ABSORPTION OF BENZOIC ACID IN SEGMENTAL REGIONS OF THE VASCULARLY PERFUSED RAT SMALL INTESTINE PREPARATION

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ABSTRACT:

Oral bioavailability is a consequence of intestinal absorption, exsorption, and metabolism and is further modulated by the difference in activities among segmental regions. The influence of these factors on the net absorption of benzoic acid (BA), a substrate that is metabolized to hippurate and is transported by the monocarboxylic acid transporter 1, was studied in the recirculating, vascularly perfused, rat small intestine preparation. Metabolism of BA was not observed for both systemic and intraluminal injections into segments of varying lengths. But, secretion of BA into lumen was noted. Absorption of BA (0.166–3.68 μmol) introduced at the duodenal end for absorption by the entire intestine was complete (>95% dose at 2 h) and dose-independent, yielding similar absorption rate constants ($k_a$ of 0.0464 min$^{-1}$). The extent of absorption remained high (92–96% dose) when BA was injected into closed segments of shorter lengths (12 or 20 cm), suggesting a large reserve length of the rat intestine. However, $k_a$ was higher for the jejunum (0.0519 and 0.0564 min$^{-1}$, respectively, for the 12- and 20-cm segments) and exceeded that for the duodenum (12-cm segment, 0.0442 min$^{-1}$) and ileum (20-cm segment, 0.0380 min$^{-1}$) at closed injection sites. The finding paralleled the distribution of monocarboxylic acid transporter isoform 1 detected by Western blotting along the length of the small intestine. Fits of the systemic and oral data (based on duodenal injection for absorption by the whole intestine) to the traditional, physiological model and to the segregated flow model (SFM) that describes partial intestinal flow to the enterocyte region showed a better fit with the SFM even though metabolite data were absent.

The intestine is well recognized for its myriad of functions—absorption, metabolism, and exsorption (for review, see Lin et al., 1999; Suzuki and Sugiyama, 2000). Net intestinal transport is due, in part, to the presence of transporters for absorption and efflux (Tsuij and Tamai, 1996). Recent advances in expression cloning of intestinal transporters have provided more definitive tools for the examination of regional distribution of the transporters (Fei et al., 1994; Shneider et al., 1995; Mottino et al., 2000; Walters et al., 2000; Ngo et al., 2001). Since the overall bioavailability is highly dependent on the intimate dynamics of metabolism, net transport, intestinal blood flow, and drug-partitioning characteristics (Doherty and Pang, 1997; Cong et al., 2000), an understanding of the roles of absorptive and exsorptive transporters at the brush-border or mucosal membrane and of the metabolic enzymes and drug and drug partitioning is of paramount importance.

Benzoate, a common preservative that is used clinically for the treatment of inborn errors in urea synthesis (Batshaw et al., 1982), was chosen for study. In rat, intestinal metabolism of benzoic acid (BA$^1$) to hippurate had been described (Strahl and Barr, 1971). Intestinal BA absorption is mediated by Mct1, the monocarboxylic acid transporter 1 (Tamai et al., 1999), that was first cloned from hamster (Garcia et al., 1994) and later the rat (Takanaga et al., 1995) intestine. Mct1 mediates the transport of other aryl acids, such as acetate (Bugaut, 1987), propionate (Harig et al., 1991), lactate (Tiruppathi et al., 1988), salicylate (Takanaga et al., 1994), and nicotinic acid (Simanjuntak et al., 1990). Mct1 was found to exist mostly on villous and not crypt cells of the duodenum and jejunum (Tamai et al., 1999). Moreover, Mct1 was also shown present at the basolateral pole of the rat jejenum (Orsenigo et al., 1999). The segmental localization of intestinal absorptive function of this transporter, however, has not been described.

Hence, we employed BA for the study of segmental absorption, metabolism, and exsorption in the vascularly perfused in situ rat small intestine preparation. In this preparation, the innate circulatory patterns and cellular architecture are preserved such that processes of absorption, metabolism, exsorption in enterocytes, and efflux of drug at the basolateral membrane may be examined concurrently. In addition, regional intestinal absorption may be studied by the injection of

$^1$ Abbreviations used are: BA, benzoic acid; Mct1, monocarboxylate transporter isoform 1; HA, hippuric acid; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; TBST, Tris-buffered saline/0.1% Tween 20; TLC, thin-layer chromatography; TM, traditional physiologically based model; SFM, segregated flow model; ANOVA, analysis of variance; ARA, amount remaining to be absorbed.
the dose into the relevant segments—whole intestine, duodenum, jejunum, or ileum—and viewed with respect to concentration dependence and drug-partitioning characteristics. The resultant data would allow proper characterization of intestinal absorptive, metabolic, and exsorptive behavior for an improved understanding of drug oral bioavailability. The applicability of the newly developed, segregated flow, intestinal model (Cong et al., 2000) that describes partial intestinal blood flow to the enteroocyte region and route-dependent intestinal metabolism—a greater extent of intestinal metabolism with oral over systemic dosing—may be further tested with the acquired data.

**Experimental Procedures**

**Materials.** Unlabeled BA and its glycine conjugate, hippuric acid (HA), were purchased from Sigma Chemical Co. (St. Louis, MO). [14C]BA (specific activity, 16 mCi/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). The radiochemical purity of BA was >99%, as judged by HPLC. All reagents used were of glass-distilled HPLC grade or of the highest purity available.

