Competitive Inhibition ELISA for the *s*-Triazine Herbicides: Assay Optimization and Antibody Characterization

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The present work describes screening and selection of rabbit antisera raised against the *s*-triazine herbicides, characterization of antibody cross-reactivity and specificity, and improvements in ELISA sensitivity. These results stem from a comprehensive synthetic approach described previously for the production of *s*-triazine haptens. Both homologous and heterologous ELISA systems were examined by using heterology based on hapten conjugation position, spacer length, and/or alkyl substitution. Sensitivity to the target analyte and to nonspecific effects was least for homologous systems. Except for extreme differences in spacer length, heterology based on conjugation position provided the largest improvement in sensitivity to the target analyte. Matrix and solvent effects depended on the ELISA system used. Specificity studies using several antibodies in competitive inhibition ELISA with over 30 inhibitors showed that conjugation position and alkyl substitutions were important determinants of antibody specificity and that the antibodies recognized the immunizing hapten better than all other inhibitors tested. The resulting assays were capable of detecting the parent and related *s*-triazines at low ppb to sub-ppb levels.

INTRODUCTION

The utility of immunoassays for the analysis of pesticide residues has been well established (Hammock and Mumma, 1980; Newsome, 1986; Harrison et al., 1988; Van Emon et al., 1989; Jung et al., 1989). The importance of synthesizing multiple haptens for the development of immunoassays has also been shown (Wie and Hammock, 1984; Harrison et al., 1989b), although this approach is not widely followed in the field of environmental analysis or analysis of crop plants (Weiler, 1990). Several immunochemical methods have been described for the analysis of *s*-triazine herbicides, all of which used a limited repertoire of haptens for the development of antibodies (Bushway et al., 1988; Dunbar et al., 1985; Huber, 1985; Huber and Hock, 1985; Robotti et al., 1986; Schlaeppi et al., 1989; Wittmann and Hock, 1989). We have previously described the synthesis and characterization of several triazine haptens (Goodrow et al., 1990) that provide expanded possibilities for the control of assay sensitivity and specificity through the use of a variety of haptens in the preparation of immunogens, plate-coating antigens, and/or enzyme–hapten conjugates. The present study describes the screening and selection of ELISA systems for further study and the characterization and optimization of the selected systems.

MATERIALS AND METHODS

Reagents. Immunochemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or ICN ImmunoBiologicals (Lisle, IL). Analytical standards for competitive inhibition ELISA were donated by Ciba-Geigy and Du Pont. The production of triazine haptens, hapten–protein conjugates, and rabbit anti-triazine antibodies has been described in detail previously (Goodrow et al., 1990). Briefly, four of the synthesized triazine haptens were conjugated to KLH, CONA, and THY carrier proteins through their carboxylic acid groups, and the conjugates were used to immunize rabbits. All results given here are for sera from terminal bleeds.

Enzyme Immunoassay and Competitive Inhibition Enzyme Immunoassay. ELISA and competitive inhibition ELISA were performed according to the methods of Goodrow et al. (1990) and Harrison et al. (1989a) in 96-well microplates (Nunc 442404). For ELISA, triazine–BSA conjugates (ELISA antigens) were adsorbed nonspecifically to the plates. Dilutions of rabbit antiserum were added, and the bound anti-triazine antibodies were quantitated by the subsequent sequential addition of enzyme-labeled goat anti-rabbit antibody and enzyme substrate. For competitive inhibition ELISA, the rabbit antiserum dilutions were preincubated with dilutions of a soluble inhibitor before they were added to the antigen-coated plate. Inhibitors were prepared as 50 mM solutions in DMSO, diluted to 1 mM working stocks in DMSO, and then diluted 20-fold with PBST for the competitive inhibition step of the ELISA. Endpoint readings of plates were made with a V_{max} microplate reader (Molecular Devices, Menlo Park, CA). All inhibition curves used for calculation of IC_{50} values were composed of a zero-dose control plus 10 nonzero standard concentrations, with duplicate or quadruplicate ELISA wells at each concentration. The software package Softmax (version 2.01, Molecular Devices) was used for fitting of sigmoid standard curves based on the four-parameter logistic method of Rodbard (1981). The calculated IC_{50} values were used for comparison of inhibition curves and determination of antibody cross-reactivity. In cases where the lower tail (high-concentration end) of the curve was not adequately defined by data points, a semilog fit of the steepest region of the curve was used to estimate the IC_{50} value, if possible.

