Hepatic uptake of hippurate: a multiple-indicator dilution, perfused rat liver study

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Yoshimura, Tsutomu, Andreas J. Schwab, Lei Tao, Ford Barker, and K. Sandy Pang. Hepatic uptake of hippurate: a multiple-indicator dilution, perfused rat liver study. Am. J. Physiol. 274 (Gastrointest. Liver Physiol. 37): G10–G20, 1998.—The hepatic transport of hippuric acid (HA), a glycine-conjugated metabolite of benzoic acid that exhibits only modest plasma albumin binding (binding association constant of 2.1 × 103 M–1), was studied in the single-pass perfused rat liver (12 ml/min), using the multiple indicator dilution (MID) technique. The venous recovery of [3H]HA on portal venous injection of a MID dose containing a mixture of a set of noneliminated reference indicators and [3H]HA revealed a survival fraction of unity, corroborating the lack of disappearance of bulk HA from plasma. When the outflow recovery was fitted to the barrier-limited model of Goesky et al. (C. A. Goesky, G. G. Bach, and B. E. Nadeau. J. Clin. Invest. 52: 991–1009, 1973), the derived influx (P(in)S) and efflux (P(out)S) permeability-surface area products were found to be dependent on the concentration of HA (1–930 µM); P(in)S and P(out)S were ~3.5 times the plasma flow rate at low HA concentration, but decreased with increasing HA concentration. All values, however, greatly exceeded the expected contribution from passive diffusion, because the equilibrium distribution ratio of chloroform to buffer for HA was extremely low (0.0001 at pH 7.4). The tissue equilibrium partition coefficient (P(i)/P(out)) or ratio of influx to efflux rate constants, k(i)/k(-1) was less than unity and decreased with concentration. The optimized apparent Michaelis-Menten constant and maximal velocity were 182 ± 60 µM and 12 ± 4 nmol·s–1·g–1, respectively, for influx and 390 ± 190 µM and 29 ± 13 nmol·s–1·g–1, respectively, for efflux. In the presence of L-lactate (20 mM), however, P(in)S for the uptake of HA (174 ± 3 µM) was reduced. Benzoic acid (10–873 µM) was also effective in reducing hepatic uptake of HA (5.3 ± 0.9 µM). These interactions suggest that MCT2, the monocarboxylate transporter that mediates the hepatic uptake of lactate and other monocarboxylic acids, may be involved in HA transport.

One conjugate (13) and sulfated estrone and bile acids and drug sulfate conjugates (11, 18, 33, 35) implicates a role for oatp or other as yet unknown transporters.

The transport of arylmonocarboxylic acids in the liver has not been studied extensively (6, 31). The hepatic transport of the simple arylcarboxylate anions aminohippurate and acetamidohippurate in rat liver perfusion experiments was inhibited by probenecid (6). Transport of the precursor, benzoate, into Caco-2 intestinal cells displayed a pH dependence, suggesting the involvement of carrier proteins (38). In the intestine, interaction was found between the transport of benzoate and L-lactate (37). Transport of L-lactate is ordinarily mediated by the monocarboxylate transporter MCT1, which is present abundantly in the intestine, erythrocytes, and cardiocytes (10, 36, 37). MCT2, which is present in the liver, is responsible for the uptake of lactate and pyruvate and is distinct from MCT1 (9). Whether the same substrate specificity for MCT1 applies to MCT2 or whether MCT2 is capable of transporting simple arylcarboxylic acids into liver cells is unknown.

In this study, we examined the hepatic uptake of hippuric acid (HA), a simple organocarboxylic acid. HA is the glycine-conjugated metabolite of benzoic acid found in almost all animal species, including humans (4). It is present in herbivorous animals, and its existence is also associated with its precursor, benzoate, a common food preservative. The fate of hippurate has been studied in conjunction with benzoate in the single-pass-perfused rat liver (5). Once formed, hippurate is neither excreted nor further metabolized by the liver; only efflux to the venous outflow occurs. Thus HA represents the simplest test compound for the study of arylcarboxylic acids. Preliminary plasma protein-binding experiments have demonstrated that HA is bound only to albumin and not to red blood cells (RBC). We used the multiple-indicator dilution (MID) technique to study the sinusoidal transfer constants for HA in the single-pass in situ perfused rat liver preparation at various steady levels of bulk unlabeled substrate. This method entails the introduction of a bolus injection into the inflowing perfusate of both [3H]HA and a set of noneliminated reference indicators against a set of background steady-state concentrations of unlabeled hippurate. We used 51Cr-labeled RBC as a vascular reference, 125I-labeled albumin and 13C]sucrose as high and low molecular weight interstitial references, respectively (14), and 2H2O as a cellular reference (30). By kinetic analysis of the outflow profile of the study
substance, [3H]HA, in relation to those of the simultaneously introduced references, we obtained estimates of parameters describing unidirectional tracer cellular influx and efflux, using the barrier-limited model of Goresky et al. (15). Competition of HA uptake by benzoate and L-lactate was further examined.

MATERIALS AND METHODS

Materials

Unlabeled HA, benzoyl chloride, and bovine serum albumin (BSA; fraction V) were purchased from Sigma Chemical (St. Louis, MO). [2-3H]glycine (sp act 43.4 Ci/mmol) was obtained from DuPont (Markham, ON). [51Cr]sodium chromate (1.61 mCi/mg) and 2H2O (>99.98% pure) were purchased from Merck Frosst (Montreal, PQ). [3H]sucrose (11.9 Ci/mmol) was obtained from New England Nuclear (Boston, MA). All reagents used were of glass-distilled high-performance liquid chromatography (HPLC) grade or the highest purity available (Fisher Scientific, Mississauga, ON).

Synthesis of [3H]HA. [3H]HA was synthesized from benzoyl chloride and [3H]glycine under aqueous and alkaline conditions (19). [3H]glycine (11.5 nmol or 500 µCi) was dissolved in 100 µl of 0.05 N sodium hydroxide. To this, 150 µl of an ethereal solution of benzoyl chloride (80 µM) were added, and 200 µl of 0.1 N sodium hydroxide were subsequently added drop by drop. After the reaction mixture was stirred for 1 h, 200 µl of 0.1 N hydrochloride and 100 µl of chloroform were added. The aqueous phase (top layer) was removed for purification by HPLC. After purification, the radiochemical purity estimated for HA by HPLC was >98%.

Distribution of HA Between Chloroform and Perfusate

The distribution of HA into Krebs-Henseleit bicarbonate solution (KHB) and chloroform was studied at HA concentrations of 3, 30, and 300 µM. Because the equilibrium distribution ratio was expected to be low, any impurity of [3H]HA, albeit representing a very small percentage of the total radioactivity, posed a complication for quantitation. For this reason, only unlabeled HA was used in the determination of the distribution ratio. The partitioning of HA in 20 ml of KHB (pH 7.4) and 20 ml of chloroform was studied. After the mixture was shaken in a capped 50-ml test tube and subsequently centrifuged, 10 ml of the chloroform phase was removed and assayed for HA. The lowest concentration of HA in chloroform (3 µM) was below the detection limit of the HPLC procedure. For the other two concentrations (30 and 300 µM), the ratio of chloroform to buffer was found to be constant (0.0001 ± 0.000015; n = 3).

