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Effects of 3-Methylcholanthrene Pretreatment on Microsomal Hydroxylation of 2-Acetamidofluorene by Various Rat Hepatomas

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

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1. The effects of 3-methylcholanthrene pretreatment on both *N*- and ring hydroxylation of 2-acetamidofluorene by microsomal preparations from various well-differentiated and poorly differentiated hepatomas, primary tumours and their host livers and kidneys were studied. 2. Well-differentiated Morris hepatomas 5123C, 5123D, 5123CTC and 7800 and their host livers had low hydroxylating activity. Pretreatment with 3-methylcholanthrene caused a several-fold increase in both *N*- and ring hydroxylation in the host livers whereas in all tumours except 5123CTC it caused a many-fold increase only in ring hydroxylation. 5123CTC tumour in addition showed a fourfold increase in *N*-hydroxylating activity. 3. Hydroxylating activities of poorly differentiated Morris hepatoma 7288CTC and Novikoff hepatoma were low and they could not be altered by 3-methylcholanthrene pretreatment. 4. Primary hepatomas produced by administration of 4-dimethylamino-3′-methylazobenzene could be stimulated to some extent on 3-methylcholanthrene pretreatment; however, primary mammary tumour produced by administration of 3-methylcholanthrene was not responsive to 3-methycholanthrene pretreatment. 5. Like host livers, kidneys of tumour-bearing animals could also be stimulated to some extent by 3-methylcholanthrene pretreatment.

Many foreign compounds, including drugs and chemical carcinogens, are oxidized by liver microsomes of all animal species studied so far (Conney, 1967). Several transplantable rat tumours such as Morris hepatomas 5123C, 5123D and 7800 have been described as minimal deviation or well-differentiated hepatomas because they have many morphological and biochemical characteristics closely resembling normal liver; these hepatomas were initially produced by oral administration of various fluorene derivatives (Morris, 1965). These, as well as poorly differentiated and primary liver tumours, have low or negligible enzyme activity to oxidize foreign compounds (Conney, Brown, Miller & Miller, 1957; Neubert & Hoffmeister, 1960; Adamson & Fouts, 1961; Conney & Burns, 1963; Hart, Adamson, Morris & Fouts, 1965; Roger, Morris & Fouts, 1967). Conney & Burns (1963) have shown that hepatoma 5123C had low azo-dye *N*-demethylase enzyme activity and that this activity could be stimulated by administration of MC.* Phenobarbital pretreatment of tumour-bearing animals has also been shown to induce drug-metabolizing enzyme activities in several liver tumours (Hart *et al.* 1965; Roger *et al.* 1967).

N-Hydroxylation of AAF was first reported by Cramer, Miller & Miller (1960*b*). They showed that *N*-hydroxy-AAF appeared in the rat urine as a conjugate (probably the glucuronide) and was found in increasing amounts as the feeding of the carcinogen progressed. The potent carcinogen AAF is metabolized by rat liver microsomes, not only to various non-carcinogenic ring-hydroxylated products, namely 1-, 3-, 5- and 7-hydroxy-AAF (Booth & Boyland, 1957; Seal & Gutmann, 1959; Cramer, Miller & Miller, 1960*a*), but also to more carcinogenic *N*-hydroxy derivatives (Irving, 1964; Lotlikar, Enomoto, Miller & Miller, 1967). Administration of MC with AAF inhibits the carcinogenicity of AAF in the rat (Miyaji *et al.* 1953; Miller, Miller, Brown & MacDonald, 1958). Pretreatment of rats with MC also causes a decreased urinary excretion of *N*-hydroxy-AAF (Miller, Cramer & Miller, 1960; Lotlikar *et al.* 1967). However, in studies *in vitro* with rat liver microsomes, both ring and *N*-hydroxylation of AAF are increased several-fold when MC is administered to animals 24h before they are killed (Cramer *et al.* 1960*a*; Lotlikar *et al.* 1967). On the other hand, after pretreatment of hamsters with MC, there is a specific and relatively

* Abbreviations: MC, 3-methylcholanthrene; AAF, 2-acetamidofluorene; *x*-hydroxy-AAF, 2-acetamido-*x*-hydroxyfluorene; 3′-methyl-DAB, 4-dimethylamino-3′-methylazobenzene.

large increase only in *N*-hydroxylation by their liver microsomes (Lotlikar *et al.* 1967). From carbon monoxide inhibition studies it has been suggested that cytochrome *P*-450 is involved in ring hydroxylation and not in *N*-hydroxylation of aromatic amines (Kampffmeyer & Kiese, 1965; Hlavica & Kiese, 1969).

