an important route for their excretion. As an example, some antibiotics are organic anions. They are readily excreted when added to the body, a fact that proved to be of nuisance in times when penicillin was scarce. Subsequently, probenecid was discovered as a potent and reversible inhibitor of organic anion renal transport and used concurrently to decrease the excretion of penicillin (Beyer, 1950).

Diuretics are another example of clinically-relevant organic anions. Some diuretics are given during renal failure, especially the loop diuretic, furosemide. They have their target of action intraluminally but they are highly protein-bound so that they do not filter readily at the glomerulus. Hence, they depend on tubule secretion to reach their site of action. Moreover, the renal extraction and natriuresis promoted by furosemide can be blocked by probenecid, a fact that is of clinical and practical importance (Hook and Williamson, 1965; Honari *et al*, 1977).

IV.3 ORGANIC CATION RENAL TRANSPORT.

This system deals with the secretion of weak bases, which are nitrogen-containing compounds, with one or more positive charges at physiological pH (Table I-2). The first

reports of organic cation secretion by the renal tubule were by Sperber (1947) and Rennick *et al* (1947).

The fact that organic cations have significant vascular effects has made research in the field of organic cation renal transport develop very slowly. To circumvent this problem, much of the early research focused on the study of endogenous organic cations such as choline and N^1-methylnicotinamide (NMN, a metabolite of vitamin B). As well, researchers have taken advantage of the technique developed by Sperber (1946). This model takes advantage of the fact that the kidney of the chicken possesses a separate portal system. After injection of a compound directly into the peritubular circulation of one kidney, the researcher is able to collect urine from both the test and ipsilateral kidneys in order to correct for substrate entry into the general circulation and subsequent filtration. However, these studies must be viewed with caution as extrapolation to the mammalian system is questionable.

For the study of the organic cation renal transport system, the ganglionic blocker tetraethylammonium (TEA) and NMN have been adopted as prototypical organic cations (Rennick *et al*, 1947; Ross *et al*, 1975). Moreover, analogous to the prototypical inhibitor of organic anion transport, probenecid, the cyanine dye 863 (Rennick *et al*, 1956) and the antimalarial drug quinine (Torreti *et al*, 1962) have been the most commonly used organic cation competitors.

The development of stop-flow, micropuncture, and microperfusion techniques has yielded data that have shown that heterogeneity of transport exists among species (Rennick *et al*, 1977; Besseghir *et al*, 1981 & 1990; Miller *et al*, 1989). As well, extensive heterogeneity of transport exists within species. For example, transport for TEA and procainamide along the rabbit proximal tubule is in the order of S1 > S2 > S3 (Schali *et al*, 1983; McKinney, 1982).

In general, the widely accepted model for organic cation renal uptake (Fig. I-4) calls for the basolateral entry of the organic cation as carrier-mediated diffusion dependent

Table I-2. List of representative compounds secreted by the organic cation renal transport system.

Endogenous	Exogenous
Acetylcholine	Amiloride
Choline	Amprolium
Creatinine	Atropine
Dopamine	Cimetidine
Epinephrine	Hexamethonium
Histamine	Isoproterenol
5-Hydroxytryptamine	Morphine
Norepinephrine	Procainamide
N^1-methylnicotinamide	Quinine
Serotonin	Quinidine
Thiamine	Tetraethylammonium
	Trimethoprim
	Verapamil

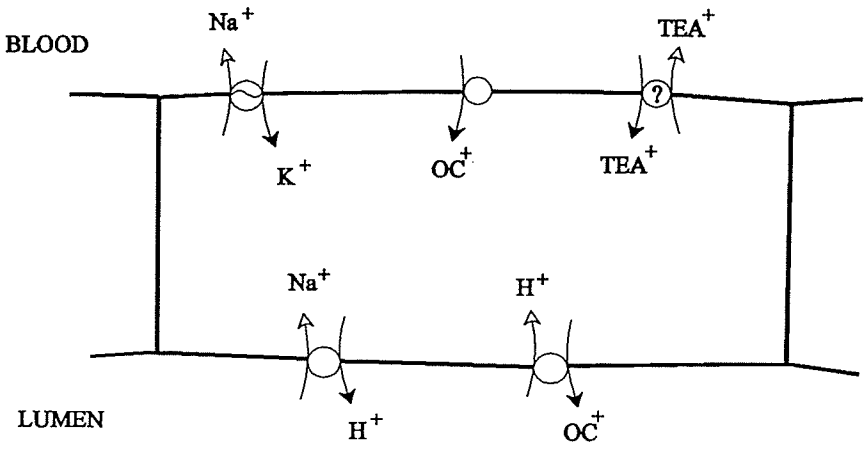


Figure I-4. Present model for the mechanism of renal transport of organic cations across the proximal tubule cell. OC^+, organic cation. TEA^+, tetraethylammonium; the TEA^+/TEA^+ exchanger has been proposed, but its existence remains controversial. For explanation of the model please see text.

on the electrochemical membrane potential created by the basolateral Na^+/K^+ ATPase (Takano *et al*, 1984; Jung *et al*, 1989; Montrose-Rafizadeh *et al*, 1989; Sokol and McKinney, 1990), but an exchanger for TEA-TEA has also been reported (Montrose-Rafizadeh *et al*, 1989; Sokol and McKinney, 1990). Exit at the luminal side has been proposed to be mediated by an organic cation-proton antiporter that uses the proton gradient created by the luminal sodium-proton exchanger (Ross and Holohan, 1983; Takano *et al*, 1984; Inui *et al*, 1985; Rafizadeh *et al*, 1986; Dantzler *et al*, 1991; Ott *et al*, 1991). The multidrug transporter, P-glycoprotein, which has the ability to exclude noncharged as well as charged hydrophobic molecules (Ford and Hait, 1990) has been implicated in the system for renal transport of organic cations. It has been proposed that both the organic cation/H^+ antiporter and the P-glycoprotein may be two aspects of the same transporter protein (Dutt *et al*, 1992).

PART V: OBJECTIVES.

The present dissertation focuses on the study of modulatory mechanisms for the organic cation renal transport system, namely, the role that endogenous anions play in the overall mechanism of transport. One aspect of this series of studies investigates the effect that the bicarbonate anion displays on the inhibitory potency of two cations previously reported to inhibit amantadine uptake.

The sequence of the investigational studies presented herein is as follows:

V.1 BICARBONATE AND ORGANIC CATION RENAL UPTAKE.

Regulation of renal handling of bicarbonate (excretion/reabsorption) is one of the major pathways by which plasma pH is controlled. The main site of bicarbonate reabsorption is the proximal tubule (Rennick, 1981; Besseghir and Roch-Ramel, 1987; Alpern *et al*, 1991) and it is at this part of the nephron where excretion of xenobiotics

such as organic cationic drugs also occurs (Rennick, 1981; Grantham and Chonko, 1991; Boom *et al*, 1992; Pritchard and Miller, 1993). Bicarbonate movement into the renal tubule cell occurs *via* the Na^+/HCO_3^- cotransporter, the Cl^-/HCO_3^- antiporter and proposed bicarbonate channels on the basolateral side. The latter two bring HCO_3^- into the cell to rectify alkali intracellular loads (Boron and Boulpaep, 1989).

Variations in pH of the glomerular filtrate will then be expected to have an effect on the exit of the drug from the cell into the tubular lumen, and hence on the excretion of cationic drugs (Besseghir and Roch-Ramel, 1987). Extensive work with luminal and basolateral membrane vesicles using the prototypical markers, tetraethylammonium and N-methylnicotinamide, have shown that the H^+ ion does indeed play a significant role in organic cation transport by kidney tubules (Holohan and Ross, 1980, 1981; Wright and Wunz, 1987; Sokol and McKinney, 1990; Katsura *et al*, 1991). The use of cell cultures has yielded qualitatively similar results (McKinney *et al*, 1988; Fouda *et al*, 1990; McKinney *et al*, 1990; Yuan *et al*, 1991). However, studies using stop-flow peritubular microperfusion of rat renal tubules have shown that excretion of the cation, cimetidine, is independent of pH (Ullrich and Rumrich, 1992).

It is surprising that, although bicarbonate is the main buffering anion present in plasma under physiological conditions, the majority of the studies have focused on the H^+ ion and not on the bicarbonate anion. Depolarization of the renal tubule cell has been reported to occur upon depletion of intracellular bicarbonate in the nominal absence of external bicarbonate (Boron and Boulpaep, 1989; Sasaki and Marumo, 1989; Seki and Frompter, 1992*a* & 1992*b*). Based on this observation, it may be expected that such bicarbonate-induced depolarization might affect the accumulation of cations by renal tubule cells. Indeed, Ullrich *et al* (1991), using stop-flow capillary perfusion, reported a moderate decrease in NMN^+ transport by rat proximal tubules in the absence of bicarbonate.

Based on these observations, the present studies were aimed at investigating more closely the role of the bicarbonate anion, independent of the H^+ ion, in the mechanism(s) for organic cation renal tubule uptake. The working hypothesis was as follows: Bicarbonate modulates amantadine uptake by the renal tubules independently of pH.

According to our hypothesis, removal of bicarbonate from the medium, at constant pH, should alter amantadine uptake. Use of non-bicarbonate buffers should yield similar transport kinetics among the anions tested.

V.2 LACTATE AND ORGANIC CATION RENAL UPTAKE.

Lactate is normally present in plasma. In mammals, the physiological enantiomer is L(+)-lactate, and its concentration rises during exercise and diet (Ahlborg *et al*, 1974; Essen, 1978; Kreisberg, 1980; Madias, 1986), lactic acidosis (Kreisberg, 1980; Madias, 1986), metabolic alkalosis (Leal-Pinto *et al*, 1973; Dawson, 1977; Emmett and Narins, 1977; Adrogue *et al*, 1978) and epileptic seizures (Orringer *et al*, 1977). It is produced from and metabolized to L(+)-pyruvate solely by the ubiquitous enzyme L(+)-lactate dehydrogenase. The major sites of metabolism are gluconeogenic tissues such as the kidney and liver (Madias, 1986), and very little is excreted in the urine (Hohmann *et al*, 1974; Oh *et al*, 1985).

The D(-) enantiomer is also found in humans but its concentration is minimal (Judge and Eys, 1962). It is converted to L(+)-pyruvate by the enzyme D-2-hydroxy acid dehydrogenase which has been reported to exist in the human body (Cammack, 1969). In mammals, D(-)-lactate is very poorly metabolized, and its removal from the body depends mainly on its excretion via the urine (Judge and Eys, 1962; Oh *et al*, 1985). Gut bacterial flora produce D(-)-lactate, and during uncontrolled growth of these enteric flora, increased amounts of D(-)-lactic acid are released into the blood stream, thereby causing D(-)-lactic acidosis (Oh *et al*, 1979; Stolberg *et al*, 1982).

Racemic lactate solutions are widely used for fluid replenishment of patients such as severely burned victims. As well, L(+)-lactate solution has also been proposed as a substitute solution for dialysis in cases of "acetate-intolerance" (Dalal *et al*, 1990; Herrero *et al*, 1994).

Lactic acid (pKa 3.86) is completely ionized at physiological pH. Lactate renal uptake can occur *via* active transport of the anion or by passive diffusion of the undissociated acid (Poole and Halestrap, 1993), the former being the most important at normal plasma pH. Two systems have been described for renal transport of lactate, one at the basolateral (contraluminal) membrane, and the other at the brush border (luminal) membrane (Poole and Halestrap, 1993).

The luminal lactate transporter has been suggested to be sodium dependent (Barac-Nieto *et al*, 1980; Jorgensen *et al*, 1984), non-selective for D(-)- or L(+)-lactate (Barac-Nieto *et al*, 1980 and 1982; Ullrich *et al*, 1982; Jorgensen *et al*, 1984) and to possess affinity for the organic anions urate and PAH (Guggino *et al*, 1983; Kahn and Weinman, 1985). Moreover, the luminal lactate transport system plays an important role in lactate re-absorption (Craig, 1946; Hohmann *et al*, 1974). The renal transporter of lactate at the basolateral membrane has been postulated to be an anion/anion exchanger (Kahn *et al*, 1985), selective for L(+)-lactate over D(-)-lactate (Barac-Nieto *et al*, 1980 & 1982; Ullrich *et al*, 1982; Jorgensen *et al*, 1984), involved in uric acid renal proximal tubule transport (Kahn *et al*, 1985; Yamamoto *et al*, 1993*a*), but separate from the PAH transporter (Kahn and Weinman, 1985; Webb *et al*, 1986; Werner and Roch-Ramel, 1991). The effect of lactate on the organic cation renal transport system has been studied to a lesser extent.

Based on preliminary findings in the previous section that bicarbonate replacement with DL-lactate (racemic) resulted in lower affinity but unchanged capacity we hypothesized that lactate modulates amantadine uptake by the renal tubules at the bicarbonate-dependent uptake sites. In the present studies, we investigated the effect of

both the pure enantiomers and enantiomeric mixtures of lactate on the mechanism for renal tubule uptake of organic cations.

Our hypothesis predicts that higher uptake amantadine rates should be observed in lactate (non-bicarbonate) buffers than in phosphate buffers. In the presence of bicarbonate, lactate should potentiate uptake if it acts synergistically with bicarbonate, or should inhibit uptake if it competes with bicarbonate for modulation of amantadine uptake.

V.3 DIASTEREOISOMERS AND ORGANIC CATION RENAL UPTAKE

It is generally believed that both anion and cation transport systems are mutually exclusive (Grantham and Chonko, 1991; Pritchard and Miller, 1993) although it has been suggested by Ullrich and Rumrich (1992) that transport sites in the tubules do not distinguish between cations or anions. Nonetheless, organic cation-organic cation as well as organic anion-organic cation interactions have been reported frequently (Besseghir *et al*, 1981; Susuki *et al*, 1986 & 1987; Boom and Russel, 1993; Katsura *et al*, 1993; Pan *et al*, 1994). Hence, the concept of heterogeneity of renal transport sites with overlapping affinities for different compounds is highly controversial.

Heterogeneity of transport is not due only to differences in transport sites, but also to stereoselectivity of such sites for different compounds. It is well known that stereospecificity of transport exists, e.g. glucose transport by the intestinal wall. This stereospecificity extends to renal handling of pharmacological agents (Hsyu and Giacomini, 1985; Ariens, 1986; Notterman *et al*, 1986; Somogyi, 1987; Mikus *et al*, 1990; Wong *et al*, 1990, 1991, 1992 & 1993; Ott and Giacomini, 1993; Higaki *et al*, 1994). We have reported previously the stereoselective inhibition of amantadine uptake by the rat proximal tubule fragments by quinine over quinidine (Wong *et al*, 1990 & 1993). However, this effect has not been studied in the absence of bicarbonate.

Therefore, the present studies were aimed at determining whether the bicarbonate effect is specific for amantadine or whether it extends to other pharmacological agents. We hypothesized that bicarbonate modulates other pharmacological agents at the bicarbonate-dependent sites. The approach to test the hypothesis was to investigate the effect of bicarbonate on the amantadine uptake inhibitory potency of the diastereoisomers, quinine and quinidine.

We expect that a decrease in the inhibitory potency should be observed for those compounds that enter the cell via the bicarbonate-dependent sites. No change will be expected in the inhibitory potency of those compounds that enter the cell via the bicarbonate-independent uptake sites.

V.4 BASOLATERAL NA^+/K^+ ATPASE AND ORGANIC CATION RENAL UPTAKE.

The widely aceepted mechanism for organic cation uptake by the renal tubule cells (Takano *et al*, 1984; Jung *et al*, 1989; Montrose-Rafizadeh *et al*, 1989; Sokol and McKinney, 1990) involves the function of the Na^+/K^+-ATPase to create an electrochemical gradient (inside negative) so that the cation enters the cells in response to the lectronegativity of the cell interior.

Many studies support such a notion as they have shown that the prototypical organic cations TEA and NMN are transported across the basolateral membrane of membrane vesicles in response to an imposed ionic gradient (Takano *et al*, 1984; Jung *et al*, 1989; Montrose-Rafizadeh *et al*, 1989; Sokol and McKinney, 1990). Furthermore, many studies have shown that the cardiac glycosides digoxin and ouabain inhibit the entry of the organic cations into the renal tubule cell (McKinney, 1984; Miller and Holohan, 1987; Besseghir *et al*, 1990), and this inhibitory effect has been attributed to the property of the glycosides to inhibit the basolateral Na^+/K^+-ATPase.

It has been suggested also that a glycoprotein present in the luminal side of the cell, known as the P-glycoprotein, is involved in the organic cation renal transport mechanism. Such a proposal is based on data showing that organic cations such as verapamil (Pedersen *et al*, 1981; Klein *et al*, 1982; Ito *et al*, 1993*c*) and quinidine (Doering, 1979; Hager *et al*, 1979; Pedersen *et al*, 1980; Mordel *et al*, 1993) modulate the excretion of the cardiac glycoside digoxin, a substrate of the P-glycoprotein (Tanigawara *et al*, 1992; Hori *et al*, 1993).

The present studies were aimed at investigating further the role of the basolateral Na^+/K^+-ATPase and the membrane electrochemical gradient in the mechanism(s) for organic cation renal uptake. Based on preliminary data from our laboratory that showed that a high extracellular concentration of potassium did not alter amantadine uptake by either tubule fragment, we hypothesized that the electrochemical potential across the basolateral membrane, and by extention, activity of the basolateral Na^+/K^+-ATPase are not rate-limiting for the influx of the organic cation into the cell.

To test this hypothesis, tissue was pre-treated with cardiac glycosides which have been reported to inhibit the Na^+/K^+-ATPase. In addition, a more direct insult to the membrane potential was performed by manipulating the extracellular concentrations of Na^+ and K^+, as well as Cl^-. According to our hypothesis, no significant change in amantadine uptake should be observed under either approach.

METHODS AND MATERIALS

PART 1: RENAL TUBULE PREPARATION.

Proximal and distal tubule fragments were purified by the Percoll gradient centrifugation method (Vinay *et al*, 1981; Gesek *et al*, 1987) as modified by Wong *et al* (1990, 1991, 1992*a*, 1993), Escobar *et al* (1994), and Escobar and Sitar (1995). Four male Sprague-Dawley rats (University of Manitoba, Winnipeg, Manitoba, Canada-Charles River breeding stock; weight range 250-300 g) were anesthetized with sodium pentobarbital (50 *mg/kg*) injected intraperitoneally. A midline longitudinal incision was made, the kidneys removed and placed in approximately 20 *m*l of ice-cold Krebs-Henseleit solution (KHS, pH 7.4) that contained (in millimolar quantities): NaCl, 118; KCl, 4.7; $MgCl_2$, 1.2; KH_2PO_4, 1.4; $NaHCO_3$, 25; $CaCl_2$, 2.5; and glucose, 11 (pH adjusted with NaOH). Throughout the rest of the procedure the kidney tissue is maintained on ice to prolong viability. After removal of the capsule, the kidneys were sliced into 4 wedges. For each wedge, the medulla was separated from the cortex approximately 1 *m*m from the cortico-medullary junction with a stainless steel single-edge industrial blade. The medullary tissue was discarded. Next, one *m*l ice-cold KHS was placed on the stage of a tissue chopper (Mickle Lab. Engineering Co. Ltd., Gomshall, Surrey, UK) and the cortical slices were placed on this stage as a layer of tissue. The tissue chopper was equipped with a double-sided stainless steel razor blade. Cortical tissue was minced twice, one cycle perpendicular to the other, at 1 *m*m intervals. Each side of the razor blade was used only once per batch of tissue. After mincing, the tissue was placed in approximately 5 *m*l of fresh ice-cold KHS. Subsequently, a KHS-collagenase solution (15 *m*l KHS, 1 *m*l 10% KHS-dialyzed bovine serum albumin [BSA] solution, 14 *m*g of collagenase [0.49 U/*m*g lyophilisate] per approximately 6 g of cortical tissue) was added to the minced tissue and this suspension was placed in a flask. The tissue suspension (final volume of 20 *m*l) was

oxygenated for two minutes with 95% O_2 / 5% CO_2 at room temperature. After oxygenation, the flask was incubated at 37°C with shaking (100 oscillations per minute) in a Dubnoff incubator (Precision Scientific Co., Chicago, IL) for 45 minutes. The tip of a Pasteur pipette was broken and replaced with a 1 ml micropippete tip. This modified Pasteur pipette was used for pipetting the tissue suspension for 5 minutes every 10 minutes in order to aid digestion. After 45 minutes, the incubating flask was placed on ice and 20 *m*l of ice-cold KHS were added to the suspension. Subsequently, the tissue suspension was filtered through a tea-strainer and an additional 10 *m*l of ice-cold KHS were used to rinse any remaining tissue from the strainer (final volume 50 *m*l). The tissue suspension was washed twice by sequential low-speed centrifugation (60 x *g*, 600 rpm, model CS centrifuge, International Equipment Company, Boston, MA, USA) at 4°C for approximately 2 minutes, and resuspension with a Pasteur pipette in approximately 40 *m*l of ice-cold KHS. After the second wash, the pelleted tissue was resuspended with a Pasteur pipette in a KHS-BSA solution (9.5 *m*l KHS, 0.5 *m*l 10% KHS-dialyzed BSA solution) and incubated at 4°C for 5 minutes. Subsequently, the suspension was centrifuged at 60 x *g* (600 rpm, model CS centrifuge, International Equipment Company, Boston, MA, USA) at 4°C for approximately 2 minutes. The final pellet was resuspended with a Pasteur pipette in an oxygenated Percoll solution (40 *m*l equal parts of Percoll and low-calcium [0.5 *m*M] double strength KHS, pH 7.4) and centrifuged for 30 minutes at 27,000 x *g* (18,500 rpm, Sorvall RC2-B automatic refrigerated centrifuge with SS34 centrifuge rotor, Du Pont Company, Wilmington, DE, USA) at 4°C. The proximal and distal tubule fractions (II and IV from bottom of tube, respectively) were collected using a Pasteur pipette and washed three times by resuspension (Pasteur pipette) in 40 *m*l of KHS and low-speed (200 x *g*) centrifugation (1050 rpm, model CS centrifuge, International Equipment Company, Boston, MA, USA) at 4°C for approximately 2 minutes. After the third wash the resulting pellets were resuspended with a 1-*m*l micropippete in the desired volume of the respective buffer to a final tubule protein concentration of approximately 4

mg/ml (3 - 5 *mg/ml*). An average yield from 6 g of cortical tissue (8 kidneys) is approximately 12 *m*g of tubule protein or approximately 2 *m*g of tubule protein / g of cortical tissue. Normally, the buffer used for resuspension was KHS. Tissue protein was determined by the Biuret reagent method as reported by Gornall *et al* (1949). Briefly, Biuret reagent (400 µl, 0.15% $CuSO_4 \cdot 5H_2O$, 0.6% $NaKC_4H_9O_6 \cdot 4H_2O$, 3% NaOH(w/v)) and an aliquot (100 µl) of the proper tubule suspension were incubated at room temperature for 30 minutes. After incubation, protein content was assessed by measuring spectrophotometric absorbance at 550 *n*m on a Spectronic 3000 Array spectrophotometer (Milton Roy Company, Rochester, NY, USA) using BSA (1 - 10 *mg/ml*) as standards.

Purity of tubule fractions was assessed by measuring the concentration of enzymes reported to be distributed selectively in renal proximal (alkaline phosphatase) and distal (hexokinase) tubules (Guder and Ross, 1984). The experimental procedure for measurement of enzyme markers was as follows: A 200 µl aliquot of tissue suspension was diluted to 1 *m*l with KHS and disrupted with a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ont, Canada) with 2 bursts of 5 seconds each at setting 5, using the P10 probe. Homogenized tissue was centrifuged at 6000 x *g* for 2 minutes (7700 rpm, Sorvall RC2-B automatic refrigerated centrifuge with SM24 centrifuge rotor, Du Pont Company, Wilmington, DE, USA) at 4°C, and the supernatant was assayed for the enzyme markers. Both tubule fractions were assayed for hexokinase and alkaline phosphatase enzymes. Alkaline phosphatase content was determined by the addition of an aliquot of supernatant (50 µl) into 1100 µl of reaction medium (1.6 *m*M p-nitrophenol phosphate, 4.4 µM zinc acetate, 18 µM magnesium chloride and 88 *m*M glycine, pH 10.5 adjusted with KOH) at room temperature. Immediately, *p*-nitrophenol formation was monitored spectrophotometrically by recording the change in absorbance at 410 *n*m at 30-second intervals for 15 cycles. Enzyme activity was calculated using a standard curve of absorbance of *p*-nitrophenol standards (0-100 µM). Hexokinase content was assessed by pre-incubation of supernatant (50 µl) in a medium (750 µl) that contained 24 *m*M

magnesium chloride, 18 *m*M D-glucose, 0.8 *m*M NADP, 14 μM EDTA (disodium salt), 2 U glucose-6-phosphate dehydrogenase (type VII) and 0.1 M Tris-HCl (pH 7.6 adjusted with NaOH at 4°C) for 20 minutes (on ice). Adenosine triphosphate (sodium salt, 50 μl) was added subsequently to a final concentration of 50 *m*M. Immediately, formation of NADPH was monitored spectrophotometrically by measuring the change in absorbance (340 *n*m) at 30-second intervals for 15 cycles, at room temperature. Enzyme activity was then calculated using a standard curve of absorbance of NADPH standards (0-100 μM).

