Immunoassay—Is There a Future Role for Nuclear Medicine?

Lynn R. Witherspoon

Ochsner Clinic and Alton Ochsner Medical Foundation, New Orleans, Louisiana

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At the 1955 annual meeting of the Society of Nuclear Medicine held in Portland, Oregon, Drs. Berson, Yalow, and co-workers presented studies (*1*), later published in greater detail (*2*), describing the discovery of insulin-binding globulin in the blood of insulin-treated diabetics. "The recognition that the binding of labeled insulin is a quantitative function of the amount of insulin present when the antibody concentration is kept fixed, and that labeled insulin can be displaced by unlabeled insulin, formed the basis of the radioimmunoassay of plasma insulin" (*3–6*). The rapid application of these principles led to the development of now numerous immunoassays used for the quantitation of ligands of clinical interest present in body fluids in trace concentrations. Measurements by radioimmunoassay involve nearly all facets of medical practice, stimulating the exploration and development of all procedures with similar applications. Although radioimmunoassays are responsible for much of the growth and evolution of nuclear medicine, they are being supplemented or supplanted by nonradioactive immunoassays in a growing number of applications. Despite their wide application and obvious contribution, radioimmunoassays will undoubtedly continue to recede in the future.

In this article, I will consider the evolution, current status, and potential future of immunoassay technology in the clinical laboratory. I will also consider current and future applications of these methods. This article is not intended to be a review; rather it is an attempt to examine the role nuclear medicine may play in the future application of these techniques. In a discussion so wide-ranging I may either unintentionally or of necessity omit techniques or applications of potential importance. In addition, while recognizing the dangers inherent in treating so much material superficially, I will attempt to document the ideas discussed so that the reader may turn to more detailed literature if stimulated to do so. With these apologies, let me attempt to place immunoassay in perspective for the nuclear medicine physician of the 1980s.

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For reprints contact: Dr. Witherspoon, Ochsner Clinic, 1514 Jefferson Highway, New Orleans, LA 70121.

Immunoassays have a number of features in common whether a radioactive or a stable label is used. Ligand assays of two general types have evolved: (a) competitive ("saturation analysis") binding assays characterized by a limited amount of binder and using labeled ligand, and (b) immunometric assays characterized by an excess of specific binder and the identification of bound ligand by a second labeled binder, most commonly, but not necessarily, a labeled antibody. Ekins has termed these assays "limited reagent" or "labeled analyte" methods in the first case and "excess reagent" or "labeled antibody" methods in the second—useful designations emphasizing the fundamental differences between the two methods (*7–9*).

Limited-reagent methods. As originally described by Yalow and Berson (*5*), competitive binding assays [e.g., radioimmunoassays (RIA)] utilize a binder (e.g., antibody) of very high affinity (K_a about 10^{12} l/mole) possessing specificity for the analyte of interest. The binder is present in limited quantity, its concentration frequently being chosen to bind 30–70% of the labeled analyte in the absence of nonlabeled ligand (*3*). Ekins has repeatedly emphasized that theoretically the most sensitive assay is achieved as the binder concentration tends to zero (*10,11*). The assay's detection limit is relatively independent of the specific activity of the labeled ligand, being a function of antibody affinity and the experimental error (i.e. error/K_a) (*9*). The least detectable dose is therefore fundamentally determined by antiserum characteristics and by the technical skill of the assayist. Extended incubation times are often required to achieve maximum sensitivity in equilibrium-limited reagent systems.

Excess-reagent methods. Wide et al. (*12*) and Miles and Hales (*13*) described immunoradiometric (IRMA) assays in which excess radiolabeled antibody was added to a small sample containing the analyte of interest. In

contrast to limited-reagent methods, virtually all the ligand of interest was bound by the radiolabeled antibody present in excess. The excess labeled antibody was removed utilizing solid-phase ligand, leaving the labeled antibody-ligand complex in solution. These IRMAs required relatively large quantities of purified ligand for use as the immunoadsorbent, not always possible to acquire. More commonly today, solid-phase nonlabeled antibody in excess is utilized to bind ligand with at least two binding sites (*14*). Bound ligand is identified either by the addition of labeled antibody ("two-site") or, after the addition of the second antibody, by the addition of radiolabeled anti-gamma globulin ("three-site"). Although the "two-site" IRMA requires relatively large amounts of ligand-specific antibody, the "three-site" assay uses the same radiolabeled anti-IgG for the detection and quantification of a variety of analytes.

The detection limits of excess-reagent methods are relatively independent of antibody affinity, since an excess of antibody is used to bind all the analyte present in the incubation mixture. Rodbard (*15*) and Ekins (*9*) have shown theoretically that the specific activity of the labeled antibody limits sensitivity in reagent-excess systems. In theory, the sensitivity of such assays might approach a single molecule, and incubation times necessary to bind all the analyte present might be very brief. In practice, sensitivity is limited by specific activity of the label, background, nonspecific binding, and experimental and misclassification errors (limited to a degree by washing) so that reagent-excess systems are now seldom more sensitive than reagent-limited systems (*16*). In systems using a solid-phase primary antibody, the antibody affinity must be relatively high due to constraints on antibody concentration achievable on solid supports. In addition, relative to liquid-phase systems, the geometry of binding is unfavorable in solid-phase systems, where the ligand and binder are not intimately mixed, resulting in lengthened incubation times and decreased ligand binding (*17*).

