

# Effect of pH on L- and D-methionine uptake across the apical membrane of Caco-2 cells

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**Abstract.** The transport systems involved in intestinal methionine (Met) absorption are described as Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent mechanisms. However, since recent studies have suggested the importance of the H<sup>+</sup> gradient as a driving force for intestinal nutrient absorption, the aim of the present work was to test whether Met transport across the apical membrane of Caco-2 cells is affected by extracellular pH. The results show that L- and D-Met uptake was increased by lowering extracellular pH from 7.4 to 5.5, in both the presence and absence of Na<sup>+</sup>. *Cis*-inhibition experiments revealed that inhibition of L-Met transport by 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH) or L-lysine (L-Lys) was higher at a pH of 5.5. Moreover, the BCH-insensitive component was not affected by pH, whereas the L-Lys-insensitive component was increased by lowering extracellular pH, thus suggesting the participation of system L. The contribution of another mechanism, sensitive to both BCH and L-Lys, was also considered. The inhibition obtained with taurine (Tau) was also higher at a pH of 5.5, thus suggesting the involvement of system B<sup>0,+</sup> on pH-stimulated component. As for D-Met uptake, the results showed higher inhibition with L-Lys and Tau at a pH of 5.5 and no effect on the L-Lys- or Tau-insensitive component. In conclusion, Met transport across the apical membrane of Caco-2 cells is increased by low extracellular pH as the result of the stimulation of two transport systems functionally identified with systems L and B<sup>0,+</sup> for L-Met and with system B<sup>0,+</sup> for D-Met.

L-METHIONINE (L-Met) is a dipolar amino acid of nutritional value. It is essential for mammals and has been shown to be limiting in poultry nutrition (12). Multiple transport mechanisms are involved in L-Met intestinal absorption: the Na<sup>+</sup>-dependent transport systems B<sup>0</sup> and B<sup>0,+</sup>, the Na<sup>+</sup>-independent mechanisms b<sup>0,+</sup> and L, and system y<sup>+</sup>, which is specific for cationic amino acids but is also able to transport neutral amino acids, including L-Met, in the presence of Na<sup>+</sup> (7, 21, 24, 26, 38, 40, 42, 46, 52). Whereas systems B<sup>0,+</sup>, b<sup>0,+</sup>, and y<sup>+</sup> are used by cationic amino acids, systems B<sup>0</sup> and L are specific for neutral amino acids (29). There are few references about the mechanisms involved in D-Met transport. The available information suggests the participation of at least systems B<sup>0</sup> and B<sup>0,+</sup> (17, 21, 54). The Na<sup>+</sup> electrochemical gradient is recognized as the primary driving force for nutrient and solute transport across the plasma membrane of most mammalian cells. However, recent studies have suggested that H<sup>+</sup> solute symport may be a characteristic of a number of mammalian transporters involved in ion-coupled transport of small peptides, monocarboxylic acids, monosaccharides, and amino acids (10, 15, 16, 23, 45). The proton amino acid transporter 1 (PAT1), the only H<sup>+</sup>-dependent transport system for amino acids described until now, recognizes not only short-chain  $\alpha$ -amino acids,  $\alpha$ -amino acids, and  $\beta$ -amino acids (3, 8) but also osmolytes such as betaine, sarcosine, and taurine (Tau), in addition to some D-amino acids [D-alanine, D-proline (D-Pro), and D-serine] (8). Results reported in this article show that both L- and D-Met transport are stimulated by lowering extracellular pH. For this reason and given that PAT1 has been reported to be unable to recognize L-Met (44), the aim of the present work was to identify the transport mechanisms involved in pH-stimulated Met transport in Caco-2 cell apical membrane.

## MATERIALS AND METHODS

**Materials.** Dulbecco's modified Eagle's medium (DMEM), trypsin, penicillin, and streptomycin were supplied by GIBCO (Paisley, Scotland). Nonessential amino acids, fetal bovine serum (FBS), bovine serum albumin (BSA), sterile phosphate-buffered saline (PBS), D-glucose, D-Pro, Tau, L-lysine (L-Lys), L- and D-Met, 2-aminobicyclo [2,2,1]heptane-2-carboxylic acid (BCH), N-ethylmaleimide (NEM), betaine, HEPES, MES, BCECF, and choline chloride (ChoCl) were supplied by Sigma (St. Louis, MO). Tissue culture supplies, including Transwells and clusters, were obtained from Costar (Cambridge, MA). L-[1-<sup>14</sup>C]Met (specific activity 55 mCi/mmol), D-[1-<sup>14</sup>C]Met (specific activity 55 mCi/mmol), and [1,2-<sup>14</sup>C]Tau (specific activity 110 mCi/mmol) were purchased from ARC (St. Louis, MO). Filtron-X was supplied by National Diagnostics (Atlanta, GA).

