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A Fluorometric Method To Measure Sublobular Rates of Mixed-Function Oxidation in the Hemoglobin-Free Perfused Rat Liver

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SUMMARY

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A new fluorometric method has been developed to measure the relative rates of the 7-ethoxycoumarin *O*-deethylase activities in periportal and pericentral regions of the liver lobule. The method utilizes a pair of micro-light guides constructed from two strands of 80 μm-diameter glass optical fibers. The micro-light guides are placed in periportal and pericentral regions on the surface of the perfused liver. The increase in tissue fluorescence due to 7-hydroxycoumarin formation from 7-ethoxycoumarin is measured in periportal and pericentral regions by illuminating tissue with light at 360 ± 50 nm and measuring fluorescence at 450 ± 50 nm. Since the steady-state tissue fluorescence due to 7-hydroxycoumarin monitored with a large light guide was found to be directly proportional to the steady-state rate of 7-ethoxycoumarin *O*-deethylation, the sublobular fluorescence of 7-hydroxycoumarin measured by micro-light guides following infusion of 7-ethoxycoumarin allows one to estimate sublobular rates of the mixed-function oxidation of 7-ethoxycoumarin. The results indicate that the rate of 7-ethoxycoumarin *O*-deethylation is approximately twice as large in pericentral regions as in periportal areas of the liver.

Immunohistochemical (1) and microspectrophotometric (2) studies have revealed that the concentration of the cytochrome P-450 induced by phenobarbital is greater in pericentral than in periportal regions of the liver lobule. The cytochrome P-450 induced by 3-methylcholanthrene, however, was more uniformly distributred over the liver lobule (1). Several hepatotoxic agents which require cytochrome P-450 for activation (e.g., acetaminophen, carbon tetrachloride) are known to cause selective injury to pericentral regions (for a recent review see ref. 3). However, enzymatic distribution in tissue alone may not determine local rates of mixed-function oxidation, since the supply of the cofactor NADPH may be rate-limiting for mixed-function oxidation in whole cells (4). Therefore, we have developed a method to determine local rates of mixed-function oxidation in periportal and pericentral regions of the liver lobule noninvasively under physiological conditions. This method utilizes a micro-light guide (5–7) constructed from two strands of 80-μm diameter optical fiber. When placed on identified sublobular regions of the liver surface, this light guide permits continuous fluorometric observation of 7-ethoxycoumarin *O*-deethylation catalyzed by the mixed-function oxidase system (8, 9). 7-Ethoxycoumarin is practically nonfluorescent under the optical conditions employed but is converted to the highly fluorescent intermediate, 7-ethoxycoumarin, by mixed-function oxidation (8). In the perfused liver, this *O*-deethylated derivative is subsequently conjugated via sulfation and glucuronidation pathways to form products which are nonfluorescent (9). Detectable amounts of 7-hydroxycoumarin accumulate in liver tissue during *O*-deethylation of 7-ethoxycoumarin in direct proportion to the rate of *O*-deethylation (Figs. 1 and 2).

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 (7). Since the diameter of these sublobular regions is several-fold greater than the tip of the micro-light guide (170 μm), it can be placed on either region to measure local fluorescence changes.

The basic experimental design to determine sublobular rates of mixed-function oxidation is as follows: (a) the tissue fluorescence increase caused by 7-hydroxycoumarin following infusion of 7-ethoxycoumarin is mea-

