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The Mechanism of Excretion of Trientine from the Rat Kidney: Trientine is not Recognized by the H^+/Organic Cation Transporter

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Abstract

Trientine dihydrochloride is used to treat Wilson's disease by chelating copper and increasing its urinary excretion. The mechanism of renal excretion of trientine has been investigated in-vivo and in-vitro.

Trientine clearance in the rat was significantly faster than creatinine clearance. When trientine and the same number of moles of copper ions were administered simultaneously to the rat, however, trientine clearance decreased to almost the same level as the creatinine clearance. To clarify this active excretion system for trientine, the uptake of trientine and a physiological polyamine compound, spermine, was investigated using rat renal brush-border membrane vesicles. Although, because trientine and spermine are organic cations, the H^+/organic cation transporter is expected to recognize these compounds, neither an outwardly directed H^+ gradient nor an inward Na^+ gradient stimulated trientine uptake. [^{14}C]Spermine uptake was, nevertheless, trans-stimulated by both unlabelled spermine and trientine and the trans-stimulating effect of spermine on trientine uptake was, furthermore, completely abolished by addition of copper ions to the incubation medium.

These results suggest that there is a specific transport system for spermine and trientine on the renal brush-border membrane. This transport system contributes to the secretion of trientine in the kidney proximal tubule but does not recognize the trientine-copper complex.

Trientine dihydrochloride, a selective chelating agent for copper, increases urinary excretion of excess copper and is used for the treatment of Wilson's disease, chronic copper intoxication in the liver, brain, and other organs and tissues (Walshe 1982). We have reported the disposition behaviour of trientine in patients with Wilson's disease (Miyazaki et al 1990) and in the LEC rat, an animal model of Wilson's disease (Iseki et al 1992), and the absorption mechanism of trientine (Tanabe et al 1996) by use of rat intestinal brush-border membrane vesicles. The bioavailability of trientine was found to be poor, because absorption of this drug was suppressed by physiological polyamines, e.g. spermine, normally present in the intestinal lumen. Spermine, in fact, caused distinct inhibition of the uptake of trientine by rat intestinal brush-border membrane vesicles. It is evident that because of their similar chemical structures trientine and spermine share a common mechanism of transport in the wall of the intestine.

There are, however, few reports on the mechanism of excretion of trientine in the kidney. Sokol & Gates (1990) reported that polyamines inhibited the transport of the organic cations tetraethylammonium and N^1-methylnicotinamide across the brush-border membrane from the rabbit kidney cortex. They concluded that polyamines are recognized and excreted by the H^+/organic cation antiporter into urine. They did not, however, directly investigate polyamine transport, and it is still not clear whether polyamines are transported across the membrane by the same H^+-antiport system. De Smedt reported that there was a Na^+-independent transport system for polyamines in LLC-PK_1 cells derived from the pig kidney (De Smedt et al 1989). It is also not clear, however, whether this transporter is the H^+/organic cation antiporter.

In this study we have investigated the uptake behaviour of polyamines by rat renal brush-border membrane vesicles in an attempt to clarify the contribution of H^+/organic cation antiporters to the transport of polyamines, including trientine.

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Materials and Methods

Chemicals

Trientine dihydrochloride was kindly donated by Tsumura (Tokyo, Japan). [^{14}C]Tetraethylammonium bromide (136·9 MBq $mmol^{-1}$) was purchased from NEN Products (Boston, MA). [^{14}C]Spermine tetrahydrochloride (4·00 GBq $mmol^{-1}$) was purchased from Amersham (Amersham, UK). Spermine tetrahydrochloride was obtained from Sigma (St Louis, MO). Other chemicals were of the highest grade available and were used without further purification.

