0022-3565/82/2221-0014$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Copyright © 1982 by The American Society for Pharmacology and Experimental Therapeutics
Vol. 222, No. 1
Printed in U.S.A.

Metabolite Kinetics: Formation of Acetaminophen from Deuterated and Nondeuterated Phenacetin and Acetanilide on Acetaminophen Sulfation Kinetics in the Perfused Rat Liver Preparation[1]

K. SANDY PANG,[2] LEWIS WALLER, MARJORIE G. HORNING and KENNETH K. CHAN

Department of Pharmaceutics, University of Houston (K.S.P., L.W.), Institute for Lipid Research, Baylor College of Medicine, Texas Medical Center, Houston, Texas (M.G.H.) and Department of Pharmaceutics, University of Southern California, Los Angeles, California (K.K.C.)

Accepted for publication March 22, 1982

ABSTRACT

The role of hepatic intrinsic clearances for metabolite formation from various precursors on subsequent metabolite elimination was investigated in the once-through perfused rat liver preparation. Two pairs of acetaminophen precursors: [^{14}C] phenacetin-d_5 and [^{3}H]phenacetin-d_0, [^{14}C]acetanilide and [^{3}H] phenacetin were delivered by constant flow (10 ml/min/liver) either by normal or retrograde perfusion to the rat liver preparations. The extents of acetaminophen sulfation were compared within the same preparation. The data showed that the higher the hepatocellular activity (intrinsic clearance) for acetaminophen formation, the greater the extent of subsequent acetaminophen sulfation. The findings were explained on the basis of blood transit time and metabolite "duration time." Because of blood having only a finite transit time in liver, the longer the drug requires for metabolite formation, the less time will remain for metabolite sulfation and the less will be the degree of subsequent sulfation. Conversely, when the drug forms the primary metabolite rapidly, a longer time will remain for the metabolite to be sulfated in liver to result in a greater degree of metabolite sulfation. Finally, the effects of hepatic intrinsic clearances for metabolite formation and zonal distribution of enzyme systems for metabolite formation and elimination in liver are discussed.

Conjugation reactions in drug biotransformation are important as the addition of polar functional groups renders the molecules more suitable for renal excretion. Normally, sulfate conjugation presents this characteristic in terms of drug detoxification (Williams, 1959). It was discovered recently, however, that sulfation of some compounds (De Baun *et al.*, 1970; Kadlubar *et al.*, 1976; Mulder *et al.*, 1977) can induce drug-mediated toxicity. The manner in which the kinetics of sulfation for xenobiotics, that undergo Phase II (conjugation) reactions subsequent to Phase I reactions, remain mostly unknown.

The liver is the major organ for drug biotransformation and conjugation reactions and much work has been done on specific compounds (Villeneuve and Sourkes, 1966; Slotkin *et al.*, 1970; Minck *et al.*, 1973; Wiebkin *et al.*, 1978; Reinke *et al.*, 1981) that are initially oxidized (Phase I reaction) and subsequently conjugated (Phase II reaction). A specific example on oxidative metabolism and subsequent sulfation, however, is provided by the O-deethylation of phenacetin in the formation of acetaminophen, which, under tracer dose/concentration conditions, is primarily metabolized to acetaminophen sulfate conjugate *in vivo* (Pang *et al.*, 1979) and *in vitro* (Pang and Gillette, 1978) in the rat. The kinetics of sulfation were examined by the comparison of the extent of acetaminophen sulfation under these tracer conditions, when [^{3}H]acetaminophen, a preformed metabolite, and [^{14}C]phenacetin, a precursor of [^{14}C]acetaminophen, were delivered simultaneously once-through the perfused rat liver preparation. The preformed metabolite was sulfated to a larger extent (67 ± 8%) than the derived metabolite (59 ± 10%) (Pang and Gillette, 1978). But, when directional flow was reversed with retrograde perfusion to the liver (Trowell, 1942), discrepancies in acetaminophen sulfation for the preformed metabolite and derived metabolite disappeared (Pang and Terrell, 1981a). These findings suggest a heterogeneous distribution of drug metabolizing enzymes in the rat liver; O-deethylation as mediated by the cytochrome P-450 system may be more abundant in the centrilobular region and sulfation as mediated by the sulfotransferases may be preponderant in the periportal region.

The role of hepatocellular activities for oxidative metabolism or intrinsic clearance to form a primary metabolite on the

Received for publication October 22, 1981.
[1] This work was supported by U.S. Public Health Service Grant GM 27323.
[2] Recipient of Research Career Development Award Grant AM 01208 from the U.S. Public Health Service.

ABBREVIATIONS: TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography.

