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Immunochemical Methods for Environmental Analysis

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Chapter 11

Immunochemical Technology in Environmental Analysis

Addressing Critical Problems

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Immunochemical technology is at a critical stage in its development for use in environmental analysis. Primary problems and issues regarding assay development and applications such as outlining common misconceptions, choice of format and choice of monoclonal or polyclonal antibodies are discussed. More far reaching concerns for the acceptance of the technology such as the roles of government and industry in assay development and standardization are also discussed. A committee to coordinate the development of immunoassay in the environmental field is proposed and its functions outlined. How some of these problems are currently being addressed are illustrated by work presented at this symposium. Work from our laboratory illustrates our approach to dealing with real world samples, more difficult target compounds and complex matrices and applying immunoassays to samples other than environmental samples.

The next few years will be critical in the development of immunochemical technology for use in environmental analysis. In this light this manuscript has three objectives. The first is to address how the critical problems facing the technology can be approached. The second is to introduce aspects of this symposium by pointing out how various laboratories are approaching these problems. Finally, this manuscript will review briefly some of the topics being addressed by this laboratory.

Evolution of Problems Facing Immunoassay

Changes in the Last Ten Years. Based on both the Miami American Chemical Society (ACS) meeting 11 years ago and the ACS meeting 10 years ago, this symposium certainly has historical significance to

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our laboratory. At the Miami ACS meeting in 1978 only two papers were presented on the immunoassay of pesticides. One was on our immunoassay for the optical isomers of the pyrethroid S-bioallethrin and the other was an immunoassay of parathion. The later study was from the laboratory of the late C. D. Ercegovich who was one of the early leaders in this field. The next year at the 1979 ACS meeting in Washington D. C. Drs. Harvey and Zweig requested that the laboratory address the potential of immunoassay for pesticide residue analysis in a symposium on *Recent Advances in Pesticide Analytical Methodology* (1). As one might anticipate, this talk drew a great deal of criticism based on many misconceptions regarding immunoassay. Some things certainly have changed in the ten years leading to this 1989 ACS meeting. Simply the increase from one paper to 27 papers on the immunochemical analysis of pesticides and other environmental chemicals illustrates a major change in the interest of pesticide chemists in immunoassay. This change has not been due to some magical improvement in immunochemical technology. In fact, immunochemical technology as it applies to the analysis of small molecules in environmental samples has not changed greatly in the last 10 years, while great changes have been made in chromatographic and spectral detection systems. The change has been in an increased awareness of the capabilities of immunoassays in the environmental field.

Misconceptions, Then vs Now. At first glance this increase in interest in immunoassay might indicate that pesticide immunoassay has matured and left its doubters and problems behind. Such is not the case. Although there is wide interest in the technology and most agricultural chemical companies have in-house expertise in immunochemical technology, immunoassays have not been used to register a single pesticide nor is an Association of Official Analytical Chemistry (AOAC) validation of immunoassays for pesticides a common phenomenon. In fact, the technology seems to have traded one set of problems for another. Acceptance of the technology was stifled for years because many scientists concluded that immunochemistry had no place in environmental chemistry based on little appreciation of its power. We now find the major problem facing the technology is that it is being over sold, in some cases as a panacea, by people who do not understand the limitations of the technology.

A major theme of this book could be the same one we advocated ten years ago. That is that immunochemistry represents a very powerful analytical tool which is applicable to many but certainly not all problems in environmental chemistry. Thus, it complements but does not replace other analytical methods. The technology is so powerful and versatile that it should be in the repertoire of every analytical chemist. Yet there must be an understanding that the technology is very useful for some compounds and some problems, but that it is no panacea. Ten years ago the challenge was to encourage understanding of the tremendous power of the technology, while today we must preach the limitations.

The major problem facing the technology ten years ago was a lack of understanding of immunochemical analysis by environmental chemists. At the time the misconceptions seemed challenging to overcome, but in many ways they are less serious than the problems that the technology faces today. One significant problem was the jargon that surrounded immunochemical technology. A major role of the scientists involved in advocating immunoassay in the environmental field over the last decade has not been to pioneer new immunochemical technology but rather to translate the jargon used in clinical immunochemistry to the jargon used in environmental chemistry. As pointed out by Ken Hunter formerly of Westinghouse Bioanalytical, our job became dramatically easier with the advent of the microcomputer which generated a standard curve for analyte vs response. Such standard curves appear more familiar to analytical chemists, reassuring them that they are dealing with real analytical chemistry and not some qualitative biological phenomenon.

Another of the major misconceptions ten years ago was that immunoassays were bioassays, and some even thought that a rabbit died each time an analysis was run. We had a generation of analytical chemists who had fought to have chromatographic methods accepted over bioassays for residue analysis, and immunochemistry seemed to offer a great leap backwards. The realization that immunoassays are physical assays which simply use biological reagents is now wide spread. The fact that the cyclodiene antibodies published by Langone and Van Vunakis in 1975 (2) are still in use, is an excellent illustration that if properly handled immunochemical reagents are very stable.

A new misconception now exists in some quarters in this decade of biotechnology. Ten years ago the biological source of antibodies tainted immunoassays as a poorly reproducible black art practiced by biologists and not by real chemists. Now in some quarters the biological source of antibodies seems to impart magical qualities to immunoassays. Some people indicate that these assays can detect biological effects, but like any physical assay, immunoassays detect molecules which may or may not be associated with biological activity. Certainly the specificity of an immunoassay can correlate with that of a receptor molecule. However, such correlations are incidental. There is an effort to apply immunoassays to all compounds and problems with no appreciation for the technology's limitations or the strengths of competing technologies.

A major challenge facing all competent analytical chemists is to make sure that the technology is advocated based on its real strengths. If the technology is over sold based either on ignorance or on a desire to advance a product for profit or one's career, there is certain to be a backlash when immunochemistry fails to provide magical results.

There also is the indication that immunoassays allow untrained analysts to run highly sensitive assays. Although immunoassays may be very forgiving and easy to perform, the quality of the data generated for any physical assay will depend upon the

integrity of the samples and the skill of the analyst. As the assays used become more difficult and the limit of detection lower, the skill of the analyst must be greater as with any analytical system.

