Role of Mixed-Function Amine Oxidase in *N*-Hydroxylation of 2-Acetamidofluorene by Hamster Liver Microsomal Preparations

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(Received 9 October 1973)

Pretreatment of hamsters with 3-methylcholanthrene (100mg/kg body wt.) 24h before death did not appreciably change the extent of *N*-oxide formation when hepatic microsomal preparations were incubated with *NN*-dimethylaniline as substrate. In contrast, the *N*-hydroxylation of 2-acetamidofluorene was increased severalfold in hepatic microsomal preparations from pretreated animals. Under these conditions there were no appreciable changes in cytochrome *P*-450 content and NADPH-cytochrome *c* reductase activity. On the basis of these comparative data, it is suggested that amine oxidase is not involved in *N*-hydroxylation of 2-acetamidofluorene.

A liver microsomal mixed-function amine oxidase has been purified which catalyses the oxidation of primary, secondary and tertiary amines (Ziegler *et al.*, 1971, 1973; Ziegler & Mitchell, 1972). This enzyme is a flavoprotein oxidase which is distinctly different from the NADPH-cytochrome *c* reductase (Masters & Ziegler, 1971).

N-Hydroxylation is an activation step in the carcinogenesis by 2-acetamidofluorene, several other aromatic amides and amines (Miller, 1970). This reaction is catalysed by an enzyme system in the liver endoplasmic reticulum in the presence of NADPH and O₂ (Irving, 1964). It was decided to determine whether mixed-function amine oxidase was involved in *N*-hydroxylation of aromatic amides.

Materials and methods

Male Syrian golden hamsters (100–120g body wt.), obtained from Huntingdon Farms, West Conshohocken, Pa., U.S.A., were maintained on a commercial diet (Wayne Lab-blox, obtained from Allied Mills, Chicago, Ill., U.S.A.) for 1 week before use in these experiments.

N-Hydroxy-2-acetamidofluorene was prepared in this laboratory (Poirier *et al.*, 1963). Other compounds were obtained as follows: 2-acetamidofluorene, Mann Research Laboratories, New York, N.Y., U.S.A.; *NN*-dimethylaniline (reagent grade), Fisher Scientific Co., Philadelphia, Pa., U.S.A.; 3-methylcholanthrene, Eastman Organic Chemical Co., Rochester, N.Y., U.S.A.; DL-isocitrate, NADP⁺, NADPH, horse heart cytochrome *c* (type III) and isocitrate dehydrogenase, Sigma Chemical Co., St. Louis, Mo., U.S.A.; *NO*-bis(trimethylsilyl)-

acetamide, Supelco, Bellefonte, Pa., U.S.A. All other chemicals were of reagent grade.

One group of hamsters was injected intraperitoneally with 3-methylcholanthrene (100mg/kg body wt.) suspended in 1ml of corn oil (CPC International, Englewood Cliffs, N.J., U.S.A.) 24h before death. Control group was injected with 1ml of corn oil. Liver microsomal fractions were prepared as described previously (Lotlikar, 1970).

The incubation medium for *N*-oxidation and dealkylation of *NN*-dimethylaniline used by Das & Ziegler (1970) was slightly modified. It contained 600μmol of glycine, 120μmol of sodium pyrophosphate buffer, pH8.4, 3μmol of NADP⁺, 60μmol of DL-sodium isocitrate, 6 units of isocitrate dehydrogenase, 30μmol of MgCl₂, 6μmol of semicarbazide and 15μmol of *NN*-dimethylaniline in a total volume of 6.0ml. The duplicate samples were equilibrated for 5min in 25ml Erlenmeyer flasks at 37°C, after which time the reaction was started by adding about 6–12mg of microsomal protein. Samples were incubated for 5min at 37°C. Formaldehyde produced was measured by the method of Nash (1953). The *N*-oxide of *NN*-dimethylaniline was determined by the method of Ziegler & Pettit (1964). A molar extinction coefficient of 8200 was used for determination of the amount of *N*-oxide formed. All of the data were corrected for blanks which were incubated with the complete system in the absence of the enzymes; after incubation 3.3M-HClO₄ was added before addition of the enzyme.

