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A Thiamine/H^+ Antiport Mechanism for Thiamine Entry into Brush Border Membrane Vesicles from Rat Small Intestine

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Abstract. Outwardly oriented H^+ gradients greatly enhanced thiamine transport rate in brush border membrane vesicles from duodenal and jejunal mucosa of adult Wistar rats. At a gradient $pH_{in}5$:$pH_{out}7.5$, thiamine uptake showed an overshoot, which at 15 sec was three times as large as the uptake observed in the absence of the gradient. Under the same conditions, the binding component of uptake accounted for only 10–13% of intravesicular transport. At the same gradient, the K_m and J_{max} values of the saturable component of the thiamine uptake curve after a 6 sec incubation time were 6.2 ± 1.4 μM and 14.9 ± 3 pmol · mg^{-1} protein · 6 sec^{-1} respectively. These values were about 3 and 5 times higher, respectively, than those recorded in the absence of H^+ gradient. The saturable component of the thiamine antiport had a stoichiometric thiamine: H^+ ratio of 1:1 and was inhibited by thiamine analogues, guanidine, guanidine derivatives, inhibitors of the guanidine/H^+ antiport, and imipramine. Conversely, the guanidine/H^+ antiport was inhibited by unlabeled thiamine and thiamine analogues; omeprazole caused an approximately fourfold increase in thiamine transport rate. In the absence of H^+ gradient, changes in transmembrane electrical potential did not affect thiamine uptake. At equilibrium, the percentage membrane-bound thiamine taken up was positively correlated with the pH of the incubation medium, and increased from about 10% at pH 5 to 99% at pH 9.

Key words: Epithelial transport — Brush border membrane — Thiamine/H^+ antiport — Thiamine intestinal transport — Cation/H^+ intestinal antiport

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Introduction

The acidic microclimate (in vitro pH 5.2–6.7) of the rat duodenal and jejunal surface can modify the intestinal transport of several substances, including weak electrolytes and vitamins (Rechkemmer, 1991). Thiamine is a quaternary amine characterized by a pyrimidine nucleus linked to a thiazole ring, which exists as a monovalent or divalent cation depending on the pH of the solution (Komai & Shindo, 1974). Endogenous and exogenous organic cations can be transported into brush border membrane vesicles (BBMVs) by organic cation/H^+ antiport systems, which have been demonstrated in the small intestine (Miyamoto, Ganapathy & Leibach, 1988) and in the kidney (Ott et al., 1991; Wright & Wunz, 1987). The purpose of the present study was to determine whether thiamine can be a substrate for an organic cation/H^+ antiport in BBMVs of rat small intestine, and to assess the features of this possible exchange mechanism. Initial experiments were designed to investigate in BBMVs the effects of differently oriented H^+ gradients and membrane potential on thiamine transport, the influence of pH on thiamine binding and the kinetics of thiamine transport at a gradient $pH_{in}5$:$pH_{out}7.5$. Subsequent experiments allowed the assessment of the stoichiometry and specificity of the thiamine/H^+ exchange mechanism in the presence of a gradient $pH_{in}5$:$pH_{out}7.5$ by using thiamine analogues and some organic and inorganic cations. The relationship between thiamine/H^+ and guanidine/H^+ antiports was evaluated by determining the inhibition constants K_i of guanidine and thiamine.

Preliminary partial accounts of these results have been presented at meetings of the Italian Physiological Society (Laforenza & Rindi, 1993, 1994; Laforenza & Verri, 1995; Laforenza, Gastaldi & Rindi, 1995).

Materials and Methods

Animals

Adult Wistar albino rats (300–400 g body wt) of either sex and reared on a complete standard diet, containing 12 μg/g thiamine, were used. The animals were killed by decapitation after 12-hr fasting with water *ad libitum.*

Preparation of BBMVs

BBMVs were prepared from duodenal and jejunal mucosa of 8–12 adult rats by using a modification of the method described by Said & Redha (1988) involving a Mg^{2+}/EGTA precipitation, which gives a lower purification, but it does not modify the proton conductance (Sabolic & Burckhardt, 1984). All procedures were carried out at 0–4°C. The enrichment of brush-border membranes was 11.3 ± 0.8 (mean ± SEM of 8 different preparations) as evaluated from the increment in sucrase activity of the final preparation as compared with the initial mucosal homogenate (*see* Casirola et al., 1988). Protein content was measured according to Lowry et al. (1951), using bovine serum albumin as a standard.

Transport Efficiency of BBMVs

The transport efficiency of the vesicular preparations was evaluated by determining the time course profile of D-glucose uptake by BBMVs after incubation with 1 mM D-[U-^{14}C]glucose (specific activity, 0.31 GBq · mmol^{-1}) under the conditions described by Casirola et al. (1988). The D-glucose uptake profile showed an overshoot at about 30 sec and equilibrium at 15–30 min, indicating that the preparations were suitable for transport studies (*data not shown:* however, a similar profile can be seen in Fig. 3*B*).

Incubation and Uptake Measurements

Vesicles were washed, suspended and pre-equilibrated for 2 hr at 5°C and then for 30 min at 25°C in solutions containing (mM): 280 D-mannitol, 2 $MgSO_4$ and 20 buffers (Tris-Hepes: pH 7.5 and 9; Mes-Tris: pH 5 and 6; Mes-Hepes: pH 4).

Ten μl of vesicle suspension were incubated at 25°C with 90 μl of solutions containing labeled thiamine or guanidine under different experimental conditions (*see* figure legends). After terminating the incubation with cold (0–4°C) stopping solution (150 mM NaCl and 1 mM Tris-Hepes, pH 7.5), the amount of thiamine radioactivity taken up by the vesicles was measured by a rapid filtration procedure (Casirola et al., 1988) using cellulose nitrate microfilters (Microfiltration System, Dublin, CA; pore diameter, 0.65 μm) previously saturated with unlabeled thiamine as described by Casirola et al. (1988). In each experiment appropriate blanks were prepared to evaluate the radioactivity of labeled thiamine nonspecifically adsorbed on the microfilter. The values of the blanks were subtracted from the total radioactivity retained on the filter. Radiometric measurements were made by using a Packard Tri-Carb model 2,000 CA liquid scintillation counter (Packard Instrument Downers Grove, IL). Unless stated otherwise, all uptake values were means ± SEM of at least triplicate determinations for each of five different preparations, each from 8–12 rats.

Short-time Incubations

For 6 sec incubation time a STRUMA short-time incubation apparatus (Innovativ-Labor AG, Adliswil, Switzerland) was used.

Statistics

The significance of the differences of the means under different experimental conditions was evaluated by using the following statistical methods: analysis of variance (ANOVA), followed by Newman-Keuls's *Q* test; Student's *t* test for paired data. All statistical tests were carried out by using a computerized program (Glantz, 1988).

