Potential of Immunochemical Technology for Pesticide Analysis

BRUCE D. HAMMOCK

Division of Toxicology and Physiology, Department of Entomology, University of California, Riverside, CA 92521

RALPH O. MUMMA

Pesticide Research Laboratory, Department of Entomology, Pennsylvania State University, University Park, PA 16802

In the fields of clinical chemistry and endocrinology, immunochemistry is often the analytical method of choice. Immunochemical methods of analysis offer many advantages including sensitivity, specificity, speed of analysis, ease of automation, cost effectiveness, and general applicability. The importance of immunochemical assays was recognized by Rosalyn Yalow sharing the Nobel Prize in Physiology and Medicine based, in part, on her pioneering work in immunoassay development (1, 2). Surprisingly, immunochemistry has found little or no practical application for the analysis of pesticides or other environmental contaminants (3). This fact is surprising because the chemical classes currently assayed by immunochemical techniques (2,4) are not fundamentally distinct from many classes of fungicides, herbicides, insecticides, nematocides, or plant growth regulators. Possibly the tremendous success of gas liquid chromatography (GLC) and ion selective detectors in the analysis of the chlorinated hydrocarbon insecticides fostered a generation of pesticide analytical chemists who were experts in and disciples of GLC. The phenomenal success of immunochemistry, specifically radioimmunoassay (RIA), was possibly analogous in fostering a generation of clinical chemists who look first to immunochemistry for the analysis of hormones and pharmaceuticals, even in cases when RIA is not necessarily the technique of choice. Biological techniques, in contrast to physical or chemical techniques for residue analysis, have been criticized by analytical pesticide chemists. It is a common misconception that immunochemical methods can be classed as biological techniques of residue analysis. Although a living organism or, at least, a cell line is required for antibody production, immunoassays using these antibodies are based on physical and chemical properties, and immunoassays can be explained in terms of the law of mass action. A tremendous immunochemical technology has developed especially in clinical chemistry, and it is time that this technology was exploited to solve new and pressing problems in environmental chemistry.

In this chapter the potential of several immunochemical techniques for residue analysis will be explored. Sufficient background methodology will be presented to allow the reader to evaluate the advantages and disadvantages of immunochemical techniques and their potential application to residue problems. A chapter of this length provides, at best, superficial treatment of immunochemical methodology and theory, but this overview, in conjunction with the included references, should offer the reader ready access to the specific immunochemical literature. Hopefully, this article will assist pesticide residue laboratories in applying existing immunochemical technology to specific problems in pesticide analytical chemistry.

The most common, but by no means the only or even the most promising, immunochemical assay for small molecules is radioimmunoassay (RIA). As an overview, an immunoassay involves chemically attaching the small molecule of interest (or a derivative of it) to a carrier protein and raising specific antibody titers to it in the serum of an animal. Very dilute antibody solutions are then used to bind the small molecule which has been radiolabeled. The competition of varying known concentrations of unlabeled material is measured and the resulting standard curve used to determine unknown concentrations (Table I). The steps leading to the development of an RIA are outlined below followed by a description of other immunochemical procedures and an analysis of the attributes and limitations of immunoassay.

Table I

Steps in the Development of an RIA

Synthesize hapten	Prepare radioligand
Couple hapten	Choose method for bound/free separation
Purify antigen	Optimize assay conditions
Characterize antigen	Develop standard curve
Immunize animal	Characterize assay
Titer antibody	Determine assay reliability
Characterize antibody	

Methodology of Antibody Formation

Hapten Synthesis. Antibody titers are raised in an experimental animal in response to an antigen or immunogen. In general, an effective antigen must be rather large and foreign to the

animal to be immunized; (proteins of greater than 10,000 mw are common antigens). By comparison, most pesticides are rather small molecules, and therefore they must first be conjugated to a protein or other large antigenic molecule before they can be used as antigens. Such small molecules which become immunogenic after attachment to a large carrier molecule are called haptens. If the pesticide has a reactive functionality suitable for conjugation it may itself be the hapten. Otherwise, a derivative of the pesticide must be synthesized suitable for attachment to the carrier. Coordination between a hapten and carrier protein may be sufficient for raising antibody titers, but covalent linkages are more reliable and definitive (4). For many pesticides, potentially useful haptens have already been described as metabolite standards or environmental degradation products.

The choice of the hapten and the conjugation procedure used may profoundly affect the ultimate sensitivity and specificity of the immunochemical assay. Generally, antibody specificity is highest for the part of the molecule distal or furthest from the carrier protein. This knowledge has frequently been utilized to develop immunoassays which will, on one hand, detect general classes of compounds which have common functionalities and to develop other assays which are highly specific. In an hypothetical system (Fig. 1), three similar molecules are represented. If molecule I is used as a hapten and it is conjugated to the protein through functionality b, the resulting antibody population is likely to cross-react with the closely related molecules II and III. Such an antibody population might find utility in developing an assay to the class of compounds represented by molecules I, II and III. Alternatively, if molecule I is conjugated through functionality a, the resulting antibody population is likely to distinguish among the three molecules and be useful for a specific assay of molecule I with minimal interference from related compounds II and III.

The importance of the site of conjugation of a hapten to a protein has been demonstrated many times with steroids and pharmaceuticals such as the barbiturates (5), and it was recently demonstrated with the insecticide S-bioallethrin (1R,3R,4'S allethrin) (Fig. 2) (6,7). The S-bioallethrin was conjugated to a carrier protein via an hydroxyl functionality of the propene side chain of the rethrelone moiety (Fig. 2A). RIA based on the resulting antibody population indicated a high degree of specificity for the absolute configuration of the chrysanthemate moiety distal from the point of conjugation and much lower specificity for the more proximal chiral center in the allethrelone moiety. In addition, the antibody could not distinguish S-bioallethrin from pyrethrin I probably because pyrethrin I has an identical configuration and differs from allethrin only in the propene side chain (6,7). If it were important to raise an antibody titer capable of distinguishing between allethrin and the pyrethrins I, the hapten could have been conjugated through its carbo-

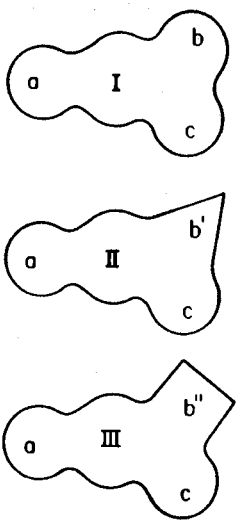
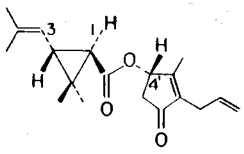
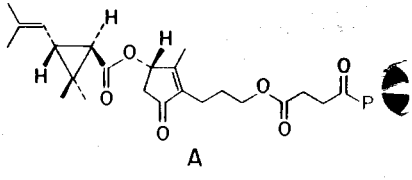


Figure 1. Illustration of importance of hapten selection on immunoassay specificity.

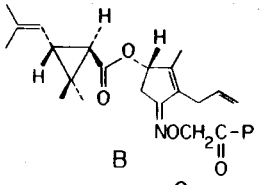
A hapten molecule (I) coupled to a protein through functionality "a" would be expected to raise an antibody titer useful for an assay of molecule I but not II or III. A hapten molecule (I) coupled to a protein through functionality "b" would be likely to raise an antibody titer useful for the assay of the class of molecules represented by I, II, or III.



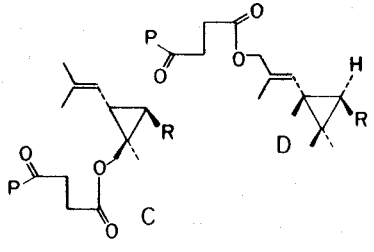
S-BIOALLETHRIN



A



B



C D

Figure 2. The structure of S-bioallethrin (1R, 3R, 4'S allethrin) and possible haptens for the formation of antigens for allethrin.

