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Solvolysis and Metabolic Degradation, by Rat Liver, of the Ultimate Carcinogen, *N*-Sulfonoxy-2-acetylaminofluorene

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SUMMARY

The synthetic ultimate carcinogen, *N*-sulfonoxy-2-acetylaminofluorene (K^+ salt, *N*-OSO_3K-2-AAF), undergoes several solvolytic and metabolic reactions that have not been reported heretofore. In aqueous media, 45–50% of *N*-OSO_3K-2-AAF is converted to 4-hydroxy-2-acetylaminofluorene, presumably by the ionic mechanism proposed previously for the formation of the *m*-amidofluorenol from *N*-acetoxy-2-acetylaminofluorene. 4-Hydroxy-2-acetylaminofluorene was not mutagenic and did not react with the nucleophile, guanosine. In aqueous media, rearrangement of *N*-OSO_3K-2-AAF to the biologically inactive *o*-amidofluorenols, 1- and 3-hydroxy-2-acetylaminofluorene, or to the respective sulfates, were only minor reactions (~3% and ~6–8%, respectively). In the presence of bovine serum albumin, *N*-OSO_3K-2-AAF was converted nearly quantitatively to the sulfates of 1- and 3-hydroxy-2-acetylaminofluorene. The rearrangement was abolished by heat denaturation of the bovine serum albumin. In the presence of rat liver homogenate or of hepatic cytosol of the rat, 30–40% of *N*-OSO_3K-2-AAF was reduced to the procarcinogen, 2-acetylaminofluorene, at the expense of 4-hydroxy-2-acetylaminofluorene. The reduction appears to be catalyzed by a low molecular weight compound in the cytosol of rat liver since denaturation of cytosolic proteins by heat had no effect on the extent of the reduction. Cytosolic reduction of *N*-OSO_3K-2-AAF to 2-acetylaminofluorene was markedly inhibited by the nucleophile, *N*-acetyl-L-methionine. This observation indicates that covalent interaction of *N*-OSO_3K-2-AAF with nucleophiles and reduction of *N*-OSO_3K-2-AAF are competing reactions.

Activation of the hepatocarcinogen, 2-AAF, involves a two-step reaction sequence. In the first step, 2-AAF is oxidized by microsomal cytochrome P-450 to the arylhydroxamic acid, *N*-OH-2-AAF (1–3). In the second step, *N*-OH-2-AAF is activated by several intracellular enzymes to reactive species (ultimate carcinogens) that form covalent adducts with cellular acceptors (2, 3). Enzymatic sulfation of *N*-OH-2-AAF by hepatic sulfotransferase generates an electrophile that interacts with cellular macromolecules (1–4), including DNA (1, 2), and is considered to be an ultimate hepatocarcinogen (1). This view was supported by experiments showing that synthetic *N*-OSO_3K-2-AAF reacts through a bimolecular mechanism with cellular receptors of high and low molecular weight (5). Similarly, N-acetoxy-2-acetylaminofluorene *N*-OAc-2-AAF, a model ultimate carcinogen, interacted with the same nucleophiles by the same mechanism, although to a lesser extent (5, 6). Although instability of *N*-OSO_3K-2-AAF in aqueous media has been established (7), the products of its degradation, either by solvolysis or by cellular metabolism, have not been identified. Studies of the reactivity of the potassium salt of *N*-acetyl-2-naphthylhydroxylamine-*O*-sulfate in aqueous media at pH 7 demonstrated an *o*-rearrangement to the sulfate ester of 2-acetamido-1-naphthol as well as formation of 2-acetamido-1-naphthol (8). A similar rearrangement has been shown to occur in the solvolysis, at neutral pH, of *N*-OAc-2-AAF (9, 10). In the case of *N*-OAc-2-AAF minor amounts (6%) of 2-AAF were also detected (10). It has been speculated that rearrangement of *N*-sulfonoxy-2-AAF proceeds more readily than that of *N*-OAc-2-AAF and may be a pathway for detoxification of this ultimate carcinogen (9, 11). The availability of synthetic *N*-OSO_3K-2-AAF made it possible to initiate studies of the reactivity of this ultimate carcinogen (5). A major objective of the present study was to determine whether, analogous to *N*-OAc-2-AAF, rearrangement of *N*-OSO_3K-2-AAF to the sulfates of the *o*-amidofluorenols, solvolysis to the *o*-amidofluorenols, and reduction to 2-AAF accounted for the rapid disappearance of *N*-OSO_3K-2-AAF from aqueous media at neutral pH. A second objective was the identification of the products of the disposi-

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ABBREVIATIONS: 2-AAF, 2-acetylaminofluorene; *N*-OSO_3K-2-AAF, *N*-sulfonoxy-2-acetylaminofluorene (K^+ salt); *N*-OH-2-AAF, *N*-hydroxy-2-acetylaminofluorene; *N*-OAc-2-AAF, *N*-acetoxy-2-acetylaminofluorene; *N*-Ac-L-Met, *N*-acetyl-L-methionine; Guo, guanosine; MBE, methyl-*tert*-butyl ether; 1-OH-2-AAF, 1-hydroxy-2-acetylaminofluorene; 3-OH-2-AAF, 3-hydroxy-2-acetylaminofluorene; 1-CH_3S-2-AAF, 1-methylthio-2-acetylaminofluorene; 3-CH_3S-2-AAF, 3-methylthio-2-acetylaminofluorene; Guo-2-AAF, *N*-(guanosin-8-yl)-2-acetylaminofluorene; 1-OSO_3K-2-AAF, 1-sulfonoxy-2-acetylaminofluorene (K^+ salt); 3-OSO_3K-2-AAF, 3-sulfonoxy-2-acetylaminofluorene (K^+ salt); MeOH, methanol; HPLC, high performance liquid chromatography; 4-OH-2-AAF, 4-hydroxy-2-acetylaminofluorene; BSA, bovine serum albumin.

tion of *N*-OSO_3K-2-AAF by homogenates and cellular fractions of rat liver, a major target of the carcinogenic action of 2-AAF. Finally, the mutagenic potential and adduct formation of the newly identified products of the solvolytic and metabolic degradation of *N*-OSO_3K-2-AAF were assayed to determine whether the carcinogenic action of 2-AAF is attributable exclusively to *N*-OSO_3K-2-AAF or whether compounds formed during the degradation of *N*-OSO_3K-2-AAF contribute to its biological action.

Experimental Procedures

Materials

N-Ac-L-Met and Guo were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Nitrofluorene, MBE, and dicyclohexylcarbodiimide were purchased from Aldrich Chemical Co. (Milwaukee, WI). [5′-^{3}H]Guo (24 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL).

Preparation of Cell Fractions of Rat Liver

Male Sprague-Dawley rats (225–250 g, Bio-Lab Corp., St. Paul, MN) were sacrificed by decapitation. For each experiment, the livers from three rats were pooled and a 25% homogenate in 0.05 M Tris-HCl buffer, pH 7.0, was prepared (6). Cytosol and microsomal fractions were obtained as described (12). Prior to use, each fraction was diluted with the buffer to give the protein concentrations listed in Tables 1, 5, 6, and 7. Protein concentrations were determined according to the method of Lowry *et al.* (13).

