Quantitative Assessment of the Mutagenic Potential of Environmental Degradative Products of Alachlor

Daniel M. Tessier* and J. Marshall Clark

Department of Entomology, University of Massachusetts, Amherst, Massachusetts 01003-2410

The *Salmonella*/microsome assay and the micronucleus test were employed to evaluate the chronic toxicity potential of environmental degradative products of alachlor. Degradative products include 2-hydroxy-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide; 2-chloro-2′,6′-diethylacetanilide; 2,6-diethylacetanilide; 2,6-diethylaniline; 2,6-diethyl-*N*-(methoxymethyl)aniline; 2-hydroxy-2′,6′-diethylacetanilide; and 2,6-diethyl-*N*-(methoxymethyl)acetanilide. 2-Hydroxy-2′,6′-diethylacetanilide and 2-chloro-2′,6′-diethylacetanilide were weakly mutagenic to *Salmonella* strain TA100, with reversion rates of 54.6 and 58.4 His^+ revertants/μmol, respectively. Reversion was dependent on bioactivation with phenobarbitol-induced microsomes. Addition of the reduced form of glutathione to the assay system resulted in decreased mutagenicity of 2-hydroxy-2′,6′-diethylacetanilide, suggesting that these products may act as toxic electrophiles that bind directly to DNA/RNA. None of the environmental degradative products tested acted as clastogens in the micronucleus test, even at near-lethal doses (ca. 500 μg/g). Only 2-hydroxy-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide cross-reacts (ca. 40%) with commercially available alachlor immunoassay test kits. The two mutagenic compounds did not cross-react.

Keywords: *Alachlor; metabolites; mutagens; Salmonella/microsome assay; immunoassay*

INTRODUCTION

Herbicides as a group account for approximately 65% of all pesticide use worldwide (Stevens and Summer, 1991). The herbicide alachlor [2-chloro-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide, Figure 1] is counted among the most used pesticides in the world. It is a selective, pre-emergent, herbicide of the chloroacetanilide family of compounds, used for annual grass and broadleaf weed control on soybean, corn, peanut, and other crops. Annual production is on the order of 130−150 million pounds, with some 95 million pounds used yearly in the United States alone (Environmental Protection Agency, 1987; Sun, 1986).

Ground water contamination by alachlor has been reported in at least 16 states, generally at low parts-per-billion (ppb) levels (Cohen et al., 1986; Parsons and Witt, 1989). Levels as high as 65 ppb, however, were reported in Ohio (Richards and Baker, 1993). Various ground water surveys have concluded that the frequency of private well contamination in alachlor-use areas is in the range of 0.06−4.9% (Natarajan and Rajagopal, 1993; Holden and Graham, 1992). Recent studies demonstrate that nonpoint source inputs are the most significant routes of contamination of ground and surface waters by herbicides in the central United States. Contaminated ground water also significantly contributes to surface water contamination (Pereira and Hostettler, 1993; Pereira et al., 1992; Pereira and Rostad, 1990; Thurman et al., 1992). It is estimated that the Mississippi River annually transports 18 tons of alachlor to the Gulf of Mexico, as just one component of a complex mixture of agrochemicals (Pereira et al., 1993).

The biotic, primarily microbial, degradation of alachlor results in a variety of aromatic amine and acetanilide compounds that have also been detected in ground and surface waters (Figure 1; Pereira and Hostettler, 1993; Pereira et al., 1990, 1992; Aizawa, 1982; Tiedje and Hagedorn, 1975). Chemical oxidative processes play a minimal role, and mineralization does not occur (Novick et al., 1986). Therefore, some of these degradative

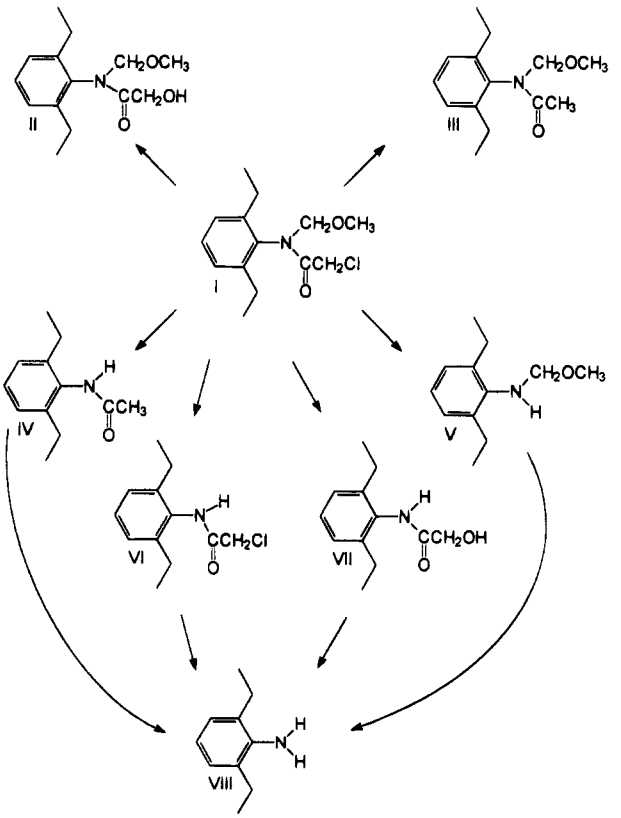

Figure 1. Environmental degradation of alachlor. **I**, alachlor; **II**, 2-hydroxy-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide; **III**, 2,6-diethyl-*N*-(methoxymethyl)acetanilide; **IV**, 2,6-diethylacetanilide; **V**, 2,6-diethyl-*N*-(methoxymethyl)aniline; **VI**, 2-chloro-2′,6′-diethylacetanilide; **VII**, 2-hydroxy-2′,6′-diethylacetanilide; **VIII**, 2,6-diethylaniline.

* Author to whom correspondence should be addressed [fax (413) 545-2115; e-mail tessier@ent.umass.edu].

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products may exist as long-term or terminal residues, and it is reasonable to assume that substantial quantities of these residues continue to accumulate in the environment. Nonetheless, there is a paucity of data regarding the relative incidence, rate of formation, stability, movement, and ultimate fate of these degradative products in the environment. It is generally accepted that degradation eventually terminates at 2,6-diethylaniline. This compound has been proven to result from the metabolic degradation of alachlor by soil fungi (Tiedje and Hagedorn, 1975) and aquatic insect larvae (Wei and Vossbrinck, 1992). It is also a product of mammalian metabolism of alachlor (Sharp, 1988). Seo et al. (1993) have shown that aromatic amines in general are activated to mutagenicity in plant cells. However, the toxicological profiles of the environmental degradative products of alachlor have not been established.

