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Uptake of Cimetidine into Syncytial Microvillus Membrane Vesicles of Human Term Placenta^{1,2}

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Accepted for publication September 22, 1995

ABSTRACT

Uptake of the H₂-receptor antagonist, cimetidine, into syncytial microvillus membrane vesicles of human term placenta was investigated to clarify whether an active transport mechanism can be responsible for the observed barrier of the human placenta for cimetidine. Imposition of an outwardly directed H⁺-gradient stimulated cimetidine uptake, resulting in a small transient overshoot. The H⁺-gradient-dependent peak uptake was decreased under voltage-clamped conditions by carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone, suggesting the presence of an organic cation-proton exchange mechanism. Uptake was partially, but significantly, inhibited by organic ca-

tion transport inhibitors, H₂-receptor antagonists and several other cationic drugs, providing further evidence for mediated uptake. H⁺-gradient-dependent cimetidine uptake was saturable and characterized by a low-affinity (K_m) of 6.3 mM and V_{max} of 17.5 nmol/mg protein/10 sec. We conclude that the system cannot play an important role in the barrier function of the human placenta in the transport of cimetidine. Rather than active transport, other factors, as for instance the degree of ionization of cimetidine at physiological pH, seem to be a more likely explanation for the low clearance of cimetidine across the human placenta.

H₂-receptor antagonists, like cimetidine and ranitidine, are frequently used therapeutic agents in the treatment of peptic ulcer in humans. Additionally, cimetidine is sometimes used in the prevention of gastric acid aspiration for women at labor under general anesthesia (McGowan, 1979).

The guanidine analogs, ranitidine (Mihaly *et al.*, 1982) and cimetidine (Mihaly *et al.*, 1983) cross the placenta in pregnant ewes at near term. Fetal plasma concentrations, however, were much lower than maternal plasma concentrations after maternal dosage. This transplacental gradient was reported to be due to the placenta itself (*e.g.*, active transport from fetus to mother, irreversible placental metabolic elimination), because fetal renal elimination did not play an important role (Mihaly *et al.*, 1983). In a follow-up study the transplacental gradient could not be explained by a low placental permeability or differential plasma protein binding of cimetidine between mother and fetus (Ching *et al.*, 1985).

Cimetidine, administered to women at labor, showed a

slow placental transfer finally achieving equal concentrations in maternal and fetal plasma. Protein binding or placental metabolism was of minor influence. Because the concentration of cimetidine in the amniotic fluid increased together with a decreasing concentration in the cord blood, a role for fetal renal elimination seemed likely (Howe *et al.*, 1981). However, because the fetal kidney begins to excrete waste products approximately 5 months after conception, the placenta could play a role in the elimination of cationic drugs before 5 months.

In the perfused human placental cotyledon, the clearance of cimetidine was low, providing evidence for a barrier function of the placenta in cimetidine transfer (Ching *et al.*, 1987). Because fetal and maternal plasma concentrations did equilibrate, active transport appeared to play an insignificant role in maintaining a barrier across the human placenta. The absence of mediated transport was confirmed by Schenker *et al.* (1987) by use of the same experimental technique. The maternal to fetal transfer of cimetidine showed no signs of accumulation, saturability or susceptibility to inhibition by structural analogs. In this study, no evidence was found also for the saturation of cimetidine uptake in microvillus membrane vesicles of human term placenta. However,

Received for publication October 20, 1994.

¹ These investigations were supported by the Netherlands Organization for Scientific Research (NWO).

² Parts of this paper were presented at the 5th Meeting of the European Placenta Group, Manchester, UK, 8-11 September 1993. Abstract: Placenta 14, A79, 1993.

ABBREVIATIONS: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; M₀, starting mince; NMN, *n*-methylnicotinamide; pH_i, pH inside the vesicle; pH_o, pH outside the vesicle; SMMV, syncytial microvillus membrane vesicles; TEA, tetraethylammonium; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; val, valinomycin.

concentrations of up to only 400 μM were used and no driving forces were applied.

More precise information concerning the driving forces, specificity and saturability of transport could provide better insight into the underlying mechanism of the low clearance for cimetidine across the human placenta. The present study was designed to investigate in more detail the molecular mechanisms of cimetidine transport across the human term placenta by using isolated SMMV.

