

Comparison of Coating and Immunizing Antigen Structure on the Sensitivity and Specificity of Immunoassays for Benzoylphenylurea Insecticides

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Several sensitive enzyme-linked immunosorbent assays (ELISA's) were developed for the benzoylphenylurea insect growth regulators diflubenzuron, BAY SIR 8514, and penfluron. The sensitivities and specificities of the assays have been investigated by using several different benzoylphenylurea derivatives as the immunogens as well as coating antigens. The following approach was used to solve the problem of bridge recognition: (a) homologous site systems, to use the same coupling site on the benzoylphenylurea molecule in the immunogen and coating antigen but to vary the bridge structure, and (b) heterologous site systems, coupling the immunogen and coating antigen from different positions on the benzoylphenylurea molecule. The heterologous systems yielded more sensitive assays, but antisera obtained from all the immunogens were used successfully in developing ELISA's for benzoylphenylureas. With these sensitive ELISA's, diflubenzuron was detected in milk at a level of 2 ppb without any sample extraction procedure, and sample cleanup led to still more sensitive assays. The reproducibility and sensitivity of the method are adequate for practical residue analysis. This work illustrates that a limited collection of antisera and coating antigens can yield a powerful library of assays capable of specifically detecting single compounds or a variety of members of a compound class.

The familiar radioimmunoassay (RIA) that was developed in 1960 by Yalow and Benson (Yalow, 1978) has provided a powerful tool for the analysis of many compounds of interest to clinical chemists and endocrinologists, including viruses, proteins, hormones, and exogenous drugs. Because of the inherent disadvantages involved with using radioactive materials (such as health hazards, disposal of radioactive waste, short half-life of ¹²⁵I, and the use of expensive counters), there has been interest in developing the nonisotopic immunoassays. Enzymes have been shown to be among the most promising of the nonisotopic labels examined to date.

Recently, solid-phase enzyme-linked immunosorbent assays (ELISA's) were described for the residue analysis of diflubenzuron and BAY SIR 8514 (Wie et al., 1982b; Wie and Hammock, 1982a) (see Table I for structures and chemical names). In this present report, ELISA's were used to compare the sensitivity and specificity of the antisera from rabbits that were immunized with different diflubenzuron-protein conjugates. Moreover, these conjugates with different hapten-carrier linkages were investigated as potential coating antigens in the assay systems. These results indicated that by having a library of antibodies and a library of coating antigens one can develop a set of immunoassays of varying selectivity and sensitivity. Thus, in this case ELISA's were developed by using haptens derivatized on the aniline side of the molecule that were capable of detecting the benzoylphenylureas as a class while others were selective for single members of the class.

MATERIALS AND METHODS

Reagents. Alkaline phosphatase conjugated goat anti-rabbit IgG was obtained from Miles Laboratory (Elkhart, IN). Ovalbumin (OA), keyhole limpet hemocyanin (KLH), and bovine serum albumin (BSA) were purchased from Calbiochem (San Diego, CA); *p*-nitrophenyl phosphate was from Sigma Chemical Co. (St. Louis, MO); isobutyl chloroformate, tri-*n*-butylamine, and *tert*-butylamine were from Eastman Kodak Co. (Rochester,

NY). Solvents were of reagent grade or better, and ethers were distilled from lithium aluminum hydride under nitrogen to remove water and peroxides.

Antisera. The anti-diflubenzuron antibodies were obtained from New Zealand white rabbits as described in a previous publication (Wie et al., 1982a). The structures and trivial names of immunizing and coating antigens used in this study are shown in Figure 1. Briefly, the rabbits were inoculated as follows: rabbits C and D with *N*-(carboxypropyl)diflubenzuron-BSA prepared by the water-soluble carbodiimide method, sera were obtained 7 months after injection; rabbit I with diflubenzuron phenoxycetate-KLH, serum was collected 17 months after immunization; rabbit J diflubenzuron phenylacetate-BSA, serum was collected 5 months after immunization; rabbit K with diflubenzuron diazonium-BSA, serum was collected 2 months after immunization; rabbit L with BAY SIR 8514 phenyl acetate-BSA, serum was collected 2 months after immunization.

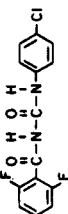
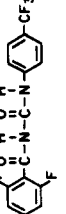
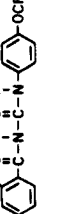
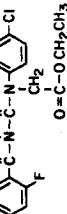
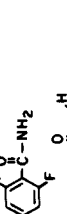
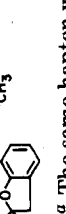
Preparation of Coating Antigens by the Mixed Anhydride Method. The haptens, such as diflubenzuron phenylacetic acid or *N*-(carboxypropyl)diflubenzuron (0.052 mmol), were dissolved in 500 μ L of dry dioxane. To this solution was added tri-*n*-butylamine (0.062 mmol) followed by isobutyl chloroformate (0.062 mmol). The resulting mixture was stirred at room temperature for 30 min and then added dropwise to a solution of OA (150 mg) or KLH (100 mg) in 15 mL of 0.2 M borate buffer, pH 8.7, and stirred at room temperature overnight. The conjugates were then dialyzed extensively in 7 mM phosphate buffer containing 0.15 M NaCl and 0.02% sodium azide (PBS). This procedure resulted in the covalent binding of approximately 2.5 mol of hapten/50 kilodaltons of protein as determined by several ELISA systems. This approximation of loading was accomplished by adding the antigen as an unknown to a minimum of two different ELISA systems. ELISA systems were used that employed antibodies that had been raised to antigens involving the use of a hapten other than the one being analyzed. The amount of hapten per unit protein was estimated from a standard curve of the free hapten in the ELISA system used and/or the hapten coupled to tyramine.