Examination of the tissue by light-microscopy was also performed as assessment of tubule integrity and purity. Light-microscopic examination was performed on unstained tubules under 100x and 430x magnification with a microscope (Bausch & Lomb Optical Co., Rochester, NY, USA). Microscopic examination of both tubule fractions revealed intact tubules of varying lengths with an apparently open lumen. Proximal tubules were long relative to distal tubules, showed their characteristic granular appearance and a yellow cytoplasm. Distal tubules showed a transparent cytoplasm, were smaller in length and width, and less granular than the proximal tubules.

Different commercial lots of collagenase with different activities and concentrations of contaminating proteases were used during the present studies. Therefore, each commercial lot of collagenase differed in terms of its potency of activity on connective tissue, a fact that has been reported previously (Peterkofsky and Diegelmann, 1971; Hefley, 1987). The assay used to determine the amount of collagenase needed from the "new" collagenase commercial lot was as follows: Comparison of the values of enzyme activities between the "old" [collagenase 1] and the "new" [collagenase 2] commercial lots of collagenase was used to determine the amount of collagenase 2 likely to produce the same effects as collagenase 1. Isolation of renal cortical tissue from eight rat kidneys was performed (*vide supra*). The tissue suspension was divided in half after mincing, and each sample was digested with either collagenase 1 or 2. The two tissue suspensions were treated separately for the rest of the purification process.

Aliquots (1 *ml*) of the suspension being digested with collagenase 2 were taken during the digestion at 15-minute intervals (total of three aliquots). The aliquot was diluted to 3 *ml* with ice-cold KHS immediately after collection and placed in ice to retard further digestion. Microscopic examination of the aliquots was performed immediately to assess separation and integrity of the tubules. At the time that visual and microscopic examination of the tubules suggested that connective tissue digestion was finished, the tissue sample was passed through the strainer. After purification of the tubule fragments by Percoll density gradient centrifugation, enzyme markers were quantified to assess purity of the fractions, and amantadine uptake assays were done on the purified tubules from both collagenase preparations to assess viability. The amount and time of incubation were adjusted based on the results obtained for collagenase 2 so that results with this "new" amount best reflected those results obtained with collagenase 1.

PART 2: TRANSPORT STUDIES

Part 2.1: Bicarbonate and amantadine uptake.

2.1.1 Transport studies in the presence of bicarbonate. Each assay tube (12 x 75 mm, Kimax® 51 borosilicate glass disposable culture tubes) contained an aliquot (50 µl) of the proper tubule suspension in the buffer that contained the proper concentration of non-labelled amantadine plus 31.3 *p*Ci of [^{3}H]amantadine (constant in all concentrations of non-labelled amantadine) in the presence or absence of any compounds to be tested. The tubes (total volume of 200 µl) were incubated with shaking (100 oscillations per minute) in a Dubnoff incubator for 30 sec at 25°C. After 30 seconds, 4 *ml* of ice-cold KHS were added and this suspension was filtered through glass filters (No. 32, Schleicher and Schuell, Inc. Keene NH) under negative pressure (450 mm Hg). The addition of 4 *ml* of buffer into the assay tube and filtration was repeated twice. Approximately 20 seconds after the last 4 *ml* had been filtered, the filters were removed from the filter box and counted in a Beckman model LS5801 scintillation counter using Ready Protein (Beckman

Instruments Inc., Fullerton CA) as cocktail. All measurements were performed in triplicate and their average used as a single measurement. Background radioactivity and non-specific uptake were determined by performing uptake studies using twice-frozen tissue, in duplicate. They were consistently 20% of control values (independent of the concentration of non-labelled amantadine used). Background radioactivity and non-specific uptake were subtracted from total radioactivity prior to determination of energy-dependent amantadine transport rates.

2.1.2 Transport studies in the absence of bicarbonate. Phosphate buffer was used as a representative solution of non-bicarbonate buffers. Sodium bicarbonate was replaced with sodium phosphate buffer (15 *m*M, pH 7.4, adjusted with NaOH) in a solution that contained (in millimolar quantities): NaCl, 135; KCl, 4.7; $MgCl_2$, 1.2; KH_2PO_4, 1.4; $CaCl_2$, 1; and glucose, 11. In other non-bicarbonate buffers, sodium bicarbonate was substituted with Tris-HEPES buffer (10 mM), acetate buffer (10 mM) or DL-lactate (racemic, 5 mM) buffer in solutions that contained (in millimolar quantities): NaCl, 135; KCl, 4.7; $MgCl_2$, 1.2; KH_2PO_4, 1.4; $CaCl_2$, 2.5 and glucose, 11 (pH adjusted with NaOH). All these buffers were iso-osmotic (osmolality adjusted with NaCl). Osmolality and pH were adjusted with NaCl and NaOH, respectively. Background radioactivity and non-specific uptake were determined by performing uptake studies using twice-frozen tissue, in duplicate. They were consistently 50% of total radioactivity (independent of the concentration of non-labelled amantadine used). Background radioactivity and non-specific uptake were subtracted from total radioactivity prior to determination of energy-dependent amantadine transport rates.

For experiments where bicarbonate was added back to non-bicarbonate buffers, the pH and osmolality of the incubating medium previous to the addition of bicarbonate were adjusted so that the final solution (buffer plus bicarbonate) was at physiological osmolality and pH (7.4). Adjustment of osmolality was done as follows: prior to the

experiment, osmolality of the phosphate buffer was measured with an osmometer (μOsmette micro osmometer, Precision Systems Inc., Natick, Mass., USA) before and after the addition of 20 μl of a sodium bicarbonate solution (25 *m*M final concentration in assay tube) to 180 μl of phosphate buffer. The increase in osmolality in the phosphate buffer due to the added sodium bicarbonate was determined. Based on such a measurement, osmolality of the phosphate buffer used for the transport assays was decreased by the same amount *via* manipulation of the sodium chloride content. Bicarbonate was added back to the buffer after oxygenation with 95-5% O_2-CO_2 gas, so that the CO_2 tension was kept constant at all bicarbonate concentrations in all buffers used. A similar approach was used to adjust the pH of the incubation medium when the assay included addition of bicarbonate to non-bicarbonate buffers. Amantadine transport (10 μM) by both tubule fragments was determined. Quinine and quinidine inhibition studies in KHS buffer were done with a different batch of tubules than those used for determinations in phosphate and phosphate-plus-bicarbonate buffers because of tissue constraints.

Part 2.2: Lactate and amantadine uptake

2.2.1 Lactate studies in the presence of bicarbonate. For experiments where lactate was added to the KHS, the pH and osmolality of the buffer were adjusted (as explained in section 2.1.2 above) prior to the addition of lactate (as a phosphate solution) so that the final incubating medium (buffer plus lactate) reflected physiological conditions (300 *m*Osmol / *k*g H_2O and pH of 7.4).

2.2.2 Transport studies in lactate buffers in the absence of bicarbonate. Phosphate buffer was used to resuspend the tissue when the absence of bicarbonate was necessary. Where different mixtures of D(-)- and L(+)-lactate were used, buffers of the

pure enantiomers were made and mixed in the proper ratios to a final total concentration of 5 *m*M. Osmolality and pH were adjusted with NaCl and NaOH, respectively.

The series of experiments where kinetic parameters were measured in KHS, DL-, L(+)- and D(-)-lactate were done separately from those done in DL-, and L(+)/D(-)-lactate enantiomeric mixtures because of tissue constraints. However, results were consistent and replicable within sets. The DL-, L(+)- and D(-)-lactate solutions used for both sets of experiments were from the same commercial lot. Composition of the racemate as well as the purities of the L(+)- and D(-)-lactate solutions were confirmed using polarimetry (Autopol III Automatic Polarimeter, Rudolph Research, Flanders, NJ, USA).

PART 2.3: Ion manipulations.

2.3.1 Potassium and calcium manipulation studies: Lower-than-physiological potassium concentrations were achieved by addition of the tissue (resuspended in normal KHS, 6 *m*M K^+) into KHS containing different potassium concentrations, e.g. 50 µl of tissue (6 *m*M) plus 150 µl of KHS (2 *m*M) to yield a final concentration of 3 *m*M K^+. The lowest potassium concentration achieved was 1.5 *m*M (1 in 4 dilution of 6 *m*M KHS) as the tissue was maintained in normal K^+ (6 *m*M, KHS buffer). The highest K^+ concentration tested was 100 *m*M.

A similar approach was followed for manipulation of calcium concentrations. Tissue was re-suspended in phosphate buffer (1 *m*M Ca^{2+}) and the lower and higher calcium concentrations were obtained by dilution into buffers (KHS or phosphate) with adjusted calcium content. The lower concentration tested was 0.25 *m*M (1 in 4 dilution of phosphate buffer into KHS). Because of the phosphate present in both buffers, the highest calcium concentration tested was 5 *m*M. Higher calcium concentrations resulted in the formation of a white precipitate. The pH and osmolality of all preceding incubating buffers were physiological (pH adjusted with NaOH and osmolality adjusted with NaCl).

2.3.2 Sodium and chloride manipulation studies. Low sodium and low chloride buffers were made by replacing sodium chloride with lithium chloride (118 *m*M), and sodium gluconate (118 *m*M), in a buffer that contained (in millimolar quantities): KCl, 4.7; $MgCl_2$, 1.2; KH_2PO_4, 1.4; $NaHCO_3$, 25; $CaCl_2$, 2.5; and glucose, 11 (pH adjusted with NaOH). Both buffers possessed a pH of 7.4 and were isotonic (300 *m*Osmol/kg H_2O; osmolality adjusted with NaCl). However, the low sodium buffer contained 25 *m*Eq/l of sodium (from $NaHCO_3$), and the low chloride buffer contained 12.1 *m*Eq/l of chloride (from KCl, $MgCl_2$, and $CaCl_2$).

PART 2.4: Na^+/K^+ ATPase studies.

2.4.1 Glycosides and amantadine uptake. Tissue was pre-incubated in KHS in the presence of the modulator to be tested (e.g. ouabain) with shaking in a Dubnoff incubator at 25°C for 10 minutes. After such time, 20 µl of an amantadine solution that contained [^{3}H]amantadine in tracer amounts was added (final concentration, 10 µM). The rest of the assay was carried out in the same manner as described in the "transport studies" section above.

Digoxin and acetylstrophanthidin are very lipophilic compounds and hence they were added to the assay tubes as an ethanol solution (95%, v/v). Therefore, the final assay tube contained 0.95 % (2 µl of an ethanol solution into 198 µl reaction buffer, 165 *m*M) ethanol. Experiments were also performed in which digoxin and acetylstrophanthidin were dissolved in dimethylsulfoxide (DMSO, final content 10% [v/v]) with results similar to those performed with ethanol as the solvent. Due to precipitation of the modulators, the highest concentration tested for digoxin and acetylstrophanthidin was 500 µM.

2.4.2 Ouabain inhibition studies with sodium and potassium concentration manipulations. Sodium chloride was replaced with LiCl (118 *m*M) in a K^+-free buffer that contained (in millimolar units): $MgCl_2$, 1.2; $NaHCO_3$, 25; $CaCl_2$, 2.5; and glucose, 11 (pH adjusted with NaOH). The lowest concentration of potassium tested was 0.375 *m*M. This low concentration was achieved by resuspending the final pellet of tissue with normokalemic/low sodium buffer in 1/4 of the necessary volume and then diluting the aliquot to be used in the assay 1 in 4 with potassium-free buffer prior to addition to the reaction tube.

PART 3: CHEMICALS.

[^{3}H]Amantadine (350 *m*Ci/*m*mol) was obtained from Amersham International (Buckinghamshire, UK); unlabeled amantadine was obtained from Dupont Canada, Inc. (Mississauga, Ontario, Canada); collagenase was obtained from Boehringer Mannheim (Laval, Quebec, Canada) and Percoll was obtained from Pharmacia Biotechnology (Baie D'urfe, Quebec, Canada); quinine, quinidine, D(-), L(+), DL sodium lactate, ouabain, ouabagenin, acetylstrophanthidin and digoxin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest grade available from commercial suppliers.

PART 4: DATA ANALYSIS.

Data are expressed as mean $\pm$ standard error (SE) of five individual experiments, unless otherwise noted. Specific uptake (non-specific uptake subtracted) of amantadine by the tubules (transport rates) is expressed as *n*mol / *m*g of tubule protein / minute. Apparent Km and Vmax values were obtained using non-linear regression fitting of the data, with the graphics program Fig.P (Biosoft®, Ferguson, MO, U.S.A), the Lineweaver-Burk (1934), and the Eadie-Hoffstee plot (1942), by measuring amantadine accumulation by the tubules at different amantadine concentrations in the incubation medium. K_i values

were determined by measuring tubule amantadine accumulation in the respective buffer under increasing concentrations of the respective inhibitor (Cheng and Prusoff, 1973). The type of inhibition was confirmed using the complimentary plots described by Dixon (1953) and Cornish-Bowden (1974) in which tubule amantadine uptake rates were measured under increasing concentrations of the inhibitors at two different concentrations of amantadine.

Theoretical rates of amantadine transport in all lactate buffers (Part II, Results section) were calculated using the Michaelis-Menten equation:

$$v = [(Vm * S) / (Km + S)] \qquad [1]$$

where, v is the theoretical amantadine transport rate in the respective lactate buffer; S is the concentration of amantadine; Km and Vmax are the measured kinetic parameters in the different lactate buffers. Theoretical rates in DL-lactate were also calculated with the kinetic parameters obtained in pure L(+)- and D(-)-lactate buffers using the following formula:

$$v_{(DL)} = [(Vm_{L(+)} * S) / (Km_{L(+)} + S)] + [(Vm_{D(-)} * S) / (Km_{D(-)} + S)] \qquad [2]$$

where, $v_{(DL)}$ is the theoretical amantadine transport rate in DL-lactate buffer; S is the concentration of amantadine; $Km_{L(+)}$, $Km_{D(-)}$, $Vm_{L(+)}$, and $Vm_{D(-)}$ are the Km and Vmax values obtained for amantadine uptake in buffers of the pure lactate enantiomers.

Statistical analysis was performed using Analysis of Variance (ANOVA). Generally, comparison of multiple groups was done only within tubules. Multiple comparisons following significant ANOVA were done using Tukey's Honestly Significant Difference (HSD) post-hoc test. Student's *t*-test was applied whenever necessary. A

value of $P<.05$ was accepted as significant. All statistical analyses were completed with the computer program SYSTAT® (Intelligent Software, Evanston, IL, USA).

RESULTS

PART I: BICARBONATE AND ORGANIC CATION UPTAKE

In KHS, apparent Km (Fig. R-1) and apparent Vmax (Fig. R-2) values for uptake of amantadine were not different between proximal and distal tubule preparations. The absence of bicarbonate from the phosphate buffer resulted in significantly decreased amantadine accumulation by both proximal and distal tubules. Apparent Km increased 3.7-fold for proximal and 2.4-fold for distal tubules. There was also a concomitant decrease of Vmax for proximal and distal tubules to 50% and 30% of those parameters measured in KHS, respectively.

Kinetic parameters were determined also in three other buffer systems: Tris-HEPES (10 mM), acetate (5 mM) and lactate (10 mM). Qualitatively, all non-bicarbonate buffer systems, except for lactate, behaved similarly (Figs. R-1 and R-2), that is, apparent Km values for amantadine uptake increased and apparent Vmax values decreased. In lactate buffer, Km increased (Fig. R-3) but there was no change in Vmax (Fig. R-4) for both tubule fragments. For proximal tubules, the lowest apparent Km value in non-bicarbonate solutions was obtained in Tris-HEPES buffer and the highest in DL-lactate buffer. The apparent Km values for phosphate and acetate were almost identical and in between those for Tris-HEPES and lactate. Apparent Vmax in phosphate buffer was 1.3- to 1.7-fold higher than Vmax in Tris-HEPES and acetate buffers for proximal tubules. For distal tubules, the apparent Km for amantadine uptake in lactate was the highest. Apparent Km for Tris-HEPES and acetate buffers were 40-50% lower than lactate, and approximately two-fold higher than phosphate.

Using the measured kinetic values, theoretical uptake rates of amantadine by proximal and distal tubules at the pharmacologically-relevant amantadine concentration of 10 μM were calculated (Fig. R-5). Rates of uptake by proximal tubules in the non-bicarbonate buffers were all significantly lower than those in KHS buffer. Calculated rates

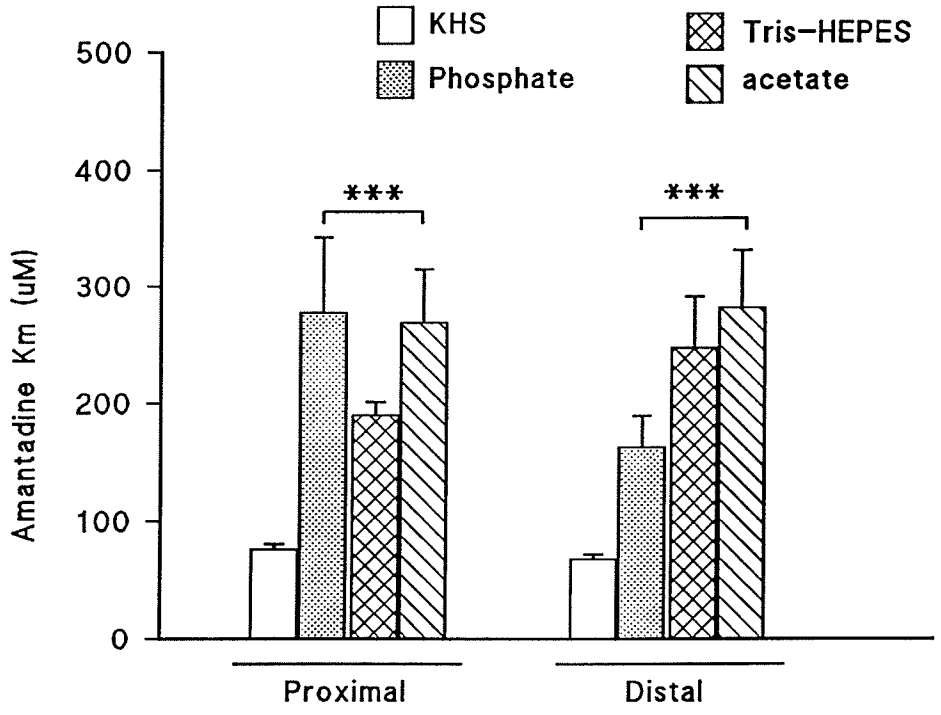


FIGURE R-1. Measured apparent Km values for transport of amantadine by proximal and distal tubules, in presence and absence of bicarbonate in the incubation medium. KHS (open), phosphate (solid), Tris-HEPES (cross-hatched) and acetate (right-hatched) buffers. All buffers were iso-osmotic and at the same pH (7.4). *** $P < .001$ when compared to its own bicarbonate control using repeated-measures ANOVA followed by Tukey's HSD post-hoc test.

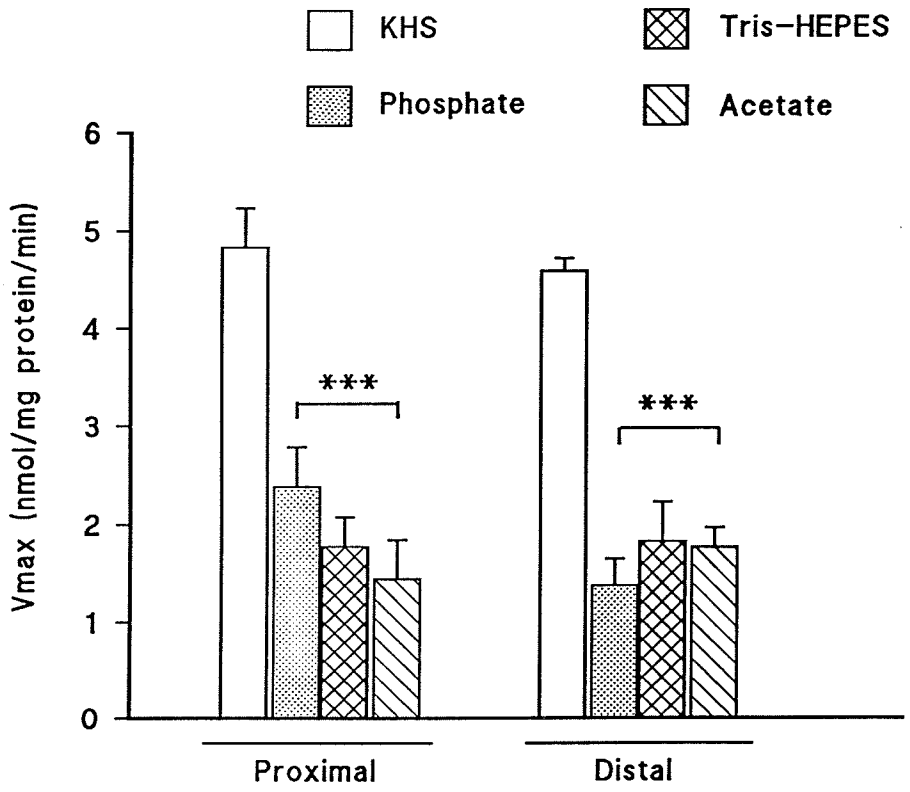


FIGURE R-2 Measured apparent Vmax values for transport of amantadine by proximal and distal tubules, in presence and absence of bicarbonate in the incubation medium. KHS (open), phosphate (solid), Tris-HEPES (cross-hatched) and acetate (right-hatched) buffers. All buffers were iso-osmotic and at the same pH (7.4). *** $p<0.001$ when compared to its own bicarbonate control using repeated measures ANOVA followed by Tukey's HSD post-hoc test.

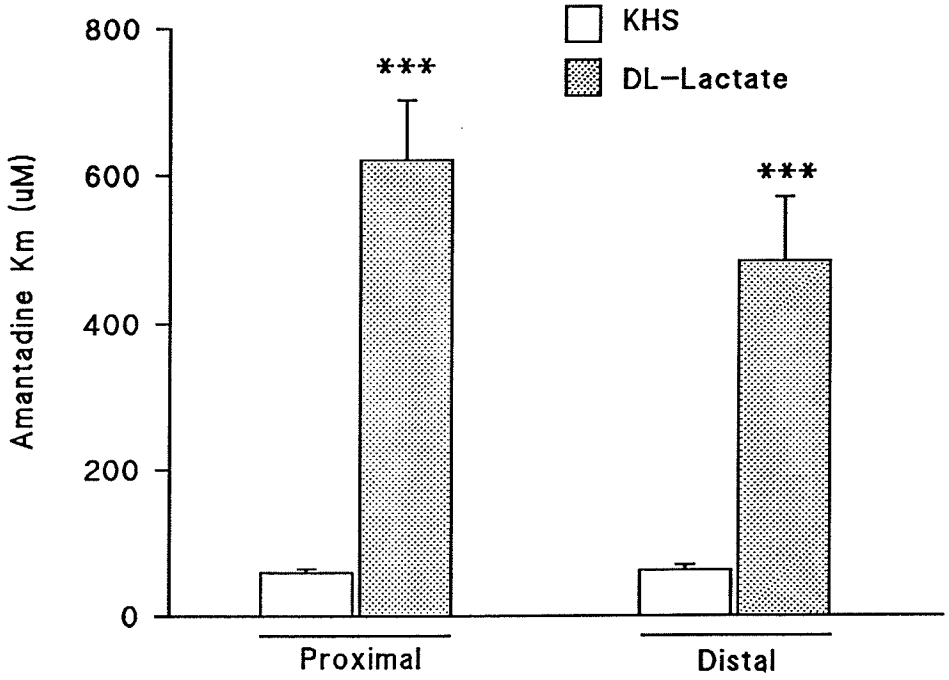


FIGURE R-3 Determined apparent Km values for transport of amantadine by proximal and distal tubules in KHS (open bars) and lactate (solid bars). *** $p<0.001$ when compared to its own bicarbonate control using two-way ANOVA, with tubule and buffer as variables, followed by Tukey's HSD post-hoc test.

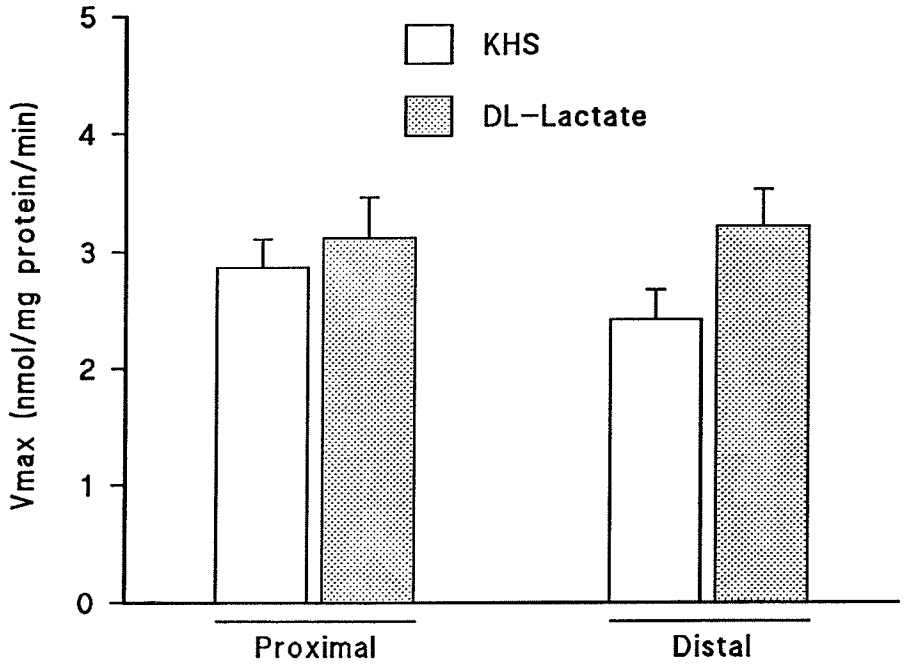


FIGURE R-4 Determined apparent Vmax values for transport of amantadine by proximal and distal tubules in KHS (open bars) and lactate (solid bars). Vmax values between KHS and lactate buffers in distal tubules did not reach statistical significance using two-way ANOVA with tubule and buffer as variables ($p<0.10$).