**Cell culture.** Caco-2 cells were kindly provided by Dr. David Thwaites (School of Cell and Molecular Biosciences, University of Newcastle upon Tyne, UK) and were cultured as previously described (23). The cells (*passages 112–121*) were routinely grown in plastic flasks at a density of  $5 \times 10^4$  cells/cm<sup>2</sup> and cultured in DMEM containing 4.5 g/l D-glucose and 2 mmol/l L-glutamine, supplemented with 1% (vol/vol) nonessential amino acids, 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in a modified atmosphere of 5% CO<sub>2</sub> in air. Cells grown to ~80% confluence were released by trypsinization and subcultured at a density of  $4 \times 10^5$  cells/cm<sup>2</sup> onto polycarbonate filters with a pore size of 0.4  $\mu$ m (Transwells; 12-mm diameter). The medium was replaced every 3 days and on the day before the experiment. Experiments were performed in cultures 19–21 days after seeding. The integrity of the cell layer was evaluated by measurement of transepithelial electrical resistance (TER) with Millicell-ERS equipment (Millipore, MA). Only cell monolayers with TER values 350  $\Omega$ cm<sup>2</sup> were used for transport experiments.

**Uptake experiments.** Transport experiments were performed as previously described (23). After TER determination, monolayers grown on filters were incubated for 5 min at 37°C in modified Krebs buffer (composition in mmol/l: 137 NaCl, 5.4 KCl, 2.8 CaCl<sub>2</sub>, 1.0 MgSO<sub>4</sub>, 0.3 NaH<sub>2</sub>PO<sub>4</sub>, 0.3 KH<sub>2</sub>PO<sub>4</sub>, 10 D-glucose, and 10 HEPES/Tris; pH 7.4 or 8.5) containing 0.2 μCi/ml of the <sup>14</sup>C-labeled substrate and 100 μmol/l of unlabeled substrate. For the experiments performed at pH 5.5 and 6.5, HEPES was replaced by MES. In some experiments, apical and basolateral NaCl was replaced by an equimolar concentration of ChoCl, and NaH<sub>2</sub>PO<sub>4</sub> was omitted. Monolayers incubated in the absence of Na<sup>+</sup> were washed in modified Krebs buffer with ChoCl instead of NaCl and NaH<sub>2</sub>PO<sub>4</sub>. After incubation, the filters were removed from the insert, dissolved in 4 ml of scintillation cocktail (Filtron-X), and counted in a Packard 1500 Tri-Carb counter (Downers Grove, IL).

**Protein assay.** Monolayers were washed in PBS and incubated overnight with 750 μl of 0.5 mol/l NaOH. The protein concentration was determined in the homogenate by the Bradford method using the Bio-Rad protein assay kit with BSA as the standard.

**Intracellular pH measurement.** Intracellular pH (pH<sub>i</sub>) was measured in Caco-2 cells grown on filters using the pH-sensitive fluorescent dye BCECF as previously described (23). After TER determination, cell monolayers were preloaded from both the apical and basolateral side, with 7.5 μmol/l BCECF (modified Krebs buffer, pH 7.4) for 40 min at 37°C. The preloaded monolayers were then washed in modified Krebs buffer (pH 7.4), placed in clusters, and incubated at an apical pH of 5.5 or 7.4 in the absence or presence of 20 mmol/l unlabeled betaine, L-Met, or D-Met. In monolayers incubated in the absence of Na<sup>+</sup>, apical and basolateral NaCl was replaced by an equimolar concentration of ChoCl, and NaH<sub>2</sub>PO<sub>4</sub> was omitted. The ratio of fluorescence of the intracellular accumulated BCECF was determined with excitation at wavelengths of 440/485 nm and emission at 535 nm using a luminescence spectrometer (Wallac 1420 Victor<sup>3</sup>; Perkin-Elmer, Boston, MA). To measure pH<sub>i</sub>, the BCECF excitation fluorescence ratios were calibrated using the K<sup>+</sup>-nigericin method, as previously described (43). The calibration curve demonstrated that the fluorescence ratios were a linear function of pH<sub>i</sub> from 6.0 to 8.0 ( $R^2 = 0.947$ ).