Such an analytical procedure may give misleading results, especially if the assay recognizes the bridge between

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Table I. I_{50} 's and Maximal Inhibitions Caused by Several Compounds in ELISA's Using Different Combinations of Antisera and Coating Antigens

inhibitor structure	system A		system B ^a		system B ^a		system C		system D		system E	
	I_{50} , ng/mL	max % inhibn by large amount, μ g/mL	I_{50} , ng/mL	max % inhibn by large amount, μ g/mL	I_{50} , ng/mL	max % inhibn by large amount, μ g/mL	I_{50} , ng/mL	max % inhibn by large amount, μ g/mL	I_{50} , ng/mL	max % inhibn by large amount, μ g/mL	I_{50} , ng/mL	max % inhibn by large amount, μ g/mL
	3.9	84 (0.2)	48	90 (2)	65	84 (1)	3.0	83 (0.2)	30	80 (1)	15	91 (1)
	6.8	76 (0.1)	190	77 (2)	630	64 (2)	8.2	60 (0.1)	ND	64	85 (1)	
	9.6	84 (0.3)	120	68 (2)	>1000	14 (1)	9.0	76 (0.1)	74	72 (1)	58	71 (4)
	>2000	40 (2)	0.74	97 (0.1)	1.7	96 (0.1)	>4000	<10 (4)	ND	460	86 (4)	
	>20000	<10 (20)	>5000	20 (5)	>3500	32 (3.5)	>20000	<10 (20)	ND	13,000	58 (4)	
	>8000	<10 (8)	>8000	<10 (8)	>3500	<10 (3.5)	>20000	<10 (20)	ND	ND	ND	

^a The same hapten was used in each case as the immunizing antigen. System B was developed by using serum from rabbit C and B' utilized serum from rabbit D. ^b Difluorobenzuron, Dimilin, TH-6040, N-[[4-(chlorophenyl)amino]carbonyl]-2,6-difluorobenzamide. ^c Penfluron, TH-6044, N-[[[4-(trifluoromethyl)phenyl]amino]carbonyl]-2,6-difluorobenzamide. ^d BAY SIR 8514, N-[[[4-(trifluoromethoxy)phenyl]amino]carbonyl]-2-chlorobenzamide. ^e N-[[[4-(4-chlorophenyl)]-1-acetoxycarbonyl]methyl]amino]carbonyl]-2,6-difluorobenzamide. ^f 2,6-Difluorobenzamide. ^g Carbofuran. The lack of cross reactivity of this insecticide is indicative of a variety of compounds evaluated.

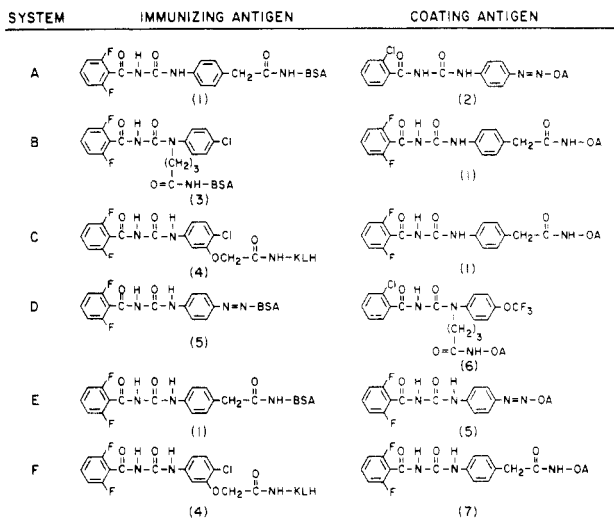


Figure 1. Structures and trivial names of benzoylphenylurea protein conjugates and combinations of different immunizing and coating antigens used in ELISA's reported here (systems A-F). See Wie et al. (1982b) and Wie and Hammock (1982a) for a further discussion of synthesis and systematic nomenclature. 1, diflubenzuron phenylacetate-protein, 2, BAY SIR 8514 diazonium-protein; 3, *N*-(carboxypropyl)diflubenzuron-protein; 4, diflubenzuron phenoxyacetate-protein; 5, diflubenzuron diazonium-protein; 6, *N*-(carboxypropyl)-BAY SIR 8514-protein; 7, BAY SIR 8514 phenylacetate-protein. Proteins routinely used include BSA (bovine serum albumin), OA (ovalbumin), and KLH (keyhole limpet hemocyanin).

the hapten and the protein. However, the different ELISA systems gave similar results in each case, and in previous studies in this laboratory, ELISA systems have indicated a loading similar to that determined chemically.

Water-Soluble Diimide Coupling Method. The requisite protein (50 mg) was dissolved in cold water (8 mL) and titrated to pH 6.5 with a pH meter by using dilute hydrochloric acid or sodium hydroxide. To each sample was added 25 mg of Morpho CDI [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-*p*-toluenesulfonate] followed immediately by an addition of 0.028 mmol of the appropriate free acid in 2 mL of dimethylformamide-water (1:5). The reaction was allowed to proceed at room temperature for 18 h and then dialyzed against four changes of 0.05 M ammonium bicarbonate followed by four changes of water. The protein conjugates were then lyophilized and stored at -20 °C as were those above.

