0026-895X/86/010088-09$02.00/0

MOLECULAR PHARMACOLOGY, 29:88-96

A New Method to Study Glutathione Adduct Formation in Periportal and Pericentral Regions of the Liver Lobule by Micro-reflectance Spectrophotometry

CRAIG HARRIS[1,2] and RONALD G. THURMAN

Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Received March 22, 1985; Accepted October 2, 1985

SUMMARY

A method was developed to measure the formation of glutathione adducts of 1-chloro-2,4-dinitrobenzene (CDNB) and 2,4-dichloro-1-nitrobenzene (DCNB) in periportal and pericentral regions of the liver lobule in the isolated perfused rat liver by surface reflectance spectrophotometry. Conjugates of DCNB and CDNB are released from livers of normal and phenobarbital-treated rats during perfusion in either the anterograde or the retrograde direction at maximal rates around 13–15 μmol/g/hr. The formation of *S*-(1-chloro-4-nitrophenyl)-glutathione and *S*-(2,4-dinitrophenyl)-glutathione by the liver decreased the amount of 366-nm light reflected from the liver surface detected with a large-tipped (2 mm) fiberoptic light guide. Initial rates of decrease in reflected light correlated highly with maximal rates of conjugate formation by the liver. Subsequently, micro-light guides were placed on periportal and pericentral regions of the liver lobule. Rates of glutathione adduct formation were calculated from the proportion of the total change in rate of reflected 366-nm light which occurred in each region and the overall rate of product formation by the liver. Changes in the reflectance signal require reduced glutathione (GSH) and were shown to originate from intracellular conjugate formation and not from adducts in the bile canaliculus. Livers from normal rats produced conjugated products from DCNB (100 μM) at maximal rates of 14 and 15 μmol/g/hr in periportal and pericentral regions of the liver lobule, respectively. With CDNB as substrate, changes in reflected light at 366 nm were detected nearly exclusively in periportal regions of the lobule in livers from normal rats. In sharp contrast, CDNB and DCNB were conjugated exclusively in periportal regions of the lobule at rates of 21–22 μmol/g/hr in livers from phenobarbital-treated rats (i.e., the reflectance signal was not altered by these substrates in pericentral areas). When CDNB and DCNB were infused into livers from phenobarbital-treated rats perfused in the retrograde direction, decreases in reflected light at 366 nm were detected initially in pericentral areas followed in about 12 min by changes in periportal regions. Maximal rates of adduct formation in both regions reached 25 μmol/g/hr during perfusion in the retrograde direction. Thus, pericentral regions indeed possess the capacity to conjugate both CDNB and DCNB. When glutathione synthesis was inhibited with L-buthionine sulfoximine treatment (6 mmol/kg), which partially depletes GSH, CDNB was conjugated in both periportal and pericentral regions of the liver lobule in livers from phenobarbital-treated rats. It is concluded that factors such as substrate uptake, intracellular binding, and GSH availability play important roles in determining the rate of GSH adduct formation in different regions of the liver lobule.

GSH protects cells against damage caused by peroxides, free radicals, and epoxides formed from the oxidation of unsaturated hydrocarbons (1–3). Intracellular GSH concentrations can be as high as 10 mM in mammalian livers but may undergo rapid depletion during conditions where reactive substrates for glutathione-dependent enzymes are generated faster than the cells' capacity to synthesize new glutathione. The hepatotoxicity due to acetaminophen, which is characterized by covalent binding of its reactive metabolites to macromolecules and cellular necrosis, is increased by depletion of GSH (4). The damage produced by acetaminophen can be largely prevented by the addition of cysteine, usually in the form of γ-glutamylcysteine or thiazolidine, which stimulates glutathione synthesis (4, 5a). Acetaminophen is one of a number of hepatotoxins which damages only those hepatocytes surrounding the central vein (4). Other hepatotoxins known to involve GSH in their detoxication pathways also cause selective damage such as the periportal and pericentral necrosis caused by allyl alcohol and carbon tetrachloride, respectively (5, 6). The reasons for zonal hepatotoxicity are not well understood, although the heterogeneous distribution of activating and detoxicating enzymes and glutathione in various zones of the liver lobule could be important factors. Immunohistochemical techniques have been used

This work was supported, in part, by Grant ES-02759 from the National Institute of Environmental Health Sciences.

[1] Graduate of the Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC 27514.

[2] Present address: Department of Pharmacology, University of Washington, Seattle, WA 98195.

ABBREVIATIONS: GSH, reduced glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 2,4-dichloro-1-nitrobenzene.

to show that cytochrome P-450 isozymes, epoxide hydrolases, glutathione *S*-transferases, and other enzymes important in biotransformation of xenobiotics are distributed differentially in periportal and pericentral regions of the liver lobule (7–9). Several reports on the distribution of ligandin and the glutathione *S*-transferases in different regions of the liver are contradictory (9–12). These reported differences may be due to differences in isozymes used for antibody preparation or species differences; however, histochemical distribution of enzymes does not take into account substrate and GSH supply. It is therefore important to study GSH adduct formation directly in systems with intact cellular structure where substrate and cofactor supply as well as enzyme activity could influence the rate of the reaction.

Recent progress has been made using miniature fiberoptic techniques to measure activation and detoxication reactions in periportal and pericentral regions of the liver lobule noninvasively from the surface of the isolated perfused rat liver (13–15). Such measurements allow the determination of flux rates in metabolic pathways in intact cells which are dependent on substrate and cofactor supply as well as enzyme activity. These studies have employed nonfluorescent substrates such as 7-ethoxycoumarin, which is metabolized in the cell to highly fluorescent 7-hydroxycoumarin (15). Reactions involving GSH, however, have not been studied using micro-fiberoptic techniques, due partly to the inavailability of suitable substrates. The spectral properties of a pair of well characterized glutathione *S*-transferase substrates, CDNB and DCNB, could theoretically be used to measure rates of glutathione conjugation in periportal and pericentral regions of the liver lobule. CDNB and DCNB undergo a red spectral shift (304 nm–340 nm) upon conjugation with glutathione.

This report describes the development of methods employing fiberoptics to measure glutathione conjugation in periportal and pericentral regions of the isolated perfused rat liver by surface reflectance spectrophotometry.

Materials and Methods

Chemicals. CDNB and DCNB were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were recrystallized from acetone:water (1:2) before use. Bovine serum albumin (fraction V) was purchased from Sigma Chemical Co. (St. Louis, MO) and was made essentially fatty acid free by charcoal filtration using Norit A (Fisher Chemical Co., Fair Lawn, NJ) according to the method of Chen (16). L-Buthionine sulfoximine was the generous gift of Dr. Alton Meister, Cornell Medical College (New York, NY). All other reagents were from common sources.

S-(2,4-Dinitrophenyl)-glutathione and *S*-(2-chloro-4-nitrophenyl)-glutathione were synthesized chemically from GSH (Sigma) according to the method of Lee *et al.* (17) from CDNB and DCNB, respectively. Each product migrated as a single spot on thin layer chromatography employing techniques described by Ryan *et al.* (18).

Animals and perfusion. Female Sprague-Dawley rats (200–350 g) were used in all perfusion experiments. All animals were allowed free access to food and water. Animals treated with phenobarbital received sodium phenobarbital (1 mg/ml) in the drinking water for 6–20 days. L-Buthionine sulfoximine (6 mmol/kg) was administered intraperitoneally in saline 3 hr before perfusion (19). Intracellular glutathione was depleted by intraperitoneal injection of diethyl maleate (0.7 g/kg) in saline 1 hr before perfusion (20).

