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Species Variations in the *N*- and Ring-Hydroxylation of 2-Acetylaminofluorene and Effects of 3-Methylcholanthrene Pretreatment.* (32086)

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Rats treated with the carcinogen 2-acetylaminofluorene (AAF) excrete major fractions of the administered compound in the urine as the *N*-, 3-, 5-, and 7-hydroxy derivatives (1-3) and in the bile as *N*-hydroxy-AAF(4). The liver endoplasmic reticulum is a major site of the enzyme systems which perform these hydroxylations, all of which require TPNH and oxygen(5-7). The activity of the hepatic aromatic ring-hydroxylases increases approximately 10-fold when weanling rats are treated 24 hours prior to assay with 1 mg of 3-methylcholanthrene (MC)(5), and administration of MC with AAF inhibits the carcinogenicity of AAF in the rat (8,9). Liver preparations from a variety of other rodent species also hydroxylate AAF (5-7).

The relative amounts of ring- and *N*-hydroxylation of AAF are of importance, since ring-hydroxylation leads to non-carcinogenic metabolites(10-12), while *N*-hydroxylation is one of the steps required for activation of the amide(11,13). This paper presents data on the relative amounts of hydroxylation at the *N*-, 3-, 5-, and 7-positions of AAF by liver microsome preparations from weanling rodents either with or without prior treatment with MC. Marked species differences were found in the relative amounts of hydroxylation of AAF at these positions before and after the administration of MC.

Materials and methods. Animals. Male weanling animals were obtained from the following dealers in Madison, Wis.: albino rats, Holtzman Rat Co.; Syrian golden hamsters, Con Olson Co.; Ha/ICR mice, A. R. Schmidt Co.; guinea pigs, O'Brien Co.; New Zealand rabbits, Willard Voss. They were fed *ad libitum* a grain diet(14) which, for the guinea pigs, was supplemented with 1 g of ascorbic acid/kg. In those experiments in which puromycin or actinomycin D was injected, food was withheld from all animals after these injections were started.

Chemicals. N-Hydroxy-AAF(11,15), 2-acetylaminophenanthrene(16), and *N*-hydroxy-2-acetylaminophenanthrene(17) were synthesized in this laboratory. 7-Fluoro-AAF and its *N*-hydroxy derivative were generously furnished by Dr. T. Lloyd Fletcher, University

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of Washington Medical School. Other compounds were obtained as follows: AAF, Mann Research Laboratories; MC, Eastman Organic Chemicals; ATP, DPNH, and TPNH, Sigma Chemical Co.; puromycin dihydrochloride, Nutritional Biochemicals Co.; actinomycin D, Cancer Chemotherapy National Service Center.

MC was injected as a solution in corn oil (10 mg/ml) 24 hours prior to killing the animals. The dose was 10 mg/100 g body weight, except for a few experiments in which rats, hamsters or rabbits received only 2 mg/100 g. Puromycin and actinomycin D were dissolved in a 0.15 M sodium chloride-0.08 M sodium phosphate buffer, pH 7.4, solution and injected according to the schedules in the footnote to Table II.

For injection AAF was suspended by homogenization in a solution which contained 1.75% of gum acacia and 0.9% NaCl; 3.0 mg of AAF was injected intraperitoneally/100 g body weight.

Assay for N- *and ring-hydroxylation.* After the rabbits were killed by air embolism or the other animals were decapitated, the livers were removed and placed in ice-cold 0.25 M sucrose solution. A 10% homogenate of liver prepared in 0.25 M sucrose solution was sedimented at 8,000 $\times$ *g* for 10 minutes to remove nuclei and mitochondria, after which the microsomal fraction was sedimented at 105,000 $\times$ *g* for 60 minutes. The surface of the microsomal pellet was washed twice with sucrose solution and then resuspended in 0.25 M sucrose solution for assay.

