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Mutagenic Activation of *N*-Hydroxy-2-acetylaminofluorene in the *Salmonella* Test System: the Role of Deacetylation by Liver and Kidney Fractions from Mouse and Rat

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SUMMARY

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The Ames bacterial mutagenesis system was used to evaluate the role of the deacetylase and sulfotransferase in the mutagenic activation of *N*-hydroxy-2-acetylaminofluorene. The mutagenicity of *N*-hydroxy-2-acetylaminofluorene mediated by liver or kidney microsomes was independent of treatment of mice or rats with microsomal enzyme inducers and, at equivalent protein concentrations, was the same whether mouse liver or mouse kidney microsomes were used. *N*-Hydroxy-2-acetylaminofluorene mutagenesis with mouse and rat liver or kidney microsomes was completely inhibited by 0.1 mM paraoxon and partially inhibited by addition of 3'-phosphoadenosine 5'-phosphosulfate when rat liver 9000 × *g* supernatant was used. Although the rates of deacetylation of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene *in vitro* were lower in kidney microsomes than in liver microsomes of either the mouse or the rat, both reactions were also completely inhibited by 0.1 mM paraoxon. The addition of ascorbic acid (10 mM) approximately doubled the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in the presence of mouse kidney microsomes but had no effect on the mutagenicity of *N*-hydroxy-2-aminofluorene in the absence of microsomal protein. Butylated hydroxytoluene (10 mM) had very little or no effect, in either the absence or presence of 10 mM ascorbic acid, on the mutagenicity of *N*-hydroxy-2-acetylaminofluorene with liver or kidney microsomes. Our data indicate that deacetylation is the most important step in the mutagenic activation of *N*-hydroxy-2-acetylaminofluorene by mouse and rat liver and kidney fractions and that the arylnitrenium ion, rather than the nitroxyl free radical, is the electrophilic species interacting with the bacterial DNA, resulting in the frameshift mutation.

INTRODUCTION

The first step in the metabolic activation of AAF[1] to a carcinogen involves *N*-hydroxylation via a cytochrome P-450-dependent monooxygenase (2). Further metabolism of

This work was presented in part at the meeting of the Federation of American Societies for Experimental Biology, Chicago, April 1977 (1).

[1] The abbreviations used are: AAF, 2-acetylaminofluorene; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; MC, 3-methylcholanthrene; BHT, butylated hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol); PB, sodium phenobarbital; PAPS, 3'-phosphoadenosine 5'-phosphosulfate.

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the *N*-OH-AAF thus formed is believed to yield the ultimate carcinogen(s) derived from AAF. Possible enzymes involved in the latter activation step include the sulfotransferase, deacetylase, transacetylase, and UDP-glucuronyltransferase (3–9). While the sulfate ester of *N*-OH-AAF appears to be the electrophilic species that is the ultimate hepatic carcinogen in the rat (3, 4), evidence obtained in our laboratory with the *Salmonella* mutagenesis test indicates that sulfation of *N*-OH-AAF by activation of rat liver sulfotransferase decreases *N*-OH-AAF mutagenicity (10). In contrast, the addition of mouse liver microsomes (11) or rat liver 9000 × *g* supernatant (12) dramatically increases *N*-OH-AAF mutagenicity but does not affect or decreases the mutagenicity of *N*-hydroxy-2-aminofluorene and 2-nitrosofluorene (12). The latter two compounds, formed after *N*-OH-AAF deacetylation, are 200–300 times more mutagenic than *N*-OH-AAF (12). These results suggest that a microsomal enzyme, possibly the deacetylase, is more important than the soluble sulfotransferase in the mutagenic activation of *N*-OH-AAF. Preliminary data indicate that in mouse liver this enzyme is not inducible by prior treatment with MC (11).

In the guinea pig, the highest microsomal deacetylation of *N*-OH-AAF is found in the liver, followed by the kidney (13), and mouse kidney microsomes appear to possess a greater potential to activate AAF into a mutagen than mouse liver microsomes (14). In this report we attempt to relate liver and kidney microsomal deacetylases, and their possible induction, to the mutagenic activation of *N*-OH-AAF *in vitro*, using the C57BL/6N and DBA/2N inbred strains of mice that have been used in our laboratory to study genetic differences in AAF mutagenesis and metabolism *in vitro* (11, 15–17). Since the *N*-OH-AAF sulfotransferase of mouse liver is very low (4), we used subcellular fractions from rat liver to study the role of the sulfotransferase in the mutagenic activation of *N*-OH-AAF. The nature of the electrophilic species responsible for the mutagenic activation of *N*-OH-AAF was examined using the antioxidants ascorbic acid and BHT.

