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Uptake Mechanism of Trientine by Rat Intestinal Brush-border Membrane Vesicles

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Abstract

The uptake characteristics of trientine by rat intestinal brush-border membrane vesicles were studied.

The uptake characteristics of trientine were similar to those of the physiological polyamines with respect to the excessive accumulation in vesicles, the pH dependency, the temperature dependency and the ineffectiveness of K^+ diffusion potential (inside negative). The initial uptake of trientine was saturable with a K_m value of 1·13 mM, which was larger than that of spermine and spermidine. Furthermore, the uptake rate of trientine was dose-dependently inhibited by spermine and spermidine. Spermine competitively inhibited the uptake of trientine with a K_i value of 18·6 μM, and it was close to the K_m value for spermine (30·4 μM).

These data suggested that the uptake of trientine was similar to that of spermine and spermidine in rat small intestinal brush-border membrane vesicles, and these polyamines seem to inhibit the absorption of trientine from the gastrointestinal tract.

In our previous investigations (Iseki et al 1991; Kobayashi et al 1992, 1993a), the main uptake mechanism of physiological polyamines (spermine, spermidine and putrescine) by rat intestinal brush-border membrane vesicles was found to be independent from sodium-dependent carrier systems, although such carrier systems for polyamine were found in cells derived from various organs (Seiler & Dezeure 1990). We also reported that polyamines are transported by means of a diffusion mechanism related to the binding into the acidic lipids of the biomembrane such as phosphatidylserine.

Triethylenetetramine 2HCl (trientine) is a strongly chelating agent for copper, and can scavenge the excessive copper ion from the body of Wilson's disease patients (Walshe 1982). The absorption behaviour of trientine after oral administration was found to be very poor and variable in the patients with Wilson's disease (Kobayashi et al 1993b) and in the Long–Evans Cinnamon rat (Iseki et al 1992) which is a model rat for Wilson's disease. This chelating agent has a similar structure to polyamines and it is speculated that the absorption mechanism of trientine from the intestinal lumen is similar to the polyamine absorption mechanism attributable to the binding to the membrane bilayers.

In the present study, in order to clarify whether trientine shares a common transport mechanism with polyamines, the uptake characteristics of this compound by rat intestinal brush-border membrane vesicles were investigated and compared with those of polyamines.

Materials and Methods

Chemicals

Trientine dihydrochloride (Tsumura & Co. Tokyo, Japan) was used as supplied. Spermine tetrahydrochloride, spermidine trihydrochloride and valinomycin were purchased from Sigma (St Louis, MO, USA). Fluorescamine was obtained from Fluka (Buchs, Switzerland). All other chemicals were of the highest grade available and used without further purification.

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Isolation of the brush-border membrane vesicles from rat small intestine

Adult male Wistar rats (200–250 g) were used in this study. Entire small intestine was excised under anaesthesia (sodium pentobarbitone; 30–60 mg kg^{-1} body weight, i.p.). Brush-border membrane vesicles (BBMVs) were isolated using the calcium precipitation method as described previously (Iseki et al 1991).

Uptake experiments

Uptake of trientine was measured by a rapid filtration technique as described previously (Iseki et al 1991). In a routine assay, 20 μL membrane suspension were added to 100 μL incubation medium kept at 37°C. The composition of the incubation media is described in the figure legends. At selected time intervals, the uptake was stopped by diluting the incubation medium with 3 mL ice-cold 10 mM *N*-2-hydroxymethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)/tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7·5) containing 150 mM KCl. The mixture was immediately filtered through a Millipore filter (HAWP, 0·45 ± 2·5 cm diameter). The filter was washed once with the same ice-cold buffer. The substrate trapped on the filter was extracted with 400 μL of buffer (500 mM KCl, 5 mM KH_2PO_4/Na_2PO_4 buffer, pH 7·5) and was measured by HPLC. As a blank, vesicle-free incubation media were handled in an identical manner.

