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The development of high sensitivity pulsed light, time-resolved fluoroimmunoassays

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Abstract - In this presentation, we review the basic concepts underlying immunoassay techniques, emphasising the distinction between "competitive" methods (which may rely on labelled antigen or labelled antibody) and "non-competitive" methods, which generally rely on labelled antibody. These two forms of assay are characterised by different sensitivity constraints, implying that, in practice, the non-competitive methods may display sensitivities several orders of magnitude greater than competitive techniques.

To exploit the sensitivity potential of the non-competitive methods, very high specific activity labels characterised by high signal/noise ratios are required. Pulsed-light time resolving fluorescence measurement techniques, using lanthanide-chelate labels, offer such characteristics. We give a brief description of this methodology which has already been exploited in a number of working immunoassays.

INTRODUCTION

Since the original independent development of the radioimmunoassay for serum insulin (1) and the "saturation assay" for serum thyroxine (2) in 1960, microanalytical methods relying on identical anlytical principles have been developed for a very wide range of substances of biological importance. These include hormones, drugs, vitamins, viruses etc. - indeed virtually all substances which occur either endogenously or exogenously in biological fluids at low concentration. These methods have formed one of the cornerstones of the great expansion in knowledge in several areas of medicine which has occurred in the past 25 years (for example, in endocrinology) an expansion which would not have been possible in the absence of microanalytical methodology permitting the detection and the measurement of the sometimes minute concentrations of bioactive substances which are found in biological systems.

The enormous impact these methods have had in medicine and other biologically-related fields (e.g. agriculture, veterinary science, environmental studies etc.) derives from the exploitation of the combination of two important attributes: the "structural specificity" characterising many protein-binding reactions (typically those occurring between antibodies and antigens, hormones and receptors etc.), and the availability of readily detectable "labels" (e.g. radioisotopes) which permit the observation of the binding reactions occurring between such substances even when they occur at exceedingly low concentrations. The first of these endows binding assay systems which the extreme specificity required for the identification of an individual substance even when it is present in relatively small amounts as low as 1 part in 10^{12}. The second confers the very high sensitivity required to detect hormones and other similar substances at the levels at which they frequently occur in many biological fluids. For example I^{125}, the radioisotope commonly used in the context of radioimmunoassay, permits observation of the reaction behaviour of molecular numbers as low as approximately 10^7 - 10^8, implying that concentrations of substances down to 10^7 molecules/ml ($\sim 10^{-14}$mol/litre) can be measured using appropriate radioiodine labelled reagents. Aside from the sensitivity which the use of radioactive isotopes confer upon assays of this type, an important attribute of radioactive measurement is its relative invulnerability to environmental interference. Radioisotopic disintegration is not affected by physical or chemical factors, and, provided counting conditions are reasonably standardised, highly accurate estimates of the radioactive content of individual samples can readily be made using cheap and easily operable equipment.

Nevertheless for some years considerable pressure has existed to find substitutes for radioisotopes for use in this context. Amongst the principal reasons for this are the widespread public perception and fear of health hazards associated with radioactivity (leading to legal constraints on the use of radioisotopes in many countries), the logistic and quality-control problems arising from the limited shelf-life of radiolabelled materials, and the expense of (automatic) radioactive sample measuring equipment. A further disadvantage of radioisotopes stems, paradoxically, from the very invulnerability of radioactive disintegration to environmental influence referred to above, implying that it is impossible to devise simple "homogeneous" assay systems in which labelled reaction products do not require separation prior to their measurement.

However, some of the arguments commonly advanced against the use of radiosotopes in the present context are of doubtful validity. For example, the amounts of radioactivity involved are exceedingly small, and the health hazards that they present in the normal hospital laboratory negligible, being often far outweighed by the potential dangers arising from carcinogens, viruses and other toxic agents which frequently form part of the laboratory environment. Likewise the costs attaching to the use of radioactive techniques are generally greatly exaggerated. Counting equipment - the only specialised apparatus normally required - is now cheap and reliable, the cost of the more sophisticated types of sample counter arising primarily from the automatic sample changing and data processing facilities which they provide. Indeed no currently visualised alternative non-isotopic methodology offering comparable sensitivity and precision to the radioisotopic techniques, and possessing similar sample handling capacity, has the slightest chance of being significantly cheaper. Meanwhile, though "non-separation" techniques are obviously attractive, and offer considerable practical advantages in certain situations, their susceptibility to interfering effects deriving from the unknown constituents of biological samples implies that they can only be readily applied, in practice, to the measurement of substances (e.g. drugs etc.) present in biological fluids at relatively high concentration.

