# CHARACTERIZATION OF CHOLINE TRANSPORT

# IN HUMAN EMBRYONIC KIDNEY (HEK-293) CELLS

By

ERICA SIOVHAN MARSHBURN

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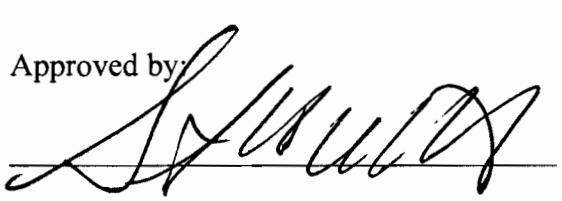

Dr. Stephen H. Wright
Department of Physiology

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**Abstract:**

Choline is an important electrolyte that is vital to cell function and reabsorption of choline in the kidney plays a significant role in regulating plasma choline concentrations. Transport across the luminal membrane in the renal proximal tubule is a key step, but the molecular identity of this process in unknown. Human Embryonic Kidney (HEK-293) cells are of renal origin and thus might provide some insight to the characteristics of this transport process. The object of this set of experiments was to determine, with reasonable certainty, which choline transporter is present in these cells and responsible for choline uptake. HEK-293 cells have been shown in previous experiments to transport choline fairly well. The profile of choline transport in HEK-293 cells was shown to be an intermediate-affinity, sodium-independent, pH sensitive process that can be inhibited by choline, MPP, amiloride, clonidine, guanidine, and quinidine, and was not blocked by TEA. This profile is inconsistent with the Organic Cation Transporter 2 (OCT2), the Multidrug and Toxin Extrusion 1 (MATE1), or the High-Affinity Choline Transporter (CHT1) and is mostly consistent with the Choline Transporter-Like Protein (CTL1). The characteristics of choline transport in HEK-293 cells were thus mostly consistent with those of CTL1 and with characteristics of choline reabsorption in the proximal tubule. CTL1 is thus a reasonable suggestion for the luminal transport step involved in choline reabsorption in the proximal tubule.

**Introduction:**

A review by Wright and Dantzler[1] describes details of transport processes in the kidney, some of which are described below. The kidneys are responsible for transport of organic anions (OA) and organic cations (OC) out of and sometimes back into the bloodstream, and these

‘organic electrolytes’ include a variety of compounds with relevance for physiological, pharmacological, and toxicological study.[1] Renal secretion of organic electrolytes plays an important role in limiting the bioavailability of toxic compounds of exogenous and endogenous origin in the human body. Renal secretion of OAs and OCs occurs principally in the proximal portion of the nephron and plays an important role in regulating plasma concentrations of these compounds. Transepithelial OC and OA transport involves different transport processes for entry and exit at the basolateral and luminal sides, respectively, of renal tubular cells.

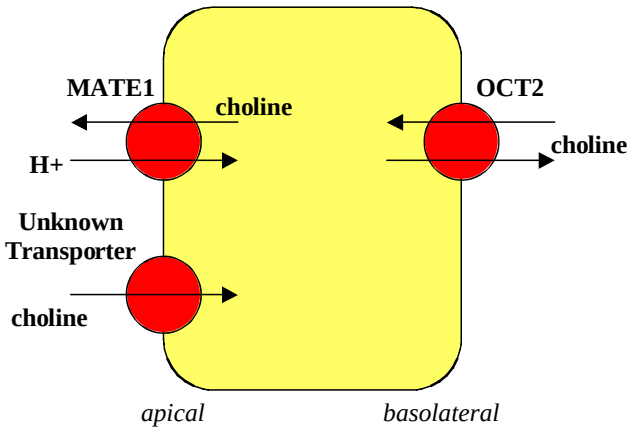
The proximal tubule is the primary site of renal OC secretion.[1] Substrates involved in this pathway include primary, secondary, tertiary, and quaternary amines with a positive charge on the amine nitrogen at physiological pH. OCs enter the cell, from the blood, across the basolateral (peritubular) membrane. This entry step is usually completed through OCT2 by means of facilitated diffusion driven by the electrical potential difference and the negative charge on the inside of the cell, or by an electroneutral exchange of OCs via an antiporter. The negative potential difference inside proximal cells accounts for the considerable accumulation of OCs inside these cells.

Exit of OCs across the luminal membrane involves a carrier mediated antiport of an OC for a proton ($H^+$).[1] This electroneutral transport of an OC for an $H^+$ via MATE1 permits OCs to exit the cell and contribute to a luminal concentration equal to or greater than that in the cytoplasm, resulting in net secretion. $OC/H^+$ exchange is the active step in the process of exiting as it depends on the movement of $H^+$ away from electrochemical equilibrium, and this state is maintained by the cooperation of processes in the luminal membrane: the $Na^+/H^+$ exchanger, and a V-type $H^+$-ATPase. Luminal $OC/H^+$ exchange is thought to be the rate-limiting step in OC

transport across proximal cell membranes. Secretion of some OCs occurs via a multidrug resistance transporter located on the apical membrane.