The Journal of PHARMACOLOGY And Experimental Therapeutics
Downloaded from jpet.aspetjournals.org at ASPET Journals on October 6, 2021
Auspex Exhibit 2016
Apotex v. Auspex
IPR2021-01507
Page 1

extents of sulfation of this metabolite, however, is unknown. The present paper is an examination of acetaminophen sulfation kinetics when acetaminophen is formed from a pair of precursors ([^{14}C]phenacetin-d_5 and [^{3}H]phenacetin-d_0; fig. 1, Structures I and II, respectively) of similar hepatic extraction ratios and from a pair of precursors ([^{3}H]phenacetin and [^{14}C] acetanilide; fig. 1, Structures II and III, respectively) of different hepatic extraction ratios. The first pair of precursors is chosen because Garland *et al.* (1976) had reported a slight kinetic isotope effect (1.61) on the O-deethylation of phenacetin in the formation of acetaminophen for the deuterated analog in rabbits. But, because the values of the extraction ratio of phenacetin were very high (~0.9), the kinetic isotope effect, if present to the same degree in the perfused rat liver preparation, may not perturb the values of the hepatic extraction ratios. The second pair of precursors is chosen because preliminary studies had shown that the hepatic extraction ratio of acetanilide (0.38–0.52) was about half the value for phenacetin (0.84–0.98). These two pairs of precursors of acetaminophen will bring out differences of acetaminophen sequential sulfation when their hepatic extraction ratios are 1) very similar and 2) drastically different.

Materials and Methods

Liver perfusion. The surgical procedure and the perfusion apparatus for normal perfusion were identical to a previously described method (Pang and Gillette, 1978). Modification of the perfusion apparatus and the surgical procedure for retrograde perfusion was identical to those described in an earlier report (Pang and Terrell, 1981a).

Male Sprague-Dawley rats (300–367 g) which were supplied by TIMCO Laboratories (Houston, TX) were used as liver donors in all studies. Only single-pass (nonrecirculating) experiments were performed. Perfusate containing tracer and constant concentrations of the precursors were delivered under constant flow (10 ml/min/liver) either by normal or retrograde flow into the rat liver preparation.

Preliminary experiments were performed to identify a system that would be devoid of drug-drug interactions when each pair of precursors was delivered simultaneously into the rat liver preparation. For example, for precursors I and II, the first-period of perfusion (40 min) entailed the perfusion of I singly and the second period (40 min) involved the simultaneous perfusion of both I and II, followed by the third and last perfusion period (40 min) of I singly. The experiment was repeated with the interchange of substrates; the three perfusion periods were: II only, I and II and II only. The criterion of constancy in drug hepatic extraction was used. These experiments were repeated at various concentrations of I and II until the metabolism of both I and II (as estimated by the hepatic extraction ratios) in the presence and absence of each other would remain a constant.

Such a system was identified for the dual delivery of [^{14}C]phenacetin-d_5 and [^{3}H]phenacetin-d_0 simultaneously to the same rat liver preparation. Studies were to be carried out by three perfusion periods (40 min each): normal, retrograde and normal perfusions at these tracer concentrations. But drug-drug interactions had resulted between [^{14}C] acetanilide and [^{3}H]phenacetin, as the hepatic extraction ratios of both compounds were decreased in the presence of each other. For studies to be carried out with [^{14}C]acetanilide and [^{3}H]phenacetin, only normal perfusion of the compounds was employed. The three perfusion periods (40 min) of the compounds singly by normal flow were: [^{14}C]acetanilide, [^{3}H]phenacetin and [^{14}C]acetanilide.

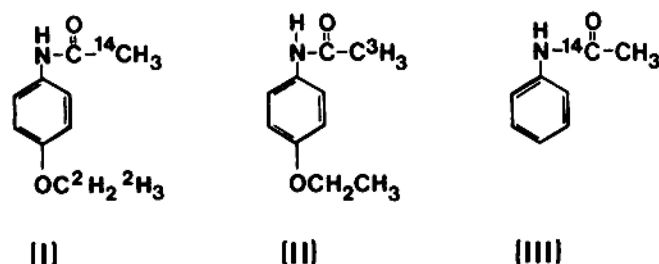

Fig. 1. Structures of [^{14}C]phenacetin-d_5 (I), [^{3}H]phenacetin-d_0 (II) and [^{14}C]acetanilide (III).

Sampling times. The mean of two determinations of the input medium taken before and after the perfusion period was used as the steady-state input drug concentration. The mean of the determinations of five consecutive 4-min samples of the outflow medium taken at 24 min after the commencement of the perfusion period was used as the steady-state output concentration (steady-state conditions were approached around 20 min after the commencement of the period for the drug and metabolites).

Bile was collected at 10-min intervals after the start of the experiment. The volumes were noted for each collection.

Assay. [^{14}C]phenacetin-d_5, [^{3}H]phenacetin, [^{14}C]acetanilide, radiolabeled acetaminophen and acetaminophen conjugates in bile and perfusate were quantified as previously described (Pang and Gillette, 1978). The sulfate, glucuronide or glutathione conjugates in plasma and in bile were determined by spotting plasma (100 µl) or bile (10 µl) on Avicel F (250 µM, Analtech, Inc., Newark, DE) thin-layer plates which contained carriers (acetaminophen, acetaminophen sulfate and glucuronide conjugates) at the origin. The plates were developed in *n*-propanolol-0.4 N NH_3 (80:20 v/v) and the radiolabeled fractions of the sulfate, glucuronide and glutathione were scraped into scintillation vials. After the addition of 1.0 ml of water and 10 ml of Aquasol (New England Nuclear, Boston, MA), radioactivity in each fraction was quantified by liquid scintillation spectrometry (Beckman LS 7500, Palo Alto, CA). [^{14}C]Phenacetin-d_5, [^{3}H]phenacetin-d_0 and [^{14}C]acetanilide in bile were determined by spotting an aliquot (10 µl) on silica gel GF TLC plates (250 µm, Analtech) that contained carriers and developing in a system of ether. The R_f values were: acetanilide, 0.53; phenacetin, 0.43; and acetaminophen, 0.29.