**Statistical analysis.** Results are means ± SE. All data were compared by one-way ANOVA followed by Student's *t*-test using SPSS software (SPSS, Chicago, IL). In all cases, *P* < 0.05 was considered to denote significance.

## RESULTS

L- and D-Met uptake (Fig. 1, A and B, respectively) were determined in the presence or absence of an imposed H<sup>+</sup> gradient (apical pH 5.5 or 7.4; basolateral pH 7.4) in both the absence and presence of Na<sup>+</sup> (ChoCl and NaCl, respectively) in the apical compartment. As expected, both L- and D-Met uptake were enhanced in the presence of the Na<sup>+</sup> gradient, and interestingly, the values were also higher in the presence of an imposed H<sup>+</sup> gradient in both Na<sup>+</sup> conditions. To further characterize this pH effect, we tested two additional pH conditions (apical pH 6.5 or 8.5; basolateral pH 7.4) for L-Met transport in the presence of Na<sup>+</sup>. The results show (Fig. 1, *inset*) no statistical differences between pH 6.5 vs. 5.5 and pH 8.5 vs. 7.4.

To confirm the lack of PAT1 involvement in L-Met transport, as previously suggested by Thwaites and Anderson (44), we performed *cis*-inhibition experiments with specific sub-

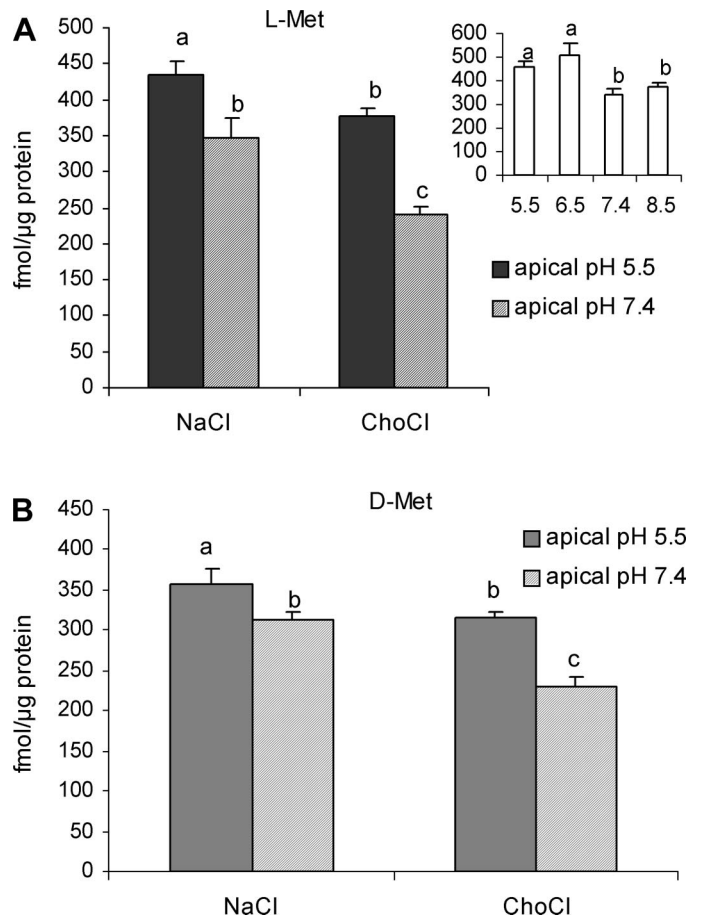


Fig. 1. L- and D-Methionine (Met) transport (A and B, respectively) across Caco-2 cell apical membrane at pH 5.5 and 7.4 in both the presence and absence of Na<sup>+</sup>. Monolayers were incubated for 5 min at 37°C in the presence of 100 μmol/l L- or D-Met and equimolar concentrations of NaCl or choline chloride (ChoCl) in the apical compartment. Two additional apical pH conditions were tested for L-Met transport in the presence of Na<sup>+</sup> (*inset*). Values are means ± SE; *n* = 6. <sup>a,b,c</sup>*P* < 0.05, mean values labeled with different letters are significantly different.

strates of this transport system that are not recognized by the other mechanisms that participate in Met uptake. The results show that 20 mmol/l D-Pro or betaine did not affect either L- or D-Met transport (Fig. 2). Moreover, if a H<sup>+</sup>-dependent transport system participated in Met uptake, a decrease in pH<sub>i</sub> would be observed during the incubation with the substrate, as is the case with betaine, a PAT1 substrate (see Fig. 3, *inset*). However, the results of pH<sub>i</sub> determination, shown in Fig. 3A, indicate that incubation with L- or D-Met in the apical compartment did not affect this variable, thus suggesting that Met transport is not coupled to H<sup>+</sup> influx. Similar results were obtained in the absence of Na<sup>+</sup> (Fig. 3B).

