Uptake of a Protein-Bound Polar Compound, Acetaminophen Sulfate, by Perfused Rat Liver

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The hepatocytic entry of acetaminophen sulfate conjugate was examined in the rat liver, perfused with red cells with and without albumin, by use of the multiple-indicator dilution technique. [^{3}H]acetaminophen sulfate was injected into the portal vein in a bolus of blood containing ^{51}Cr-labeled red blood cells (a vascular reference), sucrose (a low-molecular-weight interstitial reference) or ^{125}I-labeled albumin (a high-molecular-weight interstitial reference, included when albumin was present), and the time courses of their outflow into the hepatic venous blood were observed. The [^{3}H]acetaminophen sulfate, which binds partially to albumin, emerged between albumin and sucrose in the presence of albumin, precessed the upslope of the sucrose curve and showed a late low-in-magnitude tailing; the precession disappeared in the absence of albumin. Biliary excretion of [^{3}H]acetaminophen sulfate was less than 1% of the dose. Quantitative evaluation with a barrier-limited, space-distributed variable transit time model (including rapidly equilibrating albumin binding) accounted for the albumin effect on [^{3}H]acetaminophen sulfate behavior and demonstrated a low liver cell permeability for the acetaminophen sulfate and a small interstitial binding space for its nonalbumin-bound fraction in excess of that for sucrose, which in the absence of albumin was of similar dimensions. (HEPATOLOGY 1992; 16:173-190.)

The hepatic uptake of albumin-bound compounds by the liver has been a subject of much study, especially where the protein binding is so extensive that this limits the rate of uptake of substrate. For many such substrates, uptake rates decline much less when albumin is added to the extracellular medium than would be expected from the decline in the equilibrium unbound (or free) concentration. Among the mechanisms hypothesized to account for the phenomenon have been processes that facilitate the presentation of bound and unbound species to the liver cell surface and potential interaction with unbound species. These have included the presence of an albumin receptor on the liver cells (1); rapid dissociation from albumin (2, 3); codiffusion of free and bound substrate across an unstirred layer with a disequilibrium atmosphere adjacent to the hepatocytes (4-10), especially *in vitro;* and processes at the liver cell surface catalyzing dissociation or mediating direct transfer of bound material from protein to cell (11). These hypotheses, which were developed chiefly to account for the uptake of tightly bound endogenous substrates, such as free fatty acids, have been the object of critical scrutiny (2, 5, 7, 10, 12, 13); to complete an understanding of the uptake process, events in the cell membrane (14) and inside the liver cells (15) must be integrated into the whole. One of the questions that arises is whether, for a less tightly bound ligand, any of the hypothesized albumin effects apart from binding are quantitatively important. We examined the uptake of acetaminophen sulfate by rat liver, from this point of view.

The multiple-indicator dilution technique is an investigative tracer kinetic tool that enables one to unravel the effects of blood flow, substrate entry and cellular sequestration (removal by metabolic or excretory means) in the uptake of materials by the liver (16). With the simultaneous injection of vascular, interstitial and intracellular noneliminated reference indicators with the tracer substrate, a set of outflow profiles is obtained, which permits quantitative analysis of cell entry, efflux and sequestration. With a bolus injection of these tracers superimposed on various steady-state infusions of unlabeled substrate, the concentration dependence of the modes of entry, efflux and sequestration of the substrate may further be defined. The technique has, for instance, revealed carrier-mediated cell entry of D-galactose (17) and D-glucose (18) in dog liver, inhibition of D-glucose by stevioside (19), the slow cell entry of enalaprilat (a polar

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dicarboxylic acid metabolite of enalapril, an angiotensin converting enzyme inhibitor) in rat liver (20) and the slow entry of conjugates of 4-methylumbelliferone in rat liver (21, 22).

It appeared to us that the kinetics underlying uptake of a modestly protein bound material could be approached most easily with a compound that is protein-bound but eliminated only minimally. Previous explorations, which have used the multiple-indicator dilution approach, have indicated that labeled albumin, although gaining direct access to the interstitial space of the liver, is excluded from a proportion of the volume of that space so that despite its flow-limited distribution into its available space, it emerges with an outflow profile that is earlier and distinguishably different from that for a simultaneously injected low-molecular-weight interstitial reference that does not penetrate hepatocytes, such as labeled sucrose (16, 23). This kinetic behavior provides an effective vehicle for the examination of the uptake of a protein-bound material, especially where a proportion of the material is not tightly bound.

Acetaminophen sulfate is representative of a wide class of polar metabolites: sulfate or glucuronide conjugates generated from phenolic or carboxylic acid substrates, which are usually but not exclusively nontoxic in nature (24, 25). This polar compound is partly protein-bound (26), and does not enter RBCs or undergo metabolism or extensive biliary excretion (23, 27, 28). Recently, studies with isolated rat hepatocytes (29) have suggested the presence of identical saturable mechanisms controlling the influx and efflux of acetaminophen sulfate across the hepatocyte membrane. A similar carrier system has also been implicated in the cellular efflux of the harmol sulfate conjugate from rat liver (30). The characteristics of transport across the liver cell membrane for this type of polar conjugate and the manner in which protein binding affects its transport are otherwise largely unknown.

Utilizing the multiple-indicator dilution technique, we conducted a set of single-pass studies with acetaminophen sulfate in perfused rat liver aimed at characterizing its entry into and efflux from hepatocytes in the presence and absence of albumin binding. Tracer [^{3}H]acetaminophen sulfate conjugate was injected, together with the reference markers ^{51}Cr-labeled RBCs, ^{125}I-labeled albumin and [^{14}C]sucrose, into the rat liver either in the absence or the presence of infused unlabeled acetaminophen sulfate, at steady state. Outflow tracer dilution curves were then secured. Because the effect of albumin binding on the form of the tracer acetaminophen sulfate outflow dilution curves was of particular interest, both at the experimental and theoretical level, studies were performed in both the presence and absence of albumin in the perfusion fluid.

MATERIALS AND METHODS

[^{3}H]acetaminophen sulfate conjugate was biosynthesized from [^{3}H]acetaminophen with an isolated, saline-solution-perfused (40 ml/min) rat liver. [^{3}H]acetaminophen was introduced slowly at the input to the liver, and the subsequent outflow was allowed to recirculate to the reservoir; this mode of delivery ensured that all labeled acetaminophen underwent initial first-pass sulfate conjugate formation before recirculation. At the end of the preparation time (3 hr), the reservoir perfusate was lyophilized, extracted with methanol and subjected to thin-layer chromatography. Material was first spotted on Silica Gel GF thin-layer plate (1,000 μm; Analtech, Newark, DE) and developed in ether. The origin, which contained acetaminophen sulfate and other polar conjugates, was extracted with methanol, dried and reapplied on Avicel F (1,000 μm plate; Analtech) and developed with *n*-propranol : 0.4 mol/L NH_3 (80:20 vol/vol) (27); the fraction corresponding to acetaminophen sulfate was scraped and extracted exhaustively with methanol, filtered through glass wool and stored over Na_2SO_4. The identity of the compound was checked by incubation for 6 hr at 37° C with various enzymes in the following fashion: (a) saline solution (control); (b) arylsulfatase and D-saccharolactone, 100 mmol/L (Sigma Chemical Co., St. Louis, MO); (c) β-glucuronidase (*Helix pomatia* or bovine; Sigma Chemical Co.); and (d) glusulase (arylsulfatase and β-glucuronidase from *Helix pomatia;* Boehringer Mannheim, Pointe Claire, Quebec, Canada). This was followed by repeated dry-ice, ethyl acetate extraction for removal of the liberated [^{3}H]acetaminophen (27). A set of standards, with the labeled acetaminophen added, was incubated under similar conditions to account for recovery of the labeled acetaminophen by the extraction procedure. The enzymes, with the exception of β-glucuronidase, liberated [^{3}H]acetaminophen, confirming the presence of the labeled acetaminophen sulfate. The radiochemical purity of the prepared [^{3}H]acetaminophen sulfate was found to be >99% by both TLC and HPLC (31).

[^{3}H]Acetaminophen was purchased from Dupont (Mississauga, Ontario, Canada) (specific activity 15 mCi/mmol), and acetaminophen was obtained from Eastman Kodak (Rochester, NY). Acetaminophen sulfate, potassium salt, was kindly supplied by Dr. Emil Lin, School of Pharmacy, University of California, San Francisco. ^{51}Cr-sodium chromate (specific activity = 5,392 mBq/mg) and ^{125}I-labeled serum albumin (specific activity = 0.46 mBq/mg) were purchased from Merck Frosst (Montreal, Quebec, Canada); [^{14}C]sucrose (specific activity = 6.4 mCi/mmol) was obtained from Dupont. ^{58}Co-EDTA (1.2 mBq/mg) was custom synthesized by Merck Frosst. All solvents were of HPLC grade and were obtained from Fisher Scientific and Caledon Labs (Mississauga, Ontario, Canada).

Rat Liver Perfusion. The perfusion apparatus and rat liver perfusion were identical to those used in previous studies (23). Male Sprague–Dawley rats (250 to 350 gm; Charles River Canada, St. Constant, Quebec, Canada), yielding liver weights of 8.3 to 14.2 gm, were used. Before surgery, animals were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg). Perfusate, consisting of 20% washed, outdated human blood cells (Red Cross, Toronto, Ontario, Canada), 3% dextran (Pharmacia Fine Chemicals, Piscataway, NJ) and 1% BSA (25% in Tyrode solution; Sigma Chemical Co.) for the albumin-containing experiments, or 4% dextran for the albumin-free experiments and 17 mmol/L glucose (Abbott Laboratories, Ltd., Montreal, Quebec, Canada) in Kreb's-Henseleit bicarbonate solution, buffered to pH 7.4 and equilibrated with O_2 and CO_2 (95:5, vol/vol at 1 L/min; Matheson Gas, Mississauga, Ontario, Canada), entered the liver through the portal vein and exited through the hepatic vein at 12 ml/min; the hepatic artery was ligated. The injection port and the preparation of the injection dose were identical to those previously described (23). The hematocrit of the blood perfusate was determined for each experiment (Model MB

Microhematocrit Centrifuge; International Equipment Co., Fisher Scientific).

Multiple-Indicator Dilution Experiments. A preliminary study was performed to check adsorption of [^{3}H]acetaminophen sulfate onto the tubing of the perfusion apparatus. In sham experiments, tracer [^{3}H]acetaminophen sulfate and [^{14}C]sucrose were delivered simultaneously and perfused once through the tubings of the perfusion apparatus at 12 ml/min, without a liver in place; outflow samples were immediately collected as if they were derived from a multiple-indicator dilution experiment. After expressing the outflow concentrations in terms of the reservoir concentrations, the outflow profiles for both of the radiolabels were found to be identical. At all times, the ratios of the outflow fractions for [^{3}H] acetaminophen sulfate to [^{14}C]sucrose were found to be essentially unity, indicating that no adsorption of the tracer [^{3}H]acetaminophen sulfate to the tubing had occurred.

For the studies to be performed with albumin, the injection dose was prepared containing, in 0.1 ml, ^{51}Cr-labeled RBCs (0.11 to 0.25 μCi), ^{125}I-labeled albumin (0.78 to 1.6 μCi), [^{14}C]sucrose (0.29 to 0.5 μCi) and [^{3}H]acetaminophen sulfate (0.87 to 1.15 μCi) in a composition otherwise identical to that of the perfusate (23). In the first set of albumin-containing studies (n = 6), the rat liver was equilibrated with perfusate that recirculated at 12 ml/min for about 20 to 25 min at a temperature of 37° C. Single-pass perfusion was then begun. The dose was injected into the portal vein of the rat liver preparation while at the same time outflow samples were collected at successive 1-, 2- and 3-sec intervals for a total of 180 sec. Bile was collected at 10-min intervals for 2 hr. In the second set of albumin-containing studies (n = 7), unlabeled acetaminophen sulfate conjugate (16 to 350 μg/ml or 72 to 870 μmol/L) was delivered into the rat liver and perfused in single-pass fashion for 35 to 45 min. Three reservoir perfusate and five venous outflow samples were taken during steady state (within the last 10 to 12 min of the perfusion period, when preliminary experiments had shown constance in outflow perfusate and bile efflux) for determinations of the steady-state input and output concentrations of unlabeled acetaminophen sulfate conjugate, C_{In} and C_{Out}, respectively. Bile was collected from 0 to 15 min and at 5-min intervals thereafter to provide samples for the determination of the rate of biliary excretion of unlabeled acetaminophen sulfate. Five minutes before the termination of the perfusion period, when steady-state conditions were attained for unlabeled acetaminophen sulfate, the multiple-indicator dilution dose was injected into the inflow perfusion system of the rat liver, and outflow samples were collected as previously described.