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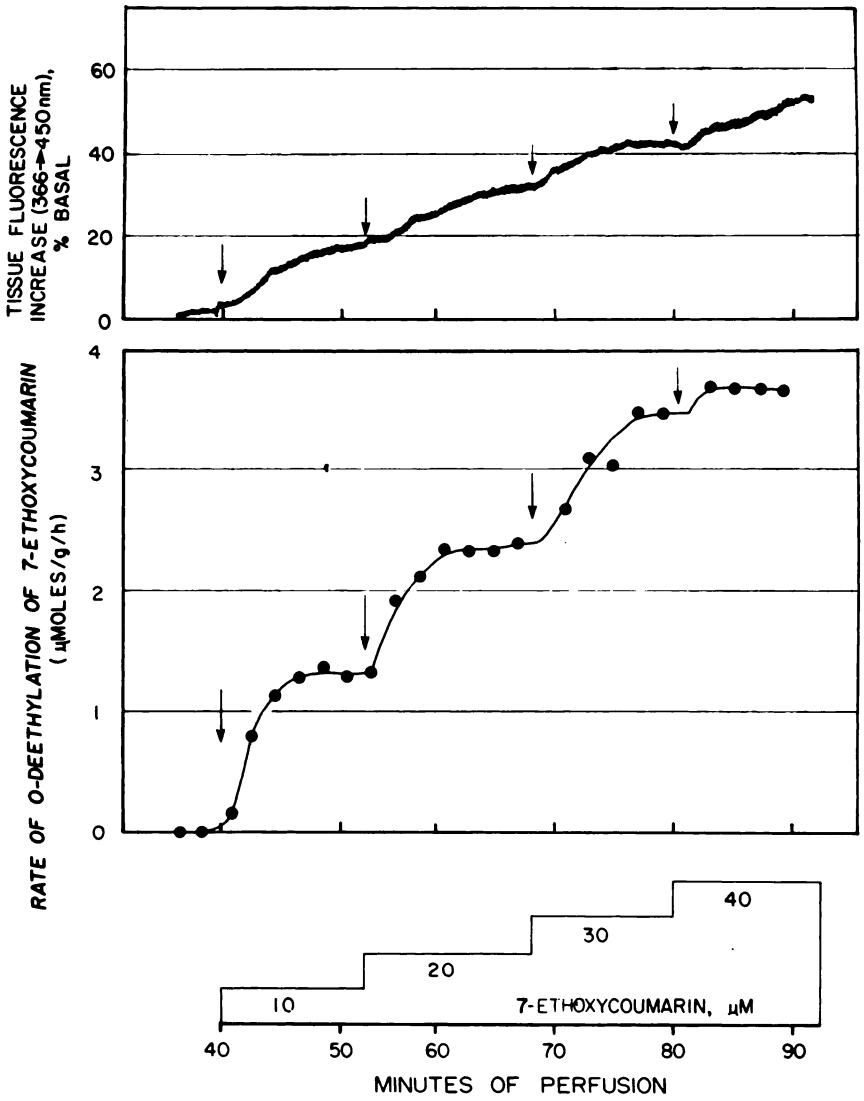

FIG. 1. *Effect of 7-ethoxycoumarin concentration on the rate of O-deethylation of 7-ethoxycoumarin and on tissue fluorescence (366 → 450 nm)*

The liver from a Sprague-Dawley female rat (228 g) treated with sodium phenobarbital (1 mg/ml of drinking water) for 5 weeks was perfused with Krebs-Henseleit bicarbonate buffer as described elsewhere (10). Infusion of 7-ethoxycoumarin is indicated by horizontal bars and *arrows*. Glucuronide and sulfate conjugates of 7-hydroxycoumarin were measured in the perfusate by incubating samples with β-glucuronidase containing sulfatase activity (Sigma Chemical Company, St. Louis, Mo.) 7-hydroxycoumarin liberated under these conditions was measured fluorometrically above 400 nm following excitation at 366 nm, employing an Eppendorf photometer equipped with a fluorescence attachment. The rate of *O*-deethylation of 7-ethoxycoumarin in micromoles per gram per hour was calculated by taking the flow rate and liver wet weight into consideration. The tissue fluorescence was measured with a Schott light guide with a tip diameter of 2 mm placed on the surface of the left lateral lobe of the liver. The excitation light (360 ± 50 nm) was from a 100-W mercury arc lamp (Illuminations Industries, Inc., Sunnyvale, Calif.) filtered with a Corning glass filter No. 5840. The emitted light (450 ± 50 nm) was detected with a photomultiplier (EMI, type 9824B) guarded with Kodak Wratten gelatin filters, Nos. 2C and 47. In this particular experiment, the output voltage of the photomultiplier was adjusted to give an anode current of 66 namp when the light guide was placed on the liver surface prior to infusion of 7-ethoxycoumarin.

sured from the liver surface with a large-tip light guide (2 mm in diameter) and is correlated with the rate of *O*-deethylation of 7-ethoxycoumarin; (b) by using two pairs of 170-μm tip micro-light guides, the fluorescence increase due to 7-ethoxycoumarin formation is measured simultaneously in both periportal and pericentral regions of the liver lobule; (c) the local fluorescence increments are then converted into local rates of *O*-deethylation employing the correlation between rates of mixed-function oxidation and tissue fluorescence increase established in Step a.