Reagents

Unlabeled thiamine chloride hydrochloride and thiochrome were obtained from Prodotti Roche, Milan, Italy; pyrithiamine bromide hydrobromide, 4′-oxythiamine chloride from Sigma Chemical, USA; amprolium from Merck, Sharp and Dohme, Pavia, Italy; EGTA (ethylenebis (oxyethylene-nitrilo) tetraacetic acid), and MES hydrate (β-morpholine-ethane sulfonic acid) from Aldrich Chimica, Milan, Italy. Omeprazole was a generous gift from Astra Hässle AB, Mölndal, Sweden. All other reagents were of analytical grade and supplied by Sigma Chimica, Milan, Italy, and British Drug House (BDH) Ltd., Poole, Dorset, England.

Labeled Compounds

D-[U-^{14}C]glucose (specific activity, 10.8 GBq · mmol^{-1}) and [^{3}H]thiamine (specific activity, 429.2 GBq · mmol^{-1}) were from Amersham International plc, Amersham, England. [^{3}H]thiamine (specific activity, 74 GBq · mmol^{-1}) and [U-^{14}C]guanidine (specific activity, 2.03 GBq · mmol^{-1}) were from Moravek Biochemical, Brea, CA.

Results

General Properties of the Thiamine/H^+ Antiport

H^+ Gradients and Time Course of Thiamine Uptake

After preequilibration (*see* Methods), BBMVs were incubated at 25°C with 1 μM [^{3}H]thiamine (specific activity, 27.75 GBq · mmol^{-1}) in the presence of differently oriented (in/out or out/in) H^+ gradients within a 4–9 pH range. The incubation was terminated by adding 3 ml of cold stopping solution at fixed time intervals between 15 sec and 30 min. Details are reported only for results obtained in the 5–7.5 pH range (Fig. 1). Results obtained at other pH values for a 15 sec incubation time are summarized in Fig. 2. The rate of thiamine uptake was influenced by the orientation of the H^+ gradient, being greatly enhanced at outwardly directed gradients ($pH_{in} < pH_{out}$) (Fig. 2). At a gradient $pH_{in}5$:$pH_{out}7.5$, the amount of thiamine taken up at 15 sec showed an overshoot which was about three times as large as the uptake observed in the absence of a pH gradient ($pH_{in} = pH_{out}$

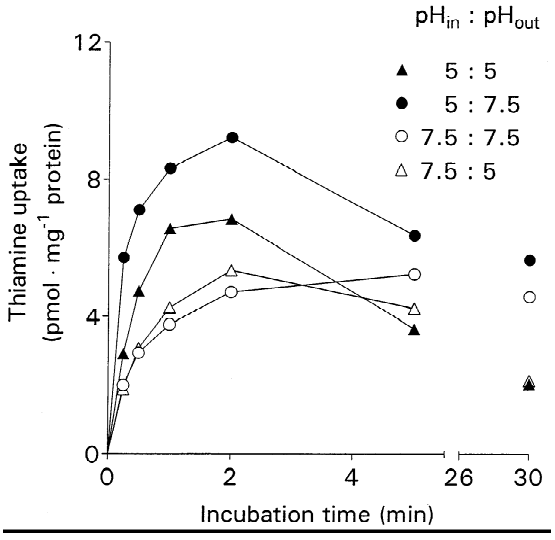


Fig. 1. Effect of pH gradients between 4 and 7.5 on the time course of thiamine uptake by rat proximal small intestinal brush border membrane vesicles. Vesicles were preincubated (2 hr at 5°C and 30 min at 25°C) in media containing (mM): 280 D-mannitol; 2 $MgSO_4$; 20 Tris-Hepes, pH 7.5 (open symbols) or Mes-Tris, pH 5 (solid symbols). Ten microliters of preincubated vesicles were then incubated at 25°C with 90 μl of solutions containing: 1 μM [^{3}H]thiamine (specific activity, 27.75 GBq · mmol^{-1}); 100 mM NaCl; 80 mM D-mannitol; 2 mM $MgSO_4$; 20 mM Mes-Tris, pH 5 (triangles) or 20 mM Tris-Hepes, pH 7.5 (circles). Symbols represent means of triplicate determinations for each of five different preparations, each from 8–12 rats. SEMs were within 10% of the mean values.

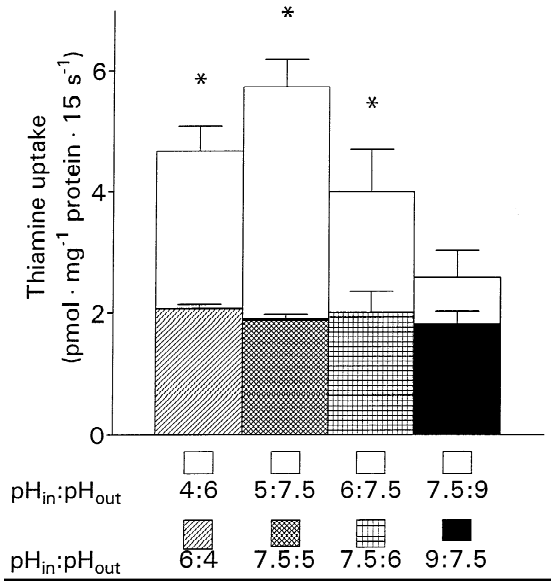


Fig. 2. Thiamine uptake by rat proximal small intestinal brush border membrane vesicles in the presence of an outwardly directed H^+ gradient (open bars) or inwardly directed H^+ gradient (filled bars). For experimental conditions, incubation media and number of experiments for each bar see Fig. 1. The pH of the incubation media was obtained with: 20 mM Tris-Hepes, pH 7.5 and 9; Mes-Tris, pH 5 and 6; Mes-Hepes, pH 4. Vertical lines above each bar represent SEM. *$P \leq 0.05$ *vs.* inwardly directed H^+ gradient (Student's *t* test).

= 7.5). At equilibrium (30 min), this difference in thiamine uptake disappeared (Fig. 1).

Transmembrane Electrical Potential

To differentiate between the effect of pH gradients and that of related changes in transmembrane electrical potential on thiamine transport, an electrical negative or positive potential was imposed across the BBMVs. To this purpose, the uptake of 1 μM [^{3}H]thiamine was measured at pH 7.5 (pH_{in} = pH_{out} = 7.5) in the presence of inorganic anions showing different permeabilities across the brush border membrane, the permeability of I^- being about 20-fold higher than that of Cl^- and K^+ (Wright & Wunz, 1987). No statistically significant differences in [^{3}H]thiamine uptake were observed over incubation times up to 2 min at different electrical transmembrane potentials (Fig. 3*A*).

The effectiveness of our procedure in generating a potential difference was shown by the enhancing effect of the I^- gradient as compared with the Cl^- gradient on the Na^+-dependent glucose uptake by BBMVs, a well recognized electrogenic transport (Fig. 3*B*).

