APPLICATIONS OF LIQUID SCINTILLATION COUNTING TO RADIOIMMUNOASSAY

Grafton D. Chase

Department of Chemistry
Philadelphia College of Pharmacy and Science
Philadelphia, Pennsylvania

I. INTRODUCTION TO RADIOIMMUNOASSAY

Events which resulted in the development of radioimmunoassay (RIA) began in the early 1950's when Berson and Yalow were investigating the metabolism of insulin by use of a radiotracer technique. In the course of their investigations they observed that virtually all diabetic patients on insulin therapy had developed anti-insulin antibodies. Although the concept of radioimmunoassay was used as early as 1924 in a qualitative way for the measurement of specific blood substances in saliva (Schiff, 1924), it was the discovery of insulin antibodies which led Berson and Yalow (1959) in the United Stated and Ekins (1960) in England to recognize the concept involving a quantitative relationship between labeled and unlabeled antigen and the specific antibody.

Radioimmunoassay represents one type of a somewhat broader classification of assays for which there is no generally accepted name but for which the terms "ligand assays", "saturation assays", "binding assays" or "competitive assays" are sometimes applied. The common denominator of these assays may be identified as a reaction involving a ligand and a binder.

The ligand is usually the substance to be assayed and may be a hormone, vitamin, drug or any other substance for which a specific binder is known. The binder is usually a specific antibody but may be a specific protein other than an antibody such as TBG or a tissue receptor. The concentration of the ligand is determined by measuring the extent of binding of the

Copyright © 1980 by Academic Press, Inc.
All rights of reproduction in any form reserved.
ISBN 0-12-549902-7

ligand with the specific binder.

To measure the extent of ligand binding a tag is used. The tag may be a radioactive nuclide but fluorescent, enzyme and other types of tag are also used. Either the ligand or the specific binder may be tagged.

The basic concept on which the standard equilibrium radioimmunoassay is predicated is a competition between untagged ligand P and tagged ligand P* for binding sites Q on a specific binder. Such a system can be represented by the equations:

$$P + Q = PQ \quad (1)$$

$$P^* + Q = P^*Q \quad (2)$$

where PQ and P*Q represent the complex formed between binder and non-radioactive and radioactive ligand, respectively.

In RIA a radioactive isotope is used to mark the ligand P*. If a state of equilibrium is attained, radioactive ligand will be distributed between the forms P* and P*Q according to mass action law. The bound fraction, P*Q, is separated from the free fraction, P*, by precipitation or other suitable means and the radioactivities, B and F, of the bound and free fractions respectively, are measured. It then follows that

$$R = \frac{B}{F} = \frac{[P^*Q]}{[P]} = K^* [Q] = K^*(q - C_b) \quad (3)$$

where q =[Q]+[PQ]+[P*Q]= total concentration of binding sites and C_b is the concentration of bound ligand. This is one form of the Scatchard equation (Scatchard, 1946).

Success of a radioimmunoassay does not depend upon equality of the equilibrium constants (Equations 1 and 2) but such an assumption simplifies the mathematics. If K = K* equation 3 can be written in the form

$$B/F = Kq = KC_b \quad (4)$$

Assuming the validity of the assumptions upon which equations 1 and 2 are based a linear Scatchard plot will result.

The objective of a radioimmunoassay is to measure the concentration p of a specific chemical substance. To do so one must relate p to radioactivity. The best approach is to evaluate the function p = f(A) where A is the observed radioactivity or radioactivity ratio, e.g., B/F or T/B. For the simple case cited the function is readily evaluated from the Scatchard

equation by equating C_b to p. It is a basic postulate that

$$\frac{\text{Total ligand concentration}}{\text{Concentration bound ligand}} = \frac{p + p^*}{C_b} = \frac{T}{B} \qquad (5)$$

where p and p* are the total concentrations of non-radioactive and radioactive ligand, respectively and T = B + F, is the total radioactivity. By use of equations 4 and 5 the concentration of non-radioactive ligand p can be related to the observed radioactivity.

$$p = -p^* + (q - R/K)\, T/B \qquad (6)$$

If R (=B/F) is small compared to K a linear relationship results when p is plotted as a function of T/B. Plots of this type are commonly called dose-response curves. By use of standard solutions the parameters of equation 6 can be evaluated and a standard curve can be constructed.