For the albumin-free experiments (n = 5), the perfusion medium contained only dextran and no albumin to preclude the binding of acetaminophen sulfate to albumin. The injection mixture contained, in 0.1 ml, ^{51}Cr-labeled red blood cells, ^{58}Co-labeled EDTA and [^{3}H]acetaminophen sulfate. ^{58}Co-labeled EDTA has recently been described to have a space of distribution in the liver identical to that of labeled sucrose, so that it serves as an alternate low-molecular-weight interstitial reference (32). Bile was collected for 2 hr at 15-min intervals.

Protein Binding, RBC Partitioning and Partition Ratio into Chloroform. The extent to which acetaminophen sulfate binds to albumin in the perfusate plasma and to rat liver proteins was studied by equilibrium dialysis (Spectropor; Fisher Scientific) and by ultrafiltration (Centricon; Amicon; 10,000 kD mol wt cutoff), respectively. Perfusate medium (containing 1% albumin) was prepared with unlabeled acetaminophen sulfate (0 to 870 μmol/L) and [^{3}H]acetaminophen sulfate (24,100 dpm/ml). In parallel, rat livers were homogenized with three times their volume of Krebs-Henseleit bicarbonate buffer; the homogenate was centrifuged at 9,000 *g* (M2-J Centrifuge, Beckman Instruments, Mississauga, Ontario, Canada) for 20 min at 0° C, and unlabeled acetaminophen sulfate (0 to 650 μmol/L) and [^{3}H]acetaminophen sulfate (31,600 dpm/ml) were added to an aliquot (1 ml) of the supernatant ("S-9 fraction"). The mixture was incubated for 2.5 to 3 hr at 37° C (preliminary studies by equilibrium dialysis indicated that equilibrium was reached within 2 hr), then centrifuged for 30 min at 100 *g* at room temperature. The concentrations of protein in the buffer side of the equilibrium dialysis cell and in the ultrafiltrate of the S-9 fraction, determined in replicate by the method of Lowry et al. (33), were found to be less than 0.2% and 4% of that of the plasma perfusate and the S-9 fraction, respectively. The unbound acetaminophen sulfate fraction was then estimated from the equilibrium ratio of the concentrations in buffer and protein sides of the equilibrium dialysis, and the ultrafiltrate to that in the original S-9 sample, after correction for the spill of protein.

The potential of acetaminophen sulfate conjugate to distribute into RBCs was examined by adding equal volumes of [^{3}H]acetaminophen sulfate in 1% albumin (~14,500 dpm/ml) to a 40% RBC/1% albumin suspension. Replicate samples were incubated at 37° C and removed at 30 min to obtain plasma. The potential concentration, at various times, of acetaminophen sulfate within RBCs, $C_{RBC,post}$, was calculated, after determination of the hematocrit (Hct) postdilution, as

$$C_{RBC,post} = \frac{C_{B,post} - (1 - Hct)C_{P,post}}{Hct} = \frac{C_{P,pre}/2 - (1 - Hct)C_{P,post}}{Hct} \quad (1)$$

where $C_{P,pre}$ is the initial plasma concentration before dilution and $C_{B,post}$ and $C_{P,post}$ are the blood and plasma concentrations at various times after the admixture of the RBCs.

The equilibrium (1-hr) distribution of acetaminophen sulfate between equal volumes of chloroform or octanol and physiological saline (pH 7.4) was determined for different concentrations of unlabeled acetaminophen sulfate (0 to 930 μmol/L) and [^{3}H]acetaminophen sulfate (53,000 dpm/ml). After centrifugation, the equilibrium partitioning of acetaminophen sulfate at its various concentrations was determined by liquid scintillation counting.

Assays. Quantification of ^{51}Cr and ^{125}I radiolabels in blood aliquots and of ^{3}H and ^{14}C activities in perfusate plasma was conducted according to previously published methods (23, 34). The potential presence of [^{3}H]acetaminophen as a metabolite of [^{3}H]acetaminophen sulfate, caused by futile cycling, was routinely sought in venous outflow and in bile by thin-layer chromatography (34) and by HPLC (31); none was found.

Unlabeled acetaminophen sulfate in plasma, bile and liver homogenates was quantified by HPLC by use of the method of Wilson et al. (31), with modifications. Because checks of plasma and bile samples with the HPLC had failed to reveal the presence of acetaminophen among all experiments, this compound was used as an internal standard for the assay. The mobile phase consisted of 0.75% glacial acetic acid in 0.1 mol/L KH_2PO_4 and was utilized at 1 ml/min through a reverse-phase column (5 μm; Ultrasphere 2.0 mm ID, 15 cm length [Beckman Instruments]). A Waters Associates HPLC (Mississauga, Ontario, Canada), equipped with a WISP model 710B 6000A pump and Model 440 detector (254 nm), was used. The retention times of acetaminophen sulfate and acetaminophen were 7.7 and 10.8 min, respectively. Deproteinization of plasma and liver homogenate (200 μl) samples was achieved by

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

Coefficients[a]	Model parameters ($ml \cdot sec^{-1} \cdot ml^{-1}$)	Physical equivalents ($ml \cdot sec^{-1} \cdot ml^{-1}$)
Influx	$k_1'\theta/(1 + \gamma_{AS})$ or $k_1 f_f \theta/(1 + \gamma_{AS})$[b]	$f_f P_{in} S/[(1 + \gamma_{AS}) V_p]$[c]
Efflux	k'_{-1} or $k_{-1} f_t$	$f_t P_{out} S/V_{cell}$[c]
Sequestration	k'_{sec} or $k_{seq} f_t$	$f_t CL^b_{cell}/V_{cell}$[d]

[a]Transfer coefficients determined from the model.

[b]$k_1' = k_1 f_f$; $k_1 = P_{in} S/V_{cell}$; f_f is the plasma free fraction; f_t is the intracellular free fraction; $\theta = V_{cell}/V_p$; and γ_{AS} is the interstitial to sinusoidal plasma space ratio for acetaminophen sulfate.

[c]$P_{in}S$ is the influx permeability cell surface area product, and V_p is the sinusoidal plasma volume. $P_{out}S$ is the efflux permeability cell surface area product, and V_{cell} is the cellular volume.

[d]CL^b_{cell} is the biliary intrinsic clearance of acetaminophen sulfate, at the input bulk concentration at which the system has been set.

an addition of four times their volumes of methanol, containing the internal standard, acetaminophen (8 μg). After evaporating the supernatant to dryness under nitrogen gas (Matheson Gas, Mississauga, Ontario, Canada), the samples were dissolved in the mobile phase for injection onto the HPLC. Bile (5 to 10 μl) was diluted to 50 μl with water, and after addition of 200 μl of methanol containing 20 μg acetaminophen, 10 to 20 μl of this was injected directly onto the HPLC. Sets of standards, containing known amounts of acetaminophen sulfate (1.5 to 23 μg/200 μl plasma and 0.43 to 6 μg/50 μl diluted bile) and appropriate amounts of the internal standard, were prepared in plasma, liver homogenate and bile and processed in a fashion identical to the samples.

Data Treatment. The outflow radioactivity for each tracer was expressed as a fraction of the injected radioactivity per milliliter of blood, and the concentrations of radiolabels at the end of the collection (180 sec) were less than 0.1% of peak values. Recoveries *(Rec)* were calculated as the product of the time integrals of the fractional recovery (sample concentration/dose) and blood flow, F, with monoexponential extrapolation to infinity,

$$\text{Rec} = \int_0^\infty \frac{F\, C(t)dt}{\text{dose}}. \quad (2)$$

The activity-time integrals (AUC), and integrals for the product of fractional recovery and time (at mid-intervals) (AUMC), were approximated as previously described (35). The mean transit times of each marker in the system (AUMC/AUC), after subtraction of the transit times of the inflow and outflow catheters, yielded the mean transit times of each indicator in the liver.

The steady-state extraction ratio, E, of unlabeled acetaminophen sulfate conjugate was expressed as

$$E = \frac{C_{In} - C_{Out}}{C_{In}}, \quad (3)$$

and the biliary excretion rate of acetaminophen sulfate at steady state was expressed as a percentage of the input rate. For [^{3}H]acetaminophen sulfate, the cumulative amount excreted into bile over 2 hr was expressed as a percentage of the injected amount.

Fitting Procedures. The background noneliminated reference set consisted of labeled RBCs, which mark out the vascular space, and the two interstitial space indicators (high-molecular-weight or labeled albumin and low molecular weight, either labeled sucrose or ^{58}Co-EDTA), which undergo flow-limited distribution (16). To characterize these, the labeled RBC curve was approximated by a variable-knot cubic spline, and the delayed wave behavior of the interstitial references was characterized by fitting the curves for each of these to the labeled RBC curve (see equations [A9] and [A10] in the Appendix), optimizing the two fitting parameters (the common large-vessel transit time t_o, and the interstitial to sinusoidal plasma space ratio, γ) by use of a nonlinear least-squares procedure. When the labeled RBC curves were not useful because of hemolysis, the relation between the two interstitial reference curves was, alternately, explored.

A theoretical model of the [^{3}H]acetaminophen sulfate outflow profiles, developed with a space-distributed model of flow-limited or barrier-limited behavior, is presented in the Appendix. As will become evident when the nature of the data is considered, it was necessary to extend the modeling to include the binding of the tracer [^{3}H]acetaminophen sulfate to albumin. Calculations with the modeling were performed on a Hewlett-Packard 9000 computer (Hewlett-Packard Co., Fort Collins, CO) using programs written in FORTRAN 77, with subroutines from the International Mathematical and Statistical Libraries (Houston, TX).

The modeling of the [^{3}H]acetaminophen sulfate curves included rapidly equilibrating protein binding, and influx, efflux and sequestration processes. The transfer coefficients for the latter are presented and defined in Table 1. The modeling, equation (A14) from the Appendix, was fitted to the acetaminophen sulfate data by varying the influx and efflux transfer coefficients, and the ratio $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$, needed to describe the appropriate interstitial space reference for the data, in relation to that for the labeled sucrose curve, and by inserting an appropriate value for the sequestration coefficient k'_{seq}. The parameters γ_{AS} and γ_{Suc} represent the interstitial to sinusoidal plasma space ratio values for acetaminophen sulfate and sucrose, respectively, and $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$ is the ratio of the total extracellular sinusoidal plasma plus interstitial space of distribution of acetaminophen sulfate to that for sucrose.

Because the proportion of acetaminophen sulfate sequestered (in this case, by biliary secretion) was very small, it was not possible to determine the proportion of tracer sequestered and the value of the sequestration parameter k'_{seq} from the outflow dilution curves alone, either from a comparison of the area under the [^{3}H]acetaminophen sulfate vascular outflow curve to that under the interstitial reference curve or, when input bulk steady values were fixed, from input and output values (concentrations were too closely similar). On the other hand, it was possible to measure accurately the proportion sequestered by biliary secretion by measurement of either cumulative excretion of tracer in bile (over the 2-hr collection) or the steady-state rate of excretion of unlabeled material, expressed as a fraction of its rate of presentation to the liver by flow. The value of the parameter k'_{seq} was therefore found as follows: within each iteration step of the fitting procedure, the outflow dilution tracer recovery, as defined by equation (A15) in the Appendix, was set equal to the observed recovery, which was taken to be (1 − the recorded proportion of acetaminophen sulfate excreted in bile). The small deviations from complete recovery caused by biliary excretion could not otherwise have been accurately approximated. When there was a minor deviation in recovery from this expected value, this had the effect of multiplying the tracer data by a constant factor, close to one. The fitted data then necessarily corre-

sponded to the observed relative biliary excretion rates.