In some ways a more difficult problem has been that the reluctance of residue chemists to embrace immunoassay has led to the development of immunoassays for environmental chemicals in metabolism, biotechnology, or clinical laboratories. Among this group of scientists there sometimes is a reverse arrogance towards the residue chemist who failed to adopt this technology. However, the assays developed outside of an analytical laboratory often use such simplistic hapten design that key recognition sites are masked. Also, there is a vast difference in matrix effects between clinical and environmental samples. The use of enzyme linked immunosorbant assays (ELISA's) or other immunoassays in a real analytical program will normally reveal new matrix problems totally unfamiliar to the clinical chemist. The experience in this laboratory is that a good analytical chemist can be trained to perform ELISA in a matter of days. However, the conversion of an immunochemist into an environmental chemist represents a major change in career and philosophy. The collection, handling, and processing of samples as well as the design of analytical studies and the handling of data are every bit as sophisticated as the preparation of monoclonal antibodies. It is critical that when the data from immunoassays are to be used for important decisions, that well designed assays are performed by trained analytical chemists (3).

When is Immunochemistry Most Applicable?

As indicated above it is very important that as advocates of this technology, we point out when it is best applied and also when it should not be applied. This topic has been covered in a variety of previous reviews (1,4-8), however we have found two figures which convey several concepts about applicability very well.

For instance in Figure 1 we represent all of the compounds for which the analyst may need methods. For some compounds such as the volatile organics in water, gas chromatography systems offer great advantages. At the other extreme there are compounds such as paraquat, the sulfonylureas or benzoylphenylureas which lend themselves wonderfully to immunoassay development. There is an important set of compounds which can be analyzed readily by several different methods. The thiocarbamates or triazines are compounds handled in this laboratory which fall into such a situation. Here the decision on the technology would depend upon the resources of the laboratory in question and the problem at hand. If the compounds were part of a multianalyte problem or if only a few samples needed to be assayed, chromatographic systems offer an advantage. In cases where field assays are needed or where a large sample load is likely, immunoassay clearly is the method of choice.

The most common question from biotechnology companies and from the agricultural chemical industry concerns which compounds

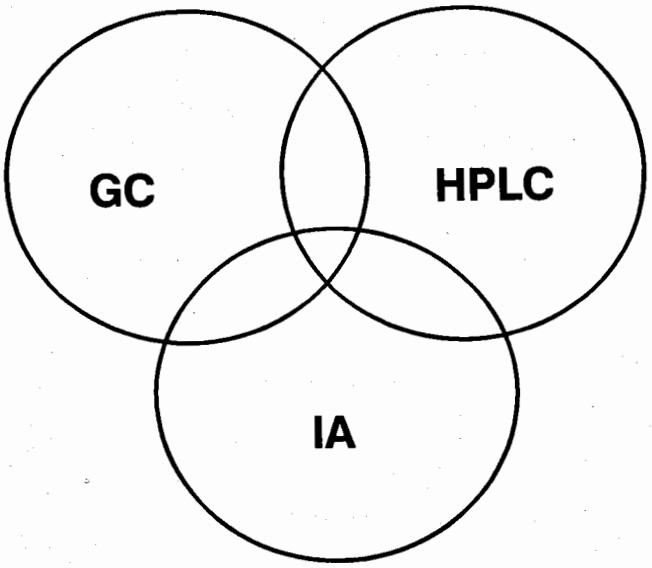

Figure 1. Applicability of immunochemistry to analytical problems. The background indicates all compounds for which analysis is needed while the respective circles indicate the subset of compounds for which gas chromatography (GC), high performance liquid chromatography (HPLC), and immunoassay (IA) are most applicable. For those compounds which can be readily analyzed by a variety of methods, the decision of which assay to use should be made based on the analytical questions to be answered.

are appropriate targets for immunoassay development. Part of this answer of course involves market analysis, which is not an appropriate topic here. It is clear that there will be a reasonable market for immunodiagnostics in the environmental field, but large obvious markets do not now exist.

With the management of the agricultural chemical industry in the past the error has been not to ask what are the proper targets. Rather immunochemical technology is ignored until other analytical methods have failed, the chemistry of the compounds has become cold, and there is tremendous pressure from marketing and registration groups for immediate analytical methods for a very difficult compound. Only then is the task of developing an immunoassay given to a new employee with few resources. This hardly represents the optimum way for a company to develop in-house expertise in immunoassay.

Immunoassays are very versatile, and if one could select but a single method, it could be the method of choice. Fortunately we have a variety of techniques available and a good analyst should know when to apply them. Table I provides some general rules for determining how difficult an immunoassay will be. The terms used are relative and possibly other dimensions to the table could be the laboratory's experience with immunoassay and the problems faced. This table does not indicate that good assays cannot be developed for hard compounds; it just indicates that the expense, skill and time required may be greater for those compounds. For instance we have developed successful immunoassays for some lipophilic, small, unstable, volatile compounds. However, such compounds would be a poor choice to use for one's first venture into immunoassay development.

Table I. Properties of Compounds which Lead to Difficulties in Immunoassay Development

PROPERTIES	
EASY	HARD
HYDROPHILIC	LIPOPHILIC
LARGE	SMALL
STABLE	UNSTABLE
NONVOLATILE	VOLATILE
FOREIGN	NATURAL

Gaining Full Use of Immunochemistry

For over a decade the data have been in the literature to support the contention that for appropriate molecules and problems immunochemical methods are far superior to competing technologies.

Yet, the methods still are not in routine use. Advocates of the technology have entertained themselves by making one immunoassay after another. Although this activity is important, we now face the more difficult challenge of validating these assays, ensuring that they are in the proper hands, and that they are used effectively. This challenge can be broken down into a number of smaller problems which do not differ greatly from problems faced with chromatographic systems. However, some of the problems will still be difficult to address. Fortunately other problems which initially will seem difficult will turn out to be no problem at all.

Should Immunoassays be Qualitative or Quantitative? This is an excellent example of a nonquestion which sometimes is discussed seriously. As discussed below, the answer is that once one has an antibody and tracer the assay can be put into either a quantitative or qualitative format depending upon the question to be addressed. Qualitative formats will be very important in the environmental arena as fast field tests. However, it is our opinion that at least until the technology is well established that qualitative tests in the environmental field should be based on reagents which have been examined in a quantitative format. Users of qualitative kits which have no quantitative data supporting them could be very embarrassed if they try to over interpret their data.

What Format Should Be Used? A great strength of immunoassay is that the same reagents can be used in many formats. We have employed Voller's ELISA format (9), but even this format has numerous variations. The format gives adequate sensitivity for most environmental questions, does not require radioactive compounds, can be optimized for speed, cost, sensitivity or other factors, and maybe most important, it has a pleasing and nonintimidating name. In addition we have advocated this format since the understanding that the same antibody can be used in numerous formats is not widespread. We feel that currently it is important to not frighten new users and regulatory agencies with formats for which they have no name recognition.

