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Mechanism of *N*-Hydroxyacetylarylamine Mutagenicity in the *Salmonella* Test System: Metabolic Activation of *N*-Hydroxyphenacetin by Liver and Kidney Fractions from Rat, Mouse, Hamster, and Man

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SUMMARY

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The mutagenicity of phenacetin and acetaminophen and their respective *N*-hydroxylated metabolites was determined in *Salmonella typhimurium* tester strains TA 98 and TA 100 in both the presence and the absence of liver and kidney metabolic activating systems from rat, mouse, hamster, and man. In TA 98 neither phenacetin, acetaminophen, nor *N*-hydroxyacetaminophen was mutagenic in either the presence or the absence of liver S-9 fractions from the 3-methylcholanthrene (3-MC)-induced rat or untreated hamster liver microsomes. The inclusion of NADPH with either the S-9 or the microsomal fractions had no effect on the mutagenicity of any of these compounds. Although *N*-hydroxyphenacetin was not directly mutagenic in TA 100, it was mutagenic in the presence of either the liver microsomal or the S-9 fractions from all three animals and in the presence of human liver microsomes. *N*-Hydroxyphenacetin was also mutagenic in TA 98 in the presence of liver microsomes from either the hamster or the mouse. Neither phenacetin, acetaminophen, nor *N*-hydroxyacetaminophen was mutagenic in TA 100 even in the presence of various metabolic activating systems. Pretreatment of the animals with either 3-MC or Arochlor 1254 or the addition of NADPH had no effect on the mutagenic activation of *N*-hydroxyphenacetin or any of the other compounds by liver and kidney fractions. *N*-Hydroxyphenacetin was also mutagenic to TA 100 in the presence of both rat and hamster kidney S-9 fractions and mouse kidney microsomes. *p*-Nitrosophenetole was mutagenic to TA 100 in both the presence and the absence of subcellular liver fractions and only slightly mutagenic to TA 98 even in the presence of metabolic activating systems. Ascorbic acid inhibited the mutation frequency (80–90%) of *N*-hydroxyphenacetin in both TA 100 and TA 98 and of *p*-nitrosophenetole in TA 100 but caused a slight increase in the mutation frequency of *N*-hydroxy-2-aminofluorene and 2-nitrosofluorene in both TA 98 and TA 100. In the presence of 1 mM ascorbic acid, *p*-nitrosophenetole rapidly decomposed with a half-life of 45 s to form azoxyphenetole and azophenetole. Paraoxon, at a 1 μM concentration, markedly inhibited both the mutagenicity of *N*-hydroxyphenacetin in TA 100 mediated by subcellular liver fractions from rat, hamster, mouse, and man as well as rat kidney S-9 fractions by 90% and the *in vitro* deacetylation of *N*-hydroxyphenacetin mediated by hamster liver microsomes. These data indicate that the initial step in the mutagenic activation of *N*-hydroxyphenacetin in the *Salmonella* system proceeds via the same mechanism as that of the known carcinogen *N*-hydroxy-2-acetylaminofluorene.

INTRODUCTION

The hepatotoxicity, mutagenicity, and carcinogenicity of *N*-acetylarylamines such as acetaminophen (*p*-hydroxyacetanilide) and AAF[1] are thought to occur follow-

[1] Abbreviations used: AAF, 2-acetylaminofluorene; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; *N*-OH-AF, *N*-hydroxy-2-aminofluorene; DMSO, dimethylsulfoxide; 3-MC, 3-methylcholanthrene.

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ing the *in vivo* metabolism of these compounds to chemically reactive intermediates (1, 2). Hepatotoxicity following large overdoses of the commonly used analgesic acetaminophen (paracetamol) has been attributed to the formation of highly reactive arylating intermediates via cytochrome *P*-450-dependent *N*-hydroxylation (2). Similarly, the first step in the metabolic activation of AAF to both a potent mutagen and a carcinogen involves *N*-hydroxylation (3). Further metabolism, however, of the initially formed *N*-OH-AAF by various enzymes, such as sulfotransferase, deacetylase, *N*-*O*-acyltransferase, and UDP-glucuronyltransferase, may be involved in the subsequent activation of *N*-OH-AAF to the ultimate carcinogenic species (4–8).

Phenacetin (*p*-ethoxyacetanilide), a component of many analgesic formulations, is also readily *N*-hydroxylated both *in vivo* and *in vitro* by liver and kidney enzymes of most rodents (9–11) and man (11). In addition to *N*-hydroxylation, phenacetin also undergoes *O*-deethylation to yield acetaminophen which may itself be subsequently *N*-hydroxylated to yield the highly reactive hepatotoxic *N*-hydroxyacetaminophen (12, 13).

Although both acetaminophen and phenacetin produce hepatic necrosis in rodents (14), only phenacetin has been adequately studied for its carcinogenic potential and has been shown to be carcinogenic in both male and female rats (15). In humans, following the chronic ingestion of large doses of analgesic preparations containing phenacetin, a high incidence of nephrotoxicity and renal pelvic tumors has been observed (16). Although the molecular species responsible for these toxicities are not known, *N*-hydroxyphenacetin and its further metabolic products have been suggested. *N*-Hydroxyphenacetin has, however, been found to be carcinogenic in rats fed this compound for extended periods of time (17).

Recently Ames and co-workers (18) have developed a simple bacterial test for determining the mutagenicity of chemical compounds. In this microbial test system approximately 85–90% of the chemical carcinogens tested were also mutagenic, indicating a strong correlation between chemical carcinogenesis and mutagenesis.

Since both phenacetin and acetaminophen undergo a similar cytochrome *P*-450-dependent *N*-hydroxylation as does the potent mutagen and carcinogen, AAF (10), we have investigated the mutagenicity of phenacetin and acetaminophen and their respective *N*-hydroxylated metabolites, *N*-hydroxyphenacetin and *N*-hydroxyacetaminophen, in the *Salmonella* test system.

MATERIALS AND METHODS

Materials. *N*-Hydroxyphenacetin was prepared by the chemical reduction of *p*-nitrophenetole in the presence of zinc dust and ammonium chloride to yield *p*-ethoxyphenylhydroxylamine (*N*-hydroxyphenetidine) (9) which without further purification was selectively *N*-acetylated with one equivalent of acetyl chloride as previously described (9). The crude product was recrystallized twice from benzene–hexane to yield pure *N*-hydroxyphenacetin, mp 103°C (literature mp 104°C (9)). Thin-layer chromagraphy (silica gel, three different solvent systems: diethyl ether–heptane, 75:25; chloroform–methanol, 95:5, and chloroform–acetone–ammonium hydroxide, 50:50:1) of *N*-hydroxyphenacetin yielded a single $FeCl_3$-positive spot in all systems. *N*-Hydroxyacetaminophen was similarly prepared by the reduction of potassium *p*-nitrophenyl sulfate to the hydroxylamine, which was *N*-acetylated, and then treated with sulfatase (Sigma Type H-2) to yield *N*-hydroxyacetaminophen as described by Gemborys *et al.* (13). *N*-Hydroxyacetaminophen was twice recrystallized from tetrahydrofuran–ethyl acetate to yield pure white plates, mp 124–126°C (dec) (literature mp 125–127°C (dec) (13)). *p*-Nitrosophenetole was prepared via an acid-catalyzed etherification of *p*-nitrosophenol with anhydrous ethanol using the procedure of Hays *et al.* (19). Recrystallization from heptane yielded *p*-nitrosophenetole as beautiful blue-green crystals, mp 33–34°C (literature mp 34–35°C (19)). *N*-OH-AAF and *N*-[*carbonyl*-^{14}C]hydroxyphenacetin (3.9 mCi/mmol) were generous gifts from Dr. Elizabeth K. Weisburger, National Cancer Institute, and Dr. Jack A. Hinson, National Heart Lung and Blood Institute, respectively. Prior to use, *N*-OH-AAF was purified by thin-layer chromatography (silica gel: chloroform–methanol, 95:5). 2-Nitrofluorene was reduced to N-OH-AF in the presence of ammonia and hydrogen sulfide. *N*-OH-AF was twice precipitated from ethanol with water to yield a light yellow powder, mp 165–170°C (dec) (literature mp 170°C (dec) (20)). *N*-OH-AF was mildly oxidized to 2-nitrosofluorene using ferric ammonium sulfate in sulfuric acid. 2-Nitrosofluorene was recrystallized twice from hexane to yield light green crystals, mp 77–78°C (literature mp 77–79°C (20)). Both *N*-OH-AF and 2-nitrosofluorene were stored under nitrogen at −20°C. Phenacetin, acetaminophen, *p*-nitrophenetole, *p*-nitrosophenol, and 3-MC were obtained from Eastman Organic Chemicals; paraoxon (diethyl *p*-nitrophenyl phosphate) was from Aldrich Chemical Company; and NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Boehringer Mannheim Biochemicals. *Salmonella* tester strains TA 98 and TA 100 were generous gifts from Dr. Bruce N. Ames, University of California (Berkeley). All other chemicals used were of the best available commercial grade.

