Synthesis of Haptens and Derivation of Monoclonal Antibodies for Immunoassay of the Phenylurea Herbicide Diuron†

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INTRODUCTION

Diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is one of a family of arylurea herbicides. The first of these—monuron, fenuron, diuron, neburon—were developed in the early 1950s and have been in common use in agriculture since the 1960s. Subsequently, numerous related compounds have been tested for herbicidal activity. Their properties and uses are summarized in review articles by Maier-Bode and Hartel (1981) and Smith and Grover (1982).

Diuron and other arylurea herbicides are used extensively throughout the United States and in many other countries. Higher concentrations are applied as soil sterilants on noncrop lands, industrial sites, and rights-of-way, and smaller amounts are used for selective pre- or post-emergence control of grasses and broadleaf weeds among vegetables, potatoes, cotton, corn, beans, cereals, and ornamentals and in orchards and vineyards. They have also been used to control aquatic plants. Runoff and leaching from agricultural use may contaminate groundwater and surface water. In 1990, 40 of 782 wells sampled in California were contaminated with 0.06–3.95 ppb of diuron from nearby legal agricultural application (Miller et al., 1990).

The arylureas have low to moderate mammalian toxicity (Smith and Grover, 1982; Ware, 1983), but parts per million (ppm) amounts of phenylureas may affect embryonic and neonatal development of some fish and aquatic invertebrates (Call et al., 1987). Little is known about diuron toxicity in humans, but the Environmental Protection Agency’s Lifetime Health Advisory Level (HAL) for diuron has been set at 10 ppb (Miller et al., 1990; Van Boven et al., 1990). This necessitates monitoring for residues in commodity and environmental matrices. The legal tolerance for diuron residues in meat and most commodities is 1–2 ppm but is as low as 100 ppb in commodities such as bananas, peaches, and nuts (U.S. Department of Agriculture, 1987; Duggan, 1988).

Standard analytical methods for detecting diuron include spectrophotometry, gas chromatography/mass spectrometry (GC/MS), and high-performance liquid chromatography (HPLC). The HPLC detection limit of 0.1–0.5 ppb of diuron in water (Smith and Grover, 1982; Goewie and Hogendoorn, 1987) is not sufficiently sensitive for the requirements of some regulatory agencies. Diuron and other substituted ureas are difficult to analyze by routine GC/MS because they are thermally unstable and decompose, although newer instrumental techniques may reduce or eliminate this problem (Mattern et al., 1989; Tamiri and Zitrin, 1987). Breakdown products (3,4-dichlorophenyl isocyanate) or thermolabile derivatives are often measured rather than the parent compound itself (Van Boven et al., 1990; Goewie and Hogendoorn, 1985; Zahnov, 1987).

A rapid and reliable immunoassay for diuron and its analogs would improve the ability of regulatory agencies to monitor use and disposal. Quantitative enzyme immunoassays (EIAs) are an ideal primary screening method for three reasons: first, EIAs overcome technical difficulties encountered with decomposition of the analytes in
GC/MS; second, many more controls, replicates, and dilutions can be run in each EIA at about 1/10 the cost per sample compared with HPLC or GC/MS.

To develop a monoclonal immunoassay for diuron, we first undertook synthesis of hapten by a scheme that would be applicable to virtually all of the phenylurea herbicides, that would present the hapten in two different ways, and that would accommodate different lengths and structures of spacer arms. We tested the different presentation to identify the best combination of immunizing and screening antigens, and from these we derived a panel of specific monoclonal antibodies (MAbs) and indirect and direct competition EIA. The EIA are accurate, cost-effective, robust, and compatible with established procedures for recovery of triazines, bromacil, and other leachable herbicides from groundwater. The MAbs show differences in the recognition of monouron that may prove to be useful in identifying which phenylurea is present in a sample. They should also be useful in immunosensors, affinity columns, and other formats for environmental monitoring and dosimetry.

MATERIALS AND METHODS

Chemicals. All organic starting materials for the hapten syntheses were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Thin-layer chromatography (TLC) utilized plastic sheets from E. Merck (Darmstadt, Germany). Plates were eluted with solvent system A (tetrabutyroluron-ethyl acetate-hexane:212:35:5 v/v/v). or other solvents, one of which was indicated, a Varian EM-390 90-MHz spectrometer (Varian, Inc., Phillipsburg, NJ, USA).

Arylurea and carbamate herbicide standards >99% pure were purchased from AccuStandard, Inc. (New Haven, CT). Reference solutions of 1 mg/mL were prepared in methanol, standardized by UV spectrophotometry, and stored at 4 °C in Teflon vials (Pierce Chemical Co.). Dilutions were prepared in PBS-Tween containing 10% methanol (0.01 M KHzPO4-KHzPO7, pH 7.4-7.8), with occasional addition of 2-mercaptoethanol. Unless otherwise specified, the EIA were performed in Immulon 2 plates (Dynatech, Chantilly, VA). Enzyme–antibody conjugates were obtained from Sigma Chemical Co. (St. Louis, MO) or Boehringer-Mannheim (Indianapolis, IN). Solvents used in this work were spectrograde, and all other chemicals were analytical reagent grade or better. Cell culture media and additives were purchased from Gibco Laboratories (New York), and fetal bovine serum was from Invitrogen, Inc. (Karlsruhe, Germany). Enzyme immunization and screening antigens, and from these we derived established procedures for recovery of triazines, bromacil, and other leachable herbicides from groundwater.

