Hepatic uptake and metabolism of benzoate: a multiple indicator dilution, perfused rat liver study

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¹McGill University Medical Clinic, Montreal General Hospital and ²Department of Medicine, McGill University, Montreal, Quebec H3G 1A4; ³Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Toronto, Toronto, Ontario M5S 2S2; and ⁴Department of Pharmacology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

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Schwab, Andreas J., Lei Tao, Tsutomu Yoshimura, André Simard, Ford Barker, and K. Sandy Pang. Hepatic uptake and metabolism of benzoate: a multiple indicator dilution, perfused rat liver study. Am J Physiol Gastrointest Liver Physiol 280: G1124–G1136, 2001.—Multiple, noneliminated references (⁵¹Cr-labeled erythrocytes, ¹²⁵I-albumin, [¹⁴C]- or [³H]sucrose, and [²H]₂O), together with [³H]hippurate or [¹⁴C]benzoate, were injected simultaneously into the portal vein of the perfused rat liver during single-pass delivery of benzoate (5–1,000 µM) and hippurate (5 µM) to investigate hippurate formation kinetics and transport. The outflow dilution data best fit a space-distributed model comprising vascular and cellular pools for benzoate and hippurate; there was further need to segregate the cellular pool of benzoate into shallow (cytosolic) and deep (mitochondrial) pools. Fitted values of the membrane permeability-surface area products for sinusoidal entry of unbound benzoate were fast and concentration independent (0.89 ± 0.17 ml·s⁻¹·g⁻¹) and greatly exceeded the plasma flow rate (0.0169 ± 0.0018 ml·s⁻¹·g⁻¹), whereas both the influx of benzoate into the deep pool and the formation of hippurate occurring therein appeared to be saturable. Results of the fit to the dilution data suggest rapid uptake of benzoate, with glycination occurring within the deep and not the shallow pool as the rate-determining step.

BENZOATE (BENZOIC ACID) is a monocarboxylate used as a food preservative and for the treatment of hyperammonemia (5). Its metabolism in humans and rats occurs exclusively by conjugation with glycine to form hippurate (hippuric acid; N-benzyolglycine) in the liver and kidney (1, 4, 6). The first step of this pathway is activation by benzyoyl-CoA ligase to form benzyoyl-CoA; a similar activation pathway also constitutes the first step of fatty acid catabolism by β-oxidation. Compounds like benzoate that do not carry hydrogen at the α- and β-positions, however, will not be degraded by β-oxidation. Rather, substitution of CoA by an amino acid may occur, such as the formation of a glycine conjugate by means of glycine N-acyltransferase (26). Both steps have been shown in rats and in cattle to proceed within the mitochondrial matrix, and energy-dependent conversion of benzoate to hippurate has been demonstrated in isolated rat and beef liver mitochondria (13).

Chiba et al. (8) suggested that hippurate synthesis from benzoate in the perfused rat liver proceeded with an overall Michaelis-Menten constant (Kₘ) of 12 µM and maximum velocity (V_max) of 101 nmol·min⁻¹·g⁻¹. The relative importance of transport vs. metabolism of benzoate on the overall glycination of benzoate in liver, however, has not been studied. Metabolism of benzoate within hepatocytes requires the bidirectional transport of substrate and metabolite across the basolateral plasma (sinusoidal) membrane and the mitochondrial outer and inner membranes. There exists information on carrier-mediated transport of benzoate across human erythrocyte (25) and intestinal Caco-2 cell membranes (35). The metabolite hippurate is not eliminated by the liver (8, 38), although avid influx and efflux occur across the basolateral membrane. Transport is most likely mediated by the hepatic monocarboxylate transporter 2 that appears to be inhibited by benzoate (12, 38). To date, however, no systematic investigation on monocarboxylate transport into mitochondria has been conducted, although the transport of pyruvate across the mitochondrial inner membrane was found to be mediated by specific carriers (7, 20). Previous studies have revealed that benzoate and hippurate are not distributed into erythrocytes, although binding to albumin exists (8, 38). In this study, we applied the multiple indicator dilution (MID) method to characterize the kinetics of hepatic transport and metabolism of benzoate to understand the roles of sinusoidal and mitochondrial transport in the overall glycine conjugation process.

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MATERIALS AND METHODS

Materials. Benzoic acid, hippuric acid, benzoyle chloride, and bovine serum albumin (fraction V) were purchased from Sigma Chemical (St. Louis, MO). $[^{14}C]$benzoic acid (specific activity, 4.07 GBq/mmol) and $[^{2-3}H]$glycine (specific activity, 1,600 GBq/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO) and DuPont Canada, (Markham, ON, Canada). Two other isotopes were used for the determination of the average input (Cin) and output (Cout) plasma concentrations of unlabeled hippurate and benzoate: $[^{51}Cr]$chromium (specific activity, 5,392 mBq/mg), and $[^{125}I]$labeled albumin (1.08 ± 0.06 mCi). The second dose contained $[^{14}C]$sucrose (183 mBq/mmol) and $[^{3}H]$hippurate (455 GBq/mmol) was obtained from NEN Life Science Products (Boston, MA). All reagents used were of glass-distilled, high-performance liquid chromatographic grade or the highest purity available (Fisher Scientific, Mississauga, ON, Canada).