Animals. Male C57Bl/6N (B6) inbred strain mice (4–6 weeks old), male Sprague–Dawley rats (100–150 g), and male Golden Syrian hamsters (80–100 g) were obtained from the National Institutes of Health Animal Supply and kept in standard hardwood bedding in plastic cages and allowed water and food (Purina Lab Chow) *ad libitum*. Animals were pretreated with a single intraperitoneal injection of either 3-MC (80 mg kg^{-1}) or Arochlor 1254 (500 mg kg^{-1}) (Analabs, Inc., New Haven, Conn.) dissolved in corn oil and killed 48 h later. Control animals received corn oil only.

Preparation of liver and kidney fractions. Animals were stunned and killed by decapitation. Following the exsanguination of animals, livers and kidneys were removed, minced, washed as free of hemoglobin as possible with phosphate-buffered saline (pH 7.2) at 4°C, and homogenized in 3 vol of phosphate-buffered saline. The 9000*g* supernatant (S-9) fraction, microsomes, and postmicrosomal supernatant were prepared and stored as described previously (21). Human livers were obtained from eight patients with total cerebral infarction who

served as kidney donors at Huddinge University Hospital, Huddinge, Sweden.[2] Liver microsomes were prepared and stored in the usual manner (21). Proteins were determined colorimetrically.

Mutagenesis assay. Mutagenesis was performed according to Ames *et al.* (18). To 2.2 ml of molten top agar containing 17 μmol $MgCl_2$, 0.125 μmol biotin, 0.215 μmol histidine, 33 μmol KCl, and 100 μmol sodium phosphate buffer, pH 7.4, at 45°C were added 0.1 ml of the bacterial tester strain (TA 98 or TA 100; 2–3 × 10^9 bacteria/ml), 0.1 ml of a solution containing the chemical to be tested dissolved in either DMSO or 50 mM acetate buffer (pH 5.0), 0.2 ml of the various subcellular fractions (microsomes, S-9, or 100,000*g* supernatant) containing 0.5–1.5 mg protein, and an NADPH generating system (2.5 μmol NADPH, 5 μmol glucose-6-phosphate, and 20 units glucose-6-phosphate dehydrogenase) when necessary. Prior to addition, kidney and liver S-9 and microsomal fractions were diluted with phosphate-buffered saline to the desired protein concentrations and filtered through a 0.45-μm Swinnex filter unit (Millipore). The concentrations of protein in the filtrates were then determined after filtration to estimate losses during this process. In assays where paraoxon was used, paraoxon was added in 0.1 ml of DMSO to the mutagenesis mixture. In assays where the pH dependence for the mutagenicity of *N*-hydroxyacetaminophen was studied, 100 μmol of sodium acetate buffer (pH 5.0–8.0) was used in place of the sodium phosphate buffer. Test compounds were added last. The colonies on each plate (histidine-independent revertants) were scored on a Count-all (Model 600) colony counter (Fisher Scientific Co., Pittsburgh, Pa.) after a 48-h incubation at 37°C. The toxicity of the test compounds to the bacteria was tested by determining the number of colonies formed in histidine-enriched (4.5 mM) agar after the bacteria had been exposed to varying concentrations of the test compounds for 30 min at 37°C and diluted to approximately 10^4/ml before plating.

Metabolism of N-hydroxyphenacetin. Incubations were carried out under conditions nearly identical to those of the standard mutagenesis assay. All incubations were performed in sterilized glass scintillation vials which contained a small disk (2.5-cm diameter, 3 mm thick) of Vogel Bonner minimal agar in the bottom of the vial. To 2.2 ml of molten mutagenesis top agar at 45°C were added 0.1 ml of bacterial suspension, *N*-[^{14}C]hydroxyphenacetin (1000 μg, 25 dpm/nmol) in 0.1 ml of DMSO, and 0.2 ml of hamster liver microsomal protein (0.8 mg) in sodium phosphate buffer. Paraoxon was added in 0.1 ml of DMSO. The complete top agar incubation mixtures were poured into the scintillation vials containing the agar disks, the vials loosely capped, and the reaction mixtures incubated at 37°C. Incubations were terminated by the rapid freezing of each vial and its contents to −70°C. The frozen vials were warmed to 45°C and 10 ml of 100 mM acetate buffer, pH 6.5, was added to each vial. After the addition of carrier *N*-hydroxyphenacetin (500 μg) and cooling to room temperature, the contents of each vial were carefully transferred to 45-ml glass-stoppered centrifuge tubes. The mixtures were then extracted with four 10-ml aliquots of diethyl ether, and the etheral extracts were combined and evaporated to dryness under nitrogen. The residue was dissolved in 200 μl of methanol, spotted onto thin-layer chromatography plates (silica gel GF, 1000 μm, 20 × 20 cm), and developed using a solvent system of chloroform–methanol (95:5). *N*-Hydroxyphenacetin (R_f 0.60) was eluted from the plates and the radioactivity of the band was measured by scintillation spectroscopy. Counts were corrected for quench (external standardization) and converted to nanomoles of *N*-hydroxyphenacetin using the specific activity of the original *N*-[^{14}C]hydroxyphenacetin.

Kinetic assays. Disappearance of *p*-nitrosophenetole and 2-nitrosofluorene was measured at 329 and 362 nm, respectively, in a Beckman Model 25 dual-beam spectrophotometer. The variation between the rates of disappearance of *p*-nitrosophenetole and 2-nitrosofluorene, calculated from the slopes of absorbance versus time curves of identical solutions determined on different days or at different times, was within 10%. Solutions of ascorbic acid were prepared in 0.1 M sodium phosphate buffer, pH 7.4, at concentrations 10–100 times those to be used in the assays and readjusted to pH 7.4. *p*-Nitrosophenetole and 2-nitrosofluorene (dissolved in 0.1 ml DMSO) were added to 9.8 ml of 0.1 M sodium phosphate buffer and the reactions were initiated by the addition of 0.1 ml of ascorbate. The rates of disappearance of *p*-nitrosophenetole and 2-nitrosofluorene were calculated from the slopes of absorbance versus time curves, excluding the zero-time point. Slopes were determined on the initial, linear portions of the curves.