Preparation of Compounds

2-AAF (14), 2-nitrosofluorene (15), *N*-OH-2-AAF (16), *N*-OSO_3K-2-AAF (5), *N*-OSO_3K-2-[*ring*-^{3}H]-AAF (5), 1- and 3-OH-2-AAF (17), 1- and 3-CH_3S-2-AAF (18), Guo-2-AAF (19), and *N*-acetyl-L-[*methyl*-^{3}H]methionine (5) were prepared by the published procedures. Melting points and spectral (UV, IR) properties were in agreement with those reported. The potassium salts of the *O*-sulfates of 1- and 3-OH-2-AAF (1- and 3-OSO_3K-2-AAF), which have not been prepared and characterized heretofore, were synthesized by the modification of the procedure of Beland *et al.* (20) that was used for the synthesis of *N*-OSO_3K-2-AAF (5). To 1-OH-2-AAF (199 mg, 0.83 mmol) and dicyclohexylcarbodiimide (870 mg, 4.2 mmol) in dimethylformamide (40 ml) was added concentrated sulfuric acid (0.05 ml, 0.90 mmol). The mixture was stirred under nitrogen (1.5 hr), filtered, and the filtrate was purged with ammonia (3 min). The filtrate was then concentrated by solvent evaporation at reduced pressure and the residue was stirred in ammonia-saturated methanol (2–3 ml) containing potassium acetate (83 mg, 0.84 mmol). Following filtration of the mixture, the solvent was evaporated at reduced pressure. The residue was washed with dry diethyl ether and taken up in methanol (8 ml). 1-OSO_3K-2-AAF (62 mg) crystallized on cooling in ice. Additional compound (62 mg) was obtained on evaporation of the methanol and crystallization of the remaining solid from ethanol. The total yield was 42%. IR absorption spectrum: ν (KBr) 1,660 (C═O), 1,250 (S═O) cm^{-1}. UV absorption spectra: λ_{max} (MeOH) 287 (ϵ, 25,500), 278 (ϵ, 25,000) nm; λ_{max} (H_2O) 274 (ϵ, 21,918) nm. Anal. Calculated for $C_{15}H_{12}NO_5KS$: C, 50.40; H, 3.39; N, 3.92; S, 8.97. Found: C, 50.43; H, 3.53; N, 3.64; S, 8.92. The preparation of 3-OSO_3K-2-AAF from 3-OH-2-AAF (203 mg, 0.85 mmol) was essentially the same as that described above for 1-OSO_3K-2-AAF with the following minor changes. After addition of potassium acetate and filtration of the mixture, evaporation of the solvent yielded an oil which solidified on addition of dry diethyl ether. The crude product was recrystallized from ethanol to give 3-OSO_3K-2-AAF (220 mg, 72% yield). IR absorption spectrum: ν (KBr) 1,674 (C═O), 1,245 (S═O) cm^{-1}. UV absorption spectra: λ_{max} (MeOH) 278 (ϵ, 21,200), 306–314 (broad peak, ϵ, 14,500) nm; λ_{max} (H_2O) 272 (ϵ, 19,975), 302 (ϵ, 13,393) nm. Anal. Calculated for $C_{15}H_{12}NO_5SK$: C, 50.40; H, 3.39; N, 3.92; S, 8.97. Found: C, 50.60; H, 3.59; N, 3.59; S, 8.74. 1- and 3-OSO_3K-2-AAF gave single peaks by reverse phase HPLC [column: 10-μm particle size, C-18 (RP10), 250 × 4.6 mm (Alltech Associates, Deerfield, IL); eluent, 40% methanol; retention time = 4 min]. Analytical thin layer chromatography (silica gel HLF Uniplates; Analtech, Inc., Newark, DE) of 1- and 3-OSO_3K-2-AAF, with chloroform/ethanol (65:35) as solvent, gave single spots, R_f = 0.25. There was no evidence for the presence of 1- or 3-OH-2-AAF in 1- or 3-OSO_3K-2-AAF, respectively. UV absorption spectra of 1- and 3-OSO_3K-2-AAF are shown in Fig. 1.

4-OH-2-AAF was prepared by solvolysis of *N*-OSO_3K-2-AAF as follows: *N*-OSO_3K-2-AAF (149 mg, 0.42 mmol) in dimethylformamide (0.5 ml) was added to the aqueous phase of a mixture of 0.05 M Tris-HCl buffer, pH 7.0 (50 ml) and ethyl acetate (200 ml). The mixture was stirred at room temperature for 2 hr. The ethyl acetate layer was separated and washed with 0.01 M sodium chloride. The ethyl acetate was then evaporated at reduced pressure. The residue solidified on standing at room temperature. A portion (35 mg) of the crude product (106 mg) was dissolved in ethyl acetate and purified by chromatography on a silica gel (100–200 mesh, 60A; Mallinckrodt, Inc., Paris, KY) column (11 × 1.1 cm) using ethyl acetate/*n*-hexane (2:1) as eluent. The second of two bands was collected and yielded 4-OH-2-AAF, m.p. 270–274° [reported: 270–272.5° (21)], after solvent evaporation. UV absorption spectrum: λ_{max} (MeOH) 280 (ϵ, 19,842), 293 (ϵ, 18,423) nm. The UV spectrum of the isolated material was identical with that of a sample of authentic 4-OH-2-AAF (21) donated by Dr. T. L. Fletcher (Hutchinson Cancer Research Center, Seattle, WA) to Dr. D. Malejka-Giganti (Veterans Administration Medical Center, Minneapolis) and made available to us. HPLC of the purified compound on Corasil II (22) (Waters Associates, Milford, MA), with ethyl acetate/*n*-hexane (4:6) as eluent gave a single peak (retention time = 28 min). Small amounts (10–25 mg) of crude 4-OH-2-AAF were also purified by preparative thin layer chromatography on silica gel plates (thickness, 1000 μm; Analtech) with chloroform/methanol (10:1) as solvent. 4-OH-2-AAF, isolated from the lower of two major bands, exhibited a UV absorption spectrum identical with that described above for the pure compound. To provide further evidence for the identity of 4-OH-2-AAF prepared by solvolysis of *N*-OSO_3K-2-AAF, 4-OH-2-AAF in pyridine was acetylated with acetic anhydride. After 1 hr the reaction

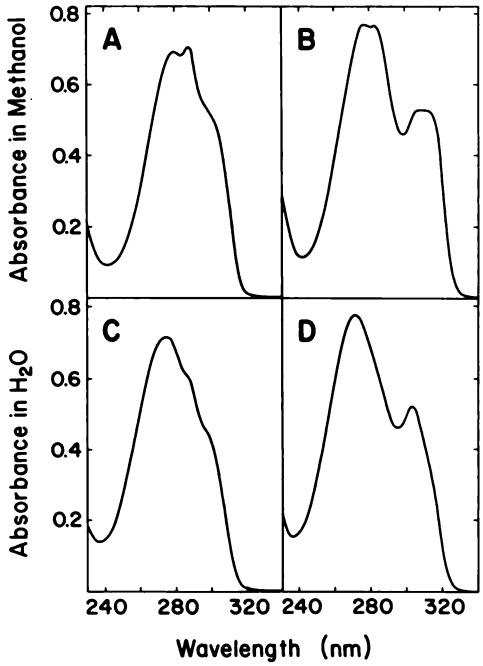

Fig. 1. UV spectra of 1- and 3-OSO_3K-2-AAF. A. 1-OSO_3K-2-AAF (in MeOH), 2.7 × 10^{-5} M; B. 3-OSO_3K-2-AAF (in MeOH), 3.7 × 10^{-5} M; C. 1-OSO_3K-2-AAF (in H_2O), 3.3 × 10^{-5} M; D. 3-OSO_3K-2-AAF (in H_2O), 3.9 × 10^{-5} M.

mixture was diluted with water and extracted with ethyl acetate. The ethyl acetate extract was washed with water. Evaporation of the ethyl acetate yielded crystalline 4-acetoxy-2-acetylaminofluorene, m.p. 234–236° [reported: 231–232° (21)]. UV absorption spectrum: λ_{max} (MeOH) 281 (ε, 36,500), 287 (ε, 39,286), 301 (ε, 31,746) nm. Reverse phase HPLC with 58% methanol gave a single peak (retention time = 21 min).