Materials and Methods

Vesicle preparation. Human placental SMMV were prepared according to a modification of the procedure described by Glazier *et al.* (1988) as method number three. Briefly, tissue obtained within 15 min after uncomplicated delivery was minced in a Waring blender and stirred for 30 min to loosen the microvilli. After MgCl_2 aggregation and differential centrifugation, both steps repeated once, SMMV were harvested and suspended in an appropriate buffer. The protein concentration of SMMV used in this study was 10.4 ± 2.2 mg/ml ($n = 23$). All subsequent steps were performed at 4°C . Vesicles were frozen in $\text{N}_2(\text{l})$ and stored at -80°C for 4 weeks at the maximum. Protein was assayed with a Coomassie blue kit (Biorad, München, Germany). Alkaline phosphatase enrichment of SMMV, measured according to Mircheff and Wright (1976), was 22-fold as compared with starting mince (M_0 : 60 ± 20 , SMMV: 1350 ± 570 $\mu\text{mol/h/mg}$, $n = 14$).

Uptake studies. Uptake of [^3H]cimetidine into SMMV was measured in quadruplicate at 37°C with a rapid filtration technique (Russel *et al.*, 1988). Uptake was started by addition of 10 μl membrane suspension to 40 μl extravascular medium containing radiolabeled compound. The transport conditions are given in the legends. At appropriate time intervals, the reaction was terminated by adding 2 ml of ice-cold stop solution with the same composition as the intravesicular medium, except for membrane binding studies in which the stop solutions were equal to the corresponding extravascular media. The samples were filtered under vacuum through a Whatman GF/F glass fiber filter and washed 3-fold. Radioactivity remaining at the filter was counted in a Beckman LS 6000 LL liquid scintillation counter. Corrections were made for nonspecific filter binding.

Data analysis. Data are presented as means \pm S.D., with n representing the number of experiments with different vesicle preparations. Statistical significance of differences in cimetidine uptake was determined with the use of two-tailed, paired Student's t -test ($P < .05$). Curve fitting was done by least squares nonlinear regression analysis with the computer program PCNONLIN (Metzler and Weiner, 1989).

Chemicals. [^3H]-L-alanine (77 Ci/mmol) was obtained from New England Nuclear (Dreieich, Germany) and [^3H]cimetidine (21 Ci/mmol) from Amersham (Aylesbury, U.K.). Cimetidine was generously donated by Smith, Kline & French Laboratories (Herts, U.K.), nizatidine by Eli Lilly & Company (Indianapolis, IN) and trimethoprim by Bergel (the Netherlands). Mepiperphenidol and famotidine were generously donated by Merck, Sharpe & Dohme (Rahway, NY). NMN and TEA were purchased from Janssen Chimica (Beerse, Belgium). All other chemicals were purchased from either Sigma (St Louis, MO), Merck (Darmstadt, Germany) or Boehringer (Mannheim, Germany). GF/F filters were obtained from Whatman Int. Ltd (Maidstone, U.K.).

Results

H^+ -gradient-dependent uptake of cimetidine. The outwardly directed proton gradient ($\text{pH}_i = 6.0/\text{pH}_o = 7.4$) stimulated cimetidine ($20 \mu\text{M}$) uptake into SMMV, resulting in a small, but significant, transient overshoot (peak *vs.*

equilibrium = 1.3) as compared with uptake in the absence of a H^+ -gradient ($\text{pH}_i = \text{pH}_o = 7.4$) (fig. 1). The difference in equilibrium uptake at 60 min is probably due to a pH-dependent increase in vesicle volume, but this should have minor effects on initial uptake values.

Effect of ionophores on H^+ -gradient uptake. To determine whether the stimulated uptake of cimetidine in the presence of a proton gradient was due to the activity of an organic cation-proton antiport system or a H^+ -diffusion potential, the effect of the proton ionophore FCCP was evaluated. FCCP causes an enhanced H^+ -flux down its concentration gradient, which lowers the availability of H^+ for a possible cation-proton exchanger and consequently decreases the uptake of the cation. The FCCP-induced H^+ -flux also renders the inside of the vesicle more negative resulting in an increase of uptake of the cation if uptake is dependent on a H^+ -diffusion potential. As shown in table 1, FCCP did not increase the H^+ -gradient-dependent initial and peak uptake rates. These results show that an inside negative H^+ -diffusion potential is unlikely to be a driving force for cimetidine uptake. However, in voltage-clamped vesicles (equal amounts of potassium at both sides of the membrane in presence of the K^+ -ionophore valinomycin), in which peak and equilibrium uptakes were not significantly altered, FCCP was able to decrease cimetidine uptake, resulting in a diminished overshoot. The decreased uptake suggests an organic cation-proton exchanger responsible for the small transient overshoot.