The acute toxicity of alachlor is relatively low (LD_{50} = 930 mg/kg, rat, oral). However, it has been classified by the U.S. EPA as a class B2 or "probable human" carcinogen, on the basis of long-term animal studies. To date, no mammalian metabolites of alachlor have been linked to its carcinogenicity, in spite of the fact that alchlor is readily metabolized. Kimmel et al. (1986) suggested that the direct-acting mutagen 2,6-diethylnitrosobenzene was an ultimate metabolite, although this conclusion was disputed by Feng and Wratten (1987). Brown et al. (1988) showed evidence consistent with DNA adduct formation by alachlor metabolites, but adducts were not isolated.

The evidence of alachlor's mobility in the environment, coupled with its oncogenicity, led to its banning in Canada in 1985 (Hoberg, 1990). In the United States and other countries, alachlor is still being used in enormous quantities as a "restricted-use pesticide". We undertook this research with the intention of gaining a better understanding of the toxicological implications of the environmental degradation of alachlor. Our approach was to perform a preliminary toxicity screen of alachlor's environmental degradative products, to determine which should be the focus of further study. At issue is the chronic exposure of humans, and aquatic and soil-dwelling organisms, to the parent compound and its degradative products through ground and surface water and soil contamination.

Chronic toxicity screening was accomplished through the *Salmonella*/microsome assay and the micronucleus test. The *Salmonella*/microsome assay determines the ability of a chemical to interact with DNA in a site-specific manner (Maron and Ames, 1983). The micronucleus test identifies the ability of a chemical to cause gross structural changes to entire chromosomes (Jenssen and Ramel, 1980). Therefore, as a general scheme for initial chronic toxicity testing, these two tests provide a reliable primary approach for assessing the genotoxic potential of environmental pollutants (Ramel, 1978).

At the present time, immunochemical technology (i.e., immunoassay) is being used for sensitive and cost-effective screening of environmental samples for the presence of alachlor (Feng et al., 1990). Commercially available immunoassays for the detection of alachlor show no cross-reactivity to the other chloroacetanilide herbicides, including acetochlor, butachlor, and metolachlor, or to triazine herbicides. There is also no cross-reactivity to the sodium oxanilate, sulfinylacetic acid, and sulfonic acid soil metabolites of alachlor. When residue levels of alachlor detected by both immunoassay and gas chromatography are compared, however, results tend to be positively biased toward the immunoassay value (Agri-Diagnostics, 1991; Feng et al., 1990). The ethanesulfonate, mercapturate, and methyl sulfide metabolites of alachlor have been reported to cross-react with commercial alachlor kits (Baker et al., 1993; Agri-Diagnostics, 1991). The degradative products of alachlor under discussion here have been detected in the environment by traditional chromatographic procedures. They have not, however, been evaluated for cross-reactivity to any of the several commercially available immunoassay kits used in the environmental detection of alachlor. We, therefore, further evaluated three commercial kits (Agri-Diagnostics, Ohmicron, and Millipore) for cross-reactivity to the toxicologically significant degradative products of alachlor, based on our initial chronic toxicity screening.

MATERIALS AND METHODS

Chemicals and Reagents. The following environmental degradative products of alachlor were synthesized and authenticated at the Gas Chromatography/Mass Spectrometry Facility, College of Food and Natural Resources, University of Massachusetts at Amherst: 2-hydroxy-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide (**II**, >99% purity); 2,6-diethyl-*N*-(methoxymethyl)acetanilide (**III**, >99%); 2,6-diethylacetanilide (**IV**, >99%); 2,6-diethyl-*N*-(methoxymethyl)aniline (**V**, >99%); 2-chloro-2′,6′-diethylacetanilide (**VI**, >99%); 2-hydroxy-2′,6′-diethylacetanilide (**VII**, >99%, Figure 1); and 2,6-diethylnitrosobenzene. Alachlor (Figure 1) was obtained from Monsanto Agricultural Co. (St. Louis, MO). 2,6-Diethylaniline (**VIII**, 98%, Figure 1), phenobarbitol, NADP, glucose 6-phosphate, glutathione (GSH), glutathion *S*-transferase (GST, EC 2.5.1.18), and dimethylbenzanthracene were purchased from Sigma Chemical Co. (St. Louis, MO). Oxoid Nutrient 2 was purchased from Oxoid USA (Columbia, MD). Bactoagar was purchased from Difco Laboratories (Detroit, MI). Other reagents and materials were obtained from common suppliers.

Bacterial Tester Strains. *Salmonella typhimurium* strains TA98 and TA100 were provided by Prof. Bruce Ames, Ph.D., Department of Biochemistry, University of California at Berkeley.

Animals. Long-Evans rats and CD-1 mice were obtained from Charles River Laboratories (Wilmington, MA), and were maintained by the Animal Care Office, University of Massachusetts at Amherst. The animals were housed in plastic tub cages and received tap water and Purina Rodent Chow *ad libitum*.

Commercial Immunoassay Kits. The Alachlor 2.0 kit was a gift of Agri-Diagnostics, Morristown, NJ. The RaPID Assay Alachlor kit was a gift of Ohmicron, Newtown, PA. The Enviroguard Alachlor Assay was purchased from Millipore Corp., Milford, MA.

Preparation of Rat Liver Microsomes. Male rats, 100–200 g, were metabolically induced by phenobarbitol exposure following the method of Marshall and McLean (1969). Briefly, their sole source of drinking water was a 0.1% solution of phenobarbitol in tap water. Rats received this oral administration for 7 days prior to sacrifice. The 100000*g* microsomal pellet from excised livers was used in subsequent *in vitro* and *Salmonella* assays. The cytochrome P_{450} content of the microsomal pellet was assayed using the method of Omura and Sato (1964). The protein content of the microsomal pellet was determined using the bicichoninic acid method with bovine serum albumin as a standard (Smith et al., 1985). Microsomal pellet fractions (ca. 33.3 mg/mL of protein) were divided into 1.0 mL cryovials, quick-frozen in dry ice, and stored at −80 °C. Subsequent determination of cytochrome P_{450} levels after storage indicated no significant reduction, even after several months.