The purpose of the present work was to determine the *N*- and ring hydroxylation of AAF by microsomal preparations of various transplantable and primary hepatomas and their host livers with and without MC pretreatment of tumour-bearing animals.

MATERIALS AND METHODS

Animals. The slow growing well-differentiated Morris hepatomas 5123C, 5123D, 5123CTC and 7800 and poorly differentiated hepatomas 7288CTC were transplanted either intramuscularly or subcutaneously in Buffalo female rats. These tumour-bearing animals were kindly provided by Dr Harold P. Morris, School of Medicine, Howard University, Washington, D.C., U.S.A. The tumours were implanted in Washington and the tumour-bearing rats were shipped to Philadelphia, where they were maintained on a commercial diet until the tumours were large enough to use. The Novikoff hepatomas were propagated in Philadelphia by subcutaneous transplantation in female rats of Sprague–Dawley strain obtained from Holtzman Rat Co., Madison, Wis., U.S.A. The primary liver tumours were produced in adult male rats of CFN strain weighing 175–200g obtained from Carworth Farms Co., New City, N.Y., U.S.A., by feeding 0.06% 3′-methyl-DAB as described by Shatton, Donnelly & Weinhouse (1962). The primary mammary tumours were produced in adult Sprague–Dawley-strain female rats by oral administration of 10mg of MC/day for 20 consecutive days for a total of 200mg as described by Gruenstein, Meranze, Thatcher & Shimkin (1966). These mammary-tumour-bearing animals were kindly supplied by Mrs Margot Gruenstein of this Institute. Adult female Buffalo rats were used as control for these studies.

Chemicals. AAF was obtained from Mann Research Laboratories, New York, N.Y., U.S.A.; 3′-methyl-DAB and MC were purchased from Eastman Kodak Co., Rochester, N.Y., U.S.A. Authentic 1-, 3-, 5- and 7-hydroxy-AAF were kindly supplied by Dr James A. Miller, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wis., U.S.A. *N*-Hydroxy-AAF was prepared as described by Poirier, Miller & Miller (1963). NADH, NADPH and ATP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were of reagent grade.

Injection of MC. Animals were injected intraperitoneally with MC (10mg/100g body wt.) suspended in olive oil (10mg/ml) 24h before they were killed. Animals bearing primary tumours with 3′-methyl-DAB were fed on regular commercial diet for a week before MC injection. Control animals were injected with olive oil.

Preparation of microsomes. After the animals were decapitated, the tissues were immediately removed and chilled in ice-cold 0.25M-sucrose solution. Tumours were then quickly freed from surrounding tissues and from necrotic material, if any. A 10% (w/v) homogenate of tissue prepared in 0.25M-sucrose solution was centrifuged at 8000*g* for 10min to remove nuclei and mitochondria. The microsomal pellet was obtained by centrifuging the mitochondrial supernatant at 105000*g* for 60min. The surface of the microsomal pellet was washed twice with 0.25M-sucrose before it was resuspended in the same medium.