Determination of membrane binding. Figure 2 shows that the uptake of $20 \mu\text{M}$ cimetidine was inversely related to medium osmolarity ($R^2 > .81$ of individual regression lines), indicating transport into an osmotically responsive intravesicular space. Because of the large variation in the uptake data between different placentas, only a rough estimate could be made of the actual intravesicular uptake, which accounted for $77 \pm 27\%$ of total uptake.

Effect of organic cations. The inhibitory effect of various organic cations on cimetidine uptake was investigated. Table 2 shows the initial H^+ -gradient-dependent uptake of $20 \mu\text{M}$ [^3H]cimetidine as percentage of representative control uptake in the presence of 1 or 5 mM inhibitor. The prototypic

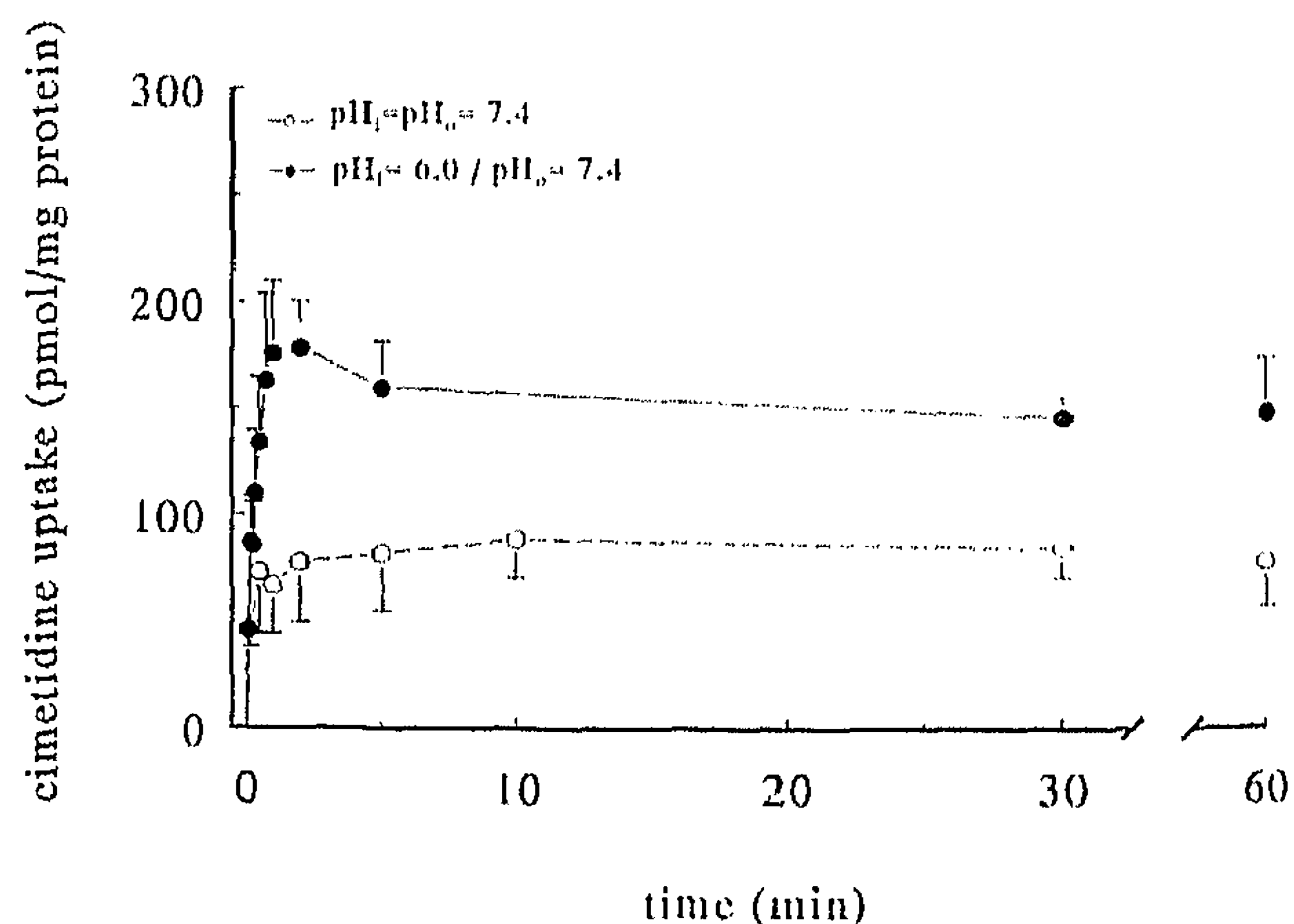


Fig. 1. Time-dependent uptake of $20 \mu\text{M}$ [^3H]cimetidine into SMMV in the presence or absence of an outwardly directed H^+ -gradient. Vesicles were suspended in 300 mM mannitol and 10 mM Mes-Tris, $\text{pH} = 6.0$ or 10 mM HEPES-Tris, $\text{pH} = 7.4$. Extravesicular medium consisted of 300 mM mannitol and 10 mM HEPES-Tris, $\text{pH} = 7.4$. Values are expressed as picomoles per milligram protein ($n = 11$).

TABLE 1

Effect of H⁺-gradients and ionophores on cimetidine uptake

Uptake of 20 μM [³H]cimetidine into vesicles suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH = 7.4, or 10 mM Mes-Tris, pH = 6.0 (A) or 100 mM mannitol, 100 mM KCl and 10 mM Mes-Tris, pH = 6.0 (B). Extravesicular media consisted of 300 mM mannitol and 10 mM Hepes-Tris, pH = 7.4 (A) or 100 mM mannitol, 100 mM KCl and 10 mM Hepes-Tris, pH = 7.4 (B). Vesicles were preequilibrated for 60 min at 37°C with 20 μM valinomycin (val) and/or 40 μM FCCP. FCCP (40 μM) was also added to the extravesicular medium. Data are presented as pmol/mg protein ($n = 3$).

Condition	Uptake		
	10 sec	60 sec	60 min
A. $\text{pH}_i = 7.4/\text{pH}_o = 7.4$	47 \pm 9	67 \pm 23	78 \pm 21
$\text{pH}_i = 6.0/\text{pH}_o = 7.4$	87 \pm 9	176 \pm 34	148 \pm 26
$\text{pH}_i = 6.0/\text{pH}_o = 7.4 + \text{FCCP}$	84 \pm 16	172 \pm 36	154 \pm 18
B. $\text{pH}_i = 6.0/\text{pH}_o = 7.4 + \text{val} + \text{FCCP}$	57 \pm 12	108 \pm 34	130 \pm 35