The incubation medium for *N*-hydroxylation of 2-acetamidofluorene contained 50mol of potassium phosphate buffer, pH7.8, 300μmol of KF, 6μmol of NADPH, 1.12μmol of 2-acetamidofluorene dissolved in 0.05ml of methanol and 1.5–3.0mg of

liver microsomal protein in a total volume of 3.0 ml. Liver microsomal proteins from control and 3-methylcholanthrene-pretreated animals were about 1.0 and 0.5 mg/ml of incubation medium respectively. After incubation for 20 min at 37°C, *N*-hydroxy-2-acetamidofluorene and other hydroxylated metabolites were extracted with diethyl ether as described previously (Lotlikar, 1970). The ether was removed under N₂ and the residue was treated with a silylating agent, *NO*-bis(trimethylsilyl)acetamide, in acetone for 1 h at room temperature. The trimethylsilyl derivative of *N*-hydroxy-2-acetamidofluorene was then separated and determined by g.l.c. A Glowall model 310 chromatograph with an argon ionization detector was used as an electron-capture detector. A 1.8 m circular glass column (4 mm internal diam.) was packed with Gas-Chrom Q (80–100 mesh) coated with 5% OV-1 (supplied by Applied Science Laboratories, State College, Pa., U.S.A.). Column, detector and flash-heater temperatures were maintained at 225°, 245° and 265°C respectively. With an argon flow rate of 50 ml/min, voltage was kept at 5 V. As a routine 1–10 µl of acetone extract was injected for determination. Synthetic *N*-hydroxy-2-acetamidofluorene after silylation had a retention time of about 5.1 min. About 0.02 nmol of trimethylsilyl ether of *N*-hydroxy-2-acetamidofluorene present in 10 µl of acetone could be easily detected by this method. There was very close agreement between this method of analysis and another method (P. D. Lotlikar & K. Wertman, unpublished work) using paper chromatography and liquid-scintillation spectrometry when 2-acetamido[9-¹⁴C]fluorene was used in the incubation. NADPH-cytochrome *c* reductase activity was assayed by the procedure of Masters *et al.* (1971). The amount of cytochrome *P*-450 in the liver microsomal material was determined by the procedure of Omura & Sato (1964). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Results and discussion

Primary, secondary and tertiary amines are oxidized by a mixed-function amine oxidase, a flavoprotein which is free of cytochrome *P*-450 and NADPH-cytochrome *c* reductase (Masters & Ziegler 1971; Ziegler *et al.*, 1971, 1973; Ziegler & Mitchell, 1972). It has been shown that the pH optimum for the mixed-function amine oxidase is about 8.4. At that pH the enzymic dealkylation of *NN*-dimethylaniline *N*-oxide is almost negligible; formaldehyde formed at that pH therefore might be due to direct attack on the amine by some other mixed-function oxidase (Das & Ziegler, 1970). The present experiments were therefore carried out at the optimum pH of the amine oxidase (Table 1). In addition to determination of *N*-oxide formation, formaldehyde was also measured. The rates of formation of these products were linear with time at two concentrations of microsomal proteins. Formation of neither *N*-oxide nor formaldehyde was changed appreciably when hamsters were pretreated with 3-methylcholanthrene.

N-Hydroxylation of 2-acetamidofluorene by liver microsomal fractions was increased severalfold when hamsters were pretreated with 3-methylcholanthrene (Table 2). Data on the increase in *N*-hydroxylation are in agreement with our previous data (Lotlikar *et al.*, 1967; Lotlikar & Wasserman, 1972) and with those reported by Matsushima & Weisburger (1972). Arrhenius (1968, 1969–70) suggested that the mixed-function amine oxidase which oxidized *NN*-dimethylaniline to its *N*-oxide was also responsible for *N*-oxidation of carcinogenic aromatic amines and amides. If the mixed-function amine oxidase were responsible for *N*-hydroxylation of 2-acetamidofluorene, then differences similar to those obtained for *N*-hydroxylation of 2-acetamidofluorene (Table 2) would have been expected for *N*-oxidation of *NN*-dimethylaniline.