The hemisuccinate of an alcohol derivative of allethrin's propene side chain (A) illustrates the use of a spacer arm between the carrier protein and the molecule of interest. Antibodies to this antigen demonstrated the greatest specificity for the chrysanthemate end of the molecule. The allethrin CMO derivative (B) was prepared at the 1' ketone. Haptens attached through a gem *dimethyl group (C) or the isobutenyl group (D) would be expected to lead to antibodies with a greater specificity for the allethrelone end of the molecule. P indicates protein.*

methoxyoxime (CMO) derivative at C-1 of the allethrelone moiety (Fig. 2B), or better through a hydroxyl substituent on a gem dimethyl group or a functionality on the isobutenyl side chain of the chrysanthemic acid moiety (Fig. 2C,2D). Several studies have emphasized that it is often important for maximal specificity to have the hapten separated from the carrier protein by a spacer arm. A hemisuccinate moiety was used for this purpose in the case of S-bioallethrin. Several of the conjugation procedures discussed in the following paragraphs insert a spacer arm between the hapten and the carrier protein due to the nature of the conjugation reagent involved, while in some cases a more deliberate attempt to insert a spacer may be made (8,9).

Hapten Coupling. The functionalities on a protein usually used for coupling haptens include NH, SH, OH, and COOH. Numerous coupling techniques have been utilized and are described in detail in the pharmacology and endocrinology literature. Coupling techniques for affinity chromatography are also often applicable to hapten-carrier coupling (10). An overview of the most widely used coupling techniques is presented below. This overview is not exhaustive; rather, it is intended to illustrate some of the many synthetic routes open to the pesticide analytical chemist. When possible, examples have been drawn from the areas of entomology or pesticide or environmental chemistry. Langone and Van Vunakis (11) used an N-hydroxysuccinimide (NHS) (12) active ester of a carboxyl substituted analog similar to aldrin and dieldrin formed by dehydration with N,N'-dicyclohexylcarbodiimide (DCC) to conjugate with human serum albumin (Fig. 3, Rn 1). Similar active ester methods have been used to conjugate to proteins carboxylic acid derivatives of allethrin (6), diflubenzuron (13), juvenile hormone (14,15), ecdysone (15), polypodine β-oxime (16), and numerous compounds of medicinal interest. The active ester can be purified (12,17,18), and it is fairly stable under acidic conditions.

Alternatively, water-soluble carbodiimides such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide methyl p-toluene sulfonate (CMC) or 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide·HCl (EDC) are available which allow a direct coupling of an amine and carboxylic acid without the isolation of an active ester (Fig. 3, Rn 2) (19,20,21). Such procedures may be very useful with rather water-soluble or unstable haptens, and the resulting cross-linking of the protein may actually increase its antigenicity (4) although solubility is commonly reduced. In designing subsequent assays one should remember that water-soluble carbodiimides and some other coupling agents may react directly with a protein and subsequent antibodies may be directed, in part, against the resulting guanidino or acyl urea derivatives (Fig. 3, Rn 3). When using immunodiffusion (discussed later) for estimation of antibody titers, this laboratory has used haptens coupled to different proteins by chemically distinct

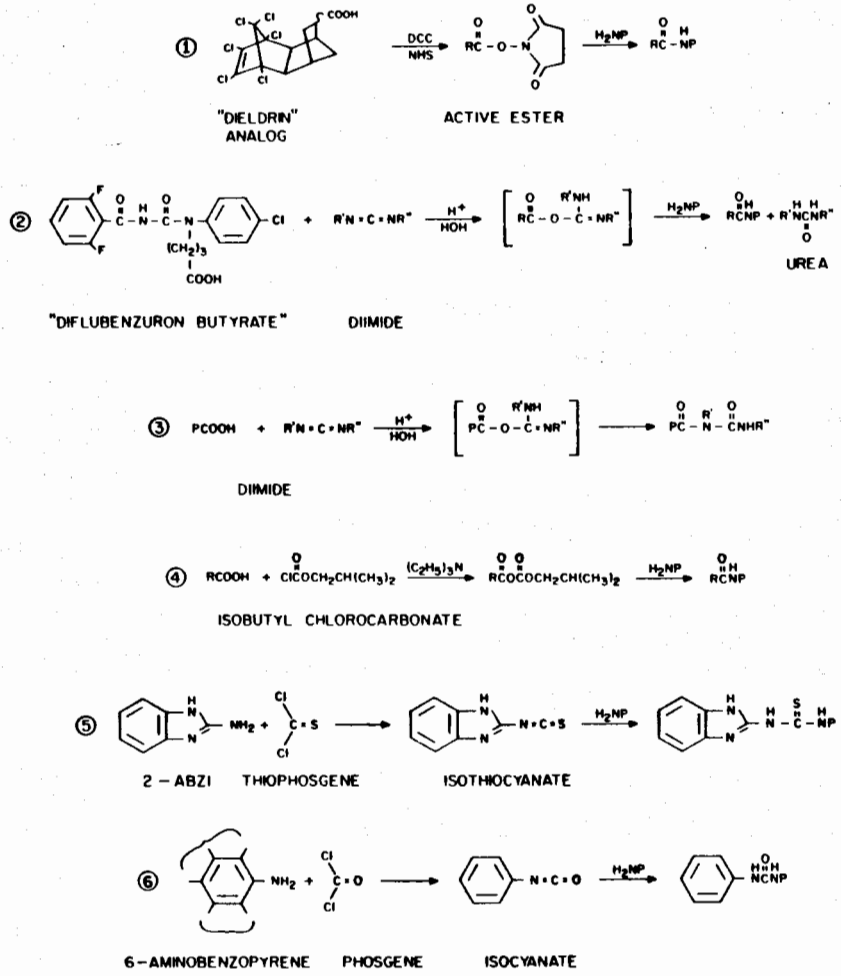


Figure 3. Some methods of hapten–protein coupling. Except for phosphorous in parathion, P indicates protein. See text for a description of reagents.

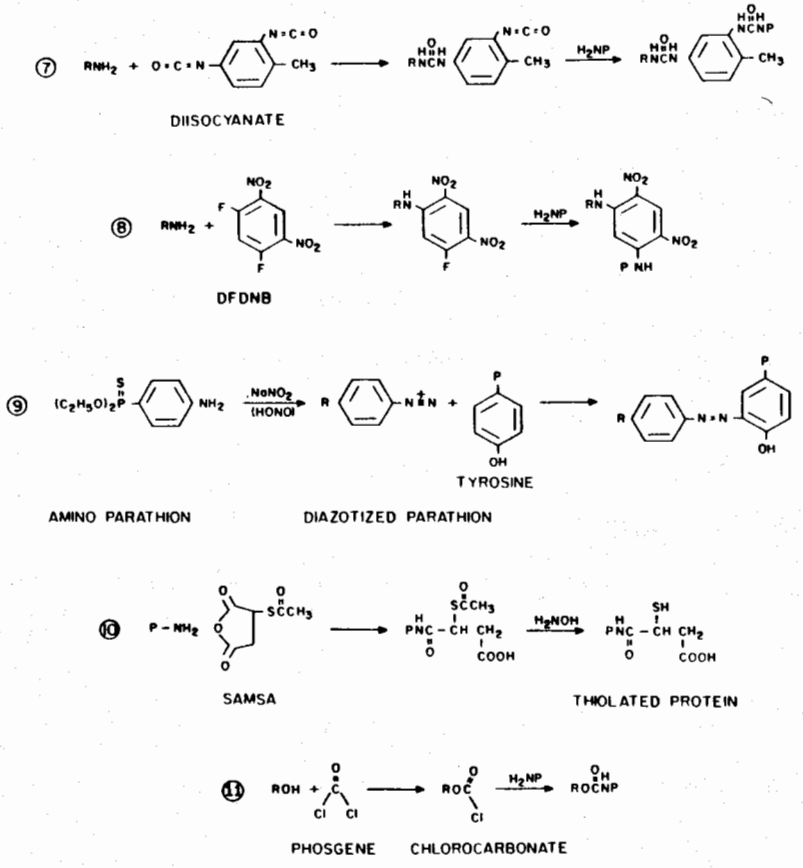


Figure 3. Continued

procedures. For instance, diflubenzuron derivatives were coupled using both the purified NHS active ester and via a water-soluble carbodiimide (Fig. 3, Rn 1,2) (13) to several different proteins.

