Development of an Enzyme-Linked Immunosorbent Assay for Carbaryl

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Seven rabbit antisera were obtained by immunization with either *N*-(1-naphthylacetyl)-6-aminohexanoic acid (**3**) or 1-(1-naphthyl)-3-(5-carboxypentyl)urea (**8**) conjugated to keyhole limpet hemocyanin (KLH). From these sera Ab2114 (against 8KLH) was used for the optimization of an enzyme-linked immunosorbent assay (ELISA) for the determination of the insecticide carbaryl. An I_{50} of 2–5 ng/mL and a detection limit of 0.2 ng/mL were obtained using *N*-(2-naphthoyl)-6-aminohexanoic acid (**5**) coupled to conalbumin as a coating antigen. No interference with naphthol, the main degradation product of carbaryl, was observed. An enzyme tracer was prepared by covalently linking hapten **5** with alkaline phosphatase. When the ELISA was performed using a format involving coating the plate with antibodies, an I_{50} of 0.4–0.6 ng/mL and a detection limit of 0.05 ng/mL were obtained. Preliminary studies with different samples showed that this immunoassay can be used for the determination of carbaryl in water, soil, body fluids, and food samples. This paper demonstrates the flexibility of using stable derivatives of a target compound such as carbaryl to generate antibodies and a sensitive ELISA for molecules containing functionalities susceptible to chemical hydrolysis and biodegradation.

INTRODUCTION

Carbamates are contact and systemic insecticides used for protection of fruits, vegetables, and field crops and as preharvest insecticides. In particular, carbaryl (1-naphthyl *N*-methylcarbamate) has been used extensively and effectively against many major agricultural pests.

Carbaryl is highly susceptible to chemical hydrolysis and to biodegradation (Aly and El-Dib, 1972; Wauchope et al., 1978; Venkateswarlu et al., 1980). Biotransformation via oxidative and hydrolytic pathways rapidly converts carbaryl into several metabolites, mainly 1-naphthol, which do not accumulate in the body but are excreted in urine, feces, and respiratory gases in a short period of time. However, some adverse effects have been reported including alterations of liver microsomal enzymes (Lechner and Abdelz-Rahman, 1985), possible subchronic neurotoxicity after long-term exposure to carbaryl used as a household insecticide (Branch and Jacqz, 1986), and changes in the immunological functions on in vitro cultures of large granular lymphocytes (Bavari et al., 1991). Carbaryl is also toxic to bees, and it seems to be more stable in honey than in other matrices (Winterlin et al., 1973; Spittler et al., 1986). In addition, the wide use of carbaryl has increased the concern about possible pollution of water supplies and soils. For these reasons the need for methods to detect small quantities of this insecticide, its metabolites, and its degradation products in food, feed, water, soil, body tissues, and fluids has been reemphasized.

Spectrophotometric (García Sánchez and Cruces Blanco, 1987; Yañez-Sedeño et al., 1988) and spectrofluorometric (Sancenón et al., 1989) methods, gas chromatography (GC) (Nagasawa et al., 1977; Wallbank, 1981; Brooks et al., 1990), and high-performance liquid chromatography (HPLC) (Spittler et al., 1986; Ward et al., 1987; Kawai et al., 1988; Strait et al., 1991) have been used for determination of carbaryl. However, derivatization and/or careful cleanup of the samples is often necessary, making the procedures difficult and time-consuming. In addition, during that process significant decomposition of carbaryl to its main degradation product, 1-naphthol, can occur. Recently enzyme immunoassays have proved to be useful for trace chemical analysis (Vanderlaan et al., 1988; Jung et al., 1989; Van Emon and Mumma, 1990; Li et al., 1991; Schneider and Hammock, 1992), because they are rapid, selective, sensitive, and inexpensive. Thus, our objective was to develop a specific enzyme-linked immunosorbent assay (ELISA) for carbaryl using chemically stable mimics. Water, soil, body fluids, and food samples spiked with carbaryl were used to demonstrate the applicability of the method.

MATERIALS AND METHODS

Chemicals and Instruments. The pesticides carbaryl, naphthaleneacetamide, naptalam, propham, propoxur, carbofuran, aldicarb, fenuron, monuron, and diuron were purchased from Chem Service (Media, PA). The ethylcarbamate derivative **9**, carbonates **10–12**, and thiocarbonates **13** and **14**, used for cross-reactivity studies, were prepared by Dr. András Székács (Huang et al., 1992). Immunochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were from Aldrich Chemical Co. (Milwaukee, WI). Analytical stock solutions were prepared in methanol and stored at −20 °C.

Thin-layer chromatography (TLC) was performed on 0.25-mm precoated silica gel 60 F254 aluminum sheets from Merck (Gibbstown, NJ). ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a QE-300 spectrometer (General Electric, 300 MHz for ^{1}H and 75 MHz for ^{13}C nuclei). Chemical shifts (δ) are given relative to TMS (tetramethylsilane) as an internal reference. Electron impact mass spectra (EIMS) were recorded on a Trio-2 apparatus (VG Masslab, Altrincham, U.K.) at 70 eV, and data are reported as m/z (relative intensity). A Hewlett-Packard 5890 gas chromatograph equipped with 15 m × 0.25 mm (i.d.) DB-5 column was interfaced to this mass spectrometer for GC–MS analyses. Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). The absorbances were read with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (405–560

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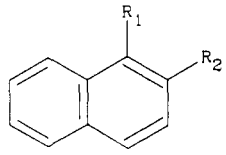