The possible stimulation of Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent amino acid transport systems by acidic pH was tested in *cis*-inhibition experiments performed on the basis of substrate specificity. BCH was used as substrate of systems B<sup>0,+</sup>, B<sup>0,+</sup>, and L (9, 41, 49), and L-Lys was used as substrate of systems B<sup>0,+</sup>, y<sup>+</sup>, and b<sup>0,+</sup>. In an attempt to investigate the participation of system B<sup>0,+</sup>, Tau was used as a possible inhibitor of the L-Lys- and BCH-sensitive component. To confirm the interaction of this β-amino acid with system B<sup>0,+</sup>,

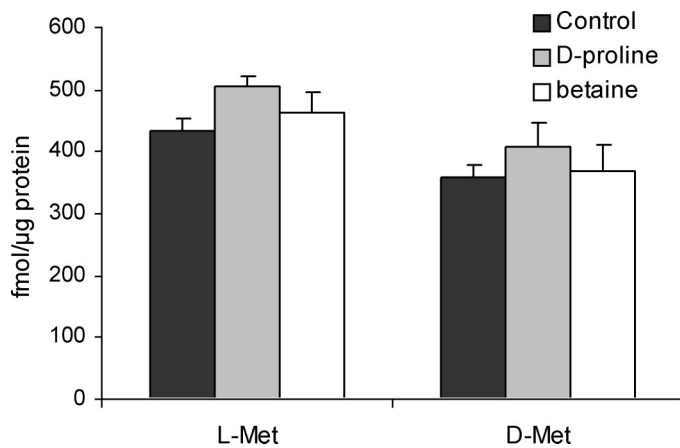


Fig. 2. *Cis*-inhibition of L- and D-Met transport by proton amino acid transporter 1 (PAT1) substrates. Monolayers were incubated at pH 5.5 in the presence of Na<sup>+</sup> and 20 mmol/l D-Pro or betaine. Values are means  $\pm$  SE;  $n = 3$ –6. Statistical analysis shows no significant differences within L- and D-Met results ( $P \geq 0.05$ ).

we tested the effect of BCH on Tau transport, and the results show a significant inhibition (control,  $1,417.0 \pm 28.9$  fmol/ $\mu$ g protein; BCH,  $920.3 \pm 43.3$  fmol/ $\mu$ g protein;  $n = 3$ ,  $P < 0.05$ ). Moreover, the participation of system  $y^+$  in L-Met transport in the absence of Na<sup>+</sup> was tested with NEM, a specific inhibitor of this transport system (11), and the results obtained show a significant inhibition (control,  $226.6 \pm 7.9$  fmol/ $\mu$ g protein; NEM,  $134.3 \pm 5.3$  fmol/ $\mu$ g protein;  $n = 10$  and 3, respectively,  $P < 0.05$ ), thus suggesting the participation of this mechanism.

In the absence of Na<sup>+</sup>, the inhibition of L-Met uptake by BCH was higher at a pH of 5.5 (Fig. 4A) and the BCH-insensitive component was not affected by pH. Regarding the effects of L-Lys, inhibition exerted by this amino acid and the remaining L-Lys-insensitive component were increased by lowering apical pH. *Cis*-inhibition experiments with Tau showed no effect at a pH of 7.4, whereas a significant inhibition was observed in the presence of an imposed H<sup>+</sup> gradient.

In the presence of Na<sup>+</sup> (Fig. 4B), the inhibition with both BCH and L-Lys was higher at a pH of 5.5. The BCH-insensitive component was not affected by lowering pH, whereas the remaining L-Lys-insensitive component was increased by lowering apical pH. Tau reduced L-Met transport in both pH conditions, and the results show that the Tau-insensitive component was higher at a pH of 5.5.