Enzyme-Linked Immunosorbent Assays (ELISA's). ELISA's were carried out according to Voller et al. (1976a,b) with slight modifications (Wie and Hammock, 1982a,b). All the reactions were performed in polystyrene cuvettes (Gilford Instrument Laboratories, Inc., Oberline, OH), and a volume of 300 μ L was employed throughout the study. The coating antigens were diluted in 0.1 M sodium carbonate buffer, pH 9.6, containing 0.02% sodium azide and added to each well. The plates were then kept overnight at 4 °C. The coated plates were washed 3 times with PBS containing 0.05% Tween 20 and 0.02% sodium azide (PBS-Tween). After addition of diluted antiserum in PBS-Tween, plates were incubated for 2 h at room temperature. After three further washes with PBS-Tween, the goat anti-rabbit IgG-alkaline phosphatase conjugate diluted in PBS-Tween (1:2500) was added and allowed to incubate at room temperature for 2 h. After another three washes, phosphatase activity was measured following the addition of a 1 mg/mL solution of *p*-nitrophenyl phosphate in 10% (w/v) diethanolamine buffer, pH 9.8. The reaction was stopped 30-40 min later by addition of 75 μ L of 3 N NaOH, and the color intensity was measured for at least

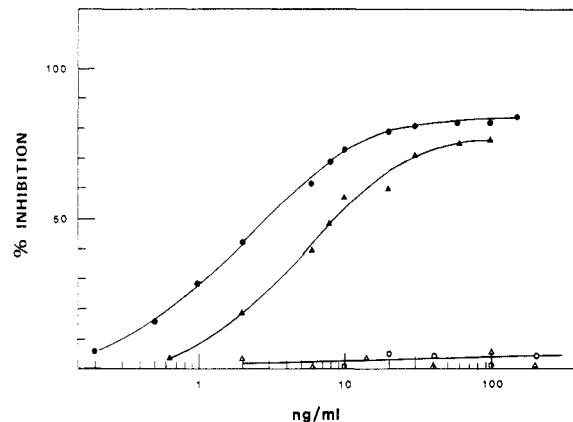


Figure 2. Typical standard curves for diflubenzuron (●), BAY SIR 8514 (▲), carbofuran (Δ), and 2,6-difluorobenzamide (○). See Table I for structures. The inhibitor concentrations are expressed in ng/mL. The ELISA system C with diflubenzuron phenoxyacetate-KLH as the immunizing antigen and diflubenzuron phenylacetate-OA as the coating antigen was used (system C, Figure 1). The serum (1/1200) from rabbit I was collected 17 months after immunization. System F (Figure 1) yielded similar curves; however, the assay showed less specificity for diflubenzuron over BAY SIR 8514.

three samples for each point in a Gilford EIA reader at 405 nm.

The preparation of inhibition curves was as follows. One millimeter of appropriately diluted anti-benzoylphenylurea serum was transferred to each of several 13 \times 100 mm culture tubes. To these antibody solutions were added increasing concentrations of hapten (i.e., diflubenzuron) dissolved in 20 μ L of acetonitrile. For the competitive assay in milk, 20 μ L of varying concentrations of diflubenzuron in acetonitrile was added to 900 μ L of milk. One part of a 10 \times PBS-Tween solution with the appropriate antibody concentration was then added. After incubation at room temperature overnight, the serum was added to the hapten-coated plates. The assays were then performed as described above. Maximum binding and background binding were assessed by adding solvent only or no antibody, respectively, to the system. Initially both whole milk and skim milk were used. Since the results were similar, only data from the analysis of whole milk are reported.

Routinely standard curves were plotted as shown in Figures 2-5. The concentration of compound yielding 50% inhibition of the color intensity (I_{50}) was determined graphically or by linear regression of the linear portion of the standard curve using a Texas Instruments 59 card programmable calculator. Alternatively, the I_{50} could be determined graphically by using probit paper or by a logit transformation on a TI 59.

Alternatively, whole milk was fortified by adding diflubenzuron in acetonitrile to give concentrations in milk ranging from 1 ppb to 10 ppt chosen so that at least three concentrations would fall on the standard curve of each ELISA. The milk samples were worked up by a modification of the method of Corley et al. (1974). Whole milk (100 g) was mixed with Na_2SO_4 (250 g) in a beaker, and then ethyl acetate (200 mL) was added. The mixture was filtered through Whatman No. 1 paper in a Büchner funnel. The procedure was repeated twice more each time with 200 mL of ethyl acetate. The combined ethyl acetate extracts were concentrated to an oily residue by rotary evaporation. The residue was dissolved in 75 mL of acetonitrile and washed 3 times with hexane (50 mL) to remove lipid. Saturated brine (75 mL) was then added to the acetonitrile and the diflubenzuron extracted with three

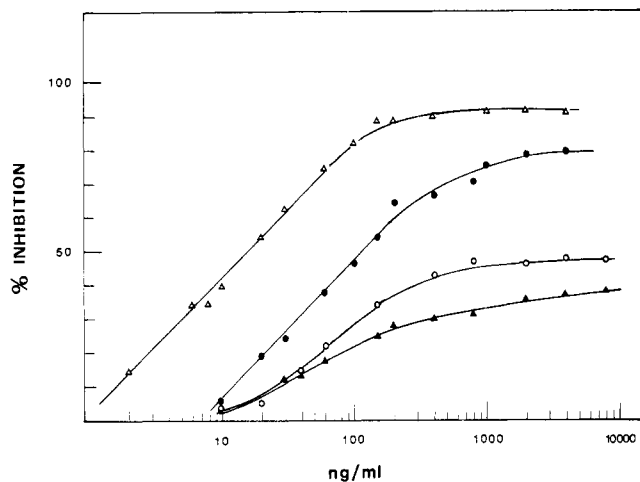


Figure 3. Effect of varying the coating antigen on the slope and sensitivities of ELISA's for anti-diflubenzuron diazonium-BSA serum obtained 8 days after secondary immunization. The concentration of antiserum and coating antigen used in each assay was optimized by checkerboard titration and the following serum dilutions were used for the four coating antigens: (a) *N*-(carboxypropyl)-BAY SIR 8514-OA [(Δ) serum dilution 1/2700]; (b) BAY SIR 8514 diazonium-OA [(\bullet) serum dilution 1/3500]; (c) diflubenzuron diazonium-OA [(\circ) serum dilution 1/3500]; (d) diflubenzuron phenylacetate-OA [(\blacktriangle) serum dilution 1/4000]. The inhibitor in each case was diflubenzuron expressed in ng/mL.