Choline is a unique compound because it is secreted like other OCs, but under physiological conditions choline is one of the few OCs that can be reabsorbed. Choline is a quaternary amine compound essential to cell function, as it contributes to the construction of cell membranes (phosphatidyl choline). Choline also functions as a methyl-group donor in methionine metabolism and in the synthesis of the neurotransmitter acetylcholine. A deficiency in choline will affect the expression of genes involved in cell proliferation, differentiation, and apoptosis. Abnormal choline transport and metabolism has been linked with neurodegenerative disorders such as Alzheimer's and Parkinson's disease.[2]

As noted above, choline is a unique OC because it is both reabsorbed and secreted. The process of choline secretion in the renal proximal tubule has been clarified, and the molecular identity of transporters involved in secretion have been established as Organic Cation Transporter 2 (OCT2) and Multidrug and Toxin Extrusion 1 (MATE1). OCT2 mediates the process of facilitated diffusion of choline across the basolateral membrane driven by the negative electrical potential within the cell. MATE1 exchanges choline for a proton at the apical membrane in an electroneutral process. These processes are displayed in a diagram below.




The transporters involved in the reabsorption of choline, however, are unknown, and that is the issue to be explored throughout this paper. The first step towards discerning what transporters are responsible for choline reabsorption, is determining which transporters choline is known to interact with. Kinetic studies have revealed three distinct systems for choline transport: (i) low affinity facilitated diffusion, (ii) high affinity sodium dependent transport, and (iii) intermediate affinity sodium independent transport. Choline interacts with a handful of known transporters throughout the body. The 'low affinity'processes include OCT2 and MATE1. OCTs are polyspecific organic cation transporters, have a low affinity for choline ($K_t$>100μM), and transport in a sodium-independent manner. [2] MATE1 is a multidrug and toxin extrusion transporter with a low affinity for choline ($K_t$>>100μM). [3] The high affinity process involves Choline Transporter 1 (CHT1). CHT1 is a high affinity ($K_t$<1μM), sodium-dependent choline transporter found primarily in the neurons and supplies choline for acetylcholine synthesis.[2] The intermediate affinity choline transport process appears to involve Choline Transporter-Like Protein (CTL1). CTL1 is an intermediate affinity, sodium-independent choline transporter-like protein expressed in different cell types and provides choline for production of the most abundant choline metabolite, the membrane lipid phosphatidylcholine.[4]

Luminal choline transport in the kidneys is found to be saturable and mediated by a transporter of intermediate affinity ($K_t$=10-20μM).[4] I hypothesize that CTL1 is responsible for choline transport in the proximal tubule, and this study aims to determine if CTL1 is responsible for choline transport in HEK-293 cells and, by extension, implicate CTL1 as a key player in the proximal tubule, and further characterize choline transport via CTL1. Only a small amount of choline filtered in the glomerulus is excreted in the urine, suggesting that homeostatic regulation of plasma choline by the renal tubule results from active reabsorption, resulting in retention of choline by the body.[5] There is also a bidirectional tubular transport system suggested for choline. Choline is found to be reabsorbed from the proximal convoluted tubules as well as from the pars recta and/or loop of Henle.[6] The uptake of choline across the brush border membrane is found to be a sodium independent process driven by the interior cell negative membrane potential, which matches well with the profile of the CTL1 transporter.[7] Choline transport in a cultured renal cell line (NRK-52E) is saturable and mediated by a single transport system, with an apparent Michaelis-Menten constant $K_t$~16.5 μM and a maximal velocity $J_{max}$~133.9 pmol/mg protein-min.[4] This profile does not fit OCT1, OCT2, or CHT1, so it suggests that the only other known choline transporter, CTL1, is potentially responsible, and coincidentally, CTL1 is expressed in kidney tissue, along with other tissues of the body.[8]

HEK-293 cells have been shown in my previous experiments to express robust choline transport and since I have previously observed choline transport in these cells, the object of my study was to determine, with reasonable certainty, which choline transporter is present in these cells and responsible for the choline uptake. In this experiment the transport of choline in HEK-293 cells will be characterized to provide a model for choline transport in the kidneys.

