Chapter 1

Analytic Immunologic Techniques

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I. Introduction

Analytic immunologic procedures have become important parts of the arsenal of techniques for describing and elucidating physiologic and developmental changes in naturally occurring antigens (Ags) from insects. Antisera, which are solutions of the natural defense secretions produced by vertebrate immune systems in response to foreign antigens, can be used as analytic reagents to make quantitative as well as qualitative measurements of these insect Ags. An antiserum contains antibodies (ABs) which are used to identify the components that are present in a complex mixture and measure their relative or absolute titers at the same time. Immunology also provides objective methods for deciding on the homology or degree of homology between similar macromolecules from different species or different stages or tissues from a single species.

This chapter will address the past and continuing use ofpolyclonal antisera of interest to insect biologists. No evaluation of the emerging monoclonal antibody (mAB) technology will be attempted, since its full implications for insect investigations are not yet known. Cautionary notes will be made, however, where a current use of polyclonal AB may or may not be replaced by mABs.

Before investigators embark on using a particular immunologic technique, they should carefully define their needs and resources. A lucid general description of antigenicity and immunity (Berzofsky, 1985; Darnell et al., 1986) is essential reading prior to attempting to understand the details of technique. The investigator should then become aware of the range of techniques available and the powers and limitations of each

technique. Reading a handbook of immunology can be confusing in the variety of available methods (Ouchterlony, 1968; Axelsen, 1983); however, there are several techniques that have been particularly useful (or popular) among insect physiologists and biochemists.

Reading a selection of recent applications of immunologic techniques to insect problems, such as the ones included in this book and the accompanying bibliographies, is a good way of delineating the range of approaches that have been useful in the past. Some familiarity with past successful immunologic approaches is recommended before attempting a novel method. Diagrams are provided here describing idealized results, but interested investigators are referred to original published results in the accompanying references. Reading a recent handbook of immunology and taking a training course in practical aspects of the subject are highly recommended for those who want to maximize the information that can be obtained with the available techniques.

Immunologic techniques can be enlightening but can be expensive in time, animals, equipment, and materials. A great deal of time and energy can be saved by seeking expert advice and training in the early stages of a study. The levels of analysis are described beginning with the least complicated and least expensive. In initial exploratory investigations, where not a great deal is known about the complexity of the system, the simplest applicable approach should obviously be tried first. The same careful and graded approach is useful as a didactic tool in a class or laboratory setting in which one wants to introduce students to the concepts of immunology in the simplest and most economical manner.

II. General Comments on the Scope of an Immunologic Approach

Several properties of polyclonal antisera do not apply to monoclonal antisera: First, the mixture of ABs found in a polyclonal antiserum recognize a variety of structural entities (determinants) on the macromolecule that is acting as an immunizing Ag (Fig. 1.1). These entities include native determinants on the Ag surface and buried "cryptic" determinants in the Ag's interior. A mAB is by definition reactive with primarily one determinant of an immunizing Ag, although its affinity to specific determinants on other Ags may be higher or lower and must be assessed by experience.

Second, the avidity (apparent affinity) of the AB population for an Ag can vary over a wide range (Fig. 1.1). Avidity is an average affinity exhibited by a complex antiserum that contains hundreds of individual

antibodies of different concentrations, each with its own affinity to the Ag. A mAB has a specific affinity for an Ag which can be described precisely by an equilibrium constant of the binding reaction.

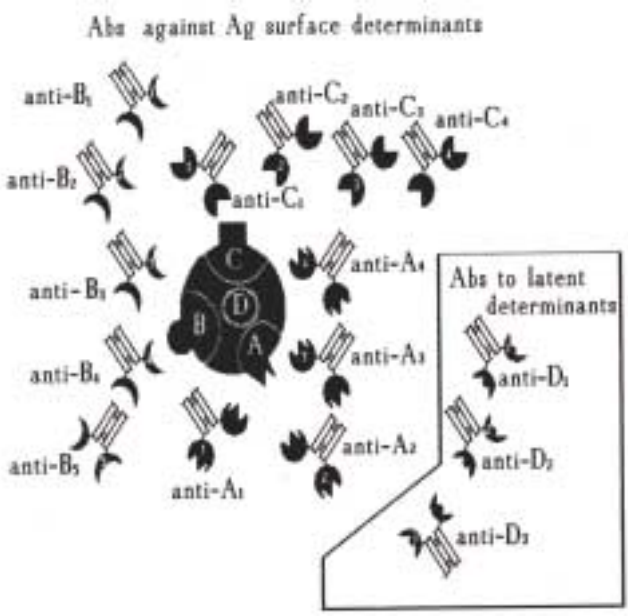

Figure 1.1. Antigenic determinants and polyclonal antisera. A polyclonal antiserum is derived from multiple clones of AB-producing cells and may have multiple distinct bivalent ABs (A_1, A_2, A_3, h_4, B1, ..., D3) reacting to each determinant (A, B, C, D) on a single native Ag, depicted in the center. Determinants A, B, and C are depicted on the surface of a polyvalent globular native protein. Cryptic internal determinant, D, may be buried within an internal core of the native protein, and antibodies reactive with this latent determinant may react with it only when the Ag is in a denatured state. Each animal immunized with the Ag may make antibodies to some or all of these determinants. Avidity of a particular polyclonal antiserum is determined by a frequency-weighted average of individual affinities of the population of secreted ABs.

Third, in the one-to-one stoichiometry of the precipitation reaction (Fig. 1.2), polyclonal antisera have no problem linking large Ags into extended networks that tend to precipitate. There is, however, a problem with monoclonal AB's becoming involved in the precipitate unless the Ag is immobilized in some other way, either to another biological structure such as another subunit, a membrane, or to an inert support such as a plastic surface or nitrocellulose membrane. Specificity allows an AB to recognize a determinant on an Ag even in the presence of excess unrelated Ag. A highly avid (high average affinity) reaction allows many semipermanent (noncovalent) bonds between Ag and AB to be formed which are not reversed frequently enough to allow the Ag to

dissolve.

One of the dangers of using monoclonal antibodies is that the AB affinity and specificity may not be high enough to form semipermanent bonds that survive washing procedures, and since there is not a substantial network of other ABs involved in an extended precipitate, the monoclonal staining of structures may be temporary. For polyclonal antisera, the stoichiometry of the precipitation reaction is usually equimolar, and thus one is able to measure specific Ag concentrations relative to the AB solution titer by identifying the equivalence concentration at which maximum precipitate is achieved, even in the presence of vast excesses of other Ags. Most polyclonal immunologic techniques take these preceding properties into account.

1. Measuring the Immunologic Complexity of a Problem

Antibodies can be used to measure and delineate the antigenic complexity of a situation. In a sense, one asks a vertebrate, such as a rabbit, how its immune system perceives a mixture of insect Ags. The rabbit's ability to recognize foreign macromolecules is another way of partitioning the complexity of an unknown system. Since insect macromolecules are almost all totally foreign to a vertebrate, the rabbit immune system usually provides a good source of antisera against individual or mixtures of insect proteins. This approach can be compared with other analytic techniques such as electrophoresis, chromatography, or enzymology. In each of these latter techniques, the concentrations of the molecules or activities to be separated are important. None of these techniques on its own is capable of a definitive identification of a particular mixture of proteins. In some cases a judicious combination of the techniques allows a strong inference concerning the identities of a mixture of molecules.

Immunologic techniques, on the other hand, are potentially capable of identifying and measuring individual Ags or mixtures of Ags all in one procedure. However, some immunologic techniques are aimed at, or limited to, measuring a single Ag; some are designed for, or adaptable to, looking at mixtures. A thorough understanding of the chemistry of the Ags under investigation and ABs in general is advisable. Be sure to read extensively about the general properties and mechanism of the particular immunologic technique one is using, its objectives, and limitations.

I prefer to start off a study with a polyclonal antiserum to the complex mixture in which the Ag of interest is normally found. For

example, if one is interested in the major Ags in a system, such as insect hemolymph or insect yolk, an exploratory study of the major Ags is often useful in indicating the most practical stage for purification of the focal Ag. Such a study will also introduce one to the major contaminants to be contended with at later stages of study.

The literature is full of examples of overstatements based on too narrow an initial focus. It is easy once one has an antiserum specific to a single protein to concentrate on that one protein and forget the remainder. For instance, a focus on the variation in titer of a particular larval-specific serum protein during the life cycle of a cockroach (Kunkel and Lawler, 1973; Kunkel, 1975) revealed a major fluctuation associated with the molting cycle; a few years later, a more comprehensive study found that all the other major serum proteins of the cockroach were fluctuating in concert (Duhamel and Kunkel, 1978). If a general antiserum to all the serum proteins had been used initially, this emphasis on the cyclical fluctuation of the single serum protein would not have occurred.

Another caution related to immunologic complexity is worth emphasizing. Certain techniques such as microcomplement fixation, dot blots, and ELISA inherently require monospecific antisera in order for their high sensitivity in measurement to be reliable. Experience with the antigenic and AB complexity of the system under study is necessary to avoid artifacts. The specificity of monospecific antisera should be certified by one or several techniques such as immunoelectrophoresis (IEP), quantitative IEP (QIEP) Ouchterlony (spotting ABs and Ags separately on agar, migration forms a precipitation line in between), or Western blot (proteins identified by placing them on nitro-cellulose and probed with antibodies).

2. Quantitative Measurement of Individual Ag Titers

A. ABs as Analytic Reagents: Physical Properties, Stability, Storage

a. Relative concentration of Ag and AB. A ratio of one AB molecule to one Ag molecule optimizes most immune precipitation assays. This is the basis of using antisera to measure the concentration of an Ag. However, this is also the basis for possible artifacts. Since an AB molecule has two combining sites, a precipitate is usually formed only if the antiserum is polyclonal and the Ag has multiple determinants on its surface or if the Ag has multiple subunits with the same determinant (Fig. 1.2). In general, monoclonal AB is not used for precipitate forming

reactions, because an Ag cannot be counted on to have multiple combining sites.

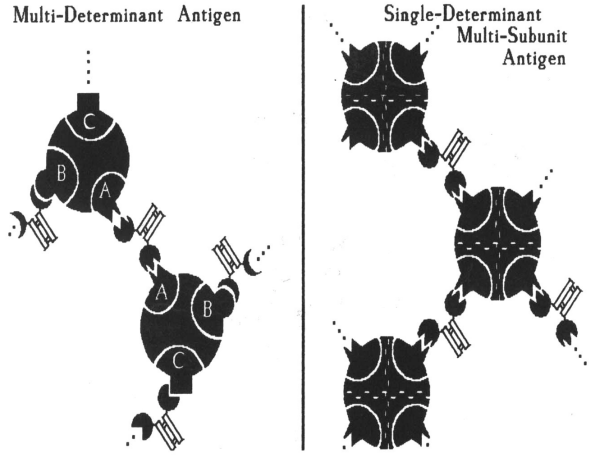

Figure 1.2. The basis of Ag precipitation by ABs is cross-linking and multivalency. An extended network of cross-linking can result in a precipitate. Every Ag is linked to another Ag through an AB, and every AB is linked to another AB through an Ag. The stoichiometry of Ag: AB to achieve complete Ag precipitation is approximately 1: 1. Minimal requirements for precipitation include either particles with multiple subunits bearing a single determinant (right) or multiple determinants on a single particle (left).

