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Reducing Equivalents for Mixed Function Oxidation in Periportal and Pericentral Regions of the Liver Lobule in Perfused Livers from Normal and Phenobarbital-Treated Rats

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SUMMARY

The supply of NADPH for cytochrome P-450-dependent mixed function oxidation from the pentose cycle and mitochondria in periportal and pericentral regions of the liver lobule was evaluated in perfused rat liver. Rates of 7-ethoxycoumarin *O*-deethylation in livers from fed, normal rats monitored with micro-light guides placed on periportal and pericentral regions were 1.2 μmol/g/hr in both regions of the liver lobule. In livers from fed, phenobarbital-treated rats, rates were 3.6 and 7.0 μmol/g/hr in periportal and pericentral regions, respectively. Following treatment of rats with 6-aminonicotinamide, an inhibitor of the pentose cycle, rates of 7-hydroxycoumarin production were approximately 0.9 μmol/g/hr in both regions of the lobule in livers from normal rats and 2.1 and 3.4 μmol/g/hr in periportal and pericentral regions, respectively, in livers from phenobarbital-treated rats. Based on the difference in rates of 7-hydroxycoumarin production in the presence and absence of 6-aminonicotinamide, we conclude that the pentose cycle supplies NADPH for 7-ethoxycoumarin metabolism at rates around 0.3 μmol/g/hr in both regions of the liver lobule in livers from normal rats and 1.5 and 3.6 μmol/g/hr in periportal and pericentral regions, respectively, in livers from phenobarbital-treated rats. Potassium cyanide, an inhibitor of mitochondrial oxidation, reduced rates of 7-ethoxycoumarin *O*-deethylation to approximately 0.6 μmol/g/hr in both regions of the liver lobule in livers from fed, normal rats and to around 0.2 μmol/g/hr after fasting or treatment with 6-aminonicotinamide. In livers from fasted, phenobarbital-treated rats, 7-hydroxycoumarin was produced at rates of 0.3 and 0.7 μmol/g/hr in periportal and pericentral regions, respectively, in the presence of KCN. Decreases in rates of 7-hydroxycoumarin production during KCN infusion indicate that the mitochondria supply about 0.7 μmol of NADPH/g/hr for 7-ethoxycoumarin metabolism in both regions in livers from normal rats and 1.3 and 2.7 μmol/g/hr in periportal and pericentral regions in livers from phenobarbital-treated rats. The sum of KCN and 6-aminonicotinamide-sensitive rates of 7-ethoxycoumarin metabolism closely approximated rates measured in the absence of the inhibitors. These data indicate that mitochondria supply 50 to 70% of the reducing equivalents for mixed function oxidation of 7-ethoxycoumarin in both regions of the liver lobule in livers from fed rats.

INTRODUCTION

Over the last several decades, it has become clear that many enzymes are distributed unequally across the liver lobule (1). For example, enzymes involved in gluconeogenesis are concentrated in cells located near the portal vein, while glycolytic enzymes and cytochrome P-450 predominate in pericentral areas (2–4). Although progress has been made in identifying the zonal distribution of many hepatic enzymes, very little information exists concerning regulation of biochemical processes in different regions of the lobule in the intact liver.

A technique employing micro-light guides to determine rates of mixed function oxidation in periportal and pericentral regions of the liver lobule has been developed recently (5). This approach, which is based on monitoring

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the conversion of 7-ethoxycoumarin to the fluorescent product, 7-hydroxycoumarin, indicated that NADPH-dependent *O*-deethylation was about twice as great in pericentral as in periportal regions of the lobule in perfused livers from both fed and fasted, phenobarbital-treated rats (5, 6). These studies were the first to measure the overall rate of a metabolic process in different regions of the liver lobule. This new approach now allows regulation of mixed function oxidation to be studied in distinct regions of the liver lobule noninvasively. For example, xylitol or sorbitol, sugars which increase the supply of NADPH, double rates of mixed function oxidation in both regions of the liver lobule, indicating that NADPH supply is an important rate determinant for drug metabolism (6).

Reducing equivalents for mixed function oxidation can be supplied either by the pentose cycle (7) or via mitochondrial oxidations (8, 9). The possibility that the supply of NADPH via the pentose cycle is greater in cells in the pericentral region is suggested by the finding that glucose-6-phosphate dehydrogenase activity is about twice as great in pericentral as in periportal regions of the liver lobule (10). Moreover, its activity is increased more in pericentral than in periportal areas after phenobarbital treatment (10). Information on the sublobular distribution of mitochondrial NADPH generation is, however, scant.

The purpose of this study was to evaluate the relative contribution of the pentose cycle and mitochondria to supply reducing equivalents for mixed function oxidation in periportal and pericentral regions of the liver lobule using the noninvasive micro-light guide approach. A preliminary account of this work has appeared elsewhere (11).