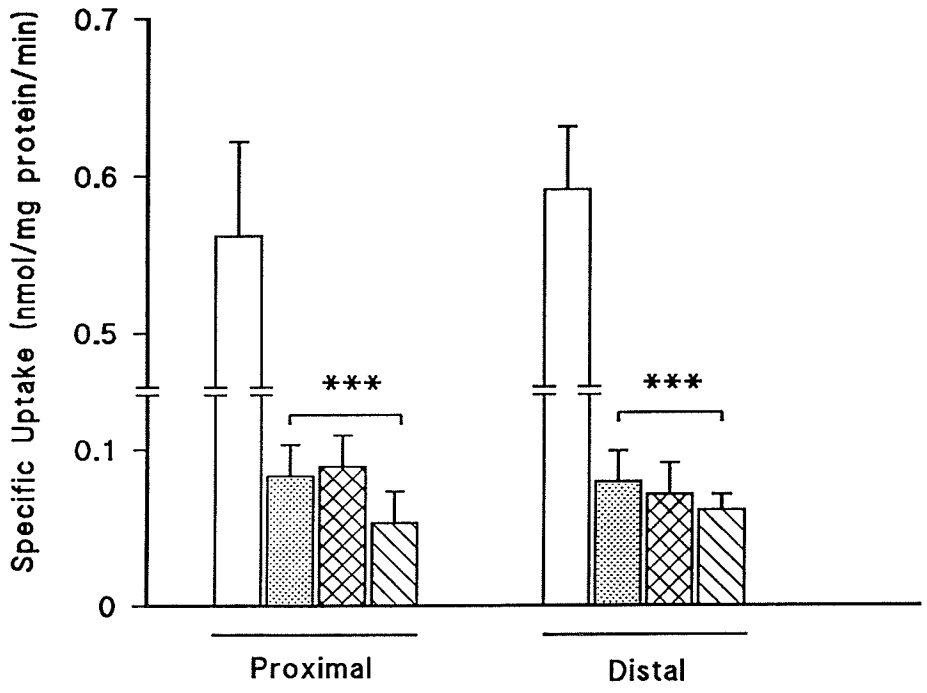


FIGURE R-5 Calculated values for amantadine transport in the different buffers using the kinetic parameters measured (refer to Figs. R-1 and R-2). Calculated transport rates in KHS (open), phosphate (solid), Tris-HEPES (cross-hatched) and acetate (right-hatched) buffers. *** $p<0.001$ when compared to bicarbonate control using repeated measures ANOVA followed by Tukey's HSD post-hoc test.

for amantadine uptake in phosphate and Tris-HEPES solutions were higher than those calculated for acetate buffer. In the same buffer, rates for uptake between proximal and distal tubules were not different. Calculated rates in lactate buffer (Fig. R-6) were 10 to 20 % of those in their respective KHS controls.

Since the data suggested that all buffers, except for DL-lactate, behaved qualitatively similarly, the following studies show only results obtained in phosphate buffer as a representative solution of the non-bicarbonate buffers. The presence of acetazolamide (ACZ) did not have an effect on amantadine uptake by either proximal (Fig. R-7) or distal (Fig. R-8) tubules in the presence of bicarbonate. In phosphate buffer there was an increase in amantadine accumulation by both tubular suspensions starting at 300 μM that did not reach statistical significance until 1 *m*M ACZ. This increased amantadine accumulation was observed to be 2.85-fold (increased to 35% of its own bicarbonate control) for the proximal and 2.44-fold for the distal tubules (increased to 33 % of its own bicarbonate control).

Amantadine (10 μM) uptake rates by tubule fragments were determined by incubating the tissue in phosphate buffer under increasing external bicarbonate concentrations up to 35 mM (Fig. R-9). The uptake rates at zero bicarbonate concentration were as predicted by our initial kinetic measurements and similar between tubules. As bicarbonate increased extracellularly, accumulation of amantadine increased. The rate of increased accumulation of amantadine was similar for proximal and distal tubules and appeared to follow the kinetics of a saturable system.

The pooled data for this set of experiments were plotted (Fig. R-9) and visual inspection of the graph suggested that distal tubules were reaching a plateau (bicarbonate saturation) before proximal tubules and before the 25 mM bicarbonate concentration. However, analysis of the individual experiments using the Michaelis-Menten approach to enzyme kinetics was performed and the calculated values for maximal bicarbonate-

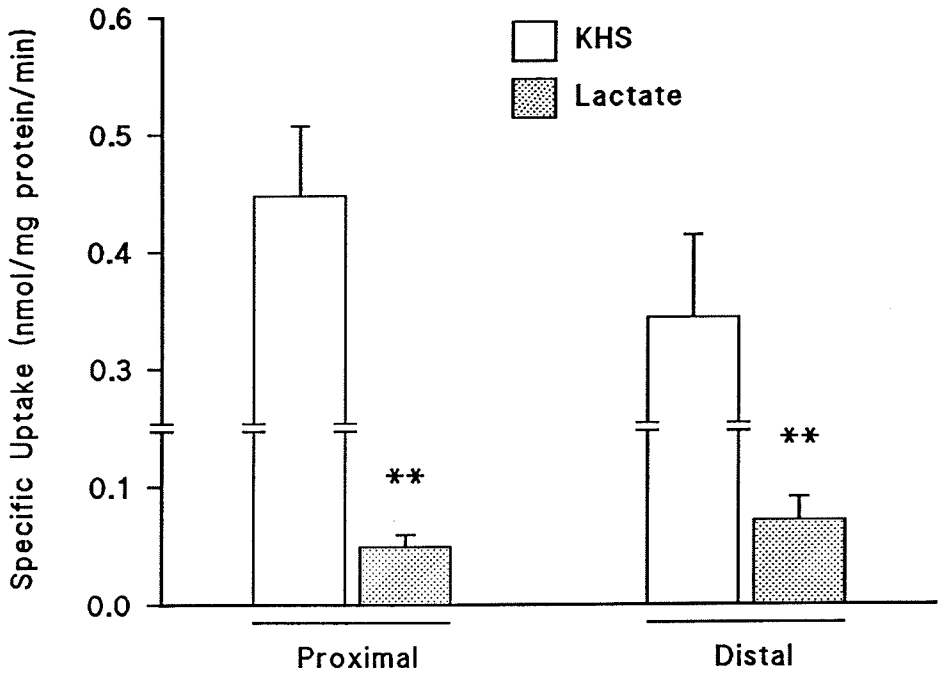


FIGURE R-6 Calculated transport rates for amantadine (10 μM) in KHS (open) and lactate (solid) buffers using the kinetic parameters measured (refer to Figs. R-3 and R-4). ** $P < .01$ when compared to bicarbonate control using repeated measures ANOVA followed by Tukey's HSD post-hoc test.

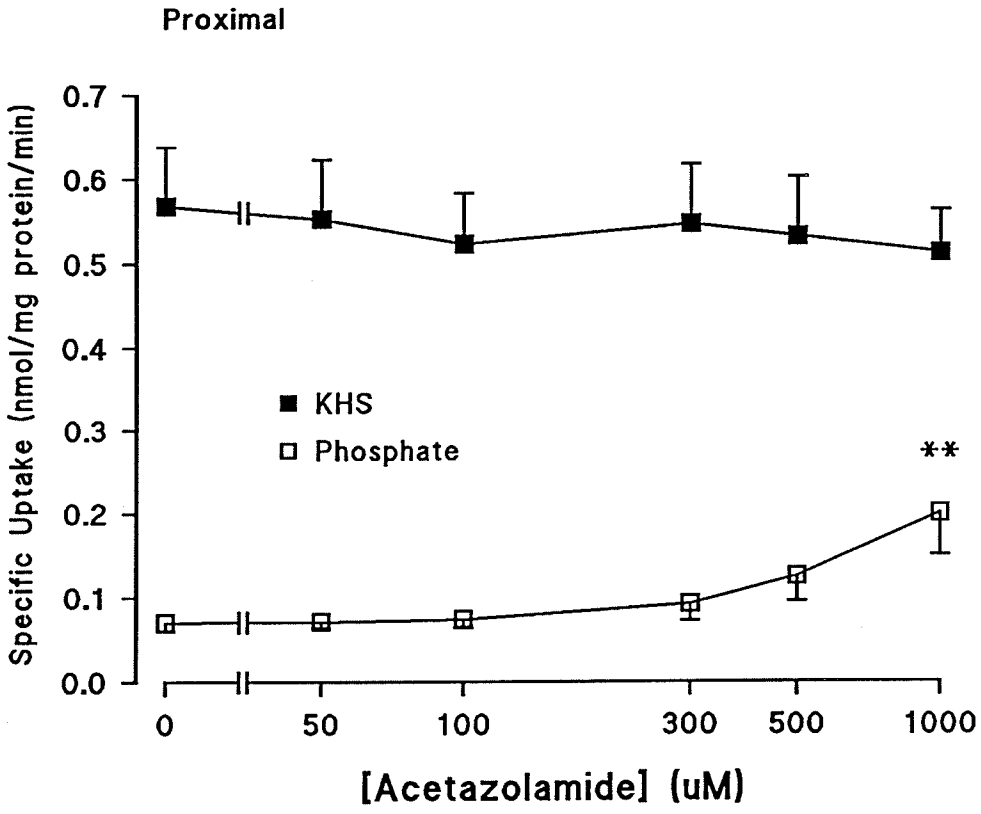


FIGURE R-7 Effect of increasing concentrations of carbonic anhydrase inhibitor, acetazolamide (ACZ), on amantadine (10 μM) transport rates in proximal tubules in KHS (■) and phosphate (□) buffers as incubation medium (n=6). Tissue was incubated for 30 seconds in the presence of ACZ previous to the addition of [^{3}H] amantadine. ** $p<0.01$ when compared to control using repeated measures ANOVA followed by Tukey's HSD post-hoc test.

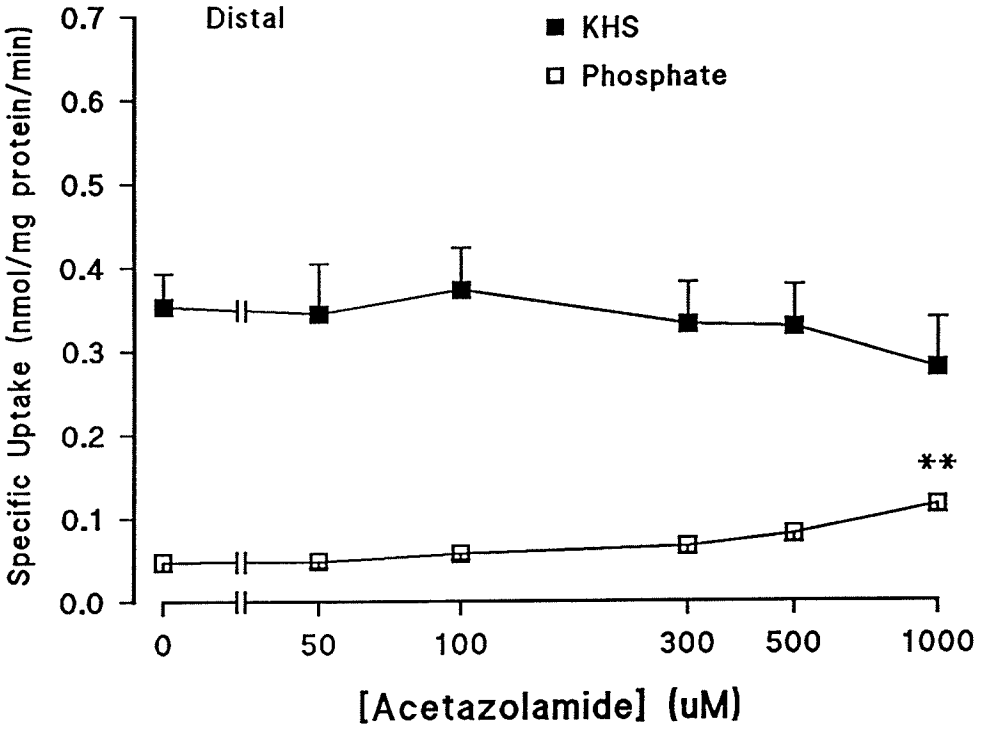


FIGURE R-8 Effect of increasing concentrations of carbonic anhydrase inhibitor, acetazolamide (ACZ), on amantadine (10 μM) transport rates in distal tubules in KHS (■) and phosphate (□) buffers as incubation medium (n=6). Tissue was incubated for 30 seconds in the presence of ACZ previous to the addition of [^{3}H] amantadine. ** $p<0.01$ when compared to control using repeated measures ANOVA followed by Tukey's HSD post-hoc test.

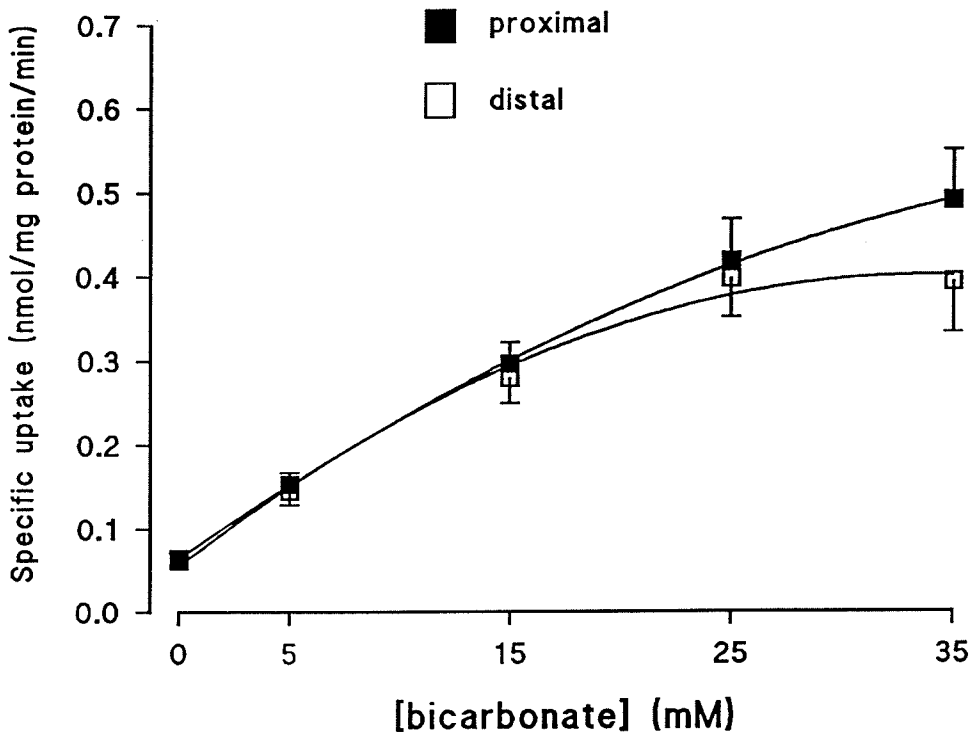


FIGURE R-9 Effect of increasing extracellular bicarbonate concentration on amantadine transport rate (10 μM) for proximal (n=7, ■) and distal (n=6,□) tubules. Tissue was incubated in the respective buffer with bicarbonate and [^{3}H] amantadine concomitantly for 30 seconds.

enhanced uptake rate at 10 μM amantadine ($V_{10\ \mu M}max$), a value analogous to Vmax of the Michaelis-Menten isotherm, were similar between tubules, 0.80 ± 0.20 for proximal and 0.73 ± 0.10 nmol/mg protein/min for distal tubules. As well, when calculation of the bicarbonate concentration that is needed to reach half maximal bicarbonate-enhanced amantadine uptake, analogous to Km, was performed for the individual experiments, similar values were obtained between tubules (22.4 ± 7.0 and 22.1 ± 4.6 mM for proximal and distal tubules, respectively).

PART II: LACTATE

Apparent Km for amantadine uptake in KHS (Fig. R-10) was lower than values measured in racemic, L(+)-, and D(-)-lactate for both tubules. For proximal tubules, Km for amantadine uptake in racemic lactate was of the same magnitude as that in D(-)-lactate. The highest Km was obtained in L(+)-lactate buffer. In distal tubules, apparent Km for uptake in racemic and L(+)-lactate buffers was similar, but higher than D(-)-lactate. Apparent Km in DL-lactate for proximal tubules was lower than for distal tubules.

Vmax for amantadine uptake in KHS and racemic lactate were not different within and between tubules (Fig. R-11). However, Vmax in L(+)- and D(-)-lactate were similar, but lower than both KHS and DL-lactate. The same qualitative results were obtained for distal tubules. Vmax in racemic lactate was similar to that in KHS, but those in L(+)- and D(-)-lactate were lower. Vmax in D(-)-lactate was higher for proximal than for distal tubules.

Kinetic parameters were measured also in buffers with 3.75D(-)/1.25L(+) and 1.25D(-)/3.75L(+) enantiomeric mixtures of lactate, using 2.5D(-)/2.5L(+) (DL racemate) as control. Apparent Km (Fig. R-12) for amantadine uptake in 3.75D(-)/1.25L(+) and 1.25D(-)/3.75L(+) mixtures for the proximal tubules did not reach statistical

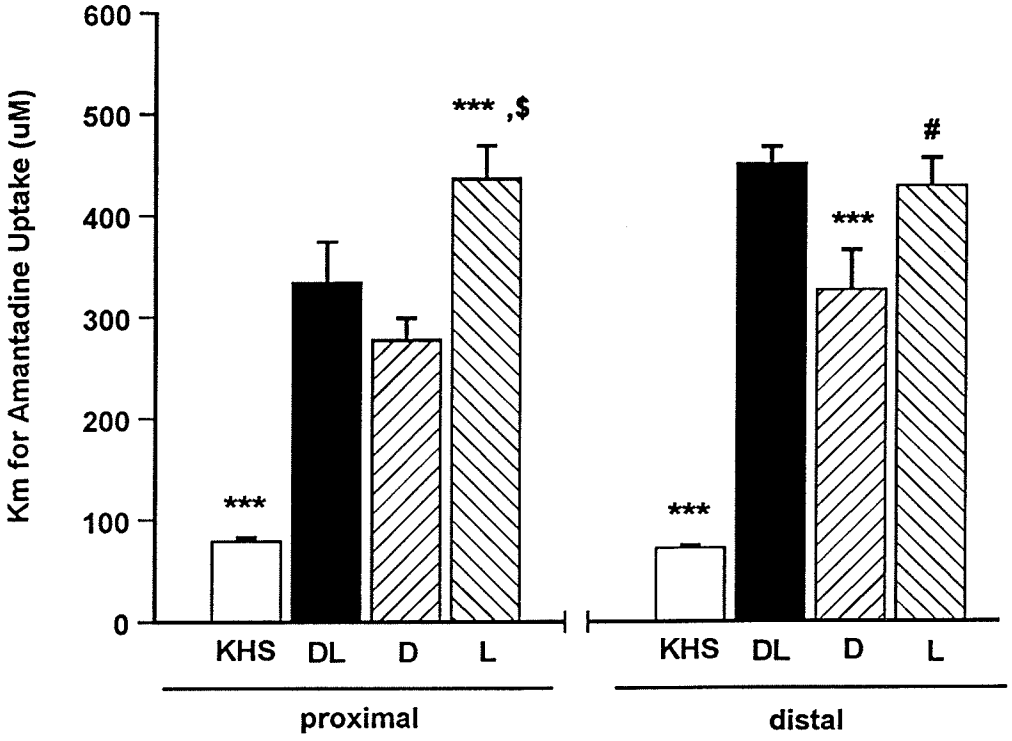


FIGURE R-10 Measured apparent Km values for amantadine uptake by proximal and distal tubules in bicarbonate buffer (Krebs-Henseleit, KHS), racemic (DL, 5 mM) and enantiomerically pure L(+)- and D(-)-lactate buffers (5 mM). All lactate buffers were bicarbonate-free, iso-osmolar and at physiological pH. ***p<.001 vs DL-lactate of proximal tubules, $ p<.001 vs D-lactate of distal tubules, # p<.001 vs D-lactate by repeated-measures ANOVA followed by Tukey's HSD post-hoc test.

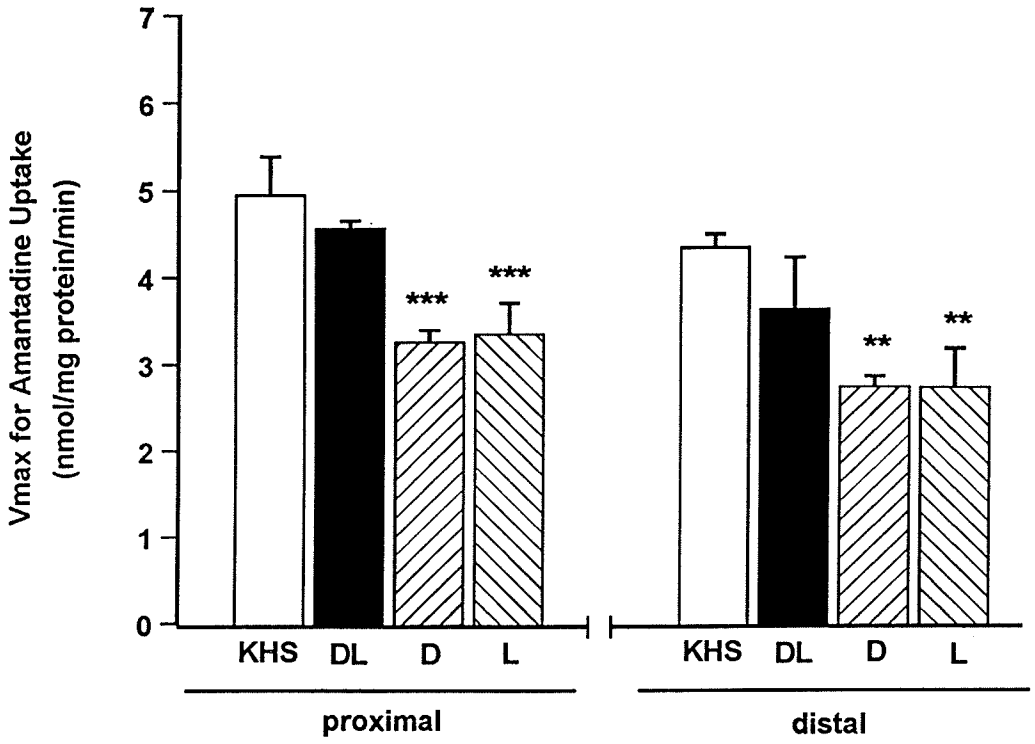


FIGURE R-11. Measured apparent Vmax values for amantadine uptake by proximal and distal tubules in bicarbonate buffer (Krebs-Henseleit, KHS), racemic (DL, 5 mM) and enantiomerically pure L(+)- and D(-)-lactate buffers (5 mM). All lactate buffers were bicarbonate-free, iso-osmolar and at physiological pH. **p<.01 vs DL-lactate, ***p<.001 vs DL-lactate by repeated-measures ANOVA followed by Tukey's HSD post-hoc test.

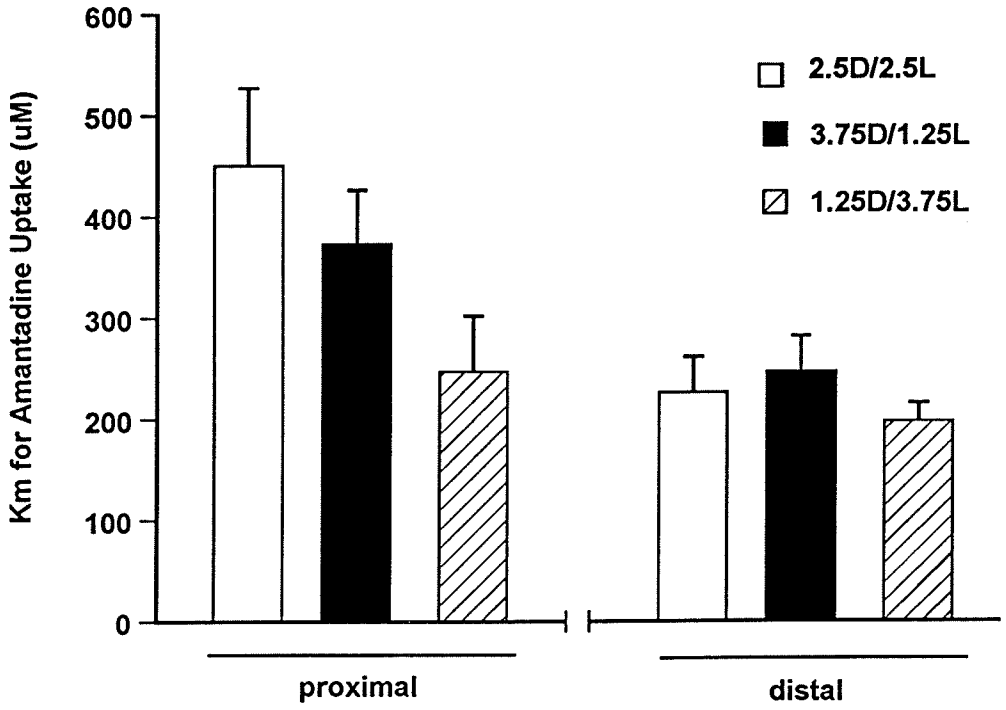


FIGURE R-12 Measured apparent Km values for amantadine uptake by proximal and distal tubules in racemic lactate (DL), and two other mixtures of the enantiomers. All buffers were bicarbonate-free and contained a total lactate concentration of 5 *m*mol / l. No significant differences were found by repeated-measures ANOVA ($P < .09$).