Apparatus for Hapten Synthesis. Melting points were determined with a Thomas Hoover apparatus and are uncorrected. Infrared spectra (IR) were determined on an IBM IR/32 FTIR spectrometer (IBM Corp., Danbury, CT). Most 1H and 13C NMR were measured (NMR) spectra, at 300.1 and 75.5 MHz, respectively, were measured on a General Electric QE-300 spectrometer (Bruker NMR, Billerica, MA); where indicated, a Varian EM-390 90-MHz spectrometer (Varian Associates, Palo Alto, CA) was used. Chemical shift values are given in ppm downfield from internal tetramethylsilane except where noted that 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid, sodium salt, was the standard. Fast atom bombardment, high-resolution (FAB-HRMS) and electron impact high-resolution (EI-HRMS) at 70 eV mass spectra were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.). FAB experiments utilized xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol as the matrix; polyethylene glycol 300 was added to the matrix as a mass calibrant. Low-resolution impact mass spectra (MS) were obtained on a Trio 2 mass spectrometer (VG Masslab, Altrincham, U.K.) or the ZAB-HS-2F instrument at 70 eV.

Hapten Synthesis. Hapten of diuron with spacer arms of three or five carbons were prepared for conjugation to carriers, enzymes, or other ligands for use as immunizing and screening agents (Figure 1). Idealized gas-phase molecular models of ethyl and the hapten were constructed in Chem3D Plus on a Macintosh Iic computer. The conformations were minimized for structural error and free energy using the Chem3D Plus implementation of Allinger's MM2 force field (Burkert and Allinger, 1982; Cambridge Scientific Computing, 1990).

Ethyl 4-[(1,4-Dichlorophenyl)amino]butanolate. Following the procedure of Wie et al. (1982), a solution of 5.24 g (20.0 mmol) of 3,4-dichlorophenylacetic acid, 9.00 g (50.0 mmol) of ethyl 4-bromobutanol, and 2.72 g (20.0 mmol) of sodium acetate trihydrate in 5 mL of ethanol was heated under reflux for 13 h. The cooled mixture was poured into 150 mL of ice/water and extracted with ether (3 × 50 mL). The combined ether extract was dried (Na2SO4) and concentrated to provide 5.35 g of a dark brown oil. This six-component (TLC) mixture was flash chromatographed on 125 g of silica gel by elution with 7.1 (v/v) hexane-ethyl acetate. The 1.24 g of crude product was recrystallized from 95% ethanol to provide 0.92 g (17%) of pure ester as white crystals: mp 71.0-72.0 °C; TLC Rf 0.62 (eluant solvent A); IR (KBr) 3934 (s, NH), 3364 (s, NH), 1713 (s, C=O, ester), 1191 (vs, CO), 1106 (m, CO) cm-1; 1H NMR (DMSO-d6) 5.72 (d, J = 8.8 Hz, 1 H, Ar-H2), 6.72 (d, J = 2.7 Hz, 1 H, Ar-H2), 6.54 (dd, J = 2.7, 8.8 Hz, 1 H, Ar-H2), 6.16 (t, J = 5.5 Hz, 1 H, NH), 4.06 (q, J = 6.1 Hz, 2 H, CH2O), 3.01 (dt, J = 5.5, 7.0 Hz, 2 H, CH2N), 2.35 (t, J = 7.4 Hz, 2 H, CH2N), 2.18 (t, J = 7.4 Hz, 2 H, C2H5), 1.18 (t, J = 7.1 Hz, 3 H, CH3), the (6.16 ppm peaks disappeared and the 3.01 ppm peaks became a single triplet with added D2O); 13C NMR (DMSO-d6) δ 172.7 (ester C=O), 149.0 (Ar-C=C), 131.4 (Ar-C=C), 121.5 (Ar-C=C), 112.4 (Ar-C), 59.8 (CH2O), 41.9 (CH2N, C2), 31.1 (CH2CO2H), 23.9 (C2), 14.2 (CH3).

(3,4-Dichlorophenyl)-[3-(ethylcarboxypropyl)]-3,3-dimethylurea. A solution of 3.65 g (5.0 mmol) of the above ester, 3.0 mL of pyridine, and 4.0 mL (43 mmol) of 3-(trimethylsilyl)propanoic-2,2,3,3-d4 acid, sodium salt (TSP), was the standard. Fast atom bombardment, high-resolution (FAB-HRMS) and electron impact high-resolution (EI-HRMS) at 70 eV mass spectra were obtained on a ZAB-HS-2F mass spectrometer (VG Analytical, Wythenshawe, U.K.). FAB experiments utilized xenon (8 keV, 1 mA) for ionization and 3-nitrobenzyl alcohol as the matrix; polyethylene glycol 300 was added to the matrix as a mass calibrant. Low-resolution impact mass spectra (MS) were obtained on a Trio 2 mass spectrometer (VG Masslab, Altrincham, U.K.) or the ZAB-HS-2F instrument at 70 eV.

