Chapter 12

The Use of the Scintillation Counter in Radioimmunoassay

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INTRODUCTION

The inclusion of radioimmunoassay and related procedures in the topics for this symposium is particularly timely, as the field has grown enormously in the past few years and still has considerable further potential. The procedures probably account for something like 90% of the use of γ scintillation counters and a substantial proportion of liquid scintillation counting at the present time. The inclusion represents something of an innovation, as topics other than liquid scintillation counting must be discussed. Radioimmunoassay (RIA) was introduced by Yalow and Berson in 1959[1] with an assay for insulin. At about the same time, Ekins[2] independently introduced a saturation analysis for thyroxine, based on similar principles.

RIA is one example of a collection of procedures for which there is not yet a universally accepted collective name. Perhaps the best suggestion to date is that of Landon[3] that they should be designated as 'binding assays'. Other examples in this category are competitive protein binding, immunoradiometric, radioenzymatic and radiomicrobiological assays. However, the same considerations apply regarding the eventual measurement of radioactivity of samples and these will form the main consideration of this presentation.

Binding assays require a substance with a limited binding capacity, which is an antibody for RIA and perhaps a natural plasma protein for competitive protein binding assays. Also required is a labelled substance similar to that to be assayed (and preferably identical with it) which would be in the form of an antigen for RIA. The tracer antigen is mixed with the sample and incubated with the antibody. The binding sites are then occupied by the labelled and unlabelled molecules in proportion to their concentration in the mixture, so that the degree of binding of radioactivity is dependent on the amount present of the substance to be assayed. Other requirements are a method of separating the bound from the free fraction of the agent and a means of determining the radioactivity in one or other of the separated components. The chief power of the method is its sensitivity – for many substances picogram quantities can be determined in 1 ml of plasma or other biological fluid. Ideally and usually another property of the system is its specificity. RIA was applied first to the determination of protein

hormones which are now assayed in very large numbers. The other big class of compounds assayed comprises the steroids in plasma and urine. More recently the method has been applied to the assay of drugs such as digoxin and morphine.

The raising of antisera is by exposure of an experimental animal to the substance to be assayed over a period and is beyond the scope of this presentation. Proteins and other large molecules may be naturally immunogenic. Smaller molecules such as steroids and drugs have to be conjugated with a protein, e.g. bovine serum albumin, before they will be recognised by a host as immunogenic. When this happens, antibody is raised to the 'hapten', i.e. the drug or steroid which is attached to the albumin, as well as to the albumin itself. Complete immunoassay kits are available but expensive. If it is desired to initiate a new assay procedure it will usually be necessary for the experimenter to raise his own antisera and possibly also to label the molecule concerned.

RADIOACTIVE LABELS

A discussion of the available labels is of importance to our considerations as they will affect counting procedures. Desirable properties are that they should be easy to substitute or attach, easy to count, cheap, have a convenient half-life and a high specific activity. The last two considerations are related and conflicting. The best labels are carrier-free but even then one cannot get away from the fact that the longer the half-life the lower the specific activity. Table 1 outlines the main labels which have been used in RIA procedures. The specific activities quoted are for carrier-free material and relate to the elements listed. Compounds may have more than one atom of radionuclide per molecule, particularly with tritium. However, although it is theoretically possible to add more than one atom of iodine to a protein, it is usually undesirable as there will be resulting damage to the labelled compound. Carbon-14 has been used as a label, but is really included in the table to indicate why it is not suitable, owing to the low specific activity. Carbon-14 compounds also tend to be more expensive than those labelled with ^{3}H. Selenium-75 has been used as a γ-emitting label for steroids;[4] at the moment, carrier-free material is not available and the specific activities attained in practice are far

Table 1. Labels used in binding assays.

Radionuclide	$T_{1/2}$	Emission	Theoretical specific activity (Ci mg-atom^{-1})
^{3}H	12y	β	29
^{14}C	5700y	β	0.06
^{125}I	60d	γ +X	2160
^{131}I	8d	β +γ	16200
^{75}Se	121d	γ +X	1070
^{57}Co	270d	γ +X	480

below the theoretical maximum. Because γ-counting is so much easier and cheaper liquid scintillation counting, considerable efforts are being made to develop the γ-labelling of steroids and drugs. In general, they cannot be labelled directly with the isotopes of iodine, but labelling of the conjugated pair is possible. By far the most common radionuclides used for RIA are ^{125}I and ^{3}H. Although ^{131}I has some advantages as far as theoretical specific activity is concerned, these advantages are not fully realisable as ^{131}I is not available at above about 30% abundance, while ^{125}I is available very close to carrier-free. In addition, the efficiency of counting for ^{125}I is usually higher, and its longer half-life makes it much more convenient.