**Intestinal Perfusion.** Perfusion apparatus and perfusate. A Two/Ten perfuser (MX International, Aurora, CO), equipped with two reservoir units, was used for recircling perfusion of the rat small intestine. Perfusate consisted of 20% of washed freshly obtained bovine red blood cells (a kind gift of Ryding Regency, Toronto, ON, Canada), 4% bovine serum albumin (Sigma Chemical Co.), 300 mg/dl glucose (Abbott Laboratories Ltd., Montreal, QC, Canada), and a complement of 20 amino acids in Krebs-Henseleit bicarbonate solution, buffered to pH 7.4 and oxygenated with carbogen (95% O2:5% CO2) and O2 (BOC Gases, Whiby, ON, Canada). After equilibration of the intestine with blank perfusate (reservoir 1) at 8 ml/min for 20 min, the study commenced upon recirculation with BA-containing perfusate (200 ml from reservoir 2) to the intestine, mimicking systemic drug administration. For oral studies, the study commenced when BA was rapidly introduced into the lumen of the designated segment during recirculation with drug-free perfusate (200 ml from reservoir 2).

Male Sprague-Dawley rats (300–400 g; Charles River, St. Constant, QC, Canada) were used as intestine donors. The rats, housed in accordance to protocols set forth by the University of Toronto Animal Committee and kept under artificial light on a 12-h light/dark cycle, were allowed access to water and food ad libitum. On the day before surgery, the rat was allowed free access to 2% glucose in drinking water but abstained from solid food for 24 h. After induction of anesthesia (intraperitoneal dose of sodium pentobarbital, 50 mg/kg), surgery was conducted as described previously (Hirayama et al., 1989). Anesthesia was maintained with an inline electrode and was adjusted to 7.4 by alteration of the inflow of temperature of the preparation at 37°C. The pH of the perfusate was monitored previously (Doherty and Pang, 2000). A heating lamp was used to maintain the temperature at 37°C—over systemic dosing—of the designated segment during recirculation with drug-free perfusate (200 ml from reservoir 2).

Intraluminal dosing. For systemic administration, BA was mixed thoroughly in perfusate of reservoir 2 to result in varying input concentrations (tracer [14C]BA of 44 ± 2.3 × 103 dpm/ml or 1.25 ± 0.06 and 432 ± 13 μM). For the studies that entailed injection of BA into the entire intestine, the dose (0.12–3.68 μmol, containing 6.5 ± 2.8 × 106 dpm in 0.4 ml of physiological saline solution, pH 7) was injected via a 1-ml tuberculin syringe directly into the lumen of the duodenum at 2 cm below the pyloric sphincter. For segmental studies, a tracer dose of [14C]BA (5.3 ± 2.9 × 106 dpm or 0.150 ± 0.084 μmol) was injected into a closed segment (12 or 20 cm, traced by silk thread) of the duodenum, jejunum, or ileum. Ligatures were placed proximally and distally of the intestinal segment for the creation of a closed loop to confine BA within the desired segment for absorption. In view of the shorter length of the duodenum, a 12-cm closed loop was chosen for study, and a similar length was also used for the jejunum; a 20-cm segment was used for both jejunum and ileum. The 12-cm duodenal loop originated at 2 cm from the pylorus; the jejunal segment (12- or 20-cm segments) was chosen at about 10 cm from the ligament of Treitz; the ileal segment (20-cm closed segment) was marked as ~22 to 2 cm from the ileocecal end. Outflow cannules were made at the ends of segments not receiving drug. Reservoir-2 perfusate samples were taken at 0, 2, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 min after the commencement of the study. The total sampling volume accounted for less than 10% of the original volume. The volume of perfusate remaining in reservoir 2 was recorded and added to the volume of perfusate sampled for mass and volume conservation considerations. At the conclusion of the experiment, the intestinal segments (injected or noninjected) were cleared of their luminal contents, cleansed by two 1-ml saline washes, and the contents were pooled. The intestine was then isolated from the carcass, gently rinsed, weighed, and homogenized for analysis of radioactivity.

**Preparation of Enterocytes and Immunoblot Analysis.** Intestinal cells were prepared according to Traber et al. (1991), with modifications. The intestine was cut into eight segments. The first was the duodenum (from duodenal end to the ligament of Treitz; 40–50 mm); the second segment of comparable length (segment 2) of the jejunum continued from the ligament of Treitz. The remaining length was equally divided into segments 3 to 8. The lumen of the strips was flushed with ice-cold PBS and filled with buffer A (96 mM NaCl, 27 mM sodium citrate, 1.5 mM KCl, 8 mM KH2PO4, 5.6 mM Na2HPO4, and 175 μg/ml PMSF) for incubation at 37°C for 15 min. After drainage, buffer B (109 mM NaCl, 2.4 mM KCl, 1.5 mM KH2PO4, 10.8 mM Na2HPO4, 1.2 mM EDTA, 10 mM glucose, 0.5 mM dithiothreitol, and 175 μg/ml PMSF) was used for filling the lumen for consecutive 4-, 4-, and 7-min incubations. The washings, which contained the enterocytes, were pooled and pelleted by centrifugation at 100g for 5 min at 4°C, resuspended in ice-cold PBS (2.1 ml/v), and kept at ~80°C until analysis. After thawing of the cells over ice, equal volumes of lysis buffer (4 mM PMSF, 2 mM EDTA, 4 mM EGTA, 0.25 mM dithiothreitol, 0.2 mM Na2CO3, and protease inhibitor cocktail (Sigma Chemical Co.; diluted 5-fold with 0.2 mM Na2CO3)) and ice-cold PBS were added and mixed. The mixture was centrifuged at 5000g for 10 min at 4°C. The supernatant was centrifuged at 27,500g for 1 h at 4°C, and the pellet was used for analyses. Aliquots of 12.5 μg of protein of the diluted, resuspended pellet sample, quantified by the method of Lowry et al. (1951), were resolved by SDS-polyacrylamide gel electrophoresis in 8% polyacrylamide gel, electrophoretically transferred to nitrocellulose membrane, and incubated with 10% nonfat milk (w/v) in TBST buffer overnight at 4°C. After three 15-min washes with TBST buffer, primary chicken-anti-Mct1 polyclonal antibodies (1:10,000; purchased from Chemicon International, Inc., Temecula, CA) were added and incubated with the blots for 1 hr at room temperature. Subsequent to three 15-min washes with TBST buffer, rabbit anti-chicken IgG horsedarid poridoxazed-conjugated affinity purified secondary antibodies (1:10,000; purchased from Chemicon International, Inc.) were added. Following three 15-min washes with TBST buffer, the immun conjugates were detected by chemiluminescence (enhanced chemiluminescence; Amersham Pharmacia Biotech, Piscataway, NJ). The intensity of the protein band was integrated using the NIH Image software (Bethesda, MD; http://rsbic.nih.gov/nih-image/) and normalized against that of a standard sample that was present in all blots.