Protein Binding of HA and Distribution into RBC

Plasma protein binding. The binding of HA to albumin was studied with ultrafiltration (10,000 mol wt cutoff, filter no. YM10; Amicon). Hippurate (0.5–500 µM) containing [3H]HA was prepared in perfusate plasma (5% BSA) and subjected to ultrafiltration at 1,000 g (M2-1 centrifuge; Beckman, Mississauga, ON) for 20 min at room temperature. The total HA concentration in plasma (Cp) was determined by HPLC and liquid scintillation spectrometry, and the unbound concentration (Cp,u) in the ultrafiltrate was quantified by virtue of the radioactivity and the specific activity of the original plasma sample. The binding constants were initially estimated by expressing the concentration ratio of bound to free, or (Cp – Cp,u)/Cp,u vs. the free HA concentration, Cp,u. Fitting of the data was subsequently performed by regression of the following expression, for one class of binding sites

\[
C_p = \frac{n[P]}{K_d + C_p + C_p,u}
\]

where \( [P] \) is the total protein concentration, \( n \) is the number of binding sites, and \( K_d \) is the binding dissociation constant, or the reciprocal of \( K_u \), the association constant for binding.

Distribution into RBC. The distribution of HA into RBC was investigated by mixing plasma perfusate (5% albumin) containing varying concentrations of HA (up to 883 µM), [3H]HA, and [14C]sucrose (a reference that does not enter RBC) with an equal volume of blank blood perfusate containing 40% RBC (vol/vol) and 5% albumin. The admixture resulted in a composition identical to that used for perfusion studies (20% RBC, vol/vol, and 5% albumin). The samples were incubated at 37°C in a rotating water bath for 30 min. Aliquots of perfusate plasma solution, before and after admixture, were assayed for [14C]sucrose and [3H]HA by liquid scintillation spectrometry, whereas unlabeled HA was assayed by HPLC. The concentration ratio of [3H]HA in RBC to the unbound [3H]HA in plasma water, \( \lambda \), was estimated by a formula developed earlier (29), and was found to be essentially zero.

Binding of HA to intracellular components (tissue binding). Tissue binding was studied in both liver homogenate (1:5 dilution) and the 9,000 g supernatant. The liver was first homogenized with 4 vol of ice-cold KHB (homogenizer by Ultraturrax T25; J anke & Kunkel IKA-Labortechnik), and then a 1:10 dilution of the homogenate was centrifuged at 9,000 g for 20 min at 4°C to provide the S9 fraction. Bulk HA and [3H]HA were added to the homogenate and S9 supernatant such that the concentrations of HA varied from 1.1 to 511 µM (10,000–20,000 dpm/ml [3H]HA); 1.0 ml of the homogenate and S9 solution was used for ultrafiltration (10,000 mol wt cutoff; Centricron; Amicon) at 1,000 g for 20 min at room temperature. Preliminary investigation showed that the leakage of liver protein in the ultrafilter, prepared in the manner outlined above, was <1% of the total protein present. Protein was evaluated by the method of Lowry et al. (24). The radioactivities in homogenate and S9 before ultrafiltration (C1) and in the ultrafiltrate (C2) were assayed by HPLC.

Rat Liver Perfusion

Male Sprague-Dawley rats weighing 274–375 g (Charles River, St. Constant, PQ; livers were 8.3–13.3 g) were used for liver perfusion. 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 allowed access to water and food ad libitum. The perfusate contained 20% freshly obtained, washed bovine RBC (Ryding Meats, Toronto, ON), 5% BSA, and 17 mM glucose (Travenol Labs, Deerpark, IL) in KHB buffered to pH 7.4. The perfusate was oxygenated with 95% O2-5% CO2 (Matheson, Mississauga, ON) and O2 (BOC Gases, Whitby, ON) and was maintained at pH 7.4 by an online flow-through pH electrode (Orion, Boston, MA). Perfusion was carried out at 37°C in a single-pass fashion as previously described (5), with perfusate (12 ml/min) entering via the portal vein and exiting via the hepatic vein. The hepatic artery was ligated.

Single-pass perfusion. Previous liver perfusion studies had confirmed the lack of removal of HA when it is formed from benzoic acid; only trace levels of hippurate were found in bile (5). Moreover, preliminary studies showed that a constancy in the perfusate outflow and biliary excretion was reached by 20
min after perfusion commenced. Single-pass studies were conducted at 12 ml/min for 60 min for all studies. Only one concentration of HA (1–930 µM) was used per rat liver. For the first set of competition experiments, HA (~200 µM) and 20 mM l-lactate were kept constant in the inflowing perfusate. For the second set of competition studies, 5 µM HA and benzoate (varying from 10 to 873 µM) were present in the inflowing perfusate; in this set of studies, the HA in the outflow was expected to exceed that entering the liver due to its formation from benzoate.

The inflow and outflow samples were collected at steady state (between 15 and 55 min), and the average (3–5 samples) was used to determine the input (C_{in}) and output (C_{out}) plasma concentration of unlabeled HA. Bile was collected from 20 min onward, at 5-min intervals. At the end of each perfusion experiment, the livers were perfused with 25 ml of ice-cold KHB, removed, weighed, quickly, and homogenized with an equivalent volume of KHB (1:1, wt/vol). The homogenates were stored at 20°C until analysis.

Multiple indicator dilution. A MID dose was introduced into the portal vein 20 min after initiation of all perfusion studies. Sham experiments (without liver) were conducted to characterize the dispersion due to the inflow and outflow catheters. MID was conducted as described previously (13). The injection mixture (0.23 ml), containing 51Cr-labeled washed bovine RBC (0.4 ± 0.14 µCi), 125I-labeled albumin (3.7 ± 1.7 µCi), [14C]sucrose (2.1 ± 2.5 µCi), [3H]HA (1.9 ± 1.1 µCi), 2H2O (0.099 ± 0.032 ml), and unlabeled HA, in a composition otherwise identical to that of the perfusate, was introduced into the inflow system by an electronically controlled HPLC injection valve. Simultaneously, outflow samples were rapidly collected at successive 1-, 2-, and 3-s intervals for a total of 180 s by a fraction collector. Bile was collected at 5-min intervals after MID injection for the next 40 min. The hematocrit of the blood perfusate and dose was determined for each experiment with the use of a hematocrit centrifuge (MB microhematocrit centrifuge; International Equipment Company Division, Fisher Scientific).