Estimates of the parameters were modified by a weighted least-squares procedure (nonlinear regression analysis) from the International Mathematical and Statistical Library until an optimal fit to the labeled acetaminophen sulfate curve was obtained. Weighting was conducted according to counting statistics noise, ensuring an error variance proportional to the magnitude of the observation (36).

Statistical Evaluation of the Fitting Results. We followed the classical (nonbayesian) interpretation of weighted least-squares parameter estimates, as discussed by Landaw and DiStefano (36). 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, the standard deviations of the fitted parameters, are then calculated for the single experiment, representing the uncertainty in the determination of the parameter from the data of the experiment (as opposed to that for parameters from different experiments, representing interindividual variability). For the parameter not obtained directly by the fitting procedure, k'_{seq}, its variance was estimated from its dependence on the other parameters and the correlation structure of the covariance of the latter (36).

RESULTS

Partition into Organic Phases and Distribution in Blood and Liver. The equilibrium distribution ratio of acetaminophen sulfate into chloroform and octanol, at pH 7.4, was negligible: 0.0058 ± 0.00052 (S.D.) and 0.019 ± 0.0015 (n = 6) for all concentrations (tracer to 695 μmol/L). Acetaminophen sulfate was found by equilibrium dialysis to bind to the 1% albumin in the perfusion medium, the plasma unbound fraction, f_f, dependent on the acetaminophen sulfate concentration, albeit in minor fashion. The unbound fraction (0.635 ± 0.043) was lowest at tracer concentrations, increased slightly over the lower concentration range and then increased at a low rate over the higher range (Fig. 1, top panel). The relation illustrated was used to derive f_f values for experiments in which acetaminophen sulfate infusion was used. Long-time ultracentrifugation, with sedimentation of plasma proteins, gave essentially identical results. A Scatchard plot of the data (Fig. 1, bottom panel) yielded an approximately linear relation corresponding to a binding constant of 570 mol/L^{-1} and an average of 4.5 binding sites for acetaminophen sulfate on each albumin molecule. The data appear adequately predicted by one class of sites. There was no detectable distribution of acetaminophen sulfate into RBCs. The unbound fraction in tissue, f_t, after correction for the dilution of liver protein, was found to be 0.733 ± 0.021. It was essentially independent of tissue acetaminophen sulfate concentration (which varied between tracer concentration and 930 μmol/L).

Multiple-Indicator Dilution Studies. Sets of characteristic indicator dilution curves from the albumin-containing (acetaminophen sulfate trace and load) and albumin-free studies are shown in Figures 2 and 3 in semilogarithmic and rectilinear forms. The semilogarithmic plots best show the delayed or tailing components of the curves, especially those that are late and low

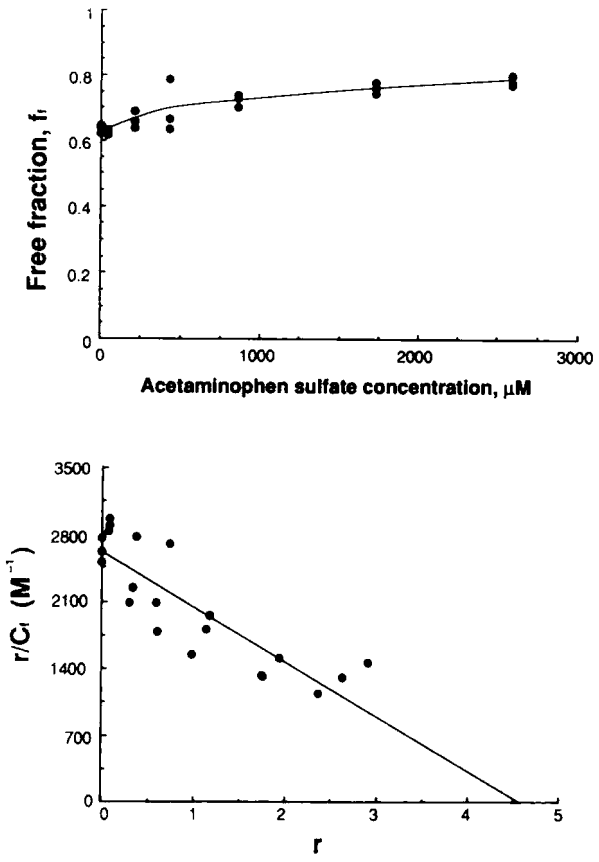

FIG. 1. *(Top panel)* Variation of the free or unbound fraction of acetaminophen sulfate in perfusate plasma containing 1% albumin, as a function of acetaminophen sulfate concentration. Zero concentration values refer to experiments performed with tracer acetaminophen sulfate only. *(Bottom panel)* Scatchard plot of the data. The parameter r is the ratio of bound acetaminophen sulfate to the total albumin concentration, and C_f is the concentration of unbound acetaminophen sulfate. The regression line yields a binding constant of 570 M^{-1} (equal to its negative slope) and a number of 4.5 binding sites per albumin molecule (equal to intersection of the line with the abscissa).

in magnitude; the rectilinear curves show the upslope relations.

The top panel of each figure shows the characteristic behavior of the noneliminated reference indicators (^{51}Cr-labeled RBCs, ^{125}I-labeled albumin and [^{14}C]sucrose). The labeled RBCs emerged first. The upslope of this curve was steepest, with the highest and earliest peak, and the downslope decayed most quickly. The ^{125}I-labeled albumin curve (a high-molecular-weight interstitial space reference) rose slightly slower, to a lower peak, later in time, and decayed with a slightly smaller slope. The [^{14}C]sucrose curve (a low-molecular-weight interstitial space reference which, in the albumin-free experiments, is substituted by its exact kinetic equivalent ^{58}Co-EDTA), characteristically separating from the ^{125}I-labeled albumin curve, showed, in relation to the labeled albumin curve, a more delayed upslope, a lower and later peak and a more prolonged downslope. The intermediate dispersion of the labeled albumin has been attributed to an access to only part of

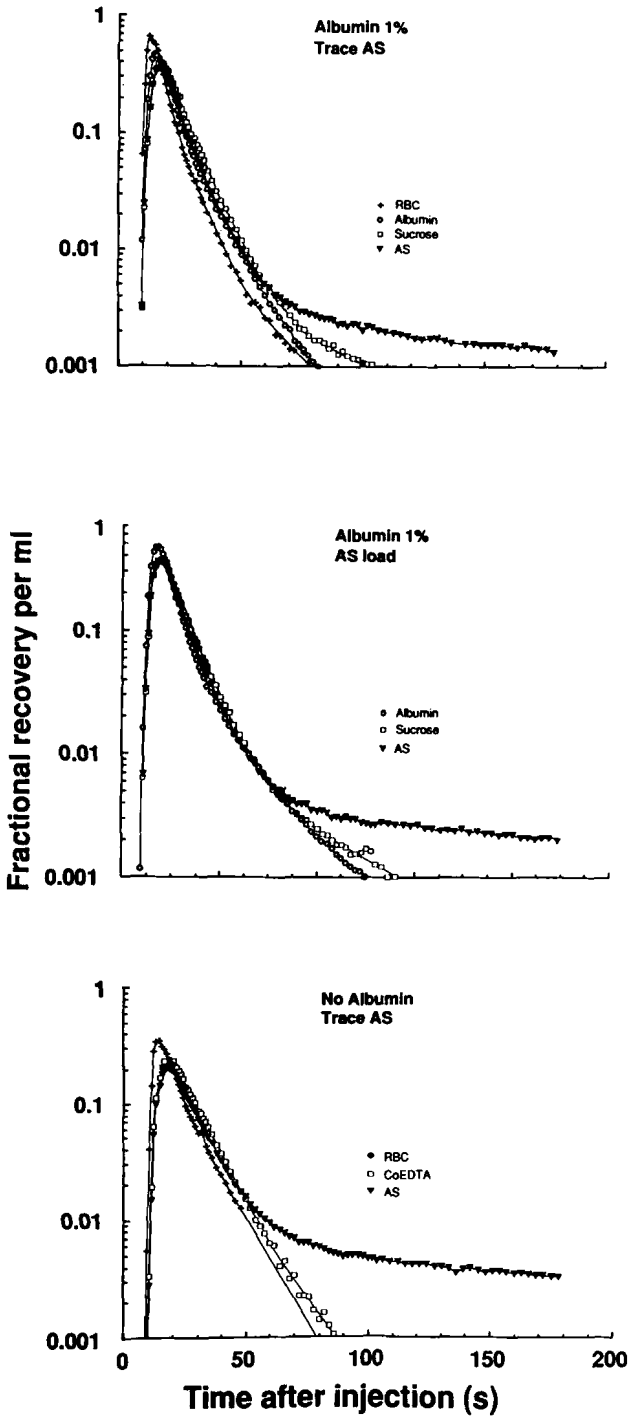

FIG. 2. Semilogarithmic outflow profiles from representative multiple-indicator dilution experiments. In the *top panel,* the perfusate contained 1% albumin; in the *middle panel,* bulk acetaminophen sulfate (464 μmol/L) was present, and the perfusate again contained 1% albumin; and in the bottom panel, no bulk acetaminophen sulfate was added, and albumin was excluded from the perfusate. AS = acetaminophen sulfate.

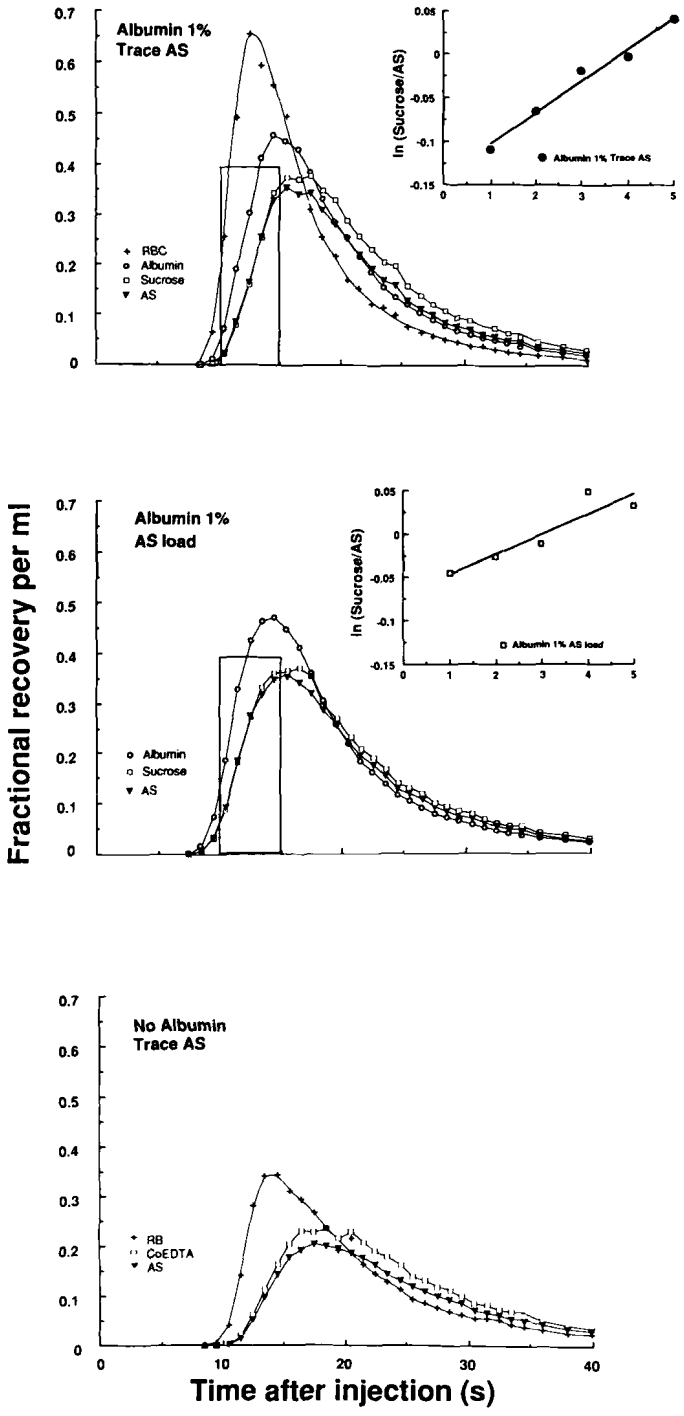

FIG. 3. Rectilinear plots of the data presented in Figure 2. The data are truncated, because the final tailing of the curves is able to be visually discerned only on the semilogarithmic presentation. *Inset panels* show natural logarithms of the ratio of sucrose: acetaminophen sulfate outflow recoveries per milliliter vs. time. When the acetaminophen sulfate curve is larger than that for sucrose, the natural logarithmic ratio is negative; when higher, it is positive, and the value zero corresponds to the point of crossover. AS = acetaminophen sulfate.

the interstitial space, consequent to the excluding effect of the resident large molecular weight interstitial polymers and the glycocalyces associated with the cell surfaces (37). The comparatively larger dispersion and longer transit time of the low-molecular-weight interstitial reference, on the other hand, occurs because this gains access to essentially the whole of the interstitial space.

