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Mechanism of *N*-Hydroxy-2-Acetylaminofluorene Mutagenicity in the *Salmonella* Test System

Role of *N-O* Acyltransferase and Sulfotransferase from Rat Liver

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SUMMARY

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N-Hydroxy-2-acetylaminofluorene *N-O* acyltransferase and sulfotransferase were purified from male Sprague-Dawley rat liver, and the partially purified enzyme fractions were used to evaluate the role of each enzyme in the metabolic and mutagenic activation of *N*-hydroxy-2-acetylaminofluorene in the *Salmonella* mutagenesis system. Partially purified *N-O* acyltransferase was approximately 5 times as active as rat liver 105,000 × *g* supernatant in its ability to activate *N*-hydroxy-2-acetylaminofluorene to a mutagen in the *Salmonella* test system. The addition of either ascorbate (2 mM) or NADPH (1 mM) markedly increased the mutagenicity of *N*-hydroxy-2-acetylaminofluorene, whereas the addition of either paraoxon (10^{-6} M) or 3′-phosphoadenosine-5′-phosphosulfate had no effect on the mutagenicity of *N*-hydroxy-2-acetylaminofluorene mediated by *N-O* acyltransferase. *N-O* acyltransferase also activated *N*-hydroxy-2-acetylaminofluorene *in vitro* to a species which formed covalent nucleic acid-aminofluorene adducts with yeast tRNA. Adduct formation was markedly inhibited by either ascorbate or NADPH but unaffected by the addition of either paraoxon or 3′-phosphoadenosine-5′-phosphosulfate. Similarly, partially purified sulfotransferase catalyzed the covalent binding *in vitro* of *N*-hydroxy-2-acetylaminofluorene to tRNA. The addition of 3′-phosphoadenosine-5′-phosphosulfate markedly increased (60-fold) the extent of adduct formation, whereas ascorbate (2 mM) and NADPH (1 mM) markedly inhibited the covalent binding of *N*-hydroxy-2 acetylaminofluorene to tRNA. The addition of 3′-phosphoadenosine-5′-phosphosulfate in combination with either ascorbate or NADPH resulted in a marked increase (50- to 70-fold) in the extent of covalent binding of *N*-hydroxy-2-acetylaminofluorene, although the total amount bound was only 10–20% of that observed with 3′-phosphoadenosine-5′-phosphosulfate alone. However, in contrast to *N-O* acyltransferase, purified rat liver sulfotransferase was completely inactive in its capacity to activate *N*-hydroxy-2-acetylaminofluorene to a mutagen in the *Salmonella* test system. The addition of either ascorbate or NADPH with or without 3′-phosphoadenosine-5′-phosphosulfate had no effect (no revertants observed) on the mutagenicity of *N*-hydroxy-2-acetylaminofluorene mediated by sulfotransferase. These data indicate that the initial step in the mutagenic activation of *N*-hydroxy-2-acetylaminofluorene in the *Salmonella* test system by rat liver 105,000 × *g* supernatant is deacetylation via *N-O* acyltransferase rather than sulfate formation via sulfotransferase.

INTRODUCTION

Previous studies on the metabolism of various aromatic amines and amides, in particular AAF,[1] have provided great insights into the mechanism(s) of chemical carcinogenesis (1, 2). AAF is readily *N*-hydroxylated both *in vivo* and *in vitro* by mammalian cytochrome P-450 mixed-function monooxygenase(s) to the proximate car-

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[1] The abbreviations used are: AAF, 2-acetylaminofluorene; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; *N*-OH-AF, *N*-hydroxy-2-aminofluorene; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PAP, adenosine3′:5′-diphosphate; DMSO, dimethylsulfoxide.

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cinogen *N*-OH-AAF (3, 4). However, further metabolism of the initially formed *N*-OH-AAF by various liver enzymes, such as the membrane-bound enzymes UDP-glucuronyltransferase and deacetylase or the cytosolic enzymes sulfotransferase and *N-O* acyltransferase, may also be involved in the subsequent *in vivo* activation of *N*-OH-AAF to the ultimate carcinogenic species (5–8).

Previous studies from this laboratory on the metabolism *in vitro* and the mutagenic activation of AAF and *N*-OH-AAF by subcellular liver fractions from rats and mice have shown that the mutagenic activation of AAF occurs via *N*-hydroxylation, followed by subsequent deacetylation of *N*-OH-AAF to *N*-OH-AF via the membrane-bound *N*-OH-AAF deacetylase. This latter reaction is the most important step in the mutagenic activation of *N*-OH-AAF in the *Salmonella* test system (4, 9–12).