**n-Octanol and Buffer Partitioning of Benzoic Acid and Acetaminophen.** Radiolabeled BA (approximately 100,000 dpm) was placed into glass tubes and dried under nitrogen. Buffers (1.95 ml at pH 1, 2, 5, 6, 7, and 8) and 50 μl of a saturated solution of unlabeled BA were mixed with the dried [14C]BA. Equivalents (2 ml) of buffer containing [14C]BA and n-octanol were placed in test tubes. Contents of the capped tubes were then rocked for 2 to 3 h with an aliquot mixer, then left to equilibrate overnight. A volume (1.5 ml) of n-octanol and 500 μl of the buffer (in triplicates) were removed for scintillation counting. The partitioning study for acetaminophen was carried out in the same manner as that described for benzoate. Aliquots of 500 μl of unlabeled acetaminophen (1 mM), a neutral compound, were added to buffers (1.5 ml) of varying pH values (1, 2, 5, 6, 7, and 8). Acetaminophen in n-octanol or buffer was analyzed after removal of 25 μl of the samples into 175 μl of n-octanol or buffer for dilution of the samples before HPLC.

**Analytical Procedures.** HPLC for benzoate and hippurate. Since BA and HA were not distributed into red blood cells (Geng and Pang, 1999), perfusate plasma (350 μl) was added to the internal standard, methoxybenzoic acid (50 μl of 16 μg/ml solution in water), deproteinized with 800 μl of acetonitrile, dried under nitrogen, and reconstituted for injection into the HPLC. A modified HPLC procedure of Chiba et al. (1994) was used—the normal run time
with a solvent gradient, consisting of 0.5% acetic acid and acetonitrile, was added to a wash period of increasing acetonitrile (from 30% and 50% over 2 min, then gradually returning to the original condition of 10% over 6 min). Radiolabeled BA and HA eluting at the various intervals were predetermined upon characterizing the radioelution at 1-min fractions (model LS 5801; Beckman Instruments). Standards for calibration curves (varying amounts of unlabeled and/or [14C]BA) were processed under identical conditions. Unlabeled and radiolabeled BA were quantified by comparing the ratios of the areas of BA to internal standard, against known concentrations of unlabeled/radio-labeled BA in the calibration curves.

Thin layer chromatographic (TLC) assay for BA and HA. In addition to HPLC, a TLC procedure (chloroform/cyclohexane/acetic acid, 80:20:10 v/v/v and Silica Gel GF 250-μm plates; Analtech, Newark, DE) was used to examine the presence of [14C]HA in the perfusate and luminal fluids. However, none was found. Since metabolites were absent in the luminal fluids and plasma, the total radioactivity of the sample was taken to represent [14C]BA. The luminal fluid was centrifuged, and the supernatant (made up to 1 ml with water) was added to 5 ml of acetonitrile, mixed thoroughly, and 3 ml of the resultant solution was subjected to scintillation counting and for TLC. To account for recovery, known amounts of [14C]BA were added to blank homogenized tissue (1 ml) and subjected to the same procedure. Recovery of the procedure was 73%.

Intestinal tissue was also analyzed. The weighed tissue was reduced to fine pieces, then homogenized (Ultra Turrax T25 homogenizer; Janke and Kunkel, IKA-Labortechnik, Staufen im Briesgau, Germany) with 2 volumes of Krebs-Henseleit-bicarbonate buffer. One milliliter of the homogenate was then added to 5 ml of acetonitrile and mixed thoroughly, and 3 ml of the resultant solution were subjected to scintillation counting and for TLC. To account for recovery, known amounts of [14C]BA were added to blank homogenized tissue (1 ml) and subjected to the same procedure. Recovery of the procedure was 57%.

HPLC assay of acetaminophen. Samples obtained from the partitioning studies were diluted 1:5 with n-octanol or buffer, and 5 μl was injected into a 10-μm Waters reverse phase, C18 μBondapak column (Waters, Milford, MA). Separation was achieved using a mobile phase consisting of 25% methanol-water flowing at 0.7 ml/min with a detection wavelength at 254 nm. The retention time of acetaminophen was 5.9 min. The ratio of the peak areas for n-octanol/buffer was obtained for all of the pH values studied.