**Materials and Methods:**

*Cell culture*

Human Embryonic Kidney (HEK-293) cells were grown at 37°C in a humidified atmosphere (5% $CO_2$) in plastic culture flasks. The medium was high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% pen-strep. Cultures were split every 3-4 days. Cells were seeded into 48-well plates at 500,000 cells/well. Uptake was typically measured 24 hours after seeding, which was sufficient for the cells to reach confluence.

*Measurement of transport*

HEK-293 cells were rinsed with room temperature (approx. 25°C) Waymouth buffer (WB) consisting of 135mM NaCl, 13mM Hepes, 2.5mM $CaCl_2 2H_2O$, 1.2mM $MgCl_2 6H_2O$, 0.8mM $MgSO_4 7H_2O$, 5mM KCl, and 28mM D-Glucose, adjusted to pH 7.4 with 5 N NaOH. The cells were then incubated in 125 µl of WB to which labeled substrate ($^3$H-choline) and appropriate test agents were added. Uptake was stopped by rinsing the cells three times with .25 ml of ice-cold WB. The cells were then solubilized in 200 µl of 0.5 N NaOH with 1% (vol/vol) SDS, and the extract was subsequently neutralized with 100 µl of 1 N HCl. Accumulated radioactivity was determined by liquid scintillation spectrometry. Rates of uptake are expressed as picomoles per milligram of protein per minute.

*Measurement of protein content*

A bicinchoninic acid (BCA) protein assay was used to determine the amount of protein in each well. 10 µl of each solubilized and neutralized well was pipetted into a 96-well plate and was incubated with 200 µl of working reagent (50 parts Reagent A and 1 part Reagent B) at 37°C for 20 minutes. Bovine serum albumin (BSA) standards were also incubated in the same way, and a microplate reader determined the absorbance of all wells. The absorbances of the

standards were then used to determine the concentration of protein per μl, and this was extrapolated to determine the overall amount of protein in each well.

**Results and Discussion:**

My first experiments involved working with choline transport in Chinese Hamster Ovary (CHO) cells, which, like HEK-293 cells, are a commonly used heterologous expression system. Previous observations had shown that wild-type CHO cells transport choline fairly well, whereas other OCs like MPP are poorly transported unless transfected with an OCT or MATE. CHO cells transfected with the OCT2 transporter expressed choline transport with an apparent $K_t$~149 μM and a $J_{max}$~62 pmol/mg-min, exhibiting a relatively low affinity, but high capacity, for choline uptake (Figure 1). However, the native choline transport of the Wild Type (WT) CHO cells that had no added transporters, while showing a much lower capacity for choline transport (i.e. lower $J_{max}$), displayed a transport process of a much higher affinity ($K_t$~23.1 μM) (Figure 1). This suggests a transporter natively expressed by CHO cells is transporting choline but was masked by addition of OCT2. Since my research was focused primarily on substrate transport in human kidneys, I decided to explore choline transport in another cell type often used in cell transport studies, HEK-293 cells. HEK-293 cells are precursor cells to kidney cells, and because they are of renal origin, any native transport expressed might have some relevance for the characterization of substrate transport in the human kidney.