Rat liver perfusion. Male Sprague-Dawley rats (Charles River Canada, St. Constant, QC, Canada; 295–367 g; livers were 9.1–13.4 g) were used for single-pass liver perfusion studies. The animals were housed in accordance with approved protocols of the University of Toronto Animal Committee, kept under artificial light on a 12:12-h light/dark cycle, and were allowed free access to water and food ad libitum. In situ single-pass liver perfusion was carried out at 37°C as previously described (8) with perfusate (12 ml/min) entering via the portal vein and exiting via the hepatic vein; the hepatic artery was ligated. The perfusate contained bovine erythrocytes (20%), freshly obtained and washed (Ryding Meats, Toronto, ON, Canada), 5% bovine serum albumin (fraction V) were purchased from Sigma Chemical, (St. Louis, MO). $[^{14}C]$benzoic acid (specific activity, 0.46 mBq/mg), and $[^{2-3}H]$water (99.98% pure) were purchased from Merck Frosst, Montreal, QC, Canada. $[^{14}C]$sucrose (183 mBq/mmol) and $[^{3}H]$sucrose (455 GBq/mmol) were obtained from NEN Life Science Products (Boston, MA). All reagents used were of glass-distilled, high-performance liquid chromatographic grade or the highest purity available (Fisher Scientific, Mississauga, ON, Canada).

Plasma outflow and bile samples (25–100 µl), supplemented with excess benzoate and hippurate, were assayed for the individual radiolabeled species by thin-layer chromatography (Silica Gel GF, 250 µm, Analtech, Newark, DE) with chloroform:cyclohexane:acetic acid (80:20:10, vol/vol/vol); the values for the distance traveled relative to the solvent front (Rf) for hippurate and benzoate were 0.15 and 0.95, respectively, and labeled sucrose and albumin did not interfere with the radiocchromatograms. The ratios associated with the authentic standards were visualized under ultraviolet light and scraped into scintillation vials. After the addition of 0.5 ml of water and 10 ml of scintillation cocktail (Ready Protein, Beckman), the radioactivity of each sample was assayed by liquid scintillation counting.

Data treatment. For the MID data, the concentration of radiolabel in the outflowing perfusate was normalized to the dose, yielding fractional recovery (or concentration/dose) (16). The fractional recovery integral (area under the curve (AUC)) was approximated by dividing the products of fractional recoveries and sample interval with monoexponential extrapolation to infinity; the integral of the product of fractional recovery and time [at mid-intervals, area under the moment curve (AUMC)] was calculated similarly (16, 30). The ratio of AUMC to AUC yielded the mean transit time (MTT). Tracer recoveries were obtained by multiplying AUCs with perfusate flow. The hippurate synthesis rate was obtained as the difference between the inflow and outflow plasma hippurate unlabeled concentrations multiplied by plasma flow, or, alternatively, as the tracer recovery of $[^{14}C]$hippurate multiplied to the steady-state delivery rate of plasma flow. Plasma flow was calculated as perfusate flow × (1 – hematocrit). These calculations were based on the findings that benzoate and hippurate do not enter erythrocytes and that biliary excretion and metabolism of hippurate are negligible (38).

Modeling of hepatic benzoate disposition. The kinetic events underlying the disposition of benzoate in the perfused rat liver are displayed schematically in Fig. 1. Benzoate and hippurate are present in the plasma space as protein-bound and nonprotein-bound (unbound) forms, and it is assumed that only the unbound form in the plasma space exchanges with that in the cellular space of hepatocytes. Furthermore, rapid equilibrium between bound and unbound forms is as-

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Fig. 1. Schematic representation of the models used to interpret benzoate uptake and biotransformation to hippurate at the level of a single sinusoid. A: model A with a single substrate intracellular pool. B and C: models B and C with 2 substrate intracellular pools and metabolite formation from shallow and deep pool, respectively. Rapid equilibrium is assumed between protein-bound and unbound forms of benzoate and hippurate in plasma and in the interstitial space, resulting in lumped intravascular pools. Hatched line: endothelial cells. See Fitting procedures for definitions of terms.
Results of recoveries, moment analyses, and transit times of outflow profiles

<table>
<thead>
<tr>
<th>Tracer recoveries</th>
<th>Tracer recoveries</th>
<th>Tracer recoveries</th>
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<th>Tracer recoveries</th>
<th>Tracer recoveries</th>
<th>Tracer recoveries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippurate injection</td>
<td>1.03 ± 0.09</td>
<td>0.99 ± 0.12</td>
<td>0.99 ± 0.08</td>
<td>1.03 ± 0.10</td>
<td>1.02 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Benzoate injection</td>
<td>1.13 ± 0.13</td>
<td>1.00 ± 0.11</td>
<td>0.98 ± 0.17</td>
<td>0.62 ± 0.23</td>
<td>0.31 ± 0.18*</td>
<td>0.93 ± 0.17</td>
</tr>
<tr>
<td>Mean transit times, s</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippurate injection</td>
<td>9.9 ± 2.5</td>
<td>16.1 ± 2.9</td>
<td>19.5 ± 2.7</td>
<td>58.5 ± 7.7</td>
<td>28.3 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>Benzoate injection</td>
<td>9.7 ± 2.7</td>
<td>16.0 ± 3.2</td>
<td>18.9 ± 4.5</td>
<td>31.6 ± 11.3</td>
<td>113.6 ± 32.1*</td>
<td></td>
</tr>
</tbody>
</table>

Values shown are means ± SD obtained from 11 animals. *P < 0.01 (paired t-test) for hippurate vs. benzoate injection.
the injected benzoate returned to the vasculature either as unchanged benzoate or as hippurate, confirming that benzoate and hippurate were not significantly excreted and that benzoate was metabolized exclusively to hippurate that was not further metabolized. Tracer [14C]hippurate recoveries decreased with increasing unlabeled benzoate concentrations, indicating saturation of benzoate conjugation. Upon fitting of hippurate formation rates to the $K_m$ equation, with the logarithmic average unbound concentration of benzoate taken as the substrate concentration (Fig. 2), the optimized maximal rate of the overall conjugation of 78 (6 nmol·min$^{-1}$·g$^{-1}$) was found to be similar to that (101 nmol·min$^{-1}$·g$^{-1}$) determined previously in steady-state rat liver perfusions (8). However, the unbound benzoate concentration at half-maximal conjugation ($K_{m,overall}$ of 2.6 ± 1.0 μM) was slightly lower than previously reported (12 μM). This small discrepancy was probably due to interanimal variations. Nevertheless, these values of the $K_{m,overall}$ were of the same order of magnitude. Rates of hippurate formation based on recoveries from the injection of [14C]benzoate furnished similar parameters ($V_{max,overall}$ of 68 ± 5 nmol·min$^{-1}$·g$^{-1}$ and $K_{m,overall}$ of 3.8 ± 1.2 μM).

