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Multi-analyte immunoassay*

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Abstract: Immunoassays rely on the molecular recognition properties possessed by antibodies to measure substances defined by a particular structure. They can therefore be defined as "structurally specific", as distinct from "functionally specific" assays, e.g. bioassays, which compare the biological effects of substances which are functionally similar, but which may differ in molecular structure.

Within the broad class of "immunoassays", two subclasses may be distinguished, differing in their design. These may be described as "competitive" and "non-competitive", respectively, reflecting their dependence on the use of optimal concentrations of antibody which are either very small or very large. It is demonstrable that "non-competitive" assays are those relying on measurement of occupied antibody binding sites following reaction with analyte; conversely "competitive" assays rely on measurement of unoccupied sites. In certain assay designs, it may be shown that fractional antibody binding site occupancy is independent of (a) antibody concentration, and (b) sample volume. Such assays may be termed "ambient analyte immunoassays". This concept has been exploited in the development of free hormone and drug assays, and currently underlines the development of salivary "dip-stick" assays in the Author's Department. The concept is also being exploited in the development of "multi-analyte" immunoassay systems, enabling the simultaneous measurement of tens or even hundreds of substances simultaneously in the same small sample. These systems depend on measurement of fractional antibody occupancy using two different labels: one labeling the "sensing" antibody, the second labeling a "developing antibody", selected to react either with occupied or unoccupied sites on the "sensing" antibody. The ratio of signals emitted by the two labeled antibodies reveals the analyte concentration to which the sensing antibody has been exposed. An array of sensing antibodies, each labeled with the same fluorescent label, is scanned (by a laser), and the fluorescent signal ratio emitted from each discrete antibody couplet in the array measured. Multi-analyte immunoassay systems of this kind are likely to totally transform medical diagnosis in the foreseeable future and are also likely to be of value in the analysis of complex protein mixtures deriving from recombinant DNA technologies.

Keywords: *Ratiometric immunoassay; fluorescence immunoassay; time-resolved fluorescence; functionally specific assay; structurally specific assay; ambient analyte immunoassay.*

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Introduction

The term "immunoassay" is used to describe the general technique wherein the substance of which the concentration in a biological fluid is to be measured (the analyte) is caused to react with a specific antibody directed against it, the analyte concentration being deduced by observation of the products of the binding reaction between them. Clearly its distinguishing feature is the use of an antibody as the essential "analytical reagent"; however, many immunoassay methodologies are very closely comparable, both in practice and principle, to other forms of "binding assay" in which other classes of specific binding protein (serum binding proteins, hormone "receptors", enzymes, etc.) are substituted for antibodies as the key analytical reagent in the system. This point is of particular importance in the context of the present Symposium since it becomes apparent, on close examination of the range of methodologies currently available for the measurement of biomolecules, that there are no clear dividing lines between them. For example, so-called "bioassays" rely on the observed interaction between the analyte and a biological "system", the latter falling within a spectrum ranging from whole animal to isolated organ, cell or cell receptor and which are primarily distinguished by the range and degree of complexity of the different receptor systems involved. An antibody simply comprises a specialised form of receptor molecule; thus it is scarcely surprising that there is little to distinguish many immunoassays from assays in which receptor replaces antibody as binding reagent. Likewise, certain immunoassays involve the use of antibodies labeled, for example, with a radioisotope, enzyme, fluorescent or chemiluminescent "marker". Such assays fall within the class of "labeled reagent" assays, originally exploited for the measurement of steroid and thyroid hormones using radioisotopically labeled organic reagents such as tritiated acetic anhydride [1], and to which the labeled antibody assays are evidently closely similar. In short, the distinction between immunoassay methods and other methods such as "bioassay" and "chemical assay" is frequently not one of analytical principle or concept, but centres solely on the special properties of antibodies as analytical reagents.

The particular usefulness of antibodies in this context principally derives from three characteristics (a) their high "structural specificity", i.e. their ability to recognise, and to bind to molecules of a particular molecular structure; (b) the relative ease with which antibodies of a defined structural specificity can be produced, originally by conventional immunisation procedures; more recently by *in vitro* hybridisation and selection techniques; (c) their generally high binding affinities, which implies that they can be employed at low concentration to measure analytes likewise at low concentration, implying very high assay sensitivity.

These properties form the basis of the ubiquitous use of immunoassay procedures throughout biomedical science for the measurement of substances of biological importance.