It is worth remarking in passing that tracers other than radioactivity have been proposed for immunoassay, such as enzymes, viruses, phage and electron spin. However, these alternatives cannot at the moment match the sensitivity of RIA, and there is more likelihood of interference upon detection.

SEPARATION OF BOUND FROM FREE AGENT

Once again the importance of the discussion under this heading relates to the way it may affect counting procedures. Table 2 lists the chief methods that have been adopted. Electrophoresis is principally of historical importance, as it was the technique adopted by Berson and Yalow in their original experiments. It is not suitable for large numbers or automation, and compared with other techniques the counting is somewhat difficult, particularly of ^{3}H-labelled compounds. In gel filtration, use is made of separation by molecular size, the higher molecular weight bound portion coming through the column first. The eluate provides liquid samples so there is no special problem of counting. However, the method is slow and tedious and is suitable only for small numbers of samples. Of much more widespread use are the techniques which result in a precipitation of either the free or the bound fraction. The double antibody technique extends the use of immunology. Another antibody is raised in a different species to the immunoglobulin used for the first antibody. When this is added to the mixture, precipitation of the bound fraction occurs. The technique is of wide application and suitable for automation. Chemical precipitation achieves the same end; commonly anmonium sulphate or ethanol are used in well-defined concentrations in order to precipitate the bound fraction. The adsorption techniques use an agent to which the free antigen is attached after incubation. The most used material is charcoal coated with dextran or albumin. Alternative materials that have been used include talc, powdered glass, Fuller's earth and ion exchange resin. The mixture is then centifruged and the supernatant decanted or sampled for counting.

Table 2. Some methods of separating antibody-bound from free antigen.

Electrophoresis	Adsorption
Chromatography	Solid phase systems:
Gel filtration	Antibody-coated tubes
Immunoprecipitation	Polymerised antibodies
Chemical precipitation	

'Solid phase' systems refer to the attachment of the antibody. In the coated tube method the antibody is merely applied to the inside of a plastic vial. The technique is undoubtedly simple, but in many people's experience lacks reproducibility. Better precision is obtained when the antibody is covalently linked to a polymer. One advantage over precipitation techniques is that centrifugation is much quicker.

During the initial validation of an assay it is often advisable to use more than one separation method. Techniques which do not lend themselves to large-scale routine use may thus be valuable at the development stage.

MEASUREMENT OF RADIOACTIVITY

It is worthwhile examining the radiations from the most commonly used label, ^{125}I. The decay scheme, electron capture followed by the emission of a single γ-ray, is deceptively simple. However, most of the γ-radiations are internally converted and subsequent re-arrangements in the atom give rise to the emission of X-rays and particulate radiation. The electron capture process itself also gives rise to similar radiations. The principal radiations are summarised in groups in Table 3. The photon radiations in the first group are the only ones with sufficient penetrating power to be measured by sodium iodide scintillation detectors. As the latter are much simpler to use, requiring no sample preparation, quench correction or continuing expense, they are almost universally preferred. The counting efficiency commonly obtained in many standard well counters is around 50%, but if the latter are designed specially for ^{125}I,

Table 3. Principal radiations from ^{125}I.

Radiation	Energy (keV)	% Abundance
γ	35	7
Kα X	27	112
Kβ X	31	24
		143
K internal conversion electron	3.7	75
L X	3.8	22
LMM Auger electron	2.9	149
		246
L internal conversion electron	31	11
M internal conversion electron	35	8
KLL Auger electron	23	14
KLX Auger electron	26	6
KXY Auger electron	30	1
		40

an efficiency of about 80% may be obtained. Nor need such a system be expensive, as thin sodium iodide suffices to absorb the low-energy radiation and consequently a minimal amount of lead shielding is required.