**Outflow profiles.** Representative outflow profiles of tracer [3H]hippurate (first injection), of tracer [14C]benzoate and its metabolite [14C]hippurate (second injection), and of the noneliminated indicators are shown in Figs. 3 and 4, respectively, for low and high concentrations of unlabeled benzoate and of 5 μM hippurate in perfusate.

**Linear superposition by use of the delayed-wave model.** The outflow profiles of the noneliminated indicators for the first and the second injection (labeled erythrocytes, albumin, sucrose, and 2H2O) in hepatic venous blood were increasingly dampened in magnitude and prolonged in time, but their shapes were otherwise similar (16, 17). MTTs of the noneliminated indicators (Table 1) and values of $t_0$ and $\gamma$ (Table 2) were virtually identical for both of the injections made at 15 and 35 min, and the values were similar to those obtained in previous perfused rat liver MID studies (16, 17, 27, 28, 30, 38). This observation attests to the stability of the liver preparation and strongly suggests constancy of the transfer constants for hippurate during the experiment.

**Model fits to outflow profiles of [3H]hippurate.** Outflow profiles obtained after injection of [3H]hippurate (Fig. 3) were similar to those obtained previously in the
absence of benzoate (38). The rising upslope of [3H]hippurate was slightly delayed with respect to that of labeled albumin, but it preceded slightly that of the labeled sucrose curve (Fig. 3), as expected due to binding of hippurate to albumin and entry into hepatocytes. The [3H]hippurate profile crossed over and then peaked lower and earlier than the labeled sucrose curve and exhibited a more delayed downslope. As the bulk benzoate concentration was increased, relatively little change was observed in the tracer [3H]hippurate curve (see Fig. 3) in relation to the curves for labeled albumin and labeled sucrose. The calculated outflow profiles showed a good fit to the [3H]hippurate data (Fig. 5). The optimal interstitial-to-sinusoid distribution ratio \( \gamma_{11} \) was slightly larger than the value of \( 1.08 \pm 0.37 \) calculated after consideration of binding of hippurate to albumin in plasma perfusate (27, 37, 38).

The transfer coefficients for transport of hippurate between plasma and hepatocytes \( k_{25} \) and \( k_{52} \); Table 3), the ratio of \( k_{25} \) and \( k_{52} \), or the equilibrium partitioning ratio \( (1.2 \pm 0.27) \), the influx permeability surface area product \( (P_{25S} \) of \( 0.052 \pm 0.019 \text{ ml}\cdot\text{s}^{-1}\cdot\text{g}^{-1} \), and the throughput component \( (48.8 \pm 6.3\% \text{ of dose; Table 3}) \) remained constant against the various unbound plasma concentrations of benzoate but were significantly smaller than those previously obtained when benzoate was absent in the perfusion fluid \( (P < 0.05) \) (38). The combined observations can tentatively be interpreted as an inhibitory influence of benzoate.

Model fits to outflow profiles of \[^{14}C\]benzoate and of formed \[^{14}C\]hippurate. Outflow profiles of tracer \[^{14}C\]benzoate showed a peak that roughly coincided in time with the \[^{3}H\]sucrose peak but was much lower in magnitude, followed by a prolonged tail (Fig. 4). Those of tracer \[^{14}C\]hippurate were much lower in magnitude and showed a peak that was considerably delayed relative to the sucrose profiles. At high benzoate concentrations, the tail of the \[^{14}C\]benzoate outflow profiles was increased in magnitude, whereas the \[^{14}C\]hippurate outflow profiles were reduced. This can be interpreted as saturation of the overall conjugation process, but the specific step or steps where this saturation occurs were not immediately apparent from inspection of the curves. A more detailed assessment of the kinetics of benzoate conjugation was obtained by modeling analysis of the experimental data.

A minimum of two intracellular benzoate pools, a shallow and a deep intracellular pool, were needed to fit the benzoate data, as can be clearly discerned by visual inspection of the fits obtained with the various models (Fig. 6). When the deep intracellular pool (compartment 4) was omitted from the model \( \text{(model A, Fig. 1A)} \), the calculated outflow profiles of benzoate and hippurate could not be fitted to the outflow data collected later than 50 s after injection (Fig. 6A). Similarly, the model denoting conversion of benzoate to hippurate in the shallow intracellular pool \( \text{(model B, Fig. 1B)} \) did not allow a good fit to the data. With this
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Fig. 3. Optimal calculated first injection outflow profiles of [3H]hippurate. Data are the same as those in Fig. 5.