Although many analytes are measured today using limited-reagent methods, a greater number of reagent kit manufacturers have attempted to exploit the potentially enhanced sensitivity and decreased incubation times inherent in excess-reagent systems. We may expect to see a wider application of excess-reagent systems in the future.

Immunoassays, whether using limited- or excess-reagent methods, require reference ligand for system calibration, labeled reagent (ligand, antibody, or reagent specific for immunoglobulins) and a highly specific binder (antibody, receptor, or other binding protein). In addition, radioassay systems, with few notable exceptions, require separation of the antibody bound from free fractions in limited-reagent systems, or from the excess radiolabeled antibody in excess-reagent systems. The use of a label whose signal is altered by proximity to the binder offers the possibility of homogenous assays not requiring a separation step.

BINDING REAGENTS

Immunoassays require a binding reagent with specificity and affinity for the ligand to be detected and quantified. Shortly after Yalow and Berson utilized IgG antibody for the quantification of insulin (*5*), other naturally occurring binding proteins were used to estimate concentrations of thyroxine (*18–20*), vitamin B_{12} (*21*), and cortisol (*22,23*). More recently, cell plasma-membrane or intracellular receptors have been utilized. Greatest present and future interest centers around the application of monoclonal antibodies and receptor systems.

Monoclonal antibodies. Perhaps second only to the elucidation of radioimmunoassay itself, development of the technique to produce unlimited IgG antibody directed against a single antigenic determinant represents a major advance in immunoassay technology. Recognizing that a number of permanent cultures of myeloma cells that secrete immunoglobin exist, Köhler and Milstein described in 1975 a modified permanent cell line that secreted antibody of predetermined specificity (*24*). These investigators developed clones (hybridomas) that secreted anti-sheep erythrocyte antibody; they were produced by the fusion of mouse myeloma cells and spleen lymphocytes derived from mice immunized with sheep erythrocytes. This technique confirms the permanence of the tumor-cell line and the genetic encoding present in a single spleen B lymphocyte for a unique immunoglobulin upon the resultant hybridoma. The method has been widely applied in a brief time, and a variety of monoclonal antibodies and assay systems that utilize monoclonal antibodies are now available commercially.

The development of hybridomas requires the fusion of appropriate tumor cells with B lymphocytes derived from an animal immunized with the antigen for which an assay is desired (*25–29*). Although other applications require human IgG, mouse hybridomas suffice to produce antibodies suitable for immunoassay. Myeloma cells and B lymphocytes derived from the spleen of an immunized mouse, when mixed in an incubation medium conducive to fusion, result in a very large number of fused cell lines, only a few of which may secrete immunoglobulins of suitable specificity and affinity for immunoassay development. Each derived cell is permitted to proliferate, after which the incubation medium is assayed for antibody. The need for a rapid, sensitive detection system for identifying hybridomas producing antibody of the desired specificity is currently best met with enzymoimmunoassays for antibody (*30,31*) or immunoradiometric assays using either labeled anti-IgG antibody or staphylococcus protein A (*26*). Clones

identified as producing antibody with desired characteristics are selected for permanent cell lines. Large amounts of antibody are recovered from the ascitic fluid resulting from implantation of intraperitoneal tumor (hybridoma) in mice. Hybridomas may demonstrate some chromosomal instability or may undergo mutation that, with time, may alter the characteristics of the immunoglobulin produced (*29*). In order to ensure sources of antisera that can be expected to be quite stable for many years, cells are frozen in liquid nitrogen (*26*). This represents a significant improvement upon polyclonal antisera, which depend upon the continued life of the animals used for their production. The use of monoclonal antibodies in immunoassays provides a new opportunity for exquisite assay specificity. The application of monoclonal antibodies in assays for infectious agents (*32*), neoplastic determinates (*33*), and hormones (*34*) where single determinant specificity is desired, is assured. Wider application of monoclonal antibodies is also to be expected because of resultant stability of test systems.

Receptors. While antibodies have been most widely used in immunoassays as specific ligand binders of high affinity, cellular ligand receptors also offer a potential source of binding material. Whereas antibodies recognize immunoreactive determinants, receptor binding at least implies ligand bioreactivity—of potentially greater clinical interest. In addition, assessment of interactions between receptors and hormones is more widely recognized as relevant to particular clinical problems (*35–38*).

Receptors have been recognized to be either associated with the target cell membrane (*39–41*) or to be present intracellularly (*38,42*). The plasma membrane has been found to contain large asymmetric glycoproteins that possess high affinity and relative specificity for polypeptide hormones, adrenergic hormones, and neurotransmitters. Binding to these receptors is reversible, with association constants (K_a) of 10^9 to 10^{10} l/mole. Activation of adenylate cyclase frequently follows specific ligand binding by a membrane receptor. Intracellular receptors have been characterized for steroid hormones, vitamin D, and thyroid hormones. Receptors for steroids and vitamin D are present in the cytosol, and translocate to the nuclear chromatin after specific ligand binding. Receptors for thyroid hormones are found in the nuclear chromatin whether or not specific ligand is present.

The interaction between ligand (L) and receptor (R) is frequently reversible and hence can be described by the law of mass action (*38,43,44*).

$$[L] + [R] \underset{K_2}{\overset{K_1}{\rightleftharpoons}} [LR]$$

At equilibrium the forward and backward rates are equal, i.e.,

$$K_1[L][R] = K_2[LR]$$

and

$$\frac{[L][R]}{[LR]} = \frac{K_2}{K_1} = K_d,$$

the equilibrium dissociation constant.
If [R] = [LR], i.e., at half saturation,

$$K_d = [L] \text{ or}$$

K_a (equilibrium association constant) = $1/[L]$.