TABLE 2. Mean transit times and distribution spaces for the noneliminated reference indicators, from the perfused (12 ml/min) rat liver experiments in which the perfusion medium contained 1% albumin and no bulk acetaminophen sulfate was added to the reservoir, and those in which the perfusion medium contained no albumin

		Mean transit times (sec)[a]			Distribution spaces		
						Interstitial space	
Experiment no.	Liver weight (gm)	Red blood cells	Albumin	Sucrose or [^{58}Co]EDTA	Vascular blood volume (ml blood per gm)	Albumin (ml plasma per gm)	Sucrose or [^{58}Co]EDTA (ml plasma per gm)
Tracer only, 1% albumin							
1	8.77	11.52	13.78	18.58[b]	0.263	0.044	0.138[b]
2	8.75	15.32	18.31	21.55	0.349	0.059	0.122
3	14.19	12.19	15.28	18.31	0.172	0.037	0.074
4	13.32	16.14	19.82	23.46	0.242	0.047	0.094
5	10.83	11.74	14.76	17.91	0.217	0.048	0.097
6	8.37	13.23	15.33	21.09	0.316	0.044	0.163
Average	10.71	13.36	16.21	20.15	0.260	0.046	0.115
± S.D.	± 2.53	± 1.95	± 2.33	± 2.22	± 0.065	± 0.007	± 0.033
Tracer only, no albumin							
14	14.12	24.57	–	30.85[c]	0.290	–	0.063[c]
15	12.31	16.71	–	20.91	0.271	–	0.058
16	16.25	14.03	–	17.37	0.173	–	0.035
17	11.97	16.77	–	23.27	0.280	–	0.094
18	9.44	12.77	–	16.71	0.270	–	0.072
Average	12.50	16.97	–	21.82	0.257	–	0.064[d]
± S.D.	± 2.80	± 4.59	–	± 5.71	± 0.048	–	± 0.021

Livers were perfused at the rate of 12 ml · min^{-1} · liver^{-1}.
[a]Corrected for the mean transit times of inflow and outflow catheters.
[b][^{14}C]Sucrose.
[c][^{58}Co]EDTA.
[d]Unpaired t test; $p < 0.05$.

The mean transit times of the noneliminated reference indicators are given in the upper part of Table 2 for the first set of albumin-containing data; from these, volumes of distribution were calculated. The vascular volume was calculated as the product of flow and the mean transit time for labeled red cells. The interstitial volume of distribution or Disse space was calculated for the two interstitial space indicators as the product of plasma flow and the difference between the mean transit time of the indicator and that of the labeled RBCs. The interstitial space of distribution for labeled albumin was always less than that for labeled sucrose. The values tabulated are of the order of those observed previously described (20, 23).

When the tracer acetaminophen sulfate was introduced in the presence of albumin but in the absence of an underlying steady-state unlabeled substrate concentration (top panels of Figs. 2 and 3), the [^{3}H]acetaminophen sulfate curves exhibited a shape quite different from that previously encountered for nonprotein-bound materials (17, 18). When the latter enter liver cells, they show a progressive diminution in upslope magnitude, in relation to that of the labeled-sucrose curve (17). Here, in contrast, the [^{3}H]acetaminophen sulfate curve precessed the labeled-sucrose curve, rising before it, in a position intermediate between the labeled-albumin and labeled-sucrose curves, but quite close to the labeled sucrose curve; the upslope thus preceded that of the labeled sucrose curve. The degree of precession is small and not easy to perceive in the illustrations. In the inset in the top panel of Figure 3, the natural logarithm of the ratio of sucrose:acetaminophen sulfate recoveries is plotted vs. time. The point of crossover corresponds to the zero value of the ordinate of the inset. Acetaminophen sulfate values were higher than those for sucrose before this and less, after it. The acetaminophen curve crosses over that for labeled sucrose near the peak, and its initial downslope decreases before that of the labeled-sucrose curve until, around 50 to 70 sec, the [^{3}H]acetaminophen sulfate curve again crosses over the labeled-sucrose curve, exhibiting a late, low-magnitude tailing. Beyond 70 sec, consistently higher outflow fractions were observed for [^{3}H]acetaminophen sulfate than for [^{14}C]sucrose, which were then virtually zero.

The form of the [^{3}H]acetaminophen sulfate curve can be qualitatively interpreted. The precession with respect to the labeled-sucrose curve is interpreted to be likely caused by the albumin binding of the acetaminophen sulfate, whereas the large early component of the [^{3}H]acetaminophen sulfate curve, which more or less follows the early part of the [^{14}C]sucrose curve, can be construed to be a component largely coming to the outflow without entering the liver cells (presumably caused by very low permeability of the liver cells for acetaminophen sulfate). The curve is smooth in shape and exhibits no early component related to the labeled albumin curve. It relates, rather, to some intermediate

TABLE 3. Results from the [^{3}H]acetaminophen sulfate indicator dilution perfused (12 ml/min) rat liver experiments, for tracer and load in the presence of 1% albumin, and for tracer when the perfusion medium lacked albumin

Experiment no.	Liver weight (gm)	Perfusate flow (ml · sec^{-1} · gm^{-1})	Hematocrit	Acetaminophen sulfate input concentration (C_{In}) (μmol/L)	Cumulative [^{3}H]acetaminophen sulfate excretion in bile		t_0 (sec)	$\frac{1+\gamma_{AS}}{1+\gamma_{Suc}}$	ΔAS_f[a] Free acetaminophen sulfate interstitial space (ml/gm)
					%	%/gm			
Tracer only, 1% albumin									
1	8.8	0.0228	0.144	–[b]	0.88[c]	0.100[c]	2.85	0.991	0.026
2	8.8	0.0228	0.140	–	0.49	0.056	1.94	0.980	0.020
3	14.2	0.0141	0.148	–	0.76	0.054	2.05	0.970	0.010
4	13.3	0.0150	0.146	–	0.82	0.062	0.82	0.970	0.010
5	10.8	0.0185	0.148	–	0.81	0.075	1.25	0.966	0.014
6	8.4	0.0239	0.132	–	0.68	0.081	3.92	0.973	0.018
Average	10.7	0.0195	0.143	–	0.74	0.071	2.14	0.975	0.016
± S.D.	± 2.5	± 0.0043	± 0.006		± 0.14	± 0.018	± 1.12	± 0.009[d]	± 0.006
Bulk acetaminophen sulfate added, 1% albumin									
7	9.2	0.0218	0.137	72	0.37[e]	0.040[e]	0.00	0.982	0.012
8	10.0	0.0200	0.144	85	0.14	0.014	2.48	0.972	0.010
9	8.8	0.0227	0.132	200	0.16	0.018	1.98	0.976	0.018
10	9.9	0.0203	0.134	239	0.63	0.064	0.00	0.985	0.014
11	10.3	0.0195	0.144	464	0.35	0.034	3.69	0.994	0.015
12	8.7	0.0229	0.140	652	0.27	0.031	0.00	0.981	0.012
13	8.3	0.0241	0.142	869	0.19	0.023	2.05	1.000	0.018
Average	9.3	0.0216	0.139		0.30	0.032[f]	2.44	0.984[d]	0.014
± S.D.	± 0.8	± 0.0017	± 0.005		± 0.17	± 0.017	± 2.36	± 0.010	± 0.003
Tracer only, no albumin									
14	14.1	0.0118	0.154	–	1.08	0.077	4.72	1.034	0.009
15	12.3	0.0162	0.154	–	1.12	0.091	1.46	1.030	0.008
16	16.3	0.0123	0.154	–	0.88	0.054	0.31	1.019	0.003
17	12.0	0.0167	0.138	–	0.77	0.064	2.42	1.028	0.008
18	9.5	0.0211	0.136	–	1.25	0.132	0.00	1.011	0.004
Average	12.8	0.0156	0.147	–	1.02	0.085	1.78	1.024[d,f]	0.006[f]
± S.D.	± 2.5	± 0.0038	± 0.010		± 0.22	± 0.035	± 1.90	± 0.009	± 0.003

Livers were perfused at the rate of 12 ml · min^{-1} · liver^{-1}.

[a] ΔAS_f = increment in interstitial space available for "free" acetaminophen sulfate above that available to sucrose.

[b] Tracer, no unlabeled acetaminophen sulfate added.

[c] Cumulative 2-hr collection of tracer [^{3}H]acetaminophen sulfate.

[d] $p < 0.01$ vs. 1.00; two-tailed t test.

[e] Steady-state fractional excretion rate.

[f] $p < 0.01$ vs. albumin-containing tracer group with no bulk acetaminophen sulfate infusion; unpaired two-tailed t test.

curve shape, one that has apparently been shaped by the albumin-binding effect. The late tailing represents tracer material returning to the vasculature late in time, after it had entered the liver cells.

A characteristic set of profiles from the set of albumin-containing experiments, in which unlabeled acetaminophen sulfate was added to the perfusate, is shown in the middle panels of Figures 2 and 3. The ^{51}Cr-labeled RBC curves showed a large downslope tailing, not previously observed, which was presumed caused by hemolysis of a proportion of the labeled RBCs in the injection dose. Because the purpose of this set of experiments was to gain insight into whether substrate handling was changed at higher concentrations, and because this knowledge could be gained without reference to the labeled RBC curves, the data were analyzed without reference to these. The relations between the profiles shown in the middle panel (those for ^{125}I-albumin, [^{3}H]acetaminophen sulfate and [^{14}C]sucrose) were generally similar to those seen in the absence of infusion of unlabeled acetaminophen sulfate.