Table 3. Optimal parameter values from fits of hippurate injection profiles

<table>
<thead>
<tr>
<th>Input Concentration of Benzoate, μM</th>
<th>γrel,H</th>
<th>γH</th>
<th>k25, s</th>
<th>k52, s</th>
<th>k25/k52</th>
<th>P25S, ml·s⁻¹·g⁻¹</th>
<th>Throughput, % of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1</td>
<td>0.38</td>
<td>-0.096 ± 0.006</td>
<td>0.890 ± 0.048</td>
<td>0.082 ± 0.003</td>
<td>0.088 ± 0.002</td>
<td>1.08</td>
<td>0.059 ± 0.002</td>
</tr>
<tr>
<td>10.4</td>
<td>0.32</td>
<td>-0.116 ± 0.005</td>
<td>1.766 ± 0.104</td>
<td>0.063 ± 0.002</td>
<td>0.070 ± 0.002</td>
<td>1.11</td>
<td>0.039 ± 0.001</td>
</tr>
<tr>
<td>56.4</td>
<td>3.23</td>
<td>-0.107 ± 0.004</td>
<td>1.239 ± 0.060</td>
<td>0.059 ± 0.001</td>
<td>0.055 ± 0.001</td>
<td>0.93</td>
<td>0.039 ± 0.001</td>
</tr>
<tr>
<td>57.7</td>
<td>3.14</td>
<td>-0.138 ± 0.004</td>
<td>0.877 ± 0.039</td>
<td>0.123 ± 0.003</td>
<td>0.148 ± 0.004</td>
<td>1.21</td>
<td>0.101 ± 0.002</td>
</tr>
<tr>
<td>167</td>
<td>9.38</td>
<td>-0.057 ± 0.007</td>
<td>0.836 ± 0.038</td>
<td>0.053 ± 0.002</td>
<td>0.094 ± 0.003</td>
<td>1.77</td>
<td>0.040 ± 0.002</td>
</tr>
<tr>
<td>170</td>
<td>11.6</td>
<td>-0.061 ± 0.001</td>
<td>0.887 ± 0.038</td>
<td>0.071 ± 0.002</td>
<td>0.092 ± 0.002</td>
<td>1.30</td>
<td>0.059 ± 0.001</td>
</tr>
<tr>
<td>179</td>
<td>10.6</td>
<td>-0.063 ± 0.005</td>
<td>0.104 ± 0.028</td>
<td>0.049 ± 0.001</td>
<td>0.077 ± 0.002</td>
<td>1.57</td>
<td>0.039 ± 0.001</td>
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<tr>
<td>463</td>
<td>40.9</td>
<td>-0.175 ± 0.008</td>
<td>1.861 ± 0.101</td>
<td>0.107 ± 0.004</td>
<td>0.095 ± 0.003</td>
<td>0.89</td>
<td>0.067 ± 0.003</td>
</tr>
<tr>
<td>464</td>
<td>38.5</td>
<td>-0.163 ± 0.008</td>
<td>1.640 ± 0.085</td>
<td>0.082 ± 0.003</td>
<td>0.079 ± 0.003</td>
<td>0.96</td>
<td>0.050 ± 0.002</td>
</tr>
<tr>
<td>817</td>
<td>103</td>
<td>-0.089 ± 0.007</td>
<td>1.023 ± 0.052</td>
<td>0.056 ± 0.002</td>
<td>0.066 ± 0.002</td>
<td>1.16</td>
<td>0.038 ± 0.002</td>
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<tr>
<td>873</td>
<td>109</td>
<td>-0.089 ± 0.005</td>
<td>1.024 ± 0.036</td>
<td>0.060 ± 0.002</td>
<td>0.067 ± 0.002</td>
<td>1.12</td>
<td>0.042 ± 0.001</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>-0.105 ± 0.040</td>
<td>1.190 ± 0.383</td>
<td>0.073 ± 0.023</td>
<td>0.085 ± 0.025</td>
<td>1.19 ± 0.27</td>
<td>0.052 ± 0.019</td>
<td>48.8 ± 6.3%</td>
</tr>
</tbody>
</table>

Values are means ± SD. γrel,H, as defined in Eq. A10; k25 and k52, cellular influx and efflux rate constants, respectively. P25S, permeability-surface area product for influx of nonprotein-bound hippurate into hepatic parenchymal cells. *Total benzoate input concentrations. Total hippurate input concentrations were kept constant at 5 μM. †Logarithmic average concentrations of non-protein-bound benzoate calculated as ln(Cout/Cin)/ln(Cout/Cin), where Cout and Cin are inflow and outflow benzoate concentrations, respectively, and f, is fraction unbound in plasma. ‡Uncertainties of parameters.

Fig. 5. Optimal calculated first injection outflow profiles of [3H]hippurate. Data are the same as those in Fig. 3.

Kinetics of benzoate glycine conjugation. Optimal parameters obtained with model C are summarized in Table 4. The parameters were only weakly to moderately correlated to each other, thus excluding complications of overparameterization of the model. The optimal interstitial to sinusoid distribution ratio of benzoate (γH) was slightly larger than the one calculated from the binding to plasma albumin (0.85 ± 0.40), as observed for hippurate (see Model fits to outflow profiles of [14C]benzoate and of formed [14C]hippurate). According to model C, the optimized permeability-surface products for uptake of unbound benzoate into the intracellular space (P12S = 0.89 ± 0.17 ml·s⁻¹·g⁻¹) were not significantly dependent on benzoate concentration (Fig. 7) and greatly exceeded the plasma flow rate (0.017 ± 0.002 ml·s⁻¹·g⁻¹). However, those for uptake into the deep intracellular pool (k34) and for irreversible metabolism of benzoate (k45) decreased with increasing benzoate concentration (Table 4; Fig. 7). This trend was also seen with the ratio of the transfer coefficients for uptake into the deep intracellular pool and return into the shallow intracellular pool (k34/k45; Fig. 7). Because of metabolic sequestration, the steady-state ratios of the benzoate content in the deep intracellular pool to that in the shallow intracellular pool was altered according to Eqs. B1–B3 in Appendix B. The proper selection of the optimal model of benzoate metabolism (model C) was thus dictated by the fit of the metabolite data. A much lower sum of squared residuals was obtained with model C than with models A and B (30× that of model C). Model C is the simplest model consistent with bidirectional transport of benzoate and hippurate between plasma and hepatocytes, irreversible metabolism, and the inclusion of a deep pool.