RESULTS AND DISCUSSION

Screening of Antisera. The assay systems (combinations of antibody and ELISA antigen) tested are summarized in Table I. In general, the strategy of Harrison et al. (1990) was applied to antibody screening. The titer of each antibody was monitored over time by using ELISA to measure binding to several triazine–BSA conjugates compared with BSA as a control antigen. Examples of results from this procedure are given in Figures 3 and 4 of Goodrow et al. (1990). All of the 17 immunized rabbits demonstrated significant titers on homologous ELISA antigens, and most of these sera also exhibited moderate to high titers on at least one heterologous ELISA antigen. The rate of success of the haptens in eliciting the production of the desired antibodies was variable. Two antisera exhibiting the highest early titers on homologous ELISA antigens proved eventually

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Table I. Summary of Results of Immunization and Screening of Rabbits[a]

immunizing hapten	no. of rabbits	hapten conjugated to BSA for ELISA								
		1a	**1b**	**2b**	**2c**	**2e**	**2g**	**2h**	**4a**	**4b**
2e	5	*	*	*	*	*	nt	*	*	*
2h	3	*	**	*	*	*	nt	*	**	**
4a	5	*	*	*	*	*	*	*	*	*
4b	4	*	**	nt	nt	**	nt	**	*	*

[a] Antibodies raised against each immunizing hapten (conjugated to KLH, CONA, or THY) were screened by ELISA on BSA conjugates made with each of the haptens indicated. Each combination of antibody and ELISA antigen that exhibited a significant titer was then also tested for sensitivity to atrazine in a competitive inhibition ELISA. An asterisk indicates useful titer and sensitivity to atrazine at the ppm level or below for that combination. Combinations not tested are indicated by nt. The most useful systems are marked by double asterisks. Hapten numbers conform to those of Goodrow et al. (1990). Structures are given in Table II.

Table II. Cross-Reactivity of s-Triazines with Anti-Triazine Sera in Competitive Inhibition ELISA[a]

inhibitor	structure			rabbit no., immunizing hapten/ELISA hapten						
	R1	R2	R3	194, **4b/2e**	268, **4b/2e**	357, **2h/4a**	355, **4a/2h**	354, **4b/2e**	841, **2e/4b**	842, **4a/2h**
atrazine	Cl	$NHCH_2CH_3$	$NHCH(CH_3)_2$	39	22	100	23	45	250	10
simazine	Cl	$NHCH_2CH_3$	$NHCH_2CH_3$	2.6	1.6	12	20	4.1	10	10
ametryne	SCH_3	$NHCH_2CH_3$	$NHCH(CH_3)_2$	100	100	5.6	65	100	20	51
simetryne	SCH_3	$NHCH_2CH_3$	$NHCH_2CH_3$	5.7	5.8	0.8	100	24	27	100
propazine	Cl	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	52	13	260	13	16	180	1.2
prometryne	SCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	220	44	11	31	40	8	4
prometon	OCH_3	$NHCH(CH_3)_2$	$NHCH(CH_3)_2$	16	9.0	14	1.9	10	12	0.5
terbutryne	SCH_3	$NHCH_2CH_3$	$NHC(CH_3)_3$	13	1.7	1.5	11	24	4	6
1a	Cl	Cl	$NHCH_2CH_3$	ND	ND	ND	ND	ND	ND	ND
1b	Cl	Cl	$NHCH(CH_3)_2$	ND	ND	ND	ND	ND	ND	ND
2a	Cl	$NHCH_2CH_3$	$NHCH_2COOH$	0.1	ND	<0.1	ND	ND	0.8	<0.1
2b	Cl	$NHCH_2CH_3$	$NH(CH_2)_2COOH$	0.3	ND	0.3	ND	ND	5	0.4
2c	Cl	$NHCH_2CH_3$	$NH(CH_2)_3COOH$	0.5	ND	1.6	ND	ND	89	0.2
2d	Cl	$NHCH_2CH_3$	$NH(CH_2)_4COOH$	0.8	ND	6.5	ND	ND	290	0.5
2e	Cl	$NHCH_2CH_3$	$NH(CH_2)_5COOH$	0.9	0.4	44	6.9	1.1	**1830**	0.5
2f	Cl	$NHCH(CH_3)_2$	$NHCH_2COOH$	ND	ND	ND	ND	ND	<0.1	<0.1
2g	Cl	$NHCH(CH_3)_2$	$NH(CH_2)_2COOH$	ND	ND	ND	ND	ND	3	<0.1
2h	Cl	$NHCH(CH_3)_2$	$NH(CH_2)_5COOH$	10	23	**700**	12	8.6	640	0.9
4a	SCH_2CH_2COOH	$NHCH_2CH_3$	$NHCH_2CH_3$	22	14	0.7	**1050**	33	15	**157**
4b	SCH_2CH_2COOH	$NHCH_2CH_3$	$NHCH(CH_3)_2$	**330**	**200**	4.4	740	**670**	23	90
cyanazine	Cl	$NHCH_2CH_3$	$NHCCN(CH_3)_2$	2.1	0.2	0.4	1.6	0.6	1.2	<0.1
cyanazine acid	Cl	$NHCH_2CH_3$	$NHCCOOH(CH_3)_2$	0.2	<0.1	0.2	<0.1	0.1	<0.1	<0.1
cyanazine amide	Cl	$NHCH_2CH_3$	$NHCCONH_2(CH_3)_2$	<0.1	<0.1	0.1	<0.1	0.1	<0.1	<0.1
hydroxycyanazine acid	OH	$NHCH_2CH_3$	$NHCCOOH(CH_3)_2$	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1
hydroxyatrazine	OH	$NHCH_2CH_3$	$NHCH(CH_3)_2$	0.7	0.3	0.9	<0.1	1.1	1.2	0.2
hydroxysimazine	OH	$NHCH_2CH_3$	$NHCH_2CH_3$	<0.1	<0.1	0.4	<0.1	0.3	<0.1	<0.1
deethylsimazine	Cl	NH_2	$NHCH_2CH_3$	0.4	<0.1	0.1	1.3	1.1	2	0.4
deethylatrazine	Cl	NH_2	$NHCH(CH_3)_2$	1.0	2.5	0.3	2.3	3.2	<0.1	0.2
deethylsimetryne	SCH_3	NH_2	$NHCH_2CH_3$	0.6	0.6	0.1	4.9	2.9	0.6	0.9
didealkylated atrazine	Cl	NH_2	NH_2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
cyanuric acid	OH	OH	OH	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ammeline	NH_2	OH	OH	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
ammelide	NH_2	NH_2	OH	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
melamine	NH_2	NH_2	NH_2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