Another necessary requisite for precipitation is that sufficient Ag be cross-linked by AB into an insoluble lattice. Outside the range of approximate equivalence, on the side of Ag excess, the probability increases of finding an AB molecule bound to two Ag molecules with no additional AB molecules available to extend a lattice. This Ag-AB Ag aggregate may be soluble (Fig. 1.3). On the side of AB excess, there tends to be less of a problem, particularly with polyclonal antisera against large Ags. The multiple determinants of a large Ag can adsorb a large number of AB molecules and still have determinants left to cross-link with another Ag molecule. After formation of a precipitate and when all the Ag is involved in a precipitate, additional AB can continue to add to the precipitate at other determinants without endangering the possibility of dissolving the precipitate by completely coating the Ag with AB on all its determinants. On a small Ag however, AB excess can lead to a soluble AB-Ag-AB complex, since if an Ag has only a few determinants, it may be completely coated with AB, be still soluble, and be blocked from cross-linking with other AB-Ag-AB complexes, since all their determinants are also covered with AB.

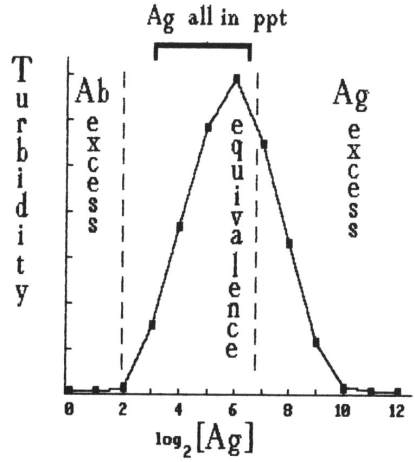

Figure 1.3. Classical Ag/AB equivalence in liquid. A fixed amount of AB is placed in a series of test tubes. A serial increasing concentration of Ag is added to successive tubes. At the low Ag concentration end of the series, no precipitate forms, because the excess of AB completely coats the determinants of the Ag, and there are no free determinants for an AB free end to cross-link with. In a region from 3 to 6 log units of Ag, all the Ag in the tubes is precipitated with available AB, generally at a higher than 1: 1 ratio. This situation is possible because many Ags have far more than two AB combining sites. As the Ag concentration is increased beyond equivalence, the frequency of soluble Ag aggregates linked by one or a few AB molecules increases. Finally, every AB molecule is linked in a soluble complex with two Ag molecules (Ag-AB-Ag), but there are no free AB molecules to cause further cross-linking.

b. Monitoring Ag synthesis and processing. Antisera can be used to follow Ag synthesis or postribosomal modification by combining selective precipitation of an Ag and pulse or continuos labeling with radioactive precursors. Several ways of using this approach can lead to substantial insight into the synthesis as well as postribosomal processing events in a protein's function (Hagedorn et al., 1978; Levenbook and Bauer, 1984; Wojchowski et al., 1986).

B. Lab and Field Ag Samples

A polyclonal immunological test for an Ag is robust in many ways, because it does not depend entirely on the Ag of interest retaining 100% of its native configuration. Some immunological tests are forgiving of many degradative phenomena such as the activity of an enzyme. In fact, immunological tests can be run on denatured material adsorbed to inert supports such as nitro-cellulose. This property considerably extends the variety of situations from which samples can be obtained, including field studies and refrigerator- or freezer-stored material. Indeed, useful

antisera have been made against fossil Siberian mammoth material which were used to show a closer affinity of mammoth to Indian elephant than to African elephant.

Ecological and taxonomic studies would be aided by the ability to take samples in the field and be assured that they will not degrade before they are analyzed in the laboratory. In this regard an organic compound, phenoxyethanol, is available that can be added to samples in the field and will sufficiently preserve protein samples at ambient temperature without deterioration of immune responses (Nakanishi et al., 1969).

C. Range of Sensitivity and Capacity of lmmunological Procedures

Immunological techniques range in sensitivity over several orders of magnitude from milligrams to nanograms of an Ag. In the least sensitive method, precipitation in liquid, the upper limit of Ag to be precipitated depends on how much AB one can afford to use per precipitation. This technique is often used where large amounts of precipitated Ag are available, such as when synthesis of a protein is being monitored. In such situations there may already be substantial previously synthesized, unlabeled protein which must also be precipitated in order to ensure that all the newly synthesized, labeled protein is found in the precipitate.

Thus, maximum sensitivity is not always the objective of an immunological technique. When maximum capacity is the objective, it is sometimes possible to extend an antiserum's capacity by using it numerous times. Binding of AB to matrices such as agarose or dextran beads is one approach to being able to reuse an AB conveniently. When maximum sensitivity of measurement is needed, several techniques are available. Very small amounts of Ag can be detected using equivalently small amounts of AB whose location and amount are then amplified and quantified by layers of secondary AB which are then visualized by enzymatic or fluorographic means. In many of the more sensitive techniques, the amount of Ag precipitated may be so small that the maximum radioactivity attainable by incorporation studies is not able to be detected above background. An investigator must have a reasonable idea about the sensitivity or capacity required to answer the questions of interest.

3. Quantification of Degree of Ag Difference

A. Intraspecific Variation

a. Processing during stages of synthesis and function. During the ribosomal synthesis and postribosomal processing of proteins, antibodies can interact with the nascent and developing structure in a preparative or analytic fashion. Antibodies to minor cellular proteins can be used to precipitate the polysomes on which the nascent chains of the protein are being synthesized, thus providing a way of purifying mRNAs of minor proteins (Shapiro et al., 1974). The processing of proteins sometimes involves destruction of determinants whose loss can be detected and followed immunologically (Storella et al., 1985; Wojchowski et al., 1986; Kunkel et al., 1987). Immunological techniques are sometimes useful in analyzing developmental steps by providing a way of purifying the protein before and after a change so that they can be analyzed by some other technique such as SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and autoradiography (Imboden and Law, 1983; Engelmann, 1986; Wojchowski et al., 1986).

b. Identification of homologous electromorphs. Seemingly related proteins or enzymes within a species can differ in electrophoretic mobility for several reasons. They may be totally unrelated, be alleles of the same gene, be developmental stages, be physiological forms, or be differential processing artifacts. Unrelated proteins would not cross-react immunologically. Mono-specific antisera are able to identify the immunologically related or distinct electromorphs of an isozyme on Western blots of the electrophoretically separated proteins onto a blotting medium such as nitrocellulose or diazotized paper (Towbin et al., 1979; Hawkes et al., 1982; Smith and Fisher, 1984). In addition to using monospecific antisera, complex antisera that have antibodies to several peptides or proteins can be affinity-purified by reacting them to electrophoretically separated peptides blotted onto nitrocellulose filters. The peptide-specific antibodies can be eluted from the filters and reused to identify or test for cross-reaction with other peptides or proteins (Olmsted, 1981).

B. Interspecific Variation

a. Phylogenetic relationships. The use of immunology to measure phenotypic differences between species is an established method of carrying out taxonomic or phylogenetic research (Champion et al., 1978; O'Brien

et al., 1985). Antibodies to serum proteins of insects cross-react most highly to closely related species, and that cross-reaction falls off with phylogenetic distance (Kunkel and Lawler, 1974). A quantitative approach to measuring the degree of cross-reaction of a drosophilid serum protein has been used te estimate evolutionary times of divergence among insects (Beverley and Wilson, 1984, 1985).

b. Homology of structure and function. Many insect Ags that have been studied have no known enzymatic activity, and therefore their homology with a protein from another species must be based on physical and chemical characteristics including their immunological cross-reactivity. When two species are sufficiently distant, a lack of cross-reacting Ag does not mean that they share no homologous Ag. The homology between Ags from different families or orders may be more sensitively measured when more conservative internal protein structures are probed with AB. Denaturation of the protein of interest on a nitrocellulose filter, for instance, has allowed the homology between arylphorins of a lepidopteran and a hymenopteran to be studied (Ryan et al., 1984.)

III. Specific Techniques

1. Obtaining Immunological Reagents

A. Choice of Animals for AB Production

Animals used for immunization with Ags include rabbit, goat, mouse, and chicken. Each has its advantages and disadvantages. The mouse is the easiest and cheapest to house but perhaps the most difficult from which to obtain large amounts of antiserum reliably. Some analytical techniques, however, need only small amounts of antiserum. With practice, large volumes of antiserum can be obtained from mice by induction of ascites fluid during the immunization procedure (Tung et al., 1976). One should be able to obtain 10-30 ml of ascites fluid per mouse and repeat that several times for some mice. In addition, mice are the basic animal for production of mABs. It is possible that mABs will replace many of the former functions of polyclonal ABs in the near future, particularly the roles involving measuring trace amounts of Ag.

The rabbit has been a mainstay of AB production for insect physiologists and biochemists; they will reliably produce 50 ml of blood weekly, indefinitely. It is yet to be determined whether considerations of

animal care costs and changing technology will displace the rabbit with the mouse or another AB source. Goats are also useful when very large amounts of antiserum are needed; one can obtain 250 ml weekly from a goat without overly stressing it (Brookes, 1986). Chickens are only rarely used in insect biochemical contexts, but they provide certain conveniences; the antibodies are harvested from the eggs they lay. The chicken continues to provide neatly packaged aliquots of its immunoglobulins for titer testing, and when the AB titer is high, one pools a series of eggs for one's final harvest. Overall, the rabbit still stands out as the most often used AB source when judged by numbers of publications.

B. Choice of Immunization Schedule

a. A standard immunization protocol. Immunization of a rabbit will be described as an example. The most frequently used protocol used in my laboratory lasts for 3 months. An initial bleeding is useful to provide control serum as needed in some techniques and for cross-matching of serotypes when pooling of sera between rabbits is anticipated. The initial injection is made with 250 µg of Ag in I ml of PBS (phosphate-buffered saline) emulsified with an equal volume of Freund's complete adjuvant. The emulsion is made by forcing the mixture through a fine tubing connecting two syringes. If the emulsion does not disperse when dropped into a dish of water, it is ready to be injected. Allowing the mixture to freeze and thaw a few times during the emulsification will help along the procedure.

The mixture is injected subcutaneously along one side of the back of the rabbit in four or five locations. The second injection is done 4 weeks later in a similar manner along the other side of the back, however, using Freund's incomplete adjuvant. Four weeks later a series of five daily injections of 50 #g Ag in 1 ml PBS are given intravenously via the marginal ear vein.