Incubation of p-nitrosophenetole with ascorbate. A solution of *p*-nitrosophenetole (100 mg; 0.67 mmol) dissolved in 1 ml of DMSO was added to 100 ml of 0.1 M sodium phosphate buffer, pH 7.4, at room temperature with vigorous shaking. Ascorbic acid (880 mg; 5 mmol) dissolved in 10 ml of 0.1 M sodium phosphate buffer, pH 7.4, was then added all at once to the solution of *p*-nitrosophenetole. The color of the reaction mixture rapidly turned from an initial blue-green to clear yellow. After approximately 15 min a light yellow precipitate began to form, and after an additional 30 min at room temperature the reaction mixture was stored at 4°C overnight. After extraction of the reaction mixture with three 100-ml aliquots of diethyl ether, the etheral extracts were combined, dried ($MgSO_4$), and concentrated *in vacuo* to yield a light brown powder. The crude powder was dissolved in hot methanol and the solution was slowly cooled to room temperature. The first crop of crystals was filtered, air-dried, and weighed to yield 15 mg of fine yellow plates, mp 156–158°C. The literature mp for azophenetole is 160°C (22). Further concentration of the mother liquid yielded 3 mg of a yellow crystalline compound. The melting point of this compound to a cloudy liquid was 134–136°C and to a clear point was 164–166°C. The literature values for the melting point for azoxyphenetole was 137.4–137.9 and 168–168.5°C (19).

Mass spectrometry. The electron impact mass spectra were determined by direct probe insertion of samples of the isolated products obtained from incubations of *p*-

[2] Consent to the use of the livers from the kidney donors for research purposes was given by the Swedish National Board of Health and Welfare.

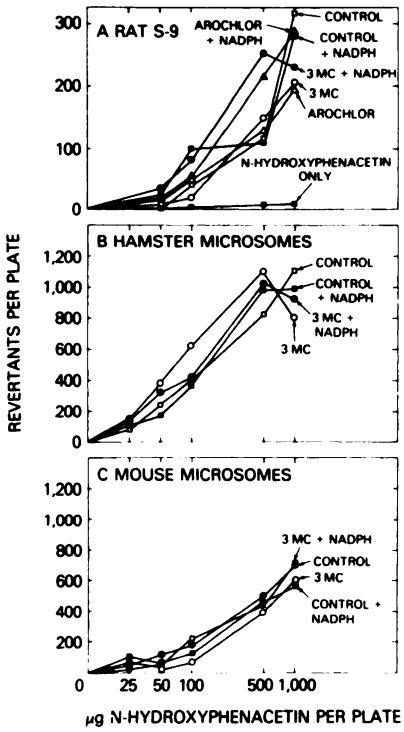


FIG. 1. *In vitro mutagenicity of N-hydroxyphenacetin in TA 100 mediated by liver S-9 fractions from Arochlor 1254- and 3-MC-treated Sprague-Dawley rats (A) and microsomal fractions from 3-MC-treated Syrian golden hamsters (B) and 3-MC-treated C57Bl/6N mice (C)*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with *N*-hydroxyphenacetin in the absence of subcellular fractions. The S-9 protein concentration was 1.5 mg per plate and the microsomal protein concentration was 0.7 mg per plate. □, Experiments without NADPH; ■, with NADPH using either untreated liver S-9 or microsomal protein; △, experiments without NADPH; ▲, with NADPH using either Arochlor-treated liver S-9 or microsomal protein; ○, experiments without NADPH; ●, with NADPH using either 3-MC-treated S-9 or microsomal protein; and ◪, mutagenicity of *N*-hydroxyphenacetin in the absence of rat liver S-9 fraction.

nitrosophenetole and ascorbic acid on a Hewlett Packard Model 5980A GC/MS equipped with a 5933A Data System (electron energy, 70 eV).

RESULTS

The mutagenicity of phenacetin and acetaminophen and their respective *N*-hydroxylated metabolites, *N*-hydroxyphenacetin and *N*-hydroxyacetaminophen, was determined in *Salmonella typhimurium* tester strains TA 98 and TA 100 in both the presence and the absence of metabolic activating systems consisting of either the S-9 or the microsomal fraction from Sprague–Dawley rat, B6 mouse, Syrian Golden hamster, and man.

In strain TA 98 neither phenacetin, acetaminophen, nor *N*-hydroxyacetaminophen was mutagenic at amounts ranging from 25 to 1000 μg per plate in either the presence or the absence of the liver S-9 fraction from the 3-MC-induced rat or untreated hamster liver microsomes (data not shown). The addition of an NADPH generating system to the standard mutagenesis assay systems had no effect on the mutagenicity of these compounds in TA 98.

Although *N*-hydroxyphenacetin was not directly mutagenic in TA 100, it was mutagenic in the presence of metabolic activating systems, either the liver microsomal or the S-9 fraction, from all three rodent species (Figs. 1A–C). *N*-Hydroxyphenacetin mutagenicity in the presence of the S-9 fraction from control rat liver was not affected by either the addition of an NADPH generating system to the mutagenesis assay mixture or the pretreatment of the animals with either 3-MC or Arochlor 1254 (Fig. 1A).

Hamster liver microsomes (Fig. 1B) were two to three times more active than mouse liver microsomes (Fig. 1C), both of which were five to eight times more active than the rat liver S-9 fraction in their ability to activate *N*-hydroxyphenacetin to a mutagen in TA 100 when expressed as revertants per milligram of protein. Similar to that which was observed in the rat, the pretreatment of either hamsters or mice with 3-MC or the inclusion of an NADPH generating system in the mutagenesis assay system had no effect on *N*-hydroxyphenacetin mutagenicity.

N-Hydroxyphenacetin, at concentrations of 500 and 1000 μg per plate, was also mutagenic in TA 100 in the presence of human liver microsomes (Table 1). Human liver microsomes (eight individuals) were considerably less active than hamster liver microsomes but about as active as rat liver S-9 fractions in their ability to activate *N*-hydroxyphenacetin to a mutagen.

Although *N*-hydroxyphenacetin was not directly mutagenic in TA 98, it was slightly mutagenic in the presence of untreated liver microsomes from either the hamster or the mouse (Table 2). *N*-Hydroxyphenacetin at the concentrations tested was not toxic to the bacteria.

Phenacetin, on the other hand, was not mutagenic in TA 100 at concentrations of 25–1000 μg per plate even in the presence of liver metabolic activating systems (liver microsomes plus NADPH generating system) from either the hamster (untreated and 3-MC or Arochlor treated) or the mouse (untreated and 3-MC treated), two species which possess very high *N*-acetyl-*N*-arylamine *N*-hy-

TABLE 1

N-Hydroxyphenacetin-induced mutagenicity in Salmonella tester strain TA 100 mediated by human liver microsomes

The mutagenesis assay was performed as described under Materials and Methods. Values are the mean number of revertants per plate observed in two experiments and have been corrected for the number of revertants obtained in the absence of microsomal protein: 77 (500 μg *N*-hydroxyphenacetin) and 86 (1000 μg *N*-hydroxyphenacetin). The microsomal protein concentration was 0.7 mg per plate.

Sample No.	*N*-Hydroxyphenacetin (μg/plate)	
	500	1000
	revertants per plate	
1	225 ± 10	346 ± 17
2	180 ± 3	294 ± 20
3	163 ± 21	319 ± 9
4	193 ± 15	276 ± 22
5	186 ± 11	304 ± 5
6	248 ± 27	330 ± 18
7	231 ± 4	388 ± 12
8	184 ± 20	340 ± 32

TABLE 2

N-Hydroxyphenacetin-induced mutagenicity in Salmonella tester strain TA 98 mediated by hamster and mouse liver microsomes

The mutagenesis assay was performed as described under Materials and Methods. Values are the mean number of revertants per plate observed in two experiments and have been corrected for the number of revertants obtained in the absence of microsomal protein: 32 (1000 μg *N*-hydroxyphenacetin). The microsomal protein concentration was 0.7 mg per plate.