D-Met *cis*-inhibition experiments with L-Lys and Tau (Fig. 5), performed in the presence of Na<sup>+</sup>, showed results similar to those for L-Met uptake, although the L-Lys- and Tau-insensitive components were not affected by extracellular pH.

## DISCUSSION

The first hypothesis about the stimulation of L- and D-Met transport in the presence of a H<sup>+</sup> gradient considered the contribution of the PAT1 system. However, *cis*-inhibition experiments with specific substrates and the results concerning pH<sub>i</sub> indicate that this transport mechanism is not involved. These results are consistent with those of Thwaites and Anderson (44), who excluded L-Met recognition by PAT1. The second hypothesis considered the activation of the Na<sup>+</sup>-dependent and Na<sup>+</sup>-independent transport systems that are already known to

mediate L- and D-Met uptake in the intestinal epithelium. In the present study, we considered the possible participation of systems B<sup>0</sup>, B<sup>0,+</sup>,  $y^+$ , b<sup>0,+</sup>, and L, all of which are expressed in Caco-2 cells (7, 14, 28, 32, 46, 51).

System B<sup>0</sup> transports neutral amino acids, including the amino acid analog BCH, and has broad substrate selectivity (41). System B<sup>0,+</sup> recognizes cationic and neutral amino acids and a wide range of substrates, including BCH and  $\beta$ -amino acids (49) and various D-amino acids (17). Although no concluding information is available about the interaction of Tau with system B<sup>0,+</sup> (previously named  $\beta$ -alanine carrier), the inhibition of Tau transport obtained with BCH led us to consider this  $\beta$ -amino acid as a good substrate to confirm the participation of this transport system. System  $y^+$  has been described as a cationic amino acid transporter and is also able to bind neutral amino acids in the presence of Na<sup>+</sup>, but with a lower affinity. Although the expression of system  $y^+$  in epithelial cells has often been restricted to the basolateral membrane (6, 20), Thwaites et al. (46) reported the participation of this transport mechanism in L-Lys transport across the apical membrane of Caco-2 cells. In addition, we have previously found a Na<sup>+</sup>-independent L-Met component sensitive to NEM, a specific inhibitor of system  $y^+$  (11), in chicken brush-border membrane vesicles (40). Those results not only confirmed  $y^+$  activity in the apical membrane but also indicated the capacity of this mechanism to transport L-Met in the absence of Na<sup>+</sup>. In this sense, Rojas and Devés (37) later described the capacity of K<sup>+</sup> and Li<sup>+</sup> to replace Na<sup>+</sup>. In the experiments performed in the chicken intestine, Na<sup>+</sup> was replaced by K<sup>+</sup>, but in the experiments reported in the present study, in Caco-2 cells, Na<sup>+</sup> was replaced by choline. In the present study, the inhibition obtained with NEM confirms the participation of system  $y^+$  on L-Met uptake in the absence of Na<sup>+</sup>.

System b<sup>0,+</sup> is a tertiary transport system that exchanges extracellular cationic amino acids and L-cystine for intracellular neutral amino acids in a Na<sup>+</sup>-independent way (30), with a higher binding affinity for the extracellular substrate (48). Finally, system L is a Na<sup>+</sup>-independent transporter that preferentially recognizes large neutral amino acids with branched or aromatic side chains, as well as BCH (9). On the basis of kinetic characteristics, two subtypes have been described, L-type amino acid transporter 1 (LAT1) and LAT2, with LAT1 having a higher affinity than LAT2 (50). Both isoforms are expressed in Caco-2 cells (13), but some authors report the restriction of their expression to the basolateral membrane in the intestinal epithelium (50). Nevertheless, a Na<sup>+</sup>-independent transport component for neutral amino acids sensitive to BCH has been detected in the apical membrane of chicken, pig, and rabbit intestine (22, 40, 42). Moreover, Berger et al. (1) reported that apical Na<sup>+</sup>-independent L-phenylalanine uptake was mediated by systems b<sup>0,+</sup> and L in Caco-2 cells. Therefore, the results showing the inhibition of L-Met uptake by BCH in the absence of Na<sup>+</sup> may confirm L activity across the apical membrane.

