J. Pharm. Pharmacol. 1995, 47: 949–956
Received January 3, 1995
Accepted June 23, 1995

© 1995 J. Pharm. Pharmacol.

Availability and Mean Transit Times of Phenol and Its Metabolites in the Isolated Perfused Rat Liver: Normal and Retrograde Studies Using Tracer Concentrations of Phenol

LINSAY N. BALLINGER, SHEREE E. CROSS AND MICHAEL S. ROBERTS

University of Queensland, Department of Medicine, Princess Alexandra Hospital, Brisbane, Queensland, 4102, Australia

Abstract

Phenolic compounds are frequently detoxified by the formation of sulphate and glucuronic acid conjugates in the liver. These conjugates are formed in the hepatocytes and then either transported into the bile or back into the blood. In this study, we examined the transport kinetics of phenol and its metabolites in the isolated perfused rat liver by monitoring the outflow profiles of these compounds after a bolus input in a single pass preparation.

Phenol was almost exclusively metabolized to phenyl sulphate (97%) at the trace concentrations used, with the amount of phenol and metabolites excreted into the bile being minimal (3·5%). The metabolite formed was rapidly transported back into the perfusate, with mean transit times of 17·4 and 12·3 s anterograde and 24·9 and 24·2 s retrograde at flow rates of 15 and 30 mL min^{-1} respectively, which were intermediate between those of Evans blue and water. The outflow concentration-time profile for phenyl sulphate formation was unaffected by the addition of another organic anion (bromosulphophthalein).

The effect of enzyme zonation on outflow concentration-time profiles was also investigated using retrograde perfusions. The transit time ratios for generated metabolite to water for anterograde perfusions (0·6) was found to be more than twice that for retrograde perfusions (0·23) at 15 mL min^{-1} and approximately 1·6 times greater at 30 mL min^{-1}, being 0·58 and 0·37 respectively. The relative ratios obtained are consistent with previous findings that normalized variance of solutes in the retrograde perfusions is greater than that for anterograde perfusions.

The liver is generally accepted as being quantitatively the major drug-eliminating organ in the body, capable of both metabolic transformation and biliary excretion. Conjugative metabolism of xenobiotics with glucuronic acid or sulphate represents an important detoxification mechanism for many compounds, including those possessing phenolic hydroxyl moieties. Glucuronidation of phenolic compounds is catalysed by the enzyme family uridine diphosphate (UDP) glucuronosyltransferase (2.4.1.17) which have molecular weights of 50–60 kDa and are tightly bound to the membranes of the endoplasmic reticulum (Burchell & Coughtrie 1989). This enzyme conjugates UDP-glucuronic acid with the hydroxyl groups of many phenolic compounds to form aglyclones. Sulphation of phenolic compounds is catalysed by the cytosolic enzyme family aryl sulphotransferase (2.8.2.1), dimeric enzymes of approximately 65 kDa molecular weight (Sekura & Jakoby 1979). In this reaction, 3′-phosphoadenosine-5′-phosphosulphate serves as the sulphate donor, yielding a phenolic sulphate ester, adenosine-5′-monophosphate and inorganic phosphate. In general, glucuronidation and sulphation of compounds increases their water solubility, decreases volumes of distribution, pharmacologically or toxicologically inactivates the compound and hence facilitates more rapid renal tubular excretion (Mulder et al 1990).

The overall process of glucuronidation or sulphation of compounds occurs in three steps: cellular uptake of the parent compound, intracellular enzymatic metabolism, and metabolite release. Studies to date on the glucuronidation and sulphation of both xenobiotics and physiological solutes have predominantly been carried out using either rat isolated hepatocytes (Brock & Vore 1984; Satofumi et al 1988) or both sinusoidal (basolateral) (Hugentobler & Meier 1986; Potter et al 1987) and canalicular membrane vesicles (Meier et al 1987; Kobayashi et al 1991; Nishida et al 1992). When the isolated perfused rat liver (IPRL) preparation has been used, the studies have usually involved steady state perfusions (Pang & Gillette 1978; Pang & Terrell 1981; Vore et al 1991). In this work, we have employed the IPRL and the impulse-response technique to study the disposition of phenol and its metabolites. Phenol was chosen due to its low molecular weight (MW 94·11) and high extraction ratio (Cassidy & Houston 1984), essentially being completely biotransformed to either the sulphate or glucuronide metabolite, with very little oxidative metabolism occurring (Weitering et al 1979). Cellular uptake of phenol is both rapid and passive due to its lipophilic character. The usual pathway of xenobiotics undergoing conjugation has been thought to be that of cellular uptake and intracellular metabolism, followed by excretion at the

Correspondence: M. S. Roberts, Department of Medicine, The University of Queensland, Princess Alexandra Hospital, Brisbane, Queensland 4102, Australia.

canalicular plasma membrane into the bile duct and subsequent recirculation via the gastrointestinal tract as the conjugated metabolite. This work shows that phenol sulphate is both rapidly formed and excreted back into the perfusate. Phenol, phenyl-β-D-glucuronide and phenyl sulphate were used as individual solutes in impulse-response injectates to provide reference profiles for metabolites generated from an injectate of phenol given by impulse-response.

Materials and Methods

Chemicals and materials

[^{14}C]Phenol (130 mCi mmol^{-1}), BCS (biodegradable counting solution) and OCS (organic counting solution) were obtained from Amersham (Arlington Heights, IL). [^{14}C]Sucrose (632 mCi mmol^{-1}), [^{3}H]water (1 mCi g^{-1}) and Econofluor were obtained from DuPont New England Nuclear (Boston, MA). Evans blue and *p*-cresol were obtained from Ajax (Sydney, NSW). Bromosulphophthalein, taurocholate, α-D-(+)-glucose, albumin (bovine-fraction V), phenyl-β-D-glucuronide, phenol and β-glucuronidase were obtained from Sigma (St Louis, MO), [^{14}C]Phenyl sulphate was prepared by single-pass perfusion through an IPRL with a high dose of [^{14}C]phenol, with subsequent validation of purity using sulphatase and glucuronidase enzyme incubation. Emulsifier Safe was purchased from Packard (Groningen, The Netherlands), Nembutal (pentobarbitone sodium) from Boehringer Ingelheim (Artarmon, NSW) and heparin (1000 units mL^{-1}) from David Bull Laboratories (Mulgrave, Victoria). Carbogen (5% CO_2 in O_2 v/v) was supplied by Commonwealth Industrial Gases (Brisbane, Queensland).

