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Chapter 18

Pesticide Immunoassay as a Biotechnology

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Immunoassays are generally applicable to environmental chemistry. They are highly sensitive, specific, and precise, which leads to rapid, cost-effective assays. In many cases, immunochemical assays are most applicable for compounds that are difficult to analyze by classical methods. For instance, GLC is best suited for compounds that are volatile, heat stable, and which have functionalities easily detected by available selective detectors. HPLC is less restrictive, but still depends on specific functionalities for detection. However, these criteria that are essential for chromatographic methodologies are not necessary for a successful immunoassay. Thus, immunoassays expand our ability to monitor compounds in a cost effective manner that are difficult to detect using the GC/HPLC technology. With trends toward more complex classical pesticides including benzoylphenyl ureas such as diflubenzuron and sulfonyl ureas such as Gleen, immunoassay may prove to be the most suitable method of analysis. Furthermore, with biological insecticides, based on classical as well as genetically engineered products such as the toxins of *Bacillus thuringiensis*, immunoassay may prove to be the only suitable physical method of analysis of expressed protein.

In the past several years, immunoassay has begun to be recognized as a useful analytical technique in pesticide residue analysis. In 1980 when Hammock and Mumma (1) pointed out the potential for this application, only a handful of laboratories were utilizing the technique. Now a number of industrial and governmental laboratories are exploring the use of this versatile technology.

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Since the award of the 1977 Nobel Prize in Medicine to Rosalyn Yallow for the development of the radioimmunoassay, immunoassay technology has had a major impact on many fields of science. A very sophisticated set of technologies has developed for the analysis of molecules which owe their great sensitivity and specificity to the biological systems that produce the reagent antibodies. All these assays are based on the high affinity, but reversible interaction of an antigen and antibody as determined by the law of mass action. However, only recently has some of this technology been applied to the environmental area. Environmental chemists slowly are beginning the realize that immunoassay is simply another analytical tool.

Advantages of Immunoassays

Any analytical technique has advantages as well as limitations. In some cases the very factors which are considered advantageous in one instance will present problems in another instance. In order to properly apply immunochemistry to analytical problems it is critical that one is aware of both these limitations and the advantages. We will try to describe here the basic advantages and how they might apply to pesticide chemistry as well as point out some of the pitfalls.

Speed of Analysis. There are a number of ways to describe the speed of analysis. One is to evaluate the assays that can be performed per man day, another is to evaluate the time required from receipt of sample to analytical result. Immunoassays score very high, regardless of which criterion of speed is applied. This tremendous speed of analysis is dependent, in turn, on the specificity and sensitivity of the immunoassay. It is important to realize that with immunoassay, as with any other analytical technique, there is a trade off among speed, sensitivity and cost. There are many immunoassay formats (2). Some require several hours to perform, others only minutes.

The actual time required to run a single sample by immunoassay is usually longer than the time required to run a single GLC analysis. However, numerous samples are usually run simultaneously with little extra time required. In most residue procedures, the major expenditure of time is in sample work up. The great advantage of immunoassay, is that in many cases, work up steps can be greatly reduced or eliminated. For example, the herbicide molinate (Figure 1) is used extensively in rice fields, and the ability to monitor levels of the pesticide in the field will assist in downstream water management. GLC workup requires extraction with an organic solvent and then analysis. We are currently developing an immunoassay which will analyze molinate in the water sample directly, with sensitivity to about 10 ppb. Similarly, the electron capture method for analysis of diflubenzuron (Figure 1) in milk has many clean up and derivatization steps. The corresponding immunoassay can quantitate diflubenzuron in milk without prior work up and with greater sensitivity (3).