Some of the radiations from ^{125}I are emitted in coincidence, and if both components are intercepted by the detector, the pulse heights will be added together, giving a signal corresponding to the sum of the two individual energies. The pulse height distribution will then show a photopeak from the X- and γ-rays at around 30 keV (the resolution of a NaI crystal is not good enough to separate the different energies in this group), together with a smaller peak at about 60 keV. The sum peak only appears if the detector geometry is good, as with a sample at the bottom of a well crystal, as the probability of interception of both constituents by the crystal varies as the square of the solid angle subtended by the detector. Whether the sum peak should be included within the channel width set on the analyser depends on the background characteristics of the system. A channel set wide enough to include both peaks will undoubtedly give higher efficiency counting but the background will also increase. If a special thin crystal is chosen, background from higher energy radiation is minimised and it may not be necessary to provide an upper threshold to the analyser, a simple discriminator sufficing. In this case the sum peak will be automatically included. It must be remembered that the photon radiations from ^{125}I are relatively easily absorbed and the use of glass tubes with appreciable heavy metal content can sometimes lead to variations of efficiency between samples.

Although a well scintillation counter provides excellent counting geometry, an alternative that comes close to it in efficiency is the use of two opposed thin flat crystals. This arrangement is adopted in at least one automatic counting system.

The chief radiation from ^{131}I is of a much higher energy than that from ^{125}I, so that separation in double isotope studies is quite easy. Some 'crossover' from the higher energy radiation is inevitably included in the ^{125}I photo-peak setting, and it is interesting to note that there is a similar peak in the ^{131}I spectrum, arising from internal conversion processes, as well as Compton scatter continuum. Selenium-75 has principal γ-radiations not too dissimilar from those of ^{131}I and so could also be readily detected and separated from ^{125}I.

An examination of Table 3 shows that, although there are no β-radiations from ^{125}I, there is an abundance of low-energy radiation which could be detected by liquid scintillation counting. Indeed, the latter gives a higher efficiency of counting than the use of the X- and γ-rays alone with a NaI crystal. However, the main interest in the liquid scintillation counting of ^{125}I is in its influence on double labelled experiments which also make use of ^{3}H. There are to be papers dealing with this subject later in the session but perhaps I can introduce it in general terms. Figure 1(a) shows the pulse height distribution from the liquid scintillation counting of ^{125}I. Two broad peaks are noticeable, corresponding to the groups with energy about 3 and 30 keV. Most of the latter come from internal conversion electrons, but some from X- and γ-ray absorption or Compton scatter in the scintillator. That the latter is a relatively small proportion can be seen from Fig. 1(b), which was obtained by placing a ^{125}I source in an inner tube suspended in the centre of a vial of scintillator. All the particulate radiation was absorbed, and the remaining photon radiation contributed 18% of the counting rate.

The similarity between the spectra of ^{125}I and ^{3}H is illustrated in Fig. 1. The pulse height distributions are not identical, and it would be theoretically possible to perform double label assays by liquid scintillation counting alone using two

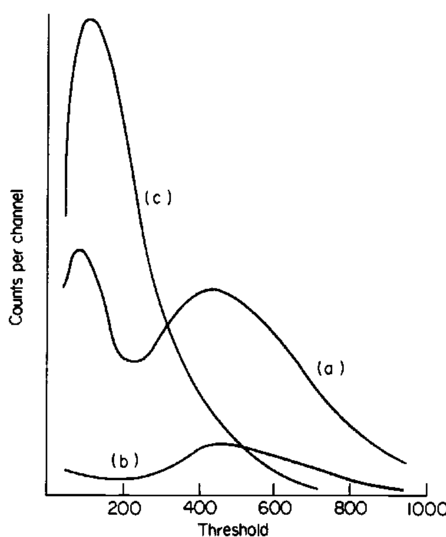

Fig. 1. Pulse height distributions from the liquid scintillation counting of ^{125}I and ^{3}H: (a) ^{125}I all radiations; (b) ^{125}I X + γ-rays only; (c) ^{3}H.

channels. However, it is much better to use γ-counting for ^{125}I and then make a correction for its contribution to the combined liquid scintillation counts.