Seven days later the rabbit is ready to be harvested for the first time. The animal is anesthetized and vasodilated with a mixture of droperidal/fentanyl injected subcutaneously (Tillman and Norman, 1983). In 20 min 50 ml of blood can be drained effortlessly by puncturing the central artery of the ear with a 20-gauge needle and collecting the stream in a 50 ml siliclad-coated centrifuge tube. The blood is incubated at 37°C for 2 h to clot and then placed in the refrigerator overnight to allow the clot to contract maximally. The clot can be removed and the sera centrifuged to remove blood cells. The sera

should be precipitated with ammonium sulfate ½ saturated and redissolved in PBS twice. This step is essential in preventing proteases, released by minor hemolysis, from progressively digesting the immunoglobulins. Further purification of the IgG fraction on DEAE cellulose is routine in some procedures but not essential for the majority of methods described in this chapter.

b. Antisera for discriminating qualitative differences. For taxonomic or homology studies, different strategies must be taken in the production of antisera. Immunization of a rabbit should be carried out for at least 10 weeks if antisera cross-reacting with the most distant of relatives are desired (Champion et al., 1978). On the other hand, if one is interested in differentiating between very closely related Ags, the earliest ABs produced after the first weeks of immunization tend to be the most discriminating.

C. Monospecific Antisera

Some immunological assays require monospecific antisera or are simpler to analyze if' monospecific antisera are used. Monospecific antisera are polyclonal antisera that are reactive with one macromolecular entity, be it a multisubunit native protein or a single polypeptide derived from a larger entity. Monospecific antisera can often be obtained by taking care to purify the Ag used for immunization and using a minimum of that Ag in the immunization procedure. Often rabbits are perverse in that they make antibodies to minor impurities in a hard-won "purified" Ag. The resultant poly-clonal, multispecific antiserum may be useless as is, but such antisera may be improved or be used to improve the situation.' For example, the unwanted antibodies may be absorbed by a solution of the Ag impurities if such a solution is available (Tanaka, 1973; Kunkel and Lawler, 1974). In this procedure, one uses a soluble or insoluble form of the offending Ags to precipitate the unwanted ABs.

In a different approach, one can use an initial multispecific antiserum and the techniques of crossed QIEP (Section III.D.2) to provide a pure Ag/AB precipitin localized in a gel which can be discretely cut out of a gel and used for immunization of another rabbit (Harboe and Closs, 1983). Still another approach is to use nitrocellulose-immobilized Ag of the desired type to prepare the corresponding AB by affinity binding to the immobilized Ag followed by elution of the specifically bound AB and use in other reactions (Olmstead, 1981; Ryan et al., 1985a).

2. Analysis of Immunological Titer

A. Ag Titer vs. AB Titer

a. Ag/AB equivalence: importance for avoidance of false negatives. A false impression of the specificity and complexity of an antiserum may be obtained if attention is not paid to the multiple Ag/AB equivalence concentrations operating in a potentially complex antiserum. Every polyclonal antiserum must be first treated as if it were a complex antiserum before it is proven to be monospecific to the Ag of interest. Each Ag will have its own concentration in a particular Ag mixture, and each corresponding AB will have its own titer in the complex antiserum. A titration curve may demonstrate that there is no one Ag/antiserum ratio in which all Ag/AB reactions are at equivalence.

Some techniques such as liquid precipitin and the Ouchterlony test are unforgiving of this situation, and false-negative conclusions about complexity may be obtained. That is, one may conclude falsely that the antiserum is more specific than it actually is, or one may conclude that a given Ag solution is simpler antigenically than it actually is. Other techniques such as QIEP or crossed QIEP are better able to deal with major imbalances in Ag/AB equivalences. QIEP can routinely accommodate 64-fold differences in Ag titer among unknown solutions whereas, using Ouchterlony or IEP, this order of difference could cause one to miss or neglect the existence of particular Ags in a complex mixture.

Avoidance of the aforementioned false-negative conclusion about complexity is important, because techniques that depend on the specificity of an antiserum such as Western blotting or in situ localization (Tanaka and Ishizaki, 1974; Raikhel, 1984; Grafet al., 1986; Peferoen et al., 1986; Raikhel and Lea, 1986) are in some cases intrinsically unable to reject false-positive reactions that they may detect. For that reason all putative monospecific polyclonal antisera and mABs to be used for in situ localization should be tested for specificity using techniques such as Western blotting of tissue extracts (Graf et al., 1986; Peferoen et al., 1986; Raikhel et al., 1986). It is clear, for instance, that the majority of mABs do not distinguish between the several *Drosophila* vitellogenin gene products (Wu and Ma, 1986).

b. Adjustment of technique and AB titer for appropriate sensitivity. The technique of choice should be appropriate for the problem at hand. If large amounts of Ag are available, then the major concern might be to

conserve valuable antiserum. If neither Ag nor AB is limiting, then the choice of technique is one of convenience and accuracy. Extremely sensitive techniques require very low amounts of Ag to be tested. At very low Ag titers Ag may be lost from a diluted solution by absorption onto glass or plastic surfaces. In some cases a less sensitive assay is the more reliable approach. A practical rule of thumb is to not use a technique that requires excessive dilution. If high dilution is essential, then all buffer solutions can be supplemented with protein such as bovine serum albumin and/or the plastic, and glass surfaces in contact with the dilute solutions may be coated with a silicone layer to minimize adsorption of proteins.

B. Measuring Ag/AB Relative Concentration, Titering

a. Classical Ag/AB equivalence titer by precipitin in liquid. A complete precipitation analysis of equivalence in liquid requires a relatively large amount of antiserum as well as Ag. In order to be quantitatively precise with this approach, one must have a reasonably monospecific antiserum and do a complete curve of precipitation from AB excess to Ag excess.
The peak of precipitate occurs close to molar equivalence between Ag and AB. This peak is localized by analyzing the amount of precipitate. The precipitate can be washed to remove contaminating molecules and then dissolved in alkali. The protein content can then be estimated by numerous techniques. In this way the titer of the Ag can be related to the titer of the AB by how much dilution was necessary to make the two solutions react maximally, i.e. at their equivalence point. If the antiserum and Ag solutions are complex, there is a high probability that the peaks of equivalence may overlap or at best form a bimodal precipitate curve, since the concentrations used are logarithmic series which compress any titer and concentration differences.

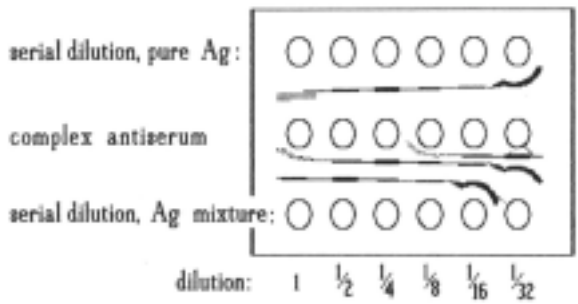

Figure 1.4. An Ag/AB equivalence test in agar is a necessary prerequisite of any successful Ouchterlony double-diffusion test. Parallel sets of wells are punched in the same agar medium in which a final "for publication" test will be done subsequently. Care is taken to space the wells identically to the future spacing of the final test pattern. A constant amount of antiserum is added to a central tier of wells. Serial dilutions of Ag are placed in parallel tiers of wells, and the Ags and ABs are allowed to diffuse together for 1 to a few days. The sharpest precipitin lines form between equivalence concentrations of Ag and AB; a pure Ag will have one or two best dilutions forming a crisp line which will intensify over the span of a few days. In a complex mixture, the equivalence dilution of each Ag may be different; initially several dilutions will show sharp lines, but with time, Ag or AB excess is indicated by a blurring of the precipitin lines. The blurring is more enhanced by staining.

b. Ag/AB equivalence in agar. Equivalence of Ag and AB in agar is determined by a combination of the concentration and diffusion coefficient of the Ag. The appropriate equivalence relationship can be determined by a serial dilution of Ag in a linear series of parallel Ouchterlony test wells (Fig. 1.4). The wells with equivalent titers of Ag and AB produce a crisp fine precipitin line which does not move with time. On either side of this equivalence relationship the excess of Ag or AB creates an unstable precipitin line which thickens and blurs with time. The majority of poor immunological illustrations of Ouchterlony reactions in research papers are due to not running this equivalence test prior to a final, "for publication" test.

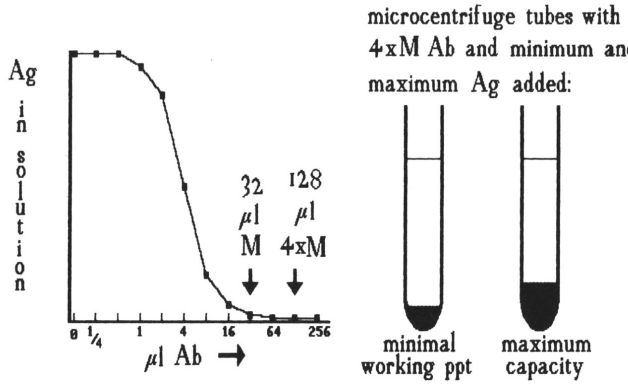

Figure 1.5. Microtitration to determine volume of antiserum and carrier Ag needed to achieve total precipitation of an Ag sample in a fluid precipitin reaction. This technique is carried out in microcentrifuge tubes. A fixed amount of carrier Ag (5-10 #g is sufficient) is added to each tube. An additional objective of this preliminary experiment is to find a convenient amount of carrier Ag providing a workable amount of precipitate that can be seen in the bottom of the tube after centrifugation, washed by resuspension in PBS, and repelleted several times without loss of precipitate. Increasing volumes of' antiserum are added to the series of tubes; precipitate is given time to form and is centrifuged out of solution. The supernatant is then tested for any Ag remaining in solution using any available micromethod, such as QIEP. The volume of antiserum that completely precipitates the carrier amount of Ag allows one to calculate an AB equivalence amount. Using a multiple of the equivalence volume of antiserum (4 x 32 = 128 #1) provides a liquid precipitin system that will always produce at least the minimal working precipitate with carrier Ag (tube on the left). This guards against AB excess. This system has the capacity to precipitate threefold more of "unknown" Ag without leaving any in solution (tube on right). If only the unknown Ag is labeled with radioactivity, the precipitate can be analyzed by diverse means to establish the nature and extent of labeling.

3. Quantitative Estimation of Concentration

A. Precipitin Techniques (minimal equipment, instructional level)

a. Fluid precipitin. Analytical estimation of precipitates using liquid phase precipitation of Ag and AB is the simplest immunological reaction to interpret. It is usually carried out using a fixed dilution of antiserum

and varying the Ag dilution from a concentration of Ag excess to a concentration of AB excess. The dilution of the Ag solution at the peak of the precipitin curve is a relative measure of the Ag titer. The higher the Ag titer in the original sample, the more it must be diluted to achieve the peak in a precipitin curve.

When the relative titer of the Ag solution is the principal information desired, this technique is not the recommended method; however, the analysis of fluid precipitins can be a valuable adjunct to the qualitative analysis of an Ag (Wojchowski et al., 1986). When one desires the approximate equivalence titer of an antiserum, it is possible to use techniques other than a large-scale liquid precipitin curve to estimate it. For instance, if the major information sought is the capacity of an antiserum to completely precipitate an Ag, one can do a microtitration from Ag excess to Ag equivalence in small, 250-µ1 micro-centrifuge tubes (Fig. 1.5). The criterion for complete precipitation would be to leave no Ag in solution. The Ag remaining in solution can be measured by a more sensitive assay such as QIEP or ELISA. Although this microtechnique does not conform to a classical liquid precipitin curve, it answers the question: How much antiserum must I add in a particular situation to ensure complete precipitation of a specified amount of Ag?