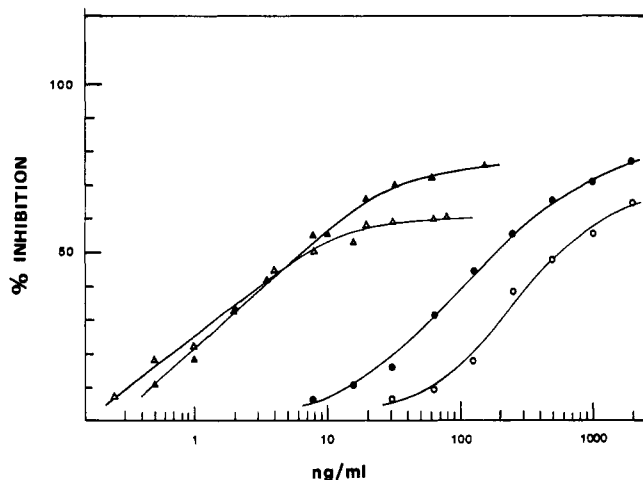


Figure 4. Standard curves for penfluron (ng/mL) in ELISA's using one coating antigen, diflubenzuron phenylacetate-OA, and several different sera from (a) rabbit I immunized with diflubenzuron phenoxyacetate-KLH (serum dilution 1/1200, system C, Figure 1) (Δ), (b) rabbit J immunized with diflubenzuron phenylacetate-BSA (serum dilution 1/1200) (\blacktriangle), (c) rabbit C immunized with *N*-(carboxypropyl)diflubenzuron-BSA (serum dilution 1/2200, system B, Figure 1) (\bullet), and (d) rabbit D immunized *N*-(carboxypropyl)diflubenzuron-BSA (serum dilution 1/2000) (\circ). See Table I for the structure of penfluron.

50-mL volumes of pentane. The pentane extract was washed with distilled water (3 \times) and concentrated by rotary evaporation and then dissolved in acetonitrile. Acetonitrile concentrations were then made up so that 20 μ L would contain the diflubenzuron fraction from 10 or 100 mL of milk equivalents. These extracts were then analyzed by using ELISA system A. Recovery was evaluated by adding [14 C] diflubenzuron to milk samples and following the distribution of radioactivity during workup. Recovery was greater than 80% in all cases.

RESULTS

The structures of coating and immunizing antigen are shown in Figure 1. Although all three protein carriers (e.g., OA, BSA, and KLH) were used for the preparation of

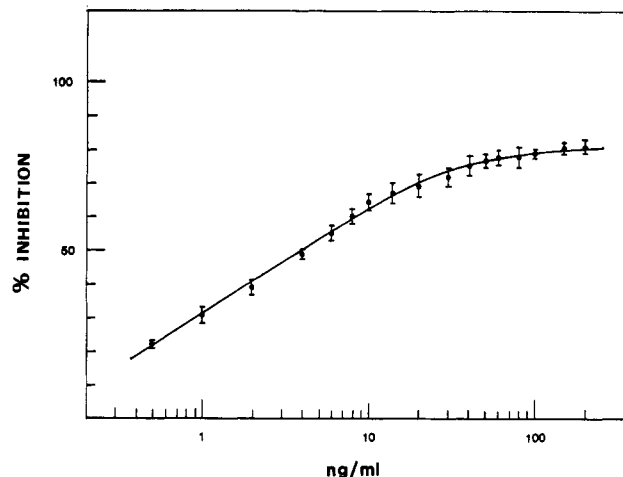


Figure 5. Standard curve for diflubenzuron in an ELISA using system A where the coating antigen is BAY SIR 8514 diazonium-OA and the serum was obtained from a rabbit J immunized with diflubenzuron phenylacetate-BSA (serum dilution 1/1200). See Table I for the structure. The curve represents an average of data generated over a period of many weeks. The standard deviation of the absorbance values was less than 3% within a single run and less than 5% among runs. There was no difference between the standard curve generated in water and two curves generated with extracts containing 10 and 100 mL equivalents of milk/mL of antibody solution added in 10 μ L of acetonitrile. At least three replicates each of three points of the linear region of the standard curve were run in each case (Ham-mock, 1982).

hapten-protein conjugates, only a selected number of compounds are reported in this study to facilitate an easy comparison of the specificity of the coating antigens and an easy comparison of the specificity of the antibody produced. For the sake of simplicity, two major categories of compounds based on the structure of diflubenzuron and BAY SIR 8514 were investigated in the present study. Two rabbits were used with each immunizing antigen; however, serum from only one of the two rabbits was employed in the development of the reported ELISA's except in the case of systems B and B' in Table I, which illustrate a difference in specificity. All of the antigen preparations used led to the production of useful antibody titers.

