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Ring- and *N*-Hydroxylation of 2-Acetamidofluorene by Rat Liver Reconstituted Cytochrome *P*-450 Enzyme System

By PRABHAKAR D. LOTLIKAR and KATHLEEN ZALESKI
Fels Research Institute and the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pa. 19140, *U.S.A.*

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The *N*- and ring-hydroxylation of 2-acetamidofluorene were studied with a reconstituted cytochrome *P*-450 enzyme system from microsomal fractions of liver from both control and 3-methylcholanthrene-pretreated rats. Proteinase treatment and Triton X-100 solubilization were two important steps for partial purification of the cytochrome *P*-450 fraction. Both cytochrome *P*-450 and NADPH–cytochrome *c* reductase fractions were required for optimum *N*- and ring-hydroxylation activity. Hydroxylation activity was determined by the source of cytochrome *P*-450 fraction; cytochrome *P*-450 fraction from pretreated animals was severalfold more active than the fraction from controls. Formation of *N*-hydroxylated metabolites with reconstituted systems from both control and pretreated animals was greater than that with their respective whole microsomal fractions.

The mammalian hepatic microsomal cytochrome *P*-450 enzyme system is involved in oxidation of steroids and foreign compounds (Lu *et al.*, 1973). The enzyme system has been resolved into three components: cytochrome *P*-450, NADPH–cytochrome *P*-450 reductase and a lipid fraction identified as phosphatidylcholine (Lu *et al.*, 1969; Strobel *et al.*, 1970).

There is unequivocal evidence that *N*-hydroxylation is an activation step, whereas ring-hydroxylation is an inactivation phenomenon, in carcinogenesis by 2-acetamidofluorene, several other aromatic amides and amines (Miller, 1970; Weisburger & Weisburger, 1973). Inhibition studies with CO have demonstrated that cytochrome *P*-450 is involved in both *N*- and ring-hydroxylation of 2-acetamidofluorene (Guttmann & Bell, 1973; Lotlikar & Zaleski, 1974; Thorgeirsson *et al.*, 1973). Induction of liver tumours in male rats by 2-acetamidofluorene is decreased to a large extent by the simultaneous administration of a small amount of 3-methylcholanthrene (Miller *et al.*, 1958). It appears that this inhibitory effect of 3-methylcholanthrene is primarily caused by induction of the inactivation process (Lotlikar *et al.*, 1967; Miller *et al.*, 1960).

In the present study, we report partial purification and solubilization of liver microsomal cytochrome *P*-450 fraction from both control and 3-methylcholanthrene-pretreated rats. These cytochrome *P*-450 fractions have been used in reconstitution experiments in studying *N*- and ring-hydroxylation of 2-acetamidofluorene.

Materials and methods

3-Methylcholanthrene was obtained from Eastman Organic Chemical Co., Rochester, N.Y., U.S.A. Bacterial protease VII, NADH, NADPH, horse heart cytochrome *c* (type III) and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. 2-Acetamidofluorene was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. 2-Acetamido[9-^{14}C]fluorene (sp. radioactivity 6.7mCi/mmol) was purchased from Tracer Laboratories, Waltham, Mass., U.S.A. All other chemicals were of reagent grade.

Male Sprague–Dawley-strain rats (200–250g body wt.), obtained from Charles River Breeding Laboratories, Wilmington, Mass., U.S.A., were maintained on a commercial diet (Wayne Lab-blox, obtained from Allied Mills, Chicago, Ill., U.S.A.) for at least 1 week before use in the present study. One group of six rats was injected intraperitoneally with 3-methylcholanthrene (100mg/kg body wt.), suspended in 1ml of corn oil, 24h before death. The control group of six animals was injected with 1ml of corn oil. Liver microsomal fractions were prepared as described previously (Lotlikar *et al.*, 1974).