***In Vitro* Metabolism with Phenobarbitol-Induced Rat Liver Microsomes.** In a final volume of 1.0 mL, 10 μL of

test compound in ethanol was incubated with either 10% or 25% (v/v) microsomal fraction in pH 7.4 phosphate buffer, in the presence of cofactors as described by Maron and Ames (1983) (4 mM NADP, 5 mM glucose 6-phosphate, 8 mM $MgCl_2$, 33 mM KCl), at 37 °C for 60 min. Negative control incubations were conducted as follows: (1) in the absence of NADP; (2) with heat-denatured (i.e., 100 °C/min) microsomes; (3) with CO-inactivated microsomes. The proteins were precipitated with ice-cold methanol and the sample filtered via 0.45 μm filters (Millipore) for analysis by reversed-phase high-pressure liquid chromatography (RP-HPLC). The system consisted of a Waters 6000 high-pressure pump and a 4.6 × 250 mm Dupont Zorbax C_{18} column at ambient temperature. The mobile phase was 50% acetonitrile in water flowing at 1.0 mL/min. Detection was by UV at 254 nm. Injection volume was 10 μL.

***Salmonella*/Microsome Assay.** The plate incorporation test format, with microsomal preincubation as described by Maron and Ames (1983), was used for all assays. The histidine requirement was confirmed in each assay, and complete genotype confirmation (i.e., histidine requirement, *rfa* mutation, *uvrB* mutation, R factor, *pAQ1* plasmid) was performed every seven to eight assays.

Each assay consisted of five doses of the test compound (0, 1, 10, 100, 1000 μg/plate; Maron and Ames, 1983; Weinstein and Lewinson, 1978; Zeiger, 1985; Clesceri et al., 1989) plus positive controls tested at four concentrations of microsomal preparation (0%, 4%, 10%, 20% v/v). The nonactivation-dependent positive control compound was 2,6-diethylnitrosobenzene. Once the mutagenicity of 2-chloro-2′,6′-diethylacetanilide was established, it was used as an activation-dependent positive control in subsequent assays. The untreated (i.e., zero dose) control consisted of test compound solvent (i.e., dimethyl sulfoxide or ethanol). The control treatment in the absence of microsomes consisted of 0.1 M phosphate buffer and cofactors. Each dose and microsome concentration combination was run in duplicate for initial screening. Positive results were repeated in triplicate. The criteria for mutagenicity adopted in the present work is the "modified twofold rule". For borderline data, these criteria assume positive results only if an increase over background reversion is shown for at least two concentrations and if at least one concentration results in a doubling over the background rate (Williams and Preston, 1983).

Glutathione S-Transferase (GST) Activity. The activity of GST (EC 2.5.1.18, Sigma) in the microsomal preparation was verified before the *Salmonella* assay was modified by addition of this enzyme to the microsomal preparation. In 1.0 mL of a 4% (v/v) microsomal preparation, the conjugation of 2-chlorodinitrobenzene (CDNB, 1 mM) to reduced gluthatione (GSH, 2.5 mM) by GST (1 unit) was monitored in a Shimadzu double-beam scanning spectrophotometer at 340 nm for 5 min (Habig, 1974). Three experimental controls were included: (1) no CDNB (only EtOH); (2) no GSH; (3) no GST.

Modification of *Salmonella*/Microsome Assay with Glutathione/Glutathione S-Transferase. GSH (final concentration 25 mM) and GST (2 units/plate) were added to a 20% (v/v) microsomal preparation. This amended microsomal preparation was then used in a *Salmonella*/microsome assay of 1000 μg/plate of 2-hydroxy-2′,6′-diethylacetanilide with strain TA100. Similar plates, with either no GST or heat-inactivated GST, were used as controls. GST was inactivated by boiling for 60 s. Plates were run in triplicate.

GSH in the absence of exogenous GST was also incorporated into assays to determine the ability of endogenous microsomal GST to attenuate the mutagenicity of 2-hydroxy-2′,6′-diethylacetanilide. GSH was added to a 20% (v/v) microsomal preparation with mutagen (1000 μg/plate) to give final concentrations of 10, 25, 50, and 100 mM. The *Salmonella*/microsome assay was then run according to standard procedures.

Micronucleus Test. The method for the micronucleus test employing acridine orange staining is described in Hyashi (1990) and CSGMT JEMS * MMS (1992). The only modification was the use of mixed sexes of mice instead of only males. Briefly, mice, 25–35 g, were injected intraperitoneally with test compound dissolved in DMSO. Peripheral blood was obtained via tail snips at 0, 24, 48, and 72 h. Samples were smeared on acridine orange-coated slides and examined by fluorescence microscopy. One thousand cells were counted from each slide preparation, with counting restricted to phase I, II, and III reticulocytes (RET). Micronuclei frequencies were calculated as (MNRET/RET) × 100, where MNRET is the number of micronucleated reticulocytes per 1000 reticulocytes (RET) (Von Ledebur and Schmid, 1973).

Experiments were conducted in triplicate for dose groups of 50, 100, and 500 μg/g of body weight. 2,6-Diethylnitrosobenzene was tested at 10, 50, and 100 μg/g of body weight because of solubility limitations. Concentrations were adjusted to limit DMSO to 5 μL/g of body weight. A known clastogen, dimethylbenzanthracene (DMBA; Salamone et al., 1980), was used as the positive control at 80 μg/g of body weight.

Cross-Reactivity of Commercially Available Immunoasssay Kits. The cross-reactivities of three alachlor immunoassays were determined for 10, 100, and 1000 μg/L aqueous solutions of five degradation products of alachlor using standard directions and formats accompanying each immunoassay kit. Samples were run in triplicate. The Ohmicron assay was read on an RPA-1 photometric analyzer (Ohmicron). Both Agri-Diagnostics and Millipore assays were read with a UVmax microplate reader (Molecular Devices, Menlo Park, CA). The concentration required to attain 50% inhibition of antibody–antigenic standard binding in the competitive assay (i.e., I_{50}), was determined from a standard curve of concentration (0.1, 1.0, 5.0 μg/L) versus absorbance. Quantitation was through an enzymatic color reaction (i.e., horseradish peroxidase). The I_{50}, least detectable dose (LDD), and percent cross-reactivity (%*R*) were calculated as follows (Weiler et al., 1986): The absorbance, measured at 450 nm, giving 50% inhibition (A_{50}) was calculated as

