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Research Article

Transcellular Transport of Benzoic Acid Across Caco-2 Cells by a pH-Dependent and Carrier-Mediated Transport Mechanism

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The pH-dependent transcellular transport of [^{14}C]benzoic acid across a Caco-2 cell monolayer is shown to be mediated by a monocarboxylic acid-specific carrier-mediated transport system, localized on the apical membrane. Evidence for the carrier-mediated transport of benzoic acid includes (a) the significant temperature and concentration dependence, (b) the metabolic energy dependence, (c) the inhibition by unlabeled benzoic acid and other monocarboxylic acids, (d) countertransport effects on the uptake of [^{14}C]benzoic acid, and (e) effects of a proteinase (papain) and amino acid-modifying reagents. Furthermore, since carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and nigericin significantly inhibited the transport of [^{14}C]benzoic acid, the direct driving force for benzoic acid transport is suggested to be the inwardly directed proton gradient. From these results, together with previous observations using intestinal brush border membrane vesicles, the pH dependence of the transcellular transport of certain organic weak acids across Caco-2 cells is considered to result mainly from a proton gradient-dependent, carrier-mediated transport mechanism, rather than passive diffusion according to the pH-partition theory.

KEY WORDS: benzoic acid; Caco-2 cell; Monocarboxylic acid; pH-dependent carrier-mediated transport; pH-partition theory; proton-coupled transport.

INTRODUCTION

A number of foreign weak electrolytes are thought to penetrate the intestinal mucosal barrier by passive diffusion of the nonionized drug species across a lipoidal membrane according to the pH-partition theory (1). This concept, however, is difficult to reconcile with the rapid absorption of organic weak acids such as benzoic acid and salicylic acid, since they are highly ionized at the pH of the intestinal lumen. Consequently, several modifications of the theory have been put forward. The first was the proposal of "virtual pH," that is, the effective pH at the intestinal surface is supposed to be 5.3, which is more acidic than the luminal pH (1). However, when the pH value at the intestinal surface was measured with a microelectrode, the "microclimate pH" was 6.6–6.9, failing to support the virtual pH hypothesis (2). Other proposals include paracellular transport of the ionized form of drugs (3,4) and the mucosal unstirred water layer effect (5,6). These explanations, however, may not apply to all cases showing deviations of the actual intestinal absorption from the pH-partition hypothesis. All of these proposals retain the assumption that organic weak electrolytes are transported across intestinal epithelia by passive diffusion.

On the other hand, a pH-dependent, carrier-mediated transport mechanism was recently demonstrated in our laboratory (7). Monocarboxylic acids, such as acetic acid and nicotinic acid, are transported across the intestinal brush border membrane (BBM) by carrier-mediated mechanisms, including proton-cotransport and pH-dependent anion-exchange systems (7–9). Since carrier-mediated transport systems for lactate (10), acetate (11), and propionate (12) in the small intestine and butyrate (13) in the colon have also been reported, it is likely that certain drugs having a monocarboxylic acid moiety as well as short-chain fatty acids are transported via a specific mechanism in addition to passive diffusion across intestinal epithelia. However, the intestinal BBM vesicles used for the studies might not adequately reflect the whole intestinal absorption processes. Net absorption through the intestinal mucosa is the outcome of complex phenomena, including BBM and basolateral membrane transport and intracellular events such as binding and translocation. Moreover, the contributions of various driving forces such as pH, inorganic ions, and membrane potential to energizing the carrier functions under physiological conditions are difficult to assess in *in vitro* BBM vesicle studies. Therefore, it is still unclear whether monocarboxylic acid carrier systems predominate as intestinal absorption mechanisms *in vivo* for certain organic weak acids.

The human colonic adenocarcinoma cell line, Caco-2, which forms monolayers of viable and polarized intestinal epithelial cells, mimics the physiological functions found in small intestinal absorptive cells (14). This cell line has sev-

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eral advantages for studies of intestinal absorption, because it forms well-differentiated intestinal epithelial cells and exhibits several functional properties of small intestine, such as carrier-mediated transport systems, receptors, and many enzyme activities (15–17). Furthermore, Caco-2 cells were originally derived from a human colon carcinoma (18) and may serve to predict *in vivo* intestinal absorption in humans (19).

The purpose of the present study was to elucidate the transport mechanism of monocarboxylic acids across intestinal epithelial cells by studying the transcellular transport in monolayers of Caco-2 cells, to confirm the carrier-mediated mechanism observed in intestinal BBM vesicles (7–9). Furthermore, we addressed the question whether the contribution of the monocarboxylic acid transport system could account for deviations from the pH-partition hypothesis. In the present study, benzoic acid was used as a model compound to examine the characteristics of the monocarboxylic acid carrier system, for three reasons. First, the intestinal pH-absorption profile of benzoic acid deviates from the pH-partition theory (20). Second, it inhibits the transport of acetic acid and nicotinic acid in intestinal BBM vesicles (7–9). Third, it is not readily metabolized during the transport process.

