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STUDIES ON THE MUTAGENICITY OF *N*-HYDROXY-2-ACETYLAMINOFLUORENE IN THE AMES–SALMONELLA MUTAGENESIS TEST SYSTEM*

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Abstract—*N*-hydroxy-2-acetylaminofluorene (NOH-2AAF) was deacetylated by Sephadex G-25-chromatographed postmicrosomal supernatant fraction to a substance (presumably *N*-hydroxy-2-aminofluorene) which was a potent frameshift mutagen in the Ames–Salmonella tester strain TA 1538. Preincubation of the supernatant fraction with paraoxon decreased the observed mutagenesis; however, the addition of several reducing agents or nucleophiles to the incubations did not alter the mutagenic response. Sulfation of NOH-2AAF decreased the mutagenesis observed under conditions of supernatant activation and increased the covalent binding of [^{14}C-acetyl]NOH-2AAF to protein. Under conditions of *N*-*O*-sulfate ester generation, ascorbate or reduced pyridine nucleotides greatly increased the number of revertant colonies. Ascorbate did not alter the rate of sulfation of NOH-2AAF, but decreased the protein covalent binding and greatly increased the reduction of the arylating species to 2-acetylaminofluorene (2AAF). Since 2AAF was not mutagenic under these conditions, these data are consistent with the concept of a free radical of 2AAF as the mutagenic intermediate.

The Ames–Salmonella mutagenicity test system, which employs bacteria as indicator organisms and mammalian enzymes for activation, has yielded an excellent correlation between known carcinogens and detected mutagens [1–3]. This procedure is of potentially great importance for the detection and characterization of environmental carcinogens and mutagens.

Since studies on 2-acetylaminofluorene (2AAF) metabolism and interaction with DNA have provided much information leading to current concepts in chemical carcinogenesis [4–7], we chose to study metabolites of 2AAF as model compounds in the Salmonella mutagenicity procedure.

This compound, 2AAF, is converted by mammalian mixed-function oxidases to *N*-hydroxy-2-acetylaminofluorene (NOH-2AAF) [8, 9]. NOH-2AAF is further metabolized by several mammalian liver enzyme systems to produce the ultimate carcinogenic species [10–12]. While sulfotransferases appear to be most important in this regard [10, 11], acyltransferase [12, 13], deacetylase [13] and glucuronyl transferase [14] have also been implicated. The role that each of these enzymatic processes plays in the observed carcinogenesis remains unclear.

Recent studies [15–18] have shown that the soluble fraction of rat liver converts NOH-2AAF to a substance which is mutagenic in the Ames–Salmonella mutagenicity test system. This mutagenic substance is presumably *N*-hydroxy-2-aminofluorene [15–17], a potent mutagenic species [1].

The addition of sulfation cofactors for the generation of the *N*-*O*-sulfate ester of NOH-2AAF by supernatant sulfotransferases caused a decrease in the mutagenic response [17]. This decrease in mutagenesis was unexpected, because the *N*-*O*-sulfate of NOH-2AAF has been thought to be a potent proximal carcinogen [4, 10, 11] since it spontaneously breaks down to yield an electrophilic species which combines with many tissue nucleophiles [8, 10, 11, 19–21] including protein, RNA and DNA. Nevertheless, it still seemed possible that part of the mutagenic effect could be mediated by the generated *N*-*O*-sulfate ester. Accordingly, we attempted to modify the mutagenesis produced under sulfation conditions by adding several nucleophiles and reducing agents.

MATERIALS AND METHODS

Chemicals. *N*-hydroxy-2-acetylaminoflurene was synthesized by reduction of nitrofluorene in the presence of zinc to the hydroxylamine, followed by acetylation with acetylchloride [22–24]. [1-^{14}C]-acetylchloride (sp. act. 3.86 mCi/m-mole) (New England Nuclear, Boston, MA, U.S.A.) was used for the preparation of ^{14}C-labeled NOH-2AAF. Identity of the NOH-2AAF was confirmed by electron impact mass spectrometry. Purity was determined by chromatography on silica gel plates in three solvent systems; benzene–acetone (80:20); ether–hexane (85 : 15) or ethyl acetate–ammonium hydroxide (conc.) (99 : 1). L-Ascorbic acid, *p*-nitrophenylsulfate, adenosine 3′ : 5′ diphosphate, glutathione, D,L-α-tocopherol acetate, methionine, triphosphopyridine nucleotide (reduced), diphosphopyridine nucleotide (reduced), deoxyribonucleic

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acid (from calf thymus), adenine, guanine, guanosine, biotin and D-histidine hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Cysteamine, 2AAF and 2-nitrofluorene were obtained from Aldrich Chemical Corp., Milwaukee, U.S.A. Guanosine-5′-monophosphate, soluble RNA and alkaline phosphatase (from calf intestine) were supplied by Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Bovine serum albumin (fraction V from bovine plasma) was obtained from Armour Pharmaceuticals, Chicago, IL, U.S.A. 7-Hydroxy-2-acetylaminofluorene (7OH-2AAF), 1-hydroxy-2-acetylaminofluorene (1OH-2AAF), 3-hydroxy-2-acetylaminofluorene (3OH-2AAF) and 5-hydroxy-2-acetylaminofluorene (5OH-2AAF) were the gracious gift of Dr. E. K. Weisburger, National Cancer Institute, Bethesda, MD.

Enzyme preparations. Adult male Sprague–Dawley rats (Charles River CDS, Wilmington, MASS.), 300–400 g were given free access to food and water. The animals were sacrificed by cervical dislocation, livers were removed and perfused with ice-cold 0.15 M KCl. Perfused livers were minced, homogenized in a Potter–Elvehjem homogenizer with 3 vol. of 0.15 M KCl buffered with 50 mM sodium phosphate, pH 7.4. The homogenate was centrifuged at 9,000 *g* for 20 min and the supernatant fractions were then centrifuged at 100,000 *g* for 60 min to sediment the microsomes. The postmicrosomal supernatant fraction was carefully removed and chromatographed on Sephadex G-25 (40 × 2.5 cm column), with 50 mM sodium phosphate buffer, pH 7.4, as eluent. The protein fraction eluted in the void volume (approximately 125 ml) and was separated from low molecular weight substances, such as glutathione and adenosine-3,5-diphosphate. The chromatographed postmicrosomal supernatant fraction was used as the enzyme source throughout these experiments. In experiments that tested the effects of paraoxon, the postmicrosomal supernatant fraction was preincubated at 37° for 20 min with 1 mM paraoxon prior to chromatography, which should remove not only glutathione and adenosine-3,5-diphosphate but also excess paraoxon from the enzyme preparation.