$$A_{50} = [0.5(\mathrm{Abs}_{max} - \mathrm{Abs}_{min})] + \mathrm{Abs}_{min}$$

where Abs_{max} and Abs_{min} were absorbance readings for the high and zero concentration standards, respectively. The corresponding I_{50} was then read directly from the plot. The LDD was calculated as the concentration resulting in at least 10% inhibition (i.e. $\mathrm{Abs}_{sample}/\mathrm{Abs}_{zero\ std} > 90\%$). The percent cross reactivity (% *R*) was calculated as

$$\%\ R = (I_{50\ \mathrm{alachlor}}/I_{50\ \mathrm{sample}}) \times 100$$

RESULTS AND DISCUSSION

***Salmonella* Assay: Bioactivation System.** The degradation of alachlor occurs principally around the nitrogen moiety (Sharp, 1988; Aizawa, 1982). The aromatic portion of the molecule is more recalcitrant to further degradation. It follows, therefore, that bioactivation of alachlor would also likely occur around the nitrogen moiety. The hepatic microsomal enzyme system used in the *Salmonella* assay consists primarily of cytochrome P_{450}-based polysubstrate monooxygenases (PSMOs). This extensive class of xenobiotic biotransforming enzymes, of which more than 20 human examples have been characterized, exhibits a range of oxidative activities to various molecular structures (Guengerich and Shimada, 1991). Because phenobarbitol is a known inducer of cytochrome P_{450} PSMOs active at nitrogen moieties (Orrenius and Ernster, 1964), this treatment was expected to sensitize the *Salmonella* assay to any possible oxidative metabolites or degradative products of alachlor at the nitrogen moiety.

At the time of sacrifice, phenobarbitol-induced rats weighed 100–200 g and had ingested an average 143 ± 25 mg kg^{-1} day^{-1} of phenobarbitol. Liver weight

Table 1. Phenobarbitol Induction of Rat Liver Enzymes[a]

	phenobarbitol intake[b]	% liver wt[c]	protein concn (mg/g)	$CytP_{450}$ (nmol/g)
control	0	5.6 ± 0.5	18.2 ± 2.9	13.4 ± 5.3
induced	143 ± 25	9.0 ± 2.7	33.3 ± 5.7	39.6 ± 16.9

[a] Average ± SD, $n = 4$ for controls and $n = 10$ for induced rats. [b] Milligrams per kilogram per day, av ± SD, $n = 9$ rats. [c] Percent of total body weight.

Table 2. Authentication of *in Vitro* Metabolism of Aromatic Amine Substrates by Phenobarbitol-Induced Microsomes

substrate standard[a]	active microsomes 25% (v/v)	inactive microsomes heat	inactive microsomes CO	inactive microsomes −NADP
2,6-DEA				
19.8 (100)	19.7 (31)[b]	19.7 (69)	19.7 (69)	19.8 (67)
	7.7 (22)			
	5.7 (1)			
	4.8 (27)			
2-NA				
7.5 (100)	7.6 (72)	7.5 (84)	7.5 (69)	7.4 (92)
	4.2 (15)			
2-ANAP				
9.9 (100)	10.0 (21)	10.1 (63)	10.1 (103)	10.1 (69)
	8.9 (2)		8.6 (0.8)	
	7.3 (0.1)			7.3 (2)
	7.0 (0.1)			7.0 (7)
	4.9 (2)		4.9 (0.5)	

[a] 2,6-DEA, 2,6-diethylaniline; 2-NA, 2-nitroaniline; 2-ANAP, 2-aminonaphthaline. [b] RP-HPLC retention times in minutes (percent peak area relative to substrate standard).

increased by 38%, protein content by 45%, and cytochrome P_{450} content by 66% over contols (Table 1).

The activity of the phenobarbitol-induced microsomal preparation was tested *in vitro* for N-oxidizing or other arylamine-directed reactions before being included in the *Salmonella* assays. In addition to 2,6-diethylaniline, two known promutagens, 2-nitroaniline and 2-aminonaphthalene, were included in microsomal incubations. Both promutagens require N-oxidation for mutagenicity (Hodgson and Gurhie, 1980). 2-Nitroaniline was chosen to represent a more water-soluble compound and 2-aminonaphthalene a more lipid-soluble compound. Since the microsomal PSMOs are membrane bound, it was reasoned that any differences in their abilities to metabolize 2-nitroaniline and 2-aminonaphthalene would indicate the relative importance of partitioning into the lipid matrix. Incubations included phenobarbitol-induced microsomal preparations and appropriate cofactors as previously described.

Table 2 presents the RP-HPLC analysis of the incubates. Since the metabolites would not be expected to have the same molar extinction coefficients as the parent compound, the response factors at the detection wavelength (254 nm) are different. The area percents, therefore, should be viewed as qualitative values only. The results of these experiments confirm that a significant amount of oxidative metabolism was occurring in the phenobarbitol-induced microsomal preparations. Retention times for all metabolite peaks were less than that of the parent compound. For the RP-HPLC system being used, this reduction in retention time indicated that they were more polar than the parent compound. Incubations in 10% microsomal preparation also resulted in relatively more of the parent compound being detected (data not shown). Heat-inactivated microsomes produced no detectable metabolites. CO-treated incubations and incubations carried out in the absence of NADPH both resulted in more parent compound being detected, but some metabolism of 2-aminonaphthalene was detected. However, together these results corroborate an enzymatic, rather than chemical, degradation of these N-containing compounds.

There were more metabolites formed when 2-aminonaphthalene, the more lipophilic substrate, was used than when 2-nitroaniline, the more hydrophilic substrate, was used. This demonstrates the importance of substrate partitioning into the microsomal lipid matrix, allowing metabolism by the membrane-bound PSMOs to occur. The lipophilicity of the environmental degradative products of alachlor may likewise determine the extent of their bioactivation by PSMOs.

Finally, some expected metabolites were not detected in our *in vitro* assay (e.g., dinitrobenzene from 2-nitroaniline). These compounds have been identified as mammalian bioactivation products in *in vivo* assays (Hodgson and Gurhie, 1980). The fact that they were not detected may reflect a limitation of the isolated *in vitro* system used above. Nevertheless, the above experiments confirm that our *in vitro* microsomal preparation was induced by phenobarbitol and that enzymatic N-oxidative metabolism was active under our experimental conditions.