The incubation medium was identical to that described by Cramer *et al*(5) except that 500 μg each of TPNH and DPNH were added per flask in place of TPN and DPN and the glucose-6-phosphate was omitted. Preliminary studies with rat, mouse, and hamster liver microsomes showed that the overall formation of hydroxylated metabolites was 20-30% greater with these levels of the reduced nucleotides than with the TPNH-generating system used by Cramer *et al*(5). KF was added at a final concentration of 0.1 M(6,7). The flasks were incubated in air for 20 minutes at 37°C, after which they were chilled and 4 ml of ice-cold 1 M sodium acetate buffer (pH 6) was added per flask. To obtain definitive data on *N*-hydroxy-AAF the contents of 18-36 flasks were combined for each analysis and extracted immediately with ethyl ether. The acidic metabolites were chromatographed on Whatman No. 1 paper with solvent systems composed of cyclohexane:t-butanol:acetic acid:water in ratios of 16:4:2:1(18) or 18:2:2:1 by volume. Optimal resolution of the 5- and 7-hydroxy metabolites was obtained with the former system, while the latter system gave better separation of *N*-hydroxy-AAF from the *ortho*-hydroxy derivatives. After elution with ethanol the metabolites were estimated by ultraviolet spectroscopy; before analysis the *N*-hydroxy-AAF was separated from AAF by extraction with 0.5 N NaOH. The identity of the *N*-hydroxy-AAF was also confirmed by its shift in spectrum on addition of 50 μl of 1 N KOH/ml of ethanol solution (cf. 17).

Reduction of N-*hydroxy-AAF* in vitro. Liver homogenates were assayed as described previously(19).

Urinary analyses. Urine samples were collected in dry-ice cooled tubes for 24 hours after intraperitoneal injection of AAF. No food was given during the urine collections, but water was available *ad libitum*. For analysis(20) the urine was incubated with β-glucuronidase and Takadiastase, after which the ether-extractable material was washed successively with 10% Na_2CO_3(21), water, 0.5 N HCl and water, chromatographed on Whatman No. 1 filter paper, eluted, and estimated as described for the *in vitro* studies.

Both the urinary analyses and the *in vitro* hydroxylation data were corrected by the following recovery data obtained by analyzing the compounds added to normal urine samples: *N*-hydroxy-AAF, 75%; 3-hydroxy-AAF, 70%; 5-hydroxy-AAF, 96%; and 7-hydroxy-AAF, 63%. Data are not presented for 1-hydroxy-AAF since the amounts were generally too low to quantitate adequately.

Results. Hydroxylation of AAF by liver microsomes of normal and MC-treated animals. The amounts of *N*-, 3-, 5-, and 7-hydroxy-AAF formed and the responses to prior administration of MC differed from species to species (Table I). The levels of ring-hydroxy-

TABLE I. Effects of Pretreatment with 3-Methylcholanthrene of the *N*- and Ring-Hydroxylation of 2-Acetylaminofluorene by Liver Microsomes.

Species	MC	No. of analyses*	mμmoles formed/g liver/20 min			
			N-Hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF
Rat	−	6	n.d.†	24 ± 5‡	20 ± 4	57 ± 12
	+§	6	37 ± 8	480 ± 100	395 ± 90	580 ± 190
Hamster	−	4	69 ± 18	47 ± 7	140 ± 19	640 ± 60
	+	8	950 ± 145	90 ± 21	186 ± 27	590 ± 130
Mouse	−	3	37 ± 6	31 ± 17	37 ± 5	120 ± 11
	+	3	236 ± 53	175 ± 33	220 ± 35	550 ± 210
Rabbit	−	4	16 ± 7	n.d.	n.d.	160 ± 85
	+	4	77 ± 15	n.d.	n.d.	200 ± 45
Guinea pig	−	4	n.d.	n.d.	43 ± 15	1150 ± 320
	+	4	n.d.	n.d.	73 ± 20	1800 ± 300

* The livers from 2–3 rats or hamsters, 6–8 mice, or 1 rabbit or guinea pig were used per analysis.

† n.d. = not detected. A small amount of ultraviolet light-absorbing material with a noncharacteristic spectrum was eluted from the paper. This could have obscured the presence of less than 7 mμmoles of *N*-hydroxy-AAF or 3-hydroxy-AAF or 17 mμmoles of 5-hydroxy-AAF/g liver/20 min.

‡ Values are expressed as the mean ± standard deviation.