MATERIALS AND METHODS

Animals and liver perfusion. Female Sprague-Dawley rats, 250–350 g, were used in this study. Where indicated, rats received sodium phenobarbital (1 mg/ml) in drinking water for at least 1 week to induce mixed function oxidase components prior to perfusion (12). Fasted rats were deprived of food for 24 hr prior to use. Rats treated with 6-aminonicotinamide received 70 mg/kg by intraperitoneal injection 4 hr before perfusion.

Livers were perfused with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°) saturated with an oxygen-carbon dioxide mixture (95:5) in a nonrecirculating system as described previously (13). The fluid was pumped (4.0 to 4.5 ml/g of liver) into the liver via a cannula placed in the portal vein and flowed past a Teflon-shielded, Clark-type oxygen electrode before being discarded. Rates of oxygen uptake were calculated from the influent minus effluent oxygen concentration difference, the flow rate, and the liver wet weight. A stock solution of 7-ethoxycoumarin was dissolved in *N,N*,-dimethylformamide which was added to the buffer to give a final concentration of 35 mM *N,N*,-dimethylformamide when 200 μM 7-ethoxycoumarin was infused into the liver. Under these conditions, *N,N*,-dimethylformamide had no effect on mixed function oxidation. Potassium cyanide, pH 7.4, was dissolved in Krebs-Henseleit buffer containing 0.5% albumin (fraction V, Sigma) and was infused into the liver at a final concentration of 2 mM in 0.003% albumin.

Determination of 7-hydroxycoumarin. Following the addition of 7-ethoxycoumarin, samples of effluent perfusate were collected every 2 min for the fluorometric determination (366 → 450 nm) of 7-hydroxycoumarin. The concentration of conjugated 7-hydroxycoumarin was determined after hydrolysis of glucuronide conjugates by β-glucuronidase (6). Rates of 7-hydroxycoumarin production were calculated from the sum of free and conjugated 7-hydroxycoumarin produced, the flow rate, and the liver wet weight.

Since rates of sulfation and glucuronidation differ across the liver lobule (14, 15), changes in 7-hydroxycoumarin fluorescence detected from periportal and pericentral regions could result from differences in local rates of conjugation. To eliminate this potential problem, sulfation of 7-hydroxycoumarin was reduced by 95% by deleting sulfate from the perfusion fluid (16). Rats were pretreated with galactosamine (400 mg/kg) by intraperitoneal injection 1.5 hr prior to perfusion. This treatment reduced glucuronidation by 85 to 100% (17) but did not affect rates of mixed function oxidation (data not shown). Following treatment with galactosamine, glucuronidation was undetectable in livers from normal rats and was less than 0.5 μmol/g/hr in livers from phenobarbital-treated rats.

Measurement of fluorescence of 7-hydroxycoumarin from periportal and pericentral regions of the liver lobule. Rates of mixed function oxidation in periportal and pericentral regions of the liver lobule were measured as described in detail previously (5, 6). Briefly, pairs of micro-light guides constructed from two strands of 70 μm-diameter glass fibers were placed simultaneously on periportal and pericentral regions on the surface of the perfused liver. Lightly pigmented regions have been identified as periportal areas and darkly pigmented spots as pericentral regions (5). Since a heterogeneous distribution of cytochrome P-450-dependent monooxygenase activity in different lobes of the rat liver has been reported (18), 7-hydroxycoumarin fluorescence was measured exclusively from the left lateral lobe with the micro-light guide in all experiments. Fluorescence due to 7-hydroxycoumarin was measured in periportal and pericentral regions of the liver by illuminating tissue with light at 366 nm and measuring fluorescence at 450 nm. Basal fluorescence of pericentral areas was lower than periportal regions (Figs. 1–3) most likely due to quenching of fluorescence by higher concentrations of cytochrome P-450 in pericentral areas (3, 4). The ratio of fluorescence signals from periportal and pericentral areas was constant over a wide range of 7-hydroxycoumarin concentrations (15); therefore, all fluorescence changes were expressed as a percentage of the basal signal to correct for slight differences in basal fluorescence (6).

Although the wavelengths employed for detection of 7-hydroxycoumarin also excite NADH and NADPH, fluorescence due to pyridine nucleotides does not inferfere with the signal because 7-hydroxycoumarin is 200 times more fluorescent than pyridine nucleotides on a molar basis (6). The change in basal fluorescence during infusion of KCN in the absence of 7-ethoxycoumarin was less than 3% of the fluorescent signal generated from 7-hydroxycoumarin; therefore, the values presented in this study are uncorrected for changes in pyridine nucleotide fluorescence.

A good correlation was observed previously between fluorescence of 7-hydroxycoumarin measured with a large-tipped light guide (2 mm diameter) and the *O*-deethylation of 7-ethoxycoumarin (5, 6). A similar correlation was observed with the micro-light guide in this study (data not shown). This correlation allows us to convert fluorescence readings measured with the micro-light guide into local rates of mixed function oxidation. Rates of 7-hydroxycoumarin formation in periportal and pericentral regions were calculated from the proportional changes in fluorescence arising from 7-hydroxycoumarin formation in the two zones of the liver lobule and from rates of mixed function oxidation by the liver (6).

