Mechanism of in Vitro Mutagenic Activation and Covalent Binding of N-Hydroxy-2-acetylaminofluorene in Isolated Liver Cell Nuclei from Rat and Mouse

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ABSTRACT

The in vitro mutagenic activation of N-hydroxy-2-acetylaminofluorene (N-OH-AAF) in the Salmonella test system (strain TA 1538) by isolated rat and mouse liver cell nuclei and the in vitro covalent binding of N-OH-AAF to nuclear nucleic acids and proteins were studied. Mutagenic activation of N-OH-AAF and 2-acetylaminofluorene by liver cell nuclei from both rat and mouse was observed. The mutagenic activation of N-OH-AAF by the mouse nuclei was 6 to 7 times greater than in the rat nuclei (2300 versus 350 revertants per 10 µg N-OH-AAF). The mutagenicity of N-OH-AAF was increased 2-fold when NADPH was included with the nuclei in the assay mixture, and this increase in N-OH-AAF mutagenicity was inhibited by a-naphthoflavone. The carboxyesterase:amidase inhibitor paraoxon (10⁻⁴ M) completely inhibited the mutagenic activation of N-OH-AAF in both mouse and rat liver nuclei. Paraoxon (10⁻⁴ M) had no effect on the mutagenic activation of N-OH-AAF in the 100,000 x g supernatant fraction from rat liver, whereas 50% inhibition of N-OH-AAF mutagenesis was observed when adenosine 3'-phosphate 5'-phosphosulfate was included in the supernatant fraction. The addition of rat liver 100,000 x g supernatant to mouse nuclei resulted in decreased mutagenicity of N-OH-AAF that could be further inhibited by paraoxon (10⁻⁵ M). On the other hand the addition of rat liver 100,000 x g supernatant to rat liver nuclei resulted in an increase in N-OH-AAF mutagenicity that was insensitive to inhibition by paraoxon. Antioxidants such as vitamin E and butylated hydroxytoluene at 10⁻² M concentration inhibited the mutagenic activation of N-OH-AAF by 20 to 30% in the mouse nuclear preparation, whereas vitamin C at the same concentration increased the N-OH-AAF mutagenesis 2- to 3-fold. Addition of cysteamine and methionine (10⁻² M) to the mouse nuclear preparation resulted in 60 and 30% inhibition, respectively, of N-OH-AAF mutagenesis.

The in vitro covalent binding of N-OH-AAF to nuclear nucleic acids and proteins was observed with both rat and mouse nuclei. The degree of covalent binding to nuclear protein and RNA was similar in both nuclei, but the binding to DNA in the rat nuclei was twice that observed in mouse DNA (21 versus 10 pmol/mg/10 min). Paraoxon (10⁻⁴ M) inhibited by more than 70% the covalent binding of N-OH-AAF to nuclear nucleic acid and proteins in both mouse and rat nuclei. Similar inhibition by paraoxon of the covalent binding of N-OH-AAF to microsomal protein was observed, whereas paraoxon had no effect on the binding of N-OH-AAF to protein in 100,000 x g supernatant fraction from rat liver.

Combining rat liver nuclei with the 100,000 x g supernatant had no significant effect on the covalent binding of N-OH-AAF to nuclear nucleic acid and protein except when adenosine 3'-phosphate 5'-phosphosulfate was included, which resulted in a significant (p < 0.05) decrease in the binding to nuclear RNA and proteins. Cysteamine and vitamin C (10⁻² M) significantly (p < 0.05) inhibited the covalent binding of N-OH-AAF to nuclear RNA and protein, whereas methionine at the same concentration had no effect.

The first step in the in vitro mutagenic activation of N-OH-AAF by isolated rat and mouse liver cell nuclei and/or the 100,000 x g supernatant fraction from rat liver and the in vitro covalent binding to nuclear nucleic acids and protein occur primarily via deacetylation by either the membrane-bound amidase or the N,O-acyltransferase in the supernatant fraction.