Animals

Female Sprague-Dawley rats (10–12 weeks old, 190–250 g, Central Animal Breeding House, Brisbane, Queensland) were maintained on standard rat food pellets, water freely available and a 12 h light-dark schedule. The study was approved by the University of Queensland Animal Experimentation Ethics Committee.

Liver perfusion

Perfusions were carried out as outlined in previous studies (Gores et al 1986). Fed rats were anaesthetized (pentobarbitone sodium, 50 mg kg^{-1}, i.p.) a ventral midline laparotomy from the front of the pelvic region to the xiphoid process of the sternum and lateral extensions were performed, and heparin (100 units) was administered via the inferior vena cava. The bile duct was cannulated with polyethylene (PE)-10 tubing, the portal vein with a 16-gauge intravenous catheter and the thoracic inferior vena cava with PE-240 tubing (10 cm in length) or a 16-gauge intravenous catheter for anterograde and retrograde perfusions, respectively. Anterograde perfusion, using the portal vein catheter as the initial point of perfusate entry, was always effected before retrograde perfusion, where inlet tubing was rapidly disconnected and reconnected such that perfusate flow through the liver was reversed. Aerated ($pO_2 > 400$ mmHg) Krebs–Henseleit bicarbonate buffer (pH 7·4), with albumin (1% w/v) and taurocholate (25 mg L^{-1}), was maintained at a temperature of 37°C in a circulating waterbath and delivered using a peristaltic pump in a nonrecirculating system at flow rates of $1{\cdot}90 \pm 0{\cdot}03$ (n = 30) and $4{\cdot}08 \pm 0{\cdot}12$ (n = 8) mL min^{-1} (g liver)$^{-1}$ (typically achieved using flow rates of 15 and 30 mL min^{-1} respectively). Liver temperature was maintained in a modified humidicrib and monitored by a thermometer transiently placed between the right and median lobes. Inflow and outflow samples were collected on ice for determination of pH, pO_2 and pCO_2 (1312 Blood Gas Manager, Allied Instrument Lab, Milan, Italy). Liver viability was assessed by macroscopic appearance, bile flow measurement, transhepatic pressure and oxygen consumption. At the completion of each experiment, the liver was excised and its wet weight determined.

Impulse-response technique

This methodology essentially follows that for multiple indicator dilution (MID) studies as employed in previous studies (Wolkoff et al 1987; St-Pierre et al 1989). The injectate used for the study of phenol metabolism consisted of approximately 1 μCi [^{3}H]water, 150 μg filtered Evans blue (Minisart N, 0·45 μm, Sartorius Chadstone, Victoria) and various trace amounts of [^{14}C]phenol (0·2–4·8 μCi) in 50 μL perfusate. The injectate for preformed metabolite studies consisted of [^{3}H]water (1 μCi), Evans blue (150 μg), 0·2 μCi [^{14}C]sucrose and 3 μg phenyl-β-D-glucuronide or phenyl sulphate in 50 μL perfusate. The injectates were delivered as boluses (impulses) via the inflow catheter, and their effluents collected at 1- to 4-s intervals over the initial 120 s. All samples were immediately stored at 4°C before analysis. Fifteen groups of experiments were performed in livers under similar conditions of buffer temperature, transhepatic pressure, perfusate pH and albumin concentration.

Sample analysis

All outflow samples and appropriate injectate and bile dilutions were analysed for total ^{3}H- and ^{14}C-activity on a 1600TR series liquid scintillation counter (Packard, Downers Grove, IL). Samples and injectate dilutions were also analysed for Evans blue absorbance using multicell plates ($\lambda = 620$ nm, Titertek Multiskan MCC). Data obtained were corrected for background either manually or automatically in all cases.

Phenol and metabolite separation

An established differential extraction procedure was substantially followed in the analysis of phenol and metabolite concentrations in the effluent, bile and appropriate injectate dilutions (Cassidy & Houston 1984). In brief, a 625 μL aliquot of OCS was added to 50 μL of sample in an Eppendorf tube which was then vortexed for 10 s. The tubes were then gently agitated for a further 30 min (SMI multi-tube vortexer American DADE, Miami, FL), and centrifuged for 10 min at 13 000 *g* (Microfuge E, Beckman, UK). To 5 mL Emulsifier Safe, a 500 μL aliquot of the supernatant (OCS containing [^{14}C]phenol only) was added and this was then assayed for activity. This method was validated using the HPLC method described later.

Metabolite characterization and impulse-response relationships

To determine the relative percentages of sulphate and

glucuronide produced from phenol in the liver perfusion, samples were incubated with β-glucuronidase before and after OCS extraction of phenol at 37°C for 30 min. Samples were then extracted with OCS and the OCS-extracted and aqueous samples were analysed as for ^{14}C counts. Inhibition of the metabolite efflux mechanism was also investigated using the perfusate with 100 μM bromosulphophthalein. In this experiment, the liver was initially perfused with Krebs buffer containing no bromosulphophthalein and a single impulse-response injectate with [^{14}C] phenol was administered. The perfusate was then changed to the Krebs buffer containing bromosulphophthalein and allowed to equilibrate before performing identical injections.