N-Hydroxyphenacetin	Revertants per plate	
	Mouse	Hamster
μg/plate		
100	25	—
500	55	126
1000	126	177
2500	165	304

droxylase activities, from the rat (untreated or 3-MC-induced S-9 fractions), or from human liver. Acetaminophen, similarly, was also not mutagenic to TA 100 at concentrations of 25–1000 μg per plate in the presence of hamster liver microsomes (untreated and 3-MC or Arochlor treated) and NADPH.

Initial studies, using both TA 98 and TA 100, indicated that *N*-hydroxyacetaminophen (25–1000 μg/plate) was not mutagenic either by itself or in the presence of S-9 liver fractions from either untreated or 3-MC-pretreated rats. The *in vitro* stability of *N*-hydroxyacetaminophen varies greatly and has been shown to be strongly pH dependent (13). Unlike other *N*-acetyl-*N*-arylhydroxylamines, such as *N*-OH-AAF and *N*-hydroxyphenacetin, *N*-hydroxyacetaminophen is very unstable in neutral solutions and rapidly decomposes. The possibility therefore existed that under the conditions of the standard mutagenesis assay, in which the pH is buffered at 7.4, *N*-hydroxyacetaminophen might be decomposing prior to interaction with the bacteria. The mutagenicity of *N*-hydroxyacetaminophen was therefore determined in TA 100 over a pH range of 5.0–8.0 in both the presence and the absence of metabolic activating systems. Over this pH range the *in vitro* half-life of *N*-hydroxyacetaminophen in aqueous sodium phosphate buffer varies from hours (pH 5.0) to 10 min (pH 8.0) (13). Under these conditions, in which the pH of the mutagenesis assay mixture was varied from 5.0 to 8.0, *N*-hydroxyacetaminophen was not mutagenic at concentrations up to 1000 μg per plate in either the presence or the absence of metabolic activating systems. The viability of the bacteria was not affected over this pH range. Toxicity studies conducted at pH 7.4 indicated that *N*-hydroxyacetaminophen was not appreciably toxic (less than 10% killing) to the bacteria at concentrations up to 1000 μg per plate in the presence of microsomal protein. However, in the absence of microsomal protein, *N*-hydroxyacetaminophen was very toxic to the bacteria (100% killing at 1000 μg per plate). The microsomal protein may therefore be protecting the bacteria from the toxic effects of *N*-hydroxyacetaminophen by preferentially reacting with it or one of its decomposition products prior to its interaction with the bacteria.

In addition to being activated to a mutagen by subcellular liver fractions, *N*-hydroxyphenacetin was also mutagenic in TA 100 in the presence of mouse kidney microsomes and Sprague–Dawley rat and hamster kidney S-9 fractions (Fig. 2). On the basis of protein concentration, both the mouse kidney (Fig. 2) and the rat liver (Fig. 1A) are more active than the rat and hamster kidneys in their ability to activate *N*-hydroxyphenacetin to a mutagen. As was seen with the rat, hamster, and mouse liver enzymes, pretreatment with either 3-MC or Arochlor had no effect on the mutagenic activation of *N*-hydroxyphenacetin by rat kidney S-9 fractions. Although *N*-hydroxyphenacetin was mutagenic in the presence of either liver or kidney S-9 and microsomal fractions (Figs. 1 and 2), it was not mutagenic to TA 100 in the presence of hamster liver 100,000*g* supernatant fractions from the hamster (Fig. 3) or from the mouse or rat (data not shown). *N*-OH-AAF, however, was quite mutagenic to TA 98 in the presence of hamster liver cytosol fractions (Fig. 3).

We have previously shown that the most important enzymatic step in the metabolic activation of *N*-OH-AAF to a mutagen in the *Salmonella* test system by liver and kidney fractions from the rat and mouse is deacetylation or deamidation (23). We therefore studied the effect of paraoxon, a known inhibitor of liver microsomal *N*-OH-AAF deacetylase (24), on the mutagenicity of *N*-hydroxyphenacetin in TA 100. Paraoxon, when added at a concentration of 10^{-5} M, completely inhibited the mutagenicity of *N*-hydroxyphenacetin mediated by both hamster and human liver microsomes, mouse liver S-9 homogenate, and rat kidney S-9 fractions (Fig. 4). At a 10^{-7} M concentration of paraoxon, the mutagenicity of *N*-hydroxyphenacetin was inhibited 50–70% when incubated with subcellular fractions from the rat kidney, mouse liver, and human liver, while the mutagenicity of

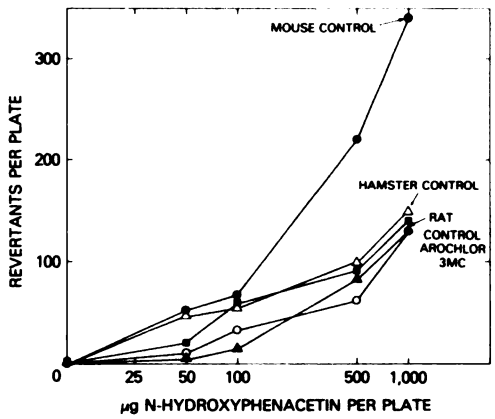


FIG. 2. *In vitro mutagenicity of N-hydroxyphenacetin in TA 100 mediated by hamster and Sprague–Dawley rat kidney S-9 homogenates and C57Bl/6N mouse kidney microsomes*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with *N*-hydroxyphenacetin in the absence of subcellular fractions. The rat kidney S-9 protein concentrations were 1.4 mg per plate and the mouse kidney microsomal protein concentration was 0.8 mg per plate. ●, Mouse kidney microsomes; ○, ■, and ▲, untreated, 3-MC-treated, and Arochlor-treated rat kidney S-9 fractions, respectively; and △, hamster kidney S-9 fraction.

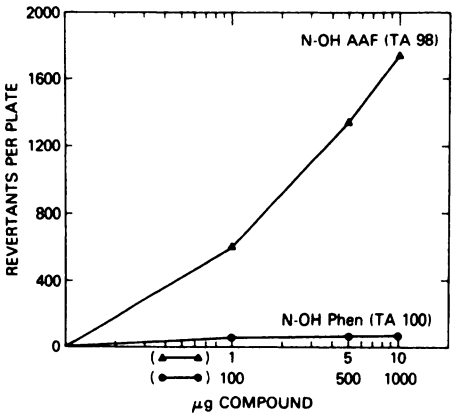


FIG. 3. *Mutagenicity of N-OH-AAF in TA 98 and N-hydroxyphenacetin in TA 100 mediated by hamster liver 100,000g supernatant fractions*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with either *N*-OH-AAF (▲) or *N*-hydroxyphenacetin (●) in the absence of any subcellular fractions. The hamster liver 100,000*g* supernatant protein concentration was 1.5 mg per plate.

N-hydroxyphenacetin mediated by hamster liver microsomes was inhibited only at concentrations of paraoxon greater than 10^{-8} M. Paraoxon alone, when tested at these concentrations, was neither mutagenic nor toxic to the bacteria in either the presence or the absence of mouse liver enzymes.

The *in vitro* metabolism of *N*-hydroxyphenacetin was similarly inhibited by paraoxon (Fig. 5). The recovery of unchanged *N*-hydroxyphenacetin from incubations performed under conditions essentially the same as those of the mutagenesis assay was taken as an approximation of the extent of metabolism of *N*-hydroxyphenacetin. Within the first hour approximately 15% of the initial amount of *N*-hydroxyphenacetin was lost, and after 24 h only 60% of the initial *N*-hydroxyphenacetin could be recovered unchanged. In the presence of either 10^{-6} or 10^{-7} M paraoxon, the amount of *N*-hydroxyphenacetin recovered after a 1-h incubation was not significantly different from that recovered from control incubations performed in the absence of paraoxon. After 24 h the metabolism of *N*-hydroxyphenacetin in the presence of 10^{-7} and 10^{-6} M paraoxon was inhibited by 18 and 60%, respectively (Fig. 5).

