The Human Organic Cation Transporter (hOCT2) Recognizes the Degree of Substrate Ionization*

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The organic cation transporter, OCT2, plays a role in renal secretion of a broad array of weak bases. To determine whether the degree of ionization of these compounds plays a role in their interaction with OCT2, we examined the influence of external pH values on the activity of the human ortholog of OCT2, as expressed in Chinese hamster ovary-K1 cells. Importantly, changing the pH value from 7.0 to 8.0 had no effect on the rate of transport of the fixed cations, tetraethylammonium and 1-methyl-4-phenylpyridinium, i.e. the pH value did not have an effect upon the transporter itself. Cimetidine (pKᵢ, 6.92), a competitive inhibitor of hOCT2, displayed a 3.5-fold increase in IC₅₀ as pH values increased from 7 to 8. hOCT2-mediated cimetidine transport decreased over this pH range, the consequence of an increase in Kᵢ and decrease in Jₘₐₓ at the higher pH value. The weak bases trimethoprim and 4-phenylpyridine showed a similar pattern of pH-sensitive interaction with hOCT2. The non-ionizable sterol, corticosterone, also inhibited hOCT2 activity, although it was neither competitive in nature nor was it sensitive to pH in the manner observed with weak bases. We conclude that the degree of ionization plays a critical role in binding of substrate to organic cation transporters.

In the mammalian kidney, the proximal tubule is the site of secretion and reabsorption of endogenous metabolites and xenobiotics, many of which are organic cations (1), i.e. compounds that possess a net positive charge at physiological pH. Excretion of organic cations (OCs)¹ is believed to occur in two steps: uptake of the OC by the proximal tubule cell across the basolateral membrane followed by the extrusion of the OC out of the cell across the luminal membrane (2–4). The transporters responsible for the movement of OCs into the proximal tubule cell are the organic cation transporters (OCT). Although the term “organic cation transporter” implies that the cationic charge is a defining element in the interaction of substrate with carrier, this is currently not believed to be the case. In a landmark study, Ullrich and Rumrich (5) used the stop-flow peritubular secretion and reabsorption of endogenous metabolites and xenobiotics, many of which are organic cations (1), i.e. compounds that possess a net positive charge at physiological pH. Excretion of organic cations (OCs)¹ is believed to occur in two steps: uptake of the OC by the proximal tubule cell across the basolateral membrane followed by the extrusion of the OC out of the cell across the luminal membrane (2–4). The transporters responsible for the movement of OCs into the proximal tubule cell are the organic cation transporters (OCT). Although the term “organic cation transporter” implies that the cationic charge is a defining element in the interaction of substrate with carrier, this is currently not believed to be the case. In a landmark study, Ullrich and Rumrich (5) used the stop-flow peritubular

* This work was supported by National Institutes of Health Grants DK58251 and HL07249. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: OC, organic cation; OCT, organic cation transporter; TEA, tetraethylammonium; MPP, 1-methyl-4-phenylpyridinium; CHO, Chinese hamster ovary.
confluent, the medium was aspirated and 1 ml of Waymouth buffer (in mM: 135 NaCl, 13 Hepes, 2.5 CaCl$_2$-2H$_2$O, 1.2 MgCl$_2$, 0.8 MgSO$_4$-7H$_2$O, 5 KCl, 28 glucose) was placed in each well for 15 min. The buffer was aspirated and replaced with 1 ml of fresh buffer for an additional 15 min. Following the incubations, 400 µl of transport buffer containing 0.75 µCi/ml [3H]TEA or 1 µCi/ml [3H]cimetidine (for cimetidine kinetic studies) and increasing concentrations of the inhibitor in Waymouth buffer was added to the wells. At intervals, the transport buffer was removed, and each well was rinsed three times with 2 ml of ice-cold Waymouth buffer containing 250 µM tetrapotassiumiammonium to stop transport. Cells were solubilized with 400 µl of 0.5 N NaOH in 1% SDS by shaking for 30 min. Solubilized cells were neutralized with 200 µl of 1 N HCl. The solution was triturated, and 500 µl was removed and placed in a scintillation vial. Six ml of liquid scintillation mixture was placed in a scintillation vial, and the amount of radioactivity was determined using a scintillation counter (Beckman model LS3801). Each individual transport experiment was done in triplicate on one plate. Experimental observations were confirmed, typically two or three times (i.e. n = 2 or 3), in separate experiments using different cell passages. All experiments were performed on passages 4–28 with no appreciable difference in the results obtained with early versus later passages (i.e. little change in $K_t$ for TEA).