Of greater conceptual significance is the distinction between "functionally specific" and "structurally specific" assays. These terms are intended to convey the notion that two entirely different forms of analytical measurement are common in biology and medicine, i.e. those measurements where the objective is to assess the *effects* of different substances, or mixtures of different substances, on a biological system, and those measurements designed to estimate the *amount*, i.e. the number of molecules, in a sample of a single substance characterised by a unique molecular structure. Though this distinction superficially appears clear cut, it is blurred in practice by the fact that many

biological substances are (initially at least) defined in terms of their presumed function, or observed biological effect, rather than in terms of their molecular structure. Thus, until such time as its exact chemical identity is established, the "measurement" or "assay" of such a substance must principally rely on the expression, in a suitable biological system, of the characteristic biological effect by which it is defined. This inevitably leads to the identification of the "amount" of the substance with one of the biological effects that it causes — an identification which is reflected in the expression of the amounts of many such substances in units of biological activity rather than of mass. Indeed, so strong is the identification of the biological effects of a substance with its amount, that definitions of international units frequently use such phraseology as "the activity contained in an ampoule . . .", although the notion that an effect can be contained in an ampoule is intrinsically absurd. Considerable confusion derives from this blurring of the distinction between the differing objectives underlying the two forms of measurement applied to biological substances; thus, although this issue has been discussed elsewhere, e.g. ref. 2, it is useful to summarise the distinction between structurally specific and functionally specific assays as a prelude to briefly reviewing current developments in the immunoassay field.

Functionally specific and structurally specific assays

The distinction between these two forms of assay was originally perceived and discussed some 40 years ago by authors such as Jerne and Wood [3] and Gaddum [4], who introduced the terms "comparative assay" and "analytical assay" respectively to describe them. The results of comparative assays can be expressed only in terms of arbitrarily defined units of the activity, or biological effect, under observation; moreover such units possess the dimensions of the particular effect observed, and cannot legitimately be employed as units of concentration. A comparison of the *activities* displayed by two samples can only numerically coincide with measurements of the *amount* or concentration of the active substance(s) they contain the same, molecularly homogenous, substance is implicated in each case. Indeed, the notion of an amount or concentration of a mixture of substances is meaningless. Furthermore, a comparative assay is valid simply if the samples compared behave identically within the system (differing only in the relative magnitude of the effects they exert); this implies that it is both necessary and sufficient that the relative potencies of the two remain constant for all minor changes in experimental conditions, e.g. changes in incubation time, temperature or pH, and for all values of the response variable, i.e. the dilution curves yielded by the two samples should be parallel. In contrast, results of analytical assays are expressed in units representative of molecular number; furthermore they are valid only if the substances present in standard and unknown samples are structurally identical. Although this necessarily implies that the substances must likewise display identical behaviour within the assay, e.g. parallelism of dilution curves etc., this is not generally a sufficient criterion of structural identity between them, and other evidence of their structural identity, e.g. similarity of chromatographic behaviour, must also be sought.

It should be particularly noted that the results of a comparison between two sample preparations in one comparative, or "functionally specific" assay system are unlikely to be identical to those yielded in another such system. For example, results of the comparison of two molecularly heterogenous preparations in an assay based on intact

animals are highly unlikely to coincide with those obtained in an assay relying on isolated cell surface receptors. Indeed the notion that an individual comparative assay is capable of measuring some general property described as the "biological activity" of a substance is false, and the assignation of biological activity units to the substance is largely illusory. Nor can comparative assays be "standardised" by the common use of an international reference preparation. In contrast, the results of all valid analytical, structurally specific, assays of a substance must necessarily coincide.

It has occasionally been claimed that immunoassays are "comparative assays", presumably on the grounds that such assays almost invariably involve comparison of an unknown sample with a set of standards. This is not so. An immunoassay is intended to measure the amount, or concentration, in a sample of a single substance characterised by a single molecular structure, and should yield the same result as any other analytical assay of that substance. Failure to do so implies that the immunoassay is analytically invalid, because, for example, it is responsive to a mixture of substances in the sample, not that it falls within the category of assays intended to compare the effects of different substances, or mixtures of substances, on a biological system. A comparative assay must not, in short, be confused with an invalid analytical assay.

Current developments in immunoassay methodology

Space does not permit a comprehensive review of current developments in the immunoassay field. These largely centre on the replacement of isotopic by non-isotopic labels, for reasons which may be grouped under four headings: (a) environmental, logistic, economic, practicality and convenience, i.e. "non-scientific"; (b) the attainment of higher sensitivity; (c) the development of "immunosensors" and "immunoprobes"; (d) the development of "multi-analyte" assay systems. In this presentation only two of these will be briefly examined.

