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Inhibitory Effect of Carbon Monoxide on the *N*- and Ring-Hydroxylation of 2-Acetamidofluorene by Hamster Hepatic Microsomal Preparations

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The effect of CO on *N*-, 3-, 5- and 7-hydroxylation of 2-acetamidofluorene by liver microsomal fractions from control and 3-methylcholanthrene-pretreated hamsters was studied. All hydroxylations were inhibited by CO, but the degree of inhibition was different for each hydroxylation. The ratios of CO to O_2 needed for 50% inhibition of the *N*-, 3-, 5- and 7-hydroxylations by control preparations were 8.0:1, 8.2:1, 4.2:1 and 7.1:1 respectively and by preparations from treated animals were 4.2:1, 8.9:1, 2.3:1 and 3.2:1 respectively. These results are discussed in terms of the possible presence of more than one type of cytochrome *P*-450 involved in hydroxylations of 2-acetamidofluorene by liver microsomal fractions from both control and pretreated hamsters.

It is well established that *N*-hydroxylation is an activation step, whereas ring-hydroxylation is an inactivation process, in carcinogenesis by aromatic amines and amides (Miller, 1970; Weisburger & Weisburger, 1973). Both ring- (Booth & Boyland, 1957; Seal & Gutmann, 1959; Cramer *et al.*, 1960) and *N*-hydroxylation (Irving, 1964) occur in the liver endoplasmic reticulum and require NADPH and O_2. Pretreatment of several species of animals with 3-methylcholanthrene elicits different amounts of induction of these enzyme systems. In hamsters, pretreatment with this inducer 24h before death produces a severalfold increase in *N*-hydroxylation of the carcinogenic aromatic amide 2-acetamidofluorene without appreciably increasing ring-hydroxylation (Lotlikar *et al.*, 1967).

Inhibition studies with CO have demonstrated participation of cytochrome *P*-450 in the *N*-hydroxylation of 2-acetamidofluorene by hepatic microsomal preparations from rats (Gutmann & Bell, 1973), mice and hamsters (Thorgeirsson *et al.*, 1973). If cytochrome *P*-450 is also involved in the ring-hydroxylation process, it is important to determine whether the same cytochrome *P*-450 which is involved in *N*-hydroxylation also participates in ring-hydroxylation of the carcinogen.

In the present study, we report the effects of various mixtures of CO and O_2 on *N*- and ring-hydroxylation of 2-acetamidofluorene by hepatic microsomal preparations from hamsters pretreated with 3-methylcholanthrene and from untreated hamsters.

Materials and methods

2-Acetamidofluorene was purchased from Mann Research Laboratories, New York, N.Y., U.S.A. 2-Acetamido[9-^{14}C]fluorene (sp. radioactivity 6.7mCi/mmol) was obtained from Tracer Lab, Waltham, Mass., U.S.A. 3-Methylcholanthrene was purchased from Eastman Organic Chemical Co., Rochester, N.Y., U.S.A. NADPH was a product of Sigma Chemical Co., St. Louis, Mo., U.S.A. Calibrated gas mixtures of CO and O_2 in N_2 were obtained from Union Carbide Corp., Linden, N.J., U.S.A. All other chemicals were of reagent grade.

Male Syrian golden hamsters (100–150g 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 at least 1 week before use in the present study. One group of hamsters was injected intraperitoneally with 3-methylcholanthrene (100mg/kg body wt.) suspended in 1ml of corn oil 24h before death. Controls were injected with corn oil alone. Liver microsomal fractions were prepared as described previously (Lotlikar, 1970).

Warburg respirometer apparatus was used for these studies. The main compartment of each Warburg flask contained 50μmol of potassium phosphate buffer, pH7.8, 50μmol of sucrose, 300μmol of KF, 1.12μmol of 2-acetamidofluorene containing 0.1μCi of 2-acetamido[9-^{14}C]fluorene dissolved in 0.05ml of methanol and 1.5–3.0mg of liver microsomal protein in a total volume of 2.8ml. The side arm contained 6μmol of NADPH in 0.2ml. After attachment to the manometer each flask was equilibrated with appropriate gas mixtures for 5min, the manometer and side arm were closed and NADPH was tipped in the main compartment from the side arm. Duplicate samples were incubated for 20min at 37°C. During incubation, flasks were covered completely with aluminium foil.