Armed with the knowledge of how much AB is necessary to precipitate an Ag in solution, one can proceed to study the synthesis and postribosomal processing of a protein. The tissue or organ site of synthesis of an Ag can be explored this way. The fat body was identified as the site of vitellogenin synthesis in the seminal paper using this approach (Pan et al., 1969). Labeling with 3H or 14C amino acids (Pan, 1977; Hagedorn et al., 1978; deBianchi et al, 1983; Brookes, 1986; Borovsky and Whitney, 1987) or 3sS-methionine (Chen et al., 1978; Brennan et al., 1982; Isaac and Bownes, 1982; Kawooya et al., 1986; Peferoen and DeLoof, 1986; Wojchowski et al, 1986) for protein synthesis, 32p for phosphorylation (Masuda and Oliveira, 1985; Della-Cioppa and Engelmann, 1987), and 2-(3H)-mannose for oligosaccharide attachment (Kunkel et al., 1980; Wojchowski et al., 1986) has been combined with immunoprecipitation and scintillation counting or fluorographic analysis of the precipitates after SDS-PAGE. This method of immune precipitation of a labeled macromolecule has become a preferred approach for analyzing protein synthesis, structure, and function.

b. The ring test. The simplest test for the presence of an Ag (or an AB) which gives a quantal answer to whether Ag is absent or present in a

solution (or whether an AB exists in an antiserum to an Ag solution) is the ring test. In this assay one overlays, in a narrow tube, a dense solution (adjusted to 5% sucrose) of Ag (or AB) with a light solution (no sucrose) of unknown to be tested. In a positive response, a precipitate accumulates at the interface of the two solutions within a half hour (Fig. 1.6). The interface is viewed on edge against a light source and compared to a control tube which has the unknown Ag (or AB) solution replaced with PBS. A strongly positive reaction produces a thick plug of precipitate within an hour.

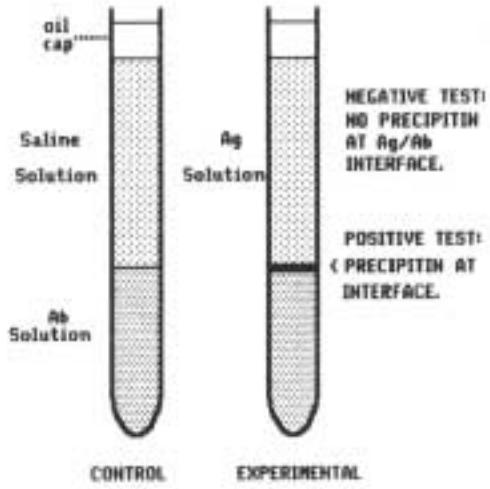

Figure 1.6. The ring test is a simple, relatively rapid test of immunological reactivity which is read by eye as positive or negative against a control tube with no reactive Ag in it. The bottom solution of AB is made more dense by addition of sucrose or glycerol. The top solution is overlaid carefully, and the position of the initially crisp interface is noted. Ag and AB diffuse together and precipitate at their liquid interface. In a "positive test" a precipitin is easily visible at the interface within a half hour.

This quick assay may be useful for screening whether serum from an immunization of a particular animal with an Ag is a success or failure, before more careful evaluation of the antiserum is attempted. It has also been used by some authors to screen chromatography fractions for an Ag prior to pooling fractions (Borovsky & Whitney, 1987). For a yes/no answer, the ring test is expensive if the antiserum or Ag is valuable since the test relies on observing a precipitate which requires relatively large volumes of Ag and antiserum compared to other assays listed below. However, if time or immunological supplies are the more important factors, this assay is useful to have available.

B. Diffusion-Based Estimation of Ag Concentration

a. Oudin one-dimensional, single diffusion. The simplest quantitative measure of Ag titer using diffusion in agarose was devised by Oudin

(Becker et al., 1951). This is a simple but elegant technique that requires very little specialized equipment. A researcher could carry out this assay on a low budget and achieve high sensitivity and accurate determinations. The theory underlying determination of the diffusion coefficient of Ags using this technique is also given by Becker and associates (1951). Students in a teaching laboratory can carry out this test with a high proportion of successes. One advantage of the technique is that it does not have to be read at any specific time after the initiation of the reaction and requires no special chemicals or dyes beyond purified agar or agarose, salt, and phosphate buffer. The only specialized equipment needed is the tubes in which the reaction is run. These tubes can be manufactured from 1/4" or *3/16"* soft glass tubing stock, available from most scientific supply houses. The manufacture of the tubes is simple, involving closing one end of each tube with a Bunsen burner or more quickly with an acetylene torch.

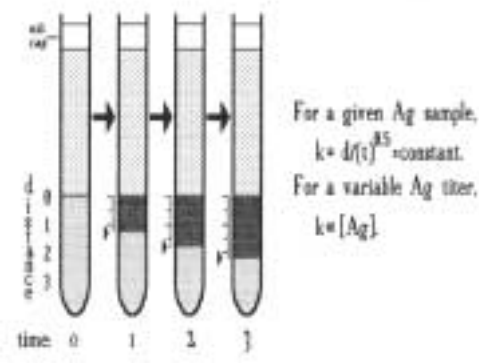

Figure 1.7. The Oudin one-dimensional, single-diffusion assay. The antiserum is placed in an ionagar or agarose gel layer and is considered to be relatively stationary. An Ag dilution, of higher concentration than the equivalence amount of AB, serves as the single dominant diffusing component. The Ag solution is added to the top of the agar at a recorded time zero, and the Ag starts diffusing into the AB-agar phase. A precipitin front is seen to advance with time. For each individual tube the distance, d, of front nigration divided by the square root of the elapsed time, t, gives a constant value, k. This k-value is proportional to the Ag concentration, and a dilution series provides a calibration curve of k versus [Ag] for determining relative or absolute titers of unknown Ag.

In the Oudin technique AB is dispersed evenly in 0.5% agarose and placed in a narrow tube. The AB in agar is overlaid with an Ag solution which must be at least more concentrated than the equivalence titer of the AB in agar. The Ag diffuses into the gel and forms a zone of visible precipitate as the free Ag meets and precipitates with its corresponding AB (Fig. 1.7). The rate at which this precipitate front moves down the tube is proportional to both the concentration and the diffusion coefficient of the Ag. The rate of front migration changes predictably,

being proportional to the square root of time. For this reason, all samples should be applied at approximately the same time, including the standards. The tubes are overlaid with mineral oil to prevent evaporation and incubated at a constant temperature in a horizontal position.

Heat shocks or prolonged temperature changes are to be avoided, as the diffusion rate is temperature dependent and the density of the precipitate will be different at different temperatures. Temperature artifacts can give the appearance of an artificial front which might be confusing in a complex antiserum. Luckily, the Ag/AB fronts move with time, but the temperature artifact "fronts" remain in place. The unknown Ag concentrations are usually determined by comparison to standard curves of known absolute or relative concentration that are run at the same time. Standard curves are highly reproducible, given constant technique and physical conditions.

If the antiserum being used is complex and the Ag solution contains several of the Ags that react with the antiserum, this technique is fraught with problems, since several fronts of precipitate will be moving along the tube and may not be able to be distinguished. The technique is limited to relatively high-titer Ag and AB solutions since the result is a visible precipitate that cannot be enhanced by protein stains.

Despite its relatively low sensitivity, the Oudin technique has been used in numerous insect studies for a variety of purposes. Prior to the advent of QIEP, the Oudin test was a preferred method of measuring the concentration of an Ag in a solution whether that solution was a serum sample (Kunkel and Lawler, 1974; Pan, 1977; Tojo et al., 1980, 1981; Teller et al., 1983), a tissue extract (Ogawa and Tojo, 1981; Tojo et al., 1980, 1981), a sucrose gradient fraction (Kunkel and Lawler, 1974), or a chromatography column fraction (Duhamel and Kunkel, 1983, 1987). The technique is still useful, since it does not require samples to be diluted, desalted, or dialyzed prior to testing, as is often necessary with QIEP.

b. Radial immunodiffusion. This technique is a two-dimensional analogue of the Oudin technique (Inglid, 1983). A plate of AB in agar is poured, and Ag wells are cut in the plate. A circle of precipitate develops around the well as the Ag diffuses out and encounters AB; the circular front grows wider with time (Fig. 1.8). It works on the same theoretical basis but does not achieve the same quantitative elegance as the Oudin, since the reservoir of Ag is usually limited, and the system runs out of Ag. This results in departure from ideal theoretical behavior, which depends on an infinite Ag reservoir. However, the depletion of Ag

provides an end point for the assay since the ring stops growing and visibly darkens at its rim signaling that it is finished expanding. The timing of this end point depends on the diffusion coefficient and concentration of the Ag and AB but usually occurs within a few days. This delay in the ability to read the assay reserves this technique for applications in which data analysis is not a priority. Ag concentration is proportional to the diameter of the precipitate front. Ag concentration is usually calculated from a standard curve established separately or from standards run with the unknown samples. Concentrations in the microgram range are measurable. A single *3¼ x 4"* plate can accommodate up to 20 unknowns.

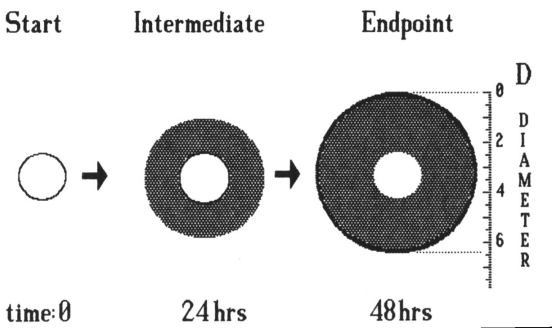

Figure 1.8. Radial immunodiffusion is a two-dimensional, single-diffusion assay similar in nature to the Oudin test. As in the Oudin test, the AB usually serves as a stationary phase, and the Ag is usually the mobile or dominant diffusing component. Antiserum is dispersed in agar and coats a slide or Petri dish. Samples of Ag are placed in wells cut in the agar surface. Ag diffuses out radially, forming a precipitate front as it advances. The system runs out of Ag, and a darker rim of precipitate develops at the edge of the final precipitate. The diameter of this disk of precipitate is determined after staining and is compared to a standard curve established for that antiserum by a previous or concurrent set of serial dilutions of known Ag titer.

This technique requires less equipment than the Oudin test; it can be performed on a microscope slide or in disposable or reusable Petri dishes. It is about as costly in antisera as the Oudin test if it is evaluated at the unstained precipitin stage. However, it is possible to read radial immunodiffusion plates after staining. Thus a substantial increase in sensitivity and economy of antiserum can be achieved by dilution of the antiserum below the level of convenient visibility of unstained precipitate. Less Ag is intrinsically required to run the assay, since the Ag reservoir is limited to the volume that fits in the sample well. Complex antisera and Ag solutions present the same problem of confusion of the precipitin fronts as with the Oudin test, although if the Ag is an enzyme for which a suitable colorimetric staining reaction is available, this assay can be both sensitive and specific (Bog-Hansen and Brogren, 1983).

