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Role of Organic Cation Transporters in the Renal Secretion of Nucleosides

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ABSTRACT. The mammalian kidney eliminates toxic substances from the body, in part via secretion by the organic cation transporters (OCT) or organic anion transporters. Nucleosides are nitrogenous heterocycles that are often secreted by human and other animal kidneys. Previous experiments have shown that 2′-deoxytubercidin (7-deazadeoxyadenosine, dTub) is secreted by the mouse kidney via a cimetidine-sensitive OCT (Nelson *et al.*, *Biochem Pharmacol* 32: 2323–2327, 1983). Experiments reported herein demonstrated that the cloned rat kidney rOCT1 transports dTub, cytosine arabinoside, 2-chlorodeoxyadenosine, and azidothymidine when expressed in the *Xenopus laevis* oocyte translation system. Although rOCT2 is 67% identical with rOCT1 in its amino acid sequence, rOCT2 does not mediate the uptake of these nucleosides. Uptake of dTub mediated by rOCT1 was pH-dependent in a manner suggesting that the positive charged moiety of dTub may be the true substrate. Protons acted as competitive inhibitors for the rOCT1-mediated uptake of dTub or tetraethylammonium (TEA), with K_i values corresponding to a pH of about 6.1. TEA and dTub mutually inhibited the uptake of one another by rOCT1, competitively, with K_i values approximately the same as their respective K_m values. These findings suggest that protons, dTub, and TEA act at a common site on rOCT1, and that rOCT1 participates in the renal secretion of dTub and other nucleosides. BIOCHEM PHARMACOL **60**;2:215–219, 2000.

KEY WORDS. organic cation; transport; AZT; TEA; renal clearance; nucleosides

A major role for the mammalian kidney is the elimination of endogenous and exogenous toxic substances from the body. It performs this task first by passively filtering small molecules in plasma via the glomerulus, and second, by actively transporting such toxins from the plasma into the lumen of the tubules through a process called "renal secretion." The secretory function is performed by two major systems, namely the organic cation and the organic anion transporters. A number of physiologically and pharmacologically important nucleosides are secreted by human and other mammalian kidneys, including dAdo† [1], and anti-tumor and anti-viral drugs such as CldAdo [2] and AZT [3]. Because dTub is a nonmetabolized, nontoxic analog of dAdo, we have used it previously to study the mechanisms for renal handling of nucleosides [4, 5]. These studies in mice showed that dTub is secreted by a Cim-sensitive OCT; thus, we hypothesized [4, 5] that there exists an OCT that recognizes and transports nucleosides, particularly dTub.

The first organic cation transporter, rOCT1, was cloned from the rat kidney [6], and it is also expressed in the rat liver and intestine. The second OCT cloned from the rat kidney, rOCT2, is 67% identical in amino acid sequence to rOCT1 and is reported to be expressed predominantly in the kidney [7]. Herein, we report that when expressed in the *Xenopus laevis* oocyte translation system, rOCT1 transports dTub and other secreted nucleosides. Interestingly, the closely related rOCT2 is not able to transport dTub and the other nucleosides in this system. Further, results suggest that the positively charged moiety of dTub is the true substrate for rOCT1, i.e. rOCT1 is not a "nucleoside transporter" but an OCT that probably plays a major role in the renal secretion of dTub and perhaps other nucleosides. Preliminary reports of this work have been presented.‡

MATERIALS AND METHODS

Materials

[^{14}C]TEA (2.4 or 3.36 mCi/mmol) was purchased from DuPont NEN. [^{3}H]dTub (9.3 Ci/mmol) was prepared by

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† *Abbreviations*: dAdo, deoxyadenosine; dTub, 2′-deoxytubercidin; araC, cytosine arabinoside; CldAdo, 2-chlorodeoxyadenosine; AZT, azidothymidine; Cim, cimetidine; OCT, organic cation transporter; araG, guanine arabinoside; TEA, tetraethylammonium; MES, 2-[*N*-morpholino]ethanesulfonic acid; and dTub^{+}, protonated form of dTub.

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Moravek Biochemicals Inc. using dTub synthesized in our laboratory as previously described [8]. [^{3}H]araC (23.3 Ci/mmol), [^{3}H]araG (2.5 Ci/mmol), [^{3}H]AZT (33 Ci/mmol), and [^{3}H]CldAdo (6.5 Ci/mmol) were purchased from Moravek Biochemicals, Inc. Other chemicals were obtained from the Sigma Chemical Co.