Protein determination. Supernatant protein was determined by the method of Lowry *et al.* [25] using bovine serum albumin as the standard.

Sulfotransferase assay. The sulfotransferase assay was essentially that of Mulder *et al.* [21], which utilizes *p*-nitrophenylsulfate and adenosine-3′ : 5′-diphosphate to generate 3′-phosphoadenosine 5′-phosphosulfate (PAPS). The incubation mixture contained (final concentrations): 0.15 M KCl, 50 mM sodium phosphate (pH 7.4); 20 μM adenosine-3′ : 5′-diphosphate; 10 mM *p*-nitrophenylsulfate; NOH-2AAF (added in 10 μl DMSO); and the postmicrosomal supernatant fraction (2.0 mg/ml) in a final volume of 3.0 ml. The mixture was incubated at 37° for various times before pouring it into a 1-cm light-path cuvette and measuring the increase in absorbance at 405 nM minus 490 nM. The rate of liberation of *p*-nitrophenol (after transfer of the sulfate group) in the absence of NOH-2AAF was subtracted from the experimentally determined rates, and the difference was used to calculate the rate of sulfation.

Mutagenesis assay. The mutagenesis assay was essentially that of Ames *et al.* [2]. However, bacteria, the postmicrosomal supernatant fraction and NOH-2AAF were preincubated at 37° for 1 hr prior to the addition of 2 ml of top agar supplemented with histidine-biotin and subsequent plating. The incubation mixtures contained in a total volume of 0.60 ml : 0.15 M KCl, 50 mM sodium phosphate (pH 7.4), bacteria (TA 1538, approximately 1×10^8 organisms), the postmicrosomal supernatant fraction, NOH-2AAF and nucleophile in the indicated concentrations. Adenosine 3′ : 5′-diphosphate (20 μM) and *p*-nitrophenyl sulfate (10 mM) were also included in mixtures designated as containing PAPS. After addition of agar, the mixtures were poured into plates and incubated at 37° for 48 hr. The number of colonies obtained represent a rate of reversion from histidine dependence to prototrophy. Results are reported as revertants/10^8 plated bacteria since addition of nucleophiles did not appear to alter backlawn growth. Where nucleophile addition produced a significant alteration in the mutation rate, survival studies were performed. The number of organisms that survived the incubation (about 20 per cent) were not significantly changed by the addition of NOH-2AAF or any of the other substances. The spontaneous mutation rate for TA 1538 was less than twenty colonies in all systems and was subtracted from the results. Assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed.

Biotransformation assay. The conditions for studying the metabolism of NOH-2AAF were identical to those described for the mutagenesis assay except that bacteria were excluded from the incubation and [^{14}C-acetyl]NOH-2AAF (20 μM) was used as the substrate. After incubation of the mixtures at 37° for 1 hr, the reactions were terminated by the addition of 10 μl of 1 M $MgCl_2$ and 2.0 ml methanol (10 mM and 77% final concentrations). The mixtures were centrifuged and the supernatant fraction was removed. The precipitate was washed with 1.5 ml methanol–water (80 : 20), and the wash was combined with the original supernatant fraction. Water (2 ml) and 100 μg of NOH-2AAF were added to the supernatant fraction and the mixture was extracted three times with 10 ml ethyl ether. The ethyl ether phase was evaporated to dryness, and the residue was dissolved in a small volume of acetone for chromatography on silica gel thin-layer plates. The solvent system used to develop the plates was benzene–acetone (80 : 20).

Chromatography of [^{14}C-acetyl]NOH-2AAF [approximately 97 per cent pure by thin-layer chromatography (t.l.c.)] indicated that, of the radioactivity spotted on the plates, about 80 per cent was recoverable upon scraping sections of the plate and liquid scintillation counting. The apparent loss of radioactivity was presumably due to a decreased efficiency in counting when the radiolabeled compound was adsorbed onto the silica gel. Adsorption may account for a small amount of streaking of the *N*-hydroxy compound during chromatography. This streaking occurred in all of the solvent systems we used, and was corrected for in metabolism studies by determin-

ing the per cent of *N*-hydroxy compound migrating at the R_f of the various metabolites.

Chromatography of extracts from incubation mixtures in which either the supernatant fraction or the sulfation cofactors were omitted yielded only one radioactive peak. The radioactivity chromatographed with authentic NOH-2AAF in three solvent systems: (1) benzene–acetone (80:20), (2) ether–hexane (85 : 15) and (3) ethyl acetate–ammonium hydroxide (conc.) (99 : 1), and was assumed to be [^{14}C-acetyl]NOH-2AAF.

Chromatography of ether extracts from incubations containing supernatant fraction plus sulfation cofactors yielded four radioactive components. These components were quantitated and subsequently characterized by chemical ionization mass spectrometry of samples from a large-scale incubation.

Component 1 had R_f values of 0.18, 0.05 and 0.68 in solvent systems A, B and C respectively. This compound was apparently neutral since it was not extracted from ether into acid or base. Chemical ionization mass spectrometry showed a parent peak at m/e of 445 and a fragment at m/e of 223, suggesting that this component is a dimer of 2AAF; however, its exact structure has not been determined.

Component 2 had R_f values of 0.36, 0.58 and 0.57 in solvent systems A, B and C respectively. These R_f values were identical to those found for authentic NOH-2AAF. Chemical ionization mass spectrometry showed a parent peak at m/e of 239 and a fragmentation pattern which corresponded to that of synthetic NOH-2AAF.