However, in the long term, ELISA is an ephemeral format. Even when streamlined and automated, it has too many steps. Certainly we should realize that it will be replaced by other systems, the most exciting of which will be biosensors. Also, other formats offer a proprietary edge in the market place which will be very important in the maturation of immunoassay systems in the environmental field. Finally, different formats will lend themselves to different environmental problems. We should continually emphasize that the same reagents can be used in many formats. Possibly in small letters we also should caution that certain antibody characteristics may be more important in one format than another, that some formats are more resistant to matrix effects, and that relative cross reactivities of compounds can change as one changes the subtle principles upon which an immunoassay works. For this reason a clear choice of formats should be made before initiating validation studies.

Clearly, ELISA is the principle format used for introducing immunoassay into the environmental field. We certainly hope that in the near future that all assays will be characterized in this format to avoid confusion.

Should the Development of Antibodies and Antigens be in the Open Literature? This question certainly is open to debate. On the negative side some companies trying to develop a proprietary niche in the environmental market may feel that they need protection and not divulge their coupling strategies and other techniques in assay development. It is very difficult to obtain patents in the area of hapten chemistry since regardless of how sophisticated we feel our individual work is, the coupling procedures are rather obvious. This is not to say that a great deal of skill and even art are not involved in hapten design and coupling, but that the technologies usually appear obvious in the eyes of patent authorities.

Possibly we suffer from an academic bias, but this laboratory strongly advocates that a general outline of immunoassay development (including the origin and characteristics of the antibody as well as the position and chemistry of hapten coupling to the antigen, and tracer or coating antigen) should be available to the user. The presence of such information in the open literature would not jeopardize a company's art since myopic details are not needed for an analyst to predict the characteristics of the assay. By keeping hapten design secret, companies can confuse many users, but their true competitors usually can discern the general methods used from the characteristics of the antibody. We strongly suggest that the time and public money which must be invested for validation studies only be invested for well characterized assays. To do otherwise would be like developing a chromatographic assay without telling the user what type of detector was being used on the gas chromatograph.

Who Should Develop Immunoassays? The answer to this question is simple - everyone. The more complex question is once these assays are developed how do we get them in the hands of users? Certainly the agricultural chemical industry should be involved in the development of assays for their products. Even if the assays are never used for registration, the assays will save companies money by being used in-house as research tools. In most companies there is such a backlog of residue samples to run that in-house assays to test formulation, plant distribution, process control and many other problems receive low priority. Immunoassays can have a major impact on these problems.

There also is another answer to the question if agricultural chemical companies should develop assays for their own compounds. That answer is that if they do not, someone else will. If the assay is developed in-house, one has control of the characteristics and distribution of the assay and hopefully the assay development is done correctly. If the assay is not done in-house the company will have no control over quality, sensitivity, or other aspects.

Ultimately it may be cost effective for a company to subcontract assay development to a clinical division in-house or to a third party. However, if this is done before the parent company has the in-house expertise to monitor assay development, this can be a very dangerous and expensive process. Often the expertise on the chemistry of the compound class is not transferred and inferior assays are developed at great expense. If it is necessary to develop early assays outside the company, it is important that the assay development is approached as a collaborative project with a group possessing an established record in the development of assays for environmental samples. Involvement of scientists from the clinical field can be very useful since they have decades of accumulated knowledge on assay formatting and development. However, the involvement of scientists with an appreciation of matrix effects, metabolism, and the regulatory questions posed is critical.

Certainly universities and government agencies should be involved in assay development. If an industrial collaboration can be established, one gains tremendous advantages with regard to chemical libraries and expertise. Universities and government agencies have done an excellent job of pioneering the development of the ELISA technology in the environmental field, but they have two major limitations. The first could be attributed to avarice, administrative incompetence in the institutions, or petty jealousies among the investigators or agencies. There certainly is little easy money to be made from immunoassays for environmental compounds in the near future. Universities and government agencies need to have a policy of providing the assays at no or low cost for research or regulatory use and some fair and systematic method of getting the reagents to third party vendors. As an example, it has taken over a dozen years of pressure from this and other laboratories before the University of California has begun the development of streamlined licensing procedures for immunochemical reagents. Hopefully this problem is being solved in other institutions.

The other problem with university and government laboratories is that they lack expertise in the variety of sophisticated formats which will be very useful in the environmental area and the methods for stabilizing and distributing reagents. Not only should these agencies provide the reagents in a standard format to interested scientists, but by providing them at a reasonable cost to the third party vendors the environmental field will gain their expertise in stabilization, packaging, formatting and marketing the assays. Which company offers the best system then can be determined in the market place.

Clearly biotechnology companies (third party vendors) should be developing kits and in some cases the assays themselves. Hopefully they can get access to the reagents available from government and academic laboratories in addition to the assays developed in-house. As discussed above, we strongly feel that it will be a good policy for these companies to quote the source of

antibodies used in their kits or to provide an overview of how the reagents were developed.

Should Monoclonal or Polyclonal Antibodies be Advocated? This subject will be treated elsewhere in this book (10-11) and has been discussed in numerous previous reviews. To most people with experience in the field, this is another nonissue where the answer will be based upon the problem at hand and the resources available. The answer to the question should not be based on the idea that monoclonal antibodies come from high technology and polyclonal from low technology. The sophistication and skill in antibody development can be just as great with either technology. The criteria for approval of a particular assay should be based on rigorous performance specifications of the final product (whether from a commercial or academic source), rather than the design of the test, an approach similar to that used in the manufacture of chromatographic columns. This way of addressing test performance renders most of the questions of antibody selection or standardization moot.

Another misconception is that monoclonals provide an unlimited antibody supply from immortal cells. Hybridoma lines are immortal only so long as they are maintained with constant selection by a skilled technician or frozen in a situation where they can be archived, maintained, and then thawed by a skilled individual. In practice the AOAC sees no difference between the validation of a pool of mono- or polyclonal antibodies used for immunoassay. Also reputable immunochemical companies treat mono- and polyclonal antibodies the same. A sufficient pool of monoclonal or polyclonal antibody is produced and stored such that the company will not have to thaw the hybridoma cells or reimmunize animals in the foreseeable future.

A serious error involves the attempt to use expensive hybridoma screening to overcome poor hapten design and handle recognition. If one is going to the expense of monoclonal production, certainly a similar investment in hapten design to reduce handle recognition is warranted. It is poor economy to use thousands of dollars of hybridoma technology to make up for the lack of a few hundred dollars of hapten design and synthesis.