Rat livers were perfused with Krebs-Henseleit-bicarbonate buffer (pH 7.6, 37°) in a nonrecirculating system as described previously (21). Substrates were dissolved in acetone (200 mM) and stirred into perfusate containing 0.0125% albumin vehicle for 20 min to allow the acetone to evaporate. Perfusion medium was saturated with 95% O_2:5% CO_2 before infusion into the liver with an oxygenator constructed from Silastic tubing as described in detail elsewhere (22). Oxygen concentration in the effluent perfusate was monitored with a Clark-type oxygen electrode. Rates of oxygen uptake were calculated from the arterial-venous differences in O_2 concentration based on flow rate and liver wet weight. Total rates of *S*-(2,4-dinitrophenyl)-glutathione and *S*-(2-chloro-4-nitrophenyl)-glutathione formation were monitored continuously in the effluent perfusate at 366 nm with an Eppendorf spectrophotometer fitted with a flow-cell cuvette. In some experiments the bile duct was cannulated with PE-10 tubing and biliary product was quantitated spectrophotometrically.

Fiberoptics. Decreases in 366-nm light reflected from the liver surface were monitored with a commercial large-tipped light guide containing approximately 100 fibers and having a tip diameter of 2 mm. This instrument monitors an area of the liver surface which contains several lobules. The liver surface was illuminated with 366-nm light from a mercury arc lamp, and reflected light was detected with a photomultiplier and was amplified and recorded as described previously (23).

Micro-light guides, having a maximum tip diameter of 300 μm, were constructed to maximize the detection of reflectance changes from periportal and pericentral regions of the liver lobule. Glass fibers approximately 70 cm in length with diameters of 80 μm were used to conduct light from the illumination source to and from the surface of the liver. Fibers were drawn through stainless steel tubing with "5-minute" epoxy to secure and protect ends from breakage. All surface ends of the glass fibers were polished to an even lustre using jewelers rouge and gamma aluminum. Pliable plastic tubing was drawn over the fibers for protection. A single 80-μm fiber directs 366 nm of light from the illumination source to the liver surface while an array of six 80-μm fibers collects the reflected light.

Spectral analysis and *in vitro* glutathione conjugation. Absorbance spectra of CDNB, DCNB, and their synthetically prepared glutathione adducts were determined with an Aminco DW-2 spectrophotometer. All samples contained either CDNB, DCNB, or the respective glutathione adduct in Krebs-Henseleit bicarbonate buffer (pH 7.4, 25°) containing 0.0125% bovine serum albumin at a concentration of 200 μM.

Rates of glutathione adduct formation were determined *in vitro* with DCNB and CDNB as substrates. Reaction mixtures contained Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°), 0.0125% bovine serum albumin, and CDNB or DCNB (100 μM) in a total volume of 2.9 ml. Reactions were initiated with the addition of 0.1 ml of glutathione (0.1–3.0 mM). Product formation was recorded for 3 min at 366 nm in an Eppendorf model 1101 M spectrophotometer. Reaction mixtures designed to approximate cellular levels of glutathione *S*-transferases contained 0.1 μg of partially purified glutathione *S*-transferase (Sigma).

Results

Absorbance spectra of CDNB, DCNB, and their glutathione adducts. CDNB and DCNB each have a single maximal absorbance peak at 250 and 253 nm, respectively. Synthetic glutathione adducts of CDNB, *S*-(2,4-dinitrophenyl)-glutathione, and DCNB, *S*-(2-chloro-4-nitrophenyl)-glutathione, however, have two absorbance peaks. The first maximum fell between 200 and 280 nm and was characteristic of the spectra obtained with GSH alone. The peak unique to the glutathione adducts absorbed maximally at 338 nm. Conjugated product formation was measured spectrophotometrically at 366 nm where absorbance of the parent compound was insignificant. The estimated extinction coefficients at 366 nm for CDNB and DCNB were 6.6 and 5.9 mM^{-1} cm^{-1}, respectively. Samples of bile collected during infusion of 100 μM CDNB had absorption spectra indistinguishable from those obtained with synthetic *S*-(2,4-dinitrophenyl)-glutathione.

Effects of glutathione concentration on enzymatic and nonenzymatic rates of glutathione adduct formation *in vitro*. Large differences in binding, catalytic properties, and rates of nonenzymatic product formation between CDNB and DCNB have been reported (24–27); therefore, we examined reaction rates under conditions approximating those encountered during perfusion. Incubation mixtures containing CDNB or DCNB (100 μM) and variable concentrations of glutathione (0.1–3.0 mM) formed adducts at maximal rates of 0.75 nmol/min. Nonenzymatic rates were slightly higher for CDNB at glutathione concentrations of 0.1–0.5 mM (Fig. 1). When partially purified glutathione *S*-transferase (0.1 μg) from rat liver was added, glutathione adducts were formed at maximal rates of 2.7 nmol/min with both substrates (Fig. 1).

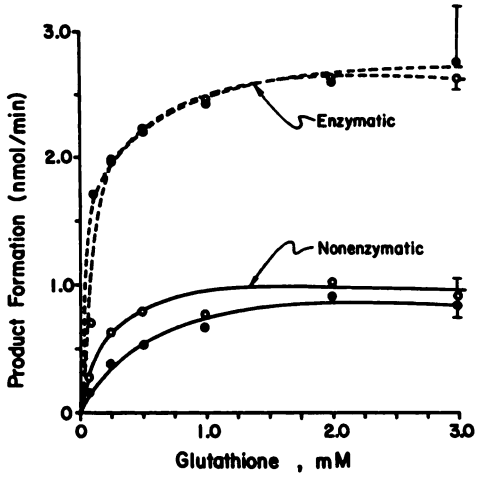

Fig. 1. Comparison of enzymatic and nonenzymatic rates of glutathione adduct formation with CDNB and DCNB *in vitro*. Incubations to determine nonenzymatic rates were performed in mixtures containing Krebs-Henseleit bicarbonate buffer (pH 7.6), 0.0125% bovine serum albumin, 100 μM CDNB (○), or DCNB (●) in a volume of 2.9 ml. Reactions were initiated by the addition of 0.1–3.0 mM glutathione in 0.1 ml of buffer. Product formation was monitored for at least 3 min at 366 nm. Some reaction mixtures (– – –) contained 0.1 μg of partially purified glutathione *S*-tranferase from rat liver.

Glutathione adduct formation in the isolated perfused rat liver. Oxygen uptake increased rapidly an average of 14 μmol/g/hr (n = 13) upon infusion of substrate (Fig. 2A). This increase was transient and returned nearly to baseline levels with a time course similar to the decrease in the rate of glutathione adduct formation. The increases in rates of oxygen uptake produced by the two substrates were not significantly different. *S*-(2,4-dinitrophenyl)-glutathione and *S*-(2-chloro-4-nitrophenyl)-glutathione were formed at maximal rates of 10–13 μmol/g/hr in the isolated perfused rat liver during infusion of 100 μM CDNB or DCNB (Fig. 2B). After initiation of infusion of substrate, rates of product formation increased until a peak was reached in approximately 20 min. Maximal rates of product formation were not maintained but declined rapidly even though substrate was infused continuously. After approximately 70 min of substrate infusion, a new, lower steady state rate of glutathione adduct formation of about 2.0 μmol product/g/hr was reached (Fig. 2B).