[a] Values given are percent cross-reactivity [(parent compound IC_{50}/test compound IC_{50}) × 100]. The compound chosen as the basis for comparison was the triazine parent (not a synthesized hapten; indicated by underlining) with the structure closest to the immunizing hapten (which is indicated by boldface). IC_{50} values are based on curves constructed by using a zero-dose control and 10 nonzero concentrations of each inhibitor, two or four replicate wells at each concentration. All curves for a single antibody were obtained in a single assay. ND indicates not done. Numbers for haptens conform to those of Goodrow et al. (1990).

to be useless for competitive inhibition ELISA, even though they also recognized some heterologous ELISA antigens well. In general, the atrazine haptens (**2h** and **4b**; structures in Table II) produced better antibodies than the simazine haptens (**2e** and **4a**). For example, all four rabbits immunized with conjugates of hapten **4b** produced antibodies with high titers in heterologous systems. In contrast, only two of the five rabbits immunized with conjugates of hapten **2e** had significant titers on heterologous antigens. The lower success rate with **2e** compared to **4b** may be related to the solubility differences noted during synthesis and conjugation (Goodrow et al., 1990), but the design of this study prevents drawing firm conclusions. This response difference seems unlikely to be due entirely to the observed solubility differences among haptens, since all immunogens produced significant antibody titers on homologous antigens. Specificity differences due to different positions of conjugation may also play a role, as will be discussed below.

Differences in antibody titer were also observed among the carrier proteins used for immunization. Most of the KLH and CONA conjugates produced acceptable responses, while the THY conjugates were less successful, likely due to their poor solubility. Only one of four THY conjugates produced titers comparable to CONA and KLH carriers with the same hapten. When poor binding of an antiserum to an ELISA antigen resulted in a low signal (absorbance for the zero-dose control below 0.200) or the

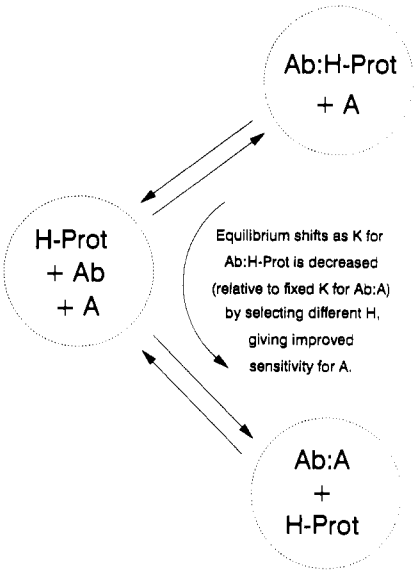

Figure 1. Rationale for the use of heterologous haptens in ELISA. Schematic representation of "quasi-equilibrium" immunoreactions in ELISA, occurring on the antigen-coated plate. Antibody is denoted Ab, analyte A, and hapten–protein conjugate H-Prot. The hapten–protein conjugate is the ELISA antigen (or plate-coating antigen). Complex formation is indicated by a colon between components, as Ab:A, which represents analyte bound to antibody. Note that a limiting concentration of antibody is required. Thus, for most assays the highest sensitivity is obtained when the affinity of the antibody for the ELISA hapten is lower than the affinity of the antibody for the analyte.