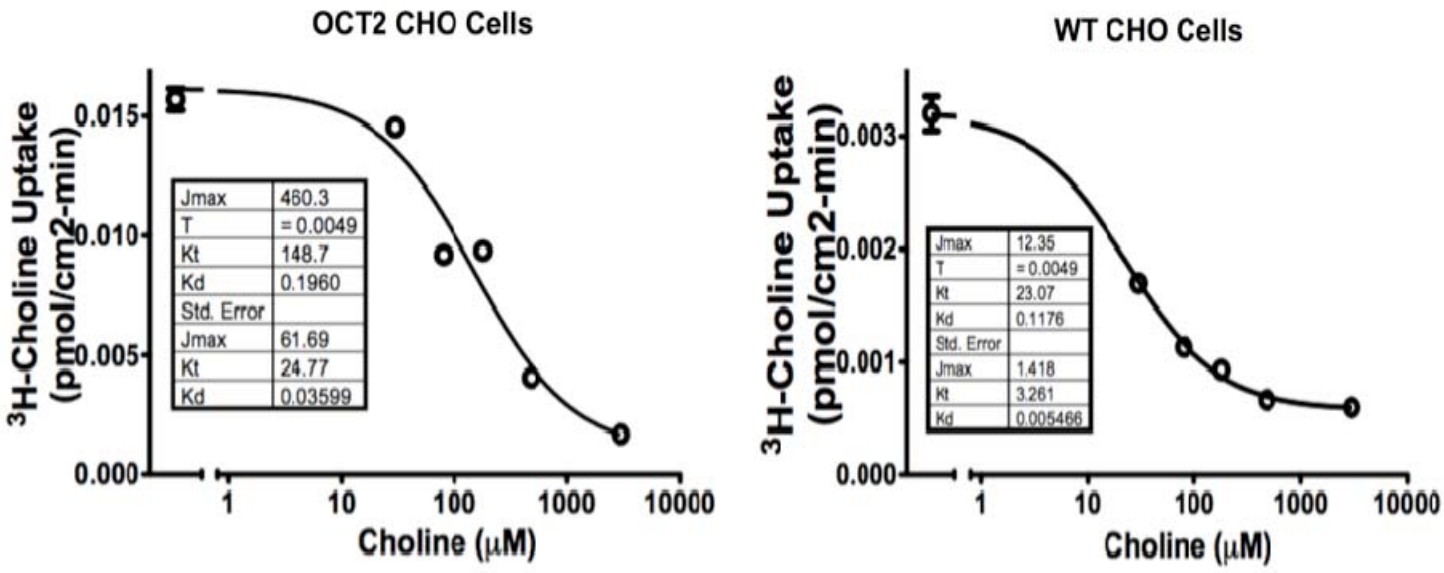



**Figure 1: Choline Transport in Chinese Hamster Ovary (CHO) Cells.** Choline transport in CHO cells transfected with OCT2 is low affinity ($K_t$=149μM). CHO WT cells also express native choline transport of a greater affinity ($K_t$=23.1μM).

***HEK-293 Cells might also express native transport and are of renal origin.***

A requirement for analyzing transport data with Michaelis-Menten kinetics, is that kinetics be based on the 'initial rate' of substrate uptake. Thus, an important step in studying transport processes is to determine a time point up to which transport is a linear process. Unlabeled choline blocked the uptake of labeled $^3$H-choline, and this process is described by the Michaelis-Menten equation for the competitive interaction of labeled and unlabeled choline:

$$J = \frac{J_{\max}[\mathrm{T}^*]}{K_\mathrm{t} + [\mathrm{T}^*] + [\mathrm{S}]} + C$$

where $J$ is the rate of $^3$H-choline transport from a concentration of labeled substrate equal to [T*]; $J_{max}$ is the maximum rate of mediated choline transport; $K_t$ is the choline concentration that resulted in half-maximal transport (Michaelis constant); [S] is the concentration of unlabeled

choline in the transport reaction, and C is a constant that represents the component of total $^{3}$H-choline uptake that was not saturated (over the range of substrate concentrations tested) and presumably reflects the combined influence of diffusive flux, nonspecific binding, and/or incomplete rinsing of the cell layer. As a higher concentration of unlabeled substrate is added to solution, it will competitively inhibit transport of the labeled substrate, effectively reducing its uptake and creating a curve that represent its kinetic profile. This kinetic profile can be used to determine the $J_{max}$ of transport, and the Michaelis constant, $K_t$, which is often used to describe the affinity a transporter has for a substrate.

Cells were incubated in WB with 1 μCi/ml of $^{3}$H-choline for 1, 2, 5, and 10 minutes (Figure 2). Separately, cells were incubated in WB with 1 mM of $^{3}$H-choline and saturating amount, 1 mM, of unlabeled choline, to demonstrate how much of the transport was blockable (Figure 2). The amount of transport that can be blocked accounts for all the transport that is carrier-mediated, and the remaining transport that wasn't blocked, represented as C, should thus be removed from consideration when discussing transporter-mediated processes. It was found that HEK-293 cells express robust choline transport that was linear up to 2 minutes, and this was the time point used for all future uptake experiments.

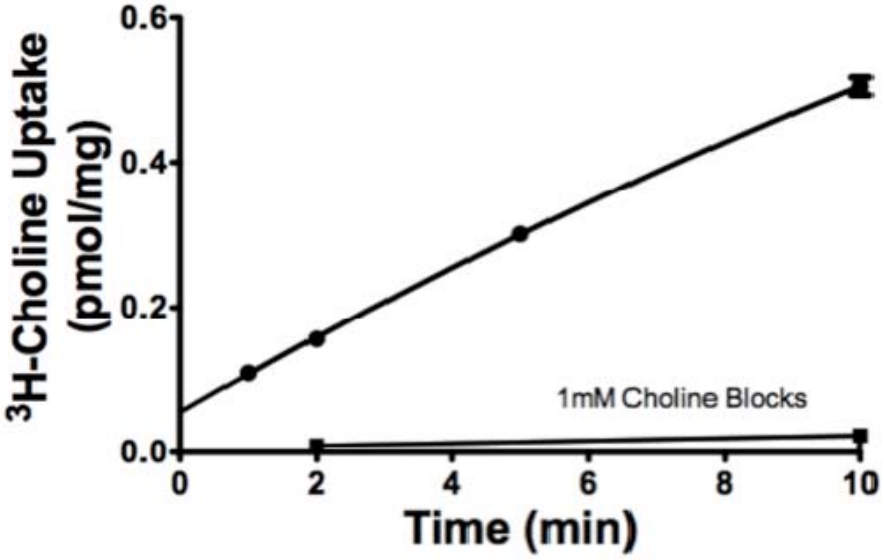



**Figure 2: Time Course for Choline in HEK-293 Cells.** Time course shows that linear transport is expressed up to 2 minutes, thus a 2-minute time point is used for all future uptake experiments. Choline block is included as a control to show blockable transport.