Several experiments were performed to verify that 7-ethoxycoumarin was most likely distributed uniformly in all regions of the liver lobule. First, when 200 μM 7-ethoxycoumarin was infused into livers from normal and phenobarbital-treated rats, concentrations of 7-hydroxycoumarin ranged from 175 to 190 μM in the effluent perfusate. Therefore, both periportal and pericentral regions of the liver lobule must be exposed to concentrations of substrate greater than 175 μM. These values are well above substrate concentrations required for maximal rates of 7-hydroxycoumarin production (37 and 68 μM in

livers from normal and phenobarbital-treated rats, respectively). Second, retrograde perfusions were performed so that local rates of 7-ethoxycoumarin metabolism could be compared at equivalent substrate concentrations. Rates of 7-ethoxycoumarin metabolism in periportal and pericentral regions were not significantly different when perfusion was in the retrograde or anterograde direction (data not shown). Thus, we conclude that "downstream" regions of the liver lobule are adequately supplied with 7-ethoxycoumarin in these experiments.

The uniform production of 7-hydroxycoumarin by the perfused liver should lead to higher concentrations of product in the "downstream" than in the "upstream" regions, and therefore, steady state fluorescence of product would not be an accurate indication of local rates of 7-ethoxycoumarin metabolism. In theory, this statement is correct; however, it was not supported by data from retrograde experiments. Steady state fluorescence of 7-hydroxycoumarin was twice as great in pericentral than in periportal regions of livers from phenobarbital-treated rats irrespective of the direction of perfusion. Thus, it appears that the product formed upstream contributes very little to the overall fluorescence signal in the downstream region and is consistent with the hypothesis that the fluorescence signal detected arises predominantly from hepatocytes and not from vascular space. Also, 7-hydroxycoumarin leaving the cell and entering the vascular space is diluted greatly by the high flow rates employed, thereby decreasing the overall fluorescent signal arising from the vascular space.

7-Ethoxycoumarin O-deethylation in isolated microsomes. Livers from normal and phenobarbital-treated rats were homogenized (0.3 g/ml) in Krebs-Henseleit bicarbonate buffer and centrifuged at 2500 × *g* for 10 min. Assays were performed in 25-ml Erlenmeyer flasks containing Krebs-Henseleit bicarbonate buffer, 0.5 mM 7-ethoxycoumarin, 1 ml of supernatant, and an NADPH-generating system consisting of 0.4 mM $NADP^+$, 30 mM isocitrate, and 0.2 unit of isocitrate dehydrogenase (Sigma), in a final volume of 2.0 ml. Incubations were initiated by the addition of the NADPH-generating solution (0.2 ml) and were terminated after 10 min by the addition of 0.5 ml of $HClO_4$ (0.6 N). The mixture was centrifuged at 2,500 × *g* for 3 min to remove precipitated protein, after which 100 μl of supernatant was added to 2.0 ml of 0.5 M Tris, pH 7.4. 7-Hydroxycoumarin fluorescence (380 → 490 nm) in the incubation mixture was measured and the concentration of 7-hydroxycoumarin was determined by comparison with authentic standards treated similarly.

RESULTS

Rates of 7-ethoxycoumarin O-deethylation in periportal and pericentral regions of the liver lobule. Micro-light guides were placed on periportal and pericentral regions of the liver lobule to monitor the conversion of nonfluorescent 7-ethoxycoumarin to fluorescent 7-hydroxycoumarin by the mixed function oxidase pathway. Fluorescence increases were detected first in periportal regions followed in less than 1 min by increases in pericentral areas. Fluorescence in both regions reached new steady state values in 6 to 10 min (Fig. 1). 7-Hydroxycoumarin fluorescence, expressed as a percentage of basal fluorescence, was similar in both regions of the lobule in livers from fed, normal rats, corresponding to rates of 1.2 μmol/g/hr in both areas (Table 1). In livers from fed, phenobarbital-treated rats, the increase in 7-hydroxycoumarin fluorescence followed a similar time course (Fig. 2); however, the increase was about twice as great in pericentral as in periportal regions of the liver lobule (7.0 versus 3.6 μmol/g/hr). When the infusion of 7-ethoxycoumarin was terminated, fluorescence returned rapidly to the baseline in both regions of the liver lobule (Fig. 2A).

Treatment of rats with 6-aminonicotinamide, an inhibitor of the pentose cycle (19), did not alter the time course of fluorescence changes (Fig. 2). However, in livers from phenobarbital-treated rats, rates were diminished from 3.6 to 2.1 in periportal regions and from 7.0 to 3.4 μmol/g/hr in pericentral regions of the liver lobule (Fig. 2B; Table 1).