Conditions for Solvolysis and Metabolic Degradation of *N*-OSO_3K-2-AAF

Isolation and quantitation of products. In the experiments in which solvolysis of *N*-OSO_3K-2-AAF was investigated, *N*-OSO_3K-2-[*ring*-^{3}H]-AAF (50 nmol) in 0.02 ml of dimethyl sulfoxide was added to 0.05 M Tris-HCl buffer, pH 7.0 (1.0 ml). In metabolic experiments (Tables 1, 2, 5, 6, and 7) the incubation mixtures contained, in addition to labeled *N*-OSO_3K-2-AAF, homogenate of rat liver or the designated cellular fraction. The protein concentration of homogenates or cellular fractions was 5 mg/ml (see Tables 1, 2, and 5) or 2 mg/ml (see Tables 6 and 7). In some experiments (see Table 7), cupric acetate monohydrate was added to incubation mixtures, at a concentration of 0.15 mM. The mixtures were incubated at 37° for 30 min. At the end of the incubations the appropriate carrier compounds were added. Proteins were precipitated by addition of ethanol (2 ml) and removed by centrifugation. To quantify the amounts of 1- and 3-OH-2-AAF, 4-OH-2-AAF, and 2-AAF, the protein-free mixtures were extracted three times with MBE (1 ml). After evaporation of MBE under nitrogen, the residues were dissolved in methanol (0.05 ml) and the compounds were isolated by reverse phase HPLC (Fig. 2a). When incubation mixtures contained major amounts of 4-OH-2-AAF in addition to the *o*-amidofluorenols, 3-OH-2-AAF was rechromatographed on Corasil II (Fig. 2b). Since 1- and 3-OSO_3K-2-AAF were not separated by reverse phase HPLC (Fig. 2a), the amounts of the isomeric sulfate esters were estimated as follows. After removal of phenols and non-polar compounds from the incubation mixture by extraction with MBE, the aqueous phase, which contained the sulfates, was taken to dryness. The residues were taken up in methanol and subjected to reverse phase HPLC. The peak which contained the unresolved sulfates (Fig. 2a) was collected. After evaporation of the eluent the sulfates were converted to the respective *o*-amidofluorenols by acid hydrolysis with 0.25 N H_2SO_4. The *o*-amidofluorenols were extracted twice with MBE (1 ml) and the ether was washed twice with water (1 ml). After evaporation of the MBE the *o*-amidofluorenols were separated by HPLC as described above. In all experiments in which *N*-OSO_3K-2-[*ring*-^{3}H]-AAF was the substrate the quantities of solvolytic or metabolic products were determined by the method of inverse isotope dilution.

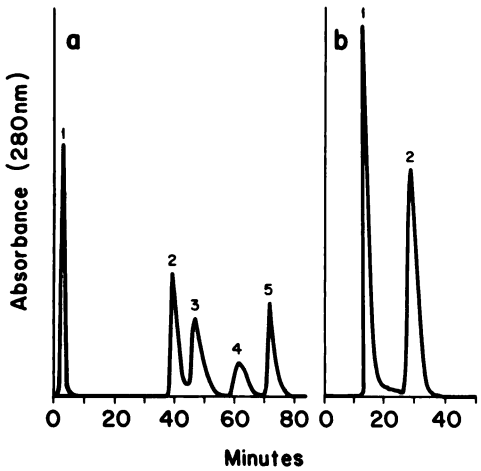

Fig. 2. A. Separation of *o*-OSO_3K-2-AAF (peak *1*), 4-OH-2-AAF (peak *2*), 3-OH-2-AAF (peak *3*), 1-OH-2-AAF (peak *4*), and 2-AAF (peak *5*) by reverse phase HPLC [C-18, 10 μm, 250 × 4.6 mm (Alltech Associates); eluent, 55% MeOH; flow rate, 0.5 ml/min]. B. Separation of 3-OH-2-AAF (peak *1*) from 4-OH-2-AAF (peak *2*) by HPLC [Corasil II (Waters Associates) eluent, ethyl acetate/*n*-hexane (35:65); 100 psi].

To test whether 1- and 3-OH-2-AAF could arise from 1- and 3-OSO_3K-2-AAF (see Table 2), the quantities of the *o*-amidofluorenols were estimated by peak height analysis of the elution profile after reverse phase HPLC of the compounds. The lower limit of detection of 1- and 3-OH-2-AAF by this method was 0.2 nmol. The amounts of 1- and 3-OH-2-AAF, produced by acid hydrolysis of 1- and 3-OSO_3K-2-AAF (see Table 2), were measured by the UV absorbance of the respective peaks of the HPLC elution profile. In these experiments, correction was made for loss of compound on the column (~25%).

Comparison of the interaction of *N*-OSO_3K-2-AAF, 1- and 3-OSO_3K-2-AAF, and 4-OH-2-AAF with nucleophiles. If 1- and 3-OSO_3K-2-AAF, in aqueous media, were to undergo dissociation to positively charged carbonium ions capable of interacting with nucleophiles or to react by a bimolecular mechanism similar to that shown for *N*-OSO_3K-2-AAF (5), the adducts formed by nucleophilic attack of sulfur atom of methionine on carbon atom 1 and 3 of the fluorene moiety would be 1- and 3-CH_2S-2-AAF. The same adducts were previously isolated and characterized as the result of the interaction of *N*-OSO_3K-2-AAF with methionine (23) or *N*-Ac-L-Met (5). In the present study, we have used the conditions established (5) for incubation of potential electrophiles with *N*-acetyl-L-[*methyl*-^{3}H]methionine, isolation of the labeled adducts, and assessment of adduct formation by inverse isotope dilution.

The electrophilic reactivity of 4-OH-2-AAF was examined by incubation of the *m*-amidofluorenol with the nucleophile, [5′-^{3}H]Guo. Guo was selected because of its demonstrated strong nucleophilicity in reactions with synthetic ultimate carcinogens (5, 6). Although the hypothetical adduct, 4-(guanosin-8-yl)-2-AAF, arising from the interaction of 4-OH-2-AAF with Guo, has not been synthesized, it is reasonable to assume that the solubility characteristics of 4-(guanosin-8-yl)-2-AAF would be sufficiently similar to those of *N*-(guanosin-8-yl)-2-AAF to permit detection by reverse phase HPLC (5, 6). 4-OH-2-AAF and [5′-^{3}H]Guo were incubated under conditions previously established for the formation *in vitro* of *N*-(guanosin-8-yl)-2-AAF from *N*-OSO_3K-2-AAF and Guo (5). Following extraction of the incubation mixture with MBE and evaporation of the solvent under nitrogen, the residue was dissolved in methanol and subjected to reverse phase HPLC. The following eluents were used in sequence: 1) 55% methanol, which elutes *N*-(guanosin-8-yl)-2-AAF, for 20 min; 2) a gradient of 55–100% methanol, over a period of 20 min; and 3) 100% methanol for 10 min. A total of 45 fractions containing 1 or 2 ml of eluate was collected, and the radioactivity of each fraction was measured following solvent evaporation.

Mutagenicity of the rearrangement products of *N*-OSO_3K-2-AAF (1- and 3-OSO_3K-2-AAF) and of 4-OH-2-AAF was measured with the use of the Ames *Salmonella* test (24), as described previously (5, 6). Triplicate plates were incubated at 37° for 48–60 hr and the number of histidine prototrophs was scored (5, 6).

Results

Instability of *N*-OSO_3K-2-AAF in Mixtures of Methanol/Water as Shown by UV Spectroscopy

Initial observations on the degradation of synthetic *N*-OSO_3K-2-AAF in methanol containing increasing amounts of water are shown in Fig. 3. In these experiments, which led us to pursue the subsequent identification of several degradation products of *N*-OSO_3K-2-AAF, decomposition of the ultimate carcinogen was followed by the decrease of the maximal absorbance of *N*-OSO_3K-2-AAF (ΔA_{276}) as a function of time and concentration of water in the medium. Whereas *N*-OSO_3K-2-AAF in dry methanol appears to be stable for at least 40 min, degradation of *N*-OSO_3K-2-AAF was markedly accelerated by increasing the content of water in the medium from 10% to

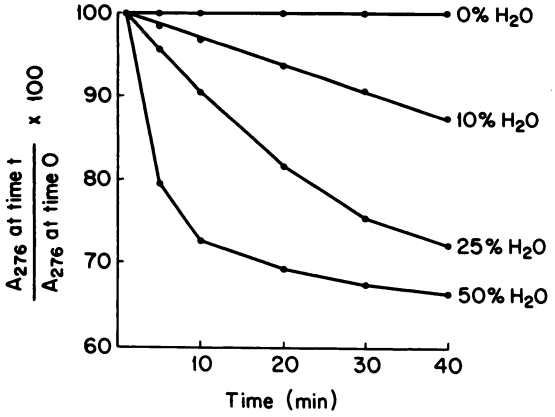

Fig. 3. Instability of *N*-OSO_3K-2-AAF in mixtures of methanol:water by UV spectroscopy. Since *N*-OSO_3K-2-AAF in MeOH absorbs maximally at 276 nm, ΔA_{276} was used as a measure of the instability of *N*-OSO_3K-2-AAF at increasing concentrations of water in the incubation system. *N*-OSO_3K-2-AAF in dry MeOH was added to the sample cuvette containing the amounts of water indicated and the UV spectrum was recorded immediately. The spectrophotometer (ACTA VI; Beckman Instruments, Inc., Irvine, CA) was placed in the Time Drive mode 1 min after addition of *N*-OSO_3K-2-AAF to the solvent and A_{276} was recorded for 40 min.