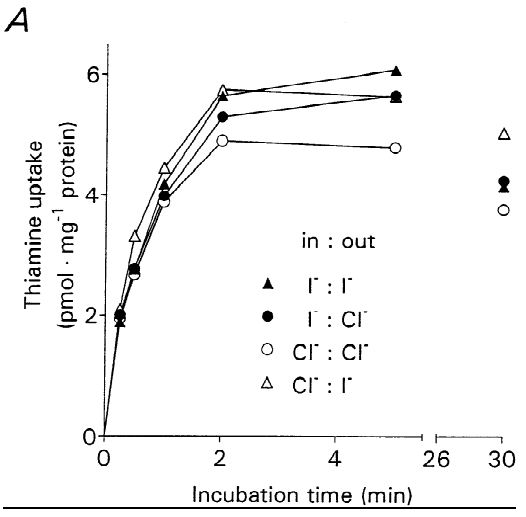
The effect of transmembrane electrical potential on thiamine transport was evaluated also at pH 5, where no binding of thiamine occurs (*see below*). Figure 4 shows no difference on thiamine uptake by making the vesicle lumen relatively negative or positive. A significant reduction ($P \leq 0.05$: ANOVA followed by Newman Keuls's *Q* test) of thiamine uptake at 1 and 2 min incubation was observed (in the absence of transmembrane electrical potential) only when iodide was present at both sides of the membrane.

Translocation and Binding at Different pHs

The effect of the pH of the incubation medium on thiamine membrane translocation and binding was evaluated by incubating the BBMVs at 25°C with 1 μM [^{3}H]thiamine in media at different pHs with increasing osmometrically (Fiske OM osmometer, Fiske Associates, Burlington, MA) controlled osmolarity.

When the values of equilibrium (20 min) vesicular uptake were plotted against the reciprocal of the osmolarities of the medium, different straight lines were obtained according to the pH of the incubation medium (Fig. 5*A*). From the values of the ordinate intercepts, the percentage of thiamine binding under iso-osmotic conditions (300 mOsmol · 1^{-1}) could be calculated (Fig. 5*B*).

The curve shown in Fig. 5*B* indicates that all thiamine taken up by BBMVs at pH 9 was membrane-bound, while all thiamine taken up at pH 4–5 was translocated



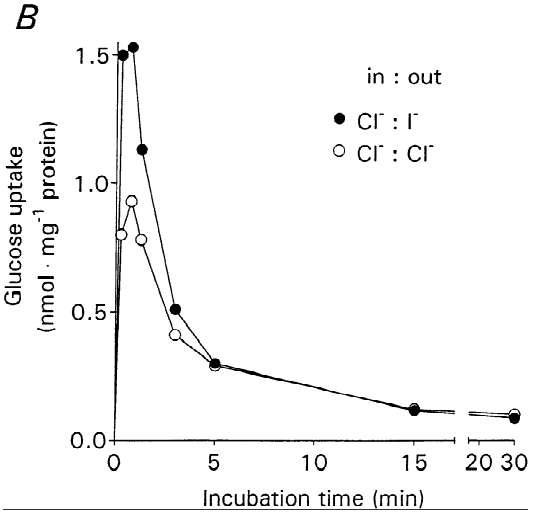


Fig. 3 (*A*) Effect of transmembrane potential on thiamine uptake by rat proximal small intestinal brush border membrane vesicles. Vesicles were preincubated as described in Fig. 1 in media containing (mM): 2 $MgSO_4$; 20 Tris-Hepes, pH 7.5; 140 KI (solid symbols) or 140 KCl (open symbols). Ten microliters of preincubated vesicles were incubated at 25°C with 90 μl of solutions containing: 1 μM [^{3}H]thiamine; 2 mM $MgSO_4$; 20 mM Tris-Hepes, pH 7.5; 140 mM KI (triangles) or 140 mM KCl (circles). Number of experiments for each symbol as in Fig. 1. SEMs were within 10% of the mean values. (*B*) Effect of transmembrane potential on glucose uptake by rat proximal small intestinal brush border membrane vesicles. Vesicles were preincubated as described in Fig. 1 in a medium containing (mM): 140 KCl; 2 $MgSO_4$; 20 Tris-Hepes, pH 7.5. Ten microliters of the vesicles were incubated at 25°C with 90 μl of solutions containing: 80 μM [U-^{14}C] D-glucose (specific activity, 0.31 GBq · mmol^{-1}); 2 $MgSO_4$; 20 Tris-Hepes, pH 7.5; 140 NaI (solid circles) or 140 NaCl (open circles). Number of experiments for each symbol as in Fig. 1. SEMs were within 10% of the mean values.

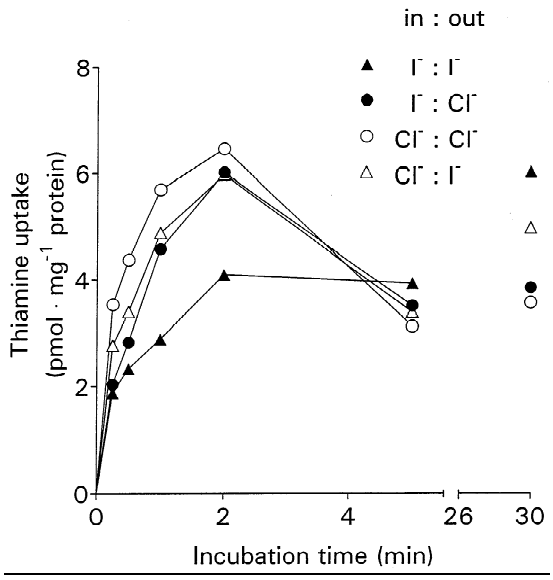


Fig. 4. Effect of transmembrane potential on thiamine uptake by rat proximal small intestinal brush border membrane vesicles. Vesicles were preincubated as described in Fig. 1 in media containing (mM): 2 $MgSO_4$; 20 Mes-Tris, pH 5; 140 KI (solid symbols) or 140 KCl (open symbols). Ten microliters of preincubated vesicles were incubated at 25°C with 90 μl of solutions containing: 1 μM [^{3}H]thiamine; 2 $MgSO_4$; 20 Mes-Tris, pH 5; 140 KI (triangles) or 140 KCl (circles). Number of experiments for each symbol as in Fig. 1. SEMs were within 10% of the mean values.

into the vesicular space without membrane binding. At intermediate pH values, the percentage of thiamine binding to the membrane increased from less than 10% at pH 5 to 99% at pH 9. Omeprazole, a gastric antisecretory organic cation (Fellenius et al., 1981), enhanced thiamine binding at pH 7.5 from 57% to 77% (Fig. 5*A*). As shown in Figs. 1 and 4, an apparent overshoot in thiamine uptake seems to occur also in the absence of pH gradient at pH 5, where no thiamine binding was observed (under equilibrium conditions). The overshoot disappeared when iodide substituted completely for chloride in both preloading and incubating media (Fig. 4). To assess the existence of a transient binding of thiamine to the membrane in the presence of chloride at $pH_{in} = pH_{out} = 5$, we determined at early incubation time (2 min: maximum of the overshoot) the extent of thiamine that was membrane-bound as above described. Under isoosmotic conditions the thiamine binding accounted for 58% ± 8 (mean ± SEM) of total uptake (Fig. 6).