Clearance study

All experiments were performed on three or four rats. Male Wistar rats, 200–250 g, were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Trientine saline solution was administered into the jugular vein at a dose of 2·5 or 10 mg kg^{-1}. The same moles of $CuSO_4$ as trientine were added to the saline to confirm the effect of copper ions on the renal excretion of trientine. After administration blood (approximately 200 μL) was collected from the femoral vein with a heparinized syringe at 30, 60, 90 and 120 min. Urine samples were collected at the same times from the bladder. Blood samples were immediately centrifuged and plasma was collected. The concentrations of trientine in plasma and urine

were determined by HPLC. The renal clearance of trientine was calculated from equation 1:

$$\text{Clearance} = (U \times V)_{1-2} \times \ln(P_1/P_2)/(P_1 - P_2) \quad (1)$$

where U is the concentration of trientine in urine, V is the volume of urine, and P_1 and P_2 are the plasma concentrations of trientine at the selected times (e.g. 30 min and 60 min). The clearance was calculated at 30 min to 60 min, 60 min to 90 min, and 90 min to 120 min, and the average of these data was used in this study. To use the plasma and urine samples at 0 and 30 min, the concentration of creatinine was determined and creatinine clearance was calculated from equation 2.

$$\text{Creatinine clearance} = (U \times V)_{0-30}/P_0 \quad (2)$$

where U is the concentration of creatinine in urine and V the volume of urine at 0 to 30 min, and P_0 is the plasma concentration of creatinine at time 0. The concentrations of creatinine in plasma at time 0 and 30 min were almost identical (data not shown).

Isolation of brush-border membrane vesicles from the rat kidney cortex
Renal brush-border membrane vesicles were prepared from the renal cortex of the rats by the Mg^{2+}/EGTA precipitation method according to Biber et al (1981) with several modifications described in our previous report (Naasani et al 1995). Brush-border membrane vesicles were washed and resuspended in the pre-loading buffer (100 mM D-mannitol, 100 mM KCl, 20 mM *N*-2-hydroxymethylpiperazine-N′-2-ethanesulphonic acid (HEPES)–tris(hydroxymethyl) aminomethane (Tris), pH 7·5 or 20 mM 2-morpholinoethanesulphonic acid–Tris, pH 5·5). Enrichment of the brush-border membrane vesicles fraction was routinely more than 10-fold in comparison with the homogenate, as revealed from assessment of the specific activity of the membrane enzyme marker, alkaline phosphatase.

Uptake study
Uptake studies were performed at 37°C using a rapid filtration technique reported previously (Iseki et al 1991). The reaction was started by mixing membrane suspension (20 μL) with incubation medium (100 μL) containing substrates. At the stated time the reaction was terminated by diluting the reaction mixture with ice-cold stop buffer (150 mM KCl, 10 mM HEPES/Tris, pH 7·5; 4 mL) then filtration through a Millipore filter (HAWP, 0·45 μm, 2·5-cm diameter). The filter was then washed once with ice-cold stop buffer (4 mL). When the [^{14}C]-labelled compound was used as a substrate, radioactivity was measured by liquid scintillation counting. In experiments to measure trientine uptake, trientine trapped on the filter was extracted with phosphate buffer (5 mM, pH 7·5; 0·5 mL) containing KCl (500 mM). Trientine was completely extracted with this buffer.

Analytical method
The detection of trientine in plasma, urine and brush-border membrane vesicles was performed by HPLC (Hitachi L-6000; Hitachi, Tokyo) with fluorimetric detection (820-FP, Jasco, Tokyo) as described previously (Miyazaki et al 1990; Tanabe et al 1996). Creatinine concentrations in plasma and urine were determined by a colorimetric method (Creatinine-Test Wako; Wako, Osaka, Japan). Protein concentration was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard.

Table 1. Creatinine clearance and renal clearance of trientine in the rat.

	Trientine dose (mg kg^{-1})	Creatinine clearance (mL min^{-1})	Trientine clearance (mL min^{-1})
Control	2·5	1·81 ± 0·06	2·32 ± 0·13**
	10·0	1·76 ± 0·13	2·08 ± 0·06*
With copper	2·5	1·85 ± 0·06	1·73 ± 0·02

Each value represents the mean ± s.d. from 3 to 4 rats. *$P < 0.05$, **$P < 0.01$, significantly different from creatinine clearance.