In practice it is found that Scatchard plots deviate from linearity when the concentration of ligand p is very great. Non-linearity results principally from non-specific binding (NSB), that is, as a result of binding reactions between the ligand and binding sites other than the specific sites. In a more detailed analysis it is also necessary to consider the influence of one antibody binding site on the other, an effect known as cooperativity (Chase, 1979).

The simple equilibrium RIA described above is only one example of a family of closely related assays which are generally considered to be radioimmunoassays or are so similar in concept that they should be mentioned in any discussion of RIA techniques.

Immunoradiometric assays (IRMA) employ a radioactively labeled antibody rather than a labeled antigen. After equilibration of labeled antibody with antigen, the excess free antibody is removed by addition of a massive quantity of immunoadsorbent. An "immunoadsorbent" is an insoluble polymer to which an antigen or binder is attached. The antibody-immunoadsorbent complex is readily removed from bound antibody by centrifugation.

"Sandwich" assays or "two-site" assays utilize two different antibodies. The first antibody is attached to a solid support such as the wall of a test tube. Being present in large amount, this antibody binds essentially all of the ligand present in the sample. A second, radioactively tagged antibody specific for the ligand is now added. Ligand becomes "sand-

wiched" between the first and second antibodies and the radioactivity remaining on the walls of the test tube serves as a measure of the quantity of ligand.

In a radioenzymatic assay (REA) the rate of formation of a radioactive product from a radioactively labeled ligand molecule is used to measure the concentration of non-radioactive ligand. The greater the amount of unlabeled ligand the less will be the specific activity of ligand resulting in a decrease in the quantity of enzymatically produced radioactive product. Case and Mezei (1978) describe an enzymatic assay for gentamicin which is typical of this type of assay.

Enzyme-linked immunosorbent assays (ELIZA) and the enzyme-multiplied immunoassay technique (EMIT) are enzymatic assays in which the radioactive tag has been replaced with an enzyme.

II. RADIOACTIVE TAGS FOR RIA

The requirements imposed on a radioactive tag for use in RIA are very stringent compared to the requirements of most tracers. In most tracer work the chemical substance being traced is usually present in migrogram quantities or more. The radioactive tracer, being perhaps six orders of magnitude less concentrated, may be present in picogram quantities. Generally these quantity restrictions create no problem with regard to the choice of radioactive tag.

In RIA, on the other hand, the substance being assayed is frequently present in picogram quantities or less. Such a quantity is itself a trace and the requirement of the radioactive tag is that it serve as a tracer for a trace. Only a relatively few radioactive nuclides meet the necessary limitations of half life and chemical properties which will allow incorporation of the nuclide into the ligand or antibody molecule.

Tritium and iodine 125 are the tags most often used for RIA. Certain assays, e.g., vitamin B-12, require the use of cobalt 57. Except for a few special cases it is not possible to use a carbon-14 tag since a sufficiently high specific activity can not be obtained owing to the long half life of carbon 14. Although iodine 131 has enjoyed widespread use as a tag for protein in years past it has been replaced almost entirely by the 60-day half life of the mass 125 isotope.

Until a short time ago RIA kits with a double tag were commercially available[a]. The ligand was tagged with iodine 125. The second tag, cobalt 57 (or sodium 22), remained in solution as a salt and served to tag the supernatant. By means of a simple calculation that part of non-specific binding caused by incomplete separation of the supernatant from the precipitae, in the course of separating free ligand from antibody-bound ligand, could be taken into account. In the author's laboratory error from this source has been reduced 50% or better by use of a second tag. It is unfortunate that the advantage of using a double tag has not been generally recognized, especially for the digoxin assay and other assays where a high degree of accuracy is required.

III. RIA SAMPLE PREPARATION FOR BETA COUNTING BY LSC

Antigen-antibody reactions occur in aqueous solution. Therefore, after separating "free" and "bound" radioactivity in a radioimmunoassay, one must frequently measure the radioactivity of an aliquot of an aqueous solution. Here we are faced with the problem of dispersing water, a polar solvent, in a non-polar toluene or xylene based scintillator system. To minimize quenching problems, especially serious when counting weak beta particles such as those emitted by tritium, it is best if a homogenous mixture of the sample and liquid scintillant results. That is, two-phase systems should be avoided if possible.

