Practical radioimmunoassay

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Preface

This book represents the efforts of faculty members of the Department of Radiology of the University of Arkansas College of Medicine. Without question, radioimmunoassay is an area of great importance in modern clinical medicine. With the widespread availability of commercial kits, a large number of laboratories (both in and out of hospitals) have offered radioimmunoassay procedures to their referring clinicians. Unfortunately, all too many of these laboratories are staffed by personnel who are inexperienced in the fundamentals, techniques, and interpretation of the results.

This book is designed to provide an elementary introduction to the broad field of radioimmunoassay and radioassay procedures. Although principles relative to the production of the reagents used for the assays are discussed, we assume that the average reader will be concerned with the use of commercial kits. Consequently, the basic thrust of the discussion has been toward the use of these kits.

Chapter 1 gives a broad preview of what will follow in the book. This chapter provides definitions and descriptions that will assist the understanding of later portions. This chapter also considers those principles of immunology as related to radioimmunoassay.

Chapter 2 deals with the chemical principles that support radioimmunoassay technology. For many readers, a considerable portion of this chapter will very likely serve to be a review of some portions of elementary chemistry. For readers who have not had sufficient training in chemistry, this chapter should be of considerable assistance for (1) reading the other parts of the book and (2) understanding the directions provided by kit manufacturers.

Chapter 3 considers the very critical problem of the separation methods used in radioimmunoassay. For radioimmunoassay to provide usable results, methods must be utilized to separate antibody-bound materials from those that are not bound. While most workers in the field are not concerned with the development of separation methods, they must understand the principles in order to utilize them in routine clinical assays.

The techniques used for the measurement of radioactivity are described in Chapter 4. This chapter provides a brief survey of the structure of matter, the interactions of radiation with matter, and radioactive decay. The chapter provides, in some detail, information about the operation of both gamma ray detecting devices and liquid scintillation counters.

Chapter 5 describes some applications of elementary statistics to radioimmunoassay procedures. This chapter considers the most important question: How long should a

sample be counted? The chapter also considers methods by which instrumentation may be checked for proper function.

Chapter 6 describes quality control methods as needed for the proper operation of a radioimmunoassay laboratory. This chapter defines the terms used for quality control and describes some specific techniques. A numerical example of a quality control method is included.

Chapter 7 describes radioassays that are based upon nonimmune principles. These are assays that are similar to radioimmunoassays but that do not require immune products. Similarities and differences as compared to radioimmunoassays are discussed.

Chapter 8 concerns principles of radiation safety as applied to the radioimmunoassay laboratory. The chapter also contains information about regulations relative to the ordering and disposal of radioactive materials. Other aspects such as contamination control in the laboratory, personnel monitoring, and radioactive material inventory are discussed.

The Appendix provides some information relative to the handling of commercial radioimmunoassay kits. In addition, the Appendix contains a listing of currently available commercial kits. Because of the rapid development of the field, very likely this will be out of date at the time of publication. It should, however, give the reader some idea of the wide variety of available kits.

We, the editors, hope that readers will communicate their feelings about errors, omissions, etc. It is our desire that the book be useful to workers in the area of radioimmunoassay. We have attempted to provide a text written in simple, nonmathematical terms. If this book in some small measure improves the performance of radioimmunoassays, the effort will have been well spent.

We thank Mrs. Norma Share for typing the manuscript. We also thank Mr. Greg Nolen for valuable technical assistance.

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Contents

CHAPTER 1

Fundamentals of radioimmunoassay

CHARLES M. BOYD
DONALD L. HERZBERG

Prior to 1960, substances present in small amounts in blood and other body fluids were exceedingly difficult to measure. Until then, chemical and bioassays of these substances, usually hormones, were somewhat crude. This led to considerable inaccuracy in the measurement of such small amounts, and results were difficult to reproduce in other laboratories. In addition, rather large samples were usually needed for the determinations. In 1960, Yalow and Berson* reported a method for the quantitative measurement of insulin based on a new technique of competitive binding assay. At approximately the same time, Ekins,† in England, reported a similar method for the determination of plasma thyroxine concentrations, which was also based on a competitive binding principle. Since 1960, no other method has been so widely applied as the "radioimmunoassay" (RIA) technique for the measurement of many substances, particularly hormones. In fact, much of the progress in endocrinology during the past decade has occurred because of the availability of these techniques.

