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Immunoassays for Trace Chemical Analysis

Monitoring Toxic Chemicals in Humans, Food, and the Environment

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Chapter 2

Hapten Synthesis for Pesticide Immunoassay Development

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The production of pesticide specific antibodies requires presentation of a conjugated form of the pesticide to an animal's immune system. This in turn demands the design of an appropriate chemical structure (a hapten) which can be covalently coupled to a carrier, but which will still elicit the production of antibodies recognizing the target analyte. The immunoassay literature does not adequately address the question of what chemical structure is required for the production of specific antibodies to low molecular weight compounds, especially in the pesticide area. Hapten design historically is considered an art, with minimal or no explanation for hapten structures which failed to produce the desired antibodies. Most failed attempts remain unpublished and thus have not shed light on the structural requirements for antibody production. We present here an examination of selected examples of successful and unsuccessful hapten designs from our laboratory and from the literature. These examples are used herein to illustrate several criteria deemed critical for successful hapten synthesis strategies.

The Development And Utilization Of Pesticide-Specific Antibodies in Immunoassays

The procedures for production of specific antibodies and their application in a competitive inhibition ELISA (Enzyme-Linked Immunosorbent Assay) are discussed in detail in the preceding chapter (Vanderlaan et al., this volume). In addition, other comprehensive overviews of the immunoassay development process in the pesticide field are available (5,7,8,19). In general, a

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synthetic antigen (immunogen or immunizing antigen) is used to immunize an animal for antibody development. This immunogen is composed of a synthesized hapten (mimicking the structure of the target compound) which is covalently attached to a carrier protein. Antibodies are produced against many sites on the immunogen, including the conjugated hapten. Antibodies thus made may recognize either the carrier protein, the hapten, or a site combining parts of both. Because several quite different results are possible from an immunization, animals or cultured cells must be tested carefully to assess the usefulness of the antibodies produced. The more common undesired results include excessive recognition of the spacer arm, recognition of the immunizing hapten without adequate recognition of the target compound, and failure of the hapten to elicit a specific response despite a successful immunization procedure.

Antibodies thus produced must be evaluated for specificity to allow analysis of the success or failure of the hapten design. The problem of antibody screening requires special consideration due to the potential for error and misinterpretation of data. Careful design of the screening protocol is especially crucial for development of monoclonal or recombinant antibodies; critical decisions must be made rapidly to preserve growing cells, but one can potentially be overwhelmed by cells producing undesirable antibodies. Antibody screening must address the following questions for either polyclonal or monoclonal procedures.

a. Does the antibody bind to the immunizing antigen? (i.e. Did the animal respond specifically to the immunogen? This test is needed only in the rare retrospective analysis of a total failure. Our screening generally starts at b; if b is successful, a is moot.)

b. Does the antibody bind to the conjugated hapten on the ELISA antigen (plate coating antigen; see Vanderlaan et al., this volume, Figure 2a) , which has a different carrier protein than the immunogen, but is otherwise identical? (i.e. Are antibodies present which recognize the hapten portion of the immunogen?)

c. Does the free immunizing hapten inhibit the binding shown in b? (i.e. Are antibodies present which recognize the unconjugated hapten structure independent of attachment to a carrier protein?)

d. Does the target analyte inhibit the binding shown in b? (i.e. Are antibodies present which recognize the analyte portion of the hapten independent of the spacer arm?)

e. Does the antibody bind to a conjugated heterologous hapten (a plate coating antigen made with a hapten related to but not identical to the immunizing hapten), and can this binding be inhibited by the target analyte? (i.e. Do the antibodies against the immunizing hapten also bind other conjugated haptens having significant differences in structure from the immunizing hapten? If the antibodies give poor sensitivity in test d, can the

problem be overcome by the use of heterologous assay systems?)

Some studies (10,21,22) have reported antibodies which meet tests b and c above, but not test d. This result indicates much stronger recognition of the conjugated or free immunizing hapten than the target analyte. Such antibodies may still yield a useful assay, as noted in test e above and the section below. Other studies (6,17) have reported antibodies which meet tests b and c above, but not tests d or e. This result indicates recognition of the conjugated or free immunizing hapten without significant recognition of the target analyte. In this case the antibodies are not useful for analysis of the target compound. We suspect both of these problems may be more frequent than is apparent from the literature, especially for small target molecules. We also suspect that more careful exploration of heterologous assay systems, facilitated by more extensive hapten synthesis, would increase the sensitivity of many existing assays. In some cases, as described below, such an approach has salvaged a functional assay from what otherwise would have been a total failure.