One approach to this problem is to use a common solvent. For example, a toluene or xylene based scintillation solution might be diluted with a more polar solvent such as dioxane or alcohol. The resulting solution should then be capable of accepting a limited quantity of an aqueous sample. One of the first such scintillators, reported by Hayes and Gould (1953), consists of diphenyloxazole (PPO) with toluene, dioxane and xylene. Kinard (1957) added dioxane and ethanol to a xylene-based scintillator containing PPO and α-naphthylphenyloxazole to give an efficient scintillator for counting aqueous tritium samples. A similar toluene/ethanol solvent system containing a mixture of the phosphors PPO, POPOP and naphthalene was suggested by Vaughn and Boling (1961). Francis and Hawkins (1967) reported that the use of 2-phenylethylamine with a toluene/PPO/POPOP scintillator will allow the addition of up to 60 per cent of water.

[a] *Beckman Instrument Company, Fullerton, California*

A second approach to the problem of aqueous sample preparation is to use a polar compound such as dioxane as the principal solvent. Farmer and Berstein (1952) first reported the use of p-terphenyl in dioxane. Later, combinations of PPO, POPOP and naphthalene in dioxane were suggested (Langham et al, 1956; Vaughn et al, 1961). The use of 2-phenylethylamine alone as a solvent with PPO and POPOP as phosphors also yielded good results (Francis et al, 1967). One of the most widely used scintillants of this type has been Bray's solution (Bray, 1960). Bray's solution is based on the use of dioxane as a solvent but also contains methanol and ethylene glycol to reduce the freezing point of the dioxane. Dioxane is now considered to be carcinogenic and its use is discouraged.

Still another approach to the incorporation of aqueous samples into non-aqueous, non-polar solvent systems has been through the use of surface active agents. One disadvantage to the use of surface active agents is that colloidal systems and emulsions frequently form. Heterogeneous systems often produce uncertainties in quench corrections (Benson, 1966). Nevertheless such systems are useful. Shapira and Perkins (1960) described the use of Hyamine 10-X and Thixcin R in a toluene-based scintillator. Triton X-100 is one of the more promising surface active agents for use as a scintillator. First reported by Meade and Stiglitz (1962) and by Patterson and Green (1965), the use of Triton X-100 as a scintillator has been the subject of numerous investigations (van der Laarse, 1967; Fox, 1968; Turner, 1968, 1969; Nadarajah et al, 1969; Lieberman et al, 1970; Whyman, 1970; Chapman et al, 1971). Other surfactants such as Triton X-114 have also been investigated. Anderson and McClure (1973) report a formulation of Triton X-114 in xylene which will accept up to 30% (v/v) aqueous sample and provides up to 47% efficiency for tritium. Another scintillation cocktail dubbed "Plasmasol" is a mixture of xylene, Triton X-100 and mono- and di-butyl phosphate (Wiegman et al, 1975). As the name Plasmasol suggests, it was designed for use with aqueous solutions containing soluble protein and is especially suited for radioimmunoassay.

In RIA it is necessary to separate the free from the protein bound activity before counting. A novel approach to separation is to partition unbound radioactivity directly into a non-polar scintillation cocktail (Castanier et al, 1970). In using this technique it is necessary that the scintillator be non-polar and hence immiscible with the aqueous phase. In addition, the free form of the radioactivity must be soluble in non-polar solvents. This separation method has been used successfully by Jowett et al (1973) for the radioimmunoassay of

aldosterone in plasma and urine and by Keane et al (1975) for the radioimmunoassay of cortisol.

The radioactivity of aqueous solutions has also been measured by use of solid state phosphors (Steinberg, 1960; Moss, 1964). A two-pase system is used: a solid phase consisting of the finely divided fluor and the liquid phase containing the radioactivity. This method is very useful in those cases where it is desired to recover the radioactive compound. In RIA, however, it is normally not necessary to recover the sample. Cost and volume are the principal considerations. To date this method has not gained widespread use in RIA.