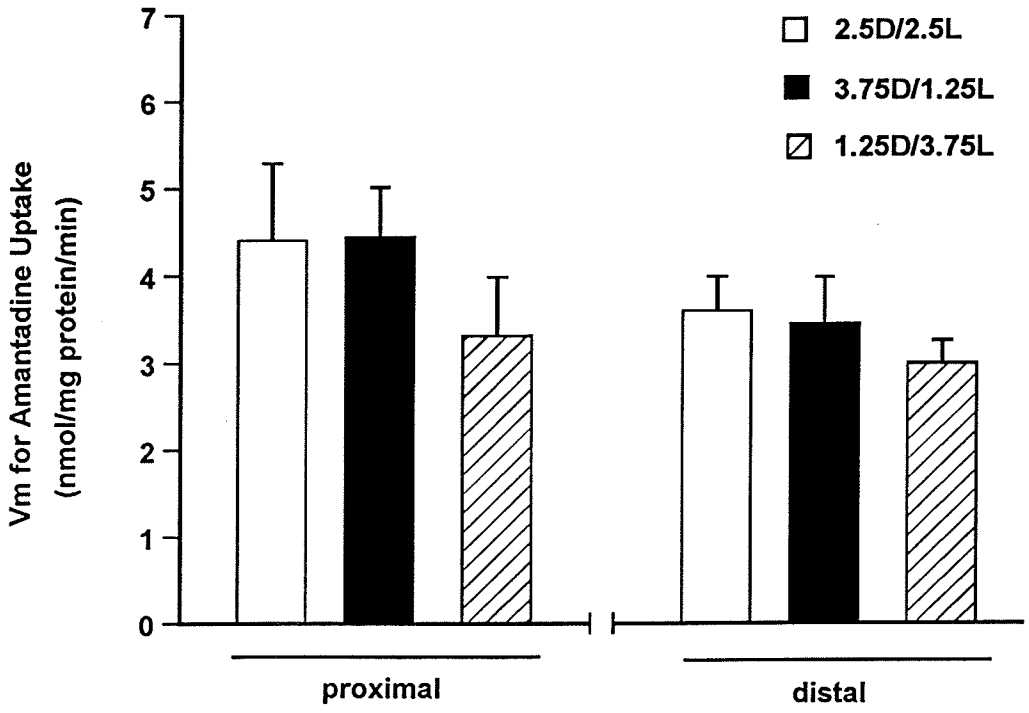


FIGURE R-13 Measured apparent Vmax values for amantadine uptake by proximal and distal tubules in racemic lactate (DL), and two other mixtures of the enantiomers. All buffers were bicarbonate-free and contained a total lactate concentration of 5 *m*mol/l. No significant differences were found by repeated-measures ANOVA ($p<.09$).

significance when compared to racemic lactate. Apparent Km for amantadine uptake by distal tubules was not different among 3.75D(-)/1.25L(+), 1.25D(-)/3.75L(+), and racemic lactate buffers. Values for Vmax (Fig. R-13) were similar for all mixtures of L(+)- and D(-)-lactate, for proximal and distal tubules.

The observed and calculated (using equations [1] and [2]; see *Methods*) uptake rates for amantadine in the different lactate buffers are presented in Table R-1 and Table R-2. For proximal tubules (Table R-1), observed rates in DL-lactate were similar to those calculated using equations [1] and [2]. Observed and calculated rates in L(+)- and D(-)-lactate buffer were similar within tubules. Moreover, observed and calculated rates in L(+)- and D(-)-lactate buffers were lower than the observed and calculated rates in DL-lactate. For distal tubules, observed uptake rates in DL-lactate buffer were similar to those calculated with equation [1]. However, values calculated for DL-lactate with equation [2] over-estimated the observed rates. In contrast, equation [1] consistently underestimated observed rates for amantadine uptake in L(+)- and D(-)-lactate buffers. Calculated and observed rates for proximal and distal tubule amantadine uptake in lactate buffers of the various enantiomeric mixtures (Table R-2) were similar, within buffer, at all amantadine concentrations.

Lactate inhibited amantadine (10 μM) uptake by proximal and distal tubules in bicarbonate buffer in a dose-dependent manner (Figs. R-14 and R-15). Complete inhibition of uptake was obtained with 20 *m*M lactate. Amantadine uptake inhibition was graded between 1 and 15 *m*M lactate and approached 60 % in both tubule fragments. The remaining 40% of uptake was inhibited abruptly by increasing the lactate concentration to 20 *m*M, independently of the enantiomer used. Ki values calculated from individual experiments are presented in Figure R-16. Ki for racemic lactate inhibition of amantadine uptake was similar to D(-)- and L(+)-lactate for proximal and distal tubules. Dixon and Cornish-Bowden plots consistently indicated that the type of inhibition was competitive.

Table R-1: Comparison of uptake rates (nmol/mg protein/min) in different incubation buffers at amantadine concentrations used for determination of Michaelis-Menten parameters.

		[Amantadine] (μM)			
Proximal		20	50	100	200
KHS	observed	0.71±0.09 ***	1.57±0.11 ***	2.16±0.22 ***	3.58±0.35 ***
DL-lactate	observed	0.32±0.11 §	0.71±0.17 §	1.16±0.08 §	2.48±0.28 §
	calculated [a]	0.32±0.04	0.73±0.09	1.29±0.15	2.05±0.25
	calculated [b]	0.37±0.06	0.83±0.14	1.47±0.24	2.38±0.39
D(-)-lactate	observed	0.17±0.04	0.44±0.08	0.58±0.08	1.48±0.28
	calculated [a]	0.22±0.02	0.49±0.04	0.85±0.07	1.34±0.11
L(+)-lactate	observed	0.17±0.04	0.36±0.09	0.56±0.05	1.12±0.17
	calculated [a]	0.15±0.02	0.34±0.05	0.62±0.09	1.04±0.15

Table R-1 (continued)

Distal		[Amantadine] (μM)			
		20	50	100	200
KHS	observed	0.57±0.13 †	1.06±0.15 ‡	1.73±0.41 #	2.21±0.18 $
DL-lactate	observed	0.18±0.03	0.45±0.06	0.82±0.11	1.60±0.25
	calculated [a]	0.15±0.02	0.36±0.06	0.66±0.11	1.11±0.19
	calculated [b]	0.28±0.06**	0.64±0.13**	1.15±0.24*	1.89±0.39
D(-)-lactate	observed	0.26±0.04	0.52±0.06	0.83±0.09	1.41±0.26
	calculated [a]	0.16±0.02*	0.36±0.05*	0.64±0.08*	1.03±0.13*
L(+)-lactate	observed	0.22±0.05	0.49±0.09	0.82±0.12	1.64±0.26
	calculated [a]	0.12±0.02**	0.28±0.05**	0.51±0.10**	0.86±0.17**

[a] using equation [1]; [b] using equation [2] (see Methods); * P<.05 vs observed; ** P<.01 vs observed; *** P<.001 KHS vs all lactate buffers; § P<.001 DL- vs L- and D-lactate; † P<.019, ‡ P<.006, # P<.007, $ P<.002 KHS vs all lactate buffers.

Table R-2: Amantadine uptake rates (nmol/mg protein/min) in lactate buffers with different enantiomeric ratios

		[Amantadine] (μM)				
Proximal		50	100	200	300	500
2.5L(+)/2.5D(-)	observed	0.42±0.05	0.76±0.07	1.46±0.14	1.54±0.16	1.89±0.36
	calculated [a]	0.41±0.03	0.75±0.07	1.16±0.11	1.65±0.20	2.19±0.31
3.75L(+)/1.25D(-)	observed	0.38±0.05	0.63±0.09	1.10±0.22	1.45±0.32	1.95±0.31
	calculated [a]	0.55±0.08	0.93±0.13	1.43±0.22	1.76±0.28	2.15±0.36
1.25L(+)/3.75D(-)	observed	0.41±0.05	0.69±0.10	1.24±0.20	1.37±0.25	2.08±0.36
	calculated [a]	0.54±0.08	0.96±0.13	1.57±0.21	1.99±0.26	2.54±0.33
Distal						
2.5L(+)/2.5D(-)	observed	0.50±0.04	0.88±0.09	1.68±0.18	1.66±0.19	2.18±0.27
	calculated [a]	0.69±0.12	1.14±0.17	1.71±0.21	2.06±0.23	2.47±0.26
3.75L(+)/1.25D(-)	observed	0.54±0.05	0.98±0.13	1.68±0.17	1.89±0.30	2.11±0.15
	calculated [a]	0.62±0.08	1.03±0.13	1.52±0.17	1.82±0.20	2.15±0.22
1.25L(+)/3.75D(-)	observed	0.55±0.05	0.92±0.11	1.68±0.20	1.92±0.27	2.11±0.33
	calculated [a]	0.59±0.07	1.00±0.12	1.54±0.19	1.88±0.24	2.29±0.31

[a] using equation [1].

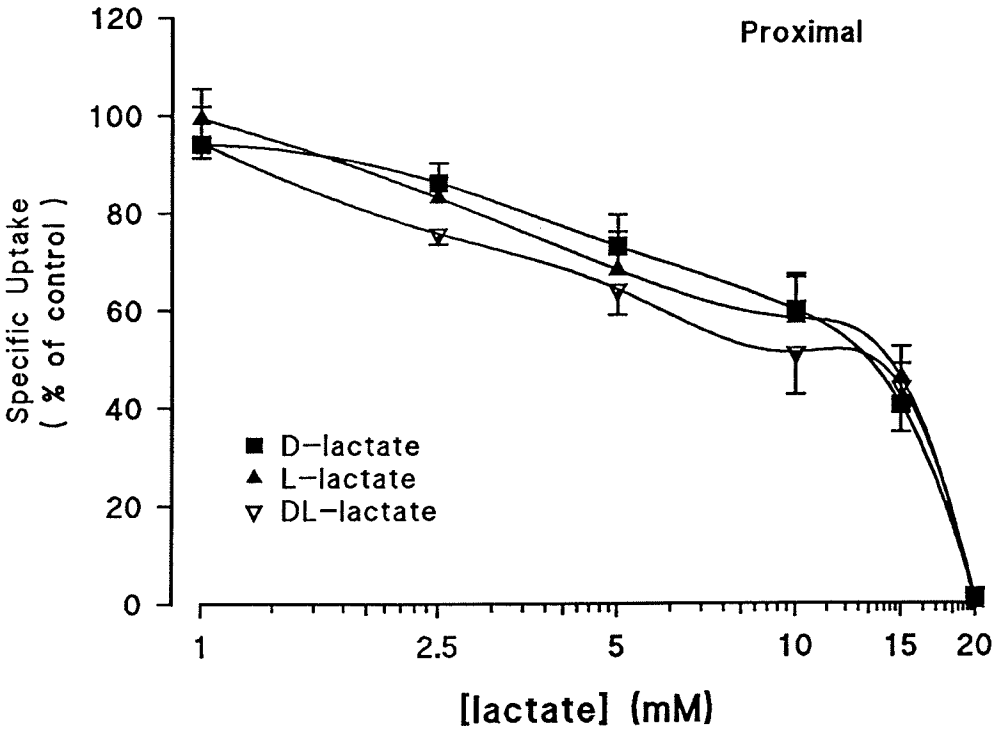


FIGURE R-14 Lactate inhibition curves of amantadine (10 μM) uptake in KHS by proximal tubules. Osmolarity and pH were kept constant at all lactate concentrations. At any one lactate concentration, amantadine transport rates were similar within tubule among the lactate mixtures.

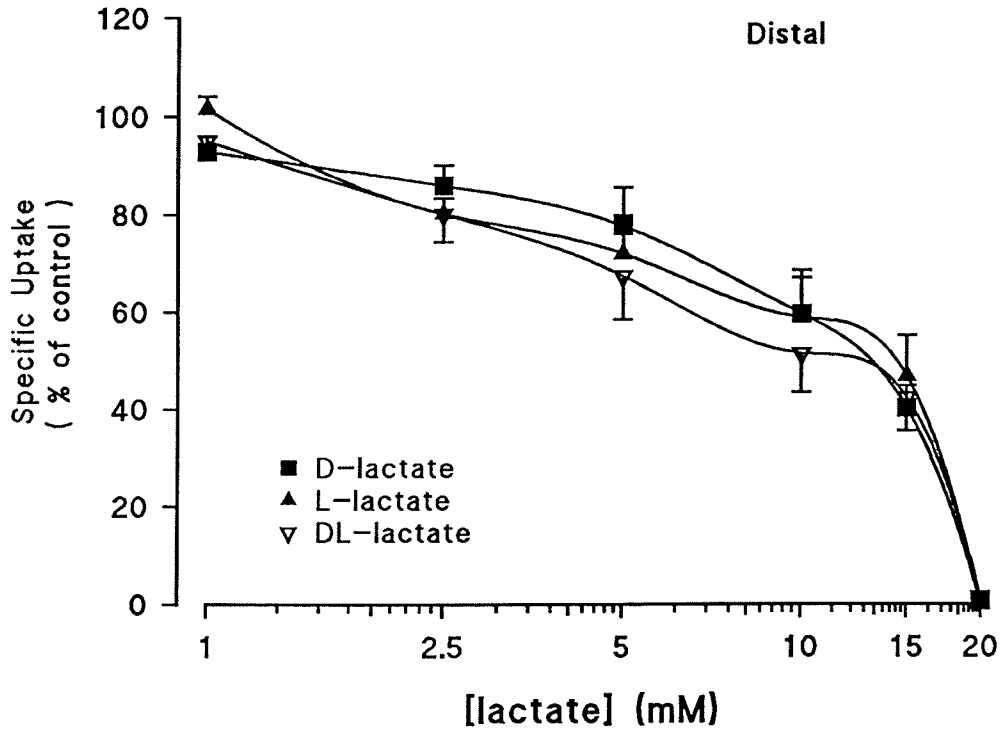


FIGURE R-15 Lactate inhibition curves of amantadine (10 μM) uptake in KHS by distal tubules. Osmolarity and pH were kept constant at all lactate concentrations. At any one lactate concentration, amantadine transport rates were similar among lactate mixtures.

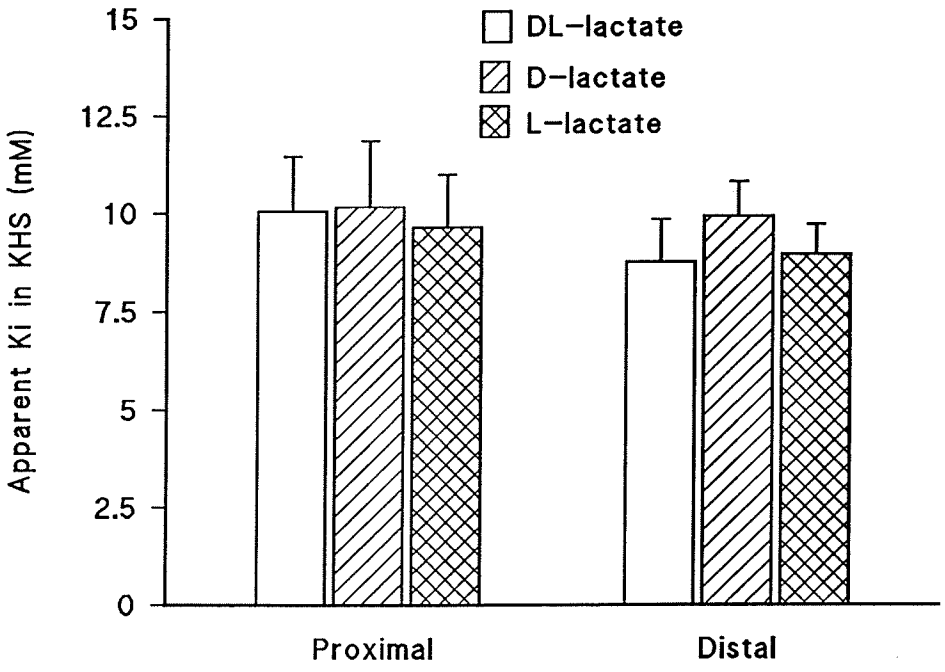


FIGURE R-16 Inhibitory constants (Ki) for amantadine proximal and distal tubule uptake inhibition by the pure lactate enantiomers and racemic lactate in KHS. No differences were found by repeated-measures ANOVA ($P<.77$ and $P<.32$ for proximal and distal tubule data, respectively).

PART III: QUININE AND QUINIDINE INHIBITION EXPERIMENTS:

Control Studies. Amantadine uptake (Fig. R-17) was lower in phosphate buffer than in KHS (10 $\pm$ 1 % and 17 $\pm$ 3 % of control, for proximal and distal tubules, respectively). Rates of uptake by the proximal tubule were higher than those by the distal tubule in KHS. Rates in phosphate buffer for amantadine uptake were not different between tubule fragments. Addition of bicarbonate (25 *m*M) to the phosphate buffer resulted in an increase in amantadine uptake to approximately control (KHS) levels. The rates in phosphate-plus-bicarbonate buffer were 80 $\pm$ 15 % and 150 $\pm$ 30 % of their respective controls (KHS) for proximal and distal tubules, respectively.

Inhibition Studies. Competitive inhibition of amantadine uptake was observed for quinine and quinidine, for both proximal and distal tubule fragments, and was independent of the buffer preparation. In KHS (Fig. R-18), apparent K_i for amantadine uptake inhibition by quinine was lower than that for quinidine for proximal tubules. For distal tubules in KHS (Fig. R-19), the apparent K_i for quinine was similar to that for quinidine. Apparent K_i values for quinidine were not different between the two buffers (KHS and phosphate).

In phosphate buffer (Fig. R-19), apparent K_i for quinine inhibition of amantadine uptake by the proximal tubules increased four-fold, compared to the value in KHS. Moreover, the apparent K_i in phosphate buffer was higher than that for quinidine in the same buffer. For distal tubules in phosphate buffer, there was no change in apparent K_i for quinine compared to KHS, but there was an increase in apparent K_i for quinidine compared to KHS. However, Ki for quinine was not different from that for quinidine within the phosphate buffer.

Competitive inhibition was also present in phosphate-plus-bicarbonate buffer. Apparent K_i values (Fig. R-20) for amantadine uptake inhibition were different between diastereoisomers in proximal but not in distal tubules. K_i for proximal tubule amantadine uptake inhibition by the diastereoisomers were compared between buffers. K_i for

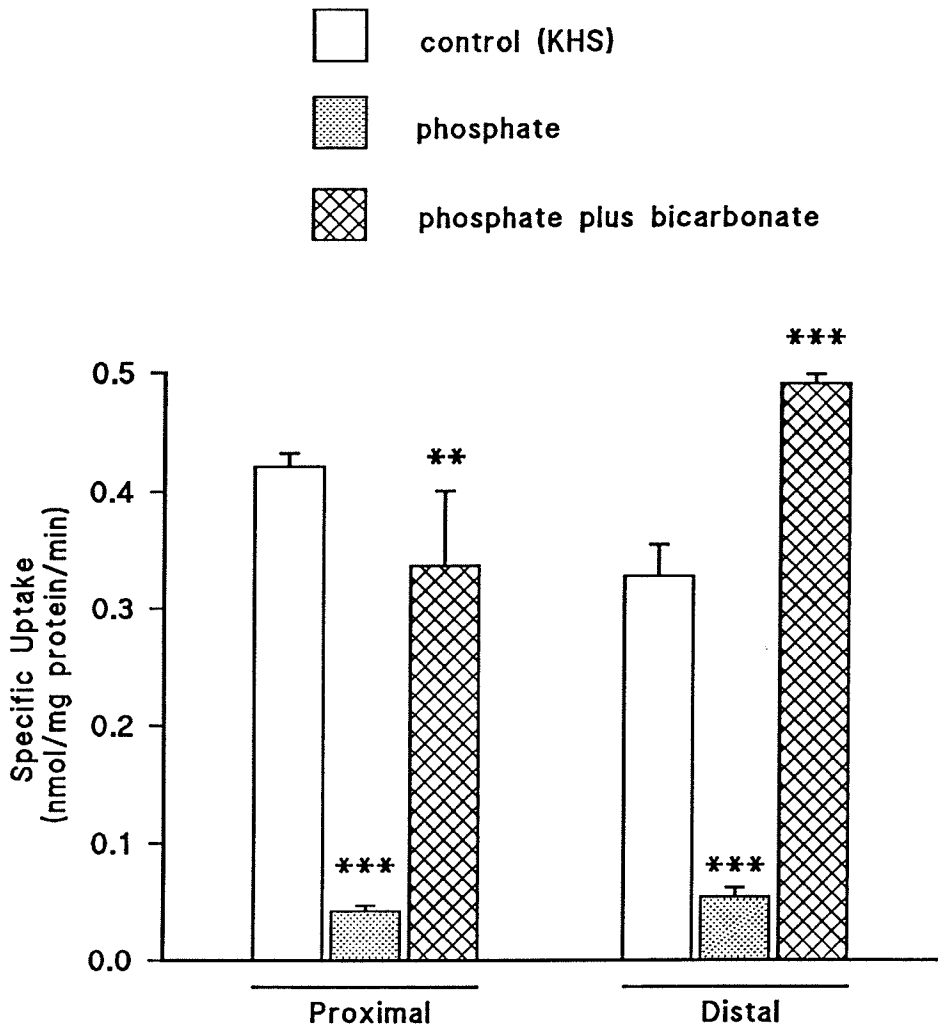


FIGURE R-17 Control rates for amantadine uptake (10 μM) by proximal and distal tubules in the different buffers used for quinine and quinidine inhibition studies Each set of experiments (buffer type) was performed in a separate batch of tubules. KHS, phosphate (solid, n=6), phosphate-plus-bicarbonate (cross-hatched, n=4). ** $p<.01$, *** $p<.001$ compared to KHS, by three-way ANOVA (tubule, buffer and drug as variables) followed by Tukey's HSD test.

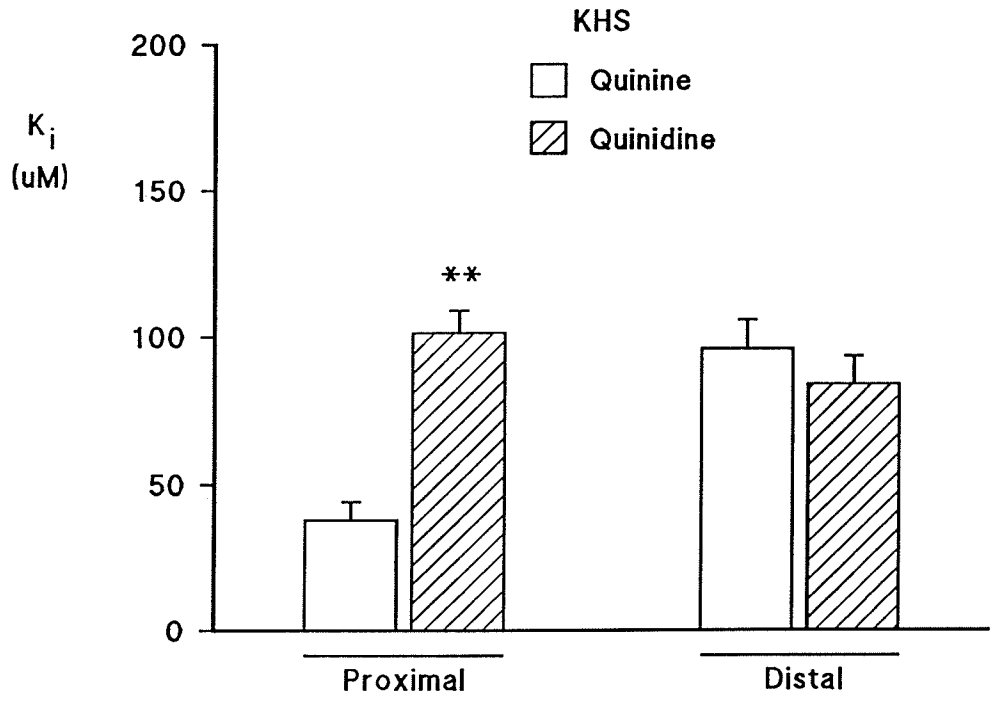


FIGURE R-18 Apparent Ki values for quinine and quinidine inhibition of amantadine uptake in KHS. Values were compared using a three-way ANOVA (tubule, buffer, and drug as parameters) followed by Tukey's HSD test. ** $p<.01$, Quinine vs Quinidine within tubule and buffer.