*Cis*-inhibition experiments revealed that the inhibition of L-Met transport by BCH was higher at a pH of 5.5, in both the presence and absence of Na<sup>+</sup>, thus suggesting that the stimulated component is sensitive to the amino acid analog. Therefore, the transport mechanisms involved could be systems B<sup>0,+</sup>, B<sup>0</sup>, or L. Moreover, the fact that the BCH-insensitive component, which may be mediated by systems b<sup>0,+</sup> and  $y^+$  in

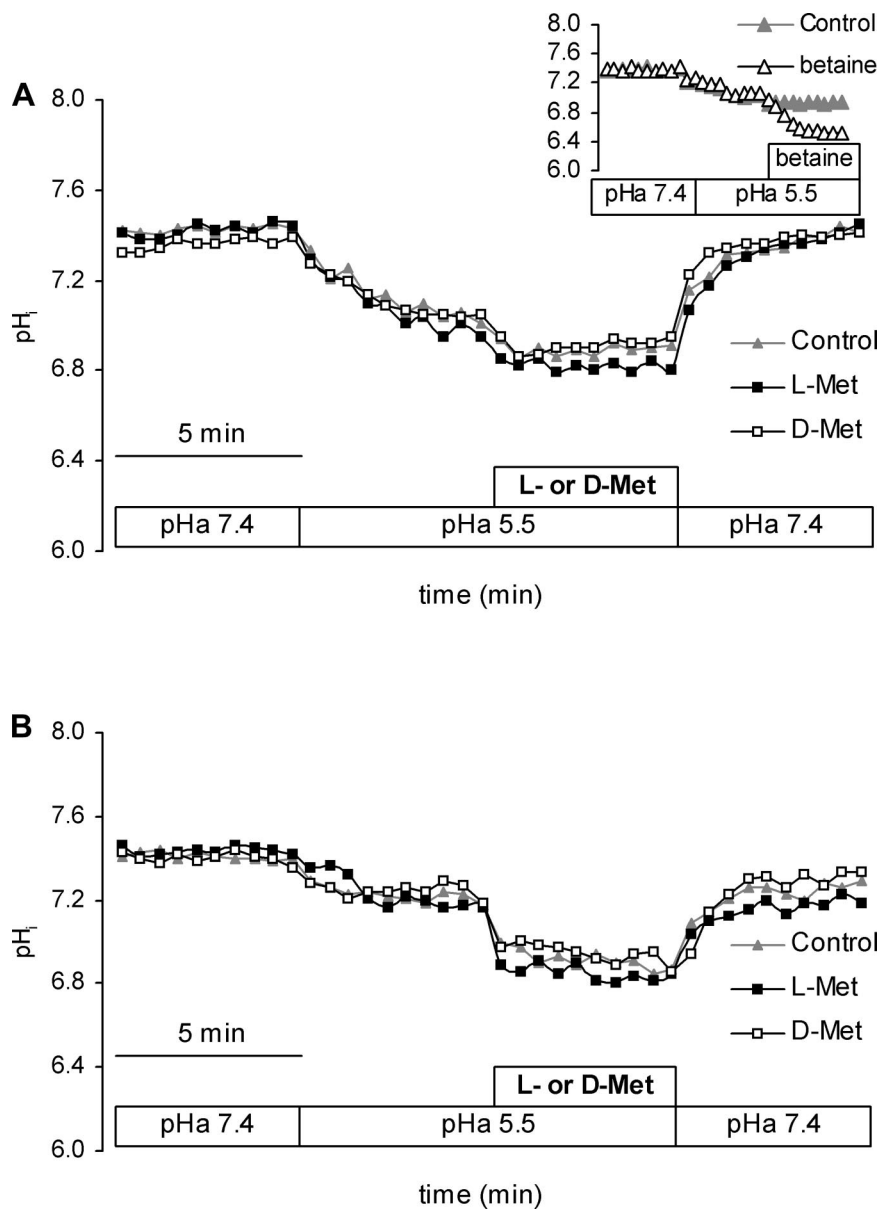


Fig. 3. Intracellular pH in Caco-2 cells loaded with the pH-sensitive fluorochrome BCECF. The preloaded monolayers were incubated in the presence and absence of  $\text{Na}^+$  (A and B, respectively) at the indicated apical pH. After 10 min, L- or D-Met (20 mmol/l) was added to the apical compartment, and after a 5-min incubation, the substrate was removed. Betaine (20 mmol/l) was also tested as a positive control of intracellular acidification (*inset*). Values are means  $\pm$  SE;  $n = 3$  (SE values were  $< 2\%$  of the mean). The statistical analysis shows no significant differences between control and L-Met- or D-Met-incubated cells ( $P \geq 0.05$ ).