Several other routes resulting in conjugation of a carboxylic acid group to an amine include reaction of the acid with an alkylchlorocarbonate (chloroformate) (Fig. 3, Rn 4). The ethyl and isobutylchlorocarbonates are commonly used; for instance, deReggi et al. (22) used ethylchlorocarbonate to make a conjugate of a succinylated ecdysterone derivative while Vallejo and Ercegovich (23) used sec-butylchlorocarbonate for the conjugation of a succinylated solanidine.

Pioneering work on immunochemical assays for pesticides involved the synthesis of haptens for DDT and malathion. Haas and Guardia (24) used the acid chlorides of malathion half ester and DDA (2,2-bis-[p-chlorophenyl]acetic acid) for conjugation while Centeno et al. (25) used the anhydrides of DDA and malathion diacid (O,O-dimethyl S-[1,2-bis-carboxyethyl]phosphorodithioate). In retrospect, more specific antibodies of a higher titer may have been obtained had a spacer arm been used.

The above methods were utilized to conjugate a carboxyl group on a hapten to an amino residue on a protein. Obviously, the above reactions could be, and have been, utilized to conjugate an amino residue on a hapten to the carboxyl residues on proteins. However, there are additional methods which have proven useful for conjugating amine containing haptens to proteins.

For instance, Lukens et al. (26) attached 2-aminobenzimidazole (2-ABZI - a degradation product of the carbamate fungicide Benomyl) to ovalbumin by reacting the amine of 2-ABZI with thiophosgene to produce the isothiocyanate followed by addition of ovalbumin (Fig. 3, Rn 5). Benzo[a]pyrene was conjugated to bovine serum albumin (BSA) by forming the isocyanate at C-6 by reaction of phosgene with the corresponding amine (27) (Fig. 3, Rn 6). Similar approaches could be applied to the development of an immunoassay for tetrachlorodibenzo-p-dioxin using the recently synthesized 1-amino-2,3,7,8-tetrachlorodibenzo-p-dioxin as a hapten (28).

The differential reactivity of the sterically hindered and unhindered isocyanate groups of tolylene-2,4-diisocyanate facilitates the stepwise conjugation of hapten (R) and protein (P) amino groups (Fig. 3, Rn 7). p,p'-Difluoro-m,m'-dinitrobenzene (DFDNB) reacts with numerous functionalities including primary and secondary amines, imidazoles, and phenols to yield mixtures of conjugated materials (Fig. 3, Rn 8). This reaction is apparently harder to control than the diisocyanate reactions, but it is much more versatile.

Aromatic amines may be converted to their diazonium salts with nitrous acid. The hapten may then be bound via azo linkages to the tyrosine (shown), histidine, lysine, and possibly arginine and tryptophane residues of the carrier protein by mixing the

protein and diazonium salt under basic conditions. This method was used in the classic immunochemical studies by Pauling et al. (29) and Landsteiner (30) and more recently to coupled aminoparathion to bovine serum albumin (BSA) (Fig. 3, Rn 9) (31). The sulfhydryl residue is commonly encountered in pesticides, and it can be utilized to conjugate a hapten to a protein via a disulfide bridge. Most proteins do not have numerous free sulfhydryl groups, so the free SH groups can be "enriched" by reacting the protein with N-acetyl-homocysteine thiolactone or more recently S-acetylmercaptosuccinic anhydride (SAMSA) (Fig. 3, Rn 10) followed by addition of the hapten (32). Thiolated proteins can be used for reaction with any compound capable of forming covalent bonds with sulfur. Glutathione or other conjugates of pesticide metabolites could also possibly be used for coupling to proteins.

Other functionalities on a hapten can be directly linked to a protein by a variety of methods or they can be converted to compounds containing a free amine or carboxyl group and then conjugated by the above methods. By reacting aldehydes or ketones with carboxymethoxylamine hemihydrochloride (CMA) the resulting oxime with a free carboxyl group can be formed as shown for the allethrin CMO derivative (Fig. 2B) (6). This procedure has also been used in coupling reactions leading to antibodies for insect molting hormones (33,34,35). The allethrin CMO derivative was found to be quite unstable, and this fact emphasizes the need for rigorous structural proof of hapten structure.

Hydroxylated pesticides are common metabolites and thus, a choice of hydroxylated materials are often available for conjugation. Exposure of metabolites with primary or secondary alcohols available to succinic anhydride in pyridine leads to a hemisuccinate as shown for allethrin derivatives in Fig. 2A. This method has been used to derivatize many compounds of biological interest including ecdysone and solanidine (15,22,23). Alternatively, hydroxyl groups can be exposed to equimolar phosgene resulting in a chlorocarbonate which will react with amino groups of proteins (Fig. 3, Rn 11) or reacted with ethyldiazoacetate followed by hydrolysis to give a carboxymethyl ether (36).

Phenols and diazotized p-aminobenzoic acid react to introduce a free carboxyl group (32). Ethylbromoacetate was used to derivatize phenolic metabolites of the insecticide diflubenzuron and model pyrethroids under anhydrous conditions. The resulting ethyl ester could be hydrolyzed in dilute methanolic base without hydrolyzing diflubenzuron. Longer spacers can be introduced by using bromopropionates and buterates, but harsher conditions are required for these less reactive bromides. The bromoacids can be used for more water-soluble haptens and chloroacetic acid has been used for estrogen (13,37). Phenols can also be coupled using other divalent reagents such as tolylene-2,4-diisocyanate or cyanuric chloride. A conjugated olefin can be reacted with 3-mercaptopropionate to also yield a free carboxylic acid at the

end of a convenient spacer arm (38).

Numerous other methods of conjugation are available and will likely be obvious to the chemist familiar with the properties of the pesticide of interest. In addition to many methods in the literature, numerous reviews give either detailed conjugation procedures (32) or references to these procedures (4,10,39). As will be discussed below, organic chemists may find the difficulty of establishing the structure(s) of the final conjugate disconcerting. In contrast to numerous papers in the literature where "recipes" for conjugation are simply followed, it is important to verify the structure of the hapten at each step of the synthesis. It may also be important to adapt the conditions of conjugation to the specific reaction in question. The stability of the hapten, the resistance of the carrier protein to denaturation, and the relative solubilities of the hapten and the carrier protein in the reaction medium should be considered.

Antigen Purification and Characterization. The antigen (hapten-carrier protein conjugate) is usually separated from low molecular weight by-products based on its large size. Dialysis is an obvious method of separation, but some lipophilic molecules pass through a dialysis membrane with great difficulty. Gel filtration provides another convenient method of separation. If the protein is not too badly denatured, repeated precipitation with an organic solvent such as ethanol is a rather certain way of removing lipophilic impurities.

The most quantitative methods of determining the moles of hapten bound per mole of carrier protein include the use of radiolabeled haptens or the monitoring of a change in the absorbance of the hapten-carrier conjugate in a spectral region where the protein itself does not strongly absorb (4). For the parathion conjugate a phosphorus determination proved to be a useful method (31). These methods are often not appropriate, so alternate methods such as the monitoring of the proteins' reactive groups (such as free amine) before and after conjugation must be employed (4,6,32,40,41). Careful controls are necessary with these procedures because self conjugation or denaturation of the protein may decrease or even increase the apparent functionalities available for binding.

There is no consensus on the optimum number of hapten molecules per carrier, but at least two molecules are required for subsequent immunoprecipitin tests. Many early studies used very high loading and useful antibody titers continue to be raised using heavily loaded antigens. Some workers feel that antibody titers with a higher average specificity for the hapten can be raised using low loading (4,42). Numerous proteins have been successfully used as carriers. Bovine and human serum albumin are very commonly used because they have numerous free amine groups and are remarkably soluble when cross-linked or even when heavily loaded with haptens. Many workers have found that mollusk

hemocyanin is phenomenally immunogenic. Although hemocyanin has been successfully used as a carrier for pesticide haptens, one often encounters solubility problems. Many other commercially available and exotic proteins have also been used as carriers. One can be relatively certain that a protein will be immunogenic if it has a molecular weight >10,000 and if it is immunochemically foreign to the animal receiving the antigen. One should also consider the ultimate use of the antibody when choosing the carrier protein. For instance, human serum albumin would be a poor carrier for a hapten if the resulting antibody were to be used to monitor human blood samples by immunodiffusion.