Compound number	R_1	R_2
1	OCH_2COOH	H
2	CH_2COOH	H
3	$CH_2CONH(CH_2)_5COOH$	H
4	$CONH(CH_2)_5COOH$	H
5	H	$CONH(CH_2)_5COOH$
6	$SO_2NH(CH_2)_5COOH$	H
7	H	$SO_2NH(CH_2)_5COOH$
8	$NHCONH(CH_2)_5COOH$	H
Carbaryl	$OCONHCH_3$	H

Figure 1. Chemical structures of the compounds used for the development of the ELISA. All of the compounds except carbaryl were attached covalently to BSA, OVA, and CONA and used as coating antigens. Compounds 3 and 8 were coupled to KLH and used for the immunization of the rabbits.

nm). The inhibition curves were analyzed with a commercial software package (Softmax, Molecular Devices) using a four-parameter logistic equation. All of the data presented from ELISA experiments correspond to the average of at least three well replicates.

Synthesis of Haptens. Compounds 3–8 (Figure 1) were synthesized as follows and used as immunizing and/or coating antigens after conjugation with proteins.

N-(1-Naphthylacetyl)-6-aminohexanoic Acid (3). A solution of 1.9 g (0.01 mol) of thionyl chloride and 1.8 g (0.01 mol) of 1-naphthaleneacetic acid in 10 mL of benzene was stirred under reflux for 4 h. The mixture was cooled and added dropwise to 2.0 g (0.015 mol) of 6-aminohexanoic acid and 3.0 g (0.03 mol) of potassium hydroxide in 100 mL of water. After 3 h at room temperature, the reaction mixture was acidified with 5 N hydrochloric acid, and 1.3 g of the desired compound was obtained as a white solid by filtration. Additionally, 0.6 g of 3 was obtained by extracting the filtrate solution with ethyl ether (2 × 40 mL). Total yield was 63%: ^{1}H NMR (DMSO-d_6) δ 1.28 (m, 6 H, CH_2), 2.38 (t, 2 H, CH_2COOH), 2.97 (dt, 2 H, CH_2 naphthyl), 7.27–8.03 (m, 9 H, aromatic $C_{10}H_7$, NH, and COOH, proven with D_2O); ^{13}C NMR (CD_3OD) δ 25.57 (C-3), 27.91 (C-4), 29.91 (C-5), 34.71 (C-2), 40.29 (CH_2CONH), 41.44 (C-6), 124.03 (naphthyl, C-2), 125.62 (naphthyl, C-3), 126.00 (naphthyl, C-6), 126.41 (naphthyl, C-7), 127.26 (naphthyl, C-8), 127.93 (naphthyl, C-5), 128.58 (naphthyl, C-4), 129.62 (naphthyl, C-9), 133.51 (naphthyl, C-10), 135.25 (naphthyl, C-1), 173.74 (CH_2CONH), 177.35 (COOH); EIMS, *m/z* (relative intensity) 299 (M^+, 17), 142 (base), 115 (20), 69 (21).

N-(1-Naphthoyl)-6-aminohexanoic Acid (4). To an ice bath cooled solution of 2.6 g (0.02 mol) of 6-aminohexanoic acid and 8.0 g (0.03 mol) of potassium hydroxide in 100 mL of water was added dropwise 3.8 g (0.02 mol) of 1-naphthoyl chloride in 20 mL of 1,4-dioxane. The mixture was stirred at room temperature for 3 h. After acidification, a solid was collected by filtration. Subsequent crystallization from ether gave 2.7 g (47%) of compound 4 as white crystals; ^{1}H NMR (DMSO-d_6) apparatus δ 1.37 (m, 6 H, CH_2), 2.20 (t, 2 H, CH_2COOH), 3.28 (q, 2 H, CH_2NH), 7.40–8.35 (m, 8 H, aromatic $C_{10}H_7$ and NH, proven with D_2O); ^{13}C NMR (CD_3OD) δ 25.67 (C-3), 27.52 (C-4), 30.05 (C-5), 34.78 (C-2), 40.67 (C-6), 124.98 (naphthyl, C-2), 125.47 (naphthyl, C-3), 126.65 (naphthyl, C-6), 126.99 (naphthyl, C-7), 128.15 (naphthyl, C-8), 128.66 (naphthyl, C-5), 130.07 (naphthyl, C-4), 130.49 (naphthyl, C-9), 134.97 (naphthyl, C-1), 135.76 (naphthyl, C-10), 172.31 (CONH), 177.42 (COOH); EIMS, *m/z* (relative intensity) 285 (M + 1, 29), 226 (24), 184 (22), 171 (19), 155 (base), 127 (89).

N-(2-Naphthoyl)-6-aminohexanoic Acid (5). To an ice bath cooled mixture of 2.6 g (0.02 mol) of 6-aminohexanoic acid and 8.0 g (0.30 mol) of potassium hydroxide in 100 mL of water was added dropwise a solution of 3.8 g (0.02 mol) of 2-naphthoyl chloride in 20 mL of 1,4-dioxane. The solution was stirred at room temperature for 4 h. A white solid was collected by filtration after acidification with hydrochloric acid. Crystallization of the solid from ether gave 4.9 g (86%) of the desired compound: ^{1}H NMR (DMSO-d_6) δ 1.43 (m, 6 H, CH_2), 2.12 (t, 2 H, CH_2COOH), 3.18 (q, 2 H, CH_2NH), 7.39–8.46 (m, 8 H, aromatic $C_{10}H_7$ and NH), 12.09 (br s, 1 H, COOH); ^{13}C NMR [CD_3OD–$(CD_3)_2SO$] δ 25.72 (C-3), 27.54 (C-4), 30.23 (C-5), 34.78 (C-2), 40.07 (C-6), 124.37–135.90 (naphthyl), 169.25 (CONH), 176.60 (COOH).