Assay for ring and N-hydroxylation of AAF. The incubation medium contained 100μmol of potassium phosphate buffer, pH7.8, 400μmol of KCl, 240μmol of nicotinamide, 600μmol of KF, 4μmol of ATP, 1.06μmol of NADPH, 1.22μmol of NADH, 0.45μmol of AAF added in 0.2ml of methanol, tissue microsomes equivalent to 60mg wet wt. of tissue, 150μmol of sucrose and water to a final volume of 6.0ml. This incubation medium is almost identical with that described by Cramer *et al.* (1960*a*) for ring hydroxylation of AAF except for the addition of KF. The presence of 0.1M-KF in the incubation system inhibits deacetylase activity (Seal & Gutmann, 1959; Irving, 1964; Lotlikar, Miller, Miller & Margreth, 1965). The flasks were incubated in air for 20min at 37°C, after which 8ml of ice-cold-1M-sodium acetate buffer, pH6.0, was added per flask. Contents of 18 flasks were combined for each analysis and extracted immediately with diethyl ether. The acidic metabolites were extracted from the ether extract into 0.5M-NaOH; finally the alkali extract was neutralized to pH6.0 and the metabolites were extracted in diethyl ether. The acidic metabolites were chromatographed on Whatman no. 1 paper with cyclohexane–2-methylpropan-2-ol–acetic acid–water (16:4:2:1, by vol.) (Weisburger, Weisburger, Morris & Sober, 1956). After chromatography the strips were air-dried and then viewed under u.v. light. Appropriate absorption zones were cut out and eluted overnight in 3ml of 95% (v/v) ethanol. The metabolites were determined quantitatively by u.v. absorption in the range 350–270nm by using a Hitachi–Coleman recording spectrophotometer. The following molar extinction coefficients with maximum wavelengths in parentheses were used for authentic hydroxy compounds of AAF: *N*-hydroxy-AAF, 23400 (287nm); 3-hydroxy-AAF, 17400 (315nm); 5-hydroxy-AAF, 25500 (283nm); 7-hydroxy-AAF, 29800 (291nm). Before spectral analysis, the *N*-hydroxy-AAF was separated from AAF by extraction with 0.5M-NaOH. All of the results presented were corrected by the following recovery data: *N*-hydroxy-AAF, 75%; 3-hydroxy-AAF, 70%; 5-hydroxy-AAF, 90%; 7-hydroxy-AAF, 63%. The amounts of 1-hydroxy-AAF were generally too low to measure adequately and the values for this metabolite have therefore been omitted. Wherever values of 10 or less nmol/20min per g wet wt. of tissue are given for any metabolite, it indicates that a small amount of u.v.-light-absorbing material with a non-characteristic spectrum was eluted.

RESULTS

Rates of both *N*- and ring hydroxylation of AAF by liver and kidney microsomes from normal rats with and without MC pretreatment are presented in Table 1. The liver of untreated control animals has much greater hydroxylating activity than has

Table 1. *Effects of MC pretreatment on the* N- *and ring hydroxylation of AAF by liver and kidney microsomes from control rats*

Adult female Buffalo strain rats weighing 200–250g were used in these experiments. Other details are as described in the Materials and Methods section. Results are given as means ±S.E.M. of determinations of four animals.

		Hydroxylation (nmol formed/20min per g wet wt. of tissue)			
Tissue	MC pretreatment	*N*-Hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF
Liver	−	25± 8	13± 2	17± 3	115± 10
	+	139±31	204±70	251±75	748±184
Kidney	−	9± 3	5± 2	2± 1	4± 1
	+	10± 2	28± 8	19± 3	65± 14

Table 2. *Effects of MC pretreatment on* N- *and ring hydroxylation of AAF by well-differentiated rat hepatomas and the host liver and kidney*

All details are described in the Materials and Methods section. Results are given as means±S.E.M.

				Hydroxylation (nmol formed/20 min per g wet wt. of tissue)			
Tissue	Rat tumour	MC pretreatment	No. of analyses	*N*-hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF
Liver	5123D	−	4	16± 6	7± 5	11± 3	23± 11
		+	2	46± 3	139±28	282± 4	453± 39
Tumour		−	4	4± 3	3± 1	6± 2	10± 4
		+	2	9± 1	90± 7	93±16	152± 14
Liver	5123C	−	4	18± 2	6± 3	10± 2	18± 5
		+	2	156±46	152±42	335±55	512±112
Kidney		−	2	6± 2	10± 3	8± 1	10± 5
		+	2	3± 1	48± 5	23± 6	49± 11
Tumour		−	5	10± 6	2± 2	5± 5	6± 4
		+	2	13± 3	85± 5	70±10	129± 9
Liver	5123CTC	−	2	11± 3	6± 1	15± 4	21± 5
		+	2	43±17	133± 4	141± 3	228± 42
Tumour		−	2	9± 4	11± 4	15± 5	11± 2
		+	2	37± 7	82± 4	59± 3	93± 10
Liver	7800	−	2	16± 3	3± 2	13± 2	25± 13
		+	4	88±16	184±48	152±10	290± 69
Tumour		−	2	7± 3	6± 1	7± 3	10± 1
		+	4	6± 2	36±10	28± 7	52± 7

kidney. Pretreatment of animals with MC increased both *N*- and ring hydroxylating activity of liver several-fold. Similar results were obtained with weanling male rats (Lotlikar *et al.* 1967). Activity in the kidney of treated animals showed several-fold increase in ring hydroxylation without affecting concentrations of *N*-hydroxy-AAF. The enzyme activities in kidney after MC stimulation were much less than in liver. Wattenberg & Leong (1962) and Gelboin & Blackburn (1964) have also reported increases in benzpyrene hydroxylase in kidney extracts after MC pretreatment of animals.