MATERIALS AND METHODS

Materials

The Caco-2 cell line was obtained from Eisai Co., Ltd. (Tokyo), and confirmed to be mycoplasma negative using the Hoechst 33258 test (Flow Laboratories Ltd., Irvine, UK). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and nonessential amino acids (NEAA) were obtained from GIBCO (Grand Island, NY). L-Glutamine, penicillin G, and streptomycin were obtained from Wako Pure Industries Ltd. (Osaka, Japan). The polycarbonate membrane and Transwell clusters, 11.2-mm diameter and 3.0-μm pore size, were purchased from Costar (Bedford, MA). Rat tail collagen (Type I) was obtained from Collaborative Research Inc. (Bedford, MA). [^{14}C]Benzoic acid (0.56 GBq/mmol) and [^{3}H]mannitol (1110 GBq/mmol) were purchased from New England Nuclear (Boston, MA). Unlabeled benzoic acid, nigericin, papain, diethylpyrocarbonate, and dithiothreitol were obtained from Sigma Chemicals (St. Louis, MO). Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were commercial products of reagent grade.

Cell Culture and Cellular Quality Assessment

Caco-2 cells were grown routinely in 75-cm^2 plastic T-flasks at 37°C in a 5% CO_2/95% air atmosphere. The culture medium consisted of DMEM, containing 10% FCS, 1% NEAA, 2 m*M* L-glutamine, 100 IU/mL penicillin G, and 100 μg/mL streptomycin. Cells reached confluency after 5–7 days in culture. All cells used in this study were between passage 48 and passage 60.

For the transport study, Caco-2 cells were seeded at a density of 1×10^5 cells/cm^2 on a polycarbonate membrane. Cells were cultured for 5 days, and the culture medium was replaced on alternate days. Cells were grown for 21 to 23 days for the transport experiment. The quality of the monolayers grown on the polycarbonate membrane was assessed by measuring the paracellular transport of [^{3}H]mannitol and the transepithelial electrical resistances (TEER) using a Millicell-ERS (Millipore, Bedford MA). TEER values were over 250 Ω · cm^2 and [^{3}H]mannitol leakage was usually less than 1.71 ± 0.01% (mean ± SE; $n = 3$) of the dose/hr/well.

For the uptake study, Caco-2 cells were seeded at a density of 1×10^5 cells/cm^2 on multidishes (Nunc, Denmark) coated with collagen. For collagen coating, 0.1 mL of 0.25% rat tail collagen dissolved in ethanol was spread onto the dishes, and the solvent was evaporated under UV light. The collagen matrix was needed to attach cells strongly to the multidishes. The matrix has no influence on the general features of the cells during differentiation (21). Cells were grown for 14 days for the uptake experiment.

Transcellular Transport and Uptake Experiments

In the transcellular transport study, the cells grown on the polycarbonate membrane were washed twice with Hank's balanced salt solution (HBSS; 0.952 m*M* $CaCl_2$, 5.36 m*M* KCl, 0.441 m*M* KH_2PO_4, 0.812 m*M* $MgSO_4$, 136.7 m*M* NaCl, 0.385 m*M* Na_2HPO_4, 25 m*M* D-glucose, and 10 m*M* Hepes for pH 7.3 or 10 m*M* Mes for pH <6.5; the osmolarity was 315 mOsm/kg). To initiate the transport experiments, HBSS (pH 7.3, 37°C) was put into the basolateral side (receiver side) and test solution was loaded onto the apical side (donor side) of a cell insert. In the standard experiment, 0.5-mL aliquots of solution were removed from the receiver side and replaced with an equal volume of fresh HBSS. In the case of the uptake study from the apical side, Caco-2 cells grown on the collagen-coated multidishes were washed with HBSS containing 25 m*M* D-glucose and 10 m*M* Hepes (pH 7.3) or Mes (pH 6.5, 6.0, 5.5). Test solution (0.25 mL) was added to initiate uptake. The cells were incubated at 37°C for the time indicated. To terminate uptake, the test solution was removed by suction and the cells were washed three times with 1 mL of ice-cold HBSS. [^{3}H]Mannitol was used as a marker for the extracellular fluid that adhered to the cells. For quantitation of the drugs absorbed, cells were solubilized with 0.5 mL Solvable (NEN).

Radioactivity was determined using a liquid scintillation counter (LSC-1,000, Aloka Co. Ltd., Tokyo). Cellular protein was measured by the method of Lowry *et al.* (22) using bovine serum albumin as a standard. Protein contents of a polycarbonate membrane and a multidish were 0.853 ± 0.02 and 0.503 ± 0.09 μg/cm^2, respectively. Details of the conditions for each experiment are given in the figure legends or table footnotes.

Treatment with Proteinase or Amino Acid-Modifying Reagents

Papain (1.0 mg/mL) was activated with 10 m*M* cysteine and 4 m*M* EDTA at 4°C for 15 min. Caco-2 cells (apical side) were incubated at 37°C for 30 min with freshly activated papain solution which contained 0.5 mg/mL papain, 5 m*M* cysteine, and 2 m*M* EDTA, adjusted to pH 6.2. The papain solution was removed and the cells were washed three times with HBSS. Monolayer leakage caused by preincubation

with cysteine and EDTA was corrected on the basis of [^{3}H]mannitol permeability.