With our standard perfusion, biliary and sinusoidal effluents were allowed to mix freely. To estimate the transport of adduct into bile, the bile duct was cannulated and glutathione conjugates were quantitated (Fig. 2A). Rates of bile flow were 75 μl/g/hr in livers from phenobarbital-treated rats and were increased to 130–150 μl/g/hr following infusion of CDNB (100 μM). Glutathione conjugates were transported nearly exclusively into the bile during the first 5 min of substrate infusion and, although continued infusion resulted in conjugates being released into the perfusate, biliary concentrations in excess of

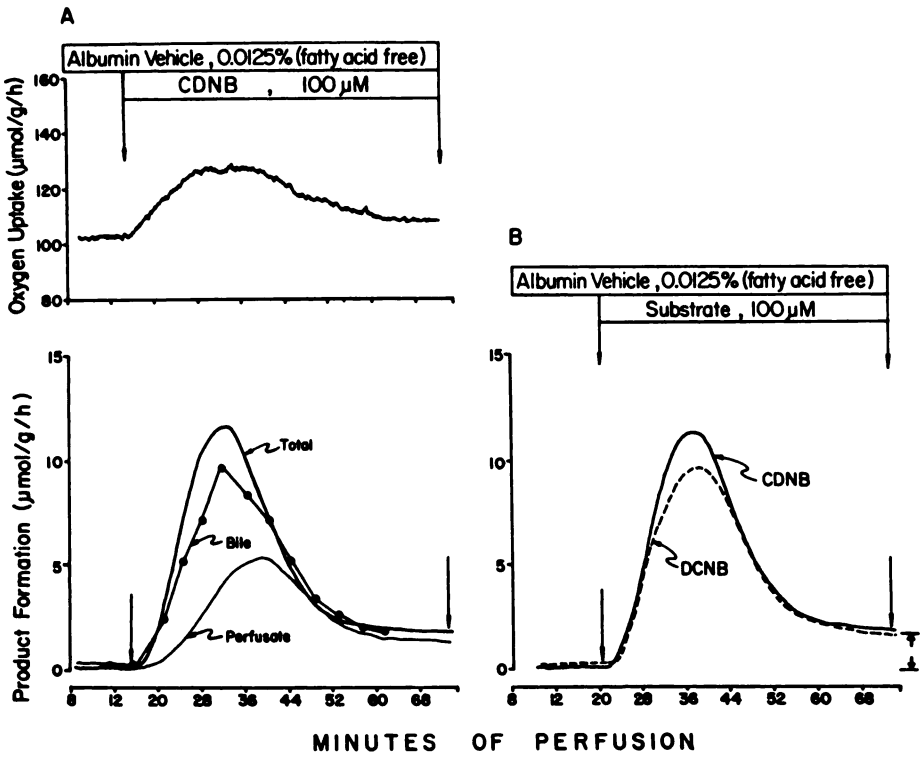

Fig. 2. Glutathione adduct formation and oxygen uptake by the isolated perfused rat liver. The effect of CDNB on oxygen uptake is depicted in A (*upper panel*). Total glutathione adduct formation is shown (A, *bottom panel*), under conditions where bile and sinusoidal effluent were allowed to mix freely (*total*) and where bile (—●—) and effluent perfusate (*bottom solid line*) were analyzed separately. A comparison of glutathione adduct formation in effluent perfusate between CDNB (——) and DCNB (– – –) is shown in B. Steady state rates of GSH adduct formation were reached approximately 50 min after the onset of substrate infusion.

54 mM were measured 18 min after the onset of substrate infusion (Fig. 2A). This represented about one-half of the total product formed. Perfusion with CDNB for longer than 90 min resulted in complete choleostasis.

Glutathione conjugation and reflectance spectrophotometry measured with a large-tipped light guide. Decreases in surface reflectance at 366 nm were initially recorded during constant infusion of substrate using a large-tipped light guide. Because CDNB adducts were washed out of the liver very slowly in these experiments, substrates were administered at various concentrations as short pulses (6 min) in order to determine the relationship between glutathione adduct formation by the liver and rates of decrease in surface reflectance at 366 nm. A typical experiment with CDNB as substrate is depicted in Fig. 3. Oxygen uptake was also increased in a stepwise manner by CDNB; maximal increases were about 24 μmol/g/hr following infusion of 100 μM CDNB (Fig. 3). Glutathione adduct formation ranged from 0.3 μmol product/g/hr at 5 μM CDNB to as high as 11.0 μmol product/g/hr following the infusion of 100 μM CDNB or DCNB (Fig. 3, *middle panel*). The rate of decrease of reflected light at 366 nm was proportional to substrate concentrations infused (Fig. 3, *bottom panel*). The initial rates of decrease in reflected light (*d* 366 nm reflectance decrease/*dt*) were highly correlated with the total rate of glutathione adduct formation by the liver (Fig. 4).

Rates of glutathione conjugation in periportal and pericentral regions of the liver lobule determined by reflectance spectrophotometry with micro-light guides. Micro-light guides were placed on the surface of the liver to detect decreases in reflected 366-nm light caused by increased absorption from intracellular glutathione adducts. Rates of product formation in periportal and pericentral regions of the liver lobule were calculated from the maximal rate of decrease in reflected light (*d* 366 nm reflectance decrease/*dt*) in each region as a proportion of the total product formed by the liver. In instances where changes were observed in periportal and pericentral regions simultaneously (Fig. 5A), the maximal rates calculated for each region at a given time point were proportional to the maximal rate of product formation by the liver at that time (14.1 and 14.7 μmol/g/hr in periportal and pericentral regions, respectively, for DCNB, Table 1). When reflectance changes were observed only in periportal areas (Fig. 5B), the maximal rate of glutathione adduct formation was nearly twice the rates of product formation by the liver on a per gram basis (18.3 μmol/g/hr) (Table 1). This calculation assumes that periportal and pericentral regions comprise about equal portions of the liver lobule (28). Infusion of DCNB (100 μM) in livers from fed, normal rats decreased surface reflectance in periportal regions of the liver lobule rapidly. This rate of decrease (2.87 μamp anode current/min) was maintained for approximately 20 min until a maximum was reached, simultaneous with the maximal rate of product formation by the whole liver (Fig. 5A). During this period, glutathione conjugates were