The activities of various well-differentiated hepatomas and their host livers are summarized in Table 2. Tumour tissues and livers of all untreated tumour-bearing rats had low hydroxylating activity; activity of the livers was higher than that of tumours. Pretreatment with MC caused several-fold increase in both *N*- and ring hydroxylation in the host livers, whereas in tumours except 5123CTC it caused a many-fold increase in ring hydroxylation only. Tumour 5123CTC showed a fourfold increase in *N*-hydroxylation activity also. Kidneys of the tumour-bearing rats also responded to MC pretreatment, like kidneys of non-tumour-bearing rats (see Table 1).

The effects of MC administration on the rates of AAF hydroxylation by poorly differentiated rat hepatomas and their host livers are given in Table 3. Host livers of untreated rats bearing 7288CTC tumour formed much greater amounts of *N*- and ring-hydroxylated products of AAF compared with

Table 3. *Hydroxylation of AAF by poorly differentiated rat hepatomas and the host liver with and without MC pretreatment*

All details are as described in the Materials and Methods Section. Values are given as means±S.E.M. of three determinations.

			Hydroxylation (nmol formed/20 min per g wet wt. of tissue)			
Tissue	Rat tumour	MC pretreatment	*N*-Hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF
Liver	7288CTC	−	89±29	120±20	113±10	249±33
		+	83±17	146± 6	202±13	609±17
Tumour		−	11± 3	4± 1	7± 2	5± 3
		+	9± 2	5± 2	6± 3	8± 5
Liver	Novikoff	−	8± 3	5± 2	12± 4	52±12
		+	14± 5	102±12	314±62	456±87
Tumour		−	6± 2	2± 1	5± 2	6± 1
		+	8± 3	5± 1	6± 3	6± 2

Table 4. *Effects of MC pretreatment on hydroxylation of AAF by primary tumours and the host tissues*

The primary liver tumours were produced in adult male rats weighing 175–200 g by administration of 0.06% 3′-methyl-DAB for 17 weeks as described by Shatton *et al.* (1962). These animals were fed on regular commerical diet for a week before MC injection. The primary mammary tumours were produced in adult Sprague–Dawley-strain female rats by oral administration of 10 mg of MC/day for 20 days. All other details are described in the Materials and Methods Section. Values are given as means±S.E.M. of three determinations.

			Hydroxylation (nmol formed/20 min per g wet wt. of tissue)			
Tissue	Rat tumour	MC pretreatment	*N*-Hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF
Liver	Liver	−	19±1	13± 3	8± 4	30± 15
		+	16±3	112±62	104±14	373±147
Tumour		−	8±3	6± 2	3± 2	7± 3
		+	6±2	25± 2	11± 5	44± 3
Liver	Mammary	−	10±2	27± 6	10± 3	31± 13
		+	28±4	127± 3	123±19	218± 24
Tumour		−	9±1	2± 1	8± 3	3± 1
		+	6±2	3± 2	16± 3	7± 4
Kidney		−	10±4	5± 2	8± 1	6± 2
		+	7±2	42± 9	38± 7	50± 18

host livers of Novikoff or well-differentiated-tumour bearing rats (see Table 1). Pretreatment of 7288CTC-tumour-bearing rats with MC increased the formation of 7- and 5-hydroxy-AAF by the liver about twofold without affecting the amounts of *N*- and 3-hydroxy-AAF. On the other hand, MC pretreatment of rats bearing Novikoff tumour caused several-fold increase in ring hydroxylation, i.e. formation of 7-, 5- and 3-hydroxy-AAF, by their livers without appreciably affecting amounts of *N*-hydroxy-AAF. Hydroxylating activities of both types of tumours were low and they could not be altered by MC pretreatment of animals.

The results presented above were obtained only with transplanted hepatomas. It was important to investigate whether hydroxylating activities of primary tumours could be altered by MC pretreatment of tumour-bearing animals. Results obtained with primary hepatomas produced by 3′-methyl-DAB and mammary tumours produced by MC are presented in Table 4. Hydroxylation of AAF by liver from both groups of tumour-bearing animals could be increased several-fold by MC administration. Hepatoma showed an increase in ring-hydroxylating activity on MC pretreatment, whereas mammary tumour was unresponsive.

DISCUSSION

The above results show a great variation in AAF-hydroxylating activities of various tumours and their host livers on MC pretreatment of animals. Host livers of all tumour bearing animals except those with tumour 7288CTC demonstrated low hydroxylating ability, which could be stimulated several-fold by administration of MC 24 h before the animals were killed.