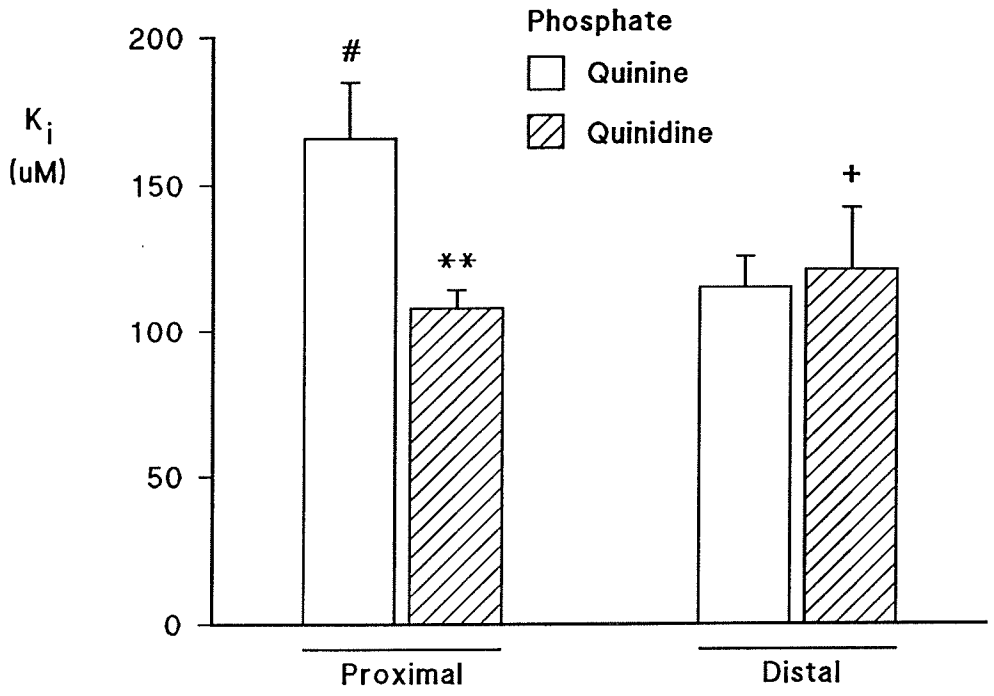


FIGURE R-19 Apparent Ki values for quinine and quinidine inhibition of amantadine uptake in phosphate buffer (n=6). Values were compared using a three-way ANOVA (tubule, buffer, and drug as parameters) followed by Tukey's HSD test. ** $p<.01$, Quinine vs Quinidine within tubule and buffer; # $p<.01$, Quinine (phosphate) vs Quinine (KHS, Fig. R-18) and Quinine (phosphate-plus-bicarbonate, Fig. R-20); + $p<.01$, Quinidine (DT, phosphate) vs Quinidine (DT, KHS; Fig. R-18).

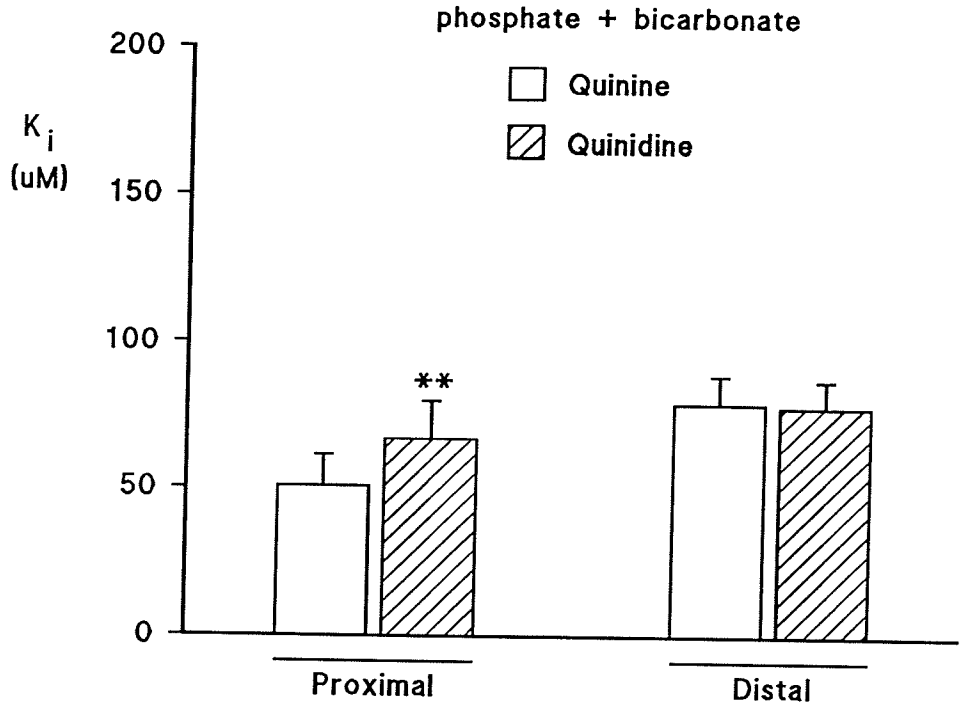


FIGURE R-20 Apparent Ki values for quinine and quinidine inhibition of amantadine uptake in phosphate-plus-bicarbonate buffer (n=4). Values were compared using a three-way ANOVA (tubule, buffer, and drug as parameters) followed by Tukey's HSD test. ** $p<.01$, Quinine vs Quinidine within tubule and buffers.

quinine in the phosphate-plus-bicarbonate buffer was lower than the control value (KHS). It was not determined whether longer tubule incubation times with the phosphate-plus-bicarbonate buffer prior to inhibition measurements would return K_i for quinidine to a value similar to that observed with KHS.

PART IV: ION MANIPULATIONS

Potassium and calcium studies.

In KHS, modifying extracellular K^+ levels (1.5 to 100 *m*M, Figure R-21) did not affect amantadine uptake by either proximal or distal tubule fragments. If the pre-incubation time in the buffer solution of the K^+ concentration being tested was increased (up to 60 min.), there was no change in amantadine uptake by the tubules. Potassium concentration manipulations in phosphate buffer were not done.

Similar manipulation of extracellular Ca^{2+} concentrations (0.25 to 5.0 *m*M) in KHS and phosphate buffer was performed. For proximal tubules (Fig. R-22) in KHS buffer, 0.25 to 2.5 *m*M Ca^{2+} did not have an effect on amantadine accumulation compared to its own KHS control (2.5 *m*M Ca^{2+}). High Ca^{2+} concentration (5 *m*M) decreased amantadine accumulation by 38 $\pm$ 6 %. In phosphate buffer, 0.25 to 1.0 *m*M Ca^{2+} did not have an effect on amantadine uptake, but 2.5 *m*M Ca^{2+} lowered amantadine accumulation by 30 $\pm$ 8 % when compared to control (1 *m*M Ca^{2+}). High Ca^{2+} (5 *m*M) lowered amantadine uptake by 46 $\pm$ 12 %.

For distal tubules in KHS (Fig. R-23), 0.25 *m*M Ca^{2+} resulted in an enhancement of amantadine uptake (13 $\pm$ 3 %). This enhancement was not observed at 1 *m*M Ca^{2+}, and at higher Ca^{2+} concentrations ($\geq$ 2.5 *m*M) amantadine uptake was decreased by 14 $\pm$ 2 % (2.5 *m*M Ca^{2+}) and 39 $\pm$ 5 % (5 *m*M Ca^{2+}). In phosphate buffer, there was no observed effect on amantadine uptake at low Ca^{2+} concentrations (0.25 and 1 *m*M), but inhibition (25 $\pm$ 5 % and 59 $\pm$ 20 %, for 2.5 and 5 *m*M calcium, respectively) was observed at higher concentrations.

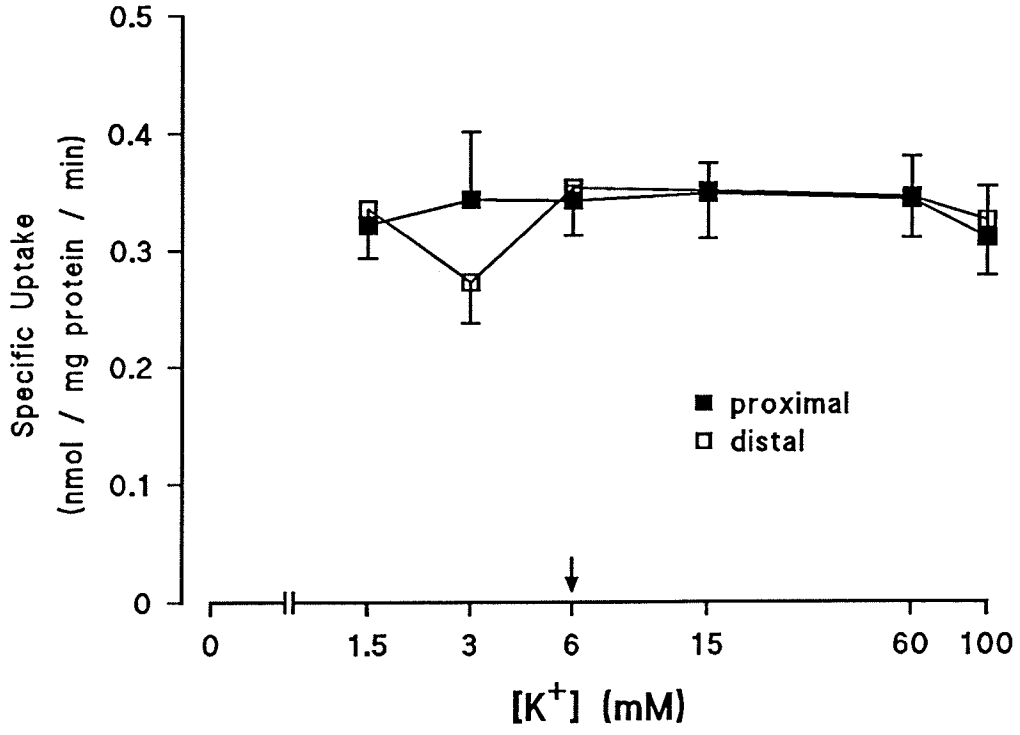


FIGURE R-21 Amantadine (10 μM) uptake rates by proximal and distal tubules in KHS (bicarbonate) buffer in varying concentrations of potassium. Arrow denotes control concentration of potassium (6 *m*M). No significant difference was encountered when rates were compared to control values (6 *m*M) by repeated-measures ANOVA followed by Tukey's HSD test ($P<.1$).

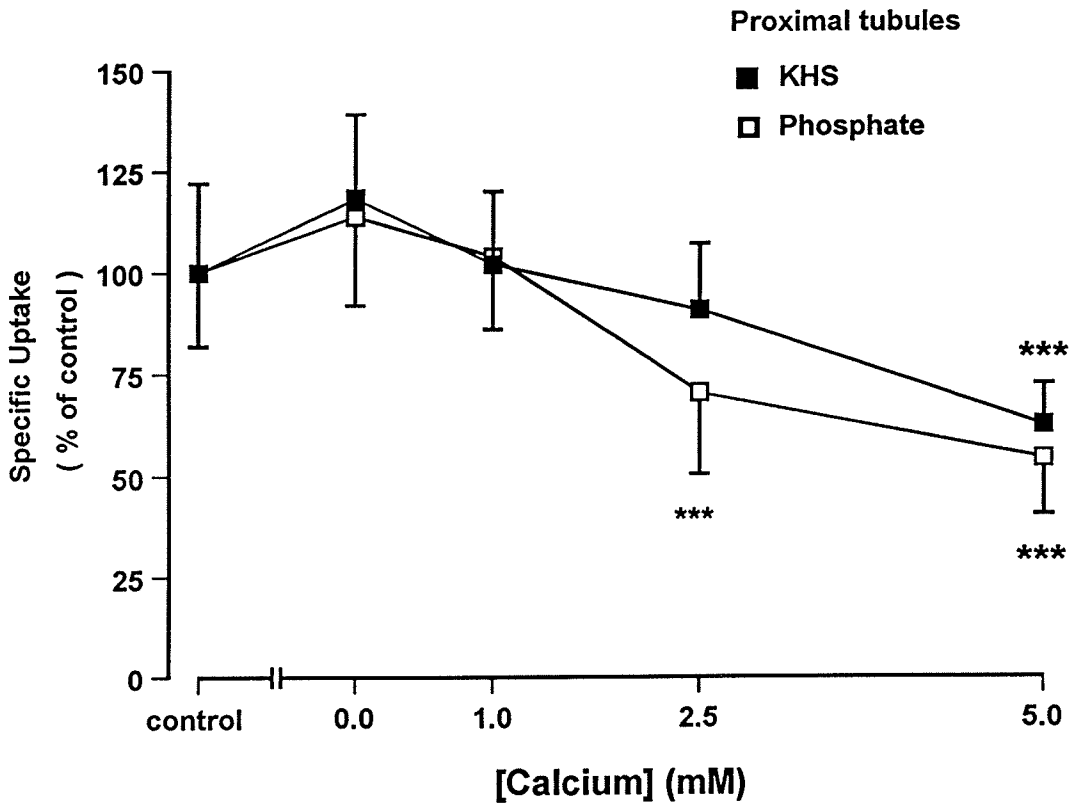


FIGURE R-22 Uptake rates (n=4) for proximal tubule uptake of amantadine (10 μ M) in varying concentrations of calcium. Control for KHS contains 2.5 *m*M calcium; control for phosphate buffer contains 1.0 *m*M calcium. All statistical comparisons were to control values using repeated-measures ANOVA followed by Tukey's HSD post-hoc test. *** $p<.001$.

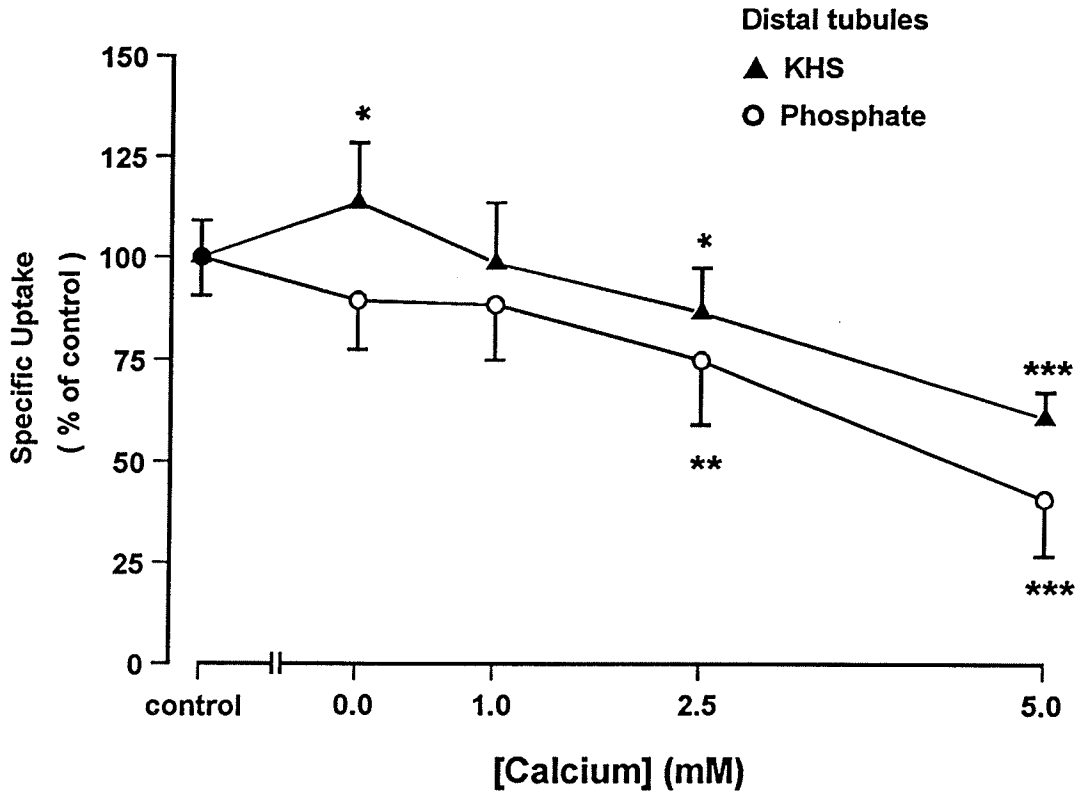


FIGURE R-23 Uptake rates (n=4) for distal tubule uptake of amantadine (10 μM) in varying concentrations of calcium. Control for KHS contains 2.5 *m*M calcium; control for phosphate buffer contains 1.0 *m*M calcium. All statistical comparisons were to control values using repeated-measures ANOVA followed by Tukey's HSD post-hoc test. * $p<.05$, ** $p<.01$, *** $p<.001$

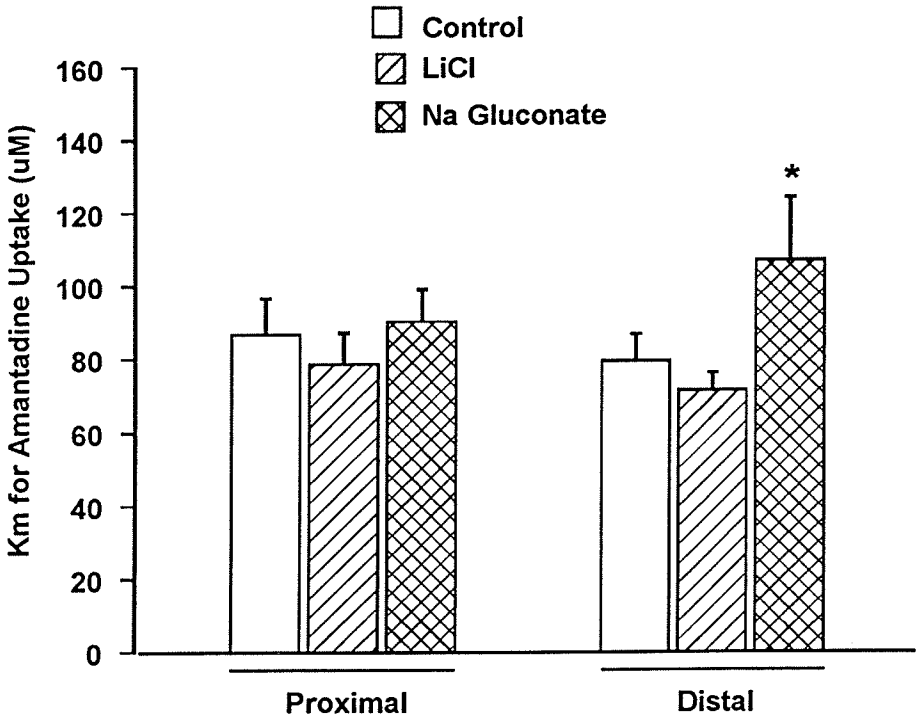


FIGURE R-24 Measured Km values for amantadine uptake by proximal and distal tubules in KHS, and low sodium and low chloride KHS buffers. LiCl: low sodium KHS that contained 25 *m*Eq/l of sodium. Na gluconate: low chloride KHS that contained 12.1 *m*Eq/l of chloride. * $P<.05$ vs control, by repeated-measures ANOVA, within tubule, followed by Tukey's HSD post-hoc test.

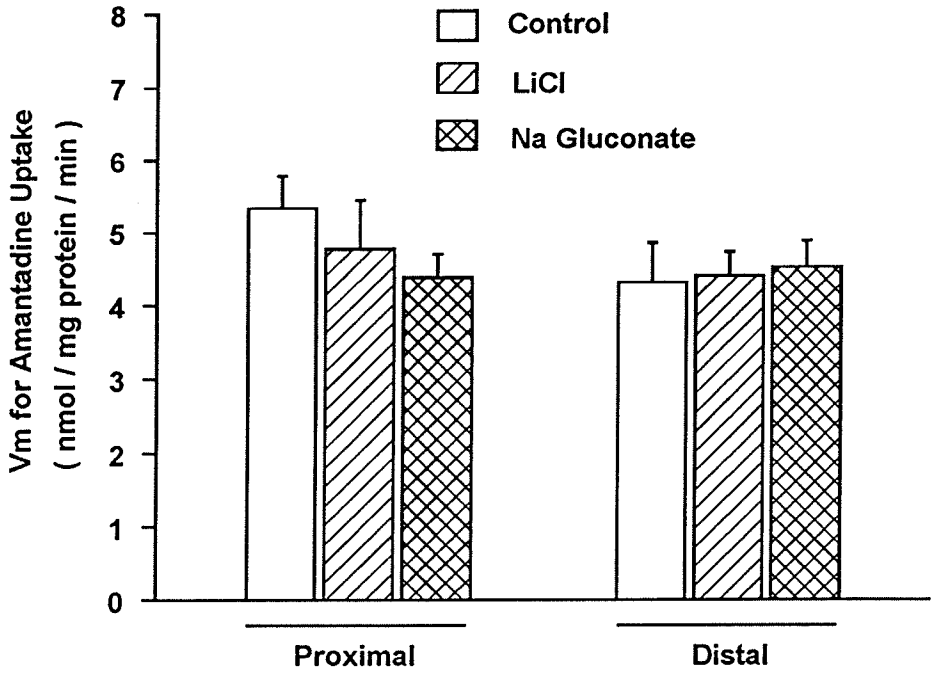


FIGURE R-25 Measured Vmax values for amantadine uptake by proximal and distal tubules in KHS, and low sodium and low chloride KHS buffers. LiCl: low sodium KHS that contained 25 *m*Eq/l of sodium. Na gluconate: low chloride KHS that contained 12.1 *m*Eq/l of chloride. No differences were found, within tubules, by repeated-measures ANOVA ($P<.41$ and $P<.95$ for proximal and distal tubule data respectively).

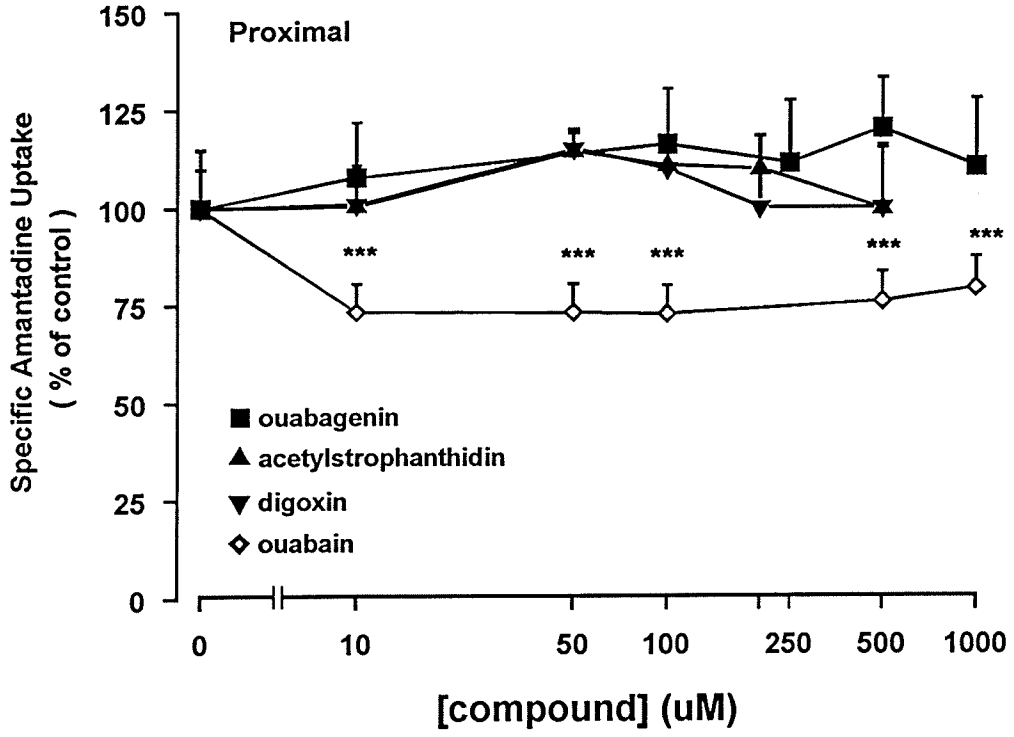


FIGURE R-26 Effect of different cardiac glycosides and ouabagenin on amantadine (10 μM) uptake by proximal tubules in KHS. Tubule tissue was incubated for 10 minutes in the presence of the modulator being tested prior to assessment of uptake. *** P<.001 vs control by repeated-measures ANOVA, within modulator, followed by Tukey's HSD post-hoc test.