The procedures for treatments with diethylpyrocarbonate (DEPC), and *N*-ethylmaleimide (NEM) were essentially the same as those described by Miyamoto *et al.* (23), and treatment with dithiothreitol (DTT) was done according to Turner and George (24). Freshly prepared DEPC solutions (0.2 m*M*) were made in 50 m*M* phosphate buffer containing 75 m*M* NaCl, pH 7.0, with ethanol (0.2%). Each reagent was added to the apical side of a Caco-2 monolayer and incubated at 37°C for 10 min. During DEPC treatment, an equal amount of ethanol (0.2%) was added to the control cells. Each preincubation solution was removed and the cells were washed three times with HBSS, then uptake was started.

Metabolism Test

Metabolism of [^{14}C]benzoic acid by Caco-2 cells was assessed by analyzing the radioactivity in the receiver side after a transport experiment. Intact and metabolized benzoic acid were analyzed by HPLC and quantitated by radioactivity measurement in the fractionated eluant. The HPLC system used to detect benzoic acid was as follows: analytical column, TSK gel ODS 80 Tm, 4.6 × 150 mm (Tosoh, Tokyo); mobile phase, a mixture of acetonitrile and 10 m*M* phosphate buffer (30/70); flow rate, 1.0 mL/min; and monitoring, at 240 nm.

Data Analysis

The slope of the initial linear portion of "permeated amount (nmol/mg protein)" versus "time (min)" curves, calculated by linear regression analysis, was defined as the permeation rate (nmol/min/mg protein). The permeability coefficient (μL/min/mg protein) was defined as the value obtained by dividing the permeation rate by the drug concentration (n*M*) on the apical side. For uptake by Caco-2 monolayers, the uptake rate (nmol/time/mg protein) was defined as the value obtained by dividing the uptake amount (nmol/mg protein) by the uptake time (10 or 30 sec) and the uptake coefficient (μL/time/mg protein) was defined as the value obtained by dividing the uptake rate by the drug concentration (n*M*) on the apical side.

To estimate the kinetic parameters of saturable transport across the Caco-2 monolayers, the transport rate (J) was fitted to the following equation, consisting of both saturable and nonsaturable linear terms, using the nonlinear least-squares regression analysis program, MULTI (25):

$$J = J_{max} \times S/(K_t + S) + k_d \times S \quad (1)$$

where J_{max} is the maximum transport rate for the carrier-mediated process, S is the concentration of substrate, K_t is the half-saturation concentration (Michaelis constant), and k_d is the first-order rate constant. All data are expressed as mean ± SE. Statistical analysis was performed using Student's two-tailed t test. A difference between means was considered significant if the P value was less than 0.05.

RESULTS

Transcellular Transport

Time Course and pH Dependence

Figure 1 shows the effect of pH on transcellular transport of [^{14}C]benzoic acid from the apical to the basolateral side across a Caco-2 cell monolayer. The pH on the apical side was changed from 5.0 to 7.3, while the basolateral pH was set at pH 7.3, which is close to the intracellular pH of Caco-2 (26). The permeated amount of [^{14}C]benzoic acid increased linearly with time after the initial lag period of a few minutes, as shown in the inset to Fig. 1. HPLC analysis, as mentioned under Materials and Methods, indicated that the permeated [^{14}C]benzoic acid was not metabolized within 90 min (data not shown). The flux of [^{14}C]benzoic acid across the Caco-2 monolayers was significantly higher than that of [^{3}H]mannitol, which reflects the paracellular permeability. Consequently, the transport of [^{14}C]benzoic acid was ascribed to transcellular permeation.

The permeability coefficient of [^{14}C]benzoic acid increased markedly with decreasing pH on the apical side to an acidic from a neutral pH. At an acidic pH, lower than pH 5.5, no further increase in permeability was observed. In the presence of 10 m*M* unlabeled benzoic acid, the transcellular transport of [^{14}C]benzoic acid was significantly reduced. The extent of the inhibitory effect of unlabeled benzoic acid increased with lowering of the pH on the apical side, suggesting that a carrier-mediated mechanism partly contributes to the transport of benzoic acid. In the following studies, transport was studied at the apical pH of 6.0. Since [^{3}H]mannitol transport was constant at various pH's (data not shown), pH-dependent transport of [^{14}C]benzoic acid cannot be ascribed to a nonspecific effect of pH change, such as membrane perturbation.