***HEK-293 Cells express robust choline transport.***

The next step in determining which transporter was responsible for this native choline transport in HEK-293 cells was to establish its kinetic profile. Cells were incubated for 2 minutes in WB containing $^3$H-choline and 0, 3, 10, 30, 100, 300, and 1000 μM of unlabeled choline. The resulting profile showed choline transport in HEK-293 cells was an intermediate-affinity process with a $K_t$~12.1 μM (Figure 3). If we remember our potential candidates for choline transporters (OCT, MATE1, CHT1, and CTL1), this $K_t$ is inconsistent with OCT or MATE1 because they have a very low affinity for choline ($K_t$>100 μM), and it is also inconsistent with CHT1 which has a very high affinity for choline ($K_t$< 1 μM). Choline transport in HEK-293 cells is an intermediate-affinity process, consistent with CTL1.

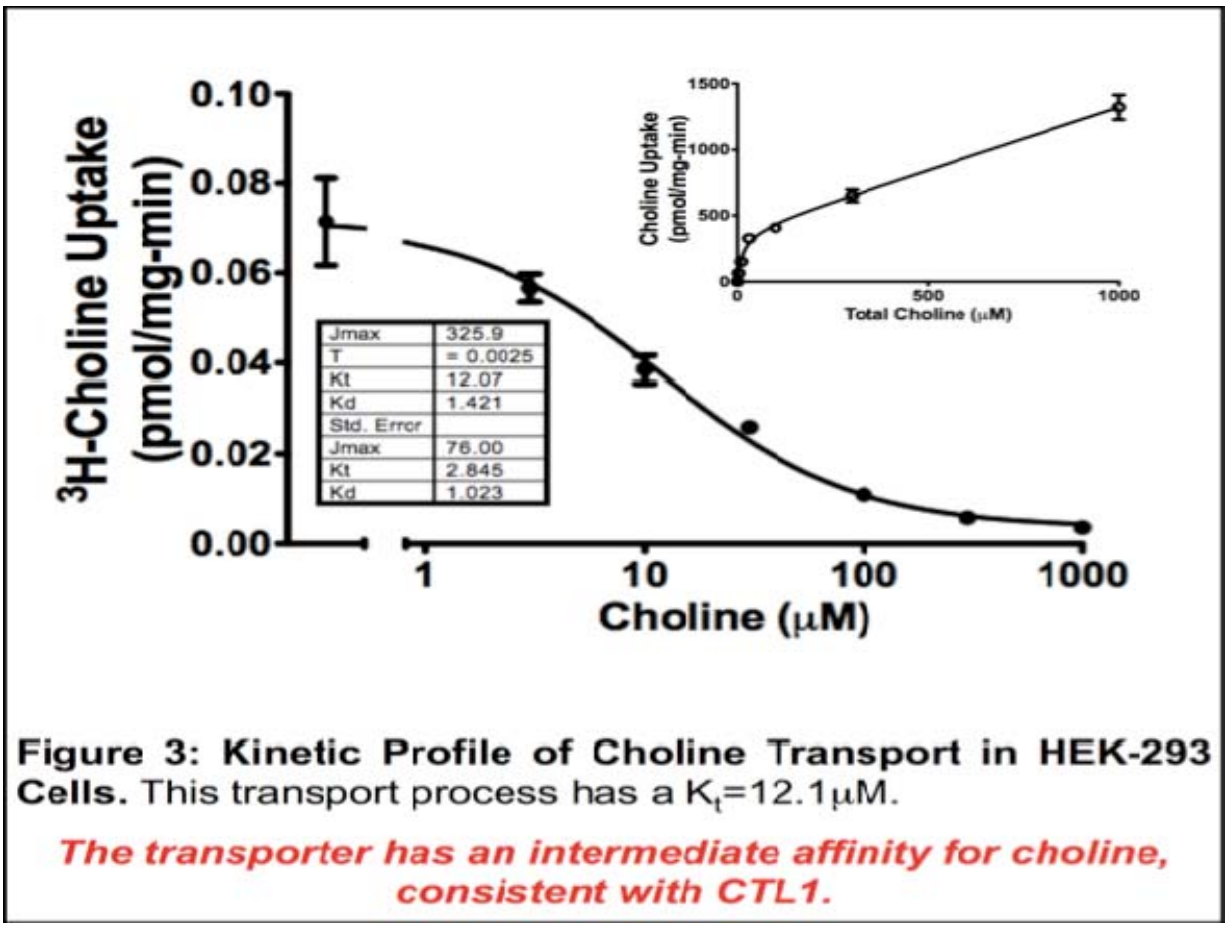



**Figure 3: Kinetic Profile of Choline Transport in HEK-293 Cells.** This transport process has a $K_t$=12.1μM.

***The transporter has an intermediate affinity for choline, consistent with CTL1.***

Sodium dependency is another factor that might help to determine the transporter responsible for choline transport in HEK-293 cells. WB was made similarly to how it is described in the methods section, but 135mM NaCl was replaced with 135 mM LiCl and was adjusted to pH 7.4 with KOH, effectively removing all sodium from the transport conditions. Cells were then incubated for 2 minutes in $Na^+$-free WB with $^3$H-choline and $Na^+$-free WB with $^3$H-choline and 1 mM of