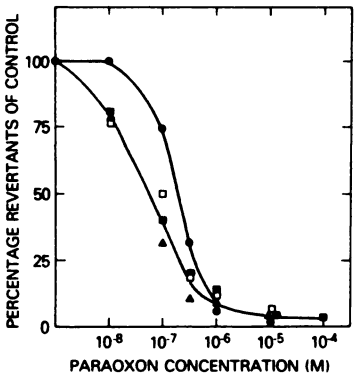


FIG. 4. *Effect of paraoxon on the mutagenic activation of N-hydroxyphenacetin in TA 100 by C57Bl/6N mouse liver S-9 fractions, hamster liver microsomes, human liver microsomes, and rat kidney S-9 fractions*

The mutagenesis assay was performed as described under Materials and Methods. The S-9 protein concentrations were 1.5 mg per plate and the microsomal protein concentrations were 0.8 mg per plate. The concentration of *N*-hydroxyphenacetin per plate was 1000 μg, and at this concentration the numbers of revertants on control plates (no paraoxon) were: hamster liver microsomes, 2500 revertants per plate; mouse liver S-9 fraction, 415 revertants per plate; rat kidney S-9 fraction, 150 revertants per plate; and human liver microsomes, 279 revertants per plate. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with *N*-hydroxyphenacetin in the absence of any subcellular fractions. ●, Hamster liver microsomes; ■, mouse liver S-9 fraction; □, rat kidney S-9 fraction; and ▲, human liver microsomes.

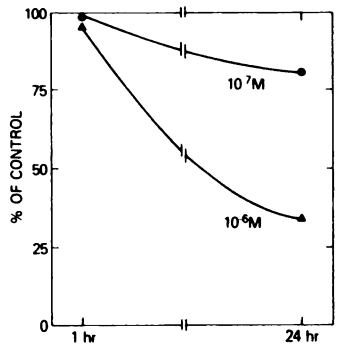


FIG. 5. *Effect of paraoxon on the in vitro metabolism of N-[^{14}C]hydroxyphenacetin*

N-[^{14}C]Hydroxyphenacetin (1000 μg, 5130 nmol, 25 dpm/nmol) was incubated with hamster liver microsomes (0.8 mg) in either the presence or the absence of paraoxon, and the amount of unchanged *N*-hydroxyphenacetin was determined. Incubation conditions and isolation and quantitation procedures were as described under Materials and Methods. Values represent the means of two separate experiments. The average percentage of recovery of *N*-hydroxyphenacetin during extraction and purification procedures was 65%. All calculations have been corrected for this extraction efficiency. In the absence of microsomal protein the amounts of *N*-hydroxyphenacetin recovered after 1 and 24 h were the same. The amounts of *N*-hydroxyphenacetin recovered from control incubation vials (with microsomes) in the absence of paraoxon were 4350 nmol (1 h) and 3005 nmol (24 h). Percentage of control (ordinate) represents the relative percentage of total metabolism of *N*-hydroxyphenacetin (e.g., 100% of control represents no inhibition of metabolism).

Attempts to prepare an analytically pure sample of *N*-hydroxyphenetidine (*p*-ethoxyphenylhydroxylamine), the deacetylated metabolite of *N*-hydroxyphenacetin, for mutagenic testing were unsuccessful. Unlike phenylhydroxylamine or its *p*-chloro analog, *p*-chlorophenylhydroxylamine, *N*-hydroxyphenetidine is quite unstable, and all attempts to isolate and purify the hydroxylamine following various attempted chemical reductions (zinc dust/ammonium chloride, ammonia/hydrogen sulfide, and aluminum amalgam/moist ether) of *p*-nitrosophenetole failed. Therefore, the *p*-nitroso derivative, *p*-nitrosophenetole (*p*-ethoxynitrosobenzene), was synthesized for mutagenic testing. In TA 98, *p*-nitrosophenetole was only slightly mutagenic at amounts ranging from 10 to 250 μg per plate in either the presence or the absence of liver microsomes from Arochlor-induced hamsters (Fig.

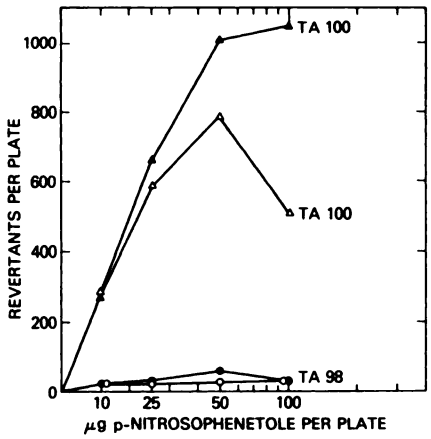


FIG. 6. *Mutagenicity of p-nitrosophenetole (p-ethoxynitrosobenzene) in TA 98 and TA 100 mediated by hamster liver microsomes*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed in the absence of *p*-nitrosophenetole. *p*-Nitrosophenetole was dissolved in DMSO prior to addition to mutagenesis assay mixtures. The microsomal protein concentration was 0.7 mg per plate. △, TA 100 without microsomes; ▲, TA 100 with microsomes; ○, TA 98 without microsomes; ●, TA 98 with microsomes.

6). In TA 100, however, *p*-nitrosophenetole was mutagenic in both the presence and the absence of hamster liver microsomes (Fig. 6). At amounts ranging from 10 to 50 μg per plate, *p*-nitrosophenetole was nearly 10 times as mutagenic as *N*-hydroxyphenacetin. At higher concentrations (>100 μg per plate), *p*-nitrosophenetole was very toxic to the bacteria in the absence of microsomal protein (100% killing at 250 μg per plate). Similar to that observed with *N*-hydroxyacetaminophen, the microsomal protein appears to protect the bacteria from the toxic effects of *p*-nitrosophenetole since 100% killing of bacteria was not seen until amounts greater than 500 μg per plate of *p*-nitrosophenetole were used (data not shown).

We have previously shown that the addition of ascorbic acid significantly increases the mutagenicity of *N*-OH-AAF mediated by either mouse kidney microsomes or purified liver cell nuclei from either the rat or the mouse in TA 1538, while no significant change in *N*-OH-AAF mutagenicity was observed when rat liver microsomes and ascorbate were combined (23, 25). In marked contrast, however, the mutagenicity of *N*-hydroxyphenacetin mediated by hamster liver microsomes in either TA 100 or TA 98 was significantly decreased in a dose-dependent fashion by ascorbic acid (Figs. 7A and B). The most pronounced effects were observed at 5 and 10 mM ascorbate, with a three- to four-fold decrease in the mutation frequency of *N*-hydroxyphenacetin as compared with controls. Similarly, the mutation frequency of the directly acting *p*-nitrosophenetole in TA 100 was also markedly decreased by the addition of ascorbic acid (Fig. 8E), while the mutagenicity in both TA 100 and TA 98 of the direct-acting deacetylated derivatives of *N*-OH-AAF, *N*-OH-AF (Figs. 8A and B) and 2-nitrosofluorene (Figs. 8C and D) was slightly increased in the presence of ascorbic acid.