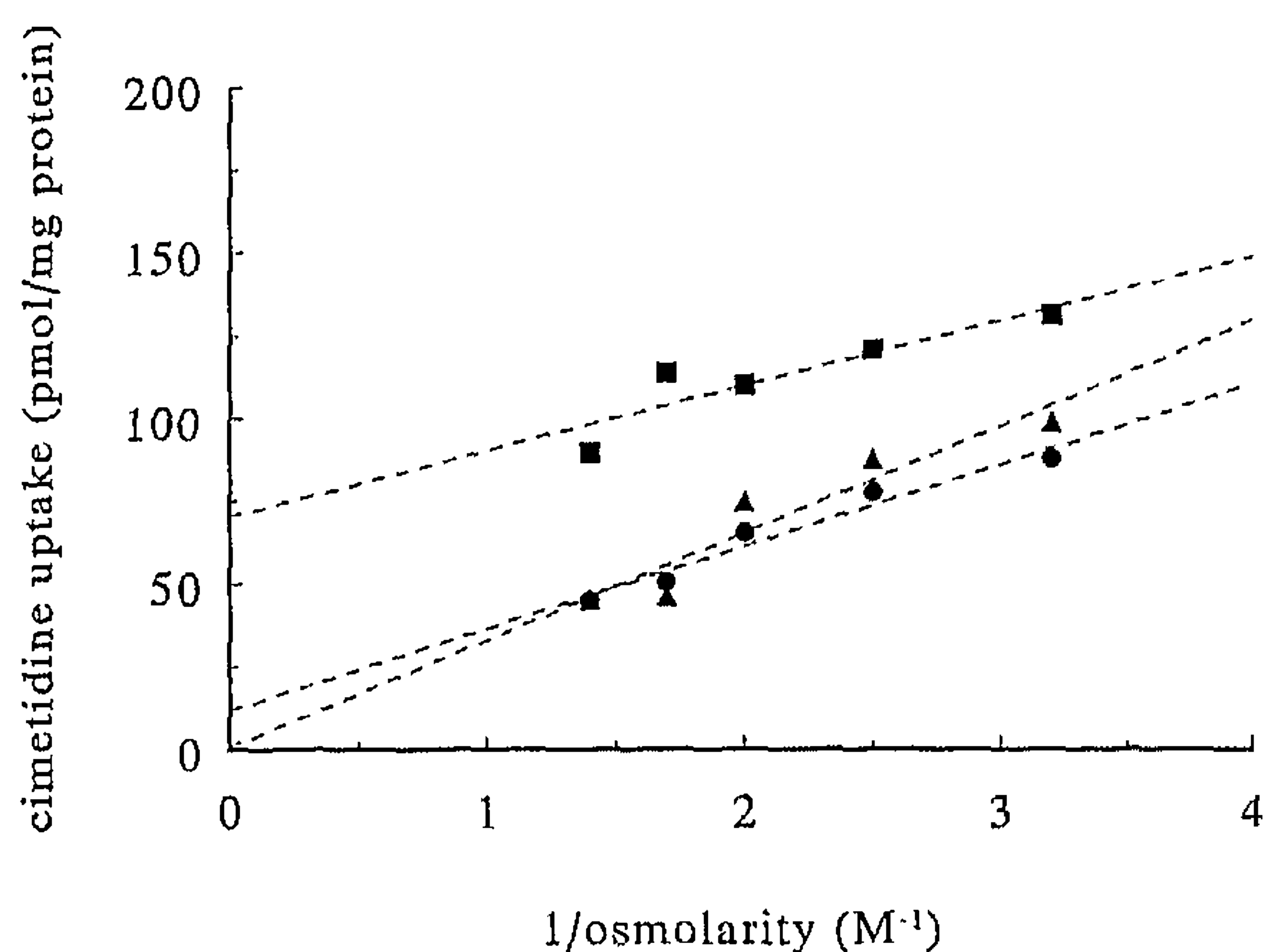


Fig. 2. Effect of increasing osmotic pressure on uptake at 30 min of 20 μM [³H]cimetidine into SMMV. Vesicles were suspended in 300 mM mannitol and 10 mM Hepes-Tris, pH = 7.4. Extravesicular media consisted of 10 mM Hepes-Tris, pH = 7.4, and increasing concentrations of mannitol. Values of three placentas are expressed individually as picomoles per milligram protein versus reciprocal osmolarity of the final concentration.

TABLE 2

Effect of various organic cations on cimetidine uptake

Effect of various organic cations (1 or 5 mM) on the 30-sec uptake of 20 μM [³H]cimetidine in the presence of an outwardly directed H⁺-gradient. Experimental conditions were similar to those given in the legend of figure 1. Uptakes are expressed relative to their own control uptakes, namely, without inhibitor at the same time and from the same placenta ($n = 4$). Mean control uptake was 112 \pm 30 pmol/mg protein ($n = 10$).

Organic Cation	mM	% Uptake
Control		100
Mepiperphenidol	1	55 \pm 19*
Amiloride	1	85 \pm 2
	5	76 \pm 15*
Trimethoprim	1	54 \pm 20*
Ranitidine	1	74 \pm 13*
Famotidine	1	69 \pm 12*
Nizatidine	1	67 \pm 14*
TEA	1	71 \pm 10*
NMN	1	74 \pm 16*
Guanidine	1	100 \pm 2
Choline	1	102 \pm 5

* $P < .05$.

organic cation transport inhibitors mepiperphenidol and amiloride and several other cationic compounds, like trimethoprim, TEA and the H₂-receptor antagonists ranitidine,

nizatidine and famotidine were able to inhibit cimetidine uptake partially, but significantly. The endogenous organic cation, NMN, decreased cimetidine uptake also, whereas choline and guanidine did not.