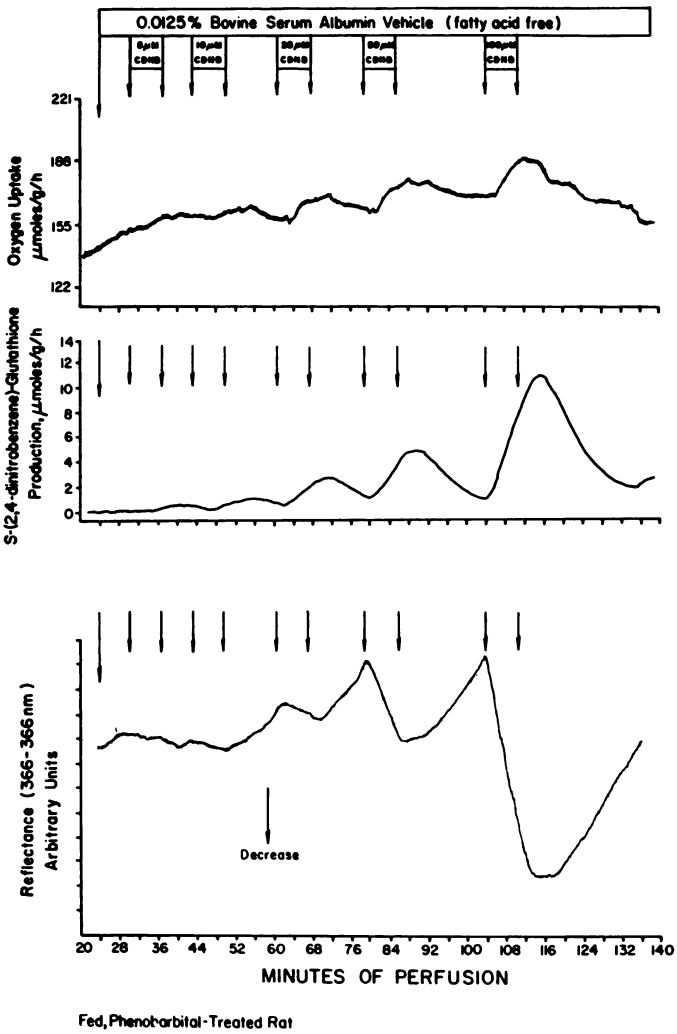

Fig. 3. Effect of CDNB concentration on oxygen uptake, *S*-(2,4-dinitrophenyl)-glutathione formation, and 366-nm reflectance measured with the large-tipped (2 mm) light guide. Perfusion conditions were described in Materials and Methods. CDNB (5–100 μM) was infused into livers from fed, phenobarbital-treated rats in 6-min pulses as indicated by the *vertical arrows*. The figure shown is representative of a single experiment. All determinations are summarized in Fig. 4 for CDNB and DCNB.

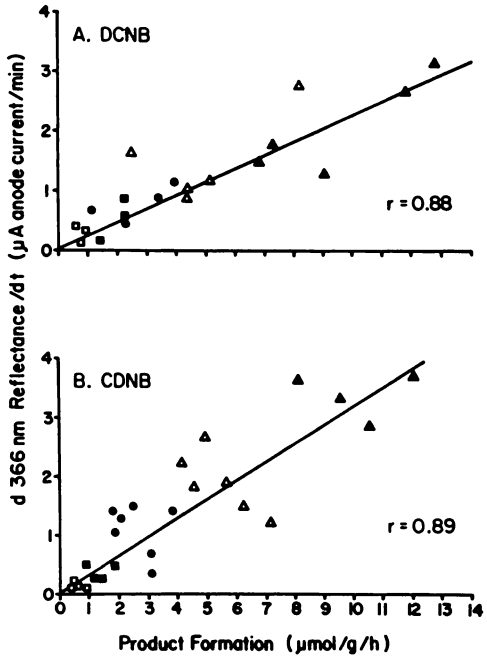

Fig. 4. Correlation between the rate of decrease in 366-nm reflectance (*d* 366-nm reflectance/*dt*) determined with the large-tipped light guide and total product formation by the liver. DCNB (A) and CDNB (B) were infused in 6-min pulses at concentrations of 5 μM (□), 10 μM (■), 25 μM (●), 50 μM (△), and 100 μM (▲). Conditions were as in Fig. 3.

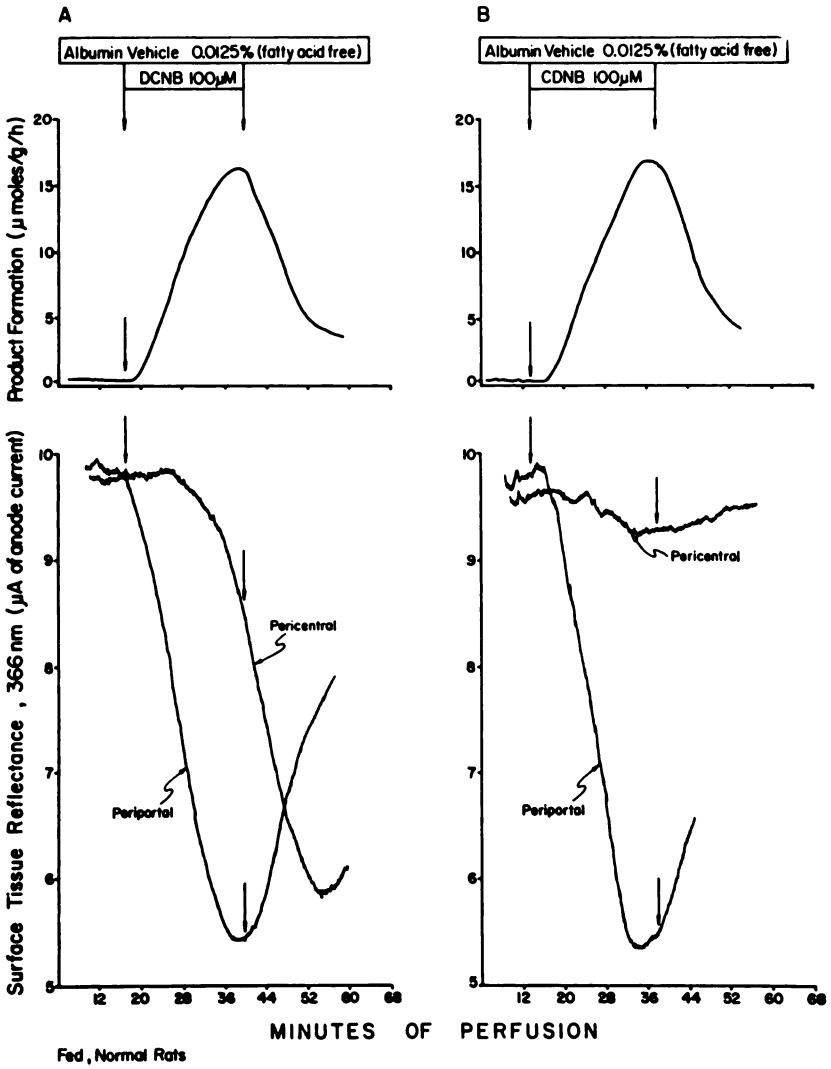

Fig. 5. Glutathione adduct formation and reflectance decreases in periportal and pericentral regions of the liver lobule determined with micro-light guides. DCNB (A) and CDNB (B) (100 μM) were infused into livers from fed, normal rats depicted by the *horizontal bars* and *vertical arrows*. Product formation was monitored continuously in the effluent perfusate. Micro-light guides were placed on periportal and pericentral regions of the liver lobule based on differences in pigmentation, and reflectance at 366 nm was monitored as described in Materials and Methods.

produced at maximal rates of 14.1 μmol product/g/hr only in periportal regions. No changes in reflectance were observed in pericentral regions of the liver lobule during the first 12 min of substrate infusion. The reflectance signal then began to decrease gradually in the pericentral region and reached a maximal rate of 3.13 μamp/min. This corresponds to a rate of product formation of 14.7 μmol product/g/hr in pericentral areas. Infusion of CDNB (100 μM) also decreased reflectance in the periportal region maximally at similar rates corresponding to rates of product formation of 18.3 μmol/g/hr (Fig. 5B). In marked contrast, reflectance decreases were barely detectable in pericentral regions of the liver lobule (rates were only 3.0 μmol/g/hr; Table 1). In contrast to livers from normal rats, livers from rates treated with phenobarbital to induce the glutathione transferases conjugated DCNB almost exclusively in periportal regions of the liver lobule (Fig. 6A). Conversely, the pattern of adduct formation with CDNB was not affected by phenobarbital treatment (Fig. 6B). In livers from phenobarbital-treated rats, rates of glutathione adduct formation with DCNB as substrate were 21.5 and 0 μmol/g/hr for periportal and pericentral regions, respectively. With CDNB as substrate, adducts were formed at rates of 20.6 and 3.0 μmol/g/hr, respectively (Table 1).