TABLE 1

Conversion of *N*-OSO_3K-2-[*ring*-^3H]-AAF to 1- and 3-OSO_3K-2-[*ring*-^3H]-AAF and to 1- and 3-OH-2-[*ring*-^3H]-AAF, by rat liver homogenates

Homogenate[a]	Metabolite formed[b]			
	1- + 3-OSO_3K-2-AAF	% of Substrate	1- + 3-OH-2-AAF	% of Substrate
	nmol		*nmol*	
–	2.9 ± 0.3	5.8	1.4 ± 0.3	2.8
+	0.8 ± 0.1	1.6	0.6 ± 0.2	1.2

[a] Protein concentration was 5 mg/ml.
[b] Values are the averages (±SD) of two experiments. Reaction conditions and methods for determining amounts of metabolites formed are described in the text.

50%. This observation confirms a previous report concerning the instability of *N*-OSO_3K-2-AAF in aqueous media (7).

Formation of 1- and 3-OSO_3K-2-AAF and of 1- and 3-OH-2-AAF from *N*-OSO_3K-2-AAF

The next part of this study was concerned with the identification of the degradation products of *N*-OSO_3K-2-AAF that might account for its disappearance from aqueous media. The first reaction examined was the rearrangement of *N*-OSO_3K-2-[*ring*-^3H]-AAF to the sulfate esters, 1- and 3-OSO_3K-2-AAF, in Tris-HCl buffer (pH 7.0) alone and in the presence of rat liver homogenate (Table 1). Occurrence of this reaction seemed likely since *N*-OAc-2-AAF had been shown to undergo rearrangement to 1- and 3-OAc-2-AAF in 40% acetone (10) and since *N*-OSO_3K-2-AAF was presumed to rearrange more readily than *N*-OAc-2-AAF (9). However, only 5.8% of *N*-OSO_3K-2-AAF rearranged to 1- and 3-OSO_3K-2-AAF in Tris-HCl buffer and even less (1.6%) was converted to the isomeric sulfate esters in the presence of rat liver homogenate (Table 1). A possible explanation for the minor amounts of the isomeric sulfates was that they were further cleaved to 1- and 3-OH-2-AAF. This explanation was ruled out by the small amounts of the *o*-amidofluorenols (1.2 and 2.8%, respectively) that were detected following incubation of *N*-OSO_3K-2-AAF in Tris buffer or in the presence of rat liver homogenate (Table 1). Moreover, neither 1-OSO_3K-2-AAF nor 3-OSO_3K-2-AAF was hydrolyzed to the *o*-amidofluorenols in the presence or absence of rat liver homogenate. Hydrolysis of the *o*-sulfate esters proceeded only in acidic media (0.25 N H_2SO_4) and required elevated temperatures (Table 2).

1- And 3-OSO_3K-2-AAF showed insignificant mutagenicity in the Ames test (24) (Table 3) compared to the standard test compounds, 2-nitroso- and 2-nitrofluorene. Even *N*-OSO_3K-2-AAF, which, for reasons previously discussed (5), exhibited low mutagenicity, was measurably more active in the Ames test than 1- or 3-OSO_3K-2-AAF. Neither of the isomeric sulfate esters formed detectable amounts of adduct with the nucleophile *N*-Ac-L-Met. In contrast, *N*-OSO_3K-2-AAF reacted with *N*-Ac-L-Met to give *o*-methylthio-2-AAF as previously described (Ref. 5; Table 3).

Stimulation of the Rearrangement of *N*-OSO_3K-2-AAF to 1- and 3-OSO_3K-2-AAF by BSA.

The data on the rearrangement of *N*-OSO_3K-2-AAF to 1- and 3-OSO_3K-2-AAF (Table 1) indicated that the extent of the reaction appeared to be significantly less in the presence of rat liver homogenate. We concluded that homogenates inhibit the conversion of *N*-OSO_3K-2-AAF, possibly by substrate binding to protein. To test this assumption, the proteins in rat liver

TABLE 2

Formation of 1- and 3-OH-2-AAF from 1- and 3-OSO_3K-2-AAF by rat liver homogenate and by acid hydrolysis

Compound	nmol Tested	H_2SO_4	Homogenate	nmol of *o*-Amidofluorenol formed[a]	
		N		1-OH-2-AAF	3-OH-2-AAF
1-OSO_3K-2-AAF	250	–	–	<0.2	
		–	+	<0.2	
		0.25[b]	–	147 ± 7	
3-OSO_3K-2-AAF	250	–	–		<0.2
		–	+		<0.2
		0.25[b]	–		147 ± 4

[a] The limit of detection of the *o*-amidofluorenols by HPLC peak height analysis was 0.2 nmol. The values are the averages of duplicate determinations.
[b] Incubations were carried out at 90° for 1 hr.

TABLE 3

Comparison of the mutagenicity and chemical reactivity of *N*-OSO_3K-2-AAF, 1- and 3-OSO_3K-2-AAF, and 4-OH-2-AAF

Compound tested	Revertants per nmol[a]	nmol of Adduct formed	
		o-CH_3S-2-AAF[b]	Guo-2-AAF[c]
N-OSO_3K-2-AAF	0.9	8.6 ± 0.3	36.0 ± 0.1
1-OSO_3K-2-AAF	<0.1	*d*	
3-OSO_3K-2-AAF	<0.1	*d*	
4-OH-2-AAF	<0.1		*e*

[a] Concentrations of test compounds *N*-OSO_3K-2-AAF, 1- and 3-OSO_3K-2-AAF, and 4-OH-2-AAF, were 20–400 μM. The values shown are the average number of reverants/nmol of compound. The average number of revertants produced by 2-nitrosofluorene (2 μM, 4 μM) and 2-nitrofluorene (20 μM, 40 μM), used as standards, were 446 and 39/nmol, respectively.
[b] Fifty nmol of *N*-OSO_3K-2-AAF, 1-OSO_3K-2-AAF, or 3-OSO_3K-2-AAF were incubated with 2500 nmol of *N*-Ac-L-[*methyl*-^3H]Met (170,000 dpm) in Tris-HCl buffer, pH 7.0 (1 ml) at 37°. After 30 min, 1- and 3-CH_3S-2-AAF were added as carrier compounds. Isolation, separation of 1- and 3-CH_3S-2-AAF by HPLC, and estimation of the amounts of adducts formed were carried out as described (5).
[c] Fifty nmol of *N*-OSO_3K-2-AAF or 4-OH-2-AAF were incubated with 2500 nmol of [5′-^3H]Guo (132,000 dpm) in Tris-HCl buffer, pH 7.0 (1 ml), at 37°. After 30 min Guo-2-AAF was added as carrier. Analysis of adduct formation is described in the text.
[d] Radioactivity of the 1- and 3-CH_3S-2-AAF peaks was <2 × background.
[e] Radioactivity detected during elution of column with 55% and 100% methanol was <2 × background.

homogenates (5 mg) were replaced by equal amounts of the homogeneous protein, BSA. Unexpectedly, conversion of *N*-OSO_3K-2-AAF to the isomeric sulfate esters was nearly quantitative (96%) under these conditions (Table 4). The catalytic effect of BSA on the rearrangement of *N*-OSO_3K-2-AAF depended on the intactness of the three-dimensional structure of BSA and was abolished by heat denaturation (Table 4). The implications of these observations on detoxification of ultimate carcinogens will be discussed below.