Receptor affinity, expressed as K_d, is equal to the free ligand concentration at half saturation.

If the total concentration of binding sites = N_{max}, then

$$N_{max} = [LR] + [R] \text{ and } [R] = N_{max} - [LR].$$

Where B is the concentration of bound ligand (= [LR]) and F is the concentration of free ligand (= [L]), then

$$[B]/[F] = [R]/K_d$$

$$= \frac{N_{max} - [B]}{K_d} = -\frac{[B]}{K_d} + \frac{N_{max}}{K_d}$$

(Scatchard equation) (*45*).

Since K_d and N_{max} are constants, a plot of [B]/[F] against [B] (Scatchard plot) ideally is a straight line with slope of $-1/K_d$ and ordinant intercept of N_{max}.

Most receptor systems depart from the simple analysis illustrated above, and in practice account must be taken for nonspecific (nonsaturable) binding, multiple binders with different affinities, and other disturbances of linearity resulting from interactions at multiple binding sites (cooperativity) (*44*). Scatchard analysis, while useful for the characterization of immunoassays, has been most widely applied in the assessment of receptor systems.

Clinically, the receptor system of greatest interest has been the estrogen-receptor status of breast cancers (*46–51*). The observation that the majority of breast cancers containing significant numbers of estrogen receptors respond to hormonal therapy, whereas few tumors devoid of estrogen receptors respond, suggests that this estimate should enter into therapy decisions for women with recurrent or residual breast cancer (*52–61*). Also necessary is evidence that cytosol estrogen receptors, identified by their ability to bind radiolabeled ligand, are biologically active (*62,63*). Biological activity may be inferred by demonstrating nuclear translocation or by demonstrating increased concentrations of progesterone receptors (*64,65*). Progesterone receptors that are independent of estrogen action have been demonstrated for one human mammary cancer (*66*). Estimates of cytosol estrogen-receptor activity in breast-cancer tissues are not beyond the capability of clinical laboratories. The need for these measurements in a timely fashion so as to aid clinical decision-making should stimulate the wider development of these procedures.

Immunoassay of receptors. Jensen has described monoclonal antibodies directed against breast-cancer estrogen receptors (*67–69*). Although the detection of immunoreactive receptors has been shown to correlate with clinical response to hormonal therapy, antibody recognition of receptor material occurs whether or not the receptor is occupied by estrogen (*68*), thus raising the question of the biological significance of such measurements. Although antibody recognition of receptor material is significantly easier, more experience with immunoassays for estrogen receptors is needed before the clinical application of these measurements is possible.

LABELS

Radiolabels. A ligand of interest is rendered radioactive by the substitution of a radioactive atom for a constituent atom (e.g., H-3, Co-57, C-14, P-32, Fe-59) or by the introduction of radioactive iodine across suitable carbon-carbon or carbon-nitrogen double bonds (e.g., in tyrosine or histidine). The iodination of insulin originally used by Yalow and Berson required the handling of 30–80 mCi of sodium iodide (I-131) in volatile solvents (*2*). In 1962 Greenwood and Hunter introduced a technique utilizing the oxidant chloramine-T, which remains in wide use today (*70*). This reaction requires much smaller amounts of radioactive iodine. The use of enzymatic oxidations has frequently resulted in greater immunoreactivity in the labeled fraction. Today, radioiodination is accomplished either by the oxidative introduction of iodine directly into the ligand or by the conjugation of preiodinated molecules to the ϵ-amino group of lysine or to the N-terminal amino acid (*71*). Haptens (e.g., steroids) are radioiodinated by the formation of a phenolic or imidazole derivative that is either pre- or postiodinated (*71*). Chromatography (column, high-performance liquid) is commonly used to extract the immunoreactive fraction from the reaction mixture. The use of monoclonal (but not polyclonal) antibody affinity columns has been shown to improve label immunoreactivity (*72*). Although the use of radiolabels has permitted immunoassay to be widely applied clinically for the detection of minute quantities of ligand, limitations on assay sensitivity inherent in the radiolabel specific activity suggest that the development of nonradioactive alternatives may be more efficacious (*8,9*). The hazards for manufacturers (but not clinical assayists) and the emotional and regulatory problems surrounding the use of radioactivity suggest that the development of nonradioactive alternatives is prudent.

While immunoassays of either excess- or limited-reagent type initially used radiolabels, nonradioactive labels are of great interest. Detection limits in limited reagent systems are not generally compromised by the specific activity of the labeled ligand since they are a function of the antibody affinity. The potential for improvement in specific activity offered by nonradioactive labels is therefore not expected to improve the detection limits of reagent-limited assay systems (*9*). The use of labels with which the signal is either attenuated or amplified by proximity to the binding reagent does, however, offer the possibility that the quantitative displacement of labeled ligand from binding reagent can be assessed without the need for physical separation of the bound fraction from the free.

In excess-reagent methods, detection limits are determined by the labeled ligand's specific activity and the background noise against which a signal must be observed (*14*). For most ligands of interest, iodination with I-125 results in labels of relatively low specific activity. Using sodium iodide crystal detectors with maximum achievable efficiency, and accepting the inevitable background activity detected by such systems, the lowest detection limits for excess-reagent assay systems are similar to those for limited-reagent assays. The possibility that nonradioactive labels of higher specific activity may result in measurably improved assay sensitivity is exciting (*8,9,14*).

