Formamidine Insecticides and Chloroacetanilide Herbicides: Disubstituted Anilines and Nitrosobenzenes as Mammalian Metabolites and Bacterial Mutagens

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The formamidines chlordimeform and amitraz and the chloroacetanilides metolachlor, alachlor, and butachlor are metabolized in intraperitoneally treated rats and by hepatic mixed-function oxidase systems to 2,4- and 2,6-disubstituted anilines that in turn are converted to the corresponding nitrosobenzenes. These metabolites are identified by gas chromatography–mass spectrometry with selected ion monitoring and by cochromatography. The Ames assay with *Salmonella typhimurium* strain TA 100 and modified S9 mix gives mutagenic potencies (revertants/nmol) as follows: the pesticides, <0.01–0.2; 4-chloro-2-methylaniline, 1.9; 2,4-dimethylaniline, 0.5; 2,6-dimethyl-, 2,6-diethyl-, and 2-ethyl-6-methylanilines, 0.01-0.02. The isolated 2,6-dialkylnitrosobenzenes are direct-acting mutagens (6–16 reverants/nmol), and the corresponding hydroxylamines and 2,4-disubstituted nitrosobenzenes and hydroxylamines, although not isolated, appear to be of similar or greater potency. The hydroxylamino and nitroso metabolites possibly contribute to the toxicological profiles of selected formamidine insecticides, chloroacetanilide herbicides, and the corresponding anilines.

The formamidine insecticides chlordimeform (**1a**) and amitraz (**1b**) are prepared from 4-chloro-2-methylaniline and 2,4-dimethylaniline, respectively. Intermediates for the chloroacetanilide herbicides are 2-ethyl-6-methylaniline for metolachlor (**6a**) and 2,6-diethylaniline for alachlor (**6b**) and butachlor (**6c**). These pesticides revert in part to the parent anilines on mammalian metabolism and environmental degradation (Aizawa, 1982; Chen and Wu, 1978; Environmental Protection Agency, 1984; Knowles and Benezet, 1977, 1981).

The formamidines **1a** and **1b** and their aniline metabolites and the chloroacetanilides **6a** and **6b** are suspected or established oncogens (Environmental Protection Agency, 1979, 1984; Food and Agriculture Organization, 1979, 1980, 1681; Schatzow, 1985; Weisburger et al., 1978). The Ames *Salmonella typhimurium* strain TA 100 assay (Maron and Ames, 1983) detects 4-chloro-2-methyl- and 2,4-dimethylanilines as mutagens on S9 activation (Zimmer et al., 1980). 4-Phenoxyanilines, which are metabolites and photoproducts of 4-nitrodiphenyl ether herbicides, are metabolically oxidized to bacterial mutagens, presumably the nitroso or hydroxylamino derivatives (Draper and Casida, 1983a,b).

This study examines the possible metabolic formation of disubstituted anilines and nitrosobenzenes by various combinations of oxidation and hydrolysis reactions of formamidine insecticides and chloroacetanilide herbicides and related compounds (Figure 1). It also considers their direct and S9-activated mutagenic activity, if any, in bacterial assays.

MATERIALS AND METHODS

Analytical Procedures. Nuclear magnetic resonance (NMR) spectroscopy was carried out with a Bruker WM 300 instrument at 300 MHz for ^{1}H. Samples were dissolved in acetonitrile-d_3, and chemical shifts (δ) are reported (ppm) downfield from tetramethylsilane. Mass spectrometry (MS) utilized a Hewlett-Packard 5985B system operated under electron-impact (EI) or chemical ionization (CI) conditions at 70 and 230 eV, respectively. CI relied on methane as the reactant gas (0.8 torr). Samples were examined by direct insertion or by gas chromatography (GC) combined with MS on a 5840A Hewlett-Packard instrument with a high-performance methyl silicone capillary column (10 m), helium as carrier gas (1 mL/min), and temperature programming (80–200 °C, 20 °C/min). In analyses with metabolite mixtures of anilines and nitrosobenzenes, selected ion monitoring (SIM) was carried out for $M^{+\cdot}$ and a major fragment in EI and for $[M + 1]^+$ and $[M + 29]^+$ in CI with dwell times of 50–200 ms optimized for detection of the nitroso derivatives.

High-performance liquid chromatography (HPLC) utilized a Beckman 344 instrument equipped with an Ultrasphere ODS (1-cm diameter) reversed-phase column eluted with methanol–water gradients.