A common misconception is that monoclonal antibodies can be used to circumvent problems of handle recognition. Although this is conceptually possible, in practice most monoclonal antibodies to small molecules demonstrate extensive handle recognition. Proper and extensive screening of numerous cell lines, as outlined above, can yield truly superior antibodies with excellent detection of the analyte (23). However, it is unreasonable to consider such a major expense unless there has been a parallel intellectual investment in good hapten design. Excellent, rugged immunochemical assays result from a combined investment in both chemistry and immunology.

Hapten Design And Utilization

The initial and critical step in the development of effective immunoassays for pesticides and other low molecular weight environmental chemicals lies in the selection of appropriate haptens which will elicit the production of antibodies demonstrating maximum specificity and sensitivity for the target molecule. It is important to distinguish between the problem of antibody production and the development of many hapten-protein conjugates for assay optimization using existing antibodies. Assay specificity and sensitivity are determined primarily by the antibody produced in response to the immunogen; thus its design and preparation are critical. However, for a given antibody, other (heterologous) haptens used in the later stages of assay design may offer critical improvements in sensitivity (10,20,22; below and e above). Recent reviews on immunoassay techniques (5,7,8,19) emphasize the importance of attention to hapten selection during assay development.

The principles of good hapten design are simple and straightforward, given a basic understanding of the process of antibody production. However, this is not an exact science. For instance, even hybridomas or inbred animals which yield antibodies recognizing the same molecule may produce antibodies with very different amino acids in their combining sites. In designing

haptens for antibody production, several general guidelines are clear. It is preferable to avoid spacer attachment at or near functional groups of the target molecule as this can reduce the number of potential sites contributing to antibody binding (nitro, amine, hydroxy, halide, etc.), either directly by chemical modification or indirectly by sterically blocking access to these groups. To enhance compound specificity, synthesis strategies should maximize exposure of unique determinants by attachment of the spacer at a position on the target molecule where members of a class have identical (or very similar) structural features. Spacers containing strong determinant groups, such as aromatic rings, conjugated double bonds, or heteroatoms should be avoided if possible, to minimize the production of spacer-specific antibodies.

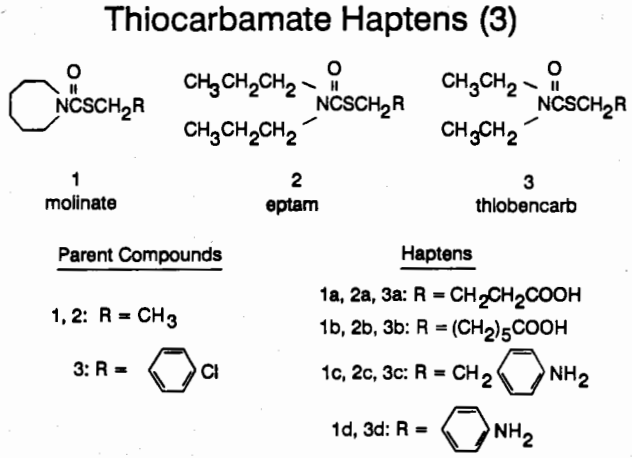
The Importance Of Negative Results

It is difficult to completely understand the immune response to a particular hapten. Development of strong data on the inability of a hapten to produce specific antibodies requires considerable resources, including many animals, time for immunization, and extensive characterization to demonstrate lack of appropriate antibodies. It is possible to reduce the difficulty of obtaining negative data through the use of mice rather than rabbits (6), a route not usually pursued except when planning monoclonal antibody production. In any case, rigorous synthetic chemistry remains critical to understanding of the final results, successful or not. The few available studies which describe unsuccessful immunizations provide valuable insight (6,17), as do those failures within our group. Based on the literature and our own experience, we have identified and examined several criteria for the successful development of immunoassays for pesticides. We present here examples illustrating and contradicting those criteria.