Radioactivity is virtually unaltered by environmental conditions, and in radiolabeled assays a false signal is generated only by radioactive contamination. Nonradioactive labels are vulnerable to environmental interferences so that the practical application of these assays thus far has resulted in few assays with sensitivity greater than that achieved in radiolabeled systems.

Nonradioactive labels. A number of stable labels have been proposed (*73*) including bacteriophages (*74,75*), erythrocyte agglutination (*76*), free radicles (*77*), enzymes (*78–82*), coenzymes, fluorescent and chemiluminescent compounds, and metals (*83*). In addition, a particle-counting immunoassay (PACIA) utilizing latex particle agglutination has been proposed. In addition to assays analogous to competitive RIA and IRMA, those alternative labels have frequently been applied in new and novel ways that minimize serum interferences and permit rapid, automated, homogenous assays.

Enzyme-labeled immunoassay (EIA). Enzyme immunoassays of two general types have been developed: those requiring separation (heterogeneous) (*84–87*) and those that do not require separation of the bound antibody from free fractions (homogeneous) (*88,89*).

Heterogeneous systems. Since first described in 1971 by Engvall and Perlmann (*90,91*), and Van Weemen and Schuurs (*92,93*), enzyme-linked immunosorbent assays (ELISA) have been developed to detect and measure either antigen or specific antibody using a number of assay designs. Immunoassays for antigen may be designed as competitive binding assays (limited reagent) or may use solid-phase antibody (analogous to IRMA). Solid-phase antigen may be used to detect specific IgG immunoglobulins. Such assays require the conjugation

of a suitable enzyme to either antigen or to antibody, analogous to a radiolabel. Because few substances that will selectively adsorb enzyme-ligand complexes have been discovered, the antibody-bound fractions have been separated from the free fractions with a second antibody or, most commonly, a solid phase has been used. Following competitive or immunometric immunoassay and separation of the bound from free fractions, substrate is introduced and enzymatic activity is quantitated. The technical aspects of these assay systems and the wide potential for their application have been the subjects of numerous reviews (*94–99*).

Heterogeneous systems by definition require a separation step, and the solid phase must be carefully washed, limiting the possibilities for automation. Although heterogeneous EIAs have been described for many ligands commonly quantitated by radioassays, commercial development has been limited. Many commercial assays are cumbersome and time-consuming due to the wash steps and the additional incubation after substrate addition, making them unattractive compared with radioassays. ELISAs may, however, be accomplished easily in large multiwell plastic trays that facilitate the wash step and, because the end point is the conversion of substrate to a colored product, are easily semiquantitated visually. The use of this technique for the detection of infectious agents or antibodies directed against them has great potential, especially in underdeveloped parts of the world where sophisticated equipment such as gamma counters or spectrophotometers are not widely available (*100,101*). Semiquantitative assays have been used widely when screening for hybridoma production of specific IgG. An enzyme-labeled pregnancy test* and an enzyme-labeled test to detect antibody directed against dog heart worms† (*D. immitis*) both use visual end points and illustrate the potential for ELISAs in outpatient physician's offices and in veterinary medicine.

Homogeneous systems. In 1972, Rubenstein et al. described a competitive immunoassay system that utilized an enzyme-ligand conjugate (*102*). When bound by antibody, the enzyme was prevented from catalyzing the conversion of substrate present in the reaction mixture. Displacement of enzyme-ligand complexes from the antibody by nonlabeled ligand was accompanied by a quantitative increase in substrate conversion. This system therefore required no physical separation of the bound from free antibody fractions and was quickly adapted to automated spectrophotometers capable of initial rate measurements. This technique has been exploited successfully by Syva Corporation (EMIT)‡ for the quantitation of small molecules present in relatively high (μg/dl) concentrations. Within a few years, Syva has captured about 20% of the total immunoassay market in the United States with about 30 assays for various drugs, representing by far the most significant clinical application of nonradioactive immunoassay to date. Enzyme-ligand complexes that convert substrate more rapidly when bound by antibody have also been described. The EMIT thyroxine assay uses such a system, the signal increasing in proportion to the concentration of the bound fraction.

Although the EMIT systems require no separation and have been automated easily (*103*), the presence of interfering substances introduced with the patient's sample limits sensitivity. ELISAs are relatively free from these interferences because the reactants are separated from serum before the introduction of substrate. While the quantitation of large molecules is possible, EMIT has been most readily applied to measurements of small molecules. Assays for thyroxine, cortisol, and digoxin have found little clinical acceptance because their protocols differ from those used for the drug assays and they have not performed as acceptably as many RIAs.

Fluorescence. Fluorescence was first used by Coons in 1941 for immunofluorescent microscopy (*104*). Fluorescence is the process of light absorption at one wave length followed by its re-emission at a second, longer wave length with a decay time of 10^{-9} to 10^{-4} sec. Although many organic molecules fluoresce, most immunoassays utilize fluorescein or umbelliferone, a derivative of 7-hydroxy coumarin (*105*). More recently, assays using chelates of rare earths have been described (*106*). Rhodamine has been used as a quencher. Like EIA, many assay systems using fluorescent labels have been described (*105,107,108*).

