RADIOIMMUNOASSAYS EMPLOYING DOUBLE ANTIBODY TECHNIQUES

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ABSTRACT

Double antibody methods are applicable to all radioimmunoassay systems. At least three approaches exist. The first, in which immunoprecipitating serum is used to precipitate soluble antigen-antibody complexes, is the most commonly used. Since the effects of interfering factors, especially those present in serum, become minimal as equilibrium is approached, care must be taken to insure that the period of incubation is of sufficient length for the immunoprecipitation reaction to reach equilibrium. In general, all efforts should focus on achieving reaction conditions which maximize stability and optimize immunoprecipitation. A second approach involves use of the first antiserum in an insolubilized form after prior precipitation with the immunoprecipitating antiserum. This approach is attractive since most non-specific factors cannot interfere with the immunoprecipitation step to a significant extent once the formation of antigen-antibody aggregates is well underway. The third approach, in which immunoprecipitating antiserum is conjugated to a solid matrix, in principle represents a more ideal method of separating bound hormone from free. If this approach is ever to be utilized, a method of conjugating antiserum to a suitable matrix such that the antibody retains most of its immunoreactivity must be devised.

* Career Development Awardee of The National Institute of Child Health and Human Development U. S. P. H. S.
Radioimmunoassays differ primarily in the method used for separation of that portion of labeled antigen which is bound to antibody from that portion which is free. The numerous procedures for accomplishing this separation vary greatly in complexity and may influence resulting assay reliability. Double antibody techniques appear to be unique since they can be applied to essentially all radioimmunoassays, including, on the one hand, assays for low molecular weight haptenic substances like steroid hormones and, on the other, high molecular weight proteinaceous hormones. Double antibody methods are flexible and can be used to provide rapid results (with some sacrifice in sensitivity), or they can be used to obtain results of unsurpassed sensitivity, precision, specificity, accuracy, and reproducibility. Under the latter conditions it is possible, with the aid of automatic gamma counting equipment and computerized data processing, for one technician to set-up and analyze 1000 determinations per week. Double antibody techniques can be used with any available antiserum and with any labeled antigen. Methods developed for an antiserum obtained from one particular species of animal are directly applicable to other antisera from the same species.

With these features, one might wonder why double antibody techniques are not used in all radioimmunoassay systems. The answers are varied: many relate to unique features possessed by a specific labeled antigen; others stem from unwillingness to alter an existing method; however, most result from insufficient understanding of double antibody techniques and their application.

New double antibody methods continue to be developed. The range of possibilities includes: a) Use of immunoprecipitating serum («second antibody») in the usual way, i.e., to precipitate soluble antigen-antibody complexes, a method which here will be referred to as «post-precipitation», b) Use of the first antiserum in an insolubilized form after prior precipitation with the immunoprecipitating antiserum, here termed «pre-precipitation», and c) The use of immunoprecipitating antiserum conjugated to a solid matrix.

Use of Post-Precipitation

The first of these methods, post-precipitation, is the one most widely used and abused. In essence, it is the application of a quantitative precipitin test to diluted reagents on a micro-scale in which the first antibody serves as an antigen for a second antibody prepared in a different species. Principles underlying this type of analysis have been known for years and were well described by Kabat & Mayer (1961). These authors point out that optimal immunoprecipitation requires an ionic strength approximating 0.15, pH between 6.5 and 8.5, antigen and antibody present in fairly high concentration at equivalence, a temperature between 0 and 37°C, and a period of incubation of
length sufficient to ensure that the reaction reaches equilibrium. Active complement, present in fresh serum, will delay specific precipitation but cause more complete precipitation of soluble complexes of antigen and non-precipitating antibody. Although the initial combination of antigen and antibody occurs within seconds, complete quantitative precipitation requires a prolonged period of incubation. The speed of precipitation is known to depend on the concentration of antibody; although two hours at 4°C may be sufficient for quantitative precipitation with undiluted, hyperimmune sera, diluted antisera require more than two days, and optimal reaction time often approaches one week. Temperature effects vary with individual antisera, some requiring low temperatures for completion of the immunoprecipitation step and others giving almost identical results at 0°C and 37°C.