The procedure for purification and solubilization of cytochrome *P*-450 from rat liver microsomal preparations was a slight modification of the procedure described for isolation of cytochrome *P*-450 from hamster liver (Lotlikar *et al.*, 1974). This procedure involved treatment of liver microsomal suspension in 0.25M-sucrose with bacterial protease

VII in a glycerol-containing buffer. The pellet after two washes in glycerol-containing buffer and re-centrifugation was suspended in 0.25M-sucrose and was treated with Triton X-100 (2mg/mg of protein) in the presence of 0.1M-potassium phosphate buffer, pH7.8, containing 10% (v/v) glycerol (5mg of protein/ml). After being stirred for 30min at 4°C, the sample was centrifuged at 105 000*g* for 1h. The pellet was discarded and solid $(NH_4)_2SO_4$ was added to the supernatant fraction to 40% saturation. After being stirred for 15min at 4°C, the solution was centrifuged at 35000*g* for 15min. The precipitate formed at the top was dissolved in 0.01M-potassium phosphate buffer, pH7.8, containing 20% (v/v) glycerol and 0.1mM-dithiothreitol; the solution was dialysed overnight against 200vol. of the same buffer. These solubilized preparations are referred to as cytochrome *P*-450 fractions, which are used for reconstitution studies with 2-acetamidofluorene as the substrate.

Cytochrome *P*-450 content was measured by the method of Omura & Sato (1964). NADPH–cytochrome *c* reductase activity was assayed by the procedure of Masters *et al.* (1971). Liver microsomal NADPH–cytochrome *c* reductase fractions prepared by the procedure of Lu & West (1972) showed activities of 1600 and 2100nmol/min per mg of protein from control and 3-methylcholanthrene-pretreated rats respectively. Such preparations did not have any detectable amount of cytochrome *P*-450. Cytochrome b_5 was assayed by the method of Omura & Sato (1964). 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-hydroxylations of 2-acetamidofluorene contained 50μmol of potassium phosphate buffer, pH7.8, 6μmol of NADPH, 1.12μmol of 2-acetamidofluorene containing 0.1μCi of 2-acetamido[9-^{14}C]-fluorene dissolved in 0.05ml of methanol, and various other microsomal fractions in a total volume of 3.0ml. Samples were incubated in air for 20min at 37°C. Extraction of hydroxylated metabolites of 2-acetamidofluorene from the incubation medium with diethyl ether, their separation by paper chromatography and quantification by radioactivity measurements were as described previously (Lotlikar *et al.*, 1974).

Results and discussion

It has been reported previously that CO-difference spectra of reduced microsomal fractions from livers of rats after 3-methylcholanthrene pretreatment showed a shift in absorption maximum of cytochrome *P*-450 from 450 to 448nm (Alvares *et al.*, 1967). Other than this shift in absorption maximum, there were no significant differences between liver microsomal fractions from control and 3-methylcholanthrene-pretreated rats during cytochrome *P*-450 purification (Table 1). After proteinase treatment of microsomal fraction, NADPH–cytochrome *c* reductase activity was decreased to a large extent in both preparations. Solubilization with Triton X-100 and $(NH_4)_2SO_4$ precipitation increased the purification of cytochrome *P*-450 about twofold. Solutions of $(NH_4)_2SO_4$ precipitates, referred to as cytochrome *P*-450 fractions, had NADPH–cytochrome *c* reductase activity of less than 5nmol/min per mg of protein. Ratios of cytochrome *P*-450 to cytochrome b_5 in these cytochrome *P*-450 fractions were much higher than those in whole microsomal fractions. These cytochrome *P*-450 fractions and NADPH–cytochrome *c* reductase fractions prepared by the procedure of Lu & West (1972) were used in reconstitution experiments. Results of such reconstitution studies with 2-acetamidofluorene as substrate are shown in Table 2. Neither the cytochrome *P*-450 fraction nor the reductase fraction alone from either control or pretreated rats was able to form significant amounts of *N*- or ring-hydroxylated metabolites. However, in the presence of both reductase and cytochrome *P*-450 fractions from control rats, some *N*- and ring-

Table 1. *Partial purification and solubilization of cytochrome P-450 from microsomal fractions of livers from control and 3-methylcholanthrene-pretreated rats*

Enzyme fraction	Treatment: Control	Treatment: 3-Methylcholanthrene	Total protein (mg)	Cytochrome *P*-450 (nmol/mg of protein)	NADPH–cytochrome *c* reductase activity (nmol/min per mg of protein)	Cytochrome b_5 (nmol/mg of protein)
Whole microsomal fraction	+		182	0.82	159	0.37
		+	202	1.13	166	0.48
Microsomal fraction after proteinase treatment	+		88	1.22	3.0	0.25
		+	87	1.42	3.3	0.29
Triton X-100-solubilized $(NH_4)_2SO_4$ precipitate	+		38	2.0	5.0	0.32
		+	43	2.2	7.6	0.20
	+		27	2.0	4.9	0.22
		+	28	2.4	4.1	0.29