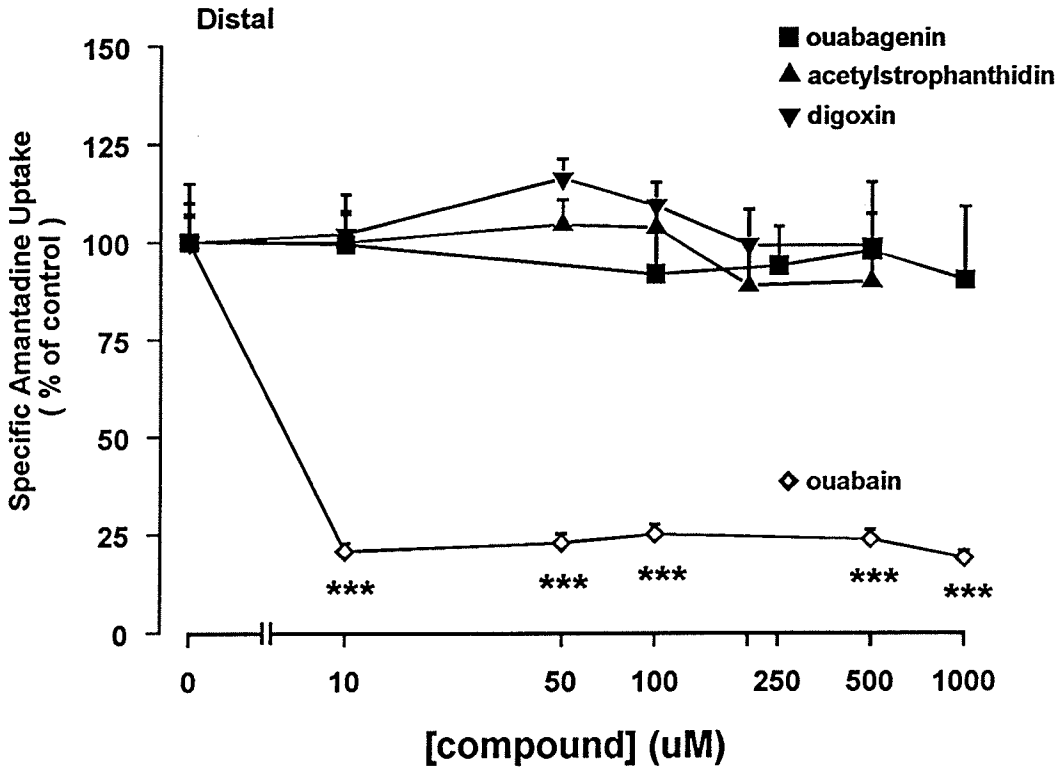


FIGURE R-27 Effect of different cardiac glycosides and ouabagenin on amantadine (10 μM) uptake by distal tubules in KHS. Tubule tissue was incubated for 10 minutes in the presence of the modulator being tested prior to assessment of uptake. *** P<.001 vs control by repeated-measures ANOVA, within modulator, followed by Tukey's HSD post-hoc test.

concentration (10-minute pre-incubation period). The possible small increase in amantadine uptake observed with ouabagenin in the proximal tubules was not observed in the distal tubule fraction.

Ouabain inhibition studies in low sodium and low potassium.

Amantadine uptake (10 μM) by proximal tubules (Fig. R-28) was not affected by low extracellular sodium or potassium, in the absence of ouabain. However, if both sodium and potassium concentrations were reduced concomitantly, amantadine uptake decreased by 40%. In the presence of ouabain (1 *m*M), amantadine uptake decreased by 23% under control conditions. A decrease in extracellular sodium did not produce additional effects. However, low extracellular potassium reversed the inhibitory effect of ouabain because rates for amantadine uptake in low potassium with and without ouabain were similar, and because the rate in the low potassium buffer in presence of ouabain was higher (150%) than the rate in normokalemic buffer in the presence of ouabain. Attenuation of the inhibitory effect of ouabain on amantadine uptake by the proximal tubules was also observed in the low sodium/low potassium buffer as the rate in this buffer was higher (125%) than the rate observed in the normokalemic/normonatremic buffer.

Distal tubular uptake of amantadine (10 μM) in KHS was not affected by low extracellular sodium, low potassium or low sodium/low potassium conditions (Fig. R-29). In the presence of ouabain (1 *m*M), amantadine uptake decreased by 80% under control conditions. Low extracellular sodium reversed the inhibitory effect of ouabain on amantadine uptake by distal tubules. Furthermore, an increase (270%) in rate of uptake was observed above the normonatremic control (no ouabain). Low extracellular potassium and low sodium/low potassium conditions also attenuated the inhibitory effect of ouabain as inhibition was only 29% and 35%, respectively. However, the increase in rate observed in the low sodium buffer was not present in the latter two conditions.

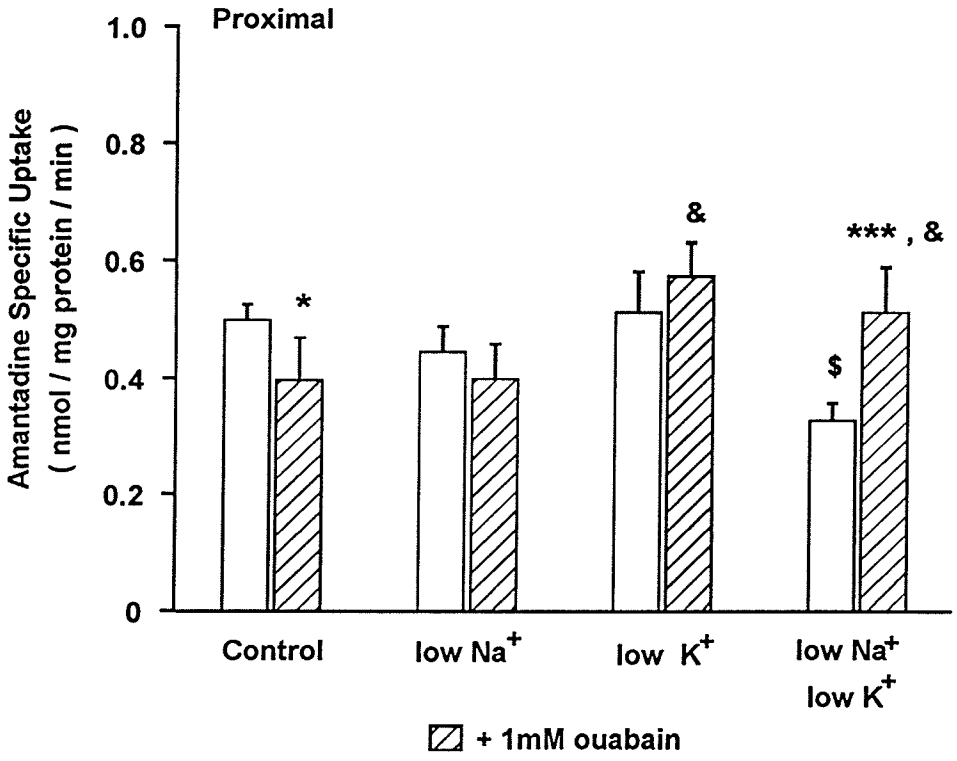


FIGURE R-28 Effect of 10-minute pre-incubation with ouabain (1 *m*M) on amantadine uptake by proximal tubules under conditions of low and normal extracellular sodium and potassium in KHS. Low sodium KHS contained 25 *m*Eq/l of sodium. Low-potassium KHS contained 0.375 *m*Eq/l of potassium. * P<.05 vs own control; *** P<.001 vs own control; & P<.05 vs control with ouabain; $ P<.05 vs control without ouabain by repeated measures ANOVA within tubule.

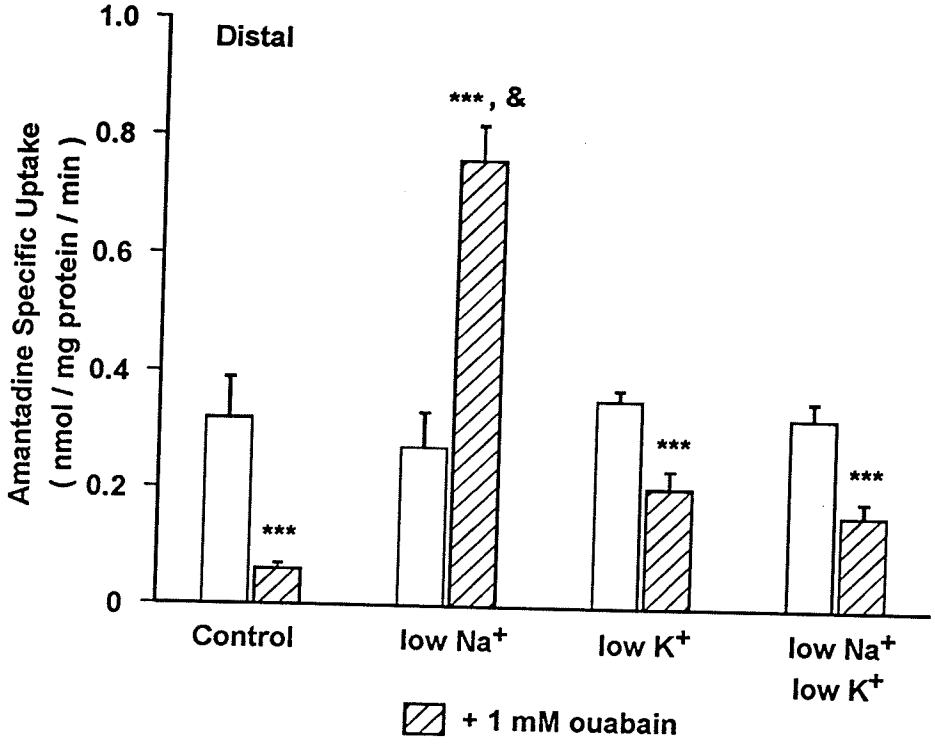


FIGURE R-29 Effect of 10-minute pre-incubation with ouabain (1 *m*M) on amantadine uptake by distal tubules under conditions of low and normal extracellular sodium and potassium in KHS. Low sodium KHS contained 25 *m*Eq/l of sodium. Low potassium KHS contained 0.375 *m*Eq/l of potassium. *** P<.001 vs own control; & P<.001 vs control with ouabain by repeated measures ANOVA, within treatment (with or without ouabain).

Ouabain studies in the absence of bicarbonate

Ouabain (1 *m*M, 10 min pre-incubation) had no effect on amantadine (10 μM) uptake by the both tubules in phosphate buffer. However, if bicarbonate was added to the phosphate buffer, ouabain inhibition increased with increasing bicarbonate pre-incubation times (Fig. R-30). For proximal tubules, inhibition by ouabain of amantadine uptake was apparent after 30 minutes of bicarbonate pre-incubation. However, inhibition of distal tubule amantadine uptake was apparent after 15 minutes of bicarbonate pre-incubation.

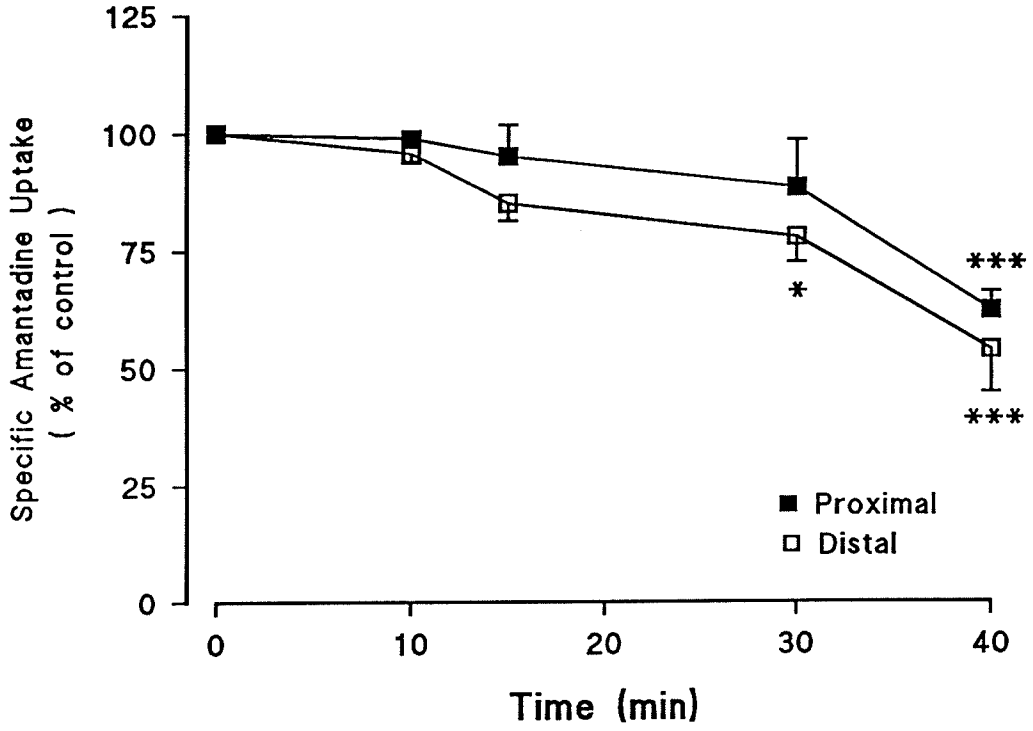


FIGURE R-30 Amantadine (10 μM) specific uptake by proximal and distal tubules after a 10-minute incubation period in phosphate buffer with ouabain (1 *m*M) and varying pre-incubation times with bicarbonate (25 *m*M). * P<.05 and *** P<.001 vs own control by repeated-measures ANOVA, within tubule.

DISCUSSION

I. METHODOLOGICAL CONSIDERATIONS.

All the present studies were performed in an *in vitro* preparation of rat renal tubule fragments. It is important to consider the following points about the protocol for amantadine uptake studies:

a) Although the purity of the tubule fractions and intactness of the tubule fragments were determined using enzyme markers and light microscopy, no functional tests were performed to determine viability of the purified tissue. Such tests could include dye exclusion assays, for example. However, maintaing the tubule protein concentration constant among preparations allowed us to determine relative viability of the tubule segments based on amantadine uptake rates, i.e. tubule fragment preparations that did not show amantadine uptake rates similar to those obtained previously in this laboratory were deemed to be unsuitable to be included in data analyses.

b) In the present studies, initial rates of uptake were measured in accordance to Michaelis-Menten-based kinetics. To fullfil such a requirement, an incubation time of 30 seconds was used in all the experiments presented herein. A 30-second incubation time is consistent with the previous report on amantadine rat renal tubule uptake from our laboratory (Wong *et al*, 1990).

c) Background radioactivity and non-specific uptake were determined using twice-frozen tissue as well as pre-incubation of the tissue in the metabolic inhibitor 2,4-dinitrophenol (2,4-DNP). The fact that uptake decreased to 20% (KHS) and 50% (non-bicarbonate buffers) of total uptake upon freezing and 2,4-DNP pre-treatment further suggests that tissue was viable during the assay.

A value of 50% of total uptake for background radioactivity and non-specific uptake in non-bicarbonate buffers may appear quantitatively unacceptable. However, it is important to keep in mind that total uptake of amantadine in the non-bicarbonate buffers is approximately 40% of total uptake in KHS. Therefore, non-specific binding and

background radioactivity are 20 % and 50% of total uptake in bicarbonate and non-bicarbonate buffers, respectively.

II. BICARBONATE AND ORGANIC CATION UPTAKE.

Amantadine uptake and its accumulation by the tubules, both proximal and distal, showed significant dependency upon bicarbonate. Ullrich *et al* (1991), showed a modestly higher uptake of N^1-methylnicotinamide (NMN) in the presence of bicarbonate in rat tubule cells using stop-flow capillary perfusion methods. Our present studies generally reflect the observations of Ullrich *et al*, but suggest that the bicarbonate anion may play a much more important role in organic cationic drug transport than previously believed. Therefore, these studies support our hypothesis and allow us to propose the existence of bicarbonate-dependent, and by extention the existence of bicarbonate-independent uptake sites. The latter appear to account for the majority of amantadine uptake. The remaining accumulation after bicarbonate removal occurs *via* the bicarbonate-independent sites and constitutes only 10-20% of control values (Krebs-Henseleit solution).

Determined kinetic parameters for amantadine uptake in the KHS solution in these studies are similar to those reported previously by our laboratory (Wong *et al*, 1990, 1991). The exception is the Vmax for proximal tubules which is lower and very similar to Vmax observed for distal tubules. The fact that the Km values were similar between our and previous preparations, suggests that the same mechanism(s) is being studied even though transporter density (Vmax) in our tissue preparation may be lower. We believe that the observed discrepancies in Vmax are due mainly to variations between different batches of collagenase used, an observation that has been previously documented (Peterkofsky and Diegelmann, 1971; Hefley, 1987). Overall, qualitative observations in all the experimental series presented in this disertation were reproducible. For example, the reported stereoselective inhibition by quinine and quinidine in proximal but not distal

tubules (see later) was confirmed in our studies (Wong *et al*, 1990, 1993). We are confident of the purity of our tubule fragments, despite their similar kinetic parameters.

Except for the results obtained in lactate buffer, the observed pattern for amantadine uptake in the absence of bicarbonate is that of increased Km (decreased affinity) and decreased Vmax (decreased maximal transport capacity). Reduced Vmax may have resulted from a change to an uptake site with decreased capacity or from a decrease in the number of uptake sites. The rise in apparent Km values describes a shift of the rate-limiting step in the uptake mechanism to a site(s) with decreased affinity for amantadine. The net effect is that uptake at this new or modified site(s) produce(s) an amantadine cell accumulation to much lower concentrations. We believe that the residual amantadine uptake occurs mainly *via* those uptake sites that are bicarbonate-independent.

In spite of the minor quantitative differences, the underlying suggestion is that one main mechanism is affected in the transport system, resulting in decreased amantadine accumulation. Lower accumulation of amantadine by the tubules can be explained by (a) decreased influx, (b) potentiated efflux or (c) both. Our studies suggest that the decreased amantadine accumulation is due mainly to a decrease in influx into the tubule cell and the reasons for our conclusions are discussed below.

According to the current model for uptake of organic cations by the renal tubule cell (Grantham and Chonko, 1991; Pritchard and Miller, 1993), entry of the cation into the cell from the basolateral (apical) side of the tubules is mainly *via* carrier-mediated transport. This occurs in response to the inward concentration gradient and to the electronegativity of the cell interior (Grantham and Chonko, 1991) so that any change in the membrane potential would be reflected in the transport rates.

Therefore, one contributing factor to the decreased amantadine accumulation in the absence of bicarbonate may be cell depolarization. Indeed, it has been shown previously with microperfused rat proximal tubules (Burckhardt *et al*, 1984; Verkman and Alpern, 1987), that in the nominal absence of basolateral bicarbonate (3 *m*M, solution pH

of 6.5) the tubular cell is depolarized so that its resting membrane potential is moved from a value of -70 *m*V to -50 *m*V. Although these studies are confounded by the fact that *p*H was not maintained constant at 7.4, the authors suggested that the change in membrane potential is mainly, but not completely, due to the extrusion of intracellular bicarbonate by the electrogenic Na^+/HCO_3^- cotransporter on the basolateral membrane. It has been proposed that this cotransporter translocates one sodium ion per three bicarbonate base equivalents out of the cell (Aronson *et al*, 1991).

Akiba *et al* (1987) measured maximal transport capacities (Vmax) of the Na^+/HCO_3^- cotransporter in basolateral membrane vesicles of the rabbit proximal tubule and found that Vmax increased as the extracellular bicarbonate concentration decreased. Therefore, it is possible that in the absence of extracellular bicarbonate this cotransporter may increase its rate of bicarbonate transport out of the cell, thereby depleting the intracellular bicarbonate pool. This bicarbonate depletion may cause a cell depolarization that contributes to a decreased amantadine entry into the cell.

The major determinant of intracellular availability of bicarbonate is the hydration of carbon dioxide to carbonic acid, which instantly dissociates into a proton and a bicarbonate anion. This step is solely catalyzed by the cytosolic enzyme carbonic anhydrase (CA). CA is abundant in the cytosol of the proximal tubules, and minimally in the distal tubule (Alpern *et al*, 1991). Therefore, it could be proposed that if intracellular production of bicarbonate is inhibited *via* inhibition of CA with acetazolamide (ACZ), then cell depolarization would concomitantly be stopped and amantadine uptake rates would not be expected to be altered from control rates.

It has been reported that ACZ (1 *m*M concentration) effectively inhibits the Na^+/HCO_3^- cotransporter in isolated rabbit S2 (Seki and Frompter, 1992*b*; Sasaki and Marumo, 1989), and the basolateral Cl^-/HCO_3^- exchanger in isolated rabbit S3 proximal tubule segments (Seki and Frompter, 1992*a*), not *via* direct interaction with the

transporters themselves, but by reducing the amount of bicarbonate available to be mobilized.

The fact that in our studies ACZ (1 *m*M) increased amantadine accumulation only by a very small extent suggests that cell depolarization is not likely to be the major reason for the observed decrease in amantadine uptake in the absence of bicarbonate. However, we did not measure membrane potential in the present studies. Therefore, our data do not rule out completely a contribution of cellular depolarization.

The ACZ experiments also address the involvement of intracellular bicarbonate in the mechanism for amantadine uptake by the renal tubules. If the intracellular bicarbonate pool played a significant role, then as explained above, the enzyme CA would have to be involved as it is the main enzyme responsible for intracellular bicarbonate availability (Alpern *et al*, 1991). Involvement of the CA-derived bicarbonate could be ruled out by measuring amantadine uptake by the tubules in the presence of ACZ, a CA inhibitor. We demonstrated that inhibition of intracellular bicarbonate generation by CA does not have a significant effect on amantadine uptake if extracellular bicarbonate availability is not a limiting factor. Further, these studies suggest that the fact that the mechanism(s) for bicarbonate handling is different between proximal and distal tubules (Alpern *et al*, 1991) may be of minimal relevance to the present work.

A point to consider is that in the intact tubule, the intracellular availability of bicarbonate would not be entirely determined by the CA system. Krapf *et al* (1987) reported that in *in vitro* microperfused rabbit proximal tubules, bicarbonate released from mitochondria, as a result of metabolic processes, would be sufficient for the Na^+/HCO_3^- cotransporter to work, albeit at a lower rate, in the absence of extracellular bicarbonate and in the presence of ACZ. This provides a possibility that, in the intact tubule, there could be still a depolarization of the cell as a result of bicarbonate removal and that this bicarbonate could be metabolically-derived.

A question arising from the ACZ experiments was whether the time of incubation during rate determination (30 sec) was sufficient for ACZ to have an effect on intracellular bicarbonate. However, ACZ studies in our laboratory with incubation times of up to 10 minutes prior to amantadine rate determination did not result in any change in the measured rates from those obtained with the 30 sec incubation time.

The conclusions proposed to explain the observations with ACZ may only apply to the proximal tubules. The explanation for the observed effect of high concentrations of ACZ on distal tubules in the absence of bicarbonate remains to be determined, as the presence of CA is minimal in this segment of the nephron. Moreover, the Na^+/HCO_3^- cotransporter has not been found yet in the distal tubules.

Since bicarbonate is intimately involved with the regulation mechanisms for intracellular pH, a point to consider is whether intracellular pH is changed when extracellular bicarbonate concentration is altered. Krapf *et al* (1987) reported that in *in vitro* microperfused rabbit proximal tubules resting cell pH was not significantly different in the presence and absence of bicarbonate. However, it has been shown using *in vivo* microperfused rat proximal tubules (Alpern and Chambers, 1986; Verkman and Alpern, 1987) that a luminal change in pH (7.4 to 6.8) caused the intracellular pH to acidify by 0.08-0.10 pH units, whereas a peritubular change in pH of the same extent caused an intracellular pH acidification of 0.25-0.35 pH units. These changes are alleviated to a major extent by the Na^+/H^+ antiporter and the Na^+/HCO_3^- cotransporter, so that the new resting intracellular pH is slightly more acidic than physiological (Akiba *et al*, 1987).

To address the concern of intracellular pH regulation, the choice of anions for the non-bicarbonate buffers included those that may have different effects on the intracellular control of pH because of access to the inside of the cell (lactate and acetate), or an inability to cross the cell membrane (Tris-HEPES). The fact that no major differences in kinetic parameters were encountered between the "external" and the "internal" buffers in these studies suggests the possibility that control of intracellular pH by the buffer may not

be critical for tubular uptake of organic cations. Since we did not measure intracellular pH in the present studies, we do not know the extent to which the individual anions buffered intracellular pH, if at all, and this requires further investigation.

The use of three other buffers ruled out the idea that the decrease in amantadine accumulation was due directly to the presence of high concentrations of phosphate. However, inhibitory anion-cation interactions that could occur between amantadine and the buffering anions, which would result in kinetic values similar to those reported here, cannot be ignored. Such observations have been reported for cimetidine in the choroid plexus and the kidney cation transport system (Suzuki *et al*, 1986 & 1987; Ullrich and Rumrich, 1992). As Tris base, which does not interact with the cation transport system in rat tubules, gives similar qualitative and quantitative data, it is unlikely that our observations are explained by inhibitory anion-cation interactions (Ullrich *et al*, 1991). Therefore, we can conclude that it is most likely the absence of bicarbonate, and not the presence of other anions, which causes the lower amantadine uptake rates in non-bicarbonate buffers.