MATERIALS AND METHODS

Materials. MC, AAF, and 2-nitrofluorene were obtained from Eastman Organic Chemicals; paraoxon (diethyl *p*-nitrophenyl phosphate), 2-aminofluorene, and ammonium pentacyanoamine ferroate disodium salt pentahydrate, from Aldrich Chemical Company; NADPH, *p*-dimethylaminobenzaldehyde, L-ascorbic acid, and BHT, from Sigma Chemical Company; Arochlor, from Analab, Inc.; and PB, from Merck & Company. *N*-OH-AAF and *Salmonella* tester strain TA 1538 were generous gifts from Dr. Elizabeth K. Weisburger, National Cancer Institute, and Dr. Bruce N. Ames, University of California (Berkeley), respectively. Prior to use *N*-OH-AAF was purified by thin-layer chromatography (silica gel; chloroform–methanol, 95:5) and crystallization (acetone–hexane). *N*-Hydroxy-2-aminofluorene was synthesized from 2-nitrofluorene as described by Lotlikar *et al.* (18) and stored under nitrogen at −20°.

Animals. C57BL/6N and DBA/2N inbred strains of mice (4–6 weeks old) and male Sprague-Dawley rats (80–120 g) were provided by the National Institutes of Health Animal Supply and maintained as before (15). Animals were treated with a single intraperitoneal dose of MC (80 mg/kg) or Arochlor (500 mg/kg) dissolved in corn oil and killed 48 hr later. PB, dissolved in 9.5 mM phosphate-buffered 0.85% NaCl, pH 7.2, was administered intraperitoneally for 3 days in successive daily doses of 40, 80, and 80 mg/kg, and the animals were killed 24 hr after the final dose.

Preparation of liver and kidney fractions. Animals were killed by cervical dislocation. All subsequent steps were carried out at 0–4°. Livers and kidneys were removed, washed in phosphate-buffered 0.85% NaCl (pH 7.2), minced, and, after several additional washes with phosphate-buffered NaCl, homogenized in 3–4 volumes of the same solution, using a Potter-Elvehjem homogenizer with a Teflon pestle. After centrifugation for 20 min at 9000 × *g*, the supernatant (S-9 fraction) was stored at −80° or further centrifuged at 105,000 × *g* for 60 min to obtain the microsomal pellet. Microsomes were resuspended in phosphate-buffered NaCl and assayed im-

mediately or resuspended in a small volume of 0.25 M potassium phosphate buffer, pH 7.25, containing 30% (v/v) glycerol and stored at −80°. Microsomes stored in this manner retained the same mutagenic activation potential for at least 4 months. Proteins were determined colorimetrically (19).

Mutagenesis. The method was that described by Ames *et al.* (20), with slight modifications. Briefly, molten (45°) top agar was mixed with 0.4 M sodium phosphate buffer (pH 7.4), 70 mM $MgCl_2$, 130 mM KCl, 0.5 mM biotin, and 0.5 mM histidine to final concentrations of 100, 8, 33, 0.045, and 0.045 mM, respectively. To 2.3 ml of this mixture were added 0.1 ml of the bacterial tester strain TA 1538 ($2–3 \times 10^9$ bacteria/ml), 0.1 ml of dimethyl sulfoxide containing the test compound (AAF, *N*-hydroxy-2-aminofluorene, or *N*-OH-AAF), and 0.1 ml of S-9 or microsomal protein. When AAF was tested, 0.05 ml of NADPH solution (20 mg/ml of phosphate-buffered NaCl) was added. Prior to addition, the S-9 or microsomal protein solutions were diluted with phosphate-buffered NaCl to the desired protein concentration and filtered using a 0.45-μm Swinnex filter unit (Millipore). The protein concentration of the filtrate was determined to estimate losses due to this procedure. After incubation for 48 hr at 37°, the number of colonies per plate (histidine revertants) was determined using a Count-all (model 600) colony counter (Fisher). The toxicity of AAF and *N*-OH-AAF to the bacteria was tested as before (11). With 10 μg/plate, less than 10% killing was observed.

Preparation of PAPS. The sulfate-activating system was purified from rat liver as described by Robbins and Lipmann (21), PAPS was synthesized according to Irving *et al.* (22), assayed using *p*-nitrophenol (21) as the substrate for sulfation, and stored in 0.1 M Tris-HCl, pH 7.4, at −20°.

Deacetylation of AAF and N-OH-AAF. Rates of deacetylation were measured as described by Jarvinen *et al.* (23). Briefly, for the assay of AAF deacetylase the final reaction mixture (2 ml) contained 1 ml of 1 M Tris-HCl buffer (pH 7.0), 0.5 ml of 1 mM AAF (in methanol–water, 1:1), and microsomal protein to a final concentration of 0.6 mg/ml. After incubation for 20 min at 37°, the reaction was terminated by adding 2 ml of a mixture (1:1) of 1% (w/v) ethanolic *p*-dimethylaminobenzaldehyde and 1 M sodium acetate buffer, pH 1.4. After 30 min at room temperature and centrifugation at $3000 \times g$ for 5 min, absorbances at 460 nm were determined. For the assay of *N*-OH-AAF deacetylase, the tubes, containing 1 ml of 0.05 M phosphate buffer (pH 6.5), 0.05 ml of 0.4% (w/v) aqueous ammonium pentacyanoamine ferroate, and 0.5 ml of microsomal protein solution (final concentration, 0.2 mg/ml), were first incubated for 2 min at 37°, after which 0.1 ml of 20 mM *N*-OH-AAF in ethanol was added. The reaction was stopped after 10 or 20 min by the addition of 2 ml of ethanol, and after 30 min at room temperature absorbances at 575 nm were measured. Under these conditions deacetylation rates were linear for both assays and were determined by reference to standard curves prepared with 2-aminofluorene or *N*-hydroxy-2-aminofluorene under conditions identical with those of the AAF deacetylase and *N*-OH-AAF deacetylase assays, respectively, but without microsomal protein. Concentrations of 13.8 μM 2-aminofluorene and 47.8 μM *N*-hydroxy-2-aminofluorene in the incubation mixture yielded absorbances of 0.275 and 0.152, respectively, with a 1-cm optical path.