HPLC analysis of phenyl-β-D-glucuronide

A simple and efficient method for simultaneous determination of both phenol and phenyl-β-D-glucuronide was developed. The mobile phase used was methanol : water: orthophosphoric acid (40:60:0·1) at a flow rate of 1 mL min^{-1} and ambient temperature through an in-line guard column (C18 μBondapak, Millipore Waters) and a reverse phase C18 column (250 × 4.6 mm, 5 micron, Brownlee). The Millipore Waters HPLC system used consisted of a dual piston pump (model 510), automatic injector (WISP 710B), ultra violet spectrophotometer (model 481) and a system interface module (SIM) integrating the data on a Powermate 1 (NEC) using a Maxima package. Under these conditions, retention times for phenyl-β-D-glucuronide, phenol and the internal standard (IS) p-cresol were approximately 4·6, 8·5 and 13·5 min, respectively. Samples were prepared by the addition of 200 μL methanol (with IS) to 100 μL sample, vortexing for 5 s then centrifugation for 10 min to pellet any precipitate. A 20 μL aliquot of the supernatant was then injected directly onto the column with the wavelength of the detector set at 254 nm. Results were analysed in terms of peak height ratios between peaks of interest and the internal standard. [^{14}C]Phenol purity was also validated using this assay by injecting a stock solution and collecting fractions for counting over 20 min at 1 min intervals.

Protein binding

The percentage of phenol and phenyl sulphate bound in perfusate (1% BSA, pH 7·4) was determined separately by equilibrium dialysis using a Spectra/Por molecular porous membrane (Spectrum Medical Industries, LA) against Krebs buffer (no BSA, pH 7·4) rolled in a water bath at 37°C for 20 h. The radiolabelled phenol or phenyl sulphate concentration on each side of the membrane was determined by scintillation counting, and the percentage bound defined as the ratio of solute in the albumin perfusate minus that in albumin free perfusate over that in albumin perfusate only.

Data analysis

Metabolite formation was calculated from the simple subtraction of extracted [^{14}C]phenol d min^{-1} from that of the total ^{14}C (phenol and metabolite) for the same sample with appropriate background corrections. The outflow profile for each solute was expressed as a fraction of the injected dose mL^{-1}. The total recoveries of the phenol and metabolites were assessed from the area under the outflow concentration–time profile (AUC) of phenol and metabolites, the measured perfusate flow rate (Q) and the amount of phenol injected (D):

$$\%\,\text{recovery} = \frac{\text{AUC} \cdot \text{Q}}{\text{D}} \tag{1}$$

where the percent recovery for phenol is defined by the AUC for unchanged phenol and the percent recovery for phenol and metabolites is defined by the AUC for ^{14}C in the perfusate. The mean transit time (MTT) for each solute was calculated from the ratio of the area under the curve (AUC) to the area under the moment curve (AUMC). In the absence of metabolism, the volume of distribution (Vd) is defined as MTT×Q. The MTT ratios were calculated as the fractions of the MTT due to distribution in tissue outside the vascular space:

$$\text{Fraction MTT} = \frac{\text{MTT}_{\text{metab}} - \text{MTT}_{\text{v}}}{\text{MTT}_{\text{w}} - \text{MTT}_{\text{v}}} \tag{2}$$

where MTT_{metab} and MTT_w are the MTT of the metabolite and water respectively, while the MTT for vascular space (MTT_v) is defined by Evans blue. All results are expressed as mean ± s.e.

Results

Typical anterograde outflow profiles (Q = 1·90 ± 0·03 mL min^{-1}(g liver)$^{-1}$) of the solutes are shown in Figs 1A and 1B. Phenol is highly extracted and a metabolite is generated with an intermediate transit time between Evans blue and water (Table 1). The profiles for the retrograde perfusions are similar in shape to the anterograde perfusions but are characterized by lower peaks and longer mean transit times for all solutes. (Figs 1C, 1D; Table 2). The percentage recoveries of Evans blue, [^{14}C]phenol, [^{14}C]metabolite, [^{3}H]water and [^{14}C]sucrose were 83.8 ± 2·5 (n = 39), 2·9 ± 0·4 (n = 24), 75·9 ± 3·2 (n = 28), 84·6 ± 3·0 (n = 41) and 83·6 ± 3·7 (n = 8), respectively. The average wet weight of the livers was 8·1 ± 0·3 g (n = 15) which represented 3·5 ± 0·1% (n = 15) of the total body weight of the animals used in this study 232·2 ± 7·0 g (n = 15). Various solvents were evaluated for a differential extraction of phenol from its metabolites in outflow samples. These included Econofluor, hexane (5% in isopropyl alcohol), BCS and OCS (containing different percentages of ether, amyl alcohol or ethyl acetate). OCS was found to yield the highest recovery of [^{14}C]phenol and subsequently chosen for the extraction procedure. The OCS extraction procedure showed a 83·0 ± 3·5% (n = 12) recovery for [^{14}C]phenol and no metabolite was shown to be extracted from the aqueous samples.

Recovery

The average ^{14}C-recovery per phenol injection from bile was 2·4 ± 0·4% (n = 25). The results obtained from all anterograde studies showed that the average Vd values of water at 1·90 and 3·66 mL min^{-1}(g liver)$^{-1}$ were 0·73 and 0·93 mL (g liver)$^{-1}$ respectively. Analysis of the effluent after a bolus injection of [^{14}C]phenol using enzymatic hydrolysis in four experiments revealed that 97·0 ± 1·4% of the radiolabelled compound recovered existed as phenyl sulphate, 0·6 ± 0·2%

