Alachlor and Its Analogues as Metabolic Progenitors of Formaldehyde: Fate of *N*-Methoxymethyl and Other *N*-Alkoxyalkyl Substituents†

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Alachlor, metolachlor, butachlor, and several analogues yield 20–60 mol % formaldehyde on incubation with the mouse liver microsomal mixed-function oxidase system under standard conditions. Studies with *N*-CD_2- and *N*-$^{13}CH_2$-labeled alachlor established that 70–80% of the metabolically generated formaldehyde is from the *N*-methylene group. The remainder is from the *O*-methyl group, a result confirmed by parallel studies with [*O*-$^{13}CH_3$]alachlor. In mechanistic terms this implies that the spontaneous decomposition of the side chain following *O*-CH_3 hydroxylation accounts for only about half of the total formaldehyde liberated from alachlor and therefore the remainder must arise via an alternative process. This need to invoke a mechanism other than *O*-CH_3 hydroxylation is supported by the observation that the *O*-*tert*-butyl and *O*-phenyl analogues of alachlor are also metabolic progenitors of formaldehyde. One plausible mechanism involves one-electron oxidation at nitrogen followed by loss of methoxy radical to form the methylene iminium ion and ultimately the *N*-hydroxymethyl derivative. The latter compound is a formaldehyde progenitor with a half-life of ~8 min in pH 7.4 aqueous solution at 18 °C.

INTRODUCTION

Alachlor (1), metolachlor (2), butachlor (3), and acetochlor are herbicidal 2-chloro-*N*-(alkoxyalkyl)-2′,6′-dialkylacetanilides (Weed Science Society of America, 1989) (Figure 1). The most important of these is 1, which is used in larger amount than any other pesticide because it is highly effective and selective in weed control in corn, soybean, and cotton. These benefits must be balanced against two adverse properties: first, 1 produces oncogenic effects in rats and mice (Environmental Protection Agency, 1987a) and there is limited evidence for the oncogenicity of 2 in rats (Environmental Protection Agency, 1987b); second, 1 and 2 are common contaminators of surface water, groundwater, wells, and drinking water in agricultural areas (Hallberg, 1989). It is therefore essential to define the products from metabolic and environmental degradation of alachlor and its analogues and to understand their toxicological importance as well as the implications of the mechanisms by which they arise.

Metabolism of 1 involves chemical modifications at seven or more sites individually or in sequence (Sharp, 1988; Feng and Patanella, 1989; Feng and Wratten, 1989; Feng et al., 1990) (Figure 2). With mammals, major portions of the doses are excreted as the glucuronide conjugate of the carbinolamide formed on O-demethylation and as other metabolites lacking the *N*-methoxymethylene substituent. In the mouse, oxidative metabolism by the microsomal mixed-function oxidase (MFO) system leads to labeling of DNA and hemoglobin from the methoxy substituent, with the *O*-hydroxymethyl intermediate or formaldehyde suggested as the derivatizing agent and candidate proximate carcinogen (Brown et al., 1988, 1989). Several

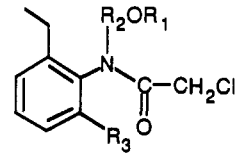

Figure 1. Structures of important 2-chloro-*N*-(alkoxyalkyl)-2′,6′-dialkylacetanilide herbicides (1, alachlor; 2, metolachlor; 3, butachlor) and related compounds examined [4, the 2′,6′-diethyl analogue of acetochlor (R_3 = CH_3); 5–9, various O-substituted alachlor analogues].

no.	R_1	R_2	R_3
1	CH_3	CH_2	C_2H_5
2	CH_3	$CH(CH_3)CH_2$	CH_3
3	$(CH_2)_3CH_3$	CH_2	C_2H_5
4	C_2H_5	CH_2	C_2H_5
5	$(CH_2)_2CH_3$	CH_2	C_2H_5
6	$CH(CH_3)_2$	CH_2	C_2H_5
7	CH_2Ph	CH_2	C_2H_5
8	$C(CH_3)_3$	CH_2	C_2H_5
9	Ph	CH_2	C_2H_5

lipophilic and relatively stable xenobiotics that serve, on metabolism, as formaldehyde (HCHO) progenitors via carbinolamides are locally or systemically active as carcinogens (Ashby and Lefevre, 1982). Although formaldehyde was not established as a metabolite of 1, it has been implied as such and could arise from at least three pathways initiated by MFO oxidations: at the *N*-methylene group (A), at the *O*-methyl group (B) (Brown et al., 1988; Feng and Patanella, 1989), or directly at nitrogen (C) (Figure 3).

This study examines 1–3 and several analogues (4–9 in Figure 1) as potential progenitors of formaldehyde in the mouse liver MFO system. Formaldehyde was analyzed as the 2,4-dinitrophenylhydrazine (DNPH) derivative (HCHO-DNPH) by using a combination of gas chromatography (GC), GC/mass spectrometry (MS) with selected ion monitoring (SIM), and high-performance liquid chromatography (HPLC) techniques. Particular attention has been given to the mechanisms by which the formaldehyde may be liberated and to the reactive species and intermediates implied in the transformations.

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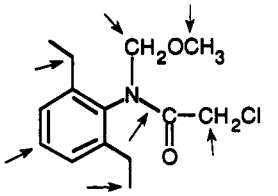

Figure 2. Sites of metabolic attack on alachlor. Conjugation with glutathione is important in metabolism of the chloromethyl and phenyl substituents.

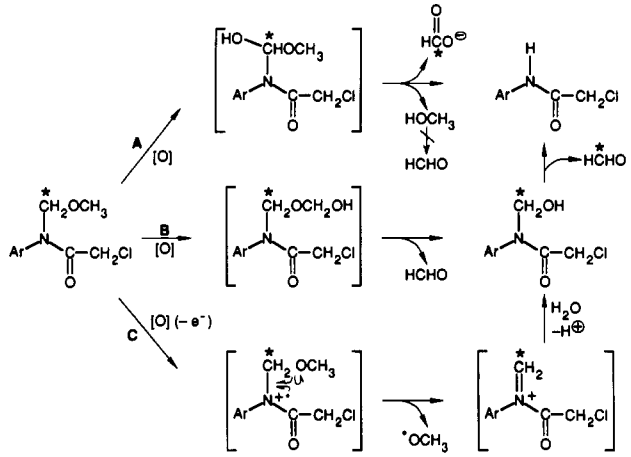

Figure 3. Alternative pathways for liberation of formaldehyde from the *N*-methoxymethyl substituent of alachlor. Ar = 2,6-diethylphenyl.

MATERIALS AND METHODS

Spectroscopy and Chromatography. NMR spectra were recorded with a Bruker AM-300 instrument (300 MHz for ^1H and 75 MHz for ^{13}C) and an ASPECT 3000 computer for $CDCl_3$ solutions containing tetramethylsilane as the internal reference in 5-mm tubes. Ultraviolet (UV) spectra were measured on a Hewlett-Packard 8452A diode array spectrophotometer and infrared (IR) spectra on a Perkin-Elmer 1600 series Fourier transform IR.