After incubation a sample (2.5ml) was withdrawn from each flask and added to 4ml of ice-cold 1M-sodium acetate buffer, pH6.0. Hydroxylated metabolites of 2-acetamidofluorene were extracted from the incubation medium with diethyl ether as described previously (Lotlikar, 1970). The hydroxylated metabolites were separated by paper chromatography on Whatman no. 1 paper with the solvent system cyclohexane–2-methylpropan-2-ol–acetic acid–water (16:4:2:1, by vol.) (Weisburger *et al.*, 1956). After chromatography appropriate zones were cut out, put into vials and eluted in 1ml of methanol for 30min. Then 10ml of Bray's (1960) solution was added to each vial and radioactivity was measured by liquid-scintillation spectrometry. Protein content of microsomal preparations was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and discussion

The present results, of severalfold increase in *N*-hydroxylation without similar increase in ring-hydroxylation of 2-acetamidofluorene by liver microsomal fractions from hamsters after 3-methylcholanthrene pretreatment (Table 1), are in agreement with our previous data (Lotlikar *et al.*, 1967; Lotlikar & Wasserman, 1972) and with those reported by Matsushima & Weisburger (1972).

A role for cytochrome *P*-450 in *N*-hydroxylation of aromatic amines and amides has been questioned for several years. The ability of mixtures of CO and O_2 in different proportions to inhibit an NADPH-dependent substrate oxidation by a microsomal fraction is usually considered to be evidence for cytochrome *P*-450 participation in that particular oxidative reaction. Kampffmeyer & Kiese (1965) reported no CO inhibition of *N*-hydroxylation of aniline and *N*-ethylaniline by rabbit liver microsomal preparations. Similarly, *N*-hydroxylation of 2-acetamidofluorene was not inhibited when liver microsomal fractions from control and phenobarbital-treated rats (Matsushima *et al.*, 1972) and control hamsters (Matsushima & Weisburger, 1972) were incubated in the presence of air and CO in the proportions 1:1. In contrast with these studies (Matsushima *et al.*, 1972; Matsushima & Weisburger, 1972) it now appears that CO and O_2 mixtures do inhibit *N*-hydroxylation of this carcinogenic compound by liver microsomal fractions from rats (Gutmann & Bell, 1973), mice and hamsters (Thorgeirsson *et al.*, 1973). Our present data (Table 1) indicate significant inhibition of *N*-hydroxylation with control hamsters in the presence of CO and O_2 in the proportions 3.7:1 and 9.4:1. These results are in agreement with those reported by Thorgeirsson *et al.* (1973). The lack of inhibition of *N*-hydroxylation reported by Matsushima *et al.* (1972) and Matsushima & Weisburger (1972) may not be due to lower proportions of CO to O_2 in their gas mixtures during incubation, as suggested by Thorgeirsson *et al.* (1973), because in the present study a significant inhibition of *N*-hydroxylation was obtained in the presence of a $CO:O_2$ mixture in the ratio 3.7:1 compared with the ratio 5:1 used by Matsushima *et al.* (1972) and Matsushima & Weisburger (1972). Both *N*- and ring- (3-, 5- and 7-) hydroxylations by preparations from treated hamsters appeared to be more sensitive to CO than those from controls (Table 1). Similar results were also obtained with ring-hydroxylations by Matsushima & Weisburger (1972).

Table 1. *Effect of CO on 2-acetamidofluorene hydroxylation by liver microsomal fractions from control and 3-methylcholanthrene-pretreated hamsters*

The following calibrated gas mixtures of CO and O_2 in N_2 were used: 4.3% O_2, 0% CO; 4.3% O_2, 4% CO; 4.3% O_2, 8% CO; 4.3% O_2, 15.9% CO; 4.3% O_2, 40.3% CO. All other details are described under 'Materials and methods'. Livers from two hamsters were used for each analysis. Results are given as means ± S.E.M. Statistical comparisons are made within control and 3-methylcholanthrene-pretreated groups: * statistical comparisons between the group containing 0% CO and other groups containing various percentages of CO with *P* values <0.05 are considered significant.