Sera from rabbits were first examined for antibody production with the following coating antigens: (a) the carrier protein only, (b) hapten coupled to carriers different from the immunizing antigen but with the same chemical linkage, and (c) hapten coupled to different carriers with a chemical linkage different from the immunizing antigen. Many other combinations of coating antigen and antiserum were investigated in the development of ELISA's, but only a limited selection of systems are shown in Figure 1 and Table I. Except in system D where the carrier immunizing antigen was KLH, a combination of the other two protein carriers (OA and BSA) was used in the present study. Systems were not pursued further if nonspecific binding of the antiserum to a carrier protein other than immunizing protein was observed. For example, sera from two rabbits immunized with BAY SIR 8514 phenylacetate-BSA were found to contain very high titers (background) when KLH and OA were used as coating antigens. Similarly, when weak binding of the antiserum to a particular coating antigen in ELISA's resulted either in the use of uneconomically high antibody titers or in inhibition curves at only very high concentrations, the system was not explored further. If much better ELISA's had not been found, there are procedures by which one often can develop useful immunoassays in spite of the above difficulties.

The optimal coating concentrations for all of the benzoylphenylurea conjugates were determined by using a checkerboard titration of both antigen and antisera. Since a plateau was reached when a concentration of greater than 4 $\mu\text{g}/\text{mL}$ coating antigen was employed in the ELISA, a solution of 5 $\mu\text{g}/\text{mL}$ protein concentration was then used for coating plates in subsequent studies.

The ability of diflubenzuron to inhibit the binding of anti-diflubenzuron phenoxyacetate-KLH to antigen coated cuvettes is demonstrated in Figure 2 (system C, Figure 2). The results are expressed as percent inhibition of a standard amount of antibody plotted against a variable concentration of hapten. Diflubenzuron exhibited strong inhibition with the 50% inhibition point (I_{50}) at a concentration of 3.0 ng/mL, and ultimately 83% inhibition could be obtained. The inhibition curve was linear over a range of hapten concentrations from 0.5 to 15 ng/mL. Inhibition studies were also performed using other haptens such as BAY SIR 8514 and penfluron (Table I), and it was found that these benzoylphenylureas were strong inhibitors for this particular antibody-antigen system. However, the closely related *N*-carboxypropyl derivative of diflubenzuron was found to be a poor inhibitor. This result could be anticipated as a portion of the antibody population elicited by the immunogen recognized not only the hapten but also the bridge. However, neither 2,6-difluorobenzamide nor carbofuran could displace the binding of antibody even at the relatively high concentration of 20 $\mu\text{g}/\text{mL}$. These results indicate that highly sensitive immunoassays can be achieved for a particular class of compounds such as the benzoylphenylureas. When the same antiserum was retained and the coating antigen slightly changed to BAY SIR 8514 phenylacetate-OA (system F, Figure 1), the working titer of the serum used was slightly reduced, the sensitivity for diflubenzuron increased somewhat, and the ability of the assay to distinguish diflubenzuron and BAY SIR 8514 decreased as expected (data not shown).

Figure 3 shows the inhibition of the anti-diflubenzuron diazonium-BSA antibodies with diflubenzuron when different coating antigens were used in the ELISA. Each of the four antigens was first titrated with the same antiserum to obtain the optimal working concentration. Although the optimal antiserum dilution was very similar in all cases, the effect of diflubenzuron in inhibiting the antibodies binding to the coating antigen was found to be significantly different with the following order: *N*-(carboxypropyl)-BAY SIR 8514-OA > BAY SIR 8514 diazonium-OA > *N*-(carboxypropyl)diflubenzuron-OA (data not shown) > diflubenzuron phenylacetate-OA > diflubenzuron diazonium-OA. The strong inhibiting effect of diflubenzuron when *N*-(carboxypropyl)-BAY SIR 8514-OA was employed as the coating antigen could be due to the tertiary N atom and the 2-chlorophenyl ring present in the molecule, which are different from that of the immunizing antigen. In this system, one can easily quantitate the amount of diflubenzuron in the low nanogram level, and the assay is linear between 2 and 200 ng/mL. In the same figure, BAY SIR 8514 diazonium-OA as the coating antigen was also found to be useful in the quantitation of the diflubenzuron although it was 8 times less sensitive than in system E. However, the inhibition curve is linear between the concentrations of 15 and 400 ng/mL. This assay enables one to determine diflubenzuron over a wide range of concentrations. On the other hand, when either diflubenzuron diazonium-OA or diflubenzuron phenylacetate-OA was used as the coating antigen, the resulting assays showed a lack of sensitivity for the determination of diflubenzuron

because of the strong binding of antibodies to these two coating antigens. The maximum inhibition for diflubenzuron under these conditions was shown to be 48 and 39%, respectively. The lowest maximum inhibition obtained for diflubenzuron diazonium-OA as the coating antigen could be directly attributable to the recognition of the linkage by the antibody as seen in many other immunoassays for small molecules.

In Figure 4, ELISA's for penfluron using diflubenzuron phenylacetate-OA as the coating antigen and sera obtained from four rabbits immunized with three different antigens are shown. Sera obtained from rabbit I and rabbit J gave very similar results for the assay of penfluron with a maximum inhibition of 76 and 60%, respectively, whereas serum from rabbit C could be used to determine the penfluron with lesser sensitivity. The difference in the inhibition curves for the two sera (rabbits C and D) that were immunized with the same antigen has also been demonstrated in the previous experiment using diflubenzuron as the inhibitor. All the above ELISA systems also demonstrated specificity for the benzoylphenylureas since a variety of other unrelated compounds such as carbofuran and 2,6-difluorobenzamide showed no inhibition even at high concentrations.