Fasting did not alter significantly rates of 7-hydroxycoumarin production in periportal and pericentral regions of livers from normal rats; however, xylitol (2 mM) infusion increased fluorescence significantly by about 30% in both regions of the liver lobule. This increase corresponded to an increase in rates of 0.3 μmol/g/hr (Fig. 3; Table 1). Stimulation of mixed function oxidation by xylitol was inhibited completely in both zones by pretreatment of rats with 6-aminonicotinamide (data not shown). Similar results were also obtained in livers from phenobarbital-treated rats (ref. 6; Table 1).

Effect of potassium cyanide on rates of 7-hydroxycoumarin production in periportal and pericentral regions of the liver lobule. Infusion of KCN (2 mM) caused a rapid decrease in 7-hydroxycoumarin fluorescence to lower steady state values in about 6 min in both regions of the liver lobule (Fig. 1). In livers from fed, normal rats, KCN reduced rates of mixed function oxidation from 1.2 to 0.5 μmol/g/hr (Table 1). After KCN infusion was terminated, fluorescence returned rapidly to the original baseline. Rates of mixed function oxidation were decreased from 3.6 to 2.3 μmol/g/hr in periportal regions and from 7.0 to 6.2 μmol/g/hr in pericentral regions by KCN (Table 1) in livers from fed, phenobarbital-treated rats.

Local rates of 7-ethoxycoumarin *O*-deethylation were virtually abolished by infusion of cyanide into livers from fasted or fed normal rats treated with 6-aminonicotinamide (Table 1). Similar results were observed in livers from fasted, phenobarbital-treated rats (Table 1).

Effect of potassium cyanide on 7-hydroxycoumarin production in vitro. Cyanide has been reported to inhibit cytochrome P-450 weakly in isolated microsomes (30). However, experiments in isolated microsomes are somewhat artificial when one considers the potential binding sites for KCN which exist in the intact cell. To attempt to address the question of the effect of cyanide on 7-ethoxycoumarin *O*-deethylation under conditions similar to those in the perfused liver, incubations were carried out in a 2500 × *g* supernatant of a 1:3 homogenate of livers from normal and phenobarbital-treated rats. Under these conditions, KCN had essentially no effect on 7-ethoxycoumarin *O*-deethylation (normal, 7% inhibition; phenobarbital-treated, no effect). Thus, it is concluded that cyanide does not affect cytochrome P-450 directly under the conditions employed in this study.

DISCUSSION

Regulation of mixed function oxidation in periportal and pericentral regions of the liver lobule in normal rats. Although the two cytochrome P-450 isoenzymes with the highest affinity for 7-ethoxycoumarin (P-450-MC and P-450-PB) and NADPH-cytochrome P-450 reductase measured *in vitro* are more concentrated in pericentral than periportal regions (3, 4, 20), rates of 7-hydroxycoumarin production measured noninvasively were similar in both regions in perfused livers from normal rats in this study

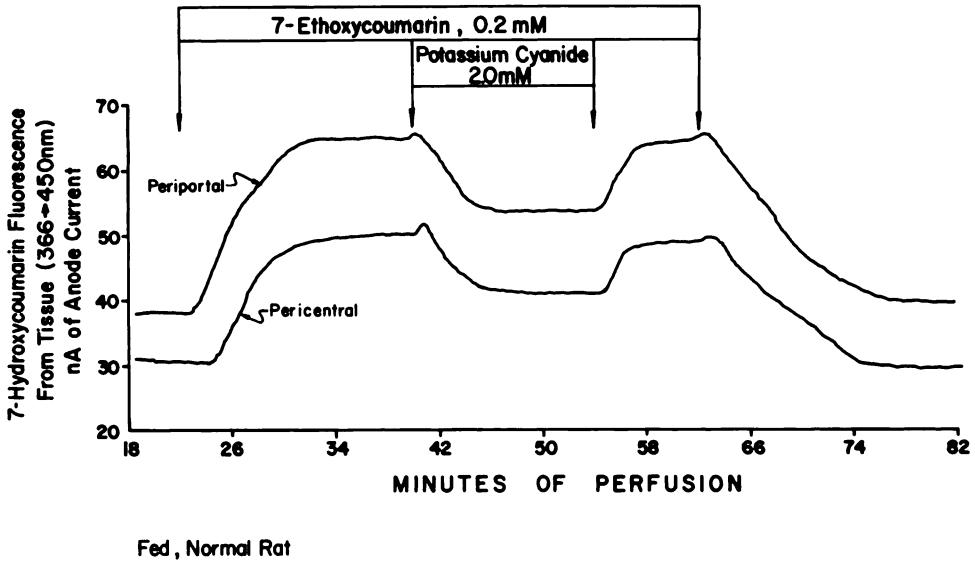

FIG. 1. *7-Hydroxycoumarin fluorescence from periportal and pericentral regions of the liver lobule following the addition of 7-ethoxycoumarin and potassium cyanide*

Two micro-light guides (tip diameter = 170 μm) were placed on two adjacent periportal regions (1 to 3 mm apart) on the left lateral lobe of a liver from a fed, normal rat. The output voltages (600–650 V) of the photomultipliers were adjusted to give similar anode currents in both channels. Subsequently, one micro-light guide was moved to a pericentral area. 7-Hydroxycoumarin fluorescence from the liver surface was determined as described in Materials and Methods. 7-Ethoxycoumarin (0.2 mM) and potassium cyanide (2.0 mM) were infused at the times indicated by the horizontal bars and vertical arrows.