Chemicals. Structures and designations for the compounds examined are given in Figure 1. The pesticides were analytical reference standards from the Environmental Protection Agency (Research Triangle Park, NC) or were obtained from Chem Services (Westchester, PA) or the manufacturer. **6d** was from Sigma Chemical Co. (St. Louis, MO). The anilines were from Aldrich Chemical Co. (Milwaukee, WI) except for the 2-ethyl-6-methyl analogue from Fluka (Hauppange, NY) and the 2-*tert*-butyl-6-methyl analogue from Ethyl Corp. (Baton Rouge, LA); they were authenticated by GC–MS and NMR (Table I). *m*-Chloroperoxybenzoic acid (MCPBA, Aldrich) was purified before use (Fieser and Fieser, 1967).

Four 2,6-dialkylnitrosobenzenes (Table I) were prepared by adding MCPBA (0.9 mmol) to the corresponding aniline (0.3 mmol) in acetonitrile-d_3 (1 mL). Within minutes a characteristic blue developed with 2-*tert*-butyl-6-methylaniline while the others gave yellow-green solutions. After 2 h the solutions were cooled (−20 °C) to precipitate most of the *m*-chlorobenzoic acid (MCBA), decanted, and inspected by GC–MS and NMR, revealing no intermediate hydroxylamine and ~90% nitroso and ~10% nitro compounds. The 2,6-dialkylnitrosobenzenes were obtained pure as solid materials on solvent evaporation after separation by HPLC or by extraction into hexane followed by chromatography through a short Florisil column developed with hexane. This procedure applied to 2-chloro-4-methylaniline and 2,4-dimethylaniline yielded only small amounts of the nitroso compounds (<5%), which were not isolated, whereas the major products were the azoxybenzenes (60–80%) isolated by thin-layer chromatography (silica gel, ether–hexane (1:1), R_f 0.50–0.52).

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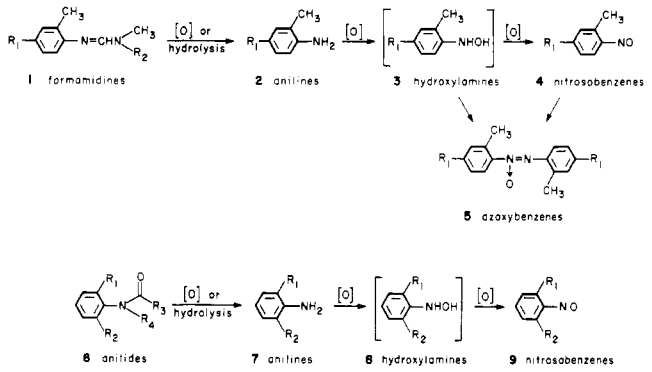

Formamidine Insecticides

chlordimeform (**1a**): R_1 = Cl, R_2 = CH_3

amitraz (**1b**): R_1 = CH_3, R_2 = CH=N-(2,4-dimethylphenyl)

Chloroacetanilide Herbicides

metolachlor (**6a**): R_1 = CH_3, R_2 = C_2H_5, R_3 = CH_2Cl, R_4 = $CH(CH_3)CH_2OCH_3$

alachlor (**6b**): R_1 = C_2H_5, R_2 = C_2H_5, R_3 = CH_2Cl, R_4 = CH_2OCH_3

butachlor (**6c**): R_1 = C_2H_5, R_2 = C_2H_5, R_3 = CH_2Cl, R_4 = $CH_2OC_4H_9$

Related Compounds

lidocaine (**6d**): R_1 = CH_3, R_2 = CH_3, R_3 = $CH_2N(C_2H_5)_2$, R_4 = H

metalaxyl (**6e**): R_1 = CH_3, R_2 = CH_3, R_3 = CH_2OCH_3, R_4 = $CH(CH_3)CO_2CH_3$

Figure 1. Designations and partial metabolic pathways for formamidine insecticides, chloroacetanilide herbicides, and related compounds. Conversions established in both ip-treated rats and hepatic MFO systems are as follows: all formamidines and anilides except **6e** to the corresponding anilines; all anilines plus the 2-*tert*-butyl-6-methyl analogue to the corresponding nitrosobenzenes.