Fluorescent indicators for immunoassay offer the potential advantages of other nonradiolabels: potentially increased sensitivity, technical simplicity, minimal health risks for manufacturers, and freedom from regulatory restrictions. Biological fluids, however, contain many compounds that are variably fluorescent. Solid-phase materials commonly used in immunoassays (i.e., glass, plastic) fluoresce naturally. Fluorescent systems may be light-sensitive, and nonspecific binding to the solid phase may present problems. Both heterogeneous and homogeneous assays have been developed, and systems based on either principle are currently in clinical use. The widest application of these assays has been in therapeutic drug monitoring where simple, rapid systems can produce clinically useful results. Homogeneous systems have adequate sensitivity for many measurements of drug concentration and are simple, some being automated. In these systems, the fluorescent signal is altered by antibody binding, so a separation step is not needed.

Heterogeneous assays. Competitive solid-phase immunoassays utilizing fluorescein-labeled antigen are available commercially for the estimation of therapeutic drug concentrations (*107,109*). Solid-phase antigen and fluorescein-labeled second antibody have been used to detect antibodies and other proteins. Heterogeneous systems overcome the background fluorescence problem

by separation of the interfering substances before the assay signal is measured. Such systems are potentially more sensitive than homogeneous systems but lack simplicity and generally require dedicated fluorometers.

Homogeneous assays. A number of approaches providing an attenuated or augmented signal have been explored, resulting in assays that require no separation of the bound from free fractions.

Fluorescence polarization. Described by Dandliker in 1964, excitation of fluorescein by polarized light results in re-emission of polarized light at a new wave length (*110–112*). Polarized molecules retain their orientation depending upon molecular size. The retained polarization of the (large) bound fraction is quantitated in the assay. This principle has been used in a commercial system that includes a dedicated fluorometer with a polarized light source for the estimation of therapeutic drug concentrations (*113–116*).

Fluorescence-quenching and fluorescence-enhancement. Emission from a fluorescent molecule is, in many circumstances, altered when it becomes part of an antigen-antibody complex. The ligand-fluorescein conjugate may be "self-quenched" with an increase in signal with antibody binding (*117*), or the signal may decrease with antibody binding (*118*). Measurement of this alteration permits assessment of the extent of antigen-antibody binding without need for physical separation of the bound from free fractions. Ullman et al. described a method they called fluorescence excitation transfer, in which the quenching compound was rhodamine (*119*). They described assays using antibody-rhodamine and antigen-fluorescein conjugates in which the antigen-antibody complex resulted in quenching, and assays in which antibodies labeled with either fluorescein or rhodamine were bridged by polyvalent antigen, resulting in quenching. Zuk et al. have described a method they called fluorescence protection immunoassay, which uses antibodies directed against the antigen of interest as well as antibodies directed against fluorescein (*120*). Formation of fluorescein-labeled antigen-antibody complexes prevents the quenching arising from binding of the labeled antigen by the antifluorescein antibody. Competitive displacement of fluorescein-labeled antigen by standard or by antigen present in patient samples results in quantitatively greater quenching.

Substrate labeled FIA (SLFIA). Developed by Burd, this system uses a conjugate of a drug and a fluorescent dye (beta-galactosyl-umbelliferone), which is a substrate for the enzyme beta-galactosidase (*121,122*). Antibody binding of the conjugate protects against cleavage by the enzyme, hence the signal is generated by the "free" fraction of unbound conjugate and is proportional to drug concentration. The signal generated in this system can be measured by any fluorometer.

While the nonradioactive immunoassay labels of greatest commercial application to date have been enzymes and fluorophores, at least two other systems have been explored experimentally. Luminescent molecules and particle-counting systems both have potential for commercial application.

Luminescence. Familiar for centuries in nature as bioluminescence, chemical excitation of suitable organic molecules that re-emit photons upon de-excitation has recently been utilized to provide labels in immunoassays (*83,123–125*). Chemiluminescence typically requires the oxidative excitation in alkali of luminol in the presence of catalyst, with detection of emitted light by a photon detector (a liquid scintillation detector can be modified for this purpose) (*126,127*). Assays using ligand or antibody conjugates of luminal have been developed (*124,128*). Unlike fluorescence, the detection process destroys the label, so that the assay cannot be "counted" again. Acridine esters requiring no catalyst have been used, resulting in fewer serum interferences (*129*). Homogeneous systems are possible, the signal being attenuated or augmented in proximity to antibody (*130–132*). Solid-phase heterogeneous systems avoid serum interferences (*133–136*). The recent advent of automated systems for injection of the oxidant and detection of the emitted light permits the wider application of luminescent systems.

Particle-counting immunoassay (PACIA). Agglutination techniques have been used for many years to identify antigen or specific antibodies. Particles used in these procedures most frequently have been erythrocytes or polystyrene beads ("latex"). The sensitivity of these procedures has been limited because they depended upon a visual end point. Cambiaso and associates used a device for counting blood cells in order to measure the agglutination of polystyrene beads with greater sensitivity (*137–139*). PACIA requires no labeling at all, but rather involves quantifying the reduction in particles resulting from agglutination. The technique is easily automated for the detection of antibodies, antigens, haptens, and immune complexes, with sensitivity approaching that of RIA (*140*). Agglutination of antibody-coated beads by rheumatoid factor (IgM), occasionally present in a patient's serum samples, can be avoided by using $F(ab')_2$. Interestingly, a PACIA with sufficient sensitivity to quantify digoxin, utilizing a mixture of specific antidigoxin-IgG and rheumatoid factor to facilitate agglutination, has recently been described (*141*).