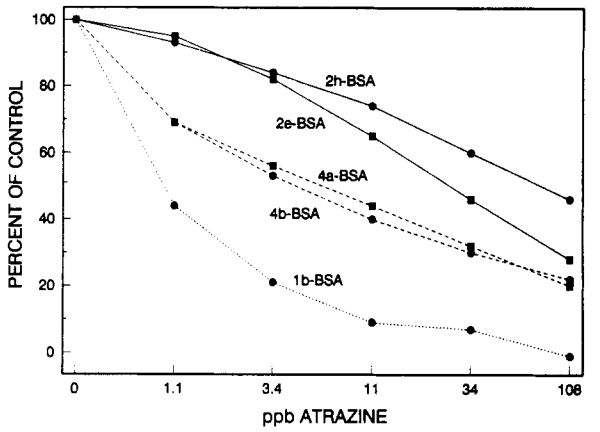

Figure 2. Effect of hapten selection on sensitivity of competitive inhibition ELISA. Inhibition of rabbit 357 serum (anti-**2h**-CONA) by atrazine was performed by using five different optimized systems, differing only in the hapten conjugated to BSA to make the plate-coating antigen used for the ELISA. Data points represent the means of triplicates. Coefficients of variation averaged 1.1%.

use of uneconomically high amounts of antiserum (dilution >1/500), the system was not explored further. Thus, only one of the seven rabbits of Table II was immunized with a THY conjugate. Systems having the highest titers on the largest number of heterologous antigens were favored for further development. These combinations of antibody and ELISA antigens were advanced to the next level screening procedure as described below. The antibodies having high titers only in homologous systems were generally not useful in competitive inhibition assays.

Assay Optimization. Optimization of individual ELISA systems, selected by the above procedure, was performed by two-dimensional titration according to the method of Gee et al. (1988). These systems were then tested for sensitivity to atrazine by competitive inhibition ELISA. Antibodies exhibiting low sensitivity (IC_{50} > 1 ppm of atrazine) even on heterologous antigens were also not tested further. Useful ELISA systems could have been developed from some of the antisera rejected at this and earlier steps by using established procedures such as ELISA signal amplification systems and affinity purification of antibodies. However, this was not necessary due to the high quality of several of the ELISA systems tested in the first competitive inhibition screen (identified in Table I by double asterisks).

Previous studies have demonstrated the value of heterologous assays in improving ELISA sensitivity for environmental compounds (Wie and Hammock, 1984; Harrison et al., 1989b). The rationale for this improvement is illustrated schematically in Figure 1. In equilibrium ELISA systems such as those used here, the binding of antibody to analyte can be favored by selecting an ELISA hapten for which the chosen antibody has a reduced affinity relative to the analyte, thus improving the assay sensitivity. The same strategy was pursued in this study through the use of the heterologous antigens summarized in Table I. Typical data showing the binding of antibodies to these heterologous haptens are given in Goodrow et al. (1990; Figures 3 and 4, conjugated haptens; Figure 6, free haptens) and Table II of this study for free haptens. The data of Table II, especially for rabbits 357 and 841, illustrate the differences in antibody binding due to hapten conjugation position, spacer length, and/or alkyl substitution. The relative binding of antibodies to these haptens is similar regardless of whether they are conjugated or free. This should allow the use of competitive inhibition ELISA data for the prediction of the sensitivity of heterologous assay systems based only on competitive inhibition ELISA results.

Homologous and heterologous systems were compared by parallel preparation of standard curves of atrazine, using one antibody with each of several haptens conjugated to BSA. The results of such a comparison (one antibody with five ELISA antigens) are given in Figure 2; similar results were obtained for other antibodies (data not shown). As expected, on the basis of the rationale of Figure 1, the homologous systems were the least sensitive. Of the three types of assay heterology examined here, the greatest improvement in sensitivity was observed for position heterologous systems, such as the **4a**- and **4b**-BSA systems of Figure 2. Both spacer length and alkyl substitution also influenced assay sensitivity, but much less than conjugation position. An exception to this observation occurs if conjugated hapten **1b** of Figure 2 is considered to be position homologous, but lacking a spacer arm. In this case removal of the spacer arm contributed the largest improvement in assay sensitivity. The same observation applied to **1a**-BSA for the few systems where the zero-dose control absorbance was sufficiently high.