TABLE 1

7-Ethoxycoumarin O-deethylation in periportal and pericentral regions of the liver lobule in perfused livers from normal and phenobarbital treated rats

Conditions were as described in Materials and Methods and in Fig. 1–3. In some experiments, rats received 70 mg/kg of 6-aminonicotinamide (6AN) 4 hr prior to perfusion. Maximal rates of 7-ethoxycoumarin *O*-deethylation in periportal and pericentral regions of the liver lobule were calculated as described in Materials and Methods. Local rates of 7-ethoxycoumarin *O*-deethylation were corrected for the small increase in NADH fluorescence observed with addition of xylitol alone (see Fig. 3). Data represent mean ± standard error from 5 to 12 livers in each group.

Nutritional state	Treatment	Addition	7-Ethoxycoumarin *O*-deethylation			
			Normal		Phenobarbital-treated	
			Periportal	Pericentral	Periportal	Pericentral
			μmol/g/hr			
Fed	None	None	1.2 ± 0.2	1.2 ± 0.1	3.6 ± 0.3	7.0 ± 0.5
		KCN	0.6 ± 0.1	0.5 ± 0.1	2.3 ± 0.2	6.2 ± 0.5
Fed	6AN	None	0.9 ± 0.1[a]	0.9 ± 0.1[a]	2.1 ± 0.2	3.4 ± 0.3
		KCN	0.2 ± 0.1	0.2 ± 0.1	1.1 ± 0.1	2.1 ± 0.2
Fasted	None	None	1.0 ± 0.1[a]	1.1 ± 0.2[a]	1.6 ± 0.2	3.4 ± 0.3
		KCN	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.1
Fasted	None	None	1.0 ± 0.1[a]	1.2 ± 0.1[a]	2.2 ± 0.7[b]	5.2 ± 0.7[b]
		Xylitol	1.3 ± 0.1	1.5 ± 0.1	3.9 ± 0.3[b]	10.8 ± 1.3[b]

[a] Not significant.

[b] Values were taken from ref. 7. All changes except those not significant were significantly different ($p < 0.01$) in two sets of comparisons: 1) fed, no treatment group compared to 6-aminonicotinamide or fasted, no treatment group; 2) no addition compared to KCN or xylitol.

(Fig. 1; Table 1). If 7-ethoxycoumarin metabolism was regulated predominantly by the activity of cytochrome P-450, one would not expect such differences between enzyme content and rates measured in the perfused liver. Two possibilities could explain these data. First, the isoenzyme pattern in normal rats has not been characterized fully (21); therefore, total activity of cytochrome P-450 isoenzymes metabolizing 7-ethoxycoumarin could be similar in both regions of the liver lobule. Alternatively, the supply of NADPH rather than mixed function oxidase components could be limiting for 7-ethoxycoumarin *O*-deethylation. This hypothesis is supported by the observation that xylitol, a sugar which increases NADPH supply (6), stimulated 7-ethoxycoumarin *O*-deethylation in both regions of the liver lobule in livers from fasted, normal rats (Fig. 3).

Pentose cycle-dependent rates of mixed function oxidation. The role of the pentose cycle in the supply of NADPH for mixed function oxidation was evaluated by using 6-aminonicotinamide treatment and fasting. Fasting depletes glycogen and glucose 6-phosphate, substrates for the pentose cycle (22). 6-Aminonicotinamide is metabolized into the 6-amino analog of $NADP^+$ which inhibits 6-phosphogluconate dehydrogenase (23). Both