Rates of glutathione adduct formation in periportal and pericentral regions of the liver lobule during perfusion in the retrograde direction. Perfusion in the retrograde direction allows high concentrations of substrate to be delivered first to pericentral areas. When perfusion was in the retrograde direction, decreases in reflectance were detectable in pericentral and periportal regions of the liver lobule. Glutathione adducts of CDNB and DCNB were formed at rates of 13.2 and 12.5 μmol/g/hr, respectively (Table 1). Reflectance decreased initially in the pericentral regions for only 8 min followed by decreases in the periportal region as shown for CDNB in Fig 7. Product formation by the liver, however, was not maximal until 23 min after the start of substrate infusion. Maximal rates of glutathione conjugation were similar for CDNB and DCNB in periportal and pericentral regions of the liver lobule in livers from phenobarbital-treated rats (Table 1).

Glutathione adduct formation in periportal and pericentral regions of the liver lobule during perfusion with calcium-free medium. Bile formation was stopped following 8 min of perfusion with calcium-free medium (29) in livers from phenobarbital-treated rats. The subsequent infusion of CDNB did not stimulate bile flow, and glutathione adducts were released into the effluent perfusate under these conditions. Maximal rates of product formation of 12.5 and 13.3 μmol/g/hr were reached 14 min after the initiation of substrate infusion

TABLE 1

Glutathione adduct formation in the isolated, perfused rat liver determined in periportal and pericentral regions of the liver lobule by reflectance spectrophotometry with CDNB and DCNB as substrates

Treatment and substrate (100 μM)	Product formation	Periportal region		Pericentral region	
		Reflectance decreases (d 366 nm/dt)	Product formation	Reflectance decreases (d 366 nm/dt)	Product formation
	μmol/g/hr	μamp anode current/min	μmol/g/hr	μamp anode current/min	μmol/g/hr
Anterograde					
Untreated					
CDNB	10.4 ± 2.7 (8)[a]	3.67 ± 1.5 (7)	18.3 ± 4.4 (7)	0.65 ± 0.8 (7)[b]	3.0 ± 3.7 (7)[c]
DCNB	14.1 ± 2.6 (3)	2.87 ± 1.1 (7)	14.1 ± 4.7 (5)	3.13 ± 1.8 (6)	14.7 ± 4.0 (7)
Phenobarbital-treated[d]					
CDNB	12.1 ± 3.4 (13)	3.43 ± 2.1 (12)	20.6 ± 6.1 (12)	0.38 ± 0.5 (12)	3.0 ± 4.1 (12)
DCNB	10.9 ± 2.4 (8)	4.19 ± 0.9 (9)	21.5 ± 5.4 (6)[e]	ND[f]	ND
Phenobarbital-treated, calcium-free buffer[g]					
CDNB	12.5 ± 2.4 (3)	3.71 ± 2.1 (4)	25.0 ± 4.7 (3)	ND	0.0
DCNB	13.3 ± 1.9 (6)	3.91 ± 2.9 (6)	23.5 ± 5.4 (6)[e]	0.62 ± 0.9 (6)[e]	2.9 ± 4.1 (6)[h]
Retrograde					
Phenobarbital-treated[d]					
CDNB	13.2 ± 0.3 (4)	0.64 ± 0.8 (5)	1.5 ± 2.9 (5)	2.61 ± 0.6 (5)	22.5 ± 5.2 (5)
DCNB	12.5 ± 2.3 (8)	ND	0.0	2.74 ± 1.8 (8)	25.0 ± 4.7 (8)

[a] All values are means ± SD. Numbers in parentheses, *N*.
[b] CDNB values significantly different from DCNB, $p < 0.05$. All statistical comparisons were made using the Student's *t* test.
[c] CDNB values significantly different from DCNB, $p < 0.01$.
[d] Rats were treated with pehnobarbital (1 mg/ml) in the drinking water 7–14 days before perfusion.
[e] Values significantly different from untreated control, $p < 0.05$.
[f] ND, no detectable changes in reflectance during the initial 10 min of substrate infusion.
[g] Rats were treated with phenobarbital. Perfusion medium was prepared as described in Materials and Methods except that calcium chloride was omitted.
[h] Values significantly different from untreated control, $p < 0.01$.

for CDNB and DCNB, respectively (Table 1). Adduct formation, measured by surface reflectance, occurred in periportal regions at rates of 25.0 and 23.5 μmol/g/hr with CDNB and DCNB as substrates, respectively. No significant decreases in reflectance were detected in pericentral regions with either substrate in the absence of calcium.

Effects of depletion of glutathione on sublobular rates of glutathione adduct formation. Following treatment of rats with diethyl maleate (0.7 g/kg) 1 hr before perfusion, rates of *S*-(2,4-dinitrophenyl)-glutathione formation were less than 1.0 μmol/g/hr with CDNB (100 μM) as substrate (data not shown). No changes in surface reflectance at 366 nm were detected in either region of the liver lobule with micro-light guides under these conditions.

Treatment of rats with L-buthionine sulfoximine, an inhibitor of glutathione synthesis (6 mmol/kg), 3 hr before perfusion decreased the rate of adduct formation 30–50%. Maximal rates were observed after approximately 12 min of perfusion and declined gradually until the original baseline was reached 80 min after the onset of substrate infusion (Fig. 8). Unlike experiments in the absence of L-buthionine sulfoximine, product was not formed at low steady state rates indefinitely (Fig. 2B). Initially, reflectance decreases corresponding to rates of 9.1 μmol/g/hr occurred only in the periportal regions. Subsequently, the liver formed glutathione adducts at approximately 5 μmol/g/hr in pericentral areas.

Discussion

Metabolism of CDNB and DCNB in the isolated perfused rat liver. CDNB and DCNB are readily conjugated with glutathione and have been used widely in the identification and characterization of glutathione *S*-transferases from various tissues and species (24–27). A red spectral shift is observed upon conjugation of both substrates with glutathione (253–338 nm), allowing easy spectrophotometric determination of adducts. The appearance of glutathione adducts can be detected effectively at 366 nm with no interference from parent substrates.

The enzymatic formation of thioether adducts from DCNB and CDNB has been shown to be specific for glutathione (24). Nonenzymatic reactions with other cellular thiols and proteins are believed to be minimal due to the rapid binding of substrates to GSH transferases.

Maximal rates of adduct formation measured in the effluent perfusate were approximately the same (13 μmol conjugate/g/hr) for both CDNB and DCNB. The maximal rate was not maintained but, rather, declined to a new steady state of about 2 μmol/g/hr in about 40 min. The rapid decline in rate is most likely due to depletion of intracellular glutathione. Glutathione content in perfused liver was approximately 4.3 μmol/g of liver.[3] Integration of the area under the product peak (Fig. 2B) showed that 5.5 μmol/g of glutathione conjugates were formed. Thus, total adduct formation and tissue contents of GSH are in close agreement. Synthesis of new glutathione could account for the slightly greater amount of product formed relative to the amount expected from static measurements of glutathione. The steady state rate of glutathione adduct formation of about 2 μmol/g/hr (Fig. 2B) is similar to rates reported for *de novo* synthesis of glutathione in isolated hepatocytes (30) and isolated perfused rat livers (29). Since pretreatment with L-buthionine sulfoximine, a highly selective glutathione synthesis inhibitor, abolished this residual steady state rate of adduct formation (Fig. 8), it is concluded that the residual rate of adduct formation is due to newly synthesized GSH. With our

[3] F. C. Kauffman, S. A. Belinsky, and R. G. Thurman, unpublished results.