GC analyses involved a Hewlett-Packard 5890 Series II chromatograph with an electron capture detector (ECD) or flame ionization detector (FID) for analysis of HCHO-DNPH and FID for determination of unmetabolized alachlor and analogues. GC/ECD utilized a DB1701 cyanopropyl silicone capillary column (30 m × 0.25 mm × 0.25 μm film thickness; J&W Scientific, Folsom, CA) with helium as the carrier gas (18 psi). Samples (1 μL) were introduced in the splitless mode at an injector temperature of 240 °C with a purge delay of 1 min. The temperature program was as follows: 180 °C for 15 min, rapid heating to 280 °C and then maintaining this temperature for 2 min. GC/FID utilized a DB5 capillary column (30 m × 0.25 mm × 0.25 μm film thickness; J&W Scientific) with helium as the carrier gas (18 psi). Samples (1 μL) were introduced as above at 280 °C with a purge delay of 1 min and with a temperature program of 80 °C for 3 min, heating to 150 °C at 70 °C/min, heating to 300 °C at 6 °C/min, and holding at 300 °C for 10 min. While FID detection was suitable for analysis of alachlor and analogues (Table I), it was not selective enough to allow quantitation of HCHO-DNPH at low levels.

MS (direct insertion) and GC/MS were accomplished with a Hewlett-Packard 5985B instrument using the Hewlett-Packard 59870C RTE-A data system. The electron energy and source temperature, respectively, were 70 eV and 200 °C for electron impact (EI)–MS or 230 eV and 130 °C for chemical ionization (CI)–MS (methane, 1.0 Torr). GC/MS was performed on a DB5 capillary column as above with helium as the carrier gas (15 psi). Samples (1 μL) were injected (splitless mode) at an injector temperature of 280 °C and with a purge delay of 1.5 min. The temperature program was 90 °C for 3 min, heating to 300 °C at 30 °C/min, and holding at 300 °C for 5 min.

HPLC analyses used a Beckman dual pump and gradient controller and a LiChrospher Si-60 (5 μm) analytical column (125 × 4 mm; E. Merck, Darmstadt, Germany). Analytes were eluted with a flow rate of 1.0 mL/min using hexane–ethyl acetate in a linear gradient from 85:15 to 75:25 over a 15-min period, followed by elution with ethyl acetate for 2 min. The eluate was monitored at 272 (for alachlor metabolites) or 338 nm (for dinitrophenylhydrazones), and peak areas were determined with a Spectra-Physics 4290 integrator. HPLC Rt values are listed in Table II. Comparison of the dinitrophenylhydrazones of acetaldehyde, propionaldehyde, butyraldehyde, benzaldehyde, and acetone with that of formaldehyde indicated that the derivatization yields and their UV extinction coefficients were essentially the same. HPLC was the preferred method for quantitation of HCHO-DNPH and other dinitrophenylhydrazones and was essential for analysis of one thermolabile compound (i.e., **11** which decomposes to **10**, as discussed later).

Preparative TLC utilized 20 cm × 20 cm × 1 mm silica gel plates developed with hexane–ethyl acetate (3:1). Visualization was by UV light.

Chemicals. Compounds **1–3** were analytical standards obtained from the Environmental Monitoring Systems Laboratory, U.S. Environmental Protection Agency (Las Vegas, NV). [*O*-$^{14}CH_3$]-**1** and compound **10** were available from or were prepared as described in an earlier study (Brown et al., 1988). Labeled precursors or substrates used were paraformaldehyde-d_2 (99.8% D), [^{13}C]paraformaldehyde (99% ^{13}C), methanol-d_4 (99.8% D), and [^{13}C]methanol (99% ^{13}C).

Permanganate Oxidation of Alachlor. A solution of **1** (0.50 g, 1.9 mmol) and $KMnO_4$ (pulverized, 0.37 g, 2.4 mmol) in *tert*-butyl alchol (25 mL) and water (10 mL) was stirred at 20 °C until the permanganate was completely decolorized (40 h). The brown precipitate was removed by filtration through a bed of Celite, and the clear, colorless filtrate was diluted with water (25 mL) and saturated NaCl solution (10 mL) and then extracted with methylene chloride (25 mL × 3). The combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure, and the residue was fractionated by preparative TLC. The major band was identified as the acetophenone from benzylic oxidation designated as 2′-$COCH_3$-**1** (18% yield): ^1H NMR δ 1.26 (apparent t, 3, J = 7.5, CH_3CH_2), 2.53 (s, 3, CH_3CO), 2.59 and 2.63 (AB of ABX_3, 2, J_{AB} = 15.0, J_{AX} = J_{BX} = 7.5, CH_3CH_2), 3.73 and 4.13 (AB, 2, J_{AB} = 13.7, CH_2Cl), 4.32 (d, 1, J = 10.4, NCHH), 5.46 (d, 1, J = 10.2, NCHH), 7.42–7.55 (m, 3, ArH); ^{13}C NMR 14.4 (CH_3CH_2), 23.2 (CH_3CH_2), 29.7 (CH_3CO), 42.9 (CH_2Cl), 57.9 (OCH_3), 79.7 (NCH_2), 126.1, 129.0, and 132.6 (aromatic CH), 135.2, 139.3, and 143.1 (other aromatic C), 168.6 (amide C=O), 200.7 (ketone C=O); EI–MS M^+ 283 (0.1), 248 (M − Cl, 39), 174 (100); UV (ethanol) λ_{max} 280 nm (ϵ = 700); IR (CCl_4) 1699 cm^{-1}; GC–FID Rt 17.6 min; HPLC see Table II. One of the minor products (1.8%) was tentatively identified by ^1H and ^{13}C NMR and EI-MS as the cyclic ether that would result from intramolecular reaction of the benzylic alcohol and the chloromethyl group. This cyclic ether was also observed as a product on borohydride reduction of 2′-$COCH_3$-**1**.

Synthesis of Alachlor and Analogues (Figure 4; Table I) via 2,6-Diethyl-*N*-methyleneaniline. 2,6-Diethylaniline (55 μL, 0.33 mmol) was reacted with paraformaldehyde (10 mg, 0.32 mmol) by heating in 4-methyl-2-pentanone (0.4 mL) containing triethylamine (20 μL, 0.14 mmol) and anhydrous Na_2SO_4 (150 mg, oven-dried and pulverized) in an open 5-mm NMR tube immersed in a bath of boiling 4-methyl-2-pentanone for 1 h with occasional agitation. The mixture was cooled and filtered through a cotton plug, and the tube and plug were rinsed with hexane (0.5 mL). GC analysis of the filtrate showed the presence of the corresponding *N*-methylene imine in 78% yield: ^1H NMR 1.12 (t, 6, J = 7, CH_3), 2.44 (q, 4, J = 7, CH_2Ar), 6.70 (t, 1, J = 7.6, H-4), 6.93 (d, 2, J = 7.7, H-3), 7.34 and 7.68 (AB, 2, J = 18.4, N=CH_2); ^{13}C NMR 14.6 (CH_3), 24.3 (CH_2Ar), 124.1 (C-4), 126.1 (C-3, C-5), 132.1 (C-2, C-6), 150.7 (C-1), and 155.6 (N=CH_2).

Alachlor (**1**) and its O-substituted analogues (**3–9**) were obtained by adding the filtrate (above) to a solution of chloroacetyl chloride (44 μL, 0.48 mmol) in hexane (0.5 mL), stirring the resulting mixture for 2 h at 20 °C, and then treating with a methylene chloride (0.5 mL) solution of the appropriate aliphatic alcohol (1.5 mL), benzyl alcohol (0.4 mL), or phenol (2.0 g) containing triethylamine (0.3 mL). After 18 h at 20 °C, the reaction mixture was washed with water (3 mL), diluted with

hexane (3 mL), washed with a second portion of water (3 mL) (or, in the case of **9**, 2 M NaOH), and dried by filtration through anhydrous $MgSO_4$. Solvent was evaporated at reduced pressure, and the excess alcohol was removed under high vacuum (and, in the case of **7**, with gentle heating). The residue was fractionated by preparative TLC to give the pure products as colorless oils (yields based on formaldehyde: **1**, 33%; **3**, 35%; **4**, 37%; **5**, 28%; **6**, 29%; **7**, 20%; **8**, 16%; and **9**, 49%). Similar results were obtained by using paraformaldehyde-d_2 (for [*N*-CD_2]-**1**), [^{13}C]paraformaldehyde (for [*N*-$^{13}CH_2$]-**1** and -**3**–**9**), methanol-d_4 (for [*O*-CD_3]-**1**), or [^{13}C]methanol (for [*O*-$^{13}CH_3$]-**1**).