X. laevis Oocyte Injection and Transport Measurements

Cloned rOCT1 [6] and rOCT2 [7] cDNAs contained in the pSPORT vector (Life Technologies, Inc.) were gifts of Dr. V. Ganapathy (Medical College of Georgia) and Dr. John B. Pritchard (Laboratory of Pharmacology and Chemistry, National Institute of Environmental Health Sciences), respectively. The 5′-capped cRNAs of these transporters were synthesized *in vitro* using the Ambion T7 mMessage mMachine transcription kit (Ambion, Inc.) as previously described [9]. *X. laevis* oocytes were collected, defolliculated manually, and injected with 20 ng (40 nL) of cRNA or with an equal volume of water per oocyte. Three days after injection, the oocytes were incubated for 4 hr in modified Barth's solution containing radiolabeled substrates (5 μCi/mL, 100 μM for [^{3}H]dTub, [^{3}H]CldAdo, [^{3}H]araC, [^{3}H]araG, and [^{3}H]AZT, and 1 μCi/mL, 300 μM for [^{14}C]TEA). When Cim was used as an inhibitor, it was present in the uptake solution at a concentration ten times that of the radiolabeled substrate. At the end of the uptake period, the oocytes were washed three times in ice-cold Barth's solution. Then they were lysed in 0.2% sodium dodecyl sulfate containing 0.2 N NaOH, and the radioactivity was determined by liquid scintillation counting. For kinetic determinations, the uptake of dTub and TEA was measured during a 1-hr incubation, since the uptake was linear for up to 2 hr (data not shown). For determining uptake at various pH levels, the following buffers were used: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES (pH 7.4), or 10 mM MES (pH 6.4 and 5.4) [9]. For efflux experiments, the substrates were introduced directly into the oocytes 3 days after injection of the cRNAs for rOCT1 or rOCT2. Specifically, 20 nL of [^{14}C]TEA (0.1 μCi/μL, 30 mM) or [^{3}H]dTub (1 μCi/μL, 100 μM) was injected. Immediately after the injection, the oocytes were washed three times in ice-cold Barth's solution and transferred to scintillation vials containing 200 μL of Barth's solution at room temperature. After a 3-hr incubation at room temperature, a 100-μL aliquot of the efflux solution was transferred to a new scintillation vial to determine the amount of substrate in the medium. The total amount of substrate injected was determined by measuring the amount of radioisotope in the efflux medium and the amount left in the scintillation vials.

Data Analyses

Statistical analyses were performed using the StatMost™ statistical analysis and graphics program (Dataxiom Software Inc.). Results are presented as means ± SEM. Significance for mean differences was determined by the unpaired Student's *t*-test. Substrate affinity and inhibition constants were determined by the method of Lineweaver–Burk [10] or Dixon and Webb [11].

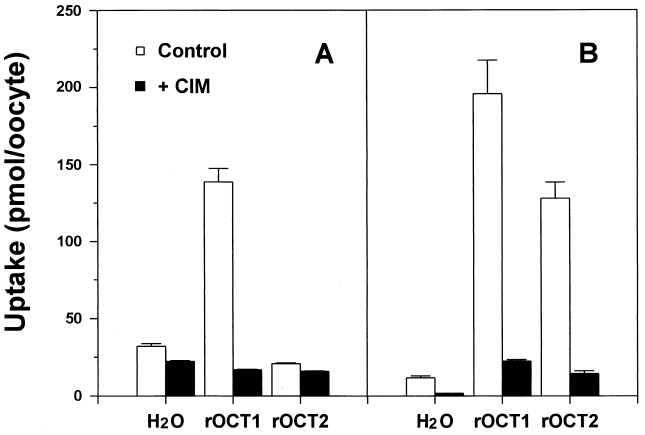

FIG. 1. Uptake of dTub and TEA into *X. laevis* oocytes injected with the cRNA of rOCT1 or rOCT2. Oocytes isolated as described in Materials and Methods were injected with 20 ng of cRNA or an equal volume of water on day 0. Three days after injection, the 4-hr uptake of [^{3}H]dTub (5 μCi/mL, 100 μM) was measured in Barth's solution in the absence (control) or presence of a 10-fold molar concentration of Cim (1 mM) (A). The uptake of [^{14}C]TEA (1 μCi/mL, 300 μM) was similarly measured in the absence (control) or presence of Cim (3 mM) (B). Mean values ± SEM for 10–15 oocytes per group are shown.