Results and Discussion

Creatinine and trientine clearance in-vivo study
To clarify whether trientine is reabsorbed or secreted in rat renal proximal tubules we measured the renal clearance of trientine in-vivo. As shown in Table 1, the renal clearance of trientine (dose 2·5 mg kg^{-1}, i.v.) was significantly greater than creatinine clearance, which is a good indication of the glomerular filtration rate. Trientine is a small (MW 146·23) extremely hydrophilic molecule. It was, therefore, considered that almost all the trientine in plasma is filtered through the renal glomerulus and that increased renal clearance will be a result of secretion from the proximal tubules. The renal clearance of trientine decreased, moreover, when the dose administered was increased to 10 mg kg^{-1}. These data suggest the existence of an active secretion mechanism for trientine in rat kidney, and it was saturable as a function of concentration. Trientine clearance decreased to a level similar to creatinine clearance when copper was co-administered with trientine. Trientine is a selective chelating agent for copper which is reported to accelerate the urinary excretion of excess copper in the body (Walshe 1982). This suggests that the mechanism of renal excretion of free trientine is different from that of the trientine-copper complex.

Effects of outward H^+ gradient and inward Na^+ gradient on the uptake of trientine and spermine
To clarify the renal secretion of trientine we investigated the mechanisms of uptake of trientine and spermine using rat renal

Table 2. Effect of an outwardly directed H^+ gradient on the uptake of tetraethylammonium by rat renal brush-border membrane vesicles.

Conditions	Uptake (pmol (mg protein)$^{-1}$) at time:		
	15 s	30 s	30 min
pH_{out} 7·5, pH_{in} 7·5	158·1 ± 20·4	126·2 ± 18·5	174·7 ± 26·8
pH_{out} 7·5, pH_{in} 5·5	1102·3 ± 59·6	537·7 ± 61·8	174·5 ± 1·7

Membrane vesicles (20 μL) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM morpholinoethanesulphonic acid/Tris buffer (pH 5·5) or 20 mM HEPES/Tris buffer (pH 7·5). Uptake studies were performed by adding a drug solution (100 μL) containing 100 mM KCl, 100 mM D-mannitol, 20 mM HEPES/Tris buffer (pH 7·5) and 120 μM [^{14}C]tetraethylammonium. Each value represents the mean ± s.e.m. of nine measurements.

brush-border membrane vesicles. It is well known that organic cations such as tetraethylammonium and N^1-methylnicotinamide are transported across this membrane by one or more H^+/organic cation antiporters (Takano et al 1984; Wright 1985). Polyamine uptake might be driven by this H^+-antiport system because polyamines have cationic amino-groups in their molecules. Table 2 shows the uptake of 100 μM tetraethylammonium by brush-border membrane vesicles. In the presence of an outwardly directed H^+ gradient the initial uptake was significantly stimulated and there was also an overshoot effect. In contrast, neither the uptake of trientine nor that of spermine was affected by the outwardly directed H^+ gradient (Fig. 1). These results suggest that the H^+/organic cation antiporter does not recognize trientine and spermine.

Na^+/polyamine symports have been reported in several mammalian cells (Seiler & Dezeure 1990) and De Smedt et al (1989) reported the occurrence of Na^+-dependent and -independent transport systems for polyamines in the renal cell line LLC-PK_1. As shown in Fig. 2, however, an inwardly directed Na^+ gradient did not alter the uptake behaviour of polyamines by rat renal brush-border membrane vesicles.

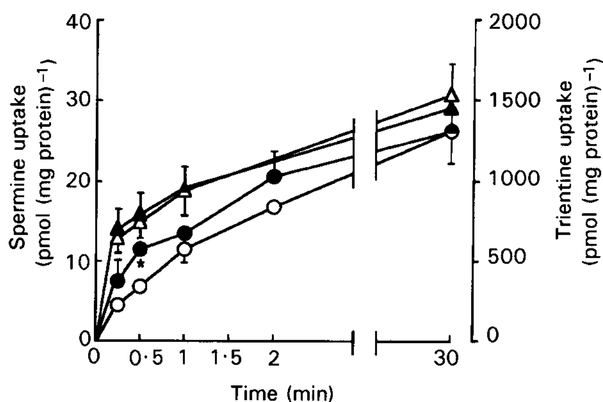

FIG. 1. Effect of an outwardly directed H^+ gradient on the uptake of trientine (○ ●) and spermine (△ ▲) by rat renal brush-border membrane vesicles. Membrane vesicles (20 μL) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM morpholinoethanesulphonic acid/Tris buffer (pH 5·5, ▲ ●) or 20 mM HEPES/Tris buffer (pH 7·5, △ ○). Uptake studies were performed by adding a drug solution (100 μL) containing 100 mM KCl, 100 mM D-mannitol, 20 mM HEPES/Tris buffer (pH 7·5) and 1·2 mM trientine or 2·4 μM [^{14}C]spermine. Each point represents the mean ± s.e.m. of nine measurements. *$P < 0·05$, significantly different from $pH_{in}7·5/pH_{out}7·5$.