Chemicals—[3H]TEA (13.2 Ci/mmol) was acquired from American Radiolabeled Chemicals (St. Louis, MO). [3H]Cimetidine was purchased from Amersham Biosciences. TEA, cimetidine, tetrapotassiumiammonium, and trimethoprim were purchased from Sigma. 4-Phenylpyridine was purchased from Aldrich Chemicals, corticosterone was purchased from Tokyo Chemical Industry (TCI, Tokyo, Japan), and MPP was purchased from Research Biochemicals International (RBI, Natick, MA). Other chemicals were acquired from standard sources and were generally the highest purity available.

**RESULTS**

Kinetic Studies of Quaternary Compounds at pH 7 and 8—Uptake of 57 nM [3H]TEA into wild-type CHO-K1 cells was comparatively low, and co-incubation with an excess of unlabeled TEA had no appreciable effect on this accumulation. Uptake of [3H]TEA into a clonal line of CHO-K1 cells stably transfected with hOCT2 was 20-fold higher than into wild type cells, and this uptake was reduced 98% by co-exposure with 2.5 mM unlabeled TEA (inset of Fig. 1). Fig. 1 shows the time course of [3H]TEA uptake into these CHO-K1 cells. Accumulation of TEA increased time dependently. Because uptake remained linear through 5 min, and therefore represented the initial rate of transport, this point was chosen to do the subsequent kinetic studies.

As a quaternary ammonium compound, the state of ionization of TEA is effectively independent of pH. Consequently, a shift in external pH from 7.0 to 8.0 should have no effect on the concentration of TEA$^-$ (although it may be expected to have an effect on the operation of hOCT2-mediated transport activity). Fig. 2 shows the effect of a shift in external pH from 7.0 to 8.0 on the kinetics of TEA transport in CHO-K1 cells stably expressing hOCT2. As shown in Equation 1, the addition of unlabeled TEA inhibited uptake of [3H]TEA by a process adequately described by the Michaelis-Menten equation for competitive inhibition (15).

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J = \frac{J_{max} [^3T]}{K_t + [^3T] + [T]} + C
\]  
(Eq. 1)

where $J$ is the rate of [3H]TEA transport from a concentration of labeled substrate equal to ["$^3T$"]; $J_{max}$ is the maximum rate of mediated TEA transport; $K_t$ is the TEA concentration that results in half-maximal transport (Michaelis constant); ["$T$"] is the concentration of unlabeled TEA in the transport reaction; and $C$ is a constant that represents the component of total TEA uptake that is 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 shown in Fig. 2, the kinetics of TEA transport were virtually identical at both pH 7.0 and 8.0 (the inset shows the relationship between uptake of total TEA, i.e. labeled plus unlabeled, and the concentration of total TEA in the medium). In five separate experiments, the $K_t$ values at pH 7.0 and 8.0 were 28.5 ± 2.7 μM and 31.6 ± 7.6 μM, respectively, with $J_{max}$ values of 7.3 ± 1.2 and 8.4 ± 1.8 pmol cm$^{-2}$ min$^{-1}$, respectively.

If, as implied from the above observation, the transport process is not sensitive to changes in external pH between 7.0 and 8.0, then competitive interaction between transport of [3H]TEA and a second quaternary ammonium substrate should also be insensitive to this change in pH. Fig. 3 shows this to be the case with respect to the inhibition of TEA transport produced by MPP. In two separate experiments, shifting external pH from 7.0 to 8.0 had no effect on the $IC_{50}$ for MPP inhibition of...
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hOCT2-mediated TEA transport: IC_{50} values of 2.3 ± 0.2 and 2.0 ± 0.1 μM at pH 7.0 and 8.0, respectively. We conclude from these observations that hOCT2, as expressed in CHO-K1 cells, is insensitive to changes in external pH between the range of 7.0 and 8.0 and that interaction of quaternary ammonium substrates with hOCT2 is not influenced by this shift in external hydrogen ion concentration.