The MS fragmentation patterns of the nitrosobenzenes differ from each other in two respects: the base peak or major fragment is M – NO for the 2,4-disubstituted compounds and 2,6-dimethylnitrosobenzenes vs. M – alkyl for the other 2,6-dialkylnitrosobenzenes (Table I); 2,6-dimethylnitrosobenzene is more thermally stable than the other 2,6-dialkylnitrosobenzenes that yield a major thermal degradation product in GC–MS (2 amu lower) (Figure 2) particularly when concentrated solutions are analyzed. The NMR of the ethyl-substituted compounds shows an interaction of the CH_2 and NO substituents evident by resolution of the methylene into two quartets in methanol-d_4.

(2,6-Dimethylphenyl)hydroxylamine and 2,6-dimethylnitrobenzene were examined in a mixture with the aniline precursor (**7**, R = CH_3) and nitrosobenzene intermediate (**9**, R = CH_3) by carrying out the MCPBA oxidation in acetonitrile-d_3 with varying amounts of MCPBA. Analyses involved integration of signals for the methyl protons appearing at δ 2.18, 2.32, 2.43, and 2.60 for the aniline, hydroxylamine, nitroso, and nitro derivatives, respectively. Examination of appropriate product mixtures gave CI–MS m/e 142 $[MH]^+$, 12 and 124 $[M - H_2O]^+$, 100 for the hydroxylamine and 156 $[M + 1]^+$, 100 and 181 $[M + 29]^+$, 8 for the nitro derivative. The other anilines (Table I) were also examined (NMR) on oxidation with 0.25 and 3 equiv of MCPBA.

Metabolism. Male albino rats (~200 g; Simonsen Laboratory, Gilroy, CA) were treated intraperitoneally (ip) with the aniline or derivative in two doses of 125 mg/kg given 10 min apart. One hour after the first dose, each rat was sacrificed and the liver removed, frozen in liquid nitrogen, and immediately homogenized in cold hexane (9 mL). The hexane extract was clarified by centrifugation (3000g, 5 min) and concentrated to 2 mL and a 1-μL aliquot examined by GC–MS–SIM.