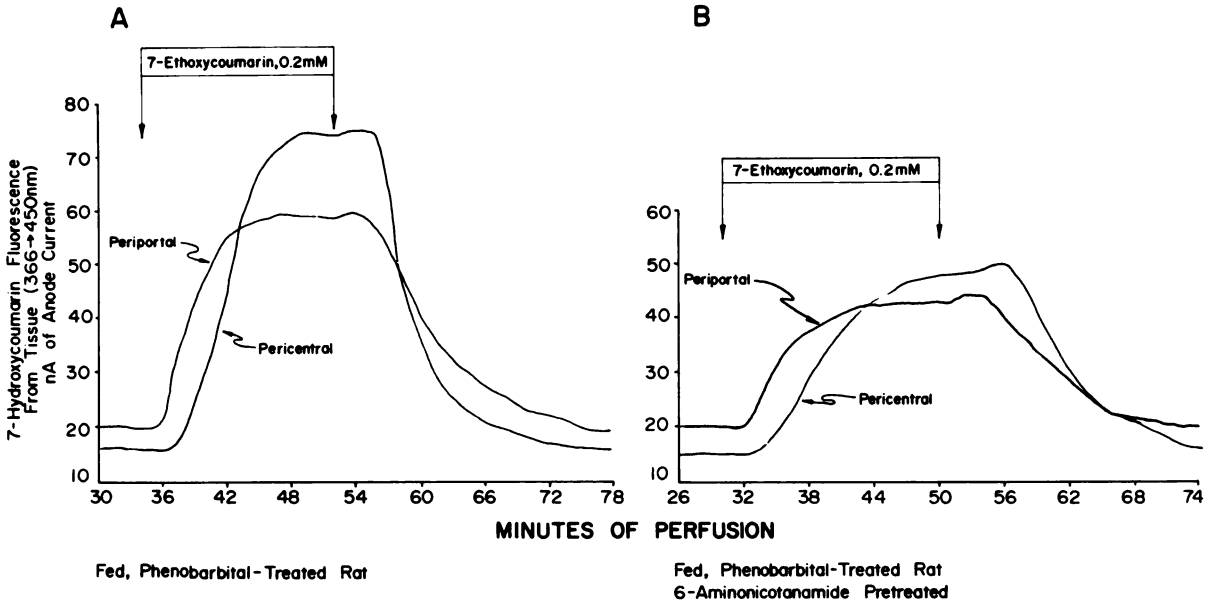

FIG. 2. *Effect of phenobarbital treatment on 7-hydroxycoumarin fluorescence from periportal and pericentral regions of the liver lobule following the addition of 7-ethoxycoumarin*

Rats received phenobarbital in their drinking water for 1–2 weeks (A). Some phenobarbital-treated rats (B) received 70 mg/kg of 6-aminonicotinamide by intraperitoneal injection 4 hr prior to perfusion. 7-Ethoxycoumarin (0.2 mM) was added at the times indicated by the horizontal bars and vertical arrows. Other conditions were as in Fig. 1.

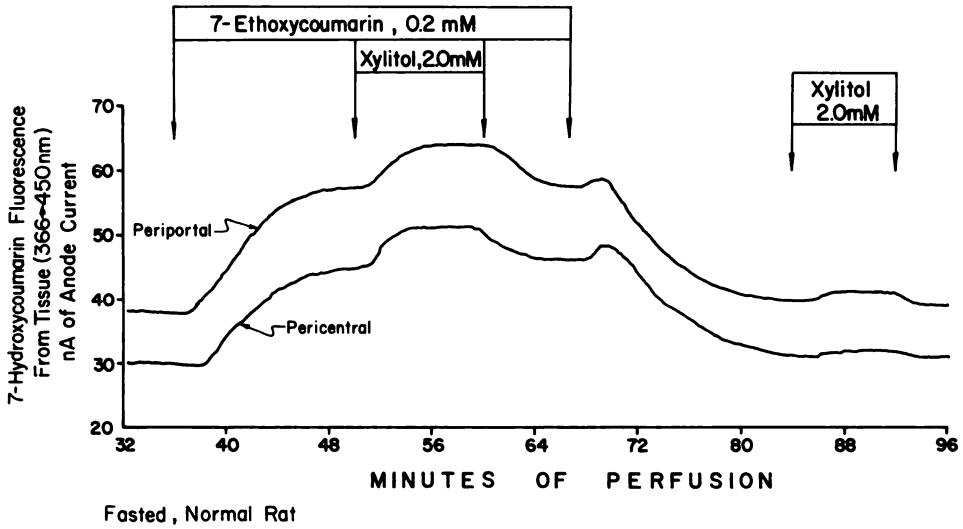

FIG. 3. *Effect of xylitol on 7-hydroxycoumarin fluorescence in periportal and pericentral regions of the liver lobule*

7-Ethoxycoumarin (0.2 mM) and xylitol (2 mM) were infused into the liver of a fasted, normal rat at the times indicated by the horizontal bars and vertical arrows. Other conditions were as in Fig. 1.

of these treatments decreased rates of NADPH generation via the pentose cycle by greater than 90% (18). When the pentose cycle was inhibited, mixed function oxidation tended to decrease from approximately 1.2 to 0.9 μmol/g/hr in both regions of the liver lobule in normal rats (Table 1). When xylitol was added, rates of 7-hydroxycoumarin metabolism were increased significantly by 0.3 μmol/g/hr (Table 1). Thus, it is concluded that the pentose cycle supplies about 0.3 μmol of NADPH/g/hr for 7-ethoxycoumarin *O*-deethylation in both regions of the liver lobule in livers from normal rats. In contrast, the pentose cycle supplied NADPH for monooxygenation at rates of 1.5 and 3.6 μmol/g/hr in periportal and pericentral regions, respectively, in livers from phenobarbital-treated rats (Fig. 4).

