Effect of Detergents on the *N*- and Ring-Hydroxylation of 2-Acetamidofluorene by Hamster Liver Microsomal Preparations

By PRABHAKAR D. LOTLIKAR and ELLEN N. DWYER

*Fels Research Institute and the Department of Biochemistry,
Temple University School of Medicine, Philadelphia, PA 19140, U.S.A.*

(Received 30 September 1976)

Effects of detergents such as cholate, deoxycholate and Triton X-100 were studied on *N*- and ring-hydroxylation of 2-acetamidofluorene by reconstituted and unresolved microsomal systems from livers of hamsters pretreated with 3-methylcholanthrene. Triton X-100 (2.5 mg/nmol of cytochrome *P*-448) inhibited *N*- and ring-hydroxylation by whole microsomal preparations by 40 and 90% respectively. Deoxycholate at the same concentration inhibited both hydroxylations completely, whereas cholate inhibited *N*- and ring-hydroxylation by 40 and 50% respectively. In reconstitution studies, the presence of Triton X-100 (0.5–1.0 mg/nmol of cytochrome *P*-448) along with unsolubilized cytochrome *P*-448 fraction and solubilized reductase fraction increased *N*-hydroxylation to an appreciable extent compared with ring-hydroxylation. Both cholate and deoxycholate at 0.5–1.0 mg concentrations had a greater stimulatory effect on ring- than on *N*-hydroxylation. Triton X-100 was better than either cholate or deoxycholate in stimulating *N*-hydroxylation activity in such a reconstituted system.

A variety of physiological and foreign compounds is oxidized by the mammalian hepatic mixed-function oxidase enzyme system (Lu *et al.*, 1973). Two components of this enzyme system, cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase, have been partially purified after solubilization with ionic detergents such as sodium cholate and deoxycholate (Lu *et al.*, 1969, 1973; Lu & West, 1972) and a non-ionic detergent, Triton X-100 (Lotlikar *et al.*, 1974).

In the carcinogenesis by 2-acetamidofluorene and several other aromatic amides and amines, a large number of studies has shown that *N*-hydroxylation is an activation step, whereas ring-hydroxylation is an inactivation phenomenon (Miller, 1970; Weisburger & Weisburger, 1973). Inhibition studies with CO have indicated that cytochrome *P*-450 is involved in both *N*- and ring-hydroxylation of 2-acetamidofluorene (Gutmann & Bell, 1973; Thorgeirsson *et al.*, 1973; Lotlikar & Zaleski, 1974). Differences in inhibition of *N*- and ring-hydroxylation of this carcinogen by CO have suggested involvement of multiple forms of cytochrome *P*-450 (Lotlikar & Zaleski, 1974). Pretreatment of hamsters with 3-methylcholanthrene has been shown to increase hepatic microsomal 2-acetamidofluorene *N*-hydroxylation activity severalfold without appreciably increasing ring-hydroxylation activity (Lotlikar *et al.*, 1967).

In the present study we report differential inhibitory effects of Triton X-100, sodium cholate and deoxycholate on *N*- and ring-hydroxylation of 2-acetamidofluorene by microsomal preparations

from livers of 3-methylcholanthrene-pretreated hamsters. We also report stimulatory effects of these detergents on hydroxylation activities by a reconstituted system from such liver preparations.

Materials and Methods

Sources for some of the chemicals used in the present study were as follows: Triton X-100, horse heart cytochrome *c* (type III), bacterial proteinase VII and NADPH, Sigma Chemical Co., St. Louis, MO, U.S.A.; sodium deoxycholate, Fisher Scientific Co., Fair Lawn, NJ, U.S.A.; sodium cholate, 2-acetamidofluorene and 2-acetamido[9-¹⁴C]fluorene (sp. radioactivity 86 mCi/mmol), Schwarz–Mann Co., Orangeburg, NY, U.S.A.; 3-methylcholanthrene, Eastman Kodak Co., Rochester, NY, U.S.A. All other chemicals were of reagent grade.