The typical enzyme linked immunosorbent assay (ELISA) method requires six hours for the total analysis of a single bank of

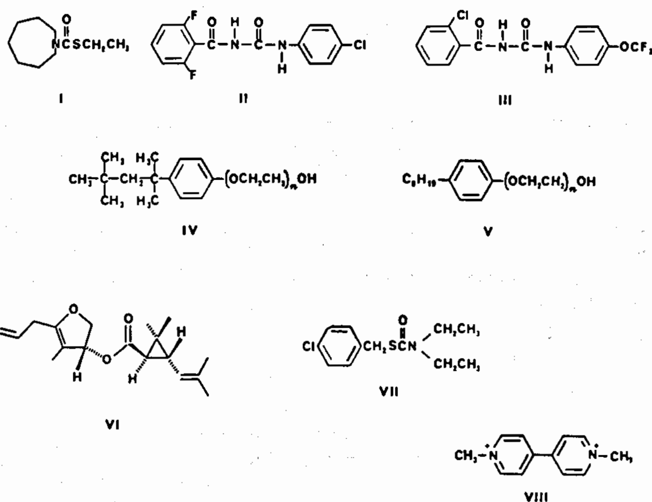

Figure 1. Structure of molinate (I), diflubenzuron (II), Bay Sir 8514 (III), Triton X (IV), Triton N (V), S-bioallethrin (VI), thiobencarb (VII) and paraquat (VIII).

samples, but this does not represent man hours required because most of the time is spent waiting; rather, it represents analysis time from start to finish. If the average retention time for a compound on GLC is three minutes, in six hours, one could optimally run 120 analyses. If each of these was a single sample at a single dilution, than 120 samples could be run. Comparably, a single operator, without automation could easily run 6-10 times this sample number by immunoassay, or, more information could be obtained about each of these 120 samples, by running more dilutions or replicates. Even if GLC is used for confirmation, immunoassays are useful for rapidly eliminating negatives and ranking samples in terms of concentration for subsequent analysis.

Ease of Automation. Solid phase assays (such as ELISA) are especially amenable to semiautomation with little investment in sophisticated equipment. Our laboratory's first ELISA reader was a system consisting of a small Gilford spectrophotometer interfaced with an Atari 400. Each cuvette holder held 50 cuvettes. Once reagents were prepared, 20-100 assays could be readily performed. Currently, we utilize a Flow Titertek Multiskan interfaced to an IBM-PC for data collection and management. With the aid of multichannel pipettors, 100-1000 assays can be performed per day in a 96 well plate format. The addition of plate washers and diluters to the scheme can further increase both speed and precision. The immunoassay format is especially amenable to robotic procedures for the handling of very large numbers of samples such as those generated by the monitoring of pesticide residues in food.

Specificity. The specificity of immunoassays can be very high. Immunoassays can readily distinguish the same functional enzyme from different but related species, or even from different organs in the same species. This may be translated as a single amino acid change in a primary sequence of over one hundred residues. For small molecules, we have demonstrated the ability to distinguish very closely related structures. For example, Triton X and Triton N (Figure 1), nonionic surfactants, require extensive sample workup and yield multiple peaks on HPLC during analysis. An immunoassay developed in this laboratory could easily distinguish between these structurally related compounds (4). We have also shown with S-bioallethrin (Figure 1) that even geometrical and optical isomers can be distinguished by immunoassay (5).

One drawback is that cross reactivity can occur to structurally related compounds. If one requires an assay specific for a parent compound, this would be a disadvantage. On the other hand, if one's goal is to detect a class of compounds or the parent and its metabolites, this cross reactivity would be advantageous. For instance, diflubenzuron is very closely related to another benzoylphenyl urea insecticide (Bay Sir 8514, Figure 1). A very high efficiency HPLC column is required to separate these compounds. We have developed several highly specific immunoassays which can distinguish between these very closely related compounds, while other assays can detect the benzoylphenyl ureas compounds as a class (3).

Sensitivity. ELISAs are not usually considered to be as sensitive as radioimmunoassays, however, sensitivities in the mid picogram/ml range have been obtained (5,6). Assays in the ng/ml range are sufficient for most analytical needs.

Sensitivity can also be considered in terms of ppb detectable in the presence of some matrix. In this case, sensitivity is a function of selectivity, and immunoassays are capable of detecting the target antigen at low concentrations even in the presence of large amounts of contaminating material. In this context, molinate is detectable at 10 ppb in water, 0.1 ppb or less in water extracts, but only 30-60 ppb in soil extracts without cleanup.

