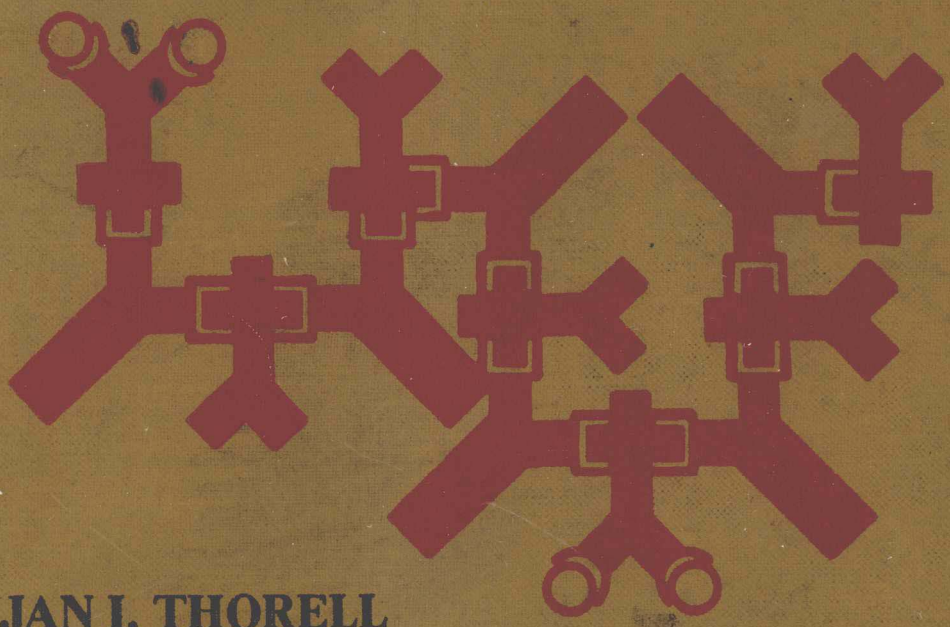
Radioimmunoassay and related techniques

METHODOLOGY AND CLINICAL APPLICATIONS



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To our families

PREFACE

During the last few years we have often been asked to recommend an introductory text on radioimmunoassay. This request has been made in many different situations: in elementary courses for medical students or medical technologists; in more advanced courses for scientists and practicing physicians; and in consultation with scientists, research trainees, and laboratory supervisors who were about to start immunoassay work for the first time. Frequently, the radioimmunoassay literature available has been too specialized. Multiauthor books and symposia, by experts writing for experts, are difficult for the beginner to utilize. On the other hand, "beginner oriented" writings, such as journal survey articles, are often too short and superficial for the serious student. Accordingly, we have written this text as both a general survey of the field and as a source of information that is detailed enough to be used as a basis for actual performance of important assays.

As we prepared this text on radioimmunoassay and related methods, we were struck by the diversity of the field. Applications of this methodology impinge on many aspects of biologic, chemical, and physical sciences. With such great diversity it is a practical impossibility to encompass all of the relevant scientific knowledge that relates to this discipline. For this reason, we have restricted the scope of this book to include the more practical aspects of this methodology that will enable practitioners in the field and interested students to obtain a thorough grounding in the basic principles of these techniques. In addition, the combination of a methodology and clinical applications in the same volume is an attempt to bridge the gap between the laboratory and the clinical.

The abundance of different methodologic variants, despite the common principles used in most methods, has made this condensation necessary. This overview does not intend to cover all possible variants reported. However, the principle of the main procedures given in the boxes is that we have performed the method in our own laboratories and, for most methods, in heavy clinical practice over a long period. This at least implies that they have worked well in our hands and should not involve unanticipated problems in actual performance.

The impact of methodologic advances on progress in medicine should not be underestimated. Radioimmunoassay serves as an important example of what a new methodology with a greatly improved sensitivity can mean in terms of advancing scientific knowledge. What of the future for this technology? We believe that the clinical utilization of radioimmunoassay is only at the threshold of an era of many new applications and developments of importance both for everyday clinical medicine and advanced biomedical research. The recent rapid advances of biochemistry are likely to help us to achieve even better binding reagents, in particular more specific and highly avid antisera. Newer labels may be employed that will improve the sensitivity of this test still further. Simpler methodologies and more rapid separation techniques will undoubtedly appear. For some clinical assays, the baton has already been passed from the researcher to the industrialist, and more practical applications to clinical medicine can be anticipated in the future.