The attainment of higher assay sensitivity

One of the most compelling reasons for the current move away from isotopically based immunoassay methodologies derives from the need to develop techniques of greater sensitivity. Radioisotopic methods are, in practice, limited to the measurement of analyte concentrations above about 10^8–10^9 molecules/ml, i.e. approximately 0.15–1.5 $pmol^{-1}$ [5]. However, in certain fields, e.g. virology, tumour detection, etc., there is an increasing requirement to detect and measure molecular concentrations below this level. The factors which determine immunoassay sensitivity have been extensively discussed [5–8], and need not be reviewed in detail here. Nevertheless, the principal concepts involved may be summarised as follows.

Principle concepts. (a) Immunoassays relying on reagent "labels" may be subdivided into two classes: (i) those relying on labeled "analyte", and observation of the labeled analyte distribution between antibody bound and unbound moieties, e.g. radioimmunoassay (RIA); and (ii) those relying on labeled antibody, and analogous observation of the labeled antibody distribution between analyte bound and unbound moieties, e.g. immunoradiometric assay (IRMA); (b) although labeled antibody methods have frequently been claimed, or assumed, to be intrinsically more sensitive than those relying on labeled analyte, this proposition is, in a general sense, untrue and the reasons commonly advanced in its support are invalid; (c) immunoassays may also be

conveniently subdivided into "limited reagent" and "excess reagent" methods, depending on whether the optimal concentration of antibody required to maximise assay sensitivity tends towards zero or infinity, respectively. (Note that the optimal antibody concentration is essentially determined by the "error structure" of the assay system, i.e. the relationship between the precision of the measurement and the various parameters governing the design of the system. If an assay is to be totally error free, no antibody concentration would be optimal, and the distinction between "limited" and "excess" reagent systems would be neither relevant nor valid.) Limited reagent systems are frequently described as "competitive"; conversely, excess reagent systems are often said to be "non-competitive". The reasons underlying this choice of nomenclature are diverse; they generally reflect the notion that, in a typical RIA, labeled and unlabeled analyte molecules "compete" with each other for a limited number of antibody binding sites. However, the term "competitive" is also applied to certain labeled antibody methods (where such a concept is inappropriate), but which are likewise distinguished by the optimal use of relatively small amounts of antibody, and which are more correctly described as of "limited reagent" design. Moreover, immunoassays in which no label of any kind is involved can also, for the same fundamental reasons, be subdivided into those of "limited reagent" (or "competitive") and "excess reagent" (or "non-competitive") design. In short, the classification of immunoassays into these two categories is unrelated to the nature of the particular reactant labeled, or indeed, whether or not a label is used. It rests entirely on the concentration of antibody which may be demonstrated, on the basis of statistical considerations, to be necessary to maximise the precision of measurement of the target analyte concentration; (d) nevertheless, in practice, all current "labeled analyte" methods are of "limited reagent" (or "competitive") design. Labeled antibody methods are "competitive" when the signal emitted by antibody binding sites unoccupied by analyte is alone detected and measured. Conversely when the signal emitted by antibody binding sites occupied by analyte is measured, the assay is optimally of "excess reagent" (or "non-competitive") design. Indeed, as discussed below, the distinction between methods involving the measurement of unoccupied and occupied antibody binding sites respectively, lies at the root of the sub-classification of immunoassays as competitive and non-competitive; (e) theoretical analysis shows that, making reasonable assumptions relating to the random errors arising in immunoassay procedures, and assuming the use of high affinity antibodies, i.e. approximately 10^{11}–10^{12} l M^{-1}, sensitivities yielded by radioisotopically based techniques, whether relying on labeled antibody (IRMA) or labeled analyte (RIA), or whether of competitive or non-competitive design, are all of closely comparable sensitivity, i.e. in the order of 10^7 molecules ml^{-1} (see Fig. 1). However, it may also be shown that, in the case of the non-competitive methods, a critical constraint on assay sensitivity is the specific activity of the label used. This constraint does not apply, in practice, to competitive assays relying on radioisotopic labels, whose sensitivity is limited by other factors. The significance of this conclusion is that, only by the use of labels possessing specific activities higher than those of the commonly used radioisotopes in assays of non-competitive design, can current sensitivity limits be breached. Conversely, the use of a higher specific activity label in a competitive assay would have no significant effect on its sensitivity; (f) non-competitive designs also display a number of other advantages deriving from the relatively high antibody concentrations on which they generally rely. These include increased reaction speeds, and hence shorter incubation times, decreased vulnerability to environmental effects, causing variations in binding