§ Weanling male animals were injected 24 hr before assay with 10 mg of MC per 100 g body weight, except for 2 experiments each with rats and hamsters and 1 experiment with rabbits in which 2 mg of MC was injected/100 g body weight. Since the results for the animals injected with the 2 levels of hydrocarbon were in the same range, they were averaged for presentation.

lation, which are relatively low in rat liver, were increased 10- to 20-fold by administration of MC. Hydroxylation by mouse liver, which is normally about twice as great as with rat liver, was increased about 5-fold by MC treatment. On the other hand, ring-hydroxylation occurred to a much greater extent with normal hamster liver than with normal rat or mouse liver and was little altered by hydrocarbon treatment. With the liver preparations from each of these 3 species the amount of 7-hydroxylation was 2-4 times that of 5-hydroxylation, and there was less 3-hydroxylation than 5-hydroxylation with mouse and hamster liver microsomes. After treatment with MC 7-hydroxylation still predominated, but the relative amounts of 3- and 5-hydroxylation were usually greater than with the liver microsomes from normal animals. Rabbit and guinea pig liver microsomes hydroxylated AAF principally in the 7-position, and MC treatment had little effect.

In general agreement with the data reported by Irving(6) the levels of *N*-hydroxylation ranged from less than 7 mμmoles/g liver/20 minutes with normal rat liver microsomes to 70 mμmoles/g liver/20 minutes with normal hamster liver microsomes. With the hepatic microsomes from these two species as well as with mouse and rabbit liver microsomes at least 5-fold increases in activity were obtained if the animals were treated with MC. *N*-hydroxylation of AAF was not observed with guinea pig liver, whether or not the animals received injections of MC.

Although 10 mg of MC/100 g of body weight was used in most of the experiments, a few experiments indicated that similar results were obtained with rats, hamsters, or mice when 2 mg of MC was injected/100 g body weight.

All of the assays were run in the presence of 0.1 M KF, which inhibits deacetylase activity(6,7,19). This amount of fluoride increased the yield of *N*-hydroxy-AAF by about 50% with microsomal preparations from MC-pretreated hamsters, but did not alter the yield of ring-hydroxy metabolites. Fluoride decreased the yield of *N*- and ring-hydroxy metabolites with rat and mouse liver microsomes by about 20%. Similar results were obtained by Cramer *et al*(5) with rat liver preparations.

The inhibition of the MC-stimulated increases in ring- and *N*-hydroxylation activities by administration of puromycin or

TABLE II. Effects of Puromycin and Actinomycin D on Increases in Hepatic Hydroxylation Activity after Administration of 3-Methylcholanthrene.*

Exp. No.	Species	MC	Drug	mμmoles formed/g liver/20 min			
				N-Hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF
1	Rat	—	—	—†	15 ± 1	6 ± 1	15 ± 0
		+	—	—	200 ± 22	123 ± 16	200 ± 14
		+	Puromycin	—	70 ± 15	43 ± 5	90 ± 20
		+	Actinomycin D	—	29 ± 5	15 ± 1	50 ± 4
2	Hamster	—	—	27 ± 3	83 ± 3	120 ± 9	460 ± 36
		+	—	173 ± 24	113 ± 12	133 ± 15	580 ± 3
		+	Puromycin	53 ± 2	96 ± 0	145 ± 29	470 ± 18
3	Hamster	—	—	60 ± 10	50 ± 6	136 ± 4	660 ± 73
		+	—	197 ± 36	67 ± 10	113 ± 26	627 ± 30
		+	Actinomycin D	80 ± 1	40 ± 5	93 ± 10	417 ± 37

* MC was injected i.p. at 0 time. Actinomycin D (6 injections of 25 μg each starting at −2 hr) or puromycin (6 injections of 3 mg each starting at −2 hr for rats or 5 injections of 2.5 mg each starting at −15 min for hamsters) was injected i.p. at 2 hr intervals. All animals were killed at 10 hr. The livers from 2 animals were pooled for each analysis; each figure is the average for 2 analyses. The contents from 18 and 36 flasks were pooled for each analysis on hamster and rat liver, respectively.