In the set of experiments conducted without albumin in the perfusate and injection solution, a trace of hemolysis was found. The occurrence is perhaps not unexpected because RBCs suspended in buffer in the absence of albumin assume a prehemolytic spherocytic shape. To obtain a RBC reference, the downslope of the RBC label curve was extrapolated on the semilogarithmic plot to exclude the tiny late component corresponding to the hemolysis, and the area was restored. In the bottom panels of Figures 2 and 3, a representative set of acetaminophen sulfate profiles is displayed. These are characteristically different from those in the top two panels. In the absence of albumin, the [^{3}H]acetaminophen sulfate profile, rather than precessing the ^{58}Co-EDTA (a low-molecular-weight interstitial ref-

Transfer of coefficients			
$\frac{k_1 f_t \theta}{(1+\gamma_{AS})} = \frac{k'_1 \theta}{(1+\gamma_{AS})}$ (ml · sec^{-1} · ml^{-1})	$k_{-1} f_t = k'_{-1}$ (ml · sec^{-1} · ml^{-1})	$k_{seq} f_t = k'_{seq}$ (ml · sec^{-1} · gm^{-1})	$P_{in} S$ (ml · sec^{-1} · gm^{-1})
0.00553	0.00554	0.000555	0.0027
0.00247	0.00659	0.000651	0.0015
0.00638	0.00585	0.000386	0.0019
0.00460	0.00653	0.000540	0.0021
0.00499	0.00639	0.000607	0.0020
0.00574	0.00789	0.000475	0.0032
0.00495	0.00647	0.000536	0.0022
± 0.00136	± 0.00081	± 0.000095	± 0.0006
0.00459	0.00556	0.000270	0.0022
0.00332	0.00172	0.000041	0.0013
0.00635	0.00821	0.000094	0.0034
0.00496	0.01061	0.000705	0.0023
0.00741	0.00487	0.000092	0.0029
0.00518	0.00942	0.000271	0.0024
0.00851	0.00490	0.000057	0.0037
0.00576	0.00647	0.000218	0.0026
± 0.00178	± 0.00309	± 0.000235	± 0.0008
0.01255	0.00567	0.000182	0.0034
0.01533	0.00904	0.000312	0.0042
0.01478	0.00973	0.000336	0.0027
0.00603	0.00267	0.000166	0.0019
0.00593	0.00736	0.000870	0.0018
0.01092[f]	0.00689	0.000373	0.0028
± 0.0046	± 0.00284	± 0.000288	± 0.0010

erence behaving identically to [^{14}C]sucrose) curve on the upslope, lagged slightly behind the ^{58}Co-EDTA curve (it was lower all along the upslope). Then, as in the top two panels, the [^{3}H]acetaminophen sulfate curve exhibited a late tailing, the return of material that had entered the liver cells. The change in the relation of the [^{3}H]acetaminophen sulfate curve to the ^{58}Co-EDTA curve in the absence of albumin reinforces the initial impression that the precession with respect to the low-molecular-weight reference in the presence of albumin is caused by the binding of the tracer acetaminophen sulfate to albumin (compare the bottom panels of Figs. 2 and 3 with the top and middle panels). The liver weights for this set were, on average, larger than those in the first set, with albumin; values for flow/(liver weight) were consequently lower, and transit times were longer. The sucrose Disse space per gram of liver was significantly lower in association with the lower flows (23).

For all the experiments, the tails of the [^{3}H]acetaminophen sulfate curves were low in magnitude and of very low slope. The slope of the tailing was so low that it could not accurately be extrapolated to infinite time; thus it was not possible to estimate a mean transit time for the labeled acetaminophen sulfate.

Biliary Excretion and Extraction from Plasma. On average, less than 1% (0.74% ± 0.14% S.D.) of the tracer dose of [^{3}H]acetaminophen sulfate was recovered in bile in 2 hr in the first set of tracer experiments (Table 3). In the second set of experiments, in which unlabeled acetaminophen sulfate was added for single-pass perfusion, virtually no difference was detectable between the steady-state input and output plasma concentrations (i.e., extractions approached zero), suggesting that the degree of removal of acetaminophen sulfate was too small to be measured accurately by difference. Bulk acetaminophen sulfate excretion, on the other hand, was measurable in bile; its biliary excretion showed some scatter but was more or less linearly related to input rate (Fig. 4). It was, on average, 0.30% ± 0.17% (S.D.) of the input rate. This suggests that there was a minor degree of saturation of the biliary excretion at the higher input levels but, with the scatter in the data, this was not clearly demonstrable. The biliary excretion data in this study, for preformed acetaminophen sulfate, are quantitatively quite different from those previously observed for the excretion of locally generated acetaminophen sulfate, the exclusive metabolite of acetaminophen at low input concentrations (27, 28). When acetaminophen was infused in concentrations of 1.5 to 40 μmol/L, in the

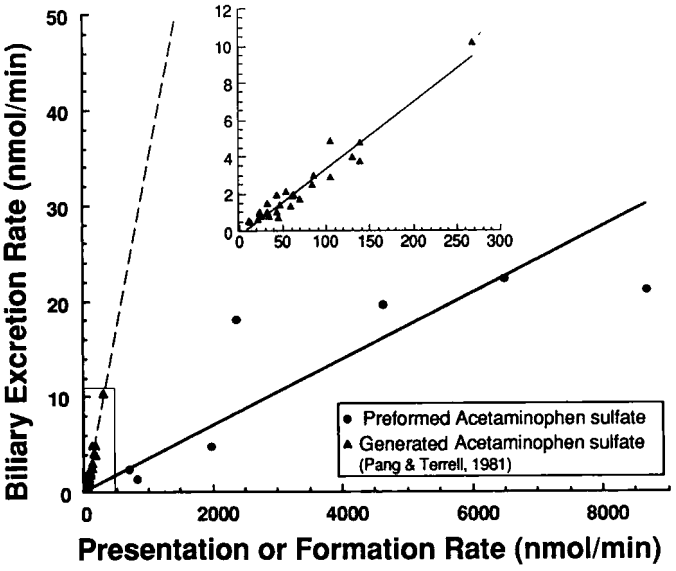

FIG. 4. Biliary excretion rate for acetaminophen sulfate, as a function of the presentation rate for acetaminophen sulfate. The *filled circles* represent data from the present set of bulk infusion experiments (where acetaminophen sulfate is presented at the hepatic input as the preformed compound). ▲ = data from Pang and Terrell (28), in which acetaminophen sulfate is generated from acetaminophen within the liver cells, during the infusion of acetaminophen.

presence of 1% albumin, the average hepatic extraction (and conversion) was 0.65 of the amount infused, and the biliary excretion averaged 3.3% of the generated acetaminophen sulfate. A plot of these data in Figure 4 illustrates that the biliary excretion rate of locally generated acetaminophen sulfate is of the order of 10 times that found with preformed acetaminophen sulfate, at comparable presentation/formation rates. In the third set of experiments, where albumin was omitted from the perfusion medium, and where no unlabeled acetaminophen sulfate was added to the perfusion medium, 1.02% ± 0.22% (S.D.) of the tracer dose, a value not different from that found in the presence of albumin, was recovered in bile over the 2-hr collection period (Table 3).

Evaluation of Indicator Dilution Results. In the first set of experiments, linear superposition of the outflow profile of the noneliminated reference indicators on the labeled RBC curve was conducted by use of the flow-limited, delayed-wave model of tracer distribution (16), as outlined in the Appendix [equations (A9) and (A10)]. This yielded values for the space ratios of interstitial to sinusoidal plasma volumes for sucrose and albumin, γ_{Suc} and γ_{Alb}, and the common transit time through the large vessels, t_0. The results were similar to those reported earlier (20, 23). The approach provides a unified description of the reference data set, when the labeled RBC curve is of good quality, and the modeling of substrate uptake has ordinarily been related to the vascular reference, by this description.

The form of the acetaminophen sulfate data, in relation to that of the interstitial references, indicates in all of the studies that most of the tracer acetaminophen sulfate passes through the liver without entering the liver cells. The indicator dilution profiles for acetaminophen sulfate were therefore analyzed with a barrier-limited model, represented by equation (A14), based on the approach previously developed to describe the hepatic uptake of tracer galactose (17), but extended to account for the effects of the partial protein binding of acetaminophen sulfate. Rapidly equilibrating partial protein binding is found to create, in the modeling, an upslope precession for acetaminophen sulfate relative to sucrose, similar to that observed experimentally. This kind of effect has not previously been explored at a theoretical level. The details are described in the Appendix. The parameters that were adjusted to obtain an optimal fit were: the extracellular space ratio $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$; and the transfer coefficients $k_1'\theta/(1 + \gamma_{AS})$, k'_{-1} and k'_{seq}, representing cellular influx, efflux and sequestration (biliary excretory) coefficients for acetaminophen sulfate (Table 1 provides definitions for these parameters).

During bulk acetaminophen sulfate infusion, hemolysis markedly distorted the labeled RBC curves, such that values for γ_{Alb} and γ_{Suc} could not be determined. The expected superposition relation of each of the reference curves on the labeled RBC curve, however, leads to another possibility, that of superposing the labeled sucrose on the labeled albumin curve to provide a t_0 estimate for the acetaminophen sulfate load experiments. Because the divergence between the two reference curves is small, estimates of t_0 in these experiments are potentially less accurate. To provide for comparison of results across the three experimental sets, one of which lacked albumin, a common parameter is needed. In each experiment, the data set contains a low molecular weight interstitial reference (labeled sucrose or ^{58}Co-EDTA) and a labeled acetaminophen sulfate curve. A fitting routine was therefore developed that exploits the relation between the two curves. This utilizes the ratio of the extracellular (i.e., vascular + interstitial) space of distribution for acetaminophen sulfate to that for sucrose, given by the expression $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$. This expression is contained within the function C_{ref}, which is developed in equation (A13), and utilized in equation (A14) to describe the acetaminophen sulfate curve. The fitting of the labeled acetaminophen sulfate curve can then be conducted without reference to the labeled RBC curve.

The relation of the form of the tracer acetaminophen sulfate curve to those of the tracer albumin and sucrose curves is of particular interest. If the albumin-bound fraction of tracer acetaminophen sulfate in the injectate had not been rapidly released, it would have come through to the outflow with the albumin to which it was bound, and each of the curves, where albumin was present, would have exhibited an early component in relation to the tracer albumin curve [see a previous analysis of the RBC carriage phenomenon (38) for an analogy]. The early carriage phenomenon did not occur, and the fit to the experimental data was systematically

good. The findings buttress the assumption underlying this analysis (that the free fraction determined experimentally in the equilibrium situation also applies to the nonsteady state encountered in this kinetic analysis). The fitted parameters are compiled in Table 3.

The fitted value for $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$ was slightly less than one when albumin was present, so that the average proportion of interstitial space occupied by acetaminophen sulfate was somewhat smaller than that for sucrose, but larger than that for albumin. Theoretically, the inferred value for γ_{AS} should have been more or less intermediate to that of γ_{Suc} and γ_{Alb} if what we have defined as the free or unbound fraction of acetaminophen sulfate in perfusate plasma occupies the same space of distribution as the labeled sucrose. In this instance, however, it is appropriate to reexamine how the various elements of the acetaminophen sulfate outflow profile have been defined. It is our assumption that the proportion of acetaminophen sulfate that is bound to plasma albumin would follow the labeled albumin in its behavior. Conversely, the unbound material, in our analysis, is that fraction not bound to the albumin, which does not follow the albumin in its behavior; this fraction is expected to distribute into the sucrose-accessible interstitial space but could also bind to the elements in the interstitial space. With the latter occurrence, the calculated interstitial space accessible to the labeled acetaminophen sulfate not bound to albumin (the "free" fraction) will be larger than that available to sucrose. From this point of view, the ratio for the interstitial to the plasma space of distribution available to acetaminophen sulfate, γ_{AS}, may be formulated as:

$$\gamma_{AS} = f_f\gamma_{AS,f} + (1 - f_f)\gamma_{Alb} \quad (4)$$

where f_f is the free fraction determined from plasma samples, $\gamma_{AS,f}$ is the interstitial space ratio for free acetaminophen sulfate and γ_{Alb} is the interstitial space ratio for albumin.

Values for $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$ are tabulated for the three different cases studied in Table 3. When no albumin is present (i.e., when $f_f = 1.0$), γ_{AS} and $\gamma_{AS,f}$ are equal, by definition, and the space of distribution available to acetaminophen sulfate is necessarily that available to the moiety we have defined as "free" acetaminophen sulfate. The average value found for $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$, in this case, was 1.024 ± 0.009 (S.D.). In the absence of albumin, the tracer acetaminophen sulfate gained access to a larger space of distribution than did the labeled sucrose. In the presence of albumin, the apparent space is smaller. When albumin is present, whether bulk acetaminophen sulfate is absent or present, no systematic difference is found. The average value for $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$ in both cases is less than one (average values were 0.975 ± 0.009 and 0.984 ± 0.010, respectively).