In keeping with the advantages of minimizing cost and assay time, Tyler et al (1973) have proposed a simplification of the RIA procedure which eliminates the need to pipet an aliquot of supernatant. They propose precipitation of the antibody-bound fraction with saturated ammonium sulfate. The protein precipitate is then dissolved in .1 ml of water and 1 ml of liquid scintillator is added directly to the solution in the original test tube. The need to use a separate counting vial is eliminated and the volume of scintillator required is only 1 ml rather than the customary 10 ml. Tissues, including proteins and amino acids, have also been solubilized by the use of formamide (Lahr et al, 1955; Pearce et al, 1956), hyamine hydroxide (Vaughn et al, 1957) alcoholic KOH (Herberg, 1960) and Triton X-100 (Meade et al, 1962). A number of commercial products are currently available so the choice of tissue solubilizers is broad. In fact, most clinical and biochemical investigators employing RIA techniques use commercial products. In a radioimmunoassay for pantothenic acid, for example, Wyse et al (1979) dissolved the antibody-bound pantothenic acid in NCS* tissue solubilizer and counted in Dimilune-30* scintillation cocktail.

IV. LIQUID SCINTILLATION COUNTING OF RADIOIODINE IN RIA

The most widely used radioactive tag in RIA is iodine 125. Iodine 125 decays by electron capture. It emits a single gamma ray having an energy of 35.48 keV. Four tellurium K x-rays with energies between 27.5 and 31.8 keV are also emitted. In addition there are L and M x-rays with energies of about 4 and 0.5 keV, respectively, as well as a variety of conversion and Auger electrons. Measurement of these relatively weak photons by

* *Trade mark*

gamma counting can lead to significant errors if proper precautions are not taken (Bakhle et al, 1964). An alternate route is to measure iodine 125 by means of liquid scintillation counting.

Yerick and Ross (1963) were among the early investigators to measure gamma-emitting nuclides by means of liquid scintillation counting. They reported a counting efficiency of only about 13% for iodine 125. Rhodes (1965) evaluated eight liquid scintillation systems for counting radioiodine and obtained a maximum efficiency of 56% measured as 100 cpm/dpm.

Auger electrons emitted as the result of iodine-125 decay are readily detected by use of liquid scintillation counting; the x-rays and gamma rays are more penetrating and tend to escape from the liquid scintillator. Iodine 125 photons, having energies of 35 keV or less, are principally detected as the result of compton interactions and to a lesser extent by photoelectric interactions. To enhance the absorption of x-rays by these processes it is necessary to increase the electron density, i.e., the number of electrons/cm^3, by increasing the average atomic number of the absorbing material, i.e., the liquid scintillator. This can be done by incorporating metal atoms in the scintillation cocktail as the salts of organic acids or by the use of complexing agents. Methods for the preparation of metal loaded liquid scintillators was reviewed by Horrocks (1962). Ashcroft (1970) obtained a counting efficiency of about 55% for iodine 125 by loading a toluene-based scintillator with tin tetrabutyl. Bransome and Sharpe (1972) obtained even higher efficiencies (up to 80%) without benefit of metal loading by use of a method they describe for selecting the proper channel settings in order to maximize counting efficiency.

Helman and Ting (1972a; 1973) and Ting (1972a) describe a method for liquid scintillation counting of iodine 125 with 70% efficiency. A microfuge tube containing the sample is inserted through a hole in the cap of a liquid scintillation vial so that the microfuge tube is immersed in the scintillation cocktail. The scintillation cocktail is loaded with lead tetrabutyl in order to increase the density and the average atomic number. It has also been shown that lead acetate can be used to increase the electron density of the scintillator.

Gamma counting by use of liquid scintillation counting has been applied to a number of radioimmunoassays. For example, LS counting of iodine 125 was used by Jordan et al (1974) for the RIA of hepatitis-associated antigen and by Helman and Ting (1972b) to the RIA of carcinoembryonic antigen.

For the radioassay of vitamin B-12 a cobalt-57 tag is usually used. Most vitamin B-12 assays are not immunoassays; a thermolabile glucoprotein, intrinsic factor, is used as a binder rather then an antibody. Such assays are therefore called "radioassays" but not "radioimmunoassays". Ting and Helman (1972c) describe an assay of this type. Gutcho et al (1973) also describe a vitamin B-12 assay and also discuss a method for optimizing a liquid scintillation counter for the measurement of cobalt 57.