RESULTS

AAF mutagenicity with mouse liver and kidney microsomes. AAF mutagenicity (5 μg of AAF per plate) with liver microsomes (1 mg of protein per plate) from C57BL/6N mice was increased more than 5-fold (from 213 to 1160 revertants/plate) after MC treatment, while that with liver microsomes from DBA/2N mice was increased less than 2-fold (from 251 to 495 revertants/plate) after MC treatment of the animals. This genetic difference in AAF mutagenicity between the two mouse strains has been observed before in our laboratory (11, 16). By contrast, when kidney microsomal protein (1 mg/plate) was used, AAF mutagenicity (5 μg of AAF per plate) was much less (45–60 revertants/plate), there was no

strain difference, and prior treatment with MC had no effect.

N-OH-AAF mutagenicity with liver and kidney microsomes. Except in the case of N-OH-AAF (10 μg/plate) mutagenesis in the presence of kidney microsomes (1 mg/plate) from DBA-2N mice (Table 1), treatment of either mouse strain with MC had no effect ($p > 0.05$, t-test) on the number of revertants obtained and there was no difference between the two strains. At comparable protein concentrations the mutagenicity with mouse liver microsomes was approximately the same as with mouse kidney microsomes (Table 1). Treatment of rats with microsomal enzyme inducers significantly affected ($p < 0.01$, one-way analysis of variance) N-OH-AAF mutagenicity (Table 1). Both MC and Arochlor treatment decreased ($p < 0.05$, Q-statistic) the number of revertants obtained in the presence of liver microsomes, and PB treatment increased ($p < 0.05$) the mutagenicity with kidney microsomes (Table 1).

Effect of paraoxon on N-OH-AAF mutagenicity. Paraoxon, when added at a concentration of 0.1 mM, completely inhibited N-OH-AAF mutagenicity mediated by liver or kidney microsomes from MC-treated C57BL/6N mice or from PB-treated rats, with the kidney enzyme being more sensitive than the liver enzyme (Fig. 1). When

TABLE 1

N-OH-AAF mutagenicity mediated by liver and kidney microsomes

The mutagenesis assay was carried out as described under MATERIALS AND METHODS. Values are mean revertants (±standard deviations) per plate observed in two to four experiments and have been corrected for the revertants obtained in the absence of microsomal protein: 28–37 (1 μg of N-OH-AAF per plate), 61–73 (5 μg of N-OH-AAF per plate), and 89–103 (10 μg of N-OH-AAF per plate).

Strain	Treatment	N-OH-AAF	Liver		Kidney	
			0.5 mg[a]	1.0 mg[a]	0.5 mg[a]	1.0 mg[a]
		μg/plate	*revertants/plate*		*revertants/plate*	
DBA/2N mouse	None	5	4355 ± 565	5810 ± 877	2905 ± 566	4605 ± 283
		10	5155 ± 502	5595 ± 919	3500 ± 99	4930 ± 368
	MC	5	6190 ± 35[b]	7100 ± 205[b]	2400 ± 170	4000 ± 42
		10	7100 ± 2942[b]	7830 ± 926[b]	3150 ± 202	6410 ± 240[c]
C57BL/6N mouse	None	5	3140 ± 658	3760 ± 290	3080 ± 509	3495 ± 14
		10	3425 ± 64	3910 ± 346	3475 ± 467	3860 ± 516
	MC	5	3230 ± 346	3375 ± 403	2880 ± 85	3170 ± 424
		10	3050 ± 573	4015 ± 255	3190 ± 474	3570 ± 580
Sprague-Dawley rat	None	1	182 ± 59	332 ± 53	46 ± 14	
		5	1237 ± 184	2401 ± 143	186 ± 28	
		10	2080 ± 184	2863 ± 155		
	MC	1	39 ± 18[d]	32 ± 8[d]	71 ± 9	
		5	100 ± 26[d]	198 ± 23[d]	257 ± 31	
		10	380 ± 36[d]	597 ± 64[d]		
	PB	1	207 ± 76	653 ± 223	76 ± 19	
		5	995 ± 51	1702 ± 138	362 ± 56[d]	
		10	2251 ± 269	2781 ± 117		
	Arochlor	1	29 ± 15[d]	38 ± 8[d]	74 ± 22	
		5	104 ± 68[d]	178 ± 32[d]	271 ± 49	
		10	508 ± 79[d]	842 ± 49[d]		

[a] Milligrams of protein per plate.

[b] Instead of 0.5 and 1.0, the protein concentrations were 0.8 and 1.6 mg/plate, respectively, for this set of values.