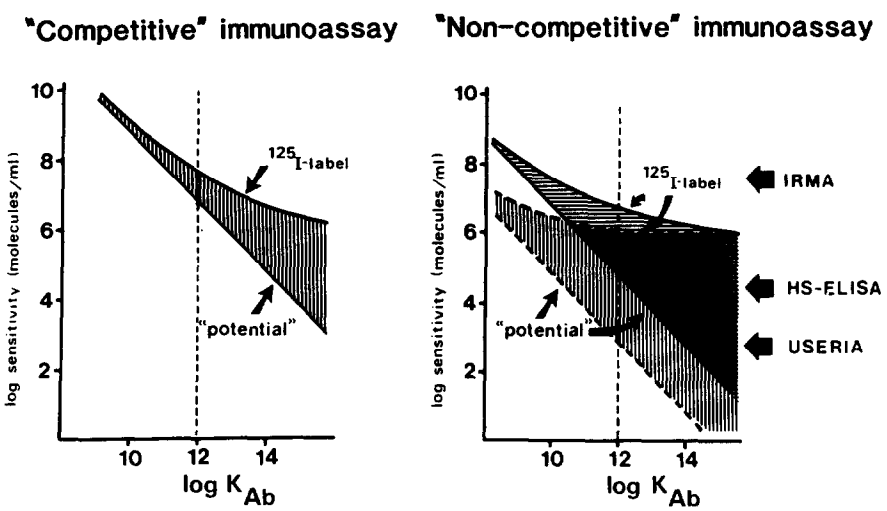

Figure 1
Curves showing the theoretically predicted relationship between antibody affinity and the sensitivities achievable using "competitive" and "non-competitive" assay strategies. The "potential" sensitivity curves assume the use of infinite specific activity labels; the sensitivities achievable using ^{125}I-labeled antigen or antibody are also shown. Shaded areas indicate the sensitivity loss due to errors in measurement of the label. Curves relating to "competitive" assays assume a 1% error in measurement of the response variable arising from "experimental" errors, i.e. errors other than those inherent in label measurement *per se*. Non-competitive curves assume "non-specific binding" of labeled antibody of 0.01% and 1% (lower and upper curves) respectively. Arrows indicate sensitivities claimed for typical non-competitive immunoassay methodologies.

affinity between antibody and analyte, lesser dependence on high antibody binding affinity, etc. Conversely, non-competitive assays are intrinsically less specific, since the structural specificity of an antibody varies depending on the relative concentrations of antibody and analyte in the system — a disadvantage which generally necessitates the inclusion, whenever possible, of an "immunoextraction" procedure, as exemplified in so-called "two-site" or "sandwich" assays. (Note: the loss of specificity inherent in non-competitive assay designs implies that they are less readily applicable to the measurement of analytes of small molecular size, which cannot be simultaneously bound by two different antibodies directed against different antigenic sites on the molecule. Such analytes are generally more appropriately measured using competitive assay methodologies, combined, if necessary with a preliminary chromatographic separation procedure.) These fundamental concepts have been set out in some detail because they lead to important new insights into immunoassay design which are relevant to assays in which (as indicated above) no label is used. Figure 2 summarises some of these insights and illustrates the following principles.

Immunoassay design principles

(a) All immunoassays fundamentally rely on observation of the "occupancy" of "specific" antibody binding sites by analyte following reaction between analyte and antibody.

(b) "Non-competitive" assays may, in practice, be defined as those in which the assay relies on observation of an observable "signal" emitted by *occupied* antibody binding sites.

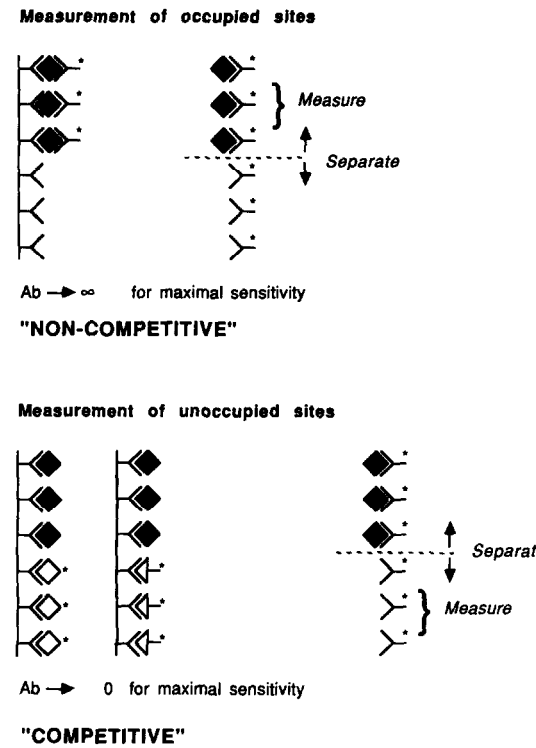

Figure 2
The distinction between "non-competitive" (above) and "competitive" immunoassays (below) reflects how antibody binding site occupancy is measured. Labeled antibody methods are "non-competitive" if occupied sites of the (labeled) antibody are measured, but are "competitive" (below right) when unoccupied sites are measured. Labeled antigen (below left) or labeled anti-idiotypic antibody methods (below centre) rely on measurement of sites unoccupied by analyte, and are therefore invariably of "competitive" design.