V. MECHANICAL INNOVATIONS TO SIMPLIFY COUNTING

Many RIA laboratories must run thousands of assays per month requiring tens of thousands of test tubes and vials. Next to accuracy, economy must be an important concern both in terms of the tubes and equipment used and the time required per assay as well. To achieve economy of time, procedures must be shortened and simplified as much as possible, consistent with good practice.

To this end Ting and Helman (1972b) describe a mini liquid scintillation vial which allows the use of only 1 to 4 ml of scintillator rather than the 15-ml volume used in the standard vial. Small vials of this type are now commercially available from a number of manufacturers.

It is a common practice to separate free ligand from protein-bound ligand by precipitation. The supernatant is often removed by decantation. In this step complete removal of the supernatant can not be achieved. It is here that a double tag, as mentioned earlier, should be used to permit application of a correction for that part of the supernatant not transferred.

If the activity of the precipitate is to be measured the supernatant is discarded and the "last" drop is blotted from the lip of the tube. If, on the other hand, the supernatant is to be counted by liquid scintillation counting it must be transferred to a counting vial. Fernandez-Pol (1975) suggests a modified liquid scintillation vial to allow transfer of the "last" drop by touching the lip of the tube to a small protuberance in the vial.

A true economy of time and tubes is achieved by counting radioactivity in the same tube in which the antigen and antibody were equilibrated. Most laboratories have standardized on

the use of 12 x 75 mm plastic or glass test tubes for RIA. By placing the tube in an adapter it can be placed directly in the liquid scintillation counter. For the counter to accept this assembly, Dixon and Cohen (1976) describe a modification of the drive mechanism of a liquid scintillation counter to accomodate a test tube 75 mm high. Hicks et al (1977) describe an alternate method which they used for the radioimmunoassay for hepatitis-associated antigen. They cut off the bottom of the Austria-125 test tube 1.5 cm from the bottom and placed the cut-off bottom in a scintillation vial for counting. Grotgen and Steinberger (1978) report that the use of 10 x 50 mm tubes eliminates the need to make any modifications.

VI. OBSERVATIONS AND CONCLUSIONS

A. *Choice of Instrument*

The choice between the purchase of a gamma counter and a liquid scintillation counter for radioimmunoassay must be made on the basis of need and economy. Many small laboratories accept the beta-counting restrictions imposed by a gamma counter for the sake of economy and for the convenience of counting with minimal sample preparation. Liquid scintillation counting, on the other hand, offers the obvious advantage of beta counting while at the same time providing the capability of gamma counting with an efficiency comparable to that of most NaI(Tl) gamma counters.

B. *Nuclides for RIA*

The requirement of high specific activity restricts the choice of nuclide principally to tritium, iodine 125 and cobalt 57. A high specific activity is required because the nuclide must serve as a tracer to a trace amount of analyte. Carbon 14 and other nuclides commonly used as tracers are generally not satisfactory for RIA applications.

On the positive side of the ledger we are reminded that the quantity of nuclide used in a radioassay can usually be adjusted to provide a convenient activity. It is therefore possible to obtain data possessing acceptable statistical accuracy with only nominal counting efficiency.

C. *Waste Disposal*

The disposal of radioactive waste continues to be a problem. The additional complications created by the chemical toxicity of toluene, dioxane and other LSC solvents impose a problem of waste disposal which may soon place severe restrictions on research involving radioactive tracers, especially where radioactivity is measured by means of liquid scintillation counting. There is need for intelligent, decisive action on the part of regulatory agencies reflecting perspective, relevance and objectivity with regard to waste disposal. Such action must be taken soon.

D. *Applications of RIA*

The most extensive use of RIA has been in the clinical laboratory for the diagnosis of metabolic malfunction and disease and for the management of therapy. For clinical applications an RIA must meet certain requirements. Accuracy, for example, should normally be +/- 10% or better. Owing to the great number of assays that must be performed, simplicity of methodology and economy are important considerations. It is desirable to use the same tube for both the reaction and for counting to reduce cost and to reduce the number of necessary operations. The use of small tubes and vials helps achieve an economic utilization of reagents and, in the case of liquid scintillator, also helps to reduce the problem of radioactive and chemically hazardous waste.