Table 1. Effect of 3-methylcholanthrene pretreatment on *N*-oxidation and dealkylation of *NN*-dimethylaniline by hamster liver microsomal fractions

All details are described under 'Materials and methods'. Liver from one hamster was used for each analysis. Results are given as means ± S.E.M. of determinations on six animals. *Statistical comparisons between control and 3-methylcholanthrene-pretreated groups with *P* < 0.05 are considered significant.

Treatment	Oxidation products (nmol formed/min per mg of protein)		
	<i>NN</i> -Dimethylaniline <i>N</i> -oxide (A)	Formaldehyde (B)	(A) + (B)
Control	6.1 ± 1.3	8.3 ± 1.8	14.4 ± 2.1
3-Methylcholanthrene	4.3 ± 0.7*	8.4 ± 0.8	12.6 ± 1.2

Table 2. Activities of various enzyme systems in hamster liver microsomal fractions prepared from control or 3-methylcholanthrene-pretreated animals

The same hamster liver microsomal preparations used for the studies described in Table 1 were also used for these studies. All other details are described under 'Materials and methods'. Liver microsomal preparation from one hamster was used for each analysis. Results are given as means $\pm$ S.E.M. of determinations on six animals. *Statistical comparisons between control and 3-methylcholanthrene-pretreated groups with $P < 0.05$ are considered significant.

Treatment...	Activity	
	Control	3-Methylcholanthrene
<i>N</i> -Hydroxylation of 2-acetamidofluorene (nmol formed/20min per mg of protein)	4.1 $\pm$ 1.5	26.3 $\pm$ 7.4*
Cytochrome <i>P</i> -450 (nmol/mg of protein)	1.5 $\pm$ 0.6	2.1 $\pm$ 0.7
NADPH-cytochrome <i>c</i> reductase (nmol/min per mg of protein)	114 $\pm$ 25	168 $\pm$ 29*

This, however, was not the case (Table 1). On the basis of these comparative data, it is suggested that amine oxidase is not directly involved in *N*-hydroxylation of 2-acetamidofluorene. In view of our present results, it is suggested that extrapolation of data on *N*-oxidation of *NN*-dimethylaniline to *N*-oxidation of carcinogenic aromatic amines and amides (Arrhenius, 1968, 1969–70) should be re-evaluated.

In contrast with previous CO-inhibition data (Kampffmeyer & Kiese, 1965; Matsushima *et al.*, 1972; Matsushima & Weisburger, 1972), it now appears that *N*-hydroxylation of 2-acetamidofluorene by hepatic microsomal fractions is inhibited by mixtures of CO and O₂ in different proportions (Gutmann & Bell, 1973; Thorgeirsson *et al.*, 1973). These results suggest a possible involvement of cytochrome *P*-450 in the *N*-hydroxylation reaction. Several investigators have reported no good correlation between changes in oxidation of many foreign compounds by hepatic microsomal fractions from several species, the cytochrome *P*-450 content and NADPH-cytochrome *c* reductase activity (Davies *et al.*, 1969; Holtzman *et al.*, 1968). In the present experiments also (Table 2), even though there was a severalfold increase in *N*-hydroxylation of 2-acetamidofluorene by hepatic microsomal fractions from hamsters after 3-methylcholanthrene treatment, there was little or no change either in cytochrome *P*-450 content or NADPH-cytochrome *c* reductase activity in these two groups of animals. Our data on cytochrome *P*-450 are in agreement with those reported by Matsushima & Weisburger (1972). Recent results indicate that there is more than one type of cytochrome *P*-450 in hepatic microsomal fractions (Lu *et al.*, 1971, 1973). Solubilization and purification of these proteins would help in elucidating the roles of these macromolecules in activation and inactivation processes involved in drug metabolism and chemical carcinogenesis.

This investigation was supported by a research grant (CA-10604) and a Career Development Award (K04-CA-42362) to P. D. L. from the National Cancer Institute, U.S. Public Health Service.

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