Component 3 had R_f values of 0.42, 0.44 and 0.83 in solvent systems, A, B and C respectively. These R_f values were identical to those found for authentic 2AAF. Chemical ionization mass spectrometry showed a parent peak at m/e of 223 corresponding to synthetic 2AAF.

Component 4 had R_f values of 0.67, 0.63 and 0.92 in solvent systems A, B and C respectively. Chemical ionization mass spectrometry showed a parent peak at m/e of 361. This molecular weight corresponds to that of a *p*-nitrophenol adduct of 2AAF. The exact structure of the substance, however, was not determined.

In systems employing [^{14}C-acetyl]NOH-2AAF in buffer or with supernatant enzymes without sulfation cofactors, small amounts of radiolabeled compound remained in the water layer after ether extraction. One peak of radioactivity, R_f = 0.56, was obtained upon thin-layer chromatography on cellulose using propanol–ammonium hydroxide (0.4 M) (80 : 20) as the developing solvent system. [^{14}C]potassium acetate had the same R_f when chromatographed in this solvent system. When the plates containing standard [^{14}C]acetate and a water-soluble compound were sprayed with acetic acid and heated, the radioactivity volatilized. On the basis of these studies, the water-soluble radioactivity is reported as acetic acid and was used as an indirect measure of the rate of hydrolysis of NOH-2AAF.

In systems containing supernatant enzymes plus sulfation cofactors, no [^{14}C]acetate was detected, but radiolabeled water-soluble conjugates of NOH-2AAF were formed. After lyophilization of the water layer, the residue was dissolved in 1.0 ml of 0.6 M Tris buffer (pH 8.0), 25 μg of alkaline phosphatase was added, and the mixture was incubated overnight. Approximately 75 per cent of the radioactive water-soluble conjugates were hydrolyzed under these conditions, as estimated from the amount of radioactivity which could be extracted into ether. After evaporation of the ether layer, the residue was separated by thin-layer chromatography on silica gel with solvent systems A, B and C; the radioactivity migrated with 7OH-2AAF, but not with 1OH-2AAF, 3OH-2AAF or 5OH-2AAF. The water-soluble radioactivity is reported as phosphate adducts of 2AAF.

Covalent binding of radiolabeled material to protein was determined by washing the protein pellet with ethyl ether–ethanol (80 : 20) until the radioactivity of the wash fluid was no more than 50 cpm/2 ml above background. The protein pellet was suspended in 1.0 ml of 1 N NaOH and dissolved with heating at 60°. Radioactivity in the solution was determined by liquid scintillation counting and the amount of metabolite covalently bound was calculated from the specific activity of [^{14}C-acetyl]NOH-2AAF.

Biotransformation assays were run in duplicate at least twice; recovery averaged 84 per cent.

RESULTS

The mutagenesis caused by various concentrations of NOH-2AAF as measured with TA 1538 is shown in Fig. 1. Without activation, NOH-2AAF is a weak

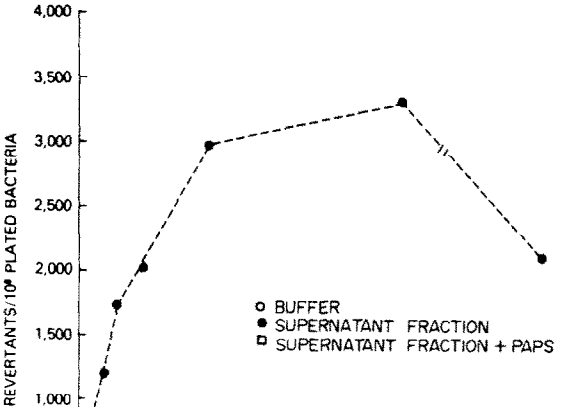

Fig. 1. Mutagenesis of NOH-2AAF for TA 1538. Varying amounts of NOH-2AAF were incubated with 1×10^8 TA 1538 in buffer (○), in buffer containing Sephadex G-25-chromatographed supernatant fraction (2 mg/ml) (●), or in buffer containing Sephadex G-25-chromatographed supernatant fraction (2 mg/ml) plus (PAPS) = 20 μM adenosine 3′5′-diphosphate and 10 mM *p*-nitrophenyl sulfate (□) as described in Materials and Methods. Supernatant protein concentration was 2.0 mg/ml. After plating and a 48-hr incubation at 37°, revertants were counted. Assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed.

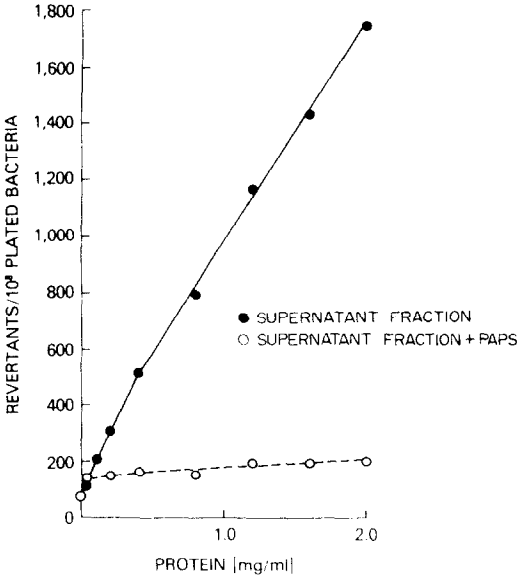

Fig. 2. Effect of supernatant protein concentration on the mutagenic activation of NOH-2AAF. Varying supernatant protein concentrations were incubated with 3 μg NOH-2AAF, with (○) or without (●) sulfation cofactor addition. After plating and a 48-hr incubation at 37°, revertants were counted. Assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed.

mutagen, 3 μg yielding only 65 colonies above background. Upon addition of Sephadex G-25-chromatographed supernatant fraction, NOH-2AAF was activated to a potent mutagen, 3 μg yielding 1750 colonies. As previously observed [17], addition of cofactors (PAPS) for sulfate ester formation of NOH-2AAF reduced the number of revertants at all concentrations of NOH-2AAF tested. Addition of *p*-nitrophenyl sulfate and adenosine-3,5-diphosphate decreased the number of revertants caused by 3 μg NOH-2AAF from 1750 to 250.