Analytical method

Trientine was determined according to our previous report (Miyazaki et al 1990) with minor modification. The samples extracted from membrane vesicles were prelabelled by fluorescamine, and then assayed by HPLC (Hitachi L-6000, Hitachi, Tokyo) equipped with a multi-wavelength fluorometric monitor

(820-FP, Jasco, Tokyo) 380 nm (excitation) and 485 nm (emission). To 200 μL sample, 400 μL 0·1 M phosphate buffer (Na_3PO_4/Na_2HPO_4, pH 9·5) and 50 μL 0·15 M trisodium ethylenediaminetetraacetate (EDTA 3Na) in the same buffer were added. Subsequently, 50 μL of 1 mM fluorescamine in acetonitrile was added and mixed vigorously for 1 min on a Vortex mixer. When the reaction was over and the excessive fluorescamine had decomposed completely (20 min), 50 μL 100 μM α-napthylamine in methanol was added to the reaction mixture as an internal standard. Twenty microlitres of the sample solution were injected into the HPLC system. A nitrile column (Nucleosil 5-CN, 250 × 4 mm i.d., particle size 5 μm, Macherey, Nagel & Co., Duren, T.F.R.), maintained at 40°C, was used for the separation of fluorescamine-labelled trientine on a mobile phase of 27% acetonitrile containing 100 mM ammonium chloride, 35 mM sodium benzene sulphonate and 6·7 mM acetic acid (pH 6·0). Protein concentration was determined by the method of Lowry et al (1951) with bovine serum albumin as the standard.

Data analysis

All experiments presented in this paper were performed in at least three different preparations and were always performed in triplicate. Statistical analysis was performed using Student's unpaired *t*-test and a $P < 0.05$ was considered significant. In a Lineweaver–Burk plot, the data were fitted by a single Michaelis–Menten term:

$$V = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where V is the initial (30 s) uptake rate, [S] is the substrate concentration, V_{max} is the maximum uptake rate and K_m is the affinity constant.

Results

Characteristics of trientine uptake by rat intestinal brush-border membrane vesicles

Time–course of the uptake of trientine (1 mM) by rat intestinal brush-border membrane vesicles is shown in Table 1. Trientine was taken up by the vesicles as a function of time, and reached equilibrium within 10 minutes in contrast to polyamine uptake (Iseki et al 1991; Kobayashi et al 1992). The equilibrium uptake of trientine was 2500 pmol (mg protein)$^{-1}$ (vesicle volume was calculated to be 2·5 μL (mg protein)$^{-1}$); this value was about 2·5 times that of D-glucose (Iseki et al 1991), which does not bind to the membrane surface. At a temperature of 4°C, the 30-s and the 30-min uptakes of trientine decreased to 32 and 40% of the uptake at 37°C, respectively (Table 2). We also studied the dependency of the uptake of trientine at 1 and 30 min on the medium pH. Table 3 indicates that both of the initial and the equilibrium uptakes of trientine were greater at lower pH than that at pH 7·5. These results were in good agreement with the uptake behaviour of polyamines (Kobayashi et al 1992).

Table 1. Time-course of the uptake of trientine (1 mM) by rat small intestinal brush-border membrane vesicles.

Time (min)	Uptake (pmol (mg protein)$^{-1}$)
0·25	433·3 ± 74·7
0·5	747·5 ± 96·8
1	1088·2 ± 90·5
2	1412·7 ± 221·2
5	1895·3 ± 235·1
10	2399·2 ± 219·8
30	2413·4 ± 274·9
60	2446·8 ± 315·6
90	2501·4 ± 312·8

Membrane vesicles (20 μL) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris (pH 7·5). Uptake studies were performed by adding an incubation medium (100 μL) containing 1·2 mM trientine, 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris (pH 7·5). Each value represents the mean ± s.e. of 9 determinations from 3 preparations.

Table 2. Effect of medium temperature on the uptake of trientine by rat small intestinal brush-border membrane vesicles.