N-(1-Naphthylsulfonyl)-6-aminohexanoic Acid (6). A solution of 4.5 g (0.02 mol) of 1-naphthylsulfonyl chloride in 20 mL of 1,4-dioxane was added dropwise over a mixture of 2.6 g (0.02 mol) of 6-aminohexanoic acid and 6.5 g (0.12 mol) of potassium hydroxide in 100 mL of water cooled with an ice bath. The solution was stirred at room temperature for 4 h, poured over cool hydrochloric acid solution, and extracted with ethyl ether (3 × 30 mL). The combined organic extracts were washed with 1 M $NaHCO_3$ (2 × 50 mL), dried with $MgSO_4$, and evaporated, giving 2.5 g (43%) of compound 6 as a white crystals: ^{1}H NMR (DMSO-d_6) δ 1.54 (m, 6 H, CH_2), 1.96 (t, 2 H, CH_2COOH), 2.71 (dt, 2 H, CH_2NH), 7.43–7.69 and 7.96–8.63 (m, 7 H, aromatic $C_{10}H_7$), 7.78 (t, 1 H, NH, proven with D_2O), 11.96 (s, 1 H, COOH, proven with D_2O); ^{13}C NMR (CD_3OD) δ 25.14 (C-3), 26.76 (C-4), 30.01 (C-5), 34.42 (C-2), 43.48 (C-6), 124.33 (naphthyl C-2), 125.98 (naphthyl, C-3), 126.40 (naphthyl, C-6), 126.91 (naphthyl, C-7), 128.81 (naphthyl, C-8), 129.21 (naphthyl, C-5), 129.74 (naphthyl, C-4), 130.75 (naphthyl, C-9), 135.48 (naphthyl, C-10), 136.55 (naphthyl, C-1), 177.28 (COOH); EIMS, *m/z* (relative intensity) 321 (M + 1, 6), 220 (32), 191 (44), 130 (36), 127 (base).

N-(2-Naphthylsulfonyl)-6-aminohexanoic Acid (7). To a cooled solution of 2.6 g (0.02 mol) of 6-aminohexanoic acid and 6.5 g (0.12 mol) of potassium hydroxide in 100 mL of water was added dropwise 4.5 g (0.02 mol) of 2-naphthylsulfonyl chloride in 15 mL of 1,4-dioxane. The mixture was stirred at room temperature for 4 h, added to 1 M hydrochloric acid, and filtered to collect 3.0 g (47%) of a white solid identified as compound 7: ^{1}H NMR (DMSO-d_6) δ 1.26 (m, 6 H, CH_2), 2.06 (t, 2 H, CH_2COOH), 2.71 (dt, 2 H, CH_2NH), 7.50–8.35 (m, 8 H, aromatic $C_{10}H_7$ and NH), 11.91 (s, 1 H, COOH); ^{13}C NMR [CD_3OD–$(CD_3)_2SO$] δ 23.90 (C-3), 25.66 (C-4), 28.82 (C-5), 33.43 (C-2), 42.53 (C-6), 126.42–136.42 (naphthyl), 176.21 (COOH); EIMS, *m/z* (relative intensity) 321 (22), 220 (74), 191 (87), 142 (16), 130 (83), 127 (base), 112 (24), 77 (30).

1-(5-Carboxypentyl)-3-(1-naphthyl)urea (8). A solution of 1.7 g (0.01 mol) of 1-naphthyl isocyanate and 0.1 g (1.30 mol) of 6-aminohexanoic acid in 100 mL of tetrahydrofuran (THF) was stirred at room temperature overnight. The following day, 2.0 g of 8 as a white solid was isolated by filtration. Subsequently, the evaporation of the solvent gave 0.9 g of pure compound after recrystallization from acetonitrile. Total yield was 96%: ^{1}H NMR (DMSO-d_6) δ 1.35 (m, 6 H, CH_2), 2.42 (dt, 2 H, CH_2COOH, 3.05 (t, 2 H, CH_2NH), 6.48 (t, 2 H, NH, proven with D_2O), 7.18–8.06 (m, 7 H, aromatic $C_{10}H_7$), 8.40 (s, 1 H, COOH, proven with D_2O); ^{13}C NMR [CD_3OD–$(CD_3)_2SO$] δ 26.26 (C-3), 27.66 (C-4), 30.96 (C-5), 36.12 (C-2), 40.01 (C-6), 122.07 (naphthyl, C-2), 123.71 (naphthyl, C-3), 125.87 (naphthyl, C-6), 126.07 (naphthyl, C-7), 126.15 (naphthyl, C-8), 127.70 (naphthyl, C-5), 127.76 (naphthyl, C-4), 128.77 (naphthyl, C-9), 155.70 (naphthyl, C-10), 135.77 (naphthyl, C-1), 158.68 (NHCONH), 178.39 (COOH); EIMS, *m/z* (relative intensity) 300 (M^+, 9), 169 (base), 143 (93), 140 (28), 115 (19).