Calculation of P_{oct} and P_{app}. According to the Henderson-Hasselbach equation, the concentration ratio of ionized to un-ionized species (C_{ionized}/C_{un-ionized}) was related to the pH and pK_{a},

\[ \text{pH} = pK_a + \frac{C_{\text{ionized}}}{C_{\text{un-ionized}}} \]

and the concentration ratio of un-ionized to ionized species (C_{un-ionized} to C_{ionized} or f) for a weak acid is \( 1/(10^{pH - pK_a}) \). Although the true partitioning ratio (P_{oct})—the ratio of C_{un-ionized} in n-octanol to C_{un-ionized,w} in water—is pH-independent, the apparent partitioning ratio (P_{app}) or concentration ratio of C_{un-ionized} in n-octanol to the sum of the un-ionized and ionized concentrations in water (C_{un-ionized,w} + C_{ionized,w}) is pH-dependent. The two parameters are inter-related in the following relationship that is universal for weak bases and acids (Ishizaki et al., 1997).

Fig. 1. Schematic presentation of TM (A) and SFM (B).
TABLE 1
Input parameters used for both TM and SFM for simultaneous fitting to data of BA after systemic and luminal dosing.

<table>
<thead>
<tr>
<th>Description</th>
<th>Symbol</th>
<th>Traditional Model</th>
<th>Segregated Flow Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracer oral dose</td>
<td>Dose&lt;sub&gt;oral&lt;/sub&gt;</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tracer IV dose</td>
<td>Dose&lt;sub&gt;IV&lt;/sub&gt;</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compartment volumes (ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reservoir</td>
<td>V&lt;sub&gt;r&lt;/sub&gt;</td>
<td>200&lt;sup&gt;b&lt;/sup&gt;</td>
<td>200</td>
</tr>
<tr>
<td>Intestinal tissue</td>
<td>V&lt;sub&gt;i&lt;/sub&gt;</td>
<td>1.62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.162&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Enterocyte layer</td>
<td>V&lt;sub&gt;en&lt;/sub&gt;</td>
<td>1.458&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Serosa and other tissues</td>
<td>V&lt;sub&gt;s,b&lt;/sub&gt;</td>
<td>0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.09&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>Intestinal blood volume</td>
<td>V&lt;sub&gt;int&lt;/sub&gt;</td>
<td>0.81&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Enterocyte blood</td>
<td>V&lt;sub&gt;en,b&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serosal blood</td>
<td>V&lt;sub&gt;s,b&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate (ml/min)</td>
<td>Q&lt;sub&gt;f&lt;/sub&gt;</td>
<td>8&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
</tr>
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</table>

The value of P<sub>app</sub> is expected to correlate with drug permeability by passive diffusion across the intestine.

Fitting of Physiological Models to Data. Data obtained for the administrations of tracer doses of BA into the reservoir and into the duodenal end for absorption by the whole intestine were used for fitting. The traditional, physiologically based model (TM) and the segregated flow model (SFM) were fit to the data. The TM views the intestinal tissue as a single compartment, and intestinal flow is not partitioned (Fig. 1A) (Cong et al., 2000). By contrast, the SFM describes the intestine as two subcompartments being perfused by separate flows, Q<sub>r</sub> and Q<sub>f</sub>, respectively, to the enterocyte and serosal layers, and the summed flow equals the intestinal blood flow, Q<sub>f</sub>, (Fig. 1B) (Cong et al., 2000); the fractional flow to the enterocyte layer is given by Q<sub>en</sub>/Q<sub>f</sub> or f<sub>en</sub>. Both models describe transport (CL<sub>a</sub>), exsorptive (CL<sub>sec</sub>), and gastrointestinal degradation/transit (CL<sub>decomp</sub>) intrinsic clearances and drug partitioning into tissue, denoted by CL<sub>d<sub>1</sub>-</sub>, CL<sub>d<sub>2</sub>-</sub>, CL<sub>d</sub>, and CL<sub>d<sub>4</sub>-</sub>; for the sake of simplicity, CL<sub>d<sub>2</sub>-</sub> was set equal to CL<sub>d<sub>1</sub>-</sub> and CL<sub>d<sub>2</sub>-</sub> to CL<sub>d</sub>. The metabolic intrinsic clearance was absent in the intestinal tissue for TM and in the enterocyte layer for the SFM since metabolism was not observed.

Mass-balanced rate equations were developed, as shown by Cong et al. (2000), for the simultaneous fitting of the systemic and oral BA data arising after tracer dose administration. The physiological constants used for fitting were adopted from Cong et al. (2000) (Table 1). The fitting program, Scientist (Micromath, Salt Lake City, UT), was used. Various weighting schemes were attempted to arrive at the optimal fits; the weighting scheme of one/prediction furnished the best fit, evidenced by the lowest residual sum of squares, the S.D. of parameter estimates, and the residual plots.

Statistical Analysis. All data were presented as the mean ± S.D., and the means were compared by use of ANOVA, with the P value of 0.05 as the level of significance.

Results

Intestinal Viability. Viability of the vascularity perfused in situ rat small intestine preparation was similar to that previously characterized in our laboratory (Hirayama et al., 1989), and there was constant perfusion pressure (54 ± 17 mm of Hg) during the study and good recovery of reservoir volume (94 ± 2.1%1 at the end of study. The hematocrit at the end of the experiment was increased only slightly by 9.5 ± 1.9% of the original values. These results were indicative of the sound viability of the intestinal preparation (Hirayama et al., 1989).