Solvent and Matrix Effects. The effects of several solvents on the baseline responses of selected ELISA systems were tested by combining antibody with buffer containing varying amounts of solvent. Significant differences in solvent tolerance were observed among ELISA systems that differed only in the hapten conjugated to BSA for ELISA (Figure 3). Similar effects on the baseline ELISA response were also observed (in increasing order of severity) for dimethyl sulfoxide, methanol, 2-propanol, dimethylformamide, and dioxane (data not shown). The relative tolerance for these solvents was similar for all of the systems tested. Similar results were seen when two blank soil extracts were combined with antibody in the ELISA to test the effect of the soil matrix on the base-

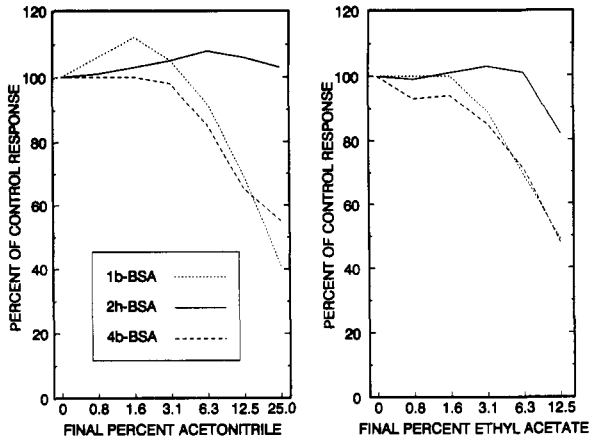

Figure 3. Effect of two solvents on baseline response of three ELISA systems using rabbit 357 serum (anti-**2h**-CONA). Data points represent the means of duplicates; coefficients of variation averaged 1.6%. Ethyl acetate was marginally miscible in PBST at 12.5%, but completely miscible at all other concentrations.

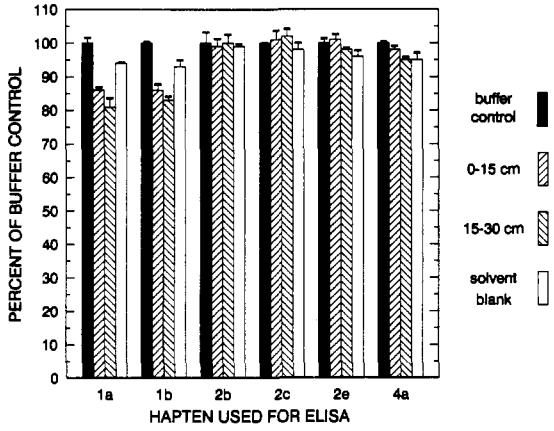

Figure 4. Effect of soil matrix on baseline response of six ELISA systems using rabbit 357 serum (anti-**2h**-CONA). Triazine-free soil from two depths was extracted with ethyl acetate. The extract was evaporated to dryness, reconstituted with buffer and then combined with antibody in the ELISA to assess the effect of the extracted materials on the ELISA baseline response. Data points represent the means of duplicates; coefficients of variation averaged 1.6%.

line ELISA response (Figure 4). In this case, the only systems seriously affected were those using **1a**- or **1b**-BSA. This experiment differs from that shown in Figure 3 in one important respect. The ethyl acetate was removed completely before ELISA analysis, so that the observed interference (Figure 4) must be due to materials extracted from the soil plus impurities remaining after solvent evaporation, rather than the solvent itself.

The combination of results shown in Figures 2–4 implies that some compromise may be required between assay sensitivity and ruggedness. This is summarized by the systems using antibody 357 and BSA conjugates of haptens **1a** or **1b**, **2e** or **2h**, and **4a** and **4b**. The conjugate **1b**-BSA provides the maximum sensitivity of the assay systems presented in Figure 2, but that system is also shown in Figure 4 (with **1a**-BSA) to have the least tolerance of the blank soil extracts. In contrast, the conjugates **2e**- and **2h**-BSA (both homologous for conjugation position) provide the least sensitivity, but with the greatest tolerance of solvents and blank soil extracts. The conjugates **4a**- and **4b**-BSA (both heterologous for conjugation position) provide intermediate assay sensitivity and tolerance to interferences. The **4b**-BSA system is as susceptible as the **1b**-BSA system to acetonitrile and ethyl acetate, but the