Conjugation to Carrier Proteins. The haptens were covalently attached to keyhole limpet hemocyanin (KLH), ovalbumin (OVA), conalbumin (CONA), or bovine serum albumin (BSA) using the mixed-anhydride method (Rajkowski et al., 1977). Briefly, the carboxylic acid haptens (0.10 mmol) were dissolved in anhydrous dimethylformamide (DMF). Tri-*n*-butylamine (0.11 mmol) was added followed by isobutyl chloroformate (0.12

mmol). The resulting mixture was stirred for 30 min and then added dropwise to a solution of the corresponding protein (100 mg) in 5 mL of borate buffer (0.2M borate–boric, pH 8.7) and stirred at room temperature for 6 h. The conjugates were extensively dialyzed in PBS (0.01 M phosphate-buffered saline solution, pH 7.4) and finally against water, lyophilized, and stored at −20 °C. Stock solutions of 1 mg/mL were prepared with PBS buffer and stored in aliquots at −20 °C.

Enzyme Tracer Synthesis. The acid **5** was conjugated to alkaline phosphatase (AP, from bovine intestinal mucosa) according to the modified active ester method (Langone and Van Vunakis, 1975). A mixture of 4.3 mg (15 μmol) of **5**, 8.6 mg (75 μmol) of *N*-hydroxysuccinimide (NHS), and 30.9 mg (150 μmol) of dicyclohexylcarbodiimide (DCC) in 325 μL of DMF was stirred at room temperature for 6 h. After centrifuging (10000 *g*, 5 min), the clear supernatant containing the active ester was added slowly to 2.0 mg of AP in 1 mL of 0.05 M tris(hydroxymethyl)-aminomethane saline solution (TBS pH 7.8). The mixture was allowed to react at 4 °C overnight. Finally, the conjugate was purified by dialysis, as described above, and stored in solution at 4 °C.

Immunization of the Rabbits. Rabbits 1338 and 1339 were immunized with 3KLH, and rabbits 1567, 1973, 1974, 1975, and 2114 were immunized with 8KLH. The immunization protocol was the same as described by Gee et al. (1988). Serum was stored at −80 °C following the addition of NaN_3 to a final concentration of 0.02%.

Analysis of the Titer. The titer of the serum from each animal was determined by measuring the binding of serial dilutions to microtiter plates coated with several concentrations of 1OVA, 1BSA, 2BSA, 3BSA, 4BSA, 4OVA, 5OVA, 5BSA, 5CONA, 6OVA, 6BSA, 7OVA, 7BSA, and 8BSA (20 to 0.06 μg/mL). Optimal concentrations for coating antigen and antisera dilution were determined as a result of these experiments. For the format using antibody-coated plates described below, optimal dilution factors for the antibody and AP conjugate were determined, as described by Jung et al. (1989), by performing two-dimensional titration experiments.

Competitive Enzyme-Linked Immunosorbent Assay. Assay conditions were optimized to provide a sensitive assay (low I_{50}) and an acceptable ratio of maximal absorbance versus noise (A/D), with an economic use of antibody and reasonable incubation periods.

(*A*) *Antigen-Coated Plates.* Microtiter plates (96 wells) were coated with 5CONA (0.5 μg/mL, 100 μL/well) in coating buffer (0.1 M carbonate–bicarbonate, pH 9.6) and incubated overnight at 4 °C covered with adhesive plate sealers. The plates were washed five times with PBST buffer (0.2 M, phosphate-buffered saline solution containing 0.05% Tween 20, pH 7.4). Serial dilutions of the analyte were added in 50-μL volume to the coated plates followed by 50 μL of the antibody (Ab2114) previously diluted 1/40000 (final dilution in the well 1/80000). After incubation at room temperature for 1 h, the plates were washed, as previously described, and 100 μL of a 1/5000 diluted goat antirabbit IgG–alkaline phosphatase solution was added. The mixture was incubated for an additional 1 h, and after another washing step, 100 μL of a 1 mg/mL solution of 4-nitrophenyl phosphate in DEA (10% diethanolamine, pH 9.8) was added. The absorbance was read after incubation for 30 min.

(*B*) *Antibody-Coated Plates.* Microtiter plates were coated with a solution of serum (1/8000, Ab2114) in coating buffer overnight at 4 °C. After a washing step, 50 μL of the standard dilutions of the analyte was added followed by 50 μL of the enzyme tracer diluted 1/4000 in PBST. The plates were incubated for 30 min at room temperature and washed, and finally 100 μL of the substrate solution was added. The absorbances were read after 1 h.

Determination of Cross-Reactivities. The compounds listed on Table III were tested for cross-reactivity by preparing standard curves in PBST and determining their I_{50} values (concentration which inhibits the binding of the antibody to the plate by 50% in the absence of analyte) in the ELISA. The cross-reactivity values were calculated as follows: (I_{50} carbaryl/I_{50} compound) × 100.

Sample Preparation for Matrix Effects Studies. (*A*) *Solvent Effect.* Carbaryl standard curves were prepared in PBST buffer containing 2, 4, 8, and 16% (v/v) acetone, acetonitrile or poly(propylene glycol) 1000. The tolerance of the assay to methanol was tested at concentrations of 1, 2, 4, 5, 8, 10, 16, 20, 30, 40, 50, 60, 70, 80, 90, and 100% (v/v) in PBST buffer.

(*B*) *Water.* Water samples from three different sources (tap water, industrial water, and Putah Creek water, Davis) were spiked with carbaryl to a final concentration of 80 ng/mL and serially diluted with PBST buffer; the carbaryl concentration was determined in a standard competition experiment.

(*C*) *Milk, Honey, and Urine.* Sample matrices were serially diluted with PBST and spiked with a constant amount of carbaryl to a final concentration of 5 ng/mL and used for ELISA determination.