(c) "Competitive" assays rely on observation of the signal emitted by *unoccupied* antibody binding sites.

Moreover, the development of "ultra-sensitive" immunoassay systems depends on adherence to the following design strategies: (a) reliance on "excess reagent" or "non-competitive" assay designs; (b) the use of non-isotopic labels displaying higher specific activities than commonly used radioisotopes; (c) the development of efficient separation systems (ensuring minimisation of reaction-product "misclassification", and hence of signal "backgrounds") and (d) reliance on dual or multi-antibody analyte recognition systems (exemplified by "sandwich" or "two-site" assays) to maintain/increase structural specificity.

These concepts have formed the basis of immunoassay development in the Author's laboratory since the early to mid-1970s. Of the candidate labels for use in this context, the use of fluorescent labels combined with sophisticated time-resolution techniques for their detection (a concept arising from discussions with J. F. Tait in 1970) appeared specially attractive for a number of reasons, including the possibility they offered of providing the enhanced specific activities and high signal-to-noise ratios required for ultra-sensitive immunoassay, as indicated above. They also permit the development of "multi-analyte" assay systems as described below.

Time-resolved fluorescence immunoassay

In pursuance of these ideas, collaboration with LKB/Wallac was initiated *ca* 1976–1977 to develop the instrumentation and technology required to develop such methods. Fortunately a group of fluorescent substances generally known as the lanthanide chelates (including, in particular, the chelates of europium and terbium) facilitate such development, possessing prolonged fluorescence decay time (approximately 10–1000 μs), large Stokes shift (approximately 290 nm) and other desirable physical characteristics which permit the construction of relatively cheap instrumentation for their measurement [9, 10]. The fluorescent properties of the lanthanide chelates may be compared with those of a conventional fluorophor such as fluorescein which is characterised by a much smaller Stokes shift (approximately 28 nm), and a fluorescent decay time and emission spectrum which do not permit it to be readily distinguished from fluorescent substances present in blood (such as bilirubin) or in plastic sample holders. The unique fluorescence characteristics of the lanthanide chelates thus permit them to be measured in the presence of a fluorescence background (deriving from extraneous sources) which, in practice, approaches zero. Figure 3 illustrates the concepts involved in pulsed-light, time-resolved, fluorescence measurement, which form the basis of the Dissociation Enhancement Lanthanide Fluoro-Immunoassay system (DELFIA) distributed by LKB/Wallac.

Although it is inappropriate to pursue this subject in greater detail, attention should also be drawn to the possibilities offered by phase-resolved fluorometry. This permits separate identification of fluorophores differing in fluorescence lifetime by their exposure to a sinusoidally modulated excitation source, and observation of their demodulated, phase-shifted, light emission [11]. This technique offers the possibility both of the development of homogenous assays, relying on a difference in fluorescence decay time of bound and free forms of the fluorescent-labeled molecule, and of discriminating between two labeled antibodies in the context of multi-analyte "ratiometric" immunoassay, as discussed below.

The fundamental immunoassay design concepts discussed above have also subsequently been exploited by a number of other manufacturers using different "high specific activity" labels in "non-competitive", "two-site", assay designs. Such labels

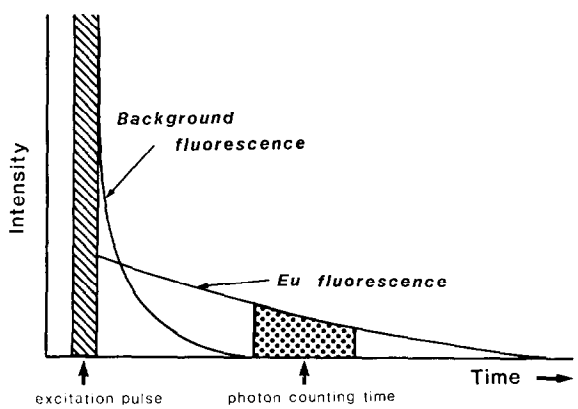

Figure 3
Basic principles of pulsed-light, time-resolved fluorescence. Fluorescence emitted by the fluorophor (typically a europium chelate), is distinguished from background fluorescence, which decays more rapidly.