Systemic Administration of Benzoic Acid. Upon recirculation of BA at low (1.2–1.9 µM; mean of 1.25 µM; n = 3) and high (414–450 µM; mean of 432 µM; n = 4) concentrations to the rat small intestine preparation, HA was not detected in either plasma or luminal fluid. There was an initial, short distribution phase, but levels of BA in perfusate remained high and constant in the reservoir perfusate (94.3 ± 1.2 and 93 ± 0.8% for low- and high-concentration studies, respectively; Table 2). The loss was almost completely attributed to the appearance of BA in lumen (4.5 ± 0.8 and 3.5 ± 1.5% for the low and high doses, respectively). After accounting for the

TABLE 2
Lack of metabolism but presence of excretion of benzoic acid in the recirculating perfused rat intestine preparations when low (1.16 to 1.3 µM consisting of labeled [14C]BA only) or high (414 to 450 µM consisting of unlabeled BA only) concentrations of BA were present in the reservoir.

<table>
<thead>
<tr>
<th>% Dose</th>
<th>1.25 µM</th>
<th>432 µM</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Reservoir perfusate</td>
<td>94.3 ± 1.2</td>
<td>93.1 ± 0.8</td>
</tr>
<tr>
<td>Luminal fluid</td>
<td>4.5 ± 0.9</td>
<td>3.5 ± 1.5</td>
</tr>
<tr>
<td>Tissue</td>
<td>0.244 ± 0.032</td>
<td>N.M.</td>
</tr>
<tr>
<td>Total recovery&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.0 ± 0.4</td>
<td>97.8 ± 0.4</td>
</tr>
</tbody>
</table>

N.M., not measured.

<sup>a</sup> Sum of amounts of benzoic acid in perfusate, luminal fluid, and tissue.

FIG. 2. Absorption of [14C]benzoic acid when tracer doses (0.119–0.214 µmol) in physiological saline solution were injected directly into the duodenum for absorption by the whole small intestine.

A, the extent of BA absorbed were reflected by the amounts of BA in reservoir perfusate versus time. The ARA plot is a semilogarithmic plot of ARA versus time. Data for regression were restricted to the first 20 min (five data points), and absorption was completed within 60 min. B, regression (——) furnished the slope, which, when multiplied by the constant 2.303, yielded the first-order absorption rate constant k<sub>f</sub> for absorption; the regressed line was extended (-----) to later times.
partitioning of BA in intestine tissue, recovery of dose was virtually complete, and similar dose recovery ($P > 0.05$) was observed for both the tracer and high dose of BA (Table 2).

**Intraduodenal Administration of Benzoic Acid for Absorption by the Entire Small Intestine.** After an intraduodenal injection of a tracer dose of BA ($0.166 \pm 0.035$ $\mu$mol comprising only of $5.8 \pm 1.2 \times 10^6$ dpm $[^{14}C]$BA; $n = 3$), the appearance of $[^{14}C]$BA in the recirculating perfusate was rapid (Fig. 2A). The extent of drug absorption at the end of 2 h of perfusion was virtually complete (96.7 $\pm$ 0.1% dose). Only a negligible accumulation of BA was observed in intestinal tissue ($<0.2%$ dose), and a minor proportion of the dose ($1.80 \pm 0.08%$ dose) was recovered from the lumen. The apparent first-order rate constant $k_a$ was obtained by plotting the difference between the amount ultimately absorbed (asymptotic value) and that at various time points—the amount remaining to be absorbed or ARA versus the time on semilogarithmic paper (Fig. 2B). The kinetics of absorption of benzoic acid was found to remain unaltered upon increasing the luminal dose to 3.68 $\mu$mol (0.4 ml of physiological saline solution). Within the dose range studied, concentration-independent absorption was observed (Table 3). There was no apparent change in the extent of BA absorption, and recoveries of BA in reservoir perfusate (94.6 $\pm$ 0.9%), lumen (2.5 $\pm$ 0.9% dose), and intestine ($<0.2%$ dose) were similar for the various doses ($P > 0.05$). Upon performance of the ARA plots, the resultant $k_a$ remained dose-independent. Again, the metabolite was not found in perfusate nor luminal fluid. Good recoveries of the dose and volume were again observed.

**Absorption of Tracer Dose of Benzoate by Various Closed Segments (12-cm or 20-cm) of the Duodenum, Jejunum, or Ileum.** Inasmuch as the lack of dose dependence in the kinetics of absorption of BA, intrasegmental injection studies were conducted with tracer doses of $[^{14}C]$BA ($5.28 \pm 2.94 \times 10^6$ dpm or $0.15 \pm 0.064$ $\mu$mol). Little difference was found in the extents of absorption, regardless of the segment and the length of the closed loop used for injection (Table 3). The total radioactivity remaining at the closed loop for injection were 2, 1.3 to 1.7, and 4.5% dose, respectively, for the duodenum, jejunum, and ileum and were not different ($P > 0.05$; ANOVA) from that for dosing of tracer $[^{14}C]$BA to the entire intestine. Again, there was no difference in the absorption rate constants, $k_a$ for BA absorption by the entire intestine, duodenum, jejunum, and ileum segments ($P > 0.05$; ANOVA), and the metabolite HA was absent in homogenized intestinal tissue, and the metabolite HA was absent in the system.