Cimetidine Inhibition Studies at pH 7 and 8—Ullrich and Rumrich (5) observed in the in vivo rat kidney that changes in external pH values (from 6 to 8) influenced neither the inhibitory influence of cimetidine on basolateral NMN transport nor the rate of cimetidine transport across the basolateral membrane of proximal tubule cells. Because over this pH range the relative concentrations of charged (cationic) and uncharged cimetidine were expected to change dramatically, they concluded that basolateral organic cation transport does not sense the degree of substrate ionization. We tested this conclusion as it pertains to the influence of external pH on the interaction of cimetidine with hOCT2. The reported pK_{a} of cimetidine varies between 6.8 and 7.0 (5, 16). We independently measured a value of 6.92 and used it for subsequent calculations of the degree of cimetidine ionization at the test pH values used in this study. At pH 7, ~46% of the molecules are protonated, whereas at pH 8, the value drops to 7.4% as calculated using the Henderson-Hasselbalch equation (17). In other words, the 10-fold decrease in [H⁺] results in an ~6-fold decrease in the concentration of protonated cimetidine. This decrease in the protonated species would likely decrease the ability of cimetidine to inhibit [3H]TEA transport if hOCT2 "sensed" the difference in the protonated (Cim⁺) over the unprotonated (Cim⁰) compound, which should be observed at pH 8 as an increase in the concentration of total cimetidine required to inhibit hOCT2-mediated TEA transport. Results of our inhibition experiments are presented in Fig. 4. The shift in pH from 7 to 8 did produce an increase in IC_{50} for cimetidine’s interaction with hOCT2. Whereas at pH 7 the IC_{50} for cimetidine inhibition of TEA was 12.9 ± 1.0 (n = 3) μM, at pH 8 the IC_{50} increased to 45.7 ± 2.9 μM. It is noteworthy, although the amount of (Cim⁺) was calculated to decrease by 6-fold between pH 7 and 8, the difference in IC_{50} values was only 3.5-fold. Using the pK_{a} value of 6.92, we also calculated the IC_{50} values on the presumption that only protonated cimetidine (Cim⁺) interacted with hOCT2. The resulting IC_{50} values were not the same, as might be expected from a calculation that used only the calculated (Cim⁺) concentration at each pH but were 5.9 and 3.4 μM at pH 7.0 and 8.0, respectively. Additional competitive inhibition studies were performed, and the results of one such experiment are shown in Fig. 5. In three separate experiments performed at pH 7, the addition of 45 μM cimetidine increased the K_{i} for TEA from 24.8 ± 2.6 μM to 127.2 ± 10.9 μM with no change in J_{max} (5.1 ± 0.4 versus 6.3 ± 0.9 pmol cm⁻² min⁻¹), resulting in a calculated K_{i} for cimetidine at pH 7 of 10.9 μM. At pH 8, the K_{i} for TEA transport was also increased by the addition of 45 μM cimetidine, from 19.6 ± 1.2 μM to 65.5 ± 5.7 μM, with no change in J_{max} (5.2 ± 0.3 versus 7.0 ± 0.8 pmol cm⁻² min⁻¹) and a calculated K_{i} of 19.2 μM. These observations support the conclusion that the degree of ionization does play a role in cimetidine’s interaction with the transporter. However, it is also apparent that the inhibitory interaction of cimetidine with hOCT2 appears to be stronger at pH 8 than can be explained by only the protonated concentration of substrate according to the Henderson-Hasselbalch equation. Nevertheless, the interaction of cimetidine with hOCT2 is influenced by external pH in a manner consistent with the conclusion that the charged substrate interacts preferentially with the hOCT2 binding site.