Identification of 4-OH-2-AAF and 2-AAF as Major Products of the Solvolysis and Metabolic Degradation of *N*-OSO_3K-2-AAF

Mechanism of formation of the two compounds. Conversion of *N*-OSO_3K-2-AAF to the isomeric sulfate esters or *o*-amidofluorenols accounted for less than 10% of degradable *N*-OSO_3K-2-AAF (Table 1). Examination of ether-soluble compounds showed 4-OH-2-AAF and 2-AAF, identified spectrophotometrically and quantified by inverse isotope dilution after separation by HPLC, to be major products of the solvolytic and metabolic degradation of *N*-OSO_3K-2-AAF (Table 5). In Tris-HCl buffer, 4-OH-2-AAF, which exhibited no significant mutagenicity or detectable chemical reactivity with guanosine (Table 3), accounted for 46% of degradable *N*-OSO_3K-2-AAF. The mechanism of formation of 4-OH-2-AAF from *N*-OSO_3K-2-AAF in aqueous media is presumably the same as that postulated for formation of the *m*-amidofluorenol from *N*-OAc-2-AAF (25). Essentially, it consists of the successive nucleophilic attack of hydroxide ions on carbon atoms 4a and 4 of the fluorene moiety of the nitrenium ion, formed from *N*-OSO_3K-2-AAF in aqueous media. Subsequent elimination of the hydroxide ion and a proton from positions 4a and 4, respectively, yields the stable *m*-amidofluorenol (25).

In the presence of rat liver homogenate, the major ether-extractable metabolite was 2-AAF, which accounted for 32% of degradable *N*-OSO_3K-2-AAF (Table 5), whereas formation of 4-OH-2-AAF was decreased by 95% (Table 5). It appears that, in the presence of rat liver cytosol, reduction of *N*-OSO_3K-2-AAF by a cellular factor(s), rather than hydroxylation of the nitrenium ion, derived from *N*-OSO_3K-2-AAF, to 4-OH-2-AAF, is the predominant reaction. Two independent metabolic pathways may account for the formation of 2-AAF from *N*-OSO_3K-2-AAF by rat liver. One is the direct reduction of *N*-OSO_3K-2-AAF, involving a single step. Alternatively, formation of 2-AAF from *N*-OSO_3K-2-AAF may involve a two-step sequence. In the first step, *N*-OSO_3K-2-AAF is hydrolyzed to *N*-OH-2-AAF. Hydrolysis of *N*-OSO_3K-2-AAF to *N*-OH-2-AAF would be analogous to the pH-dependent hydrolysis of *N*-OAc-2-AAF and other *N,O*-acetylarylamides to the respective *N*-arylhydroxamic acids (10). In the second step, the intermediate, *N*-OH-2-AAF, is reduced to 2-AAF. Reduction of *N*-OH-2-AAF to 2-AAF by a cytosolic reductase in rat liver has been demonstrated (6, 12, 15) and was confirmed in the present study (Table 6). However, when reduction of *N*-OH-2-AAF was blocked either by heat denaturation of the cytosolic reductase or by formation of a copper chelate (26, 27), formation of 2-AAF from *N*-OSO_3K-2-AAF remained unchanged (Table 6). We conclude that conversion of *N*-OSO_3K-2-AAF to 2-AAF occurs through a direct, one-step reduction, which does not involve hydrolysis of *N*-OSO_3K-2-AAF to *N*-OH-2-AAF. This conclusion is in agreement with findings of van den Goorbergh *et al* (28).

Intracellular localization of the factor which reduces *N*-OSO_3K-2-AAF. Additional experiments addressed the question whether the activity in rat liver, that reduced *N*-OSO_3K-2-AAF to 2-AAF, was identical with the cytosolic reductase that has been shown to convert *N*-OH-2-AAF to 2-AAF (12). Subcellular fractionation by the method previously used to concentrate the reductase of *N*-OH-2-AAF (12) showed that the activity that reduces *N*-OSO_3K-2-AAF was likewise associated with the cytosol (Table 7). However, whereas the reduction of *N*-OH-2-AAF was shown to be due to a cytosolic enzyme (12), heat denaturation of cytosol had no effect on the formation of 2-AAF from *N*-OSO_3K-2-AAF (Table 7). This strongly suggests that the reduction of *N*-OSO_3K-2-AAF, although catalyzed by cytosol, is a nonenzymatic reaction.

Cytosolic reduction to 2-AAF, the first step of the proposed metabolic recycling of the ultimate carcinogen, *N*-OSO_3K-2-AAF. The conversion of the ultimate carcinogen, *N*-OSO_3K-2-AAF, to the procarcinogen, 2-AAF, demonstrated in this study, raises the possibility that reduction of *N*-OSO_3K-2-AAF is the initiating step of a metabolic cycle in which *N*-OSO_3K-2-AAF may be regenerated through microsomal oxidation of 2-AAF to *N*-OH-2-AAF (1) and subsequent activation of *N*-OH-2-AAF, by cytosolic sulfation, to *N*-OH-2-AAF (1) (Fig. 4). A major factor controlling the amounts of *N*-OSO_3K-2-AAF available for a recycling process appears to be the withdrawal of *N*-OSO_3K-2-AAF by competitive interaction with cellular nucleophiles. This was demonstrated experimentally by measuring simultaneously the cytosolic reduction of *N*-OSO_3K-2-AAF and the formation of *o*-CH_3S-2-AAF in the presence of increasing amounts of the model nucleophile, *N*-Ac-L-Met (Fig. 5). In absence of *N*-Ac-L-Met, ~40% of *N*-OSO_3K-2-AAF was reduced to 2-AAF. Formation of 2-AAF declined rapidly as increasing amounts of *N*-OSO_3K-2-AAF reacted with *N*-Ac-L-Met to yield *o*-CH_3S-2-AAF. Even at low concentrations of nucleophile (2–5 mM), interaction of *N*-OSO_3K-2-AAF with *N*-Ac-L-Met was clearly the predominant reaction. At the final concentration of *N*-Ac-L-Met (20 mM), <5% of *N*-OSO_3K-2-AAF was reduced to 2-AAF. It appears

TABLE 4

Rearrangement of *N*-OSO_3K-2-[*ring*-^3H]-AAF to 1- and 3-OSO_3K-2-[*ring*-^3H]-AAF in the presence and absence of BSA

N-OSO_3K-2-AAF	BSA	Sulfate formed[a]					
		1-OSO_3K-2-AAF	% of Substrate	3-OSO_3K-2-AAF	% of Substrate	*o*-OSO_3K-2-AAF	% of Substrate
nmol	*mg/ml*	*nmol*		*nmol*		*nmol*	
50		1.8 ± 0.1	3.6	2.6 ± 0.2	5.2	4.4 ± 0.3	8.8
50	5.0	14.0 ± 0.2	28.0	33.8 ± 0.9	67.6	47.8 ± 1.1	95.6
50	5.0[b]	1.7 ± 0.1	3.4	2.8 ± 0.1	5.6	4.5 ± 0.2	9.0

[a] Values are the averages (±SD) of two experiments. Reaction conditions and methods for measuring the formation of sulfates are described in the text.
[b] BSA was denatured by heating at 95° for 10 min prior to incubation.

TABLE 5

Formation of 4-OH-2-[*ring*-^{3}H]-AAF and of 2-[*ring*-^{3}H]-AAF from *N*-OSO$_3$K-2-[*ring*-^{3}H]-AAF in the presence and absence of rat liver homogenate

N-OSO$_3$K-2-AAF	Homogenate[a]	Compound formed[b]			
		4-OH-2-AAF	% of Substrate	2-AAF	% of Substrate
nmol		*nmol*			
50	–	23.2 ± 1.5	46.4	0.8 ± 0.1	1.6
50	+	1.2 ± 0.2	2.4	15.8 ± 0.4	31.6

[a] The protein concentration was 5 mg/ml. Conditions for the reactions and methods for quantifying the formation of metabolites are given in the text.
[b] Values are the averages (±SD) of two experiments.