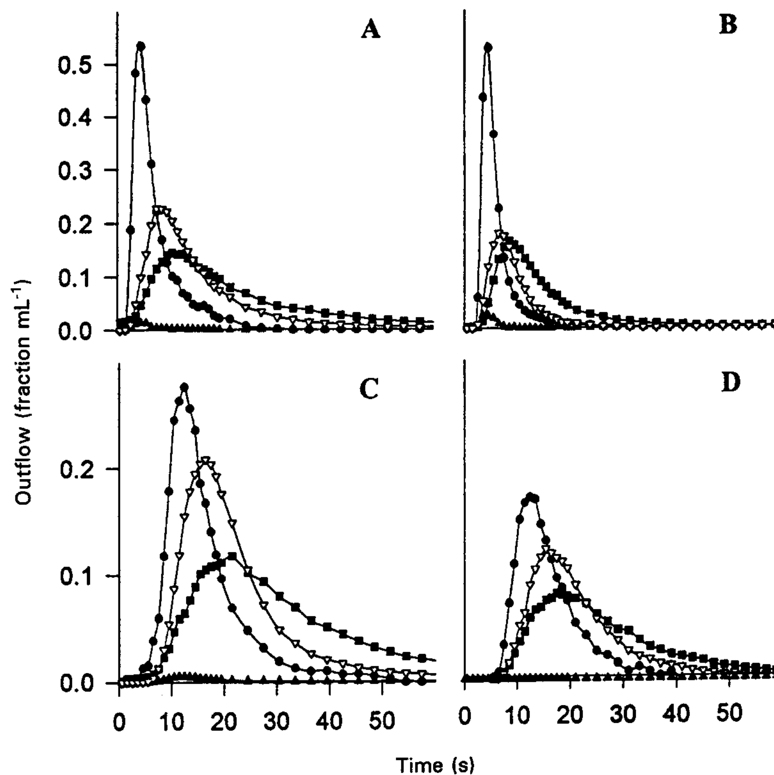

FIG. 1. Typical anterograde (A and B) and retrograde (C and D) outflow concentration–time profiles of Evans blue (●), water (■), phenol (▲) and its generated metabolites (▼) in the rat isolated perfused liver at flow rates of 1·9 ± 0·03 (A and C) and 3·66 ± 0·23 (B and D mL min^{-1}(g liver)$^{-1}$.

as phenyl-β-D-glucuronide and 1·90 ± 0·4% as unchanged phenol. Inhibition studies by the perfusion of bromosulphophthalein did not affect outflow concentration-time profiles for either phenol or its metabolites.

Metabolite profiles

Figures 2A and 2B show the outflow profiles of preformed phenyl-β-D-glucuronide and phenyl sulphate. Whilst the profile for phenyl glucuronide was similar in shape to that for the vascular reference (Evans blue), that of phenyl sulphate was slightly delayed, with slower influx and efflux portions of the curve. The mean transit times for both phenyl-β-D-glucuronide 8·1 s and phenyl sulphate 8·2 s at 1·90 mL min^{-1} (g liver)$^{-1}$ in the anterograde flow direction were both similar to that for Evans blue (8·7 s). Assuming an availability of one, the preformed metabolites of phenol can be estimated to have a Vd representing 75·3% and 80·6% of that of water at 1·90 and 3·66 mL min^{-1} (g liver)$^{-1}$, respectively, for anterograde perfusions.

Mean transit times in anterograde and retrograde perfusions

Table 2 shows the MTT for phenol and its metabolites in the tissues relative to water in both anterograde and retrograde perfusions. The MTT ratio for phenol in tissues was greatest in retrograde perfusions, 0·12 and 0·56, compared to 0·02 and 0 at flow rates of 15 and 30 mL min^{-1} (g liver)$^{-1}$ respectively. Conversely, the MTT of generated metabolites in tissues was greatest during anterograde perfusions, increasing around 37% and 21% at flow rates of 15 and 30 mL min^{-1} (g liver)$^{-1}$ respectively.

Table 1. Recovery and mean transit times (MTT) for solutes in the antero- and retrograde perfused liver in rat.

		Evans blue	Water	Phenol	Sucrose	Generated metabolites
Group I	Recovery (%)	84·5 ± 2·3 (21)	83·9 ± 2·3 (21)	2·8 ± 0·6 (13)	88·3 ± 2·2 (4)	70·5 ± 4·0 (16)
	MTT (s)	8·7 ± 0·3 (21)	23·0 ± 1·2 (21)	9·2 ± 0·6 (14)	8·3 ± 0·3 (4)	17·4 ± 1·2 (16)
Group II	Recovery (%)	73·6 ± 8·6 (7)	84·5 ± 4·6 (7)	1·8 ± 0·4 (4)	79·3 ± 9·5 (3)	82·4 ± 2·0 (4)
	MTT (s)	20·1 ± 1·4 (7)	41·0 ± 2·0 (7)	22·8 ± 3·7 (4)	20·8 ± 2·6 (3)	24·9 ± 1·6 (4)
Group III	Recovery (%)	94·6 ± 2·7 (6)	92·8 ± 5·4 (7)	5·4 ± 1·2 (4)	77·7 ± 0·0 (1)	73·5 ± 11·8 (4)
	MTT (s)	8·2 ± 1·8 (4)	15·3 ± 3·4 (7)	5·0 ± 0·3 (4)	7·8 ± 0·0 (1)	12·3 ± 3·8 (4)
Group IV	Recovery (%)	87·7 ± 11·2 (4)	77·9 ± 27·4 (4)	2·2 ± 0·4 (4)	—	93·4 ± 3·9 (4)
	MTT (s)	18·4 ± 1·4 (4)	34·5 ± 1·0 (4)	27·3 ± 5·6 (4)	—	24·2 ± 1·3 (4)

Groups I–IV are antero- and retrograde flow at 1·9 ± 0·03 mL min^{-1}(g liver)$^{-1}$ (I and II) and 3·66 ± 0·23 mL min^{-1} (g liver)$^{-1}$ (III and IV) respectively. Recovery and MTT are expressed as mean ± s.e. (n).

Table 2. MTT ratio for phenol and its metabolites in the tissues relative to water (equation 2) during both anterograde (A) and retrograde (R) perfusions at flow rates of 15 and 30 mL min^{-1} $liver^{-1}$.

Conditions			MTT ratio	
Group	Direction	Flow rate (mL min^{-1})	Phenol	Generated metabolite
I	A	15	0·02	0·60
II	R	15	0·12	0·23
III	A	30	0·00	0·58
IV	R	30	0·56	0·37

Phenol and phenyl sulphate protein binding

The percentage of phenol bound to perfusate protein was 13·6 ● 1·4 (n = 4). A higher binding was found for phenyl sulphate, the percentage bound being 59·1 ± 0·5 (n = 3).