Interaction of Other Weak Bases with hOCT2 at pH 7 and 8—We examined the interaction with hOCT2 of two other weak bases, trimethoprim and 4-phenylpyridine. Like cimetidine, the protonated concentration of trimethoprim (pK_{a}, 6.85) changes substantially over the pH range of 7.0–8.0. Whereas at pH 7 ~41.5% of the trimethoprim is protonated (trimeth⁺), at pH 8 only 6.6% is protonated. Fig. 6A shows the results of an experiment that tested the effect of shifting external pH from 7.0 to 8.0 on the kinetics of trimethoprim inhibition of hOCT2-mediated TEA uptake. In two such experiments at pH 7, the IC_{50} for trimethoprim inhibition was 60.0 ± 8.2 μM, whereas at pH 8, the IC_{50} increased to 116.5 ± 4.4 μM. As with cimetidine, the increase in the IC_{50} value at pH 8.0 was consistent with a preferential interaction of the protonated species with the hOCT2 binding site. However, the 2-fold increase in IC_{50} was not proportional to the calculated 6-fold decrease in [trimeth⁺] expected to result from the shift in external pH from 7.0 to 8.0.

4-Phenylpyridine (pK_{a}, 5.37) closely resembles the structure of MPP, the difference being a tertiary, rather than quaternary, nitrogen in 4-phenylpyridine. By calculation there is a 10-fold difference in the concentration of the protonated species at pH
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7 versus 8, although with the low \( pK_a \) of 4-phenylpyridine, the absolute concentrations are comparatively low at both pH values. Fig. 6B shows the results of an experiment that compared the kinetics of 4-phenylpyridine inhibition of hOCT2-mediated TEA uptake at pH 7 and 8. In two such experiments the IC\(_{50}\) for 4-phenylpyridine inhibition of TEA at pH 7 was 20 ± 1.3 \( \mu M \), compared with 106 ± 7.6 \( \mu M \) at pH 8. Again, the 5-fold increase in IC\(_{50}\) was less than that predicted based upon the 10-fold decrease in concentration of protonated substrate at pH 8. However, the similarity in structure between MPP, which interacted with hOCT2 in a pH-independent fashion, and 4-phenylpyridine, which showed this marked sensitivity to pH, strongly supports the contention that charge status of the substrates plays a critical role in binding to hOCT2.

Corticosterone Inhibition of TEA at pH 7 and 8—Corticosterone has been shown to interact with the organic cation transporters even though it does not possess a positive charge (6). We investigated inhibition of hOCT2-mediated TEA transport by corticosterone at pH 7 and 8 to determine whether it is a competitive inhibitor and to see if pH has an effect on a compound that is uncharged. Results from one of these experiments are shown in Fig. 7. Although corticosterone did inhibit TEA transport at both pH 7 and 8, that inhibition was neither competitive in nature nor was it sensitive to pH in the manner observed with weak bases. At pH 7, the presence of 12 \( \mu M \) corticosterone increased the \( K_i \) for TEA transport by 3-fold (from 34.0 to 100 ± 2.7 \( \mu M \; n = 2 \)), while decreasing the \( J_{\text{max}} \) by 3-fold (from 10.6 ± 1.2 to 3.4 ± 0.9 pmol cm\(^{-2}\) min\(^{-1}\)). At pH 8 the \( J_{\text{max}} \) was also decreased by 3-fold (from 13.3 ± 2.1 to 4.0 ± 0.8 pmol cm\(^{-2}\) min\(^{-1}\)), while the \( K_i \) was unchanged (65.0 ± 7.3 \textit{versus} 71.0 ± 2.4 \( \mu M \)). This inhibitory profile reflects a form of “mixed-type” inhibition (18) and suggests that the interaction of corticosterone with organic cation transporters should not be used as evidence that cationic charge is not a critical parameter for stabilization of substrate binding.