Location of the Thiamine-binding Sites

Displacement experiments were carried out according to McNamara, Pepe & Segal (1981) to investigate the side orientation of the membrane binding. Ten μl of vesicles were equilibrated for 30 min at 25°C in 90 μl of a so-

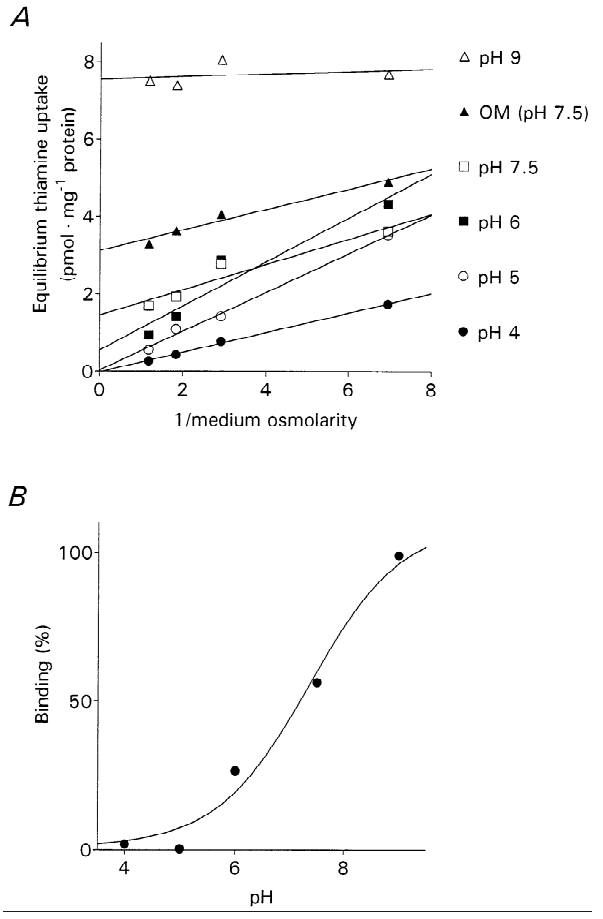


Fig. 5 (*A*) Effect of medium osmolarity and omeprazole (OM) on thiamine uptake by rat proximal small intestinal brush border membrane vesicles. Vesicles were preincubated as described in Fig. 1 in different media containing (mM): 100, 300, 500 or 800 D-mannitol; 2 $MgSO_4$; 20 buffer (Mes-Hepes, pH 4; Mes-Tris, pH 5 and 6; Tris-Hepes, pH 7.5, with and without 0.2 OM, and pH 9), and incubated for 20 min at 25°C in media containing 1 μM [^{3}H]thiamine; 20 mM of loading buffers; varying amounts of D-mannitol in order to yield the indicated osmolarity (mOsmol · l^{-1}) (given as its reciprocal). Lines were fitted by regression analysis (pH 4: $r = 0.999$, $P \leqslant 0.001$; pH 5: $r = 0.997$, $P \leqslant 0.003$; pH 6: $r = 0.962$, $P \leqslant 0.004$; pH 7.5: $r = 0.959$, $P \leqslant 0.041$; pH 7.5 with 0.2 mM omeprazole: $r = 0.983$, $P \leqslant 0.017$; pH 9: $r = 0.313$, $P \leqslant 0.687$). Number of experiments for each symbol as in Fig. 1. SEMs were within 10% of the mean values. (*B*) Effect of pH of incubation medium on thiamine binding to rat proximal small intestinal brush border membrane vesicles. Percent thiamine binding to the vesicles, under iso-osmotic conditions (300 mOsm), was calculated from the ordinate intercepts of the straight lines shown in Fig. 5*A*. The curve was obtained by fitting the points at pH values between 4 and 9 by computerized least-squares nonlinear regression (GraphPad, 1992).

lution containing: 1 μM [^{3}H]thiamine; 280 mM D-mannitol; 2 mM $MgSO_4$; 20 mM Tris-Hepes, pH 7.5. Subsequently, 5 μl of unlabeled thiamine (final concentration 50 μM) or the same volume of thiamine-free medium were added to the incubation mixture. After 6 sec the mixture was filtered as described above. After addition of unlabeled thiamine, 47% ± 2 of thiamine was displaced from vesicles. Presumably, this fraction represents the most mobile form of the vitamin. Since at pH 7.5 57% (Fig. 5) of thiamine was membrane-bound and 47% could be rapidly displaced, it can be assumed that 82% of thiamine binding occurs at the outer membrane surface.

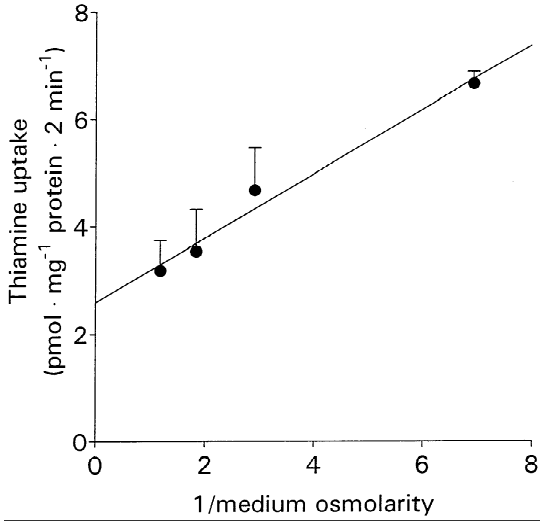


Fig. 6. Effect of medium osmolarity on thiamine uptake by rat proximal small intestinal brush border membrane vesicles. Vesicles were preincubated as described in Fig. 1 in different media containing: 100, 300, 500 or 800 mM D-mannitol; 2 mM $MgSO_4$; 20 mM Mes-Tris, pH 5, and then incubated for 2 min at 25°C in media containing: 1 μM [^{3}H]thiamine; 20 mM of Mes-Tris, pH 5; varying amounts of D-mannitol in order to yield the indicated osmolarity (mOsmol · l^{-1}) (given as its reciprocal). The fitting was calculated by regression analysis ($r = 0.9886$; $P \leqslant 0.011$). Number of experiments for each symbol as in Fig. 1.

pH Gradient and Thiamine Translocation and Binding

Since binding was influenced by the pH of the medium (Fig. 5*B*), in preliminary experiments the time course of thiamine translocation and binding in the presence of an initial outwardly directed H^+ gradient ($pH_{in}5$:$pH_{out}7.5$) was evaluated by using a procedure similar to that proposed by Bhandari, Joshi and McMartin (1988) for folate in kidney BBMVs. With this procedure [^{3}H]thiamine uptake was investigated in incubation media with increased omolarities in the presence of the above-mentioned gradient. For each incubation time, the binding component was calculated under iso-osmotic conditions from the ordinate intercepts of the straight lines resulting from the plots of the thiamine total uptake values against the reciprocal of medium osmolarity. Translocation represented the difference between total uptake and binding values. As shown in Fig. 7, for up to 15-sec incubation the proportion of bound thiamine was con-

