

# Inhibition of Glucuronidation of Benzo(a)pyrene Phenols by Long-Chain Fatty Acids<sup>1</sup>

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## ABSTRACT

Long-chain fatty acids inhibit glucuronidation of benzo(a)pyrene phenols in perfused liver; therefore, this study was designed to investigate interactions of fatty acids with  $\beta$ -glucuronidase, glucuronosyl transferase, and energy supply. In  $\beta$ -glucuronidase-deficient C3H/He mice, infusion of oleate (250  $\mu$ M) increased the release of free benzo(a)pyrene phenols from 14 to 33 nmol/g/h and decreased release of glucuronides into the perfusate from 25 to 17 nmol/g/h. Rates of accumulation of glucuronides in the liver were also diminished from 11 to 4 nmol/g/h after infusion of oleate (250  $\mu$ M). Fatty acids did not affect the release of benzo(a)pyrene metabolites into bile, and the ratio of free phenol to glucuronide production was increased from 0.57 to 1.30. A similar trend was observed in livers from DBA/2 mice that have  $\beta$ -glucuronidase. Rates of hydrolysis of benzo(a)pyrene-*O*-glucuronide were not altered in isolated microsomes by addition of oleoyl coenzyme A (CoA) or octanoyl CoA (10–100  $\mu$ M). Thus, we conclude that fatty acids do not alter glucuronidation by acting on  $\beta$ -glucuronidase.

The concentration of cofactors (UDP-glucuronic acid, UDP-glucose, and adenine nucleotides) involved in hepatic conjugation was not altered by infusion of concentrations of oleate (300  $\mu$ M) that inhibited glucuronidation in perfused livers. When oleate concentrations were increased to 600  $\mu$ M, UDP-glucuronic acid and UDP-glucose decreased 44 and 49%, respectively, and the ATP:ADP ratio declined concomitantly.

Oleoyl CoA inhibited UDP-glucuronosyl transferase noncompetitively (half-maximal inhibition, 10  $\mu$ M) in microsomes with 3-hydroxybenzo(a)pyrene or *p*-nitrophenol as substrate. In contrast, octanoyl CoA was a very poor inhibitor of transferase activity. Inhibition of the transferase by oleoyl CoA was increased markedly by treatment with detergents (Triton X-100), i.e., half-inhibition of glucuronosyl transferase was obtained with about 2  $\mu$ M oleoyl CoA. Inhibition of UDP-glucuronosyl transferase by oleoyl CoA was also increased in a dose-dependent manner by albumin, possibly due to increasing access of the CoA derivative to the enzyme. Collectively, these data indicate that fatty acids diminish glucuronidation via the formation of acyl CoA compounds that inhibit UDP-glucuronosyl transferase noncompetitively.

## INTRODUCTION

We demonstrated previously that long-chain fatty acids increased the benzo(a)pyrene phenol:glucuronide ratio in the perfused liver and suggested that they inhibit glucuronidation (1). In contrast, medium chain-length fatty acids had no effect on this process. Glucuronides of benzo(a)pyrene, which are produced at high rates and accumulate in liver (2), are not highly mutagenic *per se* (3), but hydrolysis of phenolic glucuronides of benzo(a)pyrene yields a derivative that reacts with DNA to a greater extent than either the parent phenol or the glucuronide (3, 4). Thus, stable glucuronides formed in liver may travel to other organs where they are metabolized to highly mutagenic compounds (5). Fatty acids could influence chemical

carcinogenesis by altering glucuronidation of polycyclic aromatic hydrocarbon carcinogens in the liver.

Activation of  $\beta$ -glucuronidase, inhibition of UDP-glucuronosyl transferase, or decreases in the supply of UDPGA<sup>3</sup> may all diminish glucuronidation. It is known that  $\beta$ -glucuronidase can be activated by a variety of compounds of high molecular weight, such as DNA, diamines, and suramin (6). Alterations of cytosolic calcium concentrations have also been implicated in regulating microsomal  $\beta$ -glucuronidase (7, 8) and hydrolysis of 3-hydroxybenzo(a)pyrene glucuronide (8). Although the distribution (9, 10), inducibility (10, 11), activation (12, 13), and multiplicity (14) of UDP-glucuronosyl transferases have been studied, little is known about the effects of fatty acids on glucuronidation in intact cells. This is important because nutritional state, especially carbohydrate content, plays an important role in regulating rates of glucuronidation by influencing UDPGA supply in intact cells (15). For example, oleate inhibited glucuronidation of *p*-nitrophenol by decreasing ATP and UDPGA supply (16). It is also known that the activity of UDP-glucuronosyl transferase (10, 17, 18) and microsomal  $\beta$ -glucuronidase (7) is dependent on the lipid environment. Therefore, the purpose of this study was to examine how fatty acids inhibit glucuronidation.

## MATERIALS AND METHODS

**Animals.** Female Sprague-Dawley rats weighing between 200 and 300 g and female DNA/2 or C3H/He mice weighing between 15 and 20 g with free access to laboratory chow were used in the experiments described below. Cytochrome P-450 I.A.1 was induced by injecting  $\beta$ -naphthoflavone (30 mg/kg) in corn oil i.p. once daily for 3 days before perfusion experiments.