RESULTS

Transport of Radioactively Labeled dTub and TEA by rOCT1 and rOCT2 in X. laevis *Oocytes*

TEA is a classical substrate for putative and identified OCTs, i.e. it has served as a model for organic cation secretion in various species for many years. As shown in Fig. 1B, the uptake of [^{14}C]TEA into oocytes previously injected with the cRNAs from rOCT1 and rOCT2 was enhanced, similar to the reports of others [6, 7]. In contrast to the enhanced uptake of TEA by both OCTs, the uptake of dTub was increased by rOCT1 but not by rOCT2 (Fig. 1A). The enhanced uptake of dTub by rOCT1 and of TEA by both rOCT1 and rOCT2 was inhibited by a 10-fold molar excess of Cim, suggesting a transporter-mediated process for both substrates. Selective enhancement of dTub efflux by rOCT1 was also observed in oocytes injected with this radioactive substrate, i.e. both rOCT1 and rOCT2 increased the rate of egress of TEA, whereas only rOCT1 enhanced that of dTub (Fig. 2). Together, these data suggest that TEA is a substrate for both rOCT1- and rOCT2-mediated uptake and efflux, whereas dTub is a substrate specifically for rOCT1.

Effect of pH on the Transport of dTub and TEA

To investigate whether rOCT1 recognizes the protonated, ionized form of dTub, we performed uptake experiments at

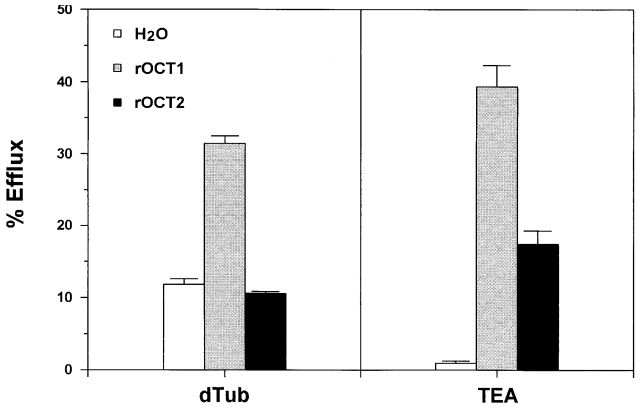

FIG. 2. Efflux of dTub and TEA by oocytes injected with the cRNAs for rOCT1 or rOCT2. *X. laevis* oocytes were injected with 20 ng of cRNA or water (control) as described in Fig. 1. Three days after injection, the oocytes were reinjected with 20 nL of [^{3}H]dTub (1 μCi/μL, 100 μM) or [^{14}C]TEA (0.1 μCi/μL, 30 mM). Efflux of radiolabeled dTub or TEA into Barth's solution was measured during a 3-hr incubation at room temperature. Results are presented as the fraction (percentage) of total radioisotope effluxed during the incubation. Mean values ± SEM for 10–15 oocytes per group are shown.