Rates of pentose cycle activity are regulated by substrate supply, enzyme activity, and the $NADP^+$/NADPH ratio (24). Glucose 6-phosphate concentrations are above the K_m for glucose-6-phosphate dehydrogenase in both regions of the liver lobule (25) and are unlikely to be the limiting factor for NADPH generation. Smith and Wills (10) reported that maximal glucose-6-phosphate dehydrogenase activity and pentose cycle-dependent NADPH generation were twice as great in pericentral as in periportal regions of the liver lobule *in vitro*. In contrast, rates of pentose cycle-dependent 7-hydroxycoumarin

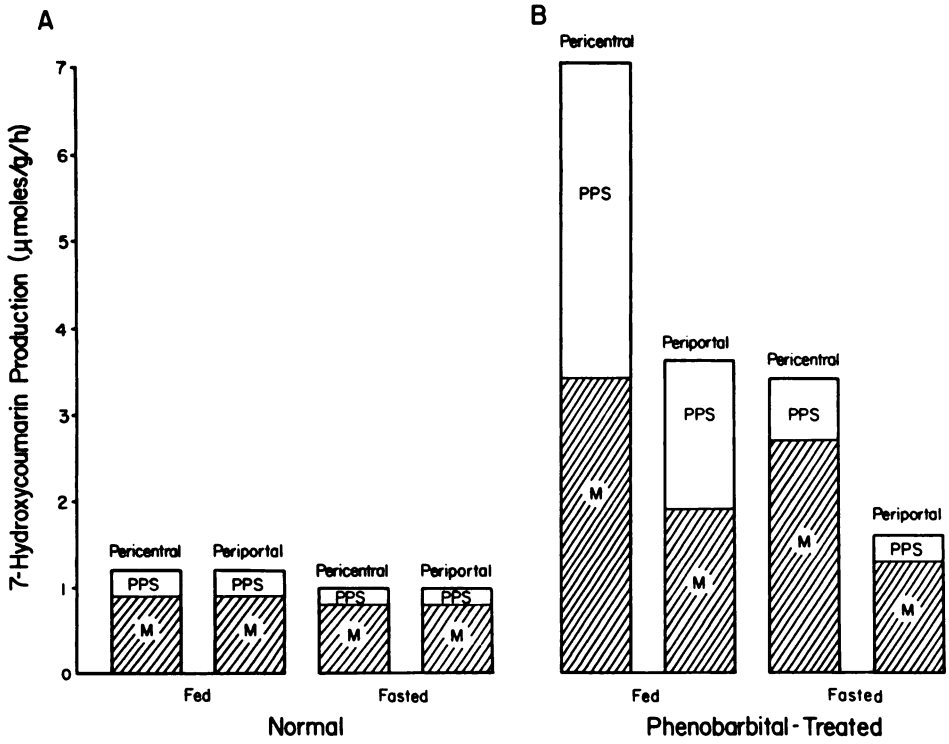

FIG. 4. *Sources of reducing equivalents for mixed function oxidation in periportal and pericentral regions of livers from normal (A) and phenobarbital-treated (B) rats*

Rates of NADPH generation by the pentose cycle (*PPS*) and mitochondria (*M*) for mixed function oxidation were determined by summing the quantitative decrease in local rates of 7-hydroxycoumarin production (Table 1) following inhibition of mixed function oxidation with cyanide and 6-aminonicotinamide.

production measured noninvasively were identical in both regions of the lobule in livers from normal rats in this study (Fig. 4; Table 1). One must be cautious in the interpretation of Smith's and Wills' data since they used somewhat artificial conditions employing liver sections, high concentrations of $NADP^+$, which should not enter intact cells, and added glucose 6-phosphate. Under these conditions, they monitored the conversion of neotetrazolium to formazan as an index of pentose cycle activity. Thus, the experimental protocol used by Smith and Wills most likely assayed maximal glucose-6-phosphate dehydrogenase activity in cells from periportal and pericentral areas, conditions which may not reflect local rates of pentose cycle activity in the intact cell accurately.

There are three alternatives to explain why we detect similar rates of pentose cycle-dependent 7-ethoxycoumarin metabolism in both regions of the liver lobule. First, rates of pentose cycle activity in the perfused liver could be higher in pericentral than in periportal regions as predicted by Smith and Wills yet could go undetected because rates of NADPH utilization by competing pathways were also greater in pericentral areas. Thus, the actual amount of NADPH available for mixed function oxidation could be similar in both regions of the liver lobule. Alternatively, differences in maximal rates of pentose cycle activity in pericentral and periportal areas could go undetected because of equal activity of specific isoenzymes of cytochrome P-450 which metabolize 7-ethoxycoumarin in both regions of the liver lobule in normal rats. Finally, rates of pentose cycle activity could be similar in both regions of the liver lobule due to regulatory factors other than enzyme activity.