[^{14}C]Phenacetin-d_5, [^{3}H]phenacetin-d_0, [^{14}C]acetanilide and radiolabeled acetaminophen in blood perfusate (1.0 ml of input and output blood) were quantified by extraction into ethyl acetate (6 ml) (Burdick and Jackson, Muskegon, MI). The extracts were evaporated to dryness under a stream of nitrogen. The residue was redissolved in ethyl acetate (200 µl) and 100 µl was spotted on the origin of silica gel TLC plates which contained unlabeled acetanilide, phenacetin and acetaminophen as carriers and developed in a system of ether. A set of standards which contained varying amounts of [^{14}C]acetanilide, [^{14}C]phenacetin-d_5, [^{3}H] phenacetin-d_0 and [^{3}H]acetaminophen in blank perfusate (1.0 ml) were extracted by the same procedure to correct for losses due to the extraction procedure.

A HPLC assay method (Wilson *et al.*, 1982) was used to validate the TLC procedure. A mobile phase of methanol: 0.1 M KH_2PO_4 in 0.75% acetic acid (7:93, v/v) was utilized at a flow of 1.0 ml/min through a guard column (Waters Associates, Milford, MA) of ODS packing, 10 µm (Whatman, Inc., Clifton, NJ), and an Ultrasphere column (Altex Scientific, Inc., Palo Alto, CA) in a liquid chromatograph (Laboratory Data Control, Riviera Beach, Fl) that was equipped with a Rheodyne injector, a Constametric III pump and a UV detector (Spectro Monitor III) at 254 nm and monitored on a linear recorder (Chart speed 20 cm/hr, Linear Instruments Corp., Irvine, CA). The retention times for the authentic standards were: glucuronide, 5 min 45 sec; sulfate, 10 min 10 sec; acetaminophen, 15 min 55 sec; glutathione conjugate, 19 min 50 sec; and acetanilide, 48 min. Phenacetin was not eluted from the column. Perfusate samples were prepared by removing the plasma by centrifugation and an aliquot (200 µl of plasma) was precipitated with methanol (500 µl). Bile samples were prepared by similar dilutions. A volume (40–50 µl) of the supernatant was injected into the HPLC system. All HPLC eluants were collected for 60 min at 1-min intervals by a fraction collector (FOXY, ISCO, Lincoln, NB). The peaks of radioactivity collection were compared to the peaks by UV detection of the authentic compounds.

Source of Materials. Unlabeled acetaminophen, phenacetin and acetanilide were purchased from Eastman Kodak Co. (Rochester, NY).

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 6, 2021

Auspex Exhibit 2016
Apotex v. Auspex
IPR2021-01507
Page 2

Unlabeled acetaminophen sulfate and glucuronide conjugates were generously and kindly supplied by Dr. Josiah N. Tam, McNeils Consumers Company (Fort Washington, PA). Acetaminophen glutathione conjugate was supplied by Dr. Bernhard Lauterberg, Baylor College of Medicine (Houston, TX).

[^{14}C]Phenacetin-d_5 (I, N-[*acetyl*-1-^{14}C]-*p*-ethoxy-d_5-aniline; specific activity, 21.4 mCi/mmol) and [^{3}H]phenacetin-d_0 (II, N-[*acetyl*-^{3}H]-*p*-ethoxyaniline; specific activity, 500 mCi/mmol) were synthesized as described in a previous report (Chan and Pang, 1982). Essentially, [^{3}H] phenacetin-d_0 was prepared by direct acylation of *p*-phenetidine with [^{3}H]acetic anhydride under the Schotten-Bauman condition and [^{14}C] phenacetin-d_5 was obtained by similar acylation of *p*-phenetidine-d_5 which was synthesized by ethylation of acetaminophen with perdeuterated ethyl iodide (Garland *et al.*, 1977). [^{14}C]Acetanilide (III) was synthesized by reacting a 5-fold excess of aniline (Eastman Kodak) with [1-^{14}C]acetyl chloride (New England Nuclear; specific activity, 57.7 mCi/mmol). The resultant [^{14}C]acetanilide was purified by acidic, basic and aqueous washed before the final extraction into ethyl acetate and was stored over sodium sulfate. The acetanilide obtained was greater than 99% radiochemically pure, as determined by TLC. [^{3}H] Acetanimophen (specific activity, 937.5 mCi/mmol) was purchased from New England Nuclear.

Results

Assay. The radiometric HPLC procedure confirmed the TLC assay that radiolabeled acetaminophen and acetaminophen sulfate conjugate were the only major metabolites in effluent perfusate resulting from the perfusion of [^{14}C]phenacetin-d_5 and [^{3}H]phenacetin-d_0 into the rat liver preparation. [^{14}C]Acetanilide was biotransformed to [^{14}C]acetaminophen, [^{14}C]acetaminophen sulfate and glucuronide conjugates. No other radioactive metabolic species were detected by the HPLC system. In bile, acetaminophen glutathione conjugate was the only other metabolite present that accompanied acetaminophen sulfate and acetaminophen glucuronide conjugates. Moreover, the amounts of radioactivity of each component in the selected samples as measured by HPLC and TLC were virtually identical.