With chromatographic analysis, assay sensitivity is usually defined as a function of peak height relative to baseline noise. With immunoassays, detection limits can be defined as limit of detection or limit of quantitation. For instance, using a highly selective antibody, the limit of quantitation of thiobencarb (a thiocarbamate herbicide used in rice culture, Figure 1) is approximately 10 ng/ml when it is based upon the linear region of the standard curve (or the mid region of a sigmoidal curve, Figure 2). The actual limit of detection relative to background noise is less than 1 ng/ml when the lower portion of the linear region of the standard curve is used.

Cost Effectiveness. The most expensive part of an assay is the analyst's time. For diflubenzuron, over 100 immunoassays can be carried out in the same time that it takes to perform 0.5 GLC based assays. This apparent advantage is obviously greatest for those compounds that require many workup steps prior to immunoassay. Even with compounds that are relatively easy to assay by classical methods, immunoassay may prove cost effective if numerous samples must be handled. Solid phase assays are relatively easy to automate completely so that many more samples can be run per unit of analyst or machine time by immunoassay than by automated HPLC or GLC.

Finally, it must be emphasized that these characteristics of the assay depend upon one another. If one wants a highly sensitive assay, it may require more cleanup, and so one sacrifices speed. If one desires an assay to detect a series of compounds, one will have to compromise specificity and expect increases in cross-reactivity. Therefore, an inexpensive assay of moderate sensitivity that can be performed in the field with no specialized equipment or highly trained personnel can be developed, but one cannot expect these assays to be highly sensitive and precise as well.

Applicability. Immunochemical technology is applicable to most analytical problems, but it can be more easily applied to some analytical problems than others. Immunoassay sensitivity ultimately depends on the K_d of the antibody-antigen complex. Since this K_d, in turn, usually depends upon summation of weak molecular interactions, immunoassays for very small molecules are rarely sensitive. Because immunoassays are performed in aqueous solutions, they are not easy to apply directly to hydrolytically unstable materials. However, one can expect success in an attempt to develop an immunoassay to a molecule of moderate to large size.