In the Appendix, an approach is given that provides a way of calculating the difference between the spaces of distribution available to free acetaminophen sulfate and sucrose. When calculated in this fashion, the space available to the free acetaminophen sulfate was, for all three sets, larger than that available to sucrose. When the excess interstitial space available to acetaminophen sulfate was expressed as an increment in the interstitial space (in milliliter equivalent plasma per gram) in excess of that available to sucrose, ΔAS_f, it was found to be significantly smaller in the third set, in comparison with the two other sets (just as the labeled sucrose interstitial space was smaller in that set). When the extra acetaminophen sulfate interstitial space was expressed as a fraction of the sucrose space, the values were 0.14 ± 0.03 and 0.10 ± 0.04 for the tracer acetaminophen sulfate experiments without and with albumin; the null hypothesis was then not rejected. Thus, it is the combination of the albumin binding together with the larger space of distribution available to "free" acetaminophen sulfate, in relation to sucrose, that accounts for the relations of the early parts of the experimental tracer acetaminophen sulfate curves to those for labeled albumin and sucrose: the rise of the labeled acetaminophen sulfate curve close to the labeled sucrose curve, rather than at an intermediate position, when albumin was included in the perfusion medium; and the lag of the labeled acetaminophen sulfate curve, on its upslope, in relation to labeled sucrose, when albumin was excluded from the perfusion medium.

Derived numerical values for the influx, efflux and sequestration coefficients, $k_1f_f\theta/(1 + \gamma_{AS})$, k'_{-1} and k'_{seq} are given in Table 3; their physical equivalents are presented in Table 1. The values for influx and efflux coefficients did not vary significantly between the two sets of albumin-containing data, those without and with bulk acetaminophen sulfate infusion. When albumin was absent from the perfusion, the value for the influx coefficient increased significantly, as expected, with the increase in the free fraction f_f; values for $k_{-1}f_t$ and $k_{seq}f_t$ were not significantly different.

The optimal calculated outflow profiles for acetaminophen sulfate showed good agreement with experimental values (the coefficient of variation for the fit averaged 0.052 ± 0.021 S.D.); an optimized fit is shown in Figure 5 for the acetaminophen sulfate data from the upper panel of Figures 2 and 3, as an example. The figure also shows that the peak portion of the outflow profile is caused by material that has not entered the liver cells, and that propagates in delayed wave fashion in the sinusoidal and interstitial spaces, but escapes entry into cells, whereas the tail portion of the curve represents material that has entered the liver cells, to return later to the vascular space (see Appendix, equation [A14]).

Based on the fitting procedure, coefficients of variation for the parameters were obtained as a dimensionless measure of the relative precision of their estimates (36). The calculated measure of the relative precision was the standard deviation, divided by the value of the parameter. For $k_1f_f\theta/(1 + \gamma_{AS})$, this averaged 0.12; for $k_{-1}f_t$, 0.17; for $k_{seq}f_t$, 0.29; and for the parameter $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$, 0.004. The precision of the estimates for the rate constants decreases as the process becomes more distant from the sinusoid. The

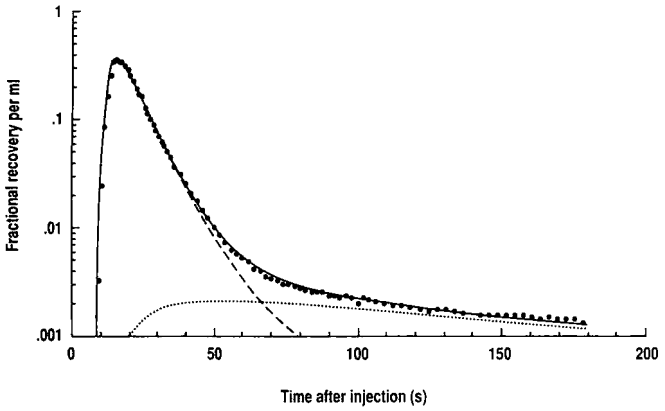

FIG. 5. Calculated outflow profiles for acetaminophen sulfate. *Points* represent the tracer acetaminophen sulfate experimental data previously shown in the *top panels* of Figures 2 and 3. The *solid line* represents the optimal fit of the model to the data. The *dashed* and the *dotted lines* represent material that has not entered the liver cells, and material that has entered the liver cells, to return later to the vascular space, as described by the first and second terms of Appendix equation (A14).

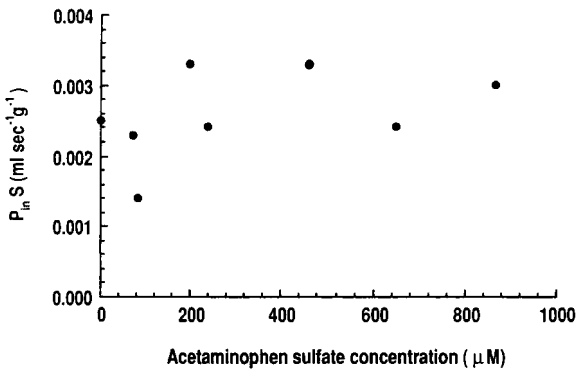

FIG. 6. Influx permeability surface area product per gram of liver, PS_{in}, as a function of the input acetaminophen sulfate concentration.

scatter in the calculated parameters between experiments is thus caused by, apart from biological variation, to a large extent, experimental variation in the data.

Values for the influx permeability surface area product of the sinusoidal plasma membranes for free acetaminophen sulfate were obtained, from the values for $k_1'f_f\theta/(1 + \gamma_{AS})$, the mean transit time for sucrose, the ratio $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$ and f_f, by use of equation (A17). The values, given in Table 3, are illustrated in Figure 6 as a function of the acetaminophen sulfate input concentration, for the bulk acetaminophen sulfate infusion experiments. The influx permeability surface area product shows no substantial evidence of saturation (i.e., decrease in magnitude), over the range of concentrations explored. The values found for the tracer acetaminophen sulfate permeability surface area product are quite low; they indicate that the hepatic sinusoidal cell membranes are very poorly permeable to acetaminophen sulfate. The values do not vary significantly between the comparable data sets. Values of particular interest are those from the studies conducted in the presence and absence of albumin, in the absence of acetaminophen sulfate bulk infusion. In comparing these two groups, the null hypothesis could not be rejected. The values for the influx permeability surface area product for acetaminophen sulfate in the presence of albumin were not different from those found in its absence.

Analysis of the results could also be conducted with average values. The ratio of the influx coefficient $k_1f_f\theta/(1 + \gamma_{AS})$ with albumin to that without albumin is 0.45; whereas, because the value for f_f is 0.635 for the tracer case in the presence of albumin and 1.00 without it, the expected ratio is 0.635. The data show, however, that the interstitial space is lower in the set without albumin, so that the effect of this needs to be appraised. If each average is multiplied by the average for the set of the ratio $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$, the ratio of the values becomes f_f times the ratio of the sinusoidal plus interstitial space without to with albumin, and the value for this is 0.409. The average ratio of the sucrose spaces with to without albumin (the inverse of the above) was 1.25; thus the estimated apparent value for f_f was 0.51. This is 0.81 times the measured *in vitro* value. With the previous approach, which utilized individual values, the ratio of the average influx permeability surface area products with to without albumin was 0.79; thus similar ratio values (0.81 vs. 0.79) arise from the two approaches. The approach utilizing the individual values provides the additional statistical insight that, although the two values differ, the null hypothesis could not be rejected; no difference is evident with and without albumin, and the two approaches correspond in a satisfying fashion. In addition, the analysis demonstrates the importance of having precise knowledge of the underlying anatomical features that vary with flow.

DISCUSSION

Lack of an Albumin Effect. These data show no evidence for facilitation of acetaminophen sulfate uptake in the presence of albumin. In terms of the unstirred layer effect, they correspond to what is expected when the unstirred layer becomes much less than the space constant for the nonequilibrium atmosphere (4). In this regime, the unstirred layer is much too small to impose a major limitation on uptake, and the massive increment in facilitation across the low albumin concentration range found with an unstirred layer much larger than the nonequilibrium atmosphere is not expected. Generally, with a minute unstirred layer, a large baseline clearance of unbound ligand is expected in the absence of albumin, with a small linear increment in this with increase in the protein concentration; whereas, when the unstirred layer is large, clearance of unbound ligand is expected to be very small in the absence of albumin, to rise sharply with the introduction of low concentrations of albumin and to increase much less steeply at higher concentrations (10). The predictions apply in either case to the situation in which dissociation is assumed not to be limiting. The facilitation effect of

the codiffusion phenomenon in the unstirred layer is, of course, expected to be largest when the albumin:ligand molar ratio is very high; it is nevertheless predicted to be quite small in an unstirred layer of dimensions expected in the intact liver even for fatty acids, for which the unbound fraction is minute (10). The lack of demonstration of an albumin effect in the uptake of acetaminophen sulfate is therefore perhaps not surprising. From this point of view, the small amount of albumin released by the once-through perfused liver will not be expected to have had a significant effect on the observation. It may be that, for substrates for which proportional uptake is very large, an albumin effect will more easily become perceptible; it would be useful, for example, to reexamine the uptake of bile acids.

Forms of the Curves: Effects of Protein Binding. The acetaminophen sulfate outflow dilution curves demonstrate that this compound is barrier-limited in its behavior in the liver. It permeates the plasma membrane of hepatocytes poorly. This is not surprising, given the high polarity of this compound, which carries one negative charge at the sulfate group, and in view of its negligible partitioning into chloroform and small partitioning into octanol.

The striking new experimental finding that has come from these studies is the observation that the effect of protein binding can be seen in the forms of the outflow dilution curves in the situation explored, that in which about one third of the compound is bound in the perfusate, at a 1% albumin concentration. The form of the early part of the dilution curve, which is composed primarily of material that has come to the outflow without entering the cells, is related to neither that expected for a completely bound compound (which would follow the labeled albumin curve), nor that for labeled sucrose, which in the first instance would have been expected to describe the behavior of the free acetaminophen sulfate, but rather to an intermediate profile.

The underlying behavior has been inferred to be one in which the dissociation and reassociation steps in protein binding are so rapid that the dissociation step is not limiting. If the latter had been limiting, tracer would visibly have been carried to the outflow with the labeled albumin, as an early component of the indicator dilution curve. This did not occur. The observation that the equilibrium albumin association is visible in the dilution curves introduces a set of expectations that, for other compounds, where binding is present in a similar proportion, it will be possible to examine experimentally the hypothesis that limited dissociation limits uptake. The likelihood of such an observation will, of course, be enhanced if the proportional net uptake of the unbound material is substantial, so that the remaining material, which is bound to albumin and not dissociating, is made apparent by the uptake process.

The effect of the protein binding is, in this case, much more simple than the delayed dissociation case; it is to make the bound acetaminophen sulfate inaccessible at the uptake surface. The influx permeability surface area product for "free" acetaminophen sulfate is thus not changed by the presence of the albumin. No additional effects beyond the binding have been demonstrated.

The Excess Interstitial Space Occupied by Acetaminophen Sulfate Not Bound to Albumin. The [^{3}H]acetaminophen sulfate not bound to albumin was found, in all three sets of experiments, with and without albumin and with and without bulk acetaminophen sulfate, to occupy a space of distribution larger than that available to sucrose. In the first set of experiments, where the size of the sucrose interstitial space could be calculated, the excess acetaminophen sulfate interstitial space was, on average, 14% ± 3% (S.D.) of the size of the sucrose interstitial space, and in the third, it was 10% ± 4% (S.D.) of the sucrose interstitial space. The excess extracellular distribution space for acetaminophen sulfate presumably represents binding. There are two possibilities: that the excess space, ΔAS_p, is associated with a cell membrane transfer process, mediating cell entry; or that the excess represents binding to elements of the interstitial space that are positively charged. If the first possibility were to account for the extra space, the effect would be at the cell surface and associated with binding during the transfer process. If the transfer process were carrier mediated, the space effect would be analogous to the enzymic space effect encountered in an analysis of the binding of monohydric alcohols to alcohol dehydrogenase *in vivo* (39). It would be, in this instance, a transporter space. Reduction in the size of the space with substrate loading would be expected, however, and this did not occur over the range of concentrations explored. A measurable transporter space has not yet been demonstrated for any other substances, and so the possibility that interstitial space binding accounts for the effect seems more likely. The premise arises from previous examinations of the equilibrium spaces of distribution in muscle and lung. The distribution of tracer inorganic sulfate and sucrose has been examined in striated muscle, where both are confined to the interstitial space (40). In rat left ventricle, the labeled sulfate and sucrose spaces were identical; in rat gastrocnemius, the sulfate space was less than the sucrose space; and in toad semitendinosus, the sulfate space was 11% larger than the sucrose space. The semitendinosus muscle values provide a parallel with our observation of a larger interstitial space available to the acetaminophen sulfate not bound to albumin than that available to sucrose. The increment appears likely because of binding to positively charged sites in the interstitial space. A converse effect is found in the lungs (41). The cationic isozyme of lactate dehydrogenase (intracellular pH = 7.9) occupies an interstitial space 1.5 times that for an anionic isozyme (intracellular pH = 5.0). A large charge effect is clearly evident.