Nevertheless, though some of the commonly offered reasons for the abandonment of radioisotopic methods are questionable, undoubted advantages would accrue if widely applicable non-isotopic labelling methods - as rugged, as simple to use, and as sensitive as the radioactive techniques - were to become available. Indeed our own involvement in this area has not primarily derived from a concern regarding the (locally minor) logistic problems associated with the use of radioisotopes. The main impetus of our own studies of non-isotopic assay methods has been the desire to develop methodologies which overcome present sensitivity constraints, thus opening up entirely new possibilities with regard to research and clinical diagnosis. Of the alternative labels which appeared to us to offer the potential for doing this, pulsed-light, time-resolved fluorescent techniques possess many attractive features, and it is this technology which we shall discuss in greater detail in the latter part of this presentation. But as a preliminary to this discussion, it is appropriate first to consider the basic principles of binding assay methods in order to clarify the circumstances in which this and other alternative labelling techniques may offer significant advantages with regard to assay performance.

BASIC PRINCIPLES OF BINDING ASSAY METHODS

An analysis of the limitations on assay performance which characterise present methodologies and the way in which these limitations may be overcome requires some understanding of the basic principles underlying analytical measurement. All analytical techniques rely on observation of the reaction between the analyte and a reagent (Fig. 1), the amount of analyte present being invariably inferred by observing the course, or products, of the reaction. Two basic strategies are available whereby the latter maybe achieved: these entail observation of the fate in the system of the analyte (Fig. 2) or alternatively that of the reagent (Fig. 3). Thus in the particular context of protein binding assays (including immunoassays), the analyte concentration may be deduced by observation of the final distribution either of the analyte or of the binding protein (e.g. antibody) between bound and residual (or "free") compartments following reaction. To facilitate observation of the distribution of analyte or reagent respectively, labelled analyte tracer or labelled reagent tracer may be added to the system (though, in principle, labelling of either of these reactants is not a fundamental prerequisite of the approaches respectively involved).

ANALYTE + ANALYTICAL REAGENT

ANALYTE–REAGENT complex + residual REAGENT + residual ANALYTE

Fig. 1. Fundamental basis of all analytical measurement.

Assuming the binding reagent involved to be an antibody, and the label used to be a radioisotope, techniques relying on analyte tracers are generally referred to as "radioimmunoassay" (RIA); those relying on antibody tracers (i.e. "labelled reagents") are conventionally described as "immuno-radiometric" assays (IRMA). Analogous terms (eg. fluoroimmunoasay (FIA); immunofluorometricassay (IFMA)) may be employed to describe techniques relying on other forms of label used to reveal the "fate" of the analyte or of the reagent in the binding reaction.

Labelled antibody (IRMA) systems, first introduced by Miles and Hales (3) and Wide (4) in the late 1960's, were originally claimed to offer greater sensitivity than the corresponding labelled analyte (RIA)

methods (3); however the validity of this assertion remained controversial for many years. For example in a detailed theoretical analysis Rodbard claimed to demonstrate that labelled antibody techniques were essentially of equal sensitivity to labelled analyte methods (5); moreover such differences in assay sensitivities obtained in practice using these two analytical approaches could justifiably be attributed to detailed physico-chemical differences in the spectrum of antibodies present in the antisera used (and to other similar factors) which prevented any valid and meaningful comparison of the relative performance of these two forms of assay. Though this controversy was never formally resolved, the notion that labelled antibody methods yield higher sensitivity has nevertheless become part of the accepted wisdom of the immunoassay field.

I. "LABELLED ANALYTE"

analyte* + antibody → analyte : antibody* (B)
+ residual analyte* (F)
+ residual antibody

Measure "fraction bound" (B) : $[Ab]_{opt} \rightarrow 0$
Measure "fraction free" (F) : $[Ab]_{opt} \rightarrow 0$

Fig. 2. Analytical method based on "analyte observation". Observation of the fate of the analyte following analytical reaction may be facilitated by inclusion of labelled analyte (denoted by asterisk) in the reaction mixture. Note that the optimal amount of reagent (i.e. antibody) to maximise assay sensitivity approaches zero, irrespective of whether the bound analyte (B) or residual (free) analyte (F) is ultimately "observed".

In reality, however, the debate relating to the relative sensitivities of RIA and IRMA methods was misplaced, and reflected a misunderstanding of a more fundamental issue. This essentially centres on the differences in optimal assay design which characterise a number of possible alternative immunoassay "strategies". In particular it should be noted that the concentration of the analyte in an immunoassay system can be deduced by the measurement (in assays relying on labelled analyte) either of the fraction of the labelled analyte which is bound or the fraction free. Nevertheless, in both cases it is theoretically demonstrable that the optimal concentration of antibody yielding maximal sensitivity tends towards zero. However, the situation is more complicated in assays in which the antibody is labelled. In such assays, the optimal concentration of antibody tends towards infinity when the bound antibody fraction is measured (assuming negligible non-specific binding of antibody) but tends towards zero when the free antibody moiety is observed.