(*D*) *Soil.* A fine, sandy, soil [for description see Wong et al. (1991)] was used for matrix effect studies. Extracts were obtained according to a classical procedure for carbaryl (Wauchope, 1978). Briefly, 2 g of soil was shaken overnight with chloroform/methanol (90/10, 100 mL). The solvent was filtered and evaporated to dryness. The residue was redissolved with 200 μL of methanol, diluted to 2 mL with PBST, spiked with carbaryl (5 ng/mL), and used for ELISA determination.

Finally, carbaryl standard curves (from 50 to 2.5×10^{-5} μg/mL) were prepared in urine, soil extract, and honey, diluted 1/25, 1/50, and 1/1000, respectively, and compared to the standard curves prepared in PBST buffer.

RESULTS AND DISCUSSION

Hapten Selection. A suitable hapten for immunization should preserve the chemical and physical properties of the target compound, but at the same time it must provide a functional group that allows its coupling to a carrier protein since small molecules are not able to stimulate an immune response (Harrison et al., 1991a). For carbaryl, one approach was the introduction of a handle through the nitrogen of the carbamate moiety. However, recognition is higher for the part of the molecule located farthest from the protein and the most characteristic part in the carbaryl molecule is the carbamate function. In addition, carbaryl is susceptible to the chemical hydrolysis of the carbamate function, producing naphthol (Aly and El-Dib, 1972; Venkateswarlu et al., 1980). Some researchers have reported the development of antibodies against the alkaloid physostigmine, a cholinergic drug possessing a carbamate function (Meyer et al., 1989; Miller and Verma, 1989). Nevertheless, in a previous study we coupled the *N*-methylcarbamate carbofuran through an oxime derivative of 3-ketocarbofuran or a hemisuccinate of 3-hydroxycarbofuran (Hammock, personal communication), and only antibodies to the corresponding phenols were obtained. In hopes that carbaryl might be more stable, we used 4- and 5-hydroxycarbaryl as starting materials since a chain could be introduced through the hydroxyl group. However, hydrolysis can occur under the basic conditions used during the covalent attachment to the protein, and therefore, immunization with these carbaryl haptens was not promising. In this context, a pH near neutral was used for the preparation of the immunogens with a physostigmine analog which also possesses the carbamate function (Meyer et al., 1989). If conjugation is successful, degradation may also occur under physiological conditions due to hydrolysis and possibly tissue enzymatic activity (Mount and Oehme, 1980).

Presumably, the characteristic electronic distribution in a molecule is one of the most important factors determining the recognition of the antibody for a compound (Harrison et al., 1991a). Hence, the idea was to immunize rabbits with other more stable derivatives showing some similarity to the carbamate functionality present in the insecticide carbaryl. It was hoped that these antibodies would partially recognize carbaryl and consequently this insecticide would be able to compete under

Table I. Results from the Titration Experiments

	coating antigen[a]															
rabbit	1OVA	1BSA	2BSA	3BSA	3KLH	4BSA	4OVA	5OVA	5BSA	5CONA	6OVA	6BSA	7OVA	7BSA	8BSA	8KLH
1338	*	*	H	H	H	H	H	*	*	L	*	*	L	H	H	H
1339	*	*	H	H	H	L	L	*	*	*	*	*	L	L	H	H
2114	*	*	H	H	H	H	H	L	H	H	L	L	*	L	H	H
1973	*	*	L	H	H	H	H	L	H	H	L	L	*	L	H	H
1974	*	*	L	H	H	H	H	L	H	H	L	L	*	L	H	H
1975	*	*	L	H	H	H	H	L	H	H	L	L	*	L	H	H
1567	*	*	*	H	H	L	*	L	L	L	L	L	*	*	H	H

[a] H, L, and * indicate the serum dilution factor range that produces absorbances of 0.5 after 30 min. H, more than 1/16000; L, between 1000 and 1/16000; *, less than 1/1000. Antisera 1338 and 1339 were raised against 3KLH and 2114, 1973, 1974, 1975, and 1567 against 8KLH.

Table II. Combinations That Gave I_{50} Values below 100 ng/mL during the Screening of Carbaryl in the Competitive Experiments

immunogen	antiserum	coating antigen	I_{50}
8KLH	1973	8BSA	18
		4OVA	15
		4BSA	33
	1974	5CONA	10
		5BSA	80
	1975	3KLH	88
	1567	5CONA	20
		5BSA	28
	2114	5CONA	2
		5BSA	2
		2BSA	17
		4OVA	12
3KLH	1338	a	a
	1339	3KLH	96
		8KLH	69
		2BSA	40

[a] Any coating antigen gave an I_{50} below 100 ng/mL.

certain coating antigen/antibody combinations (Wie and Hammock, 1982).