model (model B), the [14C]hippurate profile was predicted to exhibit a peak that was much earlier than that of the experimental data (Fig. 6B). By contrast, the model that described metabolism from the deep intracellular pool (model C, Fig. 1C) allowed an improved fit to the experimental outflow profiles of benzoate and hippurate (Fig. 6C). However, models B and C were indistinguishable when only benzoate data were fitted and the metabolite data were ignored (results not shown), as expected from theory (Appendix B). In the latter case, the optimized coefficients for benzoate transfer between the plasma and shallow intracellular pools were identical for both models and close to those listed in Table 4, whereas those representing the transfer between the shallow and the deep intracellular pools and benzoate metabolism to hippurate were altered according to Eqs. B1–B3 in Appendix B. The proper selection of the optimal model of benzoate metabolism (model C) was thus dictated by the fit of the metabolite data. A much lower sum of squared residuals was obtained with model C than with models A and B (30× that of model C). Model C is the simplest model consistent with bidirectional transport of benzoate and hippurate between plasma and hepatocytes, irreversible metabolism, and the inclusion of a deep pool.
lular pool, calculated as $k_{34}/(k_{43} + k_{45})$, were smaller than the corresponding equilibrium partition ratios for benzoate between these pools, calculated as the ratios of transfer coefficients ($k_{34}/k_{43}$) (Fig. 7). The throughput component against the various unbound concentrations of bulk benzoate used for experimentation was $11.7 \pm 5.2\%$ of the dose.

DISCUSSION

MID experiments are typical input-output experiments that can be quantitatively interpreted with concepts originating from linear systems theory. The models that are applied to these systems resemble black boxes, and the information obtained on the processes underlying the observations is, by nature, limited. However, proper interpretation of data is possible if certain restrictions, based on reasonable assumptions about the nature of the underlying physiological processes, are obeyed.

The additional data on metabolite outflow profile greatly strengthened the stance for a more detailed kinetic analysis of MID experiments, especially when the metabolite is characterized for its transport and sequestration coefficients, as in this and other studies. In MID experiments with [2-3H]lactate in perfused rat liver, the metabolite ($^{3}$H$_{2}$O) was found to be formed from a deep intracellular pool connected in series to a shallow intracellular pool. The deep pool was considered to be composed of several intermediate metabolites exchanging $^{3}$H with the two position of lactate (such as malate, fumarate, and NAD$^{+}$) (29, 32). Conversely, sulfation of acetaminophen was found to occur from the shallow and not the deep intracellular acetaminophen pool (27).
Based on the present additional data on [3H]hippurate and formed [14C]hippurate, the model with only one intracellular pool (model A) was found inadequate in describing the composite data on [14C]benzoate and [14C]hippurate at postinjection times longer than 50 s. The observation suggests that tracer benzoate present inside the hepatocyte is not represented by a single moiety that is instantly and evenly distributed throughout the hepatocyte. For [14C]benzoate, the model with two intracellular pools was found satisfactory, and the delayed appearance of the metabolite corroborates the conclusion that formation of metabolite involved a deep intracellular pool. However, in addition to this local heterogeneity of intracellular distribution of substrate, the outflow profile could be interpreted by various physiological phenomena, such as binding to intracellular proteins or other cellular binding sites (17), intracellular diffusion (24), reversible metabolism (29, 32), or subcellular compartmentalization. Similar outflow dilution profiles that were incompatible with a single intracellular pool were found with enalaprilat and sulfobromophthalein-gluthione conjugate (17, 30, 32), substances that are only excreted into bile. For these substrates, the power of discrimination of whether sequestration occurs from a shallow or from a deep pool is curtailed in the absence of additional metabolic data.

For benzoate metabolism in the rat liver, glycination occurs in the deep intracellular, pool. We propose that this pool represents the mitochondrial compartment and that the shallow intracellular pool represents the cytosolic space, a conjecture that is consistent with the presence of enzymes for hippurate synthesis (benzoyl-CoA ligase and glycine N-acyl transferase) in the mitochondria of rat and bovine livers (13, 14, 22, 36). The mitochondrial content of benzoyl-CoA in cells exposed to benzoate is unknown; however, because the maximal activity of the transferase was found to largely

![Fig. 7. Derived kinetic parameters at various benzoate plasma concentrations. P_{13S}, permeability-surface area product for influx of nonprotein-bound benzoate into hepatic parenchymal cells; k_{13}/k_{43}, equilibrium partitioning ratio between deep (mitochondrial) and shallow (cytosolic) intracellular pools; k_{43}/k_{45}, calculated ratio between the contents of the deep (mitochondrial) and shallow (cytosolic) intracellular pools; k_{45}, transfer coefficient for metabolic conversion of benzoate to hippurate. Unbound benzoate concentration is the logarithmic average unbound concentration C_u = f_u |C_u| - C_{out}/ln(C_{in}/C_{out}).](http://ajpgi.physiology.org/)