Criteria For Hapten Synthesis And Discussion Of Examples

The structures of the compounds discussed below are given in Figures 1 and 2, with references indicated under the compound names. Sites of conjugation are indicated by arrows, while dotted polygons enclose parent molecule structure which is not retained in the immunizing hapten.

Spacer Arm Location: Exposure of Determinants. The location of the spacer arm on the target molecule should be distal to important haptenic determinants to maximize their exposure for antibody binding. This point is supported by several examples. In the development of molinate haptens (3), the unique hexamethyleneimine ring was retained, leading to antibodies which were specific for molinate and recognized EPTC poorly. This must be due to the difference between the cyclized hexamethyleneimine ring of molinate and the open *N*-dipropyl group of EPTC. Assays developed for the *s*-triazine herbicides using a range of immmunizing haptens (4, Harrison et al. J. Agric. Food Chem., in press) exhibited specificity reflecting the structures of the immunizing haptens; chloro and alkylamino groups were each important determinants of specificity. In a series of papers detailing the synthesis of



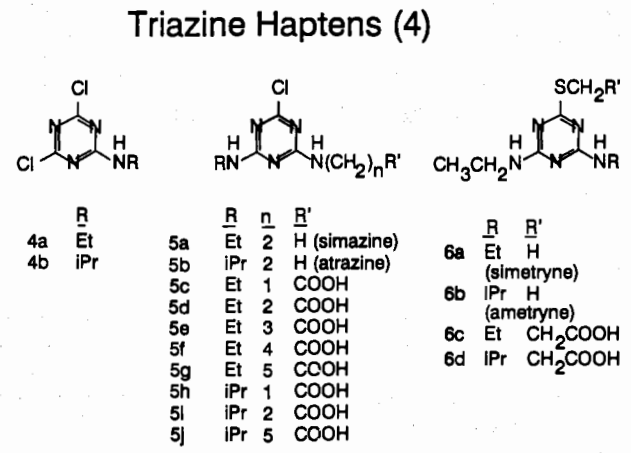


Figure 1. Structures of thiocarbamate and triazine haptens and parent compounds.