	Temperature (°C)		
Time (min)	4	25	37
0·5	5·2 ± 1·3	15·6 ± 2·2	31·0 ± 4·0
1	7·2 ± 1·5	29·2 ± 3·4	45·1 ± 3·7
30	41·4 ± 4·6	69·0 ± 3·8	100·0 ± 11·4

Medium composition is shown in Table 1. Each value is a percent of the uptake value at 37°C, 30 min (2413·4 ± 274·9 pmol (mg protein)$^{-1}$), and is the mean ± s.e. of 9 determinations from 3 preparations.

Table 3. Effect of medium pH on the uptake of trientine by rat small intestinal brush-border membrane vesicles.

	Uptake (pmol (mg protein)$^{-1}$)	
pH	1 min	30 min
5·5	1075·8 ± 56·5***	1720·8 ± 146·4***
6·0	1437·1 ± 116·1**	2196·7 ± 160·9**
6·5	1619·6 ± 159·5*	2766·7 ± 139·6
7·0	1938·4 ± 164·0	2981·8 ± 328·6
7·5	2113·4 ± 30·6	3184·4 ± 111·8

Membrane vesicles were suspended in 100 mM KCl, 100 mM D-mannitol and either 20 mM 2-morpholinoethanesulphonic acid (MES)/-Tris (pH 5·5, 6·0, 6·5) or 20 mM HEPES/Tris (pH 7·0, 7·5). Uptake studies were performed by adding an incubation medium containing 1·2 mM trientine, 100 mM KCl, 100 mM D-mannitol and either 20 mM MES/Tris (pH 5·5, 6·0, 6·5) or 20 mM HEPES / Tris (pH 7·0, 7·5). Each value represents the mean ± s.e. of 6–9 determinations from 3 preparations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with the value at pH 7·5.

Effect of a valinomycin-induced K^+-diffusion potential on the uptake of trientine by BBMVs

Our previous studies demonstrated that a K^+-diffusion potential (inside negative), generated in the presence of valinomycin, stimulated the uptake of tryptamine (Sugawara et al 1992) and disopyramide (Takahashi et al 1993), a mono-cation, but not spermidine, a polycation (Kobayashi et al 1993a). To clarify whether the uptake of trientine is classified into the monocation-type or the polycation-type, we examined the effect of K + -diffusion potential on trientine uptake by BBMVs. There were no comparable changes in trientine uptake between the presence and absence of valinomycin (Table 4). This result gives evidence that the uptake of trientine is similar to that of polyamine in respect of the ineffectiveness of K + -diffusion potential.

Table 4. Effect of a potassium diffusion potential (inside negative) generated in the presence of valinomycin on the uptake of trientine by rat intestinal brush-border membrane vesicles.

	Uptake (pmol (mg protein)$^{-1}$)	
Time (min)	Control	Inside negative
0·5	1179·3 ± 163·3	1064·5 ± 154·5
1	1797·6 ± 334·2	1440·4 ± 197·9
2	2125·0 ± 389·3	2020·4 ± 368·0
30	2931·5 ± 418·5	3080·4 ± 480·1

Membrane vesicles were suspended in 100 mM potassium gluconate, 100 mM D-mannitol and 20 mM HEPES/Tris (pH 7·5). Valinomycin in ethanol was added to the vesicle suspension at a final concentration of 6·3 nmol (mg protein)$^{-1}$ (inside negative) or the same volume of ethanol was added (control). The final ethanol concentration was less than 1 %. Uptake studies were performed by adding an incubation medium containing 1·2 mM trientine, 100 mM sodium gluconate, 100 mM D-mannitol and 20 mM HEPES/Tris (pH 7·5). Each value represents the mean ± s.e. of 9 determinations from 3 preparations.