unlabeled choline. Cells were also incubated in normal WB and $^3$H-choline and in normal WB with $^3$H-choline and 1 mM unlabeled choline. The presence of 1 mM unlabeled choline revealed how much of total $^3$H-choline transport was carrier-mediated. The results showed that removal of sodium did not eliminate mediated choline transport, but only reduced it by 31%. In other words, choline transport in HEK-293 cells is not a sodium-dependent

process, but may be sensitive to its presence or lackthereof (Figure 4). This eliminates CHT1 as a possible choline transporter in these cells, as it is sodium-dependent, and further suggests that CTL1 is the probable transporter of choline in HEK-293 cells because it transports independently of sodium, although, like the observed response to Na-removal, it too is sodium-sensitive.[10] OCT and MATE1 also transport choline with no dependence on sodium, so the results of the sodium removal experiment would also be consistent with these. However, previous experiments showed that choline transport in HEK-293 cells was via a transporter with an intermediate affinity for choline ($K_t$~16 μM) and both OCT and MATE1 have a very low affinity for choline ($K_t$>100 μM), making them less likely candidates.

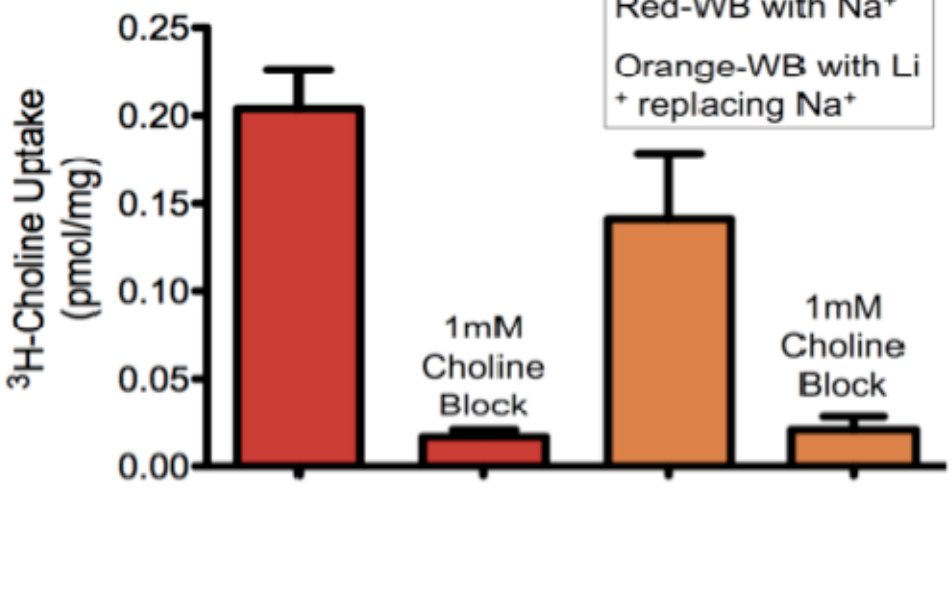



**Figure 4: Effect of Sodium Removal on Choline Transport.** $Na^+$ was replaced with $Li^+$ in Waymouth Buffer (WB) for transport and uptake was tested under these $Na^+$-free conditions. Results showed that the choline transport system did not appear to be affected by the removal of $Na^+$.