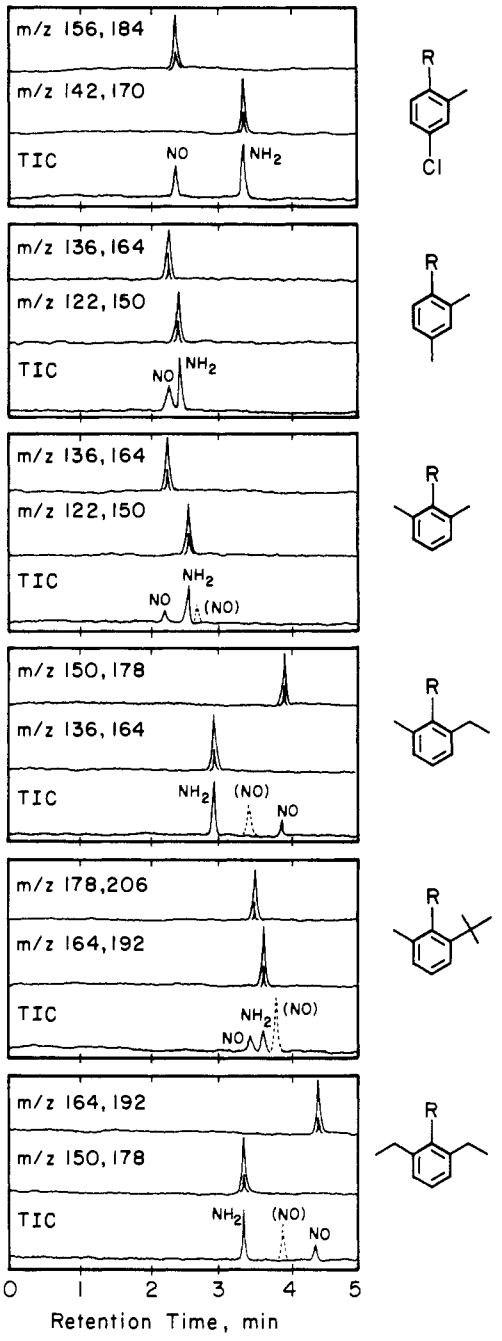

Figure 2. Gas chromatograms showing disubstituted nitrosobenzenes formed on microsomal oxidase metabolism of disubstituted anilines as detected by CI–SIM monitoring for the indicated ions. The conditions are given in Table I. CI–SIM chromatograms for the major M + 1 and minor M + 29 ions confirm the product identities and are shown superimposed for both the nitrosobenzenes and anilines. The (NO) peak arises from thermal decomposition of the corresponding nitrosobenzene by apparent loss of 2 amu. Essentially identical chromatograms are obtained with CI–SIM monitoring of in vivo metabolites of these anilines in the liver of rats, and similar chromatograms result with EI-SIM monitoring of both in vitro and in vivo metabolites. The formamidines and anilides other than **6d** yield the corresponding anilines and nitrosobenzenes but with a lower ratio of nitrosobenzenes to anilines than on direct administration of the aniline. TIC = total ion current.

Microsomal oxidase metabolism involved addition of the xenobiotic (40 μg) in ethanol (10 μL) to a mixture of rat liver microscomes (noninduced, 5 mg of protein) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) (0 or 2 μmol) in 1 mL of 50 mM sodium

Table I. Chromatographic, ^1H NMR, and MS Data for 2,4- and 2,6-Disubstituted Anilines, Nitrosobenzenes, and Azoxybenzenes

compd (Figure 1)	R_t,[a] min	EI-MS, m/z (rel intens, %) $M^{+\cdot}$	EI-MS, major frag	CI-MS, m/z $[M + 1]^+$[b]	^1H NMR (acetonitrile-d_3) chem shifts, δ
			Anilines		
2: R_1 = Cl	3.3	141 (75)	Cl (100)	142	2.08 (s, CH_3), 4.05 (NH_2), 6.60 (d, 6-H), 6.92 (dd, 5-H), 7.00 (d, 3-H)
2: R_1 = CH_3	2.3	121 (100)	CH_3 (98)	122	2.05 (s, 2-CH_3), 2.16 (s, 4-CH_3), 3.81 (NH_2), 6.52 (d, 6-H), 6.75 (d, 5-H), 6.81 (s, 3-H)
7: R_1 = R_2 = CH_3	2.4	121 (98)	CH_3 (100)	122	2.18 (s, 2,6-CH_3), 3.78 (NH_2), 6.58 (t, 4-H), 6.92 (d, 3,5-H)
7: R_1 = CH_3, R_2 = C_2H_5	2.9	135 (32)	CH_3 (100)	136	1.31 (t, CH_2CH_3), 2.22 (s, 6-CH_3), 2.58 (q, CH_2CH_3), 3.62 (NH_2), 6.74 (t, 4-H), 7.02 (t, 3,5-H)
7: R_1 = CH_3, R_2 = $C(CH_3)_3$	3.6	163 (31)	CH_3 (100)	164	1.42 [s, $C(CH_3)_3$], 2.15 (s, 2-CH_3), 4.02 (NH_2), 6.59 (t, 4-H), 6.92 and 7.10 (3- and 5-H, d)
7: R_1 = R_2 = C_2H_5	3.4	149 (22)	CH_3 (100)	150	1.18 (t, CH_2CH_3), 2.50 (q, CH_2CH_3), 3.91 (NH_2), 6.58 (t, 4-H), 6.88 (d, 3,5-H)
			Nitrosobenzenes		
4: R_1 = Cl[c]	2.4	155 (75)	NO (100)	156	2.45 (s, CH_3)
4: R_1 = CH_3[c]	2.2	135 (100)	NO (78)	136	2.39 (s, CH_3), 2.51 (s, CH_3)
9: R_1 = R_2 = CH_3	2.1	135 (79)	NO (100)	136	2.43 (s, 2,6-CH_3), 7.18 (d, 3,5-H), 7.30 (t, 4-H)
9: R_1 = CH_3, R_2 = C_2H_5	3.9	149 (60)	CH_3 (100)	150	1.23 (t, CH_2CH_3), 2.40 (s, 6-CH_3), 2.71 (q, CH_2CH_3), 7.25 (d, 3-H), 7.32 (d, 5-H), 7.47 (t, 4-H)
9: R_1 = CH_3, R_2 = $C(CH_3)_3$	3.4	177 (60)	C_5H_{12} (100)	178	1.60 [s, $C(CH_3)_3$], 1.80 (s, CH_3), 7.03 and 7.63 (3- and 5-H, d), 7.42 (t, 4-H)
9: R_1 = R_2 = C_2H_5	4.4	163 (42)	CH_3 (100)	164	1.27 (t, CH_2CH_3), 2.72 (d, CH_2CH_3), 7.33 (d, 3,5-H), 7.49 (t, 4-H)
			Azoxybenzenes		
5: R_1 = Cl	9.9	294 (53)	CH_3 (100)	295	3.31 (s, CH_3), 6.25 (d, 6-H), 7.22 (d, 5-H), 7.69 (s, 3-H)
5: R_1 = CH_3	9.1	254 (43)	CH_3 (79)	255	2.34 (s, 4-CH_3), 3.22 (s, 2-CH_3), 6.21 (d, 6-H), 6.98 (d, 5-H), 7.40 (s, 3-H)