INTRODUCTION

It is now generally accepted that most chemical carcinogens are highly reactive electrophiles, which initiate the carcinogenic process by interacting with critical nucleophilic sites in cellular macromolecules of susceptible organs. This concept is supported by experimental evidence from studies on the carcinogenicity of aromatic amines and amides, nitrosamines, and polycyclic hydrocarbons (1, 28, 32, 33, 45). Furthermore, many chemical carcinogens are found to be mutagens in microbial test systems such as the Salmonella system, indicating a strong association between chemical carcinogenesis and mutagenesis and supporting the somatic mutation theory of cancer (1, 30).

Previous studies in this laboratory on the in vitro metabolism and mutagenic activation of the precarcinogen AAF⁴ and its proximate carcinogenic metabolite N-OH-AAF by subcellular liver fractions from mice and rats led to the
conclusions that N-hydroxylation is the rate-limiting step in the mutagenic activation of AAF with subsequent deacetylation being the most important metabolic pathway in the mutagenic activation of N-OH-AAF in the Salmonella test system (8, 40–43). Moreover, we found that sulfotransferase activity, which has been closely correlated with susceptibility of AAF-induced hepatic tumors in rats (7), decreased the in vitro mutation frequency of N-OH-AAF (34, 38). Since paraaxon, a carboxylesterase:amidase inhibitor known to inhibit the deacetylation of N-OH-AAF (13), completely blocks the in vitro mutagenic activation of N-OH-AAF in liver and kidney microsomes from mice and rats, we have proposed the formation of a nitrenium ion via deacetylation as the mutagenic species of AAF and/or N-OH-AAF in the Salmonella test system (38, 42, 43). The formation of the nitrenium ion from N-OH-AF has previously been proposed as the ultimate electrophilic species in the interaction of N-OH-AF with nucleic acids (22). Mahler et al. (29) and Miller (33) have proposed the nitrenium and the amionium ions as the ultimate carcinogenic species of aryamines and acetylarylamines, respectively. These proposed ultimate carcinogens and/or mutagens are highly reactive electrophiles and in aqueous medium would be expected to react extremely quickly with proteins and other nucleophilic species in the cytoplasm and/or in the organelles where they are formed. This is consistent with the observation that sulfotransferase activity decreases the mutagenicity of N-OH-AAF in the Salmonella test system whereas the covalent binding of N-OH-AAF to cytosol proteins is increased (34). The most likely explanation is that the reactive amionium ion formed from the sulfate ester of N-OH-AAF reacts instantaneously with nucleophilic sites on cytosol proteins and never reaches the bacterial DNA. The sulfate ester of N-OH-AAF has, however, been shown to be mutagenic in the Bacillus subtilis test system when added directly with bacterial DNA (29).

The outer nuclear membrane in the rat hepatocyte is in continuum with the endoplasmic reticulum and contains qualitatively the same enzymes as are found in the endoplasmic reticulum (17). For example, liver cell nuclei from guinea pigs were shown to be capable of deacetylating N-OH-AAF, although the activity was less than 10% of that found in liver microsomes (13). Nevertheless, because of the critical location of the nuclear membranes with respect to the nuclear nucleic acids as well as the short lifetime of the proposed nitrenium ion, the deacetylase(s) in the nuclear membrane may play an important role in activation of N-OH-AAF to the ultimate carcinogenic species. The observation that following the in vivo administration of either AAF or N-OH-AAF to rats the chemical species that has been found covalently bound to DNA is N-(deoxyguanosin-8-yl)-2-aminofluorene supports the possibility that deacetylation takes place prior to arylation of DNA (23, 25).

To study further the role of deacetylation in the mutagenic and possibly also in the carcinogenic activation of N-OH-AAF as well as the mechanism of N-OH-AAF-induced mutation in the Salmonella test system, we have measured the capacity of isolated liver cell nuclei from rats and mice to catalyze the mutagenic activation of N-OH-AAF, the covalent binding of N-OH-AAF to nuclear nucleic acids and proteins, and the effect of nucleophiles and antioxidants on both the mutagenicity and the covalent binding of N-OH-AAF.