Figure 5 shows the standard curve for diflubenzuron in ELISA system A in which BAY SIR 8514 diazonium-OA was used as the coating antigen and the serum was obtained from a rabbit immunized with diflubenzuron phenylacetate-BSA. The curve represents the average of data points run over a period of several weeks, and it is apparent that the sensitivity easily reached 0.5 ppb. As discussed earlier (Wie and Hammock, 1982a), this assay is adequate for the direct analysis of diflubenzuron in environmental water samples if care is taken to adjust the pH. The specificity of this assay was examined by using several benzoylphenylureas (Table I), and they were found to inhibit the binding of antibodies with I_{50} 's of < 10 ng/mL. However, other unrelated pesticides and difluorobenzamide did not inhibit the binding even at a concentration of >10 $\mu\text{g}/\text{mL}$. Table I also outlines results from some of the ELISA systems compared in the present work. All the systems were found useful in detecting the benzoylphenylureas. A-C (Table I; Figure 1) are the most sensitive systems as a concentration of less than 1 ng/mL of benzoylphenylureas can be determined. Using the combination of two or more systems, it was also possible to distinguish two closely related compounds. For example, systems A and B or systems B and C can be employed to differentiate *N*-(carboxypropyl)diflubenzuron from other benzoylphenylureas while systems A and B could be used to distinguish diflubenzuron from BAY SIR 8514 and penfluron. Alternatively, less sensitive, but more specific ELISA's can be used to determine the concentration of diflubenzuron in the presence of BAY SIR 8514 (Wie and Hammock, 1982a). From the examples given above, sensitivity and specificity of immunoassays are therefore illustrated clearly. Only system E could be used to detect the metabolite 2,6-difluorobenzamide, and the sensitivity was at the ppm level. None of the systems detected the benzoic acids or anilines from diflubenzuron or BAY SIR 8514. However, several of the intermediates discussed in the synthesis of haptens could be used to raise antibodies for these potential metabolites (Wie et al., 1982b).

In order to detect the diflubenzuron in the milk (whole homogenized or skim milk) directly, we first performed the ELISA with system A in the presence of milk, and the results are shown in Figure 6. The results of the assay are represented as the percent absorbance vs. the per-

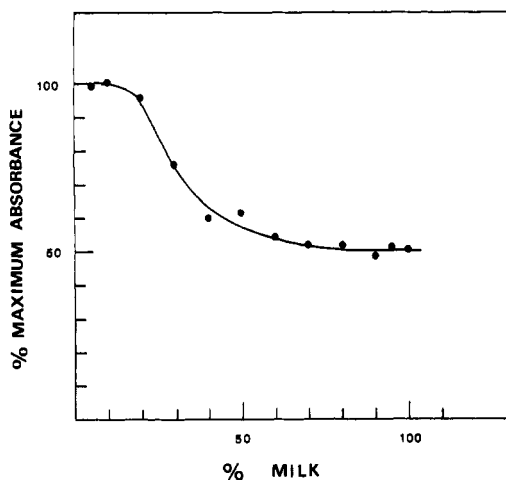


Figure 6. Effect of the milk on the binding of diflubenzuron phenylacetate-BSA antibody to BAY SIR 8514 diazonium-OA coated cuvettes. One part of serum in 10× PBS-Tween was added to 9 parts of different percentages (v/v) of milk in water. The antibody (final concentration 1/1000) was then added to the cuvette and the assay was carried out as described Materials and Methods.

centage of milk in the assay. It was surprising to note that interference was found at a ~20% (v/v) milk concentration (95% of maximum absorbance), and the maximum absorbance was reduced to 60% at a concentration of 50% milk. However, further increases in milk content did not reduce the binding of antibody and a plateau was reached at ~70% milk. To obtain the absorbance as high as the test that was performed in PBS-Tween, it was necessary to increase the antibody concentration from 1/1200 to 1/700 in this particular assay. A 10-fold concentrated solution of PBS-Tween was added to the milk to bring its level equal to the PBS-Tween concentration used in the optimization test. This procedure is important because of pH of some milk samples varied dramatically. The level of 90% milk was chosen from the plateau region of the assay as seen in Figure 6. The binding of antibodies in the presence of milk was also shown to be specific as seen in Figure 7. Using diflubenzuron as the inhibitor, it was demonstrated that a sensitive assay could be achieved with an I_{50} of 20.5 ng/mL, and a concentration of 2 ng/mL readily could be detected in whole milk with no sample workup. In the same figure, a standard curve for diflubenzuron in the absence of milk is also given. The detection of diflubenzuron in milk was 5 times less sensitive than in PBS-Tween, and this obviously was due to the high concentration of antibody used in the assay.

To improve the detection level, the diflubenzuron was extracted from milk. The initial ethyl acetate extract contained so much lipid that the resulting assay was only slightly more sensitive than analysis of whole milk. Probably the antibody is able to extract diflubenzuron from small lipid micelles in milk during the course of the assay, but the large lipid droplets from the ethyl acetate extract of milk interfered with the assay.

To avoid this problem the ethyl acetate extract of the milk was subjected to differential partitioning before the ELISA procedure. Therefore, the diflubenzuron and biomass extracted from 10 or even 100 mL of milk was added to each assay (10 or 100 milk equivalents). In this extraction procedure one could account for all of the [14 C] diflubenzuron added with over 80% of the radiolabel appearing in the final acetonitrile solution. No difference was observed among the two standard curves for diflubenzuron run with 10 mL of milk equivalents per assay