### Table 4. Optimal parameter values from fits of benzoate injection

<table>
<thead>
<tr>
<th>Input Concentration of Benzoate, (\mu M)</th>
<th>(\gamma_{in,B})</th>
<th>(k_{13}, \text{s}^{-1})</th>
<th>(k_{23}, \text{s}^{-1})</th>
<th>(k_{34}, \text{s}^{-1})</th>
<th>(k_{43}, \text{s}^{-1})</th>
<th>(k_{45}, \text{s}^{-1})</th>
<th>Throughput, % of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.1</td>
<td>-0.189 ± 0.018</td>
<td>0.516 ± 0.067</td>
<td>0.271 ± 0.016</td>
<td>0.126 ± 0.009</td>
<td>0.0651 ± 0.0035</td>
<td>0.0052 ± 0.0009</td>
<td>0.0363 ± 0.0020</td>
</tr>
<tr>
<td>10.4</td>
<td>-0.270 ± 0.017†</td>
<td>0.904 ± 0.090</td>
<td>0.304 ± 0.018</td>
<td>0.101 ± 0.008</td>
<td>0.0819 ± 0.0033</td>
<td>0.0308 ± 0.0004</td>
<td>0.0350 ± 0.0015</td>
</tr>
<tr>
<td>56.4</td>
<td>-0.205 ± 0.006</td>
<td>0.832 ± 0.064</td>
<td>0.249 ± 0.005</td>
<td>0.119 ± 0.002</td>
<td>0.0973 ± 0.0030</td>
<td>0.0066 ± 0.0007</td>
<td>0.0297 ± 0.0012</td>
</tr>
<tr>
<td>57.7</td>
<td>-0.194 ± 0.015</td>
<td>0.441 ± 0.065</td>
<td>0.206 ± 0.015</td>
<td>0.175 ± 0.013</td>
<td>0.0570 ± 0.0016</td>
<td>0.0452 ± 0.0020</td>
<td>14.3%</td>
</tr>
<tr>
<td>167</td>
<td>-0.136 ± 0.013</td>
<td>0.560 ± 0.037</td>
<td>0.195 ± 0.006</td>
<td>0.171 ± 0.007</td>
<td>0.0536 ± 0.0028</td>
<td>0.0067 ± 0.0006</td>
<td>0.1035 ± 0.0003</td>
</tr>
<tr>
<td>179</td>
<td>-0.198 ± 0.006</td>
<td>0.712 ± 0.031</td>
<td>0.293 ± 0.011</td>
<td>0.254 ± 0.012</td>
<td>0.1681 ± 0.0014</td>
<td>0.0660 ± 0.0002</td>
<td>0.1812 ± 0.0004</td>
</tr>
<tr>
<td>170</td>
<td>-0.222 ± 0.016</td>
<td>0.462 ± 0.044</td>
<td>0.218 ± 0.017</td>
<td>0.201 ± 0.016</td>
<td>0.0712 ± 0.0033</td>
<td>0.0609 ± 0.0004</td>
<td>0.0788 ± 0.0005</td>
</tr>
<tr>
<td>464</td>
<td>-0.275 ± 0.016</td>
<td>1.293 ± 0.147</td>
<td>0.425 ± 0.020</td>
<td>0.204 ± 0.008</td>
<td>0.0212 ± 0.0011</td>
<td>0.0404 ± 0.0004</td>
<td>0.0450 ± 0.0006</td>
</tr>
<tr>
<td>463</td>
<td>-0.205 ± 0.018</td>
<td>1.419 ± 0.202</td>
<td>0.472 ± 0.027</td>
<td>0.265 ± 0.007</td>
<td>0.0224 ± 0.0006</td>
<td>0.0688 ± 0.0004</td>
<td>0.0887 ± 0.0003</td>
</tr>
<tr>
<td>817</td>
<td>-0.244 ± 0.021</td>
<td>1.312 ± 0.219</td>
<td>0.668 ± 0.043</td>
<td>0.252 ± 0.009</td>
<td>0.0078 ± 0.0006</td>
<td>0.0138 ± 0.0008</td>
<td>0.074 ± 0.0005</td>
</tr>
<tr>
<td>873</td>
<td>-0.261 ± 0.013</td>
<td>0.496 ± 0.044</td>
<td>0.472 ± 0.022</td>
<td>0.207 ± 0.007</td>
<td>0.0079 ± 0.0003</td>
<td>0.0768 ± 0.0006</td>
<td>0.0888 ± 0.0006</td>
</tr>
<tr>
<td>Means ± SD</td>
<td>-0.218 ± 0.042</td>
<td>0.813 ± 0.372</td>
<td>0.342 ± 0.149</td>
<td>0.188 ± 0.056</td>
<td>0.050 ± 0.031</td>
<td>0.0685 ± 0.0026</td>
<td>0.0193 ± 0.0145</td>
</tr>
</tbody>
</table>

Values are means ± SD. \(\gamma_{in,B}\), as defined in Eq. A11; \(k_{13}\) and \(k_{33}\), shallow pool influx and efflux rate constants, respectively; \(k_{34}\) and \(k_{43}\), deep pool influx and efflux rate constants, respectively; \(k_{45}\), metabolism rate constant. \(P_{13S},\) permeability-surface area product for influx of non-protein-bound benzoate into hepatic parenchymal cells. † Total (bound and unbound) benzoate; the total input hippurate concentration was kept constant at -5 µM. ‡ Uncertainties of parameters.
exceed that of the ligase, synthesis of benzoyl-CoA is expected to be rate limiting, and the concentration should be very low (13, 36).

The spatial distribution of solutes such as benzoate and hippurate within the cell is uncertain due to the multitude of potential subcellular compartments and cellular binding sites. To appropriately estimate the apparent permeabilities and partition coefficients from transfer coefficients, the volumes of the various spaces involved have to be known. For a tentative interpretation of the intracellular benzoate kinetics, only the cytosolic and mitochondrial spaces were presently considered. The cytosol and the mitochondria occupy 58% and 18%, respectively, of the volume of hepatocytes (3). The mitochondrial-to-cytosolic concentration ratios are therefore approximately three times higher than the content ratios shown in Fig. 7. This is in agreement with considerable accumulation of benzoate inside isolated mitochondria as reported previously (14) and may reflect energy-dependent uptake of benzoate into the mitochondrial matrix and/or saturable binding of benzoate to intramitochondrial sites. A judicious examination of benzoate uptake into isolated mitochondria would be needed to elucidate the mechanism of intramitochondrial accumulation.