MATERIALS AND METHODS

Chemicals. Paraaxon (diethyl p-nitrophenylphosphate), L-methionine, cysteamine (2-aminoethanethiol), and ANF were purchased from Aldrich Chemical Company, Milwaukee, Wis.; MC was purchased from J. T. Baker Chemical Company, Phillipsburg, N. J.; L-ascorbic acid (vitamin C), BHT (2,6-di-tert-butyl-p-cresol), and dl-α-tocopherol (vitamin E) were obtained from Sigma Chemical Company, St. Louis, Mo.; N-OH-AAF was a generous gift from Dr. E. Weisburger, National Cancer Institute, Bethesda, Md.; N-acetoxy-AAF was obtained from the National Cancer Institute Chemical Depository at IIT Research Institute, Chicago, Ill.; [3H]-N-OH-AAF (8.46 Ci/mmol) was brought from New England Nuclear, Boston, Mass. and purified by thin-layer chromatography (chloroform:methanol, 97:3, v/v) to more than 99.9%; and NADPH was obtained from Calbiochem (La Jolla, Calif.). All other reagents and chemicals used were obtained commercially and were of the best grade available. Adult male C57BL/6N (hereafter called B6) mice and Sprague-Dawley rats were provided by the Veterinary Resources Branch, NIH.

Treatment of Animals and Preparation of Subcellular Fractions. Animals were kept in standard hardwood bedding in plastic cages and fed Wayne Lab-Blox and water ad libitum. An automatic 12-hr day-12-hr night cycle was maintained in the animal room. Treatment consisted of a single i.p. injection of MC (80 mg/kg body weight in corn oil) 48 hr prior to killing; controls received an equivalent volume of corn oil (1 ml/100 g body weight) alone 48 hr prior to killing. The animals were decapitated, and all preparative steps were performed at 4°. Minced hepatic tissue was washed as free as possible from hemoglobin with a buffer consisting of 500 mM 2-methyl-2,4-pentanediol (hexylene glycol), 1 mM piperazine-N,N'-bis(2-ethanesulfonic acid) disodium salt monohydrate, and 1 mM CaCl2, adjusted to pH 7.0 with 1 N HCl (48) and homogenized with 15 ml of buffer per g of liver in a glass:Teflon homogenizer. After being filtered through 4 layers of cheesecloth, the homogenate was centrifuged for 10 min at 900 × g. The pellets were then resuspended in the previously described buffer and centrifuged at 900 × g for 10 min. This washing process was repeated 3 times after which the washed pellets were resuspended with a glass:glass homogenizer in ice-cold 2000 mM sucrose, 0.1 mM MgCl2, and 10 mM Tris-HCl buffer, pH 7.5 (4 ml/g liver weight) and centrifuged at 25,000 × g for 40 min in a Sorvall SS-34 rotor at 4°. The pellet was the purified nuclear fraction.

Preparations of microsomes and 100,000 × g supernatant fractions from liver were done as previously described (40).

Mutagenesis Assay. Mutagenesis was carried out according to Ames et al. (1). To 2 ml of molten top agar at 45° were added 0.1 ml of the bacterial tester strain TA-1538 (2 to 3 × 108 bacteria per ml), 0.1 ml of DMSO containing the chemical to be tested, 0.5 ml of the various subcellular fractions containing 0.5 to 1.5 mg protein, 33 μmol of KCl, and 100 μmol of sodium phosphate buffer, pH 7.4, and with
or without NADPH (10 μmol/ml). In assays where inhibitors were used, they were added in either 0.1 ml of buffer or 0.1 ml of DMSO to the bacteria, subcellular fractions, cofactors, and top agar and mixed. The test compound was added last. The colonies on each plate (histidine revertants) were counted with a Count-all (Model 600) colony counter (Fisher Scientific Co., Pittsburgh, Pa.) after a 2-day incubation at 37°.

Covalent Binding of N-OH-AAF to Microsomal and 100,000 X g Supernatant Proteins. The incubation mixture for the assay of covalent binding of N-OH-AAF to microsomal and cytosol proteins contained, in a final volume of 3 ml (final concentrations), 250 mM of potassium phosphate buffer, pH 7.4; 5 mg enzyme protein; and 0.5 mM [3H]-N-OH-AAF (4 μCi/mmol). For experiments in which the effect of the deacetylase inhibitor paraoxon was studied, this compound was added at various concentrations in 20 μl of DMSO immediately prior to addition of substrate. When the compound was added at various concentrations in 20 μl of DMSO immediately prior to addition of substrate. When the compound was added last. The colonies on each plate (histidine revertants) were counted with a Count-all (Model 600) colony counter (Fisher Scientific Co., Pittsburgh, Pa.) after a 2-day incubation at 37°.