Kinetics of cimetidine uptake. The presence of mediated uptake was further investigated by evaluating the initial uptake of increasing concentrations of cimetidine in the presence of an outwardly directed H⁺-gradient. The relation between cimetidine concentration and uptake rate was nonlinear. Total uptake corrected for estimated nonsaturable uptake showed saturability as can be seen in figure 3. Kinetic parameters were determined by fitting an equation combining Michaelis-Menten and linear kinetics $v = V_{\text{max}} \cdot S / (K_m + S) + k \cdot S$ to the data, where v is the initial cimetidine uptake rate and S is the cimetidine concentration. Nonlinear regression analysis revealed a K_m of 6.3 \pm 4.4 mM, a V_{max} of 17.5 \pm 10.2 nmol/mg/10 sec and a k of 1.6 \pm 0.3 nmol/mg/10 sec/mM.

Discussion

In human *in vivo* studies and in the artificially perfused human placental cotyledon, no evidence was found for mediated transport of cimetidine (Howe *et al.*, 1981; Ching *et al.*, 1987). It was concluded that transfer of cimetidine across the human placental trophoblast occurs by passive diffusion. However, if diffusional transfer quantitatively exceeds uptake *via* a mediated pathway, the latter will be masked and not detected in these type of studies. Therefore, isolated membrane vesicles are a more appropriate tool to investigate whether mediated transport occurs across the plasma membrane.

Our results in human placental SMMV show that cimetidine transfer across the syncytial microvillus membrane of the human term placenta will be mainly due to passive diffusion, confirming the results obtained by others (Howe *et al.*, 1981, Ching *et al.*, 1987, Schenker *et al.*, 1987). However, a small, but significant part of total cimetidine uptake into SMMV could be attributed to mediated uptake, because uptake was pH-dependent, inhibitable by several organic cations and saturable. Because the proton ionophore, FCCP, decreased H⁺-gradient-dependent cimetidine peak uptake