Effect of pH on hOCT2-mediated Cimetidine Transport—To determine whether or not the charge of the substrate affects its ability to be transported, we measured uptake of \(^{3}H\)cimetidine. As previously mentioned, the percentage of total cimetidine-bearing charge is about 6-fold higher at pH 7 than at pH 8; so if substrate charge influences hOCT2-mediated transport, cimetidine uptake should be greater at pH 7 than at pH 8. In fact, the rate of 0.1 \( \mu M \) cimetidine was 2.5-fold higher at pH 7 than at pH 8 (results not shown). The decrease in cimetidine transport at pH 8 reflected both an increase in average \( K_i \) (from 8.6 ± 2.2 \( \mu M \) at pH 7 to 18.1 ± 5.0 \( \mu M \) at pH 8) and a decrease in average \( J_{\text{max}} \) (from 0.8 ± 0.3 pmol cm\(^{-2}\) min\(^{-1}\) at pH 7.0 to 0.4 ± 0.2 pmol cm\(^{-2}\) min\(^{-1}\) at pH 8.0) in five separate experiments.

DISCUSSION

The physiological dichotomy of renal secretion that includes apparently discrete pathways that handle “organic cations” or “organic anions” has been a central tenant of renal physiology (19, 20). Although it has long been recognized that transcellular transport of both classes of compound involves multiple parallel pathways (1), it has also been generally assumed that the central physical feature that influences interaction with one or the other of these pathways is the presence of either cationic or anionic charge. With respect to the “classical” organic cation secretory pathway, this view has been based primarily on the observation that, in addition to interaction with substrates having fixed cationic charge (e.g. quaternary ammonium compounds), the strength of interaction with weak bases is generally inversely proportional to their pK\(_a\) values. In other words, bases with high pK\(_a\) values (e.g. >9), which are predominantly protonated and, therefore, positively charged at physiological pH, generally interact more effectively with renal OC transporters than do weak bases with low pK\(_a\) values (21). Two sets of observations by Ulrich et al. in the early 1990’s challenged this long-held view. First, they demonstrated a substantial degree of “cross-over” between substrates generally assumed specific for one or the other of the classical OC or organic anion secretory pathways. Employing the stop-flow peritubular capillary microperefusion method, they showed that the basolateral OC transporter interacts with (i.e. is inhibited by) a broad range of non-cationic substrates, including a number of neutral steroid hormones and negatively charged (at physiological pH) organic acids (6). In a separate study (5), they showed that the interaction of weak bases with peritubular OC transport in rat proximal tubules is insensitive to changes in extracellular pH over the range pH 6–8. The rate of cimetidine transport into rat proximal cells was shown to be independent of pH as well. These observations lead Ulrich to propose as one of several rules for substrate interaction that the presence of cationic charge does not play a direct role in influencing binding to renal OC transporters (3).

The results of the present study indicate that charge does play an important role in stabilizing the binding of substrates to at least one OC transporter, i.e. the human ortholog of OCT2. When external pH was increased from 7 to 8, thereby decreasing the fraction of protonated (i.e. positively charged) weak base molecules in solution, interaction of those compounds with hOCT2 systematically decreased. Importantly, changing the pH value of the extracellular medium did not have an effect on the interaction of hOCT2 with the quaternary ammonium compounds, TEA and MPP, which possess a fixed positive charge independent of pH changes. Because the \( K_i \) for TEA and the IC\(_{50}\) for MPP remained unchanged as the pH value of the transport buffer was increased from 7 to 8, it can be assumed that, within this pH range, there are no structural changes in hOCT2 that significantly influence its transport activity. Furthermore, the observed changes in interaction of hOCT2 with the weak bases cimetidine, trimethoprim, and 4-phenylpyridine indicate that the change in fractional protonation of these compounds is the basis for their decreased interaction with the transporter at pH 8.

If binding of substrate to hOCT2 required that the molecule contain a charged group, one might expect kinetic calculations based on the proportion of molecules charged at each pH value to result in equal kinetic parameters (as seen for the compounds with fixed charge). This was not the case. Inhibition of transport activity exerted by all three weak bases was greater at pH 8 than predicted based on the comparatively low, calcu-
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At least three explanations for this discrepancy suggest themselves. First, it is possible that the unprotonated, as well as the protonated, form of a weak base interacts with hOCT2, albeit with a substantially reduced affinity for the receptor. If this were the case, then the shift in IC_{50}/K_a expected to occur between pH 7 and 8 would be less than that predicted on the assumption that only the charged species binds to the receptor. Second, the pH value at the membrane surface could be lower than that in the surrounding bulk. Whatever the reason, an elevated [H^+] at the membrane surface would have a proportionately greater effect on the numbers of charged weak bases in an alkaline solution than in a more acidic one and could result in the type of disproportionate inhibitory activity of weak bases at pH 8. Third, the pK_a value of a weak base as determined in bulk solution may be very different from the pK_a value within the microenvironment of the receptor. To the extent that the “effective” pK_a values of the weak bases were higher within the region of the transport receptor than in free solution, there would be a smaller difference in the fraction of protonated species in solution at pH 7 and 8 and a concomitant decrease in the predicted difference in IC_{50}/K_a values at the two pH values. Whatever the basis for the discrepancy between the observed and predicted degrees of interaction of weak bases with hOCT2, the principal point is that the degree of substrate ionization does play a central role in their ability to bind to hOCT2.