This text would not have been possible without the expertise and experience of our collaborators in the laboratory. Thanks to the keen interest of Mr. Ingvar Larsson, M.S., and Mr.

Åke Forsberg, B.A., all boxes and appendixes have been put together from our current laboratory manuals. The extensive typing was done by Mrs. Britta Thelander and Mrs. Ina Blair. During the course of our collaboration we have received valuable advice and support from Henry N. Wagner, Jr., M.D., Johns Hopkins Medical Institutions, Baltimore, Maryland; Tyra T. Hutchens, M.D., University of Oregon Health Sciences Center, Portland, Oregon; and Bertil Nosslin, M.D., Malmö General Hospital, Malmö, Sweden. We extend our gratitude to them and all the other capable workers who have contributed to this volume.

Jan I. Thorell

Steven M. Larson

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PART I

Methodology

1 INTRODUCTION

Radioimmunoassay (RIA) and related competitive protein-binding methods began a little over 20 years ago as a cumbersome research methodology in a few specialized laboratories. Since that time there has been a phenomenal proliferation of these techniques to the measurement of hormones and other substances present in minute quantities in biologic fluids. It is almost impossible to exaggerate the diversity of applications for this methodology. Virtually every branch of medical and biologic research has been affected by these techniques. In particular, endocrinology has been greatly enriched by the new knowledge that has come as a direct result of RIA methods. These methodologies are being introduced into clinical medicine at a rapid rate, and the growing commercial availability of radioassay kits promises to revolutionize the routine practice of hospital laboratories.

TERMINOLOGY

The term radioimmunoassay was coined by Berson and Yalow in describing their original methodology. This term is adequate when radioassays are described in which antibodies are used as specific binding reagents; but because many assays utilize binding reagents other than antibodies, a more general term is required. Several different terms have been proposed, including saturation analysis, competitive radioassay, competitive binding assay, displacement analysis, radiostereoassay, and competitive protein-binding procedures. Each of these terms has deficiencies when used to describe the whole class or technique. Two examples serve to indicate the deficiencies: no assay works at saturation conditions in the sense that all receptor sites are occupied; an increasing number of radioassays are not competitive.

We have adhered to the term radioligand assay for the following reasons. The word ligand emerges from the Latin word "ligare," which means "to bind." The term ligand is a common denominator for compounds participating in interactions between macromolecules and smaller molecules. All radioassay methods of the type discussed here involve the binding of a compound to some sort of specific receptor. Accordingly, the term ligand assay need not be confined to any special type of interaction occurring in the assay or to any particular type of components participating in this reaction. Also, this nomenclature will conform to that currently used in chemistry and biochemistry to describe the interaction between big and small molecules. The prefix "radio" in radioligand assay indicates that radioactive nuclides are used as the indicator for the purpose of quantitation. Radionuclides are by no means the only indicator that may be used for this purpose. Assays in which an enzyme or a fluorescent substance is used may be called enzymatic ligand assay or fluorescent ligand assay, respectively. Explanations of other terms that may be encountered during a discussion of in vitro radionuclide methodology are given in the glossary of radioligand assay terms below.

GLOSSARY OF TERMS USED IN RADIOLIGAND ASSAY

accuracy (of assay) Closeness to "true" or real value.

affinity Property of substance bound. Strength (energy) of binding to the receptor.

analyte Substance to be measured by an assay system.

antibody A protein formed as part of an immunologic response to a foreign substance. The antibody specifically combines with the foreign substance and to a variable extent with substances of similar structure.

antibody, first Term used in double-antibody assays to indicate the antibody binding the substance to be assayed.

antibody, second Term used in double-antibody assays to indicate a precipitating antibody, which binds the first antibody, in order to separate "bound" from "free" ligand.

antigen A substance that is capable of inducing formation of antibodies and that reacts specifically to the antibodies so produced.