The correlation between the tissue fluorescence increase and the rate of *O*-deethylation obtained with a large light guide involves fluorescence signals originating from many liver lobules. We assume that this correlation can be used as a calibration curve to convert fluorescence readings measured with the micro-light guide into local rates of mixed-function oxidation.

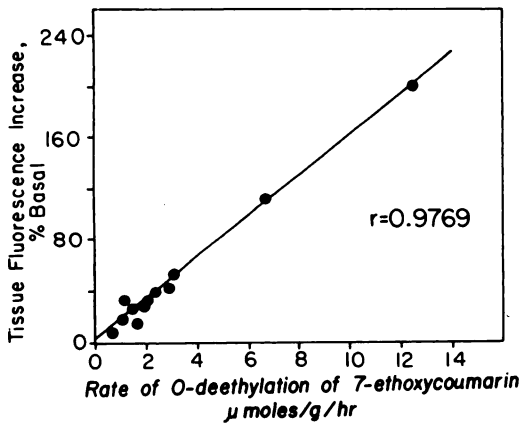

FIG. 2. *Correlation between tissue fluorescence (366 → 450 nm) increase and the rate of O-deethylation of 7-ethoxycoumarin at steady state*

Conditions were similar to those described in legend to Fig. 1, except that the concentration of 7-ethoxycoumarin infused was varied from 30 to 100 μM ($n = 6$).

When 7-ethoxycoumarin was infused into the liver in increasing concentrations, the steady-state tissue fluorescence due to 7-hydroxycoumarin (366 nm → 450 nm) measured with a large light guide increased in a stepwise fashion, as did the rate of *O*-deethylation of 7-ethoxycoumarin (Fig. 1). A linear correlation between the tissue fluorescence increase due to 7-hydroxycoumarin formation and the rate of *O*-deethylation of 7-ethoxycoumarin in the liver was observed (Fig. 2).

After placing two micro-light guides on the periportal and pericentral regions of the liver lobule, 7-ethoxycoumarin was infused. Fluorescence began to increase first in the periportal region and then in the pericentral area (Fig. 3). The maximal fluorescence increase, expressed as a percentage of the basal fluorescence, was significantly larger in the pericentral than in the periportal region (Fig. 3; Table 1). Following termination of 7-ethoxycoumarin infusion, surface fluorescence in both regions returned slowly to the baseline. The average fluorescence increase, calculated as the percentage of the basal fluo-