Several studies of insect serum proteins have found this technique economic, sensitive, and effective in quantifying changes in protein concentration during development (Irie and Yamashita 1983; Marinotti and deBianchi, 1983; deBianchi and Marinotti, 1985; Haunerland and Bowers, 1986; Trost and Goodman, 1986).

C. Electrophoretic lmmunoprecipitin Techniques

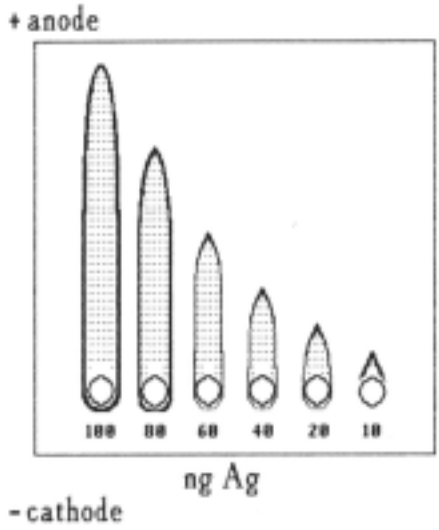

Figure 1.9. Quantitative immunoelectrophoresis (QIEP) or "rocket" IEP. The antibody is dispersed evenly in the thin layer of agarose on the slide. The quantity of Ag placed in each well can be estimated by the amount of AB area that is swept into a "rocket" of precipitin by the electrophoretically propelled Ag. This method, like radial diffusion, has a self-imposed end point which occurs when all of the Ag in the well has reacted with its equivalence amount of AB.

a. Quantitative immunoelectrophoresis, QIEP, or "rocket" IEP. The problem of quantitative determinations of complex Ag solutions was to a certain degree solved with the advent of QIEP (Laurel, 1972; Verbruggen, 1975). The advantages of this technique are its high sensitivity (ng of Ag) and its ability to quantify a large series of samples at one time. Routinely 50 samples per 3¼ x 4" slide can be run in a chamber that will hold multiple slides. The technique has an end point, as does radial immunodiffusion, and thus it will not overdevelop. QIEP adds to the method of radial diffusion the force of electrophoresis (Fig. 1.9). The equipment involved can be moderately expensive and may preclude the use of this technique for laboratories that are not suitably equipped. A standard electrophoresis power supply is required as well as a horizontal electrophoresis chamber. The electrophoresis is carried out at pH 8.3, which is close enough to the average pI of the immunoglobulins that they remain approximately stationary. As long as the Ag is mobile at that pH (and most are), this results in a rocket-shaped precipitin arc being laid down in the agarose. If the Ag is not sufficiently

mobile at pH 8.3, the proteins may have to be carbamylated prior to the electrophoresis (Dillwith and Chippendale, 1984). One of the benefits of the QIEP technique is that all of the Ag in the sample well is forced to interact with AB relatively quickly, within several hours for reasonably mobile Ag. The volume of agar that is cleared of AB by the moving Ag is equal to the equivalence volume for that amount of Ag. Although with higher concentrations of antisera the "rockets" of precipitin are immediately visible, the general routine procedure to obtain maximal sensitivity with this technique is to use a lower AB concentration in the agar and stain the rockets after drying down the agar onto the slide, This technique is about 100-fold more sensitive in measuring Ag than radial immunodiffusion, especially for large Ag molecules which are trapped by inwardly diffusing AB in the sample well at low concentrations in radial immunodiffusion. Complex antisera and Ag solutions are often resolved into different density rockets such that with a little experimentation and logic, the components of a complex system can be identified and measured at the same time.

QIEP has been used to analyze the titer of numerous insect proteins in physiological fluids or tissues. The titer of the protein vitellogenin has been followed in hemolymph and tissues of normal or experimentally treated animals for numerous insect species (Greenberg et al., 1978; Hagedorn et al., 1978; Kunkel, 1981; Buschor and Lanzrein, 1983; Koenig and Lanzrein, 1985; Kunkel and Nordin, 1985; Benford and Bradley, 1986; Oliveira et al., 1986; Zhu et al., 1986; Imboden et al., 1987). Various other serum proteins including storage proteins (deBianchi and Marinotti, 1984; Levenbook and Bauer, 1984; Ryan et al., 1985b; deKort and Koopmanschap, 1987), diapause-associated protein (Dillwith et al., 1985), and lipophorin (Venkatesh and Chippendale, 1986) are able to be monitored using QIEP-.

b. QIEP estimation of protein synthesis and processing. Using a mono-specific antiserum to an Ag of interest, it is possible to do a radioactive tracer incorporation study into an Ag which is then analyzed by QIEP in which the labeled Ag is precipitated in a rocket (Hagedorn et al., 1978; Levenbook and Bauer, 1984). To eliminate false counts from electrophoretically immobile sample components, the sample well gel is kept free of AB as in fused rocket QIEP (Fig. 1.13, wells 1 and 2). The maximum amount of precipitate in each rocket can be increased by using higher antiserum concentration in the AB gel. As a rule of thumb, each doubling of antiserum concentration halves the area of rocket created by a given Ag amount. Rockets containing 2-10 ng of protein are not

unreasonable. Care should always be taken in this technique to run the electrophoretic step long enough to remove all nonreactive substrates and proteins from the gel. The visible precipitin "rocket" is cut out of the agar gel slab on the slide after the electrophoresis step and dissolved in a scintillation solution for counting. This method compared favorably in accuracy with precipitin techniques in liquid (Hagedorn et al., 1978).

D. Quantitative Immunotransfer Technique (maximal sensitivity)

Immobilization of Ag or AB on a support such as a plastic well of a micro-titer plate or a nitrocellulose filter and subsequent probing of that immobilized protein with AB or Ag, respectively, is a rapid and sensitive technique for quantifying Ags. When the support is a filter of some sort, the technique has been dubbed dot blotting (Hawkes et al., 1982; Jahn et al., 1984). The probing of the immobilized protein may be accomplished by a fluorescence-, enzyme-, or radionuclide-linked probe. The primary probe is most often an AB or a lectin (Haunerland et al., 1986; Marinotti and Bianchi, 1986). Often the AB probe cannot be conveniently directly visualized. A second all-purpose reagent is often used such as a second AB, which recognizes the species class of the primary AB, or protein A--a bacterial protein that binds to immunoglobulins. This secondary reagent has a covalently attached reporter group such as a fluorescent molecule or a radionuclide or a chromogenic enzyme that can be localized by convenient assays. When the support is a microtiter plate and the method of developing the probe is via an enzyme-linked AB, the developing industry surrounding this technology has dubbed the technique ELISA (enzyme-linked immunosorbent assay) (see Chapter 2). This technique works best with monospecific antisera and is particularly well suited to mABs, since an artificial immobilization for the Ag is provided, obviating the importance of cross-linking.

4. Analysis of Complexity

The analysis of complexity is a particularly strong feature of immunology. With the appropriate antiserum(a), an investigator may identify and measure the titer of the major and minor Ags in an extract. This is helpful in following the changes in particular Ag titers during a physiological or developmental cycle or during purification of particular Ags. Particular techniques are more or less useful in this respect.

A. The Ouchterlony Test, Double Diffusion

Double immunodiffusion, or the Ouchterlony test, is not often the technique of choice for demonstrating complexity, but it is a powerful technique when properly applied. Unfortunately, the technique is often carried out under conditions in which the ability to measure complexity is minimized. How to maximize the visualization of complexity with Ouchterlony is described by its namesake (Ouchterlony, 1968). This maximization involves making sure that the Ag and AB reservoirs are large enough to maintain infinite pools of reactants effectively. In that instance, the place in the gel at which equivalence of Ag and AB is achieved will tend to be stationary, since precipitate for a particular Ag/AB pair accumulates at that line in the gel for the longest time. This situation can rarely be achieved with Ouchterlony run on a microscope slide in which the wells tend to be small (3-10 #1) and the distance between wells is also small. In most instances a complex antiserum used in Ouchterlony will produce a pattern of precipitin lines difficult to interpret (Fig. 1.10 (1)). Most of the Ag/AB pairs will be unbalanced and produce precipitin lines curving toward either the Ag well or the AB well. It is rare that all antibodies of a complex antiserum are sufficiently balanced to produce crisp, straight precipitin lines as in (Fig. 1.10 (2)). For maximum resolution, the well pattern shape is suggested to be rectangular; the gel should be poured in a Petri dish, and the wells placed far enough apart to allow several Ag/AB pairs to form distinct precipitin lines. The greater distances between wells make higher AB and Ag titers in the sample wells necessary to achieve visible precipitates in the gel. The resultant precipitin lines can be enhanced by a number of modifications. Placing an osmotically active component such as high-molecular-weight dextran solution in reactant wells will prevent the well from drying out by losing liquid to the gel. This will maintain for longer the apparent infinite source of reactants in the wells. The unstained precipitin reaction is more intense at colder temperatures, and the density of the precipitate can be enhanced by inclusion of PEG6000 (Verbruggen, 1975) in the agar.

A number of studies of insect Ags have used the Ouchterlony technique effectively for demonstrating antigenic complexity of particular Ag sources and testing membership of a particular Ag to a particular stage. For instance, using separate complex antisera made against female and male locust serum, it was shown that the major Ag of locust oocyte vitellin reacted only with anti-female serum (Gellissen et al., 1976). Another approach often used is to use a monospecific

antiserum against an Ag such as vitellin purified from eggs and then show via Ouchterlony that it is present in various complex mixtures such as egg extract and female hemolymph but not male hemolymph (Mundal and Law, 1978).

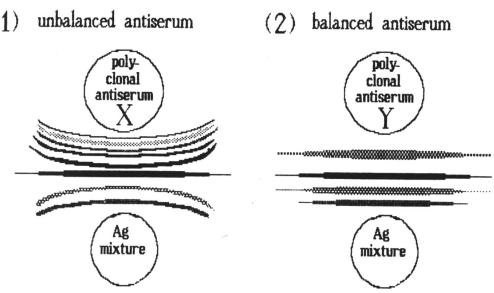

Figure 1.10. Ouchterlony double-diffusion assay. (1) Unbalanced serum. The multiple Ag/AB precipitin lines are not straight and curve away from the source of the excess component. (2) Balanced serum. The multiple Ag/AB precipitin lines are straight and crisp. This occurs when each Ag and AB are at approximate equivalence. Balanced complex antisera for Ouchterlony are difficult to achieve routinely.