RESULTS

In Vitro Mutagenic Activation of N-OH-AAF by Liver Cell Nuclei from Mice and Rats. The isolated mouse and rat liver cell nuclei were checked for purity by electron microscopy and found to be free of mitochondrial and microsomal contamination (data not shown). Chart 1 shows the dependence on nuclear protein and substrate concentrations for N-OH-AAF mutagenesis mediated by liver nuclei from MC-pretreated B6 mice. The mutation frequency of N-OH-AAF per plate was linear up to about 1.0 mg nuclear protein (Chart 1A) and up to 20 μg of substrate (Chart 1B). The bacterial toxicity at the highest concentration of N-OH-AAF used was less than 10%. Similar dependence on nuclear protein and substrate (data not shown) was obtained with nuclei from rat liver. Mutagenic activation of AAF was also observed in liver nuclei from MC-treated B6 mice (Chart 2), indicating the existence of an operational cytochrome P-450-dependent monooxygenation in the nuclear preparation. The mutagenic activation of AAF in liver cell nuclei from untreated B6 mice was very low (Chart 2). This is in agreement with earlier studies by Khandivala and Kasper (19) on MC induction of cytochrome P-450-dependent monoxygenase(s) in the nuclear membranes. To maintain the uniformity of the nuclear preparation, all the liver cell nuclei used in this study were obtained from animals pretreated with MC. The increase in N-OH-AAF mutagenicity when NADPH was included with the mouse liver nuclei was blocked by ANF (Chart 3), further indicating the participation of cytochrome P-450 in the mutagenic activation of AAF and/or AF, either of which may have been generated via reduction of N-OH-AAF and N-OH-AF to AAF and AF, respectively (26). ANF in concentrations up to 10⁻⁴ M had no effect on the mutagenic activation of N-OH-AAF by liver nuclei in the absence of NADPH. Addition of NADPH to the rat liver nuclei also increased N-OH-AAF mutagenicity (Chart 4). Paraoxon (10⁻⁸ M) almost totally blocked the N-OH-AAF mutagenesis in nuclear preparation from both mouse and rat (Chart 5). We had previously observed the same effect of paraoxon on N-OH-AAF mutagenesis when microsomes from mouse or rat liver were used for the mutagenic activation (38). Paraoxon (10⁻⁴ M) also blocked the mutagenic activation of N-acetoxy-AAF by mouse liver nuclei (Chart 6).
Mutagenic Activation of N-OH-AAF by Liver Cell Nuclei

Chart 1. N-OH-AAF mutagenicity in vitro as a function of nuclear protein concentration (A) and substrate concentration (B). The liver cell nuclei were isolated from male B6 mice 48 hr after MC treatment (80 mg/kg i.p.) as described in “Materials and Methods.” The concentration of N-OH-AAF in A was 10 µg/plate, and the nuclear protein concentration in B was 1.0 mg/plate. Points, mean of 2 experiments with determinations in triplicate. In a typical experiment, N-OH-AAF (10 µg/plate) with 1.0 mg of mouse nuclear protein gave 2460 ±380 (N = 3) revertants. Similar means ± S.D. were obtained upon repeating the experiments. In this and subsequent charts, the number of background revertants caused by N-OH-AAF at the same concentrations in the absence of any subcellular fractions has been subtracted.

Chart 2. Mutagenic activation of AAF by liver cell nuclei from control and MC-treated mice. •, liver cell nuclei from MC-treated male B6 mice; O, liver cell nuclei from untreated B6 mice. The nuclear protein concentration was 1.20 mg/plate in both cases. Points, mean of 2 experiments with determinations in triplicate.

Chart 3. ANF inhibition of NADPH-stimulated N-OH-AAF mutagenesis in mouse liver nuclei. The liver cell nuclei were isolated from MC-treated (80 mg/kg i.p.) male B6 mice as described in “Materials and Methods.” The nuclear protein concentration was 1.10 mg/plate. •, experiments without NADPH; O, experiment with NADPH and ANF. N-OH-AAF and ANF were added to the assay mixture in DMSO. Points, mean of 2 experiments with determinations in triplicate.