**Benzo(a)pyrene Phenol and Oleate.** A stock solution of 30 mM benzo(a)pyrene was prepared in acetone and stored in the dark. Before perfusion, it was diluted to 2 mM with 15% (w/v) fatty acid-free bovine serum albumin in Krebs-Henseleit bicarbonate buffer (pH 7.4) and stirred under nitrogen at room temperature for 1 h. This solution was perfused into the liver at rates that resulted in final albumin concentrations of 0.03% and benzo(a)pyrene concentrations of 4  $\mu$ M. Sodium oleate was bound to 20% bovine serum albumin, pH 7.4, and infused into the liver at concentrations indicated in the Figure and Table legends.

**Liver Perfusion.** Details of the liver perfusion technique have been described elsewhere (19). The common bile duct was cannulated with polyethylene tubing (PE-10; Clay Adams), and aliquots of bile were collected into vials at 10-min intervals. Livers were perfused with Krebs-Henseleit bicarbonate buffer (37°C, pH 7.4) saturated with an oxygen:carbon dioxide (95:5) mixture in a nonrecirculating system. Fluid was pumped via a cannula placed in the vena cava past a Teflon-shielded, Clark-type oxygen electrode before being discarded or analyzed for metabolites. Rates of oxygen uptake were calculated from the influent minus effluent oxygen concentration differences, the flow rate, and the liver wet weight.

**Analysis of Bile, Perfusate, and Liver for Phenols and Glucuronides of Benzo(a)pyrene.** Phenols of benzo(a)pyrene in perfusate, bile, and liver homogenates were measured by a modification of the Dehnen

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<sup>3</sup> The abbreviations used are: UDPGA, UDP-glucuronic acid; UDPG, UDP-glucose; F:G, ratio between free phenols and glucuronides; CoA, coenzyme A.

assay (20). Immediately upon termination of perfusion, livers were blotted, weighed, and homogenized for 30 s in 4 volumes of 0.15 M KCl using a Brinkmann Polytron. Aliquots of bile (1  $\mu$ l), effluent perfusate (1.0 ml), or liver homogenate (20  $\mu$ l) were incubated with 0.5 M Tris HCl, pH 7.4, in the presence or absence of  $\beta$ -glucuronidase (500 Fishman units) in a final volume of 1.1 ml. After incubation for 3 h (23°C), 1.0 ml was added to a 0.3-ml mixture of triethylamine:Triton X-100 (9:1) and 2.0 ml of aqueous EDTA. Fluorescence of free benzo(a)pyrene phenols was measured (435 $\rightarrow$ 522 nm) using an Aminco DW2A spectrophotometer equipped with a fluorescence attachment. Phenols of benzo(a)pyrene were quantitated based on the fluorescence of chromatographically pure 3-hydroxybenzo(a)pyrene (National Cancer Institute Chemical Carcinogen Repository, Midwest Research Institute) as a standard, and rates of production were calculated from concentrations in the effluent perfusate or bile, the rate of perfusion or bile flow, and the liver wet weight. Rates of production of phenols in liver tissue were calculated from concentrations in liver homogenates, the liver wet weight, and the time of perfusion.

**$\beta$ -Glucuronidase.**  $\beta$ -Glucuronidase was determined using benzo(a)-pyrene glucuronide(s) excreted in bile after infusing benzo(a)pyrene (20  $\mu$ M) into rat livers for 1 h. Hepatic microsomes were prepared by standard techniques of differential centrifugation, washed, and resuspended in 0.15 M KCl (21). Hydrolysis of biliary benzo(a)pyrene-*O*-glucuronide(s) was assayed using a mixture containing 50  $\mu$ l of bile, microsomes (0.75 mg protein), and fatty acyl CoA compounds. The final incubation volume was adjusted to 1.0 ml with 0.5 M Tris-HCl buffer, pH 7.4. After 1 h of incubation, liberated phenols were measured fluorometrically (20). Assays were linear with time for at least 1 h and with microsomal protein up to 1.5 mg.

**Measurement of Glucuronosyl Transferase Activity.** Glucuronidation of 3-hydroxybenzo(a)pyrene was assayed in a mixture containing 5 mM MgCl<sub>2</sub>, 2 mM UDPGA, 12.5  $\mu$ M 3-hydroxybenzo(a)pyrene, and microsomes (0.25 mg protein). The final incubation volume was adjusted to 1 ml with 0.1 M Tris-HCl buffer (pH 7.4). After 10 min of incubation at 37°C, the reaction was terminated by addition of a 0.3-ml mixture of triethylamine:Triton X-100 (9:1), and phenols were measured fluorometrically (20).

Glucuronidation of *p*-nitrophenol was assayed in a 0.5-ml mixture containing 0.2 mM *p*-nitrophenol, 5 mM UDPGA, and fatty acyl CoA compounds at concentrations depicted in the Figure legends. Assays were performed in the presence or absence of 0.05% Triton X-100. *p*-Nitrophenol liberated after 10 min of incubation at 37°C was measured spectrophotometrically at 436 nm (22).

**Measurement of Metabolites.** Metabolites were measured in HClO<sub>4</sub> extracts of livers that had been freeze-clamped with tongs chilled in liquid nitrogen (23). Samples of frozen liver weighing about 500 mg were powdered and extracted with 0.4 M HClO<sub>4</sub> as described previously (23). Protein-free extracts were neutralized with 2 M KHCO<sub>3</sub> and stored at -80°C until assayed for metabolites. UDPG was measured fluorometrically according to procedures described by Lowry and Passonneau (24).