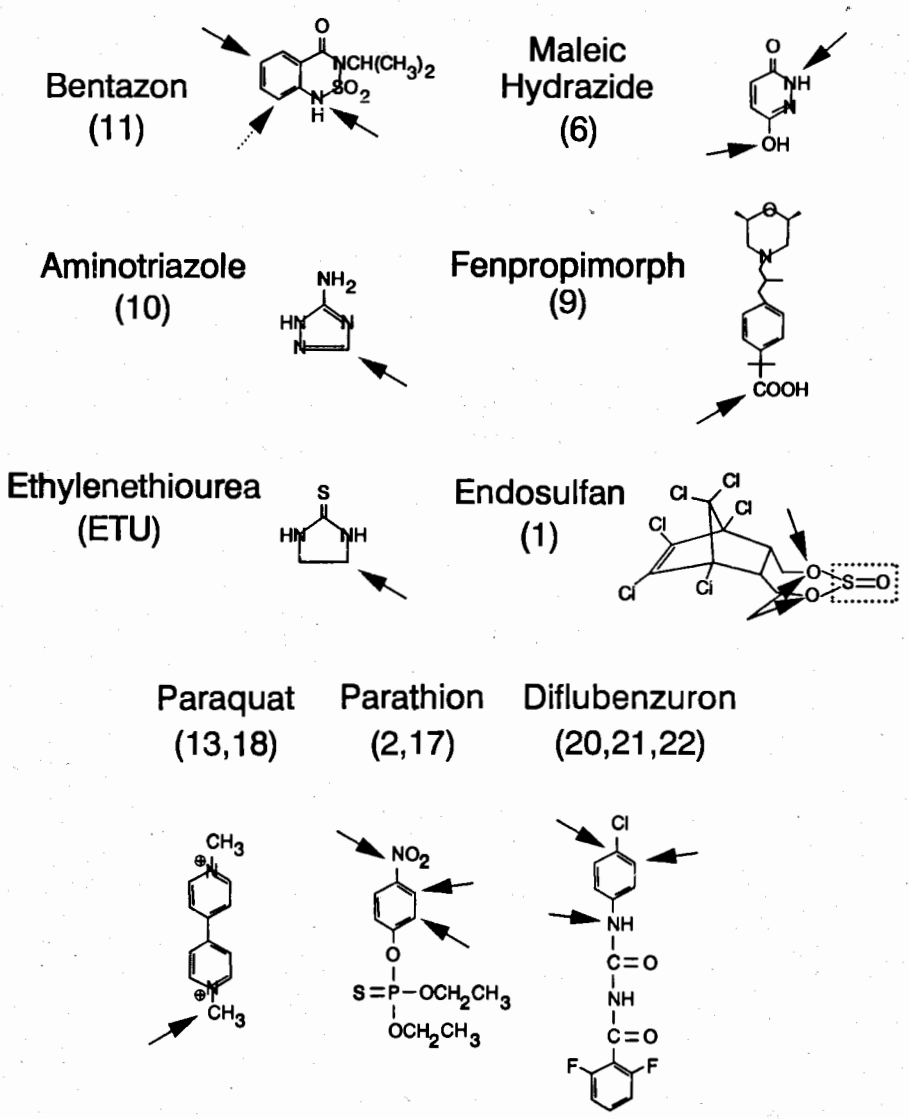


Figure 2. Structures of other compounds discussed in text. Arrows indicate sites of conjugation. Dotted polygons enclose parent molecule structure not retained in the immunizing hapten. The numbers under compound names refer to literature cited.

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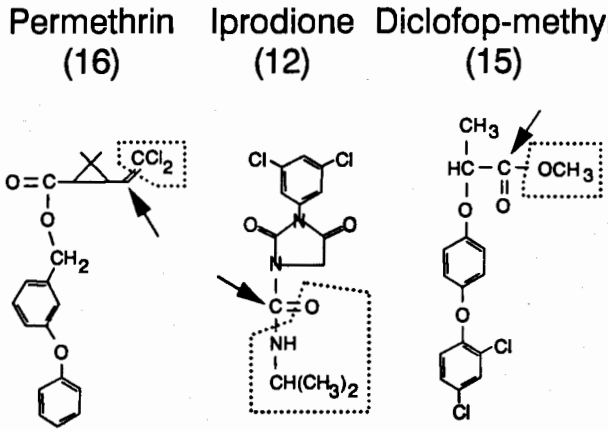

Figure 2. Continued

haptens and production of antibodies for diflubenzuron (20-22), several haptens which were derivatized distal to the 2,6-difluorobenzoylurea moiety yielded antibodies specific for this end of the molecule. An assay for fenpropimorph (9) produced highly specific antibodies because of preservation of the unique dimethylmorpholine ring. Conjugation of a permethrin hapten (16) distal to the 3-phenoxybenzyl group produced antibodies which crossreacted with several related pyrethroids because they share this portion of the permethrin structure. For iprodione (12), conjugation distal to the *m*-dichlorophenyl ring produced antibodies which were class specific, recognizing the parent, a metabolite, a hydrolysis product, and two related fungicides.

However, several exceptions to this generalization have been observed. The conjugation of an aminophenyl thiobencarb hapten by diazotization (Gee et al., this volume) produced antibodies which recognized the target compound. Conjugation of maleic hydrazide through the nitrogen of the distinctive hydrazide group (6) yielded antibodies recognizing the target compound. Preservation of the seemingly important nitro group on parathion's phenyl ring (by conjugation ortho or meta to the nitro group) led to antibodies which did not recognize parathion (17). However, useful antibodies were produced in response to diazotized aminoparathion (2; nitro group of parent compound was reduced to amino group for conjugation), but aminoparathion was also strongly recognized.