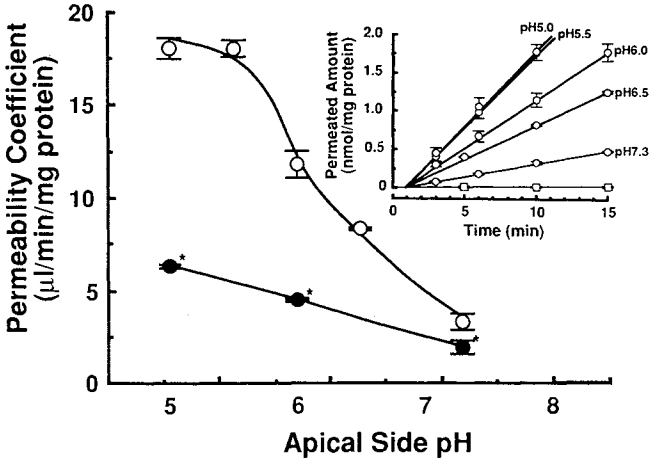

Fig. 1. pH dependence of [^{14}C]benzoic acid transport across Caco-2 monolayers. Permeability of [^{14}C]benzoic acid (10 μ*M*; ○, ●) or [^{3}H]mannitol (33 n*M*; □) was measured at 37°C by incubating Caco-2 monolayers in HBSS containing 0.952 m*M* $CaCl_2$, 5.36 m*M* KCl, 0.441 m*M* KH_2PO_4, 0.812 m*M* $MgSO_4$, 136.7 m*M* NaCl, 0.385 m*M* Na_2HPO_4, 25 m*M* D-glucose and 10 m*M* Hepes or 10 m*M* Mes at an apical-side pH of 5.0, 5.5, 6.0, 6.5, or 7.3 and at a basolateral-side pH of 7.3 in the absence (○) and presence (●) of unlabeled benzoic acid (10 m*M*). Permeability of [^{3}H]mannitol was measured at an apical-side pH of 6.0 and at a basolateral-side pH of 7.3. Each permeability coefficient was obtained by dividing the slope of the initial linear portion of the permeated amount time curve (inset) by the drug concentration. Each point represents the mean ± SE of three experiments. (*) Significantly different from the transport rate measured at the same apical-side pH without unlabeled benzoic acid ($P < 0.05$).

Concentration Dependence

Figure 2 represents the relationship between the initial permeation rate of benzoic acid and its concentration in the medium from 10 μ*M* to 20 m*M*. The result indicates that the permeability of benzoic acid consists of two components, a saturable process evident at lower concentrations and an apparently nonsaturable process observed at higher concentrations. They were analyzed according to Eq. (1) as described under Materials and Methods. The kinetic parameters obtained for benzoic acid transport were a J_{max} of 57.2 ± 3.14 nmol/min/mg protein, a K_t of 4.83 ± 0.58 m*M*, and a k_d of 0.54 ± 0.04 μL/min/mg protein. Furthermore, transcellular transport of [^{14}C]benzoic acid was remarkably temperature dependent.

Energy Dependence and Driving Force

The effects of several transport inhibitors on the permeability of [^{14}C]benzoic acid were studied to determine whether this carrier-mediated transport requires metabolic energy and is driven by the inwardly directed H^+ gradient. 2,4-Dinitrophenol (DNP) (1 m*M*), an uncoupler of oxidative phosphorylation, or sodium azide (10 m*M*), a respiratory chain inhibitor, significantly diminished the permeability of [^{14}C]benzoic acid (Table I). FCCP (50 μ*M*), a protonophore, also significantly inhibited the transport of [^{14}C]benzoic acid. On the contrary, neither the replacement of Na^+ with choline nor that of Cl^- with gluconate in the transport buffer had any effect on the permeability of [^{14}C]benzoic acid (data not shown). Neither 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), an anion-exchange inhibitor, nor 50 m*M* bicarbonate had any inhibitory effect. Therefore, anion exchange is unlikely to participate in the transport of benzoic acid across the Caco-2 monolayer. These results suggest that the transport of benzoic acid across Caco-2 monolayers is dependent on the inwardly directed proton gradient.

Structural Specificity of the Transporter

To examine the properties of the transporter responsible for benzoic acid transport across Caco-2 cells, the effects of various compounds on [^{14}C]benzoic acid permeability were studied (Table II). Many monocarboxylic acids significantly reduced the permeability of [^{14}C]benzoic acid. In contrast, taurocholic acid and a dicarboxylic acid (phthalic acid) had no effect. A neutral amino acid (L-phenylalanine), an acidic amino acid (L-glutamic acid), and a zwitterionic compound (*p*-aminohippuric acid) showed no significant reduction of the [^{14}C]benzoic acid permeability. Neither D- nor L-lactic acid had an inhibitory effect.

To study the mechanism of the inhibitions by monocarboxylic acids, their inhibitory effects were kinetically analyzed. Figure 3a shows Lineweaver–Burk plots for the effect

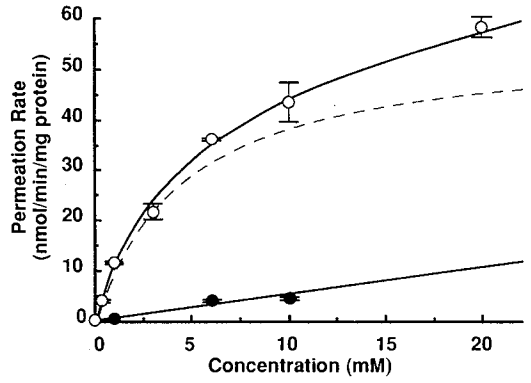

Fig. 2. Concentration and temperature dependence of [^{14}C]benzoic acid transport across Caco-2 monolayers. Permeation rate of benzoic acid was measured at 37°C (○) or 4°C (●). Incubation conditions were identical to those described in the legend to Fig. 1. The dashed line represents the permeation rate for the saturable component calculated from the kinetic parameters obtained as mentioned under Results. Each point represents the mean ± SE of three experiments.