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Ethyl 6-(3,4-Dichlorophenylamino)hexanoate. A mixture of 3.2 g (20.0 mmol) of 3,4-dichloroaniline, 8.92 g (40.0 mol) of ethyl 6-bromohexanoate, 3.00 g (22.0 mmol) of sodium acetate trihydrate, and 5.0 mL of dimethyl sulfoxide was heated at 80 °C under a nitrogen atmosphere with vigorous stirring for 13 h. At this time a TLC indicated that most of the aniline had disappeared and at least five new products had formed. The mixture was poured into 100 mL of ice/water and extracted with chloroform (3 × 100 mL). The combined chloroform extract was washed sequentially with 5.0 mL of dimethyl sulfoxide and 5.0 mL of sodium bicarbonate solution (2.0 M Na2CO3). The extract was then dried with sodium sulfate (25 mL) and saturated sodium chloride (25 mL) and dried (Na2SO4). Evaporation of the solvent left 10.76 g of an amber oil consisting of six components as determined by TLC. Flash chromatography of 1.0 g of this oil on 30 g of silica gel with elution by hexane–ethyl acetate (7:1 v/v) provided 0.51 g of crude ester as a yellow semisolid.

Recrystallization from 95% ethanol gave 0.19 g (33%) of pure ester as white crystals: mp 67–68 °C; IR (KBr) 3360 (OH), 1714 (C=O, ester), 1178 (COO−, CO32−) cm−1; 1H NMR (DMSO-d6) δ 7.22 (d, J = 8.8 Hz, 1 H, Ar-Hα), 6.67 (dd, J = 5.6, 2.6 Hz, 1 H, Ar-Hβ), 6.53 (dd, J = 7.2, 2.6 Hz, 2 H, CH2-CH2), 6.06 (t, J = 5.3 Hz, 1 H, NH), 4.05 (q, J = 7.1 Hz, 2 H, CH2O), 2.96 (dt, J = 5.6, 3.8 Hz, 2 H, CH2N), 2.29 (t, J = 7.3 Hz, 2 H, CH2O), 1.5 (m, 4 H, CH2CH2), 1.17 (t, J = 7.1 Hz, 3 H, CH3) (the 6.06 ppm peak disappeared and the 2.6 ppm peaks became a single triplet with added D2O). The bulk of the crude product was crystallized once from 95% ethanol to provide 1.94 g of slightly impure ester as pale yellow crystals. Although TLC showed traces of three other components, this material was found to be suitable for subsequent syntheses.

1-(3,4-Dichlorophenyl)-1-[5-(ethylcarboxy)pentyl]-3,3-dimethyleurea. To 0.916 g (3.00 mmol) of the hexanoate ester and 3.0 mL of dry pyridine in 10 mL of dry acetonitrile under nitrogen, 5.0 mL (4.7 g, 43 mmol) of dimethylcarbamyl chloride over 1 min. The heterogeneous mixture was agitated vigorously on a vortex mixer for 0.5 h and then allowed to stand overnight at room temperature. A small precipitate was obtained by filtration through Celite, and the filtrate was acidified with 6 M hydrochloric acid to pH 1. The resultant white precipitate was collected, washed thoroughly with water, and vacuum dried to obtain 1.73 g (95%) of crude diuron I. Recrystallization from acetone deprived it of 1.66 g (91%) of glistening white crystals: mp 161.5–162.8 °C (dec with gas evolution) [lit. (Newcombe and Collins, 1960) mp 125–125 °C; TLC Rf = 0.05 (eluent solvent A), 0.42 (eluent ethyl acetate–chloroform (5:5 v/v))].

The bulk of the crude product was crystallized once from 95% ethanol–ether (1:1 v/v) to provide 1.94 g of slightly impure ester as pale yellow crystals. Although TLC showed traces of three other components, this material was found to be suitable for subsequent syntheses.

1-(3,4-Dichlorophenyl)-1-[5-(ethylcarboxy)pentyl]-3,3-dimethyleurea. To 0.922 g (6.00 mmol), was dissolved in 12.0 mL of 1.0 M sodium hydroxide solution, whereafter 1.13 g (6.01 mmol) of powdered 3,4-dichlorophenyl isocyanate was added. The heterogeneous mixture was agitated vigorously on a vortex mixer for 0.5 h and then allowed to stand overnight at room temperature. A small amount of precipitate was removed by filtration through Celite, and the filtrate was acidified with 6 M hydrochloric acid to pH 1. The resultant white precipitate was collected, washed thoroughly with water, and vacuum dried to obtain 1.73 g (95%) of crude diuron II. Recrystallization from acetone deprived it of 1.66 g (91%) of glistening white crystals: mp 161.5–162.8 °C (dec with gas evolution) [lit. (Newcombe and Collins, 1960) mp 125–125 °C; TLC Rf = 0.05 (eluent solvent A), 0.42 (eluent ethyl acetate–chloroform (5:5 v/v))].

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(30, Cl\textsubscript{2}C\textsubscript{2}H\textsubscript{5}NCO – CO); EI-HRMS m/z calc for C\textsubscript{14}H\textsubscript{30}Cl\textsubscript{2}N\textsubscript{2}O\textsubscript{4} 332.0684, obsd 332.0685.