SEPARATION TECHNOLOGY

In competitive immunoassays, antibody-bound labeled ligand must be quantified exclusive of free ligand in order to determine the apparent ligand concentration. Similarly, in immunometric assays ligand-labeled antibody activity must be quantified exclusive of excess labeled antibody. In either system, use of a radiolabel

imposes the requirement for separation of the two phases.

"The ideal method should provide not only a clean separation of these components, but must be uninfluenced by serum and other nonspecific substances in the reaction mixture. Moreover, the method should be reproducible, technically simple, expeditious, and inexpensive" (*142*, p 303). That none is entirely satisfactory has resulted in the investigation of a large number of methods, many of which are in use today (*143,144*). There are three general approaches to this problem: (a) differential migration of bound and free labeled materials; (b) isolation of the free fraction; and (c) isolation of the bound fraction.

Differential migration of bound and free labeled fractions. This is of interest largely because the technique was used by Berson and Yalow in their initial insulin assays (*5*). Ligands are more or less adsorbed by cellulose or other materials. In general, the antibody-ligand complexes are adsorbed less well and travel with a solvent front in many chromatographic systems (*145*). Differential migration due to charge differences may be exploited by the addition of electrophoresis. These systems are generally labor-intensive and have few routine applications.

Isolation of the free fraction. This has been accomplished with a number of substances possessing relative specificity for the ligand, exclusive of the ligand-antibody complex. Charcoal has been most widely used for this purpose and has been applied principally in hapten assays. Charcoal systems are complex, follow the law of mass action, require careful optimization, and are prone to error (*146,147*).

Isolation of the bound fraction. Greater interest, both present and future, centers on systems that isolate the bound fraction. In antibody-limited immunoassays, the primary antibody-antigen complex remains in solution. The addition of anti-immunoglobulin IgG (second antibody) directed against the appropriate species has been widely used as perhaps the most specific separation method practically available (*142*). Second antibody has been used in liquid-phase systems, preprecipitated with primary antibody (*148*), insolubilized by copolymerization (*149*), and adsorbed or covalently bound to a variety of solid supports (*150–152*). Polyethylene glycol (PEG), in addition to precipitating immune complexes (*153*), has been found to facilitate second-antibody precipitation, and is frequently used in liquid-phase systems (*154*). Solid-phase systems have recently become more widely used because more uniform antibody-support systems have become technically possible, involve the least technical manipulation, may eliminate the need for centrifugation, and facilitate the trend toward immunometric assay systems. The kinetics of these systems have recently been reviewed (*144*). Solid supports have been used extensively in excess-reagent systems. The further application of solid-phase technology, for both radiolabeled and nonradioactive immunoassays, is expected.

Staphylococcus aureus protein A has been substituted for second antibody in some applications (*155–158*). This component of the bacterial cell wall has specificity for the F_c region of most mammalian immunoglobulins and offers the potential for a "universal" separant.

Recognition by Albertsson of the asymmetric distribution of hapten and antibody-hapten complexes in a two-phase liquid system led to the application of polyethylene glycol as a nonspecific immunoglobulin precipitant (*159*). Recently, the application of a magnesium sulfate/polyethylene glycol two-phase system has been described as a simple method for segregation of the antibody-bound ligand from the free (*160*). This system—termed partition affinity ligand assay (PALA)—still requires sampling the phase containing free ligand for quantification. Magnetized particles of either charcoal (*161*) or second antibody (*162,163*) have been proposed as means of simplifying the separation step and avoiding the need for centrifugation.

Thorell has recently described a truly homogeneous radioimmunoassay system (*164*). With bismuth oxide as a radiation-attenuating material, the use of a bismuth-oxide-charcoal or a bismuth-oxide-antibody-polyacrylamide matrix results in systems in which the free or antibody-bound counts are attenuated respectively. Hart and Greenwald have described an assay using tritiated and scintillant latex particles covalently coated with antigen. This system detects specific antibody that forms dimers and aggregates of the latex particles, resulting in scintillations that can be measured in a liquid scintillation counter (*165*). Radioassays using these materials may therefore be accomplished without physical separation of the two phases.

Omission of the separation step is possible for nonradioactive immunoassay when the signal generated from the labeled ligand or labeled antibody is augmented or attenuated through interaction with other assay components. Such homogeneous systems (described above) are technically advantageous and may be automated easily.

AUTOMATION

Radioimmunoassay is technically labor-intensive, involving several pipetting steps, incubations critical as to time and temperature, a separation step, and quantification of radioactivity either in the bound or the free fraction. Like other procedures in the chemistry laboratory, automation is expected to reduce the technical personnel time necessary to accomplish an assay and should improve the assay precision as well. Perhaps because of the complexity of these procedures, RIA automation has been only very marginally successful. In the

1970s at least six automated instruments were developed commercially (*166–168*). Several other systems were in various stages of development. Four remain commercially available today.

The first automated system, the Union Carbide Centria[§] (*168*), is a modular system consisting of a pipetting module, a centrifical mixer, an incubator, a separator module, and a microprocessor-controlled module for gamma counting and data reduction. Initially using column chromatography, now a second-antibody solid-phase tablet (*169*), the Centria system has limited throughput and requires nearly constant operator attention. Systems using continuous-flow techniques were developed by E.R. Squibb[**] (*170*) and Technicon[††] (*171*). A batch-mode system using small (8 × 50 mm) antibody-coated tubes, with automated pipetting, incubation, washing, counting, and data reduction is available from Micromedic[‡‡] (*166,167*). This system has been modestly successful but generally performs less well than manual assays. The Bectin Dickinson ARIA II[§§] (*172*) uses a reusable chamber within which antibody is covalently bound to a solid support. Sample and labeled ligand are introduced into the chamber. After a brief incubation, first the free, then the eluted bound fractions are rinsed into a counter for quantitation. This system has enjoyed the greatest commercial success owing to its simplicity and excellent precision. Recently, Bectin Dickinson has introduced a modified version[***] designed to increase throughput by using four counting channels. Although considerable commercial effort was expended, the large majority of radioimmunoassayists remain refractory to automation. Although it seemed that automated RIA instrumentation would play a major role in the clinical laboratory, that now appears unlikely.