TABLE 6

Formation of 2-AAF by cytosolic reduction of *N*-OH-2-AAF and of *N*-OSO$_3$K-2-AAF

N-OH-2-[*ring*-^{3}H]AAF or *N*-OSO$_3$K-2-[*ring*-^{3}H]AAF (50 nmol) was incubated in the presence or absence of cupric acetate monohydrate (150 nmol). In assays containing rat liver cytosol, the amount of protein was 2 mg. Conditions for the incubations and methods for determining the amounts of 2-AAF formed are outlined in the text.

Cu^{2+}	Cytosol	2-AAF formed (nmol) from	
		N-OH-2-AAF	*N*-OSO$_3$K-2-AAF
0	–	0.1 ± 0.0	1.2 ± 0.2
0	+	7.4 ± 0.2	18.3 ± 0.2
0	+[a]	0.2 ± 0.0	16.5 ± 1.9
150	–	0.1 ± 0.0	1.2 ± 0.3
150	+	0.3 ± 0.0	17.6 ± 2.2
150	+[a]	0.4 ± 0.0	16.9 ± 1.0

[a] Proteins were denatured by heating at 95° for 10 min.

TABLE 7

Reduction of *N*-OSO$_3$K-2-[*ring*-^{3}H]-AAF (0.05 mM) to 2-[*ring*-^{3}H]-AAF by homogenate and cell fractions of rat liver

Cell preparation	Protein	2-AAF formed[a]	*N*-OSO$_3$K-2-AAF reduced
	mg/ml	*nmol*	%
		1.0 ± 0.1	2.0
Homogenate	2.0	10.1 ± 0.6	20.2
Microsomes	2.0	1.6 ± 0.1	3.2
Cytosol	2.0	19.6 ± 0.4	39.2
Cytosol[b]	2.0	20.0 ± 0.9	40.0

[a] Values are the averages (±SD) of two experiments. Reaction conditions and methods for quantifying formation of 2-AAF are described under Experimental Procedures.
[b] Cytosolic protein was denatured by heating at 95° for 10 min prior to incubation.

that adduct formation of *N*-OSO$_3$K-2-AAF with cellular nucleophiles will effectively compete with cellular reduction of *N*-OSO$_3$K-2-AAF.

Discussion

Although there are observations on the identity of the products of the decay of the model carcinogen, *N*-OAc-2-AAF, in 40% acetone (10), there is no information on the products of the solvolytic and metabolic degradation of the ultimate carcinogen, *N*-OSO$_3$K-2-AAF. This study identifies and quantifies the major products of the solvolysis and metabolism of *N*-OSO$_3$K-2-AAF in aqueous media. Several of these compounds are structural analogues of the products isolated from the solvolysis of *N*-OAc-2-AAF (10). Similar to *N*-OAc-2-AAF, *N*-OSO$_3$K-2-AAF undergoes *o*-rearrangement to minor (<10%) amounts of 1- and 3-OSO$_3$K-2-AAF as well as to the *o*-amidofluorenols. Cellular proteins of rat liver homogenates inhibited

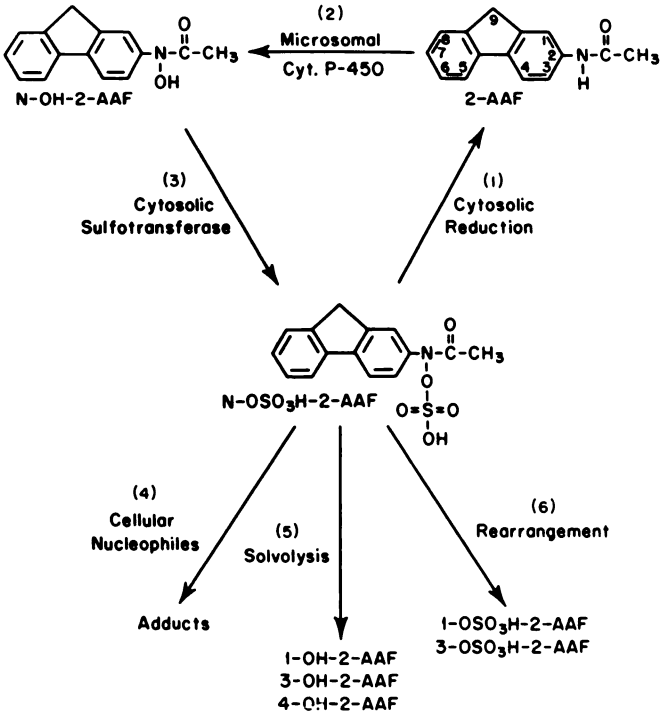

Fig. 4. Proposed scheme for the metabolic regeneration of the ultimate carcinogen, *N*-OSO$_3$K-2-AAF.

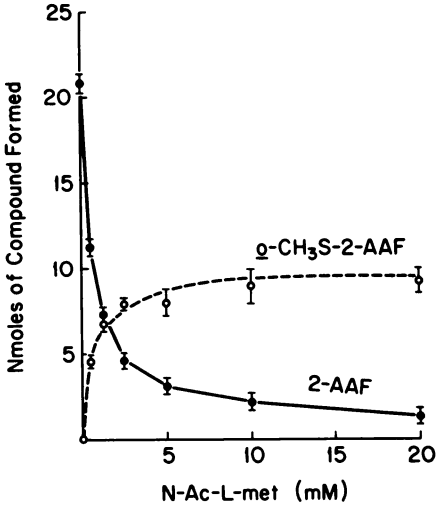

Fig. 5. Competition of the interaction of *N*-OSO$_3$K-2-AAF with *N*-Ac-L-Met and of reduction of *N*-OSO$_3$K-2-AAF to 2-AAF at increasing concentrations of *N*-Ac-L-Met. Reactions were carried out in Tris-HCl buffer, pH 7.0 (1 ml), at 37° for 30 min. Each incubation mixture contained *N*-OSO$_3$K-2-AAF (50 nmol/ml) and cytosolic protein (2 mg/ml).

the *o*-rearrangement. However, the reaction was nearly quantitative (>96%) in the presence of the model protein, BSA. Although several reports ascribe enzyme-like properties to BSA (29–32), this study provides, to our knowledge, the first experimental evidence that BSA has a catalytic effect on the detoxification of an activated arylhydroxamic acid to products that are chemically unreactive and biologically inactive. A role of serum albumin in the metabolism and/or detoxification of carcinogens is compatible with data indicating that mammalian serum albumin contains specific binding sites for carcinogens. In the case of aromatic amines, a specific binding site on rat serum albumin for 4-aminobiphenyl has been identified (33,

34). Recent data also suggest a specific binding site, on rat serum albumin, for *N*-OSO_3K-2-AAF (34). It has been postulated that site-specific interactions of *N*-sulfates of carcinogenic arylhydroxamic acids or arylhydroxylamines with serum albumin are related to the detoxification of these carcinogens (34). The greater susceptibility of analbuminemic rats, in comparison to normal rats, for induction of bladder tumors by carcinogens, such as 4-aminobiphenyl, benzidine, and 2-naphthylamine (35), also suggests a possible association of serum albumin with detoxification of carcinogenic aromatic amides and amines. Although the data of this study indicate a catalytic effect of BSA in the *o*-rearrangement of *N*-OSO_3K-2-AAF, the mechanism underlying the catalytic effect is presently unknown.