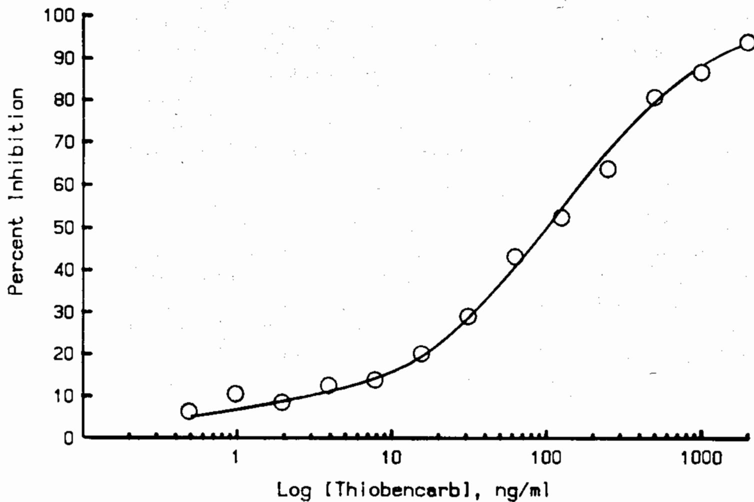

Figure 2. Typical standard curve for the thiocarbamate herbicide thiobencarb. Ninety-six well microtiter plates were coated with 0.2% glutaraldehyde for 20 min. After removing glutaraldehyde each well was coated with 0.2 ml of a 3 ug/ml solution of a hexanoic acid derivative of thiobencarb coupled to ovalbumin in 0.5M carbonate buffer pH 9.8 and allowed to incubate in the refrigerator overnight. The standard curve was prepared by incubating various concentrations of thiobencarb with a 1/3000 dilution of antiserum in phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween) overnight at room temperature. The antiserum was raised in rabbits to a p-aminophenyl derivative of thiobencarb coupled to keyhole limpet hemocyanin. The following day the plate was washed three times with PBS-Tween and the standard curve preparations added. After a two hour incubation at room temperature, 0.2 ml of a solution of goat anti-rabbit IgG conjugated to alkaline phosphatase was added. Two hours later the plate was again washed three times with PBS-Tween and a 1 mg/ml solution of p-nitrophenylphosphate in 10% diethanolamine buffer pH 9.8 was added. After a 30 minute incubation at room temperature the optical density was read at 405 nm. This is a representative of a competitive sandwich ELISA. Any antibody which binds to thiobencarb in the standard curve preparations, cannot then bind to the coating on the plate. The goat anti-rabbit antisera and p-nitrophenylphosphate serve to detect the amount of antibody bound to the plate. Thus the more thiobencarb that was in the standard curve preparation, the less antibody is free to bind to the plate and thus the less color development occurs.

This is especially true if the molecule has several easily recognizable functionalities including potential sites for formation of ionic or hydrogen bonds and regions of alternating hydrophobicity and hydrophilicity.

Small, highly lipophilic molecules may be more easily analyzed by methods other than immunoassay for a variety of reasons. However, lipophilicity alone does not preclude the development of a sensitive immunoassay. At the part per billion or trillion level of sensitivity of good immunoassays, most compounds are soluble. Even if the compound exists as a micelle or on the surface of proteins, antibodies can pull the target materials into their binding site by mass action. Antibodies vary dramatically in their sensitivity to water miscible organic solvents. However, most antibodies will tolerate a few percent, and many 30 percent or more, of solvents such as methanol and tetrahydrofuran. Thus, for a skilled analyst, simple water solubility is seldom a problem in immunoassays of even highly lipophilic materials. However, the cost of separation of a lipophilic target compound from a lipid rich matrix may greatly reduce the attraction of immunoassay development for such materials.

Role of Monoclonal Antibodies

The development of hybridoma technology is one of the most exciting events in biology because the availability of monoclonal antibodies allows many very difficult problems to be approached for the first time. However, there may be a tendency to apply the technology to problems which can be solved as well, and in some cases better, with the use of polyclonal (classical) antibodies. In evaluating the advantages and disadvantages of mono- versus polyclonal antibody technology for pesticide analysis, it is important to understand that most assay formats are not influenced by the source of the antibody (poly- versus monoclonal).

Hammock and Mumma in 1980 (1) discussed some of the advantages and disadvantages of hybridoma technology with regard to pesticide residue analysis. In the intervening years, the hybridoma field has matured sufficiently to evaluate its potential more fairly. There are numerous scientific as well as popular articles (7,8) on this rapidly evolving technology including a number of articles in this text.

The antibodies which one obtains from the serum of an animal usually consist of a population of specific types of antibodies which recognize a variety of antigenic determinants with varying degrees of specificity and affinity. In contrast, monoclonal antibodies are obtained from a cell line ultimately traceable to a single cloned cell. If there are no problems with mixed chains, the cell line will produce a single type of antibody molecule. Hopefully, this antibody will recognize a single antigenic determinant with constant affinity and specificity.

The major advantage offered by monoclonal antibodies to pesticide analysis may be as much administrative as scientific. One unresolved example of this is the patent position that one may encounter with mono- versus polyclonal assays. In theory, a cell line producing a monoclonal antibody is immortal. Thus, one moves away from the fear of the "magic rabbit" that will ultimately die

leaving a world lacking its precious serum. Realistically, the magic rabbit problem is quite rare and a reasonably good antiserum can be reproduced in another animal. However, it is rare that two antisera are totally identical in their specificity and affinity, even when they come from the same rabbit. The properties of each new bleed must be evaluated. With the expense of developing hybridoma antibodies decreasing, there may be a time when the cost of producing and maintaining a small library of monoclonal antibodies to a pesticide will be less than the cost of periodically raising and reevaluating a new antiserum, or of using the common procedure of combining many bleeds to obtain an average pool of serum. Certainly, a permanent supply of monoclonal antibodies will have an appeal to regulatory agencies, as well as to patent attorneys. However, it must be remembered that these cell lines may contain an unstable chromosome complement, and their immortality depends upon proper storage and maintenance. Realistically for an important molecule, one would work with pooled polyclonal sera stored in small aliquots or with a large amount of monoclonal antibody produced at one time and stored in small aliquots. Thus both systems usually rely on storing antibodies.