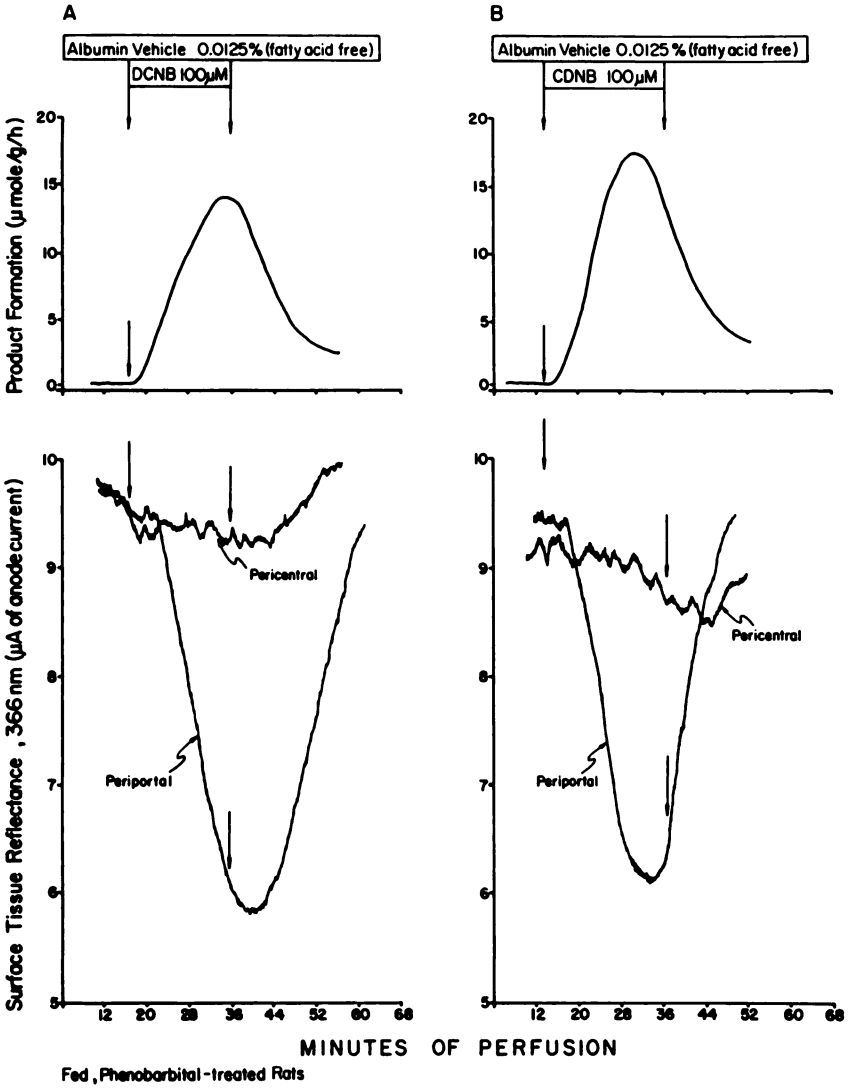

Fig. 6. Glutathione adduct formation and reflectance decreases in periportal and pericentral regions of the liver lobule. Conditions and procedures are as in Fig. 5, except that livers were from rats treated with phenobarbital (1 mg/ml) in the drinking water for 7–20 days.

perfusion conditions, substrates were delivered at a rate of 20–25 μmol/g/hr which corresponds to the peak rates seen when reflectance decreases are observed only in the periportal region. Overall product formation accounts for 60–70% of the total substrate infused. No attempts were made to measure adducts remaining in the liver following perfusion although it is believed that adducts are removed rapidly from the cells (29).

Infusion of CDNB and DCNB each increased O_2 uptake (Fig. 2). The time course of changes in oxygen uptake coincided generally with the time course of changes in rates of glutathione conjugate formation. The reason for this increase in oxygen uptake is not known, although similarities between the structures of CDNB and DCNB and dinitrophenol suggest that oxidative phosphorylation may be uncoupled.

Measurement of glutathione conjugation in periportal and pericentral regions of the liver lobule by reflectance spectrophotometry. Glutathione conjugates formed from CDNB and DCNB in the liver decrease the amount of light reflected from the liver surface due to increased absorbance at 366 nm by the accumulation of intracellular product. This conclusion is supported by a good correlation between rates of change in reflected light and rates of product formation (Fig. 4) and the fact that changes in reflected light were not observed when glutathione was depleted with diethyl maleate (Results; Ref. 20).

Unambiguous interpretation of micro-light guide data required that it be known whether conjugated product was detected in the liver sinusoids, bile canaliculi, or within the cells. Infusion of synthetic *S*-(2,4-dinitrophenyl)-glutathione (30 μM) into the liver sinusoids did not decrease the 366-nm reflectance; therefore, it is unlikley that conjugated product in the vascular space is responsible for the decreases in reflectance observed. Removal and concentration of conjugated product in the bile ducts could also account for the reflectance decreases since adduct concentrations reached greater than 54 mM and accounted for approximately one-half of the total product formed. The possibility that reflectance decreases are due to glutathione adducts in the bile also seems unlikley, however, since reflectance decreases and rates of product formation were identical in the presence or absence of bile formation (Table 1). Therefore, we conclude that the reflectance decreases monitored from the liver surface are a result of the glutathione adducts in hepatocytes.

Large differences in binding, turnover, and relative nonenzymatic rates of glutathione conjugate formation have been reported for DCNB and CDNB *in vitro* under conditions where

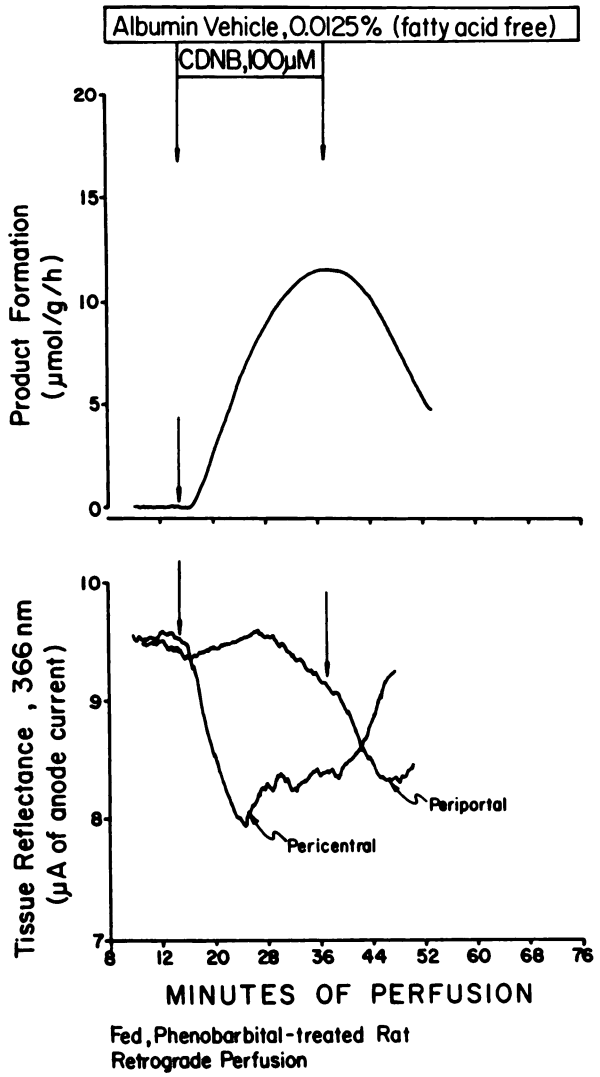

Fig. 7. Glutathione adduct formation and reflectance changes in periportal and pericentral regions of the liver lobule during perfusion in the retrograde direction. CDNB (100 μM) was infused in a liver from a fed, phenobarbital-treated rat that was perfused through the vena cava. Decreases in 366-nm reflected light were detected with the micro-light guides as described in the legend to Fig. 5.