However, *N*-OH-AAF is also activated to a mutagen in the *Salmonella* system by rat liver postmicrosomal fractions (105,000 × *g* supernatant) as we and others have previously shown (12–16). Presumably the mutagenic species which is formed is also *N*-OH-AF (13, 14). In the cytosolic fraction the mutagenic activation of *N*-OH-AAF is thought to be mediated by either, or both, *N-O* acyltransferase or sulfotransferase, both of which are also thought to be involved in the metabolic activation of *N*-OH-AAF *in vivo* to its ultimate carcinogenic species in the rat (7, 8). The relative importance of these two enzymes in the mutagenic activation of *N*-OH-AAF *in vitro* in the *Salmonella* test system is not clear. Although the sulfate ester of *N*-OH-AAF has been proposed to be the ultimate carcinogenic species in the rat liver (7), addition of sulfation cofactors (PAPS) to the standard *Salmonella* test system resulted in a marked decrease in the mutation frequency of *N*-OH-AAF mediated by rat liver cytosol (11–13, 16). These results suggest that sulfotransferase plays only a minor role, if any, in the mutagenic activation of *N*-OH-AAF. However, Andrews *et al.* (17) have proposed that an aryl nitrenium free radical derived from the sulfate ester of *N*-OH-AAF may contribute to the mutagenicity of *N*-OH-AAF in the *Salmonella* system. In an attempt to establish the relative importance of *N-O* acyltransferase and sulfotransferase in the mutagenic activation of *N*-OH-AAF in the *Salmonella* test system we have partially purified both *N-O* acyltransferase and sulfotransferase from male Sprague-Dawley rat liver and utilized the purified enzyme fractions in studies of both the metabolic and mutagenic activation of *N*-OH-AAF in the *Salmonella* test system.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (200–300 g) were obtained from the National Institutes of Health animal supply and were used for purification of both *N*-OH-AAF *N-O* acyltransferase and *N*-OH-AAF sulfotransferase. Prior to sacrifice the animals were housed in plastic cages on standard hardwood bedding and allowed water and food (Purina laboratory chow) ad libitum.

Chemicals

N-OH-AAF was a generous gift from Dr. Elizabeth Weisburger, National Cancer Institute. 9-^{14}C-Labeled *N*-OH-AAF (32 mCi/mmole) was obtained from ICN Pharmaceuticals Inc., Irvine, Calif. Labeled *N*-OH-AAF was purified by thin-layer chromatography [chloroform: methanol, 97:3 (v/v)] to more than 99.9%. PAPS was obtained from PL Biochemicals, Milwaukee, Wisc. PAP, *p*-nitrophenyl sulfate, yeast tRNA, and L-ascorbic acid were purchased from Sigma Chemical Company, St. Louis, Mo., and paraoxon (diethyl *p*-nitrophenyl phosphate) was obtained from Aldrich Chemical Company, Milwaukee, Wisc. *Salmonella* tester strains TA 98 and TA 100 were generous gifts from Dr. Bruce N. Ames, University of California (Berkeley). All other chemicals were of the best reagent grade and were obtained commercially.

Enzyme Assays

N-OH-AAF N-O acyltransferase. The incorporation of the aminofluorene moiety from 9-^{14}C-labeled *N*-OH-AAF into tRNA catalyzed by *N-O* acyltransferase was taken as a measure of acyltransferase activity. The assay was essentially that described by King with minor modification (8). 9-^{14}C-Labeled *N*-OH-AAF (84 nmoles, 3000 dpm/nmole) in DMSO (10 μl) was added to a solution of yeast tRNA (15–20 A_{258} units) in 50 mM PP_i:NaCl buffer (pH 7.0) containing 1 mM dithiothreitol to give a final volume of 0.8 ml. The reaction was initiated by the addition of a 0.1 to 0.2-ml aliquot of either rat liver cytosol or partially purified enzyme fractions. The solutions were incubated for 20 min at 37° in open 12-ml centrifuge tubes and the reaction was terminated by the addition of 1.0 ml of buffer-saturated phenol. The incubation tubes were centrifuged and a 0.5-ml aliquot of the aqueous phase was added to a 5-ml solution of 2% potassium acetate in 95% ethanol in a Millipore glass fiber (24 mm) filtration apparatus. The precipitated tRNA was washed successively with 10-ml aliquots of 70% and 95% ethanol, acetone, and diethyl ether (three times with each solvent). The dried filters were placed in scintillation vials and moistened with 0.2 ml of water. To the moistened filters was added 1.0 ml of solubilizer (NCS) and 15 ml of Aquasol (New England Nuclear Corporation, Boston, Mass.), and the radioactivity was measured by scintillation spectroscopy. Counts were corrected for quench (external standardization) and converted to nanomoles of fluorene moiety (AF) bound using the specific activity of 9-^{14}C-labeled *N*-OH-AAF. Enzymatic activity was expressed as nanomoles of fluorenyl moiety bound to tRNA per milligram of protein per 20 min.

N-OH-AAF sulfotransferase. Activity was determined spectrophotometrically as previously described (13). This assay system measures the capacity of the various enzyme fractions to generate the active sulfate conjugate on *N*-OH-AAF and reflects the net effect of PAPS formation and the transfer of the sulfate from PAPS to *N*-OH-AAF (7). *p*-Nitrophenyl sulfate was used as the sulfate donor for the synthesis *in situ* of PAPS from PAP. The incubations (3 ml) were carried out at room temperature in 1-cm light-path length cuvettes. The incubation

mixtures contained (final concentrations): 0.5 mM *N*-OH-AAF, 10 mM *p*-nitrophenyl sulfate, 20 μM PAP, 150 mM KCl, and 50 mM sodium phosphate (pH 7.4). The incubations were initiated by the addition of either cytosolic protein or purified enzyme fractions (1 mg/ml). The rates of sulfation were calculated from the rates of release of *p*-nitrophenol from *p*-nitrophenyl sulfate and were corrected from changes in absorbance (405 nm) in the absence of *N*-OH-AAF. Enzyme activity was expressed as nanomoles of *p*-nitrophenol released per milligram of protein per 5 min. Assays and controls were done in duplicate.