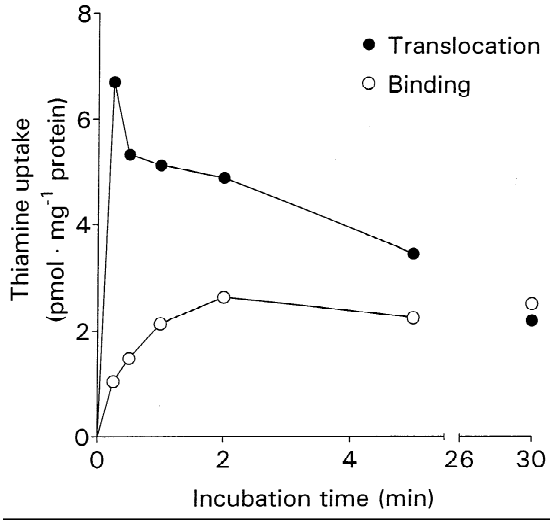


Fig. 7. Time course of thiamine binding and translocation by rat proximal small intestinal brush border membrane vesicles in the presence of a pH gradient $pH_{in}5$: $pH_{out}7.5$. Vesicles were preincubated as described in Fig. 1 in media containing (mM): 2 $MgSO_4$; 20 Mes-Tris, pH 5 with varying amounts of D-mannitol in order to yield different osmolarities (150, 550, 850 mOsmol l^{-1}), and then incubated at 25°C in media containing: 1 μM [^{3}H]thiamine; 2 mM $MgSO_4$; 20 mM Tris-Hepes, pH 7.5; and D-mannitol to yield the above-mentioned osmolarities. At each time, binding (open symbols) was determined as in Fig. 5*A* (*see also* Results); translocation (solid symbols) was the difference between total uptake, as calculated under isoosmotic conditions (*see* Fig. 5*A* and Results), and binding. Number of experiments for each symbol as in Fig. 1. SEMs were within 10% of the mean values.

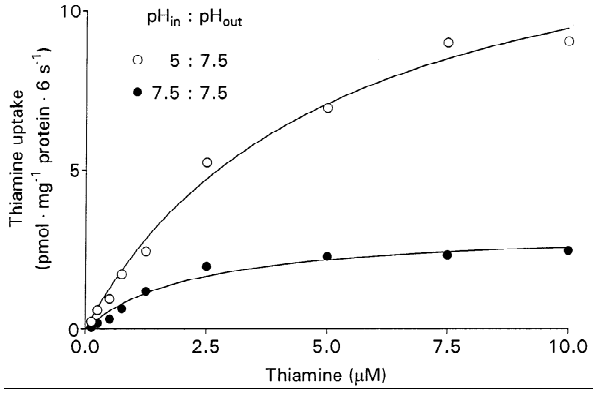


Fig. 8. Saturable component of thiamine uptake by rat proximal small intestinal brush border membrane vesicles in the presence of a pH gradient ($pH_{in}5$:$pH_{out}7.5$) (open symbols) and in the absence of a pH gradient (pH_{in} = pH_{out} = 7.5) (filled symbols). The plot was obtained graphically from total uptake curves. Thiamine uptake at 25°C was measured in vesicles preincubated as described in Fig. 1 after 6 sec incubation with different [^{3}H]thiamine concentrations; 280 mM D-mannitol; 2 mM $MgSO_4$; 20 mM Tris-Hepes, pH 7.5. Number of experiments for each symbol as in Fig. 1. SEMs were within 10% of the mean values.

stantly below 10–15% of the amount of thiamine taken up. Thus, at the short incubation times (6 sec) used for all subsequent measurements of transport, binding was virtually negligible in comparison with translocation. At longer incubation times, translocation rapidly decreased, while binding virtually reached a plateau. At the equilibrium (30 min incubation), both components were quantitatively similar.

pH Gradient and Kinetics of Thiamine Uptake

The influence of pH gradient on the kinetics of thiamine uptake was evaluated by incubating BBMVs at 25°C for 6 sec with different initial concentrations of [^{3}H]thiamine (i) in the presence of an initial outwardly directed H^+ gradient ($pH_{in}5$:$pH_{out}7.5$), and (ii) in the absence of a H^+ gradient (pH_{in} = pH_{out} = 7.5). Under both conditions a biphasic total uptake was observed which was nonlinear at low thiamine concentrations (≤2.5 μM) and linear at higher concentrations. No correction was introduced for binding, since this was virtually negligible (*see* Fig. 7). The best fit of the curves, calculated by computerized least-squares regression (GraphPad Inplot, GraphPad Software, San Diego, CA, 1992), could be resolved graphically into two components (Gastaldi et al., 1989): a linear component, expression of a nonsaturable mechanism, and a hyperbolic component, expression of a saturable mechanism displaying Michaelis-Menten-like kinetics (Fig. 8).

The apparent kinetic constants of the saturable component, calculated by computerized nonlinear regression (GraphPad Inplot, 1992) in the presence (i) and in the absence (ii) of the outwardly oriented H^+ gradient, were: Michaelis-Menten constant, K_m, (i) 6.2 ± 1.4 and (ii) 2.3 ± 0.8 μM; maximal flux, J_{max}, (i) 14.9 ± 3 and (ii) 3.2 ± 0.8 pmol · mg^{-1} protein · 6 sec^{-1}.

The values of the passive permeability coefficient K_D, calculated as the slope of the linear portion of the total uptake curves (*not reported*), were (i) 0.54 ± 0.2 and (ii) 0.49 ± 0.2 μl · mg^{-1} protein · 6 sec^{-1}. Only K_m and J_{max} values showed statistically significant differences ($P \leq 0.05$ and $P \leq 0.01$, respectively: Student's *t* test), being higher in the presence of an outwardly oriented H^+ gradient.

Peculiarities of the Thiamine/H^+ Antiport

pH Gradients and Thiamine Uptake into BBMVs: Stoichiometry of the Thiamine/H^+ Exchange

The stimulating effect of proton gradients on thiamine uptake (Figs. 1 and 2) was further investigated by incubating for 6 sec at 25°C BBMVs with an internal pH of 4 in media containing 1 μM [^{3}H]thiamine and showing a pH between 4 and 8. A sigmoid curve was obtained for