A group of 10–12 adult male Syrian hamsters (100–150 g body wt.) was injected intraperitoneally with 3-methylcholanthrene (100 mg/kg body wt.) suspended in 1 ml of corn oil 24 h before death. Preparation of liver microsomal fractions, proteinase treatment and isolation of particles after proteinase treatment were as described previously (Lotlikar *et al.*, 1974). As in the rat (Alvares *et al.*, 1967), CO difference spectra of reduced microsomal preparations from livers of hamsters after 3-methylcholanthrene pretreatment showed a shift in the absorption maximum of cytochrome *P*-450 from 450 to 448 nm (Lotlikar *et al.*, 1974). Microsomal particles

obtained after proteinase treatment are designated as unsolubilized cytochrome *P*-448 fractions. The amount of cytochrome *P*-448 in such cytochrome *P*-448 fractions as measured by the method of Omura & Sato (1964) was about 1.4 nmol/mg of protein. These cytochrome *P*-448 fractions had NADPH-cytochrome *c* reductase activity of less than 5 nmol/min per mg of protein as measured by the procedure of Masters *et al.* (1971). Liver microsomal NADPH-cytochrome *c* reductase fraction prepared by the procedure of Lu & West (1972) showed activity of about 2100 nmol/min per mg of protein. Such a reductase preparation did not have any detectable amount of cytochrome *P*-448. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

The complete incubation medium for *N*- and ring-hydroxylation of 2-acetamidofluorene contained 50 μ mol of potassium phosphate buffer, pH 7.8, 6 μ mol of NADPH, 100 μ mol of sucrose, 10% (v/v) glycerol, 1.12 μ mol of 2-acetamidofluorene containing 0.1 μ Ci of 2-acetamido[9-¹⁴C]fluorene dissolved in 0.05 ml of methanol, and microsomal protein containing 2 nmol of cytochrome *P*-448 in a total volume of 3.0 ml. It is known that glycerol stabilizes detergent-treated cytochrome *P*-450 (Ichikawa & Yamano, 1967). In the present studies, glycerol was added to the incubation medium as a protective agent for cytochrome *P*-448. Microsomal fractions were prepared and suspended in 0.25 M-sucrose; sucrose was therefore added to the incubation medium along with the microsomal fraction. Addition of sucrose did not have any effect on the hydroxylation of 2-acetamidofluorene by liver microsomal preparations. Where indicated, cytochrome *P*-448 fraction containing 2 nmol of cytochrome *P*-448, reductase fraction with NADPH-cytochrome *c* reductase activity of 600 nmol/min, 300 μ mol of KF and various amounts of Triton X-100, sodium cholate or sodium deoxycholate were also added to the incubation medium. Where indicated, fluoride was added because the presence of KF (0.1 M) in the incubation medium was shown to inhibit hamster liver microsomal deacetylase activity and to increase the formation of *N*-hydroxy-2-acetamidofluorene (Lotlikar *et al.*, 1967). Duplicate samples were pre-incubated for 30 min at 5°C before addition of 2-acetamidofluorene and NADPH. In the absence of any detergent, such a preincubation did not have any effect on the *N*- and ring-hydroxylation of 2-acetamidofluorene by either the whole microsomal preparations or a reconstituted system. Such a preincubation was required in order to observe the optimum effect of a detergent on the mixed-function oxidase system. After the addition of 2-acetamidofluorene and NADPH, samples were incubated in air for 20 min at 37°C. Extraction of hydroxylated metabolites of 2-acetamidofluorene from the incuba-

tion medium with diethyl ether, their separation by paper chromatography and determination by radioactivity measurements were as described previously (Lotlikar *et al.*, 1974).

Results and Discussion

Various detergents such as deoxycholate, cholate, Lubrol WX and Triton X-100 have been used to solubilize and purify further the membrane-bound mixed-function oxidase system of several hepatic microsomal preparations (Lu *et al.*, 1969, 1973; Mitani *et al.*, 1971; Comai & Gaylor, 1973; Lotlikar *et al.*, 1974). Resolution of this mixed-function oxidase system after solubilization with such detergents has shown that all three fractions, cytochrome *P*-450, NADPH-cytochrome *P*-450 reductase and a lipid fraction, phosphatidylcholine, are required for oxidation of several physiological substrates, such as steroids and fatty acids, and a variety of foreign compounds, including drugs and a carcinogen,