An alternative assay for the determination of sulfotransferase activity was also used in which the PAPS-dependent binding of *N*-OH-AAF to tRNA was measured (18). Incubation mixtures contained, in a final volume of 1 ml: 100 μmoles of Tris buffer (pH 7.4), 2 mg of yeast tRNA, 100 μg (0.4 μmole) of 9-^{14}C-labeled *N*-OH-AAF, and 0.65 μmole of PAPS. Reactions were initiated by the addition of either cytosolic protein or partially purified enzyme fractions, and incubations were performed at 37° for 20 min. The tRNA was isolated and processed as described for the *N-O* acyltransferase assay.

Mutagenesis Assay

Mutagenesis assay was performed according to the method of Ames *et al.* (19). To 2.2 ml of molten-top agar containing 17 μmoles of $MgCl_2$, 0.125 μmole of biotin, 0.125 μmole of histidine, 33 μmoles of KCl, and 100 μmoles of phosphate buffer (pH 7.4) at 45° were added 0.1 ml of the bacterial tester strain TA 98 (2×10^8 bacteria), 0.1 ml of a solution containing the mutagen dissolved in 0.1 ml of DMSO, and 0.2 ml of the cytosolic fractions or purified enzyme fractions. Prior to addition, cytosolic fractions or purified enzyme fractions were diluted with phosphate buffer to the desired protein concentrations and filtered through a 0.45-μm Swinnex filter unit (Millipore). The concentrations of protein in the filtrates were then determined after filtration to estimate losses during this process. In assays where paraoxon was used, paraoxon was added in 0.1 ml of DMSO to the mutagenesis mixture. In experiments where PAPS was used, PAPS was added in 0.1 ml of 150 mM phosphate-buffered saline (pH 7.4). The colonies on each plate (histidine-independent revertants) were scored on a Count-All (model 600) colony counter (Fisher Scientific Company, Pittsburgh, Pa.) after a 48-hr incubation at 37°. The toxicity of the test compounds to the bacteria was tested by determining the number of colonies formed in histidine-enriched (4.5 mM) agar after the bacteria had been exposed to varying concentrations of the test compounds for 30 min at 37° and diluted to approximately 10^4/ml before plating.

Purification of Enzyme Activities

N-OH-AAF sulfotransferase. Sulfotransferase was purified as described by Wu and Straub (20) through the hydroxyapatite chromatography stage. Sulfotransferase activities of the various fractions were monitored spectrometrically as described earlier in this paper (13).

N-OH-AAF N-O acyltransferase. Acyltransferase was purified using the procedure reported by King (8) except that the final chromatography step (Sephadex G-100) was performed under air.

RESULTS

N-OH-AAF mutagenicity with rat liver 105,000 × g supernatant fractions. As has previously been shown (11–16), *N*-OH-AAF (1–5 μg/plate) was activated to a potent mutagen by the 105,000 × *g* supernatant fraction from male Sprague-Dawley rat liver in the *Salmonella* test system (Fig. 1). The addition of either NADPH (1 mM) or ascorbate (2 mM) markedly increased the mutagenicity of *N*-OH-AAF more than 5-fold in the presence of rat liver cytosol, whereas activation of cytosolic sulfotransferase activity by the addition of PAPS (100 μg/plate) decreased the mutation frequency of *N*-OH-AAF by approximately 50%, in agreement with earlier data from this laboratory (12). Paraoxon (10^{-6} M), a potent inhibitor of both microsomal *N*-OH-AAF deacetylase activity and *N*-OH-AAF mutagenicity, had no effect on the mutagenic activation of *N*-OH-AAF by rat liver cytosol (Fig. 1). The addition of both PAPS and NADPH in combination also resulted in a marked increase in the mutagenicity of *N*-OH-AAF, although the increase observed was slightly less than that seen in the presence of NADPH alone. A similar increase in the mutagenicity of *N*-OH-AAF was also observed when PAPS and ascorbate

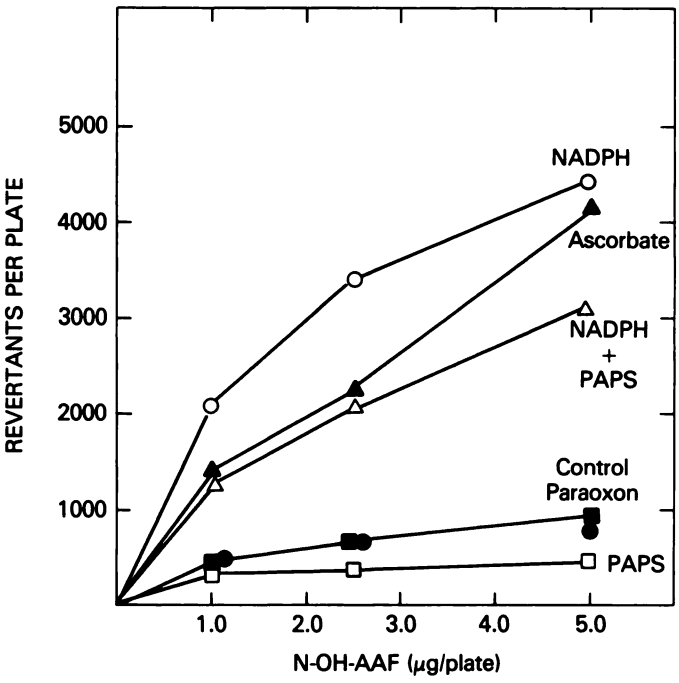

FIG. 1. *Effect of various agents on the mutagenicity in vitro of N-OH-AAF in Salmonella TA 98 mediated by rat liver 105,000 × g supernatant*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with *N*-OH-AAF in the absence of cytosolic fractions. Spontaneous revertants observed for *N*-OH-AAF at 5 μg/plate were 50–60. The cytosolic protein concentration was 1.0 mg/plate. □, experiments with PAPS (100 μg/plate); ●, cytosol only; ■, paraoxon (10^{-6} M); △, NADPH (1 mM) and PAPS (100 μg/plate); ▲, ascorbate (2 mM); ○, NADPH (1 mM).

were added to the mutagenicity assay system (data not shown).