O-Benzyl analogue **7** was also prepared by alkylation of chloroacetanilide **10**. Thus, a mixture of **10** (226 mg, 1.0 mmol), benzyl chloromethyl ether (0.37 mL, 2.7 mmol), tetrabutylammonium bromide (74 mg, 0.23 mmol), and 50% aqueous NaOH solution (1.4 mL) was stirred vigorously for 1 h at 20 °C. The mixture was extracted with ether (10 mL × 3), and the combined organic extracts were washed with water (10 mL), dried over anhydrous Na_2SO_4, and concentrated at reduced pressure. The residue was purified by preparative TLC to give **7** (162 mg, 47% yield) as a colorless oil: ^{1}H NMR δ 1.21 (apparent t, 6, J = 7.5, CH_3CH_2), 2.55 and 2.62 (AB of ABX_3, 4, J_{AB} = 15.1, J_{AX} = J_{HX} = 7.5, CH_3CH_2), 3.73 (s, 2, CH_2Cl), 4.77 (s, 2, CH_2Ph), 5.08 (s, 2, NCH_2), 7.2–7.4 (m, 8, ArH). Signals for a minor component, isomeric with the above by rotation about the amide linkage, were observed at δ 4.30 (s, CH_2Cl), 4.52 (s, CH_2Ph), and 4.96 (s, NCH_2).

Preparation and Properties of Carbinolamide 14. A mixture of *O*-benzyl analogue **7** (34 μmol) and 10% palladium on charcoal (1.9 mg) in methanol (1 mL) was stirred under a hydrogen atmosphere for 1.5 h (Figure 4). The mixture was filtered through a cotton plug and the plug washed with methylene chloride. The filtrate was concentrated under reduced pressure and the residue dissolved in hexane–ethyl acetate (85:15) and chromatographed on a microcolumn of flash silica gel (0.7 g), using the same solvent. The combined product fractions contained **11** (42% yield) and **10** (6% yield) by HPLC analysis. Repeated experiments under various conditions established that hydrogenolysis was very slow in methylene chloride, and larger amounts of catalyst led to reductive dechlorination. **11**: ^{1}H NMR δ 1.23 (t, 6, J = 7.5 CH_3), 2.5–2.7 (m, 4, CH_2Ar), 3.69 (s, 2, CH_2Cl), 4.01 (t, 1, J = 8.2, OH), 4.99 (d, 2, J = 8.1, NCH_2), 7.21 (br d, 2, J = 7.8, ArH), 7.34 (dd, 1, J = 7.0, 8.4, ArH); ^{13}C NMR 14.7 (CH_3), 24.4 (CH_2Ar), 41.5 (CH_2Cl), 75.5 (NCH_2), 127.2 (C-3′, C-5′), 129.5 (C-4′), 141.7 (C-2′, C-6′), 168.7 (C=O); no signals attributable to the amide rotamer were present in the spectrum; UV λ_{max} 228, 266, 274 (shoulder) nm.

A solution of **11** in hexane–ethyl acetate (85:15) slowly decomposed to **10** over a period of several days when stored at −10 °C. The stability of **11** in pH 7.4 100 mM sodium phosphate buffer at 18 °C was examined by UV, HPLC, and GC-FID. For the UV method a solution of **11** (0.70 μmol, contaminated with 0.30 μmol of **10**) in ethanol (100 μL) was mixed with buffer (1.0 mL), and the UV spectrum was recorded at 1-min intervals for 30 min. The absorbance at 254 nm ($\Delta\epsilon_{254}$ = 50, isosbestic point at 272 nm) increased rapidly with first-order kinetics. For the HPLC method, a solution of **11** (1.0 μmol, contaminated with 0.2 μmol of **10**) in buffer (15 mL) was incubated, and aliquots (1.0 mL) were removed at intervals, diluted with saturated NaCl solution (0.5 mL), and extracted with hexane–ethyl acetate (85:15, 1.0 mL). The organic layer was filtered through a plug of anhydrous $MgSO_4$ and a portion of the filtrate (100 μL) was analyzed by HPLC with detection at 272 nm. The proportion of **11** in the mixture of **10** and **11** decreased from an initial value of 67% in a first-order process. For GC analyses, the aqueous phase was treated with DNPH solution (0.50 mg of 70% DNPH in 0.70 mL of 2 M HCl) at 20 °C and after 30 min extracted with carbon tetrachloride (0.70 mL). The residue obtained after evaporation of the carbon tetrachloride was dissolved in ethyl acetate (0.10 mL) and analyzed by GC-FID. The peak area for HCHO-DNPH increased in a manner consistent with a first-order process. In this experiment, there was about 30% decomposition prior to the first measurement. Peak areas were expressed on a relative basis since a standard curve was not prepared.

Microsomal Preparations and Incubations. Microsomes were prepared from fresh livers of male albino Swiss-Webster mice by homogenization in 100 mM pH 7.4 sodium phosphate buffer and centrifugation of the homogenate at 10000*g* for 15 min and then of the supernatant at 105000*g* for 60 min. The microsomes were washed by resuspending in buffer and repeating the final centrifugation step, and the pellets were then suspended in buffer prior to protein determination (Bradford, 1976). Microsomes were stored at −80 °C for up to 90 days before use.

The incubation procedure was based on that of Tu and Yang (1983). Standard incubation mixtures were prepared in 2.5 cm diameter flat-bottom glass scintillation vials by sequential addition of the substrate (78 nmol) in ethanol (10 μL, followed by solvent evaporation) and portions of buffer containing NADPH (0 or 0.96 μmol) and microsomes (0.51 mg protein unless indicated otherwise) to a final volume of 1.2 mL. Samples were incubated in a shaking water bath for 1 h at 37 °C while tightly capped. Assays were performed in sets of 20–30 samples, set up at 1-min intervals to allow standardization of times through subsequent analytical steps. Reactions were terminated by addition of 25% $ZnSO_4$ solution [100 μL, containing benzaldehyde (78 nmol) as internal standard for HPLC analysis] followed by saturated $Ba(OH)_2$ (100 μL) with mixing and then cooled to 0 °C. Mixtures were clarified by centrifugation, and an aliquot (1.0 mL) was removed for formaldehyde determination.

Analyses of Formaldehyde and Other Aldehydes and Ketones as Their 2,4-Dinitrophenylhydrazone Derivatives. Formaldehyde was analyzed either by the Nash colorimetric procedure (Nash, 1953) or, after conversion to the DNPH derivative, by a procedure based on that of Tuss et al. (1982) and Lapari and Swarin (1982). For the HCHO-DNPH method, the supernatant (1.0 mL) from the microsomal incubation was added to a vial containing **1** or **5** (5.0 μg as an internal standard for GC analysis) and freshly prepared DNPH solution (0.50 mg of 70% DNPH in 0.70 mL of 2 M HCl). The samples were mixed and incubated for 30 min at 20 °C followed by extraction with carbon tetrachloride (HPLC grade, 0.70 mL) using a vortex mixer (30 s). After standing for 30 min, the organic layer was separated and either (a) used directly for HPLC analysis (20-μL aliquot) of the DNPH derivatives of formaldehyde and other aldehydes and ketones or (b) evaporated to dryness in a stream of N_2 and redissolved in ethyl acetate (0.10 mL) for GC analysis (1-μL aliquot) of HCHO-DNPH.