Automated and semiautomated instrumentation has been extensively applied to nonradioactive immunoassays. EMIT assays have been adapted to automated spectrophotometers with resultant reagent savings. Fluorometers and, more recently, luminometers—both developed for and independent of assay reagent kits—have become more widely available. The very rapid signal detection time (spectrophotometry or a single photon pulse) greatly reduces the throughput time for nonradioactive immunoassays. The relative simplicity of these assays and the availability of automated procedures account in part for their attractiveness.

PRESENT AND FUTURE IMMUNOASSAY APPLICATIONS

Currently in the United States radioimmunoassay accounts for about 70% of all immunoassay procedures.[†††] Syva's EMIT is the only stable-tracer technology that has a significant market share. It accounts for most of the rest of immunoassay testing, nearly all associated with therapeutic drug monitoring. Therapeutic drugs and thyroid testing each account for about one-fourth of all immunoassay procedures. About two-thirds of therapeutic drug measurements are accomplished using stable-tracer technology. Digoxin is still quantitated almost exclusively by radioimmunoassay due to the cumbersome technique and relatively poor sensitivity of most stable-tracer alternatives. Direct stable-tracer digoxin procedures with excellent sensitivity and precision can be expected imminently, however. A fluorescent polarization procedure appears to be clinically acceptable (*173*). If efficacious stable-tracer procedures for the measurement of digoxin and thyroxine are widely applied—as seems quite likely in the next five to ten years—then more than one-half of all immunoassay testing will be done using nonradioactive methods.

Instrument sales and development mirror this state of affairs. Gamma counters account for about 60% of present immunoassay instrumentation. While there is interest in multi-well counters, often coupled to small computers for data reduction, the market for gamma counters is otherwise nearly static. There appears to be further growth potential for the Bectin-Dickinson ARIA system, but major instrument development is associated with stable-tracer assays. Enzyme-labeled assays, especially Syva's EMIT, have been widely applied using automated spectrophotometers and centrifugal analyzers already available in chemistry laboratories for other purposes. The trend appears to be for manufacturers to supply dedicated spectrophotometers or fluorometers along with reagent kits utilizing stable-tracer labels. The most successful of these to date have been the instruments accompanying the Abbott TDX fluorescent polarization assays for therapeutic drugs.[‡‡‡] These instruments can usually be acquired through reagent-rental agreements to avoid the need for capital expenditures, making their entry into the clinical laboratory quite simple.

Further future expansion of nonradioactive testing depends upon several factors. Applications are to be expected if they address new problems. Therapeutic drug monitoring and detection of specific antibodies or antigens associated with infectious diseases are examples of these applications, and to date they represent areas of major growth for nonradioactive testing. In addition, areas of significant clinical interest that offer the opportunity for new assay applications include the measurement of free hormones [perhaps in saliva (*174*)] and free drug concentrations; the development of reliable, sensitive, and specific cancer markers; and the exploration of hormone receptors and screening procedures for genetic diseases. Efforts to displace RIA will be successful if nonradioactive procedures work better, are less expensive, and are technically easier (preferably automated). Abbott Laboratories'[‡‡‡] heat-denaturation CEA procedure has been marketed with both radiolabeled and

enzyme-labeled (ELISA) reagents. Clearly the procedure is technically easier and appears at least as efficacious clinically as the perchloric acid extraction alternative, and has captured about 70% of the total CEA sales. Even though there is no clear advantage of the EIA over the RIA procedure, about one-half of the reagents sold are enzyme labeled.

Clearly, objections to radiolabels will influence the course of future development as well. Radioactive materials are socially unacceptable, are subject to excessive governmental control, and pose waste-disposal problems. Technically, RIA reagent shelf life is limited by the tracer's physical half-life and label degradation due to radioactive decay. Radiolabeled immunoassays require physical separation of the bound-from-free fraction, require relatively long signal-counting times, and the label's specific activity may be inadequate to achieve maximum assay sensitivity. While technical problems abound for nonradioactive labels, they appear to offer attractive alternatives and may overcome these objections to radionuclides. Finally, the future of immunoassay may be determined in large measure by industry development. If the majority of manufacturers' major research and development efforts are devoted to nonradioactive immunoassay, there will be increasing market pressure to discontinue RIA in favor of non-RIA procedures.