The Permeability of Liver Cells to Acetaminophen Sulfate. The forms of the [^{3}H]acetaminophen sulfate curves and the inferences arising from their analyses indicate that permeation of this compound into liver cells is poor. Logically, the compound must be taken up to the extent that it is excreted in bile (if one excludes the

paracellular pathway), and because analysis indicates that cell efflux is taking place as well as cell entry, cell entry is larger than biliary excretion. The influx permeability surface area product for acetaminophen sulfate is quite small. It is only half that found for tracer enalaprilat, in similar experiments (20); and it is only slightly larger than that found for L-glucose, the nonmetabolizable, nonphysiologic poorly transported isomer of glucose (18). With continuing infusion of the acetaminophen sulfate, a steady-state cellular content is established; for lower (*circa* 80 μmol/L) concentrations, steady state in blood and bile is reached by 25 min and, for larger concentrations, by 15 min.

The process of cellular entry of acetaminophen sulfate was previously examined by Iida et al. (29), with isolated hepatocytes. Having explored the concentration range from 5 to 200 μmol/L, they extrapolated to a dissociation constant of 23 mmol/L, a value more than 10 times larger than the highest concentration explored. The extrapolation extends across a larger range than would usually be used for this kind of analysis, and can only be interpreted to indicate that there was little saturation across the range explored experimentally. In this study, a concentration range of 0 to 869 μmol/L was explored. No definite evidence of saturation was found. One must therefore conclude the previous inference that cell entry is mediated by a saturable carrier process is poorly supported. A passive permeability process could just as well be responsible for the cellular entry phenomenon.

It is, of course, of interest to compare the permeability of liver cells with acetaminophen sulfate with that of the inorganic sulfate ion, which serves as the precursor of the cosubstrate, 3′-phosphoadenosine-5′-phosphosulfate, for conjugate formation. Sulfate exchange, in contrast to that of chloride and phosphate, has been found to be relatively rapid, saturable (dissociation constant = 8 mmol/L) and inhibited by the anion transport inhibitors diisothiocyanostilbenedisulfonic acid and cyanocinnamate (42). At tracer concentrations, the influx permeability surface area product is of the order of 100 times that observed for acetaminophen sulfate, 50 times that for enalaprilat (20) and of the same order as that found for the carrier-mediated uptake of D-glucose at normal fasting glucose levels (18). The permeability to inorganic sulfate at tracer concentrations is, then, very high.

The permeability of the liver cells to acetaminophen, the precursor of acetaminophen sulfate, has not yet been determined, but there are data indicating that it must be very high. During one-way perfusion at flow levels similar to those used in the present set of experiments, approximately 65% of the acetaminophen entering was found to leave the liver as acetaminophen sulfate in blood, and 3.3% in bile, during steady state (27, 28). Therefore at least this proportion of the drug must have entered the liver cells. Contrast with this study where, at most, a small percentage of the acetaminophen sulfate has entered the liver cells, is stark.

The divergence between the handling of preformed and generated acetaminophen sulfate (the biliary excretion of less than 1% of label in the former instance and of about 3.3% in the latter) has been interpreted to be caused by a transport barrier for acetaminophen sulfate, in comparison with acetaminophen, at the sinusoidal (or basolateral) liver cell surface (27, 28). In this study, we have demonstrated the presence of this barrier and provided an index of the magnitude of its effect.

The Potential Steering Function Provided by Conjugation. Many drugs and some of the organic anions are metabolized in the liver to form more highly charged species. Most drugs are highly lipid soluble and have been expected to exhibit flow-limited cell entry. This kind of modification within the liver cells results in compounds which, in at least two instances (acetaminophen sulfate and enalaprilat [20]), have been demonstrated to permeate liver cell membranes poorly. The retarded efflux of the less permeable products then results in what would otherwise be unexpectedly high cellular concentrations and in protracted residence times. When the product undergoes biliary excretion, the prolonged sojourn time amplifies the proportion of the generated metabolite excreted through this pathway, as demonstrated in this study. The barrier limitation then has the function of steering a locally generated, more highly charged metabolite toward biliary excretion, after its intracellular formation from a lipophilic precursor, whereas, when the product is introduced through the bloodstream, its biliary excretion is sharply curtailed by the barrier. It appears likely that this amplification phenomenon for generated products contributes in a general fashion to the preferential biliary excretion of charged products (43, 44).

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APPENDIX: MODELING NEEDED FOR INTERPRETATION OF DATA

The model used for interpreting the data obtained from the multiple-indicator dilution experiments is a barrier-limited, space-distributed variable transit time model. This model was developed by Goresky et al. (17) to describe the uptake and sequestration of tracer galactose by the dog liver. The modeling is also similar to that recently utilized to account for the hepatic uptake of enalaprilat (20); it was extended to take into account the partial binding of acetaminophen sulfate to plasma albumin and an additional phenomenon needed to account for the forms of the curves and the binding of acetaminophen sulfate to components of the interstitial space or cellular surface.

The Single Path ("Single Sinusoid") Model. The first step in constructing the model is to describe the microevents occurring in a small section of a sinusoid and to elaborate these into a description of those in a "single sinusoid"; this will describe events along a single convection path through the sinusoidal bed, from a portal to a central venule. In a later stage, multisinusoidal behavior must be modeled to yield a description of the behavior of the whole organ.

Within the dimensions of the sinusoid, diffusion in the lateral direction may be assumed to be instantaneous; the convectional path length, however, is so long that diffusion in the longitudinal direction will not contribute significantly to transfer from entrance to exit, over the time scales involved, and it will therefore be neglected (16, 17). With these restrictions, it is possible to find an analytical solution in time and space describing the behavior of tracer within and at the outflow from a sinusoid.

Consider a low-molecular-weight tracer (herein, acetaminophen sulfate) that is partially bound to plasma proteins but does not penetrate blood cells. Let u_f be the plasma concentration of free or unbound tracer; u_b, that of protein-bound tracer; and $u = u_f + u_b$, the total tracer concentration in plasma. Similarly, v_f is the interstitial concentration of free or unbound tracer, and v_b is that of interstitial tracer bound to plasma albumin. The interstitial distribution space for the part of the tracer not bound to plasma albumin is potentially different from that for sucrose, because of differences in molecular mass and/or charge and potential binding to sites in the interstitial space or at the surface of liver cells. We therefore define a space ratio for unbound acetaminophen sulfate, $\gamma_{AS,f}$, as the ratio of the interstitial distribution space of acetaminophen sulfate not bound to plasma albumin to the sinusoidal plasma volume. It is further assumed that the interstitial distribution space of tracer bound to plasma albumin is equal to that of albumin, with the corresponding ratio being γ_{Alb}. The partial differential equation describing the behavior of such a tracer will be as follows:

$$W\frac{\partial u_f}{\partial x} + \frac{\partial u_f}{\partial t} + \gamma_{AS,f}\frac{\partial v_f}{\partial t} + \frac{\partial u_b}{\partial t} + W\frac{\partial u_b}{\partial x} + \gamma_{Alb}\frac{\partial v_b}{\partial t} = -k_1\theta v_f + k_{-1}\theta z_f \quad \text{(A1a)}$$

$$\frac{\partial z}{\partial t} = k_1 v_f - (k_{-1} + k_{seq})z_f \quad \text{(A1b)}$$

where x is the distance from the entrance at 0 to the exit at L, and t is time, W is velocity of sinusoidal blood flow (assumed constant throughout the flow path), z is the intracellular tracer concentration, z_f is the free intracellular concentration, θ is the ratio of intracellular to adjacent sinusoidal plasma volume, k_1 is the influx permeability surface area product divided by the cellular volume, k_{-1} is the efflux permeability surface area product divided by the cellular volume and k_{seq} is the cellular clearance divided by the cellular space (Table 1; 17, 20). We assume that rates of binding to and unbinding from plasma proteins are rapid compared with other events and that saturation of binding sites is not of importance (the proportion of tracer bound varies little, over the range of concentrations encountered in a single experiment). Consequently, the fraction of unbound tracer within the vascular space, $f_f = u_f/u$, will be constant and equal to that in bulk plasma, and that in tissue, $f_t = z_f/z$, was found invariant with concentration, in any case. If we assume instantaneous equilibration in the lateral direction, vascular and interstitial concentrations of bound and unbound tracers will be equal at each point along the path: $u_f = v_f$ and $u_b = v_b$. Equation (A1a) then becomes:

$$W\frac{\partial u}{\partial x} + (1 + \gamma_{AS})\frac{\partial u}{\partial t} = -k_1 f_f \theta u + k_{-1} f_t \theta_z \quad \text{(A2a)}$$

$$\frac{\partial z}{\partial t} = k_1 f_f u - (k_{-1} f_t + k_{seq} f_t)z, \quad \text{(A2b)}$$

where $\gamma_{AS} = f_f\gamma_{AS,f} + (1 - f_f\gamma_{Alb})$ is the apparent interstitial space ratio (interstitial/vascular) for total acetaminophen sulfate. Considering that the tracer, introduced instantaneously at $x = 0$, will immediately be distributed into the interstitial space, the initial conditions become:

$$u = 0 \text{ for } t < 0 \text{ or } x < 0,$$

$$u = \frac{q_s}{A(1 + \gamma_{AS})}\delta(x) \text{ and } z = 0 \text{ for } t = 0,$$

where q_s is the amount of tracer introduced into a single sinusoid at the entrance at $t = 0$ (the sinusoid is otherwise assumed to be initially empty of tracer), A is the cross-sectional area of the sinusoid (assumed to be constant along the length) and δ is the impulse or Dirac function.

With these transformations, the system of partial differential equations becomes exactly analogous to the cases previously solved (17, 20). Its solution is:

$$u = \frac{q_s}{F_s}e^{-k'_1\theta x/W}\delta(t - [1 + \gamma_{AS}]x/W) + \frac{q_s}{F_s}S(t - [1 + \gamma_{AS}]x/W)e^{-k'_1\theta x/W}e^{-(k'_{-1} + k'_{seq})(t - [1 + \gamma_{AS}]x/W)} \sum_{n=1}^{\infty}\frac{(k'_1\theta k'_{-1}x/W)^n(t - [1 + \gamma_{AS}]x/W)^{n-1}}{n!\,(n-1)!}, \quad \text{(A3)}$$

where $k'_1 = f_f k_1$; $k'_{-1} = f_t k_{-1}$; $k'_{seq} = f_t k_{seq}$; and $S(t - [1 + \gamma_{AS}]x/W)$ is a unit step function. The first term represents material that does not enter the liver cells, which propagates through the system with the velocity $W/(1 + \gamma_{AS})$. The second term represents material that enters the liver cells and escapes sequestration, to exit later through the vascular pathway.

The corresponding expressions for the reference indicators (RBCs, albumin and sucrose), developed previously (17, 20), are easily obtained from this. For a

vascular indicator such as RBCs, we set $\gamma_{RBC} = 0$ and $k_1 = 0$. This yields, from equation (A3),

$$u_{RBC} = (q_s/F_s)\delta(t - x/W). \quad (A4)$$

For an interstitial indicator (e.g., sucrose) distributed into both the vascular plasma and the interstitial space, $\gamma_{Suc} > 0$ and $k_1 = 0$. The corresponding equation will read:

$$u_{Suc} = (q_s/F_s)\delta[t - (1 + \gamma_{Suc})x/W)]. \quad (A5)$$

The labeled sucrose propagates as a delayed wave, with the velocity $W/(1 + \gamma_{Suc})$.