Assuming that some readers will have had little or no prior exposure to the field of radioimmunoassay, this chapter will provide a general overview of the field, with particular attention to the general principles involved. Certain terminology will be explained, and basic definitions will be given to help the reader understand subsequent chapters. Several of the important components of the typical radioimmunoassay system will be described, and certain important techniques will be reviewed. Some variations in the general principles have been developed and will be discussed briefly.

WHY RADIOIMMUNOASSAY?

The remarkable development of the radioimmunoassay technique is well shown by the large number of substances now measurable by applying variations of the competitive binding radioassay. Appendix A contains a partial listing of substances that are measured by radioimmunoassay.

The development of radioimmunoassay offered several advantages over previous

*Yalow, R. S., and Berson, S. A.: Immunoassay of endogenous plasma insulin in man, J. Clin. Invest. **39:**1157-1175, 1960.

†Ekins, R. P.: The estimation of thyroxine in human plasma by an electrophoretic technique, Clin. Chim. Acta **5:**453-459, 1960.

methods. *Sensitivity* provides the single most important advantage. Sensitivity is the ability of a measurement system to detect small amounts of substances, a feature derived from the ability to measure very small amounts of radioactive tracers. Many factors affect sensitivity and will be discussed below. A second advantage of radioimmunoassay procedures is the *specificity;* that is, the ability of the system to measure only the substance of interest, as in measuring only thyroxine and not any other thyroid hormones. The selectivity offered by radioimmunoassay is excellent and is continually improved as we learn more about the immune characteristics of substances and their associated antibodies. A third important advantage of radioimmunoassay is the excellent *accuracy,* which is the ability to determine the actual amount of a substance present. *Precision* refers to the reproducibility of the assay and represents the final special feature of radioimmunoassay techniques. RIA provides extremely reproducible determinations of the concentrations in replicate samples during a single assay.

From the features of sensitivity, specificity, accuracy, and precision, which are so excellent with radioimmunoassay techniques, it is quite understandable that RIA methods have become so useful. Because the technique is applicable to a variety of substances, such as hormones, and large numbers of individual sample assays can be performed almost simultaneously, even greater use for these techniques is predicted.

DEFINITIONS

Certain definitions are important for understanding radioimmunoassay and competitive binding radioassays. The first term often seen is *ligand.* Taken from the Latin word meaning "that which is bound," this term usually refers to a rather small molecule. In the radioimmunoassay principle the *antigen* is the ligand.

The *antigen** is a substance that induces antibody formation (*anti*body *gen*erator). Actually, in the RIA measurements, the production of antibodies does not constitute a part of the assay itself; however, antigens are used to produce antibodies. Antigens form one important component of the RIA system. The antigen may be unlabeled or labeled

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*The term *antigen* also refers to the material present in the patient's plasma (or other body fluid) that we wish to measure. Consequently, the term "antigen" can by synonymous with the substance under study. This seemingly clumsy nomenclature developed because the very material one wished to measure in the patient was, by necessity, used for the production of antibody by animals, such as rabbits, guinea pigs, horses, pigs, etc. As a result, the reader will see the term "antigen" when the writer actually is referring to a specific biologic material, for example, thyroid stimulating hormone (TSH) in the patient's plasma. As indicated above, purified TSH had been used to immunize animals to produce antibodies against TSH. Therefore, TSH when injected served as an "antigen" in the radioimmunoassay because it "generated" antibodies against itself.

As will be developed in detail in later chapters, labeling of the "antigen" with radioactivity allows many radioimmunoassays to work. In the example above, labeled TSH served as the ligand, because it is bound by the antibody against TSH.

The material under study, then, may appear three times in the radioimmunoassay process: (1) injected into animals to generate antibodies against itself (for example, purified TSH of porcine origin may be injected into guinea pigs), (2) purified and labeled with radioactivity in the laboratory, and (3) the very material (at an unknown concentration) that we wish to measure in the patient's specimen. Unfortunately, in many instances, the term "antigen" is used for all three cases; only in case 1 is the material a true antigen, however.