Discussion

Only a small number of studies have explored the hepatic disposition of highly extracted solutes or their generated metabolites using the impulse-response or MID technique. Most of the previous studies have been concerned with the kinetics of metabolized solutes such as diclofenac (Evans et al 1991) and diazepam (De Garcia et al 1992), or solutes removed by active transport into the hepatocytes, e.g. taurocholate (Roberts et al 1990a). Phenol was chosen for the present study due to its high reported extraction by the liver (Cassidy & Houston 1984) and that, due to their low molecular weights, its major metabolites (phenyl sulphate and phenyl glucuronide) are not appreciably eliminated in the bile. Thus, administration of phenol enabled monitoring of the appearance of phenol and its metabolites in the perfusate after impulse injections into the liver. Further, a comparison of anterograde and retrograde perfusion enabled the effects of reported zonations of sulphotransferase activity in the liver (Pang et al 1983) to be explored.

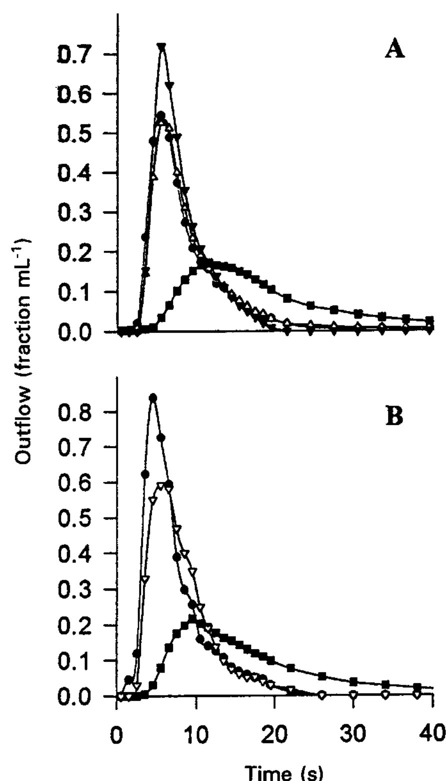

FIG. 2. Typical anterograde outflow concentration–time profiles for Evans blue (●), sucrose (△), and water (■) and the preformed metabolites of phenol, phenyl glucuronide (▼) (A), and phenyl sulphate (▼) (B) in the rat isolated perfused liver at a flow of 1·90 ± 0·03 mL min^{-1}(g liver)$^{-1}$.

Behaviour of sulphate conjugates in the liver

The anionic nature of sulphate conjugates (pKa< 1) makes them highly ionized at physiological pH, decreasing their ability to passively pass back across cell membranes once these metabolites are transported out of cells. Goresky et al (1992) used the MID technique to show that acetaminophen sulphate had a low liver cell permeability and a small interstitial binding space for its non-albumin binding fraction. Isolated hepatocyte uptake studies have suggested the existence of a saturable carrier-mediated cell entry system for acetaminophen sulphate (Stremmel et al 1993). Carrier-mediated transport systems have also been suggested to exist for other sulphate conjugates where permeability barrier limitations are present for hepatocyte entry, such as harmol sulphate (De Vries et al 1985).

Hepatic availabilities

The low availability of phenol observed for both antero- and retrograde perfusions in this work is consistent with the in-vivo findings of Cassidy & Houston (1984). The majority of the administered phenol was recovered in the form of phenyl sulphate in the perfusate, consistent with the relative dose-dependence in the formation of phenyl sulphate and phenyl glucuronide from phenol reported by Murdoch et al (1992). Two phenol sulphotransferases have been characterized in rat liver (Sekura & Jakoby 1979), both consisting of 2 subunits and reputedly similar in their substrate specificity and kinetics, but differing slightly in their amino acid composition. Using phenol as a substrate, the sulphotransferases have reported K_m values of 1·8 and 2·6 mM respectively, and V_{max} values of 100 and 110 nmol mg^{-1} min^{-1} respectively (Sekura & Jakoby 1979). The enzyme kinetic parameters show that at the tracer concentrations of phenol used in the present IR studies, any effects due to saturation of enzyme kinetics are avoided.

The recovery in the perfusate of unextracted phenol and metabolites generated from phenol (79%) was only slightly lower than that found for the non-extracted references, Evans blue (84%), sucrose (84%) and water (85%). A lower recovery of phenol had been anticipated given that up to 2·4% of the injected phenol was recovered in the bile. Thus, phenol administration enables the disposition of its major metabolite, phenyl sulphate, to be studied using impulse injections by collecting perfusate effluent fractions for phenol and phenyl sulphate analysis.

MTT of phenol and metabolites

The MTT for phenol is low and comparable to those of Evans blue and sucrose (Table 1), and consistent with the

MTT of a highly extracted solute (Roberts & Rowland 1986a). They also showed that the MTT for a solute decreases with the increase in efficiency number for elimination and is associated with a low availability.

In the simplest interpretation of volume of distribution data for generated phenyl sulphate, it is recognized that effectively all the phenol is metabolized such that the generated metabolite has an availability close to unity but a larger Vd due to the ready penetration of phenol into hepatocytes. Table 2 shows that the metabolites generated from phenol have longer MTTs than the parent compound, or preformed sulphate and glucuronide metabolites, but less than that of water. Of note was the observation that, after allowing for changes in vascular space, phenol had a much longer MTT during retrograde perfusions than in anterograde perfusions (Table 2). In contrast, the MTT for the generated metabolites in the tissue space was shorter in retrograde perfusion studies relative to anterograde perfusions.

Retrograde liver perfusions

In the present study an increase in MTT during retrograde compared to anterograde perfusions was observed for all the markers used in this study. This finding is explained by the fact that by reversing flow in the liver causes an increase in back pressure through the vascular system increasing its size and volume. This theory is supported by other work where it has been postulated that the increase in vascular back pressure is caused by the decreased compliability of sinusoid inlet sphincters, which become outlet sphincters during retrograde perfusion, resulting in distension of the vascular tree and Disse spaces (St.-Pierre et al 1989; Roberts et al 1990b).