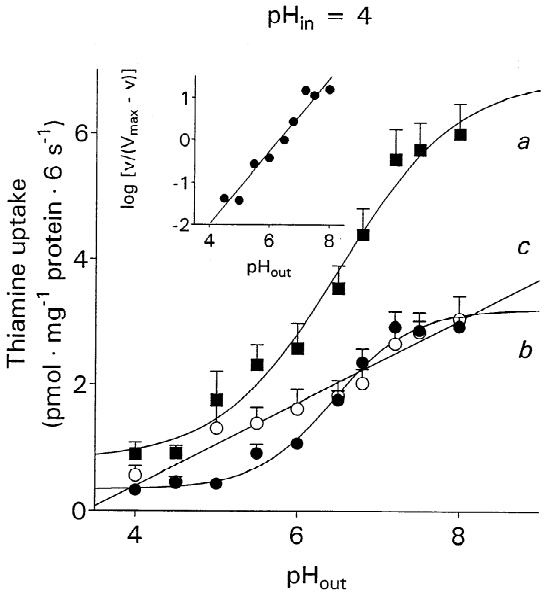


Fig. 9. Effect of extravesicular pH on thiamine uptake by rat proximal small intestinal brush border membrane vesicles. Vesicles were preincubated as described in Fig. 1 in a solution containing (mM): 280 D-mannitol; 2 $MgSO_4$; 20 Mes-Hepes, pH 4, and incubated at 25°C for 6 sec with 1 μM [^{3}H]thiamine in media containing (mM): 280 D-mannitol; 2 $MgSO_4$; 20 buffers (Mes-Hepes, pH 4 and 4.5; Mes-Tris, pH 5, 5.5 and 6; Hepes-Tris, pH 6.5, 6.8 and 7.2; Tris-Hepes, pH 7.5 and 8). The curves of total uptake (filled squares: *a*), saturable (active; filled circles: *b*) and nonsaturable (passive; unfilled circles: *c*) components, and the Hill coefficient (n_{app} = 0.9), calculated from the saturable component, were obtained by fitting the experimental points by computerized least-squares regression. The saturable component of thiamine transport was measured at 6 sec by subtracting the diffusional component (measured at 0°C) from the total uptake (measured at 25°C). Symbols represent means ± SEM of five determinations for each of five different preparations. The inset, which shows the Hill plot of the data after subtraction of the uptake values measured in the absence of a pH gradient (pH_{in} = pH_{out} = 4), can be fitted by a straight-line (r = 0.98; $P \leq 0.0001$) with a slope (Hill coefficient) of 0.85. v, uptake value at a given pH of the incubation medium; V_{max}, maximal uptake value.

the saturable component of the thiamine/H^+ antiport, maximal uptake being observed at pH 7.2–8 (Fig. 9). The curve of total uptake, its active and passive components as well as the Hill coefficient and the inhibition constant for H^+ (K_H) were obtained by fitting the experimental points by computerized least squares regression (GraphPad, Inplot, 1992) according to the Eq. (1):

$$Y = A + \frac{B - A}{1 + (10^C/10^X)^D} \quad (1)$$

where X represents the log [H^+]; A and B are the Y values corresponding to the bottom and the top of the plateau respectively; C is the X value at the middle of the curve, i.e., log (IC_{50}) (*see below*); and D is the Hill coefficient (slope factor).

From the sigmoid curve *b* shown in Fig. 9, the stoichiometry of thiamine transport with respect to internal H^+ could be estimated (Schron, 1990). The value of the Hill coefficient, calculated from the active component of thiamine transport (Fig. 9, curve *b*), was 0.9 ± 0.1 (mean ± SEM), indicating that in the pH range of 4–8 the minimum number of possible H^+ binding sites is one (Segel, 1975).

The value of the Hill coefficient was also calculated from the slope of the straight line obtained by plotting log $v/(V_{max\text{-}v})$ *vs.* the pH value of the incubation medium, where v is the uptake value at a given pH of the incubation medium, corrected for the uptake of thiamine in the absence of a pH gradient (pH_{in} = pH_{out} = 4), and V_{max} is the maximal uptake (Fig. 9: inset). V_{max} was calculated by computerized nonlinear regression as mentioned above, by plotting uptake values (corrected for uptake in the absence of a pH gradient) *vs.* the OH^- concentration in the incubation media. The resulting value (Hill coefficient) of 0.85 was similar to the value of 0.9 reported above. Therefore, since the stoichiometry of the thiamine: H^+ exchange was compatible with a 1:1 exchange ratio, thiamine transport was considered to be an electroneutral process (*see also* Fig. 3*A*).

By computerization (GraphPad, Inplot, 1992) the log (IC_{50}) value could be converted into a K_H value by using the Cheng and Prusoff equation (1973) (2):

$$K_H = \frac{IC_{50}}{1 + L/K_m} \quad (2)$$

where IC_{50} is the inhibitor concentration which causes 50% inhibition, L is the thiamine concentration and K_m is the Michaelis-Menten constant of thiamine saturable uptake. K_H values in the presence and in the absence of a pH gradient were virtually similar at 1.96 μM and 2.43 μM respectively.

Effect of Different Inorganic and Organic Cations

In the presence of a pH gradient 5_{in}:7.5_{out}, different cations were added to an incubation medium containing 1 μM [^{3}H]thiamine as reported in the legend of Fig. 8. Saturable thiamine transport was evaluated by subtracting the nonsaturable component measured at 0°C from the total transport measured at 25°C. Unlabeled thiamine and all thiamine analogues tested, except for thiochrome, inhibited the saturable component of the thiamine/H^+ antiport (Table 1). Transport was also inhibited by guanidine, its derivative phenformin (but not metformin) and some inhibitors of the intestinal guanidine/H^+ antiport, including imipramine, which was spe-

Table 1. Effect of some cations and thiamine analogues on the saturable component of the thiamine/H^+ antiport in rat proximal small intestinal brush border vesicles.

Cation	Thiamine transport (percent activity) Mean• ± SEM	Cation	Thiamine transport (percent activity) Mean• ± SEM
None (Control)	100	Organic II	
Inorganic (chloride salts)		Acetylcholine	93.3 ± 3.8
Na^+	103.7 ± 5.9	Histamine	89.4 ± 10.4
K^+	91.6 ± 11.4	Serotonin	63.6 ± 8.5
Li^+	90.4 ± 5.4	Spermidine	53.6* ± 16.3
NH_4^+	103.1 ± 11.1	Organic III	
Thiamine analogues		Guanidine	57.9* ± 10.5
4′-oxythiamine	49.0* ± 9.6	Amiloride	75.6 ± 11.8
Thiamine (unlabeled)	31.4* ± 4.8	Phenformin	37.3* ± 8.8
Amprolium	26.5* ± 2.4	Metformin	96.2 ± 7.0
Pyrithiamine	20.8* ± 6.9	Organic IV	
Thiochrome	70.3 ± 7.2	Harmaline	23.6* ± 7.2
Organic I		Clonidine	31.3* ± 6.7
Choline	92.4 ± 12.2	Imipramine	32.3* ± 2.9
Tetraethylammonium	98.2 ± 8.8	Organic V	
Creatinine	82.8 ± 8.3	Omeprazole	436.2* ± 84.2

[^{3}H]thiamine concentration, 1μM. Incubation time, 6 sec. H^+ gradient, $pH_{in}5$: $pH_{out}7.5$. For incubation media and experimental conditions see legend of Fig. 8. Cations and thiamine analogues were added to the incubation medium at an initial 0.1 mM concentration. Organic I, typical substrates of renal organic cation/H^+ antiport. Organic II, endogenous organic cations, inhibitors of the intestinal guanidine/H^+ antiport. Organic III, guanidine and derivatives. Organic IV, inhibitors of intestinal guanidine/H^+ antiport. Organic V, inhibitor of gastric (H^+-K^+)-ATPase. •, Mean of at least triplicate determinations for each of five different preparations, each from 8–12 rats. *, $p \leqslant 0.05$ *vs.* controls before transformation of data as percent activity.

cifically investigated by determining the K_i constant of the thiamine/H^+ antiport following Dixon (1953).