[c] Different ($p < 0.05$) from control (untreated) value (independent t-test).

[d] Different ($p < 0.05$) from corresponding control (untreated) value (Q-statistic).

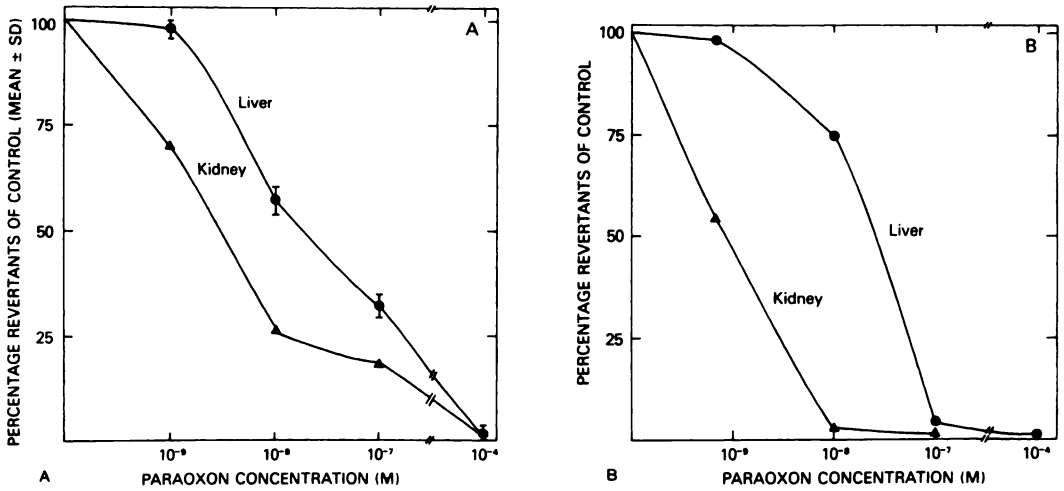

FIG. 1. *Effect of paraoxon on N-OH-AAF mutagenicity mediated by liver and kidney microsomes from MC-treated C57BL/6N mice (A) and PB-treated rats (B)*

A concentration of 5 μg of *N*-OH-AAF per plate was used. Values represent mean percentage revertants of control (uninhibited) ± standard deviations of four experiments in the case of mouse liver microsomes (●) and the means of two experiments in the case of microsomes of mouse kidney (▲), rat liver (●), and rat kidney (▲). Paraoxon was added dissolved in 0.1 ml of dimethyl sulfoxide.

tested at concentrations similar to those shown in Fig. 1, paraoxon had similar inhibitory effects on *N*-OH-AAF mutagenicity or AAF mutagenicity mediated by liver or kidney microsomes or S-9 fraction from untreated C57BL/6N and DBA/2N mice, MC-treated DBA/2N mice, and untreated, MC-treated, or Arochlor-treated rats. Paraoxon itself, when tested at 1 nM–0.1 mM, was not mutagenic in either the absence or presence of microsomes. The toxicity of paraoxon to the bacteria was tested by determining the number of colonies formed in histidine-enriched (0.9 mM) agar after the bacteria had been exposed to 0.1 μM–0.1 mM paraoxon for 5 min at 37° and diluted to approximately 2000 bacteria/ml before plating. At these concentrations paraoxon did not result in decreases in the number of colonies formed.

Effect of PAPS on N-OH-AAF mutagenicity. The addition of PAPS, at molar concentrations ranging from 25 to 75 times that of the substrate, inhibited *N*-OH-AAF mutagenicity by approximately 20% when rat kidney S-9 was used, and by approximately 50% in the presence of rat liver S-9 (Fig. 2). At these concentrations PAPS was not toxic to the bacteria.

Effect of paraoxon on deacetylation of AAF and N-OH-AAF. With liver microsomes, rates of *N*-OH-AAF deacetylation were 30–40 times higher than rates of AAF

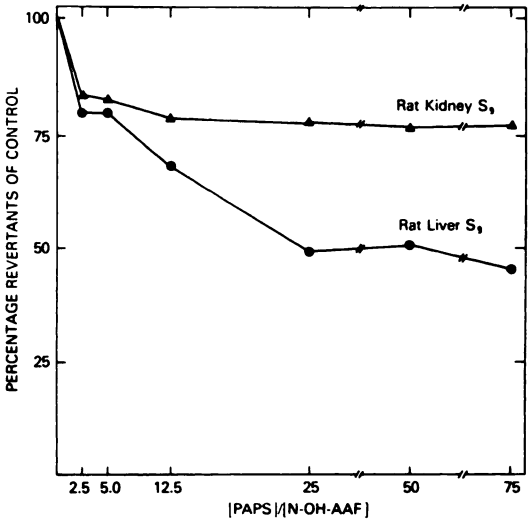

FIG. 2. *Effect of PAPS on N-OH-AAF mutagenicity mediated by liver (●) and kidney (▲) S-9 fraction from untreated rats*