In vitro covalent binding of N-OH-AAF to tRNA mediated by rat liver cytosol. In addition to activating *N*-OH-AAF to a potent mutagen, the rat liver cytosolic fraction also catalyzed the covalent binding *in vitro* of *N*-OH-AAF to yeast tRNA (Fig. 2). The extent of covalent binding of 9-^{14}C-labeled *N*-OH-AAF to tRNA was increased nearly 5-fold by the addition of either PAPS alone (40 μg/ml) or PAPS in combination with ascorbate (2 mM) to incubation mixtures containing *N*-OH-AAF and rat liver cytosol (Fig. 2). Covalent binding of *N*-OH-AAF was also increased in the presence of both PAPS and NADPH (1 mM), but the increase observed was only 50% that seen in the presence of either PAPS alone or PAPS plus ascorbate. In contrast, the addition of either ascorbate or NADPH alone resulted in a decrease in the extent of covalent binding of *N*-OH-AAF to tRNA, with NADPH exhibiting the greater inhibition (Fig. 2).

Mutagenicity of N-OH-AAF mediated by partially purified rat liver N-OH-AAF N-O acyltransferase. Using the procedure of King (8) with minor modifications, *N*-OH-AAF *N-O* acyltransferase was purified from adult Sprague-Dawley rat liver using ammonium sulfate fractionation (45–65%) of the 105,000 × *g* supernatant fraction followed by gel filtration on Sephadex G-100. Fractions which exhibited appreciable acyltransferase activity as determined by their capacity to catalyze the covalent binding of *N*-OH-AAF to tRNA as described under Materials and Methods were pooled and utilized for covalent binding studies and mutagenicity studies. Partially purified rat liver *N-O* acyltransferase markedly increased the mutagenicity of *N*-OH-AAF (5 μg/plate) when utilized in the *Salmonella* test system (500 revertants/0.1 mg of *N-O* acyltransferase protein versus 500 revertants/0.5 mg of 105,000 × *g* supernatant protein). The increase in the mutagenicity of *N*-OH-AAF was linear with respect to both protein concentration and concentration of *N*-OH-AAF (Fig. 3). Similar to that which was observed using unfractionated rat liver cytosol (Fig. 1), the addition of either ascorbate (2 mM) or NADPH (1 mM) to mutagenesis assay mixtures using purified *N-O* acyltransferase resulted in a 4- to 5-fold increase in the mutagenicity of *N*-OH-AAF (6000–7000 revertants versus 1500 revertants for control cytosol at 5 μg of *N*-OH-AAF/plate) (Fig. 3). Similarly, the addition of paraoxon had no effect on the mutagenicity of *N*-OH-AAF mediated by *N-O* acyltransferase. Although the addition of PAPS resulted in a 50% decrease in the mutagenicity of *N*-OH-AAF mediated by rat liver cytosol (Fig. 1), the addition of PAPS had no effect on the mutagenicity of *N*-OH-AAF mediated by *N-O* acyltransferase (Fig. 3).

Covalent binding of N-OH-AAF to tRNA mediated by partially purified N-O acyltransferase. Incubation of partially purified *N-O* acyltransferase with *N*-OH-AAF and yeast tRNA resulted in an approximately 15-fold increase in the extent of covalent binding of *N*-OH-AAF to tRNA (Fig. 4) as compared with that catalyzed by rat liver cytosol (Fig. 2). In contrast to that observed using unfractionated rat liver cytosol, the addition of PAPS had no effect on the covalent binding of *N*-OH-AAF mediated by partially purified *N-O* acyltransferase (Fig. 4), whereas both ascorbate and NADPH markedly inhibited *N-O* acyltransferase-mediated covalent binding of *N*-OH-AAF to tRNA. The addition of PAPS with either ascorbate or NADPH had no effect on the inhibition of binding of *N*-OH-AAF by either ascorbate or NADPH. Paraoxon (10^{-6} M) had no significant effect on the covalent binding of *N*-OH-AAF to tRNA.

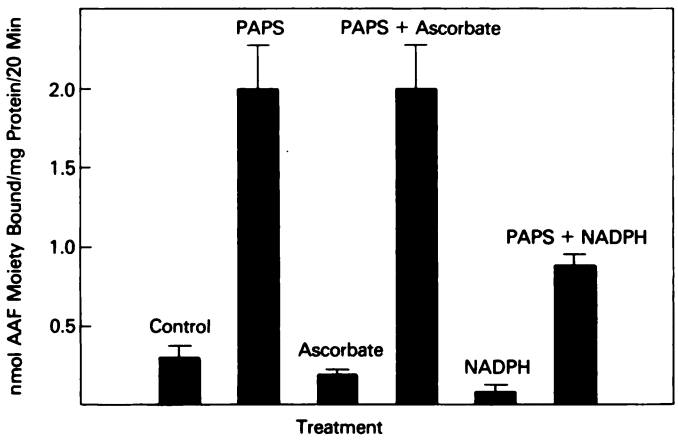

FIG. 2. *Effect of various agents on the covalent binding of N-OH-AAF in vitro to yeast tRNA mediated by rat liver 105,000 × g supernatant*

The incubation mixture, condition of assay (acyltransferase), and the isolation of tRNA-AF-bound adducts were performed as described under Materials and Methods. The concentrations of various agents were as follows: PAPS, 40 μg/ml; ascorbate, 2 mM; NADPH, 1 mM; PAPS, 40 μg/ml, plus ascorbate, 2 mM; PAPS, 40 μg/plate, plus NADPH, 1 mM. Values represent means ± standard deviation of three or more experiments.