[^{14}C]Phenacetin-d_5 and [^{3}H]Phenacetin-d_0. Constant concentrations of [^{14}C]phenacetin-d_5 (range, 80–300 × 10^2 dpm/ml or 0.18–0.68 μM) and [^{3}H]phencetin (range, 49–62 × 10^4 dpm/ml or 0.45–0.56 μM) were delivered simultaneously by normal (40 min), retrograde (40 min) and normal (40 min) perfusions into the same rat liver preparation. The sampling schedule denoted that steady-state conditions were approached during the collecting interval as identified by the constancy in hepatic venous concentrations of phenacetin and its major metabolites, acetaminophen and acetaminophen sulfate conjugate. The paired steady-state extraction ratios for d_5 (0.81–0.978) were slightly smaller than those of the nondeuterated counterpart (0.83–0.995). The report of Garland *et al* (1976), however, discussed a kinetic isotope effect of 1.61 in rabbit microsomes. Although the V_{max} and K_m values in the liver perfusion studies were not explored, the ratio of the hepatic intrinsic clearances for d_0/d_5 ranged from 1.1 to 1.2 according to the "venous-equilibration" or "well stirred" model on hepatic clearance (Rowland *et al*; 1973) and for highly extracted compound such as phenacetin-d_0 and phenacetin-d_5, these differences in hepatic intrinsic clearances will not affect the values of the steady-state hepatic extraction ratio by much.

Under these tracer concentrations, total radioactivity in bile accounted for <5% of this infused dose and the rate of the total acetaminophen species in perfusate leaving the liver at steady-state represented the rate of formation of acetaminophen; both phenacetin species were converted primarily to acetaminophen (0.92–1.05), as measured by the ratio of the concentration of acetaminophen formed in liver (total acetaminophen species in effluent perfusate) to the drop in concentration of phenacetin across the rat liver. This ratio also denoted the fraction of total hepatic elimination of phenacetin that was converted to the metabolite, acetaminophen. No trend was demonstrable, however, from the data on the formation of acetaminophen from either perdeuterated or nondeuterated phenacetin.

The extent of acetaminophen sulfation was calculated by dividing the hepatic venous concentration of acetaminophen sulfate by the sum of all acetaminophen species (unconjugated acetaminophen and conjugated acetaminophen) in hepatic venous blood, all measured at steady-state. The paired comparison of the extents of subsequent sulfation of acetaminophen, derived from two phenacetin precursors of almost identical hepatocellular activities (hepatic intrinsic clearances) in acetaminophen formation, were quite similar (P < .25). Larger extents of acetaminophen sulfation were observed during retrograde flow that were significantly higher (P < .0005) than during normal flow and the observations (table 1) were consistent with the findings reported earlier (Pang and Terrell, 1981a).

[^{14}C]Acetanilide and [^{3}H]phenacetin. Because of dual delivery of [^{14}C]acetanilide and [^{3}H]phenacetin mutually inhibited the metabolism of each other, the simultaneous delivery of these precursors was avoided. Three perfusion periods (40 min each) with the successive and single delivery by normal flow of [^{14}C]acetanilide (range 40–61 × 10^3 dpm/ml or 0.89–1.23 μM), [^{3}H]phenacetin (range, 450–610 × 10^3 dpm/ml or 0.41–0.55 μM) and [^{14}C]acetanilide were examined. For these studies, the steady-state extraction ratios were: [^{14}C]acetanilide, 0.38 to 0.52; and [^{3}H]phenacetin, 0.84 to 0.98. Biliary excretion of total radioactivity accounted for <5% of the total infused dose for both precursors and very little appeared as unchanged [^{14}C] acetanilide (6–10% of excreted amount), [^{3}H]phenacetin and [^{3}H]acetaminophen (<2% of excreted amount). The ratio of [^{14}C]acetaminophen sulfate/[^{14}C]acetaminophen glucuronide in bile was 0.48 to 1.1:1, the ratio of [^{3}H]acetaminophen sulfate/[^{3}H]acetaminophen glucuronide in bile was usually 2:1. The metabolic conversion of [^{14}C]acetanilide to [^{14}C]acetaminophen accounted for only 68 to 80% of the total disappearance of [^{14}C] acetanilide. The possibility that N-deacylation of [^{14}C]acetanilide occurred to form aniline that accompanied loss of radiolabel in the rat, a metabolic pathway that was suggested by Brodie and Axelrod (1948) in humans, was not explored. By contrast, biotransformation of [^{3}H]phenacetin to [^{3}H]acetaminophen was almost complete (91–100%). Therefore, the hepatocellular activity or hepatic intrinsic clearance (ratio of V_{max}/K_m) for the formation of acetaminophen from phenacetin was greater than that from acetanilide.