Throughout this book we will follow common convention in that the material under study will be the "antigen" (unless, of course, we are studying the *antibody,* per se).

with a tracer such as a radionuclide (^{125}I, ^{3}H, ^{14}C, etc.). The *antibody* is generally a gamma-globulin protein, which reacts with an antigen. The interaction with the antigen is specific; this specificity forms the cornerstone of the ability of radioimmunoassay techniques to measure specific substances. The term *affinity* refers to the probability of the interaction between the antigen and the antibody in a system. The term *avidity* refers to the strength of the bond between the antigen and the antibody after the complex has been formed. The latter two terms are generally related, but are somewhat different when considering the kinetics of reactions.

BASIC PRINCIPLES OF RADIOIMMUNOASSAY

Radioimmunoassay procedures are extensions of the original observations by Berson and Yalow that low concentrations of an antigenic hormone can be detected by determining the ability of these antigens to be bound by specific antibody. Further, the competition of nonlabeled hormone molecules with radioactively labeled ones for the same binding sites on the antibody causes a depression of the amount of bound labeled material in the final solution. By studying a series of standards of known amounts of unlabeled hormone, an unknown (such as insulin in a patient's plasma) can be measured because it offers a similar competition with the labeled hormone molecule for the antibody. As increasing amounts of unlabeled antigen are present, the limited binding sites on the antibody are progressively saturated. Consequently, less labeled antigen becomes bound. Incubation of the components of the system (unlabeled antigen, labeled antigen, and antibody) allows an equilibrium reaction to occur. Subsequent separation of the bound from the unbound, or "free," forms and measurement of the amount of radioactivity of either or both phases allows quantitation of the reaction that has occurred.

In more practical terms, the basic principle may be shown as in a specific determination. The actual procedure may be thought of as occurring in two major steps: (1) the establishment of the assay and (2) the assay of the unknown sample quantity.

In simplified terms the assay is established by (1) obtaining the components necessary to perform the assay, (2) allowing the components to interact under the proper circumstances, (3) separating specific components, (4) measuring those components by radioactive tracer measurement techniques, and (5) obtaining measurements of a series of known quantities of the substance for later comparison to an unknown amount in the patient sample. The components used in the assay as seen in the upper panel of Fig. 1-1 are a labeled substance that is essentially identical to the unknown substance to be measured later. We will call this the *labeled antigen*. A known amount of this labeled antigen is introduced into a solution containing an *antibody* to the antigen. At the same time, a known amount of the *unlabeled* counterpart of the antigen is also introduced into the solution. There is then a complex solution containing labeled antigen, unlabeled antigen, and antibody. These components are allowed to interact, with the antibody binding both the labeled and unlabeled antigen molecules. The resulting solution then contains two more substances, the complexed (or "bound") labeled antigen as well as the bound unlabeled antigen. A separation of the bound from the unbound components (some of each is both labeled and unlabeled) is then effected by a variety of means,