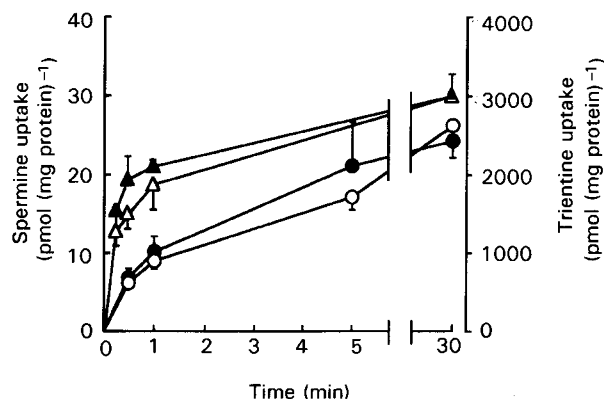

FIG. 2. Effect of an inward Na^+ gradient on the uptake of trientine (○ ●) and spermine (△ ▲) by rat renal brush-border membrane vesicles. Membrane vesicles (20 μL) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris buffer (pH 7·5). Uptake studies were performed by adding an incubation medium (100 μL) containing 100 mM D-mannitol, 20 mM HEPES/Tris buffer (pH 7·5), 1·2 mM trientine or 2·4 μM [^{14}C]spermine, and 100 mM KCl (△ ○) or NaCl (▲ ●). Each point represents the mean ± s.e.m. of nine measurements.