Choice of Animal for Antibody Production. Numerous vertebrates have been used as the source of antibodies. As techniques become more sensitive, less antibody is needed. Guinea pigs and rabbits are thus commonly used. Even mice are used, especially since the major cell lines now available for cloning antibodies are derived from mice (43). If larger quantities of antibodies are needed, one can move to either larger numbers of small mammals or to goats, sheep and larger mammals. The use of avian species is not common when haptens are used, but they may yield high, broad spectrum antibody titers against mammalian proteins. The nature of the antibodies obtained will vary somewhat with the species used. For instance, goats are known to often produce antibodies with very high affinity for haptens while guinea pigs often yield a high titer of complement.

Immunization Procedures. The antigen is usually injected into the recipient animal in Freund's complete adjuvant. This water-in-oil emulsion provides a slow release formulation for the antigen, protects the antigen, and with dead Mycobacteria, it stimulates the immune system. Subsequent booster injections are usually given in adjuvant without the Mycobacteria in order to avoid severe allergic response. The resulting antibody titer in the serum is monitored and when it has reached an acceptably high level, blood is withdrawn and the serum isolated for use in assay development. Many of the numerous immunization protocols are referenced in Parker (4) while Williams and Chase (32) give detailed instructions on the handling of animals.

Although the assays using antibodies have reached a high state of sophistication, a definitive work on immunization procedures is still lacking. It is not generally possible to reproduce the exact titer and specificity of antibodies even in apparently identical animals. This lack of reproducibility in the raising of antibody titers may have led to the reluctance on the part of pesticide analytical chemists to embrace immunochemical techniques. However, the animal is only the tool used to obtain the antibody, and once the antibody is in hand, the assays are physical in contrast to biological assays. Most radioimmunoassays use serum dilutions of 1:5,000 to 1:100,000 so

that a single rabbit will yield enough antibody for a staggering number of assays. If properly handled and frozen, antibodies may be stored for long periods. Ultimately, the serum from a single animal will be exhausted. Although it may be difficult to obtain another batch of serum of phenomenally high titer, affinity and specificity, numerous studies have shown that for most molecules one has a very high probability of obtaining useful sera following the injection of a limited number of animals (44) by standard procedures. For instance, out of 8 rabbits injected with several diflubenzuron antigens, antibody titers were detected in all rabbits against the carrier protein and in 7 against the hapten (13). Monoclonal antibody technology (see below) promises to improve the consistent availability antibodies as reagents (45).

The antibody titer is monitored in the serum by any of the numerous analytical procedures discussed below. For instance, antibodies were detected to an allethrin-hemocyanin conjugate by immunodiffusion studies using among other molecules, an allethrin-BSA conjugate (6) and passive hemagglutination was similarly used following immunization with a parathion-BSA conjugate (31). In addition to immunodiffusion, a radioimmunoassay was developed using a low specific activity ^{14}C diflubenzuron label for the monitoring of diflubenzuron antibody titers (13). In order to determine antibody titer, the serum is generally diluted until a serum concentration is reached which will bind 50% of a constant amount of hapten (44).

Antibody Characterization. In attempting to characterize the antibodies in a serum sample it should be kept in mind that one is dealing with a heterogeneous population of antibody molecules of varying specificity and affinity. There are undoubtedly antibodies present which recognize the carrier protein, but contribution of these antibodies to assay binding can be eliminated by using a different carrier or a tagged hapten. Even when only the hapten is recognized in the assay, one is dealing with a heterogeneous antibody population in a serum sample. An estimation of the average affinity constant (K_a) is often useful in the optimization of competitive binding assays (46), and one estimation of sensitivity is taken as one tenth of the reciprocal of the average binding affinity (44). Estimates of the average K_a are obtained by plotting a function of the hapten which is antibody bound vs a function of the concentration of the hapten. Such plots include Michaelis Menten curves, Scatchard plots, and Sips plots. The later two plots will also give an estimate of the heterogeneity of the antibody population (4,44,46).

The specificity of an antibody titer refers to the degree of cross-reactivity one sees with the antiserum used. By using different tagged haptens one can vary the specificity of the resulting assay; however, there is an intrinsic specificity of the antiserum which is difficult (although possible) to improve. The specificity of an antiserum is usually established by compe-

titive binding studies. Specificity is often expressed as the concentration of a substance needed to displace 50% of an antibody bound hapten. The specificity of an antiserum may be very high, requiring many-fold higher concentrations of very closely related molecules to displace the radioligand. Such specificity is the basis of the major advantages of immunochemical assays over many classical procedures; however, it may be misleading. Although an antibody may effectively discriminate among several very closely related molecules, it may bind quite tightly to an unknown molecule in an extract. Also, even a 1000X selectivity may be overcome if very high levels of even poorly reactive contaminants are present. Such problems are most common when lipophilic haptens are used. In classical GLC assays one is usually looking at a weak electrical response indicating the presence of, for instance, a mass fragment or electron capturing material. Such observations are only indicative of the presence of a pesticide if careful control runs have been performed, and similar controls are also necessary in assays in which antibodies are used.

Competitive Binding. Competitive binding provides the principle upon which most immunochemical assays are based. Enough antibody is added to a small, constant amount of radiolabeled antigen to bind 35-50% of it (the same principles apply regardless of the tag used to identify the antigen). As increasing amounts of unlabeled antigen are added, one decreases the amount of bound radiolabeled antigen which is then separated by one of a variety of techniques from the free radiolabeled antigen (Fig. 4). By monitoring the percentage bound and/or free radiolabeled antigen as the concentration of unlabeled antigen is increased, one can establish a standard curve. This standard curve can then be used to determine the concentration of an unknown (Fig. 4).

Bound/Free Separations. Usually the most time-consuming part of a radioimmunoassay involves the separation of the bound and free radiolabeled antigen. There are several promising new techniques which avoid this separation step, but the most sensitive assays still employ separation. Equilibrium dialysis is an esthetically pleasing method of separation, but it does not lend itself to the processing of a large number of samples. Gel permeation chromatography works on the same principle because the large antibody bound antigen elutes ahead of the smaller free antigen. Gel permeation is usually too slow for routine immunoassay procedure, although it forms the basis for several very rapid automated procedures. Nitrocellulose membranes will allow small antigens to pass through while retaining antibodies and they form the basis of several rapid analytical procedures. The use of dextran-coated charcoal to precipitate unbound antigen is commonly used, and it should be generally applicable to pesticides (6,7). The dextran coating on the charcoal and/or the

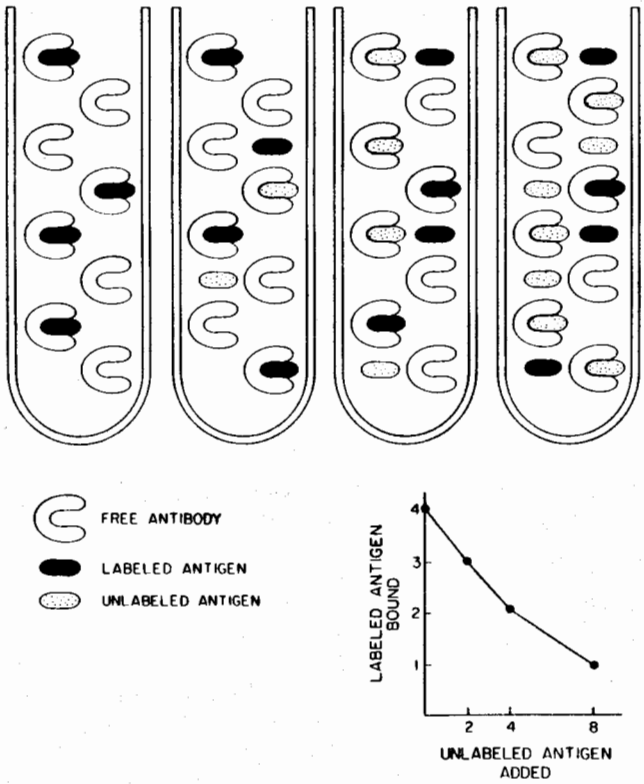


Figure 4. Illustration of the principle of competitive binding.

