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Rates of Sulfation and Glucuronidation of 7-Hydroxycoumarin in Periportal and Pericentral Regions of the Liver Lobule

J. G. CONWAY,[1,2] F. C. KAUFFMAN,[3] S. JI,[1] AND R. G. THURMAN[1,4]

Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514, and Department of Pharmacology, University of Maryland, Baltimore, Maryland 21201

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SUMMARY

Micro-light guides (tip diameter 170 μm) were placed on periportal (light) and pericentral (dark) regions of perfused liver to monitor the fluorescence (366 → 450 nm) of infused 7-hydroxycoumarin. During infusion of up to 30 μM 7-hydroxycoumarin, fluorescence of free 7-hydroxycoumarin could be detected only in periportal regions during anterograde persusion and in pericentral areas during retrograde perfusion. Over 95% of 7-hydroxycoumarin infused was converted into nonfluorescent sulfate and glucuronide conjugates. Thus, under these conditions, rates of sulfation and glucuronidation can be studied in the periportal and pericentral regions of the lobule by measuring conjugates of 7-hydroxycoumarin in the effluent perfusate via anterograde or retrograde perfusions. Sulfation predominated over glucuronidation when 2–10 μM 7-hydroxycoumarin was infused in the anterograde direction; however, with 20–30 μM 7-hydroxycoumarin, glucuronidation predominated. In contrast, rates of glucuronidation and sulfation were similar when 2–5 μM 7-hydroxycoumarin was infused in the retrograde direction; however, glucuronidation predominated at higher substrate concentrations. Thus, at low concentrations of 7-hydroxycoumarin, sulfation predominated over glucuronidation in periportal hepatocytes but not in pericentral hepatocytes. When livers were perfused with sulfate-free media, the glucuronide was the predominant conjugate formed in both periportal and pericentral hepatocytes. Taken together, these data indicate that sulfation successfully competes with glucuronidation for 7-hydroxycoumarin at low substrate concentrations. When 7-hydroxycoumarin was generated via mixed-function oxidation following the infusion of 7-ethoxycoumarin (5–20 μM), fluorescence from 7-hydroxycoumarin was detected predominantly in periportal areas during anterograde perfusions and in pericentral regions during retrograde perfusions. The predominant conjugate formed in both regions of the liver lobule during mixed-function oxidation was sulfate. Thus, rates of sulfation were significantly greater in periportal regions than in pericentral regions with 7-hydroxycoumarin was infused directly or generated indirectly via mixed-function oxidation.

INTRODUCTION

Isolated perfused liver has been used as a model to study conjugation reactions which are regulated by a number of metabolic factors, including substrate concentrations, activities of transferase enzymes, and supply of cofactors (1–3). Sulfation of phenolic substrates (1, 4–7) has been shown to be a low-capacity, high-affinity system dependent upon added sulfate, whereas glucuronidation occurs with lower affinity but has much higher capacity (1, 2). Although some workers have studied conjugation reactions in periportal and pericentral regions of the liver lobule (3, 8, 9), very little quantitative information exists concerning the rates of these processes. Studies employing hepatotoxins to destroy selectively different regions of the liver lobule suggest that glucuronyltransferases and sulfotransferases are localized predominantly in periportal hepatocytes (8–10). Pharmacokinetic modeling also suggests that sulfation of acetaminophen generated from phenacetin by perfused rat liver is greater in periportal hepatocytes than in pericentral hepatocytes (3). Although such studies provide useful information, they do not provide quantitative data on rates of conjugation in specific lobular zones of the liver.

Recently, a method to measure rates of 7-ethoxycoumarin *O*-deethylation in periportal and pericentral regions of liver was developed in our laboratory (11). This method employs micro-light guides to monitor fluorescence signals from distinct lobular zones on the liver

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[1] Department of Pharmacology, University of North Carolina.

[2] Predoctoral Fellow of the Chemical Industry Institute of Toxicology.

[3] Department of Pharmacology, University of Maryland.

[4] Recipient of Career Development Award AA-00033.

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surface (12). The fluorescence of 7-hydroxycoumarin produced via mixed-function oxidation of nonfluorescent 7-ethoxycoumarin in distinct periportal and pericentral sites can be conveniently measured with this method. It was demonstrated that maximal rates of 7-hydroxycoumarin formation were about twice as high in pericentral hepatocytes as in periportal hepatocytes in livers from fed, phenobarbital-treated rats (11).