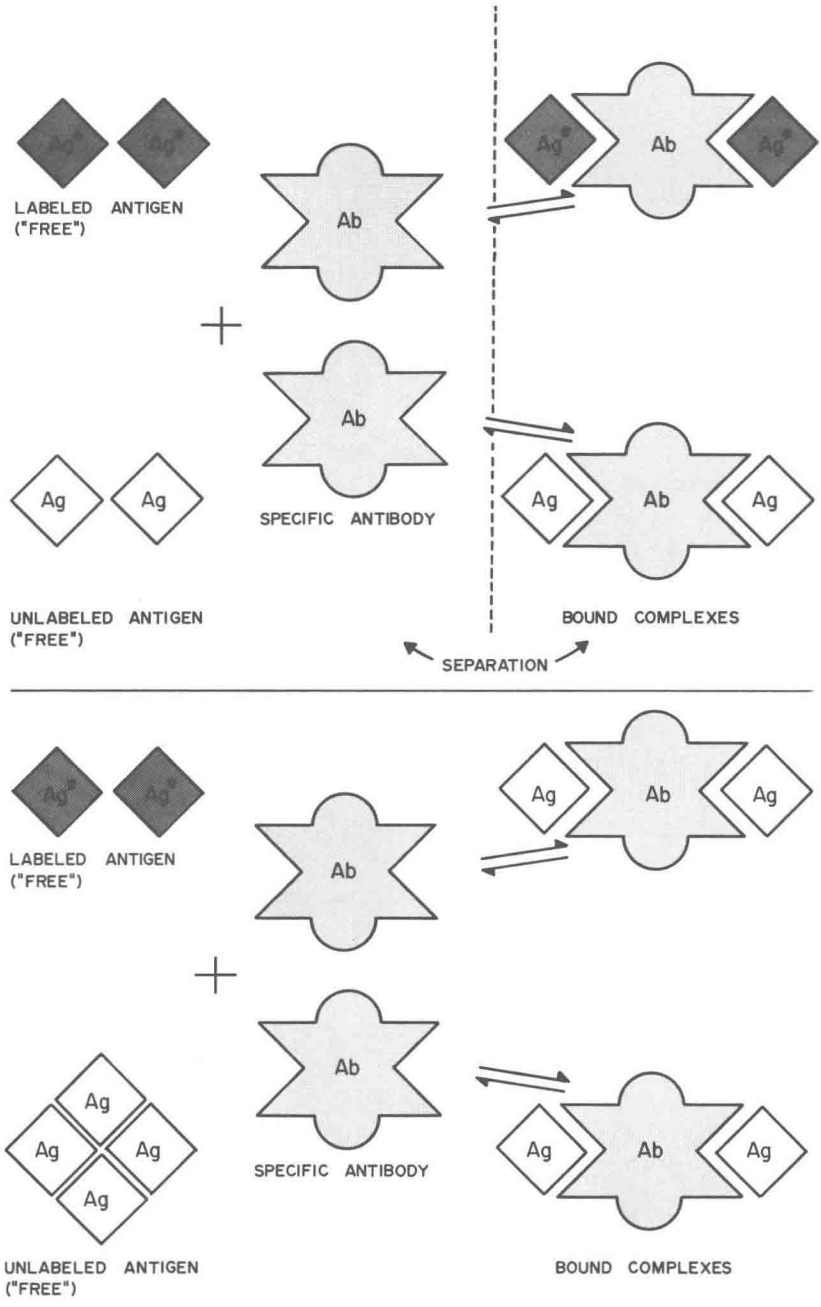

Fig. 1-1. Components and reactions in the RIA procedure. *Upper panel,* Sample antigen and identical radioactive antigen react with antibody to produce bound forms of both. Bound and free elements are separable. *Lower panel,* Excess sample antigen added to the system competes successfully with the radioactive antigen for all the antibody binding sites. The amount of radioactivity in the bound form will be reduced compared to the situation in the upper panel.

such as addition of a material that binds the remaining unbound antigens. The radioactivity present in *either* the unbound or bound forms of the substance can then be measured.

As seen in the lower panel of Fig. 1-1, adding larger (or smaller) amounts of known quantities of the original antigen solution will result in the formation of less (or more) of the labeled bound form of the substance. The excess unlabeled form "competes" successfully against the smaller amount of labeled antigen for the binding sites on the antibody. It is then easy to see that as more of the original antigen is added to the antibody, the resultant bound form will have less radioactivity and, when separated from the "free" unbound form, this difference can be measured. Since a series of known amounts can be added, a "standard curve" of the known amount present compared to the amount of radioactivity measured in the bound component can be constructed for each known quantity. For example, as seen in Fig. 1-2, if 400 ng of the antigen (not labeled) were placed in the solution with the antibody and labeled antigen, the competition for the binding sites might result in 60% of the radioactivity being present in the bound labeled form. If 800 ng were then placed in a similar solution, more displacement of the labeled antigen off the antibody would occur, resulting in perhaps only 40% of the radioactivity being present in the labeled bound form (the remainder of the labeled antigen being in the free form). Thus standards may be set up consisting of a series of known amounts of the antigens in various strengths.