A concentration of 10 μg of *N*-OH-AAF per plate was used. Values are averages of two determinations on S-9 fractions prepared from pooled organs. The abscissa is the molar ratio of PAPS and *N*-OH-AAF concentration on each plate. PAPS was added dissolved in 0.1 ml of 0.1 M Tris-HCl, pH 7.4.

deacetylation (Table 2). Activities in the liver were 1.5–8 times higher than in the kidney, and for both substrates the mouse strains had higher activity than the rat, regardless of treatment. MC treatment significantly ($p < 0.05$) decreased kidney microsomal *N*-OH-AAF deacetylation in the DBA/2N mouse and decreased ($p < 0.05$) kidney microsomal AAF deacetylation in the C57BL/6N mouse (Table 2). The effects of the various treatments on deacetylation in the Sprague-Dawley rat were analyzed by one-way analysis of variance. Treatment had a significant effect ($p < 0.01$) on liver AAF and *N*-OH-AAF deacetylation but not ($p > 0.05$) on kidney *N*-OH-AAF deacetylation. Comparisons among means were made by the *Q*-statistic. PB and Arochlor treatment significantly increased ($p < 0.05$) liver microsomal AAF deacetylation over control values, while all three treatments increased ($p < 0.05$) liver microsomal *N*-OH-AAF deacetylation (Table 2).

Paraoxon inhibited the deacetylation of both AAF and *N*-OH-AAF completely at 0.1 mM, and, in the case of kidney microsomes, at 0.1 μM (Fig. 3). Paraoxon had similar effects on deacetylation rates of liver or kidney microsomes from untreated DBA/2N or C57BL/6N mice or rats, or mice and rats treated with the various inducers listed in Table 2.

Effects of ascorbic acid and BHT on mutagenicity of N-OH-AAF and N-hydroxy-2-aminofluorene. The effects of various concentrations of ascorbic acid and BHT on *N*-OH-AAF and *N*-hydroxy-2-aminofluorene mutagenesis were analyzed by one-way analysis of variance, and comparisons between individual means were made using the *Q*-statistic (Table 3). Addition of ascorbic acid (1–10 mM) had no effect ($p > 0.05$) on *N*-hydroxy-2-aminofluorene mutagenesis in the absence of microsomal protein. At both substrate concentrations, treatment had a significant effect ($p < 0.01$) on *N*-OH-AAF mutagenesis with liver or kidney microsomes. With liver microsomes *N*-OH-AAF mutagenicity in the presence of 1–10 mM ascorbic acid or 0.01–10 mM BHT was not different ($p > 0.05$) from the control value, while 1–10 mM BHT had a small stimulatory effect ($p < 0.05$) when added together with 10 mM ascorbic acid (Table 3). With kidney microsomes the addition of 1–10 mM ascorbic acid approximately doubled ($p < 0.05$) *N*-

TABLE 2

Liver and kidney AAF and N-OH-AAF deacetylation activities of mice and rats treated with microsomal enzyme inducers

Rates were determined by reference to standard curves prepared with 2-aminofluorene (AAF deacetylation) or *N*-hydroxy-2-aminofluorene (*N*-OH-AAF deacetylation) as described in MATERIALS AND METHODS. Values represent means ± standard deviations of four determinations on microsomes prepared from pooled organs. Incubation time was 20 min in all cases except for the *N*-OH-AAF deacetylation by mouse liver microsomes, which was carried out for 10 min.

Strain	Treatment	AAF deacetylation		*N*-OH-AAF deacetylation	
		Liver	Kidney	Liver	Kidney
		nmoles product/min/mg microsomal protein			
DBA/2N mouse	None	0.51 ± 0.02	0.26 ± 0.03	24.3 ± 1.3	4.6 ± 0.7
	MC	0.54 ± 0.05	0.34 ± 0.04	24.4 ± 1.3	3.0 ± 0.7[a]
C57BL/6N mouse	None	0.61 ± 0.01	0.41 ± 0.03	29.4 ± 0.8	4.3 ± 1.3
	MC	0.55 ± 0.04	0.24 ± 0.02[a]	27.5 ± 1.6	3.6 ± 0.4
Sprague-Dawley rat	None	0.07 ± 0.02	ND[b]	2.8 ± 0.2	1.6 ± 0.8
	MC	0.08 ± 0.01	ND	3.8 ± 0.3[c]	2.4 ± 0.2
	PB	0.12 ± 0.01[c]	ND	4.6 ± 0.3[c]	1.7 ± 0.3
	Arochlor	0.10 ± 0.01[c]	ND	4.2 ± 0.1[c]	1.7 ± 0.2

[a] Different ($p < 0.05$) from control value (independent *t*-test).

[b] Level not dectable (ND) under the conditions of the assay.

[c] Different ($p < 0.05$) from control value (*Q*-statistic).