Saturation of overall benzoate conjugation was noted, as observed previously (8). From the present analysis, it may be concluded that sinusoidal transport of benzoate is not a controlling factor. From model analysis, saturation at the levels of mitochondrial benzoate uptake and metabolism is postulated to exist, and the observation is in agreement with saturation of benzoate conjugation in the isolated rat liver mitochondria (14) and the very low K_m values (between 1.1 and 7.4 μM) of beef liver mitochondrial carboxylic acid:CoA ligases toward benzoate (36). In fact, the value of K_m is similar to that of the overall glycination reaction of benzoate observed in the perfused rat liver preparation (2.6–3.8 μM for this study and 12 μM from previous studies; Ref. 8). At low benzoate concentration, a considerable proportion of benzoate entering the mitochondria appears to be metabolized, as indicated by a high k_{45}-to-k_{43} ratio. In contrast, metabolism of benzoate at higher concentrations is limited by the maximal velocity of benzoate CoA ligase. No saturation, however, was observed for transport of benzoate across the basolateral membrane of hepatocytes in the concentration range studied. Therefore, a carrier-mediated mechanism could not be confirmed. Nonsaturable transport has also been observed for D-lactate or L-lactate in similar experiment with perfused rat livers, although carrier-mediated transport was assumed in these cases (31, 32). Notably, tracer experiments under nonisotopic steady-state conditions like the ones described here provide parameters of near-equilibrium exchange that frequently result in higher K_m than experiments under zero-trans conditions (34).

The MTT of preformed hippurate (28 ± 3 s) and hippurate formed from benzoate (114 ± 32 s) differed (Table 1). The MTT of the formed hippurate is strongly influenced by the transport and metabolic (formation) coefficients and tissue binding of its precursor, benzoate, in addition to its influx and efflux transport and tissue binding parameters (33). The formation of hippurate in a deep, sequestered pool representing the mitochondria increased the residence time of the metabolite in liver and the MTT of formed hippurate.

Interpretation of MID experiments in the perfused rat liver by modeling analysis revealed rapid and nonsaturable uptake but saturable metabolism of benzoate. The approach further corroborated the mitochondrial localization of the benzoate conjugation reaction, as previously observed from in vitro experiments. Formation of hippurate in the deep pool greatly delayed the appearance of the metabolite and increased the MTT in relation to the MTT of preformed hippurate. Transport of benzoate across plasma and mitochondrial membranes of hepatocytes was found to occur in both directions simultaneously, whereas the first step of benzoate metabolism catalyzed by the benzoyl-CoA ligase reaction was saturable and appeared to be the rate-limiting step of the overall process. The present analysis thus confirms and expands the concepts previously established with in vitro experiments.

**APPENDIX A: MATRIX APPROACH TO MID EQUATIONS**

Labeled material is contained in various pools, as shown in Fig. 1C for model C. Benzoate and hippurate in blood plasma form mobile pools, with concentrations c_1 and c_2, respectively. The intracellular (presumably cytosolic) benzoate and hippurate pools, with concentrations c_3 and c_5, respectively, and the deep (mitochondrial) benzoate pool, with concentration c_4, are stationary pools. The behaviors of tracer benzoate and hippurate inside a single sinusoidal flow path and adjacent parenchymal cells (hepatocytes) are described by the following system of partial differential equations

\[
\frac{\partial c_1}{\partial t} + v_p \frac{\partial c_1}{\partial x} = \theta k_{31} c_3 - (1 + \gamma_h) k_{13} c_1 \tag{A1}
\]

\[
\frac{\partial c_3}{\partial t} = 1 + \gamma_h \frac{\partial}{\partial x} k_{13} c_1 + k_{43} c_4 - (k_{31} + k_{34}) c_3 \tag{A2}
\]

\[
\frac{\partial c_4}{\partial t} = k_{34} c_3 - (k_{43} + k_{45}) c_4 \tag{A3}
\]

\[
(1 + \gamma_h) \frac{\partial c_2}{\partial t} + v_p \frac{\partial c_2}{\partial x} = \theta k_{52} c_5 - (1 + \gamma_h) k_{25} c_2 \tag{A4}
\]

\[
\frac{\partial c_5}{\partial t} = 1 + \gamma_h \frac{\partial}{\partial x} k_{25} c_2 + k_{45} c_4 - k_{52} c_5 \tag{A5}
\]

where t is time, x is the position along the length of the path, v_p is the linear velocity of flow within the sinusoidal lumen, γ_h and γ_l are the ratios of the interstitial distribution spaces of benzoate and hippurate, respectively, to the sinusoidal plasma space, and θ is the hepatocyte-to-sinusoidal plasma volume ratio. The ratios of vascular to extravascular distribution volumes of benzoate and hippurate (γ_v and γ_l) are equivalent to γ_h and γ_l as defined previously (38). Exchange between pools is determined by transfer coefficients, with the dimension of reciprocal time, related to permeabilities and enzymatic activities. Under saturating conditions, transfer coefficients depend on the concentrations of unlabeled substrates or metabolites that may vary along the flow path due
to sequestration. To simplify the analysis, however, we will neglect variations in concentrations and treat transfer coefficients as constants (19).

The following equations relate the transfer coefficients for hepatocellular membrane passage to membrane permeabilities

$$k_{13} = \frac{P_{13S}}{f_{s} V_{p}(1 + \gamma_{H})}$$  \hspace{1cm} (A6)

$$k_{31} = \frac{P_{31S}}{f_{s} V_{cell}}$$  \hspace{1cm} (A7)

where $P_{13S}$ and $P_{31S}$ are the permeability-surface area products for exchange of nonprotein-bound benzoate across the hepatocyte cell membranes in the inward and outward direction, respectively, $V_{p}$ is the sinusoidal plasma volume, $f_{s}$ and $f_{t}$ are the fractions of non-protein-bound benzoate in plasma and cytosol, respectively, and $V_{cell}$ is the hepatocellular volume. Similar expressions hold for the hippurate permeabilities $P_{25S}$ and $P_{52S}$. The transfer coefficient $k_{13}$ represents the irreversible hepatic biotransformation activity and, when this is multiplied to the amount of intracellular precursor, yields the reaction rate.