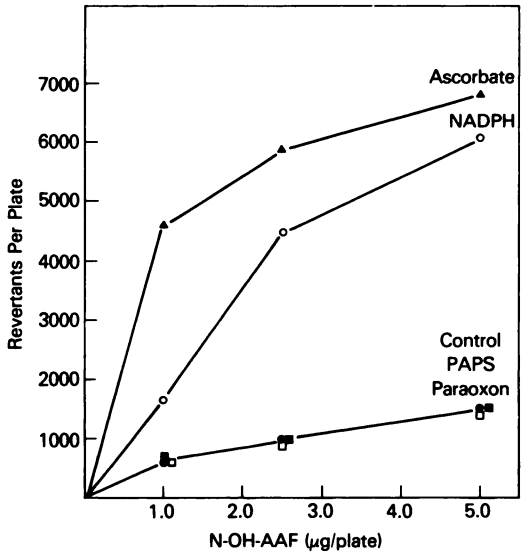

FIG. 3. *Effect of various agents on the mutagenicity in vitro of N-OH-AAF in Salmonella TA 98 mediated by partially purified rat liver N-O acyltransferase*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with *N*-OH-AAF in the absence of *N-O* acyltransferase. The protein concentration of *N-O* acyltransferase was 0.3 mg/plate in all experiments. □, Experiments with PAPS (100 μg); ■, paraoxon (10^{6} M); ●, control; ○, NADPH (1 mM); ▲, ascorbate (2 mM).

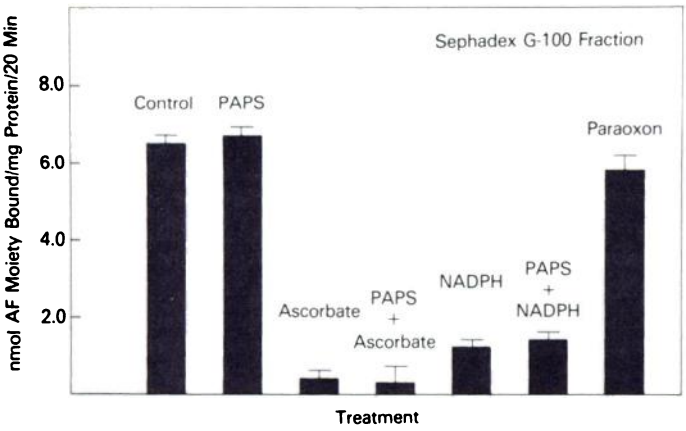

FIG. 4. *Effect of various agents on the covalent binding in vitro of N-OH-AAf to yeast tRNA mediated by partially purified rat liver N-O acyltransferase*

The incubation mixture, conditions of acyltransferase assay, and isolation of tRNA-AF bound-adducts were performed as described under Materials and Methods. The concentrations of various agents were as follows: PAPS, 40 μg/ml; ascorbate, 2 mM; NADPH, 1 mM; paraoxon, 10^{-6} *M*; and PAPS, 40 μg/ml in combination with either ascorbate, 2 mM, or NADPH, 1 mM. Values represent the means ± standard deviation of three or more experiments.

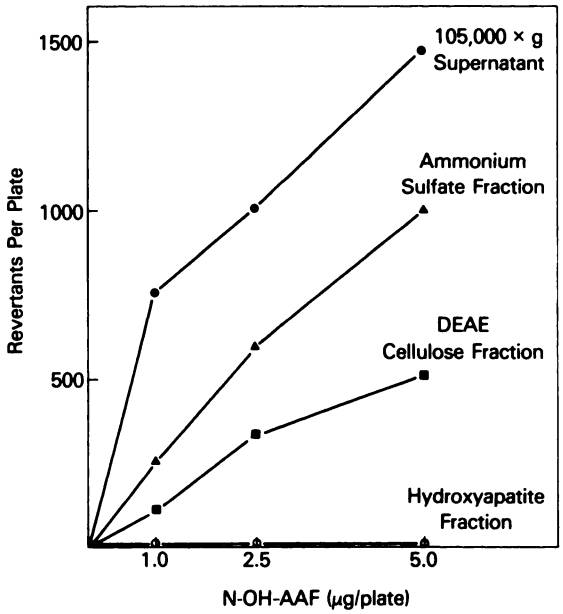

FIG. 5. *Mutagenicity in vitro of N-OH-AAF in Salmonella TA 98 mediated by partially purified fractions of rat liver sulfotransferase*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with *N*-OH-AAF in the absence of purified sulfotransferase fractions. Purification of various fractions are described under Materials and Methods. The protein concentration of each fraction was 1.0 mg/plate. ●, Experiments utilizing 105,000 × *g* supernatant; ▲, fraction obtained after ammonium sulfate precipitation; ■, fraction obtained after DEAE-cellulose chromatography; ○, experiments utilizing sulfotransferase activity following hydroxyapatite chromatography.