Chart 4. Effect of NADPH on mutagenic activation of N-OH-AAF by liver cell nuclei from rat. The liver cell nuclei were isolated from male Sprague-Dawley rats 48 hr after MC treatment (80 mg/kg i.p.) as described in “Materials and Methods.” The nuclear protein concentration was 0.85 mg/plate. •, experiment with NADPH; O, experiment without NADPH. Points, mean of 2 experiments done in triplicate.

Effect of Rat Liver 100,000 x g Supernatant on In Vitro Mutagenic Activation of N-OH-AAF by Liver Cell Nuclei. The 2 enzymes N, O-acyltransferase and sulfotransferase, which are thought to activate N-OH-AAF to its ultimate carcinogenic species in the rat liver, are localized in the cytosol (7, 20). The capacity of these enzymes for mutagenic activation of N-OH-AAF was investigated with the 100,000 x g supernatant from rat liver, since the activities of these 2 enzymes in the mouse liver are very low (7, 20) as an enzyme source with or without the isolated nuclei (Charts 7 and 8). The mutagenic activation of N-OH-AAF by the 100,000 x g supernatant was about 7 times greater than that observed in the rat nuclei. Paraoxon, in contrast to the observation in the nuclei, had no effect on the mutagenic activation of N-OH-AAF by the 100,000 x g supernatant, whereas activation of sulfotransferase by addition of PAPS decreased the mutation frequency in agreement with earlier data from this laboratory (Chart 7; Refs. 34 and 38). Addition of the supernatant to nuclei from rat liver increased the...
mutagenicity of N-OH-AAF, although to a much lesser degree than would be expected if the mutagenic activities of these 2 cell fractions were purely additive (Chart 8A). No inhibition of N-OH-AAF mutagenicity by paraoxon was observed when the nuclei and supernatant from rat were combined (Chart 8A). The mutagenic activation of N-OH-AAF by mouse liver nuclei and the supernatant from rat liver was similar (Chart 8B). Mutagenicity of N-OH-AAF was decreased when these 2 subcellular fractions were combined, although to a lesser extent than observed with the rat liver nuclei (Chart 8). In contrast to the rat liver supernatant and nuclei combination, addition of paraoxon (10⁻⁵ M) to the combination of the rat liver supernatant and mouse liver nuclei inhibited the mutagenic activation of N-OH-AAF by 50% (Chart 8).

Effect of Nucleophiles and Antioxidants on in Vitro Mutagenic Activation of N-OH-AAF by Mouse Liver Cell Nuclei. Cysteamine (10⁻² M) inhibited the N-OH-AAF mutagenesis by 70%, whereas methionine, BHT, and vitamin E at the same concentration caused 20 to 30% inhibition (Chart 9). Vitamin C (10⁻² M), in contrast to the other compounds, stimulated N-OH-AAF mutagenesis more than 2-fold (Chart 9). A similar degree of stimulation of N-OH-AAF mutagenesis by vitamin C was observed when 100,000 x g supernatant from rat liver was used as source of enzymes (data not shown).

In Vitro Covalent Binding of N-OH-AAF to Cellular Macromolecules. The degree of covalent binding to microsomal protein was about 4 times greater than the binding to soluble protein (100,000 x g supernatant). When PAPS was included with the soluble fraction, the covalent binding of N-OH-AAF to the protein was increased by 60%. Paraoxon did not inhibit the covalent binding of N-OH-AAF to the soluble proteins (Table 1). However, in contrast, paraoxon at 10⁻⁶ M inhibited the covalent binding of N-OH-AAF to microsomal proteins by 80% (Table 1) and by about 50% when the microsomal and soluble fractions were mixed and PAPS was included. Table 2 depicts the effect of paraoxon and PAPS on in vitro covalent binding of N-OH-AAF to nuclear nucleic acids and proteins and soluble proteins from rat liver when the nuclei were used alone or in combination with the soluble fraction. The degree of covalent binding to the nuclear proteins and RNA was similar, 4763 and 4860 pmol/mg/10 min, respectively, whereas the binding to DNA was 21.0 pmol/mg/10 min (Table 2). Paraoxon significantly inhibited (p < 0.05) the covalent binding of N-OH-AAF to nuclear proteins (55% inhibition), RNA (68% inhibition), and DNA (94% inhibition) when incubated with the nuclei (Table 2). Combining the nuclei and the soluble fraction resulted in no significant change in the covalent binding of N-OH-AAF to the nuclear nucleic acids and proteins, whereas addition of PAPS to this combination resulted in significantly decreased (p < 0.05) binding to the
Mutagenic Activation of N-OH-AAF by Liver Cell Nuclei