**Conjugation of Haptens to Proteins.** The diuron 1–III haptens were attached to the protein carriers bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH) using an N-hydroxysuccinimide activated ester. These conjugates were used as immunizing antigens and screening antigens in an indirect competition EIA. Using the same chemistry, the haptens were also attached to calf intestine alkaline phosphatase for use as a detecting probe in a direct competition EIA. The protein conjugation chemistry is schematized in Figure 2 and may be summarized as follows:

**Diuron–BSA and Diuron–KLH Conjugates.** The reactions were conducted in 1.5-mL microtubes containing Teflon stir bars. To 0.25 mL of the hapten–succinimide solution were added 1.0 mL of dimethylformamide (DMF, dried over molecular sieves) containing 0.202 mmol of N-hydroxysuccinimide and 0.223 mmol of 1,3-dicyclohexylcarbodiimide (DCC). The mixture was stirred at room temperature for 3.5 h and centrifuged to remove precipitated urea. The precipitate was discarded; the solution is referred to as the “active ester supernate.” Portions of the activated esters were stored at -78 °C.

Fifty milligrams of BSA (fraction V, Sigma A-6773) or KLH (tissue VIII, Sigma H-7677) was dissolved in 5.0 mL of borate buffer (0.05 M sodium borate-10H\textsubscript{2}O-0.9% NaN\textsubscript{3}-0.02% Na\textsubscript{2}HPO\textsubscript{4}, pH 7.8) in a 10-mL glass vial with a Teflon “flea” stir bar. This solution was allowed to stir vigorously, and 1.05 mL of dry DMF was added very slowly to bring the protein solution to 20% in DMF. No visible precipitation occurred during this addition. The resulting solution was stirred at room temperature and 0.25 mL of the active ester supernate was added, a few microliters at a time, very slowly over a 20-min period. A small amount of precipitate developed during this addition. The solution was stirred gently overnight at 4 °C and then dialyzed vs. 0.2 M phosphate-buffered saline (PBS), pH 7.5, in a SpectraPor membrane with pores of 12 000-14 000 MW cutoff (six changes of 4 L each). The dialyzed solution was transferred into polypropylene vials. The other BSA and KLH conjugates of the hapten active esters were prepared in essentially the same manner.

**Diuron–Alkaline Phosphatase (AP) Conjugates.** An activated ester was prepared similar to diuron I, but with a five-carbon (hexanoic acid) spacer (Figure 1, scheme A) instead of the three-carbon (butanoic acid) spacer. A solution of 10\textsuperscript{4} DEA units of alkaline phosphatase (Sigma catalog no. P00405; type VII-NT from bovine intestinal mucosa (the solution was purchased after second DC pulse, 30 ps; SSH-1 somatic hybridizer (Shmadzu Precision Instruments, Inc., Kyoto, Japan). The specific fusion conditions were as follows: temperature 36 °C; field strength, 2.30 kV/cm; first AC aligning pulse, 1 MHz, 40 V, 30 s; first rectangular DC fusion pulse, 460 V, width 20 μs; second AC aligning pulse, 40 V, 1 s; second rectangular DC fusion pulse, 460 V, width 20 μs; delay period after second DC pulse, 30 s. The decay rate for aligning and fusing pulses was 100% (instantaneous cut-off). The cultures were allowed to remain at 35 °C for 15 min after fusion to promote repair, and the cells were then resuspended in culture medium and seeded in 96-well plates at a density of approximately 3 X 10\textsuperscript{4} cells per well; 10 656 wells (111 960 cell plates) were seeded. The aminopterin was added 24 h later.

**Media and Cell Culture.** The “complete medium” for hybridoma culture was Iscove’s modified Dulbecco’s medium (IMDM), supplemented with 20% (v/v) fetal bovine serum (FBS), 10 μg/mL kanamycin sulfate, 5 X 10\textsuperscript{-6} M-mercaptoethanol, and 1 μg/mL of 1:3 iron-saturated transferrin (human type III, Sigma) and Iscove’s lipid emulsion (Iscove and Melchers, 1978; Iscove, 1984). Hybridomas were selected in complete medium containing 10% (v/v) J774.A1 macrophage-conditioned medium (Sugawara et al., 1985), 10-4 M hypoxanthine, 8 X 10-7 M aminopterin, and 3 X 10-4 M thymidine (HAT). Myelomas were adapted to grow in medium with hypoxanthine and thymidine prior to fusion, and aminopterin was added to the hybridoma medium 24 h postfuson. The hybridomas were adapted to grow in complete IMDM without emulsion, macrophage medium, or HAT after they were expanded to 24-well culture plates. Cultures were grown at 37 °C in 5% CO\textsubscript{2} and saturating humidity. Hybridomas were frozen at 10\textsuperscript{6} cells/mL in medium consisting of complete IMDM–FBS–dimethyl sulfoxide (6:3:1) using a Cryomed 910 programmable cell freezer and then stored in liquid nitrogen.