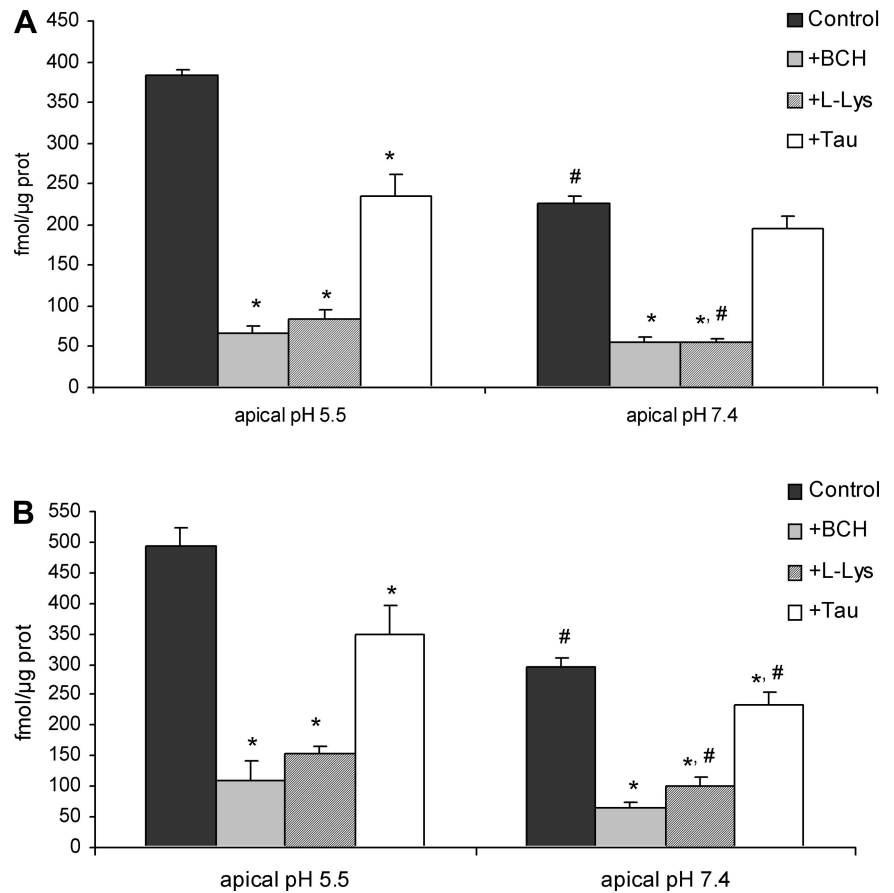
either the presence or absence of  $\text{Na}^+$ , was not affected by pH, may indicate that these transport systems are not involved in pH-stimulated L-Met transport. In this sense, Pan et al. (31) and Thwaites and Stevens (47) have observed that the activity of these transport systems in Caco-2 cells is not affected by pH in the range tested presently.

Regarding *cis*-inhibition experiments with L-Lys, the results reveal that at pH 7.4, the component insensitive to L-Lys was higher ( $P < 0.05$ ) in the presence than in the absence of  $\text{Na}^+$ , thus indicating the incorporation of system  $\text{B}^0$ . It has been reported that the transport capacity of system  $\text{B}^0$  is not affected or even reduced as extracellular pH is lowered (2, 4, 19, 27). This fact may explain the lower pH effect observed in the presence than in the absence of  $\text{Na}^+$ . Therefore, the stimulation of transport observed in the presence of  $\text{Na}^+$  after L-Lys inhibition cannot be attributed to system  $\text{B}^0$  and thus may correspond to system L. Similarly, in the absence of  $\text{Na}^+$ , this L-Lys-insensitive component, mediated only by system L, was

also higher at a pH of 5.5. In this sense, one of the functional differences between LAT1 and LAT2 is the sensitivity to pH: the activity of LAT1 is not influenced by pH (33), whereas the transport capacity of LAT2 is significantly increased by lowering extracellular pH (35, 39). Therefore, one of the best candidates for mediating pH-stimulated L-Met uptake is system L.