Quantitation of Radiolabels or Stable Isotopes

The 51Cr and 125I radiolabels in blood outflow perfusate samples (25–200 µl) and in the 1:10 diluted dose were assayed by gamma counting (Cobra II; Canberra-Packard, Mississauga, ON); the [14C]sucrose and [3H]HA in plasma perfusate (50–200 µl) and in the 1:10 diluted plasma dose were assayed by liquid scintillation counting (Scintillation Counter 5801; Beckman), as previously described (13). 2H2O was assayed by Fourier transform infrared spectrometry (model 1600; Perkin Elmer, Rexdale, ON) over a frequency interval of 2,300–2,700 cm\(^{-1}\) (30). Recovery of 51Cr, 125I, 14C, and 3H radiolabels and 2H2O in outflow samples was virtually complete.

Assay of Unlabeled HA in Plasma and Bile

The concentrations of unlabeled HA in plasma samples and bile were assayed by HPLC, as previously described (5). The HPLC method was used for the quantitation of the HA in the S9 fraction and liver homogenate.

Data Treatment

For the MID data, outflow radioactivity for each indicator was expressed as a fraction of the radioactivity of injected mixture per milliliter of blood (13). The concentration of radiolabels at the end of the collection (180 s) was <0.1% of peak values. Recoveries were calculated as the product of the time integrals of the fractional recovery and blood flow. Fractional recovery integrals were approximated by summing the products of fractional recoveries and sample intervals; fractional recovery activity-time integrals [area under the curve (AUC)] and integrals of the product of fractional recovery and time [AUC at midintervals (AUMC)] were calculated similarly (13, 32). The ratio of AUMC to AUC yielded the mean transit time.

Modeling. A scheme (Fig. 1) was developed to describe the kinetic events underlying the disposition of HA in the perfused rat liver preparation. HA in the plasma space is present as bound and unbound forms, and only the unbound HA in the plasma compartment (assumed to be the same for sinusoidal plasma and interstitial space) is assumed to exchange with that in the hepatocellular compartment. Rapid equilibrium between bound and unbound forms was assumed. It should be noted that, since albumin is excluded from part of the Disse space (14), the space of distribution for bound HA is identically diminished. Transfer rates depend on the rate constants for entry into (k1) and efflux from (k-1) the hepatocytes, as defined in Table 1. The rate constants, when multiplied by the accessible cellular water space (V_{cell}), yield the permeability-surface area products for transport (P_{app}S or P_{out}S). The rate constant for HA removal solely by excretion (k_{ex}) is virtually zero and is neglected in the modeling of the [3H]HA curve.

With these assumptions, preliminary studies showed that an adequate fit to the data was attained with the barrier-limited model of Goretsky et al. (13, 15, 32).

Superposition of noneliminated references and appraisal of influx and efflux coefficients. Superposition of the noneliminated reference indicators (labeled albumin, sucrose, and 2H2O) was performed. We used the relationship between the outflow recovery (concentration/dose) for the noneliminated tracer, C(t), or the convolution of the organ transport function h(t) with the outflow profile \[C_{cat}(t)\] of the sham experiment that defines the dispersion of the inflow and outflow catheters (for details, see APPENDIX, Eqs. A1–A3). The procedure provided values of \[t_{0}\], a common large vessel transit time, and \[\gamma\], a space ratio. For the interstitial space tracers, 125I-albumin and [14C]sucrose, \[\gamma\] is the ratio of the accessible albumin or sucrose Disse space to the sinusoidal plasma space, and for 2H2O this ratio is the sum of the accessible Disse and hepatic cellular water spaces and that in the sinusoid (in RBC and plasma). Equation A3 indicates that, after \[t_{0}\] (transit time of large vessel) and the transit time of the input and collecting systems, each point on the 125I-albumin, [14C]sucrose, or 2H2O curve is described by a convolution of the organ transport function h(t) and the outflow profile C_{cat}(t) of the sham experiment.
concentration, as binding of HA to albumin (fu) changes. The provides a value for the interstitial space ratio of a hypotheti-

Eq. A5

in plasma (fu) and very rapid exchange between bound and
diffusible substance (HA), Cdiff(1g)

point on the RBC curve, by the factor (1

curves will be delayed in time, relative to the corresponding

Table 1. Interrelationships between influx, efflux, and sequestration coefficients and their physical equivalents

<table>
<thead>
<tr>
<th>Coefficientsa</th>
<th>Model Parameter, ml·s⁻¹·ml⁻¹</th>
<th>Physical Equivalents, ml·s⁻¹·ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influx</td>
<td>k₁fu = \frac{f₁k₁θ}{(1 + γref)}</td>
<td>f₁P₁ₜₚS/((1 + γref)Vₚ) \quad \text{Eq. A5}</td>
</tr>
<tr>
<td>Efflux</td>
<td>kᵣ₁ \equiv f₁kᵣ₁ \quad \text{Eq. A8}</td>
<td>f₁P₆ₜₚS/Vₚ \quad \text{Eq. A8}</td>
</tr>
<tr>
<td>Sequestration</td>
<td>kₜₚᵦ₁ \equiv f₁kₜₚᵦ₁ \quad \text{Eq. A9}</td>
<td>f₁CLₜₚ/Vₚ \quad \text{Eq. A9}</td>
</tr>
</tbody>
</table>

PₚₜS, influx permeability-cell surface area product; Vₚ, sinusoidal plasma volume; PₚₜₚS, efflux permeability-cell surface area product; Vₜₚ, cellular volume; f₁, unbound fraction in tissue. CLₜₚ is the intrinsic clearance for removal and is negligible for hippuric acid. k₁ = PₚₜS/Vₜₚ; θ = \frac{Vₚ}{Vₚ + Vₜₚ}; γref = \frac{f₁CLₜₚ + (1 - f₁)Vₚ Alb}{f₁CLₜₚ + (1 - f₁)Vₚ Alb} and is the apparent space ratio (interstitial/total vascular) for total hippurate. f₁u₁ Fraction of unbound tracer within the vascular plasma space; Alb and Suc are ratios of interstitial space to vascular plasma water space for albumin and sucrose, respectively. a Transfer coefficients determined from the model.

curves will be delayed in time, relative to the corresponding point on the RBC curve, by the factor \((1 + γ)\), and its magnitude will be correspondingly attenuated by the factor \(1/(1 + γ)\).