Upon a closer comparison of data obtained from closed loops of same lengths, differences existed for the amounts absorbed by the jejunum versus the duodenum and the jejunum versus ileum at early time points (<0.9 min) (Figs. 3A and 4A), and for the $k_a$ for absorption (Figs. 3B and 4B; Table 4). The $k_a$ was highest for the jejunum (similar for 12- and 20-cm loops), less for the duodenum, and least for the ileum. These differences resulted in a higher ($P < 0.0005$) amount of benzoic acid left in the jejunum segment of the ileum at the end of the 2-h perfusion. However, the difference observed in the percentage of tracer doses secreted into various intestinal segments was not statistically significant, although it is noteworthy that a greater intra-animal variability existed in luminal secretions for the ileum as the injection segment. Good recoveries of the doses and volumes were again observed.

**Fitting.** The simultaneous fits of the traditional model and the segregated flow model to the systemic and duodenal BA data are shown (Fig. 5). Apparently good fits were obtained with the TM and SFM, although there was a systematic trend for the fits to the oral data. However, the fit to the SFM was slightly more superior than that for the TM as evidenced by the lesser residual sum of squares and higher correlation coefficient (Table 5). Some resemblance was observed among the fitted parameters—the ratio of the fitted distribution parameters $CL_{d1}$ and $CL_{d2}$ yielded similar ratios (4.5 and 3.5 for TM and SFM, respectively). The estimated fractional flow to the enterocyte layer was about 7% of total intestinal flow and mirrored the findings of Cong et al. (2000). This also concurred with the observations of Granger et al. (1980), who found that the proportion of blood flow perfusing the enterocytes was small. The estimated efflux intrinsic clearance ($CL_{intr}$) was necessarily higher for the SFM than for the TM since a much lower flow to the enterocyte layer prevailed. However, values of the absorption intrinsic clearance ($CL_a$) were similar for both the TM and SFM.

**n-Octanol and Buffer Partition of Benzoic Acid and Acetaminophen.** A pH dependence of BA between n-octanol and buffer was observed, showing that BA preferentially distributed into n-octanol only at low-pH values (Fig. 6). The value of $P_{oct}$ estimated according
to eq. 2 for BA was high and similar for all the pH values used (mean value of 70/11006/13). However, the apparent partition coefficient (P_app) of BA (concentration ratio in n-octanol to buffer) revealed a sigmoidal decrease with increasing pH. In comparison, the P_app of acetaminophen, a neutral and lipophilic compound that exhibits flow-limited distribution in liver (Pang et al., 1995), was pH-independent between the pH range of 3 to 7.

Western Blotting of Mct1. The distribution of Mct1, normalized to the band-intensity of the chosen standard (segment 5 of one of the intestinal preparations), was not uniform along the rat small intestine (Fig. 7). The activity was highest at segment 2 (the jejunum), slightly less for segment 1 (the duodenum), and was lower for the sixth, seventh, and eighth segments (the ileum). Since segments 1, 2, and 8, respectively, corresponded to the duodenal, jejunal, and duodenal segments used for closed-loop segmental studies, a correspondence was found between the presence of Mct1 protein and the absorptive function for BA in the closed-loop perfusion experiments.

Discussion

The perfusion studies of the rat small intestine, designed to examine processes of intestinal transport, metabolism, and secretion of BA, revealed that little distribution and metabolism had occurred and that the entire dose was recovered as unchanged BA in perfusate and lumen after systemic or oral dosing (Tables 2 and 3). Conjugated metabolites were absent in either luminal fluid or perfusate when BA was given intraluminally, and absorption of benzoic acid was rapid and almost complete at the end of 2 h of perfusion (Fig. 2). The results differed from the observation of Strahl and Barr (1971) who observed intestinal glycine conjugation of [14C]BA to [14C]HA, albeit low, in the rat intestinal slices in vitro and everted intestinal preparations. The small amounts of HA formed in these studies were materially insignificant and would not affect the overall mass balance of the system. By contrast, luminal secretion was observed and, together with the tissue and perfusate contents of BA, accounted for the entire dose of BA administered.

Absorption functionalities have been expressed as the effective permeability (P_eff), a parameter often used for estimation of the rate and extent of absorption (Amidon et al., 1995; Fagerholm et al., 1996). The parameter is dependent on several physiological characteristics, such as the surface area of the intestinal tissue and the physicochemical properties of the substrate, including lipophilicity, molecular size, hydrogen-bonding capacity, and polar surface area (Winiwarter et al., 1998). The surface area available for passive diffusion might have explained the uneven transport of benzoic acid among segments since the duodenum and jejunum possess the greatest
Heterogeneity of net intestinal uptake of BA was observed (Table 4), and Western blotting (Fig. 7) correlated to the absorptive functions of the segmental regions (Figs. 3 and 4), inferring a faster absorption for the jejunum, lesser rates by the duodenum, and the lowest for the ileum. The involvement of the transporter Mct1 should have displayed dose-dependent absorption rate constants and decreasing extents of absorption with increasing doses. However, statistically indistinguishable \( k_a \) values (about 0.0467 min\(^{-1}\)) and similar extents of absorption were observed. Although the amounts (3.68 \( \mu \)mol/0.4 ml) injected were initially present in a dose concentration as high as 9.2 mM, a value that is 3 times the \( K_m \) of Mct1 (Tamai et al., 1999), the injection segment was filled rapidly with luminal fluid that diluted surface areas due to the highest concentration of villi and microvilli in the regions and the surface area is least for the ileum (Magee and Dalley, 1986). Lipophilicity, a major determinant for predicting the extent of absorption, is often correlated with the partition coefficient when aqueous solubility is not exceeded and when the unstirred water layer is not an imposing barrier (Ungell et al., 1998). However, a \( K_a \) of 0.0464 ± 0.0010 min\(^{-1}\) was obtained for BA despite its low \( P_{app} \) (0.13) \( n \)-octanol/buffer-partitioning value at pH 7 (Fig. 6). This value of \( K_a \) was less in comparison to that for aceticaminophen (0.224 ± 0.041 min\(^{-1}\)) in similar vascularly perfused rat small intestine studies (Pang et al., 1986); aceticaminophen, a neutral lipophilic compound of good partitioning characteristics (see Fig. 6), is shown to be transported by passive diffusion into tissues in a flow-limited fashion (Pang et al., 1995). At pH 7, the \( n \)-octanol/buffer ratio for aceticaminophen (≈2) and BA (0.13) differed by about 15-fold, whereas the ratio of the \( k_a \) values differed only by about 4.83-fold.