Screening of the Sera. A tiered system was used starting with broad screening and finally resulting in one antibody/antigen combination for full evaluation. The antisera of 7 rabbits were tested against 16 coating antigens. The results of the titration experiments using the final bleedings are shown in Table I. In general, we observed that antibodies raised against 3KLH showed higher titers (**H**, the antisera can be diluted more than 16 000 times to produce absorbances ≥0.5 after 30 min) for those coating antigens possessing a methylene group (like the immunogen) or a urea function next to the aromatic ring (2BSA, 3KLH, 3BSA, 8KLH, and 8BSA). However, when the handle contains a functional group such as an amide, a sulfoxide, or an ether, a drastic decrease in the recognition occurred. In contrast, the antibodies raised against 8KLH showed higher titers for other functional groups differing from the one used to generate them. For instance, two-dimensional titration experiments using the sera of rabbits 1973, 1974, 1975, and 2114 showed high absorbances when their titers were measured in plates coated with 4BSA, 4OVA, 5BSA, and 5CONA (in addition to the 8BSA and 8KLH plates). These conjugates have an amide group directly attached to the ring. Also, high titers were observed for 3BSA and 3KLH. Nevertheless, less (L, the antisera are diluted between 1/1000 and 1/16000 to produce absorbances of 0.5 after 30 min; *, the antisera have to be diluted less than 1000 times to obtain absorbances of 0.5 after 30 min) recognition was observed for the coating antigens having the sulfonamide hapten, 6OVA, 6BSA, 7OVA, and 7BSA.

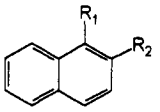
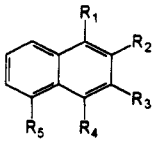
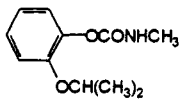
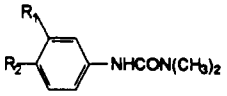
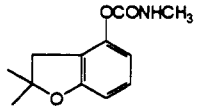
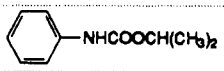
Competition Experiments. The combinations of coating antigen/antisera showing higher antibody titers were used to test for inhibition by carbaryl and its main metabolite naphthol in the ELISA, using the coating antigen method. Only some of the combinations tested were useful for the analysis of carbaryl. Naphthol was not able to compete with the coating antigen for the antibody at concentrations below 10 μg/mL. Table II shows those combinations giving an I_{50} for carbaryl below 100 ng/mL. Antiserum raised against the same immunogen gave assays with different characteristics. For example, whereas 5CONA and 5BSA can be used with the antisera Ab1974, Ab1567, and Ab2114, only 3KLH worked for Ab1975. However, carbaryl can compete using Ab1973 and 3BSA, 4BSA, and 4OVA coated plates (see Table II). On the other hand, combinations using Ab1339 always gave more sensitive assays than those with Ab1338, even though both were raised against 3KLH.

The most promising of the above combinations were compared for sensitivity to carbaryl, slope, high control absorbance, and low background. On the basis of these results, combination Ab2114/5CONA was chosen for having a high sensitivity and an excellent signal to noise ratio. This combination provided an immunoassay capable of detecting carbaryl with an I_{50} of 2–5 ng/mL and a detection limit around 0.2 ng/mL in the antigen-coated plate format.

It is known that the format can have a very strong influence on the sensitivity of the ELISA (Schneider and Hammock, 1992). On the basis of the results of the screening described above, hapten 5 was coupled to alkaline phosphatase and tested in the ELISA according to the conditions described under Materials and Methods. In this case the I_{50} was 0.4–0.6 ng/mL with a detection limit around 0.05 ng/mL. However, because of the low activity of the enzyme conjugate, absorbances of only 0.3–0.4 were obtained at antiserum dilutions of 1/8000 for Ab2114 after 1 h. In contrast, in the coating antigen format absorbances of 0.7–0.8 were obtained after 30 min at antibody dilutions of 1/80000. Both formats are suitable for analysis of carbaryl. Figure 2 shows the sigmoid curves obtained after optimization of both antigen and antibody coating formats.

Cross-Reactivities. Other carbamates (propham, propoxur, carbofuran, and aldicarb), urea (fenuron, monuron, and diuron), and naphthalene (naphthalene, naphthaleneacetamide, and naptalam) pesticides as well as carbaryl metabolites (1-naphthol and 3-hydroxy-, 4-hydroxy-, and 5-hydroxycarbaryl) were tested for cross-reactivities. Table III shows the cross-reactivity found, expressed in percentage of the I_{50} of carbaryl. In all cases the interference to the assay was negligible; only the herbicide naphthaleneacetamide showed significant cross-reactivity (84%). Similarly, the cross-reactivity by carbaryl metabolites was ≤5%. It is especially interesting to note that naphthol, the main degradation product of carbaryl, does not interfere at all in the assay, which makes this technique very useful for the detection of this insecticide. Some of the analytical methods used for carbaryl are based in its complete degradation to naphthol, which can give

Table III. Cross Reactivities for Compounds Structurally Related to Carbaryl

Chemical Structure		Compound name	Cross reactivity (%)
	R1 = OCONHCH3; R2 = H	Carbaryl	100
	R1 = OCONHCH2CH3; R2 = H	Ethylcarbamate 9	346
	R1 = H; R2 = H	Naphthalene	<0.001
	R1 = OH; R2 = H	1-Naphthol	<0.001
	R1 = CH2CONH2; R2 = H	1-Naphthaleneacetamide	84
	R1 = OCOOCH3; R2 = H	1-Methylcarbonate 10	6.2
	R1 = OCOOCH2CH3; R2 = H	1-Ethylcarbonate 11	5.5
	R1 = H; R2 = OCOOCH2CH3	2-Ethylcarbonate 12	2.8
	R1 = OCOSCH2CH3; R2 = H	1-Ethylthiocarbonate 13	10.1
	R1 = H; R2 = OCOSCH2CH3	2-Ethylthiocarbonate 14	4.5
	R1 =  ; R2 = H	Naptalam	0.05
	R1 = OCONHCH3; R2 = H; R3 = OH; R4 = H; R5 = H	3-Hydroxycarbaryl	3.7
	R1 = OCONHCH3; R2 = H; R3 = H; R4 = OH; R5 = H	4-Hydroxycarbaryl	3.6
	R1 = OCONHCH3; R2 = H; R3 = H; R4 = H; R5 = OH	5-Hydroxycarbaryl	4
		Propoxur	4.2
	R1 = H; R2 = H	Fenuron	0.009
	R1 = H; R2 = Cl	Monuron	0.02
	R1 = Cl; R2 = Cl	Diuron	0.14
		Carbofuran	3
		Propham	0.001
CH3C(CH3)2CH=NOCONHCH3		Aldicarb	4.2