B. Electrophoretic Analysis of lmmunologic Complexity

a. Immunoelectrophoresis (IEP). By far the most effective way of improving the analysis of antigenic complexity is to combine or follow an electrophoretic step or steps with an immunologic step. Classical IEP separates Ags in agarose by electrophoresis on a slide prior to placing antibodies in a trough parallel to the lane of electrophoresis. Electrophoretic pre-separation, followed by diffusion toward a reservoir of antiserum, provides separate foci of diffusion for electrophoretically separable Ags, and this improves resolution by reducing the congestion of bands seen in Ouchterlony (Fig. 1.11, top). In some cases, a complex antiserum can provide valuable evidence for the involvement of one antigen of a mixture in a physiological process such as insect serum clotting (Bohn and Barwig, 1984). Absorption of complex anti-sera with less complex Ag solutions can result in more specific antisera with names such as female-specific (Telfer, 1954; Pan and Wyatt, 1971; Tanaka, 1973; Kunkel and Pan, 1976; Mundall and Law, 1979; Ogawa and Tojo, 1981), larval-specific (Kunkel and Lawler, 1974), or egg-specific (Yamashita, 1986) antiserum (Fig. 1.11, bottom). Absorption of an antiserum involves precipitation of the majority of ABs common to a heterologous Ag source. In the most favorable situation, the heterologous source is expected to differ from the homologous source in one or a few Ags such that the absorbed antiserum may retain AB to one or only a few

distinct Ags.

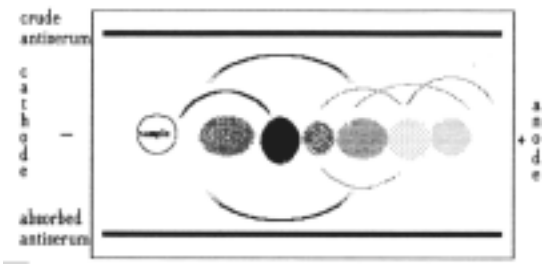

Figure 1.11. Immunoelectrophoresis (IEP). Prior electrophoresis of a mixture of antigens in agarose is combined with a subsequent step similar to Ouchterlony double diffusion. After the electrophoretic separation in the first dimension, a trough is cut in the agar parallel to the direction of electrophoresis, and antiserum is added to the trough and allowed to diffuse toward and precipitate with the electrophoretically separated Ags. A mixture of Ags is shown in this example reacting with a crude antiserum (upper trough) and an absorbed antiserum (lower trough).

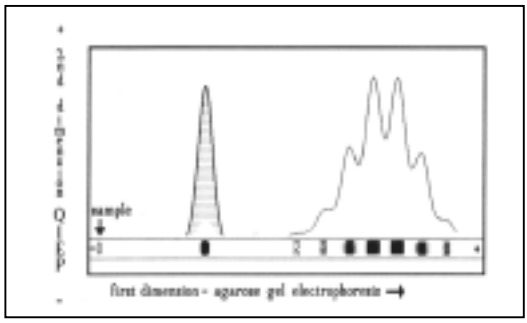

Figure 1.12. Crossed QIEP is the logical extension of IEP to QIEP. The first dimension can be run similar to IEP of Figure 1.11 above, or, for better resolution, the first dimension can be done in a vertical or horizontal agarose slab apparatus. The sliver of agarose from the first-dimension gel is mounted on a slide and supplemented with AB-containing agarose as in QIEP. The second-dimension electrophoresis of the Ags at a right angle creates AG/AB precipitin "rockets" with characteristic density of precipitate. Complex Ag solutions can be analyzed with complex antisera using this method. Fusion of adjacent rockets can be used to identify homologous electromorphs or isozymes.

b. Variations on QIEP. Immunological complexity can be dealt with in a number of ways using modifications to the basic QIEP technique according to Laurell (Laurell, 1972; Axelsen, 1983). As in IEP, one can combine the immunological detection step (QIEP) with a prior separation step such as simple slide or slab agarose electrophoresis in the first dimension to allow one to resolve Ags in the first dimension prior to the QIEP step applied at right angles (Fig. 1.12). Usually the first dimension is not done in acrylamide or media other than agarose, because there are incompatibilities of other media with agarose unless special care is taken. This technique, crossed QIEP, has been effectively used in following and evaluating the purification of proteins. If an antiserum to the crude

starting mixture is available, one can apply purification steps and used crossed QIEP to check at each successive step how much the major contaminants have been reduced (Barwig, 1985). In extremely complicated antigenic solutions such as lepidopteran serum and yolk extracts, this method has been used effectively to identify and follow the different components during development (Telfer et al., 1981).

Another type of step prior to QIEP is simple diffusion of the samples in adjacent wells in plain agar for a few hours prior to the addition of AB agar to the remainder of the slide. This approach, called fused QIEP, results in fusion of precipitin rockets of adjacent identical proteins (Fig. 1.13, wells 3 and 4). This fusion is often enough to confirm the existence of multiple Ag/AB reactions in the adjacent samples (Storella et al., 1985; Wojchowski and Kunkel, 1987). One can also analyze one set of Ags with several layers of more or less specific antisera (Wojchowski et al., 1986).

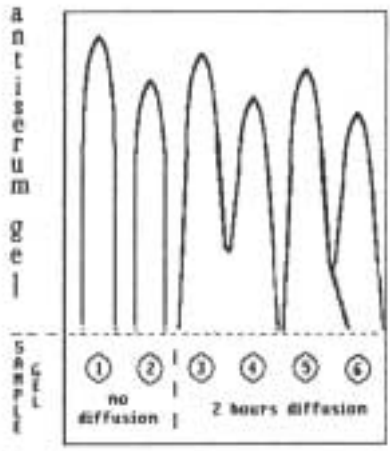

Figure 1.13. Fused QIEP is a logical extension of the Ouchterlony double-diffusion test to QIEP. The samples with potentially related Ags are placed in their individual sample wells (1-6) cut in a basal strip of agar containing electrophoresis buffer but no antiserum. The Ags are allowed to diffuse for a time determined by experiment to allow sufficient comingling of the Ags. An antiserum gel, similar to that in QIEP, is added to the remainder of the slide after 2 h of diffusion, and the samples are electrophoresed into the antiserum agar. Ags in wells 3 and 4 depict an identity reaction; they form a continuous precipitin line similar to a line of identity in the Ouchterlony test. Ags in wells 5 and 6 show partial identity by forming a "spur" of precipitate rather than a continuous line. Samples 1 and 2, placed in their respective wells just prior to elec-trophoresis, form distinct "rockets" of precipitin, untested for homology, but they leave any particulate matter contaminating the sample back in the gel surrounding the sample well.

c. PAGE electroblotting, Western blot. A powerful technique that has been added to the arsenal of techniques for analyzing complexity is the technique of electroblotting (Olmsted, 1981; Towbin et al., 1979). This combines the quantitative technique of dot-blotting with electrophoretic transfer from a one- or two-dimensional electrophoretically separated Ag sample (Fig. 1.14 (1)) onto a nitrocellulose filter. This filter can be

probed directly with a specific AB solution or a lectin solution and the Ags recognized by linked-fluorescence, linked-enzyme reactions or, linked-radioactivity methods (Fig. 1.14 (2-4)). It has been used extensively in the invertebrate literature to identify specific peptides (Brock and Roberts, 1983; Sharrock, 1983; Ryan et al., 1984, 1985a,b, 1986; Smith and Fisher, 1984; Osir et al., 1986), to identify processing pathways (Wojchowski et al., 1986; Della-Cioppa and Engelmann, 1987) and to investigate complex Ag mixtures (Cox, 1987). In addition, the antibodies can be eluted from the nitrocellulose sheets and the same sheet probed with additional antisera (Fig. 1.14 (3, 4)) (Legocki and Verma, 1980). Alternatively, the antibodies recognizing a particular spot or band can be recovered, and these "affinity-purified" antibodies can be used to probe further Western blots (Olmsted, 1981; Smith and Fisher, 1984). This latter technique allows for unlimited ability to sort out the complexity of Ag mixtures.

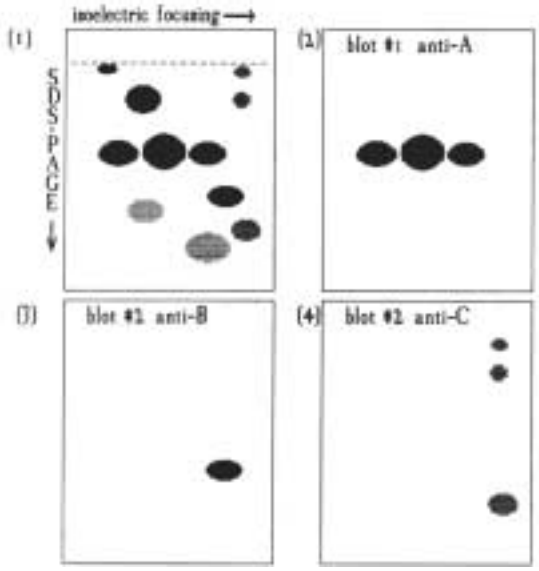

Figure 1.14. One example of PAGE elec-troblotting, the Western blot. (1) O'Farrell (1975) 2D electrophoresis of antigens by isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension. (2, 3) Separated Ags are blot or electrophoretically trans-ferred to one or more nitrocellulose membranes and probed with specific antisera. (4) Removal of the probing AB from the nitrocellulose membrane is possible, and reprobing of the still attached Ag with another AB is carried out.

5. Qualitative Differences and Similarities between Ags

A. Diffusion Techniques to Demonstrate Qualitative Homologies

a. Ouchterlony (minimum equipment, instructional level). The most frequently used technique for demonstrating identity or departures from identity in two Ags is the double-diffusion technique of Ouchterlony

(1965). This application of the Ouchterlony technique works best with monospecific antisera, since complex antisera, as suggested in Section III.D. 1.a, can be confusing to interpret. The Ag to which the antiserum was made is called the homologous Ag. An Ag' from a different source, such as a different tissue or stage or species, is termed a heterologous Ag. The Ags are placed in peripheral wells and allowed to diffuse toward a well containing an antiserum (Fig. 1.15 (1-3)). Crossing of the precipitin lines of the two systems without any indications of interaction is an indication of immunological distinctness (Fig. 1.15 (1)). The complete fusion of the precipitin lines is an indication that the homologous and heterologous Ags in the two Ag wells are identical, at least at the level of this technique's ability to discern identity (Fig. 1.15 (2)). Departures from identity are seen as "spurs" at the point of fusion of the two precipitin lines (Fig. 1.15 (3)). Such spurs are indications that a determinant on the homologous Ag is missing or dramatically changed

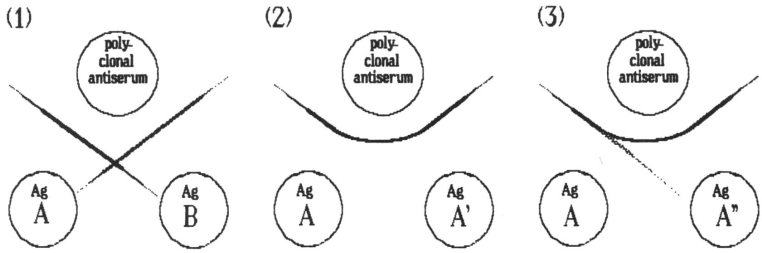

Figure 1.15. Ouchterlony double-diffusion comparison of Ags. A specific or complex polyclonal antiserum is placed in a central well in an agarosc slab and allowed to interact with Ag diffusing toward it from two peripheral wells. (1) Ouchterlony demonstration of lack of homology between antigen A and antigen B. (2) Ouchterlony demonstration of complete identity of antigen A with A'. (3) Ouchterlony demonstration of partial identity between antigen A and A"·

on the heterologous Ag. Hierarchies of spurring can be developed into a similarity matrix, and such matrices have been used to build phylogenetic trees.