[^{3}H]Acetaminophen sulfate was the only conjugated metabolite detected in the hepatic venous blood with the perfusion of [^{3}H]phenacetin. But [^{14}C]acetaminophen and glucuronide conjugates were both detected (ratio of sulfate/glucuronide conjugate was 4:1) in hepatic venous blood with the perfusion of [^{14}C]acetanilide. The extents of acetaminophen sulfation, again measured by the ratio of the steady-state output concentrations of acetaminophen sulfate conjugate to the sum of total acetaminophen species (unconjugated acetaminophen, and acetaminophen sulfate and glucuronide conjugates) were compared for this pair of precursors (table 2). Differences on the extents of acetaminophen sulfation between the precursors [^{14}C]acetanilide and [^{3}H]phenacetin were highly significant (P < .0005).

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 6, 2021

Auspex Exhibit 2016
Apotex v. Auspex
IPR2021-01507
Page 3

TABLE 1

Conversion of acetaminophen (derived from phenacetin d_0 and phenacetin-d_5) to its sulfate conjugates

A three-way analysis of variance was performed on the data. Significant differences exist between the liver preparations and for the extents of acetaminophen between normal *vs.* retrograde flows ($P < .0005$), whereas differences of the extents of acetaminophen sulfation between [14C]phenacetin-d_5 and [3H]phenacetin-d_0 were significant only at $P < .25$.

Study No.	Conversion of Acetaminophen to its Sulfate Conjugate (%)					
	(I) Normal perfusion		(II) Retrograde perfusion		(III) Normal perfusion	
	From phenacetin-d_0	From phenacetin-d_5	From phenacetin-d_0	From phenacetin-d_5	From phenacetin-d_0	From phenacetin-d_5
1	42.5	40.7	64.7	62.2	43.3	41.4
2	43.3	40.4	45.8	40.6	39.0	35.3
3	49.7	48.9	65.3	62.0	51.0	52.1
4	48.1	49.1	56.0	55.7	44.2	46.8
5	46.5	42.8	61.7	58.0	45.6	41.0

TABLE 2

Conversion of acetaminophen (derived from acetanilide and phenacetin to its sulfate conjugate

A two-way analysis of variance was performed on the data. Significant differences exist between the liver preparations and for the extents of acetaminophen sulfation between [3H]phenacetin-d_0 and [14C]acetanilide ($P < .0005$).

Study No.	% Sulfation of Acetaminophen		
	(I) From acetanilide	(II) From phenacetin	(III) From acetanilide
1	36.6	51.1	43.2
2	30.2	44.0	25.4
3	27.5	32.2	22.0
4	23.2	37.4	25.9
5	35.1	53.3	38.7

[3H]Phenacetin, the precursor with high hepatocellular activity (intrinsic clearance) for the acetaminophen formation was accompanied by a greater extent in sulfation for acetaminophen.

Discussion

Previous reports have alluded to the kinetic disposition of acetaminophen and phenacetin under tracer conditions in the perfused rat liver preparation; uneven distribution of enzymes within the liver may be responsible for differences in the extent of sulfation of acetaminophen when acetaminophen is formed by oxidative metabolism from a precursor or as preformed acetaminophen (Pang and Gillette, 1978; Pang and Terrell, 1981a). The present findings imply that in addition to the possible existence of zonal localization of drug metabolizing enzymes, the oxidative hepatocellular activity for metabolite formation also appears to influence the degree of subsequent sulfation of a metabolite.

For the precursor pairs of [14C]phenacetin-d_5 and [3H]phenacetin-d_0, which differ only slightly in enzymic capabilities in O-deethylation, differences in the extent of acetaminophen sulfation were very similar (table 1). Also, consistent with our earlier data (Pang and Terrell, 1981a), the extent of acetaminophen sulfation is greater during retrograde than during normal directional flow. For the precursor pair of [14C]acetanilide and and [3H]phenacetin which differ quite markedly in enzymic capabilities in the formation of acetaminophen, however, statistically significant differences ($P < .0005$) were observed in the extents of acetaminophen sulfation; the faster the formation of acetaminophen, the greater the extent of acetaminophen sulfation (table 2). Indeed, the phenomenon is not due to a nonlinearity in sulfation reactions either resulting from a saturation of enzyme capacity or a depletion of the cosubstrate 3′-phosphoadenosine 5′-phosphosulfate, as found in other systems (Grafstöm *et al.*, 1979), because the concentration range whereby the system is linear (≤1 μg/ml or 6.7 μM) with respect to acetaminophen sulfation (Pang and Terrell, 1981b) has not been exceeded by the sum (1.24 μM) of the tracer concentrations used in this experiment. Moreover, linearity for the oxidative pathways has been checked out by our preliminary experiments.

The apparent correlation of a faster formation and a faster sulfation of a primary metabolite may be explained by blood transit time and time required for drug conversion to primary metabolite. The blood transit time in liver is a measure of its transit in that organ and is assessed by the ratio of volume of blood in liver to hepatic blood flow [1.5 ml/(10 ml/min) in our case or 0.15 min]. The distance that a drug molecule has traveled within the liver is proportional to the time elapsed after the entry of this molecule into the liver. Because the transit time is finite, for metabolic reactions that occur sequentially, that is, a drug undergoes oxidative metabolism to form a metabolite before the metabolite is ultimately sulfated, the more time required for the Phase I biotransformation process, the less time will remain and the less likelihood there will be for subsequent metabolite sulfation. Contrastingly, the less time for metabolite formation, the more time will remain for further sulfation of the metabolite.