II. "LABELLED ANTIBODY"

analyte + antibody* → analyte : antibody* (B)
+ residual analyte
+ residual antibody* (F)

Measure "fraction bound" (B) : $[Ab]_{opt} \rightarrow \infty$
Measure "fraction free" (F) : $[Ab]_{opt} \rightarrow 0$

Fig. 3. Analytical method based on "reagent (i.e. antibody) observation". In this approach the antibody may be labelled to facilitate its observation. Note that when the bound antibody is measured, the optimal amount of antibody to maximise assay sensitivity approaches infinity.

These important differences in optimal assay design are summarised in Fig. 4. This emphasises that, of the four alternative strategies available, three involve the use of optimal concentrations of antibody tending towards zero, and thus essentially conform to the basic principles of "saturation assay". (Such techniques are also frequently referred to as "competitive".) In contrast, the fourth strategy permits the use of concentrations of antibody which are high relative to the amounts of analyte present; such systems may be described as "reagent excess" or "non-competitive". Moreover it is demonstrable that

"competitive" and "non-competitive" assay systems differ in several important respects - particularly in regard to their specificity characteristics and to the ultimate limitations on their sensitivities. In short, when discussing the performance characteristics of different immunoassay strategies, the crucial distinction is not between labelled antibody and labelled analyte methods (as represented by IRMA and RIA respectively), but between "non-competitive" and "competitive" assay systems. (Note, Rodbard's demonstration of equal sensitivity of RIA and IRMA techniques (5) - though mathematically sound - was based on the assumption that the free antibody fraction is measured i.e. Rodbard's conclusions related essentially to "competitive" labelled antibody methodologies).

OPTIMAL ASSAY DESIGN

(to yield maximal assay sensitivity)

LABELLED REACTANT	MEASURED REACTION PRODUCT	OPTIMAL [Ab]
Analyte	Free	$\rightarrow 0$
Analyte	Bound	$\rightarrow 0$
Antibody	Free	$\rightarrow 0$
Antibody	Bound	$\rightarrow \infty$

Fig. 4. Four alternative immunoassay strategies. Of these, only one (relying on observations of analyte-bound labelled antibody) is "non-competitive".

It is inappropriate to discuss in detail in this presentation the mathematical analyses relating to assay performance underlying these two forms of immunoassay. Nevertheless some key points emerge from theoretical prediction of the sensitivity limits which arise in competitive and non-competitive assay techniques.

In the case of the former, it may be shown that maximal sensitivity attainable with an antibody of affinity constant K is defined by the relative error ($\frac{\sigma_R}{R}$) in the measurement of the response variable at zero dose R (eg. "counts bound") divided by K. However, the error in the measurement of the response is made up of two components: the "experimental" error component (deriving from pipetting and other manipulations) and the "signal measurement" error (reflecting, for example, the statistical errors of radioisotope counting).

Assuming, for the sake of simplicity, that the signal measurement error is zero (as would result, in principle, from the use of a radioactive label of infinite specific activity), then the maximal attainable sensitivity is thus given by $\frac{\varepsilon}{K}$ where ε equals the overall relative error in the response deriving from "experimental" factors alone (6). For example, if we assume the experimental errors to be of the order of 1%, then the maximal sensitivity achievable using - for example - an antibody with an affinity constant of 10^{12} litres/mol is of the order of 0.01×10^{-12} mol/litre (i.e. 10^{-14} mol/litre). This fundamental relationship between maximal sensitivity and antibody affinity is plotted in Fig. 5. However, in practice, signal measurement errors augment experimental errors and hence influence assay sensitivity; thus Fig. 5 also illustrates the actual sensitivities that are theoretically predictable in an optimised assay system relying on I^{125} labelled analyte tracer and using "reasonable" sample counting times. This diagram thus reveals the extent of the "sensitivity gap" between the theoretically predicted sensitivity of a competitive assay system relying on I^{125} labelled analyte and the maximal sensitivity attainable in a corresponding system in which the signal measurement error is assumed to be zero. It reveals, inter alia, that (unless experimental errors are almost totally suppressed, i.e. that ε is reduced to 0.1% or less) little benefit is potentially derivable in a competitive assay from the use of any alternative label possessing a higher effective specific activity than I^{125} (the use of a high specific activity label implying a reduction in the "signal measurement error" which is incurred in any specified signal measurement time).