Both mono- and polyclonal antibodies have a major role and, we will see the role of monoclonal antibodies expanding. For most problems, polyclonal sera will provide adequate sensitivity and specificity faster and at a fraction of the cost of monoclonal antibodies. The idea that any monoclonal antibody will provide greater sensitivity and specificity than a polyclonal is not correct. If one is to invest in monoclonal technology it should be used to develop a large library to the hapten of choice. This library can then be screened to obtain truly superior antibodies for defined applications. For instance one can screen the library for antibodies of high specificity or antibodies which may be class specific. One also could screen the library for antibodies which will give high sensitivity or even in some cases lower sensitivity. Once antibodies are found which give optimum specificity and

sensitivity, one can rescreen for antibodies which are resistant to solvents and/or matrix effects. With proper hapten design and a large library, one can screen for the antibodies of the desired characteristics. With the development of biosensors, the availability of defined monoclonal antibodies of varying affinity and avidity will be very important. Unfortunately many hybridoma projects end with scientists finding the antibody they screened for but not the antibody that they wanted.

Although expensive in dollars and time, the investment needed for superior monoclonal antibodies is dropping. This cost may seem high initially, but it is a small investment compared to the major investment needed to characterize and validate an assay. The cost is even small compared to a modern chromatograph and work station. For many compounds this investment will be very cost effective so long as the plan is to obtain a library of superior monoclonal antibodies rather than any monoclonal antibody. Once a monoclonal antibody exists, the cDNAs coding for the respective light and heavy chains can be cloned. These cDNAs then can be engineered to provide very inexpensive antibodies which can be further tailored for applications in immunoaffinity chromatography or biosensor development. Although this added investment seems very high at this time, the technologies involved are advancing rapidly and recombinant antibodies can be anticipated to have a future role in the immunodiagnostic area (12-14). There is even the hope that one may be able to screen for antibodies in bacteria by using recombinant DNA technology (15). Development of these technologies is in the future. However, it is obvious that the field of antibody production is in for some exciting changes. Based on this potential we are placing a major effort in the area of antibody engineering.

How Should Immunoassays for Environmental Samples be Standardized? This question can be broken down into many subtopics relative to good laboratory practice, assay criteria, specifications for immunoassay readers and many more. Obviously the need for standardization will vary depending upon the uses of the assay. Also different regulatory agencies will develop differing criteria. Initially a target could be to use the criteria set forward by the AOAC and discussed in part by Hinton et al. (16) and others (17-20) at this meeting. As discussed below, if some working papers appear on standardization or a committee could be established to provide advice on standardization it would streamline acceptance by not requiring each agency to rediscover the criteria which are useful for acceptance.

In general immunoassay is not hardware intensive. However, the poor reproducibility of binding to some ELISA plates, is a recurring nightmare to analysts. While sources of intraplate variability other than the plates themselves (washing, pipetting error, thermal gradients) may contribute significantly, major differences in variability among plates have been documented (21). One of our studies (22) has identified interwell variability to be by far the largest source of variability. This variability is analogous to chromatographic baseline noise, so it is a critical

determinant of assay performance in microplate systems. Also, dimensional standardization is at present a distant dream. In our experience, at least 6 different sets of dimensional specifications are used by the few largest manufacturers of plates. Manufacturers of readers who do not make matching plates must then compromise their specifications to be able to read all of the plates on the market. As with the equipment for chromatographic systems, not all readers are identical in performance (23). It is critical that users look carefully at the specifications of the equipment purchased and have a routine system of rechecking instrument performance. It would be useful to have a committee to make manufacturers of plates and readers fully aware of the unique demands of rigorously quantitative microplate methods. This would hopefully lead to the setting of dimensional and quality standards for plates and readers. These changes could have a dramatic effect on speeding acceptance of the technology and thus expanding the market for enzyme immunoassay (EIA) plates.

What Can Industries and Regulatory Agencies Do to Advance the Technology? A major contribution that these groups can make to the advancement of the technology is to develop the in-house expertise to evaluate the strengths and limitations of the technology. As discussed above a major threat to the technology comes when it is advocated for inappropriate applications.

In the chemical industry the best way to advance the technology is to have an in-house success. This can be accomplished by selecting a chemically reasonable target and planning ahead to obtain adequate chemical support. As mentioned above, it may not be good to select a new product where there will be a great deal of time pressure on the new assay.

Agencies especially can provide a leadership role in several ways. For example the role played by the California Department of Food and Agriculture has been very positive (17), and hopefully other agencies with responsibilities at the national and international levels will take active roles as well. An important contribution is to develop strategic plans for the development of the technology and then attempt to fund work which does not lead to duplication of effort. The private sector will be greatly encouraged if agencies can provide clear procedures for the validation of assays and clear requirements for the data needed.

The most significant role that government could play is in the area of assay standardization; certainly a very active role is possible. A procedure now used by the Food and Drug Administration (FDA) could be immediately implemented by the Environmental Protection Agency (EPA). This would simply involve testing the claims of a manufacturer with regard to the specifications of their particular assay. Any leadership that agencies can provide will benefit the field greatly, and the current effort of the Las Vegas EPA laboratory will have a major impact in this area.

As discussed above getting assays into the hands of users is a major goal. This sometimes is seen as a major hurdle that is different from classical chromatographic methods. The view is that reagents may someday vanish and the assay cannot be performed.

Actually the same situation exists in the chromatographic area. For instance very few laboratories are capable of building their own gas chromatograph or mass spectrophotometer. While there is no guarantee by industry that such equipment always will be available, the market place provides an incentive for third party companies to provide such equipment. The same situation now exists with EIA readers. If regulatory agencies would suggest a system where a pool of antibody and hapten tracer is provided to them for archiving or to a large chemical or biochemical supply company, this fear regarding the availability of reagents might vanish.

A major difference between immunoassay development and the development of a chromatographic assay is that for the former a single moderate investment is needed to develop antibodies and tracers. Adapting the resulting assays to hundreds of laboratories then is relatively cheap. However, with chromatographic assays the developer can assume a heavy investment in equipment in individual user laboratories. Although in some cases the initial cost of assay development may be a little less for a chromatographic system, the total cost to society is dramatically reduced if immunoassays are developed. If government agencies can fund the initial development of a variety of assays or make the development of such assays attractive to third party companies, the rate of acceptance of the technology will increase dramatically.

How Can We Avoid "Turf Wars" in the Immunoassay Field? A major problem that the technology has faced over the last decade has been that there were too few assays. It has been difficult to justify the amount of time needed to learn the technology to analytical laboratories when there are so few applications. We still are in a situation where far too few assays exist. Certainly over the next few years additional groups entering the field will be of great assistance, and we soon will be to the point where enough assays exist for it to be attractive for a residue laboratory to devote a component of its resources to immunochemical analysis.