Rates of pentose cycle-dependent 7-hydroxycoumarin production were increased 5-fold in periportal areas and 12-fold in pericentral regions following treatment with phenobarbital (Fig. 4). The difference in rates of pentose cycle-dependent mixed function oxidation across the liver lobule may be explained in part by higher cytochrome P-450 contents in pericentral regions (4). This could produce $NADP^+$ at higher rates. Second, glucose-6-phosphate dehydrogenase activity and generation of NADPH for mixed function oxidation was increased markedly in pericentral areas by phenobarbital treatment (10). In the whole liver, phenobarbital treatment nearly doubled rates of NADPH generation via the pentose cycle[4] and increased levels of glucose 6-phosphate, 6-phosphogluconate, and ribulose 5-phosphate (26, 27). Based on our data, we cannot determine which of these alternatives is responsible for different rates of NADPH supply for mixed function oxidation by the pentose cycle; however, it is possible that phenobarbital treatment increases both cofactor supply as well as cytochrome P-450 content in both regions of the liver lobule. Consistent with this possibility, Conway *et al.* (28) observed that treatment of Ah locus-responsive mice with 3-methylcholanthrene increased NADPH turnover as well as cytochrome P-450 content.

[4] R. Scholz, personal communication.

Contribution of mitochondrial NADPH supply to mixed function oxidation. Mitochondrial oxidations also must supply NADPH, since inhibition of pentose cycle activity by fasting or treatment with 6-aminonicotinamide did not suppress rates of 7-hydroxycoumarin production completely (Fig. 4; Table 1). Cyanide, an inhibitor of oxidative phosphorylation, was used to investigate the role of the mitochondria to supply NADPH for monooxygenation (29). Under these conditions, cyanide has no effect on rates of NADPH generation via the pentose cycle (30). Cyanide decreased rates of 7-hydroxycoumarin production by approximately 0.7 μmol/g/hr in both regions of the lobule in livers from normal rats (Table 1). Thus, we conclude that mitochondria supply about 0.7 μmol of NADPH/g/hr for mixed function oxidation of 7-ethoxycoumarin. Based on the decrease in rates of 7-hydroxycoumarin production during cyanide infusion, we conclude that mitochondrial oxidations supply approximately 1.3 and 2.7 μmol of NADPH/g/hr for 7-ethoxycoumarin metabolism in periportal and pericentral regions, respectively (Fig. 4; Table 1).

Very little information exists on the distribution of metabolic pathways involved in the generation and movement of NADPH from the mitochondria into the cytosol in different regions of the liver lobule. Following phenobarbital treatment, rates of NADPH supply by the mitochondria were increased 2- and 4-fold in periportal and pericentral regions, respectively (Fig. 4). This increase in local rates of mixed function oxidation should increase $NADP^+$ supply and could theoretically stimulate mitochondrial NADPH synthesis. Alternatively, phenobarbital treatment could induce specific NADPH-generating enzymes. In support of this concept, the contents of both malic enzyme and isocitrate dehydrogenase were increased 80% by phenobarbital treatment (27).

6-Aminonicotinamide-sensitive rates of monooxygenation were calculated to be 0.3 μmol/g/hr and cyanide-sensitive rates were 0.7 μmol/g/hr in livers from normal rats. Thus, rates of 7-hydroxycoumarin production would be 1.0 μmol/g/hr (0.3 + 0.7) if the pentose cycle and mitochondria were the only sources of reducing equivalents. This value agrees closely with the rates observed in this study (1.2 μmol/g/hr; Table 1) and provides support for the use of inhibitors to quantitate rates of NADPH generation for mixed function oxidation by the pentose cycle and mitochondria in specific zones. Similar calculations apply in livers from phenobarbital-treated rats; however, the decrease in local rates of mixed function oxidation after inhibition of the pentose cycle in fed rats and the decrease in rates during cyanide infusion in fasted rats must be summed. This was necessary since cyanide did not inhibit mixed function oxidation in livers from fed rats most likely because it stimulated NADPH generation by the pentose cycle by its known activation of glycogenolysis via AMP (30, 31).

In these studies, rates of mixed function oxidation were diminished when NADPH generation was inhibited by 6-aminonicotinamide and potassium cyanide, agents which inhibit the pentose cycle and mitochondrial oxidations, respectively. It might appear that this finding was unexpected since absolute rates of NADPH generation via the pentose cycle exceeded rates of mixed function oxidation by at least 4-fold (6, 7). However, it is important to note that a large proportion of NADPH is utilized for pathways other than mixed function oxidation [e.g., fatty acid synthesis (32), the reduction of oxidized glutathione (33), flavoprotein-dependent monooxygenation (34), etc.]. Therefore, it is reasonable to suggest that all NADPH-utilizing systems compete for NADPH and are, therefore, diminished when NADPH synthesis by either the pentose cycle or mitochondrial oxidations is inhibited. Although maximal rates of NADPH synthesis via the mitochondria and pentose cycle exceed rates of monooxygenation greatly, 7-ethoxycoumarin metabolism declines because it is only one pathway competing with many other enzymes for NADPH in the cell. Thus, these studies have utilized the mixed function oxidase pathway as a simple indicator to monitor for the first time cellular rates of NADPH generation in periportal and pericentral regions of the liver lobule.

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