Spacer Arm Location: Preservation of Functional Groups. The location of the spacer arm on the target molecule should avoid attachment to functional groups, including heteroatoms, which might lead to change in polarity of the group and/or a reduction in the number of potential sites contributing to antibody binding. Coupling through a carbon atom appears preferable. Several examples can be cited to illustrate this principle. In the development of assays for the triazines (4, Harrison et al. J. Agric. Food Chem., in press), a sulfur atom was used in the spacer to mimic the size and other properties of the chloro group, allowing antibodies against thioether haptens to retain good recognition of chloro compounds while having excellent recognition of S-methyl triazines. In assays for diflubenzuron (20-22), attachment of the spacer through the ring NH produced specific antibodies, but the parent compound (target analyte) could not compete the antibody off the plate coating antigen. This problem was overcome by changing the hapten used in making the plate coating antigen. With this change, *N-methylated diflubenzuron was still recognized more strongly than* diflubenzuron, indicating the critical importance of the difference between secondary and tertiary nitrogens. The importance of this difference between secondary and tertiary nitrogens has also been observed for bentazon (11). Attachment of the spacer to the ring nitrogen produced specific antibodies, but these recognized N-methylated bentazon 100 to 1000 times better than bentazon. Similarly, in the production of antibodies to maleic hydrazide (6), conjugation through one oxygen prevented tautomerization of the remaining carbonyl to a phenol. This hapten produced antibodies which recognized the immunizing hapten, but not the phenolic parent compound. In two immunoassays for paraquat (13, 18), retention of

the positive charge on the nitrogen used for conjugation led to antibodies which bound poorly to singly charged monoquat.

The following exceptions to the above principle must be noted. In an assay for thiobencarb (Gee et al., this volume), removal of the chloro group and conjugation through that position did not prevent the production of specific antibodies. For maleic hydrazide (6), conversion of the secondary nitrogen to tertiary by conjugation through the nitrogen did not prevent the production of antibodies recognizing the parent compound. In one parathion assay (17, preservation of the nitro group on the phenyl ring (by conjugation ortho or meta to the nitro group) led to antibodies which recognized the immunizing haptens, but not parathion.

Selection of Spacer Arm. The spacer arm length and structure should be chosen carefully to reduce spacer recognition, while retaining specificity for the target molecule. Functional groups in the spacer arm should be avoided, if possible, to minimize "spacer" recognition. Alkyl spacers appear preferable; heteroatoms appear undesirable. This is borne out by several examples. In the assays for both molinate (3) and EPTC (Gee et al., this volume), antibodies against aralkyl haptens produced high titer antibodies which could not be used because spacer recognition was too strong. For thiobencarb (Gee et al., this volume) and parathion (2), the aromatic ring in the spacer was important to antibody binding, but this was desirable because it constituted part of the target analyte as well. Aminotriazole haptens (10) conjugated using the heterobifunctional reagent MBS (maleimidobenzoic acid N-hydroxysuccinimide) produced antibodies which bound the homologous hapten-protein conjugate, but did not recognize the target analyte. This result indicates recognition of the bulky and distinctive MBS spacer group. The length of the spacer arm may also be an important factor. Several examples are given below, primarily for the use of alternative haptens in ELISA with antibodies against other haptens. In one assay for the *s*-triazines (4, Harrison et al. J. Agric. Food Chem., in press), the ELISA use of a hapten with reduced length spacer, ($(CH_2)_5$ less than long alkyl spacer of immunizing hapten) increased assay sensitivity 100 fold over the homologous system. In our assays for molinate (3), thiobencarb (Gee et al., this volume), and EPTC (Gee et al., this volume), antibodies against short chain alkyl acid haptens produced no inhibition in homologous assay systems, but gave acceptable sensitivities when the spacer length of the ELISA antigen was increased by $(CH_2)_3$. Similar results were obtained for diflubenzuron (20-22), where the use of site and spacer heterologous haptens improved the assay sensitivity in most cases.

For many haptens, more complex spacers containing aromatic rings or multiple heteroatoms do not prevent the production of the desired antibodies. A bentazon hapten (11) containing a benzyl group in the spacer produced antibodies recognizing the target compound. Similarly, antibodies raised against diazotized aminoparathion recognized parathion (2). This is not surprising, since the phenyl ring of the diazotized hapten is shared by the target parathion. We have also observed the usefulness of multiple spacer types for the development of heterologous assays for improving sensitivity. In the case of the thiocarbamates, this is the only simple route for making heterologous assays. In the assays

for both molinate (3) and thiobencarb (**Gee et al.**, this volume), antibodies against alkyl acid haptens produced the best assays when ELISA conjugates (plate coating antigens) used spacer heterologous aminophenyl haptens.