Analogous theoretical considerations apply to non-competitive assay systems and are also summarised in Fig. 5. In an assay system of this kind, the maximal sensitivity obtainable is essentially defined by: a. the relative error in the estimate of the response ($\frac{\sigma_n}{\text{n.s.b.}}$) in the absence of analyte (i.e. by the error in the estimate of the "non-specifically bound" antibody), b. the fractional non-specific binding of

antibody (k) and c. the equilibrium constant of the antibody (K). In short - making certain reasonable assumptions - the limiting sensitivity of a non-competitive immunoasay (assuming infinite specific activity of the label) is given by (7) $\frac{k\sigma_n}{K \times (n.s.b.)}$.

The maximal attainable sensitivity of both competitive and non-competitive systems is thus inversely related to the affinity constant of the antibody employed, though different proportionality constants (ε (i.e. $\frac{\sigma_R}{R}$) and $\frac{k\sigma_n}{n.s.b.}$) are applicable in each case. (N.b. $\frac{\sigma_n}{n.s.b.}$ can also be represented as the relative error in the zero dose response $\frac{\sigma_R}{R}$). The differences in magnitude between these constants represents the crucial distinction between the sensitivity potential of these two forms of assay. Thus if we postulate a coefficient of variation in the non-specific binding of antibody of, say, 1%, and a fractional non-specific binding (k) also of 1%, then the maximal sensitivity of a non-competitive immunoassay system is given by $10^{-4} \times \frac{1}{K}$, or 10^{-16} moles/litre assuming the use of an antibody of affinity constant of 10^{12} litres/mol (a value which represents the maximal affinity generally seen in practice).

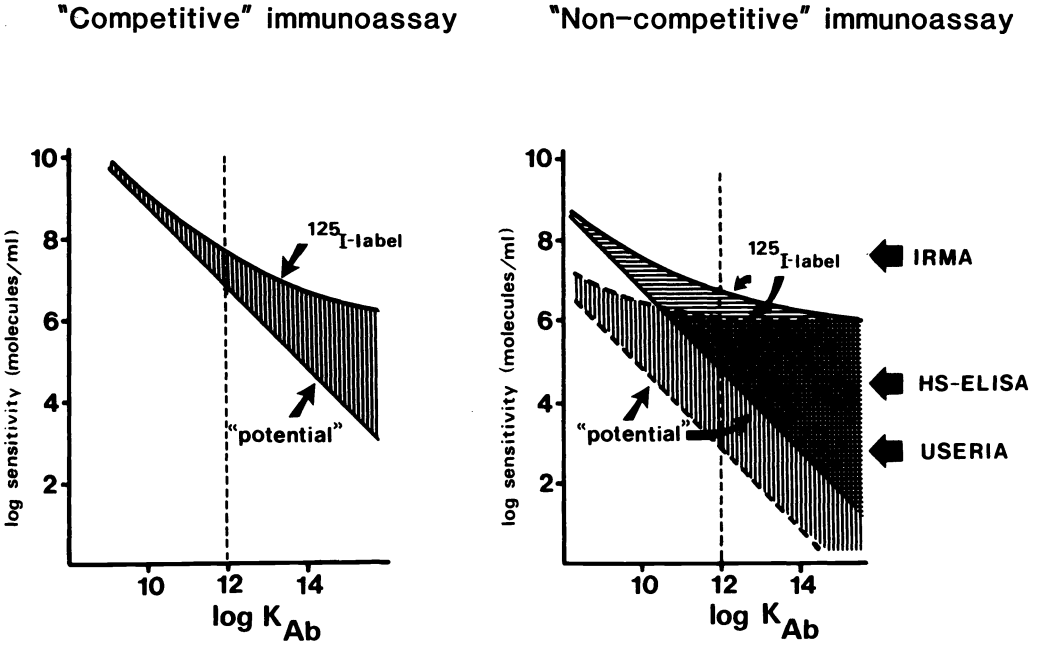

Fig. 5. "Potential" (or "maximal") sensitivities of competitive and non-competitive immunoassay systems as a function of the antibody equilibrium constant (K). The value for ε assumed for competitive systems = 0.1 (1%); values for k of 0.01 (1%) (upper curve), 0.0001 (0.01%) (lower curve) have been assumed for non-competitive systems. $\frac{\sigma_n}{n.s.b.}$ = 0.01 (1%) in both cases.