The effect caused by the absence of bicarbonate seems to be reversible, as its addition to the incubating medium causes an immediate increase in amantadine accumulation. Therefore, we are led to the conclusion that the bicarbonate interaction is most likely at the extracellular surface. In addition, although no saturation was reached in the present studies, this bicarbonate-enhanced amantadine uptake appears to follow saturation kinetics, for both proximal and distal tubules (see Fig. R-9), suggesting that the mechanism by which it occurs may involve a basolateral protein. Based on such a proposal, Michaelis-Menten-based enzyme kinetics were applied to the data. Maximal uptake rates at apparent bicarbonate saturation ($V_{10\ \mu M}max$) were not different between proximal and distal tubules suggesting that bicarbonate may modulate the same fraction of uptake sites in both tubule fragments. As well, the fact that the bicarbonate concentrations necessary to reach half maximal bicarbonate-enhanced uptake ($Km_{10\ \mu M}$)

did not differ between the tubules, suggests that the uptake sites upon which bicarbonate is acting may be similarly regulated. The $Km_{10\ \mu M}$ values approximated the physiological bicarbonate concentration (25 *m*M), at the basolateral membrane, for both tubules.

Therefore, in the *in vivo* situation at physiological bicarbonate concentrations, the organic cation uptake system in the renal tubules may already be working at half saturation with respect to the bicarbonate-dependent component and would have a "reserved capacity" for uptake in case of plasma bicarbonate concentration fluctuations. A pathological condition in which a rise in plasma bicarbonate occurs is that of metabolic alkalosis (Alpern *et al*, 1991). Our experimental data suggest the possibility that such an increase in plasma bicarbonate concentration may result in increased amantadine excretion due to increased tubule transport.

The measured $Km_{10\ \mu M}$ and $V_{10\ \mu M}max$ for bicarbonate in the present studies possess high variability. As bicarbonate concentrations become low, non-bicarbonate amantadine uptake contributes increasingly to the measured rates at the lower part of the curves. This residual uptake confers high variability to the measured Michaelis-Menten kinetic parameters. We believe that such analysis is acceptable because at higher bicarbonate concentrations the contribution to amantadine accumulation by the much less efficient non-bicarbonate dependent uptake system(s) is not quantitavely significant.

Therefore, it seems that organic cation uptake by the tubules depends on both bicarbonate and drug concentration. Our studies suggest that at the pharmacologically-relevant amantadine concentration of 10 μM (Aoki and Sitar, 1988) and under the same conditions of bicarbonate concentrations, both proximal and distal tubules would have similar uptake rates if compared on the basis of tubular protein content. As well, these studies further support the hypothesis that bicarbonate modulates proximal and distal tubule amantadine uptake at the basolateral membrane in a similar manner.

It is possible that in the present studies, decreased amantadine accumulation is due to an increase in efflux into the luminal side. Such a model may contribute to the

explanation for decreased tubular accumulation of amantadine in the absence of bicarbonate. Although the issue of whether the tubule lumen is patent or closed is controversial, we believe that in our tubule preparation the lumena are open (Gesek *et al*, 1987). The role of the luminal membrane remains to be assessed. However, the rapid increase in amantadine uptake upon addition of bicarbonate to the phosphate solution is consistent with a bicarbonate effect on the basolateral membrane rather than on the luminal membrane. Also, since the time of incubation used in these studies is small (30 sec), we are led to the tentative conclusion that our uptake data reflect events occurring primarily at the basolateral membrane, and contribution of the luminal membrane to our observations is unlikely to be quantitatively important.

Nonetheless, if luminal bicarbonate is involved, under normal physiological conditions one would expect the higher (control) amantadine uptake rates by proximal tubules as compared to distal tubules because this is the section of the nephron where most of the bicarbonate present in the glomerular filtrate is reabsorbed (Alpern *et al*, 1991). Conversely, the lower rates (those observed in bicarbonate-free solutions) would be expected in distal tubules, where levels of luminal bicarbonate are minimal. This hypothesis correlates well with *in vivo* observations that distal tubules make a smaller contribution to organic cation secretion as compared to proximal tubules (Rennick, 1981; Grantham and Chonko, 1991; Pritchard and Miller, 1993).

Finally, our lactate buffer results suggest that during conditions of increased lactate concentrations, as it occurs during lactic acidosis (Kreisberg, 1980; Madias, 1986), metabolic alkalosis (Leal-Pinto *et al*, 1973; Dawson, 1977; Emmett and Narins, 1977; Adrogue *et al*, 1978) and ischemia (Alpern *et al*, 1991), amantadine uptake may be compromised. It is debatable whether this effect is due to the increase in the concentration of the lactate anion itself or the conditions it represents, e.g. acidosis. Since the effects we observed between lactate and acetate were different, we believe that it is possible that reduced amantadine uptake is a direct effect of the lactate anion. However,

this interpretation is speculative since we did not perform a direct comparison (lactate and acetate). This aspect of amantadine uptake requires further experimental studies.

However, it is important to note that the fact that maximal transport capacity (Vmax) did not decrease in the lactate buffers is suggestive of competitive-type of inhibition. That is, a possible explanation for the unaltered Vmax is that lactate interacts with the same number of sites as bicarbonate, quite possibly, at the bicarbonate-dependent uptake sites. However, this conclusion is tentative and it forms the basis for the next experimental series described in the present dissertation (*vide infra*).

The same inhibitory effect, by lactate, is observed for the transport of organic anions, where it is lactate itself which produces a change in kinetic parameters (Pritchard and Miller, 1993), suggesting a possible link between the two transport systems. This interpretation may be confounded further by the complexity of the lactate transport sites involved in its excretion and reabsorption by the renal tubules (Craig, 1946; Hohmann *et al*, 1974; Barac-Nieto *et al*, 1980 & 1982; Ullrich *et al*, 1982; Guggino *et al*, 1983; Jorgensen and Sheikh, 1984; Kahn and Weinman, 1985; Kahn *et al*, 1985; Webb *et al*, 1986; Werner and Roch-Ramel, 1991; Yamamoto *et al*, 1993*a*) and by any inhibitory effects that lactate may have on renal tubule cell metabolism, most importantly glycolysis and ATPase function (Relman, 1972; Dawson, 1977; Madias, 1986; Eiam-Ong *et al*, 1994).

Separate and conceptually similar observations to our results with lactate have been reported for the renal transport of drugs in patients suffering from cystic fibrosis. In this disease, a defect on transport of chloride (Quinton, 1990; Clarke *et al*, 1992), increases renal excretion of xenobiotics such as trimethoprim (Hutabarat *et al*, 1991), an organic cation, and ticarcillin (Wang *et al*, 1993), an organic anion. Wang *et al* (1993) demonstrated an increase in apparent affinity (lower Km) without a change in apparent capacity (Vmax) for the excretion kinetics of ticarcillin. Therefore, although our lactate studies have been carried out in a non-physiological buffer, it is possible that an effect by

lactate on amantadine uptake, perhaps because of its cellular transport, may still be observed in a more physiological solution (i.e. Krebs-Henseleit buffer) and/or in the *in vivo* situation.

It is important to emphasize that although all the observations among the different buffers reported here are qualitatively similar between proximal and distal tubule fragments, the mechanism by which distal tubules transport cations is poorly understood. We have shown that distal tubules do not demonstrate chiral selectivity between the cationic stereoisomers quinine and quinidine. In contrast, preferential inhibition by quinine over quinidine was observed for amantadine uptake by proximal tubules (*vide infra*, Wong *et al*, 1990 & 1993). Our hypothesis is therefore that the mechanism(s) for amantadine uptake is(are) heterogeneous between these tubules fragments

SUMMARY I:

Lower accumulation of amantadine by the tubules may be due to a shift of uptake from the bicarbonate-dependent to the bicarbonate-independent mechanism(s), the latter possessing lower affinity and lower maximal transport capacity for amantadine uptake.

The fact that the amantadine uptake sites may be subdivided into bicarbonate-dependent and -independent sites provides further support for the long-held belief that the organic cationic drug transport system(s) is(are) heterogeneous with respect to uptake sites.

The bicarbonate interaction occurs at the extracellular level and the saturability of the effect suggests that the bicarbonate anion itself may be directly involved. Such involvement could be similar to that in the organic anion transport system in which the basolateral Cl^-/HCO_3^- exchanger functions as an organic anion/organic anion co-transporter (Blomstedt and Aronson, 1980; Guggino *et al*, 1983; Kahn and Aronson, 1983; Suzuki *et al*, 1987).

It has been shown that extracellular pH variations, with the concomitant bicarbonate concentration variations, have a direct effect on intracellular mechanisms, mainly intracellular pH regulation, and that these variations have a direct effect on organic cation excretion (Holohan and Ross, 1981; McKinney, 1984; Wright, 1985; Rafizadeh *et al*, 1986 & 1987; Sokol *et al*, 1985 & 1988; Katsura *et al*, 1991; Yuan *et al*, 1991). We believe we have been able to separate both of these conditions by maintaining the pH of the incubating medium constant.

III. LACTATE AND ORGANIC CATION UPTAKE.

In the previous section we proposed the existence of bicarbonate-dependent amantadine uptake sites. We showed that uptake decreased in the absence of bicarbonate, independently of the anion used to replace it. In addition, we reported also that racemic lactate was unique among the anions tested as a modulator of amantadine uptake in that it inhibited affinity but not capacity for amantadine energy-dependent uptake.

In the present section we pursued the effect of lactate on amantadine uptake by the tubule fragments. The present studies show that affinity for amantadine uptake is decreased when racemic lactate (5 *m*M) is used to replace bicarbonate, but maximal transport capacity (Vmax) remains unchanged.

The difference in affinities for amantadine uptake among all lactate buffers in the absence of bicarbonate suggests that uptake sites at which lactate is acting may select for one or the other lactate enantiomer. In addition, affinity for amantadine uptake in racemic (DL) lactate buffer was similar to that in D(-)- for proximal and to that in L(+)-lactate buffer for distal tubules. Such observations suggest that L(+)-and D(-)-lactate have a more predominant effect in proximal and distal tubules, respectively. We propose that the sites upon which lactate is acting may be divided into sub-types of sites, L(+)- *vs* D(-)-lactate-responsive/dependent sites, within and between tubules.

Although the decrease in Vmax obtained in the enantiomerically pure buffers (no bicarbonate) is suggestive of an inhibitory effect by lactate on amantadine uptake, we believe that a more plausible explanation of these results is as follows: since the value of Vmax depends chiefly on the number of uptake sites present in the tissue, the decrease in Vmax suggests that the number of uptake sites modulated by either L(+) or D(-)-lactate is less than those modulated by bicarbonate (bicarbonate-dependent sites) and racemic lactate. Such a proposal seems logical only if the uptake sites affected by DL-lactate are separated into L(+)-responsive and D(-)-responsive sites. In the presence of both enantiomers (racemate), and in the absence of bicarbonate, all the lactate-responsive sites will be modulated for amantadine uptake, and the measured kinetic parameters would reflect the characteristics of both types of lactate-responsive sites. In the presence of either pure enantiomer, lactate would act as an inefficient replacement modulator for amantadine uptake by the tubules at those sites with higher affinity for the specific enantiomer. Hence, only a proportion of all the lactate-responsive sites are modulated for amantadine uptake, reflected by a lower Vmax than that measured in racemic (DL) lactate buffer.

However, the fact that in the absence of bicarbonate the kinetic measurements in buffers of different enantiomeric ratios of lactate were similar among the mixtures used suggests that the mechanism for modulation of tubule amantadine uptake is complex. It appears that the lactate-responsive sites are selective, rather than specific, for the lactate enantiomers and that minimal presence of the L(+)-enantiomer is able to mask D(-)-lactate modulation of amantadine uptake by tubule fragments. Interestingly, the lowest concentration of L(+)-lactate used (1.25 mM or 25% of the mixture) would be close to the physiological concentration of L(+)-lactate (Poole and Halestrap, 1993), thus an interaction of lactate with the organic cation transport mechanism(s) may be expected at this concentration.

The proposal that two types of lactate-responsive sites exist and that both are modulated in the presence of DL-lactate is supported by the fact that rates of uptake for proximal tubules in the DL-lactate buffer calculated with equations [1] and [2] are similar. However, distal tubule data were qualitatively different from proximal tubule data, suggesting that the mechanism(s) of lactate modulation of amantadine uptake is different between tubules. The reason for the under-estimation by formula [1] of the observed distal tubule uptake rates in the pure enantiomer buffers eludes us at the present time.

It is worth noting that uptake rates in racemic and enantiomerically pure lactate buffers were consistently higher than 20% of rates in bicarbonate buffer, that portion of the energy-dependent amantadine tubule uptake which we have previously described as bicarbonate-independent uptake (Escobar *et al*, 1994; Escobar and Sitar, 1995). At least three possible hypotheses may be proposed to explain such an observation (Fig. D-1), although it is important to emphasize that a combination of all three hypotheses is possible to explain the mechanism for lactate modulation of amantadine uptake:

- (A) lactate modulates uptake sites that are physically different than the bicarbonate-dependent and bicarbonate-independent uptake sites, i.e. lactate-dependent sites.
- (B) our working hypothesis that lactate is an inefficient modulator of amantadine uptake via the bicarbonate-dependent uptake sites.
- (C) lactate enhances uptake capacity at the bicarbonate-independent uptake sites.

These hypotheses (Figure D-1) make the following assumptions:

- lactate interacts with only one type of uptake site. Such an assumption may not be physiologically valid. However, it simplifies our model.
- lactate modulates the extracellular side of the basolateral membrane. As explained before (*vide supra*), we believe that our uptake studies are mainly a reflection of the

events occurring at the basolateral membrane. However, as explained later in this section, lactate may also act intracellularly to modulate amantadine uptake.

- in the absence of bicarbonate, amantadine uptake occurs *via* the bicarbonate-independent uptake sites (③) which possess lower affinity and lower capacity than the bicarbonate-dependent uptake sites, as demonstrated before (*vide supra*).

According to hypothesis **(A)**, in the presence of lactate and in the absence of bicarbonate, uptake of amantadine would occur via the lactate-dependent (①), and the bicarbonate-independent (③) uptake sites. The overall observed effect would be higher uptake rates than in the absence of bicarbonate and lactate (phosphate buffer). If hypothesis **(B)** is correct, the lactate-dependent uptake sites (①) do not exist, amantadine uptake occurs via the bicarbonate-dependent sites (②) which are lactate-responsive, i.e. respond to modulation by lactate, and the bicarbonate-independent uptake sites (③). Transport rates would be lower than rates obtained in KHS because lactate is a less efficient modulator than bicarbonate. Hypothesis **(C)** proposes also that lactate-dependent uptake sites (①) do not exist, and that lactate modulates the bicarbonate-independent uptake sites (③). In the presence of lactate and in the absence of bicarbonate, amantadine uptake would be modulated only *via* this type of site.

It cannot be determined which of these three hypotheses is most likely to explain the observations in lactate buffer based solely on studies in the absence of bicarbonate. Differences among the three hypotheses become apparent if both lactate and bicarbonate are present. If hypothesis **(A)** is correct (Fig. D-1), in the presence of bicarbonate and lactate, amantadine uptake would occur *via* sites (①), (②), and (③). An apparent increase in uptake rates would be observed. If hypothesis **(B)** is correct, amantadine

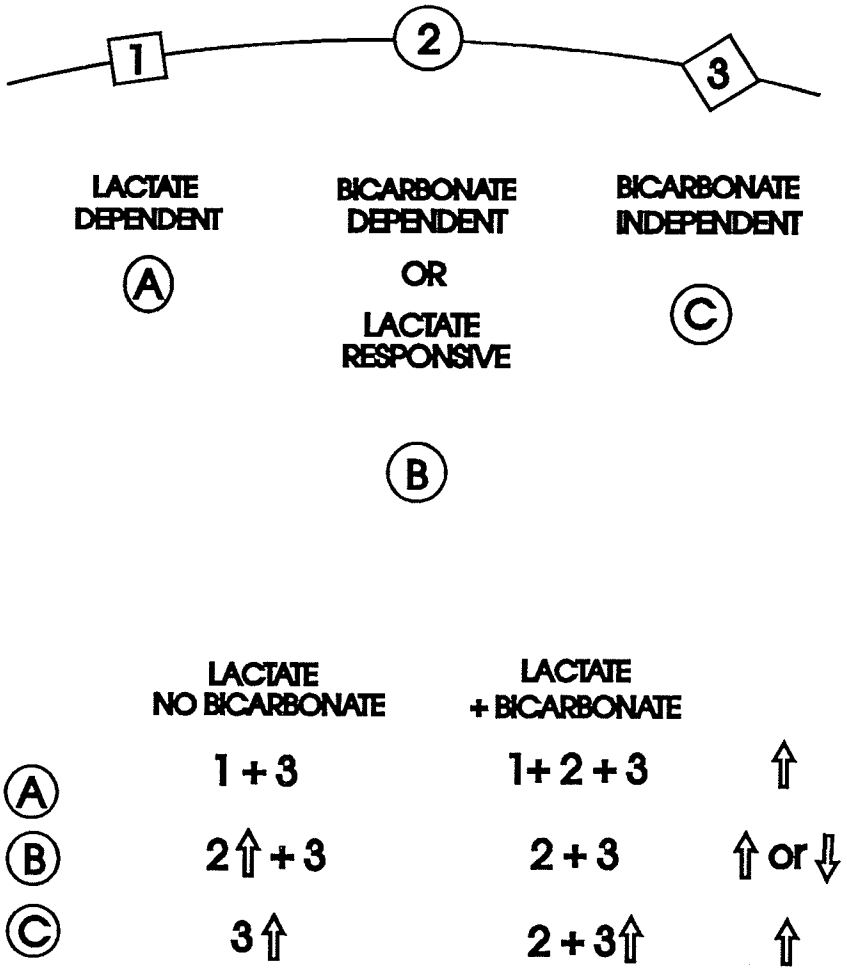


Figure D-1. Illustration of three hypotheses that may explain modulation by lactate of amantadine renal tubule uptake. Numbers denote transporters, and circled letters denote hypotheses. For every hypothesis, the illustration shows which transporters are believed to mediate amantadine uptake. ⇧ and ⇩ beside a number denotes enhanced or decreased uptake *via* that transporter respectively. Arrows on the right-hand side denote the predicted rate of amantadine uptake for the condition of "lactate + bicarbonate" compared to bicarbonate (KHS) buffer.

uptake would occur *via* sites (②) and (③). If lactate and bicarbonate act synergistically for modulation of sites, an apparent enhancement of uptake would be observed. On the other hand, if competition for modulation of the bicarbonate-dependent sites (②) occurs between lactate and bicarbonate, the end result may be an apparent inhibition of amantadine uptake since lactate is a less efficient modulator than bicarbonate. Hypothesis **(C)** would predict that uptake of amantadine in the presence of bicarbonate and lactate would be higher than that observed in bicarbonate buffer alone.

The fact that lactate had no apparent effect in phosphate buffer suggests that hypothesis **(C)** may not be likely and that lactate exerts its action on a site other than the bicarbonate-independent sites. Moreover, since the inhibitory effect of lactate appears to be of the competitive type at lactate concentrations of less than 15 *m*M, it suggests that our working hypothesis (hypothesis **(B)**), rather than **(A)**, may be the more plausible explanation for the modulation of amantadine uptake by lactate. Therefore, the site of action of lactate may be directly on the bicarbonate-dependent amantadine transporter just as we hypothesized.

Although the complete inhibition of amantadine uptake at a lactate concentration of 20 *m*M would suggest interaction at the bicarbonate-independent sites (hypothesis **(C)**), we propose that such an observation may reflect instead the triggering of an intracellular mechanism which completely turns off energy-dependent amantadine uptake. Indeed, it has been reported that during conditions that acidify the renal intracellular milieu, such as lactic acidosis and respiratory acidosis, there is a decrease in the rate of glycolytic reactions (Relman, 1972; Dawson, 1977, Madias, 1986), and renal ATPase function (Eiam-Ong *et al*, 1994). It remains to be determined whether lactate inhibition of amantadine tubule uptake in phosphate buffer may be time-dependent due to the slow activation of the intracellular event we propose. At the present time, we cannot infer as to the site, intracellular or extracellular, at which lactate inhibits amantadine uptake so

abruptly between the concentrations of 15 and 20 *m*M (Figs. R14 & R-15). Further studies will be necessary to delineate such a mechanism.

Overall, the similarity in uptake rates in enantiomerically pure lactate buffers (no bicarbonate) at concentrations of amantadine lower than 200 μM, and the lack of stereoselective inhibition of amantadine (10 μM) uptake by lactate in the presence of bicarbonate suggest that no stereoselective effect of lactate will be observed until high concentrations of amantadine (>200 μM) are reached. Although a stereoselective effect by lactate on amantadine uptake in KHS was not evident in our studies, Yamamoto *et al* (1993*a*) reported that L(+)-lactate infusion did not affect the excretion of the oxypurines, allopurinol and its metabolite oxypurinol, whereas infusion of racemic lactate decreased their excretion (Yamamoto *et al*, 1993*b*). These reports are consistent with a stereoselective effect of D(-)-lactate on the excretion of purine bases. Therefore, an interaction of lactate with other cations appears to be possible.

SUMMARY II:

We provide evidence that lactate may mediate amantadine uptake by renal proximal and distal tubules at the bicarbonate-dependent uptake sites by serving as a replacement for bicarbonate, but with lower efficiency.

We propose that in the absence of bicarbonate, the existence of sites that are L(+)-lactate-responsive and those that are D(-)-lactate-responsive becomes apparent, and that these sites are heterogeneously distributed between tubules.

In the presence of bicarbonate, lactate competes for modulation of the bicarbonate-dependent uptake sites and that this competition is reflected as graded inhibition of energy-dependent amantadine uptake.

Complete inhibition at a lactate concentration of 20 *m*M may reflect the triggering of an intracellular mechanism that turns off energy-dependent amantadine uptake by the tubules.

Our findings suggest that *in vivo* studies would be helpful to determine whether adjustments in drug therapy may be indicated during pathophysiological conditions where circulating lactate concentration is altered.

IV. BICARBONATE EFFECT ON INHIBITION OF ORGANIC CATION UPTAKE BY DIASTEREOISOMERS.

In the present studies, we report for the first time that bicarbonate modulates stereoselectivity of inhibition of renal uptake of amantadine by renal tubules. As an extension of our inhibition studies, we conclude that the presence of bicarbonate enhances amantadine uptake selectively at the site that is quinine-sensitive, in proximal tubules, but in contrast, enhancement occurs at the quinidine-sensitive uptake site in the distal tubules.

Control Uptake Studies: In order to determine whether amantadine uptake in the different buffers (bicarbonate, phosphate and phosphate-plus-bicarbonate) were consistent with our previous observations, rates of amantadine uptake without any quinine or quinidine were compared. Replenishment of bicarbonate to the phosphate buffer increased amantadine uptake rates to values that approximated control levels. However, these studies showed much higher amantadine uptake than control upon replenishment of bicarbonate to the phosphate buffer. This discordance is most likely a reflection of the variability of amantadine uptake, since the phosphate-plus-bicarbonate data were obtained from a different batch of tubules than those data obtained in the KHS buffer. However, the effect of tubule pre-incubation time in the buffer with the added bicarbonate on amantadine uptake remains to be assessed as an alternative explanation.

Inhibition Studies: Quinine and quinidine inhibited amantadine accumulation in a competitive fashion independently of the presence of the bicarbonate anion.

Stereoselective inhibition of quinine over quinidine in the proximal but not distal tubules confirmed studies reported previously by our laboratory (Wong *et al*, 1990, 1993). However, bicarbonate modulated the inhibitory potencies of quinine and not quinidine for the proximal tubule; and for quinidine and not quinine in the distal tubules. Stereoselective inhibition was present in the proximal tubules both in the presence and absence of bicarbonate, with quinine being more potent than quinidine in KHS and the opposite potency observed in phosphate buffer. Moreover, since the inhibitory potency of quinidine is not affected upon removal of bicarbonate, we may conclude that the effect of bicarbonate is selective for the site that transports quinine and amantadine, i.e. the "quinine-sensitive site" in the proximal tubule.

When bicarbonate (physiological concentration, 25 *m*M) is added back to the phosphate buffer all the inhibitory potencies on amantadine tubule uptake returned to control levels. Such an observation provides further support to our hypothesis that the effect of bicarbonate on amantadine uptake sites is reversible and that the bicarbonate-dependent amantadine uptake site is quinine-sensitive in proximal, and quinidine-sensitive in distal tubules. However, quinidine becomes more potent in the proximal tubules compared to its control (KHS). This observed increased in inhibitory potency by quinidine of amantadine uptake may reflect inhibition occurring also at the quinine-sensitive site as a result of non-selective organic cation transport upon sudden re-activation of this transporter.

Overall, these conclusions support our hypothesis that bicarbonate modulates uptake other organic cations as well as amantadine. Also, they lend support to the suggestion that uptake sites in the tubules possess overlapping affinities for different compounds. Moreover, they may describe a new modulatory mechanism for transport of chiral compounds so that under physiological conditions, tubules would secrete selectively a specific conformation. Therefore, it would seem important to determine how

pathological conditions that alter bicarbonate levels, such as metabolic acidosis and alkalosis (Alpern *et al*, 1991), affect transport of pharmacological agents.

Although both quinine and quinidine inhibited amantadine uptake in a competitive manner independent of bicarbonate presence, there was no observed stereoselective inhibition by quinine and quinidine in any of the buffers, for the distal tubules. However, the fact that the inhibitory potency of quinidine is decreased upon bicarbonate removal suggests that bicarbonate seems to regulate amantadine distal tubule uptake at the "quinidine-sensitive site". Moreover, such a conclusion together with the fact that uptake sites in the distal tubules do not exhibit stereoselectivity whether bicarbonate is present or absent, would suggest that there exists at least two types of bicarbonate-dependent amantadine uptake sites: one that is quinine-sensitive in the proximal tubules, and one that is quinidine sensitive in the distal tubules. This evidence suggests that uptake sites are heterogeneous between tubule fragments.