Post-precipitation, double-antibody methods require care analogous to that employed for quantitative precipitin tests. Since the amount of first antibody to serve as antigen is present in extremely low quantities, and since each assay tube has a slightly different composition due to the presence of variable amounts of unknowns, it follows that all efforts should focus on conditions which maximize stability and optimize immunoprecipitation. This implies that the above principles, outlined by Kabat & Mayer (1961), should be followed with meticulous care.

Such considerations seem to have been ignored by many investigators employing post-precipitation radioimmunoassays. A number of studies have been reported based on the use of highly diluted first antibody without non-immune globulin added to increase the antigenic bulk. Since, as might be expected, serum influenced the results, these and other investigators have tried a number of variations in an attempt to minimize the non-specific effects of serum. These efforts have included attempts to inactivate complement by heating samples and by adding chelating agents (Morgan et al. 1964; Sheldon & Taylor 1965; Grant 1968). In addition, neutral antigen-antibody precipitates have been added to aid in collection of the precipitate (Quabbe 1969), »hormone-free« serum has been added such that all tubes contained the same volume of serum (Odell 1969; Goodfriend 1969; Thorell 1969), the possibility of cross-reactions between the gamma globulin in the first antibody and the gamma globulin in the sample has been studied (Hales & Randle 1963; Morgan et al. 1964; Kuzuya & Samols 1964; Welborn & Fraser 1965), and samples of serum have been diluted such that serum components do not interfere (Morgan & Lazarow 1963; Welborn & Fraser 1965; Melani et al. 1968).

Although each of these modifications have been reported to reduce the interfering effects of serum on the immunoprecipitation step and thereby have permitted reductions in the incubation time of this step, it does not appear that the major goal of some investigators has been to obtain maximal precision, accuracy and freedom from interference of all potential factors including
those in serum. Attainment of this goal should include serious consideration of the role of time on the rate of completion of the immunoprecipitation step. The observations reported by Kabat & Mayer (1961), our data (Midgley 1969), and the data in essentially all published reports concerning the role of time on the completion of the precipitation step have indicated that this step requires days, not hours, to insure completion. As incubation time was increased, the effects of non-specific interfering factors decreased (Soeldner & Slone 1965; Welborn & Fraser 1965; Grant 1968). The effects of interfering factors are increased as reaction conditions depart from those which are optimal at equilibrium and are minimized by prolonging the immunoprecipitation step so that it reaches equilibrium. Since different samples alter the incubation mixture in various, unpredictable ways, it would seem desirable to employ optimal conditions at all times.

It would be impossible to describe the ideal set of reaction conditions, although a number of factors can be mentioned. Since immunoprecipitation is dependent to some extent on concentration of antigen (here equivalent to the first antibody with its bound hormone), and since this concentration is extremely low, non-immune globulin of the same species as the first antibody must be added. This may be done at different times. Some investigators have added carrier globulin with the second antibody, others have added it as part of the diluent for the sample, while we and others have included it as part of the diluent for the first antibody. We have chosen to dilute our full-strength initial antiserum 400 fold in a buffer containing 0.05 M EDTA, 0.01 M sodium phosphate, and 0.14 M sodium chloride, pH 7.0. The EDTA was included as a chelating agent to minimize differences in complement and as a means for increasing the total buffering capacity in our incubate. All subsequent dilutions of first antibody have been made in a large pool of non-immune serum diluted 400 fold in the same buffer. Two tenths of a ml of this mixture are added to each tube and incubation with immunoprecipitating antiserum is carried out for 72 hours at 4°C. Thus, the amount of globulin in each tube is held constant at the amount present in a 1:400 dilution of non-immune serum. By insuring that the second antibody is at equivalence with this amount of globulin, then any appropriately diluted antiserum of the same species can be utilized without rechecking on the conditions for equivalence. The use of 0.2 ml of a 1:400 dilution of non-immune serum was chosen because it provided a reasonable bulk of precipitate, gave precise results, and did not require a large amount of second immunoprecipitating antibody. Furthermore, larger amounts of precipitate were found to slip down the walls of round-bottomed, disposable culture tubes during decantation which thereby necessitated the use of an aspiration step. We found the latter to be slightly less precise and to be more time consuming, especially with larger volumes of supernatant fluid. To facilitate decantation with an incubation mixture con-
taining 1% protein, we have found that tubes with an internal diameter greater than or equal to 10 mm are optimal. Further, we have found that addition of 3 ml of cold buffer to the incubation mixture just prior to centrifugation can obviate the need for a wash step. The major limitation of the procedure outlined above is the danger of using dilutions of first antibody close to 1:400. This assumes that the globulin concentration in the immune serum is at an identical concentration to that in the non-immune serum. Often this is not the case. Failure to consider this point can lead to an apparent decrease in bound labeled hormone as first antibody concentrations approach 1:400.