In spite of the numerous projects in need of scientists working on them, the situation seems to be evolving where several laboratories are working on the same compounds. With some major problems such as the dioxins and dibenzofurans or triazines this clearly is justified. The variety of isomers and metabolites which need to be analyzed as well as the political importance of the class of compounds require the input of several laboratories. In addition a common group of compounds targeted by several laboratories will facilitate comparison of differing technologies. With other projects the resources could be better utilized without duplication, but at this stage of development in the technology, it certainly helps the technology to have procedures repeated independently in several laboratories. This situation clearly is no different from classical methodology where hundreds of "new" analytical methods have been published for DDT, but it is a situation where we need to avoid nonproductive duplication.

Some duplication can be avoided by the agencies that fund the research. Those of us who run soft money laboratories often are in

the position of providing the assays for which we receive funds. When different agencies need the same assay, unless there is a major effort at coordination, several laboratories may receive funds to develop assays for the same compound. The chemical industry also should realize that if a regulatory agency does not have access to their assays, that they will have to fund development of a duplicate assay separately. When the chemical industry realizes that it is to their benefit to make assays for their compounds available, then there will be few cases of academic and government laboratories developing assays which already exist in industry. Immunochemical companies who sell qualitative kits or assays where the methods used for assay production are not available to the public should realize again that regulatory agencies or government laboratories may have to develop competing assays.

A burden certainly will fall on academic laboratories for the next few years to ensure that a spirit of collaboration exists among the laboratories in the field. The questions is not who develops the 'first' assay for a compound or the 'best' assay but rather that the field advances and assays get into the hands of users. The technology is complex enough that the field will benefit from different methods even on the same compound. Hopefully with widely used assays such as those for the triazines the laboratories involved will exchange haptens and antibodies and jointly use a library of reagents to generate the multilaboratory data needed for validation.

All laboratories now have the obligation, not only to develop assays, but to get the assays into the hands of users. Rather than racing to develop new assays, possibly we should judge our success based on our ability to transfer the technology successfully to user laboratories. We routinely send reagents to other laboratories in the field. We try to send these reagents with a detailed protocol as well. Not that this is the best way to run the assay, but it represents a method that will give reliable results in the hands of both experts and neophytes.

There is a Need for a Committee to Coordinate the Development of Immunoassays in the Environmental Field. A committee such as the one outlined above now exists in Europe. In the following paragraphs we suggest that such a committee may be of benefit in the United States. However, there is a caution that the committee comes with a variety of problems. For the purpose of this paper the acronym for this Committee for the Evaluation of Immunoassay in Environmental Chemistry will be the palindrome CEIEC.

Possible Roles of CEIEC. The major role of CEIEC would be to act as a cautious advocate for the overall technology rather than a single assay. It also could act as a clearinghouse for information and people dealing with immunoassay and a way for United States researchers to coordinate with scientists internationally. A major goal would be to serve as a forum for discussion of problems relevant to the entire field. Such a committee could encourage

investment in the field in general from both the public and private sector. Possibly the most urgent role for such a committee would be to facilitate and coordinate validation efforts for the technology in general as well as for specific assays.

Problems with CEIEC. In advocating the establishment of such a committee one must consider that it can have both real and perceived problems. CEIEC could be seen as an unfair advocate of one technology or company over another. There is a danger that some regulatory agencies would see it as usurping their roles. This would have to be a very carefully drawn line. The committee could act faster than an agency since its actions would not be legally binding, but the committee's life could be very short if it was seen as a threat to existing agencies. Certainly the committee would have to avoid the charge that it was raising funds or advancing the reputation of one laboratory at the expense of another. Clearly the goal of CEIEC would be to expand rather than to restrict representation which could lead to an unwieldy organization. Its management would present a political tight rope where policies considered good for the field would have to be enforced by mutual acceptance rather than regulation. In this light the committee would be similar to some of the industrial groups trying to avoid pesticide resistance problems. Finally, as a scientific community we must ask if the benefits of such an organization will truly outweigh the added administrative load and even potential dangers should the committee be run in a negative way.

Recent Work in the Immunoassay Area

The 198th ACS meeting certainly is a landmark meeting in the immunoassay field. For the first time at this meeting we have seen reports from a variety of major agricultural chemical companies about the in-house efforts in immunodiagnostics (11,24-29) as well as collaborative validation studies between a biotechnology company and a university (30) and a contract laboratory (31). In addition to the development of polyclonal based systems, there is an increased interest in the development of monoclonal antibodies for environmental chemicals (10,11,32). Deschamps and Hall (32) presented a nice comparison of the relative attributes of mono- vs polyclonal based systems for the herbicide picloram.

It is reassuring in the Agrochemical Division to see presentation of results on veterinary drugs (27) and environmental compounds which are not pesticides as well as to see the entrance of synthetic chemists into the area (33). As the targets selected for immunoassay development become more difficult, chemical expertise in hapten design will become more critical. This meeting was notable for the first report of the use of computer aided design in analysis of hapten presentation (10). Different animals often have completely different antibody combining sites to the same antigen. Thus, one can anticipate an element of art and luck in hapten design. However, one can stack the odds on the side of a

favorable assay by the application of careful hapten design. A nice example of clever hapten design was presented by Mei and Yin (34) on coupling the carboxylic acid of methoprene. In general in the juvenile hormone field the simplistic approach of attaching the acid directly to a lysine has been used. This work used 4-hydroxybutanoic acid to minimize handle recognition. A similar approach was used with alachlor where a sulfur was used to mimic a chlorine (25). This assay illustrates both a strength and limitation of immunoassay. The hapten design indicates that the assay will detect some major degradation products of alachlor in addition to the parent. Since these workers have described how their assay was made, the characteristic can be used to advantage in exposure studies (29) or the interfering materials easily removed.

The Dupont work (24,28) in addition to several other studies provides an excellent correlation between immunochemical and classical methods. The work also provides a useful caution that with such sensitive assays extreme care is needed in sample handling. Excellent correlations also were obtained between classical and immunochemical methods with clomazone (26). A caution common to both assays is that neither correlates perfectly with bioassay. This is a reminder that immunoassays are physical assays with no magical biological properties. An interesting validation study using triazine antibodies indicated that high pressure liquid chromatography (HPLC) detected atrazine in a sample while immunoassay did not. When the sample was further analyzed by gas chromatography-mass spectrometry, the ELISA results were confirmed (11). This certainly does not indicate that ELISA is superior to HPLC, but that the different methods complement each other and can be used to cross check each other.

As with our work with a variety of compounds, the work from Dupont on triazines (28) illustrates that the same antibody can give assays of vastly different specificity and sensitivity if it is used with a different coating antigen. This again illustrates the importance of a laboratory developing a library of antibodies and antigens for a whole class of compounds and the respective metabolites, rather than a piece meal approach to assay development.

