FUNDAMENTALS OF RIA

AND OTHER LIGAND ASSAYS©

A PROGRAMMED TEXT

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JEFFREY C. TRAVIS, Ph.D.

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Scientific Newsletters, Inc., 2421 W. Broadway,
P. O. Box 4546, Anaheim, California 92803

Radioassay Publishers, Division of Scientific Newsletters, Inc.
2421 West Broadway, P.O. Box 4546, Anaheim, CA 92803

INSTRUCTIONS

To use this text, use a piece of paper to cover material below the frame being studied. Write down the answer to the questions and check correct answer below dotted lines. When a series of concepts are covered, an entire group of answers appears at the end of a concept.

FOREWORD

The need for a comprehensive, basic text in RIA, where the concepts are presented in an easy-to-understand form, prompted Dr. Travis to write this programmed text.

Although radioassay techniques have revolutionized clinical testing and will continue to have an impact, other related techniques, such as enzyme, fluoro, and viro-immunoassay, will find a place in the armamentarium of the clinical laboratory. Since the basic concepts underlying these ligand assays are the same, they have been incorporated in the appropriate sections, thus making this book a leader in the field.

Not only can Fundamentals of RIA and Other Ligand Assays serve as a learning text, but the appendix incorporating radioisotope theory and statistical error in counting, nuclear regulations and radiation safety, glossary, review questions and references can be used as a handy reference.

This text will be of value not only to those entering the field of ligand assays but to those already in the radioassay field.

Sincerely,

Edith Helman,
Publisher

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I. INTRODUCTION AND BACKGROUND

Radioimmunoassay (RIA), or more appropriately competitive protein binding assay (CPB, radioligand assay), profoundly influenced evolution of the modern clinical laboratory. Where clinical testing once was limited to measurement of substances in gram, milligram, and occasionally microgram amounts (10^{-6} gm), the advent of RIA methodologies enabled for the first time measurement of substances present in extremely minute amounts in biological fluids. The enlightened combination of both radioisotopes and specific high affinity binding proteins, such as antibodies, made possible the sensitivity and specificity necessary to accurately measure physiological concentrations of substances at the microgram, nanogram, and picogram (10^{-6}, 10^{-9}, and 10^{-12}gm, respectively) levels; i.e., one-trillionth of a gram!

Today, in assays such as the Estrogen Receptor Assay (ERA), binding capacities are measured in terms of femtomols (10^{-15}M) bound per gram of tissue containing the specific receptor. Most importantly, development of radioligand assays enabled these determinations in biological fluids to be made quickly, easily, and economically relative to laborious classical chemical determinations. It made possible routine and specific clinical analyses of hormones, vitamins, drugs, and other substances which occur in minute concentrations but which, nevertheless, are significant in pathology.

Berson and Yalow introduced the technique of radioimmunoassay in 1960 for the hormone insulin, which culminated almost a decade of pioneering work on the nature of antibody binding, production, and interaction with labeled and unlabeled antigens.(1) They showed that the addition of increasing amounts of insulin to a mixture containing a *fixed* amount of antibody (in excess) and labeled antigen (tracer) caused a gradual decrease in the amount of tracer which was bound to antibody although the absolute amount of bound insulin increased. They recognized that binding of labeled antigen had a quantitative relationship to the amount of unlabeled insulin present when the concentration of antibody and labeled antigen in the reaction mixture were kept constant. This formed the basis of competitive protein binding theory.

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1. Yalow, R S and Berson, S A, *J Clin Invest* 39:1157, 1960.

Gaining acceptance for their theories often required overcoming many obstacles and prejudices. One example described by Drs. Yalow and Berson is illustrative and is informative for the student of RIA to appreciate the development of this methodology.

When first attempting to publish their findings that insulin-treated diabetics had insulin-binding antibodies, the first journal to which they submitted their manuscript rejected it with a comment to the effect that "everyone knows that insulin does not make antibodies."(2) This should be considered in the context of today's knowledge of the potential antigenicity of virtually any substance.

So great an impact on clinical laboratory analysis was Berson & Yalow's introduction of the radioassay technique, which in their first published assay used immunological binding to form protein-bound complexes, that the term radioimmunoassay (RIA) became descriptive for all competitive protein binding techniques, even those which did not employ antibodies as the binding reagent. Today, current acceptance of the more precise term "radio-ligand" assays is being promoted and is discussed in the text. In addition, other types of nonradioactive "labels" such as enzymes, viruses, and fluorescent compounds are available for "tagging" antigens. Thus, it is perhaps more correct to refer to these assays generally as ligand-binding assays.

The rapid advancement of commercial availability of ligand-binding assay test kits, most commonly RIA kits, have made possible routine clinical determination of a plethora of substances in microconcentration in blood and other body fluids. This text is designed to enable a student to learn the basic principles and practical applications of this exciting area of laboratory technology through self-teaching, programmed instruction.

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2. Yalow, R S and Berson, S A, Chapter 1 in *Principles of Competitive Protein Binding Assays,* Odell & Daughaday, ed., J. P. Lippincott Co., Philadelphia, 1971, pg 1.