UDPGA was measured by high-performance liquid chromatography using a Whatman SAX-12.5 column (particle size, 5  $\mu$ m). UDPGA in neutralized tissue extracts was eluted with a linear gradient of phosphate buffer (0.5 $\rightarrow$ 0.2 M KH<sub>2</sub>PO<sub>4</sub>, pH 5.5) and KCl (0.1 $\rightarrow$ 0.15 M) at a flow rate of 0.5 ml/min. UDPGA was detected at 254 nm with an average retention time of 13.9 min.

Adenine nucleotides were separated by high-performance liquid chromatography utilizing a C<sub>18</sub>- $\mu$ Bondapak column (4.6  $\times$  250 nm; particle size, 5  $\mu$ m). Adenine nucleotides in neutralized tissue extracts were eluted isocratically with 0.15 M KH<sub>2</sub>PO<sub>4</sub> (pH 5.5) at a flow rate of 1 ml/min and were determined at 254 nm. Average retention times for ATP, ADP, and AMP were 6.2, 7.7, and 15.8 min, respectively.

## RESULTS

**Effects of Oleate on Formation and Export of Phenols and Glucuronides of Benzo(a)pyrene from Livers of C3H/He and DBA/2 Mice.** Because the expression of  $\beta$ -glucuronidase in mice is determined by a single gene (25), mutants exist. DBA/2 mice,

which are homozygous for the dominant G allele, exhibit high  $\beta$ -glucuronidase activity, whereas C3H/He mice are  $\beta$ -glucuronidase deficient (25, 26). Therefore, livers from these 2 strains of mice were used to determine the influence of  $\beta$ -glucuronidase on glucuronidation of benzo(a)pyrene and to explore the effects of fatty acids on the hydrolase.

Maximal steady-state rates of release of free phenols and glucuronides of benzo(a)pyrene from livers of C3H/He mice into perfusate were approximately 14 and 25 nmol/g/h, respectively (Fig. 1, lower panel). Rates of release in livers from DBA/2 mice were similar (Table 1). Therefore, it is clear that  $\beta$ -glucuronidase does not influence glucuronidation of benzo(a)pyrene. The infusion of oleate into livers from both C3H/He and DBA/2 mice increased the release of free phenols into the effluent perfusate and slightly decreased the release of glucuronides. Thus, the F:G increased 2- to 3-fold in the perfusate (Table 1). Release of free phenols and glucuronides into bile in livers from both strains was approximately 10 and 18 nmol/g/h, respectively (Fig. 1, upper panel)—values that were not affected by the infusion of oleate. In contrast, oleate reduced rates of accumulation of glucuronides in intrahepatic stores (11, control; 4.5, oleate) in both strains without affecting rates of accumulation of free phenols (7.1, control; 7.9, oleate). Consequently, the F:G ratio also increased 2- to 3-fold in the liver. Production of total free phenols was elevated significantly from rates of 31 to 51 nmol/g/h by infusion of oleate in livers from both strains, and overall rates of glucuronide production were decreased from 53 to 36 nmol/g/h. Thus, overall rates of mono-

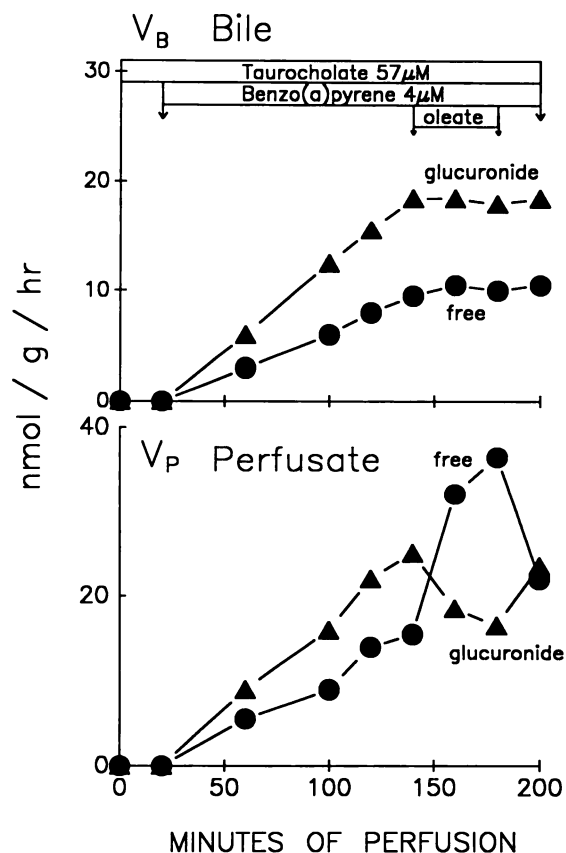


Fig. 1. Effects of oleate on the production, storage, and export of benzo(a)pyrene phenols in perfused liver from  $\beta$ -naphthoflavone-treated C3H/He mice. Benzo(a)pyrene was infused into perfused mouse livers at a final concentration of 4  $\mu$ M, and oleate was infused at a final concentration of 250  $\mu$ M.  $V_B$ , rate of benzo(a)pyrene phenol (free + glucuronide) export via bile;  $V_P$ , rate of export via perfusate;  $\Delta$ — $\Delta$ , glucuronide;  $\bullet$ — $\bullet$ , free.

Table 1 Effect of oleate on accumulation, storage, and export of free phenols and glucuronides of benzo(a)pyrene from  $\beta$ -naphthoflavone-treated C3H/He and DBA/2 mice

Livers were perfused as described in "Materials and Methods." Benzo(a)pyrene was infused into the liver at a final concentration of 4  $\mu$ M, and oleate was perfused at a final concentration of 250  $\mu$ M. Every group was compared with appropriate control using a matched pair *t* test. Benzo(a)pyrene phenol production rates are shown in means  $\pm$  SD (nmol/g/h). *n* = 4 in each group.