The purpose of the present study was to develop a method with which to quantitate rates of conjugation of fluorescent 7-hydroxycoumarin to the nonfluorescent sulfate and glucuronide in periportal and pericentral areas of perfused liver. The data indicate that rates of conjugation of 7-hydroxycoumarin are different in periportal and pericentral regions of liver and that sulfate formation predominates in periportal regions. Preliminary accounts of this work have appeared elsewhere (13).

METHODS

Animals. Female Sprague-Dawley rats weighing 200–350 g were pretreated with phenobarbital (1 g/liter) in the drinking water for 10–20 days prior to perfusion experiments (14). All rats had free access to laboratory chow.

Liver perfusion. Details of the nonrecirculating perfusion technique have been described elsewhere (15). The perfusate was Krebs-Henseleit bicarbonate buffer (pH 7.4) saturated with a gas mixture of oxygen (95%) and carbon dioxide (5%). For experiments with sulfate-free media, magnesium sulfate was replaced on a molar basis with magnesium chloride. 7-Hydroxycoumarin and 7-ethoxycoumarin (Sigma Chemical Company, St. Louis, MO.) were dissolved in the perfusate by gentle heating. For perfusions in the anterograde direction, the perfusate was pumped into the portal vein (3–4 ml/g/min), and effluent perfusate was collected via a cannula placed in the inferior vena cava. For retrograde perfusions, the direction of flow was reversed. Effluent perfusate flowed past an oxygen electrode before it was either collected for metabolite measurements or discarded. The oxygen tension in the effluent perfusate was determined continuously with a Clark-type platinum electrode to monitor tissue viability.

Sulfate and glucuronide conjugates of 7-hydroxycoumarin in the perfusate were hydrolyzed enzymatically as described elsewhere (1) and were measured fluorometrically (366 → 450 nm) with a quartz light guide (tip diameter 2 mm) coupled to a Johnson Foundation fluorometer. The fluorescence of samples was compared with that of authentic 7-hydroxycoumarin standards incubated under identical conditions.

Micro-light guide technique. The construction of micro-light guides for the detection of 7-hydroxycoumarin fluorescence from the liver surface has been described previously (11, 12, 16). The micro-light guide consists of two strands of glass fiber (diameter 80 μm) which are held together at the tip with epoxy glue.

We have demonstrated previously by infusion of India ink that light areas and dark spots visible on the surface of the hemoglobin-free perfused rat liver correspond to periportal and pericentral regions of the liver lobule, respectively (12). Since the diameter of these regions is several-fold greater than that of the tip of the micro-light guide (12), it is easy to place the light guide on either region to measure fluorescence. Fluorescence changes in periportal and pericentral regions of the liver were monitored simultaneously by placing micro-light guides on light and dark areas of the left lateral lobe of the perfused liver with micromanipulators. One strand of the micro-light guide was connected to a 100- W mercury arc lamp and the other strand to a photomultiplier. The liver was illuminated with 366-nm light, and fluorescence was detected at 450 nm (16).

Microscopic examination of the lobular distribution of 7-hydroxycoumarin. The left lateral lobe of the liver was frozen during perfusion by pressing an aluminum mallet chilled in liquid nitrogen gently on the lobe and then immersing the liver in liquid nitrogen. This process preserved the histological structure of the lobe. Blocks of tissue (3 mm^3) were dissected in the frozen state, mounted on a cryostat chuck, and cut into 20-μm sections. Frozen sections were lyophilized at −40°. Fluorescence of 7-hydroxycoumarin was detected in the dried sections by using a Nikon microscope equipped with an epi-illuminator.

RESULTS

Uptake of 7-hydroxycoumarin by periportal and pericentral hepatocytes. Figure 1 illustrates a typical experiment in which micro-light guides were placed on periportal and pericentral regions of the liver. When 10 μM 7-hydroxycoumarin was infused via the portal vein, increased fluorescence was evident only in periportal tissue. When the 7-hydroxycoumarin infusion was increased to 20 μM, fluorescence was increased in periportal tissues but still could not be detected in pericentral tissues. At 30 μM, a further increase in fluorescence in periportal regions was observed concomitant with a slight fluorescence increase in the pericentral area. When the infusion of 7-hydroxycoumarin was terminated, fluorescence returned to baseline. When perfusions were performed in the retrograde direction, concentrations of less than 5 μM 7-hydroxycoumarin could be detected in pericentral regions but not in periportal regions (Fig. 2). The retrograde infusion of 30 μM 7-hydroxycoumarin produced a large increase in fluorescence in pericentral cells but only a small signal in periportal tissue. When the infusion of 7-hydroxycoumarin was terminated, the fluorescence in both regions returned to baseline (Fig. 2).