The "I-125-label" curves represent the predicted sensitivities attainable assuming labelling of analyte or antibody with I-125 (1 atom/molecule) and 'reasonable' values for counter efficiency and counting time etc. Note that for affinity constants below 10^{12} litres/mol (the upper limit, in practice, of antibody affinity), the sensitivity loss arising from the use of I-125 is small in the case of competitive systems, but may approximate three orders of magnitude in the case of non-competitive techniques. Note also that a non-competitive IRMA may display a sensitivity 1-2 orders of magnitude greater than a competitive RIA when based on antibody of identical affinity. Arrows indicate typical assay sensitivities reported for non-competitive IRMA, and for analogous enzyme labelled antibody assays relying on fluorogenic (HS-ELISA (12)) and radioactive (USERIA (11)) substrates.

Figure 5 illustrates two "maximal sensitivity" curves for non-competitive assays based respectively on the assumption of 1% and 0.01% non-specific binding of labelled antibody. Also shown in Fig. 5 are curves relating assay sensitivity to the antibody affinity constant assuming the antibody used to be labelled with I^{125}. It is evident that in a non-competitive assay system there exists a large sensitivity loss with respect to that theoretically achievable as a result of the use of I^{125} as a label. In short, one of the important implications of the analyses summarised in Fig. 5 is that non-competitive systems are capable of yielding assay sensitivities some orders of magnitude greater than competitive assays assuming, inter alia, that (a) the fractional non-specific binding of antibody (and other "noise-creating" effects) can be reduced to a very low level, and (b) that labels displaying a much higher specific activity than I^{125} can be identified and utilised.

Though the full sensitivity potential of non-competitive immunoassay systems can only be fully exploited by using non-radioisotopic antibody markers, some advantage nevertheless derives from the adoption of this assay design even when I^{125} labels are used, as is also revealed in Fig. 5. This Figure demonstrates that - depending on the affinity constant of the antibody used (and the assumptions made regarding non-specific binding of antibody, the magnitude of pipetting and other experimental errors etc.) - it is generally advantageous to employ a non-competitive approach, the sensitivity attainable being of the order of 10 times greater (or more) than that of the corresponding competitive design using antibody of identical affinity.

Experimental support for the overall validity of the theoretical predictions summarised in Fig. 5 is provided by:

(a) the observation that no radioimmunoassay (or comparable competitive non-isotopic immunoassay system) has yielded a sensitivity significantly higher than 10^{-14} Moles/litre (ie approximately 10^7 molecules/ml)

(b) that (non-competitive) immunoradiometric assays using monoclonal antibodies appear to yield sensitivities up to an order of magnitude greater than corresponding (competitive) radioimmunoassays based on the same antibody

(c) the use of labels displaying much higher effective specific activities than radioisotopes (e.g. in consequence of enzyme multiplication of the signal) can, in non-competitive assay designs, yield assay sensitivities extending to below 10^3 molecules/ml.

OTHER ADVANTAGES OF NON-COMPETITIVE IMMUNOASSAY DESIGNS AND/OR THE USE OF LABELLED ANTIBODIES

Detailed theoretical analysis of the design and sensitivity constraints characterising non-competitive and competitive immunoassay systems - summarised in the preceeding discussion - reveals that sensitivity improvements of several orders of magnitude are potentially achievable by the adoption of non-competitive immunoassay strategies. Other advantages which stem particularly from the use of labelled antibodies per se (regardless of assay design) derive from:

(a) their greater stability;

(b) the possibility of using "universal" labelled reagents (i.e. labelled anti-IgG);

(c) the avoidance of the technical problems occasionally involved in the preparation of labelled analyte tracers.

Further advantages deriving from non-competitive assay designs include:

(a) a shortening of assay incubation times in consequence of the permissible use of relatively high concentrations of antibody;

(b) a reduced dependence upon errors in the pipetting of reagents;

(c) a reduced dependence upon any minor variations on antibody binding affinity arising from inter sample variability of incubation milieux;

(d) the possibility of using dual antibody two-site (or "sandwich") systems.

In summary, the use of labelled antibodies in non-competitive immunoassay systems can be predicted to lead to assays of increased speed, sensitivity, specificity (by reliance on "two-site" assay designs), ruggedness and overall reliability. The main disadvantages of this approach are (a) the need to isolate and label relatively pure labelled antibodies, (b) the high relative consumption of antibody, (c) some increase (in certain protocols) in the number of incubation steps required. Some, at least, of these disadvantages have been obviated by the advent of monoclonal antibody production methods.

In order to fully exploit the advantages of non-competitive assay designs, however, it is clearly necessary to identify labels of higher specific activity than that of commonly used radioisotopes.