This concept that an oxidative product has a "duration time" within the liver that may be governed by the time required by the biotransformation process for its formation is illustrated by a simulation. Simple mass-balance equations were written to denote the rate of change drug (equation 1) and its primary (equation 2) and terminal (sulfated) metabolites (equation 3) in liver:

$$V_{L_{(D)}} \frac{d[D]}{dt} = QC_{In} - CL_{int_{(D \to M_I)}} [D] \tag{1}$$

$$V_{L_{(M_I)}} \frac{d[M_I]}{dt} = CL_{int_{(D \to M_I)}} [D] - CL_{int_{(M_I \to M_{II})}} [M_I] \tag{2}$$

$$V_{L_{(M_{II})}} \frac{d[M_{II}]}{dt} = CL_{int_{(M_I \to M_{II})}} [M_I] \tag{3}$$

where V_L denotes the volume of distribution of drug in liver, Q the hepatic venous blood and C_{In} the input drug (D) concentration. The square brackets denote liver concentrations of drug (D), primary metabolite (M_I) or terminal (sulfated) metabolite (M_{II}); CL_{int} denotes the hepatic intrinsic clearance and subscripts ($D \to M_I$) and ($M_I \to M_{II}$), respectively, denote the metabolic conversions of drug to primary metabolite and pri-

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 6, 2021

Auspex Exhibit 2016
Apotex v. Auspex
IPR2021-01507
Page 4

mary metabolite to terminal (sulfated) metabolite. For the sake of convenience, V_L and Q were designated as unity and M_I and M_{II} were the only metabolites formed in the sequence of $D \rightarrow M_I \rightarrow M_{II}$. The intrinsic clearance for metabolite formation, $CL_{int_{(I)\rightarrow M_I}}$, were assigned values of 0.4, 0.1 and 0.025 ml/sec. The intrinsic clearance for terminal (sulfated) metabolite formation, $CL_{int_{M_I \rightarrow M_{II}}}$ was fixed at 0.05 ml/sec.

The concentrations of drug and metabolite species in liver against time (time elapsed after drug entry) were simulated by use of a computer program, MLAB (Knott and Reece, 1977). Differing profiles (concentration *vs.* time in liver) for drug, primary metabolite and sulfated metabolite result along the flow path (time) in the liver (fig. 2). If the liver were of infinite length and the blood transit time infinite, the sulfated metabolite (M_{II}) will be the only species detected in hepatic venous blood for a single passage of drug. But the liver is of finite length and the blood of finite transit time. The concentrations of drug and metabolite species at the end of the flow path in liver (the value of blood transition time) becomes the effluent concentrations and will have different concentrations, depending on the intrinsic clearance for metabolite formation (fig. 2).

This simulation can be presented in an alternate fashion by expressing the ratio of the concentration of the terminal metabolite to the sum of all metabolites, that is, $[M_{II}]/([M_I] + [M_{II}])$ at various times (fig. 3). It can be seen from this plot, which expresses the extent of sequential sulfation of the primary metabolite *vs.* the time elapsed after the entry of drug into the liver, that the faster the formation of the primary metabolite (higher intrinsic clearance for metabolite formation), the greater the extent of subsequent metabolite sulfation.

The simulations (figs. 2 and 3) are indeed oversimplistic views of the sequential sulfation of a primary metabolite which is itself formed *via* oxidative metabolism from various precursors because uneven distribution of drug metabolizing enzymes may exist in the rat liver lobule. The real trend for metabolite sulfation, however, will not deviate qualitatively from the predicted curves (fig. 2 and 3) as long as the relative "median distance" for the enzyme systems for primary metabolite formation and metabolite sulfation remain constant (fig. 4). This median distance is conceptually shown by the distribution and the medians of two enzymic systems, A and B; the median is a measure of the "center" of enzymic distribution, the point on the scale of observations on each side of which there are equal areas of the enzyme system, and the median distance for either enzymes A or B is described as the distance between the median of the enzymic system and the point of entry of blood to the liver (fig. 4). For different precursors that form a common primary metabolite with different hepatocellular activities and different enzymic systems, and the primary metabolite is eliminated *via* a common conjugation pathway, for example, sulfa-

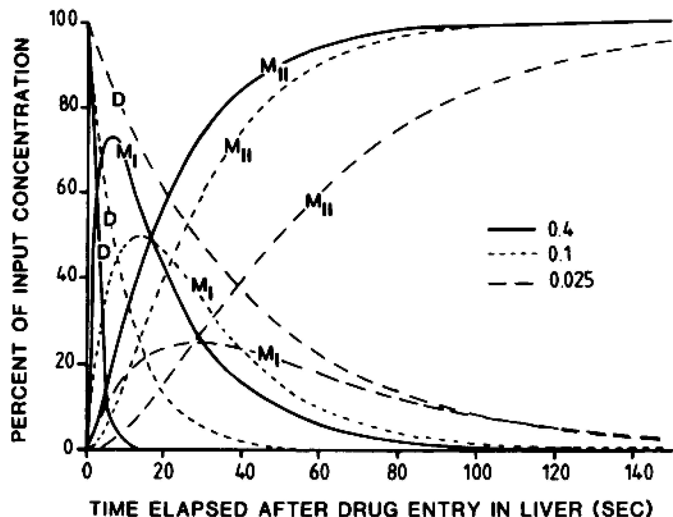

Fig. 2. A simulation of the concentrations of drug (D) and its primary (M_I) and terminal (M_{II}) metabolites in liver. The hepatic intrinsic clearances for M_I formation from D are varied (0.4, 0.1 and 0.025 ml/sec), whereas the hepatic intrinsic clearance for M_I elimination (sulfation) is kept constant (0.05 ml/sec). The drug decays with the elapse of time, whereas the primary metabolite follows a biphasic upswing and downswing curve and the sulfated metabolite accumulates.