† Levels of N-hydroxy-AAF formed by rat liver were too low for accurate estimation.

actinomycin D (Table II) indicated that both increases in activity resulted from the synthesis of additional enzyme. Because of the low levels of hepatic N-hydroxylase activity obtainable in rat liver microsomes within 10 hours after injection of MC only ring-hydroxylation data are reported for these experiments. In MC-treated rats actinomycin D reduced the amount of ring-hydroxylation by liver microsomes to 15-25% and puromycin reduced it to about one-third the level obtained in the absence of an inhibitor. With hamster liver microsomes actinomycin D and puromycin treatments reduced the increase in N-hydroxylation activity on MC-treatment to less than 20% that obtained in MC-treated hamsters not treated with these drugs. Ring-hydroxylation by hamster liver microsomes was not appreciably altered by MC treatment alone or in the presence of puromycin or actinomycin D.

Determination of the hydroxylation of AAF by MC-pretreated hamster liver at concentrations of AAF from 3.75×10^{-5} to 7.5×10^{-4} M and preparation of Lineweaver-Burk plots gave approximate K_m's of 1×10^{-7}, 8×10^{-6}, 3×10^{-5}, and 7×10^{-5} M for N-, 3-, 5-, and 7-hydroxylation.

In preliminary studies with MC-pretreated hamster liver microsomes similar amounts of AAF and 7-fluoro-AAF were N-hydroxylated, while only 5-10% as much N-hydroxy-2-acetylaminophenanthrene was formed. With rabbit liver Irving(6) found that 7-fluoro-AAF was N-hydroxylated 2-3 times as well as AAF.

In confirmation of Irving's results with rabbit liver(6) microsomal preparations from the livers of MC-treated hamsters could be stored at 0°C for 7 days without loss of N-hydroxylation activity.

Reduction of N-*hydroxy-AAF* in vitro. In contrast to the increases in N- and ring-hydroxylation with liver microsomes from MC-treated rats and hamsters, MC treatment did not alter the capacities of hamster or rat liver homogenates to reduce N-hydroxy-AAF (Table III).

Urinary metabolites of AAF in normal and MC-treated animals. In view of the marked *in vitro* differences in the capacities of hamster and rat liver microsomes to N- and ring-hydroxylate AAF and the differences in the effects of administration of MC on these enzyme systems, the urinary metabolites of AAF were compared in normal and MC-treated rats and hamsters (Table IV). The overall urinary patterns of both species were relatively little affected by MC treatment, as compared to the several-fold increases in some of the metabolites which were found *in vitro*. However, it is of interest that while MC treatment decreased the urinary excretion of N-hydroxy-AAF in the rat, the excretion of this metabolite was greater in MC-treated than in control hamsters.

TABLE III. Lack of Effect of Pretreatment with 3-Methylcholanthrene on Reduction of *N*-Hydroxy-2-Acetylaminofluorene by Rat or Hamster Liver Homogenates.

Species	MC* mg/100 g	No. of analyses	mμmoles formed/g liver/20 min (mean ± s.d.) AAF	AF
Rat	0	10	2100 ± 450	1100 ± 230
"	2	2	2100 ± 125	950 ± 25
"	10	4	2400 ± 500	980 ± 400
Hamster	0	4	9300 ± 1400	9300 ± 1300
"	2	2	8300 ± 1500	10000 ± 630
"	10	4	9000 ± 930	9000 ± 1200

* Injected i.p. 24 hr before assay.

Discussion. The considerable differences in the capacities of hepatic microsomes from various species to ring- and *N*-hydroxylate AAF may offer at least a partial explanation of the different susceptibilities of the species to carcinogenesis by AAF(1,12). Thus, though hamster liver microsomes are more active than rat liver microsomes in *N*-hydroxylation of the carcinogen, they also have a greater capacity for ring-hydroxylation. The latter reaction, in addition to the greater activity of hamster liver homogenates for the reduction of *N*-hydroxy-AAF, may result in lower effective levels of *N*-hydroxy-AAF in hamster tissues as compared to rat tissues. The greater urinary excretion of conjugates of *N*-hydroxy-AAF by the hamster as compared to the rat (Table IV) argues against this concept. However, as shown by Wiseman *et al*(4) the much greater excretion of *N*-hydroxy-AAF in the bile as compared to the urine of the rat makes the urinary excretion of *N*-hydroxy-AAF an uncertain indicator of its tissue levels; the biliary excretion of *N*-hydroxy-AAF by the hamster has not been investigated.