[a] Temperature program (80–200 °C, 20 °C/min), 10-m high-performance methyl silicone capillary column. [b] All compounds give (m/z, relative intensity) $[M + 1]^+$ (100), $[M + 29]^+$ (8–10), and $[M + 41]^+$ (2–5). [c] Compounds not purified so aromatic protons obscured by MCBA and other products.

phosphate pH 7.4 buffer. After incubation for 30 min at 37 °C, cooling, and addition of NaCl (~ 1 g), the mixture was extracted with hexane (1 mL × 2) followed by direct GC–MS–SIM analysis of a 1-μL aliquot.

Mutagenicity Assays. *S. typhimurium* strains TA 97, TA 98, TA 100, and TA 102, provided by B. N. Ames (Department of Biochemistry, University of California, Berkeley), were used for mutagenesis assays by standard procedures (Maron and Ames, 1983) except for a 4-fold increase in the amount of S9 fraction and a 2-fold increase in the amount of salts and cofactors [$MgCl_2$, KCl, glucose 6-phosphate, and nicotinamide adenine dinucleotide phosphate (NADP)]. Controls in one series of experiments involved NADP deletion. Comparison compounds in the TA 100 assay gave 660 revertants/0.1 μg of nitroquinoline *N*-oxide direct and 1400 revertants/5 μg of 2-aminofluorene with the standard S9 level and 500 revertants/5 μg using 4 times the normal amount of S9 fraction.

2,4-Disubstituted azoxybenzenes (**5**, R_1 = Cl or CH_3) were analyzed not only by the standard procedure above without S9 fraction but also by a modified method to detect volatile and unstable mutagens. The bacteria were incubated on the plates (Barber et al., 1983) for 5 h prior to introducing the test compound either directly into the agar as above or via the vapor phase from a source separated from the bacteria. The latter procedure involved turning the assay plate upside down and adding the test compound in acetonitrile (25 μL) onto a sterile filter paper disk (6-mm diameter) in the cover plate. The assay dish was sealed immediately with parafilm and incubated for 48 h. Control plates (25 μL of acetonitrile) showed normal background revertants.

A chemical alternative to the S9 bioactivation procedure was examined by reacting the anilines and their derivatives with MCPBA, quenching the excess bactericidal oxidant, and directly assaying the mutagenicity of the mixture. Compounds **1a**, **1b**, **6a–6e**, and the corresponding anilines or related compounds (0.1 mmol) were treated with purified MCPBA (0.3 mmol) in acetonitrile (1 mL). After 1 h at 4 °C for the anilines or 2 h at 25 °C for the derivatives, the residual peracid was destroyed by adding excess Me_2SO (28 mmol) followed immediately by the mutagenesis assay. NMR revealed that Me_2SO quenching does not affect the oxidation products. This procedure was varied to find the optimal oxidant ratio for maximum mutagenic activity and to analyze the product composition by mixing the aniline (0.4 mmol) and purified MCPBA (0.1–1.2 mmol) in acetonitrile-d_3 (4 mL) at 4 °C. After 1 h at 4 °C one portion of the reaction mixture was examined for mutagenic activity by quenching with Me_2SO and assay as above. The remaining portion was directly examined by NMR and MS to approximate the product composition.