The data of this study indicate that about 55% of *N*-OSO_3K-2-AAF is transformed in aqueous media to 1- and 3-OSO_3K-2-AAF, 1- and 3-OH-2-AAF, 2-AAF, and 4-OH-2-AAF, which appears to be the major (46%) solvolysis product. 4-OH-2-AAF was previously isolated, in low (10%) yield, as a product of the decomposition of *N*-OAc-2-AAF in aqueous media (25). The *m*-amidofluorenol is presumed to arise from a nucleophilic attack of a hydroxyl ion on the nitrenium ion that results from the dissociation of *N*-OAc-2-AAF or *N*-OSO_3K-2-AAF, respectively (25). On the basis of yield, it would appear that *N*-OSO_3K-2-AAF dissociates in aqueous media more readily than *N*-OAc-2-AAF. This conclusion agrees with the demonstrated greater electrophilicity of *N*-OSO_3K-2-AAF in comparison to that of *N*-OAc-2-AAF (5). In addition to the above compounds, the dimer, 1-(*N*-2′-fluorenylacetamido)-2-acetylaminofluorene, has been reported as a solvolysis product of *N*-OH-2-AAF that was sulfated enzymatically to *N*-OSO_3K-2-AAF (36). Formation of the dimer accounted for 22% of *N*-OH-2-AAF subjected to enzymatic sulfation (36). A dimer, tentatively designated as 3-(*N*-2′-fluorenylacetamido)-2-acetylaminofluorene, was obtained in 25% yield from the solvolysis of *N*-OAc-2-AAF (10). A compound exhibiting a UV absorption spectrum identical to that of 1-(*N*-2′-fluorenylacetamido)-2-acetylaminofluorene (36) was also observed in the present study. However, a quantitative estimate of its formation from *N*-OSO_3K-2-AAF was not obtained. Assuming a 25% yield of dimer (10, 36), the major portion (~80%) of the products of the solvolysis of *N*-OSO_3K-2-AAF appears to be identified. 1-(*N*-2′-Fluorenylacetamido)-2-acetylaminofluorene exhibited extremely low mutagenicity (36) and, on the basis of structural considerations, is unlikely to react with nucleophils. The present evidence indicates that the solvolysis products of *N*-OSO_3K-2-AAF, identified thus far, are non-mutagenic and chemically unreactive. It seems likely that carcinogenic potential is associated exclusively with *N*-OSO_3K-2-AAF and not with the compounds formed during its solvolysis.

An unexpected finding of this study was that reduction of *N*-OSO_3K-2-AAF to 2-AAF, which is negligible (2%) in aqueous media, was the predominant reaction (46%) in the presence of rat liver cytosol, replacing hydrolysis of *N*-OSO_3K-2-AAF to 4-OH-2-AAF as the major reaction. Reduction of *N*-OSO_3K-2-AAF may be the result of the nucleophilic attack of a hydride ion on the nitrenium ion arising from dissociation of *N*-OSO_3K-2-AAF in aqueous solvents. Mechanistically, it may be similar to the cytosolic reduction of *N*-OH-2-AAF which was postulated to involve attack of a hydride ion on a nitrenium ion (12). The major difference between the cytosolic reduction of *N*-OSO_3K-2-AAF and that of *N*-OH-2-AAF appears to be the nature of the catalyst. The reduction of *N*-OH-2-AAF was shown to be an enzymatic reaction catalyzed by a group of acidic proteins separable by ion exchange chromatography on DEAE-cellulose (12). The enzymatic reduction of *N*-OH-2-AAF was confirmed in the present study by heat denaturation of cytosolic proteins which abolished formation of 2-AAF. In contrast, reduction of *N*-OSO_3K-2-AAF, which is not affected by denaturation of cytosolic proteins, appears to be a nonenzymatic reaction that may be carried out by a low molecular weight compound in cytosol. In support of this view are data which indicate that an electrophilic species, presumed to arise from *N*-OH-2-AAF by enzymatic sulfation, was reduced to 2-AAF by ascorbic acid (37, 38). It has also been shown that reduction of *N*-OSO_3K-2-AAF, in the presence of RNA or DNA, may be increased by glutathione or cysteine (39). Identification of the factor(s) in the rat liver cell which will reduce synthetic *N*-OSO_3K-2-AAF to 2-AAF, and which may include thiol compounds, ascorbic acid, and cofactors of oxidation-reduction reactions (NADH, NADPH), will be the subject of further investigation.

As mentioned above, cytosolic reduction of *N*-OSO_3K-2-AAF to 2-AAF may be the first step of a metabolic cycle by which *N*-OSO_3K-2-AAF is regenerated through *N*-hydroxylation of 2-AAF and subsequent enzymatic sulfation of the arylhydroxamic acid. Metabolic recycling of an ultimate carcinogen would enhance the chances for interaction of the ultimate carcinogen with acceptors, a step critical for the initiation of transformation. Although recycling of an ultimate carcinogen has, to our knowledge, not been suggested or reported, there are, in addition to *N*-OSO_3K-2-AAF and other activated arylhydroxamic acids, a number of aromatic nitrogen-containing carcinogens that may be subject to such a process (40). The present information does not permit an estimate of the amounts of *N*-OSO_3K-2-AAF that are recycled. Additional experiments are required to determine the quantities of *N*-OSO_3K-2-AAF that complete the proposed regenerative cycle following reduction to 2-AAF.

One factor affecting reduction of *N*-OSO_3K-2-AAF is the scavenging of *N*-OSO_3K-2-AAF by cellular nucleophiles. Competition between adduct formation and reduction was demonstrated in this study. However, the amounts of nucleophilic acceptor, that were selected for the purpose of demonstrating a clear-cut effect, were 10- to 400-fold greater than the quantities of *N*-OSO_3K-2-AAF in the incubation systems. The question whether the hepatic cell, and specifically the hepatic cytosol, contains the excess of reactive nucleophiles required to shift reduction of *N*-OSO_3K-2-AAF to covalent adduct formation remains open for further investigation.

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References

1. Miller, E. C., J. A. Miller, E. W. Boberg, K. B. Delclos, C.-C. Lai, T. R. Fennell, R. W. Wiseman, and A. Liem. Sulfuric acid esters as ultimate electrophilic and carcinogenic metabolites of some alkenylbenzenes and aromatic amines in mouse liver, in *Carcinogenesis* (E. Huberman and S. H. Barr, eds.), Vol. 10. Raven Press, New York, 93–107 (1985).
2. Miller, E. C., and J. A. Miller. Mechanisms of chemical carcinogenesis. *Cancer (Phila).* **47**:1055–1064 (1981).
3. Miller, E. C., and J. A. Miller. The metabolic activation and reactivity of carcinogenic aromatic amines and amides, in *Chemical and Viral Oncogenesis. Proceedings of the 11th International Cancer Congress* (P. Bucalossi, U.