***Salmonella* Assay: Mutagenicity Determination.** Alachlor and five environmental degradation products of alachlor (**II**, **IV**, **VI**, **VII**, **VIII**; Figure 1), all of which have been identified in ground water, were evaluated for mutagenicity with the *Salmonella*/microsome assay. 2,6-Diethylnitrosobenzene, the putative oxidative product of 2,6-diethylaniline, served as a direct-acting mutagenic positive contol (Kimmel et al., 1986). Background reversion for TA100 was consistently in the range of 95−150 colonies/plate. This is in agreement with published values of 46−184 colonies/plate (Maron and Ames, 1983). Background reversion for TA98 was 20−36 colonies/plate, with published values of 5−63 colonies/plate (Maron and Ames, 1983).

Two environmental degradation products of alachlor, 2-chloro-2′,6′-diethylacetanilide (**VI**) and 2-hydroxy-2′,6′-diethylacetanilide (**VII**), were mutagenic to strain TA100 (Figure 2). 2-Chloro-2′,6′-diethylacetanilide produced a statistically significant ($p < 0.05$, t-test) and toxicologically relevant (e.g., modified twofold rule) increase in the reversion of TA100 over controls at 100, 1000, and 5000 μg/plate (top panel, Figure 2). The maximal reversion rate was 58.4 revertants /μmol when incubation was carried out in a 20% (v/v) microsomal preparation. Linear regression analysis confirmed a dose−response relationship, over three concentration ranges (i.e., 100, 1000, and 5000 μg/plate), for the 10% ($r^2 = 0.85$) and the 20% ($r^2 = 0.96$) microsomal preparations. The reversion rates occurring at doses of 1000 and 5000 μg/plate, with 0% and 4% microsomal preparations, were statistically significant ($p < 0.05$, t-test) over control values. There was no difference in reversion rates between the 0% and 4% microsomal preparation concentrations. Reversion rates increased, however, as the concentrations of microsomal preparation were increased to 10% and 20% (v/v). Therefore, reversion was enhanced by, but not dependent on, microsomal activation. Because of its close structural relationship to the other compounds under consideration, a 1000 μg/plate dose of 2-chloro-2′,6′-diethylacetanilide was used as the activation-dependent control in subsequent assays. Differences in reversion rates between the 0% and 20% microsomal concentrations were moderate (1.14-

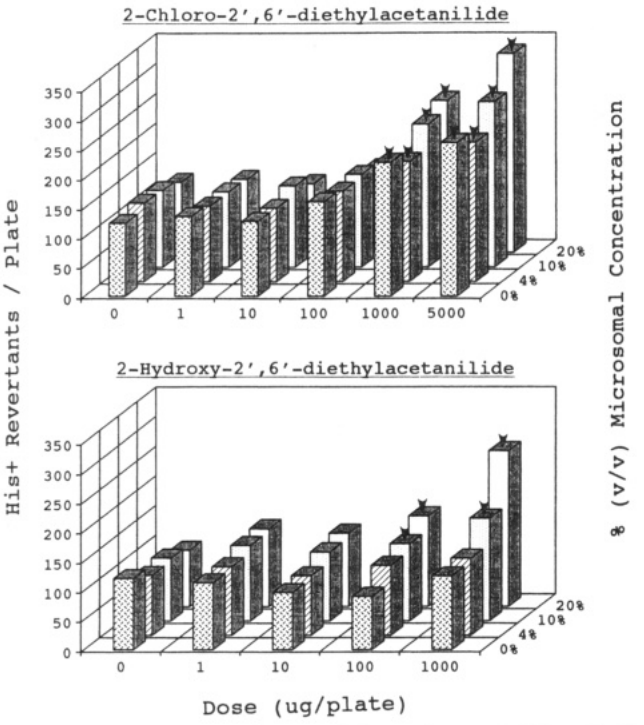

Figure 2. Reversion of strain TA100 due to 2-chloro-2′,6′-diethylacetanilide (top) and 2-hydroxy-2′,6′-diethylacetanilide (bottom) at 0%, 4%, 10%, and 20% (v/v) microsomal preparation. Arrowheads indicate values statistically significant from controls (t-test, $p < 0.05$, $n = 5$).

fold) but reproducible and statistically significant ($p < 0.05$, t-test).

Although 2-chloro-2′,6′-diethylacetanilide was very soluble in the DMSO carrier, some precipitation was observed at the 1000 and 5000 μg levels when added to the aqueous culture/microsome mix. Upon addition of the 45 °C top agar, the precipitate redissolved at the 1000 μg level and partially redissolved at the 5000 μg level. This solubility limitation may indicate that the reversion rates reported are low estimates because cellular absorption of compound may have been limited due to precipitation. Alternatively, the precipitation may have resulted in some bacteria being exposed to lethal concentrated doses. This also would decrease the reversion rate by decreasing the number of living bacteria able to undergo a mutation event.

2-Hydroxy-2′,6′-diethylacetanilide also produced a statistically significant ($p < 0.05$, t-test) and toxicologically relevant (e.g., modified twofold rule) increase in the reversion of TA100 over controls at 100 and 1000 μg/plate (bottom panel, Figure 2). The maximal reversion rate was 54.6 revertants/μmol when the incubations were carried out in 20% (v/v) microsomal preparation. A dose–response relationship was observed over two concentration ranges (100 and 1000 μg/plate) for plates incorporating 10% and 20% microsomal preparation. The reversion rate for the 20% microsomal concentration (i.e., 54.6 revertants/μmol) was higher than for the 10% concentration (i.e., 36.0 revertants/μmol). Reversion rates at 0% and 4% microsomal concentrations were not statistically different from control values. The mutagenicity of this compound is therefore dependent on microsomal bioactivation.

2,6-Diethylacetanilide also reverted TA100. However, reversion was statistically significant from controls ($p < 0.05$, t-test) only at one of the tested doses (i.e., 1000 μg/plate) and at only the highest microsomal concentration (20% v/v, data not shown). These data, therefore, do not meet the modified twofold rule. It is mentioned, however, because of the relation to 2-hydroxy-2′,6′-diethylacetanilide and 2-chloro-2′,6′-diethylacetanilide. All three degradative products are secondary acetanilides, and this structure–activity relationship may shed light on a common mechanism of mutagenicity. The overall level of mutagenicity increases in the order 2,6-diethylacetanilide < 2-hydroxy-2′,6′-diethylacetanilide < 2-chloro-2′,6′-diethylacetanilide. The strength of the acetyl moieties as leaving groups follows in the same order, namely −H (2,6-diethylacetanilide) ≪ −OH (2-hydroxy-2′,6′-diethylacetanilide) < −Cl (2-chloro-2′,6′-diethylacetanilide). If these moieties are removed by homolytic cleavage, electrophilic species are produced. Electrophiles in turn interact with nucleophilic macromolecules, most notably DNA and RNA, resulting in alkylated or otherwise structurally altered forms of these macromolecules (Williams and Weisburger, 1986). The mechanism of mutagenicity of these compounds as toxic electrophiles is thus implicated in this structure–activity relationship.