The uptake of 0.5 and 1 μM [^{3}H]thiamine (*see* Fig. 8 legend for incubation medium) was determined at 6 sec in the presence of varying amounts of imipramine (25, 50, 75 and 100 μM). Imipramine inhibition was both competitive and noncompetitive (K_i value of 0.5 mM), being the point of intersection of the two straight lines (obtained by plotting 1/*J vs.* inhibitor concentrations) below the *X* axis. Interestingly, 1 mM imipramine inhibited both the saturable (77 ± 3.9% inhibition, mean of 5 different experiments) and the nonsaturable (90 ± 6.6% inhibition, mean of 5 different experiments) components of the thiamine/H^+ antiport. All inorganic cations tested, at an initial 0.1 mM concentration, failed to inhibit thiamine uptake. It is noteworthy that omeprazole was the sole compound which enhanced (approximately four times) the saturable component of the thiamine transport.

Since most organic cations that inhibit thiamine uptake are weak bases, the possibility that they act indirectly by dissipating the H^+ gradient should be considered. Therefore, separate experiments were conducted to evaluate the effect of two of them, imipramine and harmaline, on thiamine uptake under equilibrium exchange conditions. The uptake of 1 μM [^{3}H]thiamine (*see* Fig. 8 legend for incubation medium) was determined at 6 sec under equilibrium conditions (unlabeled thiamine in = out = 1 mM in the absence of pH gradient, $pH_{in} = pH_{out} = 7.5$) in the presence of 0.1 mM imipramine or harmaline. Imipramine and harmaline inhibited significantly ($P \leqslant 0.05$) the exchange process by about 41% and 48% respectively (controls: 1.07 ± 0.34; imipramine: 0.63 ± 0.25; harmaline: 0.56 ± 0.2 pmol · mg^{-1} protein · 6 sec^{-1}; means of five different experiments ± SEM). This result supports the hypothesis of a direct interaction of the inhibitors with the thiamine transporter.

Guanidine/H^+ and Thiamine/H^+ Antiports

The relationship between the saturable components of the thiamine/H^+ and guanidine/H^+ antiports was investigated at a gradient $pH_{in}5$:$pH_{out}7.5$ by using three different approaches: (i) evaluation of the potency of unlabeled thiamine and its analogues 4′-oxythiamine, amprolium and pyrithiamine in inhibiting the guanidine/H^+ antiport; (ii) determination of the inhibition constant (K_i) of guanidine for the thiamine/H^+ antiport and (iii) determination of the K_i of thiamine for the guanidine/H^+ antiport.

The relative inhibiting potencies of unlabeled thiamine and its analogues for the guanidine/H^+ antiport were: pyrithiamine > thiamine > 4′-oxythiamine > amprolium (Fig. 10).

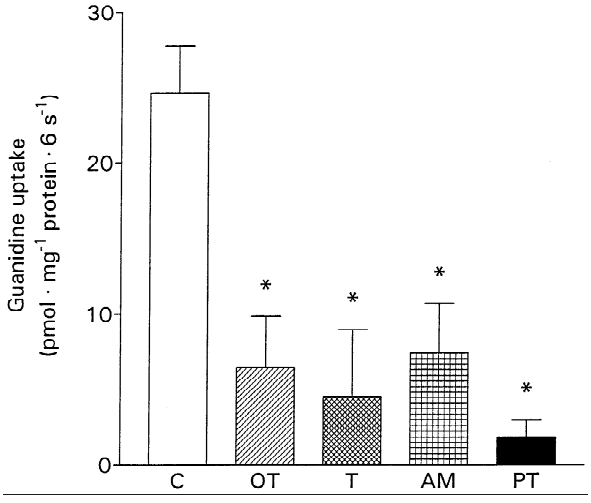


Fig. 10. Potency of thiamine and its analogues 4′-oxythiamine, amprolium and pyrithiamine in inhibiting saturable guanidine transport in rat proximal small intestinal brush border membrane vesicles in the presence of a gradient $pH_{in}5$:$pH_{out}7.5$. For incubation media and experimental conditions see Fig. 8. Thiamine and analogues were added to the incubation medium at an initial concentration 10 times as high as that (50 μM) of [U-^{14}C]guanidine (specific activity, 1.02 GBq · mmol^{-1}). The saturable component of guanidine transport was measured at 6 sec by subtracting the diffusional component (measured at 0°C) from the total uptake (measured at 25°C). Bars represent means ± SEM of five replicate determinations for each of five different preparations. C, controls; OT, 4′-oxythiamine; T, unlabeled thiamine; AM, amprolium; PT, pyrithiamine. *, $P \leq 0.05$ *vs.* OT, T, AM and PT (ANOVA followed by Newman Keuls's *Q* test).

The inhibition of the thiamine/H^+ antiport by guanidine and the inhibition of the guanidine/H^+ antiport by thiamine were determined by measuring the uptake at 6 sec of [^{3}H]thiamine (0.5 and 1 μM) (*see* Fig. 8 legend for incubation medium) in the presence of an increasing amount of unlabeled guanidine (1, 5, 7.5 and 10 mM) and the uptake of [U-^{14}C]guanidine (25 and 50 μM) in the presence of an increasing amount of unlabeled thiamine (0.5, 2, 3.5 and 5 mM) (Dixon, 1953). Guanidine and thiamine inhibited competitively (intersection of the straight lines above the X axis) the uptake of labeled thiamine and guanidine respectively, and had similar K_i values (10 and 7.8 mM, respectively).

Discussion

The functional efficiency of BBMV preparations, based on the time course of D-glucose uptake (*see* Fig. 3*B*), and their purity, based on the enrichment of sucrase activity, were similar to those reported in the literature and considered suitable for transport studies. The presence of a H^+ gradient across the vesicle membrane enhanced [^{3}H]thiamine uptake only when the direction of the gradient was from inside to outside ($pH_{in} < pH_{out}$) (Figs. 1 and 2). The transport rate increased with increasing gradient (Fig. 2), suggesting that the entry of thiamine into BBMVs (and hence into the enterocyte) is associated with a countertransport of H^+ (thiamine/H^+ antiport). In line with this evidence, Moseley et al. (1992) recently showed that thiamine transport in the liver is dependent on a sinusoidal membrane antiportal H^+ exchange mechanism distinct from the cation/H^+ and Na^+/H^+ antiports.

An unexpected overshoot in thiamine uptake was observed also in the absence of a pH gradient ($pH_{in} = pH_{out} = 5$). This overshoot, which was determined by a transient binding of thiamine (about 60% increment at 2-min incubation; Fig. 6) to the membranes, disappeared when iodide was present in both the vesicle suspending medium and the incubation medium (Fig. 4).

These observations, which presently are not easy to understand, deserve further studies to be fully explained.

Although thiamine exists as a monovalent cation at pH 7.5 (Komai & Shindo, 1974), its uptake was not influenced by changes in membrane electrical potential (Fig. 3*A*), suggesting that thiamine transport by BBMVs is an electroneutral process, as previously reported for intestinal intact tissue (Hoyumpa et al., 1975), basolateral membrane vesicles (Laforenza, Gastaldi & Rindi, 1993), and erythrocytes and ghosts (Casirola et al., 1990). Electroneutral H^+/organic cation antiport systems have been demonstrated previously in intestinal (Miyamoto et al., 1988) and renal BBMVs (Wright & Wunz, 1987; Ott et al., 1991) from different animal species, including man. Both thiamine binding to BBMVs and translocation were greatly influenced by external pH. At equilibrium (20 min incubation), within a pH range of 4–6, [^{3}H]thiamine was fully translocated into the vesicular space.