Formaldehyde was quantitated by adding [^{13}C]formaldehyde (15, 30, 45, 60, and 75 nmol) in place of substrate and incubating with microsomes in the presence of NADPH. A linear least-squares fit (correlation coefficient 0.998 in a typical experiment) gave a slope which was used to convert peak area ratios (HCHO-DNPH compared with benzaldehyde–DNPH derived from benzaldehyde as the internal standard) (GC/FID or HPLC) to nanomoles of formaldehyde. A solution of [^{13}C]formaldehyde (1.50 mM) was prepared for this purpose by heating [^{13}C]-paraformaldehyde with 2 M HCl (0.50 mL) at 100 °C for 5 min. The solution was diluted with water (20 mL), adjusted to pH 7.4 with concentrated Na_2HPO_4 solution, diluted with water to a final volume of 50 mL, and then standardized by the Nash colorimetric procedure (Nash, 1953).

Four assays without substrate were performed in each set to estimate the background or endogenous formaldehyde: two assays with added NADPH and two without. The average formaldehyde value for the first pair was subtracted from all assays using NADPH, and the average of the second pair was subtracted from all assays in which NADPH was omitted. The background level was significant in the presence of NADPH and microsomes but was very low in the absence of NADPH.

Analyses of Free Formaldehyde Using Formaldehyde Dehydrogenase (FDH) and Stability of Formaldehyde and Methanol to the Incubation Conditions. In this study the total formaldehyde [i.e., that determined by the analytical procedure which includes exposure to $Ba(OH)_2$, $ZnSO_4$ and HCl] is subdivided into "free formaldehyde" and "bound formaldehyde". Free formaldehyde is that which is susceptible to oxidation by FDH to formate (Ando et al., 1979); bound formaldehyde is that which is not. Substrate (**1**, **2**, **3**, or **4**, 78 nmol) [or [^{13}C]-formaldehyde (78 nmol) or [^{13}C]methanol (78 nmol) as controls] was incubated with microsomes and NADPH (0 or 0.96 μmol) either in the presence or in the absence of FDH [0.042 units of lyophilized powder from *Pseudomonas putida* (Sigma Chemical

Co., St. Louis, MO) and 0.96 μmol of NAD]. Reaction mixtures were subjected to the standard derivatization procedure and analyzed by HPLC. All microsomal incubations were for 60 min. For controls, formaldehyde and methanol were added at 0, 1, 5, 21, 41, or 60 min after the start of the incubation. These analyses showed that under the standard microsomal incubation conditions with NADPH, formaldehyde undergoes no more than a 20% loss and that methanol is not a source of formaldehyde (Figure 5). They also show that, in the presence of FDH, formaldehyde available early in the incubation period is completely destroyed but that added or generated later is only partially affected.

Isotope Distribution in Formaldehyde as a Metabolite of [*N*-CD_2]-, [*O*-CD_3]-, [*N*-$^{13}CH_2$]-, and [*O*-$^{13}CH_3$]Alachlor and Several [*N*-$^{13}CH_2$]Analogues. The isotope distributions of HCHO-DNPH's derived from [^{2}H]alachlor and [^{13}C]alachlor and its analogues were analyzed by GC/MS-CI-SIM by monitoring the ion clusters (which always contained the $[M + H]^+$ base peak) at *m/z* 211, 212, 213, and 214 and 210, 211, 212, and 213, respectively. To avoid sampling errors, at least 20 spectra (Matthews and Hayes, 1976) were recorded over the HCHO-DNPH GC peaks (Rt = 9.4 min). Standard samples were run under the same conditions in each experiment to determine the contribution of labeled and unlabeled HCHO-DNPH to each of the observed ions; these values were used to calculate the isotrope distribution in the assay samples.

Extent of NADPH-Dependent Metabolism of Alachlor and Its Analogues. The DNPH/GC/FID method allows quantitation not only of formaldehyde but also of recovered substrates. NADPH-dependent metabolism is calculated as the percentage loss of substrate compared to an assay in which NADPH is omitted. On the other hand, absolute recoveries of substrates are determined by comparing GC peak area ratios of recovered substrate to internal standard (1 or 3) with the same ratios for standard solutions of substrates to internal standard. For substrates **1**, **2**, **3**, **5**, **6**, **7**, **8**, and **9** the absolute recoveries of substrates were 98, 82, 31, 68, 69, 9, 74, and 7% in the absence of NADPH and 9, 3, 4, 4, 3, 3, 4, and 2% in the presence of NADPH, respectively. To assess the stability of substrates toward the DNPH derivatization procedure (2 M HCl), their recovery after 30 min in DNPH solution at 20 °C was determined relative to that from the same volume of buffer. Substrates **1**, **6**, **7**, **8**, and **9** were recovered in 95, 82, 32, 72, and 25% yields, respectively. Under these same conditions the yield of formaldehyde as a substrate decomposition product, determined by GC-FID quantitation of the HCHO-DNPH formed, was less than 1% in each case.

Methanol as a Potential Metabolite of [*O*-$^{14}CH_3$]-1. Microsomal incubation mixtures as above but with [*O*-$^{14}CH_3$]-**1** (3×10^6 dpm, 78 nmol) as substrate were incubated with NADPH (0 or 0.96 μmol) for 60 min at 37 °C. Methanol (5 μL) was then added and the mixture filtered through a reverse-phase microcolumn (Baker Spe C_{18}) (which served to remove alachlor), and the vial and column were washed with saturated NaCl solution (0.50 mL). The column was sucked dry, and the eluate was made basic (NaOH, 110 μL of 2 M solution) and shaken with a methylene chloride (2.5 mL) solution of 3,5-dinitrobenzoyl chloride (57.5 mg) and tetra-*n*-butylammonium bromide (2.0 mg) for 2 h at 18 °C. The organic layer was separated and eluted through a microcolumn of flash silica gel (0.7 g) with methylene chloride. The eluate was concentrated and purified by preparative TLC to yield methyl 3,5-dinitrobenzoate (18 mg, 64% yield based on added methanol) which was recrystallized from ethanol–water as white sheets (14 mg). Liquid scintillation counting (with quench correction) indicated that 1.6% of the substrate could be accounted for as [^{14}C]methanol. In the absence of NADPH this was 0.03%.

The extent of substrate metabolism was estimated by washing the reverse-phase column (above) with a methanol solution (4 mL) of unlabeled **1** (20 mg), concentrating the eluate and, after preparative TLC, counting the fraction corresponding to **1**. Substrate **1** was recovered in 28% yield from incubation mixtures containing NADPH and in 90% yield from those without.

RESULTS

Synthesis and Characterization of Labeled Alachlor and Alachlor Analogues. The Olin (1971) procedure for preparation of *N*-alkoxymethylchloroacetanilides was adapted to microscale synthesis (Figure 4) in a one-pot process with yields of 16–49% from 1 to 10 mg of paraformaldehyde. The NMR spectra of chloroacetanilides **1** and **3–9** in $CDCl_3$ (Table I) showed them to occur as a mixture of rotamers, isomeric about the hindered amide linkage (Chupp et al., 1969; Ratts and Chupp, 1974), with a *Z*:*E* ratio of about 94:6 for all except the *O*-*tert*-butyl analogue, which favors the *Z* conformer to a lesser extent (87:13). In aqueous solution at pH 7.4, the *Z*:*E* ratio for **1** is reduced to 69:31. For carbinolamide **11**, the NMR spectrum shows the OH and NCH_2 resonances as a sharp triplet and doublet, respectively, with no signals being observed for the other rotamer. This suggests that the molecule is locked in the *Z* conformation by intramolecular hydrogen bonding between the OH and the amide carbonyl oxygen. Internal hydrogen bonding may also explain the relative stability of **11** in nonhydroxylic solvents (see below). The mass spectra (EI) of the various isotopically labeled alachlor samples (Table I) establish that the alachlor fragment ions at *m/z* 238, 237, 202, 188, 160, and 146 all retain the NCH_2 group. A plausible fragmentation pathway involves initial loss of methanol with hydrogen abstraction from the benzylic position (237), followed by cyclization with loss of chlorine (202) or chloromethyl radical (188); the *N*-methylene lactam (188) then loses CO (160) or NCO (146).