Table 1. *Inhibitory effects of detergents on N- and ring-hydroxylation of 2-acetamidofluorene by microsomal preparations from livers of 3-methylcholanthrene-pre-treated hamsters*

In addition to the complete incubation medium, all samples contained 0.1 M-KF during incubation. Where indicated, detergent concentration is expressed as mg of detergent added/nmol of cytochrome *P*-448. Other details are described in the Materials and Methods section. In the absence of any detergent, control microsomal preparations formed 19 ± 1.5 and 50 ± 6.0 nmol of *N*- and ring-hydroxy-2-acetamidofluorene/20 min per nmol of cytochrome *P*-448 respectively. Results of control microsomal preparations and detergent effects on such preparations are given as means $\pm$ S.E.M. of three analyses. Results for detergent effects are expressed as percentages of the control.

Detergent (mg)	2-Acetamidofluorene hydroxylation (% of control)	
	<i>N</i> -	Ring
—	100	100
Triton X-100		
0.5	89 $\pm$ 7	49 $\pm$ 3
1.0	74 $\pm$ 5	15 $\pm$ 5
2.5	61 $\pm$ 4	11 $\pm$ 3
Sodium cholate		
0.5	81 $\pm$ 9	86 $\pm$ 10
1.0	78 $\pm$ 4	72 $\pm$ 7
2.5	64 $\pm$ 8	52 $\pm$ 5
Sodium deoxycholate		
0.5	69 $\pm$ 6	51 $\pm$ 4
1.0	48 $\pm$ 5	24 $\pm$ 3
2.5	3 $\pm$ 1	4 $\pm$ 2

Table 2. *Effect of detergents on 2-acetamidofluorene hydroxylation by microsomal fractions from livers of 3-methylcholanthrene-pretreated hamsters*

Unsolubilized cytochrome *P*-448 fractions were obtained after proteinase treatment of liver microsomal preparations. Other details are described in the Materials and Methods section. Where indicated, cytochrome *P*-448 fraction containing 2 nmol of cytochrome *P*-448 and reductase fraction containing 600 nmol/min of NADPH-cytochrome *c* reductase were added. KF (0.1 M) was not present during incubation in these samples, except when whole liver microsomal preparation was used. Where indicated, detergent concentration is expressed as mg of detergent added/nmol of cytochrome *P*-448. In the absence of any detergent, whole microsomal preparations (control) formed 19 ± 1.5 and 50 ± 6.0 nmol of *N*- and ring-hydroxy-2-acetamidofluorene/20 min per nmol of cytochrome *P*-448 respectively. Results of whole microsomal preparations are given as means $\pm$ S.E.M. of three analyses. Results of reconstitution studies are averages of three analyses and are expressed as percentages of the control; experimental variations were less than 10% of the average of three analyses.

Addition of fraction		Detergent (mg)	2-Acetamidofluorene hydroxylation (% of control)	
Cytochrome <i>P</i> -448	Reductase		<i>N</i> -	Ring-
+			0.3	0.3
+	+		3.2	5.5
		+Triton X-100		
+	+	0.5	20	13
+	+	1.0	37	14
+	+	2.5	19	4.8
		+Sodium cholate		
+	+	0.5	6.8	11
+	+	1.0	13	19
+	+	2.5	7.1	13
		+Sodium deoxycholate		
+	+	0.5	7.6	14
+	+	1.0	11	17
+	+	2.5	9.2	3.6

benzo[*a*]pyrene (Lu *et al.*, 1969, 1973; Strobel *et al.*, 1970). In addition to solubilizing the mixed-function oxidase system, it has been shown that detergents such as Triton X-100, deoxycholate and cholate could also partially replace the lipid fraction in the hydroxylation of benzo[*a*]pyrene by the reconstituted liver microsomal system from rats pretreated with 3-methylcholanthrene (Lu & West, 1972).