Retrograde perfusions are frequently used to probe enzyme zonation. Of relevance to this study is the observation that sulphation activity is generally thought to be higher in the periportal region of the acinus than in the centrilobular region, although conflicting evidence can be found in the literature between some in-vivo and isolated hepatocyte studies. For example, the sulphation of acetaminophen has been suggested to be periportal (Pang & Terrell 1981; Araya et al 1986), whereas the isolated hepatocyte studies of Anundi et al (1993) suggest that sulphation of acetaminophen occurs in the perivenous region. Homma et al (1992) have suggested that the sulphation of phenol in female rat livers occurs more in the perivenous rather than the periportal region. Further evidence examining the sulphation of other substrates, such as harmol (Pang et al 1983), gentisamide (Morris et al 1988) and salicylamide (Xu & Pan 1989) suggest metabolism occurs in the periportal region. The majority of the evidence relating to the glucuronidation of substrates suggest metabolism throughout the sinusoids (El Mouelhi & Kauffman 1986; Morris et al 1988; Xu & Pang 1989), with a few studies suggesting metabolism in the perivenous region alone (Conway et al 1987). The zonation of harmol sulphotransferase using the overall metabolite profile changes with the alteration from anterograde to retrograde perfusions has been demonstrated (Pang et al 1983; Thurman et al 1986). Enzyme zonation would only be expected to affect metabolite MTTs and availabilities in the presence of a permeability barrier to those metabolites. If the metabolites were excreted into the perfusate and no further distribution occurred, the sinusoid is simply considered to consist of two zones with differing enzyme activity and distribution, with overall availability being the product of the individual availabilities in these two regions, irrespective of direction of flow (Roberts & Rowland 1986b; Roberts et al 1988). Similarly, the overall MTT is defined by the sum of transit times in each of the regions, again irrespective of direction of flow and the extent of distribution in the metabolizing region.

Enzyme zonation of phenol metabolism

Combining the data from anterograde and retrograde perfusions, we can conclude that the sulphation of phenol occurs mainly in the periportal region. The MTT for an extracted solute decreases with an increase in extraction (Roberts et al 1990a). Selective sulphation of phenol in this periportal region during anterograde perfusions leads to metabolism of inflowing phenol before any significant tissue distribution can take place (Fig. 3), resulting in a lower MTT and apparent volume of distribution than would be observed for a uniform enzyme distribution. Conversely, in retrograde perfusions, phenol entering a sulphation deficient perivenous region would distribute into tissues before reaching the metabolizing enzymes in the periportal region (Fig. 3). A longer MTT and larger distribution volume is observed for phenol in retrograde perfusions and the generated metabolites have a much shorter MTT and smaller volume distribution. Hence, the differences in MTT for phenol and its generated metabolites during anterograde and retrograde perfusions are consistent with sulphation of phenol occurring mainly in the periportal region.

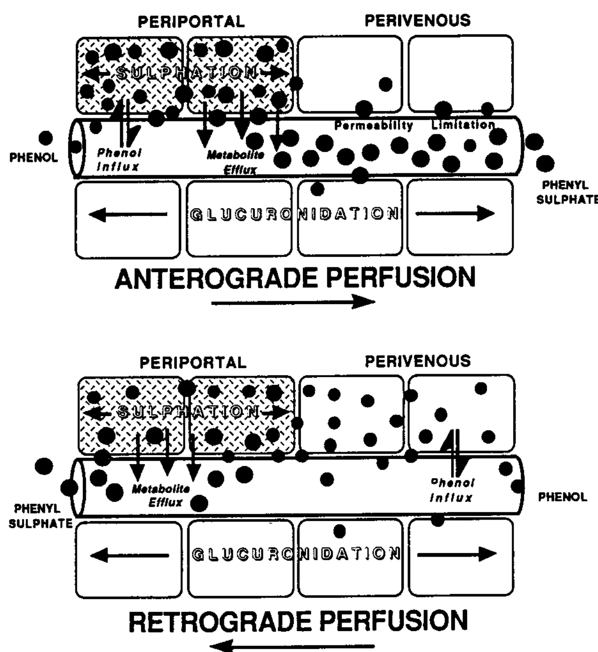

FIG. 3. Diagrammatic representation of the suggested enzyme zonation in the liver sinusoid showing the differences in distribution of phenol amd metabolites between vascular and extravascular spaces in the periportal and perivenous regions during anterograde and retrograde perfusions.

Distribution of preformed metabolites
The MID curves for preformed metabolite injections showed that phenol glucuronide has an identical profile to that of the vascular marker Evans blue, suggesting that most of this solute is mainly restricted to the vascular space during passage through the liver. Phenyl sulphate, however, had a profile slightly delayed to the vascular marker (Fig. 2), indicating the presence of limited hepatocyte permeability.

Inhibition of hepatocyte efflux mechanisms with bromosulphophthalein
The existence of a unidirectional transport efflux mechanism for intracellularly generated metabolites from the cytoplasm to the perfusate is supported by the finding that preformed metabolites do not undergo significant distribution into hepatocytes. The efflux of metabolic conjugates has been observed in other tissues including enalaprilat in the kidney (Schwab et al 1990) and 1-napthyl-β-D-glucuronide in the brain (Leininger et al 1991). An organic anion transport mechanism for harmol sulphate from hepatocytes back into the blood has been suggested (De Vries et al 1985). Carrier mediated transport systems have been implicated for the excretion of some bile acids and sulphate esters (Eng & Javitt 1983; Stremmel et al 1993). Studies in mutant rats have also pointed to the presence of an anionic transport system in the canalicular membrane for the excretion of sulphate conjugates of bile acids (Kuipers et al 1988; Takikawa et al 1991). However, it has been suggested that drug sulphate conjugates do not share this carrier (Pang et al 1994), which would account for their non-appearance in the bile samples analysed. In an attempt to inhibit the metabolite efflux mechanism of the hepatocyte, bromosulphophthalein, an ionized diagnostic trimethylmethane dye that is rapidly extracted by hepatocytes and excreted into the bile as a glutathione conjugate (Combes 1960), was added to the perfusate. The lack of effect of bromosulphophthalein on outflow concentration–time profiles for all solutes tested suggests that the efflux mechanism used by the bromosulphophthalein conjugated metabolites is not shared by the metabolites of phenol. Significant biliary excretion of bromosulphophthalein was observed, although not quantified, by the overall purple colouration of the bile samples collected. Hirom et al (1972) suggested that a molecular weight threshold of 325 ± 50 existed for the efflux of organic anions from hepatocytes into the bile for rats. The molecular weights of phenol and its sulphate conjugate are 94 and 174, respectively, which does not favour their biliary excretion. Bromosulphophthalein has a molecular weight of 792 and seems to be preferentially excreted into the bile by a system not shared with smaller sulphate conjugates. Therefore, even though bromosulphophthalein has been widely used as an organic anion in membrane transport elucidation, its use in the determination of the possible efflux mechanism of the metabolite phenyl sulphate from hepatocytes would seem limited.