include enzymes, which can be used as the basis of enzyme amplification systems of the kind developed by IQ Bio, Cambridge, UK [12], or as catalysts of chemiluminescent reactions (as developed by Whitehead *et al.* [13] and marketed by Amersham International, Amersham, UK) or reactions yielding fluorescent [14] or radioactive [15] products. They also include chemiluminescent markers such as the acridinium esters developed by McCapra *et al.* [16] and utilised by Woodhead and his colleagues [17], now marketed by Ciba-Corning Diagnostics Corporation, MA, USA. Common to all "ultra-sensitive" immunoassay methodologies relying on such alternative labels is their dependence on a non-competitive, labeled antibody, assay strategy whenever appropriate; however, for the reasons indicated above, competitive methods continue to be generally employed for the measurement of analytes of small molecular size, e.g. therapeutic drugs, steroid and thyroid hormones, etc. Nevertheless the convenience (from a manufacturing viewpoint, and for other technical reasons) of generally relying on labeled antibodies has meant that, even in these cases, the use of labeled antibody techniques is increasingly preferred.

In vitro techniques of monoclonal antibody production

The advent of the *in vitro* hybridoma techniques of monoclonal antibody production pioneered by Köhler and Milstein [18] has also been of great importance in the present context. The ability to produce a selected antibody directed against a single antigenic site (or "epitope") in unlimited amounts and in relatively pure form obviously facilitated the exploitation of the concepts discussed above, which, up to this time, had been impeded by the technical difficulties associated with the isolation and purification of the labeled antibodies on which they depend. Though, as indicated above, methodologies relying on labeled antibodies can be of either competitive (limited reagent) or non-competitive (excess reagent) design, the particular importance of the hybridoma techniques has always primarily rested, in the Author's opinion, on the means they offer for the easier development of non-competitive "sandwich" assay methodologies. Amongst the principal reasons for this view are the following: (a) the requirement inherent in such techniques for relatively large amounts of antibody in comparison with "limited reagent" methods; and (b) the lesser dependence of non-competitive methods on the possession by the antibody (or antibodies) used of a high affinity constant.

Thus an immunoassay of equal sensitivity to a competitive method can be constructed using a non-competitive design and a labeled antibody of considerably lower affinity, the permitted affinity difference being dependent, *inter alia*, on the efficiency of the separation system employed. This conclusion is of considerable practical importance since, given the laborious nature of the techniques involved, the probability of identifying stable antibody secreting clones yielding antibodies possessing the high affinities demanded by competitive methods, and with appropriate specificity characteristics, is relatively low. For this reason, monoclonal antibodies are, in practice, generally of lower affinity than those produced by conventional *in vivo* techniques, although this disparity is likely to be progressively eroded. Thus hybridoma based monoclonal antibody production methods have never appeared to offer particular advantages in the development of competitive, labeled antigen, methods such as RIA, although there are, in principle, no reasons precluding their use in this context. Nor is it impossible that certain selected monoclonal antibodies might prove to yield improved specificity, as compared with a polyclonal mixture, when used in assays of conventional competitive

design. These reservations are not intended to deny the existence of certain logistical advantages in the use of monoclonal antibodies in competitive labeled antibody designs, such as are now increasingly relied on by manufacturers for the assay of analytes of small molecular size.

Ambient analyte immunoassay

Another important concept in the present context is that embodied in an analytical approach which may be described as "ambient analyte immunoassay" [19]. This term is intended to describe a type of immunoassay system which, unlike conventional methods, measures the analyte concentration in the medium to which the antibody is exposed, being essentially independent of sample volume. This concept is illustrated in Fig. 4, and relies on the physico-chemical proposition that, when a "vanishingly small" amount of antibody (preferably, but not essentially, coupled to a solid support) is exposed to an analyte containing medium, the resulting (fractional) occupancy of antibody binding sites reflects the ambient analyte concentration. Clearly binding of analyte by antibody depletes the amount of analyte in the surrounding medium, but provided the proportion so bound is small, i.e. less than, for example, 1% of the total, such disturbance can be ignored.

Ambient analyte immunoassay methodology exhibits some important and distinctive features. The first is that the system "response" essentially constitutes the ratio of occupied (or, alternatively, unoccupied) to total antibody binding sites. Secondly, within the limits implied above, the amount of antibody used in the system is irrelevant. For example, the introduction of 10, 100, or 1000 antibody molecules into a medium containing millions or billions of analyte molecules will result, in each case, in virtually identical fractional antibody binding site occupancy, this being determined solely by the ambient analyte concentration in the medium and the affinity constant of the antibody used (see Fig. 5).