inaccurate results since naphthol may result from environmental chemicals other than carbaryl. In addition, naphthol could itself be a contaminant. Additionally, experiments with naphthalene carbonates (10–12) and thiocarbonates (13 and 14) demonstrated specificity toward the carbamate function (cross-reactivities <10%). The high cross-reactivity of compound 9 (346%) is due to handle recognition since a long substituent on the nitrogen makes this compound more similar to the immunogen (8KLH).

Matrix Effect Studies. The effects of different kinds of matrices on the responses of the ELISA were tested to demonstrate the ruggedness of the assay and potential applications. Water from three different sources (tap water, industrial water, and Putah Creek water, Davis), soil extract, urine, milk, and honey were selected for these experiments. Direct analysis was possible for the three kinds of water samples tested; however, some preparation of the sample is necessary prior to the analysis of urine, soil extract, and especially honey and milk prior to determination of low levels of carbaryl. Dilution of the samples is a simple alternative to circumvent unwanted effects produced by matrix.

Figure 3 shows the effect of the dilution on the reliability of the measurement. In urine, values near the spiked amounts were found after a dilution greater than 1/50. Soil had to be diluted at least 1/25, and honey had to be diluted over 1000 times to have an accurate measurement. Finally, a strong interference from milk components on the ELISA was observed (i.e., effects can be seen at dilutions higher than 1/50000), and therefore a cleanup step would be necessary for routine sample analysis. However, since most of the matrix effects seen lead to an enhancement in the sensitivity, an alternative approach would be to run the standards in the presence of the matrix.

The determination of the origin of these interferences was not the objective of this work. Nevertheless, features such as pH, protein, sugar, and salt concentration must be considered. Additionally, solvents employed during extraction procedures can also significantly affect the characteristics of the ELISA. Figure 4 shows the effect of methanol, acetone, and acetonitrile on the sensitivity of the ELISA. A significant decrease in the sensitivity was observed when increasing amounts of solvent were added to the buffer assay. The same behavior was observed for poly(propylene glycol). Methanol caused the least effects of the solvents tested and seemed to be tolerated when present at concentrations below 10% (v/v). In all cases, the decrease in the sensitivity occurred with a simultaneous decrease in the signal to noise ratio due to the slower color development. However, according to results reported in the past for other ELISA systems developed in this laboratory (Gee et al., 1988; Harrison et al., 1991b; Li et al., 1991; Lucas et al., 1991), different assays show diverse direction and magnitude of response to factors present in the matrix such as solvent, pH, protein, or salt concentration.

To assess the potential of the ELISA for the analysis of carbaryl, standard curves were constructed with various matrices. Water was analyzed without dilution as no matrix effect was observed. Urine, honey, and soil extract were previously diluted to 1/50, 1/1000, and 1/32, respectively. Figure 5 shows that the curves generated in the presence of matrices are parallel to that in buffer. Using

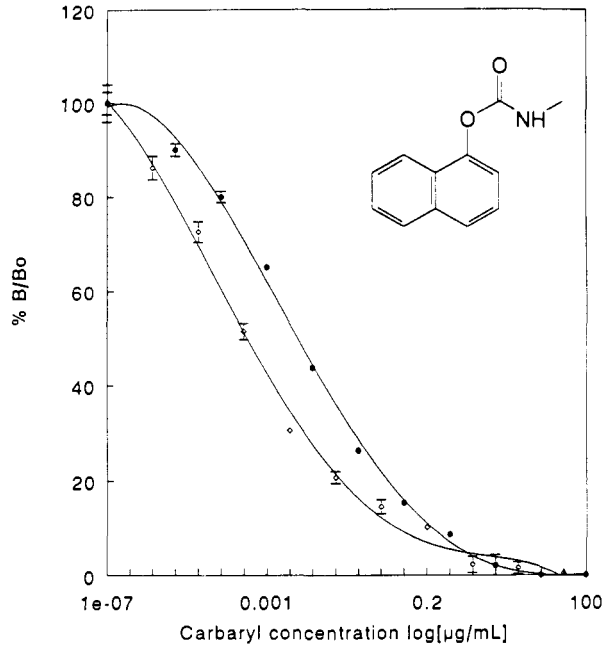


Figure 2. Standard curves of carbaryl in PBST buffer using different formats. For the coating antigen format Ab2114 was diluted 1/80000 and was used in combination with 5CONA. For the antibody coating format the same antibody was diluted 1/8000 and hapten **5** was coupled to alkaline phosphatase. The I_{50} was 5 ng/mL for the coating antigen format and 0.4 ng/mL for the antibody coating format. Each point corresponds to the average of two different plates where three wells were used for every concentration of carbaryl. The average of the coefficients of variation is 1.1%. B, averaged absorbance measured for every concentration on the carbaryl standard curve; B_0, averaged absorbance measured when carbaryl concentration is zero. (●) Antigen coating format; (◊) antibody coating format.