Chart 8. N-OH-AAF mutagenesis in vitro. Effect of combining liver cell nuclei from rat (A) and mouse (B) with 100,000 x g supernatant (Supernat) fraction of rat liver. Isolation of liver cell nuclei and 100,000 x g supernatant fraction as described in "Materials and Methods." The nuclear protein concentration was 1.00 mg/plate for both mouse and rat, and the supernatant concentration was 2.10 mg/plate. O, number of revertants with nuclei alone; D, number of revertants with the supernatant fraction alone; •, number of revertants with nuclei and the supernatant combined; A, number of revertants with nuclei and the supernatant combined treated with paraxon (10^-4 M). Points, mean of 2 experiments done in triplicate.

Chart 9. Effect of antioxidants and nucleophiles on N-OH-AAF mutagenicity in mouse liver nuclei. Isolation of liver cell nuclei as described in "Materials and Methods." The nuclear protein concentration was 1.20 mg/plate. O, number of revertants in nuclei alone; •, •O, O, A, A, effect of vitamin (Vit.) C, vitamin E, BHT, methionine (Meth.), and cysteamine (Cysta.), respectively, on the number of revertants. The concentration of each compound was 10^-2 M in the assay system. Points, mean of 2 experiments done in triplicate.

nuclear proteins and RNA but in no significant change in the binding to DNA. Paraoxon at the same concentration (10^-4 M), which inhibited the covalent binding of N-OH-AAF to nuclei alone, had no effect on the binding when the nuclei and soluble fraction were combined (Table 2). DMSO at concentration up to 3.2 x 10^-2 M had no significant effect on the covalent binding of N-OH-AAF to nuclear nucleic acids and protein.

The results of treating mouse liver nuclei with cysteamine, methionine, vitamin C, paraxon, and DMSO on the in vitro covalent binding of N-OH-AAF to nuclear nucleic acids and protein are shown in Table 3. Cysteamine (10^-2 M) and vitamin C (10^-2 M) significantly inhibited (p < 0.05) the binding to nuclear protein and RNA but had no significant effect on the binding to nuclear DNA. Paraoxon (10^-4 M) significantly (p < 0.05) inhibited the binding to both nuclear RNA and DNA as well as to nuclear protein in an agreement with observations in the rat nuclei (Table 2).