It has been argued that while a long immunoprecipitation step is ideal, in practical terms results are desired more rapidly. This view is often expressed by investigators who employ a 4 day first incubation step («equilibrium conditions»). They feel that more than one additional day for immunoprecipitation is not practical. However, it is possible to obtain comparable results with a shorter first incubation period. By employing non-equilibrium conditions one can complete the first period in a maximum of two days, use three days for immunoprecipitation, and thus complete the assay in the same five days. We employ a one day incubation at 4°C of first antibody and sample followed by a second day with labeled hormone added. To obtain the same proportion of antibody-bound labeled hormone a higher concentration of first antibody is required, but the inhibition curves are essentially superimposable when compared to those obtained under equilibrium conditions if the appropriate antibody concentrations are chosen.

If more rapid results are desired, they can be obtained, but at a sacrifice in sensitivity and specificity. Thus, the first two steps of the non-equilibrium reaction can be performed in a few minutes of time and the second immunoprecipitation step can be terminated after a few hours. The short second step necessitates a considerably higher concentration of antibody to bind a reasonable amount of labeled hormone which decreases sensitivity. The very short immunoprecipitation step contains all the dangers referred to above, but when unknowns and standards are closely similar, ideally differing only in content of hormone, this technique can be used with care. For example, with an appropriate incubation schedule, peaks in serum LH can be located in this fashion with a total assay time of four hours.

Use of Pre-Precipitated Antibody

This technique appears to have been described first by Hales & Randle (1963) in an attempt to avoid interference by factors in serum. It is of interest that they employed no carrier non-immune globulin with their diluted anti-
serum. In their methods the first antibody was allowed to react with the immunoprecipitating antiserum for 16–24 hours before labeled hormone and standard or unknown were added. Incubation was then continued for 22 hours under equilibrium and non-equilibrium conditions.

This approach, in which the immunoprecipitating step is allowed to proceed to near completion under identical conditions in all tubes, is particularly attractive. Although immunologic reactions obey the law of mass action and are reversible, the association constants are sufficiently high that it is unlikely that minor interfering factors can have a significant effect once the formation of the antigen-antibody aggregates is well underway. Although this approach has been used (Wilde et al. 1967; Jorgensen 1969), surprisingly it has not been studied extensively. Part of the reason undoubtedly lies in the need to know the number of tubes desired in an assay at least one day in advance of actually adding the sample. Another explanation may relate to sensitivity and utilization of antibody. We have found that pre-precipitation techniques require considerably more antibody (5 to 10 fold) to bind the same proportion of labeled hormone and that under these conditions the assays had lower sensitivity and precision. Part of our difficulty may stem from having included carrier non-immune globulin with the first antibody so that a larger precipitate resulted, the major portion of which consisted of non-immune globulin. The practicability of this approach needs to be studied in more detail.