An increasing amount of unlabeled antigen displaces a constant amount of labeled antigen from a constant amount of antibody. Separation of antibody bound and free material results in a standard curve that can be used to determine the amount of unlabeled antigen in unknown samples.

presence of nonspecific sera greatly reduce the precipitation of antibodies and may increase the ease with which a charcoal suspension is handled. Florisil may sometimes be substituted for charcoal. Although techniques which bind the antigen are generally applicable to lipophilic molecules, they will shift the bound/free equilibrium with time. Thus, assays are often more time-dependent with antigen binding rather than antibody binding techniques.

The binding of a charged antibody to ion exchange resins or hydroxyapatite is also commonly used for separation. The antibody can be precipitated using polyethylene glycol or ammonium sulfate and/or a second antibody such as goat anti-rabbit leaving unbound antigen in solution. More recently a surface protein, protein A, on the surface of some Staphylococcus aureus cells has been found to specifically bind and precipitate many antibodies. Solid phase systems in which the antibody is coated on tubes or attached to polyacrylamide or dextran particles lend themselves to very rapid analysis. Larger amounts of antibody are generally required for solid phase assays and some additional effort in assay optimization is often needed.

There are several lines of research which may lead to very sensitive, rapid immunochemical assays which do not require separation of free and bound antigen. The ELISA procedure discussed below does require separation, but direct inhibition of a hapten-substituted enzyme by antigen binding (EMIT procedure) may alleviate a separation requirement. A sensitive method for the analysis of 2-ABZI has been demonstrated based on fluorescence polarization (26). Binding can also be measured without separation by attaching electron spin resonance (ESR) probes to antigens and monitoring the ESR band width of the nitroxide signal. Metal tagged haptens which are then analyzed by atomic absorption spectrometry show some promise (47,48). Lasers are increasing the sensitivity of turbidity methods but, at best, these methods are of moderate sensitivity.

Optimization of the Assay. As discussed earlier, competitive binding assays are based on the law of mass action where the affinity of the antibody for an antigen is $K_a = Ab \cdot Ag/(Ab)(Ag)$ and when 50% of the total antigen is bound $K_a = 1/(Ab)$. If an antibody population were homogeneous, the mathematics used to describe the binding would be rather straightforward. However, the heterogenicity of the antibody population complicates the situation so that there is no mathematical treatment that will completely describe all antibody-antigen interactions. The mathematical bases of immunoassay have been discussed by a number of workers (49-54), and work in this direction is continuing with a trend towards the development of computer programs universally adaptable to data from a variety of immunoassays (53-56). One can optimize an assay based on a logical progression of experiments using the physical constants intrinsic to the assay (46,

56), or one can approach the optimum assay conditions empirically by determining the amount of antibody needed to give 35-50% binding of ∿10,000 CPM of the radiolabeled antigen at experimentally determined incubation times (4,46). Most assays are run under conditions approaching or at equilibrium. However, the theoretical assay sensitivity and assay speed can be increased by using nonequilibrium conditions. Nonequilibrium conditions are often used in automated procedures.

Numerous methods exist for plotting competitive binding data. In choosing a method, one should keep in mind that the selection of an optimum plotting technique may simplify data handling or facilitate quality control evaluations, but plotting methods cannot enhance the intrinsic sensitivity or accuracy of an assay. The amount of antibody bound ligand is usually measured because small changes in antigen concentration will yield larger relative changes in the antibody bound radioactivity than in the unbound radioactivity. In theory, assay precision should be enhanced by monitoring both bound and free antigen, but this course is seldom followed.

Parker (4) pragmatically suggests plotting counts precipitated on the ordinate against the logarithm of the total unlabeled antigen concentration on the abcissa rather than spending an inordinate amount of time in selecting the "optimum" plotting procedure. The standard curve can usually be made more linear by using logit, probit, or arc sine functions (52). Unless automated data reduction is used, such plots provide adequate standard curves for most assays. There are numerous commercial products for RIA data reduction as well as a variety of published programs.

With constant random error, the precision of an RIA increases as the slope of the dose-response curve increases and decreases as the error increases with constant slope. Future availability of monoclonal antibodies may greatly increase the steepness and improve the shape of the resulting dose-response curve. As with any analytical techniques, it is crucial to appreciate the confidence intervals which one has at various points of the dose-response curve, in addition to the many measurement and collection errors which may be made before the immunoassay is employed.

Choice of Radioligand. A ^{14}C radiolabel will probably exist for most pesticides which will be considered for radioimmunoassay development. Such an intrinsic radiolabel will prove very valuable in titering antisera and possibly in numerous other steps from antigen synthesis through assay development. Unfortunately, for the actual assay, the commonly available ^{14}C radiolabels may not be of high enough specific activity. The theoretical limit on the specific activity of a single carbon atom is ∿63 mCi/mmole, and few pesticides have a specific activity of over 50 mCi/mmole even when they are labeled in

numerous positions. Although many factors influence radioimmunoassay sensitivity, assay sensitivity generally increases with the square root of the specific activity of the radioligand. Thus, ^{3}H and ^{125}I are commonly used. ^{3}H may be incorporated into the structure of a pesticide directly (an intrinsic radioligand). High levels of incorporation are possible by a wide variety of procedures, and carrier free tritium will yield about 29 Ci/mmol/atom incorporated. Isotope effects are much more common with ^{3}H than with ^{14}C, and seemingly trivial radiosyntheses may become very difficult when high specific activities are desired.

It is not necessary that the tracer or radioligand is structurally identical to the pesticide of interest. The same considerations used in deciding where to attach a hapten to a protein should be applied to attaching a hapten to either a commercially available labeled compound or to a compound which is easily labeled in a subsequent step. For instance, the conjugation of the hemisuccinate of S-bioallethrin to commercially available ^{3}H tyramine (p-[2-aminoethyl]phenol) led to a useful radioligand (6,7).

The most common isotope used in radioimmunoassays is ^{125}I. Incorporation of a single atom of ^{125}I will result in a specific activity of ∿2400 Ci/mmol. Since introduction of an iodine will usually cause a tremendous change in a hapten, the iodine is usually introduced on a separate moiety such as histamine, tyrosine, or tyramine which is attached to the hapten. ^{125}I offers many advantages over ^{3}H as a tracer. It is relatively easy and inexpensive to introduce, and its high specific activity leads to greater theoretical assay sensitivity. As a gamma emitter it is seldom subject to quench, and it can be efficiently detected with a solid scintillation counter. Solid scintillation counters are usually less expensive than liquid scintillation counters of similar sophistication. ^{125}I does not require the use of scintillation solution which makes assays easier, cheaper, and faster. However, as a gamma emitter which can undergo bioaccumulation, ^{125}I must be handled very carefully, and its 2-month half-life (vs 12 yrs for ^{3}H) necessitates repeated radiosyntheses. An additional problem is that many laboratories new to radioimmunoassay do not have solid scintillation counters even though liquid scintillation counters are commonly available.

There are numerous commercial adapters which increase the efficiency of liquid scintillation counters for gamma emitters. A modification of a suggestion by Beckman Instruments has proven quite useful in this laboratory. Thin-walled glass tubes were permanently attached through a hole in the cap of a standard scintillation vial with epoxy-cement. The vial was filled with a standard scintillation solution such as Omniflour® or 1.5% butyl PBD in toluene containing, in addition, tetraethyl or tetrabutyl lead. For the conditions in our laboratory, 3% v/v of tetraethyl lead in the scintillation solution resulted in >55% counting efficiency for ^{125}I on the ^{3}H window of a Beckman LS230 for

samples added in 6 x 50 mm glass test tubes. The scintillation vials were permanently sealed under N_2 in order to avoid the decomposition of the solution and the release of toxic vapors. There are several other suggestions for the counting of ^{125}I in liquid scintillation systems (57,58). Iodine is often introduced ortho to the phenol of tyrosine or a tyrosine-like material or into histamine attached to a hapten under mild oxidizing conditions. Chloramine T (N-chloro-p-toluenesulfonamide sodium salt) or lactoperoxidase-H_2O_2 are often used as oxidizing agents. A solid phase chloroamide has been recently reported (59). These procedures are only suitable if the molecule is stable to oxidizing conditions. Alternatively, a separate molecule may be labeled with iodine and then attached to the hapten (18). Some such compounds are commercially available and detailed procedures are available from most suppliers of radioactive iodine.