II. PRINCIPLES AND THEORY OF LIGAND BINDING ASSAYS

Radioimmunoassay, displacement analysis, saturation analysis, competitive protein binding, radioligand assay are all names used to describe those assays which are based on the technique (first described by Berson and Yalow) in which unlabeled (present in excess) and labeled molecules of the same species compete for a limited number of sites on a specific binding protein. Once equilibrium has been obtained, the percentage of total label which is bound is inversely proportional to concentration of unlabeled substrate (the substance to be measured) which has been added to the reaction mixture.

This plethora of names is often confusing to those relatively inexperienced in ligand assays. For example, RIA has become almost a generic term for radioassays employing a *binding protein* and *radio-labeled binding agents (ligands),* irrespective of whether an immune or nonimmune system radioligand assay is being described.

Technically, *all* so-called saturation or equilibrium analyses which employ a binding protein and whose labeled and unlabeled ligands compete for limited binding sites are correctly termed "competitive protein binding assays" or CPB. If the binding protein employed is an antibody and the label is a radioisotope, the CPB assay is then termed a *radioimmunoassay.* In practice, however, it has become commonplace to refer to radioimmune binding assays as RIA and to limit the use of CPB terminology to those assays employing a plasma protein as its specific binding agent. As we shall see, the generic term "ligand binding assay" (ligand referring to "anything that binds") is being promoted as the descriptive term of all variations of this group.

No response required

A. GENERAL BACKGROUND AND THEORY

1. The necessity for sensitive techniques such as RIA arose because the concentration of hormones (and drugs and

vitamins) in biological fluids is on the order of 10^{-7}–10^{-11}M/L. Three basic types of measurement are available for quantitating minute substances in blood: *chemical, bioassay,* and *ligand binding analyses.*

a. Hormones are present in blood in minute concentrations on the order of _____.

b. Name three common types of analytical methods used to measure minute concentrations of hormones and drugs: _____, _____, and _____.

---

a. 10^{-7}–10^{-11}M/L
b. chemical, bioassay, radioligand

---

2. Chemical methods routinely used in the clinical lab (usually *spectrophotometry)* rarely can measure hormone concentrations accurately at levels much below *milligram* amounts. More sensitive techniques such as *spectrofluorometry* can assay levels on the order of *micrograms* but are limited to those compounds that fluoresce or that can be reacted with something which causes them to fluoresce. Also, these assays are subject to significant interferences (i.e. quench, contaminated glassware, etc.). More sensitive chemical techniques such as *gas-liquid chromatography* are technically too cumbersome for routine clinical use.

a. Assays which proved unsuitable for routine clinical quantitation of hormones in blood due to insensitivity, interferences, or technical difficulty included (i) _____, (ii) _____, and (iii) _____.

---

a. (i) spectrophotometry
(ii) spectrofluorometry
(iii) GLC

---

3. For many years *bioassay* (approximation of hormone concentration by the extent of its effect on a test animal or biological tissue) alone had sufficient sensitivity for quantitation of minute hormone concentrations. Unfortunately, bioassay was very *expensive* because it required maintenance of *live animals or tissue,* was very *tedious* and *time-consuming* and suffered from *lack of specificity.*

a. Give four major drawbacks of bioassay for routine clinical measurement of hormones in blood: (i)_____, (ii)_____, (iii) _____, and (iv) _____.

---

a. (i) expensive
(ii) tedious
(iii) time-consuming
(iv) lack of specificity

---

4. Ligand assays have the distinct advantage over these earlier procedures because they can accurately measure hormone concentrations at *microgram, nanogram,* and even *picogram* (10^{-6}, 10^{-9}, 10^{-12}gm respectively) levels and with good *specificity.* They are relatively easy to perform from a technical standpoint and many analyses can be performed *quickly* relative to time-consuming chemical and bioassay analyses.

a. 10^{-6} to _____gm concentrations of hormones in blood can be routinely measured using _____.

b. Ligand assays have the advantage of speed and_____ _____ over earlier techniques.

---

a. 10^{-12}
radioligand assays
b. specificity

---

5. At this point, certain terms should be briefly defined. An *antigen (Ag)* is anything that when injected into an animal will stimulate an immune response, i.e., production of an antibody or antibodies which will bind antigens. An *antibody (Ab)* is a *gamma globulin* produced as an immune response against a foreign substance or antigen and which specifically binds that substance.

   a. A substance which elicits antibody formation against it (that will bind with it) is called an _____.

   b. Antibodies are part of the _____ globulin fraction of serum proteins.

---

   a. antigen (Ag)
   b. gamma

---

6. A *ligand* is "anything that will bind" to an appropriate binding reagent and usually refers to both labeled tracer *and* unlabeled substrate in a CPB assay. A *hapten* is an organic molecule (200-1000 mw) which is *nonimmunogenic* (nonantigenic) itself but which elicits an immune response when attached to protein. In this way, nonantigenic small molecules such as steroid hormones and drugs can (as haptens conjugated to protein) stimulate antibody production with specificity for the hapten molecule itself.

   a. The radiolabeled and unlabeled substrate in a CPB assay which binds to the specific binding molecule employed is called a _____.

   b. A small molecule which is not, itself, antigenic, is called a _____.