To substantiate the role of toxic electrophiles in the mutagenic potential of alachlor, we designed a series of experiments involving glutathione *S*-tranferases (GSTs). GSTs are a class of detoxifying enzymes with a wide range of activity (Jakoby, 1978). One important function of GST is the conjugation of electrophilic toxins with the reduced form of glutathione (GSH), resulting in polar and excretable products. If the two mutagenic degradation products of alachlor are indeed acting as toxic electrophiles, then the addition of a GST/GSH conjugating system to the *Salmonella* assay should attenuate their mutagenicity.

Initial experiments were designed to determine that exogenous GST was active when added to the microsomal preparation used in the *Salmonella*/microsome assay (Tessier, 1994). The conjugation of 2-chlorodinitrobenzene (CDNB), a known GST substrate, was spectrophotometrically determined. These determinations indicated that the microsomal preparation contained measurable *endogenous* GST. Control experiments were run in the absence of both exogenous GST and exogenous GSH. Results of these experiments verified that spontaneous hydrolysis of CDNB was not occurring (i.e., the conjugation was truly occurring by the action of the endogenous GST). If so, simply adding GSH to the standard assay should inhibit mutagenicity (i.e., the mutagens would be activated by the microsomal PSMOs to electrophilic species but then be conjugated by microsomal GST to exogenous GSH before they could cause mutations). GSH is water soluble and therefore is not an endogenous component of the microsomal preparation. Because of this, GST does not normally influence microsomal activation of promutagens in the assay.

When GSH was added to a standard *Salmonella*/microsome assay of 1000 μg/plate of 2-hydroxy-2′,6′-diethylacetanilide using strain TA100, the mutagenic potential of this compound was attenuated in a dose-dependent manner (Figure 3). The addition of exogenous GST (ca. 2 units/plate) to microsomes fortified with 25 mM GSH did not greatly affect the inhibition rate compared to that of nonfortified microsomes. This may be due to a greater titer of the endogenous GST enzyme or a lack of substrate specificity of the exogenous enzyme.

Strain TA100 detects base-pair substitutions (Maron

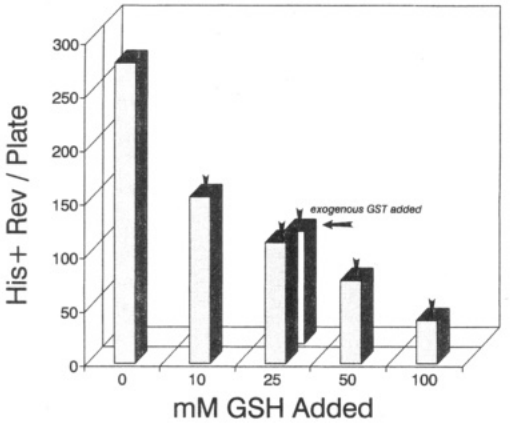

Figure 3. Effect of glutathione addition on the reversion of strain TA100, due to 1000 μg/plate 2-hydroxy-2′,6′-diethylacetanilide, with 20% microsomal preparation. Arrowheads indicate values statistically different from 0 mM GSH control (*t*-test, $p < 0.05$).

Table 3. Reversion of Strain TA98 Due to 2-Chloro-2′,6′-diethylacetanilide, 2-Hydroxy-2′,6′-diethylacetanilide, and 2,6-Diethylacetanilide

	no. of TA98 His$^+$ revertants ± SD microsomal preparation[a]			
dose (μg/plate)	0%	4%	10%	20%
2-Chloro-2′,6′-diethylacetanilide				
1000	31 ± 2	31 ± 1	29 ± 2	nt[b]
100	29 ± 2	33 ± 4	29 ± 2	nt
10	27 ± 3	28 ± 2	33 ± 1	nt
1	33 ± 3	25 ± 4	32 ± 1	nt
0	35 ± 2	34 ± 2	22 ± 3	nt
2-Hydroxy-2′,6′-diethylacetanilide				
1000	26 ± 1	24 ± 4	25 ± 1	24 ± 4
100	29 ± 4	30 ± 2	16 ± 3	27 ± 4
10	27 ± 2	24 ± 8	31 ± 5	18 ± 0
1	28 ± 0	25 ± 5	32 ± 2	27 ± 2
0	28 ± 7	27 ± 4	25 ± 4	26 ± 2
2,6-Diethylacetanilide				
1000	15 ± 3	18 ± 3	25 ± 2	20 ± 5
100	17 ± 4	23 ± 0	27 ± 5	23 ± 2
10	22 ± 0	20 ± 1	18 ± 3	29 ± 6
1	19 ± 3	26 ± 4	28 ± 1	22 ± 4
0	19 ± 2	19 ± 2	26 ± 4	18 ± 3

[a] Percent (v/v) microsomal preparation. [b] nt, not tested.

and Ames, 1983). In view of this, we postulate that 2,6-diethylacetanilide, 2-chloro-2′,6′-diethylacetanilide, and 2-hydroxy-2′,6′-diethylacetanilide covalently bind to DNA and prevent proper base pairing at the mutation site. Brown et al. (1988) also presented data consistent with DNA adduct formation due to 2-chloro-2′,6′-diethylacetanilide, in both *in vitro* and *in vivo* systems. Interestingly, these compounds were not mutagenic to strain TA98 (Table 3). This strain detects frameshift mutagens, which are typically planar, polycyclic molecules that bind to DNA base pairs. The binding stabilizes deformations in the DNA molecule and leads to mutation (Maron and Ames, 1983). The lack of response of strain TA98 to the compounds in question is in keeping with their nonplanar, monocyclic structure and corroborates the contention that these mutagens act by causing base-pair substitutions.