Table I. Inhibitory Effect of Metabolic Inhibitors and Ionophores on [^{14}C]Benzoic Acid Transport[a]

Condition	Relative permeability (% of control)[b]
+1 m*M* DNP (−glucose)	66.5 ± 2.35*
+1 m*M* DNP (−glucose + 3-O-methylglucose)	53.9 ± 2.31*
+10 m*M* NaN_3 (−glucose)	20.3 ± 1.61*
+0.1 m*M* DIDS	99.9 ± 3.23
+50 m*M* bicarbonate	97.8 ± 1.57
+50 μ*M* FCCP (0.5% EtOH)	81.0 ± 2.62*

[a] Caco-2 cells were preincubated with DNP or NaN_3 for 20 min. The amount of permeated [^{14}C]benzoic acid was measured at 37°C for 15 min by incubating Caco-2 cells in HBSS (apical pH, 6.0; basolateral pH, 7.3) with each reagent. The other incubation conditions were the same as described in the legend to Fig. 1.

[b] Each value represents the mean ± SE of three experiments.

* Significantly different from the control value by Student's *t* test ($P < 0.05$).

Table II. Inhibitory Effect of Various Compounds on [^{14}C]Benzoic Acid Transport[a]

Inhibitor	Concentration (m*M*)	Relative permeability (% of control)[b]
Acetic acid	10	68.4 ± 2.18*
Nicotinic acid	10	89.6 ± 0.72*
Phenylacetic acid	10	62.5 ± 2.82*
Pravastatin	20	89.3 ± 2.19*
Propionic acid	10	53.9 ± 1.60*
Salicylic acid	10	64.9 ± 0.47*
Valproic acid	10	32.5 ± 5.10*
p-Aminohippuric acid	10	98.6 ± 1.21
Taurocholic acid	0.25	91.2 ± 2.33
L-Glutamic acid	10	98.9 ± 2.83
D-Lactic acid	10	103.0 ± 3.76
L-Lactic acid	10	97.1 ± 5.20
L-Phenylalanine	10	95.0 ± 0.61
Phthalic acid	10	113.5 ± 4.08

[a] The amount of permeated [^{14}C]benzoic acid was measured at 37°C for 15 min by incubating Caco-2 cells in HBSS buffer (apical pH, 6.0; basolateral pH, 7.3) in the presence of each inhibitor. The other incubation conditions were the same as described in the legend to Fig. 1.

[b] Each value represents the mean ± SE of three experiments.

* Significantly different from the control value by Student's *t* test ($P < 0.05$).

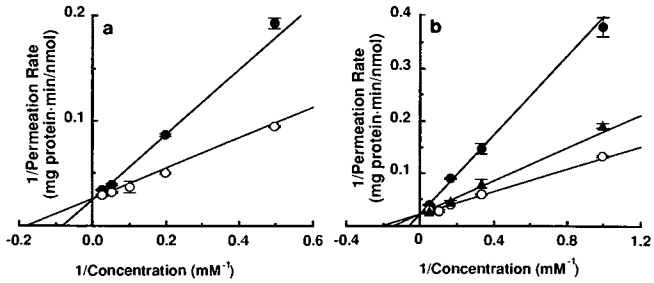

Fig. 3. Lineweaver–Burk plots for the transport of benzoic acid across Caco-2 monolayers. (a) Permeation rate was measured in the absence (○) or presence of 10 m*M* acetic acid (●). Incubation conditions were the same as described in the legend to Fig. 1. Each point represents the mean of three experiments. (b) Transport was measured in the absence (○) or presence of 10 m*M* salicylic acid (▲) or 10 m*M* valproic acid (●). Incubation conditions were the same as described in the legend to Fig. 1. Each point represents the mean ± SE of three experiments.

of acetic acid on benzoic acid permeation rates after subtraction of the nonsaturable component. Similarly, Fig. 3b shows the effects of salicylic acid and valproic acid on the permeation rate of benzoic acid. All three monocarboxylic acids competitively inhibited the transport of benzoic acid. The inhibition constants, K_i, for acetic acid, salicylic acid, and valproic acid were 16.7 ± 2.64, 20.4 ± 3.10, and 3.94 ± 0.17 m*M*, respectively.

Effect of Papain Treatment

Figure 4 shows the effect of proteinase (papain) treatment of Caco-2 cells on [^{14}C]benzoic acid permeability across the cell monolayer. [^{14}C]Benzoic acid permeability was reduced to 25% of the control value by 30 min of preincubation of Caco-2 cells with papain. This result suggests that a membrane protein digested by papain is required for the transport of benzoic acid.

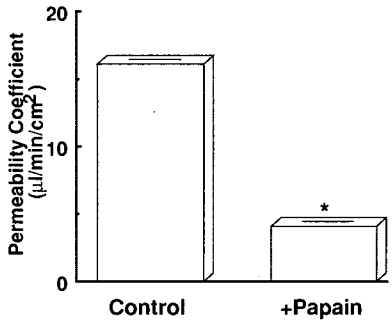

Fig. 4. Effect of papain treatment of the transport of [^{14}C]benzoic acid across Caco-2 monolayers. After preincubation of Caco-2 cells with 0.5 mg/mL activated papain in 50 m*M* phosphate buffer containing 5 m*M* cysteine and 2 m*M* EDTA, pH 6.2, at 37°C for 30 min, the permeability coefficient was measured under the same conditions as described in the legend to Fig. 1. In the control experiment, Caco-2 cells were preincubated in the same manner without papain. Each point represents the mean ± SE of three experiments. (*) Significantly different from the uptake rate measured without papain ($P < 0.05$).