Several of the papers presented demonstrate that the same antibody can be used in a variety of different formats (31-32). This characteristic will become increasingly important. Certainly the same assay can be used both for analysis of environmental samples and in the analysis of human body fluids as a biomarker approach (29,35); in the latter application immunoassay offers numerous advantages.

Many of the problems now faced by immunoassay mentioned above and in other articles (3,4,36) clearly are being addressed by scientists in academic, governmental and industrial laboratories. With the level of expertise that is now evident in the field, one can have confidence that the problems will be solved and that immunochemical assays will assume their rightful role as one of the tools of the modern environmental chemist.

Representative Work from This Laboratory

Assay development can be described in a timeline such as shown in Figure 2. We will illustrate points on this timeline with examples from our laboratory.

An example of an assay developed in our laboratory which is well along the timeline is the one for the herbicide molinate. This compound is relatively volatile and has a relatively hydrolytically unstable thiocarbamate bond. Four haptens were synthesized by a thiol replacement reaction with thiocarbamate sulfones which left the hexahydroazepine ring unmodified. Two of the haptens had alkyl chain spacers terminating in a carboxylic acid. The other two had p-aminophenyl spacers. Antibodies against an alkyl chain derivative conjugated to keyhole limpet hemocyanin (KLH) were used in an indirect competitive ELISA format with a p-aminophenyl hapten conjugated to conalbumin as the coating antigen. This assay had a limit of detectability of about 3 ppb and an I_{50} of approximately 100 ppb. A laboratory dissipation study was conducted and samples analyzed by liquid scintillation counting and ELISA. Samples were either added directly to liquid scintillation cocktail and counted or diluted in buffer and mixed with antibody for the ELISA determination. This pilot study confirmed that the ELISA could quantitatively measure molinate in samples, with the advantage of not needing extraction prior to analysis. Details of the hapten synthesis, assay development and optimization were reported by Gee et al. (37).

To further validate the assay for use with environmental samples, water samples spiked with molinate were extracted and analyzed by ELISA and GC. Recovery comparisons were made between ELISA and GC for both liquid-liquid and solid phase extraction methods. Recoveries were greater than 90% for levels as low as 1ppb for all analysis and extraction method comparisons (38). This study also described the utility and compatibility between solid phase extraction and ELISA for measuring low concentrations of molinate. As much as 10% acetonitrile/propylene glycol (1:1) or 5% methanol had no effect on the molinate assay. Details of this study were reported by Li et al. (38).

Subsequently we have completed an extensive validation study using field samples which contained high concentrations of molinate following an aerial application. These samples were analyzed by ELISA directly after buffering and confirmed by GC analysis of split samples. One of the most valuable lessons from this validation study was the importance of the various quality control considerations (22). From sigmoidal standard curves, 20-60 percent of the control absorbance was determined experimentally to be the region of greatest precision. Thus sample concentrations arising from data generated outside this area would be less reliable. Control charts were constructed for both positive and negative control samples as a means of evaluating assay performance over the study period. Such charts can be useful indicators of changes in the assay that may affect reported results and are a commonly used

tool in clinical chemistry. These control data were also run through a nested analysis of variance. The largest relative error contributions arose from well to well (or well replicate) variability. Other details of this study such as data handling and other sources of procedural error can be found in Harrison et al. (22).

We view this series of studies as an essential prototype for the entire development and validation process. For example, we have also completed a similar study for molinate using an improved format to analyze low concentration samples obtained from the Sacramento River and associated drainage canals. We are also using this prototype in the development and validation of our assays for triazines (39). Our experience in the validation of the molinate assay, especially our understanding of the quality control problems, has been crucial to our successful transfer of the triazine assays to the California Department of Food and Agriculture (CDFA) and other laboratories for routine application to environmental samples.

The same assay which was transferred to CDFA has been used by our laboratory to demonstrate the usefulness of immunoassay for screening water samples. In this study, 75 well water samples were analyzed by GC and immunoassay for triazines. The background level of the immunoassay was 15 ppt, determined by repeated solid phase extraction and analysis of reagent water blanks; the highest level detected was approximately 0.3 ppb. The coefficient of variation for a single sample run 6 times was 10%. The variability of the two methods was comparable based on analysis of 18 paired samples; the mean coefficient of variation was 11% for the ELISA and 13% for the GC. The most valuable attribute of this application is the low false negative rate. None of the 40 samples having the lowest concentrations by ELISA were positive by GC. This assay is now being used in a large scale field test by CDFA as well as in a worker exposure study.

An important extension of our large validation studies involves the use of data bases from field studies in the development of improved statistical methods for a variety of problems in quantitative applications of immunoassays. These problems include the preparation and analysis of calibration curves, treatment of "outliers" and values below detection limits, and the optimization of resource allocation in the analytical procedure. This last area is a difficult one because of the multiple level nested designs frequently used in large studies such as ours (22). We have developed collaborations with David Rocke and Davis Bunch (statisticians and numerical analysts at Davis) in order to address these problems within the context of working assays. Hopefully we also can address the mathematical basis of using multiple immunoassays as biochemical "tasters" to approach multianalyte situations.

As mentioned above and in various reviews (1,4,6-8), hapten synthesis is the first and probably one of the most important steps in assay development. The most general "rules" in hapten design are to locate the spacer attachement distal to important haptenic

determinants to maximize their exposure for antibody binding; spacers containing strong determinant groups should be avoided to minimize the production of spacer specific antibodies; functional groups used for coupling must be compatible with target molecule functional groups to avoid cross-linking or modifying the target during conjugation; consideration of hapten and target stability under conjugation, immunization and assay conditions; selection of hapten to improve solubility or at least avoid solubility problems and to minimize the number of synthetic steps by using commerically available materials or, in some cases, direct coupling to the target. We have described and examined these basic criteria for hapten synthesis in some detail using examples from our laboratory and the literature (40).

The significance of these criteria is demonstrated routinely in our laboratory. In some cases, however, where the development of an immunoassay may be difficult, the choice of the hapten to be synthesized may depend greatly on the ultimate use of the assay with samples. For example, bentazon, a rice herbicide, is a small molecular weight (MW 240) molecule with an unique acidic secondary sulfonamide (pKa 3.4). An N-derivatized bentazon compound was synthesized. This changed the molecule from an acidic secondary sulfonamide to a neutral tertiary sulfonamide. Antisera raised against this N-derivative conjugated to KLH showed 2-orders of magnitude greater binding to N-ethyl bentazon than to bentazon (Figure 3). An assay such as this could be useful in assessing bentazon concentrations after the sample has been ethylated. Sample derivatization prior to GC analysis is a commonly used technique. With compounds for which immunoassay development is difficult, due to the presence of multiple reactive groups, antibodies against derivatized compounds is an alternative.