Mutagenicity of N-OH-AAF mediated by partially purified N-OH-AAF sulfotransferase. Purification of rat liver *N*-OH-AAF sulfotransferase activity was performed as described by Wu and Straub (20). During the various purification steps, sulfotransferase activity was followed spectrometrically as described under Materials and Methods (13). Fractions exhibiting significant sulfotransferase activity were pooled and the sulfotransferase activities of the pooled fractions at the various purification steps were determined. An over-all purification of approximately 55-fold was obtained after hydroxyapatite chromatography. Partially purified sulfatransferase exhibited an enzymatic activity of 145 versus an activity of 2.7 obtained using the 105,000 × *g* supernatant fraction. Using the various partially purified fractions of sulfotransferase activity, the capacity of each fraction to catalyze the mutagenic activation of *N*-OH-AAF was determined, and the results are summarized in Fig. 5. The protein concentration of each partially purified enzyme fraction was 1.0 mg/plate. At each successive purification step of rat liver sulfotransferase activity, the capacity to activate *N*-OH-AAF to a mutagen was decreased. The mutagenicity of *N*-OH-AAF (5 μg/plate) mediated by the ammonium sulfate fraction was only 67% of that mediated by the 105,000 × *g* supernatant fraction, whereas the DEAE-cellulose fraction was 3 times less active as the 105,000 × *g* supernatant fraction. The final fraction obtained following hydroxyapatite chromatography and the fraction with the greatest sulfotransferase activity was completely inactive in its capacity to activate *N*-OH-AAF to a mutagen in the *Salmonella* test system (Fig. 5). Addition of ascorbate, NADPH, or PAPS had no effect (no revertants observed) on the mutagenicity of *N*-OH-AAF mediated by the hydroxyapatite fraction of rat liver sulfotransferase.

In vitro covalent binding of N-OH-AAF to tRNA mediated by partially purified rat liver N-OH-AAF sulfotransferase. In the absence of PAPS, the capacity of partially purified sulfotransferase (hydroxyapatite fraction) to catalyze the covalent binding of *N*-OH-AAF to tRNA (Fig. 6) was not significantly different from that of the 105,000 × *g* supernatant fraction. However, following the addition of PAPS, there was a 60-fold increase in the extent of the covalent binding of *N*-OH-AAF to tRNA. Similar to that observed with both the rat liver 105,000 × *g* supernatant fraction and the partially purified *N-O* acyltransferase fraction, both ascorbate (2 mM) and NADPH (1 mM) caused a marked inhibition in the extent of covalent binding of *N*-OH-AAF to tRNA mediated by rat liver sulfotransferase. The addition of PAPS in combination with either ascorbate or NADPH resulted in a marked increase (50- to 70-fold) in the extent of covalent binding of *N*-OH-AAF, although the total amount bound was only 10–20% of that observed with PAPS alone.

DISCUSSION

The proximate carcinogen, *N*-OH-AAF, is activated in target organs via various enzymatic and possibly nonenzymatic processes to yield highly reactive electrophilic intermediates capable of covalent interaction with critical cellular macromolecules (DNA, RNA, protein, etc.) and thereby initiating the carcinogenic process (2, 6, 8, 16, 21, 22). Similarly, the *in vitro* mutagenic activation of *N*-OH-AAF in the *Salmonella* test system may

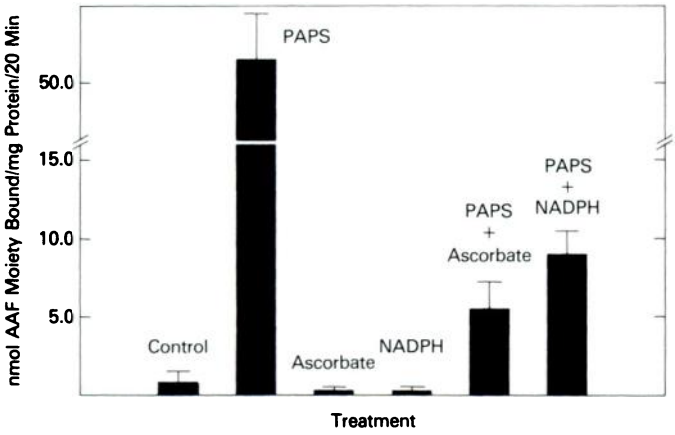

FIG. 6. *Effect of various agents on the covalent binding in vitro of N-OH-AAF to yeast tRNA mediated by partially purified rat liver sulfotransferase*

The incubation mixture, conditions of sulfotransferase assay (PAPS-dependent covalent binding), and isolation of tRNA-AF-bound adducts were performed as described under Materials and Methods. Sulfotransferase activity obtained following hydroxyapatite chromatography was utilized in all experiments. The concentrations of various agents were as follows: PAPS, 40 μg/ml; ascorbate, 2 mM; NADPH, 1 mM; and PAPS, 40 μg/ml in combination with either ascorbate, 2 mM, or NADPH, 1 mM.