Concentration dependence and inhibitory effects of spermine and spermidine on the uptake of trientine by BBMVs

The initial uptake rates of spermine and spermidine by the BBMVs were found to be saturable in spite of the absence of ionic gradients (Iseki et al 1991; Kobayashi et al 1992). Therefore, we investigated the dependency of the initial uptake of trientine on its concentration. The initial uptake of substrates by BBMVs is composed of two factors, the binding to the outer membrane surface and the transport across the membrane (Kobayashi et al 1993a). Consequently, the initial uptake rate of trientine was corrected by subtracting the uptake values at 4°C (the binding to the membrane surface) from the uptake values at 37°C for 30 s. The uptake rate of trientine exhibited a saturable behaviour (Fig. 1), and the kinetic calculation resulted in a K_m value of 1·13 mM, and a V_{max} value of 2803 pmol (mg protein)$^{-1}$/30 s (Fig. 2).

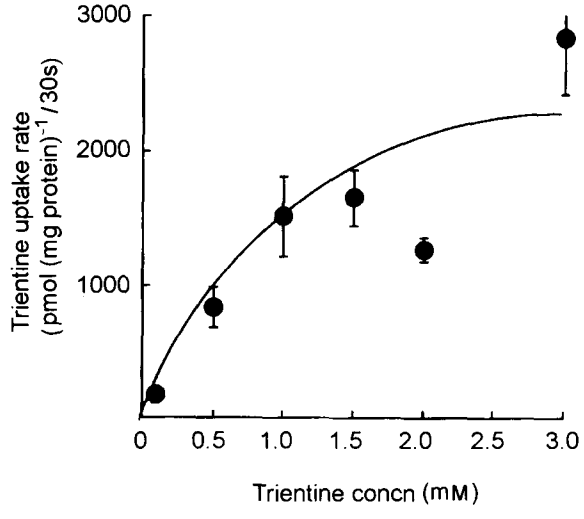

FIG. 1. Concentration dependence of the uptake rate of trientine by rat small intestinal brush-border membrane vesicles. Membrane vesicles (20 μL) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris buffer (pH 7·5). Uptake studies were performed by adding an incubation medium (100 μL) containing 20 mM HEPES/Tris buffer (pH 7·5), 100 mM D-mannitol, 100 mM KCl and various concentrations of trientine. Data were calculated by subtracting the uptake at 4°C from that at 37°C. Each point represents the mean ± s.e. of 9 determinations from 3 preparations.

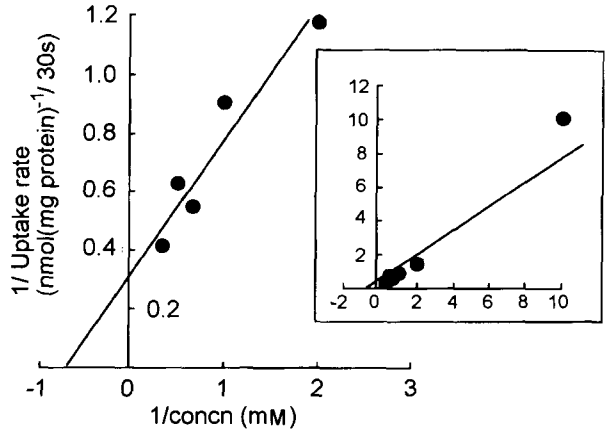

FIG. 2. Lineweaver–Burk plot of the uptake of trientine by rat small intestinal brush-border membrane vesicles. Data are calculated from Fig. 1. A linear regression program has been used to fit data, the intercepts of line with x and y axis are: $-K_m^{-1}$ ($K_m = 1·13$ mM) and V_{max}^{-1} ($V_{max} = 2803$ pmol (mg protein)$^{-1}$/30 s).

Furthermore, the initial uptake, the equilibrium uptake and the binding to the membrane surface of trientine was significantly inhibited by spermine and spermidine at lower concentration (Table 1). The inhibitory effects of spermine, which has four cationic amine groups, were stronger than those of spermidine. In addition, Dixon analysis showed that spermine competitively inhibited the initial uptake rate of trientine (Fig. 3). The concentration needed to produce half of the inhibitory effect (K_i) was a value of 18·6 μM, which was approximately the same as the K_m value of spermine uptake (30·4 μM, Kobayashi et al 1993a). These data suggest that the uptake mechanism of trientine by rat intestinal BBMV is identical to that of spermine and spermidine (Table 5).

