Basic Principles of Radioimmunoassay Testing: A Simple Approach

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This is the first article in a new four-part CE series on radioimmunoassay. After reading and studying this paper, the reader should be able to: 1) describe the fundamental concepts of radioimmunoassay techniques; 2) discuss the various components in radioimmunoassay testing; and 3) understand the reaction principles.

This article reviews the principles of radioimmunoassay (RIA) technique, describing both its conventional concepts and current state-of-the-art. The approach is to discuss the various components involved in RIA testing, and how they interact in the reaction (test) tube. Also included are various conditions that affect these reactions and how to deal with them in a normal working environment.

The historic background leading up to today's concept of RIA will not be addressed since the infancy may best be reviewed in the classic references by Yalow and Berson (*1–3*). This article will approach this subject by looking at what is involved in setting up an RIA procedure. Most "radioimmuno-assayists" (a term coined around 1978 with the founding and development of the Clinical Ligand Assay Society) today use a wide variety of commercially available kits. Those unique individuals who have the expertise, a stringent reagent Q.C. program, and time to develop their own RIA reagents will not find this article of great use. It is designed primarily to help the routine RIA technologist who is pipetting from pre-packaged, prediluted, precontrolled kits. It is written for those who would like to know more about from where those little bottles of "colored water" come, what went into their preparation, what do they contain, and, most of all, what kind of "magic" is occurring when one mixes them together in a test tube. The term "magic" is used because there is a lot occurring in that test tube which cannot be observed.

This article is divided into three parts. Part I deals with components and is subdivided into four sections (primarily because there are four basic steps in setting up an RIA procedure): a) sample or standard; b) label or labeled antigen; c) primary binder; and d) separation step. Part II deals with the reactions occurring in the test tube when these components from Part I are combined. The principles to be discussed are RIA, competitive protein binding (CPB), sequential saturation analysis, immunoradiometric assays (IRMA), and radiolabeled binding assays. Part III is a brief discussion on how enzyme and fluorescence immunoassays compare with the RIA assays.

PART I—THE COMPONENTS

Sample or Standard

More often than not, the first pipetting step in any RIA procedure is to add either the patient sample or standards into their respective test tubes. Each will be discussed individually.

Sample

The sample is the unknown and in almost all radioassay procedures, with rare exception, the only variable. It shares the term of unlabeled antigen with the standard, the difference of course being that the standard has known concentrations whereas the sample concentration is unknown. Just about any sample type can be analyzed in an RIA procedure, but most kits are generally designed for serum or plasma only. One must be aware of the protein dependency of the kit's components. Not just any specimen type may be analyzed. It must contain a protein concentration similar to that found in serum. Other biological fluids on which radioassay procedures are frequently ordered are urine, spinal fluid, and most body fluids. There are, of course, the receptor assays on tissue biopsies and actual tumor tissue. In routine RIA procedures, spinal fluid and urine specimens are frequently submitted for analysis. These are major problems because their protein content is roughly 300–1,000 times less than that of serum (*4*). One approach to compensate for the protein content of these specimens is to prepare a dilution of the body fluid, generally 1:2, using the blank serum or a pooled serum. Blank serum means that it does not contain any of the constituent being measured. The result will need to be multiplied by two. Most reagent or kit manufacturers have not established reference ranges (normals) for body fluids other than for serum or plasma. The exception, of course, would be if the kit is designed specifically for measurement of a specific body fluid. A myelin basic protein kit is designed for cerebrospinal fluid only.

Standard

A *primary standard* is prepared from a constituent that can be extracted and purified from a tissue source or can be synthesized. It is a standard that can be weighed out and an appropriate dilution made and used in any assay. Some primary standards that are readily available in purified form (and relatively inexpensive) are: the thyroid hormones, T_3 and T_4, cortisol, and other adrenal and most gonadal steroids.

A *secondary standard* is used when the primary standard needed is not easily synthesized or purified from tissue extracts

and not readily available. It generally consists of a pool containing the desired analyte which is analyzed against the primary standard. Then, the value that is obtained from the primary standard curve is assigned to that pool. This pool then becomes a secondary standard and is typical of what is included in most kits. Examples of compounds that are not readily available in purified form, against which the secondary standards are calibrated, include the peptide hormones: FSH, ACTH, TSH, HCG, prolactin, and growth hormone. Kit manufacturers will maintain a supply of this primary standard by which they can calibrate their secondary standards. Most kits of this type contain secondary standards.

When discussing standards, the matrix should be considered. Matrix refers to the diluent used to dilute the standard. Standards, whether primary or secondary, are diluted in some form of protein base or matrix. (Most RIA reactions are protein dependent.) Ideally, the matrix to use would be that of human serum for assays where direct pipetting is done from an unextracted sample. This matrix must be relatively free of the analyte to be measured. Human-free serum is frequently prepared by literally dumping Norit charcoal into a hepatitis-free serum pool. It is mixed overnight, followed by filtration of the pool to remove the charcoal. This process, referred to as "stripping," will remove most components or analytes or effectively reduce their concentration so that they may be used as a free diluent or a free serum. The most common serum matrix is human serum. Second to this are human serum components, such as human serum albumin. Other popular, but less desirable, matrices are bovine serum albumin, horse serum, and bovine gamma globulin. The matrix may simply be a buffer. What is important is that the matrix closely approximate the binding characteristics, constituents, and pH of the patient sample. As an example, for the assays that require an extraction step and reconstitution in a buffer, the standards will be diluted in that same buffer. If the matrix does not approximate that of the patient's sample, an accuracy problem may arise. The concentration of the standard may not bind the same as that in the patient's sample. Thus, the level of the patient's serum may not be as accurate as it should be. Manufacturers, however, will generally compensate for this problem by adjusting the concentration of the standards to correspond to expected patient sample ranges. This could wreak havoc with an assay by changing the matrix they use. This change can markedly affect the accuracy of the analyte being measured, and more than likely result in a shift in quality control ranges.