antiserum Serum from an immune animal, contains various antibodies.

autologous Term of immunologic relationship, from same individual.

avidity Property of binder, strength (energy) of binding of ligand.

competitive inhibition Describes inhibition of binding of radioligand to receptor by ligand.

competitive protein binding Synonym for radioligand (assay), emphasizing importance of protein receptors in in vitro assays that utilize competitive inhibition.

competitive radioassay Synonym for radioligand assay, emphasizing competition between radioligand and ligand for a specific receptor binding site as the basis for in vitro radioassay.

damage Jargon term used to indicate denaturation of the radioligand. This may occur during the process of radiolabeling—"labeling damage" (in the case of labeling by means of radioiodination, "iodination damage"). If it happens during the incubation period of an assay, it is often referred to as "incubation damage" or during storage of the radioligand as "storage damage."

displacement analysis Synonym for radioligand assay, emphasizing the principle of displacement of radioligand by stable ligand as the basis for in vitro radioassay.

hapten A substance that is not immunogenic in itself, but becomes immunogenic when complexed to another compound. The antibody produced will bind the noncomplexed hapten also; for example, steroids and digoxin.

heterologous Xenogeneic, from an individual of another species.

homologous From another individual of same species: *syngeneic* if genetically identical, *allogeneic* if genetically different.

immunogen A substance capable of inducing an immune response when introduced into an immunologically competent host.

immunoglobulins A group of serum proteins of which the following classes are known: IgG, IgM, IgA, IgD, IgE. They emerge as the effect of an immunoresponse and have the property in common to specifically bind the antigen. IgG is quantitatively dominant and is the usual binding antibody in RIAs.

immunoradiometric assay Ligand to be measured is assayed directly by combination with specific labeled antibodies rather than in competition with a labeled derivative for a limited amount of antibody.

incubation damage Alteration in binding characteristics of a ligand during the incubation period of an assay (usually applied to radioligand). This may result, for example, from aggregation of molecules, enzymatic degradation, radiation damage, or deiodination.

incubation period, first Ambiguous term used to describe (1) period of preincubation of ligand and binding agent before addition of radioligand (disequilibrium assays) or (2) period of incubation between ligand, radioligand, and binding agent before addition of precipitating (second) antibody (double-antibody assays).

incubation period, second Ambiguous term used to describe (1) period of incubation after addition of radioligand (disequilibrium assays) or (2) period of incubation with precipitating (second) antibody (double-antibody assays).

iodination damage *See* damage.

ligand The substance that is bound. In radioassays usually the hormone or other moiety that is to be assayed.

precision (of assay) Degree of agreement of repeated measurements of a quantity. Usually expressed as the coefficient of variation for repeated measurements of the same sample: intraassay if it refers to precision within an individual assay run, interassay if it refers to results from different sets of assays.

quality control In general terms, the analytical and other steps that must be taken to ensure that results of assays are reliable and representative for the true concentration of the material to be assayed.

radioimmunoassay Radioligand assay in which the receptor is an antibody.

radioligand Radioactive form of ligand.

radioligand assay General term for in vitro radioassay based on receptor-ligand binding.

radioreceptor assay Term usually used when a cell-

associated receptor (membrane, cytoplasm, nucleus) is used as the binder for a radioligand assay. May also be used less commonly as a general term for in vitro radioassay based on receptor-ligand binding.

radiostereoassay Synonym for radioligand assay, emphasizing the importance of tertiary protein structure and specific complementary orientation of ligand and receptor in in vitro radioassay.

receptor A substance (a protein) that specifically binds a certain compound (ligand). In radioligand assays most commonly an antibody but includes other binders such as plasma transporting proteins, enzymes, or cell-associated binding substances (membrane receptor, intracellular receptor).

saturation analysis Synonym for radioligand assay, emphasizing the progressive saturation of available receptor-binding sites with ligand, and the subsequent nonavailability of receptor sites for binding of radioligand, as the basis for in vitro radioassay.

sensitivity Minimum quantity detectable. Depends in part on assay precision.

specificity Ability to assay a single substance in heterogeneous mixtures. For RIA, the capacity to discriminate antigens of similar structure.

standard A substance, usually chemically identical to the substance to be assayed, that is added to certain reference tubes in an assay series to serve as a yardstick for quantitation of the contents in the samples. It is not necessarily of maximum purity. An International Standard is a particular preparation of a substance that has been adopted by the World Health Organization as a common reference for quantity of this substance.

titer Measurement of antibody concentration. For RIA, the titer is frequently defined in terms of the dilution of antiserum that will bind 50% of added radioligand.