Table 5. Inhibitory effects of various concentrations of spermine and spermidine on the uptake of trientine (1 mM) by rat small intestinal brush-border membrane vesicles.

	Binding	Transport	
	(%)	0·5 min	30 min
Control	100·0 ± 6·3	100·0 ± 8·8	100 ± 2·8
Spermine			
1 μM	73·3 ± 5·7	84·2 ± 5·0**	103·4 ± 6·3
10 μM	67·8 ± 2·8*	60·9 ± 17·1***	73·1 ± 10·8***
100 μM	53·3 ± 2·8**	23·0 ± 9·8***	36·9 ± 4·6***
Spermidine			
100 μM	75·7 ± 22·1	86·3 ± 13·3	71·4 ± 2·0***
500 μM	26·9 ± 10·6***	64·5 ± 3·2*	57·3 ± 1·9***

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 20 mM HEPES/Tris buffer (pH 7·5), 100 mM D-mannitol, 100 mM KCl, 1·2 mM trientine and either 0, 1·2, 12 or 120 μM spermine or 0, 120 or 600 μM spermidine (final concentrations of spermine and spermidine are indicated in the Table). Binding indicates the uptake at 4°C and 30 s and transport indicates the uptake at 37°C less the binding value. The control values were 409·7 ± 25·8 (binding), 1293·3 ± 113·8 (transport, 0·5 min) and 2258·9 ± 63·2 (transport, 30 min). Values are the mean ± s.e. of 9 determinations from 3 preparations. *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with control.