2,6-Diethylnitrosobenzene was strongly mutagenic to both the TA98 and TA100 strains in the presence and absence of activating microsomes (Figure 4). The maximal reversion rate for strain TA100 was 2688 revertants/μmol with activation and 1915 revertants/μmol without. At low doses, higher reversion rates

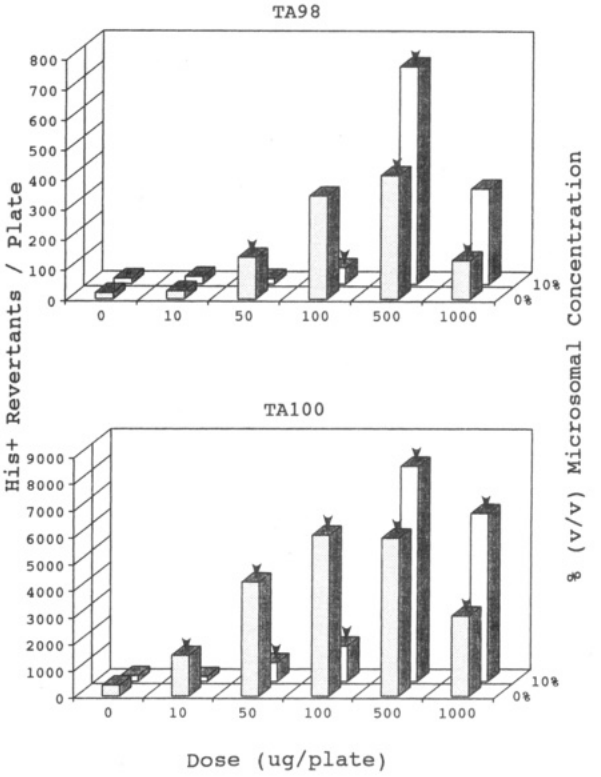

Figure 4. Reversion of strains TA98 and TA100 due to 2,6-diethylnitrosobenzene at 0% and 10% microsomal preparation. Arrowheads indicate values statistically different from control (*t*-test, $p < 0.05$).

occurred with no microsomal activation. This may indicate deactivation of this compound by microsomal PSMOs at low levels. Alternatively, simple partitioning of the mutagen into the microsomal lipid matrix may have offered some protection from mutagenicity. The reversion rates for strain TA98 were 233 revertants/μmol with, and 132 revertants/μmol without, activation. A similar phenomenon of higher mutagenicity without activation at low doses was observed. Reversion over background was observed with as little as 10 μg/plate for strain TA100 and 50 μg/plate for strain TA98.

2,6-Diethylnitrosobenzene has been reported to exist in solution as a mixture of monomeric and dimeric forms (Wratten et al., 1987), which may account for its generality in inducing mutagenesis in both tester strains. In its monomeric form, the free nitroso moiety gives the molecule the functionality of an alkylating point mutagen, as evident from its effect on strain TA100. Two nitroso groups can react to form the dimer. This eliminates the alkylating functionality of the monomer but forms a large bicyclic molecule that can intercalate into DNA. Mutagenicity to the frameshift-sensitive TA98 strain supports this contention. These data verify the mutability and sensitivity of both tester strains of *Salmonella* used in assaying the environmental degradative products of alachlor.

Alachlor, hydroxyalachlor, and 2,6-diethylaniline were not mutagenic to either the TA98 or TA100 strains, either with or without microsomal activation (Table 4).

Micronucleus Test. Genotoxicity may occur through two primary mechanisms: (1) mutagenesis and (2) clastogenesis. While mutagenesis refers to small changes in nucleic acid sequence in the DNA, clastogenesis describes gross changes or damage to the chromosome as a whole. Many compounds that are mutagenic are also clastogenic (Jenssen and Ramel, 1980).

Table 4. Reversion of Strains TA98 and TA100 Due to Alachlor, Hydroxyalachlor, and 2,6-Diethylaniline

	no. of His$^+$ revertants + SD			
	TA98		TA100	
dose (μg/plate)	+MP[a]	−MP	+MP	−MP
	Alachlor			
1000	28 ± 4	22 ± 1	150 ± 0	154 ± 9
100	29 ± 2	21 ± 2	171 ± 21	126 ± 5
10	29 ± 4	29 ± 1	158 ± 11	147 ± 4
1	31 ± 4	30 ± 2	130 ± 9	150 ± 4
0	20 ± 1	25 ± 6	161 ± 5	154 ± 20
	Hydroxyalachlor			
1000	27 ± 6	21 ± 2	99 ± 6	toxic
100	27 ± 0	21 ± 3	104 ± 3	99 ± 13
10	39 ± 3	21 ± 5	93 ± 6	128 ± 5
1	26 ± 5	15 ± 5	93 ± 2	107 ± 0
0	30 ± 5	19 ± 2	108 ± 4	111 ± 15
	2,6-Diethylaniline			
1000	toxic	toxic	127 ± 1	94 ± 6
100	25 ± 2	26 ± 0	121 ± 20	102 ± 2
10	22 ± 5	25 ± 5	113 ± 16	85 ± 6
1	28 ± 3	25 ± 6	111 ± 36	88 ± 4
0	24 ± 0	21 ± 4	107 ± 18	81 ± 4

[a] +MP/−MP, with/without 20% (v/v) microsomal preparation. Data are shown for high microsomal concentration only.

Table 5. Frequency of Micronucleated Mouse Reticulocytes Due to Alachlor and Selected Degradation Products[a]

	high dose	24 h[b]	48 h	72 h
untreated		0.20 ± 0.14[c]	0.20 ± 0.28	0.10 ± 0.14
DMBA (control)[d]	80 mg/g	0.35 ± 0.10	2.00 ± 0.95	2.60 ± 0.58
2-chloro-2′,6′-diethylacetanilide	500	0.10 ± 0.10	0.43 ± 0.25	0.10 ± 0.10
2-hydroxy-2′,6′-diethylacetanilide	500	0.23 ± 0.15	0.20 ± 0.10	0.25 ± 0.07
2,6-diethylnitroso-benzene	100	0.15 ± 0.10	0.08 ± 0.10	0.00
alachlor	500	0.30 ± 0	0.15 ± 0.07	0.45 ± 0.21

[a] Results are for high dose only: no dose−response relationship observed. [b] Hours after intraperitonial injection in DMSO carrier. [c] (MNRET/RET) × 100; 3000 cells counted (3 animals × 1000 cells/animal). [d] DMBA, dimethylbenzanthracene.