Experiments with fluorescence microscopy were carried out to verify that free 7-hydroxycoumarin was not present in pericentral regions of the liver lobule during anterograde perfusions at concentrations of less than 30 μM. When 20–30 μM hydroxycoumarin was infused, a bright fluorescence could be detected only in periportal regions of the liver lobule (Fig. 3). Retrograde perfusions with 20–30 μM 7-hydroxycoumarin gave fluorescence signals only in pericentral regions, whereas higher concentrations of 7-hydroxycoumarin gave fluorescence over the entire lobule (data now shown).

Rates of sulfation and glucuronidation of 7-hydroxycoumarin in periportal and pericentral regions of the liver. In all experiments, concentrations of 7-hydroxycoumarin of 20 μM or less caused increases in surface fluo-

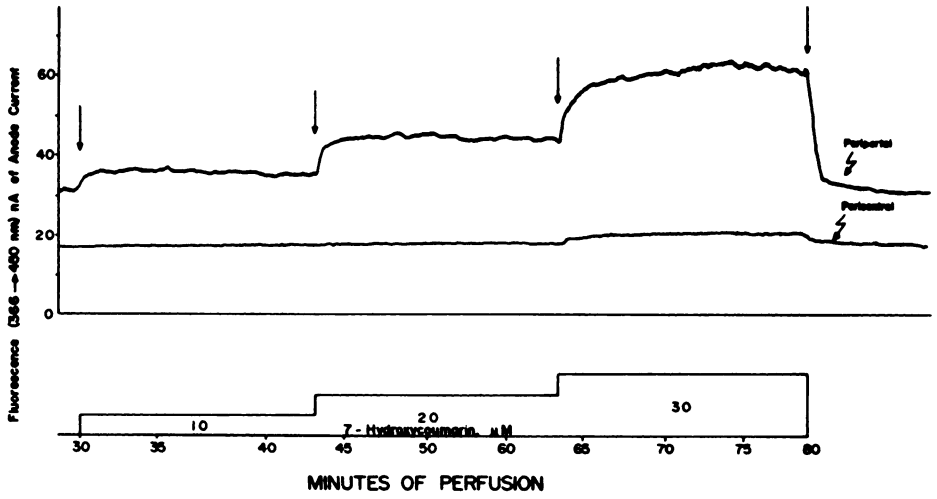

FIG. 1. *Fluorescence increase upon infusion of 7-hydroxycoumarin in periportal and pericentral regions of liver perfused in the anterograde direction*

Two micro-light guides were placed in periportal and pericentral regions (1 mm apart) on the left lateral lobe of the liver. Periportal and pericentral regions were identified by differences in native pigmentation as well as by differential responses to anoxia (7). 7-Hydroxycoumarin was dissolved in Krebs-Henseleit bicarbonate buffer and infused as indicated by horizontal *bars* and *arrows*. Typical experiment; fed, phenobarbital-treated rat.

rescence only in the region of the liver lobule first exposed to perfusate (i.e., periportal tissue if flow was in the anterograde direction and pericentral tissue if flow was retrograde). Because the micro-light guide can detect less than 5 μM 7-hydroxycoumarin from the surface of the tissue, it is assumed that the conjugates formed during the infusion of up to about 30 μM 7-hydroxycoumarin in the anterograde direction represent those formed exclusively by periportal tissue. Likewise, conjugation of up to 30 μM 7-hydroxycoumarin via perfusions in the retrograde direction reflects conjugates formed predominantly by pericentral cells. Under these conditions, free 7-hydroxycoumarin could not be detected in the effluent perfusate, and 95%–100% of infused 7-hydroxycoumarin was converted into glucuronide or sulfate conjugates.