**Hybridoma Production.** The mice were euthanized, and splenocytes were recovered by standard asceptic techniques. Red blood cells were lysed by incubation of the spleen cells in 0.017 M Tris-HCl, pH 7.2-0.17 M NH\textsubscript{4}Cl, and the lymphocytes were recovered by centrifugation (800g, 10 min) through 1 mL of sterile fetal bovine serum. These cells were counted and washed twice by suspension in electofusion buffer (0.25 M glucose–0.1 mM calcium acetate-0.1 mM magnesium acetate-1.0 mM KH\textsubscript{2}PO\textsubscript{4}, pH 7.4) and resuspended by centrifugation in fusion buffer. Hybridomas were prepared by electrical fusion, with P3X63Ag8.653 myelomas as fusion partners. The myelomas were collected by centrifugation (800g, 10 min, room temperature), resuspended at 10\textsuperscript{6} cells/mL in electofusion buffer containing 0.6 unit/mL of freshly dissolved dispase (neutral protease from Bacillus polymyxa; Boehringer Mannheim), incubated for 15 min at room temperature, and then recovered by centrifugation. The treated cells were resuspended and washed twice by centrifugation in fusion buffer. All of the splenocytes were added to myelomas to give a ratio of five splenocytes per myeloma. Aliquots (0.8 mL) of the cell suspension were then fused in the FTC-03 chamber of an SSH-1 somatic hybridizer (Shimadzu Precision Instruments, Inc., Kyoto, Japan). The specific fusion conditions were as follows: temperature 36 °C; field strength, 2.30 kV/cm; first AC aligning pulse, 1 MHz, 40 V, 30 s; first rectangular DC fusion pulse, 460 V, width 20 μs; second AC aligning pulse, 40 V, 1 s; second rectangular DC fusion pulse, 460 V, width 20 μs; delay period after second DC pulse, 30 s. The decay rate for aligning and fusing pulses was 100% (instantaneous cut-off). The cultures were allowed to remain at 35 °C for 15 min after fusion to promote repair, and the cells were then resuspended in culture medium and seeded in 96-well plates at a density of approximately 3 X 10\textsuperscript{4} cells per well; 10 656 wells (111 960 cell plates) were seeded. The aminopterin was added 24 h later.

**Immunization and Monitoring of Mice.** Pairs of mice of three strains (Swiss Webster, Biozzi, and B10.Q) were immunized with KLH conjugates of haptens I–III. The initial immunizing doses consisted of 50 μg of conjugate (as carrier protein) in 0.1 mL of physiological saline, and one mouse dose (approximately 50 μg) of Ribi adjuvant (MPL + TDM emulsion, Ribi Immunochem Research, Inc., Hamilton, MT). Booster doses given 7 and 2 days after the first dose consisted of 25 μg of conjugate protein in saline with Ribi adjuvant. Immunizations were subcutaneous, in three or four sites on the back of the mouse. Sera were taken on the 29th day after the first injection. Titters were determined by indirect EIA and sera further analyzed by competition EIA. Mice selected for hybridoma production were “hyperimmunized” by intravenous (tail vein) injection of 50 μg of conjugate in 0.05 mL of saline 3 days prior to cell fusion and about 45 days after the first immunizations. To lessen the risk of autoantibody formation, delayed-type hypersensitive mice were given a subcutaneous injection of antihistamine and antivasopasmin drugs 1 h before the intravenous hyperimmunizing boost (Karu, 1993).
source. The next day, the mice received approximately 10⁶ log-phase hybridoma cells in 0.5 mL of sterile IMDM salt solution. Ascites fluids were harvested by peritoneal tap with an 18-gauge needle from the seventh day after the cells were introduced.

**MAb Subtyping and Purification.** Immunoglobulin subclass was determined by EIA using a commercial kit (Southern Biotechnology Associates, Birmingham, AL). IgG antibodies were purified by affinity chromatography of protein A or protein G sepharose (Pierce or Pharmacia).

**Enzyme Immunoassays.** The EIAs were done essentially as described by Volier et al. (1976). A standard indirect EIA (immobilized hapten conjugate) was used to evaluate the response of mice to the various immunizing conjugates and to screen the MABs. This format as well as a direct (immobilized antibody) format was used for competition EIAs.

For the indirect EIAs, conjugates were adsorbed to EIA plates in "coating buffer" (0.015 M Na₂CO₃-0.035 M NaHCO₃-0.003 M NaNO₃, pH 9.6). The diluent for antibodies and antibody-analyte mixtures was PBS-Tween-BGG-methanol (0.01 M K₃PO₄-K₂HPO₄, pH 7.4-0.15 M NaCl-0.02% NaN₃-0.08% Tween 20-10% methanol-0.1% bovine γ-globulin). The detecting antibody in indirect EIAs was alkaline phosphatase conjugated to goat anti-mouse IgG. The substrate solution for color development was p-nitrophenyl phosphate (Sigma 104 substrate tablets) 1 mg/mL in 10% (w/v) diethanolamine-HCl, pH 9.8-0.4 M MgCl₂-3 mM NaN₃. Individual EIA plates were tightly covered with "coating buffer" (0.015 M Na₂CO₃-0.035 M NaHCO₃-0.003 M NaNO₃, pH 9.6). The diluent for antibodies and antibody-analyte mixtures was PBS-Tween-BGG-methanol (0.01 M K₃PO₄-K₂HPO₄, pH 7.4-0.15 M NaCl-0.02% NaN₃-0.08% Tween 20-10% methanol-0.1% bovine γ-globulin). The detecting antibody in indirect EIAs was alkaline phosphatase conjugated to goat anti-mouse IgG. The substrate solution for color development was p-nitrophenyl phosphate (Sigma 104 substrate tablets) 1 mg/mL in 10% (w/v) diethanolamine-HCl, pH 9.8-0.4 M MgCl₂-3 mM NaN₃. Individual EIA plates were tightly covered to prevent evaporation during incubation periods. Between steps, the plates were washed three times with PBS-Tween from a squeeze bottle and dried by rapping on lint-free towels. "Blocking solutions" to prevent adventitious binding were dissolved in PBS-Tween and filtered (0.45 μm) to remove aggregates before use.