[3H]HA outflow dilution curves. We used the relationship between the outflow recovery (concentration/dose) for the diffusible substance (HA), Cdiff(t), or the convolution of the organ transport function, hdiff(t), with the outflow profile of the inflow and outflow catheters, Cdiff(t) (see APPENDIX, Eq. A10), and that of the noneliminated reference, suroce(Cₚₜₚₚ(t), see Eq. A1). A quantitative analysis of the [3H]HA outflow profile was carried out with a model developed previously (15, 32). Because binding of HA to RBC is negligible, the hypothetical reference that described the extracellular behavior of [3H]HA was constructed based on the unbound fraction of HA in plasma \(f₁u₁\) and very rapid exchange between bound and free forms (Eq. A5). A similar strategy was used for salicylamide sulfate (39) and the glutathione conjugate of bromosulfophthalein (13). The calculated parameter γref (Eq. A5) provides a value for the interstitial space ratio of a hypothetical reference that, outside the cells, behaves in a manner identical to that for HA. It is expected that change with HA concentration, as binding of HA to albumin \(f₁u₁\) changes. The unbound fraction \(f₁\) is calculated with the known binding parameters \(Kₙₐ\) and \(n\) obtained from the binding studies (40)

\[
f₁ = \frac{- (1 + nKₙₐ[Pₜ] - Cₚₜₚₚ) + \sqrt{(1 + nKₙₐ[Pₜ] - Cₚₜₚₚ)^2 + 4Cₚₜₚₚ/Kₙₐ}}{2Cₚₜₚₚ/Kₙₐ}
\]

(2)

The theoretical reference transport function may then be appropriately related to that for sucrose (Eq. A7) in describing the extracellular behavior of HA. The coefficient for intracellular sequestration, \(kₜₚᵦ₁\), was set to zero. The organ transport function for HA was then calculated with Eq. A8. The rate coefficients for influx, \(f₁k₀₁\), and efflux, \(kₑ₁\), defined in Table 1 were provided by the fitting procedure. The first term represents material that propagates through the system without entering the liver cells, or the throughput component. The second term represents material that enters the liver cells and returns later and exits via the vascular pathway, or the returning component. As defined by Goresky et al. (15), \(k₁\) and \(kₑ₁\) are the permeability-surface area products for influx and efflux across the hepatocyte membrane, respectively, per milliliter of cell water \(Vₚₜₚ\) (see Table 1).

To obtain \(k₁\), the product \(f₁k₀₁\) is divided by \(f₁\) (Eq. 2) and the space distribution ratio, \(θ\), or the ratio of \(Vₚₜₚ\) to the extracellular distribution space for HA (Eq. A9). Alternatively, the influx permeability product \(PₚₜS\) was obtained with Eq. A11. Normally, the influx parameters are related to the logarithmic average of the unbound input and output concentrations (12, 13, 40). Because there was a lack of hippurate elimination, the unbound concentration in the plasma \(Cₚₜₚₚ\) is constant throughout the sinusoids and is given by \(f₁Cₚₜₚₚ\), the product of the unbound fraction in input plasma \(f₁\) and the steady-state input concentration \(Cₚₜₚₚ\), or \(f₁Cₚₜₚₚ\), the product of the unbound fraction in output plasma \(fₚₜₚₚ\) and the steady-state output concentration \(Cₚₜₚₚ\).

The efflux rate constant, \(kₑ₁\), was obtained by dividing \(kₜₚᵦ₁\) by \(f₁\) the unbound fraction of HA in liver tissue. The unbound tissue concentration \(Cₚₜₚₚ\) was calculated from the tissue partition equilibrium ratio, \(k₁/kₑ₁\), which equals \(Cₚₜₚₚ/Cₚₜₚₚ\).

Superposition and MID fitting procedures. From the fractional outflow recovery curve of the vascular reference (the labeled RBC curve), the transport function of the injection and collection system of the outflow profile for the sham experiments conducted with injection of an MID dose into the inflow and outflow catheters, without the presence of a liver, was deconvoluted (13). A linear flow-limited transformation of the deconvoluted RBC curve was then carried out to generate a calculated first pattern for each diffusible reference, by selection of trial values for the ratio of the extravascular to vascular distribution spaces and \(f₁\), the common large-vessel transit time. The resulting curve was convoluted with the system transport function. The generated diffusible reference curve (for labeled albumin, sucrose, or water) was compared with that obtained experimentally, and the parameter values were repetitively refined until a best fit was obtained using a least-squares procedure (International Mathematics Statistical Library, Visual Numerics, Houston, TX). The classical weighted least-squares approach to parameter estimates, as discussed by Landaw and DiStefano (22), was used as the criterion for fitting. A weighting strategy was carried out according to counting statistics noise, assuring an error variance proportional to the magnitude of the observation (7, 22). The Jacobian matrix (matrix of sensitivities) obtained from the fitting program was used to calculate variances and covariances of the fitted parameters. The square roots of the variances and the standard deviations of the fitted parameters for each experiment represent the uncertainty in the parameter estimate.

With these values in hand, a similar process was used to gain best fit values for influx and efflux coefficients for HA. The outflow dilution data were fitted to Eq. A8 by variation of \(f₁k₀₁\) and \(kₑ₁\), as defined previously (13, 32), using the same fitting procedure as above. The tracer HA outflow profile was further resolved into throughput and exchanging (returning) components. The concentration of HA on HA concentration was taken into account in that the nonlinear binding to albumin over the concentration range was considered (the fraction of unbound HA increased from 0.39 to 0.56 when the input concentration of HA varied from 1 to 930 µM).

Statistics

All data are means ± SD. Student's t-test statistic was used, and a P value ≤ 0.05 was viewed as significant.
RESULTS

Protein binding of HA. The binding of HA to albumin was concentration dependent. One class of binding site was found (n = 1.03), with a $K_a$ of $2.1 \pm 10^{-3}$ M$^{-1}$ (Fig. 2). Within the concentration range used for the MID studies, the unbound fraction of HA in plasma varied from 0.39 to 0.56. The extent to which HA was bound to liver homogenate or S9, however, did not vary with HA concentration (1.1 to 500 µM); values for the unbound fraction of HA in diluted homogenate and S9 were $1.6 \pm 0.012$ and $0.99 \pm 0.02$ (n = 5), respectively. The values suggest a lack of binding of HA to tissue proteins.

Hepatic extraction and biliary excretion of HA. When perfusate containing increasing bulk plasma concentrations of HA (1.3 to 930 µM) was used for perfusion, the steady-state hepatic extraction ratio of unlabeled HA remained virtually zero. Only trace amounts of HA were found in bile; the biliary excretion of HA was $0.35 \pm 0.14$% of the total dose. Given the extremely low excretion rate of HA, the use of a model without sequestration appeared justified.

Linear superposition by use of the delayed-wave model. Recoveries of labeled RBCs, albumin, sucrose, and $^2$H$_2$O, including $[^3]$HHA, in hepatic venous blood were complete within experimental errors. Representative outflow profiles for the labeled substances injected into the portal vein of the liver are shown in Figs. 3 and 4. The labeled RBC emerged first and reached the highest and earliest peak; the RBC outflow curve had the steepest upslope, and the downslope decayed most rapidly. The $^{125}$I-albumin curve rose slightly less quickly and decayed with a slightly reduced slope, showing a lower and later peak. In comparison to the labeled albumin curve, the $[^1]$C-sucrose curve showed a slightly more delayed upslope, a slightly lower and later peak, and a more prolonged downslope. The greatest dispersion was seen with $^2$H$_2$O, whose upslope and downslope were very delayed and whose peak occurred much later with a much lower magnitude, due to its permeation of the cellular as well as vascular and interstitial spaces.