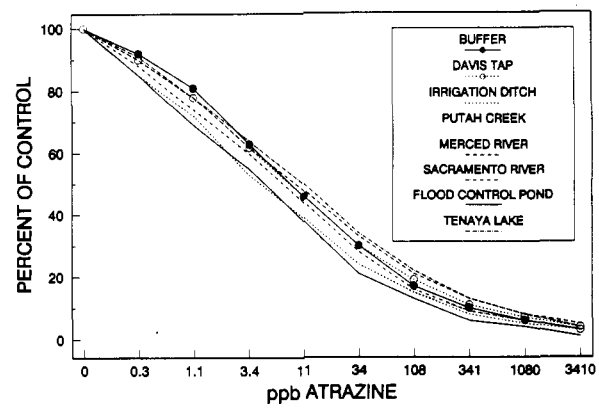

Figure 5. Effect of eight different environmental water matrices on competitive inhibition ELISA. Inhibition of rabbit 357 serum (anti-**2h**-CONA) by atrazine spiked into water samples was performed. The plate-coating antigen used for the ELISA was **4a**-BSA. The water source is indicated for each curve. Data points represent the means of quadruplicates; coefficients of variation averaged 1.1%. The respective IC_{50} values (from top) were 8.0, 7.6, 4.3, 3.0, 8.4, 6.5, 4.5, and 9.5.

4a-BSA system is more tolerant of the blank soil extracts. This example emphasizes the need for careful consideration during the assay optimization process of the end use of the assay, the type of sample to be analyzed, and the user's analytical requirements. Many of the reagent combinations examined provided adequate sensitivity for the analysis of triazines in complex matrices. However, careful selection and evaluation is needed to obtain the desired combination of sensitivity and ruggedness. Our ability to accomplish such a goal in this study derives directly from the library of haptens prepared for ELISA development (Goodrow et al., 1990).

To assess the potential of these ELISA systems for the analysis of field samples, standard curves were constructed by diluting atrazine in various environmental water samples and combining with an equal volume of diluted antibody in the competitive inhibition ELISA. The water samples were from a wide variety of sources near Davis, CA: Tenaya Lake and Merced River in Yosemite National Park (pristine), locally typical agricultural irrigation water (deep groundwater sampled after some surface flow, high mineral and organic content), flood control pond (combined residential and agricultural runoff highly concentrated by evaporation), and Putah Creek (a small local oxbow lake of minimal flow, turbid with algae). The resulting standard curves, with IC_{50} values ranging from 3.0 to 9.5 ppb, are shown in Figure 5 for one ELISA system. The three curves that differed visibly from the buffer control curve were the irrigation well, Putah Creek, and flood control pond curves. This effect is not surprising, considering the high solute and/or particulate loads of these water samples. The results obtained for other ELISA systems, all of which were less sensitive for atrazine, showed even less deviation from the buffer control (data now shown). ELISA systems more sensitive for atrazine were not tested with these water samples. It is important to note that this is a worst case experiment, using very complex and unfiltered water samples, a high proportion (50%) of sample in the competitive inhibition step, and no buffering of the sample. In a similar situation where ELISA was used for the direct analysis of molinate in unfiltered rice field water, buffering of the sample before analysis produced accurate and reliable results (Harrison et al., 1989a). A similar strategy of simple sample processing, using filtration or sedimentation, possibly with buffering, should allow this system to be applied for the direct analysis of *s*-triazines in complex water samples.

Table III. Effects of Selected Structural Changes on Relative Recognition of *s*-Triazines by Anti-Triazine Antibodies[a]

type of structural change		ring position (Table II), structural change (from/to)	compounds compared (from/to)	rabbit no., immunizing hapten 194, **4b**	357, **2h**	355, **4a**	268, **4b**	354, **4h**	841, **2e**	842, **4a**
change in size of alkyl group	decrease	R3, iPr to Et	atrazine to simazine	−15	−8.6	−1.2	−14	−11	−2.5	+1.0
			ametryne to simetryne	−18	−7.0	−1.5	−17	−4.2	+1.4	+2.0
	increase	R2, Et to iPr	atrazine to propazine	+1.3	+2.6	−1.7	−1.7	−2.8	−1.4	−8.3
			ametryne to prometryne	+2.2	+2.0	−2.1	−2.3	−2.5	−2.6	−14
		R3, iPr to tBu	ametryne to terbutryne	−7.9	−3.7	−6.2	−59	−4.2	−5.5	−8.3
dealkylation	monodealkylation	R2, iPr to H	atrazine to deethylsimazine	−97	−1000	−18	−220	−41	−170	−24
		R2, Et to H	atrazine to deethylatrazine	−39	−330	−10	−8.8	−14	*	−50
			simazine to deethylsimazine	−6.5	−120	−15	*	−3.7	−66	−25
	didealkylation	R2 and R3, Et/iPr to H	reduced cross-reactivity to <0.1% for all antibodies							
steric/polar		R3, iPr to 2-CN(iPr)	atrazine to cyanazine	−19	−250	−14	−110	−75	−210	*
		R1, SCH_3 to OCH_3	prometryne to prometon	−14	+1.2	−16	−4.9	−4.0	+1.6	−6.6
		R1, SCH_3 to Cl	ametryne to atrazine	−2.6	+18	−2.9	−4.5	−2.2	+13	−5.3
			simetryne to simazine	−2.2	+15	−5.1	−3.6	−5.9	+3.6	−10
			prometryne to propazine	−4.1	+23	−2.3	−3.4	−2.5	+23	−3.0
		R1, Cl to OH	atrazine to hydroxyatrazine	−55	−110	−230	−73	−41	−200	−59
			simazine to hydroxysimazine	*	−29	*	*	−14	*	*