Homology between an Ag derived from different stages or tissue sources within a species can often be established with this technique (Pan, 1977; Chino and Yazawa, 1986; Venkatesh and Chippendale, 1986). This can take the form of absolute identity claims which are often overstated, since the Ouchterlony test sometimes does not detect differences of as much as 5% amino acid sequence difference. It is often stated, for instance, that the vitellogenin and vitellin of a particular

species are immunologically identical based on a lack of spurring in the Ouchterlony double-diffusion assay. Such low-resolution lack of differences will survive until proved or disproved by a more discriminating test. The immunological method of choice for detecting minimal departures from identity is microcomplement fixation (MC/F).

Homology between Ags from different insect species has often been demonstrated with the Ouchterlony test. The simple existence of homologous Ag in other insects is relatively easily surveyed using the Ouchterlony technique (Kunkel and Lawler, 1974; deBianchi et al., 1983; Dillwith et al., 1985). Some proteins such as vitellogenins have changed rapidly during evolution (Kunkel and Nordin, 1985), and thus when cross-reaction is found, it is evidence for a relatively close phylogenetic relationship. Thus the observations of a strong identity reaction between vitellogenins of *Leucophaea* and *Nauphoeta* (Imboden et al., 1987) and between *Bombyx* and *Philosamia* (Izumi et al., 1980) are particularly important to physiological experiments concerning the species pairs. Homology between particular purified arylphorins of two well-studied "model" species, *Hyalophora cecropia* and *Manduca sexta*, was determined with antisera to each others' protein (Telfer et al., 1983). Two serum proteins of the little-studied lepidopteran *Papilio polyxenes* (Ryan et al., 1986) were shown to be homologous to two well-studied serum proteins of *Manduca sexta*. Arylphorins of the corn earworm and tobacco hornworm were shown to be homologous using the available *Manduca* AB (Haunerland and Bowers, 1986). In this way, once the homologies between proteins are established, someone working on a relatively unstudied species can tap into the experience of past researchers on properties and purification techniques developed using proteins of well-studied "model" species. Lack of an immunological cross-reaction with the Ouchterlony test should be viewed cautiously. Anticock-roach LSP's lack of a reaction with cricket serum (Kunkel and Lawler, 1974) is a function of phylogenetic distance to be expected in such an ancient divergence. Similarly, *Hyalophora* and *Manduca* arylphorins do not cross-react with *Calliphora* arylphorin (Telfer et al., 1983), nor does *Drosophila melanogaster* LSP_2 cross-react with similar proteins as far removed as *Ceratitus* (Mintzas and Reboutsicas, 1984). Of course these negative results are not interpreted as proof that a homologously related and functioning protein does not exist in distant relatives unless immunological evidence is available that immunological cross-reaction does extend that far. In some instances, other immunological techniques can be used to establish more distant relationships (see Section III.E.2.c below) (Ryan et al., 1984).

b. Oudin demonstration of homology and physical property differences. A monospecific antiserum can be used to measure the degree of homology between two reactive Ags using the Oudin test (Hayden and Becker, 1960). If two Ags have identical physical characteristics and differ only in amino acid sequence, the degree of immunological cross-reaction can be estimated by comparing the density of the precipitin front formed by each Ag with the same AB in Oudin tubes. This is a simple procedure that can be carried out with any gel-scanning apparatus, and it results in quantitative comparisons that are parallel to the measurements made by MC'F (Kunkel and Lawler, 1974; Kunkel and Nordin, 1985).

The Oudin single-diffusion assay itself is also able to detect the physical differences or changes in Ags that affect the Stoke's radius and the diffusion coefficient (Becker et al., 1951). Such changes must be relatively large to be detected, such as the difference between a monomer and a dimer of an Ag. When the Oudin test does detect substantial differences in the diffusion coefficient of two Ags, the use of Oudin tests to compare degree of cross-reaction is counter indicated. However, it should be noted that this counter-indication applies to complications created with other immunological techniques as well. It is possible in some of these cases to force all of the Ag into a uniform monomeric state for the purpose of quantitative comparisons (Beverley and Wilson, 1982).

B. Electrophoretic Techniques for Demonstrating Qualitative Differences

a. Immunoelectrophoresis (IEP). IEP has been used effectively to demonstrate electrophoretic mobility differences of an Ag (Bohn and Saks, 1986). When performed in purified 1% agarose, mobility difference can be interpreted as differences in charge, since globular macromolecules up to 1 megadalton do not interact with the gel matrix. If the buffer pH for the electrophoretic step is adjusted to allow an estimation of the pH of reversal of antigen direction in the electrophoretic field, the isoelectric points of Ags can be determined (Kunkel and Pan, 1975).

b. Fused QIEP. Immunologic identity or similarity can be demonstrated in QIEP in a technique analogous to Ouchterlony spur formation. As in Ouchterlony, attention to the possibility of artifacts is essential. Using this procedure, the loss or change of a determinant on a

protein during a physiologic, developmental, or evolutionary step may be detected. When the Ags are substantially changed, a decrease in the density of the precipitin line for the heterologous Ag may be noticeable in addition to the precipitin spur (Fig. 1.13, samples 5 and 6).

c. Crossed QIEP. Electrophoretic mobility of homologous Ags differs by substantial amounts owing to allelic differences or postribosomal processing. Isozymes or electromorphs may be tested for immunological cross-reaction by allowing them to separate sufficiently in one-dimensional electrophoresis such that they produce separate staining bands in their first dimension. By electrophoresing them into an antiserum dimension, one can test if they are immunologically distinct proteins or if they are different mobility forms of a related protein (Fig. 1.12). Such a technique demonstrated seven immunologically related, random oligomeric structures of the hexameric insect serum protein LSP (Duhamel and Kunkel, 1983). A similar study of *Manduca* demonstrated that three electromorphs of vitellogenin are immunologically identical (Imboden and Law, 1983). Of course such electromorphs could have up to 5% amino acid sequence difference and still not give rise to a spur difference.

d. Western blot assay, homologies. The combination of electroph0retic separation and immunological identification is combined in the Western blot technique (Towbin et al., 1979). This technique has been used in several cases to establish homology between insect proteins from different tissues and stages of development. The stages of vitellin peptide processing were established by Purcell and co-workers (1986) by making a monospecific antiserum to later-stage peptides and using those ABs to probe earlier-stage precursor peptides. The homologies between serum and tissue proteins from different species (Brock and Roberts, 1983; Ryan et al., 1985a; Kozma and Bownes, 1986) and even between different insect orders (Ryan et al., 1984; Robbs et al., 1985) have been demonstrated using Western blotting. Homology is not a trivial problem, because insects have been shown to have many distinct serum proteins with different native electrophoretic mobilities and different but similar molecular weights (Wyatt and Pan, 1979). The reuse of the original protein blot onto nitrocellulose with different probing antibodies is a substantial help in sorting out the complexity of the gels (Legocki and Verma, 1980). Also, the salvage of AB from blots of specific Ag spots on 1D or 2D native or SDS PAGE gels is extremely useful in assigning homologies between Ags.

C. Quantification of Qualitative Differences

a. Quantitative precipitin reactions. The use of liquid precipitin reactions to compare Ags is quite sensitive, and its results have been shown to parallel the more demanding and more sensitive MC'F technique, but the liquid precipitin technique is wasteful of Ag and AB solutions. The technique done properly requires that a complete precipitin curve from AB excess to Ag excess be done for each AB/Ag pair (Leone, 1947). The homologous Ag/AB pair will give the largest integral of area under the precipitin curve. This technique is relatively easy to do in a teaching lab using a simple spectrophotometer, such as a Bausch & Lomb Spectronic-20, to measure turbidity of the liquid precipitin reaction. The reactions develop relatively quickly, and a comparison between two Ags can be carried out easily in the space of one laboratory period. However, it is not recommended as a general research approach unless the Ags and ABs involved are available in large supply.

A great deal more detailed information about the degree of immunological relatedness between Ags can be obtained using Ouchterlony double-diffusion spur hierarchies (Ouchterlony, 1968), which are interpretable in terms of shared determinants and Ven diagrams (Moore and Goodman, 1968). Thus if two different techniques or processes destroy a protein determinant of a particular Ag, comparing the two modified forms with Ouchterlony can demonstrate whether or not they have been modified within the same determinant. Three related biological species with cross-reacting Ag can be ordered by how recently they shared a common Ag, evidence for their phylogenetic relationship. For instance, the Dictyoptera share an Ag, LSP (Kunkel and Lawler, 1974), which allows the relative phylogenetic affinities of the cock-roaches, termites, and preying mantids to be investigated. It is clear from the strength of the spurs in those group comparisons that termite LSP is more closely related to cockroach LSP than might earlier have been suspected and that preying mantid LSP is more distantly related than that of termites.

There is a multidimensionality in the spur data from Ouchterlony that is not available through other comparison techniques. Besides estimating the proportion of shared determinants with a related Ag, the Ouchterlony test allows one to compare whether the shared determinants with two other related Ags are the same. The quantification via size of spurs has been shown to be highly correlated with MC'F (Moore and Goodman, 1968). There is also the possibility of quantifying the

precipitin spur reactions by densitometric scanning of precipitins (Butler and Leone, 1968). This technique has been used to demonstrate the affinity of lepidopteran vitellins within the speciose genus *Catocalla* (Kunkel et al., 1976), concluding that vitellin cross-reaction may be useful in differentiating closely related species.

b. Microcomplement fixation (MC'F). The ability of MC'F to detect single amino acid differences between two homologous proteins has established it as the immunological technique of choice in detection and quantification of qualitative differences between Ags. The technique uses dilute Ag solutions that need not be pure and monospecific antisera. The AB concentration used to achieve a peak of complement fixation with dilutions of the homologous Ag is used as a reference to calculate the necessary increase in AB concentration to obtain an equivalent fixation curve for a related Ag. The log of the required increase in concentration is termed the immunologic distance (ID) and is proportional to the percentage difference in amino acid sequence. Immunologic cross-reaction disappears in a series of well-studied Ags with departures in amino acid sequence approaching 30-40% (Champion et al., 1974).

Although single amino acid mutations in enzymes have been detected consistently with MC'F (Champion et al., 1974), it must be added that various postribosomal modifications such as peptide cleavage or phosphorylation may also have large effects on MC'F, and lack of identity is therefore not proof that a heterologous Ag recognized as different by MC'F represents a distinct gene product.