Mice	Addition	Rates of production of benzo(a)pyrene phenols + glucuronides (nmol/g/h)											
		Perfusate (V <sub>P</sub> )			Bile (V <sub>B</sub> )			Liver (V <sub>S</sub> )			Total V <sub>T</sub>		
		F	G	F:G	F	G	F:G	F	G	F:G	F	G	F:G
C3H/He	None	14.3 $\pm$ 4.2	24.8 $\pm$ 2.4	0.58	9.7 $\pm$ 4.3	18.2 $\pm$ 4.9	0.51	7.1 $\pm$ 2.0	11.1 $\pm$ 2.0	0.68	31.7 $\pm$ 10.0	53.3 $\pm$ 8.2	0.57
	Oleate	33.4 $\pm$ 9.3 <sup>a</sup>	17.3 $\pm$ 9.8 <sup>a</sup>	1.93	10.1 $\pm$ 4.4	14.8 $\pm$ 3.0	0.56	7.8 $\pm$ 2.1	4.5 $\pm$ 1.0 <sup>b</sup>	1.77	51.2 $\pm$ 10.9 <sup>a</sup>	36.5 $\pm$ 12.1 <sup>a</sup>	1.30
DBA/2	None	15.4 $\pm$ 3.3	32.2 $\pm$ 7.6	0.48	8.9 $\pm$ 2.2	12.7 $\pm$ 2.1	0.71	7.0 $\pm$ 1.6	12.5 $\pm$ 4.0	0.56	31.4 $\pm$ 4.8	57.3 $\pm$ 7.9	0.55
	Oleate	41.0 $\pm$ 11.6 <sup>a</sup>	17.1 $\pm$ 2.9 <sup>a</sup>	2.4	9.4 $\pm$ 2.8	13.4 $\pm$ 3.4	0.70	6.3 $\pm$ 2.4	7.3 $\pm$ 1.0 <sup>a</sup>	0.86	56.6 $\pm$ 13.8 <sup>a</sup>	37.8 $\pm$ 12.5 <sup>a</sup>	1.50

<sup>a</sup> *P* < 0.01.

<sup>b</sup> *P* < 0.05.

Table 2 Effects of oleate on adenine nucleotide contents in perfused rat liver

Rat livers were perfused as described in "Materials and Methods." Benzo(a)pyrene was infused at final concentrations of 4  $\mu$ M, and oleate was infused at final concentrations of 300 or 600  $\mu$ M. Livers were freeze-clamped and tissues were stored at  $-80^{\circ}\text{C}$ . Adenine nucleotides ( $\mu$ mol/kg) were determined by high-performance liquid chromatography as described in "Materials and Methods."

Addition	ATP	ADP	AMP	ATP:ADP
None	2874 $\pm$ 288 <sup>a</sup>	822 $\pm$ 127	378 $\pm$ 98	3.49
Oleate, 300 $\mu$ M	2322 $\pm$ 286 <sup>b**</sup>	774 $\pm$ 119	390 $\pm$ 140	3.00
Oleate, 600 $\mu$ M	1254 $\pm$ 300 <sup>**</sup>	930 $\pm$ 376	495 $\pm$ 77	1.35 <sup>*</sup>

<sup>a</sup> Mean  $\pm$  SE.

<sup>b</sup> \* *P* < 0.05; \*\* *P* < 0.01, for comparison with appropriate controls.

oxygenation of benzo(a)pyrene were not affected by oleate in perfused mouse liver. However, the F:G ratio was increased 2- to 3-fold by oleate in both strains, indicative of diminished rates of glucuronidation.

The effect of octanoyl and oleoyl CoA on rates of hydrolysis of benzo(a)pyrene glucuronide cannot be explained by an action of  $\beta$ -glucuronidase. In the absence of acyl CoA, benzo(a)pyrene-*O*-glucuronide was hydrolyzed at rates of approximately 1 nmol/mg microsomal protein/min, and addition of oleoyl or octanoyl CoA (10 to 100  $\mu$ M) had no significant effect on  $\beta$ -glucuronidase activity (data not shown).

**Effect of Fatty Acids on UDP-Glucuronic Acid in Perfused Rat Liver.** In perfused rat liver, basal UDP-glucuronic acid content was 252  $\mu$ mol/kg and was unaltered by infusion of low concentrations of oleate (300  $\mu$ M). With higher concentrations of oleate (600  $\mu$ M), however, UDP-glucuronic acid decreased markedly to 112  $\mu$ mol/kg. Similar tendencies were observed with UDP-glucose, a precursor of UDP-glucuronic acid (data not shown).

Adenine nucleotides were also measured since the formation of UDP-glucuronic acid is dependent upon a constant supply of ATP (27). In the absence of oleate, ATP, ADP, and AMP levels in liver were 2874, 822, and 378  $\mu$ mol/kg, respectively. Oleate (300  $\mu$ M) decreased ATP content slightly to 2322  $\mu$ mol/kg but had no effect on ADP or AMP. In contrast, a higher concentration of oleate (600  $\mu$ M) decreased ATP levels to 1254  $\mu$ mol/kg and decreased the ATP:ADP ratio nearly 3-fold (Table 2). Under these conditions, total adenine nucleotides were decreased from 4074 to 2679  $\mu$ mol/kg (Table 2).