Figure 2 shows the effect of supernatant protein concentration on the mutagenic activation of NOH-2AAF. Increasing concentrations of supernatant protein up to 2.0 mg/ml caused a linear increase in the number of revertants obtained after addition of 3.0 μg NOH-2AAF. In the presence of the PAPS-generating system, however, only small amounts of protein (about 0.25 mg/ml) were needed to obtain the small mutagenic response observed with this system. Subsequent studies used a supernatant protein concentration of 2.0 mg/ml.

Other workers have attributed the supernatant-mediated mutagenesis to an esterase which catalyzes the deacetylation of NOH-2AAF to NOH-2AF [15–17]. In accord with this view, pretreatment of the supernatant fraction with paraoxon, which inhibits esterases but not sulfotransferases, markedly decreased both the mutagenesis and the formation of acetate (Table 1). However, in the presence of PAPS, no acetate was released from NOH-2AAF and the paraoxon pretreatment did not further decrease the number of revertants.

If the mutagenicity of the NOH-2AAF metabolites was caused by highly reactive intermediates, substances that inhibit the covalent binding of the intermediates by reacting with them should decrease the incidence of mutagenicity. In the presence or the absence of PAPS, however, the number of transformations was not markedly changed (< 20 per cent) by the addition of guanine derivatives, RNA, DNA, glutathione, 2-AF or 2AAF (Table 2). Moreover, methionine, cysteamine or α-tocopherol did not change the number of transformations in the presence of PAPS. Furthermore, ascorbic acid as well as reduced pyridine nucleotides increased rather than decreased the number of transformations, particularly in the presence of PAPS. In the absence of NOH-2AAF, the addition of ascorbate to the supernatant fraction with or without sulfation cofactors did not alter the spontaneous mutation rate (data not shown).

Figure 3 shows the effects of increasing concentrations of NADPH, ascorbate or glutathione on the mutagenesis observed under sulfation conditions. Very low concentrations of NADPH relative to ascorbate were necessary to produce maximal stimulation. However, at concentrations of 2 mM, the stimulatory effects of the two substances were nearly the same. With increasing concentrations, glutathione slightly increased the number of revertant colonies.

Several studies were undertaken to clarify the

Table 1. Effect of paraoxon on NOH-2AAF-induced mutagenesis and biotransformation*

Addition(s)	Revertants/10^8 plated bacteria	[^{14}C]acetic acid generated (nmoles)
NOH-2AAF (3 μg)	65	0.00
NOH-2AAF + supernatant fraction	1793	0.95
NOH-2AAF + paraoxon-supernatant fraction	1180	0.49
NOH-2AAF + supernatant fraction + PAPS	250	0.00
NOH-2AAF + paraoxon-supernatant fraction + PAPS	240	0.00

* NOH-2AAF (3 μg), bacteria (10^8 TA 1538) and Sephadex G-25-chromatographed postmicrosomal supernatant or paraoxon-supernatant fraction (enzyme preincubated with paraoxon (1 mM) for 20 min at 37° prior to Sephadex G-25 chromatography) in 0.60 ml of 0.15 M KCl–0.05 M sodium phosphate buffer (pH 7.4) were incubated at 37° for 1 hr prior to addition of 2 ml of top agar supplemented with histidine-biotin and subsequent plating. Revertants were scored after a 48-hr incubation at 37°. Biotransformation assays were performed as described above except that bacteria were eliminated from incubation mixtures and [^{14}C-acetyl]NOH-2AAF was used as the substrate. After a 1-hr incubation at 37°, reactions were terminated and incubation mixtures analyzed for the presence of [^{14}C]acetic acid as described in Materials and Methods. Biotransformation and mutagenic assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed.

Table 2. Effects of various agents on the mutagenesis of NOH-2AAF*

Agent	Revertants/10^8 bacteria		
	Buffer	Supernatant fraction	Supernatant fraction + PAPS
No addition	65	1750	252
Guanine (50 μM)	91	1740	196
Guanosine (50 μM)	135	1870	298
Guanosine-5′ phosphate (1.8 mM)	78	1800	285
RNA (0.3 mg/ml)	95	1610	275
DNA (0.2 mg/ml)	130	2040	225
Glutathione (1.0 mM)	85	1700	280
Methionine (10 mM)	92		280
Cysteamine (1 mM)	50		277
D,L-α-Tocopherol acetate (100 μg/ml)			260
2-Aminofluorene (20 μM)	73	1870	255
2-Acetylaminofluorene (200 μM)	70	1800	260
NADPH (1 mM)		2600	2800
NADH (1 mM)		2380	2490
Ascorbic acid (1.9 mM)	92	2896	2910

* NOH-2AAF (3 μg), bacteria (10^8 TA 1538) with or without Sephadex G-25-chromatographed postmicrosomal supernatant fraction ± sulfation cofactors (PAPS) and agents (at the indicated final concentration) in 0.60 ml of 0.15 M KCl–0.05 M sodium phosphate buffer (pH 7.4) were incubated at 37° for 1 hr prior to addition of 2 ml of top agar supplemented with histidine-biotin and subsequent plating. Revertants were scored after a 48-hr incubation at 37°. Assays were performed in duplicate at least twice and approximately a 5 per cent variation among duplicates was observed.

mechanism by which ascorbate enhanced the mutagenesis caused by NOH-2AAF under sulfation conditions. Addition of agar at various times during the incubation in the presence or absence of ascorbate did not change the number of mutations (Fig. 4, panels a and b). Thus, possible reactions of components of agar with the NOH-2AAF metabolites did not appear to play an important role in mutagenesis. However, when the supernatant fraction and the PAPS-generating system were preincubated with bacteria for various times before the addition of ascorbate (Fig. 4, panel c), the stimulatory effect of ascorbate was greatly diminished. Thus, it seems unlikely that the chemically reactive intermediate was converted to a stable product which in turn is activated by ascorbate. Further, when the supernatant fraction and the PAPS-generating system were incubated with ascorbate for various times prior to the addition of bacteria (Fig. 4, panel d), the stimulatory effect of ascorbate was decreased. Thus, it seems unlikely that ascorbate reacted with the reactive intermediate to form a relatively stable mutagenic product. Instead it seems that ascorbate produced its stimulatory effect by increasing the formation of a transient metabolite of NOH-2AAF.