However, the increase in L-Met uptake at pH 5.5 cannot only be explained by the stimulation of system L. In fact, the higher inhibition obtained with L-Lys by lowering extracellular pH suggests the contribution of a transport mechanism also able to interact with cationic amino acids. The best candidate for mediating this component may be system  $\text{B}^{0,+}$ , which recognizes both BCH and L-Lys as substrates. Tau is a sulfonic  $\beta$ -amino acid transported by systems TauT and PAT1 (25, 36, 47), and as suggested in this report, it seems also to be recognized by system  $\text{B}^{0,+}$ . Given that L-Met is not recognized by TauT or by PAT1, system  $\text{B}^{0,+}$  would be the only mecha-

Fig. 4. *Cis*-inhibition of L-Met transport by 2-aminobicyclo[2,2,1]heptane-2-carboxylic acid (BCH), L-lysine (L-Lys), and taurine (Tau). Cells were incubated at pH 5.5 and 7.4 in the absence and presence of Na<sup>+</sup> (A and B, respectively) and 20 mmol/l of the unlabeled substrates. Values are means ± SE; n = 6. \*P < 0.05, mean differences from the respective control. #P < 0.05, mean differences between pH 5.5 and 7.4.



nism sharing L-Met and Tau as substrates. As expected, at a pH of 7.4, L-Met uptake was not affected by Tau in the absence of Na<sup>+</sup>, whereas it was significantly reduced in the presence of Na<sup>+</sup>. Moreover, the transport component inhibited by Tau in the presence of Na<sup>+</sup> was higher at a pH of 5.5, thus suggesting the participation of system B<sup>0,+</sup>. The higher uptake through the Tau-insensitive component at pH 5.5 would again reflect the higher activity of system L in these conditions.

Regarding D-Met uptake, *cis*-inhibition experiments in the presence of Na<sup>+</sup> revealed that the transport component inhibited

by L-Lys and Tau was higher at a pH of 5.5, thus again suggesting the participation of system B<sup>0,+</sup>. Nevertheless, because there was no effect of pH on either L-Lys- or Tau-insensitive components, system L does not appear to participate in D-Met stimulation.

All these results demonstrate the involvement of B<sup>0,+</sup> in pH-stimulated Lys- and BCH-sensitive Met transport. However, there is no available information about the sensitivity of this transport mechanism to pH. In fact, H<sup>+</sup> permeation at low pH appears to be a common feature of many Na<sup>+</sup>-coupled transporters (5). In this sense, system SGLT1, considered the archetype of Na<sup>+</sup>-solute cotransporters, can also function as a H<sup>+</sup>-sugar cotransporter (18). SGLT1 shows an affinity for H<sup>+</sup> that is ~500 times greater than for Na<sup>+</sup>, whereas the affinity for glucose is ~25 times lower in the H<sup>+</sup>-coupled compared with the Na<sup>+</sup>-coupled mode (18, 34, 53). A similar effect of pH on L-Met and D-Met uptake may be expected, although in this case the H<sup>+</sup> does not seem to be cotransported given that no intracellular acidification has been found.

In conclusion, L- and D-Met transport is increased by acidic extracellular pH as the result of stimulation of two transport systems functionally identified with systems L and B<sup>0,+</sup> for L-Met and with system B<sup>0,+</sup> for D-Met.

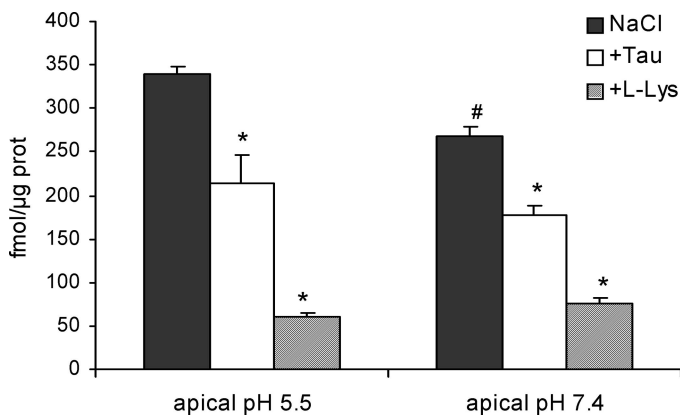


Fig. 5. *Cis*-inhibition of D-Met transport by L-Lys and Tau. Cells were incubated at pH 5.5 and 7.4 in the presence of Na<sup>+</sup> and 20 mmol/l of the unlabeled substrates. Values are means ± SE; n = 3. \*P < 0.05, mean differences from the respective control. #P < 0.05, mean differences between pH 5.5 and 7.4.

#### GRANTS

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