The rapid and almost complete absorption of BA, a weak organic acid with \( pK_a \) of 4.19, and the higher \( k_a \) for BA than expected from the ratio of the \( P_{app} \) values between BA and aceticaminophen implicates the presence transporter function. Indeed, Tamai et al. (1999) demonstrated transport of benzoic acid with a high \( K_m \) of about 3 mM by the proton-driven monocarboxyate transporter 1 in transfected cells versus mock cells. Immunohistochemical studies had revealed that Mct1 was present throughout the gastrointestinal tract, from the stomach to the large intestine. In the small intestine, Mct1 was found localized on the basolateral membrane of immature crypt cells and on the brush-border membrane of mature cells of the villi, and Mct1 transport activities expressed in MDA-MB231 cells appeared to be bidirectional and asymmetric. There was observable efflux and a much more rapid absorption of BA.

**TABLE 4**

Summary of volume recoveries, extents of absorption, luminal recoveries, and absorption rate constants obtained after injection of tracer doses of \(^{14}C\)benzoic acid into various intestinal segments

<table>
<thead>
<tr>
<th>Preparations/Segments Injected</th>
<th>Rat Weight</th>
<th>Hematocrit</th>
<th>Volume Recovery</th>
<th>Amount in Reservoir Perfusate</th>
<th>Amount in Luminal Fluid</th>
<th>Total Recovery</th>
<th>Absorption Rate Constant* ( k_a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Segment Injected</td>
<td>Segment(s) Not Injected</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>g</td>
<td>%</td>
<td></td>
<td>% dose</td>
<td></td>
<td>min(^{-1})</td>
<td></td>
</tr>
<tr>
<td>Duodenum (n = 3)</td>
<td>319</td>
<td>0.151</td>
<td>92.9</td>
<td>96.7</td>
<td>1.76</td>
<td>0.157</td>
<td>98.6</td>
</tr>
<tr>
<td>(Whole length)</td>
<td>±0.37</td>
<td>±0.002</td>
<td>±0.9</td>
<td>±1.1</td>
<td>±0.08</td>
<td>±0.111</td>
<td>±1.0</td>
</tr>
<tr>
<td>Duodenum (n = 5)</td>
<td>396</td>
<td>0.153</td>
<td>93.7</td>
<td>94.6</td>
<td>1.98</td>
<td>0.762</td>
<td>97.4</td>
</tr>
<tr>
<td>(Length = 12 cm)</td>
<td>±0.11</td>
<td>±0.004</td>
<td>±1.1</td>
<td>±3.0</td>
<td>±1.28</td>
<td>±0.239</td>
<td>±0.022</td>
</tr>
<tr>
<td>Jejunum (n = 4)</td>
<td>321</td>
<td>0.155</td>
<td>95.6(^{a,c})</td>
<td>95.5</td>
<td>1.33</td>
<td>0.185(^{a})</td>
<td>0.058</td>
</tr>
<tr>
<td>(Length = 12 cm)</td>
<td>±0.29</td>
<td>±0.006</td>
<td>±0.8</td>
<td>±0.4</td>
<td>±0.26</td>
<td>±0.007</td>
<td>±0.023</td>
</tr>
<tr>
<td>Jejunum (n = 5)</td>
<td>300</td>
<td>0.151</td>
<td>94.9</td>
<td>93.7</td>
<td>1.68</td>
<td>0.137</td>
<td>0.029</td>
</tr>
<tr>
<td>(Length = 20 cm)</td>
<td>±0.18</td>
<td>±0.006</td>
<td>±1.3</td>
<td>±1.3</td>
<td>±0.33</td>
<td>±0.041</td>
<td>±0.007</td>
</tr>
<tr>
<td>Ileum (n = 7)</td>
<td>301</td>
<td>0.156</td>
<td>91.8(^{b})</td>
<td>91.1(^{a})</td>
<td>5.04(^{a,b})</td>
<td>0.586</td>
<td>0.128</td>
</tr>
<tr>
<td>(Length = 20 cm)</td>
<td>±0.17</td>
<td>±0.009</td>
<td>±2.5</td>
<td>±1.1</td>
<td>±1.80</td>
<td>±0.595</td>
<td>±0.065</td>
</tr>
</tbody>
</table>

ANOVA: P > 0.05, P > 0.05, P > 0.05, P > 0.05, P > 0.05, P > 0.05, P > 0.05, P > 0.05

\(^{a}\) Sum of amounts in reservoir perfusate, lumen, and tissue.

\(^{b}\) Obtained from ARA plot.