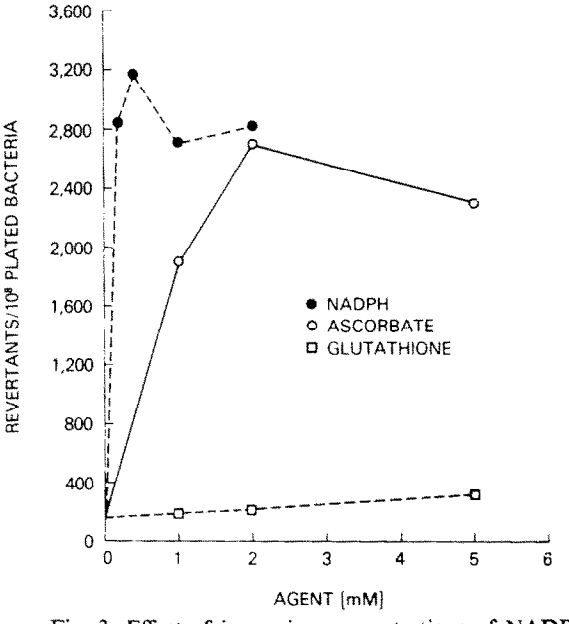

Fig. 3. Effect of increasing concentrations of NADPH, ascorbate or glutathione on NOH-2AAF mutagenesis under sulfation conditions. Varying agent concentrations NADPH (●), ascorbate (○) or glutathione (□) were incubated with 3 μg NOH-2AAF and Sephadex G-25-chromatographed postmicrosomal supernatant fraction (2 mg/ml) + PAPS (20 μM adenosine 3′ : 5′-diphosphate + 10 mM *p*-nitrophenylsulfate) for 1 hr at 37° prior to plating and scoring of revertants as described in Materials and Methods. Assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed.

Ascorbate did not affect the rate of *N*-*O*-sulfate ester generation as measured by the *p*-nitrophenol produced during the reaction. As shown in Fig. 5a, most of the sulfation occurs in the first 20 min and is essentially complete after 30 min. Ascorbate (1.9 mM) did not alter the sulfation of NOH-2AAF at any of the time periods measured. Nevertheless, ascorbate markedly decreased the amount of radiolabeled compound covalently bound to protein (Fig. 5b). Most of the covalent binding of the radiolabel occurred during the first 20 min of incubation. Ascorbate decreased the covalent binding at each time period, and after 60 min, an 80 per cent inhibition in covalent binding was observed. Ascorbate also decreased the protein covalent binding of [9-^{14}C]-NOH-2AAF to an extent similar to that obtained for the ^{14}C-acetyl radiolabel (data not shown).

As shown in Fig. 6, ascorbate caused the formation

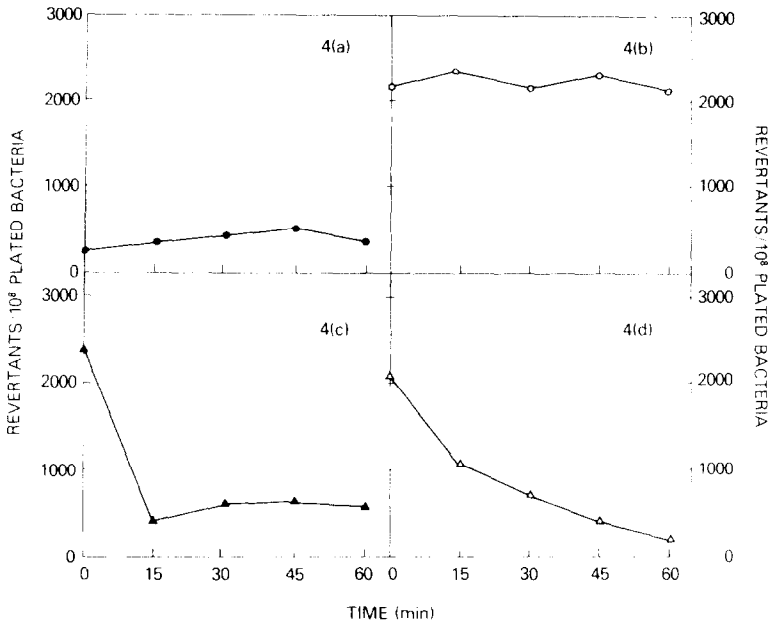

Fig. 4. Time course of NOH-2AAF mutagenesis under sulfation conditions. NOH-2AAF (3 μg) and sulfation cofactors were incubated with supernatant protein (2.0 mg/ml) for the indicated times prior to chilling and agar addition with subsequent plating. Key: (a) ●——●, supernatant fraction + PAPS + TA 1538; (b) ○——○, supernatant fraction + PAPS + ascorbate (1.9 mM) + TA 1538; (c) ▲——▲, supernatant fraction + PAPS + TA 1538 with ascorbate added at the indicated times; and (d) △——△, supernatant fraction + PAPS + ascorbate with TA 1538 added at the indicated times. Assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed.