These concepts were originally exploited in the original development of what has come to be known as "two step" free hormone immunoassay [20], but it is clear that they are of far wider application, and can, in particular, be utilised in the construction of immunosensors and immunoprobes. One such example is a probe for the measurement of salivary steroids, currently being developed and tested in the Author's Department. Comprising a small antibody coated plastic "dip-stick" comparable in size and shape to a

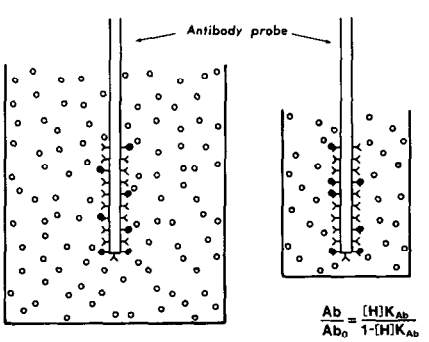

Figure 4
Basic principle of "ambient analyte" immunoassay (AAI). The fractional occupancy (F) of a vanishingly small amount of antibody (of affinity K) is determined by the analyte concentration in the medium ([An]).

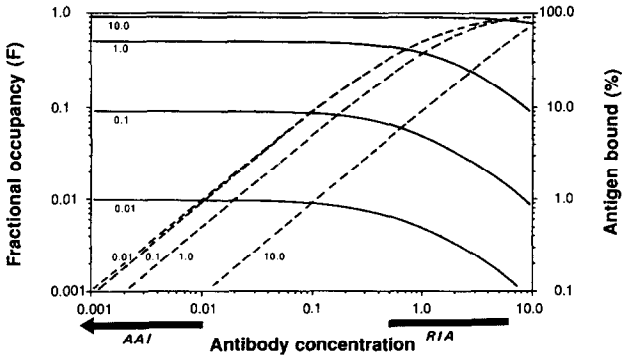

Figure 5
Fractional antibody binding site occupancy (F) plotted as a function of antibody binding site concentration for different values of analyte (antigen) concentration [An]. The percentage binding of analyte to antibody (b) is also shown. All concentrations are expressed in units of $1/K$. Note that for antibody concentrations of less than $0.01/K$ (approximately), percentage binding of analyte is <1%, and fractional binding site occupancy is essentially unaffected by variations in antibody concentration extending over several orders of magnitude, being governed solely by [An]. Note that radioimmunoassays and other "competitive" immunoassays are commonly designed using antibody concentrations approximating $0.5/K$–$1/K$ or above (implying $b_0 > 30\%$), in accordance with the precepts of Berson and Yalow, e.g. ref. 21.

clinical thermometer, this device is intended to permit the measurement of salivary steroid levels without necessitating the collection of saliva. Following exposure of such an "immunoprobe" to a test medium, it is removed, and antibody occupancy subsequently determined. This can be effected in two ways: (i) by exposure of the probe to, for example, a labeled antibody capable of reacting with occupied binding sites; or (ii) by its exposure to a labeled substance (such as labeled analyte or labeled anti-idiotypic antibody) capable of reacting with unoccupied sites. In conformity with the concepts and terminological definitions discussed above, a probe relying on measurement strategy (i) may be described as "non-competitive"; likewise a probe relying on strategy; (ii) constitutes a "competitive" probe. Differentiation between these two forms of probe is important because, *inter alia*, the particular approach adopted dictates such design features as the amount and affinity constant of the antibody coated on the probe surface.

Multi-analyte, ratiometric, immunoassay systems

Work in the Author's laboratory is currently directed towards a further objective based on the concepts discussed above, i.e. the development of a random access, multi-analyte, immunoprobe system capable of measuring, in the same small sample, any number of individual analytes from selected analyte "menus", e.g. a hormone menu, a viral antigen menu, an allergen menu etc. The possibility of measuring tens or hundreds of analytes, or molecular variants of the same functionally defined substance, in the same sample is both technically feasible and potentially of great importance. The fundamental concepts underlying the approach involved may be briefly indicated.

As discussed above, the notion of ambient analyte assay simultaneously introduces two extremely important and novel concepts: (i) that an estimate of analyte concentration can be based upon the use of an infinitesimal amount of "sampling" antibody; and (ii) that such an estimate derives from a direct measurement of the fractional antibody occupancy by the analyte, irrespective of the exact amount of antibody used. It should be

emphasised that the latter proposition is valid only in the context of ambient analyte assay, and is not true in current conventional immunoassay systems, in which fractional antibody occupancy depends both upon the amount of antibody in the system, and sample volume. In short, exposure of a small number of antibody molecules (in the form, for example, of a "microspot" located on a solid support) to an analyte-containing fluid results in an antibody binding site occupancy which reflects the analyte concentration in the medium. Following such exposure, the antibody bearing probe may be removed and exposed to a "developing" solution containing a high concentration of an appropriate second antibody directed either against a second epitope on the analyte molecule if this is large, i.e. the occupied site, or against unfilled antibody binding sites in the case of analytes of small molecular size.