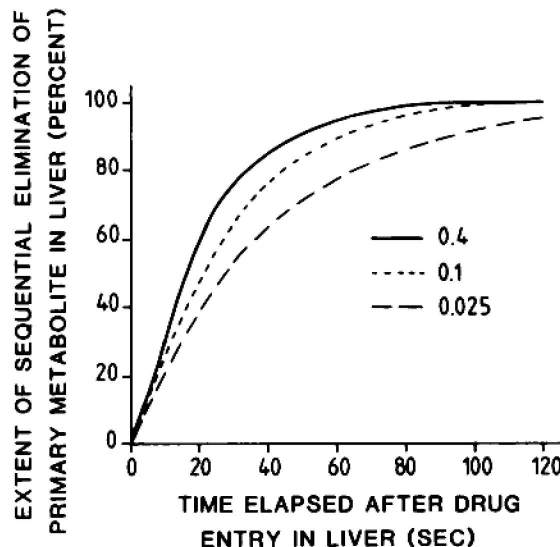

Fig. 3. The extent of subsequent elimination of a primary metabolite as a function of the time elapsed after drug entry into the liver and the hepatocellular activities for primary metabolite formation (0.4, 0.1 and 0.025 ml/sec); the hepatocellular activity for primary metabolite sulfation is kept constant (0.05 ml/sec). The simulations are transformed data from figure 2.

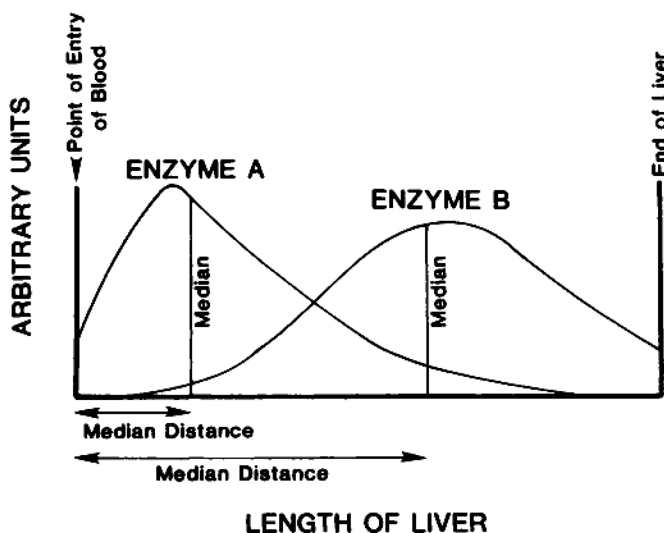

Fig. 4. The distribution of enzymic activities for enzyme systems A and B and their median values along the length of the liver. The distance between the median and the point of entry of blood is the median distance.

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 6, 2021

Auspex Exhibit 2016
Apotex v. Auspex
IPR2021-01507
Page 5

tion, and when the difference between the values of the median distance for these enzymic processes remain relatively constant, the same skewedness may appear on the family of curves (figs. 2 and 3). Also, when the drug, D, forms primary metabolites other than M_I, and M_I forms terminal metabolites other than M_{II}, as exemplified by acetanilide metabolism during its single passage through the rat liver in which other primary and terminal metabolites (acetaminophen glucuronide) are formed, an analogous situation will result, as long as a common sequence such as $D \rightarrow M_I \rightarrow M_{II}$ is identified.

An understanding of this concept of metabolite duration time is of paramount importance to metabolite kinetics and to drug-mediated toxicity due to bioactivation. The same metabolite species may exert differential pharmacologic/toxic action depending on the nature of the precursors, *viz.*, the hepatocellular activities for the formation of the metabolite and when administered as a preformed metabolite.

Acknowledgments

The authors wish to thank: Ms. Mary Lacy for the preparation of the manuscript; Mr. Robert Masters for artwork and photography; Dr. Josiah N. Tam, McNeils Consumers Products, Inc., for his generous supply of acetaminophen sulfate and glucuronide conjugates; Dr. Richard N. Stillwell, Institute for Lipid Research, Baylor College of Medicine, for computer simulations; and Dr. James R. Gillette, National Institutes of Health for stimulating and constructive suggestions.

References

BRODIE, B. B. AND AXELROD, J.: The fate of acetanilide in man. J. Pharmacol. Exp. Ther. **94**: 29–38, 1948.

CHAN, K. K. AND PANG, K. S.: Synthesis of singly ^{2}H-, ^{3}H-, and ^{14}C- and doubly labeled acetaminophen, phenacetin, and p-acetanisidine. *In* J. Labeled Compounds and Radiolabeled Pharmaceuticals, in press, 1982.