***Choline transport in HEK-293 cells is not sodium dependent.***

At this point, the data reasonably suggested that CTL1 could be the transporter for choline in HEK-293 cells, and so further experimentation was done comparing known characteristics of choline transport via CTL1 and choline transport in HEK-293 cells. Choline transport via CTL1 has been found to be sensitive to pH, transporting less choline at a pH higher or lower than physiological pH (~7), and transporting more effectively in the range of physiological pH. Choline transport in HEK-293 cells was similarly effected, further suggesting CTL1 as a reasonable option for the choline transporter in these cells (Figure 5).

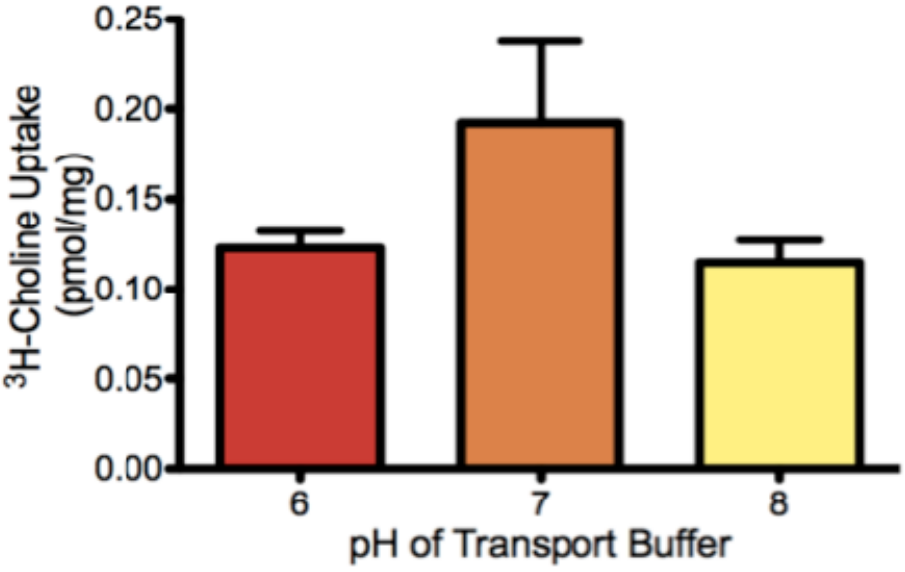



**Figure 5: pH Effects on Choline Transport.**
Transport is greatest ~pH 7, or physiological pH. Higher or lower pH hinders transport.

***Mechanism for choline transport is pH sensitive and most effective at a physiological (pH~7).***

I then looked at the effects of various inhibitors on choline transport in HEK-293 cells. Cells were incubated for 2 minutes in WB with $^3$H-choline and 1mM of unlabeled choline, MPP, amiloride, quinidine, TEA, clonidine, or guanidine. Labeled choline transport was inhibited by

unlabeled choline, MPP, amiloride, clonidine, guanidine, and quinidine (Figure 6). Choline transport was not inhibited by TEA (Figure 6). TEA and MPP are active substrates for OCT2, so if OCT2 was responsible for choline transport, both of these substrates would have eliminated almost all transport, which was not the case. Past studies characterizing CTL1 found that TEA and MPP do not inhibit choline transport, while unlabeled choline, amiloride, clonidine, guanidine, and quinidine do.[9] The results are thus mostly consistent with other profiles of CTL1, further confirming my hypothesis that CTL1 is responsible for this choline transport.