substrates are far in excess (25–27). There were, however, no differences in product formation for the two substrates in the perfused liver (Fig. 2, Table 1) and *in vitro*, where conditions were chosen to approximate the relative protein and substrate concentrations encountered during liver perfusion (Fig. 1). Sublobular patterns of adduct formation were not the same for DCNB and CDNB in the normal rat.

In contrast to DCNB, reflectance did not decrease in pericentral regions in livers from rats when CDNB was infused, although rates of product formation were identical with both substrates (Table 1).

The rapid but transient decrease in reflectance seen when livers from phenobarbital-treated rats were perfused in the retrograde direction (Fig. 7) indicates clearly that the lack of reflectance changes in the pericentral region is not due to the inability of these cells to form glutathione adducts. The limited duration (8 min) of decrease in reflectance in the pericentral region and the onset of the reflectance decrease in the periportal region prior to the peak in product formation suggests that a smaller glutathione pool may be depleted rapidly in the pericentral region when substrate is infused. This view is supported by the observation that glutathione concentrations were higher in periportal than in pericentral regions of the lobule (31). Furthermore, the distribution of glutathione within the liver lobule has been shown not to be a simple linear concentration gradient from the portal tract to the central vein. Hepatocytes located within 100 μm of the central vein (3–4 cells) appeared to contain much less glutathione than did those in other regions of the liver lobule. This observation may explain why only very small decreases in reflectance were observed in pericentral regions during perfusion of CDNB in the anterograde direction, even though enough substrate was delivered to convert all of the glutathione present in periportal and pericentral regions into adducts. Attempts to increase substrate concentrations above 100 μM to evaluate the saturation of the glutathione conjugation process in periportal regions of the liver lobule were unsuccessful since high concentrations caused swelling of the liver which interfered with the optical signal.

L-Buthionine sulfoximine diminished the capacity of periportal regions to exclusively conjugate CDNB, most likely by

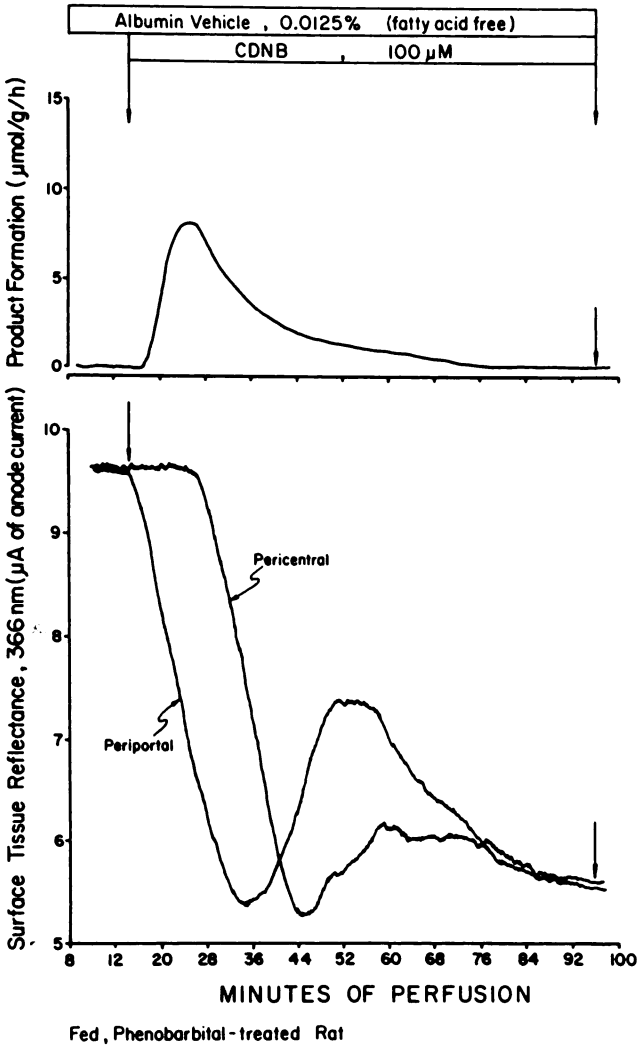

Fig. 8. Glutathione adduct formation and reflectance decreases at 366 nm in periportal and pericentral regions of the liver lobule during perfusion of livers from rats treated with L-buthionine sulfoximine (6 mmol/kg) 3 hr before perfusion. CDNB (100 μM) was infused continuously in the liver of a fed, phenobarbital-treated rat.

decreasing glutathione content across the liver lobule. Under these conditions reflectance decreases occurred in both periportal and pericentral regions of the liver lobule and the rate of product formation was decreased to nearly 50% of controls (Fig. 8). This suggests that a slower substrate turnover due to reduced glutathione levels may result in more free substrate reaching the "downstream" regions, as indicated by reflectance decreases in the pericentral regions of the liver lobule. Glutathione content, however, cannot be used to explain the different pattern of reflectance decrease seen with DCNB in the normal liver (Fig. 5). The cellular uptake characteristics of CDNB and DCNB could provide a more reasonable explanation, although such parameters have not been measured directly. CDNB is more lipophilic than DCNB and could pass more easily through the membrane in "upstream" or periportal regions. Less efficient uptake of DCNB could result in more free substrate being delivered to the "downstream" or pericentral region.

Treatment of rats with phenobarbital did not alter the lobular metabolism of CDNB (Fig. 6). Conjugation of DCNB, however, was altered dramatically by phenobarbital treatment. Reflectance decreases which were observed in both regions in livers from normal rats (Fig. 5) were observed only in periportal regions in livers from phenobarbital-treated rats (Fig. 6). Phenobarbital treatment increases liver mass and elevates intracellular levels of glutathione *S*-transferases (32, 33). These changes may increase the binding and catalysis of DCNB to adducts in periportal regions of the liver lobule and thereby prevent substrate from reaching pericentral regions (Fig. 6). This increase in turnover could act to offset the slow partitioning of DCNB into the cells resulting in reflectance decreases only in the periportal region of the liver lobule.

Removal of conjugated product from the cell does not appear to affect rates of conjugation or the distribution of adduct formation in periportal and pericentral regions of the liver lobule. Release of adducts from the liver has been shown to be rapid and linear (29) in the perfused liver. It is possible, however, that high intracellular concentrations of conjugated product could bind to proteins involved in glutathione metabolism and thereby limit the uptake and further catalysis of substrate.

In this study, the measurement of glutathione conjugation in periportal and pericentral regions of the liver lobule by reflectance spectrophotometry has been demonstrated for the first time. Sublobular rates of glutathione adduct formation of CDNB and DCNB are most likely regulated by differences in substrate uptake, intercellular binding, and glutathione availability. Reflectance spectrophotometry for the measurement of glutathione conjugation may be a useful method to understand sublobular mechanisms of hepatotoxicity involving glutathione.