occur via both enzymatic and nonenzymatic pathways (9–16). Previous studies using only the 105,000 × *g* supernatant fraction suggested that the mutagenic activation of *N*-OH-AAF in the *Salmonella* test system occurred via process(es) other than that catalyzed by sulfotransferase, since a sharp decrease in the mutagenicity of *N*-OH-AAF was observed when sulfation cofactors were added to rat liver cytosol and *N*-OH-AAF (13). The results from the current study using partially purified rat liver sulfotransferase as the mutagenic activating system for *N*-OH-AAF have confirmed those results. Purified rat liver sulfotransferase was completely inactive in its capacity to activate *N*-OH-AAF to a mutagen in the *Salmonella* test system (Fig. 5). Addition of cofactors which markedly increase the mutagenicity of *N*-OH-AAF mediated by either rat liver cytosol (Fig. 1) or purified *N-O* acyltransferase (Fig. 3), namely NADPH and ascorbate, had no effect on the mutagenic activation of *N*-OH-AAF by sulfotransferase, indicating that the aryl nitrenium free radical derived from the *N*-OH-AFF sulfate ester does not contribute to the mutagenicity of *N*-OH-AAF (17). However, sulfotransferase was quite active in its ability to activate *N*-OH-AAF of a species capable of covalent interaction with tRNA when PAPS, the endogenous sulfate donor, was added to sulfotransferase.

Purified *N-O* acyltransferase, however, was quite active in the mutagenic activation of *N*-OH-AAF (Fig. 3, ref. 17). Addition of PAPS, which causes a decrease in the mutation frequency of *N*-OH-AAF mediated by unfractionated rat liver cytosol, as well as addition of paraoxon, a known carboxyesterase:amidase inhibitor (11, 12), has no effect on the mutagenic activation of *N*-OH-AAF mediated by *N-O* acyltransferase (Fig. 3). The mutagenicity of *N*-OH-AAF mediated by either purified *N-O* acyltransferase (Fig. 3) or unfractionated rat liver cytosol (Fig. 1) is increased to similar extents by the addition of either ascorbate or NADPH. Both *N-O* acyltransferase and sulfotransferase, however, are capable of activating *N*-OH-AAF to a reactive species capable of covalent interaction with yeast tRNA, probably via different mechanism.

Although the mechanism of *N*-OH-AAF mutagenic activation mediated by *N-O* acyltransferase is not known, evidence indicates that deacetylation of *N*-OH-AAF via *N-O* acyltransferase, and possibly free radical formation similar to the mechanism proposed for the mutagenic activation of *N*-OH-AAF by rat liver microsomal and nuclear fractions, is responsible for the activation of *N*-OH-AAF (12). Rat liver *N*-OH-AAF *N-O* acyltransferase is a sulfhydryl-dependent enzyme that catalyzes the intramolecular acetyl transfer of *N*-OH-AAF to form *N*-acetoxyaminofluorene (8). Weeks *et al.* (15) have shown that the mechanism of metabolic activation of *N*-OH-AAF to an intermediate capable of forming adducts with nucleic acids could be dissociated from that which induced mutations in *Salmonella* TA 1538. The addition of varying concentrations of either GMP or 2-aminofluorene to incubations containing *N*-OH-AAF, tRNA, and *N-O* acyltransferase resulted in a 70% inhibition of adduct formation, whereas similar additions to the *Salmonella* test system had no effect on the mutagenicity of *N*-OH-AAF mediated by *N-O* acyltransferase (15). Therefore, if *N*-acetoxyaminofluorene were the mutagenic intermediate formed from *N*-OH-AAF, then the addition of either 2-aminofluorene or GMP would be expected to decrease the mutagenicity of *N*-OH-AAF.

An additional, although minor, pathway for the mutagenic activation of *N*-OH-AAF may involve free radical formation. Using various peroxidases, H_2O_2, and *N*-OH-AAF, Bartsch and Hecker (22) suggested a nitroxyl free radical route for the carcinogenic activation of *N*-OH-AAF in which two nitroxyl free radicals dismutate to *N*-acetoxy-2-acetylaminofluorene and 2-nitrosofluorene. Walker and Floyd (21) have proposed a similar activation mechanism for *N*-OH-AAF. No one to our knowledge, however, has shown the formation the nitroxyl free radical catalyzed by either microsomal or cytosolic preparations. Stier *et al.* (23), however, detected the formation of a free radical from incubation mixtures containing AAF, rabbit liver microsomes, and an NADPH-generating system which exhibited an ESR spectrum nearly identical with the one obtained by Bartsch and Hecker (22) for the chemically prepared nitroxide free radical derived from *N*-OH-AAF. 2-Nitrosofluorene is a potent, direct-acting frameshift mutagen in *S. typhimurium,* whereas *N*-acetoxy-2-acetylaminofluorene is only weakly mutagenic. The mutagenicity of *N*-acetoxy-2-acetylaminofluorene is increased several-fold by the addition of either rat liver S-9 or cytosolic fractions (14) or the inclusion of mouse liver nuclei in the *Salmonella* test system (12). Since ascorbate markedly increases the mutagenicity of *N*-OH-AAF mediated by either microsomal preparations or purified *N-O* acyltransferase, a possible explanation may be that ascorbate (or NADPH) is reducing any nitroxyl free radicals formed from *N*-OH-AAF back to *N*-OH-AAF, which is then deacetylated to form the highly mutagenic *N*-OH-AF via either the membrane-bound deacetylase or the soluble *N-O* acyltrans-