The dose is introduced at the origin ($x = 0$) of the initially tracer-free sinusoid. The system of differential equations therefore needs to be solved with the following initial condition, at $t = 0$

$$c_{1,s}(0) = \frac{q_{0}}{P_{s} \delta(t)}$$  \hspace{1cm} (A8)

where $q_{0}$ is the amount of tracer initially applied to the entrance of the sinusoid, $P_{s}$ is sinusoidal blood flow, and $\delta$ is the impulse function. All other concentrations are set to 0 at $x = 0$.

We use an eigenvalue method previously developed by Schwab et al. (27, 29) for our calculations. For this purpose, the transfer coefficients are collected into the compartmental matrix $A$

$$A = \begin{pmatrix}
-k_{13} & 0 & k_{31} & 0 & 0 \\
0 & -k_{25} & 0 & 0 & k_{52} \\
k_{13} & 0 & -[k_{31} + k_{34}] & k_{34} & 0 \\
0 & 0 & k_{34} & -[k_{43} + k_{45}] & 0 \\
0 & k_{25} & 0 & k_{45} & -k_{52}
\end{pmatrix}$$  \hspace{1cm} (A9)

Because benzoate and hippurate show extracellular distribution spaces similar to that of sucrose due to the low vascular protein binding, the latter is used as a reference tracer. In accordance with this, the average relative velocity along the sinusoids is defined to be 1 for sucrose and 0 for hepatocytes.

The average velocity of benzoate in plasma contained in the combined sinusoidal and the interstitial spaces relative to sucrose is $(1 + \gamma_{Suc})/(1 + \gamma_{H})$, and that of total hippurate is $(1 + \gamma_{Suc})/(1 + \gamma_{H})$. Analogous to what was previously developed (37, 38), we introduce the derived parameters

$$\gamma_{rel,H} = \frac{1 + \gamma_{H}}{1 + \gamma_{Suc}} - 1$$  \hspace{1cm} (A10)

and

$$\gamma_{rel,B} = \frac{1 + \gamma_{B}}{1 + \gamma_{Suc}} - 1$$  \hspace{1cm} (A11)

such that the value of $\gamma_{Suc}$ is not needed for this evaluation. The relative velocities are collected in the diagonal matrix $W$

$$W = \begin{pmatrix}
\frac{1 + \gamma_{Suc}}{1 + \gamma_{B}} & 0 & 0 & 0 & 0 \\
0 & \frac{1 + \gamma_{Suc}}{1 + \gamma_{H}} & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0
\end{pmatrix}$$  \hspace{1cm} (A12)

Concentration terms are collected in a vector, $u$, whose elements are amounts per unit sucrose distribution space, normalized to the injected dose

$$u = \frac{1}{q_{0}(1 + \gamma_{Suc})} \begin{pmatrix}
[1 + \gamma_{H}]k_{1} \\
[1 + \gamma_{H}]k_{2} \\
\theta_{3} \\
\theta_{4} \\
\theta_{5}
\end{pmatrix}$$  \hspace{1cm} (A13)

With these definitions, the system of partial differential equations can then be written concisely as

$$\frac{\partial u}{\partial t} + W \frac{\partial u}{\partial x} = Au$$  \hspace{1cm} (A14)

where $\tau = (1 + \gamma_{Suc})\beta/\beta_{p}$. The initial conditions become

$$u = \delta(x)u_{0}, \hspace{0.5cm} t = 0$$  \hspace{1cm} (A15)

where

$$\begin{pmatrix}
1 \\
0 \\
0 \\
0
\end{pmatrix}$$

These equations are used to calculate the response of the whole liver to an impulse input, as explained in detail elsewhere (27, 29). We used an algorithm that is based on the representation of the impulse response of the reference indicator (labeled sucrose) by a sum of exponentials. The observed sucrose outflow profile $C_{Suc}(t)$ is the convolution of the impulse response with the catheter transport function $h_{cath}(t)$

$$C_{Suc}(t) = h_{cath}(t) * \frac{1}{F} \sum_{i=1}^{n} \alpha_{i} e^{{\gamma_{i}t}}$$  \hspace{1cm} (A17)

where $F$ is hepatic perfusate flow and $*$ is the convolution operator. The parameters $\alpha_{i}$ and $\gamma_{i}$ are obtained by the method of moments with exponential depression (11, 21) after numerical deconvolution (2).

The outflow profiles of the substrate (benzoate) and metabolite (hippurate) are then the first and second elements of the vector

$$C(t) = h_{cath}(t) * W \sum_{i=1}^{n} \alpha_{i} e^{\gamma_{i}t} - \gamma_{i}Wu_{0}$$  \hspace{1cm} (A18)

The use of a matrix as an exponent has been explained elsewhere (27). Approximating the catheter transport func-
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tion as a piecewise polynomial of third degree (9), each
element of \( C(t) \) is a sum of terms obtained by convolution of a
piecewise polynomial with an exponential function. An
algorithm for analytical evaluation of the convolution inte-
grals was developed (not shown).

The transfer coefficients for influx and efflux at the baso-
lateral membrane, metabolism, and exchange between shal-
low and deep intracellular pools were varied until an optimal
simultaneous fit for benzoate and hippurate was found. The
hepatocyte influx permeability surface area product is ob-
served. The algorithm used for deconvolution was obtained from the
National Simulation Resource, Department of Bioengineering, Uni-
versity of Washington, Seattle, WA.

Present address of Tsutomu Yoshimura: Department of Drug
Metabolism and Pharmacokinetics, Eisai Tsukuba Research Labo-
ratories, Tsukuba, Ibaraki 300-2635, Japan.

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