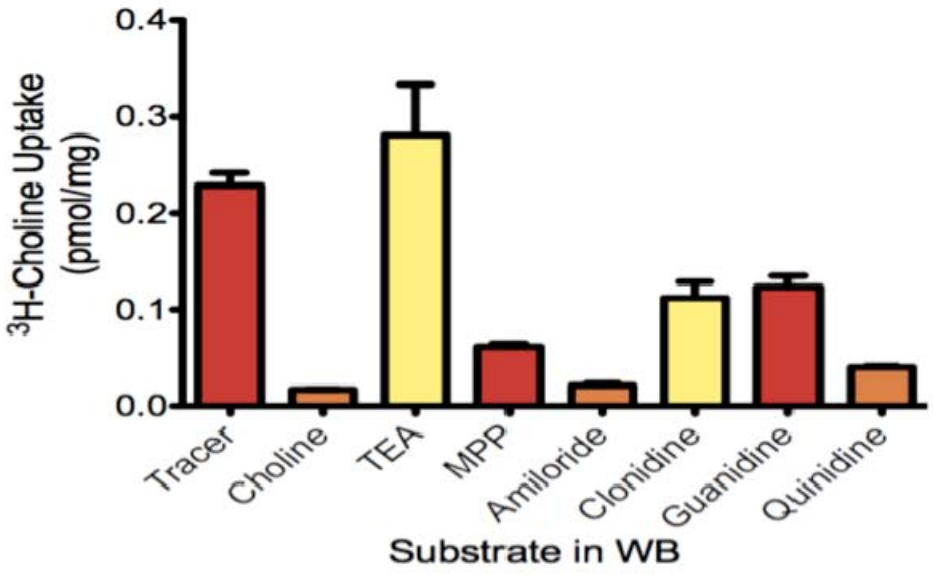
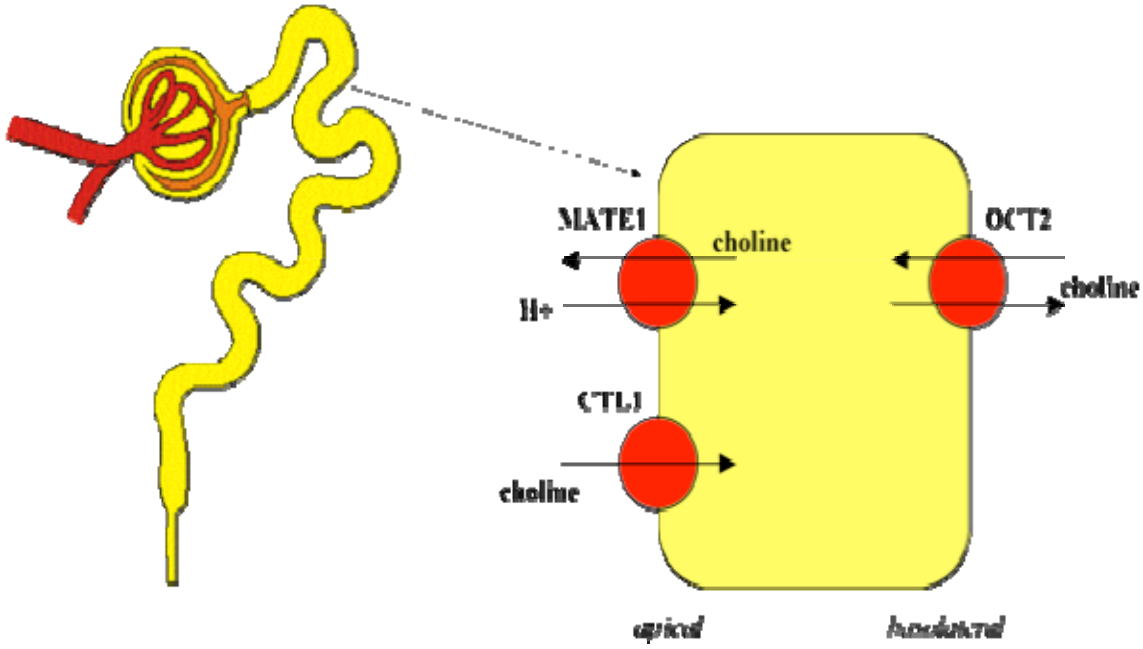



**Figure 6: Effects of Other Substrates on Choline Transport.** TEA, clonidine, and guanidine did not appear to inhibit choline transport, but choline, MPP, amiloride, and quinidine did.

***The choline transport mechanism is inhibited by choline, MPP, amiloride, and quinidine. TEA, clonidine, and guanidine do not appear to inhibit choline uptake.***

The profile of choline transport in HEK-293 cells is thus an intermediate-affinity, sodium-independent, pH sensitive process that can be inhibited by choline, MPP, amiloride, clonidine, guanidine, and quinidine, and is not blocked by TEA. This profile is inconsistent with

OCT2, MATE1, or CHT1 and is mostly consistent with CTL1. The proposed mechanism of choline transport in epithelial cells of the renal proximal tubule is thus:




The characteristics of choline transport in HEK-293 cells are mostly consistent with those of CTL1 and with characteristics of choline reabsorption in the proximal tubule. CTL1 is thus a reasonable suggestion for the luminal step in choline reabsorption in the proximal tubule.

**Future Studies:**

Further experimentation to confirm this hypothesis might include isolation of mRNA from HEK-293 cells, running PCR with primers for OCT2, MATE1, CHT1, and CTL1, and submitting the samples the elctrophoresis to determine which of these transporters are present, if any, in these cells. One might also transfect HEK-293 cells with CTL1 using a Lentivirus, lipofectamine, or electroporation, and then measure the transport and examine the characteristics of transport that CTL1 is responsible for. Then one should knock down the CTL1 gene using

shRNAi, and observe transport without CTL1. If my hypothesis is correct, all choline transport should be eliminated with this step and CTL1 can be confirmed as the transporter of choline in HEK-293 cells.

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