Table 2. *Requirements of various microsomal fractions from livers of control and 3-methylcholanthrene-pretreated rats for 2-acetamidofluorene hydroxylation*

All details are described under 'Materials and methods'. Where indicated whole microsomal fractions from liver of control and 3-methylcholanthrene-pretreated rats containing 3.0nmol of cytochrome *P*-450 and NADPH–cytochrome *c* reductase activity of about 510nmol/min were used. Similarly, where indicated fractions containing 3.0nmol of cytochrome *P*-450 and NADPH–cytochrome *c* reductase activity of 600nmol/min were added.

Cytochrome *P*-450 fraction added		Reductase fraction added		Hydroxy derivatives of 2-acetamidofluorene formed (nmol/20min)	
Control	3-Methylcholanthrene-pretreated	Control	3-Methylcholanthrene-pretreated	*N*-	Ring-
+				0.6	0.5
	+			0.6	0.7
		+		0.3	0.1
			+	0.1	0.1
+		+		2.3	1.9
+			+	1.7	1.3
	+		+	16	32
	+	+		12	23
Whole microsomal fraction (control)				1.4	10
Whole microsomal fraction (3-methylcholanthrene-pretreated)				6.0	57

hydroxylation occurred. A similar amount of hydroxylation was obtained when the reductase fraction from treated instead of control rats was used in the presence of cytochrome *P*-450 fraction from control rats. Cytochrome *P*-450 fraction from 3-methylcholanthrene-pretreated animals in the presence of reductase fraction from either control or treated animals formed appreciable amounts of *N*- and ring-hydroxylated metabolites. These results indicate that, even though both cytochrome *P*-450 and reductase fractions are required for hydroxylating activity, the degree of hydroxylating activity is determined by the source of cytochrome *P*-450 fraction. In the present studies, the cytochrome *P*-450 fraction from 3-methylcholanthrene-pretreated rats is severalfold more active than the cytochrome *P*-450 fraction from controls. These results are similar to those of our earlier data for hamster preparations (Lotlikar *et al.*, 1974). Lu *et al.* (1973) in their reconstitution experiments also demonstrated different specificity in the cytochrome *P*-450 fractions from livers of phenobarbital- and 3-methylcholanthrene-treated rats with regard to benzphetamine demethylation and 3,4-benzo[*a*]pyrene hydroxylation.

In the present studies, *N*-hydroxylated metabolite formation with reconstituted preparations from both control and treated animals was much higher than that with their respective whole microsomal fractions (Table 2). van der Hoeven & Coon (1974) have obtained similar higher activities with reconstituted rabbit liver preparations while studying hydroxylation of benzphetamine and ethylmorphine. As in the studies by van der Hoeven & Coon (1974), in our system also cytochrome *P*-450 may be the rate-limiting component only in the reconstituted system and not in the whole microsomal fractions. In contrast with the *N*-hydroxylated metabolite, formation of ring-hydroxylated metabolites in a reconstituted system from treated animals was about 50% of that in the whole microsomal fraction. It is possible that the presence of small amounts of Triton X-100 in the cytochrome *P*-450 fraction might have been responsible for this inhibitory effect. We have observed that addition of Triton X-100 to whole microsomal fraction does inhibit ring-hydroxylation to a large extent (P. D. Lotlikar & K. Zaleski, unpublished work).

In contrast with other investigators (Lu *et al.*, 1973; van der Hoeven & Coon, 1974), in our present studies a requirement for exogenous lipid by reconstituted preparations could not be easily demonstrated (results not shown). It has been shown that Triton X-100 could not be removed completely in spite of extensive dialysis (Lu & Levin, 1974). Under such conditions Triton X-100 might substitute for phospholipid for 2-acetamidofluorene hydroxylation, as reported by Lu & West (1972).

It has been demonstrated that cytochrome b_5 is not an obligatory component in the NADPH-dependent hydroxylations of various compounds (Lu *et al.*, 1974). It appears that differences in hydroxylations observed in our present studies with reconstituted systems from control and 3-methylcholanthrene-pretreated rats could not be readily explained on the basis of their cytochrome b_5 content.

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