Intracellular Uptake

pH Dependence

The effect of apical pH on the intracellular uptake of [^{14}C]benzoic acid was studied (Fig. 5). The uptake was linear for more than 30 sec (data not shown). The 10-sec uptake of [^{14}C]benzoic acid increased with decreasing pH on the apical side. In the presence of 10 m*M* unlabeled benzoic acid, the uptake rate of [^{14}C]benzoic acid decreased significantly. The uptake was also remarkably reduced at 4°C. These results suggest that a carrier-mediated transporter is functioning at the apical membrane.

Concentration Dependence

Figure 6 shows the concentration-dependent uptake of benzoic acid over 30 sec at the apical membrane. The results observed for the intracellular uptake are consistent with transcellular transport that proceeds by both saturable and nonsaturable processes. The evaluated kinetic parameters J_{max}, K_t, and k_d were 96.2 ± 7.84 nmol/30 sec/mg protein, 1.28 ± 0.234 m*M*, and 2.37 ± 0.445 μL/30 sec/mg protein, respectively.

Effect of the Proton Gradient

To clarify the significance of the proton gradient, the effect of nigericin, which exchanges protons with potassium ions across the membrane, was examined. In this study the cells were pH-clamped by incubating them in a buffer containing 135 m*M* KCl and nigericin. As shown in Fig. 7, [^{14}C]benzoic acid uptake was substantially reduced at pH 6.0 in the presence of nigericin.

To evaluate further the role of the pH gradient across the apical membrane, cells were acid-loaded by exposure to 30 m*M* NH_4Cl (NH_4 prepulse) for 20 min. The uptake of [^{14}C]benzoic acid by the cells was examined in the buffer containing tetramethylammonium (TMA) in place of sodium, to inactivate the Na^+/H^+ exchanger, so that the intra-

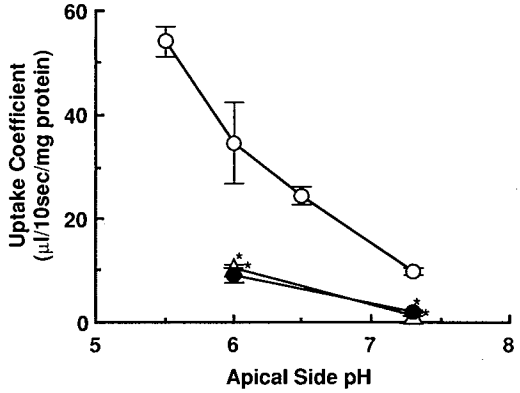

Fig. 5. pH and temperature dependencies of [^{14}C]benzoic acid uptake. Uptake coefficient for Caco-2 cells was measured at 10 sec in the absence (○) and presence (●) of unlabeled benzoic acid (10 m*M*) at 37°C and, at 4°C (△). Incubation conditions were the same as described in the legend to Fig. 1. Each point represents the mean ± SE of four experiments. (*) Significantly different from the uptake coefficient measured at the same apical-side pH without unlabeled benzoic acid and from that measured at 37°C ($P < 0.05$).

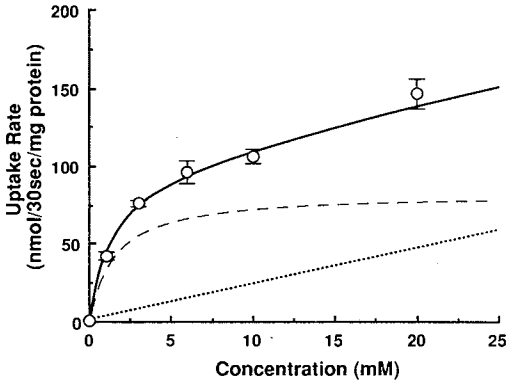

Fig. 6. Concentration dependence of [^{14}C]benzoic acid uptake rate by Caco-2 monolayers. Uptake by Caco-2 cells was measured at 30 sec during incubation at 37°C and pH 6.0. Incubation conditions were identical to those described in Fig. 1. The dashed line represents the uptake rate for the saturable component calculated from the kinetic parameters obtained as mentioned under Results. The dotted line represents nonsaturable uptake. Each point represents the mean ± SE of four experiments.

cellular pH was stable and lower than the ordinary pH (27). By inducing an acidic intracellular pH with NH_4Cl, [^{14}C]benzoic acid uptake was reduced. Therefore, the ability of the cells to transport benzoic acid intracellularly is dependent on the presence of an inwardly directed proton gradient.

Countertransport

Figure 8 shows the uptake of [^{14}C]benzoic acid by Caco-2 cells preloaded with 25 m*M* unlabeled benzoic acid, 25 m*M* salicylic acid, 25 m*M* phthalic acid, or 50 m*M* bicarbonate. By preloading with unlabeled benzoic acid or salicylic acid, [^{14}C]benzoic acid uptake was significantly enhanced. On the contrary, the uptake by Caco-2 cells loaded with 25 m*M* phthalic acid or 50 m*M* bicarbonate was not enhanced. Therefore, it is strongly suggested that carrier-mediated transport is involved in benzoic acid uptake by Caco-2 cells and that the transport system is shared with salicylic acid.