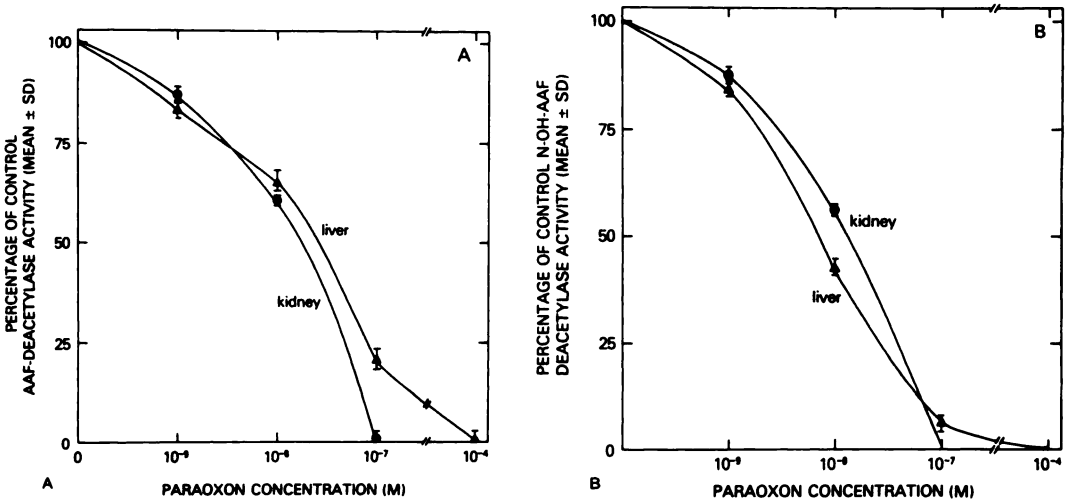

FIG. 3. *Effect of paraoxon on microsomal AAF deacetylase (A) and N-OH-AAF deacetylase (B) of liver (▲) and kidney (●) from untreated DBA/2N mice*

Values (percentage of control, Table 2) represent means ± standard deviations of four determinations on microsomes prepared from pooled organs. Paraoxon was added dissolved in 0.1 ml of absolute ethanol.

TABLE 3

Effects of ascorbic acid and BHT on mutagenicity of N-OH-AAF and N-hydroxy-2-aminofluorene

See MATERIALS AND METHODS for details of the mutagenesis assay and preparation of liver and kidney microsomes from MC-treated C57BL/6N mice. Ascorbic acid was added dissolved in the phosphate buffer normally added to the mutagenesis mixture. BHT, dissolved in dimethyl sulfoxide, was added to microsomes (0.1 ml of dimethyl sulfoxide per milliliter of microsomal suspension) 10 min prior to mixing with the other components of the mutagenesis mixture. Values are mean revertants per plate (±standard deviations) observed in two experiments.

Addition	Concentration mM	Liver microsomes[a]		Kidney microsomes[b]		No microsomal protein	
		0.5 μg[c]	1.0 μg[c]	0.5 μg[c]	1.0 μg[c]	0.2 μg[d]	0.5 μg[d]
		revertants/plate		*revertants/plate*		*revertants/plate*	
None		829 ± 15	1475 ± 90	225 ± 27	445 ± 40	2761 ± 169	3640 ± 38
Ascorbic acid	1.0	1086 ± 3	1915 ± 69	416 ± 21[e]	606 ± 50	2330 ± 383	4152 ± 194
	10.0	909 ± 66	1922 ± 2	578 ± 32[e]	810 ± 96[e]	2471 ± 127	4049 ± 849
BHT	0.01	633 ± 53	1246 ± 188	158 ± 16	377 ± 50		
	1.0	602 ± 24	1077 ± 180	216 ± 24	289 ± 42		
	10.0	666 ± 45	1421 ± 239	244 ± 16	257 ± 45		
Ascorbic acid	10.0						
+BHT	0.01	720 ± 48	1910 ± 8	362 ± 24	917 ± 45		
	1.0	1312 ± 91[e]	2214 ± 284	578 ± 24	897 ± 26		
	10.0	1479 ± 135[e]	2181 ± 94	578 ± 16	964 ± 201		

[a] The concentration of microsomal protein was 0.4 mg/plate.
[b] The concentration of microsomal protein was 0.2 mg/plate.
[c] Micrograms of *N*-OH-AAF per plate.
[d] Micrograms of *N*-hydroxy-2-aminofluorene per plate.
[e] Different ($p < 0.05$) from control values (*Q*-statistic).

OH-AAF mutagenicity, while 0.01–10 mM BHT, alone or in combination with 10 mM ascorbic acid, had no effect ($p > 0.05$, Table 3).

DISCUSSION

It has been shown (11) that the mutagenic activation of *N*-OH-AAF by mouse liver fractions is independent of cyto-

chrome P-450, indicating that enzymes other than monooxygenases are involved. The most likely activating enzyme for *N*-OH-AAF is the microsomal deacetylase, since the rate of revertants is approximately doubled when the microsomal fraction is used instead of the S-9 fraction (11). This report substantiates the latter supposition, in that the enzyme, in both the mouse and the rat, responsible for the activation of *N*-OH-AAF into a mutagen *in vitro* can be completely inhibited by 0.1 mM paraoxon (Fig. 1), a known inhibitor of liver microsomal *N*-OH-AAF deacetylase (13). Similarly, the deacetylation of AAF and *N*-OH-AAF *in vitro* is completely inhibited by 0.1 mM paraoxon (Fig. 3). Inhibition by paraoxon of carboxyesterase/amidase (EC 3.1.1) of pig or ox liver *in vitro* has been shown before (24). Other recent evidence (25) indicates that *N*-hydroxy-2-aminofluorene is the mutagenic intermediate in *N*-OH-AAF mutagenesis mediated by rat liver supernatant. Moreover, *N*-hydroxy-2-aminofluorene has been shown to be 380 times more mutagenic than *N*-OH-AAF (26).