Conclusion. Phenol is rapidly metabolized after administration in trace bolus doses to the isolated perfused liver yielding low availabilities. The major metabolite formed, phenyl sulphate, is restricted in its entry into hepatocytes as a preformed metabolite is but rapidly transported back into perfusate from hepatocytes after generation from phenol. The results for retrograde perfusions are consistent with the observed alterations in physiology associated with a change in direction of perfusions. The relative tissue distributions of phenol and its generated metabolite during anterograde and retrograde perfusions suggest that the sulphation of phenol occurs predominantly in the periportal region of the liver sinusoids.

Acknowledgements
The authors wish to acknowledge the financial support of the National Health and Medical Research Council and the Lions Northern New South Wales and Queensland Kidney and Medical Research Foundation.

References

Anundi, I., Lähteenmäki, T., Rundgren, M., Moldéus, P., Lindros, K. O. (1993) Zonation of acetaminophen metabolism and cytochrome P-450 2E1-mediated toxicity studied in isolated periportal and perivenous hepatocytes. Biochem. Pharmacol. 45: 1251–1259

Araya, H., Mizuma, T., Horie, T., Hayashi, M., Awazu, S. (1986) Heterogenous distribution of the conjugation activity of harmol in isolated rat liver cells. J. Pharmacobiodyn. 9: 218–222

Brock, W. J., Vore, M. (1984) Characterization of uptake of steroid glucuronides into isolated male and female rat hepatocytes. J. Pharmacol. Exp. Ther. 229: 175–181

Burchell, B., Coughtrie, M. W. H. (1989) UDP-glucuronosyltransferases. Pharmacol. Ther. 43: 261–289

Cassidy, M. K., Houston, J. B. (1984) In vivo capacity of hepatic and extrahepatic enzymes to conjugate phenol. Drug Metab. Dispos. 12: 619–624

Combes, B. (1960) Conjugation of sulphobromophthalein sodium with glutathione in thio ether linkage by the rat. J. Clin. Invest. 39: 1214–1216

Conway, J. G., Kauffman, F. C., Tsukuda, T., Thurman, R. G. (1987) Glucuronidation of 7-hydroxycoumarin in periportal and pericentral regions of the lobule in livers from untreated and 3-methyl-cholanthrene-treated rats. Mol. Pharmacol. 33: 111–119

De Garcia, J. M., Evans, A. M., Rowland, M. (1992) Application of the axial dispersion model of drug elimination to the kinetics of diazepam in the isolated perfused rat liver. J. Pharmacokin. Biopharm. 20: 171–193

De Vries, M. H., Groothuis, G. M. M., Mulder, G. J., Nguyen, H., Meijer, D. K. F. (1985) Secretion of the organic anion harmol sulphate from the liver into blood. Biochem. Pharmacol. 34: 2129–2135

El Mouelhi, M., Kauffman, F. C. (1986) Sublobular distribution of transferases and hydrolases associated with glucuronide, sulfate and glutathione conjugation in human liver. Hepatology 6: 450–456

Eng, C., Javitt, N. B. (1983) Chenodeoxycholic acid-3-sulphate; metabolism and excretion in the rat and hampster and effects on hepatic transport systems. Biochem. Pharmacol. 32: 3555–3558

Evans, A. M., Hussein, Z., Rowland, M. (1991) A two-compartment dispersion model describes the hepatic outflow profile of diclofenac in the presence of its protein binding. J. Pharm. Pharmacol. 43: 709–714

Gores, G. J., Kost, L. J., LaRusso, N. F. (1986) The isolated perfused rat liver: conceptual and practical considerations. Hepatology 6: 511–517

Goresky, C. A., Pang, K. S., Schwab, A. J., Barker, F., Cherry, W. F., Back, G. G. (1992) Uptake of protein-bound polar compound, acetaminophen sulphate, by perfused rat liver. Hepatology 16: 173–190

Hirom, P. C., Millbur, P., Smith, R. L., Williams, R. T. (1972) Species variations in the threshold molecular-weight factor for the biliary excretion of organic anions. Biochem. J. 129: 1071–1077

Homma, H., Nakagome, I., Matsui, M. (1992) Differential localisation of sulphotransferase isoenzymes in rat liver. Biochem. Biophys. Res. Commun. 183: 872–878

Hugentobler, G., Meier, P. J. (1986) Multispecific anion exchange in basolateral (sinusoidal) rat liver plasma membrane vesicles. Am. J. Physiol. 251: G656–G664

Kobayashi, K., Komatsu, S., Nishi, T., Hara, H., Hayashi, H. (1991) ATP-dependent transport for glucuronides in canalicular plasma membrane vesicles. Biochem. Biophys. Res. Commun. 176: 622–626

Kuipers, F., Enserink, M., Havainga, R., van der Steen, A. B. M., Hardonk, J., Fevery, M. J., Vonk, R. J. (1988) Seperate transport systems for biliary secretion of sulfated and unsulfated bile acids in the rat. J. Clin. Invest. 81: 1593–1599

Leininger, B., Ghersi-Egea, J., Siest, G., Minn, A. (1991) In vivo study of the elimination from rat brain of an intracerebrally formed xenobiotic metabolite, 1-naphthyl-β-D-glucuronide. J. Neurochem. 57: 1163–1168

Meier, P. J., Valantinas, J., Hugentobler, G., Rahm, I. (1987) Bicarbonate sulfate exchange in canalicular rat liver plasma membrane vesicles. Am. J. Physiol. 253: G461–G468