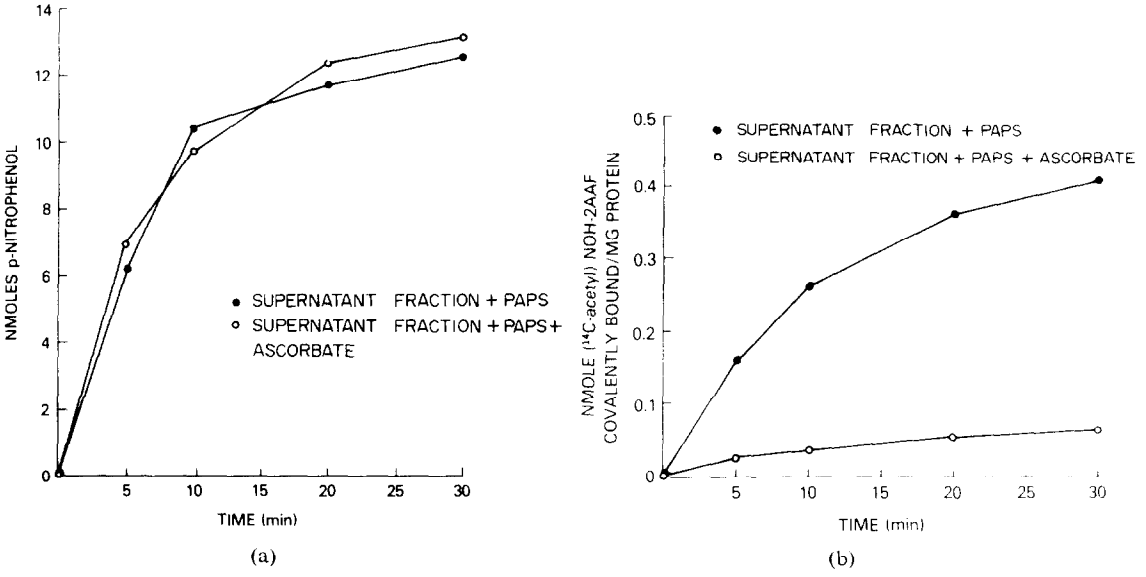

Fig. 5. (a) Time course of sulfation of NOH-2AAF. Incubation mixtures contained (final concentrations): 0.15 M KCl, 50 mM sodium phosphate (pH 7.4); 20 μM adenosine-3′ : 5′-diphosphate; 10 mM *p*-nitrophenyl-sulfate; 6 μM NOH-2AAF (added in 10 μl dimethyl sulfoxide); and postmicrosomal supernatant fractions (2.0 mg/ml) ± ascorbic acid (1.9 mM) in a final volume of 3.0 ml. The mixture was incubated at 37° for various times prior to pouring it into a 1-cm light-path cuvette and measuring the increase in absorbance at 405 nM minus 490 nM. The liberation of *p*-nitrophenol in the absence of NOH-2AAF was subtracted from the experimentally determined value and the difference was used to calculate the amount of sulfation. Assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed. (b) Time course of covalent binding of [^{14}C-acetyl] NOH-2AAF. Incubation mixtures were identical to those described in Fig. 5(a) with the exception that [^{14}C-acetyl]NOH-2AAF (25 μM) was used as the substrate. After incubation for the indicated times, reactions were terminated and protein covalent binding was determined as described in Materials and Methods. Assays were performed in duplicate at least twice. Approximately a 5 per cent variation among duplicates was observed.

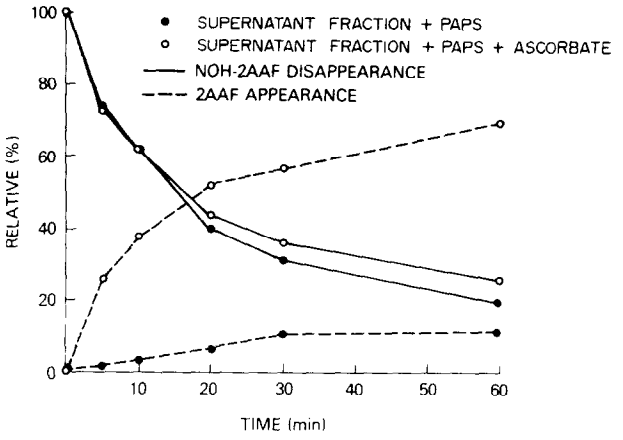

Fig. 6. Time course of disappearance of NOH-2AAF and appearance of 2AAF. Incubation mixtures contained (final concentrations): 0.15 M KCl, 50 mM sodium phosphate (pH 7.4); 20 μM adenosine-3′ : 5′-diphosphate; 10 mM *p*-nitrophenylsulfate; 25 μM [^{14}C-acetyl]-NOH-2AAF; and postmicrosomal supernatant fraction (2.0 mg/ml) ± ascorbic acid (1.9 mM) in a final volume of 0.60 ml. After a 1-hr incubation at 37°, reactions were terminated and incubation mixtures analyzed for the presence of [^{14}C-acetyl]NOH-2AAF and [^{14}C-acetyl]AAF as described in Materials and Methods. Key: (——), disappearance of [^{14}C-acetyl]NOH-2AAF; (– – – – –), appearance of [^{14}C-acetyl]2AAF.

of large amounts of 2AAF in the incubation medium without altering the disappearance of NOH-2AAF. At the end of the 1-hr incubation, the amount of 2AAF formed in the presence of ascorbate was seven times that formed in its absence. Thus, it appears that ascorbate antagonized covalent binding to protein by reducing the arylating species to 2AAF.

Table 3 summarizes the effects of ascorbate on the pattern of products formed from [^{14}C-acetyl]NOH-2AAF. Little nonenzymatic breakdown of NOH-2AAF occurred in the presence or absence of ascorbate. Addition of supernatant fraction without the sulfation cofactors catalyzed the deacetylation of NOH-2AAF as measured by the liberation of acetic acid. The ability of ascorbate to increase the loss of acetate label under these conditions may account for the increase in mutagenesis observed upon incubation of NOH-2AAF with supernatant fraction in the absence of sulfation cofactors (Table 2). The PAPS-generating system without ascorbic acid markedly increased the disappearance of NOH-2AAF, the covalent binding to protein and the appearance of the dimer, the phosphate conjugates, the *p*-nitrophenol adduct, and 2-AAF. But, in accord with the data in Table 1, the formation of acetic acid was blocked. The rate of formation of the dimer of 2AAF and the *p*-nitrophenol adduct paralleled the sulfation of NOH-2AAF (data not shown). In a separate experiment we determined that dimer formation occurs at an appreciable rate only in the presence of 2AAF, and appears to be a condensation product of the arylating species and 2AAF (data not shown). Neither the dimer nor the *p*-nitrophenol adduct (present in amounts up to 1.0 μg) was mutagenic in TA 1538 with or without addition of postmicrosomal supernatant protein sulfation cofactors (data not shown). The rate of appearance of phosphate ester adducts of 2AAF also paralleled the sulfation of NOH-2AAF (data not shown). After incubation of the conjugate with alkaline phosphatase 7OH-2AAF was the only 2AAF derivative identified in both control and ascorbate incubation systems.