Label

In an RIA technique, a label has many synonymous terms such as tracer, trace, labeled antigen, tagged material, the tag, the hot stuff, etc. Generally, it is a purified form of the analyte that one attempts to measure in the patient's sample. The difference is that it has gone through a labeling or tagging process by which a radioactive molecule has been attached. There are numerous methods available for protein purification prior to tagging and/or immunization (5). As was mentioned earlier in the section on standards, steroids can be obtained in rela-

tively pure form and generally do not require further purification prior to immunization or labeling.

One of the more frequently used isotopes for labeling in a clinical laboratory is iodine-125. Tritium (H-3) is less frequently used. Cobalt-57 was at one time used exclusively in the vitamin B-12 assays, since the cyanocobalamine molecule, B-12, contains a cobalt molecule in which it is easy to substitute a cobalt-57 atom. Recent advances in dual labeled kits, such as FSH/LH, utilizes Co-57 attached to LH and I-125 attached to FSH.

Iodination

Iodination of various compounds used in an RIA laboratory falls under two generalized categories. One is a direct iodination, which requires an oxidation reduction reaction that attaches the iodine-125 directly onto an aromatic group of the molecule (6–8). The second method, indirect iodination, attaches the iodine-125 to the aromatic group of a hapten or ligand such as tyrosine, and then this ligand is attached onto the analyte to be tagged (8).

All methods for iodination require purification of the newly tagged material by removing from the mixture any untagged antigen and all of the free iodine that did not tag to the analyte. There are a number of procedures that can be used to purify the newly labeled compound, such as Sephadex filtration, chromatography, electrophoresis, and dialysis (9–14).

Tritiation

Most laboratories do not do their own tritiated labeling primarily because of the commercial availability of low cost, pre-labeled tritiated compounds, mostly steroids. The labeling is accomplished by a reduction reaction involving the antigen (steroid) to be tagged with tritium gas (15). One aspect that must be considered with respect to purchasing labeled compounds, especially tritiated steroids, is that even though H-3 has a 12.3 yr half-life, it does not mean that the integrity of the tagging mechanism is stable for 12.3 yr on the shelf.

It is always recommended that the purity of the tritiated steroid be confirmed by chromatography prior to use (16,17). Manufacturers of H-3 steroids always report the radiochemical purity at the date of manufacture and also indicate the rate of degradation. In addition, they periodically confirm the purity every 3 to 6 mo. Degradation occurs because the H-3 (pure beta particle) is in direct contact with the steroid to which it is tagged, and some radiation damage is directed to the molecule itself. With time, the H-3 will become detached from the steroid compound to which it is labeled. By performing the purity quality control on a regular basis, it has been the experience of this investigator that problems with the tracer will be detected before manufacturer notification. This detection could prevent assay reruns and patient redraws due to faulty labeled antigen. Further, one can monitor the rate the label is coming off of the steroid and determine when to purchase freshly tagged steroid. For some assays, such as free testosterones, the purity of the tritiated compound is checked weekly. Fresh H-3 steroids are routinely obtained at least every 6 mo.

Advantages of Iodinated over Tritiated Labels

1. Less of the purified antigen needs to be labeled and less antigen mass improves the sensitivity of an assay.
2. Higher specific activities of radioiodinated steroids can be produced since more radioactivity per molecule of antigen is obtained.
3. Decreased mass of antigen in the assay requires less antiserum when compared to tritiated steroids.
4. Higher specific activity can also be used, thus permitting higher counts and perhaps shorter counting times (18).
5. The ease of handling is enhanced because, for most assays, it is not necessary to decant into another tube (or vial).
6. There is no need to handle hazardous organic solvents associated with liquid scintillation cocktail.
7. The need for liquid scintillation quench correction is obviated with iodinated sample counting.

One disadvantage of using an Iodine-125 label is, of course, its short half life (59.7 days) as compared to the half-life of 12.3 yr for tritium. As a result, radiolabeling is done more frequently when iodine-125 is used as the tag.

Purified Antigen for Labeling

The ideal labeled antigen is the same purified antigen that was used in the immunization process to produce the specific antibodies. When the antigens are identical, the RIA kinetics of the system will have the highest affinity for the antibody sites of any antigen available. This "ideal" situation is, of course, extremely rare. An unexpected low binding may be caused by the fact that the affinity of the antibody for the labeled antigen is less than acceptable. One cause could be the damage that has occurred to the antigen during tagging. Traumatization during tagging can structurally change the sites the antigen once had for the antibody. This must be considered when binding problems occur, especially with a fresh tag. The quality of the iodine used in tagging is also important. Even kit manufacturers occasionally encounter bad batches of iodine. The initial tag may be fine, but may deteriorate within 24 to 48 hr. During the assay, it will appear as though not enough tracer is binding to the antibody (low counts). Alternatively, it could also effectively "alter" the standard concentration. Because the iodine-125 is no longer attached to its respective antigen, the antigen is now "cold" or an unlabeled antigen exactly like the standard.

Primary Binder

In RIA procedures, the primary binder is a specific antibody. This antibody has antigenic sites specifically for the antigen or the analyte to be measured. Production of antibodies requires immunization of a particular animal with the purified antigen or analyte, called an immunogen. The more commonly used animals are rabbits, sheep, goats, monkeys, mice, rats, chickens, and donkeys. The type of antibody to be produced will govern what kind of immunization schedule is best (4). Most, but not all, immunizations require that a second or "booster" injection follow 4 to 6 wk after the initial injection so that an adequate titer of antibody is achieved (19,20). The RIA technique is used to determine the presence and/or titer of an antibody. A radioactive tracer for the analyte measure-

ments is added to varying dilutions of antibody to see if the tracer binds. Then, determination as to what dilution of the antiserum will bind to approximately 50% of the working tracer used in the assay can be made (21).