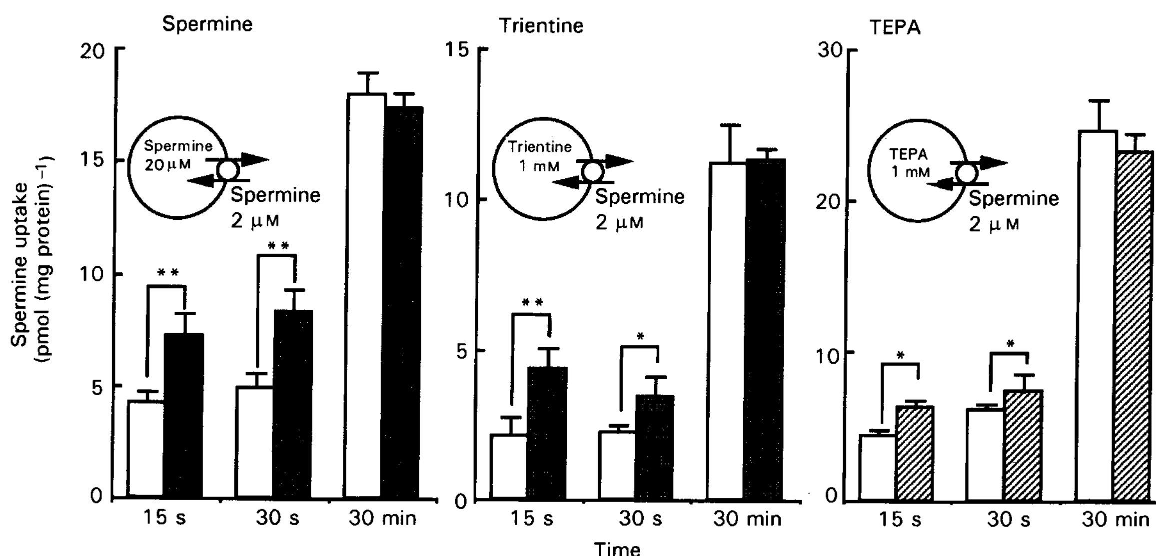

FIG. 3. Effect of pre-loading of spermine, trientine and tetraethylenepentamine (TEPA) on [^{14}C]spermine uptake by rat renal brush-border membrane vesicles. Membrane vesicles (20 μL) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris buffer (pH 7·5). Uptake studies were performed by adding an incubation medium (20 μL) containing unlabelled-spermine, trientine, tetraethylenepentamine (pre-load group) or nothing (control). After 30 min, drug solution (120 μL) containing [^{14}C]spermine and unlabelled-spermine, trientine, tetraethylenepentamine (control) or nothing (pre-load group) was added to the suspension of brush-border membrane vesicles. Each column represents the mean ± s.e.m. of nine measurements. *$P < 0·05$, **$P < 0·01$, significantly different from control (unhatched columns).

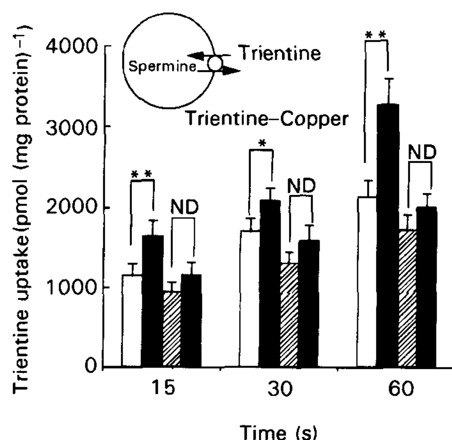

FIG. 4. Effect of pre-loading of spermine on the uptakes of trientine (■) and trientine–copper complex (■) by rat renal brush-border membrane vesicles. Each column represents the mean ± s.e.m. of nine measurements. $*P < 0.05$, $**P < 0.01$, significantly different from control (□ and ▨, respectively). ND = no significant difference.

Trans-stimulation effect on the uptake of [^{14}C]polyamine

Trans-stimulation studies were performed to clarify whether the uptake of trientine is achieved by a carrier-mediated mechanism. As shown in Fig. 3, the initial uptake of 2 μM [^{14}C]spermine was significantly greater in vesicles pre-loaded with unlabelled 20 μM spermine (at 15 s; control, $4{\cdot}20 \pm 0{\cdot}50$ pmol (mg protein)$^{-1}$; pre-loaded spermine, $7{\cdot}25 \pm 0{\cdot}97$), 1 mM trientine (control, $2{\cdot}09 \pm 0{\cdot}62$; pre-loaded trientine, $4{\cdot}33 \pm 0{\cdot}71$), or tetraethylenepentamine (control, $4{\cdot}36 \pm 0{\cdot}36$; pre-loaded tetraethylenepentamine, $6{\cdot}26 \pm 0{\cdot}46$) than that in the control vesicles. Fig. 4 shows that the initial uptake of 1 mM trientine was also stimulated by pre-loading with 50 μM spermine (at 15 s, control, $1145{\cdot}7 \pm 148{\cdot}3$; pre-loaded spermine, $1627{\cdot}3 \pm 191{\cdot}9$). In order to understand in more detail the effect of copper on the secretion of trientine, we further investigated and compared the trans-stimulation uptake of trientine into spermine-pre-loaded brush-border membrane vesicles in the presence or absence of copper ions. As shown in Fig. 4, copper ions completely abolished the stimulatory effect of intravesicular spermine on the uptake of trientine (at 15 s, control, $924{\cdot}9 \pm 136{\cdot}8$; pre-loaded spermine, $1153{\cdot}3 \pm 165{\cdot}5$). We confirmed that the effect of copper ions on the trans-stimulation of trientine was not a result of direct damage to the brush-border membrane vesicles by showing there were no difference between the uptake profiles of spermine in the presence or absence of copper ions in the incubation media. These data suggest that there is a polyamine carrier on the rat renal brush-border membrane which recognizes spermine, trientine and tetraethylenepentamine, but not trientine–copper complex.

In conclusion, there is a transporter for polyamine compounds on the rat renal brush-border membrane which plays an important role in the secretion of trientine into the renal proximal tubules but does not recognize the trientine-copper complex. The mechanisms of transport of these polyamines across the basolateral membrane remain unclear.

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