Prior or simultaneous administration of ethionine,

puromycin or actinomycin D with either MC or phenobarbital prevents the stimulation of microsomal oxidative enzymes (Conney, Miller & Miller, 1957; Conney, Davison, Gastel & Burns, 1960; Gelboin & Blackburn, 1964; Lotlikar *et al.* 1967). These and amino acid-incorporation studies (Gelboin & Blackburn, 1963) indicate that MC, phenobarbital and several other inducers (Conney, 1967) cause induction or synthesis of new microsomal enzyme proteins.

It has been shown that several well-differentiated tumours, namely Morris hepatomas 5123C, 5123D and 7800, have either little or no ability to metabolize various drugs (Conney & Burns, 1963; Hart *et al.* 1965; Lotlikar *et al.* 1965; Roger *et al.* 1967). The present results are in agreement with these earlier findings. Pretreatment of animals with MC caused several-fold increase in the hydroxylating activities of tumours compared with the tumours of untreated animals. These stimulations are much more pronounced than those observed with phenobarbital treatment (Hart *et al.* 1965; Roger *et al.* 1967). In the present experiments only one tumour, Morris hepatoma 5123CTC, could *N*-hydroxylate AAF when tumour-bearing animals were pretreated with MC. This appears to be the first example of *N*-hydroxylation of AAF by any tumour tissue studied so far. The present results suggest that the activities of oxidative enzymes are low or diminished in well-differentiated tumours, but that the enzyme activities can be increased in these tumours by certain inducers such as MC.

In contrast with well-differentiated hepatomas, poorly differentiated hepatomas could not be stimulated by MC pretreatment. Thus, in my experiments, both tumours 7288CTC and Novikoff were resistant to MC effect. Hart *et al.* (1965) observed that pretreatment of Novikoff-tumour-bearing animals with phenobarbital did not have any effect on the metabolism of aminopyrine, neoprontosil or *p*-nitrobenzoic acid by a supernatant fraction from the Novikoff hepatoma. They reported, however, that the side-chain oxidation of hexobarbital was stimulated in this tumour to a slight but statistically significant extent. It appears that various substrates have different affinities for the oxidative enzymes. In the present studies, host liver of 7288CTC-tumour-bearing animals had high *N*- and ring-hydroxylating activity before MC pretreatment. Administration of MC increased the activity about twofold. One possible explanation for the high activity in the host liver before MC pretreatment could be that the tumour might have acted as an inducer for the host liver.

Conney & Burns (1963) reported that MC pretreatment was unable to induce the synthesis of the azo-dye *N*-demethylase system in a primary hepatoma produced by 3′-methyl-DAB administration. Hart *et al.* (1965) also demonstrated that phenobarbital pretreatment of animals did not appear to stimulate aminopyrine demethylation by primary liver tumours produced by DAB administration. However, they observed that the metabolism of hexobarbital by these primary tumours was stimulated to some extent. In the present studies, primary hepatoma produced by 3′-methyl-DAB was stimulated to some degree on MC pretreatment. No stimulation could be observed with primary mammary tumour produced by MC administration.

After pretreatment of animals with MC, the relatively specific and large increase in *N*-hydroxylation by hamster liver microsomes, compared with the relatively small increase in *N*-hydroxylation and large increase in ring hydroxylation by rat liver microsomes under similar conditions, suggested that MC treatment might affect carcinogensis by AAF differently in the two species (Lotlikar *et al.* 1967). The work of Enomoto, Miyake & Sato (1968) supports this hypothesis. They demonstrated that, compared with the marked inhibition of AAF carcinogenesis in rats by MC administration (Miller *et al.* 1958; Miyaji *et al.* 1953), no inhibition of tumour induction by AAF was obtained on simultaneous administration of MC to hamsters. In the present experiments, tumours when stimulated followed the hydroxylation pattern of rat liver and not of hamster liver. These studies suggest that some of the control mechanisms (induction with MC) that are present in normal liver are still functional in well-differentiated tumours but are apparently lost in poorly differentiated hepatomas.

The present studies demonstrated that, in addition to liver, kidneys of tumour-bearing animals could be easily stimulated by MC pretreatment. Wattenberg & Leong (1962) and Gelboin & Blackburn (1964) have also reported such stimulatory effects of MC on benzpyrene hydroxylase in rat liver, kidney, small intestine, lungs and several other tissues.

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