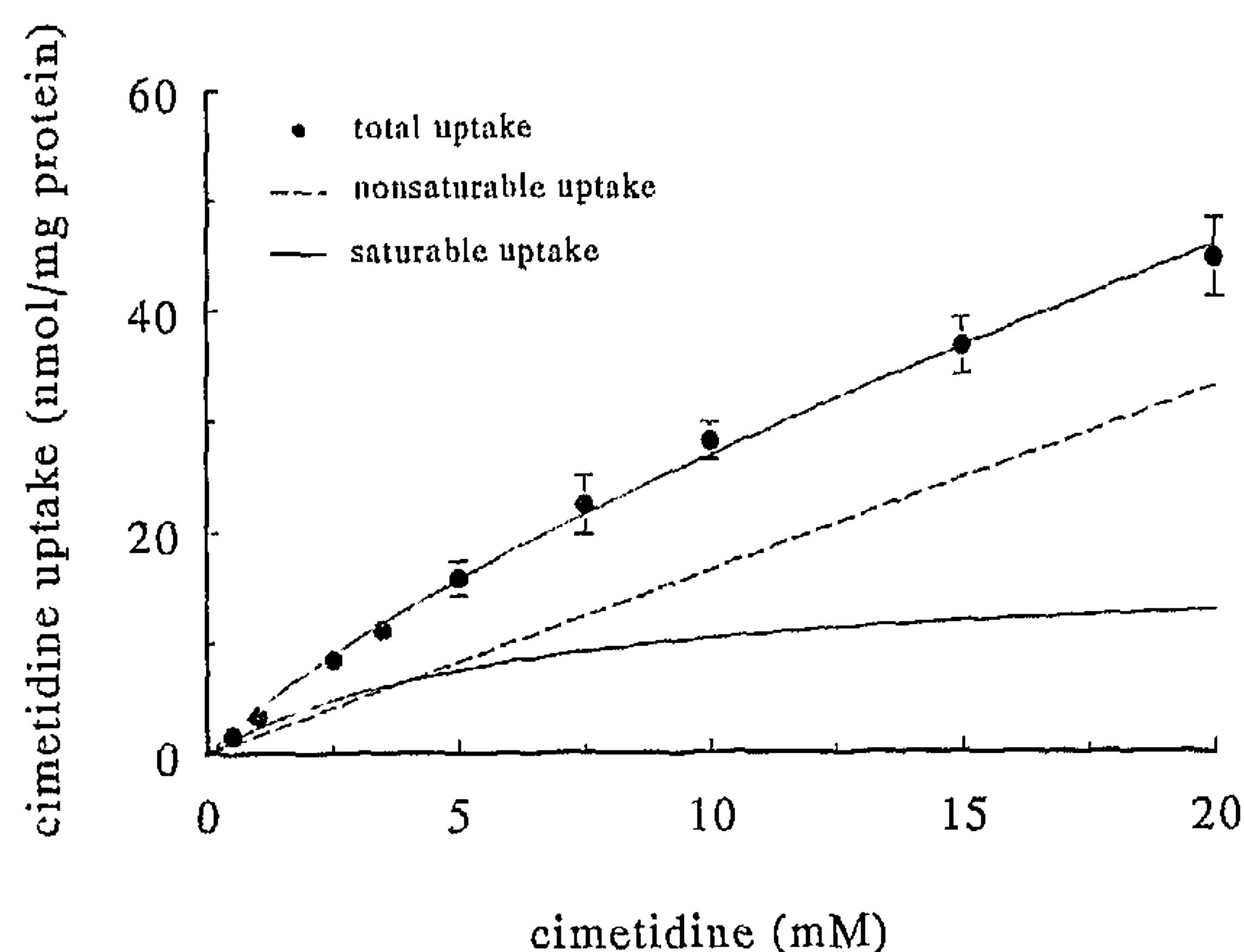


Fig. 3. Concentration-dependent uptake of [³H]cimetidine at 10 sec into SMMV in the presence of an outwardly directed H⁺-gradient. Experimental conditions were the same as described in the legend of figure 1. Cimetidine concentrations ranged from 10 μM to 20 mM. Values are expressed as nanomoles per milligram protein ($n = 3$).

under voltage-clamped conditions, we suggest the presence of an organic cation-proton antiport system for cimetidine in human placental SMMV, responsible for the small part of mediated uptake. Cimetidine itself has been reported to interfere with the human placental Na^+/H^+ -exchanger present in the syncytial microvillus membrane, in a reversible manner and competitive with respect to Na^+ (Ganapathy *et al.*, 1986). It seems possible that cimetidine, in the presence of a H^+ -gradient, is transported by this Na^+/H^+ -exchanger. The inhibition of cimetidine uptake we observed in presence of the Na^+/H^+ -exchange inhibitor amiloride would support such a view. However, this cationic drug is not a selective inhibitor, because it also inhibits organic cation transport in human placental and rabbit renal cortical brush-border membrane vesicles (Ganapathy *et al.*, 1988; Miyamoto *et al.*, 1989). The inhibition of guanidine uptake into renal cortical brush-border membrane vesicles by cimetidine (Miyamoto *et al.*, 1989) has not been found in human placental brush-border membrane vesicles (Ganapathy *et al.*, 1988). Because guanidine did not inhibit cimetidine uptake significantly in our experiments, the guanidine analog cimetidine is not likely to be transported by the guanidine-proton antiport system present in human placental SMMV (Ganapathy *et al.