ALTERNATIVE HIGH SPECIFIC ACTIVITY LABELS

Table 1 summarises the relative specific activities of some different classes of label which have been utilised for immunoassay purposes. The conversion of many molecules of substrate by a single enzyme molecule implies an amplification of the specific activity of enzyme-labelled antibody molecules assuming high sensitivity of detection of the reaction product. Examples of high sensitivity non-

TABLE I. General indication of relative specific activities of commonly used labels. Note the low specific activity of I-125. Note also that an enzyme label, by 'amplifying' the number of detectable events (e.g. using a radioactive substrate) may greatly enhance the effective specific activity of the label and hence the sensitivity of non-competitive assay systems.

Specific activity of I-125
1 detectable event/sec/7.5 x 10^6 labelled molecules
Specific activity of enzyme label
determined by enzyme "amplification factor" and detectability of reaction product
Specific activity of chemiluminescent label
1 detectable event/labelled molecule
Specific activity of fluorescent label
many detectable events/labelled molecule

competitive enzyme-labelled antibody techniques which have exploited this phenomenon are the USERIA technique of Harris et al (8) and the method of Shalev et al (9), relying on radioactive and fluorogenic substrates respectively (see Fig. 5). Chemiluminescent labels - in spite of quantum efficiencies generally considerably lower than 100% - are also, in principle, capable of yielding higher specific activities than radioactive isotopes, and hence higher (non-competitive) immunoassay sensitivities. (A recent example of such an assay which may have far-reaching consequences in relation to the routine diagnosis of thyroid disease is the high-sensitivity measurement of thyroid-stimulating hormone (TSH) which permits the measurement of the sub-normal levels of this hormone which are seen in hyperthyroidism (10).)

Fluorescent labels are potentially capable of yielding very high specific activities since each labelled molecule may be induced to yield many photons in response to exposure to a high energy light input. Fluorescent markers also possess a number of other attractive features, including the possibility of confirmatory measurements on the same sample, and the facility they offer for observation of their spatial distribution on a solid surface (thus permitting the ready development of multiple immunoassays on the same sample as discussed briefly below). The principal problems associated with conventional fluorescent measurements are the background fluorescence generated by many biological substances, plastics, solvents etc. and the bleaching effects deriving from continuous exposure of the fluorophore to high intensity light. These effects conspire to limit the assay sensitivities which are attainable by the use of such methods. However, these disadvantages may be largely obviated by recourse to pulsed-light, time-resolving techniques as discussed in the following section.

PULSED-LIGHT, TIME-RESOLUTION FLUORESCENT MEASUREMENT TECHNIQUES

The possibility of employing pulsed-light time-resolution fluorometric immunoassay techniques was first considered in our own laboratory following discussions with J.F. Tait in 1970 who, at this time, proposed the development of a pulsed-laser, time-resolving fluorescent microscope for use in cytological studies. However, because of the cost of the equipment required, studies on the development of such a technique could not be pursued. Subsequently, discussions with Dr. E. Soini of LKB-Wallac Oy in the mid-1970s revealed that the rare earth chelate fluorophores offered an opportunity for the development of pulsed-light, fluorescent techniques employing time-resolving fluorometers of sufficient cheapness to fall within the range of small clinical chemistry laboratories. Following development of a prototype instrument by LKB-Wallac, a basic methodology for the attachment of europium chelates to antibodies, and a convenient technique for the measurement of the fluorescent signals were developed collaboratively between our own laboratory and LKB-Wallac Oy which have provided the basis for the subsequent development of a range of pulsed-light time-resolved fluoroimmunoassays.

The basic method exploits the nature of fluorescence. When a fluorophore is excited by pulsed radiation, fluorescence is emitted following each incident pulse with an intensity which decreases exponentially with time in a characteristic manner (11). Moreover, as shown in Fig. 6, an electronically "gated" detection system may be used to accumulate photons emitted over any selected time interval immediately following extinction of the incident light source. Such a system may, in principle, be employed to identify the fluorescent signals emitted by fluorophores characterised by different decay

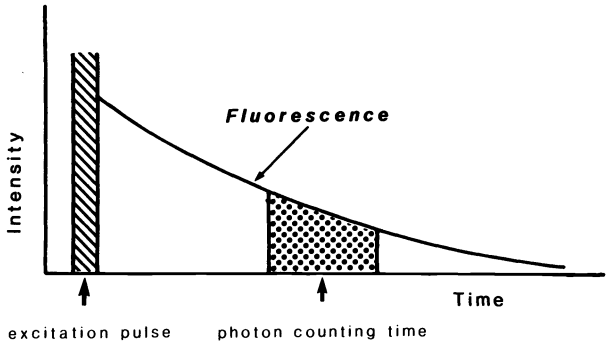

Fig. 6. Following an excitation pulse, fluorescent photons may be counted over any predetermined counting time.