---

   a. ligand
   b. hapten

---

7. The *unlabeled substrate* in a ligand assay is the "substance in the specimen which is *to be quantitated* in the assay" and is called the *analyte.*

   a. In a ligand assay, that substance in unknown concentration in the specimen which is to be measured is called the _____.

   b. The analyte is synonymous with _____ substrate.

---

   a. analyte
   b. unlabeled

---

8. A *ligand assay* is a general term applied to *any binding assay* characterized by the following general equation:

$$\text{Ligand} + \text{Binding Reagent} \rightleftharpoons \text{L} - \text{BR complex}$$

   a. A binding assay is referred to as a _____ assay.

---

   a. ligand

---

9. A *competitive protein binding assay (CPB)* is a ligand assay in which the *binding reagent* is a *protein* with a *limited number* of available binding sites for which *both* labeled and unlabeled ligands *compete.*

   a. A competitive ligand assay employing a protein as binding reagent is termed a _____.

---

   a. CPB

---

10. The substance used to monitor the count of a binding assay is called the *tracer* (also *marker, label).* The label may be a *radioisotope, enzyme, fluorescent substance* or *virus.*

    a. The _____ label is used to monitor the count of a ligand assay.

    b. List 4 types of labels: (i) _____, (ii) _____, (iii) _____, and (iv) _____.

---

a. label
b. (i) radioisotope
   (ii) enzyme
   (iii) fluorescent substance
   (iv) virus

Introduction of ligand assays gave the clinical laboratorian the means by which substances in very low concentration in biological fluids could be measured for routine use in pathology. To more completely understand this relatively new technology we need to look at the essential component of all ligand assays, that is, the binding reactions involved.

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11. *Protein Binding* has been defined as "the association between a protein and another substance (which may itself be a protein) that results from the action of many weak, shortrange forces at a multiplicity of sites on each of the reacting molecules."

No response required

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12. *Extent of Binding* $\propto$ *[affinity] [capacity]* where affinity is the *strength of binding* and capacity refers to the *number of available binding sites.*

    a. _____ is the strength of binding between a binding protein and the molecule it binds _____.

    b. The number of binding sites refers to _____.

    c. Extent of binding in a CPB $\propto$ _____.

---

a. Affinity, (Ligand)
b. capacity
c. [Affinity] [Capacity]

---

13. Binding forces involved in CPB reactions are *hydrogen binding, ionic forces,* and *van der Waal's forces.* Since these are short-range forces, the three-dimensional configuration or "fit" of one molecule into another is important. The ability of these reactants to "fit" together *(complimentarity)* is closely related to the specificity and affinity of binding.

    a. Short-range binding forces at work in radioligand assays are _____, _____, and _____.

---

a. H binding, ionic forces, van der Waal's

---

14. Although the exact nature of ligand binding in these assays is still obscure, the reactants are theoretically considered to reach *equilibrium* at the end of the incubation step (although as we shall see, many ligand systems presently in use do not reach equilibrium for a variety of reasons). Equilibrium is that stage in a reaction where the concentration of the reactive species is no longer changing. The equilibrium equation is:

$$L + BP \underset{k2}{\overset{k1}{\rightleftharpoons}} L\text{-}BP \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where L = Ligand (Analyte) BP = Specific Binding Protein
L-BP = the bound complex

a. Write the equilibrium equation for protein binding assays

_____

---

a. $L + BP \underset{k2}{\overset{k1}{\rightleftharpoons}} L\text{-}BP$

---

15. Equation (1) obeys the law of mass action and by definition:

$$\frac{[L\text{-}BP]}{[L]\ [BP]} = \frac{k1}{k2} = K_a = \frac{\text{Equilibrium or Affinity}}{\text{Constant}} \quad \ldots \quad (2)$$

where the brackets indicate molar concentrations.

Therefore:

K_a is in units of $(M/L)^{-1}$ or L/M.

a. K_a = _____ Constant

b. Units for K_a are _____.

---

a. Equilibrium or Affinity
b. L/M

---

16. A definition of the equilibrium constant (K_a) is that volume, in liters, into which a gram molecule of protein can be diluted so that 50% binding of a minimal amount of tracer ligand will result.

The law of mass action is based on the assumption that all binding sites function independently of each other and with equal affinity for both labeled and unlabeled ligands. In practice, however, binding reagents may discriminate between labeled and unlabeled ligands for various reasons.

No response required

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17. Most plasma proteins have a *single binding site* of *constant affinity*. Antibodies may have *multiple binding sites* with *varying affinities* for the same ligand, i.e., *heterogeneity* of binding sites.

    a. Plasma binding proteins possess a _____ binding site of _____ affinity.

    b. Antibodies have _____ binding sites and _____ affinities.

---

a. single, constant
b. multiple, varying