The testing of a variety of chemical carcinogens in the *Salmonella*-microsomal test system has shown that more than 85% of carcinogens are also mutagens (27, 28). Consequently it is to be expected that the enzymes involved in the mutagenic activation of chemical carcinogens also mediate the activation of these compounds to ultimate carcinogens *in vivo*. The involvement of deacetylation in carcinogenesis *in vivo* by AAF or *N*-OH-AAF is not well defined at present. Although the activity of rat liver *N*-OH-AAF sulfotransferase correlates well with susceptibility to hepatic carcinogenesis by *N*-OH-AAF (4) and this carcinogenesis can be enhanced by administration of sodium sulfate (6), other evidence indicates that enzymes other than the sulfotransferase may contribute to the initiation of liver tumors as well as tumors occurring in extrahepatic tissues. For instance, analysis of hepatic DNA adducts following administration of AAF and *N*-OH-AAF to rats indicates that the acetyl group is lost from AAF and *N*-OH-AAF before or during arylation of DNA (29–31). Such a mechanism was also suggested from work *in vitro* (32, 33). Moreover, *N*-OH-AAF induces tumors in tissues lacking *N*-OH-AAF sulfotransferase (4, 22).

While our data do not totally exclude the possibility that the sulfotransferase is involved in part in the mutagenic activation of *N*-OH-AAF, this soluble enzyme is certainly less important for this process than the microsomal deacetylase, since the addition of PAPS decreased *N*-OH-AAF mutagenicity mediated by rat liver S-9 fraction (Fig. 2). Furthermore, *N*-OH-AAF sulfotransferase activity of mouse liver is very low (4), and *N*-OH-AAF mutagenicity is approximately doubled when mouse liver microsomal fraction is used instead of the S-9 fraction (11). The inhibition of *N*-OH-AAF mutagenicity by PAPS (Fig. 2) confirms our earlier observation with the use of a PAPS-generating system (10). It has been shown (34) that a number of esters, including the sulfate of *N*-OH-AAF, are mutagenic in the *Bacillus subtilis* system of Freese and Strack (35). In this system the reaction of the previously formed esters with the transforming DNA takes place in the absence of metabolism and permeability barriers such as exist in the *Salmonella* test system (26). It therefore seems likely that the decrease in *N*-OH-AAF mutagenicity, observed when PAPS-activated sulfotransferase (10) (Fig. 2) is included in the *Salmonella* test system, is not due to lack of mutagenicity of the sulfated *N*-OH-AAF per se, but rather to the extreme instability of this ester in aqueous solution, which prevents it from reaching the bacterial DNA. Consistent with this explanation is the finding that the sulfate ester of *N*-OH-AAF is not carcinogenic when injected subcutaneously (4).

Other evidence (5, 7) indicates that *N*-OH-AAF activation to form DNA adducts in the presence of rat liver cytosol is mediated by an *N*-*O*-acetyltransferase. This soluble enzyme is virtually absent from mouse liver (5) and therefore probably does not play a role in *N*-OH-AAF mutagenesis mediated by mouse liver microsomes. Its activity in mouse kidney is unknown, but rat kidney contains 3–5 times less than rat liver (7). In the mutagenesis test *N*-acetoxy-AAF is weakly active in the absence of

mammalian enzymes (26) but is approximately as mutagenic as *N*-OH-AAF in the presence of rat liver postmicrosomal supernatant (25). Also, the addition of *N*-OH-AAF *N*-*O*-acetyltransferase purified from rat liver increases *N*-OH-AAF mutagenicity (36).