In our previous studies, cytochromes *P*-450 and *P*-448 were partially purified from rat and hamster liver microsomal preparations after proteinase treatment and Triton X-100 solubilization. Such cytochrome *P*-440 and *P*-448 fractions were able to *N*-hydroxylate 2-acetamidofluorene very efficiently in the presence of deoxycholate-solubilized and purified reductase fraction (Lotlikar *et al.*, 1974; Lotlikar & Zaleski, 1975). However, we were unable to reconstitute deoxycholate- or cholate-solubilized cytochrome *P*-450 or *P*-448 fraction under similar conditions (P. D. Lotlikar, unpublished work).

The present study was designed to compare the effects of the detergents Triton X-100, cholate and deoxycholate on *N*- and ring-hydroxylation of 2-acetamidofluorene by liver microsomal preparations. It was found that addition of these detergents at 0.5–2.5 mg/nmol of cytochrome *P*-448 to the incuba-

tion medium had differential inhibitory effects on *N*- and ring-hydroxylation of 2-acetamidofluorene by liver microsomal systems from hamsters pretreated with 3-methylcholanthrene (Table 1). Thus Triton X-100 at 0.5 mg/nmol inhibited ring-hydroxylation by about 50% without appreciably affecting *N*-hydroxylation. Even at 2.5 mg/nmol Triton X-100 inhibited *N*-hydroxylation by only 40%. Similar results were obtained with cholate with regard to *N*-hydroxylation. However, in contrast with Triton X-100, cholate, even at 2.5 mg/nmol, inhibited ring-hydroxylation by only 50%. Of the three detergents tested, cholate as found to be the least potent inhibitor and deoxycholate was the most potent inhibitor of both *N*- and ring-hydroxylation. It has also been observed that *N*-hydroxylation by liver microsomal preparation from control hamsters is inhibited to a greater extent by Triton X-100 than is that from hamsters pretreated with 3-methylcholanthrene (P. D. Lotlikar, unpublished work). In previous studies, Triton X-100 has been found to be a very potent inhibitor of *N*-demethylation of both ethylmorphine (Lu & Levin, 1974) and dimethylnitrosamine (P. D. Lotlikar, unpublished work). The present *N*-hydroxylation data appear to be the first example where Triton X-100 has not been found to be a potent

inhibitor of the microsomal mixed-function oxidase system.

In previous reconstitution studies, dimethylnitrosamine demethylation activity of unsolubilized cytochrome *P*-450 fraction in the presence of solubilized reductase fraction was about 40% of that obtained with whole liver microsomal preparations from hamsters (Lotlikar *et al.*, 1975). In contrast, in the present study, unsolubilized cytochrome *P*-448 fraction in the presence of reductase fraction yielded only a small amount of *N*- and ring-hydroxylation activity (Table 2). The presence of 0.5–1 mg of Triton X-100/nmol increased *N*-hydroxylation to an appreciable extent compared with ring-hydroxylation. A higher concentration of Triton X-100 had an inhibitory effect on both types of oxidations. Both sodium cholate and deoxycholate at 0.5–1 mg/nmol had greater stimulatory effects on ring-hydroxylation than on *N*-hydroxylation.

In previous reconstitution experiments, where cytochrome *P*-448 was partially purified after proteinase treatment and Triton X-100 solubilization of microsomal preparations, it was found that *N*-hydroxylation activity was completely restored, whereas ring-hydroxylation activity was restored only to about 20–30% of the activity of whole microsomal preparations (Lotlikar *et al.*, 1974; Lotlikar & Zaleski, 1975). However, in the present report (Table 2) *N*-hydroxylation activity was restored to about 40% of the activity of the whole microsomal system; this might be due to the inhibitory effect of Triton X-100 present in the incubation medium (Table 1).

Differential inhibition of *N*- and ring-hydroxylation of 2-acetamidofluorene by CO (Lotlikar & Zaleski, 1974) and by various detergents such as Triton X-100, cholate and deoxycholate (present data, Table 1) suggest possible involvement of multiple forms of cytochrome *P*-450. Similarly, the above data and the differential stimulatory effect on these hydroxylation reactions by these detergents (present data, Table 2) also suggest different localizations of these various cytochromes *P*-450 in the endoplasmic membrane.

This research was supported in part by a research grant (CA-10604) and a grant (CA-12227) to the Fels Research Institute from the National Cancer Institute, U.S. Public Health Service.

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