SUMMARY III:

Amantadine accumulation is reduced in the absence of bicarbonate. These data further support our previous proposal (Section I) that amantadine accumulation by both tubule fragments is mediated by bicarbonate-dependent and bicarbonate-independent uptake sites.

These apparently different uptake sites may be dissected by their sensitivity to competitive transport inhibitors, i.e. in the proximal tubules, the bicarbonate-dependent uptake site is also quinine-sensitive, and the non-bicarbonate-dependent uptake site is also quinidine-sensitive. These results indicate a possible new tool for exploring and characterizing the different mechanisms for transport of organic cations across the renal tubule cell. Studies may now be pursued in which a relatively selective inhibitor of a specific uptake site can be used, e.g. quinine to study the bicarbonate-dependent amantadine uptake site in the proximal tubule.

V. EFFECT OF ION MANIPULATIONS ON ORGANIC CATION UPTAKE

Potassium and Calcium Studies. In our previous report (Escobar *et al*, 1994), we challenged the suggestion that one of the possible explanations for the decreased accumulation of amantadine by the tubules under zero extracellular bicarbonate was cell depolarization resulting from intracellular bicarbonate efflux. This cell depolarization may create an opposing force to amantadine influx across the basolateral membrane. In the present studies we did not measure tubule cell membrane potential. However, in our studies, high extracellular K^+ did not affect amantadine uptake by the tubules. Therefore, these studies provide further indirect evidence against the proposal that cell depolarization plays a major role in the decrease of amantadine uptake in the absence of bicarbonate, as hyperkalemic solutions depolarize proximal tubule cells (Biagi *et al*, 1981; Burckhardt *et al*, 1984; Smith *et al*, 1988; Yang *et al*, 1988).

Low K^+ concentrations (down to 1.5 *m*M) in the incubation medium had no effect on amantadine accumulation. This observation is important as low extracellular K^+ (0.5-1.0 *m*M) is known to inhibit the Na^+/K^+-ATPase enzyme by 50% (Skou, 1962 & 1965; Jorgensen, 1980). Since this ATPase is mainly responsible for maintaining the negative intracellular potential, we hypothesize that the Na^+ pump may not play a major role in amantadine uptake by renal tubules in the presence of extracellular bicarbonate. However, in our studies, the extracellular K^+ concentration was not low enough to completely inhibit all Na^+/K^+-ATPase activity (Skou, 1962 & 1965; Jorgensen, 1980), and in the absence of such direct measurements, our interpretation is tentative.

The next ion studied was calcium. These experiments were necessary to assess whether the lower Ca^{2+} concentration (1 *m*M) in the phosphate buffer contributed to low amantadine uptake. It was observed that for proximal tubules, only high Ca^{2+} concentrations (2.5 *m*M for phosphate buffer is "high" Ca^{2+} concentration) decreased

amantadine accumulation. For distal tubules, the results were qualitatively different, as an enhancement of amantadine uptake was observed at low Ca^{2+} concentrations. Although the increase observed at low Ca^{2+}, and the decrease seen at 2.5 *m*M were statistically significant, we do not believe them to be physiologically important since the changes are modest (13 and 14 %, respectively). However, in the absence of direct intracellular Ca^{2+} measurements, it is uncertain as to the exact effects of extracellular Ca^{2+} manipulations on intracellular Ca^{2+} concentrations.

High extracellular calcium (5 *m*M) decreased amantadine accumulation by both tubule fragments equally. We believe that a likely explanation for such an observation is that of cell death due to swelling as intracellular Ca^{2+} is increased (McCarty and O'Neil, 1992). It is quite possible that the low uptake rates by the tubules may be due to effects of calcium precipitation, although during the assays no precipitate was observed. A search of the literature revealed no publications on the direct effect of calcium on organic cation transport. Although calcium channel blockers such as verapamil have been shown to inhibit competitively organic cation transport in renal tubules (Ott and Giacomini, 1993; Pan *et al*, 1994; Sitar *et al*, 1995), the involvement of the calcium channel blocking activity on renal handling of organic cations is not known.

The possibility that moderate changes in extracellular Ca^{2+} concentrations may play a significant role in the long-term situation cannot excluded. Such a role may involve alteration of intracellular sodium levels *via* the Na^{+}/Ca^{2+} exchanger present on the basolateral side of renal tubules (Mandel and Murphy, 1984; Yang *et al*, 1988). Therefore, Ca^{2+} interaction with the mechanism of organic cation transport by renal tubules will need further studies.

Sodium and Chloride studies.

The observation that a decrement of the sodium concentration in the incubating medium did not alter kinetic parameters for amantadine uptake by either tubule fragment

in the presence of bicarbonate suggests that amantadine uptake sites are not sodium-sensitive. We cannot rule out the possibility that sodium is required for uptake, as extracellular sodium in our studies was not completely abolished. Moreover, as we did not manipulate intracellular sodium, we cannot rule out the possibility that it is rate-determining. Sodium-dependent efflux of organic cations has been shown for the luminal membrane in which the Na^+/H^+ exchanger is present (Holohan and Ross, 1981; Hawk and Dantzler, 1984; McKinney, 1984; Wright, 1985; Rafizadeh *et al*, 1987; Jung *et al*, 1989; Katsura *et al*, 1991). However, as explained previously, we believe that our studies reflect events occurring mainly at the basolateral membrane. As a result we cannot extend our observations to the extrusion of the organic cation into the luminal space and further studies will be necessary.

In addition, low extracellular concentration of sodium has been reported to alter membrane potential by inhibiting the basolateral Na^+/K^+ ATPase (Skou, 1962 & 1965; Jorgensen, 1980). Therefore, based on the classical model of organic cation uptake by the renal tubule cell (Grantham and Chonko, 1991; Pritchard and Miller, 1993), such a maneuver would be expected to increase amantadine tubule accumulation. However, the fact that no change in tubule accumulation of amantadine was observed in our low sodium studies supports further our proposal that the electrochemical gradient across the basolateral membrane does not play a limiting role in the mechanism for organic cation uptake by renal tubule fragments.

The results obtained with low extracellular chloride suggest that chloride flux may be involved in the mechanism of organic cation uptake by the distal but not the proximal renal tubules. Moreover, the fact that affinity, but not maximal transport capacity, was altered is consistent with a competitive type of inhibition and therefore it suggests that the chloride ion may be directly involved with the uptake of organic cations. If chloride movement across the basolateral membrane is involved, then it is possible that the $Na^+/Cl^-/HCO_3^-$ cotransporter and the Cl^-/HCO_3^- antiporter present in the renal basolateral

membrane (Krapf *et al* 1987; Sasaki and Marumo, 1989; Seki and Frompter, 1992*a* & 1992*b*) are intimately involved with the mechanism for organic cation renal uptake. This proposal fits with our previous observation that amantadine uptake is bicarbonate-dependent and it may further support the direct involvement of the bicarbonate anion.

SUMMARY IV:

Manipulation of extracellular concentrations of sodium and potassium suggests that the negative electrochemical gradient across the basolateral membrane does not play a significant role in the mechanism for organic cation transport by renal tubule fragments. These studies challenge the overall model for organic cationic drug transport as well as previous reports for other organic cations which suggest that transport and accumulation of organic cations by renal tubules occurs in response to the electrochemical gradient (Montrose-Rafizadeh *et al*, 1987; Wright and Wunz, 1987; Sokol and McKinney, 1990; Grantham and Chonko, 1991; Pritchard and Miller, 1993).

Availability of extracellular calcium is not rate-limiting for organic cation uptake by the renal tubules. However, at concentrations of 5 *m*M, calcium is inhibitory.

The amantadine uptake sites in both tubule fragments appear be sodium-insensitive.

Heterogeneity of uptake sites between tubules is supported by the observation that proximal tubule amantadine uptake is insensitive to extracellular chloride fluctuations whereas those uptake sites present in the distal tubules appear to be sensitive to chloride ion changes.

VI. BASOLATERAL RENAL Na^+/K^+ ATPASE AND ORGANIC CATION UPTAKE.

Based on the classical model for organic cation uptake by the renal tubule cells (Grantham and Chonko, 1991; Pritchard and Miller, 1993), alterations to the membrane

potential across the basolateral membrane should alter organic cation uptake. This membrane potential is maintained by the basolateral Na^+/K^+ ATPase. Therefore, inhibition of this ATPase would be expected to alter organic cation flux across the basolateral membrane.

Our studies with the Na^+/K^+ ATPase inhibitors, digoxin and acetylstrophanthidin, suggest that such a proposal may not extend to the mechanism for renal uptake of amantadine. In other words, the fact that of the cardiac glycosides tested only ouabain inhibited amantadine tubule uptake suggests that Na^+/K^+ ATPase does not play a significant role in the mechanism. Moreover, it provides further indirect evidence in support our hypothesis that the membrane potential is not necessary for the influx of the organic cation.

Other possibilities cannot be ignored. It is possible that the Na^+/K^+ ATPase present in rat tissue is insensitive to inhibition by cardiac glycosides or that different sub-types of the pump with different sensitivities to glycoside inhibition exist so that even with concentrations of the glycosides used herein reported to inhibit the Na^+/K^+ ATPase (Rasmussen *et al*, 1985; Larsson *et al*, 1986; Soltoff and Mandel, 1986; Avison *et al*, 1987), not all of its activity was inhibited. Both of these proposals have been suggested previously (Jorgensen, 1980; Sweadner, 1985). Another possibility is that the difference in inhibitory potencies for ouabain between the tubules is related to the fact that Na^+/K^+-ATPase activity is higher in the proximal than in distal tubules (Jorgensen, 1980) and so at the same glycoside concentration, less Na^+/K^+-ATPase activity will be inhibited in the proximal tubules reflected as lower inhibition of amantadine uptake. Both possibilities would suggest that the Na^+/K^+-ATPase may indeed play a significant role in the mechanism(s) for amantadine uptake. Therefore, further studies where the activity of the pump is measured directly may be necessary to determine its exact role in the mechanism for amantadine renal uptake.

It is worth mentioning that digoxin has been reported to accumulate (Koren *et al*, 1983; Wernke and Cacini, 1990), and to be secreted by the renal tubule cells (Steiness, 1974; Halkin *et al*, 1975), possibly *via* the P-glycoprotein present in the luminal side of the renal tubule cell (De-Lannoy *et al*, 1992; De-Lannoy and Silverman, 1992; Ito *et al*, 1992; Tanigawara *et al*, 1992; Hori *et al*, 1993; Ito *et al*, 1993*a* & 1993*b*; Okamura *et al*, 1993). Moreover, the P-glycoprotein has been implicated in the transcellular transport of several drugs including the organic cation cimetidine (Charuk *et al*, 1994; De-Lannoy *et al*, 1994; Dutt *et al*, 1994; Pan *et al*, 1994). As well, since the excretion of digoxin and other substrates for the P-glycoprotein is affected by the presence of the organic cations such as quinidine (Doering, 1979; Hager *et al*, 1979; Pedersen *et al*, 1980; Mordel *et al*, 1993) and verapamil (Pedersen *et al*, 1981; Klein *et al*, 1982; Ito *et al*, 1993*c*) they have been suggested to be modulators of P-glycoprotein.

If P-glycoprotein had a significant involvement in the mechanism(s) for amantadine tubule uptake addition of digoxin would be expected to decrease efflux of amantadine into the luminal space and cause an increase in amantadine tubule accumulation. Since digoxin did not have any apparent effects on amantadine tubule accumulation it suggests that the P-glycoprotein may not be a part of the mechanism(s) for amantadine uptake into the cell. However, the present studies were not aimed at investigating the role of the P-glycoprotein in the mechanism for amantadine uptake and further studies may be required.

The fact that ouabain did inhibit amantadine uptake suggests that it may do so by a different mechanism than inhibition of the Na^+/K^+ ATPase. Ouabain inhibited only 20% of the energy-dependent proximal tubule uptake of amantadine. In contrast, 80% of the distal tubule amantadine uptake was inhibited, and the remaining distal tubule uptake (the ouabain-insensitive amantadine uptake) was similar to the bicarbonate-independent uptake of amantadine. Therefore, it appears that only 25% of the bicarbonate-dependent amantadine uptake by the proximal tubules is ouabain-sensitive but that 100% of the bicarbonate-dependent uptake by the distal tubules is ouabain-sensitive. Such an

observation suggests further that there may exist sub-types of bicarbonate-dependent uptake and that those present in the distal tubules possess more homogeneity. In addition, the bicarbonate-dependent amantadine uptake sites in the distal tubules appear to be similar to only a proportion (25%) of those sites present in the proximal tubules.

Low extracellular sodium and low extracellular potassium have been shown to inhibit the Na^+/K^+ ATPase (Skou, 1962 & 1965; Jorgensen, 1980), although it has been suggested that minimal presence of potassium is sufficient to maintain pump activity (Jorgensen, 1980). Therefore, the present studies with manipulation of sodium and potassium in the presence and absence of ouabain were performed in order to determine whether the site of action of ouabain was indeed the Na^+/K^+ ATPase. If ouabain inhibits amantadine uptake *via* its actions on a site other than the Na^+/K^+ ATPase, then any alterations on uptake of amantadine as a result of inhibitory effects of low sodium and/or low potassium on the pump should be additive to that of ouabain. On the other hand, no further effects on tubule amantadine accumulation should be observed with ouabain if inhibition of the Na^+/K^+ ATPase is the site of action for ouabain and low extracellular sodium and/or low extracellular potassium.

Low extracellular sodium had no effect on amantadine uptake by either proximal or distal tubules. Therefore, these results suggest that the mechanism for ouabain inhibition of amantadine proximal tubule uptake is, to a large extent, sodium-independent. In contrast, ouabain inhibition of amantadine uptake by the distal tubules appears to be sodium-dependent as a complete reversal of inhibition was observed in low extracellular sodium. The increase in amantadine distal tubule accumulation past control levels under the low extracellular sodium conditions may be explained by a facilitated influx or a decreased luminal efflux of amantadine. We cannot discern between those possibilities and further studies may be required.

The binding site for ouabain on the Na^+/K^+ ATPase has been reported to be at the potassium binding site (Skou, 1962 & 1965; Jorgensen, 1980). Therefore, it would be

expected that in conditions of low extracellular potassium there would be an enhancement of ouabain inhibition of the Na^+/K^+ ATPase. If this ATPase plays a significant role in the mechanism of organic cation tubule uptake, the expected result would be a decreased amantadine tubule accumulation. Therefore, the reversal of ouabain inhibition of proximal and distal tubule amantadine uptake under low extracellular potassium conditions appears paradoxical and supports further the idea that the site of action for ouabain is not the Na^+/K^+ ATPase. Furthermore, it appears that the mechanism for ouabain inhibition of amantadine uptake is potassium-dependent. The proportion of the ouabain-inhibited uptake by both tubules reversed by low extracellular potassium was similar (35% and 45% of control values for proximal and distal tubules, respectively) suggesting that the potassium-sensitive part of the ouabain-inhibited amantadine uptake may be similar between tubules. The exact mechanism by which low potassium attenuates the inhibitory potency of ouabain and promotes increased amantadine accumulation is unknown to us at the present time.

Low potassium together with low sodium decreased proximal tubule amantadine uptake suggesting that sodium and potassium act synergistically to enhance amantadine flux into the cell under normal conditions. Moreover, it appears that the extracellular absence of one cation can be compensated for by the presence of the other. Therefore, contrary to the interpretation of results reported in previous sections, these data suggest that sodium and potassium indeed may be required for amantadine tubule uptake, although they may not be rate-limiting. Our data suggest also that the mechanism for ouabain interaction with the amantadine uptake system is able to compensate for the combined low extracellular potassium and low extracellular sodium-promoted decrease. The increased uptake caused by ouabain in the absence of extracellular sodium and potassium suggest that possibly ouabain may modulate efflux of the organic cation at the luminal membrane. However, further studies may be necessary to elucidate such a mechanism.

We propose that ouabain inhibits amantadine uptake *via* an unique but uncharacterized mechanism that may not involve the basolateral Na^+/K^+ ATPase. Studies in our laboratory with tritiated ouabain agree with studies by Ito *et al* (1992) which suggest that ouabain does not enter the renal tubule cell. Therefore, the mechanism by which ouabain inhibits amantadine uptake includes most probably an extracellular event or the triggering of an intracellular event *via* extracellular means.

The presence of bicarbonate is necessary for ouabain to exert its inhibitory effect as ouabain does not inhibit the bicarbonate-independent tubule specific uptake of amantadine. This point is supported by the fact that addition of bicarbonate to the incubation medium resulted in the return of the inhibitory effect of ouabain. The fact that a certain pre-incubation time is needed for the inhibition to be observed strongly suggests that the mechanism by which ouabain inhibits amantadine uptake involves an intracellular event.

SUMMARY V:

Na^+/K^+ ATPase activity is not rate-limiting for organic cation renal uptake. Therefore, the present studies argue against the classical mechanism in that they challenge the proposal that the main driving force for organic cation entry into the cell is highly dependent on the electrochemical gradient.

Ouabain inhibits amantadine uptake *via* an unique mechanism that may involve an intracellular event that does not involve the access of ouabain to the intracellular space. Moreover, this mechanism may not involve the basolateral Na^+/K^+ ATPase.

Sodium and potassium may act synergistically to enhance amantadine flux into the cell. As well, the extracellular absence of one cation can be compensated for by the presence of the other. Therefore, both cations may be required, but not rate-limiting, for amantadine tubule uptake.

VII. REVISED MODEL FOR ORGANIC CATION UPTAKE BY THE RENAL PROXIMAL TUBULE.

In light of the present dissertation, the classical model for organic cation uptake by the renal proximal tubule (Grantham and Chonko, 1991; Pritchard and Miller, 1993) may need to be revised.

Figure D-2 depicts our revised model to explain the mechanism for amantadine uptake by the proximal renal tubule cell based on our present findings and those reported previously (Wong *et al*, 1990 & 1993; Escobar *et al*, 1994; Escobar and Sitar, 1995). We propose that amantadine specific uptake into the cell is energy-dependent since tissue treatment with 2,4-dinitrophenol and freezing decrease tubule uptake. Moreover, since uptake studies follow Michaelis-Menten transport kinetics, we propose that specific uptake is mediated by a saturable component of the basolateral membrane. Our findings with low and high extracellular potassium, and low extracellular sodium suggest that the influx of the organic cation does not occur in response to the electronegativity of the cell interior. Instead, our revised model proposes either a primary or secondary active uptake step at the basolateral membrane that is non-electrogenic. This uptake step appears to be sodium-, potassium-, and chloride-insensitive to a very large extent.

Our acetazolamide (ACZ) experiments suggest that intracellular bicarbonate pools do not play a significant role in the presence of unlimited extracellular bicarbonate (Escobar *et al*, 1994). Therefore, bicarbonate exerts its action extracellularly at the basolateral membrane, quite possibly directly on the transporter. It remains to be determined whether bicarbonate interacts with any component of the luminal membrane. Bicarbonate handling by the renal tubules is complex. For proximal tubules, HCO_3^- flux across the basolateral membrane is intimately involved with chloride flux. However, the fact that the transporters seemed to be chloride-insensitive suggests that although the Cl^-/HCO_3^- cotransporter and the Cl^-/HCO_3^- antiporter present in the renal basolateral membrane may be involved, they do not play a quantitatively significant role. We cannot

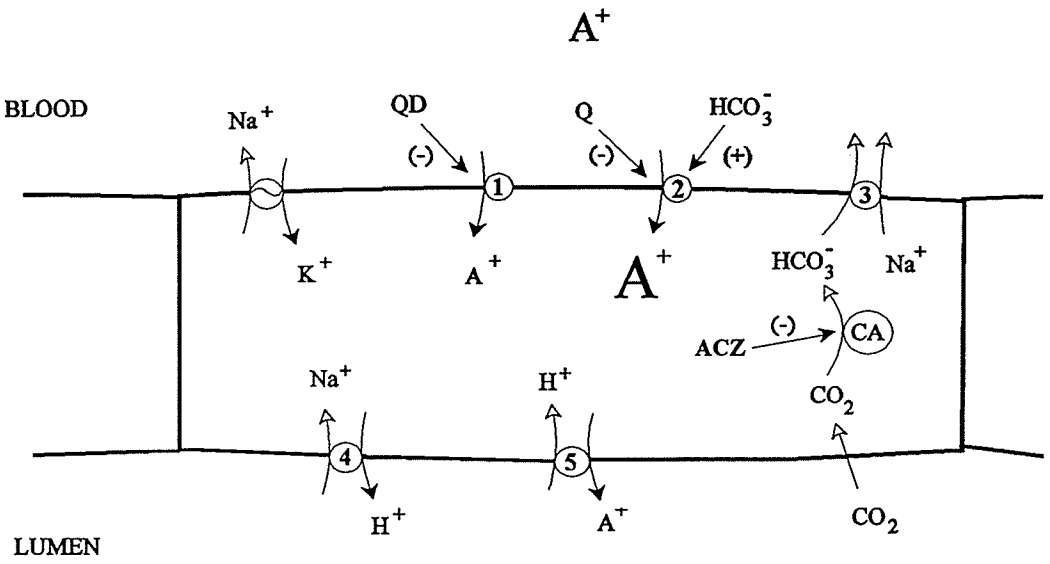


Fig. D-2: Revised model for amantadine uptake by proximal rat renal proximal tubules. **1-5**, Proposed transporters: **1**, Bicarbonate-independent amantadine transporter; **2**, bicarbonate-dependent amantadine transporter; **3**, Na^+/HCO_3^- co-transporter; **4**, Na^+/H^+ antiporter; **5**, H^+/Organic $Cation^+$ antiporter. **QD**, quinidine; **Q**, quinine; **ACZ**, acetazolamide; **CA**, carbonic anhydrase; **(+)** refers to activation; **(-)** refers to inhibition; A^+, amantadine (size reflects concentration due to respective transporter). For explanation please refer to text.

rule out the involvement of other transporters such as the Na^+/HCO_3^- cotransporter (site 3) and further studies are necessary.

Entry of the cation into the cell is mediated by uptake sites at the basolateral membrane that may be sub-divided into: site 1, non-bicarbonate-dependent uptake sites (low affinity, low capacity); and site 2, bicarbonate-dependent uptake sites which possess high affinity and high capacity (Fig. D-2). The majority of the energy-dependent amantadine accumulation in the presence of bicarbonate (approx. 80 %) is accomplished *via* the more efficient uptake sites (site 2). We propose that lactate also is able to modulate these sites (site 2) and dissects them further into L(+)- and D(-)-lactate responsive sites. Removal of extracellular bicarbonate will result in decreased amantadine accumulation that is mediated mainly *via* the less efficient bicarbonate-independent uptake sites (site 1).

The bicarbonate-dependent and -independent uptake sites in the proximal tubules may be dissected further by the fact that the former sites seem to be sensitive to quinine, whereas the latter sites appear to be sensitive to the presence of quinidine. Exit from the cell is mediated by a H^+/organic cation antiport (site 5) which utilizes the H^+ gradient (out>in) created by the Na^+/H^+ exchanger (site 4)(Holohan and Ross, 1981; Wright, 1985; Jung *et al*, 1989).

It has been proposed that one of the main driving forces for entry of the organic cation into the cell is the electrochemical gradient created by the Na^+/K^+ ATPase present in the basolateral membrane. However, based on our results with the cardiac glycosides, digoxin and acetylstrophanthidin (Section V), we suggest that sodium pump activity is not rate-limiting in the mechanism for organic cation uptake by the renal tubules.

Our model explains why upon bicarbonate removal from the medium the inhibition kinetics of quinine, but not quinidine, are observed to change. If extracellular bicarbonate is replenished, and there is no cell damage, amantadine uptake *via* the bicarbonate-

dependent uptake site is restored, accumulation is increased, and quinine inhibition is enhanced.

Although we have evidence that the mechanism for amantadine uptake across the distal tubule cell is similar, we may not assume that it is exactly the same. Consequently, renal distal tubule handling of amantadine awaits further exploration. However, present and previous studies support the notion that many of the steps in the mechanism proposed for proximal tubule cells, are also operational in the distal tubule cells. Most importantly, our present studies suggest that there are indeed bicarbonate-dependent and bicarbonate-independent uptake sites in the distal tubules. The bicarbonate-dependent sites (Table D-1) appear to be quinidine-sensitive, in contrast to the proximal tubules which are quinine-sensitive, since removal of bicarbonate affects only the inhibitory potency of quinidine. These uptake sites may be sodium- and potassium-independent but they may have a higher sensitivity to chloride fluctuations.

Table D-1. Summary of sub-classification of the bicarbonate-dependent (Krebs-Henseleit buffer) amantadine uptake sites in the renal cortical tubule fragments.

PROXIMAL	DISTAL
Quinine-sensitive	Quinidine-sensitive
D(-)- > L(+)-lactate responsive	L(+)- > D(-)-lactate responsive
25% ouabain sensitive	100% ouabain sensitive
Na^+ and K^+-insensitive	Na^+ and K^+-insensitive
Cl^--insensitive	Cl^--sensitive
5 *m*M Ca^{2+} inhibitory	5 *m*M Ca^{2+} inhibitory

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