In a conventional RIA procedure, the antibody concentration should be limited so that the antibody will be saturated by one-half of the tracer in the absence of unlabeled antigen (standard). If a higher concentration of antibody will reduce the sensitivity in the lower range of your standard curve, then the converse is also applicable that a lower concentration of antibody will reduce the sensitivity in the higher range of the standard curve (Fig. 1). Thus, 50% of trace binding is optimum. To create an optimum condition for the binding of the assay, adjust the antibody titer to correspond to the preferred concentration range. Diluting the antibody will improve the sensitivity in the low range, whereas increasing the concentration of the antibody will improve the sensitivity in the high range.

In the immunization process, following the injection, the immunogen seeps out from the emulsion. The host animal considers this antigen to be foreign and will respond by producing antibodies (22). The spectrum of antibodies released into circulation will be capable of binding the purified antigen in the assay with varying degrees of affinity(23). An obtained antibody titer represents a mean titer for the diversified population of antibodies produced by the host organism. Antibodies that bind the antigen most effectively are said to have the highest affinity. Those with low affinity may have a high affinity for another segment of the antigen molecule which may resemble a similar compound, and thus
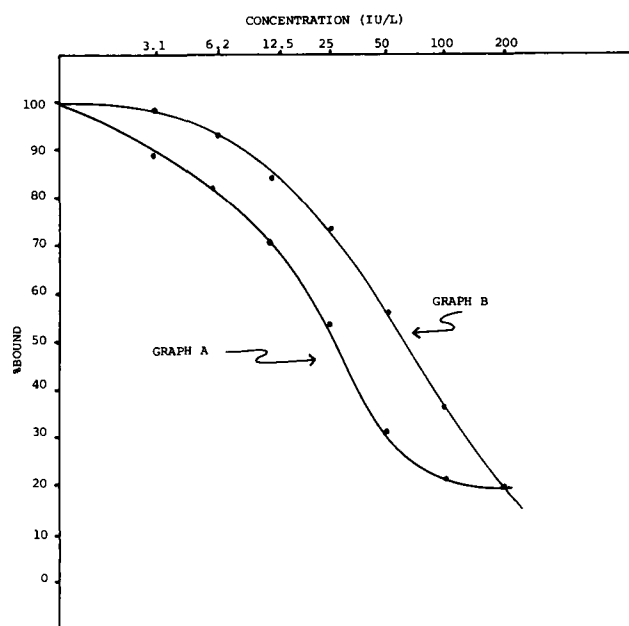

FIG. 1. Graph A represents an RIA standard curve generated when a working antibody titer is further diluted 1:2. Note the improved low-end sensitivity and loss of high-end sensitivity. Graph B represents a standard curve generated when the concentration of antibody is 2 times the normal working titer. Note the loss of low-end sensitivity and greatly improved high-end sensitivity.

may have a high affinity for another similar compound (cross-reactivity). When the antibody population consists of such an aggregate of antibodies, it is called a polyclonal antiserum.

How well the antibody detects what it is designed to detect determines the specificity of the antiserum. If it will bind with other similar compounds, it is said to cross-react with these other compounds. If the antibody has high cross-reactivity to other related compounds, it is considered to have low specificity. An antibody with high cross-reactivity to structurally related compounds may not be desirable or may require that special steps be taken in the RIA prior to using this antibody to remove cross-reacting or interfering substances from the patient's sample. An example of this would be the extraction steps that are sometimes required with certain RIA procedures and/or the chromatography (purification step of the patient sample prior to analysis). Another approach to improving the specificity of an antiserum is to supersaturate the antibodies present in the antiserum with an excessive amount of the antigen with which it cross-reacts. By doing so, all of the binding sites for this antigen are occupied prior to its use in the assay, thus eliminating any observable cross-reactivity. This process is referred to as absorption of the antibody (22).

Recent advances in the field of antibody production have virtually eliminated antibody cross-reactivity at the level of the cell production. Monoclonal antibodies have been in use in clinical labs for almost 3 years. In brief, the production of monoclonal antibodies begins with immunization of the mouse, quite similar to the immunization of a polyclonal antibody. The spleen cells of mice with the highest titer antibodies are fused with mouse myeloma cells in the presence of polyethylene glycol. The rapidly dividing fused myeloma spleen cells are called hybridomas. Those hybridoma cells with the desired antibody purity and specificity can then be injected into the peritoneal cavity of mice who will, in turn, produce extremely large quantities of this antibody, which accumulate in the ascites fluid of the mouse. The success rate, however, is low. Approximately five of the desired hybridoma antibodies are produced out of the 200,000 fused hybrid cells that can be produced from the billions of spleen cells from a single animal. The entire culture and selection process takes about 4 mo (24,25). The advantages of monoclonal antibodies are that they are highly specific and can be maintained in cell culture indefinitely. This advantage eliminates one of the significant problems in RIA, that is, antibody lot changes which frequently result in differences of affinity and specificity from

lot to lot. Once the monoclonal antibody is produced and cultured, it is all but guaranteed that this same antibody will be present in all kits. Even though the lots of all the other reagents change, the antibody lot will remain the same.

Binding Proteins

The primary binder in a radiolabeled assay need not be an antibody. For certain assays, it is simply a natural protein that is known to bind with high affinity to the analyte to be detected. Historically, a number of radiolabeled assays use protein binders instead of antibodies. Assays of this type are referred to as CPB assays. Table 1 lists some of these procedures and their corresponding protein binders. Of the assays listed, only vitamin B-12 and folic acid are still widely used CPB assays. The others have been replaced by RIA techniques as antibodies for each analyte became available and as the quality of antibody was improved.

Intrinsic factor is used exclusively in vitamin B-12 assays as the primary binder. In vivo, intrinsic factor is found in the cell membranes of the gut. In order for ingested vitamin B-12 to be absorbed from the gut into the blood stream, it must first bind to intrinsic factor for passage across the gut membrane. Around 1979, controversy surrounding the impurity of the intrinsic factor used in vitamin B-12 kits sparked an intense study by the National Committee for Clinical Laboratory Standards (NCCLS). It was determined that the impurities in the intrinsic factor (called "R" proteins) would bind other B-12 analogs present in serum, in addition to vitamin B-12. As a result, binding this could mask the true vitamin B-12 status of a patient (26). All vitamin B-12 kits currently on the market contain purified intrinsic factor, or at least have the "R" protein sites blocked.