An indirect competition EIA was used to quantify the phenylureas and to compare assay conditions and recognition by different antibodies. Subsaturating amounts of diuron-BSA conjugate and hybridoma culture supernate were determined by indirect EIA. Immuno 2 wells were coated overnight at 4 °C with a limiting amount of diuron-BSA conjugate (20-250 ng of carrier protein) in 0.1 mL of coating buffer. Standards (0.05-200 ppb) or unknowns were mixed with the limiting dilution of antiserum or hybridoma culture fluid in the diluent and incubated overnight at room temperature in tightly sealed polycarbonate tubes. The coated wells were washed, and aliquots (0.1 mL) of the antibody-analyte mixture were applied. After 2 h at room temperature, the wells were washed and the remainder of the assay performed as described above. When competitive binding occurred, color development was inversely proportional to the amount of phenylureas in the sample.

For the direct competition EIA format with hybridoma culture supernate or ascites fluids, wells were coated with 20-200 ng of affinity-purified goat antimouse IgG in the coating buffer at 4 °C overnight and then incubated for 2 h at room temperature with the fluid containing MAB. This EIA could also be done by coating wells directly with 20 ng of affinity-purified MAB. The plates were then washed with PBS-Tween, and 0.1 mL of the fluid containing the analyte and amount of diuron I-AP conjugate was added for 1 h at room temperature. After this step, the plates were washed again, alkaline phosphatase substrate was added, and the rate of color development was recorded.

**Data Analysis.** Color development was measured on a Multiskan EIA reader (Flow Laboratories) interfaced with a Macintosh computer, and the rates of the reaction (ΔA/min x 10⁹) were calculated by linear regression. Competition EIA dose-response curves were fitted using the four parameter logistic model, and the data were analyzed as described previously (Schmidt et al., 1990).

**RESULTS**

**Synthesis of Haptens and Conjugates.** The structures and synthetic routes for the two synthesis schemes we used are shown in Figure 1. In scheme A, the diuron molecule was derivatized at the terminal nitrogen of the urea moiety with a methylene carbon chain to provide adequate separation from the carrier protein. In the second series of haptens (scheme B), the spacer arm was attached at the internal nitrogen of the urea moiety. Haptens with methylene spacers of three and five carbons were made by following scheme A, and a hapten with a three-carbon methylene spacer was made by following scheme B. We refer to these as the diuron I, diuron II, and diuron III haptns, respectively (Figure 1, bottom row).

These strategies were designed to provide two different presentations of diuron, or any similar aryureas, with spacer arms of virtually any length and structure. Our scheme A resembles one originally used by Newsome and Collins (1980). Scheme B was designed to preserve both the dichlorophenyl and dimethylurea moieties as potential epitopes. Molecular modeling of the energy-minimized idealized structures indicated that derivatization which replaces the hydrogen on the internal nitrogen of diuron is likely to somewhat alter the electronic and steric properties. In the energy-minimized conformational models at the bottom of Figure 1, the carbonyl oxygen in the diuron I hapten lies at an angle to the dichlorophenyl ring. This oxygen is nearly coplanar with the ring in diuron II and diuron III.

The activated diuron esters shown in Figure 2 retained their reactivity after storage for several months at -70 °C. The active esters were also made efficiently in a modified coupling reaction using the water-soluble N-hydroxysulfosuccinimid and 1-ethyl-3-(3’-dimethylaminopropyl)-carbodiimide, essentially as described by Staros et al. (1986) and Klibanov et al. (1989). The KLH and BSA conjugates could be stored at 4 °C for more than a year in phosphate-buffered saline containing 0.02% NaN₃, with no loss of activity.

**Responses of Mice to the Diuron Conjugates.** To test the response of each hapten, pairs of Swiss Webster, Biozzi, and B10.Q mice were immunized with KLH conjugates of haptens diuron I, I, or III. Hapten-specific antibody titer was determined on EIA plates coated with diuron-BSA conjugates that had spacers different from those of the immunizing conjugate. In this assay, all three Diuron-KLH conjugates evoked high-titer anti-hapten responses in all of the mice. Serum dilutions of 1000-10000-fold contained sufficient antibody to give a strong response in an indirect competition EIA. The antisera differed dramatically in their ability to bind free diuron in the competition EIAs (Table 1). Sera from mice immunized with diuron II-KLH gave the most sensitive competition EIAs, with half-maximal inhibition (Id₅₀ values of 27-247 ppb, on EIA plates coated with diuron I-BSA. No significant competitive binding of diuron occurred when diuron I was the immunizing hapten and diuron III was the screening hapten or vice versa. On the basis of these results, one Biozzi mouse and one Swiss Webster mouse immunized with diuron II-KLH were selected for hybridoma production.