**Effects of Acyl CoA on Glucuronosyl Transferase Activity in Isolated Microsomes.** Since decreased glucuronidation could reflect inhibition of glucuronosyl transferase by fatty acids, we examined the effect of acyl CoA compounds and fatty acids on glucuronosyl transferase activity in isolated microsomes. Basal rates of glucuronidation of 3-hydroxybenzo(a)pyrene were about 2.5 nmol/mg/min and were decreased by both oleoyl

CoA and octanoyl CoA (Fig. 2). Inhibition was greater with the longer-chain derivatives and was noncompetitive (half-maximal inhibition = 10  $\mu$ M oleoyl CoA; Fig. 3). Coenzyme A *per se* (50  $\mu$ M), oleate (300  $\mu$ M), or octanoate (300  $\mu$ M) had no effect on glucuronosyl transferase activity.

UDP-glucuronosyl transferase(s), which consists of a family of closely related but functionally heterogeneous enzymes, exhibit(s) broad substrate specificity (14, 28). To ascertain whether acyl CoA inhibited glucuronidation of benzo(a)pyrene specifically, we examined the effect of acyl CoA on the glucuronidation of the model substrate *p*-nitrophenol by microsomes. Rates of glucuronidation of *p*-nitrophenol were also decreased dramatically from 9.5 nmol/mg/min to approximately 6.5 and 2.9 nmol/mg/min after the addition of 10 and 50  $\mu$ M oleoyl CoA, respectively (Fig. 4). Half-maximal decreases occurred with 12.5  $\mu$ M oleoyl CoA. As with 3-hydroxybenzo(a)pyrene, octanoyl CoA was much less potent (Fig. 4A).

Experiments were also performed with detergent-activated enzyme. When Triton X-100 was added to microsomes, glucuronosyl transferase activity increased 6- to 7-fold as expected (Fig. 4B). Oleoyl CoA also decreased rates of glucuronidation in detergent-treated preparations; however, half-maximal inhi-

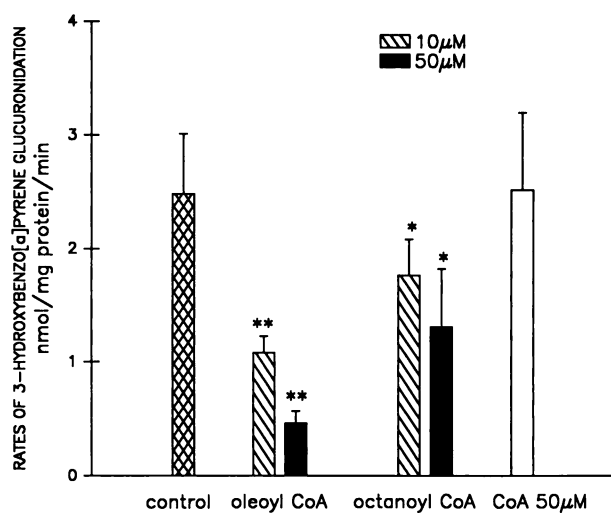


Fig. 2. Effects of acyl CoA on rates of glucuronidation of 3-hydroxybenzo(a)pyrene by hepatic microsomes. Glucuronidation of 3-hydroxybenzo(a)pyrene was assayed in a 1-ml mixture containing 5 mM MgCl<sub>2</sub>, 2 mM UDPGA, 12.5  $\mu$ M 3-hydroxybenzo(a)pyrene, 0.1 M Tris-HCl buffer (pH 7.4), about 0.25 mg microsomal protein, and acyl CoA concentrations as shown. Reactions were terminated after 5 min by adding fluorescence cocktail, and liberated 3-hydroxybenzo(a)pyrene was measured fluorometrically. Data are mean  $\pm$  SEM (bars). \* *P* < 0.05; \*\* *P* < 0.01 for comparison with controls.

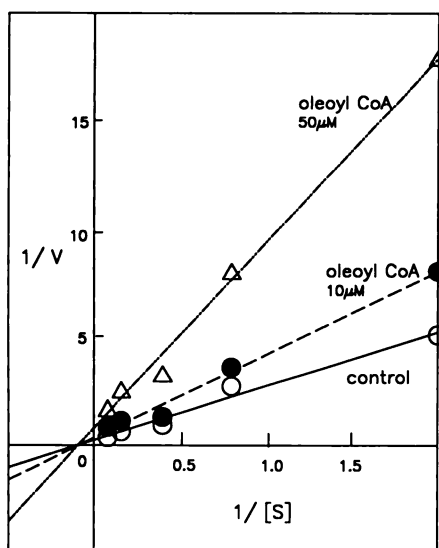


Fig. 3. Inhibition of glucuronidation of 3-hydroxybenzo(a)pyrene by oleate in hepatic microsomes. Conditions as in Fig. 2.  $\circ$ — $\circ$ , Control;  $\bullet$ — $\bullet$ , 10  $\mu$ M oleoyl CoA;  $\Delta$ — $\Delta$ , 50  $\mu$ M oleoyl CoA;  $v$ , velocity;  $[S]$ , concentration of substrate.