Folic acid, historically, was first measured by microbiological techniques (as was vitamin B-12). This method for folate was replaced by a CPB technique after it was found that a purified protein in milk called beta lactoglobulin would act as a binder. This has been employed in folate radioassays ever since (27).

It is important to understand the relationships between the three components, sample, label, and primary binder, involved in the reaction mixture. In a conventional RIA procedure, the sample or standard competes with the tracer for the limited number of antibody binding sites. When these components are first introduced to each other, there is a first order reaction in the direction of binding to the (until now occupied) antibody sites. This reaction continues until equilibrium is established

TABLE 1. Competitive Protein Binding

Test Name	Protein Binder
Progesterone	Progesterone binding globulin
Testosterone	Sex binding globulin
Cortisol	Transcortin (cortisol binding globulin)
T_4	Thyroxine binding globulin (TBG)
Vitamin B-12	Intrinsic factor
Folic acid	Beta lactoglobulin

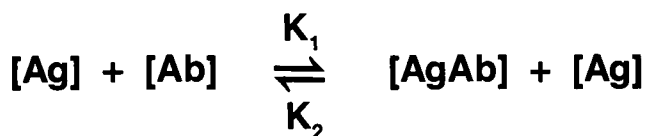$$[Ag] + [Ab] \underset{K_2}{\overset{K_1}{\rightleftharpoons}} [AgAb] + [Ag]$$

FIG. 2. This equation depicts the equilibrium reaction between antigen and antibody in a conventional RIA procedure. Ag=antigen (labeled or unlabeled); Ab=1° antibody; AgAb=antigen-antibody complex formed during the reaction; K_1=the rate of formation constant, association, or affinity constant; K_2=dissociation constant. An in-depth discussion of this equation can be found in ref. 37.

▲ = labeled antigen (tracer), ○ = patient (HCG), ⅄ =antibody molecule
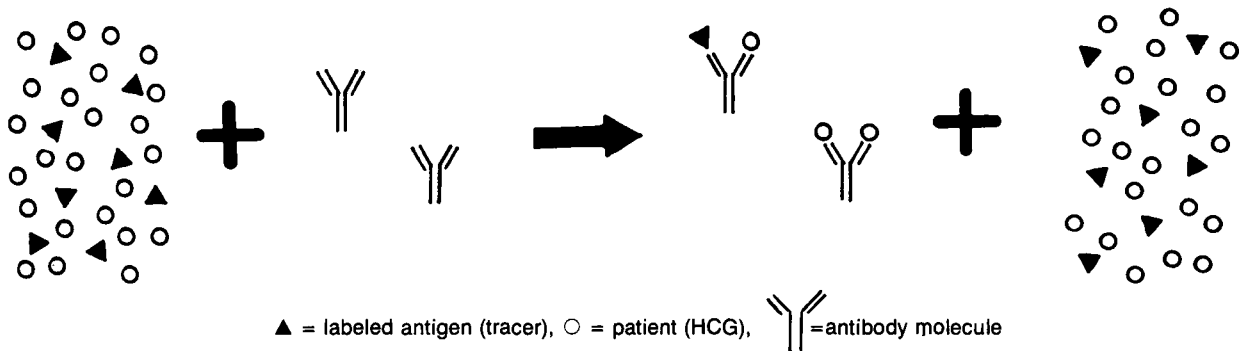
FIG. 3. A schematic representation of an RIA assay where the patient is in a high concentration relative to the concentration of tracer. After equilibrium, there will obviously be mostly patient bound to the antibody and not much tracer. When the antibody bound fraction is counted, the counts will be low.

between what is bound to the antibody (the free fraction) (Fig. 2) (28,29). The time it takes for equilibrium to be established varies from method to method, because of the affinity and specificity of the antibodies and the optimum conditions established for the reaction to reach equilibrium. Another way to visualize equilibrium is that the proportions of the tracer-versus-patient bound to the antibody are equal to the proportions of tracer-versus-patient not bound to the antibody. The trend is toward speed without sacrificing quality. To meet this challenge of "faster" assays, manufacturers have begun a trend toward shorter incubations. Some assays have been redesigned so that complete equilibrium is not achieved. Instead, the reaction is stopped prior to equilibrium, but supposedly without sacrificing the necessary sensitivity. The success or failure of this type of assay to maintain sensitivity can be demonstrated by setting up two to three parallel runs and extending the incubation interval two to three times beyond what is prescribed. Make note of the higher binding (zero cpm/total activity) and in most cases improved sensitivity.

The basic RIA concepts can be applied to a practical situation. If a patient has an elevated HCG concentration and this sample is analyzed by conventional RIA procedures as explained above, there is so much patient HCG present after equilibrium is established that it will displace most of the tracer from the antibody. This results in a relatively high level of patient HCG bound to the antibody; thus, there will not be much tracer bound to the antibody (Fig. 3). If you were to

remove the antibody from the reaction system and count the radioactivity, the radioactive counts would be low. The converse is also true; if you have a patient with little or no HCG, after the equilibrium is established, what will be bound to the antibody will be mostly, or all, tracer. Thus, when the bound fraction is counted, the radioactive counts will be high (Fig. 4).

The inverse relationship between a patient concentration and radioactive bound counts has been clearly explained. The key point is that the inverse relationship exists *only* when the antibody *bound* phase is counted in a conventional RIA procedure. Other modifications of the above procedures will be discussed in the section on binding reactions.