Selection of Coupling Chemistry. In practice, the selection of coupling chemistry is closely linked to the points discussed in the previous section. The choice of chemical reaction for the conjugation of hapten to protein must take into consideration the reactivity of the hapten molecule's other functional groups. Most of the compounds described in this review used alkyl COOH or aryl NH_2 groups for conjugation.The differences in stability among azo, amide, ester, and disulfide linkages illustrate the range of possibilities available with different conjugation reactions. The easiest (and most common) approach is to select the simplest and most stable conjugation chemistry which is appropriate for the most easily produced hapten(s). This is not always the best strategy. The stability (chemical and biological) of the hapten molecule during conjugation and subsequent use must allow for the *in vivo* production of antibodies and *in vitro* production and use of hapten-protein conjugates. Functional group protection strategies may be required during conjugation. For example, in attempting to produce stable aminotriazole conjugates (10), chromophore groups were used to protect the reactive primary amine, providing colorimetric monitoring of conjugation; these groups were then removed after conjugation. In the preparation of bentazon conjugates (11), dimerization of the activated hapten occurred during the conjugation procedure, due to reactivity of the unprotected secondary nitrogen. One possible solution would be to use a spacer which can cyclize with the secondary nitrogen, temporarily protecting it while providing another functional group for conjugation; after conjugation, ring opening would restore the secondary nitrogen, leaving an alkyl spacer. Our work on ethylenethiourea (ETU) is an example where normal hapten conjugation protocols did not produce verifiable conjugates. Because the unique portion of this molecule is also highly reactive, special precautions were required for verification of conjugation.

Solubility of Hapten and Conjugate. The solubility of hapten and conjugate must also be considered, due to the nonpolarity of most pesticides. Unusual measures may be required to overcome problems of hydrophobicity. For endosulfan (1), periodate conjugation (aqueous $NaIO_4$) of the diol was difficult due to poor water solubility of the hapten. However, the N-hydroxysuccinimide active ester method could be performed using over 50% dimethylformamide as a cosolvent. In diflubenzuron antigen preparation (20-22), the use of diazotization resulted in a reactive intermediate which is charged and thus very soluble in aqueous systems. In addition, differences exist among the possible carrier proteins. In assays for the *s*-triazines (4, Harrison et al. J. Agric. Food Chem., in press), the solubility of all haptens was improved by using 20% dimethylformamide as a cosolvent in aqueous conjugation reactions. The solubility of all bovine serum albumin (BSA) conjugates was better than keyhole limpet hemocyanin (KLH) conjugates, due to solubility differences intrinsic to the two proteins. Thus KLH

conjugates were used for immunization (emulsification for injection mitigates solubility problems) and BSA conjugates were used for ELISA.

Ease of Synthesis. The ease of hapten synthesis is also important, especially in minimizing the number of synthetic steps required. For most of the assays covered here, generally two or three steps were required from commercially available starting materials. It is also convenient to use readily available starting materials if possible. For examples, the direct conjugation of reactive dichlorotriazines yielded ELISA antigens useful for analysis of the *s*-triazines (4, Harrison et al. J. Agric. Food Chem., in press). Direct conjugation of acid metabolites produced specific antibodies against both fenpropimorph (9) and diclofop-methyl (15). We must emphasize however that synthetic expediency at the expense of the other considerations we have discussed above is likely to create more problems than it solves.

Further Discussion of Thiocarbamates and Triazines

The haptens shown in Figure 1 were synthesized for the thiocarbamates molinate, EPTC, and thiobencarb. Two haptens for thiobencarb (3a-3b) contained S-alkyl spacers (in place of the *p*-chlorobenzyl group) with a terminal carboxylic acid group for conjugation, while two haptens contained aralkyl spacers (3c-3d) and were conjugated via a diazonium salt. The thiobencarb haptens with alkyl spacers elicited adequate antibodies, but the aralkyl haptens produced antibodies with superior specificity for thiobencarb, since hapten 3d is nearly identical to the target compound. This antibody could be used in both heterologous and homologous assays for the parent thiobencarb with acceptable sensitivity.