Ascorbate did not markedly change the amount of NOH-2AAF that disappeared in incubation mixtures containing the PAPS-generating system, but greatly increased the amount of 2AAF formed during the incubation. Thus, ascorbate reduced the electrophilic species produced by sulfation back to 2AAF as evidenced by: (1) the large amount of 2AAF generated, (2) the decrease in protein binding and (3) the decrease in the amount of the adducts produced by reaction with nucleophiles.

Table 3. Metabolism of [^{14}C-acetyl]NOH-2AAF*

Compound	Buffer	Buffer + ascorbate	Supernatant fraction	Supernatant fraction +ascorbate	Supernatant fraction +PAPS	Supernatant fraction +PAPS+ ascorbate
NOH-2AAF	100.0	96.6	86.0	73.3	24.1	23.7
2AAF	0.0	3.4	4.5	12.5	11.2	62.4
Dimer	0.0	0.0	0.0	0.0	22.1	1.4
p-Nitrophenol adduct	0.0	0.0	0.0	0.0	7.8	0.8
Acetic acid	0.0	0.0	9.4	14.1	0.0	0.0
Phosphate adducts	0.0	0.0	0.0	0.0	30.8	11.0
Covalently bound material	0.0	0.0	0.1	0.1	4.0	0.7

* [^{14}C-acetyl]NOH-2AAF (16.0 nmoles; 3.9 mCi/m-mole) was incubated in the presence or absence of ascorbic acid (1.9 mM final concn) in 0.60 ml of 0.15 M KCl–0.05 M sodium phosphate buffer (pH 7.4) with or without Sephadex G-25-chromatographed postmicrosomal supernatant fraction (2.0 mg/ml) ± PAPS (20 μM adenosine 3′ : 5′diphosphate + 10 mM *p*-nitrophenyl sulfate) for 1 hr at 37°. Reactions were terminated and incubation mixtures analyzed for metabolites as described in Materials and Methods. Assays were performed in duplicate at least twice and approximately a 5 per cent variation among duplicates was observed. Values indicate the percentage of [^{14}C-acetyl]-NOH-2AAF recovered as the respective component. Average recovery in these experiments was 84 per cent of the incubated radioactivity.

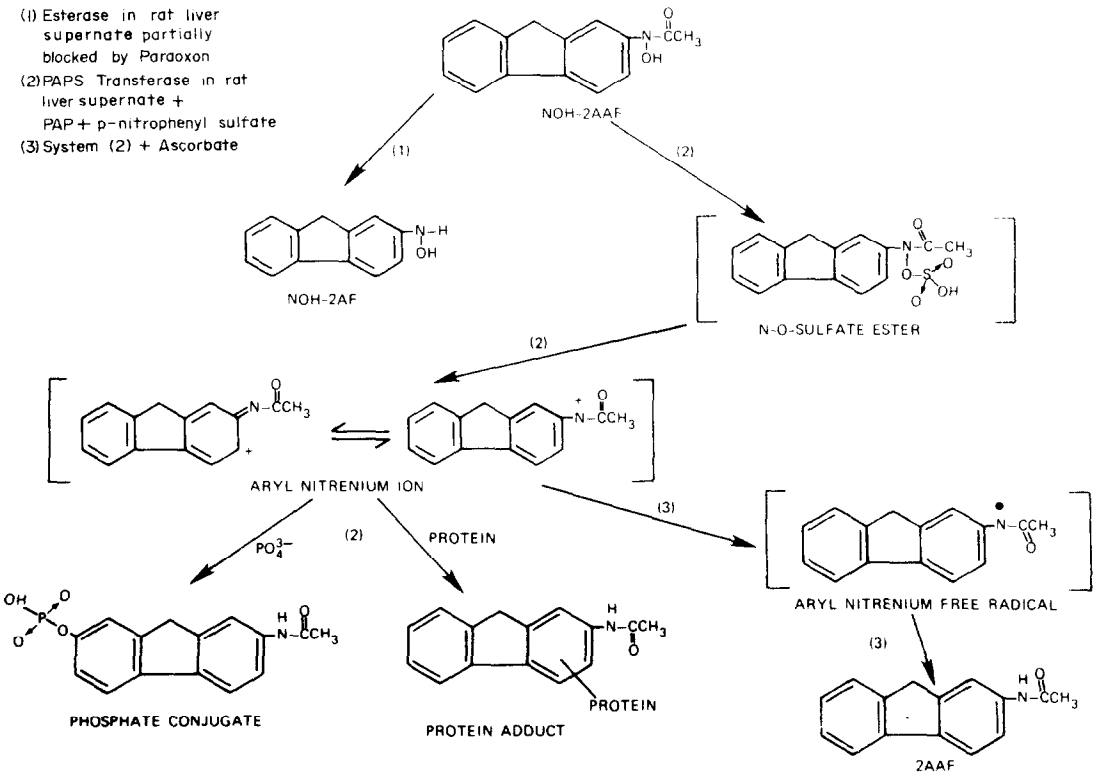

Fig. 7. Proposed mechanism for the metabolic activation of NOH-2AAF to a frameshift mutagen. Numbers in parentheses indicate metabolic pathways discussed in the text.