The consideration of the liquid scintillation counting of ^{3}H alone in connection with RIA is primarily concerned with sample preparation. The subject does not warrant detailed consideration here as the techniques used are in general no different from those already discussed in this and previous symposia. If the separation procedure results in an aqueous solution, such as the decanted bound fraction from a charcoal precipitation or the free fraction from a double antibody separation, there is no difference from other aqueous sample counting. Standard procedures that have been used include dioxane-based scintillator, solubiliser, emulsion or gel systems. It is worth remembering that if only a small aqueous volume is required, a correspondingly small volume of scintillator suffices and the mixture can be placed in a disposable plastic tube held in a standard vial with no loss in efficiency but a gain in economy.

Another useful technique, introduced by Abraham *et al.*,[5] and applicable to non-polar substances such as progesterone, is simply to mix the aqueous solution with a toluene-based scintillator and allow the mixture to equilibrate. The activity is extracted into the scintillator and can be counted reproducibly in the two-phase system. It is even possible to rely on partition into liquid scintillant without removal of an ammonium sulphate precipitate.[6]

If it is desired to assay a precipitate, such as the bound fraction from a double-antibody or chemical precipitation, two courses are open. The precipitate can be suspended in a gel scintillator. This places reliance on the reproducibility of self-absorption of the β-rays which is a problem with heterogeneous systems, as no method of quench correction can allow for variations. An alternative is to extract the label from the precipitate and treat it as a liquid sample. Once again, small volumes often suffice.

It is not usual to apply quench corrections when ^3H is used for RIA, as conditions are so standard that the degree of quenching is considered the same for each sample. However, it has been shown that in some circumstances quenching is somewhat variable, and that correction is worthwhile. This factor should always be investigated in any new technique.

If separation has been by electrophoresis or chromatography, the simplest way of measuring ^3H is to cut up the paper or cellulose acetate and suspend the strips in liquid scintillator. The best efficiency and reproducibility are obtained if this process results in the complete elution of the active material into the scintillator, but reasonable counting can be performed if there is no removal from the medium.

AUTOMATION

The rapid growth and wide applicability of RIA, the large number of samples generated and the relatively complex data processing required all call for automation. Scintillation counters themselves have already led the way, but there is also a need for the automation of dispensing, dilution, addition of reagents and separation. Equipment such as the Analmatic or Micromedic systems lend themselves well to some of the procedures, but there is still some transfer of trays or samples to and from centifruge and counter required. The traditional chemical auto-analyser system using continuous flow is not well suited to processes requiring finite and possibly lengthy incubation or counting, although efforts are being made to develop such a technique for RIA. The fact that counting time may place a limit on the number of samples processed has been recognised in some commercial systems which provide multiple detectors.

The most fully automatic system dedicated to RIA is that developed by Bagshawe[7] and now commercially available. Special mechanical modules handle the dispensing, dilution and addition, but an important feature is that separation of the precipitate is by filtration. The glass fibre filter pads are mounted on a plastic tape, simplifying passage through a detector module containing five opposed pairs of counters. Another feature is the inclusion of a computer, which controls all the modules, stores the sample information, acts as a scaler for counting and performs the calculations and curve fitting necessary to provide a direct output of agent concentration.

Automated equipment is expensive and in considering its justification attention must be paid to the work load and to the costs of alternative methods. In any case, it is a salutory exercise to assess the component costs of any assay procedure. Any estimates must be dependent on local circumstances, and the following are offered just as an example.

Antiserum is cheap enough not to be a significant factor in considering costs. A labelled tracer might cost up to 1p per assay and materials for separation another 1p. These items can be compared with the cost of commercial kits, which range from about 10–100p per assay. Of course, one is then paying for development, quality control and convenience.

Costs of radioactivity counting must include a figure for the capital equipment, based on write-off over a suitable period, costs of servicing and number of assays performed. It is likely that the figure will be 1-2p per assay. A vial might cost 1-10p and, for ^3H-assays, scintillator another 1-10p. The overall total of about 4-25p does not include any salaries of technical staff. These might account for 5-20p per assay,

so a substantial investment in automation can readily be justified if there is sufficient work load. Nor are other laboratory overheads included, and these must obviously be borne in mind when comparing any estimates with the cost of having assays performed commercially, currently in the range of £2–£10 per assay.

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DISCUSSION

R. P. Parker: In view of the wide variations in sample volume, tube size and detector size that are catered for in commercial equipment, would you comment on the relative merits and accuracy of using the various amounts of sample, etc?