In theory, one could screen a large number of clones, and find those that are producing antibodies of exceptionally high affinity and specificity. In practice, the screening effort required to obtain a few positive clones is often so major that the further effort of finding those producing the optimum antibody is not undertaken. Designing a rapid screen for a producer of an optimum antibody is obviously much more difficult than simply finding producers. Some part of the screening effort may be reduced because of automation, yet even then, the monoclonal antibody found may not prove superior to a good serum. Murine antibodies are not well known for high affinity, and the optimum clone resulting from a major screening program may yield an antibody with a K_d which is not as low as the $K_d$ave of a rabbit serum. At this time, there appears to be an attempt to solve problems in immunochemistry using thousands of dollars of screening and hybridoma technique that could be solved by hundreds of dollars of planning and hapten synthesis. Even after extensive screening, poor selection of hapten structures or poor screening strategies can result in an unworkable monoclonal based assay.

The defined specificity of a monoclonal antibody is unique among biological reagents, yet an antiserum may offer greater specificity. A single interfering substance which binds to the monoclonal antibody could lead to complete cross reactivity in the resulting immunoassay. This same interference may only be a minor problem in a polyclonal system where it would bind to only a small subpopulation of total antibodies reacting with the molecule of interest. Obviously, the optimum situation would result from the employment of a library of well characterized monoclonal antibodies as either a mixture, (analyzing for several components in one assay) or a series of assays (analyzing one sample in several assays).

Monoclonal antibodies do offer some advantages now, and many advantages in the future. For an institution wishing to limit the sensitivity of a technique, the defined specificity and lower

affinity offered by hybridoma technology could offer a promising approach. The reactions of a monoclonal antibody are easier to handle mathematically than those of a polyclonal mixture. This fact, coupled with a known kinetic k_a and k_d, makes monoclonal antibodies much easier to use in many assay formats, especially with some "biosensors". It is important to realize that hybridoma technology is advancing. With automated systems, it is possible to screen large numbers of clones and thus develop a library of antibodies of varying specificity and sensitivity. Such libraries can be invaluable, especially with complex antigens. Finally, techniques are being developed using multiple mouse strains to realize the potential of hybridoma technology to produce antibodies of extraordinary specificity and sensitivity.

Until immunoassay is more widely accepted in the field of agricultural chemicals, it seems questionable that the added expense of hybridoma technology is warranted scientifically for routine analytical problems. However, it seems certain that hybridoma technology will dominate the immunoassay field in the future.

Biological and Genetically Engineered Pesticides

Up to this point, the advantages of immunochemical assays in pesticide analyses have been discussed with an emphasis placed on classical pesticides. However, development of the new biotechnology of molecular genetics may have a dramatic impact on pesticide chemistry and place immunochemical assay in an indispensable position in pesticide analysis. Cognizant of the powerful tools of molecular biology, researchers and the pesticide industry are rapidly developing new classes of pesticides. These "new" pest control agents are usually of a biochemical and biological nature (eg. peptides, microbial toxins, and micro-organisms). For instance, fermentation technology has already given us avermectin and ivermectin. While a complex exotoxin from *Bacillus thuringiensis (BT)* is not allowed in current formulations, it has been considered for development as an insecticide itself. These materials are far more complex than the compounds normally analyzed by GLC and HPLC. Although their analysis can certainly be approached by classical means, immunoassays seem particularly advantageous with these molecules. One can anticipate the development of more such complex molecules, and the need for analytical technology to deal with them. Furthermore, given the general public's attitude toward "genetic engineered" microbes, it is certain that the vast majority of products from this type of research will instead be peptides and proteins. For such materials, immunochemical methods are unsurpassed.