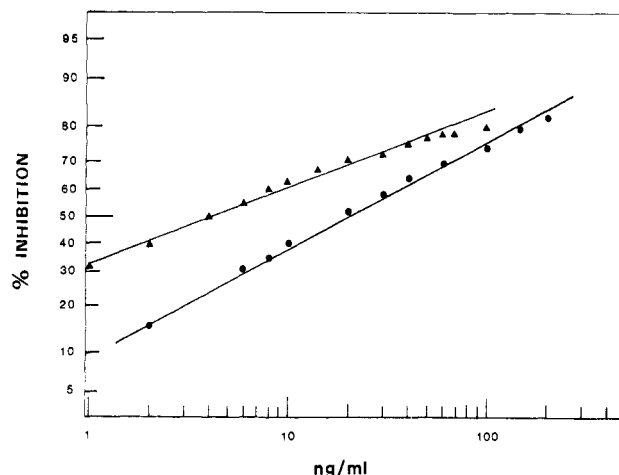


Figure 7. Standard curve for and sensitivities of an ELISA for the insect growth regulator diflubenzuron in whole milk (●) and PBS-Tween buffer (▲). Inhibition is expressed on a probit scale to increase the linear region of the standard curve. Before transformation to a probit scale, the curve for diflubenzuron in buffer was very similar to the curve shown in Figure 2.

and 100 mL of milk equivalents per assay and the standard curve for diflubenzuron shown in Figure 5 by using ELISA system A. In other words, the lines generated from the milk extracts were parallel to the standard curve and statistically indistinguishable from it at at least three points. Also, the diflubenzuron concentration estimated from the ELISA was identical with that estimated from the radioactivity in the final acetonitrile sample and the diflubenzuron added to the original milk. The linear region of the standard curve extends well into the picogram range. However, assuming a sensitivity of only 1 ng, one is capable of analyzing below the 100-ppt level using 10 mL of milk and below 10 ppt using 100 mL of milk. Because of the above data, the limited number of extraction steps involved, and the high, reproducible recovery, the assay procedure is anticipated to be very accurate. It must be cautioned that the unique polarity of diflubenzuron allows a tremendous cleanup to be effected by this simple partition method. Such a dramatic increase in sensitivity should not be anticipated for most pesticides.

DISCUSSION

In order to induce specific antibodies to a small molecule, the hapten should be coupled covalently to the carrier molecule with a spacer arm sufficiently long to allow T-cell recognition. Unfortunately, at least a proportion of the antibody population elicited by any particular hapten conjugate recognizes not only the hapten and carrier protein but also the bridge. In consequence, a coating antigen sharing the same bridge often has a higher affinity for the antibody than does the free hapten. For example, it was concluded from the studies on parathion immunoassay development that due to the small size of parathion, the induced antibodies preferentially bound the parathion derivatives that contained the bridging group structures and the various precursors of these derivatives (Vallejo et al., 1982), although as shown by this and other studies, successful assays based on homologous systems are possible (Hunter and Lenz, 1982; Hunter et al., 1982). Similarly, in the present study, diflubenzuron could not inhibit the antibodies obtained from a rabbit immunized with *N*-(carboxypropyl)diflubenzuron-BSA while *N*-(carboxypropyl)diflubenzuron-OA was used as the coating antigen in the ELISA. However, it is possible to develop a useful assay by using diflubenzuron phenylacetate-OA as the coating antigen with this antibody as shown in our

previous study (Wie and Hammock, 1982a). It is demonstrated also by the use of anti-diflubenzuron phenoxyacetate-KLH antiserum that one can detect diflubenzuron at a concentration of <0.5 ppb (Figure 5). Moreover, this assay system is particularly suitable for the detection of a class of compounds such as the benzoxyphenylureas since penfluron and BAY SIR 8514 also could be detected at the low nanogram level. The specificity of the assay was demonstrated by the observation that *N*-carboxypropyl derivatives of diflubenzuron and BAY SIR 8514, carbofuran and difluorobenzamide, and a variety of other compounds were poor inhibitors of this ELISA. From the view point of residue analysis, it is useful to develop an ELISA that can detect a class of compounds, thus facilitating rapid screening of a large number of samples. Other systems (Table I, systems B-D) also are useful in measuring benzoxyphenylureas as a class. Moreover, it is possible to distinguish one benzoxyphenylurea from the others by the combination of two or more systems and analysis using simultaneous algebraic equations. The same principle was demonstrated further by the results shown in Figure 4 where penfluron inhibits the binding of four different antisera to a same coating antigen, diflubenzuron phenylacetate-OA. A similar degree of inhibition by penfluron was found for diflubenzuron phenylacetate-KLH and diflubenzuron diazonium-BSA sera, but the serum from rabbits immunized with *N*-(carboxypropyl)diflubenzuron-BSA demonstrated higher affinity to the coating antigen than the two previous antisera. It could be noted in this report that all of the immunizing antigens used contained the 2,6-difluorobenzoyl moiety. Greater specificity and sensitivity for compounds such as BAY SIR 8514 could be obtained by using the 2-chlorobenzoyl moiety. Also, all haptens were coupled via the aniline moiety, giving greatest specificity for the benzoyl portion. A further study utilizing attachment through the benzoyl moiety should yield immunoassays capable of distinguishing benzoxyphenylureas differing in the chemistry of the aniline moiety as well.

In Figure 3, the use of one antiserum and several different coating antigens in the development of an ELISA is demonstrated clearly. Both *N*-(carboxypropyl)-BAY SIR 8514-OA and BAY SIR 8514 diazonium-OA were found to be useful as coating antigens in the assay, but the binding of *N*-(carboxypropyl)diflubenzuron-OA was stronger than free diflubenzuron so that a poor dose-response curve was observed. The tertiary N atom present in the diflubenzuron molecule of the coating antigen did not lead to significant interference with recognition, but further modification of the molecule by converting the 2,6-difluoro- to a 2-chloro-substituted phenyl for the coating antigen was needed to optimize the ELISA. These results further illustrate that careful consideration of chemical linkage and its position in the preparation of hapten-protein conjugates may lead to valuable immunoassays for small molecules.