Separation Step

The final step in an RIA procedure is removing the antibody bound fraction from the unbound or free fraction so that the radioactivity can be counted. This is referred to as a separation

TABLE 2. Common Separation Techniques

Liquid Phase Separation	Solid Phase Separation	
	Liquid Reagent	Solid Reagents
Dextran-coated charcoal Resin	Second antibody Polyethylene glycol Second antibody/ polyethylene glycol "Micro" beads	"Macro" beads Coated tubes

▲ = labeled antigen (tracer), ○ = patient (HCG), ⅄ =antibody molecule
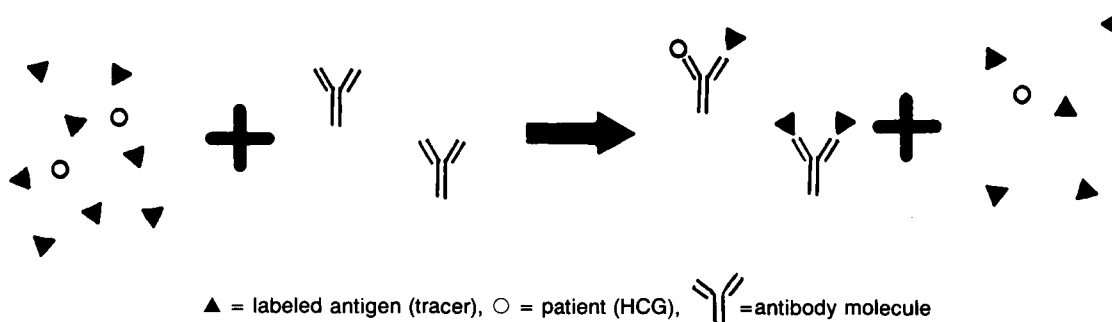
FIG. 4. An identical assay as in Fig. 3, except now the patient concentration is very low relative to the tracer. After equilibrium, mostly tracer will be bound to the antibody, resulting in high radioactive counts in the bound fraction.

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step. There are two general categories under which separation techniques can be classified: a) liquid phase separation; and b) solid phase separation (Table 2).

The designation liquid or solid phase separation refers to the location of the primary antibody after the separation step is completed. In the liquid phase separation, the primary antibody is liquid or part of the supernatant. Solid phase means that the primary antibody is in some sort of solid form at the completion of the assay. There are two subgroups in the solid phase category: liquid reagents, which result in a pellet, and large solid matrices, which require only a wash and/or decantation.

Liquid Phase Separation

Dextran Coated Charcoal. Charcoal, by itself, is a clarifying, decolorizing, filtering agent (30). Alone, it is used as a stripping agent for serum. If you were to add it to the assay tubes, it would absorb everything—both the bound and free fraction. When the charcoal particles are coated with dextran (a polysaccharide), it forms a mesh around the charcoal particles, thus, limiting the size of the molecules that can be adsorbed by the charcoal. When added to the RIA reaction mixture, it will adsorb only the tracer or patient analyte not bound to antibody. The antibody bound fraction is too large to be adsorbed by the dextran-coated charcoal (DCC), thus, following a centrifugation step, the primary antibody is located in the liquid phase or supernatant. The charcoal (free phase) is in the pellet. Once DCC is added to the reaction mixture, it is generally allowed to incubate 10 min at 4 °C for optimum adsorption. Centrifugation is then carried out, also at 4 °C. If not carried out at this temperature, the charcoal will not pack well in the bottom of the tube, with some of it remaining on the sides. A word of caution: Do not allow the DCC to remain in the reaction mixture any longer than specified. Prolonged exposure to DCC can result in the DCC stripping the antigen (labeled or unlabeled) from the antibody (31).

Resin. The action of resin is very similar to that of DCC, but it has been used less frequently. T_3 resin uptakes are one of the few tests still utilizing the substance.

Solid Phase Separation—Liquid Reagents

Polyethylene Glycol. PEG is a water soluble polymer with a basic formula $H(OCH_2CH_2)nOH$, where $n \geq 4$ (30). PEG is known to precipitate proteins, although the exact mechanism is not fully understood. It is thought to be related to a breakdown of the hydroxy group on the PEG in the formation of complexes. Factors that control effective use of PEG are: 1) percent of PEG; 2) protein content in the assay tube; 3) pH; and 4) the salt content of the buffer. The optimum conditions will vary with the analyte being measured. Alone it is a fast, clean separation of bound and free in which it quickly precipitates the bound fraction (32). Most procedures require that it be pipetted and centrifuged at 4 °C. Its disadvantage is its nonspecificity. Depending on the nature of the proteins in the assay tube, it can precipitate most large proteins in the reaction vessels, not just the antibody in question.

Second Antibody (Precipitating Antibody). The second antibody is simply an antibody against the primary antibody,

i.e., if the first antibody was produced in rabbits, this 1° antibody will be a rabbit gamma globulin. To produce a second antibody to this first antibody, ordinary rabbit gamma globulin is used as an immunogen and injected into another species, i.e., sheep, goats, etc. This second animal will then produce an antibody to rabbit gamma globulin. When this antirabbit gamma globulin is reacted with the primary antibody (being a rabbit gamma globulin), it forms a huge complex with the primary antibody, which then renders itself insoluble and precipitates out of solution. The advantages are that it is the cleanest, most sensitive form of separation available. However, it is a slow process. Depending on the quality and affinity of the second antibody, incubations with the reaction mixture could vary from 2–24 hr.

Second Antibody/PEG. This method is a successful attempt to combine the sensitivity and precision of the second antibody with the speed of PEG. Virtually all second antibody separations now incorporate PEG. Once this reagent is added, incubations range from 1–30 min depending on the assay.

Microimmobilized Bead with Primary Antibody. In this system, the primary antibody is bonded to a microscopic bead (glass, sephadex, or plastic). In the separation step, the immobilized microbeads containing the bound fraction are centrifuged. The free fraction is then decanted.

Microimmobilized Bead with Second Antibody. In this case, the second antibody has been covalently linked to a solid particle. After incubation and centrifugation, the free fraction is discarded and the bound fraction counted.

Solid Phase Separation—Solid Reagents

Macroimmobilized Beads with Primary Antibody. In this case, the primary antibody is coated onto a large bead. One bead is added per test tube. The separation step involves washing the free antigen—labeled and unlabeled—away from the bead and counting the bead. What is bound to the bead is not disturbed by the washing process.