RESULTS

Peracid Oxidations. Acetonitrile-d_3 is the solvent of choice for the MCPBA oxidations for three reasons: the anilines react readily in this solvent and give a less complex product mixture than in chloroform-*d*; there is no interference in the relevant NMR regions; it is not bactericidal and does not affect mutagenesis assays in amounts up to 25 μL. Treatment of the 2,6-dialkylanilines with 0.25–3 mol equiv of MCPBA gives sequentially the hydroxylamino, nitroso, and nitro derivatives, as illustrated with 2,6-dimethylaniline in Figure 3. The hydroxylamines decompose on attempted isolation and were therefore used as a mixture with the parent aniline and nitroso derivatives. Three equivalents of MCPBA are optimal for producing and isolating the more stable nitrosoaryl com-

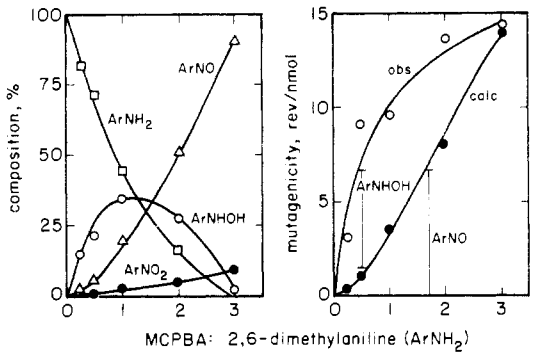

Figure 3. Composition and mutagenicity of reaction mixtures of 2,6-dimethylaniline with various ratios of *m*-chloroperoxybenzoic acid. The "calculated mutagenicity" curve is based on the potency and amount of 2,6-dimethylnitrosobenzene present. Ar = 2,6-dimethylphenyl. The difference between the calculated and observed mutagenicities is attributed to the hydroxylamine.

pounds. In contrast, the 2,4-disubstituted anilines form the hydroxylamino and nitroso derivatives only as transient products in combined yields of ~10% while the major components (~90%) at all MCPBA molar ratios (0.25:1 to 2:1) are the azoxybenzenes (Table I).

Alachlor reacts with equimolar MCPBA within 24 h at 25 °C to give a mixture of >17 products (GC–MS), one of which is proposed to be desmethoxymethyl-alachlor (225, $M^{+\cdot}$).

Detection of Anilines as Pesticide Metabolites and Nitrosobenzenes as Aniline Metabolites. The GC–MS–SIM method is satisfactory for analysis of in vivo and in vitro metabolite mixtures for the anilines and nitrosobenzenes that are characterized by GC–MS–SIM, both EI and CI, and by cochromatography (Table I, Figure 2). No attempt was made to detect other possible metabolites.

The livers of rats treated with the formamidines and chloroacetanilides contain small amounts of administered compound, about 1–5% of the corresponding aniline relative to the administered dose, and usually the nitroso derivative at the borderline sensitivity limit (0.1 ng/μL). Metalaxyl (**6e**) is an exception, giving no detectable aniline or nitroso compound. On direct administration of the aniline the nitroso yields are ~0.1%, an amount easily evident by the GC–MS–SIM procedure.

The microsomal in vitro studies reveal liberation of the corresponding aniline from formamidines **1a** and **1b** and anilide **6d** in up to 10% yield, but the corresponding nitroso derivatives were not obtained in single determinations. Chloroacetanilides **6a–6c** give aniline yields of 1–5%, and in about one-third of the experiments the nitrosobenzene is also evident at near the detection limit of the method. The anilines always give detectable amounts of the corresponding nitrosobenzenes in yields as follows: 1–2% of 2-ethyl-6-methyl-, 2,6-diethyl-, and 2-*tert*-butyl-6-methylnitrosobenzenes with ~5% degradation products and ~30% aniline recovery; 0.1–0.5% of 4-chloro-2-methyl- and 2,4- and 2,6-dimethylnitrosobenzenes with 20–50% aniline recovery.

Mutagenicity of Anilines and Their Derivatives (Table II; Figure 3). The TA 100 strain is more sensitive than the TA 97, TA 98, and TA 102 strains to the 2,6-dialkylnitrosobenzenes (assayed as MCPBA oxidation mixtures) so all subsequent considerations refer to the TA 100 results. Not any of the anilines examined or their derivatives (**1a**, **1b**, **6a–6e**) are detected as direct-acting mutagens, i.e. <0.01 revertant/nmol. On S9 fortification, the 2,4-disubstituted anilines are more potent mutagens than the 2,6-dialkylanilines with activities (revertants/nmol) of 0.5–1.9 and 0.01–0.02, respectively. The only