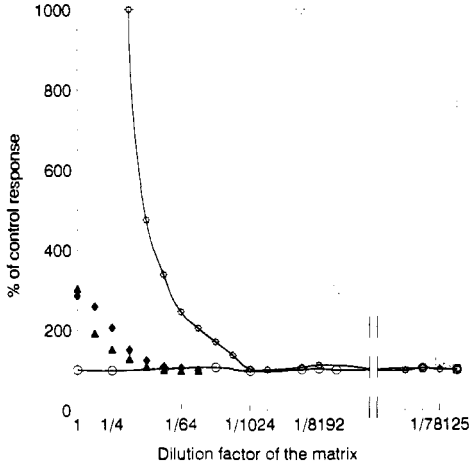


Figure 3. Effect of the dilution of the matrix on the reliability of the ELISA. The samples were serially diluted with PBST and spiked with carbaryl to a concentration of 5 ng/mL. The amount measured is expressed as a percentage of the spiked amount (control in buffer). No negative effects in the direct measurement of water samples was observed. The adverse effect disappears after the samples are diluted over factors of 1/50 for the urine, 1/25 for the soil extract, and 1/1000 for honey. Milk showed a strong matrix effect which had to be eliminated by employing sample cleanup procedures. Datum points are the average of three wells, and the average of the coefficients of variation is 1.2%. (♦) Urine; (▲) soil extract; (☆) honey; (□) whole milk; (○) PBST buffer.

the coating antigen format, over 5, 160, 250, and 5000 ng/mL (values were based on a measurement in the midpoint of the curve according to the dilution factor applied) can be detected, respectively, in the water, soil extract, urine,

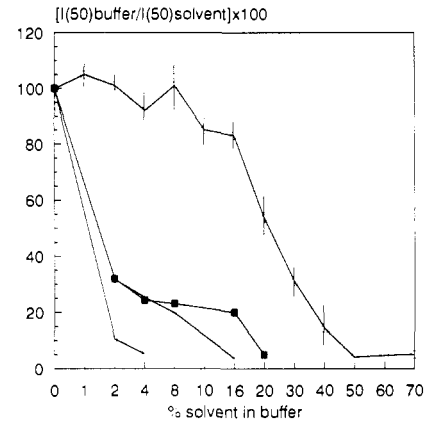


Figure 4. Presence of small amounts of organic solvents in the ELISA alters the characteristics of the assay. Acetone, acetonitrile, and poly(propylene glycol) 1000 produce a dramatic negative effect on the sensitivity of the carbaryl assay. Of the solvents tested, only methanol seemed to be tolerated when the concentration remained below 10% (v/v) in the assay buffer. Datum points represent the average of three wells. Coefficients of variation averaged are less than 2%, and the standard deviations are shown for methanol. (☆) Poly(propylene glycol) 1000; (+) acetone; (■) acetonitrile; (○) methanol.

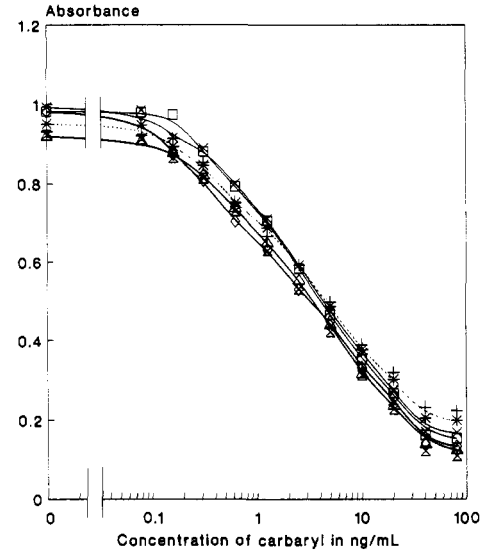


Figure 5. Standard curves obtained after serial dilution of carbaryl in different matrices. Water samples were used without dilution for determination. Honey, urine, and soil extract were previously diluted 1/1000, 1/50, and 1/32, respectively. All of the curves obtained were parallel to the one obtained in PBST buffer, demonstrating the reliability of the assay when performed with the mentioned matrices. The values correspond to an average of three wells, and the average of coefficients of variation is 1.8%. (□) PBST buffer; (+) tap water; (*) industrial water; (◊) creek water; (×) honey; (△) urine; (▽) soil extract.

and honey samples selected for this study without prior sample workup.

When samples showing very strong matrix effects have to be analyzed, an alternative is to employ the ELISA as a detection system for HPLC or TLC. On the other hand, the intermediate polarity (log P) of many carbamates suggests that significant cleanup can be obtained by a single solid-phase extraction or differential partitioning techniques. Optimizing such procedures is beyond the scope of this study. However, in this paper we show that moderate levels of organic solvents have a clear influence in the assay, and therefore this is a factor to be considered when the samples are extracted or purified before analysis.

It is worth mentioning that a previous paper (Harrison et al., 1991b) reported that different antibody/coating

antigen combinations tolerate the interferences from matrices to different degrees. Consequently, the performance of similar studies with other combinations of antibodies and coating antigens (see Table II) constitutes another option for solving the problems derived from the matrix effect.

Conclusions. The ELISA described for the measurement of carbaryl will allow the detection of this insecticide in contaminated samples. This assay will provide an easy and fast tool for the screening of carbaryl without the interference of naphthol, its main degradation product. In addition, the fact that a urea derivative has provided antibodies for the detection of carbaryl in a competitive enzyme immunoassay opens the door for the development of similar assays for other carbamate pesticides or a more general application to a variety of hydrolytically unstable compounds.

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