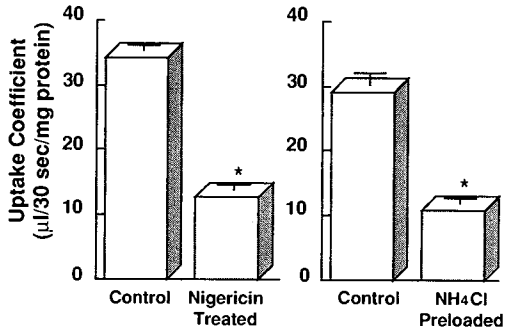

Fig. 7. Effect of proton gradient on [^{14}C]benzoic acid uptake. Caco-2 cells were pretreated with 10 µg/mL nigericin for 5 min at pH 6.0 or preloaded with 30 m*M* ammonium chloride for 20 min at pH 7.2. Each point represents the mean ± SE of four experiments. (*) Significantly different from the uptake coefficient measured without nigericin or NH_4Cl ($P < 0.001$).

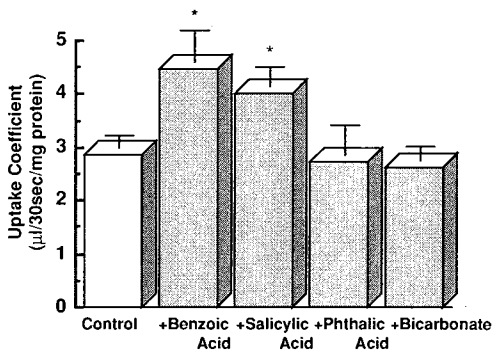

Fig. 8. Countertransport in [^{14}C]benzoic acid uptake. Caco-2 cells, ATP-depleted by 1 m*M* DNP, were preloaded with 25 m*M* benzoic acid, 25 m*M* salicylic acid, 25 m*M* phthalic acid, or 50 m*M* bicarbonate for 15 min. Each point represents the mean ± SE of four experiments. (*) Significantly different from the uptake coefficient measured without preloaded compounds ($P < 0.05$).

Effect of Amino Acid-Modifying Reagents

Table III shows the effect of treatment of the apical membrane of Caco-2 cells with DEPC, NEM, or DTT on [^{14}C]benzoic acid uptake. By pretreatment with these amino acid modifying reagents, [^{14}C]benzoic acid uptake was significantly reduced. Since Caco-2 cells pretreated with DEPC, NEM, or DTT were used for the uptake assay after being washed three times with HBSS buffer to remove unreacted modifying reagents, direct interaction of benzoic acid with these reagents is unlikely. These inhibitory effects are presumed to be caused by specific interactions with the carrier protein responsible for benzoic acid transport. Therefore, the transporter of benzoic acid is likely to require histidyl and/or thiol residues for its function.

DISCUSSION

When transcellular transport of [^{14}C]benzoic acid across a Caco-2 cell monolayer was studied by changing the pH on the apical side, the transport rate increased with decreasing pH, as shown in Fig. 1. The apparent pK_a value evaluated from the pH profile of the permeability coefficient according to the pH-partition hypothesis, which states that only the undissociated form of a weak electrolyte can permeate the

Table III. Effects of Amino Acid-Modifying Reagents on the Uptake of [^{14}C]Benzoic Acid[a]

Inhibitor	Concentration (m*M*)	Relative uptake (% of control)[b]
DEPC	0.2	20.6 ± 1.37*
NEM	0.5	59.3 ± 7.58*
DTT	10	86.5 ± 1.59*

[a] After preincubation with 0.2 m*M* DEPC, 10 m*M* DTT, or 0.5 m*M* NEM, uptake of [^{14}C]benzoic acid was measured at 37°C for 30 sec by incubating Caco-2 cells in HBSS buffer (pH 6.0). The other incubation conditions were the same as described in the legend to Fig. 1.

[b] Each value represents the mean ± SE of four experiments.

* Significantly different from the control value by Student's *t* test ($P < 0.05$).

membrane, shows a deviation from the true pK_a value of 4.2. This observation is consistent with that made previously in *in situ* rat small intestinal absorption (20). Furthermore, in the presence of unlabeled benzoic acid, the [^{14}C]benzoic acid permeability coefficient was significantly reduced. With a lower pH on the apical side, a higher inhibitory effect was observed (Fig. 1). It is difficult to explain these observations in terms of simple diffusion according to the pH-partition theory. Therefore, the mechanisms underlying such a pH dependence were examined in the present study.

The present study establishes the existence of a carrier-mediated transport system for benzoic acid in Caco-2 cells. The evidence may be summarized as follows: (a) the initial permeation rate of benzoic acid was composed of both saturable and nonsaturable processes (Fig. 2); (b) metabolic inhibitors, sodium azide and DNP, reduced the permeability significantly, indicating the energy dependence of the transport process (Table I); (c) unlabeled benzoic acid and some monocarboxylic acids showed significant inhibitory effects on the transport of [^{14}C]benzoic acid (Figs. 1 and 3 and Table II); (d) reduction of the incubation temperature from 37 to 4°C decreased the initial permeation rate remarkably (Fig. 2); (e) preloading of unlabeled benzoic acid or salicylic acid enhanced the initial uptake of [^{14}C]benzoic acid, showing a transstimulation or countertransport effect (Fig. 8); and (f) the permeability coefficient or the cellular uptake was significantly reduced when Caco-2 cells were digested by a proteinase, papain, or treated with amino acid-modifying reagents (Fig. 4 and Table III). These results, from a to f, satisfy the criteria for a carrier-mediated transport mechanism; the validity of the carrier-mediated mechanism is supported especially by the significant effects of both papain digestion and amino acid modifications of Caco-2 cells, because both experiments are targeted on the proteins on the external surface of Caco-2 cells. The significant reduction in the permeability coefficient and the cellular uptake provide direct evidence for the participation of a membrane protein, i.e. carrier protein, in the transport of benzoic acid.