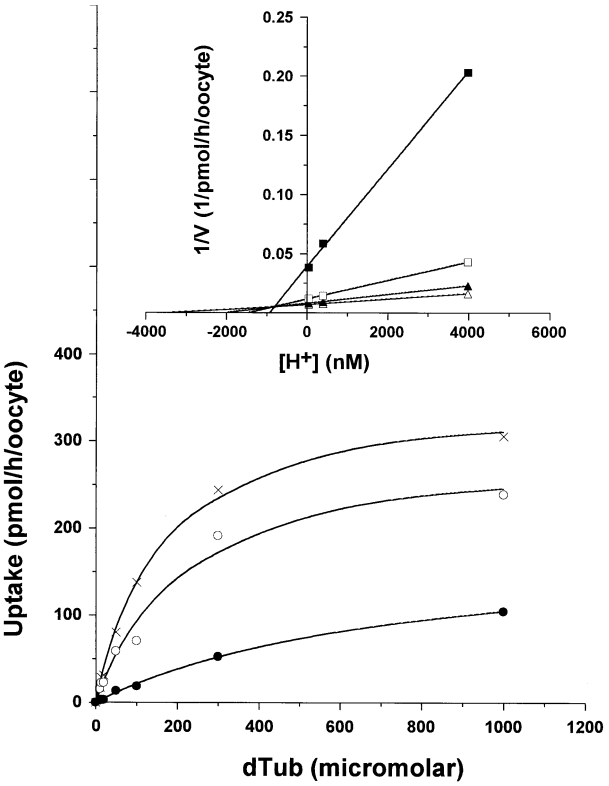

FIG. 3. Influence of pH on the uptake of [^{3}H]dTub by oocytes injected with the cRNA for rOCT1. *X. laevis* oocytes were injected with 20 ng of cRNA for rOCT1 or water (control) of equal volume. Three days after injection, uptake of dTub was measured using [^{3}H]dTub (5 μCi/mL, 100 μM). Incubation was performed at room temperature for 1 hr at pH 7.4 (●), pH 6.4 (○), or pH 5.4 [X]. The data shown represent the uptake in cRNA-injected oocytes minus the uptake in control oocytes. Inset: Dixon plot for the dTub uptake at the three different proton concentrations in which the positively charged moiety of dTub was the assumed substrate (calculated from the Henderson–Hasselbalch equation using a pK_a value of 5.3 for dTub) [2]. Key: [dTub$^+$] = 1 μM (■), [dTub$^+$] = 5 μM (□), [dTub$^+$] = 10 μM (▲), and [dTub$^+$] = 15 μM (△).

various external hydrogen ion concentrations (Fig. 3). The pK_a for dTub is 5.3 [4]; therefore, the ionized fractions at pH 5.4, 6.4, and 7.4 are approximately 50, 10, and 1%, respectively. In water-injected controls, the uptake of dTub increased linearly with increasing concentrations of dTub at each pH, suggesting passive diffusion in the absence of expressed rOCT1 (data not shown). The amount of uptake in water-injected oocytes averaged 1/8 and 1/50 of that in rOCT1 cRNA-injected oocytes for dTub or TEA, respectively. After correction for the uptake in water-injected controls, saturable uptake of dTub by rOCT1 was apparent at pH 5.4, 6.4, and 7.4 in a manner consistent with the substrate being the protonated form of dTub (Fig. 3). The enhanced uptake at reduced pH was not as might be expected from the pK_a value for dTub, quantitatively, probably because external protons inhibit rOCT1 and rOCT2 [12]. The effect of pH on dTub uptake is consequently complicated by this inhibition. As shown by the inset to Fig. 3, a Dixon plot is consistent with protons serving as competitive inhibitors of the dTub uptake. This inset expresses the dTub concentration as the ionized or protonated species, calculated using its pK_a of 5.3 [4] and the Henderson–Hasselbalch equation [10]. As summarized in Table 1, TEA also competitively inhibited dTub uptake by rOCT1 in the oocyte system, having a K_i value of 286 μM at pH 5.4. Likewise, dTub$^+$ is a competitive inhibitor of TEA uptake with a K_i value of 19 μM. For the results shown in Table 1, the mutual inhibition of the uptake of dTub and TEA was determined at pH 5.4 to increase the concentration of the putative true substrate, i.e. dTub$^+$. The estimated K_i values for proton inhibition of dTub or TEA uptake were 0.84 and 0.86 μM, corresponding to a pH value of about 6.1. Since protons competitively inhibit the uptake of dTub and TEA, the apparent K_m values are a function of pH. Therefore, it is likely that the K_m estimates obtained for dTub and TEA in the proton inhibition experiments, i.e. 10 and 63 μM (Table 1), may more closely approximate the true K_m values for these substrates.

Uptake of Other Nucleoside Analogues by rOCT1 in the Xenopus *Oocyte System*

The results thus far suggest that dTub is a substrate for rOCT1 but not rOCT2. To determine whether this observation is unique to this nucleoside analogue, we tested other nucleosides as potential substrates for rOCT1 and rOCT2 (Fig. 4). Uptake of araC, CldAdo, and AZT was increased significantly (3.5-, 1.8-, and 1.6-fold, respectively) by rOCT1; however, like dTub, rOCT2 did not enhance the uptake of these nucleoside analogues. Two of these clinically important nucleoside analogues have been shown to be secreted by the human kidney [2, 3]. Renal clearance of CldAdo approximates renal plasma flow in

TABLE 1. Kinetic constants for rOCT1-mediated uptake of dTub and TEA in *X. laevis* oocytes*

Substrate†	Inhibitor†	K_i‡ (μM)	K_m‡ (μM)	V_{max}‡ (pmol/hr/oocyte)
dTub§	TEA§	286	33	133
TEA§	dTub§	19	251	164
dTub	H^+	0.84	10	281
TEA	H^+	0.86	63	216

*Uptake of radiolabeled dTub or TEA into the oocytes was measured 3 days after injecting the cRNA for rOCT1 as described in Materials and Methods. Incubations were performed at room temperature for 1 hr.