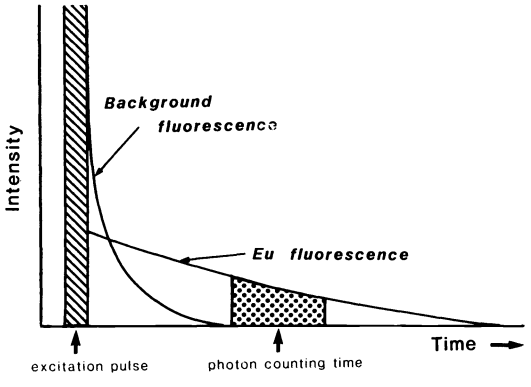

Fig. 7. Short-lived background fluorescence is allowed to fall to an insignificant level before counting of the long-lived fluorescence from the fluorophore of interest.

times in a manner which is closely analogous to the approach which has often been employed to resolve mixtures of radioisotopes on the basis of their differing half-lives (12,13). However, the simplest and most satisfactory situation clearly exists when a sample contains two fluorophores, one of which displays a fluorescence decay time very much longer than the other. In these circumstances it is possible to measure the signal originating from the fluorophore displaying the longer decay time by permitting the more rapidly decaying fluorescence to die away to an insignificant level before commencing photon measurement (see Fig. 7). This simple principle of "time resolution" can be exploited to reduce or effectively eliminate background fluorescence if this is characterised by a much shorter lifetime than that of the fluorophore of interest as shown in Fig. 7.

The fluorescence associated with serum proteins and many other common organic substances is characterised by lifetimes of the order of 10 nanoseconds (the fluorescence lifetime being defined as the time required for the fluorescence emission to decay to $\frac{1}{e}$ of its initial intensity following excitation). The lifetimes of some of the fluorophores which are commonly employed in immunochemistry are listed in Table 2. Clearly the simple time resolution method illustrated in Fig. 7 cannot readily be exploited to distinguish between background fluorescence and that characterising many of the fluorophores, such

TABLE 2. Fluorescence lifetimes of some common fluorophores.

	Fluorescence decay time
Nonspecific background	10 ns
Fluorescein isothyiocyanate (FITC)	4.5 ns
Dansyl chloride (DNS-Cl)	14 ns
N-3-pyrene maleimide (NPM)	100 ns
Rare Earth Chelates	1 μs-ms

as FITC or DNS-CL, listed in Table 2; indeed even in the case of NPM (which possesses a lifetime of 100 nanoseconds) a significant contribution might be expected from background sources if a substantial proportion of the specific fluorescence deriving from the fluorophore per se were to be encompassed within the selected measurement interval of the detection system. In contrast, the fluorescent lanthanide chelates listed in the Table form a relatively unique group of fluorophores because of their much extended fluorescence decay times, which are some 3-4 orders of magnitude longer than those characterising most background fluorescence. The prolonged decay times of these compounds essentially stem from the delays incurred in the internal transfer processes whereby light energy

absorbed by the organic moiety is conveyed to the chelated rare earth atoms which constitute the essential source of the emitted fluorescent photons. Meanwhile, a second useful characteristic of the lanthanide chelates is their large Stokes shift - that is the difference in the wave lengths of the fluorescent and (optimal) exciting radiations. For example, europium emits fluorescence in a narrow band of wavelengths at around 613 nm; maximal excitation of the chelate occurs using incident light of a wavelength of 340, implying a shift of approx 270nm. The combination of a large Stokes shift and an extended fluorescence decay time thus provides the basis for the construction of relatively simple and inexpensive pulsed light, time-resolving fluorometers capable of yielding exceptionally high signal/noise ratios which are the essential prerequisite of high sensitivity fluorometric measurements.

Our main experience relates to europium-(III) chelates although most of the remarks we make here apply to terbium (III). When europium (III) is chelated to certain β-diketones it forms a highly fluroescent product with the long lifetimes and large Stokes shift referred to earlier. The effective fluorophore is the tris chelate (see Fig. 8). When the chelation of the metal ion is completed with a suitable Lewis base - usually referred to as a synergist (Fig. 9) it has increased quantum yield and is a stable fluorophore in aqueous systems.

We have not been able to produce practical immunoassays based on antibodies directly labelled with fluorophores of the kind shown in Fig. 9. We achieve an equivalent end indirectly. The antibody is covalently coupled with a suitable bifunctional chelating reagent which strongly chelates the metal ion. The only considerations at this stage are that the labelled antibody should retain its essential immunochemical properties and the metal ion be kinetically inert throughout storage and during immunoreaction. Following reaction with antigen the labelled antibody is exposed to an appropriately formulated β-diketone "cocktail" which results in transference of the metal ion into the highly fluorescent chelate (Fig. 9) (12).