Superposition of the noneliminated reference indicator curves onto the labeled RBC curve with the deconvolution procedure provided the optimized parameters $t_0$ and $\gamma$ (Table 2). The average $t_0$ value ($3.7 \pm 1.3$ s) was similar to those obtained for other perfused livers (12, 13). The $\gamma$ values for the interstitial substances (for labeled albumin and labeled sucrose) and for $^2$H$_2$O ($\gamma_{Alb} = 0.65 \pm 0.22$, $\gamma_{Suc} = 1.1 \pm 0.5$, and $\gamma_{H_2O} = 5.2 \pm 1.5$) were similar to those estimated in a similar fashion in other perfused rat liver preparations (12, 13). After the approximation of the AUMC and AUC for the noneliminated indicators with cubic splines, the average mean transit times were estimated by moment analysis and were converted to their respective volumes after multiplication by appropriate flows (Table 2). The mean transit times for the noneliminated indicators were generally similar to those obtained in other perfused rat liver MID studies.

Evaluation of MID results: outflow profile for HA. The rising upslope of $[^3]$HHA was slightly delayed with...
respect to that of labeled albumin, but it slightly preceded the labeled sucrose curve (Fig. 3). The $[^{3}H]HA$ curve crossed over the labeled sucrose curve, then peaked lower and earlier than the labeled sucrose curve, as expected, due to binding of HA to albumin. During its decay, the $[^{3}H]HA$ curve again crossed over the labeled sucrose curve and exhibited a more delayed downslope (Fig. 4). Fits to representative experiments are shown in Fig. 4. The tracer $[^{3}H]HA$ outflow profile was further resolved into the throughput and exchanging (returning) components. The throughput component increased from 40 to 60% of the total dose over the unbound concentration range studied (Fig. 5).

The optimized parameters obtained by fitting $\gamma_{rel}$, the influx coefficient $f_{u}k_{1}$, and the cellular efflux coefficient $k_{8}$ are summarized in Table 3. $P_{in}$ estimated with Eq. A11 was 3.5 ± 0.6 times that of the plasma flow rate (0.017 ± 0.002 ml·s$^{-1}$·g$^{-1}$) at the lower HA concentration used, and these values decreased with increasing concentration, demonstrating saturability (Fig. 6A). The corresponding $k_{1}$ values were also concentration dependent (Fig. 6B). Fitting these values to $V_{max}/(K_{m}f_{u})$ yielded the apparent constants for uptake: with $P_{in}$, $K_{m} = 162 ± 53$ µM and $V_{max} = 19 ± 6$ nml·s$^{-1}$·g$^{-1}$, and with $k_{1}$, $K_{m} = 182 ± 60$ µM and $V_{max} = 12 ± 4$ nml·s$^{-1}$·g$^{-1}$ (mean ± SD of parameter estimate); a slight but insignificant difference in these estimates existed due to the reliance of $k_{1}$ on $V_{cell}$ and subsequently $f_{u}$. Saturation was also displayed for $P_{out}$ and $k_{1}$; the latter was equal to the efflux coefficient, since the unbound fraction in tissue, $f_{u}$, was found to be unity (Fig. 7; Table 3). Fitting these values to the estimated tissue unbound concentration, $C_{t,u}$, yielded

![Fig. 4](http://ajpgi.physiology.org/)

**Fig. 4.** Semilogarithmic presentation of tracer $[^{3}H]HA$ and noneliminated reference outflow profiles at various steady-state plasma concentrations of hippurate; data are same as those in Fig. 3. Outflow profiles were resolved into throughput (dotted area) and returning components. Reference curve to which the HA curve is related is outlined; the corresponding space of distribution is slightly smaller than that for labeled sucrose due to binding to albumin.

**Table 2.** Mean transit times of noneliminated reference indicators and their distribution volumes obtained with superposition and splining procedures for hippuric acid in multiple-indicator dilution studies in perfused rat liver

<table>
<thead>
<tr>
<th>Mean Transit Times, s$^{*}$</th>
<th>Distribution Space, ml/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>Albumin</td>
</tr>
<tr>
<td>----</td>
<td>---------</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>± 3</td>
<td>± 4</td>
</tr>
</tbody>
</table>

Data are means ± SD; n = 21. RBC, red blood cells; $V_{sin}$, sinusoidal blood volume; $V_{f,Alb}$, albumin Disse space; $V_{f,Suc}$, sucrose Disse space; $\gamma_{H,2O}$, ratio of interstitial space to vascular plasma water space; $t_{p}$, large vessel transit time; $\theta$, ratio of cellular water to sinusoidal plasma space, calculated as $\theta = \gamma_{H,2O} \times [f_{p}(1 - Hct) + f_{r}(Hct)]/[f_{p}(1 - Hct)] - \gamma_{Suc}$, where $f_{p}$ and $f_{r}$ are the water content of plasma and RBC, respectively, and Hct is hematocrit. *Corrected for transit times of inflow and outflow catheters, from the splining procedure.
very similar kinetic constants ($K_m = 330 \pm 140$ and $390 \pm 190 \mu M$; $V_{max} = 42 \pm 16$ and $29 \pm 13 \text{nmol} \cdot \text{g}^{-1} \cdot \text{s}^{-1}$) for efflux. The tissue equilibrium partitioning ratios, $k_2/k_1$, were slightly lower than unity, and the values were similar to that found in the liver of the hairless guinea pig (24). The values were highest at the lower HA concentrations (average value of $0.82 \pm 0.19$) but gradually decreased with increasing concentration (Fig. 8).

Interactions with L-lactate and benzoate. The hepatic transfer of HA in the presence of L-lactate (20 mM) and benzoate (from 10 to 873 µM) is summarized in Table 4. The values of $P_{in}S$ for HA uptake at ~180 µM (for controls see Table 3, preparations 11–13) were statistically different from those in the presence of L-lactate, although the changes in $P_{out}S$, $k_1$ and $k_{-1}$ were not significant. In the presence of benzoate (10 µM), the outflow HA concentrations increased to 13 µM, whereas for benzoate concentrations >200 µM, HA outflow concentration varied from 81 to 103 µM, due to HA formation from the various concentrations of benzoate. The accrued HA concentration was expected not to evoke changes in transport, since the influx and efflux parameters had remained rather constant at input HA concentrations <200 µM (see Figs. 6 and 7). The changes observed were therefore induced by benzoate. $P_{in}S$ and $P_{out}S$ and $k_3$ and $k_{-1}$ for HA uptake were decreased in the presence of benzoate (see Table 3, preparations 1–10 for controls).

**DISCUSSION**

Hippuric acid, similar to its precursor benzoic acid (5), is found to exhibit poor binding properties to albumin ($n = 1$, $K_b = 2.1 \times 10^3 \text{M}^{-1}$) and does not distribute into RBC. As expected with these binding constants, the unbound fraction of HA increased only slightly, from 0.39 to 0.56, over the wide input plasma concentration range of HA studied (1–930 µM). Binding of HA to intracellular (tissue) components was also found to be absent.