\(^{c,d,e}\) Significantly different from whole intestine.

\(^{f}\) Statistical difference between duodenum and jejunum (length = 12 cm).

\(^{g}\) Statistical difference between jejunum (length = 20 cm) and ileum.

**TABLE 5**

Optimized parameters from the simultaneous fitting of systemic and intraduodenal data of benzoate from the recirculating, vascularly perfused rat small intestine with the traditional model (Fig. 1A) and the segregated flow model (Fig. 1B) obtained with the fitting procedure (based on the best weighting scheme of one per prediction)

Averaged data (expressed as % dose) for intravenous (n = 3) and intraduodenal (n = 4) injections of tracer BA were fitted simultaneously with mass-balanced equations for the TM and SFM (see Cong et al., 2000 for details of the equations).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Traditional Model</th>
<th>Segregated Flow Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL(_{1a}) (ml/min)</td>
<td>1.84 ± 0.71(^{a})</td>
<td>16.3 ± 50(^{a})</td>
</tr>
<tr>
<td>CL(_{12}) (ml/min)</td>
<td>0.41 ± 0.077</td>
<td>4.7 ± 14.8</td>
</tr>
<tr>
<td>CL(_{in}) (ml/min)</td>
<td>60 ± 1640</td>
<td>66 ± 1158</td>
</tr>
<tr>
<td>CL(_{out}) (ml/min)</td>
<td>69 ± 1877</td>
<td>107 ± 1859</td>
</tr>
<tr>
<td>CS(_{cry}) (ml/min)</td>
<td>~0</td>
<td>~0</td>
</tr>
<tr>
<td>( k_a )</td>
<td>0.070 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>MSC(^{c})</td>
<td>4.9</td>
<td>5.3</td>
</tr>
<tr>
<td>RSS</td>
<td>646</td>
<td>505</td>
</tr>
<tr>
<td>Coefficient of determination</td>
<td>0.995</td>
<td>0.997</td>
</tr>
</tbody>
</table>

\(^{a}\) Standard deviation of parameter estimate.

\(^{b}\) The greater the number, the better the fit.

MSC, model selection criterion; RSS, residual sum of squares.
uptake studies involving Mct1 (Tamai et al., 1999) posed as a stagnant system in which the influence of peristalsis and reserve length was absent and would not affect transport of BA.

Other examples on heterogeneity of intestinal transporters have been demonstrated. Expression of the proton-coupled oligopeptide transporter (Pept1) (Fei et al., 1994) and nucleoside transporter (Ngo et al., 2001) was more abundant in the proximal intestine. For P-glycoprotein substrates, the net mucosal to serosal absorption was greater for the jejunum/ileum (Collett et al., 1999). Gottoh et al. (2000) demonstrated dominance in mRNA expression of multidrug resistance-associated protein 2 (Mrp2) in the jejunum, followed by the duodenum and ileum, with very little in the colon, as confirmed by Mottino et al. (2000). The excretion of the glutathione conjugate 2,4-dinitrophenyl-S-gluathione by multidrug resistance-associated protein 2 was greatest in the jejunum and correlated to the mRNA expression (Gottoh et al., 2000).

The present studies with the in situ-recirculating, vascularly perfused small intestine preparation provided information on both drug absorptive and secretory capacities and on segmental absorption and exsorption of the intestine. The fit to the TM to the data was not as good as the SFM, which suggests that only 7% of intestinal blood flow to the enterocycle layer (Table 5). Fits to the data were, however, associated with high coefficients of variation, and \( CL_s \) and \( CL_{sec} \) were approximated with little assurance. Unfortunately, metabolism of hippurate was too low to be monitored, and proper selection of the intestine model was much hampered despite the slightly better fit of the SFM to data. It is speculated that model discrimination between the TM and the SFM would be much improved if metabolite data were present (Cong et al., 2000; Schwab et al., 2001). The same was found in modeling BA metabolism in rat liver when metabolic data were not used to refine the model (Schwab et al., 2001).

A rapid and uneven absorption of BA was observed among the segmental regions, being highest in the jejunum and slightly lower in the ileum. The absorption pattern of BA paralleled the distribution of Mct1, the monocarboxylic acid transporter 1 (Fig. 7). It is surmised that future studies on model substrates that display differential absorption, metabolism, and exsorption by the various segmental regions—duodenum, jejunum, and ileum—would allow for the integration of these events and the examination of their overall influence of these events on drug bioavailability with improved certainty.

### References


Geng W and Pang KS (1999) Differences in excretion of hippurate, as a metabolite of benzoate

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**Fig. 6.** Partitioning of benzoic acid, a weak acid, and acetaminophen, a neutral compound, between n-octanol and buffers of varying pH values.

**Fig. 7.** A, Western blots of Mct1 along the length of the small intestine (divided into eight segments).

B, the length of the midpoints of the segments from the pyloric sphincter were plotted against the normalized intensities of the bands (mean ± S.D.; \( n = 4 \)). See text for details.
and as an administered species in the single pass isolated perfused rat kidney explained. *J Pharmacol Exp Ther* **280**:597–606.  
Magee DF and Dalley AF II (1986) *Digestion and The Structure and Function of the Gut* Karger, Basel, Switzerland.  
Magee DF and Dalley AF II (1986) *Digestion and The Structure and Function of the Gut* Karger, Basel, Switzerland.  
Magee DF and Dalley AF II (1986) *Digestion and The Structure and Function of the Gut* Karger, Basel, Switzerland.  
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