†Concentrations of substrates used were as folows: dTub: 10, 13.3, 20, 50, 100, 300, and 1000 μM; TEA: 10, 41.7, 100, 417, 1000, and 2000 μM. Concentrations of inhibitors used were: dTub: 0, 10, 50, and 200 μM; TEA: 0, 10, and 100 μM; H^+: pH 5.4, 6.4, and 7.4

‡Kinetic constants were determined by the methods of Lineweaver–Burke and Dixon [10, 11]. In all cases, inhibition was competitive. K_i and K_m values for dTub were calculated for the protonated form using the Henderson–Hasselbalch equation [4, 10].

§Experiment was performed at pH 5.4 to increase the concentration of the putative true substrate or inhibitor, $dTub^+$.

children with acute leukemia [2]. AZT and its glucuronide metabolite are eliminated principally in urine via both glomerular filtration and tubular secretion. Six hours following i.v. administration, 18–29% of the dose is excreted in urine as unchanged drug, and 45–60% is excreted as its metabolite [13]. In mice, Cim failed to alter the disposition and renal clearance of AZT significantly, suggesting that renal secretion is not rate-limiting for its elimination in mice (unpublished data). The uptake of araG was not influenced by either rOCT1 or rOCT2 under these experimental conditions (Fig. 4). The renal clearance of araG exceeded that of inulin in mice, strongly suggesting its secretion by the kidney (unpublished observation).

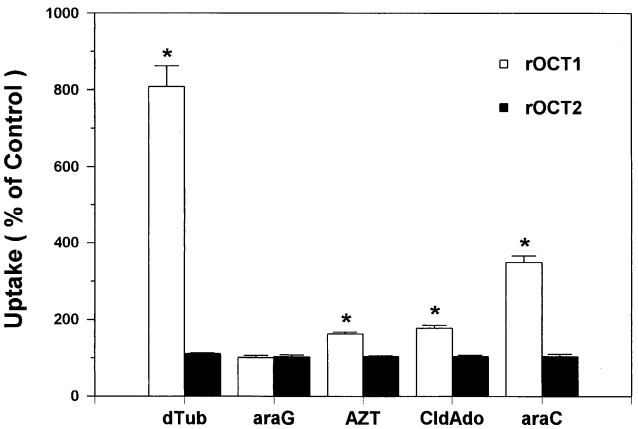

FIG. 4. Uptake of various nucleosides into oocytes injected with the cRNAs for rOCT1 and rOCT2. *X. laevis* oocytes were injected with 20 ng of cRNA or water (control) on day 0. Three days after injection, uptake of nucleosides into the oocytes was measured as described in Materials and Methods using ^3H-labeled nucleosides as potential substrates (5 μCi/mL, 100 μM). The oocytes were incubated in radiolabeled nucleosides at room temperature for 4 hr, and the total nucleoside uptake was determined as described in Materials and Methods. Results are presented as percent uptake values in cRNA-injected oocytes versus vehicle controls (100% for dTub was 12 ± 0.2 pmol/oocyte, for araG was 7.5 ± 0.2 pmol/oocyte, for AZT was 9 ± 0.2 pmol/oocyte, for CldAdo was 19.2 ± 0.9 pmol/oocyte, and for araC was 2 ± 0.2 pmol/oocyte). Mean values ± SEM for 10–15 oocytes/group are given. Key: (*) significantly different from water-injected controls ($P < 0.05$), by Student's *t*-test.