To gain insight into the mechanism of inhibition of the mutagenicity of *N*-hydroxyphenacetin and *p*-nitrosophenetole by ascorbic acid, varying concentrations of *p*-nitrosophenetole were incubated in the presence of ascorbic acid and the disappearance of *p*-nitrosophenetole was monitored colorimetrically at 329 nm. As can be seen from the results summarized in Fig. 9A, *p*-nitrosophenetole was rapidly degradated, with a half-life ($t_{\frac{1}{2}}$) ranging from 30 s for 0.033 mM *p*-nitrosophenetole to 45 s for 0.26 mM *p*-nitrosophenetole in the presence of 1 mM ascorbate. These concentrations of *p*-nitrosophenetole correspond to concentrations of 12.5 and 100 μg per plate of *p*-nitrosophenetole in the standard mutagenesis assay. Similarly, the disappearance of 2-nitrosofluorene was monitored colorimetrically at 362 nm, and like 2-nitrosophenetole, 2-nitrosofluorene was rapidly degraded by 1 mM

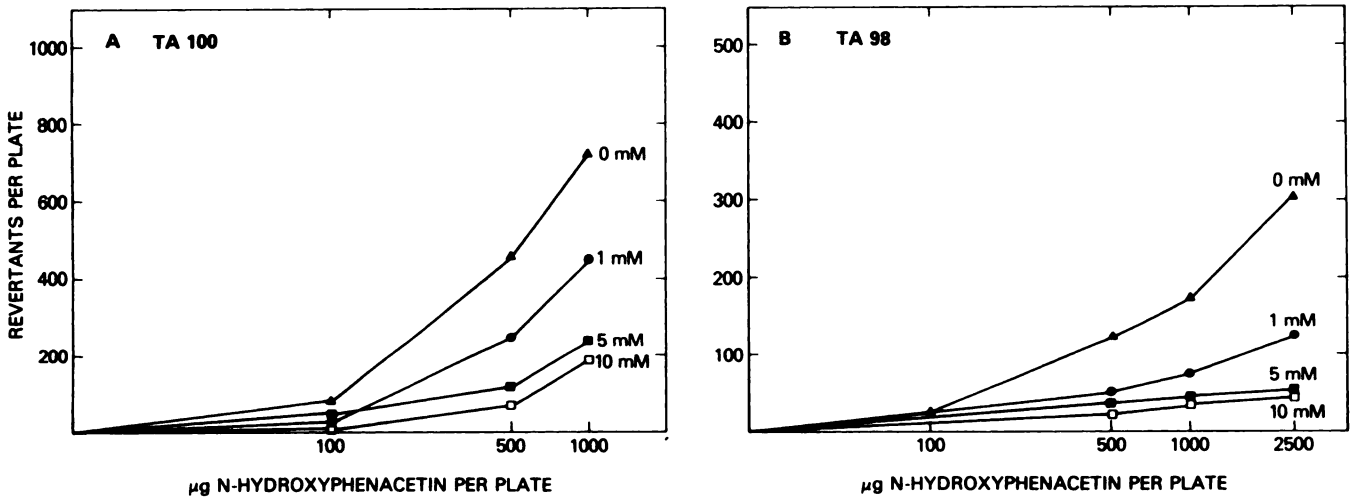


FIG. 7. *Effect of ascorbic acid on the mutagenicity of N-hydroxyphenacetin in TA 100 (A) and in TA 98 (B) mediated by hamster liver microsomes*

The mutagenesis assay was performed as described under Materials and Methods. Points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed with *N*-hydroxyphenacetin in the absence of any subcellular fractions. The microsomal protein concentration was 0.5 mg per plate. Concentration of ascorbic acid: ▲, 0 mM (control); ●, 1 mM; ■, 5 mM; and □, 10 mM.

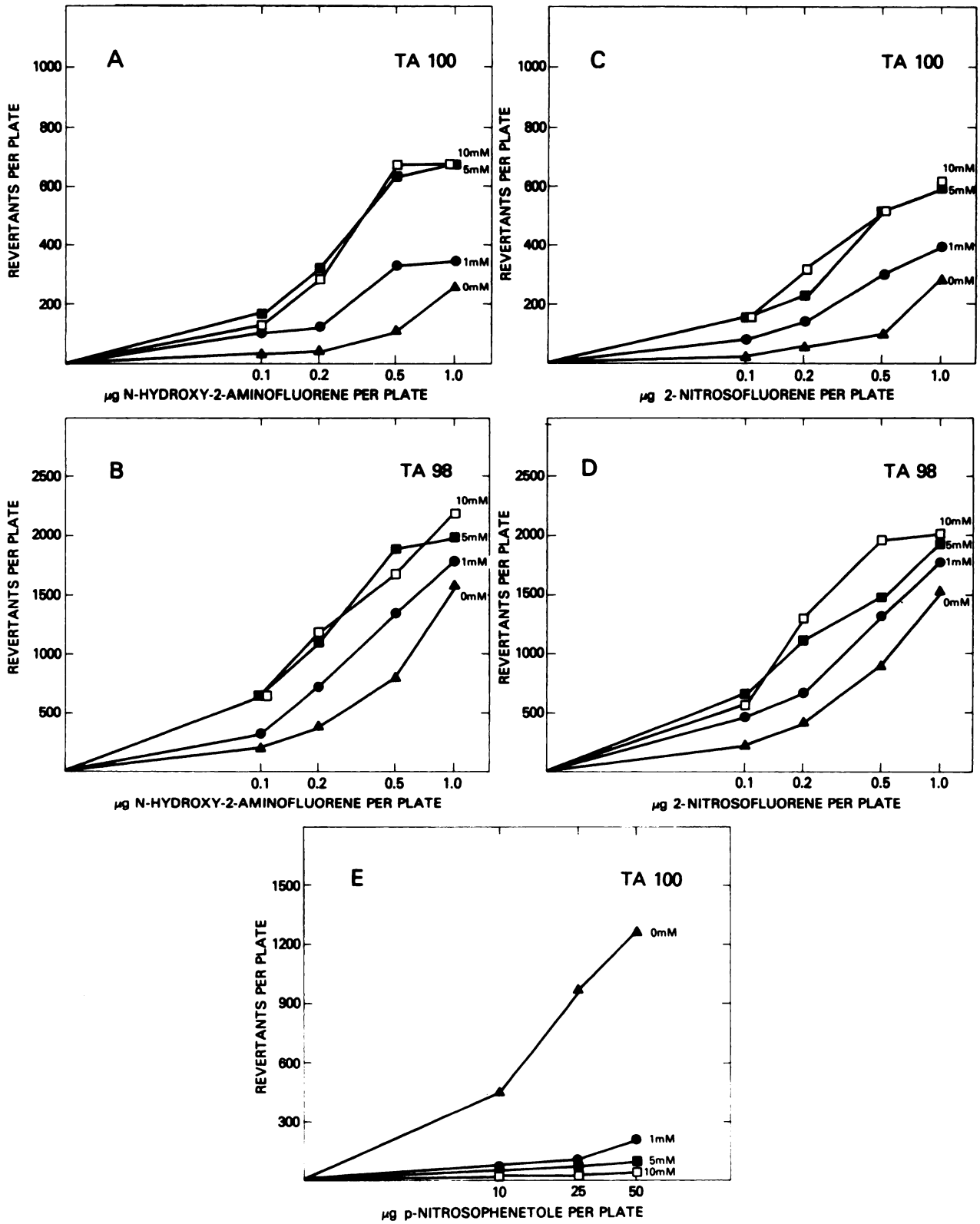


FIG. 8. *The effect of ascorbic acid on the mutagenicity of N-OH-AF 2-nitrosofluorene and nitrosophenetole*

(A) Mutagenicity of *N*-OH-AF in TA 100. (B) Mutagenicity of *N*-OH-AF in TA 98. (C) Mutagenicity of 2-nitrosofluorene in TA 100. (D) Mutagenicity of 2-nitrosofluorene in TA 98. (E) Mutagenicity of *p*-nitrosophenetole in TA 100. The mutagenesis assays were performed as described under Materials and Methods. In all curves the points are the mean number of revertants per plate observed in two experiments and have been corrected for spontaneous (background) revertants observed in the absence of test compounds. Concentration of ascorbic acid: ▲, 0 mM (control); ●, 1 mM; ■, 5 mM; and □, 10 mM.