DISCUSSION

The proximate carcinogen N-OH-AAF is activated in target organs via several enzymic and possibly nonenzymic pathways to the ultimate carcinogen(s) capable of covalent interaction with critical cellular macromolecules and thereby initiates the carcinogenic process (2, 3, 9, 10, 20, 33). Similarly, the in vitro mutagenic activation of N-OH-
Salmonella test system may also occur via several enzymic and nonenzymic pathways (2, 8, 39, 42). In this study we have shown that purified liver cell nuclei from rat and mouse can activate N-OH-AAF to both a mutagen(s) in nuclear nucleic acids and protein from mouse liver cell nuclei are described under "Materials and Methods." Pretreatment aoxon had no effect on the mutagenic activation of N-OH-AAF by both rat and mouse liver cell proteins. Furthermore, we have shown that the mutagenic activation of N-OH-AAF by subcellular liver fractions from mouse and rat in the mutagenesis assay (39). The liver cell nuclei activate N-OH-AAF to both a mutagen(s) in nuclear nucleic acids and protein from mouse liver cell nuclei are described under "Materials and Methods." Pretreatment aoxon had no effect on the mutagenic activation of N-OH-AAF by both rat and mouse liver cell proteins. Furthermore, we have shown that the mutagenic activation of N-OH-AAF by subcellular liver fractions from mouse and rat in the Salmonella test system, whereas N-acetoxy-AAF is by itself a very weak mutagen (30, 39). The mutagenicity of N-acetoxy-AAF is, however, increased severalfold when either the 9000 x g or 100,000 x g liver fractions are included in the mutagenesis assay (39). The liver cell nuclei activate N-acetoxy-AAF, and this activation is inhibited by paraoxon (Chart 6). The mutagenicity of N-acetoxy-AAF in this system is, however, 50% less than that of N-OH-AAF. The mutagenic activation of N-OH-AAF in the 100,000 x g supernatant fraction from rat liver has been shown previously (24, 39). The mutagenic activation of N-OH-AAF by the supernatant, in contrast to both the liver microsomes and nuclei, is not inhibited by paraoxon but is decreased by about 50% when PAPS is included in the assay system (Chart 7; Ref. 34). Although the mechanism of N-OH-AAF mutagenic activation by the supernatant fraction is not totally understood, the available evidence indicates that deacetylation of N-OH-AAF by acetyltransferase and perhaps by free radical formation is responsible for the activation whereas sulfotransferase does not play a part in the mutagenic activation of N-OH-AAF (2, 21). This conclusion is further supported by the data obtained when the mutagenesis testing of N-OH-AAF is performed by combining the rat and mouse nuclei and the supernatant from rat (Chart 8). Both nucleophiles and antioxidants are known to decrease the carcinogenicity of AAF and many other chemical carcinogens (11). The effect of these compounds, however, on the mutagenicity of chemical carcinogens in the various test systems is less well known. Protection from radiation-induced mutation by a variety of nucleophiles is, however,
The mutagenicity of N-OH-AAF in the Salmonella-nuclei test system is reduced by the nucleophiles methionine and cysteamine and by the antioxidants vitamin E and BHT, whereas vitamin C, itself an antioxidant, markedly increased the mutagenicity of N-OH-AAF (Chart 4). The mechanism by which vitamin C induced an increase in N-OH-AAF mutagenicity is not clear at this point. A possible explanation is that vitamin C reduced the nitroxyl free radical of N-OH-AAF back to the parent hydroxamic acid, which in turn was deacetlylated by either the membrane-bound amidase or the soluble acyltransferase to generate the highly mutagenic hydroxylamine.

The testing of a variety of chemical carcinogens in the Salmonella: microsomal (or 9000 x g supernatant) test system has shown that more than 85% of these carcinogens are also mutagens (30). The possibility therefore exists that the same enzymes involved in the mutagenic activation of chemical carcinogens also mediate the in vivo activation of these compounds to ultimate carcinogens. For assessment of the relative roles of the deacetylase, acyltransferase, sulfotransferase, and free radical formation in the mutagenic and perhaps also in the carcinogenic activation of N-OH-AAF, we compared the mutagenic activation of N-OH-AAF by isolated rat liver cell nuclei alone or in combination with the 100,000 x g supernatant fraction to the extent of covalent binding of N-OH-AAF to nuclear nucleic acids and proteins under the same conditions (Charts 8 and 9; Tables 1 and 2).

Paraoxon inhibits, in agreement with its effect on the N-OH-AAF mutagenesis, the covalent binding of N-OH-AAF to both nuclear proteins and the nucleic acids, whereas the addition of the 100,000 x g supernatant to the nuclei does not increase, in contrast to the N-OH-AAF mutagenesis, the covalent binding of N-OH-AAF to nuclear protein or nucleic acids (Chart 8A; Table 2). The combination of nuclei, 100,000 x g supernatant, and PAPS results in an increase in the covalent binding of N-OH-AAF to the soluble protein and a decrease in the binding to nuclear protein and RNA but no change in the binding to the nuclear DNA. The same combination decreases the mutagenicity of N-OH-AAF whether the nuclei are omitted (Chart 7) or combined with the supernatant and PAPS (data not shown). These data indicate that a paraoxon-sensitive amidase localized in the nuclear membrane as well as in the microsomes is capable of activating N-OH-AAF in vitro to both a mutagen and/or an electrophile capable of covalent binding to nuclear nucleic acids and protein. Sulfotransferase, however, does not appear to play a role in the in vitro activation of N-OH-AAF into either a mutagen and/or an electrophile capable of covalent interaction with nuclear nucleic acids or protein. King et al. (21, 44) have studied the in vitro mutagenic activation of N-OH-AAF by rat liver N,O-acetyltransferase and have concluded that mutagenic activation of N-OH-AAF by the N,O-acetyltransferase occurs via deacetylination. When rat nuclei and the supernatant are combined, both the N-OH-AAF mutagenesis and the extent of covalent binding of N-OH-AAF to the nuclear nucleic acids and protein are not affected by addition of paraoxon, although mutagenesis and covalent binding of N-OH-AAF by nuclei alone are inhibited by paraoxon (Charts 5 and 8A; Table 2). A similar interaction but to a lesser degree is observed when the supernatant is combined with mouse nuclei and rat liver microsomes (Chart 8B; Table 1). The mechanism of interaction between the 100,000 x g supernatant fraction and the membrane-bound amidase(s) located in organelles such as microsomes and nuclei is unclear at this point. It is, however, possible that the inhibition of paraoxon-sensitive amidase activity is caused by high-affinity endogenous substrates in the supernatant fraction. The mutagenic activation of N-OH-AAF by the combination of nuclei and the supernatant may therefore be catalyzed primarily by the N,O-acetyltransferase, an enzyme insensitive to paraoxon inhibition (C. M. King, personal communication).