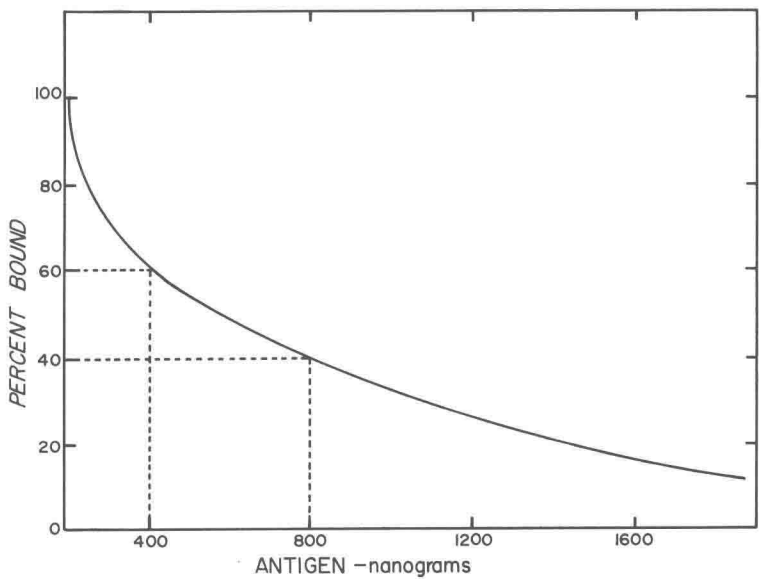

Fig. 1-2. Example of standard curve of RIA reaction. Antigen "bound" to antibody (as a percent of the antigen maximally bound) is compared to the antigen concentration. By construction of a curve using standards and by measuring the relative amount of radioactivity displaced from the antibody by the sample antigen (% radioactivity in bound form), the nanograms of unknown antigen present in the sample can be found.

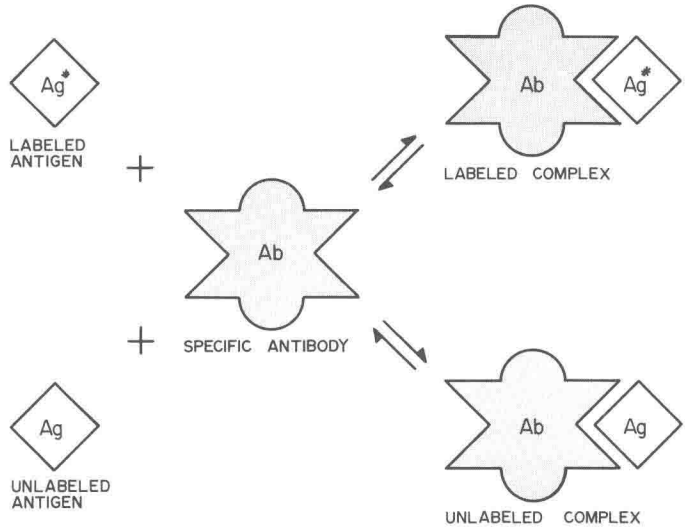

Fig. 1-3. Basic principle of the competitive binding reaction in radioimmunoassay. The labeled antigen (Ag*) competes with the unlabeled antigen (Ag) for the binding sites on the specific antibody, resulting in bound forms, one labeled (Ag*Ab) and one unlabeled (AgAb).

Following establishment of the assay and the standard curve just described, the patient sample may then be placed in the solution, representing an unknown amount of the antigen. If, after proper reaction of the components and their separation, 40% of the bound radioactive antigen were present in the solution, the unknown must represent 800 ng, as the same 40% bound form was found when the standard solution containing that amount (800 ng) was added.

A classical representation of the competitive binding reaction is shown in Fig. 1-3, where the labeled antigen (Ag*) and the unlabeled antigen (Ag) compete for the binding sites on a specific antibody (Ab), with resultant complexes being either labeled (Ag*Ab) or unlabeled (AgAb) bound forms. By maintaining a known amount of labeled antigen, a stable amount of specific antibody, and then separating the bound forms from the unbound forms, relative amounts of the unlabeled antigen can be determined.