Not all of our assay development work is successful and we have found it instructive to analyze our negative results in some detail. This is especially important for failures of hapten design, where no useable antibodies to the target compound are obtained. There are a number of strategies for attaching the hapten to a carrier molecule. One is to attach the spacer arm to the protein, then attach the hapten to the free functional group of the spacer arm (41). We have found that this conjugation strategy failed to produce high affinity antibodies for both amitrole (Figure 4) and bentazon, yielding instead antibodies which primarily recognize the spacer. Similar data have been obtained by others (42-43). These examples emphasize the value of the approach to antibody screening described by Harrison et al. (40) in understanding negative data.

In optimizing an assay during development the nature of the interaction of the analyte with the antibody is particularly important. Assays usually are carried out under physiological conditions and frequently no effort is made to optimize for pH, ionic strength, or other factors. These factors can directly affect the assay by modifying the presentation of the soluble analyte to the antibody or changing the interaction of the antibody and the conjugated hapten used in the assay. For example, assays for some compounds show a distinct pH dependence. In an indirect competitive

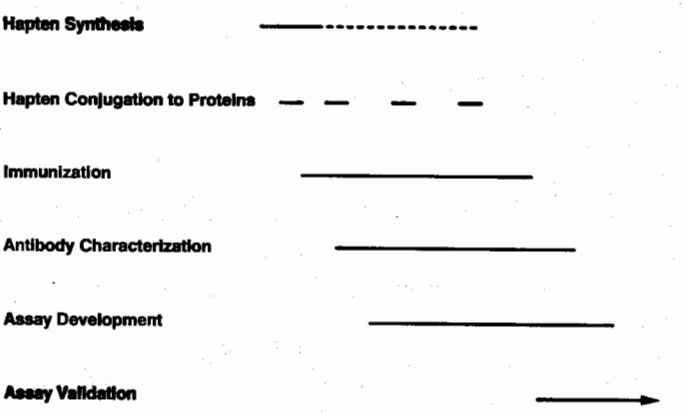

Figure 2. Timeline illustrating the relationship among the various assay development and implementation steps. It is critical that hapten preparation occur first. However existing assays can be improved by rational improvements in reagents or format. Once a validation study is undertaken, it is important to use a constant format and reagent set.

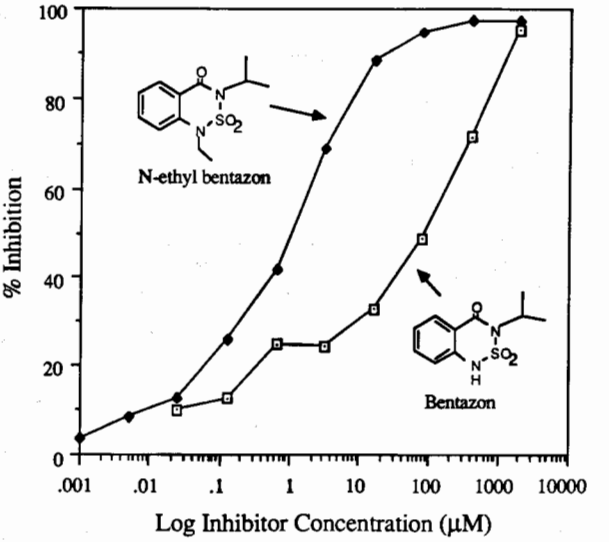

Figure 3 . Relative sensitivity of a rabbit antibody to bentazon and methylated bentazon. A rabbit antisera against an N-derivatized bentazon had better recognition of methylated bentazon than bentazon. Coating antigens were Bz(6)-O-MPAA-BSA and Bz-succ-BSA for methyl bentazon and bentazon respectively. These curves indicate that one may find a much more sensitive assay for a derivative than for the parent compound. As in chromatographic analysis, it may be advantageous to run immunoassays on derivatives and an immunoassay such as this could easily be used to quantitate derivatized bentazon samples.

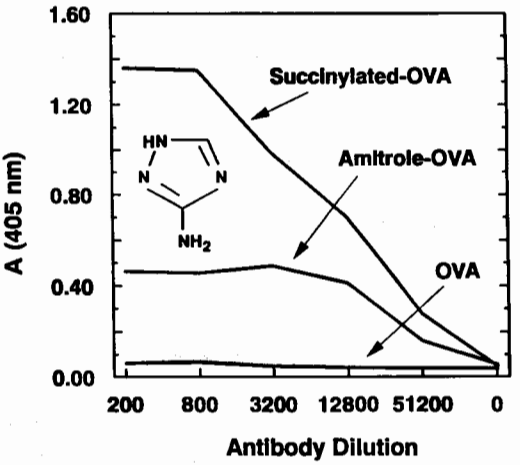

Figure 4. Rabbit antibody specificity for different coating antigens after the second bleeding. Binding of the anti-aminotriazole antibody to the homologous coating antigen, amitrole-succinylated ovalbumin (OVA), compared to the native protein, OVA, and succinylated OVA as coating antigens. The antibodies show low binding to the homologous antigen but high recognition of the succinylated protein. These data show a common problem when raising antibodies to very small molecules. The antibodies from this bleed have a low affinity for the aminotriazole hapten while the hemisuccinate used as a spacer on the succinylated protein is antigenic.

ELISA, the sensitivity of the assay for the herbicide glyphosate was improved by at least one order of magnitude when the assay was conducted at pH 5.8, rather than 7.3 (Figure 5). Glyphosate (phosphonomethylglycine) has several Zwitterionic forms, so it is not surprising that careful optimization of pH led to a dramatic assay improvment as did a shift away from phosphate buffers. This work by Dr. Jung is a dramatic demonstration of how a series of optimizations can improve the sensitivity of assays several orders of magnatude. Along the same line, Sharp et al. (24) have reported that 0.01M $CaCl_2$ greatly improves the sensitivity of some, but not all assays for chlorsulfuron. Although optimization of sensitivity is important, it is also necessary to recognize that there may be a tradeoff between the increased sensitivity and assay ruggedness.