CHARACTERISTICS OF RADIOLIGAND ASSAY

Receptors that bind radioactive substances were first used to assess the binding capacities of proteins such as thyroxine-binding proteins and transferrin. Competition between radioligand and ligand in the unknown sample for a specific receptor-binding site was first used from 1956 to 1960 as a means of measuring ligand concentration by Berson and Yalow and associates. This concept has been called the principle of competitive inhibition. These investigators deserve much of the credit for developing the basic techniques for radioligand assays in general. Their initial studies were of insulin metabolism in diabetes. They were able to achieve ^{131}I labeling of insulin in specific activities high enough to permit adequate assay

Table 1-1. Ligand-receptor combination in radioligand assays

Antibodies		Plasma transporting proteins		Tissue receptors	
Ligand	**Antibody to**	**Ligand**	**Receptor**	**Ligand**	**Receptor**
Protein hormones	Protein hormone (sometimes conjugated)	Thyroxine	TBG	Vitamin B_{12} Nucleotides	Intrinsic factor Nucleotide binding
Steroid hormones	Steroid-protein complex	Vitamin B_{12}	Transcobalamin	Estrogen	Uterus cytosol receptor
Enzymes	Enzyme	Testosterone	Testosterone-binding globulin	ACTH	Adrenal cortex Cell membrane receptor
Nucleotides	Nucleotide-protein complex	Cortisol	Corticosteroid-binding globulin	Insulin	Liver, placenta Cell membrane receptor
Virus	Virus, in animal or human				
Drugs	Drug-protein conjugate				

sensitivity. They also devised methods for separation of the unbound radioligand from that bound to specific antibody. This work resulted in the first RIA, which was for serum insulin, in 1960. The use of a nonimmune serum-binding protein for a competitive assay was independently developed by Ekins, who devised a method for measurement of thyroxine using the thyroxine-binding properties of specific binding globulins in human plasma. The subsequent development of a series of RIAs for other hormones and biologically important substances was greatly facilitated by the work of Hunter and Greenwood in 1962, who developed the chloramine-T method for readily labeling proteins with high specific activities of ^{131}I.

Radioligand assay methods have in common a binding reagent (receptor) that selectively binds the substance to be measured (ligand). Antibodies have been the most extensively used type of receptor (RIA), but the specific binding properties of other proteins have also been used (Table 1-1).

The revolution in the measurement of biologic substances created by radioligand assays was primarily an effect of a dramatic increase in *sensitivity* in comparison with other chemical methodologies. This sensitivity is related to the