Rates of sulfation and glucuronidation of 7-hydroxycoumarin by the liver were calculated employing influent minus effluent concentration differences, liver weight, and flow rate. Figure 4A and B depicts the rates of sulfate and glucuronide conjugates of 7-hydroxycoumarin

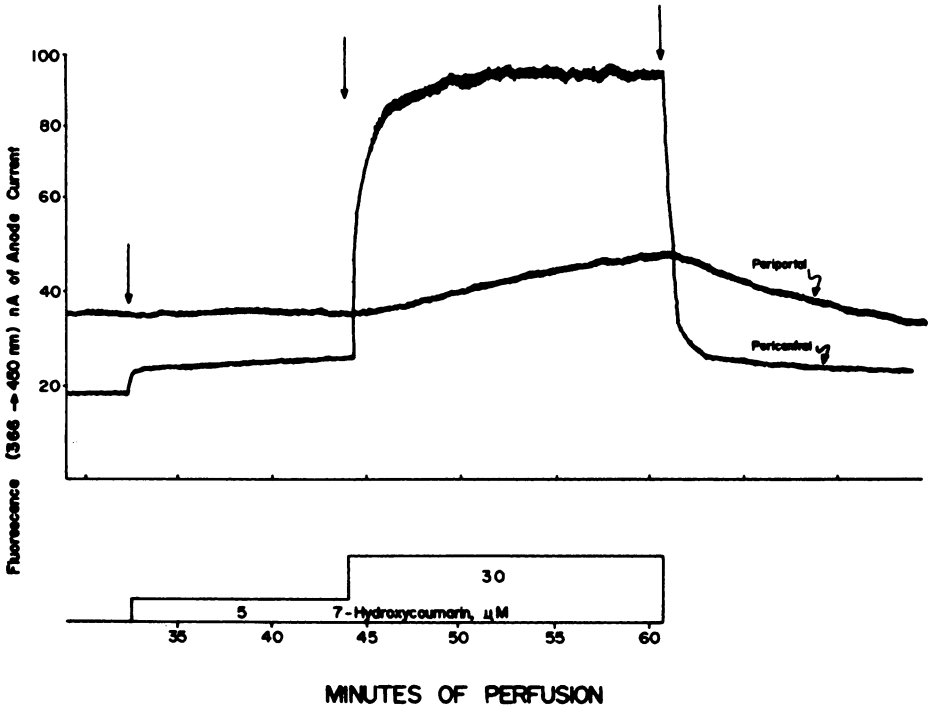

FIG. 2. *Periportal and pericentral 7-hydroxycoumarin fluorescence from retrograde perfusion*
Conditions as in Fig. 1 except that flow was via the vena cava.

FIG. 3. *Light micrograph of freeze-dried tissue section from rat liver perfused in the anterograde direction with 20 μM 7-hydroxycoumarin*
Livers were frozen during perfusion using an aluminum mallet chilled in liquid nitrogen to preserve liver histological structure. Sections (20 μm) were cut from the frozen liver and lyophilized at −40°. Periportal areas (*light*) are flourescent and pericentral regions (*dark*) are not (×80).

formed during anterograde and retrograde perfusions. In periportal tissue (anterograde perfusions), sulfation predominated over glucuronidation at 7-hydroxycoumarin concentrations of 2–10 μM (Table 1). At higher substrate concentrations, glucuronidation predominated (Fig. 4A).

The pattern of conjugation was quite different in pericentral hepatocytes (retrograde perfusions; Fig. 4B). At low 7-hydroxycoumarin concentrations (2–5 μM), rates of sulfation and glucuronidation were equivalent (Fig. 4B; Table 1); however, at higher substrate concentrations, rates of glucuronidation were significantly greater than rates of sulfation in pericentral regions (Fig. 4B). At low substrate concentrations (2–10 μM), the ratio of sulfation to glucuronidation was much greater in periportal regions than in pericentral regions, respectively, as determined from anterograde and retrograde perfusions (Table 1).

Sulfation can be markedly suppressed by omitting sulfate from the perfusate (1). When livers were perfused with sulfate-free media, sulfate conjugation was minimal (Fig. 5). Under these conditions, 7-hydroxycoumarin did not produce fluorescence changes in pericentral regions during anterograde perfusions or in periportal regions during retrograde perfusions (data not shown). Thus, rates of glucuronidation were equivalent in periportal and pericentral regions in the absence of sulfate (Fig. 5). Rates of glucuronidation were significantly higher ($p < 0.05$) than those observed when the sulfation system was operative (compare Figs. 4 and 5).