For the clinical laboratory it is recommended that liquid scintillation counters be equipped with the capability of automatic quench correction. The liquid scintillator employed must be capable of accepting an aqueous sample containing ammonium sulfate or other salts. Many commercial liquid scintilators are available which perform this function effectively for both tritium and iodine 125-tagged assays.

A less frequently used but more demanding application of RIA is in the analytical laboratory where this technique is being used for the quality control of certain pharmaceutical products and biological substances. Here a high degree of accuracy is required, i.e., one or two per cent. Simplicity and economy, while important, are not a major consideration. Laboratories conducting such analyses are frequently equipped with computer facilities capable of processing raw data for quench correction. Often involved in research, analytical laboratories usually have liquid scintillation counting facilities available for biochemical tracer work.

E. Automation and Data Reduction

For many years gamma counters, equipped with microprocessors, have been programmed by the manufacturer to reduce RIA data. To date, microprocessors on liquid scintillation counters have been programmed for quench correction but not for data reduction. However, the reduction of RIA data by microprocessor for LSC appears to be imminent. The following brief comments are presented in the hope that certain fallacies and errors may be avoided and that other desirable features might be included (Ekre, 1971).

1. Features To Be Avoided in RIA Data Reduction

a. Procedures which obscure information

- Normalization
- Log transforms - "compresses" error
- Subtraction of a constant value for non-specific binding - a violation of the mass action law
- Use of arbitrary coordinates - yields meaningless paramters
- Logit transforms - "compress" error

b. Procudures which introduce error

- Use of arbitrary coordinates which result in a curved plot
- Use of straight lines to connect data points on a non-linear plot
- Assuming linearity of a non-linear plot - e.g., a logit-log plot
- Use of "pooled" sera for standards to simulate non-specific binding of patient's serum
- Assuming that all assay data can be reduced in the same way

2. Features To Be Included in RIA Data Reduction

a. Procedures which provide information

- Methods which evaluate the concentration of the antibody and the radioactive antigen
- Methods which evaluate the binding constant of the specific binder
- Methods which signal changes in the assay parameters
- Methods which measure the extent of non-specific binding and signal when it is excessive
- Methods which submit to dimensional analysis

b. Procedures which minimize error

Fitting a theoretical relationship for which the function is known

Proper weighting of data to minimize the effect of an error in a single point

REFERENCES

Anderson. L., and McClure W. (1973). *Anal. Biochem.* 51, 173.

Ashcroft, J. (1970). *Anal. Biochem.* 37, 268.

Bakhle, Y., Prusoff, W., and McCrea, J. (1964). *Science* 143, 799.

Benson, R. (1966). *Anal. Chem.* 38, 1353

Berson, S., and Yalow, R. (1959). *J. Clin. Invest.* 38, 1996.

Bransome, E., Jr., and Sharpe, S., III. (1972). *Anal. Biochem.* 49, 343.

Bray, G. (1960). *Anal. Biochem.* 1, 279.

Case, R., and Mezei, L. (1978). *Clin. Chem.* 24, 2145.

Castanier, M., and Scholler, R. (1970). *C.R. Acad. Sci. Ser.* D271, 1787.

Chapman, D., and Marcroft, J. (1971). *Int. J. Appl. Rad. Isotopes.* 22, 371.

Chase, G. (1979). *The Ligand Quaterly.* 2, No. 3

Dixon, R., and Cohen, E. (1976). *Clin. Chem.* 22, 1746.

Ekins, R. (1960). *Clin. Chim. Acta.* 5, 453.

Ekre, C. and Foote, W. (1971). *J. Animal Sci.* 33, 819.

Farmer, E., and Berstein, I. (1952). *Science* 115, 460.

Fernandez-Pol, J. (1975). *J. Nuc. Med.* 16, 963.

Fox, B., (1968). *Int. J. Appl. Rad. Isotopes.* 19, 717.

Francis, G., and Hawkins, J. (1967). *Int. J. App. Rad. Isotopes* 18,223.