*, 1988). Cimetidine did inhibit membrane potential-dependent choline uptake into SMMV (van der Aa *et al.*, 1994). In contrast, choline was not able to inhibit cimetidine uptake significantly in the present study. Therefore it is also unlikely that cimetidine is transported by the choline carrier.

The affinity of the system is low, as can be concluded from a K_m of 6.3 mM characterizing the concentration-dependent uptake of cimetidine. The clinical relevance of this low-affinity system with respect to the use of H_2 -receptor antagonists during pregnancy seems therefore limited in terms of a possible accumulation into the trophoblast or extensive elimination from fetal to maternal circulation. Such a low-affinity transport system in microvillus membranes would only make sense in combination with a high-affinity system in basal membranes. From *in vivo* and perfused cotyledon experiments no evidence is available for the presence of such a system (Howe *et al.*, 1981; Schenker *et al.*, 1987).

We therefore conclude that the system cannot be an important factor in the barrier function of the human placenta in the transport of cimetidine as observed by Ching *et al.* (1987). Thus, active transport can indeed be ruled out in maintaining such a barrier. Other factors, as for instance the degree of ionization of cimetidine ($\text{pK}_a = 6.8$) at physiological pH, are probably of more importance to the low clearance of cimetidine across the human placental trophoblast.

Acknowledgments. We gratefully acknowledge the Department of Gynaecology and Obstetrics, University Hospital St Radboud, Nijmegen for their assistance in obtaining placentae.

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