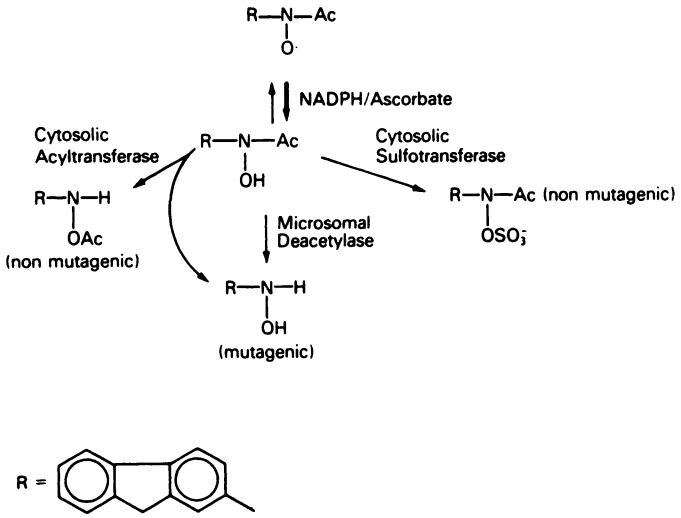

FIG. 7. *Proposed mechanism for the metabolic and mutagenic activation of N-OH-AAF in the Salmonella test system*

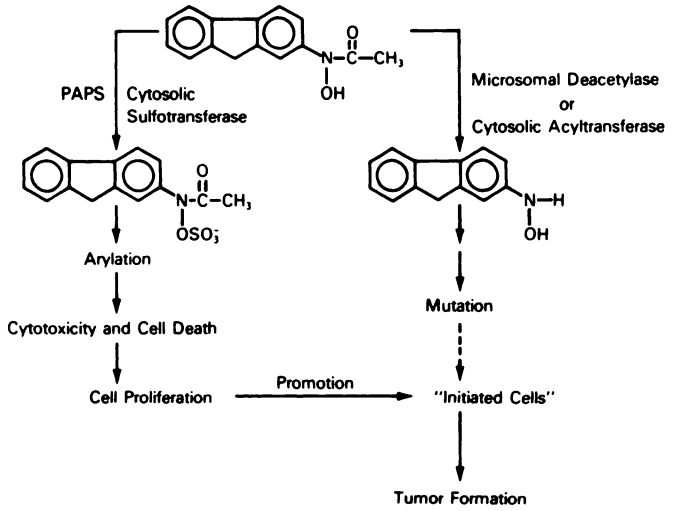

FIG. 8. *Proposed role of sulfotransferase in N-OH-AAF-induced carcinogenesis*

ferase (Fig. 7). In the hydrogen peroxide peroxidase model system of Floyd *et al.* (24), ascorbate inhibited *N*-OH-AAF oxidation and is preferentially oxidized, presumably by reducing any nitroxyl free radicals formed back to *N*-OH-AAF.

The role of free radical formation from *N*-OH-AAF in the mutagenic activation *in vitro* as well as the covalent binding to tRNA is not clear. Both ascorbate and NADPH cause a marked increase in the mutagenicity of *N*-OH-AAF while at the same time cause a marked decrease in the covalent binding of *N*-OH-AAF to yeast tRNA. Since both ascorbate and NADPH react via one-electron reductions with free radicals, the possibility exists that either ascorbate or NADPH may reduce any nitroxide free radicals derived from *N*-OH-AAF back to the parent hydroxamic acid which could then serve as a substrate for deacetylation via *N-O* acyltransferase (Fig. 7). The net effect would be an increase in the formation of the highly mutagenic *N*-OH-AF. Ascorbate in itself has no effect on the mutagenicity of *N*-OH-AF (11, 25). Furthermore, Andrews *et al.* (17) have shown that ascorbate causes an increase in the release of acetate from *N*-OH-AAF when *N*-OH-AAF and ascorbate are incubated in the presence of rat liver 105,000 × *g* supernatant fractions. Further mutagenic activation of the initially formed *N*-OH-AF to either a nitrenium free radical or a nitrenium ion within the bacteria, similar to that which we have proposed for the mutagenic activation of *N*-OH-AAF via the membrane-bound deacetylase, is also possible (12).

Although sulfotransferase is not involved in the mutagenic activation of *N*-OH-AAF in the *Salmonella* test system, the sulfate ester of *N*-OH-AAF is directly mutagenic in the *Bacillus subtilis* system (26). The susceptibility of rats and mice to hepatic carcinogenesis by *N*-OH-AAF correlates well with liver sulfotransferase activity, and carcinogenesis can be enhanced by the administration of sodium sulfate (27). The sulfotransferase contributes significantly to the *in vitro* covalent binding of *N*-OH-AAF to cytosolic proteins (14) and yeast tRNA (Fig. 6), but it does not contribute significantly to the covalent binding of *N*-OH-AAF to nuclear DNA, RNA, or proteins *in vitro* (12). Since presumably the process of *N*-OH-AAF-induced carcinogenesis in the liver involves both initiation and promotion, we would like to propose that the role of *N*-OH-AAF sulfotransferase in *N*-OH-AAF-induced liver carcinogenesis is that of a promoter (Fig. 8, ref. 28). *N*-OH-AAF is metabolized via sulfotransferase to the highly reactive and cytotoxic sulfate ester which causes cell death and induces subsequent cellular proliferation (29, 30). This cellular proliferation may in turn promote the process of carcinogenesis of "initiated" cells which have been transformed by *N*-OH-AF or subsequent metabolites of *N*-OH-AF.

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