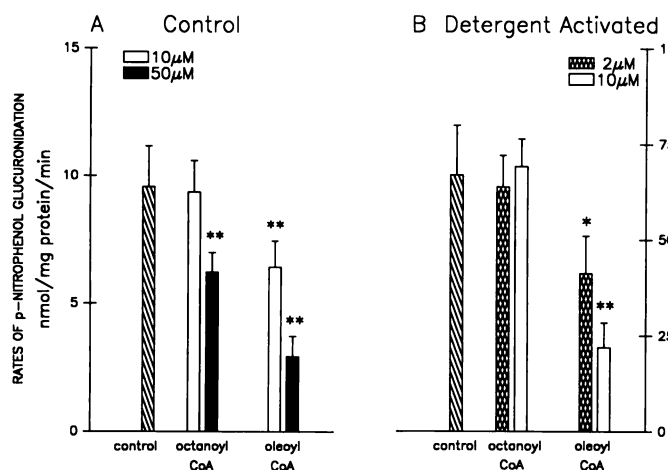


Fig. 4. Effects of acyl CoA on rates of glucuronidation of *p*-nitrophenol in hepatic microsomes. Glucuronidation of *p*-nitrophenol was measured in a 0.5-ml mixture containing UDPGA (5 mM), *p*-nitrophenol (0.2 mM), phosphate buffer (0.05 M, pH 7.1) with (detergent activated) or without (control) Triton X-100 (0.05%), and 0.2 mg of microsomal protein. Acyl CoA concentrations are as shown. *p*-Nitrophenol was measured as described in "Materials and Methods." Data are mean  $\pm$  SEM (bars). \*  $P < 0.05$ ; \*\*  $P < 0.01$  for comparison with respective controls.

bition occurred with much lower concentrations of oleoyl (<2  $\mu$ M) than octanoyl CoA.

Because long-chain acyl CoA compounds have high affinities for protein (29), and binding proteins exist in abundance in intact cells, the effect of protein binding on the inhibition of glucuronosyl transferases by acyl CoA was studied. In the absence of acyl CoA, rates of glucuronidation of *p*-nitrophenol were about 10 nmol/mg/min. Rates were not affected by addition of albumin over the range of 0.1–0.5% (data not shown). In contrast, rates of glucuronidation of *p*-nitrophenol in the presence of oleoyl CoA were decreased in a dose-dependent manner by 0.25 and 0.50% albumin (Fig. 5), indicating that binding to albumin enhanced the inhibition of glucuronosyl transferase activity by oleoyl CoA.

## DISCUSSION

Benzo(a)pyrene is monooxygenated via aryl hydrocarbon hydrolase, and the resulting phenols undergo glucuronidation via UDP-glucuronosyl transferase in reactions dependent upon UDPGA. Concomitantly, benzo(a)pyrene-*O*-glucuronide can be cleaved via  $\beta$ -glucuronidase. In intact cells, the ratio of free phenol:glucuronide is dependent on the balance between these 2 processes and could be influenced by fatty acids in a number of ways. Fatty acids could: (a) increase the activity of  $\beta$ -glucuronidase; (b) decrease cofactor supply; or (c) inhibit glucuronosyl transferase.

**$\beta$ -Glucuronidase.** First, there were no differences in total rates of metabolism of benzo(a)pyrene in liver from C3H and DBA mice (Table 1). Second, long-chain fatty acids, which activate  $\beta$ -glucuronidase, did not affect the ratio of free benzo(a)pyrene to its glucuronide in liver from  $\beta$ -glucuronidase-deficient compared with normal mice. Fatty acids increased the ratio of free phenols to glucuronides in livers from  $\beta$ -glucuronidase-deficient C3H/He mice to about the same extent as in livers from DBA/2 mice that have normal  $\beta$ -glucuronidase activity (Table 1). Thus, it is concluded that  $\beta$ -glucuronidase is not involved in inhibition of glucuronidation by oleate in the perfused liver. In support of this idea, we observed that acyl CoA compounds had no effect on hydrolysis of benzo(a)pyrene glucuronides by isolated microsomes (Fig. 2).

**Cofactor Supply.** To evaluate whether lower rates of glucuronidation in the presence of fatty acids were due to decreases in UDPGA supply, UDPGA and UDPG were measured in the presence and absence of fatty acids. Previous studies demonstrated that UDPGA supply, an obligatory cofactor for glucuronidation, was reduced by fasting (30) and could become an important rate determinant at high concentrations of fatty acids (15). Formation of UDPGA might also be influenced by fatty acids in other ways. For example, UDPGA is formed in liver from UDPG in a reaction that is catalyzed by UDPG-dehydrogenase and requires  $NAD^+$  (27) as cofactor. Since fatty acid metabolism decreases  $NAD^+$ , UDPG-dehydrogenase could be inhibited indirectly. However, because UDPG content in perfused liver was actually decreased by oleate, it is unlikely that UDPG-dehydrogenase was inhibited.

Formation of UDPGA also requires a constant supply of

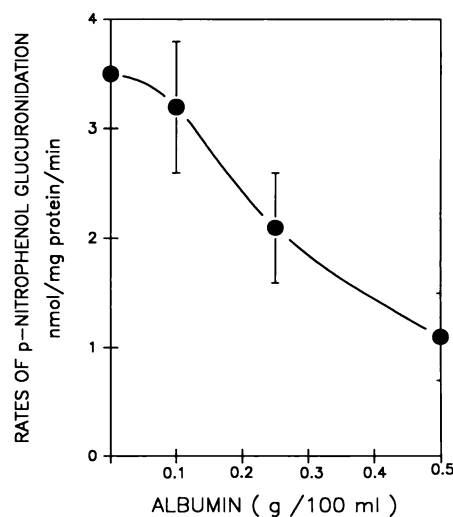


Fig. 5. Effect of albumin on the inhibition of glucuronidation of *p*-nitrophenol by oleate. Conditions as in Fig. 4 except that albumin was added at a final concentration of 0.1–0.5% as indicated. Data are mean  $\pm$  SEM (bars).