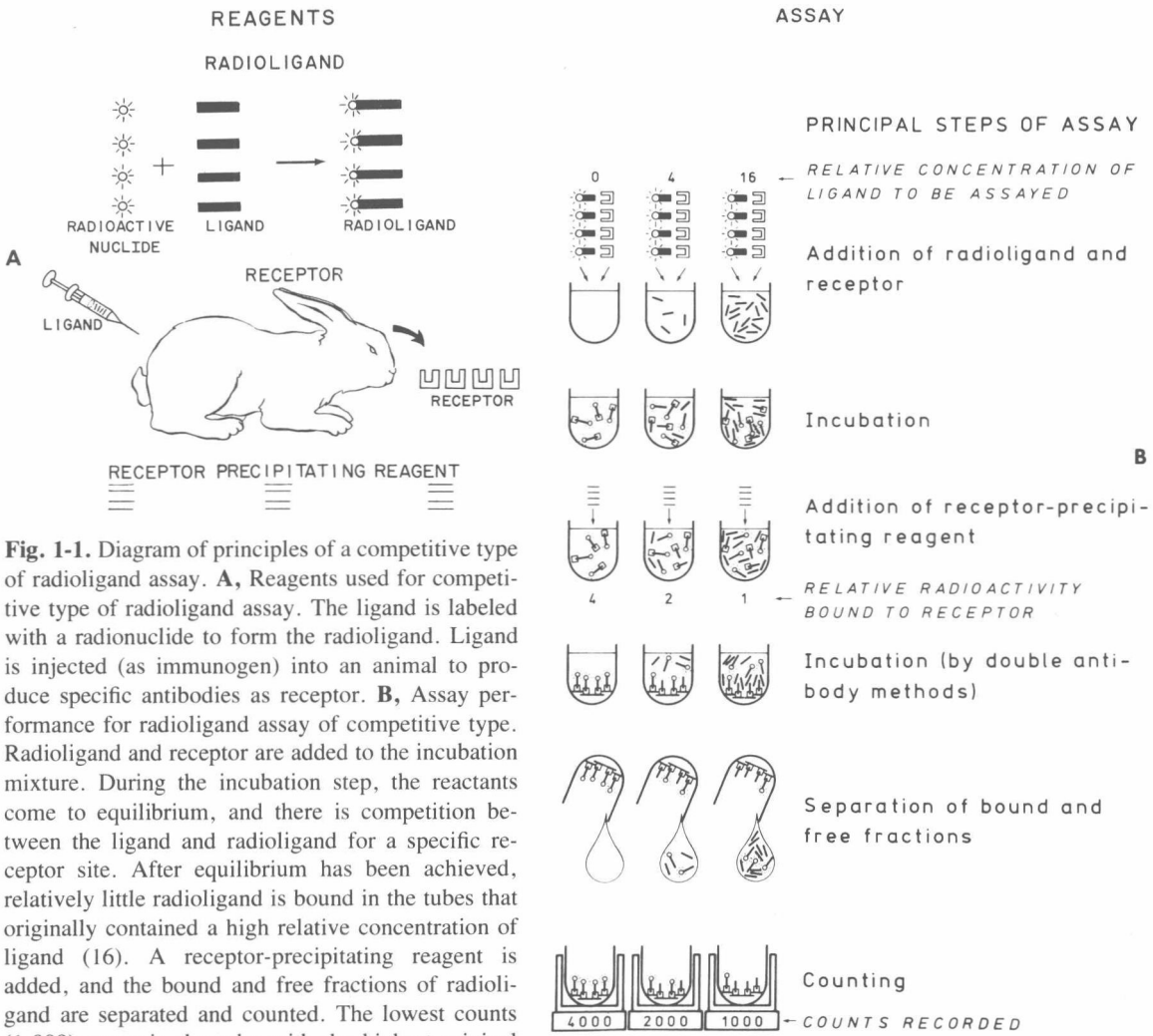

Fig. 1-1. Diagram of principles of a competitive type of radioligand assay. **A,** Reagents used for competitive type of radioligand assay. The ligand is labeled with a radionuclide to form the radioligand. Ligand is injected (as immunogen) into an animal to produce specific antibodies as receptor. **B,** Assay performance for radioligand assay of competitive type. Radioligand and receptor are added to the incubation mixture. During the incubation step, the reactants come to equilibrium, and there is competition between the ligand and radioligand for a specific receptor site. After equilibrium has been achieved, relatively little radioligand is bound in the tubes that originally contained a high relative concentration of ligand (16). A receptor-precipitating reagent is added, and the bound and free fractions of radioligand are separated and counted. The lowest counts (1,000) occur in the tube with the highest original relative concentration of radioligand (16).

extremely sensitive methods for the detection of radioactivity. For example, it is possible to measure the radioactive isotope ^{125}I in quantities as small as 10^{-14} g (or approximately 10^{-12} M) with good precision. Since the serum concentration of most hormones is on the order of 10^{9} to 10^{10} M, detection of hormone levels at the lowest physiologic concentration is now possible. The radioactive tracer used in these assays is called the radioligand.