Alachlor, 2-chloro-2′,6′-diethylacetanilide (**VI**), 2-hydroxy-2′,6′-diethylacetanilide (**VII**), and 2,6-diethylnitrosobenzene were evaluated with the micronucleus test to determine if they were also acting at the chromosomal level as clastogens (Table 5). These compounds failed to induce micronucleii formation at doses ranging from 10 to 500 μg/g, including near-lethal doses. Except for 2,6-diethylnitrosobenzene, administration of the high dose resulted in acute toxic responses in the test mice (i.e., rapid onset of convulsions that lasted from 20 to 40 min followed by collapse and immobility that lasted up to 24 h). Those mice that did not die recovered to normal activity by the end of the third day. The high dose for 2,6-diethylnitrosobenzene was 100 μg/g due to solubility limitations. These data indicate that the genotoxicity of these test compounds is likely limited to the gene-locus or "point" mutations demonstrated by the *Salmonella*/microsome assay. The positive control, DMBA, was administered at a dose of 80 μg/g to verify the procedure. The micronucleii frequency of 0.35% at 24 h was in agreement with published data (Salamone et al., 1980).

Immunoassay Cross-Reactivity. Three commercially available alachlor immunoassay test kits were evaluated for cross-reactivity to environmental degradative products of alachlor. With the exception of hydroxyalachlor (**II**), negligible cross-reactivity was observed for 2,6-diethylacetanilide (**IV**), 2-chloro-2′,6′-diethylacetanilide (**VI**), 2-hydroxy-2′,6′-diethylacetanilide (**VII**), and 2,6-diethylaniline (**VIII**, Table 6).

Table 6. Cross-Reactivity of Alachlor Degradative Products to Commercially Available Immunoassay Kits

	% cross-reactivity	I_{50} (ppb)	LDD (ppb)
Ohmicron			
alachlor	100	0.73	0.05
2-hydroxy-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide	39.4	1.85	0.33
2-chloro-2′,6′-diethylacetanilide	0.3	250	90
2,6-diethylacetanilide	<0.1	>1000	1000
2- hydroxy-2′,6′-diethylacetanilide	<0.1	>1000	100
2,6-diethylaniline	<0.1	>1000	1000
Agri-Diagnostics			
alachlor	100	1.7	0.50
2-hydroxy-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide	40.5	4.2	10
2-chloro-2′,6′-diethylacetanilide	0.4	420	100
2,6-diethylacetanilide	<0.2	>1000	1000
2-hydroxy-2′,6′-diethylacetanilide	<0.2	>1000	1000
2,6-diethylaniline	<0.2	980	100
Millipore			
alachlor	100	0.48	0.50
2-hydroxy-2′,6′-diethyl-*N*-(methoxymethyl)acetanilide	>4.8	<10	<10
2-chloro-2′,6′-diethylacetanilide	<0.05	>1000	1000
2,6-diethylacetanilide	<0.05	>1000	1000
2- hydroxy-2′,6′-diethylacetanilide	<0.05	>1000	1000
2,6-diethylaniline	<0.05	>1000	1000

Hydroxyalachlor cross-reacted by 39.4% in the Ohmicron assay and by 40.5% in the Agri-Diagnostics assay. A cross-reactive response was also observed in the Millipore kit, but the extreme slope of the concentration versus response curve prevented an accurate determination of the I_{50} and % R (Tessier, 1994). Since the half-life of this degradative product is unknown, we cannot at this point determine whether or not its cross-reactivity is a factor in the positive bias of these assays versus chromatographic analysis.

The I_{50} values for 2,6-diethylacetanilide (**IV**), 2-chloro-2′,6′-diethylacetanilide (**VI**), 2-hydroxy-2′,6′-diethylacetanilide (**VII**), and 2,6-diethylaniline (**VIII**) reveal a 300- to >1300-fold selectivity for the target analyte, alachlor. The lower limits of detection (LLD) are 2000−20 000 times higher than for alachlor. Therefore, the potential for these compounds to be detected by, and therefore bias, alachlor immunoassay is insignificant.

Commercially available immunoassay technology for the detection of environmental residues of alachlor will also detect some environmental degradative products of alachlor. This cross-reactivity is reflected in the positive bias of alachlor residue levels determined by immunoassays, as compared to those measured by traditional chromatographic methods (Baker et al., 1993; Agri-Diagnostics, 1991). However, the two mutagenic degradative products, 2-chloro-2′,6′-diethyl-acetanilide (**VI**) and 2-hydroxy-2′,6′-diethylacetanilide (**VII**), are not cross-reactive and so are not detected. To date, immunoassay technology is used primarily to screen environmental samples for pesticide residues to limit more involved testing to a smaller sample set. However, screening in this way may cause toxicologically important degradative products of alachlor to pass unnoticed. Thus, the lack of immunoassay technology directed specifically at the degradative products of alachlor limits the environmental fate monitoring of this herbicide and proper assessment of its toxicological ramifications.

Conclusions. The environmental, primarily microbial, degradation of the herbicide alachlor results in a variety of aromatic secondary amine compounds. Two

of these products, 2-hydroxy-2′,6′-diethylacetanilide and 2-chloro-2′,6′-diethylacetanilide, are mutagenic in the *Salmonella*/microsome assay. Both compounds appear to act as point mutagens. They do not cause frameshift mutations. Their mutagenicity is attenuated by conjugation to the reduced form of glutathione by glutathione *S*-transferase. The mechanism of mutagenicity is therefore postulated to be as toxic electrophiles. They do not behave as clastogens in murine clastogenicity assays.

Given (1) alachlor's extensive use, (2) its lack of mineralization, and (3) its detection in ground water and surface water in the low parts per-billion range, it is reasonable to assume that a substantial quantity of degradation products, including two that are mutagenic, is present in the environment. This should be of concern regarding chronic exposure of soil-dwelling and aquatic organisms and humans through drinking water.

Immunoassay technology used in the environmental monitoring for alachlor does not detect its mutagenic degradative products. Future research should address the relative incidence, movement, and persistence of 2-hydroxy-2′,6′-diethylacetanilide and 2-chloro-2′,6′-diethylacetanilide in the environment. Immunoassays directed specifically at these mutagens will assist in this endeavor. Likewise, further studies on the exact mechanism of bioactivation and mutagenesis will determine whether other chloroacetanilide herbicides might also pose the same risks.

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