[a] Values given are ratios of IC_{50} values (the same values are used for calculating the cross-reactivity values of Table II). Thus, each value represents the absolute magnitude of the difference in recognition caused by the designated structural change, while plus or minus signs indicate the increase or decrease in recognition. For example, the chaange from SCH_3 to OCH_3 (prometryne to prometon) decreased recognition by antibody 194 14-fold, while the same change improved recognition by antibody 841 nearly 2-fold. Values which cannot be calculated because of missing IC_{50} values are indicated by an asterisk.

Specificity of Antisera. The specificity of selected individual ELISA systems was evaluated by performing competitive inhibition ELISA using over 30 *s*-triazines as inhibitors. The relative cross-reactivity values for these systems, calculated as ratios of IC_{50} values, are given in Table II. Haptens **1a** and **1b** were not tested by competitive inhibition ELISA because of their demonstrated covalent binding to protein under conditions similar to those used in the competitive inhibition step of the ELISA (Goodrow et al., 1990). In all cases the inhibitor recognized best was the immunizing hapten (Table II, boldface cross-reactivity value). The cross-reactivity data in Table II for rabbits immunized with the same hapten (**4b**: 194, 268, and 354; **4a**: 355 and 842) illustrate the variability of the rabbit immune response even under controlled conditions. This variability reinforces the need for immunizing a sufficient number of animals and careful screening of the resulting antibodies.

Because these cross-reactivity studies used a large number of compounds that are very similar in structure, antibody specificity can be summarized by examining the changes in relative recognition due to isolated structural changes. The effect of selected structural changes was estimated from the relationships between pairs of inhibitors from Table II. Ratios between IC_{50} values were calculated, giving the relative change in recognition caused by a single structural change, and are presented in Table III. Specificity data were similar for several other sera not listed in Tables II and III.

One significant result shown by Table III is that a major difference exists in specificity of the antibodies produced by the two families of haptens. This difference is reflected in the Table III values indicating the change in relative recognition with a change from Cl to SCH_3. The antibodies made against haptens **2e** and **2h** (357 and 841; conjugation position R3 from Table II) both lose a large amount of recognition when the Cl is changed to SCH_3. In contrast, recognition by the other antibodies, made against haptens **4a** and **4b** (194, 355, 268, 354, 842; conjugation position R1 from Table II), is improved with this change. It is significant that this structural change produces a larger magnitude effect for anti-**2e** or anti-**2h** antibodies than for anti-**4a** or anti-**4b** antibodies. The antibodies made against haptens conjugated through mercaptopropanoic acid spacers (**4a,b**) recognize the *S*-methyl-*s*-triazines well, but they also still strongly recognize the 2-Cl parent compounds such as atrazine, likely due to the fact that the sulfur of the spacer effectively mimics the Cl which it replaces (Goodrow et al., 1990). In contrast, the antibodies made against haptens conjugated through *N*-alkyl acid spacers and retaining the Cl (**2e,h**) lost recognition due to the steric effect of adding a methyl group, as well as changing the Cl to sulfur. The importance of the sulfur to recognition by these two antibodies (357 and 841) appears to be small, as shown by the Table III data for prometryne to prometon (little effect for change of SCH_3 to OCH_3). This was not case for the other five antibodies of Table III, which strongly preferred sulfur to oxygen, as would be expected since their immunizing haptens were thioethers (**4a,b**).

Several other structural changes listed in Table III have large effects on relative recognition by antibodies. The greatest and most universal effect on recognition came with the loss of both alkyl groups. The loss of Cl by hydroxylation also produced a universally significant effect, but this seemed to be more important for the antibodies made against haptens conjugated through *N*-alkyl acid spacers (357, 841). Loss of either *N*-alkyl group alone reduced recognition significantly for all antibodies, though the magnitude of the effect varied considerably. Similarly, the effects of changes in *N*-alkyl group size were highly variable. Table III also shows that modification of an *N*-alkyl group by the addition of an uncharged polar group (atrazine to cyanazine) diminished antibody recognition dramatically.