Conjugation of 7-hydroxycoumarin generated from

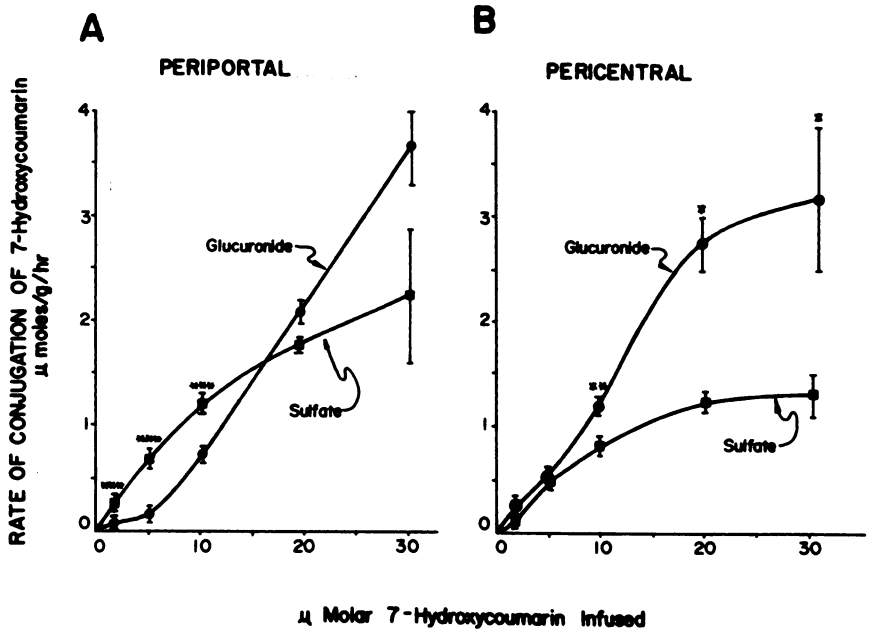

FIG. 4. *Rates of sulfation and glucuronidation in periportal (A) and pericentral (B) regions of perfused rat liver*

Eight livers were perfused in experiments similar to that shown in Fig. 1 in the anterograde direction (A) and eight in the retrograde direction (B) as shown in Fig. 2. To ensure viability, no liver received more than three concentration steps of 7-hydroxycoumarin. Under these conditions, no free 7-hydroxycoumarin could be detected in the effluent perfusate. Perfusate was collected and assayed for glucuronide and sulfate conjugates of 7-hydroxycoumarin (see Methods). The mean ± standard error of the mean for 5–11 data points is indicated for each concentration of 7-hydroxycoumarin. Comparisons were made by employing a matched-pairs *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$ for comparisons between sulfation and glucuronidation in the same lobular region.

mixed-function oxidation of 7-ethoxycoumarin. Nonfluorescent 7-ethoxycoumarin is *O*-deethylated to fluorescent 7-hydroxycoumarin by mixed-function oxidases (11). Infusion of 5–10 μM 7-ethoxycoumarin via anterograde perfusion produced an increase in 7-hydroxycoumarin fluorescence only in periportal regions of the liver lobule (Fig. 6). With retrograde perfusions, 7-hydroxycoumarin fluorescence was detected predominantly in pericentral regions (data not shown). Under these conditions, both sulfate and glucuronide conjugates of 7-hydroxycoumarin were collected during the infusion of 7-ethoxycoumarin during anterograde and retrograde perfusions (Table 2). During the infusion of 5–20 μM 7-ethoxycoumarin, rates of sulfation always exceeded rates of glucuronidation regardless of the direction of perfusion. During anterograde perfusions, the sulfate to glucuronide ratio in the perfusate decreased from 10 to 3 as 7-ethoxycoumarin concentrations were increased. In contrast, this ratio remained relatively constant during the infusion of various concentrations of 7-ethoxycoumarin in the retrograde direction (Table 2).

TABLE 1

Rates of conjugation of 7-hydroxycoumarin during infusion of 7-hydroxycoumarin

Conditions a in Figs. 1 and 2. Values are means ± standard error of the mean of number of determinations in parentheses.

7-Hydroxycoumarin infused	7-Hydroxycoumarin conjugated		Ratio sulfate/ glucuronide
	Sulfate	Glucuronide	
	μmoles/g/hr		
Anterograde perfusion			
2 μM	0.36 ± 0.028[a]	0.006 ± 0.005[a] (5)	>20[b]
5 μM	0.71 ± 0.075[a]	0.15 ± 0.036[a] (10)	6.11
10 μM	1.25 ± 0.13[a]	0.64 ± 0.09[a] (12)	1.90
Retrograde perfusion			
2 μM	0.17 ± 0.032	0.20 ± 0.004 (5)	1.65
5 μM	0.37 ± 0.045	0.42 ± 0.067 (9)	0.92
10 μM	0.67 ± 0.065	1.26 ± 0.161 (7)	0.60

[a] $p < 0.01$, anterograde versus retrograde perfusions.
[b] Ratio was arbitrarily defined as >20 because of the near absence of glucuronidation.