DE BAUN, J. R., SMITH, J. Y. R., MILLER, E. C. AND MILLER, J. A.: Reactivity *in vivo* of the carcinogen N-hydroxy-2-acetylaminofluorene: Increase by sulfation. Science (Wash. DC), **167**: 184–186, 1970.

GARLAND, W. A., HSIAO, K. C., PANTUCK, E. J. AND CONNEY, A. H.: Quantitative determination of phenacetin and its metabolite by GLC-chemical ionization mass spectroscopy. J. Pharm. Sci. **66**: 340–347, 1977.

GARLAND, W. A., NELSON, S. D. AND SASAME, H. A.: Primary and β-secondary deuterium isotope effects in the O-deethylation of phenacetin. Biochem. Biophys. Res. Commun. **72**: 539–545, 1976.

GRAFSTRÖM, R., ORMSTAD, K., MOLDÉUS, P. AND ORRENIUS, S.: Paracetamol metabolism in the isolated perfused liver with further metabolism of a biliary paracetamol conjugated by small intestine. Biochem. Pharmacol. **28**: 3573–3579, 1979.

KADLUBAR, F. F., MILLER, J. A. AND MILLER, E. C.: Hepatic metabolism of N-hydroxy-N-methyl-4-aminoazobenzene and other N-hydroxy arylamines to reactive sulfuric acid esters. Cancer Res. **36**: 2350–2359, 1976.

KNOTT, G. AND REECE, D.: MLAB, An Online Modeling Laboratory, 7th ed., Division of Computer Research and Technology, National Institutes of Health, Bethesda, 1977.

MINCK, K., SCHUPP, R. R., ILLING, H. P. A., KAHL, G. F. AND NETTER, K. J.: Interrelationship between demethylation of p-nitroanisole and conjugation of p-nitrophenol in rat liver. Naunyn-Schmiedeberg's Arch. Pharmacol. **279**: 347–360, 1973.

MULDER, G. J., HINSON, J. A. AND GILLETTE, J. R.: Generation of reactive metabolites of N-hydroxy-phenacetin and glucuronidation and sulfation. Biochem. Pharmacol. **26**: 189–196, 1977.

PANG, K. S. AND GILLETTE, J. R.: 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, 1978.

PANG, K. S. AND GILLETTE, J. R.: Sequential first-pass elimination of a metabolite derived from its precursor. J. Pharmacokinet. Biopharm. **7**: 275–290, 1979.

PANG, K.S., STROBL, K. AND GILLETTE, J. R.: A method for the estimation of the fractional conversion of a precursor to a metabolite in the rat *in vivo* with phenacetin and acetaminophen. Drug Metab. Dispos. **7**: 366–372, 1979.

PANG, K. S. AND TERRELL, J. A.: Retrograde perfusion to probe the heterogeneous distribution of hepatic drug metabolizing enzymes in rats. J. Pharmacol. Exp. Ther. **216**: 339–346, 1981a

PANG, K. S. AND TERRELL, J. A.: Sulfate conjugation kinetics of acetaminophen by the perfused rat liver preparation. Biochem. Pharmacol. **30**: 1959–1965, 1981b.

REINKE, L. A., BELINSKY, S. A., EVANS, R. K., KAUFFMAN, F. C. AND THURMAN, R. G.: Conjugation of *p*-nitrophenol in the perfused rat liver: The effect of substrate concentration and carbohydrate reserves. J. Pharmacol. Exp. Ther. **217**: 863–870, 1981.

ROWLAND, M. BENET, L. Z. AND GRAHAM, G. G.: Clearance concepts in pharmacokinetics. J. Pharmacokinet. Biopharm. **1**:123–136, 1973.

SLOTKIN, T. A., DISTEFANO, V. AND AU, W. Y. W.: Blood levels and urinary excretion of harmine and its metabolites in man and rats. J. Pharmacol. Exp. Ther. **173**: 26–30, 1970.

TROWELL, O. A.: Urea formation in isolated perfused liver of the rat. J. Physiol. (Lond.) **100**: 432–458, 1942.

VILLENEUVE, A. AND SOURKES, T. L.: Metabolism of harmaline and harmine in the rat. Rev. Can. Biol. **25**: 231–239, 1966.

WIEBKIN, P., FRY, J. R., JONES, C. A., LOWING, R. K. AND BRIDGES, J. W.: Biphenyl metabolism in isolated rat hepatocytes: Effect of induction and nature of conjugates. Biochem. Pharmacol. **27**: 1899–1907, 1978.

WILLIAMS, R. T.: Detoxification Mechanisms, Williams and Wilkins, Baltimore, and Chapman and Hall, London, 1959.

WILSON, J. M., SLATTERY, J. T., FORTE, A. J. AND NELSON, S. D.: Analysis of acetaminophen metabolite by high-performance liquid chromatography with UV and amperometric detection. J. Chromatogr. **227**: 453–462, 1982.

Send reprint requests to: Dr. K. Sandy Pang, Department of Pharmaceutics, University of Houston, 1441 Moursund St., Texas Medical Center, Houston, TX 77030.

Downloaded from jpet.aspetjournals.org at ASPET Journals on October 6, 2021

Auspex Exhibit 2016
Apotex v. Auspex
IPR2021-01507
Page 6