**Properties of the Hybridomas.** On the basis of the results of the serum tests, the hybridoma supernates were

<table>
<thead>
<tr>
<th>immunizing conjugate (KLH)</th>
<th>screening conjugate (BSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>940-NC*</td>
</tr>
<tr>
<td>II</td>
<td>27-247</td>
</tr>
<tr>
<td>III</td>
<td>2790-NC</td>
</tr>
<tr>
<td></td>
<td>475-1660</td>
</tr>
</tbody>
</table>

* NC, no competition with up to 2 ppm of diuron.
SCHEME A

\[
\text{Cl}\text{Cl}\text{N=CCl} + \text{CH}_3\text{NH}(\text{CH}_2)_n\text{COOH} \rightarrow \text{Cl}\text{Cl}\text{N=CCl}(\text{CH}_2)_n\text{COOH} \quad (n = 3 \text{ or } 5)
\]

SCHEME B

1) (\text{CH}_3)_2\text{NCCl}

2) HCl, H\text{H}_2\text{O}

\[
(n = 3 \text{ or } 5)
\]

Figure 1. (Top) Structure and conformation of diuron. (Center) The two hapten synthesis schemes detailed under Materials and Methods. (Bottom) Energy-minimized conformations of the three haptens used for immunization and screening.

Figure 2. Formation of the N-hydroxysuccinimide active esters of the haptens, and the subsequent conjugation to protein carriers, as described under Materials and Methods.

initially screened for antibodies that bound to diuron-I-BSA. Table 2 summarizes some properties of the eight MAbs that most sensitively recognized diuron. Two MAbs were derived from the Swiss Webster mouse, and the remaining six MAbs came from the Biozzi mouse. All of the MAbs were of the IgG subclasses, which can be easily purified to near homogeneity with commercially available affinity media. The most sensitive competitive binding of diuron by all of the MAbs occurred when the coating antigen was diuron I-BSA, as with the original mouse sera.
Table 3. Relative Cross-Reactivity of MAbs with Other Phenylureas and Similar Aryl Carbamates

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MAb 21</th>
<th>MAb 60</th>
<th>MAb 195</th>
<th>MAb 202</th>
<th>MAb 275</th>
<th>MAb 481</th>
<th>MAb 488</th>
<th>MAb 520</th>
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<tbody>
<tr>
<td>diuron</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>linuron</td>
<td>40.0</td>
<td>61.1</td>
<td>3.5</td>
<td>7.4</td>
<td>31.3</td>
<td>2.7</td>
<td>8.7</td>
<td>3.0</td>
</tr>
<tr>
<td>monuron</td>
<td>2.4</td>
<td>1.4</td>
<td>3.4</td>
<td>3.5</td>
<td>1.7</td>
<td>3.2</td>
<td>5.2</td>
<td>2.8</td>
</tr>
<tr>
<td>fenuron</td>
<td>NC</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>NC</td>
<td>NC</td>
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</tr>
<tr>
<td>siduron</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>tebuthiuron</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
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<td>NC</td>
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<tr>
<td>propham</td>
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<td>NC</td>
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<tr>
<td>barban</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* NC, no competition up to 5 ppm.

The cross-reactivity of each MAb with other phenylurea moieties is the primary recognition feature. However, the urea moiety also influences binding; linuron differs from diuron only by having one N-methoxy and one N-methyl group. None of the eight MAbs showed any significant binding of 1,2-dichlorobenzene or 1,3-dichlorobenzene (data not shown). The metabolites 3,4-dichloroaniline, 3,4-dichlorophenylurea, and 1-(3,4-dichlorophenyl)-3-methylurea in amounts up to 1 ppm did not compete with diuron I-AP for binding to MAb 481 in the direct EIA (data not shown). None of the MAbs reacted with the triazine herbicides atrazine and simazine or with bromacil (data not shown). Thus, the MAbs appear to be suitable for quantifying diuron in the presence of other leachable herbicides that may be found in environmental samples.

Comparison of Direct and Indirect Competition EIAs. All eight MAbs competitively bound diuron in the direct competition EIA using diuron I-AP conjugate but with less sensitivity than in the indirect EIA. Cross-reactivities of the other phenylureas were not significantly different in the two assays.

The precompetition and competition steps of the indirect EIA could be as short as 2 h, and the competition step in the direct EIA could be as short as 1 h without affecting the I50. Bovine γ-globulin was superior to bovine serum albumin, gelatin, and nonfat dry milk as a blocking agent in these assays. Coefficients of variation of 10% or less were routinely obtained using hybridoma culture fluid, ascites fluid, or purified IgG.

Figure 3. Effect of methanol on the indirect competition EIA. Limiting dilutions of the indicated MAb and various amounts of diuron were incubated at room temperature overnight in PBS-Tween containing the indicated amounts of methanol. These mixtures were applied to wells coated with 25 ng of diuron I-BSA, and the indirect competition EIA was conducted as described under Materials and Methods.

The effect of methanol was tested on the indirect competition EIA, because methanol is an efficient eluant for phenylureas and triazines bound to C18 solid-phase extraction resin. Figure 3 illustrates how the I50 value and the rate of color development for six of the MAbs were affected by the presence of methanol in the competitive binding step of the indirect competition EIA. MAb 488 appeared to be the best choice for routine assays. It had the second lowest I50 in the absence of methanol and an acceptable decrease of sensitivity (increase of I50) and assay color development rate when the competition step contained up to 15% (v/v) methanol. MAb 21 functioned...
very well in EIAs containing as much as 15% methanol, but it was less sensitive than MAb 488.