Use of Immunoprecipitating Antibody Conjugated to a Solid Matrix

To our knowledge, this method has not been reported. In principle it should represent a more ideal method for separation of bound from free hormone. The first reaction could be allowed to proceed in a soluble state for any time period desired. The reaction could then be terminated by addition of an insoluble form of immunoprecipitating antibody. Non-specific factors should not interfere with the secondary reaction. The major problem with this approach will be to develop an efficient method for conjugating the antiserum to a suitable matrix such that the antibody retains most of its immunoreactivity.

Miscellaneous Topics

Double-antibody techniques in general appear to be relatively free of problems with pre-existing »damaged« labeled hormone. Double-antibody methods do not depend on properties of the labeled hormone other than immunoreactivity.
The ability of the hormones to bind to some solid adsorbent is not required. With double-antibody techniques, only the immunoreactive hormone is examined, the »damaged« fraction does not bind to antibody and is not included in the precipitate. Thus, a fairly large fraction of damaged labeled hormone could be present and have little effect on the assay other than result in a slight decrease in binding by diluted first antibody and little or no measurable effect on binding with excess antibody. Some forms of »damaged« hormone can result in a higher background in control tubes containing precipitated, non-immune serum. However, this component usually remains low relative to the amount of labeled hormone bound to antibody, and can be subtracted. The major effect of subtracting a high background is a decrease in precision. Preliminary studies in our laboratory have indicated that a large portion of the labeled hormone remaining in control tubes containing precipitated non-immune serum is adsorbed to glass rather than bound to the precipitate.

Pre-precipitated antibody techniques are ideally suited for obtaining rapid results and for automation. The presence of bound and free labeled hormone in two different physical states facilitates separation by a variety of techniques including filtration, dialysis, sedimentation, and centrifugation. These separation methods can be incorporated readily into the existing automatic equipment for chemical analysis. Furthermore, the existence of antibody in a pre-precipitated form eliminates one of the most time-consuming steps in the double antibody technique. By way of example, Abraham (1969) reported on the use of dextran-coated charcoal to assess »damage« in freshly labeled hormones. He demonstrated a close correlation between results by this technique and results obtained by a pre-precipitated double antibody technique and results obtained by a pre-precipitated double antibody technique and claimed the advantage of dextran-coated charcoal to be speed (approximately 60 min). However, in our laboratory, by using tubes containing pre-precipitated antibody, stored frozen, it has been possible to obtain a direct assessment of immunoreactivity of labeled hormone with a one hour incubation and a 20 minute period of centrifugation.

Before terminating this discussion, one note of caution should be raised. Antibodies have been described in serum protein fractions other than classical 7S or IgG immunoglobulin. If an antiserum contains specific antibodies in one immunoglobulin fraction, such as IgG, and non-specific antibodies in another fraction, such as IgA, then resulting assay specificity could depend to some extent on which of these two immunoglobulin fractions was at equivalence with the immunoprecipitating antibody. Thus, it is conceivable that specificity could vary with the properties of the immunoprecipitating antiserum. Although we have found no evidence to indicate that the problem exists to a significant extent, being forewarned is being prepared.

253
REFERENCES

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DISCUSSION

Hunter: There is no doubt that anyone of us can select a hormone-free plasma on the basis that in our system it gives no different curve from the standard curve in diluent. What is required, however, is to select a group of sera which one honestly believes to be hormone-free, to put all that large group of sera into the assay and see if one can then reproduce the standard curve in the presence of each of these sera. We have studied a group of animal sera, and although one could select one animal, in this case the sheep, to show curves not different from the standard curve in diluent, one could select others, for example the horse or the rabbit, in which great differences were found between the standard curve in serum and in diluent.

Midgley: I agree.

Odell: In my opinion, the immunoabsorbed serum is not an appropriate hormone-free serum for the following reasons: one takes the very serum one is going to run the immunoassay with and precipitates all of the substances that react with that anti-serum. Part of them are hormones and part of them are non-hormonal materials. Then one takes the serum which is freed from all these materials and ascertainment
whether it reacts in the immunoassay. Almost by definition, one has made it non-reactive in the immunoassay.