ATP, and fatty acids decrease the content of ATP in tissue by uncoupling oxidative phosphorylation (31). It is known that activation of ATPase by fatty acids is dependent upon their chain length (32). We found that a high concentration of oleate (600  $\mu$ M) decreased UDPGA and UDPG in liver (see "Results") concomitant with decreases in ATP (Table 2). On the other hand, this phenomenon was not observed at low concentrations of oleate, during which glucuronidation was inhibited.

**Glucuronosyl Transferase.** Inhibition of glucuronidation of hydroxylated metabolites of benzo(a)pyrene by long-chain CoA compounds is best explained by their direct interaction with glucuronosyl transferase. In support of this possibility, rates of glucuronidation of 3-hydroxybenzo(a)pyrene (Fig. 2) and *p*-nitrophenol (Fig. 4) in isolated microsomes were decreased noncompetitively by oleoyl CoA (Fig. 4). The new finding that acyl CoA compounds inhibit glucuronosyl transferase has important implications for the regulation of conjugation in the intact liver.

The more potent inhibition of glucuronosyl transferase by long- versus short-chain acyl CoA may be due to their greater lipophilicity. UDP-glucuronosyl transferase is embedded in the membrane of the endoplasmic reticulum (10, 12, 33), and its activity is dependent upon its lipid surroundings. The phospholipid bilayer most likely plays an important role in regulating access of lipophilic substrates or inhibitors to the transferase, an integral membrane protein (10). In support of this idea, it is known that inhibition of glucuronidation of *p*-nitrophenol increases with increasing chain length of aliphatic alcohols (10). In the present study, addition of detergent not only increased the activity of UDP-glucuronosyl transferase by 6–7-fold but it also increased its sensitivity to acyl CoA compounds. Thus, long-chain acyl CoA compounds inhibit UDP-glucuronosyl transferase activity directly.

Long-chain acyl CoA compounds have high affinity for protein (29), and inhibition of monooxygenation by long-chain acyl CoA compounds can be blocked by albumin (1). In the presence of acyl CoA, albumin enhanced rather than diminished the inhibition of glucuronosyl transferase in a dose-dependent manner, an effect that was not observed in the absence of acyl CoA. One possible mechanism for this phenomenon is that protein binding increases the delivery of acyl CoA to the transferase in the membrane. A direct effect on the enzyme seems unlikely, however, since albumin inhibited glucuronosyl transferase in the presence of fatty acids in detergent-activated preparations (data not shown).

We conclude that addition of long-chain fatty acids diminishes glucuronidation of benzo(a)pyrene phenol by inhibiting glucuronosyl transferase directly.