Physicians whose primary nuclear medicine responsibilities include radioimmunoassay will clearly be confronted with this evolution in immunoassay technology. While I have attempted to outline the scope of this evolution, a more detailed understanding of nonradioactive techniques is necessary if alternatives to RIA are to be judged on their merits. Except for signal generation and detection, all immunoassays are based on similar principles, so that professionals with experience in radioimmunoassay will have little trouble understanding alternative procedures. As with evolution in medical imaging, decisions regarding alternative methods tend to become political or territorial rather than being based on what is most efficacious medically. Decisions about what changes in immunoassay methods are to be made, and when, will have to be confronted. Measurements of the same ligand by radioimmunoassay in the nuclear medicine area and by nonradioactive immunoassay in clinical chemistry will not be tolerable. If nuclear medicine professionals currently responsible for radioimmunoassays wish to continue to be so involved in the future, the need for plans to accommodate these changes in immunoassay methods must be recognized. A needed first step is recognition that radioimmunoassay is but a subset of clinical immunochemistry, and that integration of the radioimmunoassay effort with the remainder of immunochemistry is essential. It is important for nuclear medicine physicians to communicate regularly with the pathologists and clinical chemists responsible for nonradioactive immunoassay. We hope that past efforts and present interests can be respected; for example, there is no particular reason why the laboratory responsible for thyroid testing for many years should not continue to do this, even using nonradiolabeled reagents if that best serves an institution's needs. Failure to establish this communication and to develop the necessary relationships will likely mean the abrupt transfer of many procedures accomplished by immunoassay from the nuclear medicine laboratory to another laboratory using nonradioactive techniques.

Finally, reagent manufacturers, while attracted to high-volume (and high-profit) procedures, will feel little incentive to replace RIA with non-RIA reagents for a large number of infrequently requested hormone assays. The transition from RIA, while perhaps inexorable, will progress relatively slowly. Laboratories doing only RIA will be confronted with the loss of their high-volume procedures (i.e., digoxin, thyroxine), and be left with a number of low-volume "service" procedures.

CONCLUSIONS

It seems clear that nonradioactive immunoassays will ultimately supplant radioimmunoassays in the clinical laboratory. This change will occur relatively slowly and may not be complete within this decade. Evolution in immunoassay technology will include a trend towards immunometric assays using solid phases and less sensitive, homogeneous, automated assay systems. Monoclonal antibodies will be more frequently used in ligand assays, as will be estimations of hormone receptor number and affinity. Nonradioactive labels, including enzymes, fluorophores, and possibly chemiluminescent compounds, will ultimately replace radioactive labels. Agglutination or agglutination-inhibition systems using latex particles in particle-counting systems offer a sensitive "nonlabeled" approach to immunoassay. Nuclear medicine professionals must be aware of these impending changes in immunoassay technology and plan appropriately for their future implementation.

FOOTNOTES

* Tandem™-Visual HCG Pregnancy Test, Hybritech, Inc., San Diego, CA 92121.

† Dirotect™-Canine Heartworm Test Kit, Mallickrodt, Inc., Bohemia, NY 11716.

‡ EMIT™, Syva Company, Palo Alto, CA 94303.

§ Union Carbide Corporation, Pleasantville, NY 10570.

** Gammaflo™, E.R. Squibb & Sons, Inc., Princeton, NJ 08540.

†† Technicon Star™, Technicon Instruments Corp., Tarrytown, NY 10591.

‡‡ Concept 4™, Micromedic Systems, Horsham, PA 19044.

§§ ARIA II™, Becton Dickinson Immunodiagnostics, Orangeburg, NY 10962.

*** ARIA HT™, Becton Dickinson Immunodiagnostics, Orangeburg, NY 10962.

††† Future Directions for Nonisotopic Immunoassays, a conference

sponsored by Robert S. First, Inc. Tarrytown, NY, May 10-11, 1982.

‡‡‡ Abbott Laboratories, Diagnostics Division, North Chicago, IL 60064.

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SNM Conjoint Congress

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In cooperation with the Computer and Instrumentation Councils and Technologist Section, the Society of Nuclear Medicine invites physicians, basic scientists, and technologists to attend the 3rd Conjoint Congress. Join us February 1–6, 1984 in Orlando, Florida to examine one of the most challenging developments in nuclear medicine: **The Technology of NMR**. Examine NMR instrumentation, imaging techniques for spacial encoding, problems of pulse sequencing, image processing and display, spectroscopy, and system evaluation.

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Schedule

Wednesday, February 1	8:00 a.m.–10:00 p.m.	Technologist Section Committee Meetings
Thursday, February 2	8:00 a.m.– 5:00 p.m.	Technologist Section National Council Meeting
Friday, February 3	8:00 a.m.–10:00 p.m.	SNM Committee Meetings
	1:00 p.m.– 5:00 p.m.	Technologist Section Educational Program
Saturday, February 4	8:30 a.m.– 5:00 p.m.	SNM Board of Trustees Meeting
	8:30 a.m.– 5:00 p.m.	Technologist Section Educational Program
Sunday, February 5	8:30 a.m.– 5:00 p.m.	Computer & Instrumentation Councils' Symposia on NMR
	8:30 a.m.–12:00 p.m.	Technologist Section Educational Program
Monday, February 6	8:30 a.m.– 5:00 p.m.	Computer & Instrumentation Councils' Symposia on NMR

Seeking New Journal Editor

The second term of the current editor of the Journal of Nuclear Medicine will end in January 1985. The Society's Publications Committee is charged with overseeing the process by which a new editor will be selected to replace Frank H. DeLand, MD.

According to the Bylaws of the Society of Nuclear Medicine, the JNM Editor will be nominated by the Publications Committee and appointed by a majority vote of the Board of Trustees. The Publications Committee will be interviewing candidates in February 1984 and hopes to present a nominee to the Board for approval in June 1984 to allow a smooth transition between editors.

The editor will serve a five-year term (beginning in January 1985) and may be reappointed to serve an additional five-year term.

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