Conclusions. The synthesis of numerous *s*-triazine haptens (Goodrow et al., 1990) has served here as a foundation for the development of antibodies that recognize many related *s*-triazines. We have used some of these haptens and antibodies to design immunoassays that have low to sub-ppb sensitivity for atrazine and related compounds. We have shown the potential usefulness of these assays for the detection and measurement of *s*-triazine herbicides at levels commonly found in environmental samples. These

assays have demonstrated that conjugation position and *N*-alkyl substitution strongly influence antibody specificity. The assays described have also verified the usefulness of heterologous haptens in the design of sensitive ELISA systems. The data presented here indicate that sensitivities to both analyte and interferences are linked to some degree. Thus, it is important to routinely evaluate this connection in the course of assay development so that the best compromise between sensitivity and ruggedness is obtained; the final use of the assay must be considered in this decision.

An area not yet explored for the triazines is that of modifying assay specificity through the use of different haptens for ELISA, as described by Wie and Hammock (1984). A polyclonal antiserum is a collection of antibodies that recognize the hapten, the spacer arm, and the carrier protein in different orientations and with varying specificity. Only a small portion of the antibodies present in the serum actually are responsible for the baseline signal in the ELISA. This portion is determined by the hapten used to make the ELISA antigen, which is not recognized equally by all antibodies in a serum. Thus, the use of an ELISA antigen made with a different hapten may select a different subpopulation of hapten-specific antibodies capable of producing a different assay specificity. In this way a library of antisera and ELISA antigens such as described here could produce a number of immunoassays of varying specificity for members of the compound class. Surprisingly, a similar approach often results in a dramatic improvement in assay sensitivity and/or specificity even with an apparently homologous monoclonal antibody population.

An important application of assay of varying specificities, such as those described here, would be to perform quantitative determinations of each component of mixed samples. An example often seen among our environmental samples is the simultaneous occurrence of simazine and atrazine. Measurement by ELISA using two (or more) systems of different specificities for simazine and atrazine would give two (or more) apparently different answers for the total triazine content. The relative cross-reactivity values of the systems (such as shown in Table II) would then be used to calculate the relative contributions of the compounds detected. An example of such a pairwise combination might be 194 (Table III, over 10-fold better recognition of atrazine than simazine) and 355 (Table III, less than 2-fold difference). Similar combinations would enable one to analyze mixtures of the 2-chlorotriazines, such as atrazine, and the 2-SCH_3-triazines, such as ametryne. One pairwise combination that would be appropriate for this analysis is 357 (over 10-fold difference between Cl and SCH_3) and 194 (approximately 3-fold difference). Many other such permutations are possible using the antibodies described here.

Subsequent papers continuing this work will cover the production and characterization of monoclonal antibodies, optimization of assay sensitivity and ruggedness using the library of haptens described here, and practical utilization of the assays developed. The resulting assays have proven to be very sensitive and rugged for the analysis of *s*-triazines and metabolites in a variety of matrices, including water, soil, and human body fluids. Some of these assays recently have been transferred to the California Department of Food and Agriculture and several other laboratories for routine use in the analysis of *s*-triazines in environmental water samples and other matrices as a precursor to the official validation process.

ABBREVIATIONS USED

BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; IC_{50}, analyte concentration required for 50% inhibition; KLH, keyhole limpet hemocyanin; THY, thyroglobulin; CONA, conalbumin; PBST, phosphate-buffered saline plus Tween 20.

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Registry No. **1a**, 3440-19-5; **1b**, 3703-10-4; **2a**, 68228-19-3; **2b**, 82784-46-1; **2c**, 125454-26-4; **2d**, 125454-27-5; **2e**, 125454-28-6; **2f**, 68228-20-6; **2g**, 125454-29-7; **2h**, 98849-84-4; **4a**, 125454-30-0; **4b**, 125454-31-1; atrazine, 1912-24-9; simazine, 122-34-9; ametryne, 834-12-8; simetryne, 1014-70-6; propazine, 139-40-2; prometryne, 7287-19-6; prometon, 1610-18-0; terbutryne, 886-50-0; cyanazine, 21725-46-2; cyanazine acid, 36576-43-9; cyanazine amide, 36576-42-8; hydroxycyanazine acid, 36576-44-0; hydroxyatrazine, 2163-68-0; hydroxysimazine, 2599-11-3; deethylsimazine, 1007-28-9; deethylatrazine, 6190-65-4; deethylsimetryne, 4147-58-4; diodealkylated atrazine, 3397-62-4; cyanuric acid, 108-80-5; ammelide, 645-92-1; ammelide, 645-93-2; melamine, 108-78-1.