A negligible extraction ratio of HA was observed, confirming the previous observation that HA was poorly excreted by the perfused rat liver preparation (5). To gain insight into the transfer processes, we estimated the transfer coefficients and transfer rate constants from the MID experiments, since the events underlying the handling of hippurate were revealed in its outflow behavior in relation to the outflow behavior of other reference materials. We found that the transport parameters for influx and efflux (transfer clearances or rate constants) were relatively constant for HA input concentrations below 200 µM but eventually decreased with increasing concentration. The transfer processes are, however, quite rapid for hippurate: $P_{in}S$ was three to four times that of the plasma flow rate at low concentrations and one to two times that of the plasma flow rate at higher concentrations. Efflux was equally as fast (Table 3). The lack of hepatic excretion of HA is due purely to its poor candidacy for excretion and is not a result of poor penetration.

Consistently, the magnitude of $P_{in}S$ (or influx clearance per gram liver) and $K_1$ (Fig. 6) and their efflux counterparts $P_{out}S$ and $K_{-1}$ (Fig. 7) was reduced with rising concentrations. The increasing throughput component (Fig. 5) and the declining partition coefficient (Fig. 8) conform to the assumption that a carrier

**Table 3.** Optimized parameter values derived for hippuric acid in rat liver perfusion studies, from the fitting procedure

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>$C_{in}$, µM</th>
<th>$P_u$, g</th>
<th>$P_v$, g</th>
<th>$f_j$, s</th>
<th>$k_1$ or $k_{-1}$, s</th>
<th>$P_{in}S$, ml·s⁻¹·g⁻¹</th>
<th>$k_1$, s⁻¹</th>
<th>$k_{-1}$, s⁻¹</th>
<th>$k_1$ or $k_{-1}$, s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3</td>
<td>0.38</td>
<td>-0.02</td>
<td>0.95</td>
<td>2.4</td>
<td>0.047</td>
<td>0.119</td>
<td>0.057</td>
<td>0.103</td>
</tr>
<tr>
<td>2</td>
<td>4.4</td>
<td>0.38</td>
<td>-0.14</td>
<td>0.75</td>
<td>4.8</td>
<td>0.127</td>
<td>0.169</td>
<td>0.070</td>
<td>0.123</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>0.38</td>
<td>-0.24</td>
<td>0.17</td>
<td>3.6</td>
<td>0.100</td>
<td>0.108</td>
<td>0.055</td>
<td>0.086</td>
</tr>
<tr>
<td>4</td>
<td>8.3</td>
<td>0.38</td>
<td>-0.10</td>
<td>1.51</td>
<td>7.2</td>
<td>0.158</td>
<td>0.111</td>
<td>0.080</td>
<td>0.146</td>
</tr>
<tr>
<td>5</td>
<td>18</td>
<td>0.38</td>
<td>-0.13</td>
<td>0.54</td>
<td>5.0</td>
<td>0.116</td>
<td>0.139</td>
<td>0.058</td>
<td>0.092</td>
</tr>
<tr>
<td>6</td>
<td>18</td>
<td>0.38</td>
<td>-0.02</td>
<td>0.76</td>
<td>0.076</td>
<td>0.123</td>
<td>0.060</td>
<td>0.116</td>
<td>0.123</td>
</tr>
<tr>
<td>7</td>
<td>49</td>
<td>0.39</td>
<td>-0.04</td>
<td>1.16</td>
<td>4.6</td>
<td>0.071</td>
<td>0.110</td>
<td>0.045</td>
<td>0.085</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>0.39</td>
<td>-0.23</td>
<td>0.73</td>
<td>6.3</td>
<td>0.122</td>
<td>0.126</td>
<td>0.052</td>
<td>0.086</td>
</tr>
<tr>
<td>9</td>
<td>89</td>
<td>0.40</td>
<td>-0.06</td>
<td>1.15</td>
<td>5.1</td>
<td>0.118</td>
<td>0.146</td>
<td>0.076</td>
<td>0.125</td>
</tr>
<tr>
<td>10</td>
<td>90</td>
<td>0.40</td>
<td>-0.08</td>
<td>0.79</td>
<td>3.8</td>
<td>0.086</td>
<td>0.129</td>
<td>0.062</td>
<td>0.103</td>
</tr>
<tr>
<td>11</td>
<td>178</td>
<td>0.41</td>
<td>-0.12</td>
<td>1.01</td>
<td>5.8</td>
<td>0.107</td>
<td>0.131</td>
<td>0.061</td>
<td>0.090</td>
</tr>
<tr>
<td>12</td>
<td>184</td>
<td>0.41</td>
<td>-0.04</td>
<td>0.54</td>
<td>2.9</td>
<td>0.104</td>
<td>0.104</td>
<td>0.054</td>
<td>0.101</td>
</tr>
<tr>
<td>13</td>
<td>184</td>
<td>0.31</td>
<td>-0.22</td>
<td>0.80</td>
<td>2.8</td>
<td>0.146</td>
<td>0.146</td>
<td>0.056</td>
<td>0.146</td>
</tr>
<tr>
<td>14</td>
<td>252</td>
<td>0.25</td>
<td>-0.11</td>
<td>0.88</td>
<td>6.1</td>
<td>0.097</td>
<td>0.150</td>
<td>0.031</td>
<td>0.150</td>
</tr>
<tr>
<td>15</td>
<td>416</td>
<td>0.46</td>
<td>-0.037</td>
<td>0.68</td>
<td>3.0</td>
<td>0.048</td>
<td>0.064</td>
<td>0.024</td>
<td>0.057</td>
</tr>
<tr>
<td>16</td>
<td>475</td>
<td>0.47</td>
<td>-0.041</td>
<td>1.02</td>
<td>6.0</td>
<td>0.167</td>
<td>0.127</td>
<td>0.055</td>
<td>0.118</td>
</tr>
<tr>
<td>17</td>
<td>799</td>
<td>0.53</td>
<td>-0.067</td>
<td>1.12</td>
<td>5.3</td>
<td>0.043</td>
<td>0.068</td>
<td>0.020</td>
<td>0.032</td>
</tr>
<tr>
<td>18</td>
<td>793</td>
<td>0.54</td>
<td>-0.14</td>
<td>0.78</td>
<td>4.4</td>
<td>0.035</td>
<td>0.050</td>
<td>0.016</td>
<td>0.026</td>
</tr>
<tr>
<td>19</td>
<td>814</td>
<td>0.54</td>
<td>-0.039</td>
<td>0.85</td>
<td>4.6</td>
<td>0.057</td>
<td>0.087</td>
<td>0.025</td>
<td>0.043</td>
</tr>
<tr>
<td>20</td>
<td>899</td>
<td>0.56</td>
<td>-0.038</td>
<td>0.80</td>
<td>4.0</td>
<td>0.052</td>
<td>0.082</td>
<td>0.024</td>
<td>0.042</td>
</tr>
<tr>
<td>21</td>
<td>930</td>
<td>0.56</td>
<td>-0.043</td>
<td>0.92</td>
<td>4.5</td>
<td>0.052</td>
<td>0.084</td>
<td>0.026</td>
<td>0.039</td>
</tr>
</tbody>
</table>
protein is involved, since saturation is evident (12, 13). The alternate mechanism of passive diffusion, however, is untenable, since the distribution ratio (equilibrium partitioning of drug into chloroform and buffer at pH 7.4) was extremely low (0.0001). A similarly low value was also obtained by Lanman et al. (23). The $K_m$ for influx is quite high (160–180 µM), and this explains why the transport remained virtually first order for input plasma concentrations of 200 µM (corresponding to 100 µM unbound concentration). The $K_m$ for efflux is even higher (330–390 µM). The rate of distribution of hippurate into the tissue thus appears to be limited by the sinusoidal permeation of HA molecules from blood to tissue.