DISCUSSION

The various pathways of NOH-2AAF metabolism are shown in Fig. 7. The potent mutagenic response obtained upon incubation of NOH-2AAF with rat liver post-microsomal supernatant fraction in the absence of a PAPS-generating system appears to be due to deacetylation producing NOH-2AF (pathway 1). Paraoxon antagonized the mutagenesis, and inhibited the formation of acetate (Table 1). Although the inhibition was not complete, it is noteworthy that other esterase inhibitors of the cytosol esterases (e.g. fluoride [26]) incompletely inhibited NOH-2AAF decaetylation. By contrast, the addition of 2-aminofluorene (2AF) or (2AAF), which inhibits the transacylation of NOH-2AAF [12, 13], did not alter the supernatant-mediated mutagenesis. While transacylation apparently does not account for the mutagenesis observed under these conditions, the participation of acyltransferase cannot be ruled out in this study, however, since Weeks *et al.* [27] recently reported that 2AF does not alter the acyltransferase-mediated activation of NOH-2AAF to a mutagen in the Ames–Salmonella tester strain TA 1538. In the presence of the supernatant fraction without the sulfation cofactors, ascorbate slightly increased the mutagenesis, presumably by increasing the rate of deacetylation (Table 3).

In the presence of the PAPS-generating system and PAPS sulfotransferases in the rat liver supernatant fraction, NOH-2AAF was rapidly converted to the *N-O*-sulfate ester (pathway 2). In fact, the rate of formation of the *N-O*-sulfate ester was so rapid that little if any of the NOH-2AAF was deacetylated (Tables 1 and 3). Although the addition of the PAPS-generating system markedly decreased the number of revertants to about 250 (Tables 1 and 2; Figs. 1 and 2), the identity of the metabolite that causes these mutations is not clear. This number of mutations can occur with as little as 0.02 μg *N*-hydroxy-2-aminofluorene (data not shown), an amount far below the sensitivity of the assay for deacetylation. If the mutations were caused by *N*-hydroxy-2-aminofluorene, however, treatment of the soluble enzyme with paraoxon should have caused a further decrease in the number of mutations (Table 1). On the other hand, it also seemed possible that the mutations were caused by a breakdown product of the *N-O*-sulfate ester. The *N-O*-sulfate ester undergoes spontaneous heterolytic cleavage [6, 8, 28–30] to produce an arylating moiety shown in Fig. 7 as an aryl nitrenium ion with one of many possible resonance structures. This highly electrophilic intermediate reacts with many tissue nucleophiles including RNA, DNA and protein [8, 10, 11, 19–21]. Nucleophiles present in high concentrations in incubation mixtures also react with the electrophilic intermediate of the *N-O*-sulfate ester. The two products pictured are a phosphate conjugate and a protein adduct. However, the electrophilic aryl nitrenium ion also formed a conjugate with *p*-nitrophenol generated during the sulfate transfer reaction. Moreover, relatively large amounts of a dimer of 2AAF were produced. Small amounts of 2AAF itself were generated during the reaction, presumably via hydrogen abstraction from the

solvent [30, 31]. However, the addition of methionine, glutathione or guanosine-5-phosphate, in concentrations that are known to inhibit the covalent binding of the electrophilic intermediate to protein [32], did not cause a further decrease in the number of mutations (Table 2). Thus, the mechanism of mutagenesis under these conditions remains unclear.

Ascorbate addition (pathway 3) greatly decreased the amount of covalent binding to protein and adduct formation (Table 3) without affecting either the rate of *N*-*O*-sulfate ester generation (Fig. 5a) or the disappearance of NOH-2AAF (Fig. 6). Since large amounts of 2AAF were detected in incubation systems containing ascorbate, it seems likely that ascorbate acts primarily by reducing the electrophilic aryl nitrenium ion to 2AAF. In regard to this mechanism, Scribner and Naimy [27–29] have shown that the *N*-*O*-acetoxy ester of 2AAF breaks down to yield an aryl nitrenium ion that is reduced by ascorbate to 2AAF. Since ascorbate is known to undergo one-electron oxidation–reduction reactions, the reduction of the aryl nitrenium ion probably occurs by two one-electron steps through an aryl nitrenium free radical. Since the end product was 2AAF, a non-mutagenic compound in TA 1538, the ascorbate-dependent increase in mutagenesis may be caused by this aryl nitrenium free radical. Studies on the time course of NOH-2AAF mutagenesis (Fig. 4) show that ascorbate produces its effect by acting on a metabolite of NOH-2AAF and that this metabolite is unstable. When ascorbate was added after most of the NOH-2AAF had been converted to the *N*-*O*-sulfate ester, the increase in mutagenesis was diminished. Moreover, preincubation of NOH-2AAF with the PAPS-generating system and ascorbate before the addition of the bacteria also greatly diminished the stimulatory effect. These results are consistent with the concept that an unstable intermediate such as the proposed aryl nitrenium free radical is responsible for the large increase in mutagenesis.

Since glutathione, cysteamine or vitamin E did not increase the mutagenesis of NOH-2AAF under sulfation conditions, it seems possible that the rate of hydrogen abstraction from these compounds by the arylating species is less than that for ascorbate under these assay conditions. Biotransformation of NOH-2AAF after addition of these compounds was not investigated in these studies nor were the effects of NADPH or NADH.

It is especially noteworthy that the mutagenic metabolites formed by the supernatant fraction alone or by the system supplemented with both PAPS and ascorbate do not covalently bind to protein as readily as the proposed aryl nitrenium ion formed in the sulfation system. Indeed, as discussed above, it is not even certain that any of the mutagenesis that occurs in the sulfation system is due to a chemically reactive metabolite. In accord with this view, Weeks *et al.* [27] found that while guanosine monophosphate could decrease the acyltransferase-mediated covalent binding of NOH-2AAF to nucleic acids, it was ineffective in reducing the observed mutation frequency. Although the reason for the apparent inverse relationship between mutagenicity and covalent binding is not clear, it may be important that both the sulfate conjugate and the proposed aryl nitrenium ion are charged molecules. Thus, the rate at which these substances penetrate lipid barriers including the bacterial wall may be slower than their rates of inactivation. In regard to this concept, it may be important that the proposed aryl nitrenium free radical is neutral and therefore may penetrate bacterial walls more rapidly than the ion.

Whatever the reason for the lack of correlation between mutagenesis and chemical reactivity of NOH-2AAF metabolites, it becomes evident that the most chemically reactive metabolite formed in the system is not necessarily the most mutagenic metabolite. Ultimately the understanding of what types of compounds modify mutagenesis in the Ames–Salmonella test system may lie in determining the effects of these agents on the interaction of mutagen with the bacterial genome.

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