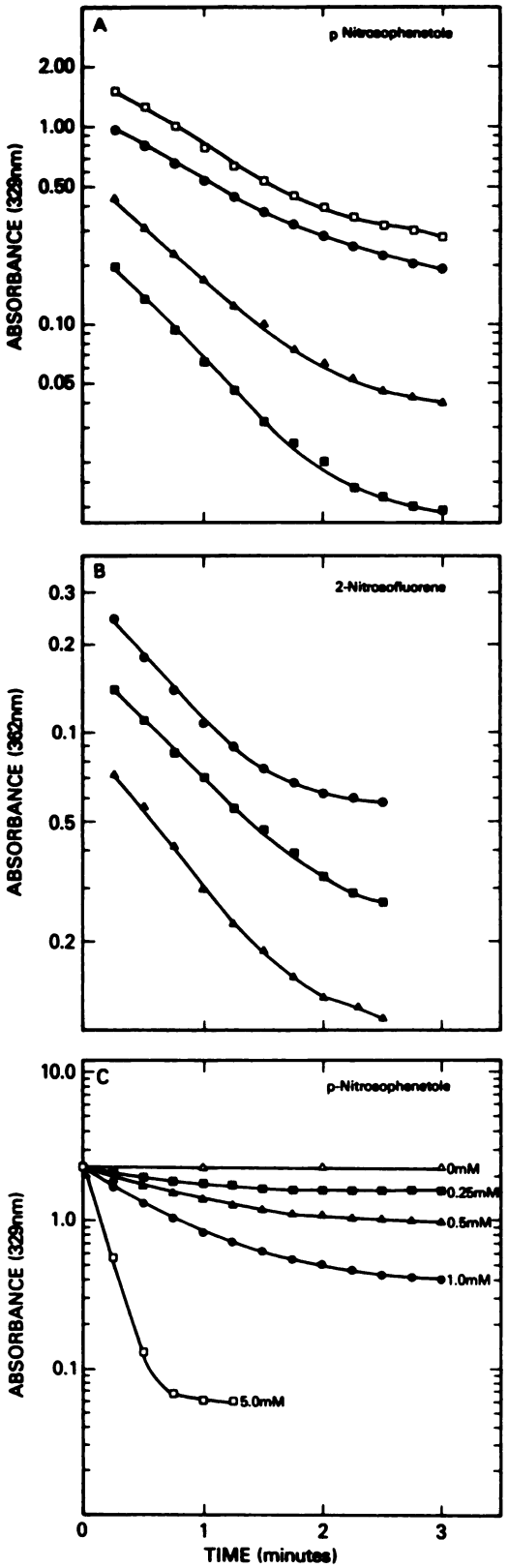


FIG. 9. *Effect of ascorbic acid on the decomposition of p-nitrosophenetole and 2-nitrosofluorene*

Solutions of *p*-nitrosophenetole and 2-nitrosofluorene were incubated with ascorbate at 22°C in 0.1 M sodium phosphate buffer (mutagenesis buffer), pH 7.4, and the absorbances of *p*-nitrosophenetole (329 nm) and 2-nitrosofluorene (362 nm) were measured. (A) Effect of varying the initial concentrations of *p*-nitrosophenetole. The initial concentrations of *p*-nitrosophenetole were: □, 0.26 mM; ●, 0.13 mM; ▲, 0.065 mM; and ■, 0.033 mM. The concentration of ascorbate was 1 mM. (B) Effect of varying the concentrations of 2-nitrosofluorene. The initial concentrations of 2-nitrosofluorene were: ●, 0.028 mM; ■, 0.014 mM; and ▲, 0.007 mM. The concentration of ascorbate was 1 mM. (C) Effect of varying the initial concentrations of ascorbate were: □, 5.0 mM; ●, 1.0 mM; ▲, 0.5 mM; ■, 0.25 mM; and △, no ascorbate. The concentration of *p*-nitrosophenetole was 0.26 mM.

ascorbate. The $t_{\frac{1}{2}}$ for the decomposition of 2-nitrosofluorene was very similar to that for the decomposition of 2-nitrosophenetole, being about 45 s (Fig. 9B). Incubation of a single concentration of *p*-nitrosophenetole (0.26 mM) with varying concentrations of ascorbic acid revealed that the rate of decomposition of *p*-nitrosophenetole was greatly dependent upon the initial concentrations of ascorbate. The $t_{\frac{1}{2}}$ for the decomposition of 0.26 mM *p*-nitrosophenetole in the presence of 5 mM ascorbate was about 6 s whereas in the presence of 1 mM ascorbate the $t_{\frac{1}{2}}$ of *p*-nitrosophenetole was 45 s (Figs. 8A and C).

Incubation of *p*-nitrosophenetole with ascorbic acid yielded two products which were subsequently identified as azophenetole and azoxyphenetole from physical and mass spectral data (Figs. 10A and B).

DISCUSSION

The mutagenic testing of a wide variety of chemical compounds in the *Salmonella* test system has shown that approximately 85–90% of the chemical carcinogens tested were also mutagens, indicating a strong correlation between *in vitro* mutagenesis and *in vivo* carcinogenesis (18). It might therefore be expected that the same enzymes involved in the metabolic activation of various xenobiotics to mutagens may also be involved in the *in vivo* activation of the same compounds to carcinogens.

Other workers have reported that both phenacetin and *N*-hydroxyphenacetin are mutagenic *in vitro* in the *Salmonella* tester strain TA 100 (26). However, the results of our studies indicate that only *N*-hydroxyphenacetin is mutagenic *in vitro* in TA 100, while phenacetin, acetaminophen, and *N*-hydroxyacetaminophen do not appear to be mutagenic in this strain. Furthermore, it appears that the mutagenic activation of *N*-hydroxyphenacetin in the *Salmonella* system by subcellular tissue fractions from rat, hamster, mouse, and man proceeds via the same mechanism by which the known carcinogen *N*-OH-AAF is activated to a mutagen *in vitro* (23). However, unlike *N*-OH-AAF, which can also be activated to a mutagen in strains TA 98 and TA 1538 by the liver cytosolic enzyme, *N*-*O*-acetyltransferase (25, 27, 28), *N*-hydroxyphenacetin is not activated to a mutagen by rat, hamster, or mouse liver 100,000*g* supernatant fractions.

The inhibition of the mutagenicity of *N*-hydroxyphenacetin by paraoxon is paralleled by a similar decrease in the disappearance, presumably via deacetylation, of *N*-hydroxyphenacetin *in vitro* following incubation of *N*-hydroxyphenacetin with hamster liver microsomes and paraoxon (Fig. 5). Presumably the parallel inhibition of mutagenesis and decrease in the metabolism of *N*-hydroxyphenacetin reflect a decrease in the formation of the more proximate mutagenic species, either the hy-