Figure 6 shows the class recognition of one of the triazine antibodies produced in our laboratory. Immunoassays for the triazines will be very interesting due to the existance of numerous structural analogs in this important class of herbicides. Although most degradation products lack herbicidal activity, they can be important analytical targets as indicators of human or environmental exposure. The antibody in Figure 6 recognizes triazines having a -Cl or $-SCH_3$ in the 2 position of the ring, such as atrazine (I_{50} = 6.5 ppb), simazine (IC_{50} = 54 ppb) and ametryne (I_{50} = 130 ppb), regardless of minor changes in the N-alkyl substitution pattern. The monodealkylated or 2-hydroxy metabolites are also recognized, though to a lesser degree (I_{50} > 3500 ppb). We have obtained similar results for several other rabbit antibodies and five mouse monoclonal antibodies. Such antibodies can be used for direct analysis of triazines by ELISA, separation of related triazine species by immunoaffinity chromatography, or removal of triazines from contaminated samples. The relative recognition of the various triazines and their metabolites depends on the hapten used to produce the antibody. Variables we have explored thus far in our work on the triazines include position of conjugation, spacer length, and alkyl group substitution pattern (39-40). Use of a library of antibodies and coating antigens can result in either class or compound specific assays. A series of related assays can be used to screen samples for certain substitutions, aiding identification of the immunoreacting compounds.

We have also applied ELISA to several biological pesticides including the endotoxin of Bacillus thuringiensis kurstaki (Btk). In this application to a macromolecular analyte, we have used a double antibody sandwich ELISA for Btk to measure the amount of ELISA reactive material in formulations of the pesticide. Figure 7 shows the use of an ELISA standard curve of gel purified Btk endotoxin to measure the immunoreactive material in dilutions of two Btk formulations. It has been demonstrated that ELISA can serve as a quick quality control check for formulations of Bacillus thuringiensis israelensis (44). Such examples indicate that immunoassays will be increasingly important as biologicals and products of recombinant DNA research impact our field (44).

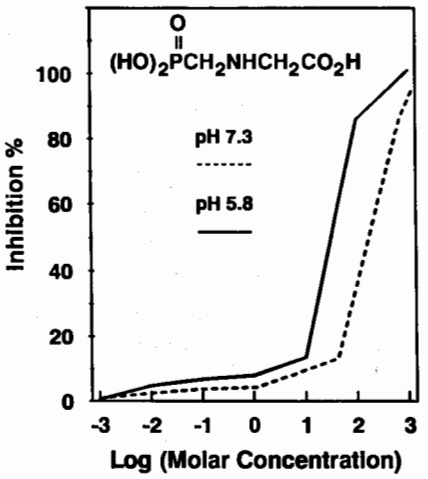

Figure 5. Inhibition of anti-glyphosate antibody by glyphosate. Inhibition curves for polyclonal anti-glyphosate antibodies raised in rabbits were conducted in 50 mM TRIS buffer at pH 5.8 and pH 7.3. The curves show an increased affinity between antibody and glyphosate at the lower pH buffer, illustrating that for some compounds, optimization for pH is critical. Careful optimization of assay conditions is especially important as the molecule becomes smaller, for zwitterionic materials, and for analytes where hydrogen bonding may play a major role in antibody binding.

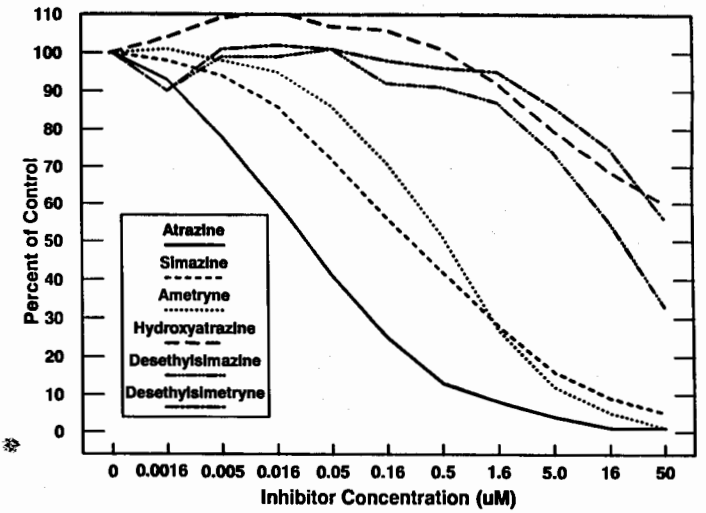

Figure 6. Competitive inhibition ELISA results. Recognition of atrazine and several related compounds, including metabolites, by a rabbit antiserum raised against a conjugate of an atrazine hapten and a carrier protein. This antibody recognizes triazines having either a -Cl or -SCH_3 in the 2 position of the ring, such as atrazine and ametryne, regardless of minor changes in the N-alkyl substitution pattern. By careful design of the immunizing and coating antigen, one can vary the properties of the resulting assay to detect a single compound or a predictable set of related compounds.

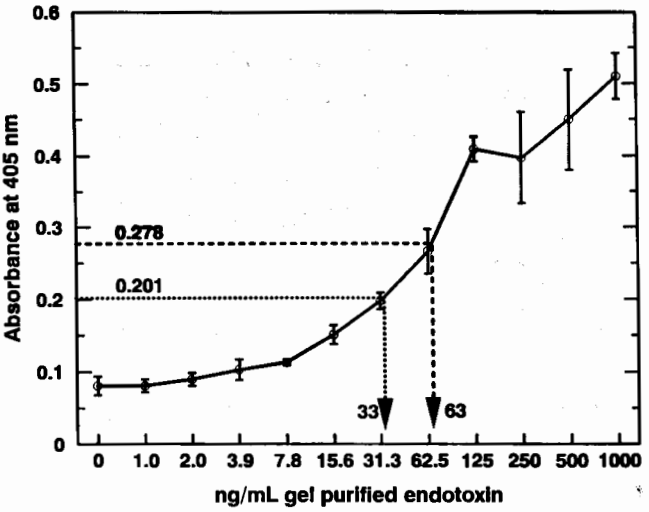

Figure 7. Standard curve of gel purified 60 kd protein endotoxin of Btk was generated using a double antibody sandwich ELISA. The arrows indicate dilutions of two Btk formulations; absorbance values were used to determine the endotoxin concentrations of the formulations, based on the standard curve. The formulation dilutions gave curves that were virtually superimposable on the standard curve. Such similarity in shape and slope indicate that the antibody is likely binding to a specific determinant common to the purified Btk and the Btk in the formulation. In general immunoassays for biopolymers are much easier to develop than assays for small molecules. However, only recently has an interest in trace analysis of such materials begun to develop in the environmental field. Thus, sample cleanup and handling is not as sophisticated as with small molecules.

Summary

Immunoassays are now being seen as useful supplements to classical chromatographic analytical systems. In the near future we will see also an integration between immunochemical and classical procedures as immunochemical methods are used to prioritize or clean up samples before chromatography or as a post-column detection system. If "Green" initiatives in several countries pass we will see two striking trends. The first will be a shift of agricultural production to other areas and the second will be an acceleration in the development of biological methods of pest control. Immunochemistry offers tremendous advantages for inspection of the large increase in imported food and may be the only viable analytical method for many biological pesticides.

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