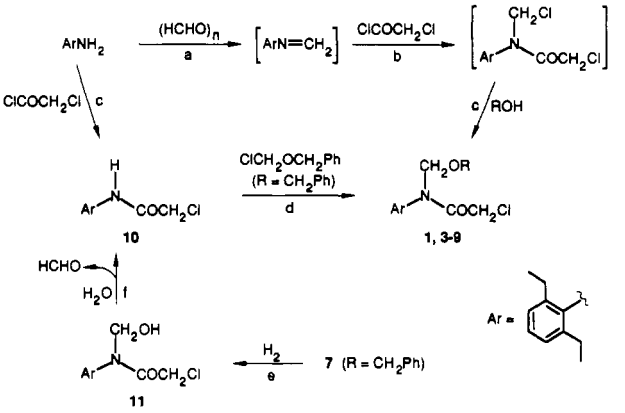

Figure 4. Synthesis routes for alachlor, isotopically labeled alachlor, and their analogues (see Figure 1 for structures of **1–9**). Reaction conditions: a, $(C_2H_5)_3N$, Na_2SO_4, 4-methyl-2-pentanone; b, hexane; c, $(C_2H_5)_3N$, CH_2Cl_2; d, $(n\text{-}C_4H_9)_4N^+Cl^-$; e, Pd/C, CH_3OH; f, pH 7.4.

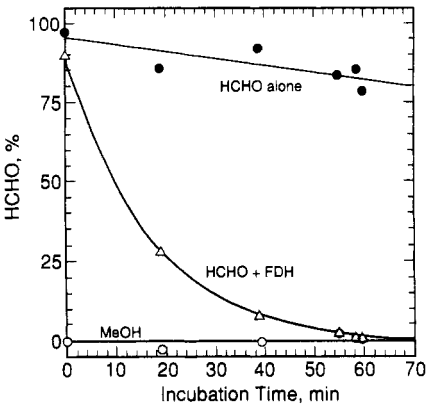

Figure 5. Effect of formaldehyde dehydrogenase on recovery of formaldehyde and evaluation of methanol as a potential source of formaldehyde on incubation with the mouse liver microsome–NADPH system. Incubation was for 60 min in each case with formaldehyde or methanol added at varying times during this period.

Chemical Oxidation of Alachlor. No reaction was

Table I. Characterization of Alachlor Analogues with Various *N*-Alkoxyalkyl Substituents

ArN(COCH$_2$Cl)R			EI-MS (m/z)		NMR chemical shift, multiplicity, and coupling constant[b]						
						CH$_2$Cl		NCH$_2$		OCα	
no.	R	Rt,[a] min	M$^+$	major fragments		major	minor	major	minor	major	minor
1	CH_2OCH_3	15.3	269 (10)	188 (87) 160 (100)	^{1}H	3.70 s	4.34 s	4.96 s	4.85 s	3.49 s	3.34 s
					^{13}C	42.0	–[c]	80.5	–	58.0	–
1	$^{13}CH_2OCH_3$ 99.3% ^{13}C[d]	15.3	270 (14)	189 (100) 161 (96)	^{1}H	3.70 s	4.33 s	4.96 d, 157.2	4.84 d, 154.9	3.49 d, 5.5	3.34 d, 6.1
					^{13}C	42.1	–	80.6	83.0	58.0	–
1	$CH_2O^{13}CH_3$ 98.4% ^{13}C[d]		270 (9)	188 (85) 160 (100)	^{1}H	3.70 s	4.33 s	4.96 d, 5.3	4.84 d, 2.7	3.49 d, 142.2	3.34 d, 142.1
					^{13}C	42.1	–	80.6	–	58.0	56.3
1	CD_2OCH_3[d]		271 (11)	190 (100) 162 (69)	^{1}H	3.70 s	4.34 s	(4.94[e]) (s)	–	3.50 s	3.34 s
					^{13}C	42.1	–	–	–	57.9	–
1	CH_2OCD_3[d]		272 (14)	188 (85) 160 (100)	^{1}H	3.70 s	4.34 s	4.96 s	4.85 s	(3.46[e]) (s)	(3.30[e]) (s)
					^{13}C	42.1	–	80.5	–	–	–
3	$^{13}CH_2O(CH_2)_3CH_3$	18.8	312 (4)	189 (44) 176 (100) 161 (65)	^{1}H	3.69 s	4.34 s	5.00 d, 157.1	4.88 d, 154.6	3.67 dt, 3.1, 3.6	3.41 dt, 3, 7
					^{13}C	–	–	79.4	81.6	–	–
4	$^{13}CH_2OC_2H_5$	16.1	284 (15)	189 (77) 176 (72) 161 (100)	^{1}H	3.70 s	4.35 s	5.01 d, 157.0	4.89 d, 154.7	3.73 dq, 3.1, 7.0	3.48 dq, 3, 7
					^{13}C	–	–	79.0	81.4	–	–
5	$^{13}CH_2O(CH_2)_2CH_3$	17.4	298 (11)	189 (50) 176 (100) 161 (65)	^{1}H	3.70 s	4.35 s	5.01 d, 157.0	4.89 d, 154.7	3.64 dt, 3.1, 6.7	3.38 dt, 3, 6
					^{13}C	–	–	79.3	81.6	–	–
6	$^{13}CH_2OCH(CH_3)_2$	16.5	298 (3)	189 (11) 176 (100) 161 (20)	^{1}H	3.69 s	4.34 s	5.01 d, 156.6	4.90 d, 154.5	3.98 d hep, 3.9 6.0	–
					^{13}C	42.24	–	77.0	79.7	–	–
7	$^{13}CH_2OCH_2Ph$	24.7	346 (0.5)	191 (37) 161 (9) 91 (100)	^{1}H	3.73 s	4.30 s	5.08 d, 157.6	4.95 d, 155.3	4.77 d, 4.1	4.52 d, 4.8
					^{13}C	–	–	78.8	80.9	–	–
8	$^{13}CH_2OC(CH_3)_3$ 99.2% ^{13}C[d]	17.3	312 (2)	189 (6) 176 (100) 161 (16)	^{1}H	3.67 s	4.36 s	4.99 d, 155.2	4.89 d, 153.8	–	–
					^{13}C	42.20	41.12	72.5	75.1	–	–
9	$^{13}CH_2OPh$ 99.5% ^{13}C[d]	23.5	332 (0.5)	239 (100) 189 (0.5) 163 (91)	^{1}H	3.74 s	4.37 s	5.56 d, 159.3	5.48 d, 157.8	–	–
					^{13}C	41.93	–	76.5	79.4	–	–

[a] Conditions described as GC-FID under Materials and Methods. [b] Percentage of major rotamer (CONRR', Z) based on relative integrated intensities of the signals observed (compound, substituent observed, %): (N-$^{13}CH_2$)-1, NCH_2, 94.4%; (*O*-$^{13}CH_3$)-1, NCH_2, 94.4%; (*O*-$^{13}CH_3$)-1, OCH_3, 69% in buffer in which ^{13}C shifts were δ 57.2 (major) and δ 56.3 (minor) (vs acetone at δ 30.0); **3**, NCH_2, 92.7%; **5**, NCH_2, 93.7%; **8**, CH_2Cl, 87%; **9**, NCH_2, 92.4%. Absorbance not observed (e.g., ^{13}C of minor rotamers with ^{13}C as natural abundance) or not observable. [d] Calculated from relative integrated intensities of $^{12}CH_n$ and $^{13}CH_n$ signals (^{1}H). For [*N*-CD$_2$]-1 and [*O*-CD$_3$]-1, the residual ^{1}H signal could not be detected. [e] ^{2}H NMR chemical shift ($CDCl_3$ = 7.26).