Coated Tubes. In this widely used technique, the primary antibody is bound to the walls of the assay tube (generally 12 × 75 mm). The advantage is thus saving centrifugation time. The free fraction is decanted and what is bound to the primary antibody on the wall of the tube is counted. One disadvantage is a reduction in precision when compared to that of a liquid separation step. This reduction is negligible, however, being only 2–3% less (higher CVs).

PART II—BINDING REACTIONS

RIA

The basic reactions in a conventional RIA procedure have been previously discussed.

CPB

This technique has been previously mentioned in the section on primary binders. It is very similar to that of RIA. There are two types of CPB systems: a) one is identical to RIA in that the sample (or standard) and tracer compete for available binding sites on the binding protein (except there is only one binding site per protein binding molecule, as opposed to two

■ = Primary binder, ⬡ = Tracer, ○ = Patient
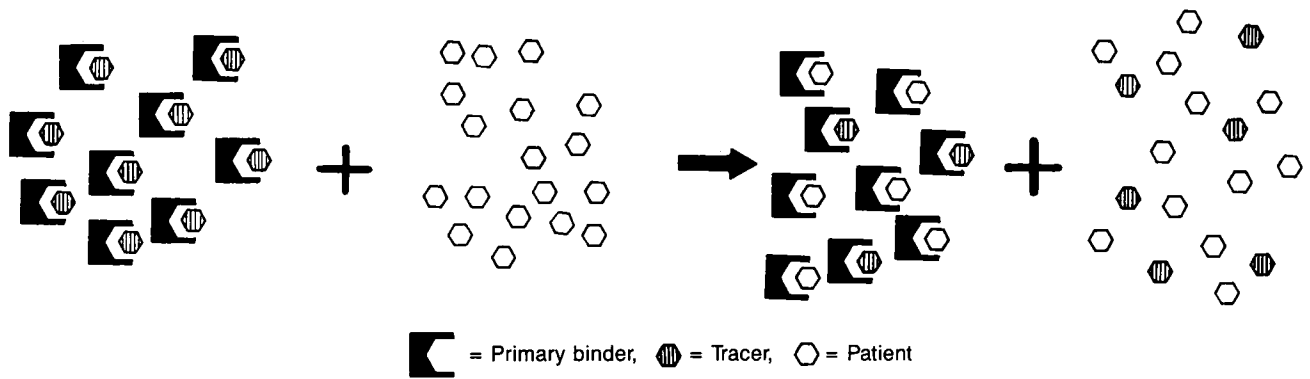
FIG. 5. A schematic representation of a competitive protein binding assay. The primary binder is first saturated with tracer, then the patient competes with and displaces the tracer in proportion to its (the patient's) concentration.

binding sites on each antibody molecule); and b) the protein binder is first saturated with the tracer. The sample (or standard) is then added, and then displaces the tracer in proportion to the relative concentration of the sample or standard (Fig. 5).

Sequential Saturation Analysis

In an RIA procedure where the tracer may have a higher affinity for the antibody binding sites than the material to be detected in the patient sample, the technique of sequential saturation analysis may be employed. In these assays, the patient sample or standard is allowed to react with the antibody first. In this case, the concentration of antibody is high relative to the concentration of antibodies used in a routine RIA procedure. In the sequential saturation analysis, all of the patient sample or standard will bind to the antibody, leaving many unoccupied antibody sites. After a suitable incubation period, the tracer is added. It will then occupy all of the binding sites not occupied by the patient or standard (Fig. 6). The second incubation is crucial since over-incubation could result in complete displacement of the patient sample or standard, as the tracer has a higher affinity for the antibody. Thus, the separation step must be initiated without delay following the tracer incubation.

This approach has been used in numerous other assays since it has been determined that allowing the patient sample to have first exposure to the antibody binding sites improves sensitivity in the low ranges of the standard curve.
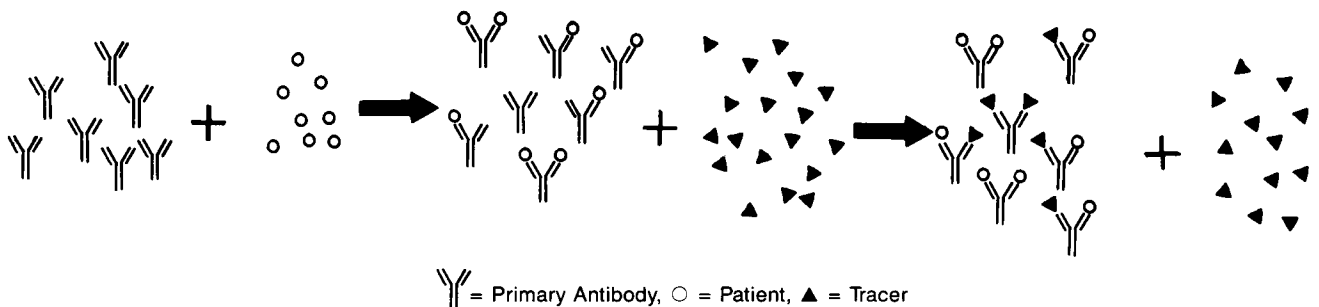
Immunoradiometric Assay

Immunoradiometric assay (IRMA) is the term for what was referred to as "sandwich technique." Many monoclonal assays utilize this technique because the fewer number of pipetting steps reduces hands-on time. It is most often employed in macro-bead technology where the primary antibody is coated onto the macroscopic bead. The patient sample or standard is first permitted to react with the antibody. The remaining unwanted patient sample consitutents are then washed away. Another primary antibody is added, but this antibody is labeled with a radioactive isotope, generally iodine-125. This labeled antibody will bind to the patient's antigen, which is already bound to the primary antibody on the bead. Following incubation, the labeled antibody not bound is washed away (Fig. 7). In this type of procedure, the counts are directly proportional to the patient sample concentration. More patient sample bound to the primary antibody on the bead will result in more labeled antibody bound to the patient sample sites.

Recent advances in IRMA technology and monoclonal antibodies have eliminated the second wash step.