The term *competitive binding* is an appropriate name for the above system. Since an immune system composed of antigen and antibody is used in this competitive binding, the term *radioimmunoassay* is also appropriate. Competitive protein binding analysis (CPBA) is a specific type of assay using this principle, but nonimmune components are used in the method. This method will be explained later; however, the basic principles underlying CPBA are quite comparable to radioimmunoassay (RIA).

Note that the labeled antigen (or labeled ligand) need not be absolutely identical to the competing unlabeled material, as long as the degree of saturation of the binding antibody or the competition for the binding sites is somewhat similar. As radiolabeling of many molecules alters them significantly, nonspecific binding factors become important.

To summarize the basic principle, Fig. 1-3 shows that as more unlabeled antigen (Ag) is added to the system containing a similar labeled antigen (Ag*) more of the binding sites are occupied on the antibody (Ab) by that antigen. Consequently, fewer sites are available to the labeled antigen (Ag*) for binding. The labeled complex (Ag*Ab) then will appear in lesser amounts relative to the unlabeled complex (AgAb). There is a *reciprocal* relationship between the amount of unlabeled complex added to the system and the amount of radioactivity in the labeled or "bound" complex. This relationship and the subsequent measurement and comparison of the bound and free elements of the system form the basic principle of radioimmunoassay.

COMPONENTS OF THE RADIOIMMUNOASSAY SYSTEM

As indicated, three basic components form the basis of the radioimmunoassay system: (1) equilibration or incubation, (2) a method of separation of bound from free elements, and (3) counting of the radioactivity in the components. Interpretation of the results requires analysis of the amount of radioactivity of the bound and free forms of the patient's plasma (unknown) relative to concentrations of standards.

Standards that are either identical or similar to the unlabeled antigen (ligand) are most important in that they must also behave like the unknowns in the system. Nonidentity with the substances to be measured occur because of (1) natural variations, such as "big" insulin, fragments of hormones, and other substances, (2) species differences, (3) artifacts produced by degradation, and (4) synthetic errors in the manufacture of artificial substances. The stability of the antigen used as a standard becomes critical because the standards are often used in assays over a long period of time. There are interfering substances, which may prevent nonidentity of the standards with the unknowns. These include factors such as heparin (and other drugs), urea, bilirubin, buffers, temperature effects, and pH.

The second major component of the RIA system is the *labeled antigen*. Important criteria for this reactant in the RIA system include: (1) high purification of the antigen and (2) its ability to be radiolabeled without loss of immunoreactivity. Since many antigens are polypeptides that contain tyrosyl residues, they can be fairly easily labeled with iodine by a variety of methods; some of these methods are described in other chapters. Often ^{125}I is used as the tracer, since a higher specific activity* with iodinated antigens can be obtained than when using either tritium or ^{14}C. As compared to ^{125}I, ^{131}I has some theoretical advantages for radiolabeling (such as a shorter half-life). However, isotopic abundance is often low, counting characteristics are poor compared to ^{125}I, and there is more molecular radiation damage (self- or auto-irradiation) associated with its use.

The third major element in the system is the *specific antibody*. The main criterion for a suitable antibody concerns its specificity for antigen interaction. This specificity is influenced by the heterogeneity of antibodies for the same antigen; that is, cross-reactivity

*Specific activity—activity per unit mass of substance. Units such as mCi/m mole, cpm/μg, etc. are frequently used.

for similar substances. Since the antibodies are produced in a variety of animals, species variation may be important. It should be emphasized that the radioimmunoassay system depends on an antigen-antibody reaction and not upon the biologic activity of the antigen or ligand. Other influences on the interaction include antigenic damage that occurs with time (as by enzymes) and nonspecific binding of other ligands.

A specific concentration of the antibody must be used in the RIA system. The dilution of antibody is often chosen so that the antibody binds approximately 50% of the labeled antigen. This results in a bound to free ratio (B/F) of the elements of 1:1. Since the antibody is generally used in a very dilute solution, a high titer is not considered important in the selection of the antibody. However, the *affinity* of the antibody is extremely important and will be discussed later.

In addition to the usual gamma-globulin antibody, there has been recent use made of tissue binders, which are also specific for binding certain antigens. Although not true antibodies, in the RIA system they function in a similar manner. These binders generally have high affinity for the antigens and recognize the active portion of the antigen.