Table II. HPLC Retention Times for Alachlor and Analogues and DNPH Derivatives of Aldehydes and Ketones

compd	Rt,[a] min	compd	Rt,[a] min
alachlor and analogues (272 nm)		DNPH derivatives (338 nm)	
1	3.8	HCHO	7.1
10	5.5	CH_3CHO	6.1 (6.6)[b]
11	10.5	$(CH_3)_2CO$	5.4
2'-$COCH_3$-1	13.8	C_2H_5CHO	3.7 (4.2)[b]
		n-C_3H_7CHO	2.8 (3.3)[b]
		PhCHO	3.0

[a] Conditions described under Materials and Methods. [b] Values in parentheses are for minor isomers in ~1:4 ratio.

observed when alachlor was treated with 1 equiv of MCPBA in $CDCl_3$ at room temperature. However, with aqueous permanganate benzylic oxidation occurred, leading to a mixture of 2'-$COCH_3$-1 and the cyclic ether (see Materials and Methods). The occurrence of the latter compound implies the intermediacy of 2'-$CHOHCH_3$-1.

Reaction of Alachlor and Alachlor Analogues by NADPH-Independent Processes. With the exceptions of **3**, **7**, and **9**, substrates were recovered in moderate to good yields (68–98%) after incubation with mouse liver microsomes in the absence of NADPH followed by formaldehyde derivatization and extraction. The losses of **7** and **9** are substantial (91–93%) but can be accounted for by their instability to the DNPH/HCl derivatization step. Formaldehyde is not released during this chemical decomposition, nor is it released from the benzylic oxidation product (2'-$COCH_3$-1) during the derivatization procedures.

Alachlor Metabolites Other than Formaldehyde. An experiment aimed at detecting initial metabolites that might be lost through subsequent metabolic or chemical alteration involved 5 min of incubation in the MFO system followed by immediate extraction with ethyl acetate–hex-

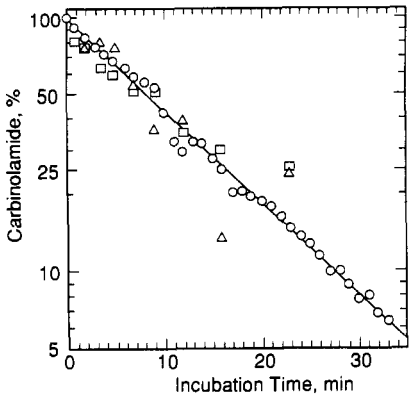

Figure 6. Stability of carbinolamide **11** on incubation in aqueous solution at pH 7.4 and 18 °C determined by analysis of **10** and **11** involving direct UV spectroscopy (○) or extraction and HPLC (□) (normalized to 100% at $t = 0$) or by analysis of the relative amount of formaldehyde by solvent partitioning and the DNPH/GC-FID procedure (△).

ane and HPLC analysis. In this situation, 35% NADPH-dependent metabolism occurred and the only observed metabolite (4.6% yield) was chloroacetanilide **10**. Compounds specifically sought but not detected (<1% yield) were 2′-$COCH_3$-**1** and carbinolamide **11**.

Stability of Carbinolamide 11 in Aqueous Solution at pH 7.4. The half life of **11** is a few hours in methanol or ethanol, decomposing to secondary anilide **10**. In aprotic solvents it is much longer, and **11** can be purified by silica gel TLC using hexane and ethyl acetate. The decomposition of **11** to **10** in aqueous solution at pH 7.4 and 18 °C follows first-order kinetics (Figure 6) with a half-life of about 8 min, as determined directly by UV spectroscopy at 254 nm or by HPLC analysis of an organic extract. This breakdown of **11** is paralleled by the concomitant formation of formaldehyde, analyzed from the aqueous phase by the DNPH/GC-FID procedure.

Formaldehyde as a Metabolite of Alachlor and Selected Analogues. In preliminary studies the formation of formaldehyde from incubation of **1** with mouse liver microsomes was evaluated by using the Nash and DNPH/GC-FID methods by comparing **1** with several known formaldehyde progenitors at the same concentration. The results indicated that **1** was a better source of formaldehyde than hexamethylphosphoramide and nicotine with formaldehyde yields (Nash procedure) of 26, 8, and 5 mol %, respectively, and 0.38 mg/mL microsomal protein. Alachlor was also better than *p*-nitroanisole and diuron, with formaldehyde yields (DNPH/GC-FID method) of 24, 13, and 11 mol %, respectively, at 0.21 mg/mL microsomal protein. In each case the formation of formaldehyde was NADPH dependent. In a separate experiment with **1**, addition of the MFO inhibitor piperonyl butoxide (12 μM) to the incubation mixture prevented the liberation of formaldehyde.

The best method for quantitation of metabolically formed formaldehyde and its acid-labile progenitors is the DNPH/HPLC procedure. Alachlor and its ethoxy analogue (**4**) yield 66–81 mol % formaldehyde under the standard assay conditions, whereas butachlor (**3**) and metolachlor (**2**) are less favorable substrates with 50 and 22 mol % yields, respectively (Table III).

Free formaldehyde is completely degraded within 60 min on addition of FDH to the incubation mixtures (Figure 5; Table III) thereby providing a method to differentiate free and bound formaldehyde. On this basis, formaldehyde liberated from **2** is completely in the free form in contrast to that from **1**, **3**, and **4**, which give only 34–62% of the

Table III. Effect of Formaldehyde Dehydrogenase on Yield of Formaldehyde from Alachlor and Three Analogues after Incubation with Mouse Liver Microsomes and NADPH

ArN($COCH_2Cl$)R		HCHO,[a] mol %	
no.	R	without FDH	with FDH
Ar = 2,6-diethylphenyl			
1	CH_2OCH_3	81, 74	43, 33
4	$CH_2OCH_2CH_3$	78, 66	30, 19
3	$CH_2O(CH_2)_3CH_3$	55, 45	33, 29
Ar = 2-ethyl-6-methylphenyl			
2	$CH(CH_3)CH_2OCH_3$	25, 18	0.9, −0.8
control (HCHO)		100, 100	1.5, −0.4

[a] HPLC analysis as DNPH derivative relative to that of benzaldehyde added as the internal standard. Values from two separate experiments given in the same order.

Table IV. Isotope Distribution in Formaldehyde Derived from [*N*-CD_2]Alachlor and [*O*-CD_3]Alachlor after Incubation with Mouse Liver Microsomes and NADPH

ArN($COCH_2Cl$)R[a]		isotope distribution,[b] %		
desig	R	HCHO	DCHO	DCDO
N-CD_2	CD_2OCH_3	18 (31)	7 (6)	75 (63)
O-CD_3	CH_2OCD_3	94 (95)	1 (1)	5 (4)

[a] Ar = 2,6-diethylphenyl. [b] Preliminary results based on one experiment. GC/MS-SIM analyses as DNPH derivatives. Values in parentheses are prior to correction for endogenous formaldehyde (analyzed by the DNPH/GC-ECD procedure).

formaldehyde in a form metabolized by FDH. The remainder of the formaldehyde from these compounds either is released late in the incubation (so it is not completely oxidized by FDH) or is present in a bound but acid-labile form.