Albumin enters only part of the interstitial space because of a polymer exclusion phenomenon (16). It behaves in a manner similar to sucrose, with a virtually identical t_0 but a smaller apparent space ratio (i.e., $\gamma_{Alb} < \gamma_{Suc}$). With the proportion of the acetaminophen sulfate that is bound to albumin, if $\gamma_{AS,f}$ is approximately of the magnitude of γ_{Suc}, γ_{AS} will be smaller than γ_{Suc}. As a consequence, the acetaminophen sulfate not entering the liver cells, which is propagating in delayed wave fashion in the sinusoidal plasma and interstitial spaces, with the velocity $W/(1 + \gamma_{AS})$, will emerge at the outflow before the labeled sucrose.

In the previous development, we have defined the distribution space for free and bound acetaminophen sulfate separately, and have assumed the concentration of each within its equilibrium interstitial space to be equal to that in the plasma space. One could also have developed the description by assuming that albumin is partitioned within the interstitial space. Although the latter corresponds more closely to the physical events, both descriptions are equivalent.

Construction of Outflow Profiles from the Whole Liver. The tracer concentrations at the outflow of the whole liver will be flow-weighted averages of the outflow concentrations from all of the single paths. Because different paths have different transit times, this average is computed to take into account the distribution of transit times among the paths. As before (16, 20), we assume that all paths share a common large-vessel transit time, t_0.

Let $g(\tau)d\tau$ be the fraction of total organ blood flow emerging from paths with sinusoidal transit times between τ and $\tau + d\tau$, such that $g(\tau)$ is a distribution with the integral of this function equal to 1. The outflow concentration of the whole organ will then be

$$C(t) = \int_0^\infty u(W\tau, t - t_0)g(\tau)d\tau, \quad (A6)$$

where $u(x,t)$ is the total tracer concentration at location x and time t.

The ratio of the amount of tracer arriving at a single sinusoid, q_s, to blood flow at the exit of a single sinusoid, F_s, will be equal to the ratio of total tracer dose injected, q, to total organ flow, F; thus

$$\frac{q_s}{F_s} = \frac{q}{F}.$$

For the vascular reference indicator, labeled RBCs, substitution of equation (A4) into equation (A6) with $C(t) = C_{RBC}(t)$ and $u(x, t) = u_{RBC}$, yields

$$C_{RBC}(t) = \frac{q}{F}\int_0^\infty \delta(t - t_0)g(\tau)d\tau = \frac{q}{F}g(t - t_0). \quad (A7)$$

For the low molecular weight interstitial indicator-(sucrose), substitution of equation (A5) into equation (A6) with $C = C_{Suc}$ and $u(x, t) = u_{Suc}$, yields

$$C_{Suc}(t) = \frac{q_s}{F_s}\int_0^\infty \delta(t - t_0 - [1 + \gamma_{Suc}]\tau)g(\tau)d\tau = \frac{q}{F}\frac{1}{1 + \gamma_{Suc}}g\left(\frac{t - t_0}{1 + \gamma_{Suc}}\right). \quad (A8)$$

Because the outflow profiles for both the vascular reference and the interstitial indicators are related to the transit time distribution, g, they can be related to each other, as follows,

$$C_{Suc}(t) = \frac{1}{1 + \gamma_{Suc}}C_{RBC}\left(\frac{t - t_0}{1 + \gamma_{Suc}} + t_0\right). \quad (A9)$$

Similarly, for the higher molecular weight interstitial reference, labeled albumin, with a reduced space of distribution in the interstitial space,

$$C_{Alb}(t) = \frac{1}{1 + \gamma_{Alb}}C_{RBC}\left(\frac{t - t_0}{1 + \gamma_{Alb}} + t_0\right). \quad (A10)$$

For acetaminophen sulfate, substitution of equation (A3) into equation (A6) yields:

$$C_{AS}(t) = \frac{q}{F}\left\{\frac{g\left(\frac{t - t_0}{1 + \gamma_{AS}}\right)}{(1 + \gamma_{AS})}e^{-\frac{k'_1\theta}{1 + \gamma_{AS}}(t - t_0)} + e^{-(k'_1 + k'_{seq})(t - t_0)}\int_0^\infty g(\tau)e^{-(k'_1\theta - [k'_{-1} + k'_{seq}][1 + \gamma_{AS}])\tau}\sum_{n=1}^{\infty}\frac{(k'_1k'_{-1}\theta\tau)^n(t - t_0 - [1 - \gamma_{AS}]\tau)^{n-1}}{n!(n - 1)!}d\tau\right\}. \quad (A11)$$

In order to evaluate this equation, the distribution of sinusoidal transit times, $g(\tau)$, has to be known. This can be derived, as previously indicated, from the curve for one of the reference indicators. Because the outflow profile for sucrose is closest to that of acetaminophen sulfate (see Figs. 3 and 4), equation (A8) will be regarded as most suitable for this purpose. The development is therefore continued, utilizing $C_{Suc}(t)$ to define g.

Equation (A11) is simplified by defining a new function

$$C_{ref}(t) = \frac{q}{F}\frac{1}{1 + \gamma_{AS}}g\left(\frac{t - t_0}{1 + \gamma_{AS}}\right), \quad (A12a)$$

which is related to g in the following fashion,

$$g(\tau) = \frac{F}{q}(1 + \gamma_{AS})C_{ref}[(1 + \gamma_{AS})\tau + t_0]. \quad (A12b)$$

The new function C_{ref}, is equal to the coefficient of the exponential in the first term of equation (A11). This corresponds to the behavior expected of a hypothetical reference material that is distributed into an interstitial space defined by the space ratio γ_{AS}, but that does not enter the liver cells.

The new hypothetical reference is defined, in turn, in relation to the labeled sucrose curve, from equation (A8), by the relation

$$C_{ref}(t) = \frac{(1 + \gamma_{Suc})}{(1 + \gamma_{AS})} C_{Suc}\left(\frac{(t - t_0)(1 + \gamma_{Suc})}{(1 + \gamma_{AS})} + t_0\right). \quad \text{(A13)}$$

This function can then be utilized to simplify the resulting expression and to provide better insight into the relations involved. Substituting equation (A13) into equation (A11), and changing the integration variable τ to $\tau' = (1 + \gamma_{AS})\tau + t_0$, we find

$$C_{AS}(t) = C_{ref}(t)e^{-\left(\frac{k'_1\theta}{1+\gamma AS}\right)(t - t_0)} + e^{-(k'_{-1} + k'_{seq})(t - t_0)} \int_{t_0}^{\infty} C_{ref}(\tau')e^{-\left(\frac{k'_1\theta}{1+\gamma AS} + k'_{-1} + k'_{seq}\right)(\tau' - t_0)} \cdot \sum_{n=1}^{\infty} \frac{\left(\left[\frac{k'_1\theta k'_{-1}}{1 + \gamma_{AS}}\right][\tau' - t_0]\right)^n (t - \tau')^{n-1}}{n!(n-1)!} d\tau'. \quad \text{(A14)}$$

The first term represents that material that has not entered the liver cells, and which propagates in delayed wave fashion in the sinusoidal and interstitial spaces, but escapes entry into cells, whereas the second represents material that has entered the liver cells to return later to the vascular space.

Computationally, the parameters to be determined by fitting this expression to the tracer acetaminophen sulfate curve include the ratio $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$, which automatically defines C_{ref}, and the transfer coefficients for influx, efflux and sequestration ($k_1'\theta/[1 + \gamma_{AS}]$), k'_{-1} and k'_{seq}). The coefficient $k_1'\theta/(1 + \gamma_{AS})$ is equivalent to $k_1 f_f \theta/(1 + \gamma_{AS})$, which is defined as the free fraction times the influx permeability surface area product divided by the summed sinusoidal plasma plus average interstitial or Disse space for acetaminophen sulfate.

The recovery of acetaminophen sulfate in hepatic venous perfusate, as derived from the relation between the RBC and sucrose curves and previous expressions, is given by (20, 45),

$$\text{Rec} = \frac{F}{q}\int_0^{\infty} C_{Suc}(\tau')\exp\left[-\left(\frac{k'_1\theta k'_{seq}}{[1 + \gamma_{Suc}]k'_{-1}k'_{seq}}\right)(\tau' - t_0)\right]d\tau', \quad \text{(A15)}$$

where $\tau' = (1 + \gamma_{AS})\tau + t_0$.

Calculation of Permeability Surface Area Products. Because the quantities k_1' and θ are always linked together, they cannot be determined separately; only their product is identifiable. From this product, the permeability surface area product can be determined. According to the definition of the rate constant, k_1', the influx permeability surface area product for the uptake of acetaminophen sulfate, $P_{in}S$, can be calculated as:

$$P_{in}S = k_1 V_{cell} = k_1' V_{cell}/f_f,$$

where V_{cell} is the intracellular volume. According to the definitions of θ and γ_{AS}, this is equivalent to

$$P_{in}S = \frac{k'_1\theta V_p}{f_f} = \left(\frac{k'_1\theta}{[1 + \gamma_{AS}]}\right)\left(\frac{V_p[1 + \gamma_{AS}]}{f_f}\right), \quad \text{(A16)}$$

where V_p is the volume of sinusoidal plasma adjacent to the liver cells, and V_p $(1 + \gamma_{AS})$ is the apparent distribution volume of acetaminophen sulfate in the sinusoids. The distribution volume of sucrose within sinusoids is the sum of the plasma and interstitial spaces $(V_p + V_I)$; it is calculated as

$$V_p(1 + \gamma_{Suc}) = F_p(\bar{t}_{Suc} - t_0), \quad \text{(A17)}$$

where $F_p = F(1 - \text{hematocrit})$ is the plasma flow rate per gram, $\bar{t}_{Suc}$ is the mean transit time for sucrose in the liver and the latter minus the transit time of large vessels (t_0) is the mean transit time of sucrose across the sinusoidal bed. Because, from this, the volume of distribution of acetaminophen sulfate can be calculated, the permeability surface area product can be expressed as

$$P_{in}S = \left(\frac{k_1 f_f \theta}{1 + \gamma_{AS}}\right)F_p(\bar{t}_{Suc} - t_0)\frac{(1 + \gamma_{AS})}{f_f(1 + \gamma_{Suc})}, \quad \text{(A18)}$$

where all of the terms needed are known.

Calculation of the Volume of Distribution of Interstitial Acetaminophen Sulfate in Excess of That of Sucrose. This quantity is derived from the space ratios for free acetaminophen sulfate and sucrose, as follows

$$\Delta AS_f = V_p(\gamma_{AS,f} - \gamma_{Suc}).$$

Elimination of $\gamma_{AS,f}$ from equation (A16) and equation (A4) yields:

$$\Delta AS_f = V_p/f_f(\gamma_{AS} - [1 - f_f]\gamma_{Alb} - f_f\gamma_{Suc}),$$

which can be rearranged into

$$\Delta AS_f = \frac{V_p[1 + \gamma_{Suc}]}{f_f}\left[\left(\frac{1 + \gamma_{AS}}{1 + \gamma_{Suc}} - 1\right) - (1 - f_f)\left(\frac{1 + \gamma_{Alb}}{1 + \gamma_{Suc}} - 1\right)\right].$$

Substitution according to equation (A17) yields:

$$\Delta AS_f = \frac{F_p[\bar{t}_{Suc} - t_0]}{f_f}\left[\left(\frac{1 + \gamma_{AS}}{1 + \gamma_{Suc}} - 1\right) - (1 - f_f)\left(\frac{1 + \gamma_{Alb}}{1 + \gamma_{Suc}} - 1\right)\right]. \quad \text{(A19)}$$

The expression $(1 + \gamma_{AS})/(1 + \gamma_{Suc})$ is obtained from fitting the acetaminophen sulfate data to the model, as shown above, and the expression $(1 + \gamma_{Alb})/(1 + \gamma_{Suc})$ is obtained from the linear superposition of the albumin upon the sucrose curve. Plasma flow is expressed in milliliters/second/gram.