The relatively specific and large increase in *N*-hydroxylation by hamster liver microsomes after pretreatment of the animals with MC, as compared to the relatively small increase in *N*-hydroxylation and large increase in ring-hydroxylation by rat liver microsomes under the same conditions, suggested that MC treatment might affect carcinogenesis by AAF differently in the two species. Recent data of Enomoto *et al*(22) support this hypothesis. Thus, as compared to the marked inhibition of AAF carcinogenesis in rats by MC administration(8,9), no inhibition of tumor induction by AAF was obtained on simultaneous administration of MC to hamsters.

Comparisons of the relative amounts of *N*-, 3-, 5-, and 7-hydroxylation by hepatic microsomes from the various species and the effects of MC administration on each of these activities provide evidence that individual systems are responsible for *N*-, 3- and 5-, and 7-hydroxylation. Thus, the relatively specific increase in *N*-hydroxylation as a consequence of the administration of MC to the hamster differentiates the *N*-hydroxylation system from any of the ring-hydroxylation systems. Comparisons of the ratios of 3-, 5-, and 7-hydroxylation by the hepatic microsomes from various species show that 7-hydroxylation activity varies independently of 3- and 5-hydroxylation. In particular, guinea

TABLE IV. Urinary Metabolites of 2-Acetylaminofluorene in Control and Methylcholanthrene-Treated Rats and Hamsters.

Species	MC	% of dose (mean ± s.d.) excreted as *N*-Hydroxy-AAF	3-Hydroxy-AAF	5-Hydroxy-AAF	7-Hydroxy-AAF
Rat	−	4.0 ± 0.1	5.4 ± 2.4	9.3 ± 3.3	20 ± 10
	+	1.7 ± 0.3	7.7 ± 1.1	20 ± 3.9	10 ± 2
Hamster	−	13 ± 1.3	2.3 ± 0	7.3 ± 1.3	59 ± 3
	+	21 ± 4.9	3.3 ± 0.3	5.9 ± 1.5	20 ± 3

AAF (3 mg/100 g body weight) was injected i.p. 24 hr after i.p. injection of MC (10 mg/100 g body weight). Urine was collected for 24 hr. Each figure is the average for 2 analyses, each of which was carried out on the pooled urine from 4 weanling animals.

pig liver microsomes form about 25 times as much 7-hydroxy-AAF as 5-hydroxy-AAF, while this ratio is less than 4 for rat, hamster, and mouse liver microsomes. These data do not provide evidence that 3- and 5-hydroxylation are carried out by separate systems. Kiese(23) has recently reviewed the information in this area and has also concluded that *N*- and ring-hydroxylation of aromatic amines occur through different enzyme systems. Likewise, Weisburger *et al*(24) and Seal and Gutmann(7) suggested earlier that *ortho* and *para* hydroxylation of AAF were carried out by different systems.

Summary. 1. Assays for *N*- and ring-hydroxylation of 2-acetylaminofluorene (AAF) by hepatic microsomes from normal and 3-methylchloranthrene (MC)-treated rats, hamsters, mice, rabbits, and guinea pigs showed marked differences in these enzyme activities in normal animals of the different species and in the effects of MC-treatment. The microsomes of all species, except the guinea pig in which *N*-hydroxylation could not be detected, showed increases in *N*-hydroxylation on MC-treatment; the largest increase was observed with hamster liver microsomes. Only rat and mouse liver microsomes showed large increases in ring-hydroxylation activity on MC-treatment. Increases in *N*-hydroxylation activity of hamster liver microsomes and in ring-hydroxylation activity by rat liver microsomes after MC treatment were inhibited by administration of actinomycin D or puromycin. 2. MC-treatment of rats or hamsters did not affect the reduction of *N*-hydroxy-AAF by liver homogenates. 3. MC administration caused a decrease in urinary excretion of *N*-hydroxy-AAF by rats and an increased urinary excretion by hamsters.

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