Grotjan, H., and Steinberger, E., (1978). *Clin. Chem.* 24, 1193.

Gutcho, S., Johnson, J., and McCarter, H. (1973). *Clin. Chem.* 19, 998.

Hayes, F., and Gould, R. (1953). *Science.* 117 480.

Helman, E., and Spiehler, V. (1974). *Clin. Chem.* 20, 516.

Helman, E., and Ting, P. (1972a). *Beckman Clinical Brief* #4.

Helman, E., and Ting, P. (1972b). *Beckman Clinical Brief* #9.

Helman, E., and Ting, P. (1973). *Clin. Chem.* 19, 191.

Herberg, R., (1960). *Anal. Chem.* 32, 42.

Hicks, E., Oei, T., Nordschow, C., and Hughes, N. (1977). *Transfustion.* 17, 503.

Horrocks, D. (1962). *Packard Tech. Bull.* No. 2.

Jordan, W., Spiehler, V., Haendiges, R., and Helman, E. (1974). *Clin. Chem.* 20,733.

Jowett, T., Slater, J., Piyasena, R., and Ekins, R. (1973). *Clin. Sci.* 45, 607.

Keane, P., Stuart, J., Mendez, J., Barbadoro, S., and Walker, W. (1975). *Clin. Chem.* 21, 1474.

Kinard, F., (1957). *Rev. Sci. Instr.* 28, 293.

Lahr, T., Olsen, R., Gleason, G., and Tabern, D. (1955). *J. Lab. Clin. Med.* 45,66.

Langham, W., Eversole, W. Hayes, T., and Trujullo, T. (1956). *J. Lab. Clin. Med.* 47, 819.

Lieberman, R., and Moghissi, A. (1970). *Int. J. Appl. Rad. Isotopes.* 21, 319.

Manns, J. and MacKenzie, E. (1969). *Can. J. Physiol. Pharmacol.* 47,490.

Meade, R. and Stiglitz, R. (1962). *Int. J. Appl. Rad. Isotopes.* 13, 11.

Moss, G. (1964). *J. Nuc. Med.* 5, 69.

Nadarajah, A., Leese, B., and Joplin, G. (1969). *Int. J. Appl. Rad. Isotopes.* 20, 733.

Patterson, M., and Greene, R. (1965). *Anal. Chem.* 37, 854.

Pearce, E., DeVenuto, F., Fitch, W., Fircshein, H., and Westphal, U. (1956). *Anal. Chem.* 28, 1762.

Rhodes, B. (1965). *Anal. Chem.* 37, 995.

Scales, B. (1967). *Int. J. Appl. Rad. Isotopes.* 18, 1.

Schiff, F. (1924). *Klin. Wochenschr.* 3, 679.

Shapira, J., and Perkins, W. (1960). *Science.* 131, 414.

Steinberg, D., (1960). *Anal. Biochem.* 1, 23.

Ting, P. (1972a). *Beckman Clinical Brief* No. 6.

Ting, P., and Helman, E. (1972b). *Beckman Clinical Brief* No. 7.

Ting, P., and Helman, E. (1972c). *Beckman Clinical Brief* No. 16.

Turner, J. (1968). *Int. J. Appl. Rad. Isotopes.* 19, 557.

Turner, J., (1969). *Int.J. Appl. Rad. Isotopes.* 20, 499.

Tyler, J., Hennam, J., Newton, J. and Collins. W. (1973). *Steroids.* 22, 871.

van der Laarse, J. (1967). *Int. J. Appl. Rad. Isotopes.* 18, 485.

Vaughn, B., and Boling. E. (1961). *J. Lab. Cli. Med.* 57, 159.

Vaughn, B., Steinberg, D., and Logan, J. (1957). *Science.* 126, 446.

Whyman, A. (1970). *Int. J. Appl. Rad. Isotopes.* 21, 81.

Wiegman, T., Woldring, M., and Pratt, J. (1975). *Clin. Chim. Acta.* 59, 347.

Wyse, B., Wittwer, C., and Hansen, R. (1979). *Clin. Chem.* 25, 108.

Yerick, R. and Ross, H. (1963). *Oak Ridge Radioisotope Conf.*, p 20.