The same philosophies which apply to the choice of a radiolabel for radioimmunoassay generally apply to the attachment of any tracer or indicator molecule. These indicators may include such things as a fluorescent, electron spin resonance, metallic, or enzymatic markers. If an intrinsic radiolabel is not used, the method by which the label is introduced may effect the assay specificity and sensitivity, just as the choice of hapten does (Fig. 1). If the same hapten derivative is used for preparing the antigen and the radioligand, the resulting antibody may have a higher affinity for the radioligand than the molecule to be assayed. This situation will reduce the theoretical sensitivity of the assay (see Figure 5).

Enzyme-linked Immunosorbent Assay. A promising alternative to the RIA procedure is an enzyme-linked immunosorbent assay (ELISA) which depends upon the conjugation of a functional enzyme to either an antigen or antibody. The amount of enzyme present in a competitive binding assay is quantitated instead of the amount of radiolabeled compound. The concentration of the enzyme can be determined through its subsequent reaction with a substrate which results in a measurable spectroscopic change.

Conjugation of enzymes to antigens or antibodies were first developed for histochemical techniques and were used for localization of antigens and antibodies in tissue sections (60). Enzymes were quickly adapted for immunoassays, and Engvall and Perlmann (61) developed a procedure for the quantitation of an antigen. Alkaline phosphatase was conjugated via glutaraldehyde to rabbit IgG (antigen). Sheep antibody against rabbit IgG was coupled to microcrystalline cellulose by cyanogen bromide and the amount of antigen binding to the antibody was a direct relationship with the amount of phosphate ester cleaved by the coupled enzyme in a given period of time. This technique was widely adapted for the quantitation of various proteins and infectious agents (62-69).

A number of enzymes have been used with immunoassays. These

include lactic dehydrogenase, mushroom tyrosinase, glucose oxidase, acid phosphatase, alkaline phosphatase, and horse-radish peroxidase. The latter two enzymes have received most of the attention and peroxidase is usually preferred because of its low cost (70) although other reactions may be more sensitive and reproducible (68,69).

Bi- or multi-functional reagents have been used to link enzymes to other proteins. These include various carbodiimides, bisdiazotized amines, cyanuric chloride and glutaraldehyde. Enzymes linked to rabbit IgG antibodies from sheep, goats, and horses are commercially available and greatly facilitate the ELISA procedure. The preparation of the antigen and the development of the corresponding rabbit antibody (IgG fraction) have been described previously.

The ELISA procedure recently has been used for the analysis of parathion (31). Since this procedure has considerable potential a more detailed description of the analysis of parathion is in order. The conjugation procedure using amino parathion (AP) was described earlier (Fig. 3, Rn 9), and this conjugate was then administered to rabbits for development of a population of specific antibodies (Ab_1) against BSA or AP. Ab_1 demonstrated immunological activity only for the hapten when AP was conjugated to rabbit serum albumin (RSA). This antigen (RSA-AP) was rendered insoluble via attachment to the polystyrene surface of microtiter plates under basic conditions (Fig. 6.1).

Following the removal of excess antigen the specific antiserum (Ab_1) was allowed to react with the surface bound antigen (Fig. 6.2). After washing away excess antiserum, an enzyme (E, horse-radish peroxidase) conjugated to goat γ-globulin (Ab_2), produced against rabbit γ-globulin of the antiserum (Ab_1), was added (Fig. 6.3). The binding of the enzyme complex to the solid phase was a measure of the amount of bound RSA-AP and Ab_1. The enzyme concentration was measured spectrophotometrically by means of its catalyzing the oxidation of hydrogen peroxide in the presence of 5-aminosalicyclic acid in a given period of time (Fig. 6.4).

Analysis of parathion by this technique is based on the competition between the free form of parathion (P) and its conjugated form (AP) for the binding sites on the first antibody (Ab_1) (Fig. 6.5). Due to this competition, there is a decrease in the binding of the conjugated form as the concentration of the free form (P) increases. Complete inhibition of the binding of Ab_1 for BSA-AP may result when P is present in greater quantity than Ab_1. The concentration of the parathion in an unknown sample can then be determined by comparing the degree of antibody inhibition caused by the addition of the sample extract with that resulting from the addition of known amounts of the same substance. For parathion analysis no cleanup of the extracts of fruits or vegetables were necessary.

Various parameters of the ELISA procedure need to be

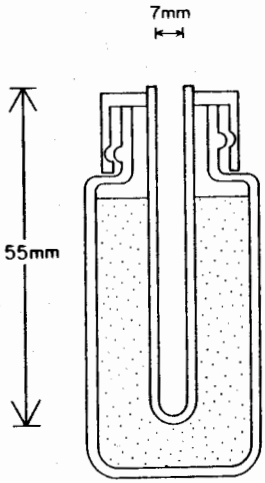


Figure 5. Cross section of a scintillation vial illustrating a system for counting ^{125}I in a liquid scintillation system.

The sample to be counted is inserted into the 7 × 55 mm glass well immersed in heavy metal charged scintillation cocktail in a permanently sealed vial.

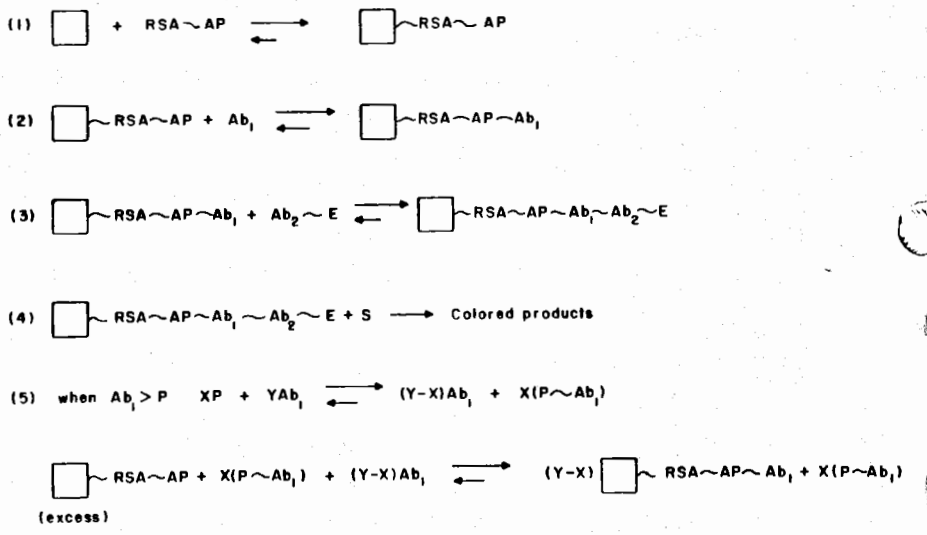


Figure 6. Schematic representation of ELISA.

(□) polystyrene surface; (RSA) rabbit serum albumin; (AP) conjugated aminoparathion; (Ab_1) first antibody (rabbit anti-parathion); (Ab_2) second antibody (goat anti-rabbit); (E) enzyme (horse-radish peroxidase); (S) substrate; and (P) free hapten (parathion).

optimized for each analysis. These parameters include the concentration of antibodies, time of incubations, temperature, and the composition of the washing fluids. Optimum time of incubations for analysis of parathion ranged from 2-3 hours. A solution of 0.9% NaCl and Tween 20 was determined to be most suitable for washing the microtiter plate in all steps of the procedure.

The antiserum exhibited high specificity for the functionalities of parathion, e.g., 58 ng/ml of parathion produced 50% inhibition of the oxidation of 5-aminosalicyclic acid. Changing of the ethyl groups to methyl groups as in methyl parathion (2000 ng per 50% inhibition) or replacement of the sulfur atom with oxygen as in paraoxon (1850 ng per 50% inhibition, 23), greatly reduced the competitive binding. Amino parathion did exhibit a significant cross reaction (275 ng per 50% inhibition, 23) but p-nitrophenol bound poorly to the antibody (5000 ng per 50% inhibition). The lower limit of detection of parathion by the ELISA procedure was found to be 5.0-10.0 ng/ml which corresponded to 0.025-0.05 ppm in crude extracts of fruit, vegetables, and human serum.