DISCUSSION

Development of a new fluorescence method to determine rates of conjugation in periportal and pericentral hepatocytes. A simple, noninvasive method for comparing dynamic metabolic events in different zones (e.g., periportal and pericentral) of the liver lobule has been developed. Micro-light guides placed on periportal and pericentral regions of the liver have previously been used to monitor pyridine nucleotides (12) and 7-hydroxycoumarin fluorescence (11). Several important applications of this technology have been made. For example, differences in inflow oxygen tension at which periportal and pericentral pyridine nucleotides were reduced when oxygen was removed from the perfusate have been used to estimate the hepatic lobular oxygen gradient (12, 17). Chronic treatment with ethanol increased this gradient (17). In another study, the fluorescence from the surface of the liver of 7-hydroxycoumarin generated from the mixed-function oxidation of 7-ethoxycoumarin correlated well with rates of mixed-function oxidation by the liver. 7-Hydroxycoumarin fluorescence from periportal and pericentral regions of the liver detected with micro-light guides was used to show that maximal rates of 7-ethoxycoumarin *O*-deethylation were about twice as high in pericentral regions as in periportal regions in livers from fed, phenobarbital-treated rats (11).

In the present study, this micro-light guide has been used to define conditions which allow rates of conjugation by periportal or pericentral hepatocytes to be studied

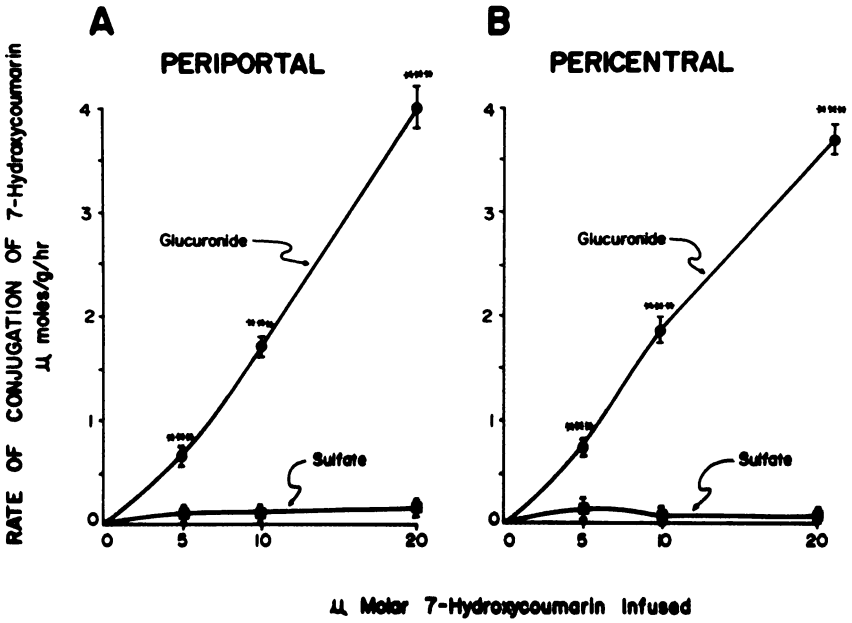

FIG. 5. *Rates of sulfation and glucuronidation in periportal (A) and pericentral (B) regions of rat livers perfused with sulfate-free medium* Seven livers were perfused with Krebs-Henseleit bicarbonate buffer deficient in sulfate in experiments similar to that shown in Fig. 1 in the anterograde direction (A) and five in the retrograde direction (B) as shown in Fig. 2. Perfusate was collected and assayed for glucuronide and sulfate conjugates of 7-hydroxycoumarin (see Methods). The mean ± standard error of the mean for five to seven data points is indicated for each concentration. ***$p < 0.001$ for comparisons between sulfation and glucuronidation in the same lobular region.