## REFERENCES

- Zhong, Z., Gao, W., Kauffman, F. C., and Thurman, R. G. Effect of fatty acids on formation, distribution, storage and release of benzo(a)pyrene phenols and glucuronides: studies with the isolated perfused rat liver. *Cancer Res.*, 49: 2012–2016, 1989.
- Kari, F. W., Kauffman, F. C., and Thurman, R. G. Effects of bile salts on rates of formation, accumulation and export of mutagenic metabolites from benzo(a)pyrene produced by the perfused rat liver. *Cancer Res.*, 45: 1621–1627, 1985.
- Kari, F. W., Thurman, R. G., and Kauffman, F. C. Characterization of mutagenic glucuronide formation from benzo(a)pyrene in the nonrecirculating perfused rat liver. *Cancer Res.*, 44: 5073–5078, 1984.
- Kinoshita, N., and Gelboin, H. V.  $\beta$ -Glucuronidase catalyzed hydrolysis of benzo(a)pyrene-3-glucuronide and binding to DNA. *Science (Washington DC)*, 199: 307–309, 1978.
- Wall, K., Gao, W., te Koppele, J. M., Kwei, G. Y., Kauffman, F. C., and Thurman, R. G. The liver plays a central role in the mechanism of polycyclic aromatic hydrocarbons. *Carcinogenesis (Lond.)*, 12: 783–786, 1991.
- Levy, G. A., and Marsh, C. A. Preparation and properties of  $\beta$ -glucuronidase. *Adv. Carbohydr. Chem.*, 14: 381–428, 1959.
- Sokolove, P. M., Wilcox, M. A., Thurman, R. G., and Kauffman, F. C. Stimulation of hepatic microsomal  $\beta$ -glucuronidase by calcium. *Biochem. Biophys. Res. Commun.*, 121: 987–993, 1984.
- Whittaker, M., Sokolove, P. M., Thurman, R. G., and Kauffman, F. C. Stimulation of 3-benzo(a)pyrenyl glucuronide hydrolysis via calcium activation of microsomal  $\beta$ -glucuronidase. *Cancer Lett.*, 26: 145–152, 1985.
- Gram, T. E., Hansen, A. R., and Fouts, J. R. The submicrosomal distribution of hepatic uridine diphosphate glucuronosyltransferase in the rabbit. *Biochem. J.*, 106: 587–591, 1968.
- Kasper, C. B., and Henton, D. Glucuronidation. In: W. B. Jakoby (ed.), *Enzymatic Basis of Detoxification*, pp. 3–28. New York: Academic Press, 1980.
- Bock, K. W., Josting, D., Lilienblum, W., and Pfeil, H. Purification of rat-liver microsomal UDP-glucuronosyltransferase. Separation of two enzyme forms inducible by 3-methylcholanthrene or phenobarbital. *Eur. J. Biochem.*, 98: 19–26, 1979.
- Binham, P., Berry, C., and Hallinan, T. Compartmentation of uridine diphosphate glucuronosyltransferase: effect of uridine diphosphate *N*-acetylglucosamine on the apparent *V* of glucuronidation. *Biochem. Soc. Trans.*, 6: 178–180, 1978.
- Zakim, D., and Vessey, D. A. Membrane dependence of uridine diphosphate glucuronosyltransferase. Effect of the membrane on kinetic properties. *Biochem. Soc. Trans.*, 2: 1165, 1974.
- Chowdhury, J. R., Chowdhury, N. R., Falany, C. N., and Tephly, T. R. Isolation and characterization of multiple forms of rat liver UDP-glucuronate glucuronosyltransferase. *Biochem. J.*, 233: 827–837, 1986.
- Reinke, L. A., Belinsky, S. A., Evans, R. K., Kauffman, F. C., and Thurman, R. G. Conjugation of *p*-nitrophenol in the perfused rat liver: the effect of substrate concentration and carbohydrate reserves. *J. Pharmacol. Exp. Ther.*, 217: 863–870, 1981.
- Danis, M., Kauffman, F. C., Evans, R., Holtzclaw, W. D., Reinke, L. A., and Thurman, R. G. Inhibition of *p*-nitroanisole *O*-demethylation in perfused rat liver by oleate. *Biochem. Pharmacol.*, 34: 609–616, 1985.
- Zakim, D., and Vessey, D. A. The effect of lipid-protection interaction on the kinetic parameters of microsomal UDP-glucuronosyl transferase. In: A. Mardones (ed.), *The Enzymes of Biological Membranes*, Vol. II, Biosynthesis of Cell Components, pp. 443–446. New York and London: Plenum Publishing Corp., 1976.
- Graham, A. B., and Wood, G. C. The phospholipid dependence of UDP-glucuronosyltransferase. *Biochem. Biophys. Res. Commun.*, 37: 567–575, 1969.
- Scholz, R., Hansen, W., and Thurman, R. G. Interaction of mixed-function oxidation with biosynthetic processes. I. Inhibition of gluconeogenesis by aminopyrine in perfused rat liver. *Eur. J. Biochem.*, 38: 64–72, 1973.
- Dehnen, W., Tomingas, R., and Roos, J. A modified method for the assay of benzo(a)pyrene hydroxylase. *Anal. Biochem.*, 53: 373–383, 1973.
- Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J., Narashimulu, S., Cooper, D. Y., and Rosenthal, O. Drug interactions with hepatic microsomal cytochrome. *Mol. Pharmacol.*, 2: 187–190, 1966.
- Zakim, D., and Vessey, D. A. Techniques for the characterization of UDP-glucuronosyltransferase, glucose-6-phosphatase and other tightly-bound microsomal enzymes. *Methods Biochem. Anal.*, 21: 1–36, 1973.
- Kauffman, F. C., Evans, R. K., and Thurman, R. G. Alterations in nicotina-mide and adenine nucleotide systems during mixed-function oxidation of *p*-nitroanisole in perfused livers from normal and phenobarbital-treated rats. *Biochem. J.*, 166: 583–592, 1977.
- Lowry, O. H., and Passonneau, J. V. A flexible system of enzymatic analysis. New York: Academic Press, 1972.
- Paigen, K. The effect of mutation on the intracellular location of  $\beta$ -glucuronidase. *Exp. Cell Res.*, 25: 286–301, 1961.
- Thorpe, S. R., Fiddler, M. B., and Desnick, R. J. Enzyme therapy IV: a method for determining the *in vivo* fate of bovine  $\beta$ -glucuronidase in  $\beta$ -glucuronidase deficient mice. *Biochem. Biophys. Res. Commun.*, 61: 1464–1470, 1974.
- Miettinen, T. A., and Leskinen, E. Glucuronic acid pathway. In: W. H. Fishman (ed.), *Metabolic Conjugation and Metabolic Hydrolysis*, pp. 157–237. New York and London: Academic Press, 1970.
- Bock, K. W., Bock-Hennig, B. S., Lilienblum, W., Pfeil, H., and Vol, R. F. Role of UDP-glucuronosyltransferase in the inactivation of benzo(a)pyrene. *Adv. Exp. Med. Biol.*, 136: 53–73, 1982.
- Wilkinson, T. C. I., and Wilton, D. C. Studies on fatty acid-binding proteins. *Biochem. J.*, 238: 419–424, 1986.
- Belinsky, S. A., Kauffman, F. C., and Thurman, R. G. Effect of nutrition on monooxygenation and conjugation in the liver. In: J. Hathcock (ed.), *Nutritional Toxicology II*, pp. 41–62. New York: Academic Press, 1986.
- Borst, P., Loos, J. A., Christ, E. J., and Slater, E. C. Uncoupling activity of long-chain fatty acids. *Biochim. Biophys. Acta*, 62: 509–518, 1962.
- Pressman, B. C., and Lardy, A. Effect of surface active agents on the latent ATPase of mitochondria. *Biochim. Biophys. Acta*, 21: 458–466, 1956.
- Bock, K. W., and White, I. N. H. UDP-glucuronosyltransferase in perfused rat liver and in microsomes: influence of phenobarbital and 3-methylcholanthrene. *Eur. J. Biochem.*, 46: 451–459, 1974.