The large difference in rates of hepatic *N*-OH-AAF deacetylation between the mouse and the rat (Table 2) is paralleled by similar large differences between *N*-OH-AAF mutagenesis with mouse liver microsomes and that with microsomes from MC- or Arochlor-treated rats (Table 1). However, *N*-OH-AAF mutagenesis with liver microsomes from untreated and PB-treated rats approaches that with mouse liver microsomes (Table 1), and the large species difference in *N*-OH-AAF mutagenesis mediated by kidney microsomes (Table 1) is accompanied by only a small species difference in kidney microsomal *N*-OH-AAF deacetylase (Table 2). Also, while *N*-OH-AAF mutagenicity mediated by mouse liver and kidney microsomes is approximately the same (Table 1), the *N*-OH-AAF deacetylase activities are 6–7 times lower in the kidney than in the liver of the mouse (Table 2). Similar but less pronounced lack of agreement occurs in rat liver and kidney (Tables 1 and 2). It is possible that the relative amounts of mutagenic and nonmutagenic molecular species formed after *N*-OH-AAF deacetylation differ between liver and kidney and between mouse and rat (18). Such differences would not be reflected in rates of deacetylation, since ammonium pentacyanoamine ferroate rapidly forms a stable complex with the *N*-hydroxy-2-aminofluorene liberated and thereby prevents it from further metabolism (13). Moreover, this complexing reagent possibly activates the *N*-OH-AAF deacetylase, a process dependent on its concentration in the reaction mixture, animal species, organ, and possibly animal treatment (13). Treatment of rats with MC or Arochlor, both inducers of hepatic cytochrome P-448, drastically decreased *N*-OH-AAF mutagenicity with rat liver microsomes (Table 1). This effect is not due to a decrease in availability of *N*-OH-AAF by partial reduction to AAF, as has been observed in other systems (18), since addition of excess NADPH did not restore *N*-OH-AAF mutagenicity to values obtained with microsomes from untreated rats (data not shown). Whether the decrease in *N*-OH-AAF mutagenesis with liver microsomes from MC-treated rats is related to the protective effect of MC against hepatic AAF carcinogenesis in this species *in vivo* (37) is an intriguing, though entirely speculative, possibility. Nevertheless, even though deacetylation rates (Table 2) cannot be correlated quantitatively with mutation frequency (Table 1), both processes are inhibited in both liver and kidney microsomes of each species (Figs. 1 and 3) by similar concentrations of paraoxon. Species differences in hepatic *N*-OH-AAF deacetylase, such as those between rat and mouse (Table 2), have also been observed between rat and guinea pig (13, 23); in the guinea pig the kidney, after the liver, has the highest *N*-OH-AAF deacetylation activity (13). While our data on the importance of mouse kidney in the metabolic activation of AAF to a mutagen seem at variance with those of Brusick *et al.* (14), a direct comparison is difficult since the mouse strain, protein concentration, and bacterial tester strain used were different in the present study.

Although MC treatment of the responsive C57BL/6N mice results in increased kidney microsomal P-450, but not in a blue shift of the Soret maximum, which is characteristic of liver microsomal P-450 after such treatment (38), no increase in kidney microsome-mediated AAF mutagenicity is observed (see RESULTS). This finding is consistent with observations in the rabbit. While MC treatment results in increased hepatic microsomal P_1-450 (or P-448) and AAF *N*-hydroxylase in this species, the latter enzyme is not induced in kidney microsomes (39) and there is no increase in the electrophoretic band of 54,000 mol wt characteristic of rabbit liver microsomes after MC treatment (40).

Liver and kidney microsomal carboxyesterases/amidases are not inducible by inducers of other microsomal enzymes (24). Arochlor is a possible exception, since levels of nonspecific rat liver esterases are doubled after treatment with this inducer (41).

The observed effects, in most cases of minor importance, of various inducers on AAF or *N*-OH-AAF deacetylation (Table 2), which are possibly related to the particular substrate or animal strain used, are not reflected in effects on *N*-OH-AAF mutagenicity (Table 1).

We conclude that in liver and kidney fractions from mouse and rat the most important enzymatic step in the metabolic activation of *N*-OH-AAF to a mutagen in the *Salmonella* test system is the deacetylation reaction. The exact nature of the electrophilic species involved in the interaction with nucleic acids is not known. On the basis of studies *in vitro,* Kadlubar *et al.* (42) suggested that urinary bladder tumor induction by *N*-hydroxyarylamines may be mediated by the formation, under acidic conditions, of the highly reactive electrophilic arylnitrenium ion after hydrolysis of the *N*-glucuronide. Using various peroxidases, H_2O_2, and *N*-OH-AAF, Bartsch and Hecker (43) proposed the nitroxyl free radical route of *N*-OH-AAF activation, two nitroxyl radicals dismutating to form 2-nitrosofluorene and *N*-acetoxy-AAF. Such a mechanism was also proposed by Floyd and co-workers (44–46), even though cyanide, a known inhibitor of peroxidase, does not inhibit the horseradish peroxidase-H_2O_2-catalyzed oxidation of *N*-OH-AAF (44). In this system ascorbate inhibits *N*-OH-AAF oxidation and is oxidized preferentially (45), presumably by reducing the nitroxyl free radical back to *N*-OH-AAF.

In the *Salmonella* mutagenesis system, ascorbic acid increases *N*-OH-AAF mutagenicity in the presence of kidney microsomes while the antioxidant BHT has very little or no effect, either by itself or in the presence of 10 mM ascorbic acid (Table 3). Thus it seems that the *N*-hydroxy-2-aminofluorene-derived nitrenium ion, rather than the nitroxyl free radical, is the more important electrophilic species involved in *N*-OH-AAF mutagenesis and that the stimulatory effect of ascorbic acid on *N*-OH-AAF mutagenicity mediated by kidney microsomes is the result of increased reduction of any nitroxyl free radical back to *N*-OH-AAF, in turn resulting in increased formation of nitrenium ion after deacetylation. It is possible that the formation of the nitroxyl free radical from *N*-OH-AAF is favored to a greater extent in kidney than in liver microsomes, leading to the greater effect of ascorbic acid on mutation frequency in kidney microsomes (Table 3). Further studies on the relative importance of the nitroxyl free radical and the nitrenium ion in the mutagenic activation of *N*-OH-AAF are in progress.

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