Morris, M. E., Yuen, V., Pang, K. S. (1988) Competing pathways in drug metabolism. II. Enzymic systems for 2- and 5-sulfoconjugation are distributed anterior to 5-glucuronidation in the metabolism of gentisamide by the perfused rat liver. J. Pharmacokin. Biopharm. 16: 633–656

Mulder, G. J., Coughtrie, M. W. H., Burchell, B. (1990) Conjugation reactions in drug metabolism. In: Mulder, G. J. (ed.) Glucuronidation. Taylor and Francis, London, pp 51–105

Murdoch, R. T., Ghabrial, H., Smallwood, R. A., Morgan, D. J. (1992) Effect of malaria on phenol conjugation pathways in the perfused rat liver. Biochem. Pharmacol. 43: 1229–1234

Nishida, T., Gatmaitan, Z., Roy-Chowdhry, J., Arias, I. M. (1992) Two distinct mechanisms for bilirubin glucuronide transport by rat bile analicular membrane vesicles. J. Clin. Invest. 90: 2130–2135

Pang, K. S., Gillette, J. R. (1978) Kinetics of metabolite formation and elimination in the perfused rat liver preparation: differences between the elimination of preformed acetaminophen and acetaminophen formed from phenacetin. J. Pharmacol. Exp. Ther. 207: 178–194

Pang, K. S., Terrell, J. A. (1981) Conjugation of acetaminophen by the perfused rat liver preparation. Biochem. Pharmacol. 30: 1959–1965

Pang, K. S., Koster, H., Halsema, I. C. M., Scholtens, E., Mulder, G. J., Stillwell, R. N. (1983) Normal and retrograde perfusion to probe the zonal distribution of sulphation and glucuronidation activities of harmol in the perfused rat liver preparation. J. Pharmacol. Exp. Ther. 224: 647–653

Pang, K. S., Schwab, A. J., Goresky, C., Chiba, M. (1994) Transport, binding and metabolism of sulfate conjugates in the liver. Chem.-Biol. Interact. 92: 179–207

Potter, B. J., Blades, B. F., Shepard, M. D., Thung, S. M., Berk, P. D. (1987) The kinetics of sulfobromophthalein uptake by the rat liver sinusoidal vesicles. Biochim. Biophys. Acta 898: 159–171

Roberts, M. S., Rowland, M. (1986a) A dispersion model of hepatic elimination: 1. Formulation of model and bolus considerations. J. Pharmacokin. Biopharm. 14: 227–260

Roberts, M. S. Rowland, M. (1986b) A dispersion model of hepatic elimination: 3. Application to metabolite formation and elimination kinetics. J. Pharmacokin. Biopharm. 14: 289–308

Roberts, M. S., Donaldson, J. D., Rowland, M. (1988) Models of hepatic elimination; comparison of stochastic models to described residence time distribution, enzyme heterogeneity and systemic recycling on hepatic elimination. J. Pharmacokin. Biopharm. 16: 41–84

Roberts, M. S., Fraser, S., Wagner, A., McLeod, L. (1990a) Residence time distributions of solutes in the perfused rat liver using a dispersion model of hepatic elimination: 1. Effect of changes in perfusate flow and albumin concentration on sucrose and taurocholate. J. Pharmacokin. Biopharm. 18: 209–234

Roberts, M. S., Fraser, S. Wagner, A., McLeod, L. (1990b) Residence time distributions of solutes in the perfused rat liver using a dispersion model of hepatic elimination: 2. Effect of pharmacological agents, retrograde perfusions, and enzyme inhibition on Evans Blue, sucrose, water and taurocholate. J. Pharmacokin. Biopharm. 18: 235–258

Satofumi, I., Takashi, M., Noriko, S., Masahiro, H., Shoji, A. (1988) Transport of acetaminophen conjugates in isolated rat hepatocytes. Drug Metab. Dispos. 17: 341–344

Schwab, A. J., Barker, F., Goresky, C. A., Pang, K. S. (1990) Transfer of enalaprilat across rat liver cell membranes is barrier limited. Am. J. Physiol. 258: G461–G475

Sekura, R. D., Jakoby, W. B. (1979) Phenol sulphotransferases. J. Biol. Chem. 254: 5658–5663

St-Pierre, M. V., Lee, W. F., Schwab, A. J., Goresky, C. A., Pang, K. S. (1989) The multiple indicator dilution technique for characterization of normal and retrograde perfusions in the once-through rat liver preparation. Hepatology 9: 285–296

Stremmel, W., Tiribelli, C., Vyska, K. (1993) The multiplicity of sinusoidal membrane carrier systems of organic anions. In: Tavoloni, N., Berk, P. D. (eds) Hepatic Transport and Bile Secretion. Physiology and Pathophysiology. Raven Press, New York, pp 225–244

Takikawa, H., Sano, N., Narita, T., Uchida, Y., Yamanaka, T., Horie, T., Mikani, T., Tagaya, O. (1991) Biliary excretion of bile acid conjugates in hyperbilirubinemic mutant Sprague-Dawley rats. Hepatology 14: 352–360

Thurman, R. G., Kauffman, F. C., Baron J. (1986) In: Thurman, R. G., Kaufmann F. C., Jungermann, K. (eds) Regulation of Hepatic Metabolism: Intra- and Intercellular Compartmentation. Plenum, New York, pp 321–382

Vore, M., Durham, S., Yeh, S., Ganguly, T. (1991) Hepatic clearance and biliary secretory rate maximum of taurocholate in the recirculating and single pass isolated perfused rat liver. Biochem. Pharmacol. 41: 431–437

Weitering, J. C., Krijgsheld, K. R., Mulder, G. J. (1979) The availability of inorganic sulphate as a rate limiting factor in the sulphate conjugation of xenobiotics in the rat? Biochem. Pharmacol. 28: 757–762

Wolkoff, A. W., Johansen, K. L., Goeser, T. (1987) The isolated perfused rat liver: preparation and application. Anal. Biochem. 167: 1–14

Xu, X., Pang, K. S. (1989) Hepatic modelling of metabolite kinetics in sequential and parallel pathways: salicylamide and gentisamide metabolism in perfused rat liver. J. Pharmacokin. Biopharm. 17: 645–671