The procedure gave good reproducibility as expressed in the coefficient of variation (CV%) of results of between-run (6.2-8.6) and within-run (4.8-6.5) variations. Accuracy of the ELISA procedure was tested by comparing results of parathion analysis in extracts of fortified and field samples with results obtained by a GLC method. Correlation coefficients ranged in almost all cases between 0.93-0.99.

The ELISA procedure for the analysis of parathion as described above requires nearly eight hours, although many samples can be simultaneously assayed. However, incubation times can be shortened to one-half hour, in most cases, resulting in only a 10% reduction in sensitivity. Also the polystyrene microtiter plates containing bound RSA-AP can be mass produced and stored in a freezer. Since the enzyme-linked antibody can be purchased, the limiting factor of the applicability of the ELISA procedure, as well as the RIA procedures, for other pesticides is the development of the antiserum to the pesticide.

The ELISA procedure shares many of the advantages of RIA, and it has additional advantages of requiring only inexpensive equipment and of being well adapted to automated or partially automated methods. For instance, Ruitenberg et al., (71) has mechanized the ELISA procedure for screening of 4000 sera samples daily. A number of disadvantages of the ELISA procedure also can be sited. These include the nonstability of the developed color requiring daily analysis (not necessary in the RIA method), nonlinearity of color development, and less sensitivity than some other immunochemical methods.

Other Immunoassay Methods. Other immunoassay methods can be used to quantitate the hapten; these include homogeneous enzyme

immunoassay (EMIT), radial immunodiffusion, immunoelectrophoresis and passive hemagglutination tests. These techniques are often used to characterize the antibody, but they also can be used to quantitate the hapten through inhibition experiments. The EMIT procedure involves direct inhibition of enzyme activity when an antibody binds to a hapten conjugated near the enzyme's active site. Thus, it is particularly useful with small molecules, and it is very rapid because no separation steps are required (69). This technique is promising, but it has not been widely used and it is often of lower sensitivity than ELISA.

Radial immunodiffusion procedures are varied but all depend upon the diffusion of the antigen or antibody in a gel producing a precipitate which is proportional to the quantity of reactants (often sensitive to 25 ng protein with visual methods (32,72, 73)). Modifications using radiolabeled antigens or antibodies may increase the sensitivity fifty-fold (74,75). If the antibody is first mixed with the hapten, the concentration of free unbound antibody will decrease proportionately and result in a decrease in the precipitate formed with the antigen which can be observed visually or with radiolabeled methods.

The hemagglutination test is often used to express antibody titer, but it also can be used to quantitate the hapten. This test is based on the fact that erythrocytes, when treated with a dilute solution of tannic acid, acquire the property of being able to adsorb protein (the conjugated antigen). Such protein-coated red blood cells are agglutinated by specific antiserum directed against the hapten. The agglutination titer is expressed as the reciprocal of the highest dilution of the serum that causes agglutination of the red blood cells. The antiserum can be incubated with the sample extract containing the specific hapten prior to conducting the hemagglutination test. The amount of unbound antiserum is reduced by the amount equivalent to the free hapten and consequently the dilution of antiserum necessary to produce agglutination is inversely proportional to the amount of hapten in the sample (32).

Monoclonal Antibody Technology. Based on pioneering work by Köhler and Milstein (76) a new technology is evolving which may greatly improve the availability, specificity, and sensitivity of antisera. Monoclonal antibody technology has been the subject of a technical compendium (43) and a nontechnical review (45). Simplistically, spleen lymphocytes immunized *in vivo* or *in vitro* are fused with a myeloma cell line, and the resulting hybrids are selected on the basis of nutritional requirements and then cloned. Those clones producing monoclonal antibodies of the desired specificity are injected into mice where the resulting ascites tumor fluid may contain gram quantities of a monoclonal antibody. Alternatively, antibodies can be collected directly from a cell culture medium. Since clones are used, it is not necessary for the antigen to be highly pure and antibodies

selective for optical isomers conceivably could be obtained from a racemic hapten. Although in its infancy, monoclonal antibody technology may offer many advantages. As earlier discussed, the serum of an animal contains a large population of antibodies with varying specificities and affinities. With monoclonal antibodies one or more antibody types of high affinity and optimum specificity can be selected and propagated for use in immunoassays. Immunoassays using monoclonal antibodies are characterized by a very steep dose-response curve which translates as greatly enhanced assay precision. Although the clonal hybrids may have an unstable chromosome complement, with proper technical care the lines could be considered "immortal". Thus, a single, uniform antibody reagent of defined specificity could be provided to many laboratories from frozen cell lines which are occasionally thawed to produce antibodies. On the negative side, monoclonal technology is new and has not been widely applied to the analysis of haptens. Relatively simple cell culture facilities are needed and since culturing must currently be done in antibiotic-free medium, a high level of technical skill is necessary. Several companies have recently entered the field, and it may soon be possible to obtain antibodies on a contract basis. Some of the potential advantages and disadvantages of monoclonal technology as applied to residue analysis are listed in Table II.

Table II

Potential Advantages and Disadvantages of Monoclonal Antibodies as Applied to Pesticide Residue Analysis

Advantages	Disadvantages
Cell lines "immortal"	Chromasome complement often unstable
Very high specificity possible	Specificity too great
Steep dose-response curve (very precise assays)	Dose-response curve too steep (small linear region)
Technology advancing rapidly	New technology
Large amount of antibodies	Not widely used with haptens
Extraordinary high titer	Simple cell culture facilities needed
Uniform antibodies	Antibiotic-free medium used
Activate *in vivo* or *in vitro*	Precipitation assays difficult
Pure antigen not necessary	

Attributes and Limitations of Immunoassay

Immunoassay Sensitivity. Yalow (2) points out that as little as 0.1 picogram (0.05 picomolar) gastrin can easily be detected by immunoassay in a milliliter of incubation medium. Immunoassays to small lipophilic molecules are generally less

sensitive than those to proteins and peptides, and molecules having several polar functionalities separated by nonpolar areas often lend themselves most readily to highly sensitive radioimmunoassays (4). With steroids, sensitivities on the order of picogram are not uncommon (44) and useful assays for steroids and drugs have been devised at much lower sensitivities. As discussed earlier, the actual assay sensitivity depends upon the affinity of the serum, the incubation volume, and the amount of tracer and antibody used (which translate, in part, to the specific activity of the tracer).

The sensitivity of the overall analytical procedure depends upon many factors obviously including the type of sample to be analyzed and the skill of the analytical chemist. If an immunoassay is used to measure the amount of pesticide in a water sample by adding the water sample directly to the immunoassay, very high sensitivity may not be obtained although the assay will require very little time to perform. Alternatively, if the water sample is extracted and the immunoassay is employed only after several highly efficient cleanup steps, phenomenal sensitivity may be obtained at the expense of a large investment in time. In some situations, immunochemical methods may decrease the limit of detectability of a pesticide residue (77), but more importantly they may, in some cases, decrease the time and cost needed to reach a level of detectability as has been demonstrated with parathion (31).

Specificity. The specificity of an immunoassay is related in some respects to the sensitivity. The high specificity of immunoassays often allows samples to be analyzed with a minimum of cleanup. The remarkable specificity of antigen-antibody interactions has been reviewed in the classic text by Landsteiner (30). More recently, Al-Rubae (31) demonstrated a high level of specificity in an ELISA procedure for parathion. As previously discussed, parathion could be easily distinguished from methyl parathion and p-nitrophenol and the assay demonstrated little or no cross reactivity with a number of other pesticides. A radioimmunoassay for S-bioallethrin showed no cross reaction for any of several other pyrethroids tested (except pyrethrin I), and it was capable of distinguishing S-bioallethrin (1R,3R,4'S) from the other 7 optical and geometrical isomers of allethrin. Since biological activity and biodegradation may depend upon the configuration of an insecticide (6,7) the ability of immunoassays to distinguish among chiral isomers (78,79) may become of great importance to future pesticide metabolism and residue investigations. The high potential specificity of immunochemical methods may prove very useful, in conjunction with other methods, in the confirmation of the presence of residues. Monoclonal technology is likely to allow pesticide analysis based on immunochemistry to be even more specific and sensitive.