Subsequently, an estimate of binding site occupancy of the "sampling" (solid-phase) antibody may be derived by measurement of the ratio of signals emitted by the two antibodies forming the dual antibody "couplets". This can be conveniently achieved by labeling each of the antibodies used with different markers; for example, a pair of radioactive, enzyme or chemiluminescent markers. Fluorescent labels are particularly useful in this context because, by the use of laser scanning techniques, they readily permit arrays of different antibody "microspots" distributed over a surface, each directed against a different analyte, to be individually examined, thus enabling multiple assays to be simultaneously carried out on the same small sample (see Fig. 6). Nevertheless, the same principles are clearly applicable using other forms of label.

It is premature to discuss here the technical details of the systems of this kind, based, for example, on laser-based "confocal" microscopy, which are currently under development; it is sufficient simply to draw attention to the possibilities they offer. Laser beams can be focussed onto very small areas, so that the number of individual assays which can be located within a multianalyte array is restricted primarily by the density with which the different sampling antibodies can be packed onto a suitable surface. Meanwhile it should be noted that, as the surface area exposed to the laser beam is reduced, the background signal deriving from the solid support, from "non-specifically bound" antibody and from other similar extraneous sources, correspondingly falls. This

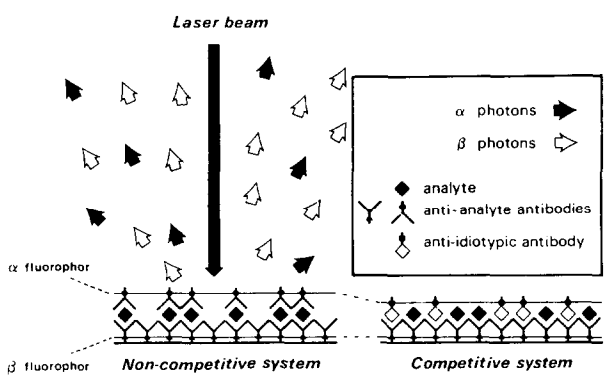

Figure 6
Basic principle of dual label, ambient analyte, immunoassay relying on fluorescent labeled antibodies. The ratio of α and β fluorescent photons emitted reflects the value of F (see Fig. 5) and is solely a function of the analyte concentration to which the probe has been exposed. The ratio is unaffected by the amount or distribution of antibody coated (as a monomolecular layer) on the probe surface.

implies that the ratio of the two fluorescence signals can be successfully measured from very small areas, as indeed, is often done in other biological applications.

Differentiation of the fluorescent signals yielded by the two fluorophores can, of course, be readily achieved using a combination of physical techniques exploiting the differences in wavelength and/or decay time of the two emissions, for example, by the use of a pulsed or sinusoidally modulated laser source, and time- or phase-resolved detectors combined with appropriate wavelength filters. This aside, the technology involved closely resembles that employed in compact disk recorders and other similar data-storage devices, the obvious difference being the light emitted from each of the discrete zones forming the antibody array is fluorescent rather than reflected, and yields chemical rather than physical information.

The development of multi-analyte assay systems of this kind can be anticipated to bring about fundamental changes in medical diagnosis and many other biologically related areas. For example, it is both conceivable and within the range of present technology that immunoprobes will be developed capable of measuring every hormone (or iso-hormonal component), together with other endocrinologically related substance within a single small sample of blood, providing data which, when analysed with the aid of computer based "expert" pattern recognition systems, will reveal endocrine deficiences only dimly perceived using current "single analyte" diagnostic procedures. Such systems also provide a possible solution to a need frequently voiced by manufacturers, i.e. the development of "random access" immunoassay methodology, permitting the selection of any desired test or combination of tests from an extensive menu. Clearly the accommodation of a large range of individual immunoassays on a small immunoprobe, comparable in its overall physical dimensions with a few drops of blood, would totally transform the logistics of immunodiagnostic testing. Yet a further possible application of great potential importance relates to situations, in medicine, in the pharmaceutical and food industries, agriculture, environmental research etc., in which samples must be screened for the presence of individual constituents from amongst a wide range of structurally distinct substances. Such a possibility is clearly of particular importance within the context of the present Symposium, in which a need is clearly evident for simple methodologies capable of analysing complex mixtures of polypeptides and proteins. Nevertheless, notwithstanding the great structural specificity of many antibodies, immunological assays can never provide conclusive proof of the structural identity of an individual substance under examination. Thus in many situations, such as in the analysis of the products of recombinant DNA techniques, such assays should be combined with other physico-chemical techniques to minimise, as far as is possible, the chance that molecularly distinct substances escape individual identification.

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