Isotope Distribution in Formaldehyde as a Metabolite of [*N*-CD_2]- and [*O*-CD_3]-1 (Table IV). Preliminary analyses of the DNPH derivatives by GC/MS-SIM revealed that approximately 75% of the formaldehyde produced from [*N*-CD_2]-**1** contained two deuterium atoms, whereas only 5% of the formaldehyde from [*O*-CD_3]-**1** appeared as DCDO. The latter result is not significantly different from that obtained for unlabeled formaldehyde. Although this preliminary result clearly shows that the *N*-methylene group is the major carbon source for the metabolically generated formaldehyde, it was not considered quantitative; quantitation was subsequently achieved by using [^{13}C]alachlor, as described below.

Formaldehyde Yield from [*N*-$^{13}CH_2$]- and [*O*-$^{13}CH_3$]-1. GC/MS-SIM analyses of the DNPH derivatives revealed that 78–80% of the formaldehyde came from the *N*-methylene substituent and 20–22% from the *O*-methyl group (Figure 7; Table V). In the presence of FDH [N-$^{13}CH_2$]-**1** leads to essentially 100% $H^{13}CHO$ (Table V).

Fate of the Methoxy Group of 1. On the basis of studies with [*O*-$^{14}CH_3$]-**1**, methanol is not a significant metabolite from this carbon center since no more than 1.6% of the radiocarbon appeared as [^{14}C]methanol (analyzed as the 3,5-dinitrobenzoate) under conditions in which the NADPH-dependent metabolism of **1** was 69%. As a control, it was established that methanol is not converted to formaldehyde in the standard microsome–NADPH incubation mixture (Figure 5).

Effect of Alkoxy Substituents on Yield and Isotope Distribution in Formaldehyde as a Metabolite of Alachlor and Analogues. Alachlor and selected analogues with various O-substituents undergo extensive NADPH-dependent metabolism (~65 to >95%) with

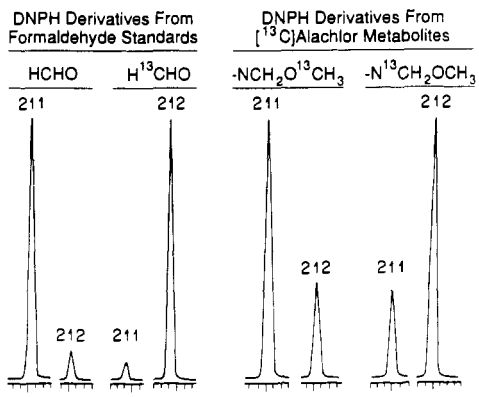

Figure 7. Isotopic composition of formaldehyde as a metabolite of [*N*-$^{13}CH_2$]- or [*O*-$^{13}CH_3$]alachlor analyzed by GC/MS-SIM as the DNPH derivative.

Table V. Isotope Distribution in Formaldehyde from [*N*-$^{13}CH_2$]Alachlor and [*O*-$^{13}CH_3$]Alachlor after Incubation with Mouse Liver Microsomes and NADPH and the Effect of Formaldehyde Dehydrogenase on the Product from [*N*-$^{13}CH_2$]Alachlor

parameter	amount,[a] % H^{12}CHO	amount,[a] % H^{13}CHO
Comparison of Labeling Position[b]		
N-$^{13}CH_2OCH_3$	20 ± 2	80 ± 2
N-$CH_2O^{13}CH_3$	78 ± 1	22 ± 1
Effect of FDH with *N*-$^{13}CH_2OCH_3$[c]		
no FDH	26, 20	74, 80
with FDH	3, −2	97, 102

[a] GC/MS-SIM analyses as DNPH derivatives. [b] Mean and standard error of four separate experiments. [c] Values from two separate experiments given in the same order.

formaldehyde as a significant to major product (20–60 mol %) (Figure 8; Table VI). Acetaldehyde and acetone are also NADPH-dependent metabolites of *O*-ethyl compound **4** and *O*-isopropyl compound **6**, respectively (Figure 8), with yields of ~10% each, as is benzaldehyde (~3%) from **7**. In contrast, propionaldehyde and butyraldehyde were not detected (<1%) as metabolites of **5** and **3**, respectively.

The formaldehyde yields are greatest from alachlor and its *O*-ethyl, *O*-*n*-propyl, and *O*-isopropyl analogues (**1** and **4–6**), lower from the *O*-*n*-butyl, *O*-*tert*-butyl, and *O*-benzyl derivatives (**3**, **8**, and **7**, respectively), and lowest from the *O*-phenyl compound (**9**) and metolachlor (**2**). In each case the formation of formaldehyde was NADPH dependent. For **1**, 74–80% of the total formaldehyde originates from the *N*-$^{13}CH_2$ moiety (Table VI). The values for ^{13}C in H^{13}CHO-DNPH from [*N*-$^{13}CH_2$]-**3** to [*N*-$^{13}CH_2$]-**9** fall in the range 91–98%, and the experimental deviation can be attributed to the contribution of endogenous unlabeled formaldehyde; the slightly lower values of 91–94% ^{13}C for compounds **3**, **7**, and **9** with respect to the other derivatives are associated with lower metabolism and therefore a greater contribution from endogenous formaldehyde (Table VI).

DISCUSSION

This study focuses on the *N*-alkoxyalkyl substituent of the chloroacetanilide herbicides which has been reported as a major site of initial or ultimate metabolic attack in several of the mammals examined (LeBaron et al., 1988; Sharp, 1988). This substituent group is of special interest because it appears to contribute more in percentage terms than does the aromatic ring to labeling of DNA and protein in mice and because it has been postulated to be metabolically degraded to formaldehyde (Brown et al., 1988). Thus, an understanding of the metabolic fate of this portion of the molecule and the mechanisms involved in its degradation may be applicable to a variety of compounds that contains this structural feature.

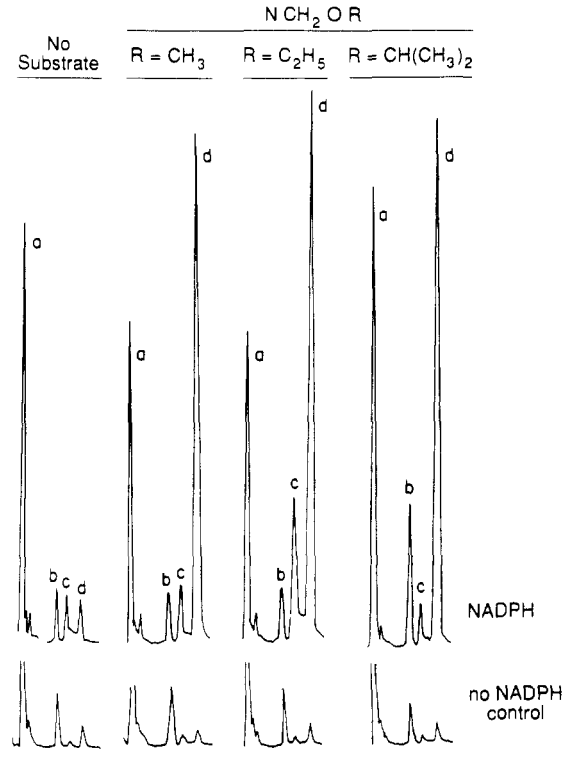

Figure 8. HPLC chromatograms of DNPH derivatives corresponding to formaldehyde, acetaldehyde, and acetone as metabolites of alachlor and its *O*-ethyl and *O*-isopropyl analogues after incubation with mouse liver microsomes alone or with NADPH. Benzaldehyde is the internal standard. HPLC conditions and Rt values are given in Table II. DNPH derivatives: a, PhCHO (int std); b, $(CH_3)_2CO$; c, CH_3CHO; d, HCHO.