The other main feature of this methodology is *specificity*. Most biologically active compounds are built up by the common elements C, O, H, and a few others. The uniqueness of these compounds is therefore determined not so much by their overall composition but by the order in which the elements are put together. It is the architecture of the compounds that determines their function. A unique structure will give the molecule a unique external configuration. This is also the basis for the assay specificity. The ligand is bound by the receptor at a specific receptor-binding site. This binding site has a configuration that is complementary to a particular area on the surface of the ligand. The binding between ligand and receptor can be viewed as the fitting of a key into a lock. The ability of a receptor to recognize the external configuration is therefore the basis for identification of the particular compound in a biologic fluid. Antibodies have been produced that have such high specificity that they can distinguish between two peptides that differ by as little as one atom, for example, thyroxine and triiodothyronine. The fit between receptor and ligand also influences the strength of the bond, a factor that along with the specific activity of the radioligand determines the sensitivity of the assay.

In addition, radioassays have been refined to the point that they are relatively easy to perform on a mass scale with a high degree of precision. These techniques lend themselves relatively well to automation. In this regard we are already beginning to see in the clinical laboratory the fruit of a decade or more of rapid commercial development.

PRINCIPLES OF RADIOLIGAND ASSAY

In order to establish a radioligand assay, three prerequisites must be met: (1) a receptor must be available that specifically binds the substance (ligand) to be measured; (2) the ligand must be labeled with a radioactive nuclide (radioligand); and (3) separation must be achievable between the ligand bound to the receptor and the ligand that is unbound. The radioligand assay may be divided into two general categories: competitive and noncompetitive radioligand assay.

Competitive assays

The competitive assay is still the most common type. The principles of such methods are shown in Fig. 1-1. In this case, the specific

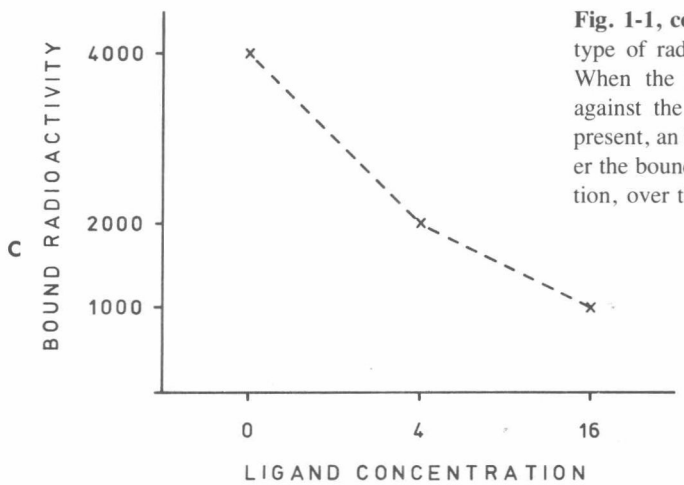

Fig. 1-1, cont'd. C, Standard curve for competitive type of radioligand assay. Data from Fig. 1-1, **B.** When the bound radioactivity counted is plotted against the relative ligand concentration originally present, an inverse relationship is observed. The lower the bound counts the greater the ligand concentration, over the range 0 to 16.

receptor is an antibody directed against the ligand to be measured. The ligand is injected into a species of animal that sees this substance as foreign and produces a specific antibody against the ligand. The radioligand is produced by coupling of radioactive nuclide to the ligand as a marker for the ligand substance to be measured (Fig. 1-1, *A*). The principal steps of assay are illustrated in Fig. 1-1, *B*. The amount of antibody used (in this simplified example) has a binding capacity that approximately corresponds to the amount of radioligand present. If there is any native (unlabeled or "cold") ligand present, it will compete with the radioligand molecules for the limited number of binding sites. As the concentration of native ligand increases, less and less radioactive material will be bound to the receptor. Thus the greater the concentration of native ligand in the biologic specimen, the lower the amount of ra-