**Midgley:** This comment relates almost exclusively to the primary antigen-antibody reaction, not to the immunoprecipitation step. When one uses a hormone-free plasma prepared by immunoabsorption, only the hormones are removed, not the non-specific factors which interfere with the double antibody immunoprecipitation step. Yet the latter is probably the major reason for adding serum.

**Stevens:** You have demonstrated the effects of EDTA on the second antibody precipitation step of the method. Do you know whether there are any effects of EDTA on the first antigen-antibody reaction?

**Midgley:** No. We added EDTA, tested it in a number of radioimmunoassay systems and did not notice any change in immunological activity in any of the samples of serum or standards tested. The addition of EDTA did increase the buffering capacity of our tubes quite considerably, since our buffer consisted solely of 0.01 molar phosphate.

**Odell:** We have very systematically evaluated the possibility of using pre-precipitating antigen-antibody combinations, because they do have theoretical advantages and we have the possibility of their use for TSH, LH and FSH. We found that we do not get good precision, nor even very reliable equilibrium displacement of the radioactive hormone that is bound in this mixture before the assay takes place. We came to the tentative conclusion that there might be a very different ability to displace competitively the radioactive hormone pre-bound for very small polypeptides, such as insulin, as opposed to larger hormones, such as gonadotrophins.

**Midgley:** In the pre-precipitated state, it is possible that antibody molecules are at a disadvantage because they are partially interlocked in the precipitate. This would mean that affinity for labelled antigen would be considerably less.

**Odell:** Yes, in addition, the stereoindrance by these large molecules may be really significant, as compared to a little hapten.

**Wide:** I would like to comment on two points made by Dr. Midgley. Firstly, the problem of adsorption of unbound hormone onto cellulose acetate membranes may be minimized by washing with a solution containing a high concentration of protein such as albumin or horse serum. Secondly, when a pre-precipitation step is employed, the antiserum may be pre-incubated in bulk and aliquots lyophilized in ampoules for later use, thus anticipation of the quantity required at any particular time is unnecessary and the assay may be completed within 24 h of receiving the specimen.

**Eshkol:** We found that non-specific adsorption to cellulose acetate filters (Oxoid and Millipore) could be prevented by immersing them prior to their use in a 2–5 % solution of bovine serum albumin.

**Midgley:** Bovine albumin is often contaminated with bovine luteinizing hormone. That is the reason why we stopped using it. We have switched to a more inexpensive protein, namely lyophilized or fresh egg white.

**Saxena:** Dr. Midgley, in your paper, on page 7, you indicate that with double antibody technique only the immunoreactive hormone is examined. The damaged fraction
does not bind to the antibody and is not included in the precipitate. You say that after incubation you may have 35 or 40% damage. You start with 10,000 counts/min of intact label, and if 30% is lost for the immunoreaction, how do you account for the effect of the loss of this labelled tracer on the assay?

**Midgley:** I did not say that in our system we have 30% damage. I said that even if one started with 30% iodination damage, the damaged component would not react with the first antibody and therefore would not appreciably influence the resulting assay. The starting bound/free ratio would be lower.

**Odell:** Damage of iodine preparations in the double antibody techniques may show up in three different ways: a) the damaged hormone may fail to react with the first antibody, an apparent lowering of the B/F would appear, b) subtle alterations in affinity of binding to the first antibody may occur, c) damaged hormone may adhere to gamma globulins used as carrier in the buffer and be thus precipitated in the second antibody. Such labelled hormone would appear as a "high blank" in control tubes containing no first antibody.

**Midgley:** We have found that labelled rat prolactin occasionally gives a very high background, as manifested by a high count rate in control tubes containing precipitated non-immune globulins. We initially presumed that this represented adherence of the rat prolactin to the globulins, but actually found that it was due to adherence to the glass tube. The precipitate could be removed from these tubes without greatly decreasing the bound radioactivity, and comparable binding was found in the tubes containing only labelled rat prolactin plus our diluent (phosphate buffered red saline with 1% egg white).