separately in the intact perfused liver. During anterograde perfusion, infusion of up to about 30 μM 7-hydroxycoumarin produced stepwise increases in fluorescence detected from periportal but not from pericentral regions of the liver lobule (Fig. 1). Opposite results were obtained during retrograde perfusions (i.e., 7-hydroxycoumarin was detected in pericentral regions but not in periportal regions; Fig. 2). Since less than 5 μM 7-hydroxycoumarin could be detected from the liver surface (Fig. 2), we conclude that 7-hydroxycoumarin must enter hepatocytes very rapidly. Were this not the case, the micro-light guide placed on the downstream regions (i.e., the pericentral region during anterograde perfusion) would have detected fluorescence of 7-hydroxycoumarin. Thus, conditions have been established to detect 7-hydroxycoumarin predominantly in periportal or pericentral regions of the liver lobule during anterograde and retrograde perfusions, respectively (Figs. 1 and 2). This was confirmed by fluorescence microscopy of thin sections of liver (Fig. 3). Since essentially all of the 7-hydroxycoumarin infused was conjugated, it is concluded that conjugates formed by the liver at concentrations of less than 30 μM 7-hydroxycoumarin in anterograde perfusions arise predominantly from periportal hepatocytes. Analogously, conjugation by pericentral hepatocytes can be studied by retrograde perfusion at low concentrations of 7-hydroxycoumarin.

Characterization of glucuronidation and sulfation in periportal and pericentral regions of the liver. It is well documented that the enzymes of mixed-function oxidation are unevenly distributed over the liver lobule (9, 11, 18); however, much less information is available on enzyme systems involved in conjugation reactions. One approach has been the selective destruction of periportal

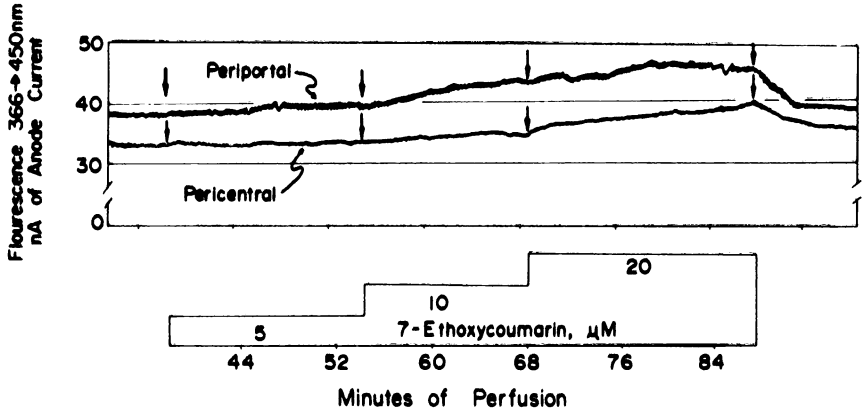

FIG. 6. *Periportal and pericentral 7-hydroxycoumarin fluorescence from the O-deethylation of 7-ethoxycoumarin* Conditions as in Fig. 1, except that 7-ethoxycoumarin was infused. Typical experiment.

TABLE 2

Rates of conjugation of 7-hydroxycoumarin during infusion of 7-ethoxycoumarin

Conditions as in Fig. 6. Values are means ± standard error of the mean of determinations made in four to eight livers.

7-Ethoxycoumarin infused	7-Hydroxycoumarin conjugated		Ratio sulfate/glucuronide	7-Hydroxycoumarin formed
	Sulfate	Glucuronide		
	μmoles/g/hr			*μM*
Anterograde perfusion				
5 μM	0.32 ± 0.039[a]	0.033 ± 0.009	11.2 ± 1.68	1.74
10 μM	0.66 ± 0.046	0.17 ± 0.054	6.6 ± 1.61	3.71
20 μM	1.11 ± 0.094	0.58 ± 0.211	3.2 ± 0.75	7.23
Retrograde perfusion				
5 μM	0.18 ± 0.037	0.06 ± 0.093	2.8 ± 0.39	1.61
10 μM	0.50 ± 0.057	0.15 ± 0.021	3.4 ± 0.39	4.22
20 μM	0.88 ± 0.141	0.41 ± 0.075	2.4 ± 0.52	7.28

[a] $p < 0.05$, anterograde versus retrograde.

or pericentral hepatocytes by site-specific toxins. James *et al.* (9) used allyl alcohol to destroy periportal regions, and bromobenzene to damage pericentral areas prior to assaying for microsomal glucuronyltransferase activity. They observed higher specific activity of glucuronyltransferase in microsomes from bromobenzene-treated rats than in those from allyl alcohol-treated rats, consistent with a predominant periportal localization of glucuronyltransferase activity. However, enzyme distribution in tissue may not determine local rates of glucuronidation per se, since the supply of the cofactor UDP-glucuronic acid may be an important determinant of rate (1, 2). With low substrate concentrations under conditions where sulfation occurs, glucuronidation of 7-hydroxycoumarin by pericentral regions exceeded that by periportal areas (Fig. 4; Table 1). In the absence of sulfate, rates of glucuronidation in the two regions were equivalent (Fig. 5). Thus, under these latter conditions, where 7-hydroxycoumarin is most likely limiting, the lobular distribution of glucuronidation as measured quantitatively in these studies is different from the distribution of glucuronyltransferase activities following treatment with selective hepatoxins (9). At higher (20–30 μM) substrate concentrations, glucuronidation occurred at about equal rates in both regions of the liver lobule (Fig. 4). These studies demonstrate clearly the utility of the new method described above (Figs. 1–3), since it allows direct determination of rates of conjugation predominantly in pericentral and periportal regions of the liver lobule.