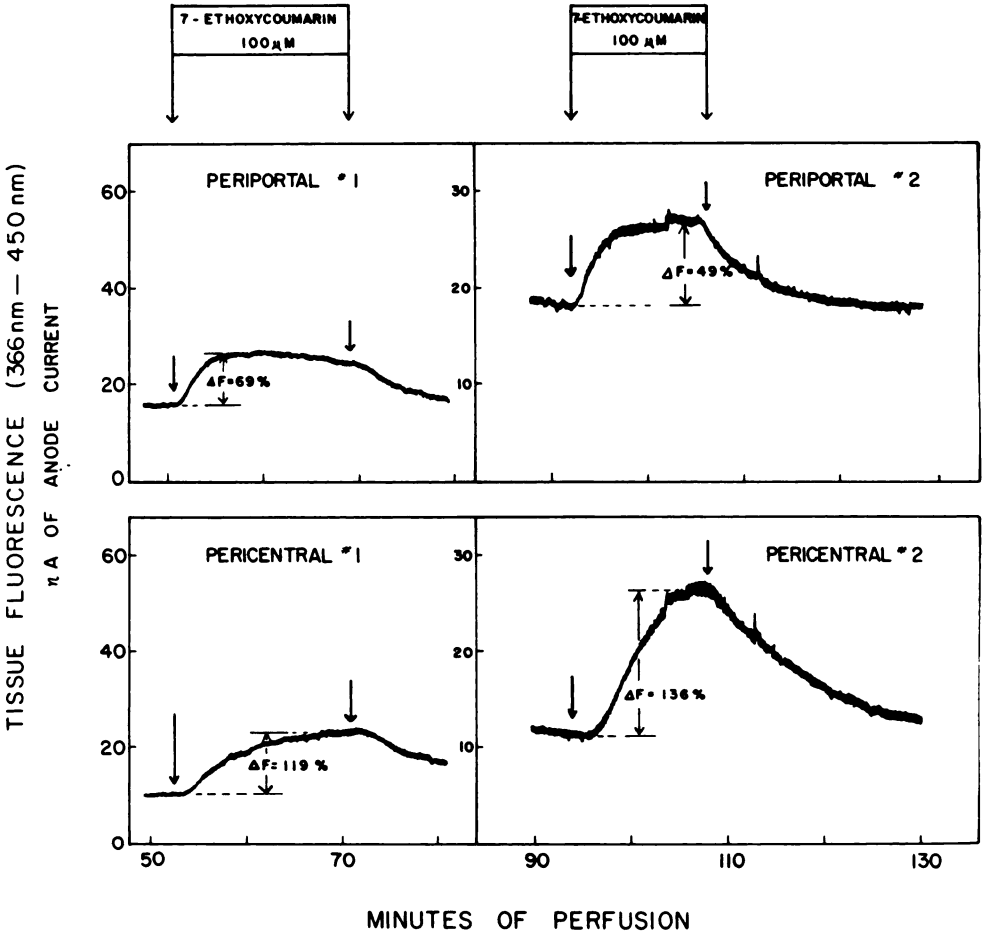

FIG. 3. *Fluorescence increase (366 → 450 nm) upon infusion of 7-ethoxycoumarin in periportal and pericentral regions of the liver lobule*

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 the liver. 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. The infusion of 7-ethoxycoumarin is indicated by the horizontal bars and *arrows*. The fluorescence increments are expressed as a percentage of the basal fluorescence intensity of the liver before the infusion of 7-ethoxycoumarin was initiated. The figure depicts two consecutive measurements (#*1* and #*2*) from one liver. Other details of the experiment are similar to those given in the legend to Fig. 1.

TABLE 1

Periportal and pericentral rates of mixed-function oxidation of 7-ethoxycoumarin by perfused rat liver

Experiments were carried out as described in the legend to Fig. 3. Rats (260–310 g) were pretreated with phenobarbital for 5–6 weeks prior to experiments. The lower basal fluorescence in the pericentral area relative to the periportal region is probably due to fluorescence quenching by higher concentration of cytochrome P-450 in the pericentral area. The anode current (nanoamperes) from the photomultiplier (EMI Type 9824B) was measured at an output voltage of 750–850 V. Data represent mean ± standard error of the mean (five measurements from four livers).

	Sublobular region	
	Periportal	Pericentral
Basal tissue fluorescence (namp) (366 → 450 nm)	37 ± 3	24 ± 3[a]
Tissue fluorescence increase upon infusion of 7-ethoxycoumarin (namp)	30 ± 6	37 ± 6
Tissue fluorescence increase as % of basal fluorescence	79 ± 14	148 ± 11[a]
Local rate of 7-ethoxycoumarin *O*-deethylation (μmoles/g/hr)	4.7 ± 0.9	9.0 ± 0.7[a]

[a] $p < 0.01$ when data from periportal region were compared with those from the pericentral region.

rescence, was found to be approximately twice as large in the pericentral region as in the periportal region (Table 1). By using the correlation in Fig. 2 as a calibration curve, the rate of mixed-function oxidation in the periportal and pericentral regions was estimated. Rates of mixed-function oxidation obtained in this manner are about 2-fold greater in the pericentral region than in the periportal region (Table 1). However, this result does not address the question of whether or not the NADPH supply is rate-limiting for mixed-function oxidation in periportal or pericentral tissue. Xylitol, which increases the supply of NADPH and stimulates mixed-function oxidation of *p*-nitroanisole (11), increased the fluorescence signal due to 7-ethoxycoumarin in pericentral but not in periportal tissue by about 100% (data not shown). This suggests that NADPH supply may rate-limit mixed-function oxidation in pericentral but not in periportal regions of the liver lobule.

In conclusion, we have described a fluorometric method to measure sublobular rates of mixed-function oxidation of 7-ethoxycoumarin. With this technique, we have obtained the first direct physical evidence that the rate of mixed-function oxidation in the pericentral regions is approximately twice as large as in the periportal area. This finding is consistent with the histochemical (1) and microspectrophotometric (2) findings that cytochrome P-450 is concentrated more in the pericentral than in the periportal regions and may provide a possible explanation for the pericentral hepatotoxicity of many drugs (3). In addition, this paper describes a general type of experiment which may be applied to the determination of rates of a wide variety of metabolic reactions in sublobular regions of the liver if appropriate fluorochromes can be found.

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