That carrier proteins are involved in the transsinusoidal transfer of HA was evident, since the uptake and efflux displayed saturation. The anion transport protein oatp expressed in HeLa cells (34) was, however, not involved in hippurate or benzoate uptake (K. S. Pang and A. W. Wolkoff, unpublished observations). There was demonstrable competition by L-lactate and benzoate (10–873 µM), which depressed $P_{in}$ and $P_{out}$. The reduction in PS products for HA influx and efflux by L-lactate suggests the putative role of the hepatic monocarboxylate transporter, MCT2 (9, 10); the natural substrate is likely to be L-lactate (8). The interaction between L-lactate and benzoate has been thoroughly studied in the cloned and expressed MCT transporter, MCT1, in hamster and rabbit intestine (9, 10, 36, 37). Reduction of L-lactate but not D-lactate transport by benzoate and inhibition by α-cyanocinnamide were

---

Fig. 6. Plots of influx permeability-surface area product ($P_{in}S$) (A) and influx rate constant ($k_1$) (B) as functions of unbound plasma concentration of HA. Lines are fitted lines that yield similar $K_m$ and $V_{max}$ for HA influx. See text for details.

Fig. 7. Plots of efflux permeability-surface area product ($P_{out}S$) (A) and $k_{-1}$ (B) as functions of calculated tissue unbound concentration of HA. Lines are fitted lines that yield similar $K_m$ and $V_{max}$ for HA efflux. See text for details.

Fig. 8. Plot of the theoretical tissue partition coefficient, $k_1/k_{-1}$, as a function of unbound plasma concentration of HA.
observed (37). Carrier-mediated transport of benzoic acid was found to occur within Caco-2 cells; a pH dependence was further identified (38). Fast disappearance of benzoate from peritoneal fluid, suggestive of carrier-mediated uptake by the peritoneum, was recently reported (28). All of these findings point to transport of benzoic acid by MCT. The present data suggest that hepatic transport of the carboxylates by this transporter may be extended to hippurate in the rat liver.

APPENDIX

The model used for interpreting the data in this study was the barrier-limited, space-distributed, variable transit time model developed by Goresky et al. (15). It describes the relationship between the dose-normalized outflow profiles for the substance under study, \( C_{\text{infl}}(t) \), and those of the interstitial reference substances (sucrose or albumin). Because HA binds to plasma proteins but not RBC, a hypothetical reference transport function was constructed, as follows (32), is defined with respect to the proportion bound to labeled albumin and that which is unbound. In the absence of binding and uptake, the latter would behave like labeled sucrose.

The single path (or single sinusoid) model. The sinusoid is thought to be a single pathway with adjacent sheets of hepatocytes, from which the irreversible biliary excretion occurs. Within the sinusoid, with the small lateral dimensions, diffusion in the lateral direction is assumed to be instantaneous; the sinusoid is so long, however, that diffusion in the longitudinal direction will not contribute significantly to transfer from entrance to exit over the time scale involved, and this is therefore neglected. With these assumptions, space can be described by a single variable, \( x \), denoting position along the sinusoid flow path, and it is possible to find an analytical solution in time and space describing the behavior of tracer within the vasculature and tissue and at the outflow from a sinusoid.

To evaluate the experimentally obtained outflow profiles, the dispersion of the injected bolus by the injection apparatus and the inflow and outflow catheters must be considered, as previously described in detail (13). For example, the experimental sucrose curve, \( C_{\text{suc}}(t) \), is the convolution of the organ sucrose transport function (catheter-corrected outflow profile or impulse response), \( h_{\text{suc}}(t) \), with the outflow profile obtained from the apparatus in the absence of a liver, \( C_{\text{cath}}(t) \)

\[
C_{\text{suc}}(t) = (C_{\text{cath}} * h_{\text{suc}})(t)
\]

where \( * \) is the convolution operator. Similarly, for RBC

\[
C_{\text{RBC}}(t) = (C_{\text{cath}} * h_{\text{RBC}})(t)
\]

The transport functions were computed from the experimental data for \( C_{\text{suc}}(t) \), \( C_{\text{RBC}}(t) \), and \( C_{\text{cath}}(t) \) by deconvolution, using an algorithm obtained from the National Simulation Resource in Mass Transport and Exchange, University of Washington, Seattle, Washington (1, 2).

The parameters of linear superposition according to the flow-limited model of Goresky et al. (15), i.e., the interstitial to vascular distribution spaces, \( \gamma \), and the common long-vessel transit time \( t_o \), were found by first calculating the RBC transport function, \( h_{\text{RBC}}(t) \), by deconvolution as mentioned above and then calculating the organ sucrose transport function, \( h_{\text{suc}}(t) \), from the organ RBC transport function, \( h_{\text{RBC}}(t) \), according to the following equation

\[
h_{\text{suc}}(t) = \frac{1}{1 + \gamma} h_{\text{RBC}} \left[ \frac{t - t_o}{1 + \gamma} + t_o \right]
\]

A calculated sucrose outflow profile is then found by convolution according to Eq. A1, which is then fitted to the experimental outflow profile, \( C_{\text{suc}}(t) \), by a nonlinear least-squares procedure.

Uptake, release, and sequestration of hippurate was evaluated by use of the barrier-limited space-distributed variable transit time model developed by Goresky et al. (15). This model allows the determination of the mass transfer coefficients (Table 1) by comparing the outflow profile of the substance under study (HA) with appropriate reference indicators that are not taken up by hepatocytes. Because HA binds to albumin and is partly excluded from the interstitial space, none of the experimental reference indicators is directly useful for the modeling process, and a theoretical reference transport function was constructed, as follows