Veronesi, and N. Casanelli, eds.), Vol. 2. Excerpta Medica, Amsterdam, 3–8 (1975).
4. Weisburger, J. H., R. S. Yamamoto, G. M. Williams, P. H. Grantham, T. Matsushima, and E. K. Weisburger. On the sulfate ester of *N*-hydroxy-*N*-2-fluorenylacetamide as a key ultimate hepatocarcinogen in the rat. *Cancer Res.* **32**:491–500 (1972).
5. Smith, B. A., J. R. Springfield, and H. R. Gutmann. Interaction of the synthetic ultimate carcinogens, *N*-sulfonoxy- and *N*-acetoxy-2-acetylaminofluorene, and of enzymatically activated *N*-hydroxy-2-acetylaminofluorene with nucleophiles. *Carcinogenesis (Lond.)* **7**:405–411 (1986).
6. Smith, B. A., H. R. Gutmann, and J. R. Springfield. Interaction of nucleophiles with the enzymatically-activated carcinogen, *N*-hydroxy-2-acetylaminofluorene, and with the model ester, *N*-acetoxy-2-acetylaminofluorene. *Carcinogenesis (Lond.)* **6**:271–277 (1985).
7. Maher, V. M., E. C. Miller, J. A. Miller, and W. Szybalski. Mutations and decreases in density of transforming DNA produced by derivatives of the carcinogens 2-acetylaminofluorene and *N*-methyl-4-aminoazobenzene. *Mol. Pharmacol.* **4**:411–426 (1968).
8. Boyland, E., and D. Manson. The biochemistry of aromatic amines. The metabolism of 2-naphthylamine and 2-naphthylhydroxylamine derivatives. *Biochem. J.* **101**:84–102 (1966).
9. Calder, I. C., and P. J. Williams. The thermal *ortho*-rearrangement of some carcinogenic *N,O*-diacetyl-*N*-arylhydroxylamines. *Chem.-Biol. Interact.* **11**:27–32 (1975).
10. Underwood, G. R., and R. B. Kirsch. The solvolysis of *N*-acetoxy-2-acetylaminofluorene and *N*-acetoxy-4-acetylaminobiphenyl: delicate balance between nitrenium ion formation and hydrolysis. *J. Chem. Soc. Chem. Commun.* 136–138 (1985).
11. Irving, C. C. Conjugates of *N*-hydroxy compounds, in *Metabolic Conjugation and Metabolic Hydrolysis* (W. H. Fishman, ed.), Vol. 1. Academic Press, New York, 53–119 (1970).
12. Gutmann, H. R., and R. R. Erickson. The conversion of the carcinogen *N*-hydroxy-2-fluorenylacetamide to *o*-amidophenols by rat liver *in vitro*. An inducible enzymatic reaction. *J. Biol. Chem.* **244**:1729–1740 (1969).
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
14. Chow, Y. M., H. R. Gutmann, and E. Kaplan. Effect of the structural isomer *N*-3-fluorenylacetamide on microsomal binding and hydroxylation of the carcinogen *N*-2-fluorenylacetamide. *Biochem. Pharmacol.* **30**:1253–1261 (1981).
15. Lotlikar, P. D., E. C. Miller, J. A. Miller, and A. Margreth. The enzymatic reduction of the *N*-hydroxy derivatives of 2-acetylaminofluorene and related carcinogens by tissue preparations. *Cancer Res.* **25**:1743–1752 (1965).
16. Westra, J. G. A rapid and simple synthesis of reactive metabolites of carcinogenic aromatic amines in high yield. *Carcinogenesis (Lond.)* **2**:355–357 (1981).
17. Weisburger, E. K., and J. H. Weisburger. *ortho*-Hydroxy derivatives of the carcinogen 2-acetylaminofluorene. *J. Org. Chem.* **19**:964–972 (1954).
18. Lotlikar, P. D., J. D. Scribner, J. A. Miller, and E. C. Miller. Reaction of esters of aromatic *N*-hydroxy amines and amides with methionine *in vitro*: a model for *in vivo* binding of amine carcinogens to protein. *Life Sci.* **5**:1263–1269 (1966).
19. Kriek, E., J. A. Miller, U. Juhl, and E. C. Miller. 8-(*N*-2-Fluorenylacetamido)guanosine, an arylamidation reaction product of guanosine and the carcinogen *N*-acetoxy-*N*-2-fluorenylacetamide in neutral solution. *Biochemistry* **6**:177–182 (1967).
20. Beland, F. A., D. W. Miller, and R. K. Mitchum. Synthesis of the ultimate hepatocarcinogen, 2-acetylaminofluorene *N*-sulphate. *J. Chem. Soc. Chem. Commun.* 30–31 (1983).
21. Pan, H.-L., and T. L. Fletcher. Derivatives of fluorene. IX. 4-Hydroxy-2-fluorenamine; new 3,4-benzocoumarin derivatives. *J. Org. Chem.* **25**:1106–1109 (1960).
22. Gutmann, H. R. Isolation and identification of the carcinogen *N*-hydroxy-2-fluorenylacetamide and related compounds by liquid chromatography. *Anal. Biochem.* **58**:469–478 (1974).
23. DeBaun, J. R., E. C. Miller, and J. A. Miller. *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: its probable role in carcinogenesis and in protein-(methion-*S*-yl) binding in rat liver. *Cancer Res.* **30**:577–595 (1970).
24. Maron, D. M., and B. N. Ames. Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.* **113**:173–215 (1983).
25. Scribner, J. D. Conversion of the carcinogen *N*-acetoxy-2-acetamidofluorene to 4-hydroxy-2-acetamidofluorene. *J. Am. Chem. Soc.* **99**:7383–7384 (1977).
26. Gutmann, H. R., and P. Bell. *N*-Hydroxylation of arylamides by the rat and guinea pig. Evidence for substrate specificity and participation of cytochrome P_1-450. *Biochim. Biophys. Acta* **498**:229–243 (1977).
27. Enomoto, M., P. Lotlikar, J. A. Miller, and E. C. Miller. Urinary metabolites of 2-acetylaminofluorene and related compounds in the Rhesus monkey. *Cancer Res.* **22**:1336–1342 (1962).
28. van den Goorbergh, J. A. M., J. H. N. Meerman, H. de Wit, and G. J. Mulder. Reaction of 2-acetylaminofluorene-*N*-sulfate with RNA and glutathione: evidence for the generation of two reactive intermediates with different reactivities towards RNA and glutathione. *Carcinogenesis (Lond.)* **6**:1635–1640 (1985).
29. Taylor, R. P., S. Berga, V. Chau, and C. Bryner. Bovine serum albumin as a catayst. III. Conformational studies. *J. Am. Chem. Soc.* **97**:1943–1948 (1975).
30. Taylor, R. P. Enzyme-like activities associated with albumin, in *Albumin Structure, Function* (V. M. Rosenoer, M. Oratz, and M. A. Rothschild, eds.). Pergamon Press, Oxford, 183–201 (1977).
31. Kokubo, T., T. Uchida, S. Tanimoto, M. Okano, and T. Sugimoto. A hydrolytic enzymelike behavior of bovine serum albumin in hydrolysis of *p*-nitrophenyl esters. *Tetrahedron Lett.* **23**:1593–1596 (1982).
32. Kurono, Y., T. Kondo, and K. Ikeda. Esterase-like activity of human serum albumin: enantioselectivity in the burst phase of reaction with *p*-nitrophenyl α-methoxyphenyl acetate. *Arch. Biochem. Biophys.* **227**:339–341 (1983).
33. Skipper, P. L., M. W. Obiedzinski, S. R. Tannenbaum, D. W. Miller, R. K. Mitchum, and F. F. Kadlubar. Identification of the major serum albumin adduct formed by 4-aminobiphenyl *in vivo* in rats. *Cancer Res.* **45**:5122–5127 (1985).
34. Kadlubar, F. F., D. A. Casciano, C. C. Weis, P. L. Skipper, S. R. Tannenbaum, and B. Ketterer. Detoxification of carcinogenic aromatic amines by reaction with serum albumin. *Proc. Annu. Meet. Am. Assoc. Cancer Res.* **26**:107 (1985).
35. Honma, Y., T. Kakizoe, H. Komatsu, T. Niijima, T. Sugimura, and S. Nagase. Agglutination assay of bladder cells by concanavalin A proved the high susceptibility of analbuminemic rats to bladder carcinogens. *Cancer Lett.* **19**:7–11 (1983).
36. Andrews, L. S., L. R. Pohl, J. A. Hinson, C. L. Fisk, and J. R. Gillette. Production of a dimer of 2-acetylaminofluorene during the sulfation of *N*-hydroxy-2-acetylaminofluorene *in vitro*. *Drug Metab. Dispos.* **7**:296–300 (1979).
37. Andrews, L. S., J. A. Hinson, and J. R. Gillette. Studies on the mutagenicity of *N*-hydroxy-2-acetylaminofluorene in the Ames-*Salmonella* mutagenesis system. *Biochem. Pharmacol.* **27**:2399–2408 (1978).
38. Andrews, L. S., J. M. Fysh, J. A. Hinson, and J. R. Gillette. Ascorbic acid inhibits covalent binding of enzymatically generated 2-acetylaminofluorene-*N*-sulfate to DNA under conditions in which it increases mutagenesis in *Salmonella* TA-1538. *Life Sci.* **24**:59–64 (1979).
39. Meerman, J. H. N., and R. B. Tijdens. Effect of glutathione depletion on the hepatotoxicity and covalent binding to rat liver macromolecules of *N*-hydroxy-2-acetylaminofluorene. *Cancer Res.* **45**:1132–1139 (1985).
40. Beland, F. A., R. H. Heflich, P. C. Howard, and P. P. Fu. The *in vitro* metabolic activation of nitro polycyclic aromatic hydrocarbons. *ACS Symp. Ser.* **283**:371–396 (1985).

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