Besides the ability of the immunoassay to detect a group of compounds, the specificity of this assay can be very impressive. Immunoassays for diflubenzuron have been shown to distinguish it from the very closely related compound, BAY SIR 8514 (Wie and Hammock, 1982a). Similarly, RIA's for the insecticide *S*-bioallethrin have shown that immunoassay can distinguish the eight optical and geometrical isomers of allethrin with over a 100-fold distinction between the insecticidal, 1*R*,3*R*,4'*S* compound and some related optical and geometrical isomers (Wing and Hammock, 1979). The ability to distinguish closely related compounds is also well-illustrated with the Triton X and

Triton N nonionic surfactants. The ELISA can detect all members of the Triton X nonionic series (Triton X 45, 100, 114, 305, 405) while showing minimal cross reactivity for the closely related Triton N series, the corresponding Triton X or N free phenol, or other ionic or nonionic surfactants (Wie and Hammock, 1982b). Hence, one can design a degree of specificity into an immunoassay such that an ELISA system can be used to rapidly screen classes of compounds while other ELISA's can be very specific for a particular compound. It also is possible to distinguish the parent materials from their metabolites by immunoassay while developing other assays for both parent and metabolites.

The potential sensitivities of immunoassays are well-known, and the assay of diflubenzuron in the milk was chosen as an example since it represents a difficult substrate for residue analysis. As indicated in Figure 7, direct analysis of milk for diflubenzuron with ELISA provides the necessary sensitivity (2 ppb). Furthermore, two partitioning steps allow one to quantitate diflubenzuron below a level of 10 ppt. Now it is important that the accuracy and reproducibility of immunochemical procedures be determined on actual analytical samples evaluated in two or more laboratories. The extraction/partition procedure, which allows analysis at below 10 ppt, is far more laborious than the direct assay shown in Figure 7 yet much more rapid than the gas-liquid chromatography or high-pressure liquid chromatography assays for this class of compounds. As with any physical assay, sensitivity can be increased at the expense of added analysis time and reagent costs. The advantage of immunoassay is that for appropriate targets the requisite sensitivity can be obtained at much lower cost than with classical techniques.

In classical residue analysis the minimum detectable amount usually is determined from a chromatographic peak that is statistically different from the noise on a sample background trace. Such a criterion could be applied to immunoassays, especially if the assay were being extended to its absolute limit of sensitivity. In the assays reported here, the sensitivities were high enough that one could practically define sensitivity in terms of the linear region of the standard curve. For instance, in the direct analysis of diflubenzuron in milk shown in Figure 7, one could reproducibly detect an inhibition of 5% and distinguish it from background values. This value yields a limit of detection below 500 pg/mL of milk. However, in this study only the linear portion of the curve was used (a minimum of 15% inhibition), yielding a sensitivity of approximately 2 ng/mL of milk. This latter course of action commonly is used with immunoassays for several reasons. Not only does it simplify calculation, but this approach allows one to run several dilutions of an unknown and thus determine if the concentration/inhibition line of the unknown is parallel to that of the standard curve. This approach not only gives greater confidence in the quantitative data obtained but also provides qualitative information.

The other major advantage offered by immunochemical analysis is speed. Most of the expense in an analysis is involved in sample cleanup. The sensitivity and specificity of immunoassays tend to minimize the need for such cleanup procedures and thus may tremendously reduce the cost of analysis. Moreover, solid-phase immunoassays are very easy to automate. For instance, an inexpensive interface between the Gilford EIA reader and an Atari 400 computer in this laboratory is being used for semiautomated data acquisition and analysis. By use of more sophisticated systems with minimal technician input, hun-

dreds of samples can be analyzed per man day and the resulting data analyzed automatically. The speed of the immunoassay can be improved further by using a variety of techniques such as direct ELISA as shown in our previous work on diflubenzuron (Wie and Hammock, 1982a).

Immunoassays have numerous potential applications to environmental chemistry, but immunochemical technology is most likely to find rapid application to those compounds where analysis by classical procedures is very difficult or expensive. The benzoylphenylurea insect growth regulators represent such an intractable residue problem. The compounds are nonvolatile and can only be derivatized for GLC after a lengthy and tedious series of wet chemical procedures. The extinction coefficients are too low for rapid, sensitive analysis by HPLC methods, and these methods are quite laborious when the compounds must be extracted from a complex matrix. In marked contrast, the immunoassay procedures developed in this laboratory are much more sensitive and reproducible at a fraction of the cost. The unique polarity of these compounds allows simple partition procedures to be used for rapid cleanup of the parent material. Although development of immunoassays for the major metabolites of the benzoylphenylureas should be trivial, there may be limited need for such assays since metabolites possibly can be estimated as a function of parent detected. Immunoassay may offer some advantages over classical procedures even when those classical procedures are straight forward. Once the technology is transferred to a residue laboratory, it is likely to be applied to a still wider variety of analytical problems such as microbial agents (Wie et al., 1982a) and products of genetic engineering.

In this study it is demonstrated that the sensitivity of a solid-phase immunoassay can be increased greatly by using heterologous immunoassay systems. Work on benzoylphenylureas also illustrates that even a limited collection of antisera and coating antigens can yield a powerful library of assays for residue analysis (Wie et al., 1982b; Wie and Hammock, 1982a,b).

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Registry No. Diflubenzuron, 35367-38-5; BAY SIR 8514, 64628-44-0; penfluron, 35367-31-8; *N*-[[[(4-chlorophenyl)[1-(acetoxycarbonyl)methyl]amino]carbonyl]-2,6-difluorobenzamide, 82049-98-7; 2,6-difluorobenzamide, 18063-03-1.

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