Radiolabeled Binding Assays

In these assays, the presence of an antibody or a receptor—not an antigen—is detected. A radiolabeled antigen is added to the patient sample. If the antibody, receptor, or binder is present, the labeled antigen will bind to it. Following incubation and separation, the presence and quantitation of

Υ = Primary Antibody, ○ = Patient, ▲ = Tracer

FIG. 6. A schematic representation of a sequential saturation assay. The patient first reacts with an excess of antibody; later tracer is added which occupies all the antibody sites not occupied by the patient. There is an inverse relationship between the counts bound to the antibody and the patient concentration.
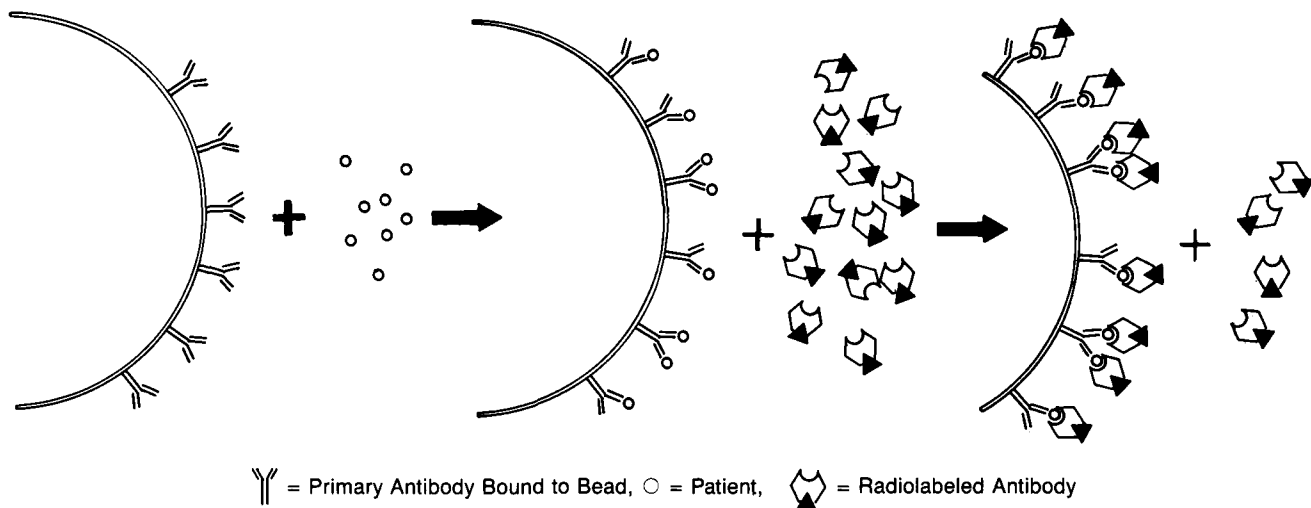
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FIG. 7. One example of an IRMA technique utilizing a "macro" bead. The patient is first reacted with excess antibody on the bead. A radio-labeled *antibody* is then added which binds only to any patient already bound to the primary antibody. Excess labeled antibody is discarded and the bead is then counted. The amount of radioactive counts on the bead is *directly* proportional to the patient concentration.

the bound fraction will determine if the antibody, binder, or receptor is present. In some of the radiolabeled binding assays, the results are expressed as a percent of the radioactivity bound. The more radioactivity present, the more antibody present. Testing for insulin antibodies, anti-DNA antibodies, and B-12 binders is generally expressed this way.

Other assays, such as breast tumor receptor assays, require more precise quantitation of the exact concentration of receptor present. In these procedures, by adding known concentrations of the radiolabeled antigen, one can quantitate the concentration of the binder by using the method of Scatchard ("Scatchard's analysis") (33,34).

One additional reaction type that deserves special attention because it does not fall into the above categories is T_3 uptake assay, which has remained essentially the same since about 1959. In this assay, the patient sample is mixed with radio-labeled T_3. The primary binder is the patient's own thyroxine binding globulin (TBG). Therefore, there are two variables: the patient's thyroid hormone concentration and the patient's TBG concentration. The labeled T_3 will occupy all available binding sites on the patient's TBG molecules. It will compete with the patient's own T_3 on TBG, but will not displace the patient's T_4 due to the T_4s high affinity to bind TBG much more than that of T_3. A separation step (resin) follows, which absorbs all of the labeled T_3 not bound to the TBG. The bound fraction is discarded and the free fraction is counted. The results are expressed as a percent of radioactive T_3 taken up by the separating agent, such as resin. Thus, an elevated T_3 uptake result means either a high T_3 concentration or a low TBG concentration. A low T_3 uptake result would of course mean the opposite.
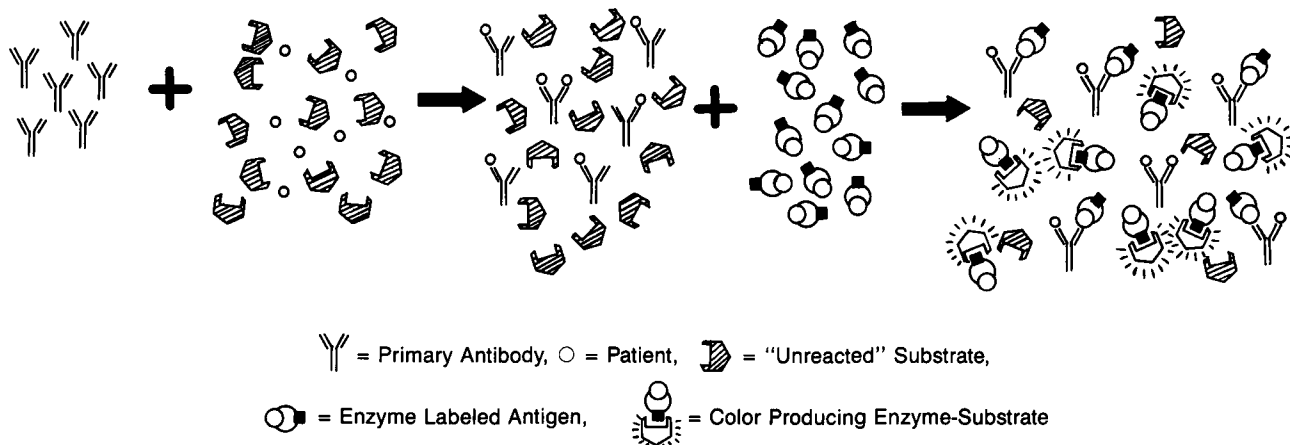
PART III—ALTERNATIVES TO RIA

Since the mid-1960s, when labeled immunoassays first became available, radioisotopes have been the label of choice, providing the greatest precision, sensitivity, and specificity for microanalytic procedures to date.