Although proteins and peptides resulting from research in biotechnology followed by fermentation are unlikely to present environmental contamination problems, a whole new concept in pollution may arise from this area of research. Since nucleic acids have the potential to reproduce in the environment, we do have the potential to pollute some segments of a "wild" gene pool with man-made or contrived genes. In the last year, the protein toxin of *BT kurstaki* has been cloned into, and expressed in plants

(e.g. tobacco, cotton), and Monsanto is still trying to test the same toxin in the field following expression in an alternate species of bacterium (personal communication). The crude mixture from a genetically engineered bacterium containing the *BT* toxin gene already has been field tested in California. In situations where foreign genes are released into the environment, immunochemical procedures are necessary to monitor the expression, as well as the products of expression in the environment. Immunoassays could be used directly on suspect biological material, or on the protein produced in a cell free translation system using the DNA from the suspect materials. Thus, immunoassays become complementary to the genetic probes, which will be necessary to monitor the actual gene.

Immunoassays not only represent a biotechnology themselves, but they may well be the best way to analyze for products of biotechnology research. In this regard, immunoassays for the whole crystal toxin of the *BT kurstaki* and *israelensis* strains have been developed (9-13). These assays all demonstrated good correlation with biological assays for the toxins, and they are routinely used for monitoring production and quality control in the fermentation process. Adaptation of these assays to actual residue procedures will be a logical alternative for monitoring these "new" biological pesticides.

Immunoassay vs. Bioassay in the Detection of Biological and Genetically Engineered Pesticide. It has been rather widely assumed that the biological assay is the only way to perform residue analysis on biological insecticides in the field. However, residue analysis either by biological or by immunochemical assay for a toxin is analogous in that one desires a selective detector. This is achieved in bioassay by using test animals that are susceptible to the toxic materials, and in immunoassay by an antibody which selectively recognizes antigenic determinants on the target molecule. Therefore both bioassays and immunoassays can be as specific as the design of the respective assay itself.

On the other hand, sensitivity is a different matter. In bioassays, the test animal acts as a detector and a concentrator simultaneously. For example, the mosquito larvae bioassays for *BT israelensis* δ-endotoxin is extremely sensitive because of the feeding behavior of the mosquito larvae. The animal selectively ingests particulate matter and thus accumulates the crystal toxin, even when exposed to very low toxin concentrations. An LC_{50} of 200pg/ml was reported for the purified crystal toxin (14). Sensitivity of immunochemical assays operates on a different principle. All enzyme immunoassays are basically amplification systems in which enzyme conjugated to the antibody or the antigen is used to drive an enzyme reaction which amplifies a weak signal (eg. low concentration). Therefore the sensitivity of an immunoassay is ultimately limited by the binding constant (10^8 to 10^{13}) between the antibody and the antigen. Although various well established techniques (eg. fluorescent immunoassay, Avidin/Biotin systems, radio-labelled immunoassays, etc.) can be used to further enhance the detection limit of an immunoassay, it still may not detect biologically significant levels of many peptides or proteins in environmental samples.

As with any other analytical residue procedure where the sensitivity of an assay is insufficient, further sample workup is required. However, microbial degradation, heat sensitivity, pH sensitivity, and solubility should all be taken into consideration when one is attempting to concentrate or extract biological materials during sample workup.

<u>Dot-Blotting</u>. In our experience, the *Dot blot technique* is most simple and versatile in sample workup for biological materials. The dot-blot assay takes advantage of the fact that pure nitrocellulose strongly adsorbs proteins, nucleic acids and cellular compounds. Antigens can be quickly immobilized onto a nitrocellulose membrane, and then be analyzed with any of the many EIA procedures. Concentrations of the antigen are revealed by the intensity of color development on the membrane. Such color development can be determined qualitatively, or quantitatively, if one chose, by instruments available commercially. An added advantage of the dot-blotting technique is that multiple sample application is possible. Repeated applications further concentrate the antigen on the membrane, and thus extends the detection limits for solutions of lower concentration (Figure 3). This, theoretically, can increase the detection limit indefinitely. Thus the dot-blotting technique essentially achieves what biological assays have to offer. It acts simultaneously as a concentrator and a detector.