Table II. Mutagenicity of Disubstituted Anilines, Formamidine Insecticides, Chloroacetanilide Herbicides, and Related Compounds

compd	revertants/nmol[a] S9	revertants/nmol[a] MCPBA[b]
2,4-Disubstituted Anilines and Formamidine Insecticides		
4-chloro-2-methylaniline	1.9[c]	1.4
2,4-dimethylaniline	0.5[c]	9.8
chlordimeform (**1a**)	0.2[c]	0.2
amitraz (**1b**)	<0.01	0.7
2,6-Dimethylaniline and Derivatives		
2,6-dimethylaniline	0.01	14.5[d] (15.6)
lidocaine (**6d**)	<0.01	<0.01
metalaxyl (**6e**)	<0.01	<0.01
2-Ethyl-6-methylaniline and Chloroacetanilide Herbicide		
2-ethyl-6-methylaniline	0.01	6.8[d] (8.9)
metolachlor (**6a**)	<0.01	<0.01
2,6-Diethylaniline and Chloroacetanilide Herbicides		
2,6-diethylaniline	0.02[c]	5.0[d] (5.7)
alachlor (**6b**)	<0.01	<0.01
butachlor (**6c**)	0.05[c,e]	<0.01

[a] Each compound alone (without activation) is not detected as a mutagen (<0.01 revertant/nmol). Comparable values (revertant/nmol) for 2-*tert*-butyl-6-methylaniline are <0.01 with S9 and 1.3 with MCPBA. [b] Reaction mixtures with a 3:1 molar ratio of MCPBA and the aniline. Data in parentheses are for the pure 2,6-dialkylnitrosobenzenes. [c] Mutagenicity <0.01 revertants/nmol on deleting NADP from the S9 mix. [d] Mutagenicity (revertants/nmol) for the MCPBA reaction mixtures with strains TA 97, TA 98 and TA 102, respectively, are as follows: 2,6-dimethylaniline, 3.3, 0.7, and 2.1; 2-ethyl-6-methylaniline, 1.2, 0.4, and 1.0; 2,6-diethylaniline, 1.5, 0.4, and 1.0. [e] Moriya et al. (1983) also report 0.05 revertant/nmol.

derivatized anilines detected as mutagens are **1a** and **6c**, giving 0.2 and 0.05 revertants/nmol, respectively. Each of these activities of the anilines or their derivatives is dependent on NADP(H). These assays used 4 times the normal level of S9 mix, which increases the potency, i.e. at the normal S9 level the potencies of 4-chloro-2-methylaniline, 2,4-dimethylaniline, and **1a** are <0.1 revertant/nmol.

MCPBA at 3 mol equiv activates each of the anilines and formamidines but not the anilides as mutagens. The 2,4-disubstituted compounds, assayed as mixtures with small amounts of nitroso- and hydroxylamino derivatives but predominantly the azoxybenzenes, give 1.4–9.8 revertants/nmol. The mutagenicity of MCPBA oxidation mixtures from 2,6-dimethyl-, 2,6-diethyl-, and 2-ethyl-6-methylanilines are similar to those of the corresponding nitrosobenzenes (5.7–15.6 revertants/nmol). 2-*tert*-Butyl-6-methylaniline gives a less mutagenic oxidation mixture than those derived from the other anilines.

Although not isolated for individual assay, the mutagenicity of the hydroxylamines from oxidation of 2,6-dialkylanilines can be extrapolated from the activity of mixtures of known composition (NMR) in which the only mutagens are the hydroxylamino and nitroso compounds, e.g. 20 revertants/nmol for the hydroxylamine from 2,6-dimethylaniline (Figure 3). Similar experiments and extrapolations with 2,6-diethyl- and 2-ethyl-6-methylanilines establish that the corresponding nitroso- and hydroxylamino compounds are within 3-fold of each other in mutagenic potencies.

Mutagenicities for the individual components in the MCPBA reaction mixtures are not easily interpretable in the case of the 2,4-disubstituted anilines since the nitroso and hydroxylamino compounds are present in low yields and are unstable, the reaction leading instead to large

amounts of azoxybenzenes, which as pure compounds exhibit low activity (<0.1 and 0.9 revertants/nmol for 2,4-dimethyl- and 4-chloro-2-methylazoxybenzenes, respectively). However, they are more active with the preplating assay both directly and by volatilization exposure, i.e. (revertants/nmol) 2,6-dimethylazoxybenzene 1.2 direct and 1.6 by volatilization and 4-chloro-2-methylazoxybenzene 5.4 direct and 3.2 by volatilization.