References

1. Sies, H., and E. Cadenas. Biological basis of detoxication of oxygen free radicals, in *Biological Basis of Detoxication* (W. B. Jakoby, ed.). Academic Press, New York, 181–201 (1983).
2. Levine, W. G. Glutathione and hepatic mixed-function oxidase activity. *Drug Metab. Rev.* **14**:909–930 (1983).
3. Hernandez, O., and J. R. Bend. Metabolism of epoxides, in *Metabolic Basis of Detoxication* (W. B. Jakoby, J. R. Bend, and J. Caldwell, eds.). Academic Press, New York, 207–228 (1982).
4. Mitchell, J. R., D. J. Jollow, W. Z. Potter, D. C. Davis, J. R. Gillette, and B. B. Brodie. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmacol Exp. Ther.* **187**:211–217 (1973).
5. Rouiller, Ch. Experimental toxic injury of the liver, in *The Liver*, (Ch. Rouiller, ed.), vol. II. Academic Press, New York, 335–476 (1964).

5a. Meister, A. Selective modification of glutathione metabolism. *Science (Wash. D. C.)* **220**:472–477 (1983).

6. Recknagel, R. O. Carbon tetrachloride hepatotoxicity. *Pharmacol. Rev.* **19**:145–168 (1976).
7. Baron, J., J. A. Redick, and F. P. Guengerich. Immunohistochemical localizations of cytochromes P-450 in rat liver. *Life Sci.* **23**:2627–2632 (1978).
8. Redick, J. A., T. T. Kawabata, F. P. Guengerich, P. A. Krieter, T. K. Shires, and J. Baron. Distributions of monooxygenase components and epoxide hydratase within the livers of untreated male rats. *Life Sci.* **27**:2465–2470 (1980).
9. Baron, J., and T. T. Kawabata. Intratissue distribution of activating and detoxicating enzymes, in *Biological Basis of Detoxication* (W. B. Jakoby, ed.). Academic Press, New York, 105–135 (1983).
10. Bannikov, G. A., V. I. Guelstein, and T. A. Tchipshera. Distribution of basic azo-dye-binding protein in normal rat tissues and carcinogen-induced liver tumors. *Int. J. Cancer* **11**:398–411 (1973).
11. Fleischner, G. M., J. B. Robbins, and I. M. Arias. Cellular localization of ligandin in rat, hamster, and man. *Biochem. Biophys. Res. Commun.* **74**:992–1000 (1977).
12. Redick, J. A., W. B. Jakoby, and J. Baron. Immunohistochemical localization of glutathione *S*-transferases in livers of untreated rats. *J. Biol. Chem.* **257**:15200–15203 (1983).
13. Ji, S., J. J. Lemasters, and R. G. Thurman. A fluorometric method to measure sublobular rates of mixed-function oxidation in the hemoglobin-free perfused rat liver. *Mol. Pharmacol.* **19**:513–516 (1981).
14. Conway, J. G., F. C. Kauffman, S. Ji, and R. G. Thurman. Rates of sulfation and glucuronidation of 7-hydroxycoumarin in periportal and pericentral regions of the liver lobule. *Mol. Pharmacol.* **22**:509–516 (1982).
15. Ji, S., J. J. Lemasters, and R. G. Thurman. A non-invasive method to study metabolic events within sublobular regions of hemoglobin-free perfused liver. *FEBS Lett.* **113**:37–41 (1980).
16. Chen, R. F. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242**:173–181 (1967).
17. Lee, C.-H., L. Johnson, R. H. Cox, J. D. McKinney, and S.-M. Lee. Mouse liver glutathione *S*-transferases: biochemical and immunological characterization. *J. Biol. Chem.* **256**:8110–8116 (1981).
18. Ryan, A. J., and J. R. Bend. The metabolism of styrene oxide in the isolated perfused rat liver: identification and quantitation of major metabolites. *Drug Metab. Dispos.* **5**:363–370 (1977).
19. Griffith, O. Depletion of glutathione by inhibition of biosynthesis. *Methods Enzymol* **77**:59–63 (1981).
20. Plummer, J. L., B. R. Smith, H. Sies, and J. R. Bend. Chemical depletion of glutathione *in vivo*. *Methods Enzymol.* **77**:50–59 (1981).
21. Scholz, R., W. Hansen, and R. G. Thurman. Interaction of mixed function oxidation with biosynthetic processes. I. Inhibition of gluconeogenesis in perfused rat liver by aminopyrine. *Eur. J. Biochem.* **38**:64–75 (1973).
22. Berry, M. N., R. L. Hamilton, E. M. Severinghaus, and M. C. Williams. A simple and inexpensive membrane "lung" for rat liver perfusion, in *Regulation of Hepatic Metabolism* (F. Lundquist and N. Tygstrup, eds.), Alfred Benzon Symposium VI. Munksgaard, Copenhagen, 790–795 (1974).
23. Chance, B., V. Legallis, J. Sorge, and N. Graham. A versatile time-sharing multichannel spectrophotometer, reflectometer, and fluorometer. *Anal. Biochem.* **66**:498–514 (1975).
24. Habig W. H., M. G. Pabst, and W. B. Jakoby. Glutathione *S*-transferases: the first step in mercapturic acid formation. *J. Biol. Chem.* **249**:7130–7141 (1974).
25. Habig, W. H., and W. B. Jakoby. Assays for differentiation of glutathione *S*-transferases. *Methods Enzymol.* **77**:398–405 (1981).
26. Mannervik, B., and H. Jensson. Binary combinations of four protein subunits with different catalytic specificities explain the relationship between six basic glutathione *S*-transferases in rat liver cytosol. *J. Biol. Chem.* **257**:9909–9912 (1982).
27. Keen, J. M., W. H. Habig, and W. B. Jakoby. Mechanism of the several activities of the glutathione *S*-transferases. *J. Biol. Chem.* **251**:6183–6188 (1976).
28. Lemasters, J. J., S. Ji, and R. G. Thurman. Sublobular measurement of metabolism with probes in the isolated perfused rat liver, in *Regulation of Hepatic Metabolism: Intra- and Intercellular Compartmentation* (R. G. Thurman, F. C. Kauffman, and K. Jungermann, eds.). Plenum Press, New York, in press.
29. Wählander, A., and H. Sies. Glutathione *S*-conjugate formation from 1-chloro-2,4-dinitrobenzene and biliary *S*-conjugate excretion in the perfused rat liver. *Eur. J. Biochem.* **96**:441–446 (1979).
30. Reed, D. J., and S. Orrenius. Role of methionine in glutathione biosynthesis by isolated hepatocytes. *Biochem. Biophys. Res. Commun.* **77**:1257–1264 (1977).
31. Smith, M. T., N. Loveridge, E. D. Wills, and J. Chayen. The distribution of glutathione in the rat liver lobule. *Biochem. J.* **182**:103–108 (1979).
32. Schulte-Heimann, R. Induction of liver growth by xenobiotic compounds and other stimuli. *Crit. Rev. Toxicol* **3**:97–120 (1974).
33. Kaplowitz, N., J. Kuhlenkamp, and G. Clifton. Drug induction of glutathione *S*-transferases in male and female rats. *Biochem. J.* **146**:351–356 (1975).

Send reprint requests to: Dr. Ronald G. Thurman, Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.