<table>
<thead>
<tr>
<th>Interactant</th>
<th>( r_{\text{ref}} )</th>
<th>( t_{\text{ref}} )</th>
<th>( k_{\text{ref}} ) or ( f_k )</th>
<th>( k_{\text{in}} )</th>
<th>( P_0 )</th>
<th>( P_\text{out} )</th>
<th>( k_{\text{in}} )</th>
<th>( k_{\text{out}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Input HA conc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>174 ( \mu ) M L-lactate</td>
<td>-0.053</td>
<td>1.15</td>
<td>5.1</td>
<td>0.069</td>
<td>0.109</td>
<td>0.037</td>
<td>0.058</td>
<td>0.070</td>
</tr>
<tr>
<td>20 mM L-lactate</td>
<td>-0.085</td>
<td>1.13</td>
<td>6.0</td>
<td>0.106</td>
<td>0.127</td>
<td>0.047</td>
<td>0.067</td>
<td>0.090</td>
</tr>
<tr>
<td>5.2 ( \mu ) M benzoate</td>
<td>-0.080</td>
<td>1.75</td>
<td>10.6</td>
<td>0.088</td>
<td>0.088</td>
<td>0.034</td>
<td>0.055</td>
<td>0.054</td>
</tr>
<tr>
<td>+9.9 ( \mu ) M benzoate †</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>+172 ( \mu ) M benzoate †</td>
<td>-0.067</td>
<td>0.93</td>
<td>3.7</td>
<td>0.051</td>
<td>0.116</td>
<td>0.036</td>
<td>0.058</td>
<td>0.071</td>
</tr>
<tr>
<td>+844 ( \mu ) M benzoate †</td>
<td>-0.056</td>
<td>1.12</td>
<td>4.9</td>
<td>0.049</td>
<td>0.078</td>
<td>0.034</td>
<td>0.047</td>
<td>0.056</td>
</tr>
<tr>
<td>+9.9 ( \mu ) M benzoate †</td>
<td>-0.052</td>
<td>0.82</td>
<td>4.3</td>
<td>0.048</td>
<td>0.090</td>
<td>0.031</td>
<td>0.053</td>
<td>0.054</td>
</tr>
<tr>
<td>+9.9 ( \mu ) M benzoate †</td>
<td>-0.056</td>
<td>0.97</td>
<td>4.6</td>
<td>0.062</td>
<td>0.086</td>
<td>0.044</td>
<td>0.055</td>
<td>0.070</td>
</tr>
<tr>
<td>+9.9 ( \mu ) M benzoate †</td>
<td>-0.053</td>
<td>1.10</td>
<td>12</td>
<td>0.050</td>
<td>0.063</td>
<td>0.027</td>
<td>0.075</td>
<td>0.023</td>
</tr>
<tr>
<td>+9.9 ( \mu ) M benzoate †</td>
<td>-0.089</td>
<td>1.10</td>
<td>6.9</td>
<td>0.067</td>
<td>0.072</td>
<td>0.035</td>
<td>0.047</td>
<td>0.053</td>
</tr>
</tbody>
</table>

Parameters were obtained from curve fitting, \( \gamma_{\text{ref}}, \) \( f_k, \) and \( f_k^{-1} \), transfer coefficients for influx and efflux, respectively (see definitions in Table 1). NS, not significant. P values for input HA concentrations are vs. HA controls, preparations 1–13 (see Table 3). P values for output HA concentrations are vs. HA controls, preparations 1–10 (see Table 3). *Output HA concentrations were 13 \( \mu \) M in these preparations. †Output HA concentrations were 81–103 \( \mu \) M in these preparations. © Controls chosen according to output HA concentrations.
$$h_{\text{ref}}(t) = \frac{1}{1 + \gamma_{\text{ref}}} \cdot h_{\text{RBC}} \left( \frac{t - t_0}{1 + \gamma_{\text{ref}} - 1} \right)$$  \hspace{1cm} (A4)$$

where $\gamma_{\text{ref}}$ is the ratio of extravascular to vascular distribution space of HA. The value of this ratio is

$$\gamma_{\text{ref}} = \gamma_{\text{Suc}} f_u + \gamma_{\text{Alb}} (1 - f_u)$$  \hspace{1cm} (A5)$$

where $f_u$ is the unbound fraction of HA in plasma.

Because sucrose was used as a reference indicator, the appropriate reference transport function was calculated as follows (32)

$$h_{\text{ref}}(t) = \frac{1}{1 + \gamma_{\text{ref}}} \cdot h_{\text{Suc}} \left( \frac{t - t_0}{1 + \gamma_{\text{ref}} - 1} \right)$$  \hspace{1cm} (A6)$$

where

$$\gamma_{\text{ref}} = \frac{1}{1 + \gamma_{\text{ref}} - 1}$$  \hspace{1cm} (A7)$$

From above, the ratio $(1 + \gamma_{\text{ref}})/(1 + \gamma_{\text{Suc}})$ or the ratio of the total sinusoidal plasma plus interstitial spaces of distribution for HA, in relation to that for labeled sucrose, further defines $(1 + \gamma_{\text{ref}})$, which was used to describe the appropriate interstitial space reference for the data. The organ transport function for HA was then calculated according to the barrier-interstitial space reference for the data. The organ transport function for HA, in relation to that for labeled sucrose, further defines $(1 + \gamma_{\text{ref}})$, which was set to zero in Eq. A5.

The value of this ratio is obtained from (32)

$$\gamma_{\text{ref}} = \left[ \frac{\text{fu}}{\text{fu} + \text{fu}} \right] \cdot \text{fu}$$  \hspace{1cm} (A8)$$

where $k_1$ and $k_{-1}$ are transfer coefficients for entry into and efflux from the hepatocytes, and $k_{\text{sec}}$ is the sequestration coefficient describing removal of HA, which was set to zero in the present case; $r' = (1 + \gamma_{\text{ref}})\cdot r$ and $r = x/V_f$, where $x$ is the distance and $V_f$ is the linear velocity of sinusoidal blood. The ratio of cellular to extracellular distribution spaces for HA, $\theta'$, is obtained from

$$\theta' = \frac{V_{\text{cell}}}{(1 + \gamma_{\text{ref}}) \cdot V_{\text{Suc}}(1 + \gamma_{\text{ref}})}$$  \hspace{1cm} (A9)$$

where $\theta$ is the ratio of $V_{\text{cell}}$ to plasma water space ($V_p$) and $\gamma_{\text{ref}}$ is the ratio of the extravascular to the vascular distribution space of HA (Eq. A5). $V_{\text{Suc}}$ is sinusoidal sucrose space, and $V_{\text{cell}}$ and $V_{\text{Suc}}$ are estimated from their mean transit times.

The calculated HA outflow profile, $C_{HA}(t)$, is obtained by convolution of $h_{HA}(t)$ with the outflow profile obtained from the apparatus in the absence of a liver, $C_{\text{cath}}(t)$

$$C_{HA}(t) = (C_{\text{cath}} \cdot h_{HA})(t)$$  \hspace{1cm} (A10)$$

This was fitted to the experimental data for $C_{HA}(t)$ by varying the parameters $f_u k_1 \theta'$, $k_{-1}$, and $\gamma_{\text{ref}}$. From the fitted value of $f_u k_1 \theta'$, $k_{-1}$ was calculated using $\theta'$, obtained using Eq. A8 and Eq. A10. Finally, $P_{in}$ was obtained from the fitted value of $f_u k_1 \theta'$, as follows

$$P_{in} = f_u k_1 \theta' V_{\text{Suc}} (1 + \gamma_{\text{ref}})$$  \hspace{1cm} (A11)$$

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