DISCUSSION

Two formamidine insecticides (**1a** and **1b**) and three chloroacetanilide herbicides (**6a**–**6c**) are oxidized or hydrolyzed to 2-alkylanilines with 4-chloro, 4-methyl, or 6-alkyl substituents. 2,6-Dimethylaniline is also formed on hydrolysis of the local anaesthetic and antiarrythmic drug lidocaine (**6d**) (Nelson et al., 1977). The current study not only confirms formation of the relevant aniline from each compound, both in vivo and in vitro, but also identifies the corresponding nitrosobenzene as a further metabolite of each aniline (Figure 1). Metalaxyl (**6e**) appears to be an exception since no 2,6-dimethylaniline is detected. To facilitate analyses, the formamidines, anilides, and anilines are administered ip at high doses that may not be relevant to low-level exposures. The anilines are consistently and clearly evident in the liver and enzyme systems when administered directly or when formed metabolically from the formamidine or anilide. The nitrosobenzenes are always detected under the same circumstances on metabolism of the anilines but are not consistently evident from the formamidines or anilides, possibly due to the lower levels of anilines available at any given time. Three other features probably contribute to the low apparent yields of nitrosobenzenes: the nitroso compounds are thermally labile and partially decompose during GC; they are analyzed in the presence of a large excess of aniline, e.g. 20-fold, resulting in considerable loss on reaction between these compounds; the phenylhydroxylamines or nitrosobenzenes may react with liver components at a rapid rate relative to that at which they are formed. Hydroxylamines from MCPBA oxidation mixtures are not detected by GC–MS under the conditions used, so even if present in vivo their reactivity and thermal instability would rule against their analysis.

The 2,6-dialkylanilines are readily oxidized with MCPBA to hydroxylamino and nitroso derivatives, only the latter of sufficient stability for easy isolation. The 2,4-disubstituted anilines give only small amounts of hydroxylamino and nitroso derivatives with MCPBA but instead give large amounts of azoxybenzenes. 2,6-Dialkylanilines do not yield azoxybenzenes under these conditions probably due to steric hindrance. Our preliminary observations (NMR, GC–MS) suggest that the isolated azoxybenzenes dissociate in part to the nitrosobenzenes and anilines in dilute acetonitrile solutions.

The formamidines, anilides, and anilines are not detected as direct bacterial mutagens in the *S. typhimurium* TA100 assay. Even on S9 fortification the 2,6-dialkylanilines and the anilide derivatives are of low activity (i.e. <0.01–0.2 revertants/nmol). The 2,4-disubstituted anilines and formamidines are more potent on S9 fortification (0.2–1.9 revertants/nmol) except for amitraz (**1b**) (<0.01 revertants/nmol). The mutagenicity of the 2,6-dialkylanilines on MCPBA oxidation and probably on S9 activation is attributable to the hydroxylamino and nitroso derivatives as direct-acting mutagens. Similar relationships may apply to the 2,4-disubstituted anilines although the evidence is less direct because of the ease of azoxybenzene formation. The MCPBA-activated mutagenicity of the formamidines probably results from formation of the corresponding nitrosobenzenes, based on analogy with peracid treatment of *N*-(benzylidene)methylamine yielding nitrosomethane (Taylor et al., 1976).

The anilines considered here vary over a large range in their acute, subacute, and chronic toxicity and in the types of lesions produced (Short et al., 1983; Weisburger et al., 1978). Nitroso and hydroxylamino metabolites undoubtedly contribute to their toxicological profiles and that of pesticides and drugs derived from these anilines.

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Registry No. **1a**, 6164-98-3; **1b**, 33089-61-1; **2a**, 95-69-2; **2b**, 95-68-1; **4a**, 65063-95-8; **4b**, 38974-06-0; **5a**, 99766-49-1; **5b**, 99766-50-4; **6a**, 51218-45-2; **6b**, 15972-60-8; **6c**, 23184-66-9; **6d**, 137-58-6; **6e**, 57837-19-1; **7** (R_1 = *t*-Bu, R_2 = Me), 13117-94-7; **7** (R_1 = R_2 = Me), 87-62-7; **7** (R_1 = R_2 = Et), 579-66-8; **9** (R_1 = Et, R_2 = Me), 99766-46-8; **9** (R_1 = R_2 = Me), 19519-71-2; **9** (R_1 = R_2 = Et), 99766-47-9; **9** (R_1 = Bu, R_2 = Me), 99766-48-0; mixed-function oxidase, 9040-60-2.

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