Three of the possible pathways for metabolism of the *N*-methoxymethylene substituent of **1** are given in Figure 3. In pathway A hydroxylation of the *N*-methylene substituent is expected to liberate formic acid and methanol but not formaldehyde. Although formic acid was not analyzed, little or no methanol was detected, whereas large amounts of formaldehyde are formed. Since methanol is stable to the incubation procedures and formate in this system would not be reduced to formaldehyde, pathway A either is minor or is not involved. Pathway B centers on *O*-methyl hydroxylation leading to formaldehyde liberation in the subsequent spontaneous decomposition of the side chain. On the basis of the isotope ratios in the liberated formaldehyde, this pathway can account for ~40–60% of the formaldehyde formed from **1**. It is probably also a contributing path with some of the analogues: for **2** in liberating formaldehyde and for the *O*-ethyl, *O*-isopropyl, and *O*-benzyl analogues **4**, **6**, and **7**, in yielding acetaldehyde, acetone, and benzaldehyde, respectively. However, the yields of higher aldehydes or ketone relative to formaldehyde in these cases is too low. In addition, pathway B cannot be involved in the metabolism of the *O*-*tert*-butyl and *O*-phenyl analogues **8** and **9**, since the O-substituent is not susceptible to α-oxidation, yet these compounds are metabolic sources of formaldehyde. Therefore, at least one other pathway must be operating. A third NADPH-dependent (and piperonyl butoxide sensitive) oxidation mechanism (pathway C) involves one-electron oxidation at nitrogen. This overcomes some of the deficiencies associated with path-

Table VI. Effect of Alkoxy Substituents on Yield and Isotope Distribution in Formaldehyde after Incubation of [*N*-$^{13}CH_2$]Alachlor and Several Analogues with Mouse Liver Microsomes and NADPH

ArN($COCH_2Cl$)R			HCHO yield mol %[b]		
no.	R	metabol,[a] %	without NADPH	with NADPH	% ^{13}C[c]
Ar = 2,6-diethylphenyl					
1	$^{13}CH_2OCH_3$	92, 90	0 ± 0	57 ± 8	74, 80
4	$^{13}CH_2OCH_2CH_3$	95, 94	0 ± 0	60 ± 4	96, 98
5	$^{13}CH_2O(CH_2)_2CH_3$	95	1 ± 1	51 ± 4	95, 97
3	$^{13}CH_2O(CH_2)_3CH_3$	87, 88[d]	0 ± 0	38 ± 3	94, 94
6	$^{13}CH_2OCH(CH_3)_2$	95, 96	1 ± 0	58 ± 7	96, 99
7	$^{13}CH_2OCH_2Ph$	~65[d]	3 ± 1	37 ± 6	92, 92
8	$^{13}CH_2OC(CH_3)_3$	>95, 96	1 ± 0	40 ± 6	94, 97
9	$^{13}CH_2OPh$	>68, 73[d]	0 ± 0	20 ± 4	90, 92
10	H	71, 62	1 ± 1	−1 ± 1	
Ar = 2-ethyl-6-methylphenyl					
2	$CH(CH_3)CH_2OCH_3$	95, 97	0 ± 0	20 ± 2	

[a] NADPH-dependent loss of substrate (see Materials and Methods) determined by GC-FID analysis of the DNPH derivatization extract given as results of two separate experiments. [b] HPLC analysis as DNPH derivative relative to that of the internal standard of benzaldehyde (two experiments) or acetone-d_6–DNPH (one experiment) given as mean and standard error of three separate experiments. [c] Values from two separate experiments given in the same relative order. [d] Estimates, due to extensive NADPH-independent loss (see Materials and Methods).

way B since it rationalizes the release of formaldehyde from the *N*-methylene substituent without involving initial oxidation at the *O*-alkyl or *O*-aryl group. This has literature precedent in studies with cytochrome P-450, in which N-oxidation is accompanied by the ejection of an alkyl radical (Augusto et al., 1982; Lee et al., 1988). An analogous reaction for alachlor would extrude a methoxy radical, leaving the *N*-methylene iminium ion. Addition of water would yield the unstable carbinolamide **11**, which would decompose liberating formaldehyde. (Interestingly, the base peak in the CI/MS spectra of **1** at m/z 238 can be assigned as the *N*-methylene iminium ion, although in this situation it is clearly formed by a different process.) One weakness with pathway C is that very little methanol is observed relative to the large amount of formaldehyde formed. However, it is plausible that the implied highly reactive methoxy radical may be trapped by a protein or other cellular constituent before it can abstract a hydrogen atom. In summary, it appears that pathway C dominates with respect to the initial site of oxidation of the *N*-alkoxymethyl substituent of alachlor and several analogues, whereas pathway B may be more important for overall formaldehyde yield in the special case of alachlor since it liberates 2 mol of formaldehyde from the *N*-methoxymethyl substituent.

The *N*-methoxymethyl group of **1** is metabolized to both free and bound formaldehyde. The *O*-methyl group gives 20% of the formaldehyde, and this is in a free form destroyed by FDH. The *N*-methylene group gives 80% of the formaldehyde, some free and some bound under the experimental conditions. One possibility for the nature of the bound formaldehyde is as an adduct formed by reaction of formaldehyde or the methylene iminium ion with protein; comparable reactions may contribute to the observed labeling of DNA and hemoglobin (Brown et al., 1988). Alternatively, any metabolite modified elsewhere in the molecule in such a manner that the modification renders the *N*-alkoxymethyl or *N*-aryloxymethyl substituent unstable to hydrolysis in the strongly acidic medium used for analysis would be categorized as bound formaldehyde; however, the high formaldehyde yields rule against this possibility as a major contributor. A more likely candidate as the source of bound formaldehyde is carbinolamide **11** since it is a probable intermediate in the present in vitro studies and is known to be an in vivo metabolite which is excreted as its *O*-glucuronide (Brown et al., 1988; Feng and Patanella, 1989). Although **11** cleaves to **10** and formaldehyde in aqueous medium at physiological pH within minutes of its metabolic formation, in vivo it may be somewhat protected from hydrolysis by localization in membrane lipids. In the present study, **11** could not be detected by HPLC–UV, even when organic solvent extracts were analyzed.

Alachlor and several of its analogues are formaldehyde progenitors. Their oncogenic effects are conceivably due to this metabolite, which is a known carcinogen (Zimmerman, 1988) and which forms DNA–protein cross-links (Wilkins and MacLeod, 1976) and binds to DNA (McGhee and von Hippel, 1977; Le Botlan, 1989). However, alternative explanations are also possible, and an electrophilic intermediate such as the *N*-methylene iminium ion, if formed, may contribute to the overall toxicological properties of **1** and related compounds.

ABBREVIATIONS USED

CI, chemical ionization; DNPH, 2,4-dinitrophenylhydrazine; ECD, electron capture detector; EI, electron impact; FDH, formaldehyde dehydrogenase (EC 1.2.1.46); FID, flame ionization detector; GC, gas chromatography; HCHO, formaldehyde; HCHO-DNPH, the 2,4-dinitrophenylhydrazone of formaldehyde; HPLC, high-performance liquid chromatography; IR, infrared; MFO, mixed-function oxidase; MS, mass spectrometry; SIM, selected ion monitoring; UV, ultraviolet.

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