Conversely, at pH values higher than 9, at which thiamine is undissociated and the thiazole ring is open (Komai & Shindo, 1974), [^{3}H]thiamine was entirely bound to the vesicular membrane (Fig. 5*A* and *B*). At intermediate pH values thiamine exists as a monovalent cation, and its translocation rate and membrane binding showed an inverse and direct relationship, respectively, with the pH value. However, in the presence of a pH gradient 5_{in}:7.5_{out}, the quantitative relationship between translocation and binding depended on incubation time (Fig. 7). With short incubation times, under conditions that approximate initial velocity (pH gradient intact), the binding component was virtually negligible and translocation accounted for about 90% of total uptake. With long incubation times, under conditions that approximate equilibrium (pH gradient dissipated), about 55% of the thiamine taken up was membrane-bound (Fig. 7), an extent similar to that reported in Fig. 5*A*, pH 7.5. More-

over, separate experiments showed that most of [^{3}H]thiamine bound to membrane vesicles can be easily displaced by unlabeled thiamine, suggesting that thiamine binding occurs at the outer side of the vesicles (McNamara, Pepe & Segal, 1981).

The transport of thiamine through the thiamine/H^+ antiport ($pH_{in} < pH_{out}$) showed a well defined saturable component whose kinetic constants were much higher than those recorded in the absence of H^+ exchange (Fig. 8). In particular, a pH gradient of 2.5 units ($pH_{in}5$:pH_{out} 7.5) increased thiamine maximal flux (J_{max}) about 5 times and reduced the affinity value (as indicated by an increase in K_m) about 3 times. Since passive permeability was virtually unaffected (as indicated by unaltered K_D coefficients), the conclusion can be drawn that the H^+ gradient energized thiamine transport. Interestingly, an outwardly directed H^+ gradient has been found to increase the J_{max} of Na^+ (Gunther & Wright, 1983) and tetraethylammonium (Wright & Wunz, 1987) in BBMVs from rabbit jejunum and kidney by the same order of magnitude as that found in our study for thiamine. In the above models, however, K_m values were not affected by the H^+ gradient.

Altogether, our early (Casirola et al., 1988) and present findings indicate that the saturable component of thiamine transport into BBMVs is a Na^+-independent uphill process (Fig. 1), which can utilize the energy supplied by a H^+ gradient (Fig. 8), and involves a 1:1 stoichiometry exchange of thiamine with H^+ (Fig. 9).

The competitive inhibition between H^+ and thiamine indicates that H^+ has an affinity for binding sites similar to that of thiamine either in the presence (K_H 2 μM; K_m 6.2 μM) or in the absence (K_H 2.4 μM; K_m 2.3 μM) of a pH gradient. On the basis of the differences in sensitivity to inorganic monovalent cations and substrate specificity (Table 1), the thiamine/H^+ antiport in BBMVs appeared to be distinct from the Na^+/H^+ antiport (only harmaline and clonidine inhibited significantly both systems) and from the renal organic cation/H^+ antiport (Gunther & Wright, 1983). The thiamine/H^+ antiport, however, appears to share some substrate specificity with the intestinal guanidine/H^+ antiport (Miyamoto et al., 1988) (Table 1). In fact, thiamine was able to inhibit competitively the guanidine/H^+ exchange (K_i = 7.8 mM), while guanidine could inhibit the thiamine/H^+ exchange (K_i = 10 mM), suggesting some similarity of the two H^+ antiport systems. Some thiamine analogues could also inhibit the guanidine/H^+ antiport (Fig. 10). Of the two antidiabetic guanidine derivatives (biguanides), phenformin and metformin, only phenformin inhibited markedly the intestinal thiamine/H^+ antiport (Table 1) and thiamine absorption. These differential effects of the two guanidines might contribute to explain why phenformin (but not metformin) can produce lactic acidosis (Assan, Heuclin & Girard, 1987), a syndrome frequently related to thiamine deficiency (Campbell, 1984). Imipramine, a potent inhibitor of guanidine/H^+ exchange (Miyamoto et al., 1988), also inhibited markedly the saturable component of the thiamine/H^+ antiport (K_i = 0.5 mM). However, imipramine also inhibited the nonsaturable component (*see* Results) of the thiamine/H^+ exchange, suggesting a possible alteration in membrane structure as reported for bacterial membranes (Tanji et al., 1992). With respect to the organic cations which were found to inhibit the thiamine/H^+ antiport, their action appears to involve a direct interaction with the thiamine transporter and not merely dissipation of the proton gradient.

Omeprazole, a substituted benzimidazole and weak organic cation which blocks gastric acid secretion by inhibiting membrane (H^+-K^+)ATPase (Fellenius et al., 1981), is structurally similar to the tricyclic form of thiamine (Brown, 1990). Therefore, it has been speculated that these two compounds could compete for a common membrane binding site (Brown, 1990).

In our experiments, however, omeprazole caused a 4-fold increase in the rate of thiamine entry into BBMVs (Table 1), a finding which seems to exclude a competitive inhibitory action on thiamine uptake. Moreover, omeprazole increased by 30% thiamine binding to BBMVs at pH 7.5. The effects of omeprazole could be due to its ability to reduce free sulfhydryl groups at both pH 5 and 7.4, as shown in isolated hog gastric membrane (Im et al., 1985), thus altering the functional properties of the membranes and their interaction with thiamine. If this occurred also in intestinal BBMVs, conceivably the alteration could enhance the activity of the thiamine/H^+ antiport as well as thiamine binding. However, further experiments are required to clarify fully the effects of omeprazole, even though we can exclude any direct reaction of this compound with thiamine (Laforenza & Rindi, *unpublished observations*). All thiamine analogues, except for thiochrome, were strong inhibitors of the intestinal thiamine/H^+ antiport. This substrate specificity differs from that of the hepatic thiamine/H^+ antiport, which is unaffected by pyrithiamine and amprolium, and is inhibited by choline and tetraethylammonium (Moseley et al., 1992), to which, on the other hand, the intestinal thiamine/H^+ antiport was insensitive (Table 1).

Assuming that Aronson's calculations for the Na^+-H^+ exchanger (Aronson, 1985) can be applied to our case, the thiamine/H^+ antiport mechanism allows both the intestinal absorption and the secretion of thiamine, depending on whether the value of the ratio $[thiamine]_{out}$:$[thiamine]_{in}$ is higher or lower than that of the ratio $[H^+]_{out}$:$[H^+]_{in}$.

Currently the thiamine ratio is higher and intestinal absorption can take place. When thiamine ratio becomes lower, as may happen during very high amounts of vi-

tamin are ingested, thiamine may also be secreted into the lumen as Polin et al. (1961) showed in the chick.

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