Enzyme Immunoassays

In the mid- to late-1970s, a new form of immunoassay emerged utilizing enzyme "tags" rather than radioisotopes (35). In these enzyme immunoassay (EIA) procedures, an enzyme (horseradish peroxidase, glucose-6-phosphate dehydrogenase, and alkaline phosphatase, to mention a few) can be "tagged" to either antigen or antibody in the presence of a substrate that is specific for the enzyme. If a separation step is required in the immunoassay, it is referred to as being heterogeneous; if none is required, it is homogeneous. (Note: All RIA procedures require a separation step and are, therefore, heterogeneous.) The enzyme and substrate are selected so that their combined effect is the production of color (Fig. 8). Therefore, there is a direct relationship between the color development and the patient concentration. Quantitation is carried out by measuring the change in absorbance over a period of time as the enzyme labeled antigen binds to the antibody. This technique is widely used in drug analysis—especially in cases of drug abuse (35,36).

There are advantages and disadvantages to using EIA as compared to RIA, depending on the analyte to be detected. For drugs, EIA has the advantage of speed. Results can be available in seconds (once reagents are prepared and calibrators read). The same curve can be used over again throughout the day (37). RIA procedures take much longer to perform. A separation step is always involved. A standard curve should be run with each batch. Enzyme reagents have a longer shelf-life, 1-2 yr, as compared to RIA kits whose shelf-life is dictated by the half-life of the isotope. Most iodine-125 reagents have a 30-60 day shelf-life. EIAs, however, do not appear to be as sensitive as RIAs. This is due, perhaps, to nonspecific inhibition or steric hindrance (28). Because of the state-of-the-art of spectrophotometers, the calculation time for EIAs was at one time considerably less. The state-of-the-art of gamma counters for RIA testing is meeting this challenge, with the multiwell (20 well) gamma counters accompanied by com-

$\mathcal{Y}$ = Primary Antibody, ○ = Patient, ⬡ = "Unreacted" Substrate,

⬡■ = Enzyme Labeled Antigen, ⬡ = Color Producing Enzyme-Substrate

FIG. 8. A schematic representation of a homogeneous EIA procedure. The patient and substrate are first mixed with an excess of primary antibody. Enzyme labeled antigen is then added and occupies all antibody binding sites not occupied by the patient. The substrate reacts only with enzyme labeled antigen *not* bound to antibody to produce a color reaction. Here, there is a direct relationship between color development and patient concentration.

puterized data reduction systems.

The statement that enzyme assays eliminate or reduce the cost of radioactive waste disposal is not completely valid. The regulations set forth by the Nuclear Regulatory Commission (NRC) state that liquid I-125 can be poured down the sink as long as the monthly average does not exceed 0.15 μCi/gallon of water being emptied from the facility into the sewer system (*38*). The limiting factor here is how much water is leaving the building as waste. Solid I-125 waste must be monitored by a Geiger counter. If found not to exceed background radiation in millirads per hour, it can be disposed of as non-radioactive waste. The majority of laboratories would fall well under these guidelines and virtually no disposal expense is incurred. Similarly, tritium may now be poured down the sink as long as it does not exceed 0.05 μCi/ml of water being disposed of from a facility into the sewer system (*39*). This is approximately 189 μCi/gallon of water and does not include H-3 dissolved in organic solvent. Liquid scintillation vials containing H-3 in organic solvent can now be incinerated in an incinerator equipped to handle combustible materials. The vials must first be crushed, then the tritiated solvent jet-sprayed into the incinerator. A costly alternative would be to have the sealed LS vials picked up, transported, and buried by an agency designed for that purpose.

Flourescent Immunoassay Procedures

Fluorescent immunoassay (FIA) procedures are basically similar to RIA in that the standard or patient sample compete with the fluorescein tagged label for a limited number of antibody binding sites (*28,35,40*). Following a suitable separation step (heterogeneous assay), the free unbound label is measured by a fluorometer. The patient concentration is directly proportional to the amount of fluorescein present, since the assay is measuring the free fraction.

Fluorescent polarization immunoassays (FPIA) are gaining a wide acceptance in drug measurement (*41,42*). As in FIA, patient sample or standard competes with the fluorescent tagged label for limited antibody sites. Measurement is based on the inverse relationship between the amount of fluoresence and patient concentration.

CONCLUSIONS

Presenting a broad overview of the concepts of RIA testing would require an article of even greater length than this one. In an effort to conserve space, and keep it "simple," this article has discussed the following four components in an RIA procedure: a) production techniques and selected aspects of quality control for various component types and sources; b) interaction of components in the test tube to produce an RIA test; c) an encompassing look at some of the more widely used reactions available; and d) a comparison of available techniques as alternatives to RIA.

Although this article is not a compendium, it does provide a view of the basic principles of RIA, and it is written with the intention of providing a solid foundation for more in-depth studies.

Locating an all-encompassing text on immunoassay principles for more in-depth reading is quite difficult, but the following titles come closest to meeting this criteria: a) the 1983 two-volume text edited by Ashkar entitled *Radiobioassays* (*37*); b) the 1971 edition of *Principles of Competitive Protein Binding Assays*, edited by Odell and Daughaday (*5*); and c) Odell and Franchimont's 1983 edition of *Principles of Competitive Protein Binding Assays* (*43*). These references provide more detailed discussions of the principles emphasized in this article.

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