Sulfation has been shown to be a low-capacity, high-affinity system (1, 19). With excess sulfate, the entry of sulfate into the cell is much faster than rates of sulfation (20). Pang and Terrell (3) utilized the uneven distribution of mixed-function oxidation to study the lobular distribution of sulfation in perfused liver. These studies were based on the reasonable assumption that phenacetin is converted into acetaminophen via mixed-function oxidation to a greater extent in pericentral hepatocytes than in periportal hepatocytes. Under these conditions, more acetaminophen sulfate was detected in the effluent perfusate with retrograde perfusions than with anterograde perfusions (3), leading the authors to conclude that sulfation occurs to a greater extent in periportal hepatocytes than in pericentral hepatocytes. This conclusion is supported generally by direct measurement of rates of sulfation of 7-hydroxycoumarin in the studies reported here (Fig. 4; Table 1). Rates of sulfation are twice as high in periportal hepatocytes as in pericentral hepatocytes (Table 1; Fig. 4). Thus, these data are consistent with the hypothesis that either sulfotransferases or cofactor supply (e.g., 3′-phosphoadenosine 5′-phosphosulfate or the enzymes needed for its synthesis) predominate in periportal hepatocytes.

Competition between sulfation and glucuronidation in periportal and pericentral regions of the liver. It is known that sulfation and glucuronidation can compete for substrate (1). For example, when perfusate was switched from one containing sulfate to one deficient in sulfate, sulfation was rapidly diminished whereas glucuronidation was enhanced (1). In these experiments (Fig. 4), sulfation markedly exceeded glucuronidation at low concentrations in periportal hepatocytes but not in pericentral hepatocytes (Table 1). We therefore asked whether this was due to competition between the two metabolic processes for substrate. Since glucuronidation was significantly greater during perfusion with sulfate-free medium (i.e., in the absence of sulfation), we conclude that the higher rate of sulfation observed in periportal regions is due to competition for substrate (Fig. 5). Competition was less dramatic between these two important metabolic systems in pericentral hepatocytes (compare Figs. 4B and 5B). At the present time, we cannot explain why the sulfation system is more successful at competing for substrate in periportal regions than in pericentral regions of the liver lobule. However, the sulfation system can compete more successfully for 7-hydroxycoumarin in both regions, since it has a much lower apparent K_m as derived from perfusion experiments (about 8 μM) than does glucuronidation (about 90 μM) (data not shown).

Generation of 7-hydroxycoumarin via mixed-function oxidation of 7-ethoxycoumarin. When 7-ethoxycoumarin was added to the liver, about half of it was rapidly converted into 7-hydroxycoumarin conjugates (Table 2). It is reasonable to assume that the remainder of the 7-ethoxycoumarin infused leaves the liver unmetabolized. Furthermore, perfusion of 7-ethoxycoumarin in the an-

terograde direction (Fig. 6) produced 7-hydroxycoumarin fluorescence from periportal regions but not pericentral regions of the liver lobule (Fig. 6). With retrograde perfusion, fluorescence was detected only in pericentral areas (data not shown). At these low concentrations of 7-hydroxycoumarin formed, we assume that it is predominantly conjugated at the lobular site of generation, since 7-hydroxycoumarin was detected only in the region first exposed to flow. Thus, mixed-function oxidation appears to be rate-limiting for conjugation reactions in both regions when the substrate for conjugation arises via metabolism. It is highly likely, therefore, that conjugates are formed predominantly in the region of the liver lobule where they are generated. In support of this hypothesis, the patterns of the ratio of sulfate to glucuronide were similar in any given lobular region irrespective of whether the substrate was 7-hydroxycoumarin (Table 1) or 7-ethoxycoumarin (Table 2).

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Send reprint requests to: Dr. R. G. Thurman, Department of Pharmacology 231H, University of North Carolina, Chapel Hill, N. C. 27514.