DISCUSSION

To our knowledge, these are the first MAbs developed for the ary lurea herbicides. The hapten structures were selected for evaluation as immunizing and/or detecting conjugates on the basis of our previously reported criteria (Harrison et al., 1991). In fact, the results of this study strongly support the hapten design criteria specified by Harrison et al. Of the six possible combinations of hapten-spacer arm conjugates that we tested as immunizing and screening antigens, one combination resulted in a competition EIA that was 100–2000-fold more sensitive than the others. This combination employed the diuron II hapten as immunizing antigen. This hapten consists of an electronically innocuous three-methylene spacer group on the terminal nitrogen of the urea moiety, distal to the major determinant groups. This exposes for antibody manufacture a near-perfect mimic of the target molecule with respect to electronic, steric, and hydrophobic properties. A structurally different mimic, diuron I, used the same spacer arm, but it was attached to the internal nitrogen. Diuron I hapten was recognized less well by the MAbs and thus proved to be the best screening hapten.

Newsome and Collins (1990) raised polyclonal sera in rabbits to two phenylurea haptens and were able to format a competition EIA of similar sensitivity for diuron and related ary lureas. However, they were unable to evoke a response in rabbits to their N’-(3,4-dichlorophenyl)-N-methyl-N-butylurea hapten, which was presumably the same as our diuron II hapten, that gave us the most sensitive competition EIA. This difference led us to more closely examine the synthesis, purification, and analysis of our diuron II. We repeated the synthesis five times with slight variations, and we recrystallized the product from different solvents. The melting points of our materials, 161.5–162.5 °C, remained substantially above Newsome and Collins’s reported value of 123–125 °C. In contrast to their result, we found diuron II to be virtually insoluble in dichloromethane, which therefore was unsuitable for recrystallization. To more thoroughly characterize the structure and confirm the identity of our material, we used IR, 13C NMR, and HRMS. Our 1H NMR was in fair agreement with the results reported by Newsome and Collins, except that we observed a downfield carboxylic acid OH peak that they did not report. Because Newsome and Collins (1990) did not report an IR spectrum, there is no evidence that their hapten had a carboxylic acid group. We speculate that their material may have cyclized, thus preventing its conjugation to the carrier protein.

Like Newsome and Collins, we observed the most sensitive competition with free herbicide using a heterologous coating hapten. During development of our MAbs, additional, polyclonal rabbit antisera that react with diuron, monuron, and linuron were prepared using haptens similar to those used to derive our MAbs (Schneider et al., 1992). The schemes we describe here allow synthesis of these and other ary lurea hapten's with spacers of virtually any degree of rigidity and chain length. These should prove useful for deriving polyclonal or monoclonal antibodies and formatting assays with more specificity for other ary lureas.

The direct competition EIA is faster and simpler than the indirect EIA, and it had I50 values similar to those of the indirect EIA for four of the eight MAbs. The effect of methanol on the rate of color development and the increase in I50 for diuron was different for every MAb. Tolerance of up to 15% methanol facilitates the assay of samples recovered from solid-phase extraction columns. Since we can concentrate residues from a groundwater sample 400-fold by solid-phase extraction, the extract taken into the EIA may be as much as 60-fold more concentrated than the original groundwater. If the assay detection limit is defined as the concentration required for 10% inhibition (approximately 0.8 ppb for diuron in the indirect EIA), then the practical detection limit for samples is on the order of 0.01 ppb.

In California, the Department of Pesticide Regulation is required to monitor five major leachable herbicides found in the state’s groundwater: simazine, atrazine, prometon, bromacil, and diuron. Since all of these compounds can be determined by conventional GC or HPLC, EIA would only be cost-effective if it could be used as a screening method for all five compounds. We recently demonstrated that we could use multivariate statistical methods to identify the three individual triazines (simazine, atrazine, and prometon) from their patterns of cross-reaction with triazine MAbs we derived previously (Kauvar et al., 1992; Cheung et al., 1993). Differences in the cross-reactivity for different phenylurea analogs may similarly make it possible to use three or more of the diuron MAbs to distinguish individual urea herbicides. An EIA recently developed for bromacil may complete the suite of tests that could be run concurrently, eliminating the need for instrumental analysis as a primary screen for these leachable herbicides in groundwater (Szurdoki et al., 1992). A validation of the extraction method and EIA for diuron in groundwater is presented in an accompanying paper.

ACKNOWLEDGMENT

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LITERATURE CITED


Ware, G. W. Pesticides: Theory and Application; Freeman: New York, 1983; p 308.


Registry No. Supplied by Author: Diuron, 330-54-1; linuron, 330-55-2; monuron, 150-68-5; siduron, 1989-88-6; fenuron, 101-42-8; tebuthiuron (Spike), 34014-18-1; propham (IPC), 122-42-9; barban (carbyme), 101-27-9; atrazine, 1912-24-9; simazine, 122-34-9; prometon, 1610-18-0; bromacil, 314-40-9. The hapten we call diuron II was assigned CAS 66320-26-1 in the work of Newsome and Collins (1990).

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