Sometimes options for spacer arm location are limited, as is the case for EPTC (Figure 1). Many thiocarbamates contain the S-ethyl moiety; thus only the dipropylamino group is unique to EPTC. Hence the spacer attachment for all our EPTC haptens (2a-2c) was at the sulfur, distal to the dipropylamino group. These haptens elicited good titer antibodies, but the best EPTC assay was 100 fold less sensitive than the best assays for molinate or thiobencarb. It appears that this deficiency is due to the EPTC structure itself. In light of this result, it is interesting to note the structural similarity of molinate and EPTC; symmetric opening of the molinate hexamethyleneimine ring yields EPTC. We suspect that the critical difference between these two compounds is the added rotational freedom of the two n-propyl groups of EPTC. While the relative assay sensitivities suggest that the alicyclic hexamethyleneimine group is superior to the aliphatic dipropylamino group for the production of antibodies, to our knowledge no systematic study of the advantages of ring systems exists.

In the triazine series (Figure 1), the best immunizing hapten for making triazine-class specific antibodies was compound 6d. This hapten has the spacer attached at the 2-position and contains 4-ethylamino and 6-isopropylamino groups. Hapten 6c, the 6-ethylamino analog, was slightly less effective, but this may be related to the decreased solubility and accompanying differences in conjugation and *in vivo* antigen presentation. It is not clear at present which is

the best immunizing hapten for producing simazine specific antibodies. The most sensitive monoclonal antibody (1-2 ppb for atrazine) was made in response to compound 6d. The immunizing hapten producing the best specificity for atrazine was 5j (containing 2-chloro and 4-isopropylamino groups, with the spacer arm at the 6-position). Only 8% cross reactivity with simazine was noted for the best rabbit antibody made against this hapten, a surprising result considering that the structures differ only by one methylene group. Antibodies against compound 5g (containing 2-chloro and 4-ethylamino groups, with the spacer arm at the 6-position) were also very specific for atrazine, as expected. However, their cross-reactivity for simazine was approximately 100%, but with reduced sensitivity compared to the antibodies against compound 5j.

The recognition of variable spacer arm length triazine haptens was evaluated using competitive ELISA. Using antibodies made against haptens 5j and 6d, a clear decrease in recognition was observed as the spacer arm was shortened. Based on these results, the n = 1, 2, and 3 derivatives of simazine (5c-5e) and the n = 1 and 2 derivatives of atrazine (5h-5i) were chosen for conjugation to alkaline phosphatase and BSA. The ultimate goal of this approach is to produce more sensitive heterologous assays by exploiting the reduced affinity of the antibodies for the conjugated haptens, while retaining specificity for the triazine class (4, Harrison et al. J. Agric. Food Chem., in press).

Conclusions

1. The importance of the above criteria, especially spacer recognition and preservation of parent molecule functional groups, appears to increase as the size of the target molecule decreases and as the number of clearly recognizable functionalities decreases (6,10).
2. Some molecules may be inadequate for the production of specific antibodies due to size, reactivity, or structure. Limitations due to size may be manifested only as decreasing assay sensitivity with decreasing molecular size.
3. Sometimes it is impossible or very difficult to avoid sacrificing a useful determinant group in hapten synthesis; exploration of multiple haptens is crucial in such cases (6).
4. Multiple haptens should be prepared for each target compound, including different conjugation positions and spacer lengths and structures, if possible. While it may be unnecessary to use all of the synthesized haptens for antibody production, heterologous systems should be carefully explored to optimize assay sensitivity while retaining specificity and ruggedness.
5. The strategy of synthesizing a library of potential haptens during the early phase of agricultural chemical product development would be a valuable corporate policy because it would facilitate the later development of immunoassays. An added benefit is that these derivatives would offer new compounds for screening as potential pesticides or serve as metabolite standards.
6. The ability to generate class or compound specific antibodies depends greatly on the class/compound structure and the number of

closely related compounds which might be encountered in routine analysis. The use of multiple haptens employing different conjugation positions and substitution patterns allows exploration of class/compound specificity.

7. When designing assays for larger molecules, immunizing haptens containing less than the complete parent structure may yield antibodies which adequately recognize the target molecule (12, 16). Such haptens are more likely to produce class specific antibodies due to non-recognition of the omitted structure.
8. Study of the literature on low molecular weight drugs (14 for review) may offer further insight (valproic acid, phenobarbital, caffeine, nicotine, etc.).
9. Understanding of hapten-antibody interaction would benefit from physical-chemical study of binding (x-ray, NMR, etc.), with the realization that for most molecules there will be many possible antibody combining site structures.

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