Pretreatment	Gas phase $CO:O_2$ ratio	No. of analyses	2-Acetamidofluorene derivative	Hydroxylation (nmol formed/20min per mg of protein)			
				N-Hydroxy	3-Hydroxy	5-Hydroxy	7-Hydroxy
Control	0	8		1.70±0.54	0.43±0.27	3.0±1.5	15.5±5.7
	0.9:1	8		1.75±0.68	0.50±0.35	3.1±1.1	13.0±5.2
	1.9:1	6		1.40±0.37	0.35±0.13	1.8±0.41	11.1±4.5
	3.7:1	8		1.20±0.25*	0.38±0.24	1.5±0.30*	10.2±2.1*
	9.4:1	8		0.73±0.30*	0.20±0.07*	1.0±0.37*	6.7±3.3*
3-Methylcholanthrene	0	8		15.7 ±2.9	1.4 ±0.40	5.2±1.5	27.6±3.9
	0.9:1	8		13.2 ±3.8	1.4 ±0.52	3.4±1.4*	22.4±5.5
	1.9:1	7		11.1 ±1.5*	1.1 ±0.43	2.6±0.92*	18.0±2.8*
	3.7:1	7		8.1 ±1.4*	0.91±0.38*	2.2±0.48*	12.2±3.2*
	9.4:1	8		4.9 ±1.8*	0.75±0.21*	1.2±0.53*	7.2±1.5*

Table 2. *Gaseous inhibition constants for 2-acetamidofluorene hydroxylations by hamster liver microsomal preparations*

Results which were statistically significant from Table 1 were used for calculations of gaseous inhibition constants. The gaseous inhibition constant (K) is the ratio of CO to O_2 needed for 50% inhibition. It is calculated from the partition equation (Warburg & Kubowitz, 1929)

$$K = \frac{n}{1-n} \times \frac{[CO]}{[O_2]}$$

where n is the ratio of the rate of hydroxylation in the presence of CO to the rate of hydroxylation in the absence of CO. The concentrations of CO and O_2 are the percentages of each in various gas mixtures used for hydroxylation. Results are given as means±S.E.M., for the numbers of determinations given in parentheses. *Statistical comparisons between control and 3-methylcholanthrene-pretreated groups for each hydroxylation with P values <0.05 are considered significant. †Statistical comparisons between N-hydroxylation within control and 3-methylcholanthrene-pretreated groups and other hydroxylations with P values <0.05 are considered significant. ‡ Statistical comparison within the control group between N-hydroxylation and 5-hydroxylation gave a P value >0.05 but <0.1; the P value >0.05 is considered not significant.

	Gaseous inhibition constant (K)	
Reaction assayed	Control	3-Methylcholanthrene-pretreated
N-Hydroxylation	7.97±0.93 (2)	4.23±0.23* (3)
3-Hydroxylation	8.15 (1)	8.85±1.90† (2)
5-Hydroxylation	4.19±0.49 (2)‡	2.28±0.48*† (4)
7-Hydroxylation	7.13±0.01 (2)	3.24±0.23*† (3)

Calculations of the results presented in Table 1 in terms of gaseous inhibition constants show striking differences between control and pretreated animals (Table 2). The constants for N-, 5- and 7-hydroxylation in the control group (but not that for 3-hydroxylation) are about twice as much as those for the respective hydroxylations in the pretreated group. There were no significant differences in constants for various hydroxylations within the control group. However, differences in constants were significant among the 3-methylcholanthrene-pretreated group. Conney *et al.* (1968) observed different degrees of inhibition for hydroxylation of testosterone at positions 6β, 7α and 16α when testosterone was incubated with rat liver microsomal preparations in the presence of CO–O_2 mixtures. Like the suggestion by Conney *et al.* (1968), the present data also indicate the presence of more than one type of cytochrome P-450 in the hamster liver preparations.

Recently, solubilization and partial purification of these cytochromes have been achieved. In their reconstitution experiments, Lu *et al.* (1971, 1973) have shown different specificity in the cytochrome P-450 fractions from livers of control, phenobarbital- and 3-methylcholanthrene-pretreated rats with respect to benzphetamine demethylation, hydroxylation of 3,4-benzo[a]pyrene and testosterone and the oxidation of several other substrates. Similarly, in our reconstitution studies with hamster liver microsomal fractions we have demonstrated that the N-hydroxylation activity of the cytochrome P-450 fraction from 3-methylcholanthrene-pretreated animals is different from and severalfold greater than that of the cytochrome P-450 fraction from controls (Lotlikar *et al.*, 1974). Three spectrally distinguishable forms of cytochrome P-450 have now been isolated from rat liver microsomal fractions (Comai & Gaylor, 1973). However, there was no evidence in those studies as to the presence of catalytic activity in the purified preparations. Characterization of these proteins and their catalytic activity would help in understanding the mechanisms of oxidation by these macromolecules.

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