DISCUSSION

The apparent secretion of dAdo and reabsorption of Ado were first observed in a child lacking adenosine deaminase and in cancer patients treated with the deaminase inhibitor deoxycoformycin [1]. Secretion of dAdo was confirmed in mice, and the analogue dTub was used subsequently to determine the potential mechanism(s) for the secretion [4]. Although there are at least five known nucleoside transporters [14, 15], renal secretion of dTub was proven to occur by a Cim-sensitive OCT using classical pharmacological methods [4, 5]. On the other hand, the renal secretion of dAdo did not seem to occur by the same mechanism: Cim did not block its secretion *in vivo* (in fact, secretion was enhanced) [5], and dAdo failed to inhibit the uptake of classical OCT substrates by mouse kidney slices [4]. Secretion of dAdo was inhibited completely by nitrobenzylthioinosine and dipyridamole in mice [16], suggesting that an inhibitor-sensitive nucleoside transporter plays a role in its renal secretion. In summary, although dTub originally was selected to mimic the renal secretion of dAdo, the mechanism(s) for dTub and dAdo secretion appears to be distinct in the mouse.

rOCT1 and rOCT2 are the first OCTs to have been cloned and are members of a family of transporters belonging to the major facilitator superfamily [17, 18]. We observed in this study that rOCT1 was an effective transporter of dTub in the *X. laevis* oocyte translation system (Figs. 1 and 2), confirming our hypothesis [4, 5] that there is an OCT capable of transporting this secreted nucleoside. To our knowledge, this is the first report of a cloned OCT that transports dTub and other nucleosides. The affinity (K_m) and transport maximum (V_{max}) values for dTub approached or exceeded those of the classical OCT substrate, TEA (Table 1). Although only about 1% of dTub is ionized at physiological pH, the rate of transport of the total dTub into the oocytes approached that of TEA, i.e. the estimated "true substrate" $dTub^+$ is well below its K_m at this pH (Fig. 3, Table 1). The similar K_m and K_i values for TEA and dTub uptake and inhibition (Table 1) are consistent with their interacting at a common site, a site possibly shared with protons based upon the competitive nature of their mutual inhibition. We previously reported that rOCT2 failed to transport dTub and other nucleosides when expressed in NIH3T3 cells [19] or *X. laevis* oocytes [9]. rOCT1 failed to mediate the uptake of either dAdo or Ado (data not shown), which is consistent with the previous suggestion that dTub and dAdo are handled differently in the kidney.

Similar to the *es* nucleoside transporter, rOCT1 is thought to be a "facilitated diffusion" transporter [18], i.e. incapable of establishing a transmembrane gradient and operating indepen-

dently of an energy source. Thus, it is difficult to envision a mechanism whereby this carrier alone could effect vectorial, active transport, characteristic of renal secretion. The presence or intracellular localization of the *es* nucleoside transporter in the kidney is not known; however, there is evidence indicating that OCT1 is a basolateral OCT [12]. Whether another carrier, perhaps located at a site different from that of OCT1, plays a role in dTub renal secretion is also not known. In this regard, we have observed that dTub is a substrate for the putative organic cation/proton antiporter as measured in pig kidney cortex brush border membrane vesicles (data not shown). Also, we recently described an energy-dependent OCT in opossum kidney proximal tubule cells that is almost certainly distinct from OCT1 or OCT2, and this carrier might participate in the renal secretion of dTub [9].

Since rOCT1 and rOCT2 are structurally quite homologous [7], the finding that rOCT1 but not rOCT2 is capable of transporting dTub and other nucleosides is of interest. This finding is also in sharp contrast to the recent report of rOCT1 and rOCT2 in stably transfected MDCK cells [12], i.e. the affinities for a number of non-nucleoside OCT substrates and inhibitors were similar for both carriers. These authors also noted that acidification of the extracellular medium decreased the uptake of TEA at the basolateral membrane [12]. As demonstrated herein, external protons act as competitive inhibitors for the rOCT1-mediated uptake. Although this inhibition may occur via a change in membrane potential [17], it appears to be a mediated process involving rOCT1, since it is competitive with dTub or TEA.

In summary, rOCT1 recognizes a number of nucleosides not utilized by rOCT2, suggesting considerable variation in their active sites for these substrates, and rOCT1 appears to be a likely participant in the renal secretion of dTub and perhaps, of other nucleosides. The different substrate selectivity between closely related transporters has been utilized to locate the "binding sites" with chimeric constructs, as recently reported for the nucleoside transporters rCNT1 and rCNT2 [20], and for hCNT1and hCNT2 [21]. A similar approach should be possible in the study of rOCT1 and rOCT2 in the future to identify the specific region responsible for the transport of dTub by rOCT1.

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