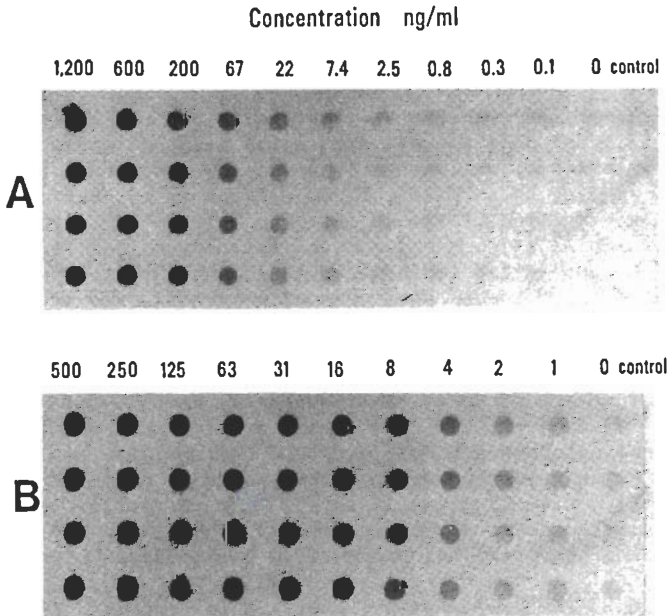

Figure 3. Dot-blotting of *BT israelensis* δ-endotoxin (A) one sample application vs. (B) five sample applications.

Applications to Environmental Health

Pest Management. The capability of immunochemical technology to provide reliable, low cost analytical data to individuals lacking intensive training and sophisticated equipment will probably be its greatest contribution. Due to the expense of current analytical methods, industry and government normally only ask those analytical questions for which an answer is required by regulatory agencies. Individuals who can make the best practical use of these analytical data to increase the agricultural productivity seldom have access to such information. The ability to determine deposit, coverage, residue, and if a pesticide even reached the microenvironment of the target host are obviously critical for the effective use of an agricultural chemical. In a more pragmatic sense, any chemical which fails to reach the target could be considered a pollutant. However, the analytical capability to answer such questions is far out of reach for most pest management scientists.

Rapid Field Assays. The high speed and simplicity of many immunoassay formats allow them to be performed in the field. Therefore it is likely that the immunochemical technology will do more than supplement existing technology. It will greatly extend our power to monitor environmental health. For example, farmers can insure that a herbicide is at a safe level before planting; applicators can be assured that drift is limited and that the chemical reached the target; farmworkers can be assured that a field is safe to enter, and wholesalers and regulatory agencies will know whether residue levels are acceptable.

Human Exposure. Immunoassays are especially useful in monitoring materials in human body fluids. When immunoassays are developed for key environmental pollutants or indicator compounds, the toxin itself can be used as a marker of human exposure by monitoring urine or blood samples. Paraquat (Figure 1) is a commonly used herbicide which is responsible for the majority of deaths attributed to pesticides in the U.S. An immunoassay for paraquat is being used for clinical diagnosis of poisoning, and we have found that crude blood, urine and lymph can be analyzed directly in an immunoassay for paraquat with greater sensitivity than other published techniques. This same immunoassay has been used in a worker exposure study. Here, the immunoassay and GLC data compared favorably (6), however, the immunoassay offered the advantage of directly analyzing human body fluids and the antibody could even be used to extract paraquat from filter patches, eliminating the acid extraction step. Such rapid assays make it possible to carry out the pharmacokinetic studies necessary to evaluate the significance of occupational exposure to pesticides.

Released Time. Pesticide residue analysis as practiced today is both equipment and labor intensive. In many laboratories, the experienced scientists who manage the laboratories are so involved with handling the day to day problems caused by an ever increasing work load that they lack the time to apply their skill and

creativity to long term problems. A very important result of implementing immunochemical technology in a residue laboratory could be the release of equipment, monetary resources and especially human resources from the routine residue analysis to the many pressing problems in analytical and environmental chemistry.

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