The role of free radical formation from N-OH-AAF, either directly or indirectly by forming, e.g., 2-nitrosofluorene and N-acetoxy-AAF from the nitrosoyl free radical (3, 4, 9, 10) in the in vitro mutagenic activating as well as covalent binding to the nuclear nucleic acids and protein is unclear. Cysteamine, for example, inhibits the mutagenesis of N-OH-AAF by the liver nuclei to the same extent as does the covalent binding of N-OH-AAF to nRNA and protein but has no effect on the covalent binding of N-OH-AAF to nuclear DNA (Chart 9; Table 3). Vitamin C, on the other hand, increases the mutagenicity of N-OH-AAF and decreases the covalent binding to nRNA and protein but has no effect on the extent of covalent binding to nuclear DNA (Chart 9; Table 3). Although cysteamine would presumably react both with a free radical, such as the nitroxyl radical, and an electrophile, such as the nitrenium ion, and therefore not discriminate between these 2 species, vitamin C is not expected to react with the nitrenium ion. Since vitamin C reacts via 1 electron reduction with free radicals, the possibility exists that the covalent binding of N-OH-AAF to nuclear proteins and nucleic acids occurs in part via free radical formation, whereas the mutagenic species of N-OH-AAF is primarily an ionic one, most probably the nitrenium ion. The latter proposition is supported by the observation that vitamin C has no effect on the mutagenicity of N-OH-AF in the Salmonella test system (38). We therefore conclude that the first step in the in vitro mutagenic activation of N-OH-AAF by isolated rat liver cell nuclei and/or 100,000 x g supernatant as well as the covalent binding of N-OH-AAF to nuclear nucleic acids and protein occurs primarily via deacetylation by either the membrane-bound amidase or by the acyltransferase in the supernatant fraction (Chart 10). The formation of the nitrenium ion from N-OH-AF at neutral pH is very slow, and it is only at acidic pH that N-OH-AF reacts via the nitrenium ion to any extent with nucleophilic sites on nucleic acids and proteins (22). It therefore seems likely that the final step in the mutagenic activation of N-OH-AF takes place within the bacteria via the formation of either the nitrenium ion or a free radical.

The susceptibility of rats and mice to hepatic carcinogenesis by N-OH-AAF correlates well with the activity of liver sulfotransferase (7, 46), and this carcinogenesis can be enhanced by the administration of sodium sulfate (46). Rat liver sulfotransferase does not appear to contribute to either the in vitro mutagenic activation or to the in vitro covalent binding of N-OH-AAF to nuclear nucleic acids and protein. It is therefore tempting to speculate, on the basis of the data presented here, that the role of sulfotransferase in the hepatocarcinogenesis of N-OH-AAF is due to the
cytotoxic effects of the sulfate ester, which in turn leads to cell proliferation and thereby promotes the "initiated" cells.

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Mutagenic Activation of N-OH-AAF by Liver Cell Nuclei


Mechanism of in Vitro Mutagenic Activation and Covalent Binding of N-Hydroxy-2-acetylaminofluorene in Isolated Liver Cell Nuclei from Rat and Mouse

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