Another observation that has been used to support the conclusion that charge does not play a role in binding of substrates to renal OC transporters is that a number of neutral steroids are highly effective inhibitors of peritubular OC transport (6). We examined the mechanistic basis of the interaction of the steroid corticosterone with hOCT2. Although we confirmed that corticosterone is an extremely effective inhibitor of hOCT2 (IC_{50} of <10 μM), it is not a competitive inhibitor. The reduction in transport activity produced by acute exposure to corticosterone was the result of a large decrease in J_{max} as well as a modest increase in K_c (Fig. 7). There are many possible mechanisms to account for this “mixed type” profile of inhibition (18) including interactions at the binding site. However, it cannot be assumed that corticosterone is interacting with the transporter at the same site as TEA or that it is necessarily a substrate for the OCTs. Consequently, inhibitory interactions of non-charged substrates with renal OC transporters should be interpreted with caution.

The importance of charge in stabilizing substrate interaction with hOCT2 contrasts sharply with the absence of such influence evident in the aforementioned studies in intact rats (5, 6). At least three issues could be involved in this discrepancy. First is the possible influence of species differences. Marked species differences have been noted with respect to both quantitative (IC_{50}) and qualitative (actual transport) differences in OC interactions with human, rat, mouse, and rabbit orthologs of OCT1 (24). Thus, substrate charge may play a less important role in binding to rOCT2 than it does to hOCT2. Second, the
interaction reported in Ullrich’s (5, 6, 21) studies might reflect interactions with OCT homologs other than OCT2. In the human, OCT2 appears to be the only OCT homolog expressed at appreciable levels in the basolateral membrane of human proximal tubules (13). In the rat kidney, however, OCT1 is expressed at substantial levels in the S1 and S2 segments of rat proximal tubules, whereas OCT2 is restricted to the S2 and S3 segments (7). The microperfusion method employed in the studies of Ullrich et al. (5, 6) would be expected to access both S1 and S2 segments as exposed at the surface of the cortex. Thus, the results of Ullrich’s experiments may have reflected, or been dominated by the characteristics of rOCT1, rather than rOCT2. Finally, the possible discrepancy between the results reported here compared with those observed by Ullrich et al. (5) may reflect the different techniques used to make the measurements. The in vivo microperfusion method relies on the rapid delivery of very small volumes of test solution to the local environment of the selected proximal tubules. The substantial buffering capacity of the extracellular compartments through which the test solution must pass before reaching the transport surface may have limited the degree of change in the external pH value to which the transporters and substrates were actually exposed. This confounding point would not have been an issue in the present studies employing confluent monolayers of cells grown in culture wells.

In summary, the interaction of weak bases with hOCT2 was markedly influenced by the pH of the extracellular medium, an effect almost certainly reflecting the influence of pH on the net charge of these molecules. In contrast, changes in external pH had no effect on the interaction of substrates having fixed cationic charge. In addition, the inhibitory interaction of a neutral steroid with hOCT2 was not competitive, suggesting that such interactions may not involve the binding of a neutral molecule at the OCT2 binding site. These results suggest that OCT2 does detect the degree of ionization of its substrates and that the presence of cationic charge stabilizes the binding of substrate to the transport receptor.

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J. Biol. Chem. 2002, 277:22491-22496.
doi: 10.1074/jbc.M203114200 originally published online April 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M203114200

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