MC'F has not been applied extensively to insect problems, but when applied it has resulted in some interesting conclusions. A major serum protein of drosophilids has been studied with MC'F suggesting far reaching conclusions for the mechanism of evolution of the Hawaiian Drosophilinae (Beverley and Wilson, 1985; Lewin, 1985). This same protein is found in all dipterans and could be a basis for a unified protein-clock time scale of dipteran evolution (Beverley and Wilson, 1984). Similarly, a major serum protein of cockroaches, LSP (Kunkel and Lawler, 1974), related to the arylphorins of holometabolous insects, was studied with MC'F (Kunkel and Nordin, 1985) in a broad selection of cockroaches showing LSP to have a long and conservative history including a strong relationship to a termite serum protein. Thus immunological relatedness studied with this particular Ag argues that termites occupy a position within the order Dictyoptera equivalent to families of cockroaches. Although some taxonomists would argue that the evolution of sociality gives termites as a group the right to occupy

their own order, a phylogenetic perspective concentrating on the time and branch points of divergences might consider termites to have originated as a social family of cockroaches. Using antisera against this and similar proteins, one might be able to unify the phylogenetic study of arthropods in general. The use of immunologic distances provides another quantitative measure with which to study organismal evolution.

IV. Conclusions

The techniques described in this chapter by no means exhaust the variety of possible immunological approaches to the study of insect Ags. They are rather a selected list of the frequently and personally applied approaches that have, in my view, had a useful impact on the field of insect biochemistry and physiology. Even among the techniques discussed, there are an abundance of variations that may be useful for answering a particular question. Such variations can be gleaned from the general literature on immunological techniques (see Axelsen, 1983) after one has a firm understanding of the basic principles. Each year new techniques and products become available, and the person familiar with the general procedures is more adept with new techniques or variants of old methods.

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Figure 1.1. Antigenic determinants and polyclonal antisera. A polyclonal antiserum is derived from multiple clones of AB-producing cells and may have multiple distinct bivalent ABs (A1, A2, A_3, h_4, B1, ..., D3) reacting to each determinant (A, B, C, D) on a single native Ag, depicted in the center. Determinants A, B, and C are depicted on the surface of a polyvalent globular native protein. Cryptic internal determinant, D, may be buried within an internal core of the native protein, and antibodies reactive with this latent determinant may react with it only when the Ag is in a denatured state. Each animal immunized with the Ag may make antibodies to some or all of these determinants. Avidity of a particular polyclonal antiserum is determined by a frequency-weighted average of individual affinities of the population of secreted ABs.

Figure 1.2. The basis of Ag precipitation by ABs is cross-linking and multivalency. An extended network of cross-linking can result in a precipitate. Every Ag is linked to another Ag through an AB, and every AB is linked to another AB through an Ag. The stoichiometry of Ag: AB to achieve complete Ag precipitation is approximately 1: 1. Minimal requirements for precipitation include either particles with multiple subunits bearing a single determinant (right) or multiple determinants on a single particle (left).

Figure 1.3. Classical Ag/AB equivalence in liquid. A fixed amount of AB is placed in a series of test tubes. A serial increasing concentration of Ag is added to successive tubes. At the low Ag concentration end of the series, no precipitate forms, because the excess of AB completely coats the determinants of the Ag, and there are no free determinants for an AB free end to cross-link with. In a region from 3 to 6 log units of Ag, all the Ag in the tubes is precipitated with available AB, generally at a higher than 1: 1 ratio. This situation is possible because many Ags have far more than two AB combining sites. As the Ag concentration is increased beyond equivalence, the frequency of soluble Ag aggregates linked by one or a few AB molecules increases. Finally, every AB molecule is linked in a soluble complex with two Ag molecules (Ag-AB-Ag), but there are no free AB molecules to cause further cross-linking.

Figure 1.4. An Ag/AB equivalence test in agar is a necessary prerequisite of any successful Ouchterlony double-diffusion test. Parallel sets of wells are punched in the same agar medium in which a final "for publication" test will be done subsequently. Care is taken to space the wells identically to the future spacing of the final test pattern. A constant amount of antiserum is added to a central tier of wells. Serial dilutions of Ag are placed in parallel tiers of wells, and the Ags and ABs are allowed to diffuse together for 1 to a few days. The sharpest precipitin lines form between equivalence concentrations of Ag and AB; a pure Ag will have one or two best dilutions forming a crisp line which will intensify over the span of a few days. In a complex mixture, the equivalence dilution of each Ag may be different; initially several dilutions will show sharp lines, but with time, Ag or AB excess is indicated by a blurring of the precipitin lines. The blurring is more enhanced by staining.

Figure 1.5. Microtitration to determine volume of antiserum and carrier Ag needed to achieve total precipitation of an Ag sample in a fluid precipitin reaction. This technique is carried out in microcentrifuge tubes. A fixed amount of carrier Ag (5-10 #g is sufficient) is added to each tube. An additional objective of this preliminary experiment is to find a convenient amount of carrier Ag providing a workable amount of precipitate that can be seen in the bottom of the tube after centrifugation, washed by resuspension in PBS, and repelleted several times without loss of precipitate. Increasing volumes of' antiserum are added to the series of tubes; precipitate is given time to form and is centrifuged out of solution. The supernatant is then tested for any Ag remaining in solution using any available micromethod, such as QIEP. The volume of antiserum that completely precipitates the carrier amount of Ag allows one to calculate an AB equivalence amount. Using a multiple of the equivalence volume of antiserum (4 x 32 = 128 #1) provides a liquid precipitin system that will always produce at least the minimal working precipitate with carrier Ag (tube on the left). This guards against AB excess. This system has the capacity to precipitate threefold more of "unknown" Ag without leaving any in solution (tube on right). If only the unknown Ag is labeled with radioactivity, the precipitate can be analyzed by diverse means to establish the nature and extent of labeling.

Figure 1.6. The ring test is a simple, relatively rapid test of immunological reactivity which is read by eye as positive or negative against a control tube with no reactive Ag in it. The bottom solution of AB is made more dense by addition of sucrose or glycerol. The top solution is overlaid carefully, and the position of the initially crisp interface is noted. Ag and AB diffuse together and precipitate at their liquid interface. In a "positive test" a precipitin is easily visible at the interface within a half hour.

Figure 1.7. The Oudin one-dimensional, single-diffusion assay. The antiserum is placed in an ionagar or agarose gel layer and is considered to be relatively stationary. An Ag dilution, of higher concentration than the equivalence amount of AB, serves as the single dominant diffusing component. The Ag solution is added to the top of the agar at a recorded time zero, and the Ag starts diffusing into the AB-agar phase. A precipitin front is seen to advance with time. For each individual tube the distance, d, of front nigration divided by the square root of the elapsed time, t, gives a constant value, k. This k-value is proportional to the Ag concentration, and a dilution series provides a calibration curve of k versus [Ag] for determining relative or absolute titers of unknown Ag.

Figure 1.8. Radial immunodiffusion is a two-dimensional, single-diffusion assay similar in nature to the Oudin test. As in the Oudin test, the AB usually serves as a stationary phase, and the Ag is usually the mobile or dominant diffusing component. Antiserum is dispersed in agar and coats a slide or Petri dish. Samples of Ag are placed in wells cut in the agar surface. Ag diffuses out radially, forming a precipitate front as it advances. The system runs out of Ag, and a darker rim of precipitate develops at the edge of the final precipitate. The diameter of this disk of precipitate is determined after staining and is compared to a standard curve established for that antiserum by a previous or concurrent set of serial dilutions of known Ag titer.

Figure 1.9. Quantitative immunoelectrophoresis (QIEP) or "rocket" IEP. The antibody is dispersed evenly in the thin layer of agarose on the slide. The quantity of Ag placed in each well can be estimated by the amount of AB area that is swept into a "rocket" of precipitin by the electrophoretically propelled Ag. This method, like radial diffusion, has a self-imposed end point which occurs when all of the Ag in the well has reacted with its equivalence amount of AB.

Figure 1.10. Ouchterlony double-diffusion assay. (1) Unbalanced serum. The multiple Ag/AB precipitin lines are not straight and curve away from the source of the excess component. (2) Balanced serum. The multiple Ag/AB precipitin lines are straight and crisp. This occurs when each Ag and AB are at approximate equivalence. Balanced complex antisera for Ouchterlony are difficult to achieve routinely.

Figure 1.11. Immunoelectrophoresis (IEP). Prior electrophoresis of a mixture of antigens in agarose is combined with a subsequent step similar to Ouchterlony double diffusion. After the electrophoretic separation in the first dimension, a trough is cut in the agar parallel to the direction of electrophoresis, and antiserum is added to the trough and allowed to diffuse toward and precipitate with the electrophoretically separated Ags. A mixture of Ags is shown in this example reacting with a crude antiserum (upper trough) and an absorbed antiserum (lower trough).

Figure 1.12. Crossed QIEP is the logical extension of IEP to QIEP. The first dimension can be run similar to IEP of Figure 1.11 above, or, for better resolution, the first dimension can be done in a vertical or horizontal agarose slab apparatus. The sliver of agarose from the first-dimension gel is mounted on a slide and supplemented with AB-containing agarose as in QIEP. The second-dimension electrophoresis of the Ags at a right angle creates AG/AB precipitin "rockets" with characteristic density of precipitate. Complex Ag solutions can be analyzed with complex antisera using this method. Fusion of adjacent rockets can be used to identify homologous electromorphs or isozymes.

Figure 1.13. Fused QIEP is a logical extension of the Ouchterlony double-diffusion test to QIEP. The samples with potentially related Ags are placed in their individual sample wells (1-6) cut in a basal strip of agar containing electrophoresis buffer but no antiserum. The Ags are allowed to diffuse for a time determined by experiment to allow sufficient comingling of the Ags. An antiserum gel, similar to that in QIEP, is added to the remainder of the slide after 2 h of diffusion, and the samples are electrophoresed into the antiserum agar. Ags in wells 3 and 4 depict an identity reaction; they form a continuous precipitin line similar to a line of identity in the Ouchterlony test. Ags in wells 5 and 6 show partial identity by forming a "spur" of precipitate rather than a continuous line. Samples 1 and 2, placed in their respective wells just

prior to elec-trophoresis, form distinct "rockets" of precipitin, untested for homology, but they leave any particulate matter contaminating the sample back in the gel surrounding the sample well.

Figure 1.14. One example of PAGE electroblotting, the Western blot. (1) O'Farrell (1975) 2D electrophoresis of antigens by isoelectric focusing in the first dimension followed by SDS-PAGE in the second dimension. (2, 3) Separated Ags are blot or elec-trophoretically transferred to one or more nitrocellulose membranes and probed with specific antisera. (4) Removal of the probing AB from the nitrocellulose membrane is possible, and reprobing of the still attached Ag with another AB is carried out.

Figure 1.15. 'Ouchterlony double-diffusion comparison of Ags. A specific or complex polyclonal antiserum is placed in a central well in an agarosc slab and allowed to interact with Ass diffusing toward it from two peripheral wells. (1) Ouchterlony demonstration of lack of homology between antigen A and antigen B. (2) Ouchterlony demonstration of complete identity of antigen A with A'. (3) Ouchterlony demonstration of partial identity between antigen A and A".