The final component is the *patient sample,* which is really the antigen (unlabeled) to be measured in the serum or plasma, and is analogous or identical to the unlabeled standard antigen discussed above. Certain separation and purification steps may be necessary before the sample antigen is truly representative of the desired substance to be measured. For example, thyroxine is bound to serum proteins and in many systems must be separated from those proteins prior to its measurement. This is so because the proteins themselves would act as binders in competition with the thyroxine antibody, and they also would present the thyroxine molecule in a form different from the standards used to establish the assay.

THE REACTION

The elements given in Fig. 1-3 are incubated together to allow a reaction to occur. The competition between labeled and unlabeled ligands is allowed to occur simultaneously until equilibrium is reached between the ligands and the binder (antibody). Because of this equilibrium reaction, the RIA method is often referred to as "equilibrium saturation." Most competitive binding assays are of the equilibrium type.

The sensitivity obtained by radioimmunoassay, while dependent somewhat on the specificity of the antibody as well as antigenicity of the antigen, depends primarily upon the *equilibrium constant* (or affinity constant). This constant characterizes the energy of the antigen-antibody reaction and is usually of such great degree that a high sensitivity results. Additional details are given in Chapter 2.

SEPARATION OF BOUND FROM FREE REACTANTS

There are several methods used for separating the bound component from the free component. These are often selected rather arbitrarily, as determined by empirical trials to find which produce the best separation with the least effort while maintaining maximal sensitivity. The methods include differential migration (by chromatography, electrophoresis, chromatoelectrophoresis, gel filtration, etc.), precipitation of the bound form

(such as by a double antibody technique), and absorption of the free phase by charcoal, resin, polypropylene tubes, etc. Other methods include dialysis and ultrafiltration to accomplish the separation.

The separation technique should have no effect on the antigen-antibody reaction. There are some advantages as well as disadvantages of each system for each separation. Since it is often difficult to predict which separatory technique will be best for any single system, empirical trials are usually necessary.

MEASUREMENT OF RADIOACTIVITY

The radioactivity of the bound radioactive components and unbound radioactive components is measured by standard techniques. Gamma counting is used in the case of those tracers emitting gamma rays, such as ^{125}I and ^{131}I. Liquid scintillation counting is used to count the same components when ^{3}H and ^{14}C or other beta-emitting radionuclides are used in the assay system. Because of the high specific activity of the labeled antigen or ligand, high count rates are often easily obtainable. This allows even greater precision in the counting statistics.

ANALYSIS OF DATA

The measured radioactivity reflects the relative amounts of radioactive bound fraction and radioactive unbound fraction; the results are usually plotted as a ''dose-response'' curve. In each system certain effects and implications are best seen with specific coordinate systems. Generally, these coordinate systems are chosen to produce the maximal sensitivity for the assay when low levels are being measured, and an overall broad range of sensitivity when a wide range of values may be present in the unknowns. The specific plot is also selected to give an accurate reflection of the true quantity of substance being measured, especially at the lowest levels. Plots must allow precision for repeated measurements. The ordinate and abscissa may be in various combinations of linear, logarithmic, or other transformations. Attempts at linearization of the curves or alterations to allow computer generation of the curves may increase their utility. While curve manipulation may increase sensitivity, accuracy, and precision of the measurements, it does not truly alter the basic results of the measurement.

VARIATIONS OF THE BASIC SYSTEM

Although the competitive protein binding or competitive binding radioassay seems very similar to the radioimmunoassay, the ligand in the competitive protein binding assay (CBPA) is bound to a protein rather than an antibody. Consequently, we are not dealing with the ligand as an antigen as such, but as a chemical substance. There are specific binding proteins for many hormones, such as corticosteroid-binding globulin and thyroxine-binding globulin. The principles of saturation and competitive binding, however, are exactly the same as with the radioimmunoassay presented above. There are a few basic technical differences between the radioimmunoassay and competitive protein binding assays. Since RIA systems usually have higher affinity constants as compared to the CBPA, RIA systems have greater sensitivity for measuring low levels