Although one must ultimately rely upon the immune system of

an animal to determine the specificity of a given antibody population, methods were discussed earlier which can be used to predict the antibody specificity when a given hapten is used. Antisera, which will detect parent pesticide plus toxic metabolites, could be used in combination with one or more highly specific antisera to quantitate several molecules of interest. An assay of moderate specificity will be of greater use in some analytical applications than a highly specific assay. The more general assay may be very useful in screening for the presence of a class of compounds or the presence of a specific functionality in a metabolite. Such assays can be used very effectively by coupling them with chromatographic techniques such as thin-layer chromatography (TLC) or open column chromatography. The use of immunochemical tests as sensitive detectors for high performance liquid chromatography certainly offers promise in pesticide analysis. A nice example of such a procedure is the analysis of N,N-dimethylindolealkylamines in biological fluids (80).

Speed of Analysis. The speed with which many immunochemical analyses can be completed illustrates a major advantage of immunochemical procedures. Immunochemical assays are most time and cost effective when the sample load is large. Parker (4) estimated that a single technician could perform 100-5000 radioimmunoassays per day with little or no assay automation in comparison to 20-40 GLC assays (3). Numerous inexpensive systems are available to decrease analysis time. These systems may include solid phase separation techniques, automatic dispensers, test tube racks which will fit directly into a centrifuge and/or scintillation counter, and data handling systems. Alternatively, there are fully automated systems based on RIA or ELISA which require very little operator attention and which handle 25-240 samples/hr. Gochman and Bowie (81) have outlined the basis of operation and summarized the features of automated RIA systems and extensive literature is available from the manufacturers.

As with many analytical procedures, the most time-consuming part of the assay is sample preparation. The high specificity and sensitivity of immunoassays may tremendously reduce the workup needed before actual analysis of the sample. For example, analysis of allethrin in milk by the accepted analytical procedure based on electron capture GLC required 4-8 hour per sample in our hands (82). Similar sensitivity and higher specificity could be realized using an immunochemical assay requiring 15-30 minutes per sample. With some loss of sensitivity, immunoassays may be very rapid. Turbidity measurements can be made so quickly and quantitatively that they may be very useful for field analyses of pesticides. Such rapid procedures might prove very useful in determining pesticide coverage on specific areas of a plant immediately after application, detecting drift, or monitoring the safety of a field for worker reentry.

In this chapter we have discussed the advantages of immuno-

chemical methods as a supplement to more classical analytical techniques. Possibly among the most important contributions of immunochemistry to future pesticide analysis will be its use as a tool to open new areas of pesticide analytical chemistry. If very rapid, inexpensive assays can be developed, pesticide analysis may be increasingly employed to enhance effective pesticide use rather than as simply an enforcement or residue tool.

Cost Effectiveness. As with the other advantages of immunochemical analysis, cost may be quite variable. Reagent costs for several automated systems have been estimated at under $1.25 per sample. The cost is obviously much lower for less sophisticated assay systems, especially if some reagents are prepared in house. A major consideration is the expense of new instrumentation. For dedicated or automated instrumentation for either RIA or ELISA procedures, the cost may be $50-100,000. However, most analytical laboratories already have the basic instrumentation needed for immunoassays. Moderate sensitivity can be obtained through the use of numerous procedures such as radial immunodiffusion and hemagglutination. These procedures require no expensive equipment or reagents and they may be very useful in areas where equipment acquisition or maintenance is a problem.

The expense of an analytical procedure depends upon much more than the cost of the final analysis. Much of the expense of an assay is related to sample preparation, and for many applications immunoassays have tremendously reduced the time needed for sample preparation. Another consideration is the amount of time needed for the development of an assay. The additional expertise which must be developed in an analytical laboratory before immunoassays can be used with confidence may seem formidable, and waiting for an animal to develop antibodies may lead to unacceptable delays in assay development. On the other hand, once a usable antibody titer is obtained, the development of a workable assay is usually straightforward. It is also likely, if immunoassays become accepted for some aspects of pesticide analysis, immunoassay kits or at least critical reagents will become commercially available. Such kits already exist for many pharmaceutical products and hormones, and numerous companies will supply antibodies to a user supplied hapten on a contract basis (83).

Applicability. Parker (4) points out that one can assume that workable radioimmunoassays can be developed "with all except the smallest or most unstable molecules." Once a useful antibody titer is obtained, often only very small changes in a generalized procedure are needed to obtain a workable assay. Although immunoassays would appear to be generally applicable to pesticide analytical problems they may be most useful in solving specific problems which appear intractable when classical procedures are used. Immunoassays are often most sensitive and specific when

several polar functionalities exist. Such compounds may be rather nonvolatile or heat labile and difficult to analyze by classical methods. Although sensitive, specific immunoassays have been developed for nonpolar compounds, such compounds may be most readily analyzed by GLC procedures. For laboratories not interested in the development of their own antisera, each Fall "Lab World" (83) lists suppliers of immunochemical reagents.

Problems with Immunoassays. As with any analytical technique, there are numerous problems associated with the use of immunochemical technology. Most of these problems are common to any analytical procedure, but some are relatively unique to immunoassay and have been covered by Parker (4). The parameters which should be monitored to maintain quality control of the assay have been discussed by Rodbard et al. (50). A major concern discussed earlier is cross reactivity or interference, especially if it is unexpected. One can guard against this problem by employing well characterized antiserum, by using sample blanks, and by running standard curves in the presence of extracts. One must rely upon the equipment and reagents used in analytical procedures. Antibodies are certainly not as stable as many chemical reagents; however, the guaranteed shelf life of many commercial lyophilized preparations is over 5 years at 4°C. The integrity of the reagents must be periodically reestablished, especially if the assays are only performed sporadically.

Immunoassays lend themselves to the processing of a large number of samples. The same number of control and standard assays are required whether one or a large number of samples are assayed. For an analytical laboratory faced with analyzing a large number of samples for the presence of a few pesticides, immunochemical procedures are likely to offer many advantages over some more clasical analytical methods. If the same laboratory were faced with quantitating the residues of a large number of differing chemicals in a few samples, immunochemical procedures are likely to be less cost and time efficient than an equally sensitive GLC based assay.

Possible Contributions of Immunochemical Methods to Pesticide Analysis. As Ercegovich (3) pointed out, it is unlikely that immunochemical methods will replace current, established analytical methods of pesticide analysis. However, the analytical chemist who carefully compares the attributes and deficiencies of immunochemical methods of analysis with other procedures is likely to find applications for which immunochemical methods offer distinct advantages.

In many cases, those compounds which are most difficult to assay by classical procedures because of numerous polar functionalities and poor volatility are the very compounds which lend themselves most readily to immunochemical analysis (4). One can also predict that the number of pesticides marketed with a high

degree of optical purity will increase, and immunochemical methods lend themselves to the analysis of chirality at the residue level (6,7). Thus, there will probably be some pesticides for which immunochemical methods will provide the future enforcement procedures of choice for residue analysis.

It is envisioned that immunochemical procedures can be more commonly used as a supplement to classical methods of pesticide analysis. Since samples can often be analyzed without expensive and time-consuming cleanup procedures usually required of most methods, the immunological assays can rapidly screen many samples at significantly lower cost. When the immunoassays indicate that samples contain appreciable pesticide residues, the samples can be further analyzed by GLC or other methods. Alternatively, an immunochemical assay may provide a confirmatory test. Specific antisera also can be used to concentrate pesticides and to clean up extracts by means of affinity chromatography procedures, thereby, permitting greater sensitivities of GLC, HPLC or other methods. It is expected that immunochemical and especially the ELISA procedures may contribute to field reentry and human exposure problems where simple, rapid, inexpensive procedures are desired. Finally, the possible usage of these methods in developing countries could be of practical importance due to the simplicity of the procedures, the ease with which they are interfaced with thin-layer analysis, and the use of relatively simple laboratory apparatus.

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