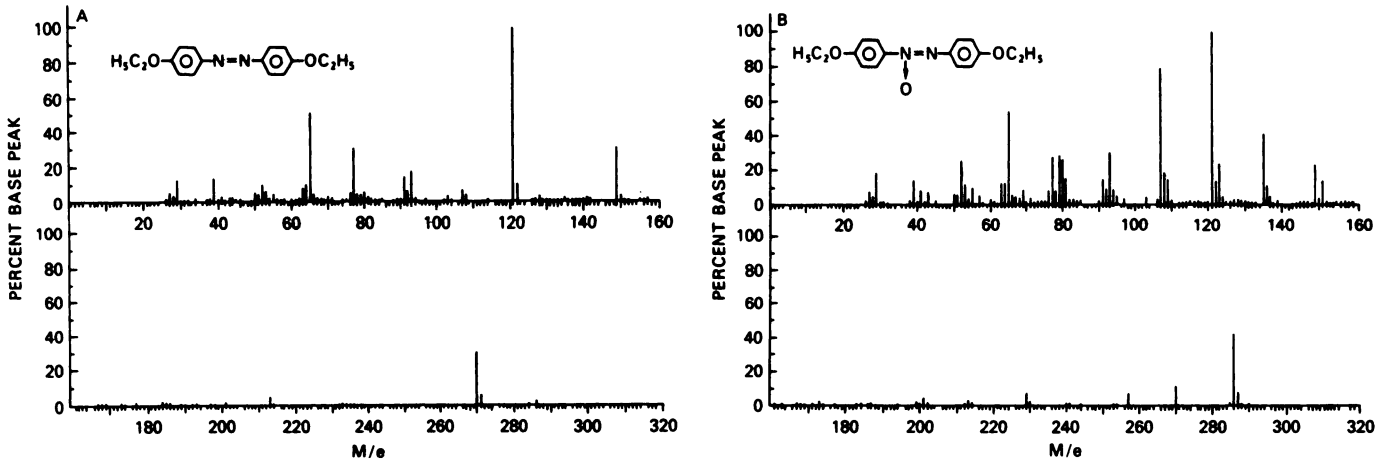
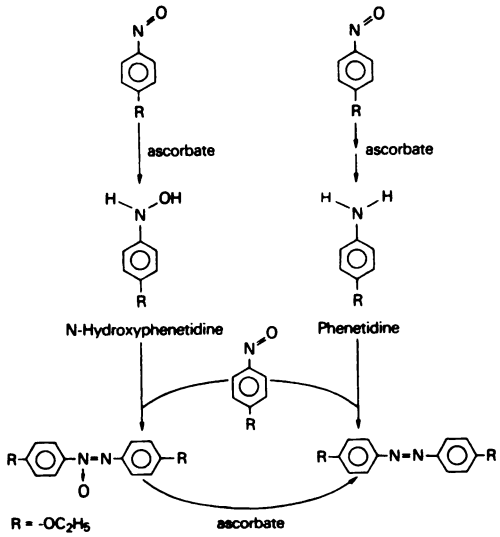


FIG. 10. *Electron impact mass spectra pf azophenetole (A) and azoxyphenetole (B)* Products were isolated and identified as described under Materials and Methods.

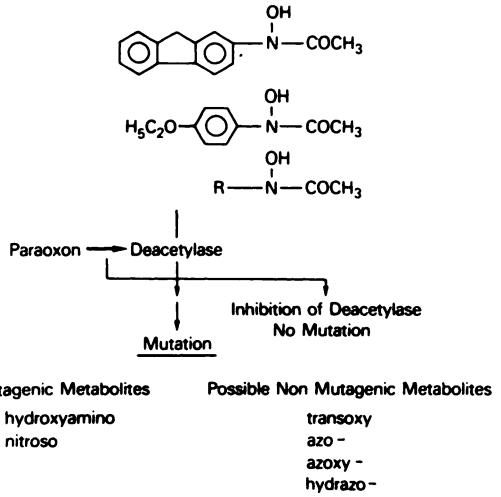
droxylamine or the nitroso derivative. The *p*-nitroso derivative, *p*-nitrosophenetole, is a direct-acting mutagen which is nearly 10 times as potent a mutagen as *N*-hydroxyphenacetin, which requires metabolic activation (Fig. 6). The *in vitro* metabolism studies show that nearly 40% of the *N*-hydroxyphenacetin is metabolized during a 24-h incubation period. It is, however, not known what proportion of the metabolized *N*-hydroxyphenacetin is converted to the mutagenic species (the hydroxylamine and/or nitroso derivatives), and a substantial part of the metabolized *N*-hydroxyphenacetin may be converted to nonmutagenic metabolites such as azophenetole and azoxyphenetole.

Ascorbic acid causes a decrease in the mutation frequency of both *N*-hydroxyphenacetin and *p*-nitrosophenetole in TA 100 while increasing the mutation frequency of *N*-OH-AAF (23, 25, 29), *N*-OH-AF, and 2-nitrosofluorene in strains TA 98 and TA 100 (Fig. 8). Guttenplan has shown that ascorbate inhibits *N*-methyl-*N*-nitrosoguanidine (MNNG)- and dimethylnitrosamine (DMN)-induced mutagenesis in TA 1530 (30). With MNNG, ascorbate was shown to lead to a direct decomposition of MNNG and deactivation of MNNG as a mutagen. Although absorbate inhibited DMN-induced mutagenesis, no direct decomposition of DMN could be shown (30). Both 2-nitrosofluorene and *p*-nitrosophenetole undergo rapid transformations, with a disappearance $t_{\frac{1}{2}}$ of 45 s, in the presence of 1 mM ascorbate at pH 7.4, although the mutagenicity of 2-nitrosofluorene is slightly increased in the presence of ascorbate (Fig. 8). The incubation of nitrosophenetole with ascorbate results in the formation of azophenetole and azoxyphenetole. The formation of these two compounds (and possibly others) probably occurs as a result of the initial reduction of *p*-nitrosophenetole by ascorbate to yield *N*-hydroxyphenetidine which can either react with another molecule of *p*-nitrosophenetole to yield azoxyphenetole or be further reduced to phenetidine (Scheme I). Azophenetole can be formed via the reduction of azoxyphenetole by another molecule of ascorbate or via the condensation of phenetidine with *p*-nitrosophenetole (Scheme I). Azoxyphenetole is neither mutagenic nor toxic to TA 100 at concentrations ranging from 10 to 1000 μg per plate in either the presence or the absence of hamster liver microsomes from Arochlor 1254-pretreated animals (data not shown). Although 2-nitrosofluorene decomposes at a similar rate as does *p*-nitrosophenetole in the presence of 1 mM ascorbate, subsequent reactions of the presumably initially formed *N*-OH-AF with 2-nitrosofluorene may not occur to the same extent as does the analogous reaction between *p*-nitrosophenetole and *N*-hydroxyphenetidine, thereby explaining the differential effect of ascorbic acid on the mutagenicity of *p*-nitrosophenetole and 2-nitrosofluorene.

As we and others have shown, ascorbate increases *N*-OH-AAF mutagenicity in the presence of both mouse kidney microsomes (23) and rat liver 100,000*g* supernatant fractions (25, 29). In this system the mutagenic species derived from *N*-OH-AAF appears to be the *N*-OH-AF-derived nitrenium ion rather than the nitroxyl free radical derived directly from *N*-OH-AAF (25). Ascorbate reduces in a one-electron redox process any nitroxyl free radicals derived from *N*-OH-AAF back to *N*-OH-AAF, which in turn may result in an increase in the formation of nitrenium ions after deacetylation.



SCHEME I



SCHEME II

At present it is not possible to assign a similar function to ascorbate in the mutagenic activation of *N*-hydroxyphenacetin mediated by hamster liver microsomes. It is, however, quite clear that the initial step in the metabolic activation of both *N*-OH-AAF and *N*-hydroxyphenacetin by liver and kidney fractions from rat, mouse, hamster, and man is deacetylation (Scheme II). The *N*-hydroxyphenacetin-induced mutagenesis in TA 100 is completely blocked by paraoxon, a known inhibitor of *N*-OH-AAF liver deacetylase (24) and of *N*-OH-AAF mutagenesis (23). Subsequent activation, however, of the initially formed *N*-hydroxyphenetidine from *N*-hydroxyphenacetin to the corresponding nitrenium ion similar to that which we have proposed for the activation of *N*-OH-AF requires additional studies. However, it does appear that subsequent reactions involving *N*-hydroxyphenetidine and its nitroso analog may be important in the metabolic deactivation of the initially formed hydroxyamine from *N*-hydroxyphenacetin since *N*-hydroxyphenacetin-induced mutagenesis is inhibited in the presence of ascorbate.

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