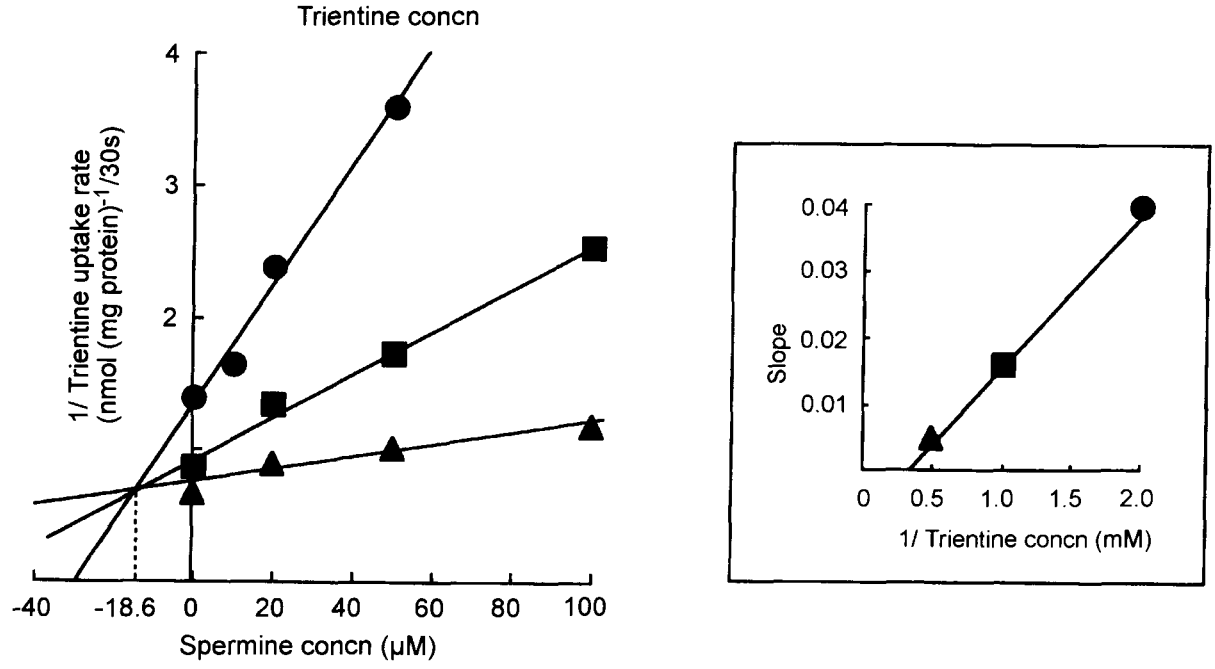

FIG. 3. Dixon plot analysis of the inhibitory effect of spermine on the uptake of trientine by rat small intestinal brush-border membrane vesicles. Membrane vesicles (20 μL) were suspended in 100 mM KCl, 100 mM D-mannitol and 20 mM HEPES/Tris buffer (pH 7·5). Uptake studies were performed by adding an incubation medium (100 μL) containing 20 mM HEPES/Tris buffer (pH 7·5), 100 mM D-mannitol, 100 mM KCl, various concentrations of spermine and 0·6 mM (●), 1·2 mM (■) and 2·4 mM (▲) of trientine (final concentrations of trientine are 0·5, 1 and 2 mM, respectively). Each value represents the mean of 9 determinations from 3 preparations. Inset is a replot of the slopes of the Dixon plot.

Discussion

Trientine has four amine groups and its chemical structure is very similar to spermine, a physiological polyamine. Many researchers have reported that polyamines are transported into the cells by specific carriers depending on an inward Na^+-gradient (Seiler & Dezeure 1990). However, our recent studies have shown only a weak effect on an inwardly Na^+-gradient (Iseki et al 1991; Kobayashi et al 1992), and there was no effect on the uptake of trientine (data not shown). Therefore, the predominant transport process of polyamines in rat intestinal brush-border membrane seemed to be passive diffusion which is dependent on the electrostatic binding to the acidic phospholipids such as phosphatidylserine, described previously (Iseki et al 1991). In the present study, to clarify the transport mechanism of trientine across BBMVs, we characterized the uptake of trientine from a viewpoint of the similarity to the transport of the physiological polyamines. The uptake characteristics of trientine were found to be similar to those of spermine and spermidine with respect to the excessive accumulation into the vesicles, the pH dependence, the temperature dependence, and the ineffectiveness of a valinomycin-induced K+ diffusion potential.

Dixon plot analysis demonstrated that spermine competitively inhibited the uptake of trientine with a K_i value of 18·6 μM, which was similar to the K_m value for spermine, suggesting that trientine uptake participates in a common system with spermine. It is likely that the uptake of trientine and polyamine into the BBMVs is driven by the binding of substrates to the acidic charges enriched in the inner layer of the membrane bilayer. The final uptake value of trientine was less than that of spermine and spermidine, although trientine uptake was 2-3 times greater than that of D-glucose. Additionally, the saturation parameter of trientine uptake ($K_m = 1·13$ mM) was relatively large in comparison with those of spermine (30·4 μM) and spermidine (148·1 μM) (Kobayashi et al 1993a). These results seem to be due to the fact that the net charge of trientine (2·11) is smaller than that in spermine (3·62) and spermidine (2·96) at pH 7·5.

It is known that millimolar levels of polyamines exist in the gastrointestinal tract (Osborne & Seidel 1990). These polyamines are synthesized by enterocytes and microflora in the small intestine. Therefore, it is considered that trientine absorption from the intestinal lumen can be affected by the presence of physiological polyamines. Furthermore, the concentration of polyamine in the small intestinal lumen was variable when the enterocytes or microflora were damaged (eg, ulcer, stress, antibiotic drug administration). We have confirmed that the absorption of trientine from the intestine of Wilson's disease patients was poor and very variable (Kobayashi et al 1993b).

The present study demonstrates that the uptake of trientine by rat intestinal brush-border membrane vesicle shares with polyamine a common transport mechanism which includes a charge-interaction between the polycation and the negative-charge of the inner membrane layer. This mechanism provides an explanation for the poor and variable absorption of trientine in Wilson's disease patients.

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