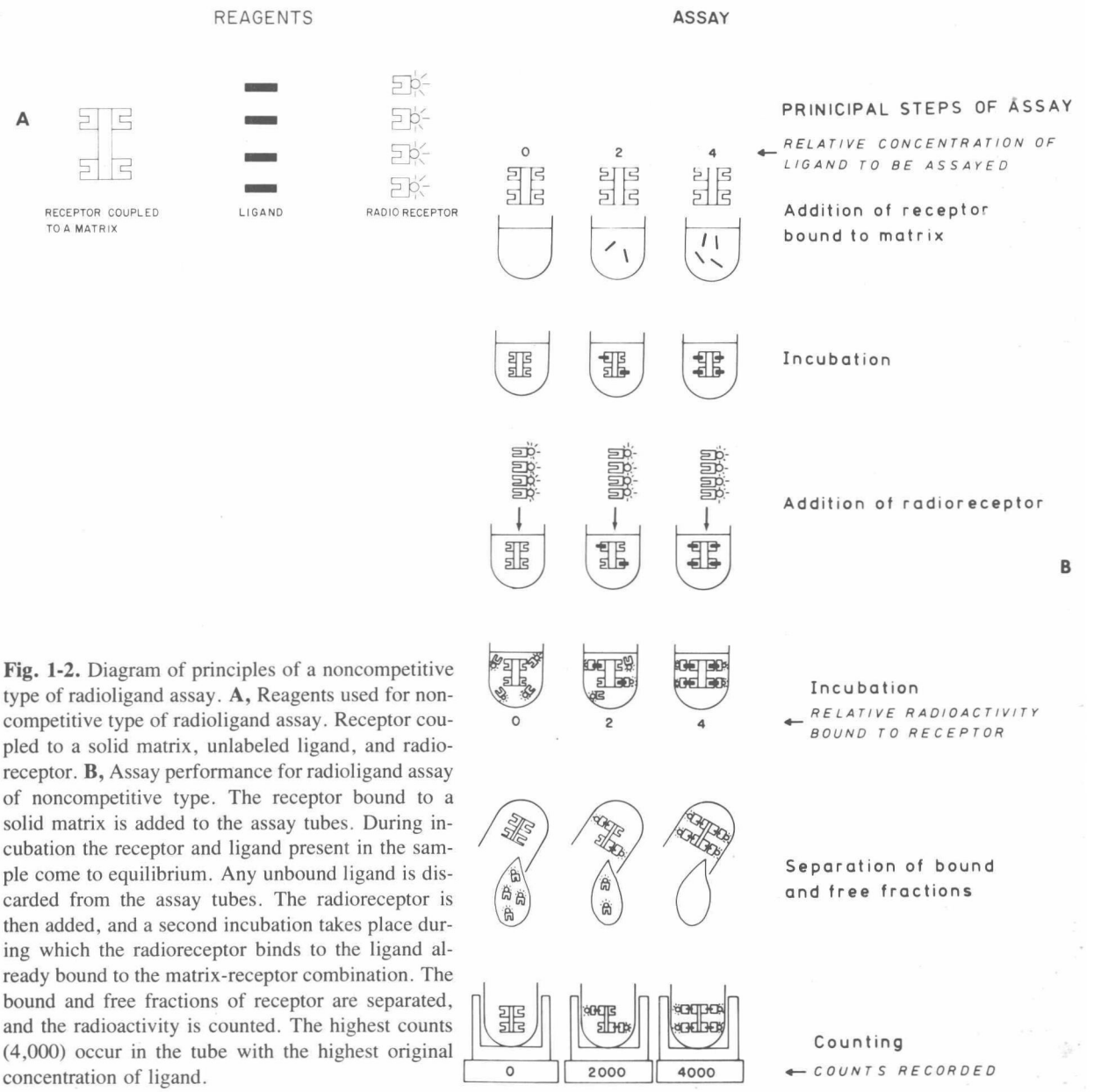

Fig. 1-2. Diagram of principles of a noncompetitive type of radioligand assay. **A,** Reagents used for noncompetitive type of radioligand assay. Receptor coupled to a solid matrix, unlabeled ligand, and radioreceptor. **B,** Assay performance for radioligand assay of noncompetitive type. The receptor bound to a solid matrix is added to the assay tubes. During incubation the receptor and ligand present in the sample come to equilibrium. Any unbound ligand is discarded from the assay tubes. The radioreceptor is then added, and a second incubation takes place during which the radioreceptor binds to the ligand already bound to the matrix-receptor combination. The bound and free fractions of receptor are separated, and the radioactivity is counted. The highest counts (4,000) occur in the tube with the highest original concentration of ligand.