Though this clearly entails an additional operation, the elution of europium from antibody into solution occurs in a few seconds or minutes, and necessitates minimal sample manipulation (i.e. the addition of a small volume of the chelating "cocktail" to each antibody-containing sample tube). (Amongst other advantages, this procedure reduces inter sample variation in the measurement of the fluorescent signal using currently available fluorometric equipment.) A number of assays based on this approach have recently been described (e.g. 15, 16, 17).

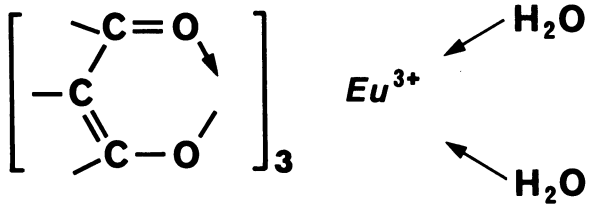

Fig. 8. Europium ions chelated to β-diketone make good fluorophores with long life-times suitable for simple time-resolved fluorescence measurements. The fluorescence is however quenched by solvent molecules.

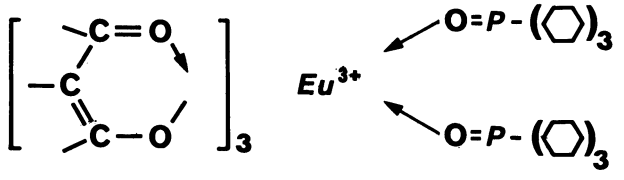

Fig. 9. When Eu-β-diketone fluorophores are completed with suitable Lewis bases, such as tri-octyl phosphine oxide (TOPO) they become environmentally insensitive even in aqueous media.

Assuming reagents become available which enable antibodies and other molecules to be labelled with highly fluorescent chelates of the kind shown in Figs. 8 and 9, a number of important new developments will be made possible, for example, high sensitivity homogenous assays. Indeed, the feasibility of these has already been demonstrated by Dr. Miller and his colleagues (18) although present techniques possess low sensitivity.

Thus present developments in this field almost certainly represent merely the first stage in the evolution of new immunoassay technologies which are likely ultimately to revolutionise the entire field of microanalysis. For example, another possibility offered by fluorescent techniques is that of the simultaneous measurement of many analytes in the same sample by the introduction of a small probe on which is deposited an appropriate array of antibody spots (each of differing specificity) (Fig. 10). Subsequent exposure of the antibody array to a mixture of fluorescent antibodies, followed by scanning

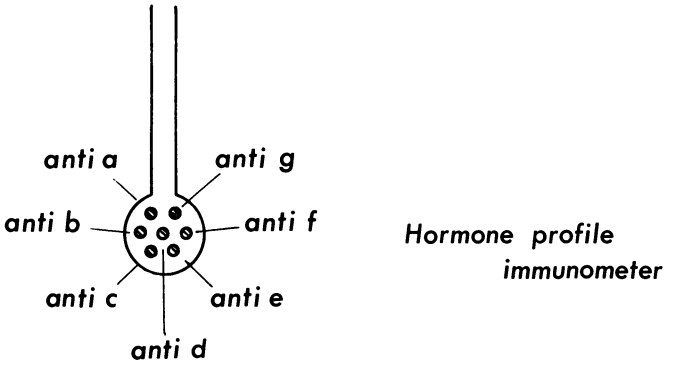

Fig. 10. Proposed "hormone profile immunometer": a small plastic surface on which an array of antibody spots of different specificities is deposited. Measurement of the fluorescence emitted from each spot permits simultaneous analysis of a complete spectrum of different analytes.

of the distribution of fluorescence using a finely focussed pulsed-light beam, will yield a complete analysis of the analyte composition of the sample. Such an approach could be employed, for example, to obtain a complete hormone profile, or to distinguish particular viral or bacterial antigens etc. Obviously the development of a multiple analyte assay system of this kind cannot be readily accomplished using either radioactive markers or, indeed, many of the other non-isotopic labels (e.g. chemiluminescent and enzyme labels) whose reactions cannot be spatially resolved in the manner that fluorescent techniques make possible.

This possibility is one of several reasons for our own particular interest in fluorescence techniques, and for our original decision to pursue, in collaboration with LKB-Wallac, this new form of technology. However there is no doubt that chemiluminescent and enzyme labels also possess many very attractive features, and that immunoassay techniques based on their use stand a very good chance of featuring prominently in the armamentarium of the clincal chemist in the next few decades.

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