P. Tothill: The efficiency of a well counter varies considerably with height and therefore with volume. Thus it is important to standardise the volume of the samples. If this is small, as is common, then it is best for a narrow sample container to be used and a small diameter well. Indeed, the sample volume of either precipitate or supernatant can be kept to no more than 1 ml in the great majority of RIA procedures and this could lead to simplification and economy of γ-counters designed specially for RIA work, and to savings on scintillator and vials in liquid scintillation counting.

R. Evans: Although it is true that in a well scintillation counter the portion of the sample that is within the well will be counted with higher efficiency than any portion of the sample outside the well, nevertheless one can improve sensitivity of

measurement by using a larger sample extending outside the well. A further possible benefit in this case is that small variations in sample volume will affect the counting result much less critically than will the same volume variation in a sample entirely within the well.

P. Tothill: I agree that the plot of count rate against volume reaches a plateau where changes of volume are not critical, but I doubt whether such large volumes are available from RIA procedures.

N. Harding: For some of our experiments we would like absolute activity estimates of ^{125}I. For this purpose both standards and methods of estimating both liquid and crystal counting efficiencies are required. Would Dr. Tothill comment on the best methods available for estimating the efficiency of counting ^{125}I?

P. Tothill: We have only measured efficiencies using manufacturers' quoted activities on ordinary commercial ^{125}I and I would refer Dr. Harding to the Radiochemical Centre for ^{125}I standards. One can use the relationship between the areas under the sum peak and the singles photopeak as an absolute determination of ^{125}I in a well counter, and it appears to give reasonable results. There was a paper in *Nucleonics* some years ago.

A. McNair: Iodine-125 can be accurately standardised by a double crystal coincidence method measuring coincidences between the X-rays. Standards are readily available, those from the Radiochemical Centre being guaranteed to have an accuracy of better than ± 2% overall uncertainty. In order to standardise a liquid scintillation counter it would probably be necessary to use a ^{125}I standard and derive some quench correction curves.

D. W. T. Lee: Does not the peak occurring in the ^{125}I region of the ^{131}I γ-spectrum partially consist of γ-rays from $^{131}Xe^{m}$ (half-life 12d) which results from the decay of the ^{131}I? This xenon can build up in the solution so that for dual labelling experiments the crossover of ^{131}I into the ^{125}I channel can vary with time.

P. Tothill: I do not know the decay scheme of $^{131}Xe^{m}$ and whether it includes radiation within the counting window for ^{125}I. Certainly some of the ^{131}I γ-rays are internally converted and the resulting X-rays account for most of the peak in the 30 keV region.

[**Editor**: 0.6% of the ^{131}I disintegrations result in $^{131}Xe^{m}$ with a γ-ray emission at 160 keV; the remainder result in stable ^{131}Xe. The contribution to the spectrum in the 30 keV region is therefore negligible.]

F. E. L. ten Haaf: I should like to make a comment regarding the counting efficiency of large samples that extend beyond the well of a well-type γ-counter. As the counting efficiency is high within the well and low outside, with large samples one is measuring the specific activity of the sample rather than the total activity. If the samples are of unequal volume (and height), this will lead to errors in the determination of the sample activity.

P. Tothill: I can only agree and emphasise that the volume used must be known and either kept fixed or corrections applied for variations.

G. W. Pearson: I should like to point out that in view of the considerable amounts of radioactive iodine that are involved in labelling procedures, there can be radiation protection problems.

P. Tothill: This is a very important point, and particular care must be taken owing to the volatile nature of iodine. Provided the correct precautions are used, including operation in a fume cupboard, there should not, however, be any undue hazard in using radioactive iodine for protein labelling. Monitoring is important and any portable scintillation counter held against the neck will indicate a significant thyroid accumulation.

R. P. Parker: We have found whole body counting a useful check on the possibility of contamination or ingestion of radioactive iodine by personnel. Iodine-131 in particular can be detected at amounts considerably below the maximum permissible body burden. Routine checks by this method have been of particular value in training new staff.

W. R. Carr: In view of the criticism of plastic vials for liquid scintillation counting, has Dr. Tothill any comment to make concerning their use in RIA procedures?

P. Tothill: In our experience, modern vials appear to behave quite satisfactorily, although earlier versions showed loss of toluene and deformation.