Since the carrier-mediated transport of benzoic acid showed significant pH dependence, a further study was conducted to identify the direct driving force for the transport. The activation at acidic pH can be accounted for by H^+ cotransport, an OH^- exchange system, or a possible change in affinity to the carrier protein depending on the extracellular pH. The absence of significant inhibition by DIDS, an inhibitor of anion exchange, suggests that an OH^- exchange system with benzoic acid is probably not involved (Table I). Furthermore, no significant effect of bicarbonate ion was observed. Recent studies using intestinal BBM vesicles have demonstrated HCO_3^- exchange transport of short-chain fatty acids (9,11–13). However, in benzoic acid transport across a Caco-2 monolayer, the contribution of such an anion-exchange transport seems negligible. On the other hand, FCCP and nigericin exhibited significant inhibitory effects. Moreover, intracellular acidification by means of an ammonium chloride prepulse significantly reduced benzoic acid transport (Fig. 7). Accordingly, it is most likely that the direct driving force for benzoic acid transport is an inwardly directed proton gradient.

It is noteworthy that the structural specificity of the carrier responsible for benzoic acid transport across Caco-2 monolayers was very similar to that observed using intestinal BBM vesicles in the H^+ cotransport of acetic acid (7), nicotinic acid (8), and lactic acid (10). The transport of [^{14}C]benzoic acid was significantly inhibited specifically by monocarboxylic acids but not by a dicarboxylic acid, amino acids, or a bile acid. These results demonstrate that the transporter for benzoic acid is specific for certain organic anions having one monocarboxylic acid moiety in the molecule. Furthermore, we established that the benzoic acid transport is competitively inhibited by acetic acid, salicylic acid, and valproic acid (Figs. 3a and b). Therefore, all of these organic acids are suggested to be transported in Caco-2 cells via an H^+ cotransport system which is at least partially common to that carrying benzoic acid. It is interesting that the absence of any significant effect of D- and L-lactic acid on benzoic acid permeability is inconsistent with the significant and stereospecific inhibitory effect of this endogenous acid previously observed in the uptake of acetic acid (7) or nicotinic acid (8) by BBM vesicles. Further studies are needed to clarify the structural specificities of monocarboxylic acid transporters.

The rate-limiting process in the overall transcellular transport is likely to be the uptake process at the apical membrane of Caco-2 cells, for the following reasons: (a) the initial uptake of benzoic acid was accomplished in a saturable manner with an apparent K_m of 1.28 m*M*, which is very close to the K_m value (4.83 m*M*) obtained by measuring the transcellular permeation rate (Fig. 6); (b) unlabeled benzoic acid caused inhibition of [^{14}C]benzoic acid uptake (Fig. 5); (c) the initial uptake rate was reduced at 4°C compared with that at 37°C; (d) intracellularly imposed unlabeled benzoic acid or salicylic acid stimulated the uptake of [^{14}C]benzoic acid (Fig. 8); and (e) amino acid modifying reagents showed a significant inhibitory effect on the apical uptake of [^{14}C]benzoic acid (Table III), suggesting that the transport carrier protein is on the apical membrane of the Caco-2 monolayer. All of the characteristics obtained by measuring uptake from the apical side are consistent with those observed for transcellular transport. Therefore, the carrier is considered to be situated on the apical side and presumably regulates the permeation rate of monocarboxylic acids across Caco-2 cells, although the existence of a similar carrier in the basolateral membrane can not be excluded.

Modifications of histidyl and thiol residues greatly reduced the transport activities. These amino acid residues have been shown to be essential for the function of many transport systems driven by H^+ gradients (23). Studies with renal BBM vesicles have demonstrated that histidyl residues are located at the Na^+–H^+ exchanger (28), which also accepts H^+ as a substrate (29). For H^+-gradient dipeptide transport in rabbit intestinal BBM, a histidyl residue is essential (30). Therefore, it is probable that histidyl residues have an important role in transport proteins in which proton movement occurs.

We conclude that the transcellular transport of benzoic acid across a Caco-2 monolayer is performed by a carrier-mediated transport system specific for monocarboxylic acids. Our results also suggest that the overall transport is regulated at the process of apical membrane permeation in Caco-2 cells, which is in accordance with the characteristics of the H^+-cotransport system in the BBM of rat and rabbit

small intestines. Moreover, monocarboxylic acid drugs such as benzoic acid, salicylic acid, and valproic acid appear to share the transport system. From these results, the pH dependence of the transcellular transport of weak organic acids across Caco-2 cells is ascribed mainly to the carrier-mediated, H^+-cotransport mechanism. Therefore, intestinal absorption of many acidic drugs with a monocarboxylic moiety is likely to be performed by H^+ cotransport on a specific pH-dependent carrier, not by passive diffusion according to the pH-partition theory.

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