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REVIEW

Homogeneous Immunoassays

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Index Entries: Homogeneous immunoassays using spectrophotometric and fluorescence methods; cofactor and prosthetic group labels; enzyme and enzyme modulator labels; fluorescent probe and fluorogenic enzyme substrate labels; fluorescent quenching or enhancement immunoassays; fluorescence protection immunoassay; fluorescent excitation transfer immunoassay; fluorescence polarization and correlation immunoassays; immunoassay using total internal reflection spectroscopy; substrate-labeled fluorescent immunoassays; spin immunoassay.

Introduction

Specific binding assays may be categorized as either heterogeneous or homogeneous procedures, depending upon whether the assay requires a separation of antibody bound from free analyte. Homogeneous techniques do not require this step because the activity of the label is modulated by antibody binding. The difference in activity thus generated between the two forms of the label is great enough to allow one form to be measured in the presence of the other. During the past ten years there has been a number of homogeneous immunoassays reported based on a wide variety of labels. In this article we will review the principles upon which these procedures are based, concentrating more heavily on the recently reported methods.

Spectrophotometric Methods

Enzyme Labels

The homogeneous immunoassay technique known as EMIT® uses enzyme labels and is widely applied to the quantitation of biologically important substances (*1*). The enzymes most prominently used as labels in this procedure have been

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lysozyme (*2*), glucose-6-phosphate dehydrogenase (*1*), malate dehydrogenase (*3*), and β-galactosidase (*4*). Here the analyte, which is present in unknown concentration and a constant amount of a conjugate, consisting of the analyte covalently bound to one of the enzymes above, compete for a limited number of antibody binding sites. Since the amount of antibody in the assay is insufficient to bind all the analyte and conjugate, at equilibrium the conjugate is distributed between an antibody bound and a non-antibody bound (free) form. This distribution is controlled by the level of analyte from the specimen, so that the amount of conjugate in the free or bound form is related in a dose-dependent manner to the analyte. Since binding by antibody modulates the activity of the conjugate, the bound and free forms express enzyme activity to greatly different extents. This characteristic of the conjugate thereby allows one form of the conjugate to be measured in the presence of the other, and eliminates the need for separation of the two forms prior to measurement of the label. EMIT assays have been developed for drugs of abuse, therapeutic drugs, and for several hormones (*1*).

A similar approach has been used to generate homogeneous assays for staphylococcal enterotoxin B (SEB) (*5*) and human IgG (*4*). A conjugate of the enterotoxin and β-amylase was used to monitor a competitive protein binding (CPB) assay for SEB. Here, antibody binding inactivated the conjugate so that only the free form was capable of expressing enzyme activity. This activity, in turn, could be directly related to the concentration of SEB in the specimen. Likewise, antihuman IgG antibody, a conjugate of human IgG and β-galactosidase and a macromolecular substrate for the enzyme were combined to produce a homogeneous immunoassay for IgG. In this instance it was necessary to prepare a macromolecular substrate for the enzyme to ensure that the enzyme activities expressed by the bound and free forms of the conjugate would be distinguishable.

Another homogeneous technique has been developed that is based on the use of coupled enzyme reactions (*6*). Here, one enzyme, e.g., glucose 6-phosphate dehydrogenase, of a pair that reacts in sequence is covalently bound along with the analyte to a solid phase. The second enzyme, e.g., hexokinase, is then used to label an anti-analyte antibody. The solid-phase reagent and the labeled antibody are incubated with a sample containing the analyte. During the incubation period, the enzyme labeled antibody is distributed between a catalytically inactive solution phase (high molecular weight inhibitors and also scavengers may be added to preferentially reduce solution-phase enzyme activity) and a catalytically active (coupled reaction) solid phase. The distribution of the enzyme-labeled antibody between the two phases can be modulated by the level of unlabeled analyte. Thus the amount of coupled enzyme activity associated with the solid phase can be related to analyte concentration. This assay has been applied to the measurement of human IgG.

Cofactor Labels

A number of enzyme cofactor–analyte conjugates have been used to monitor homogeneous immunoassays. Nicotinamide adenine dinucleotide (NAD) has been derivatized and covalently coupled to biotin or 2,4-dinitrophenol (DNP) to form

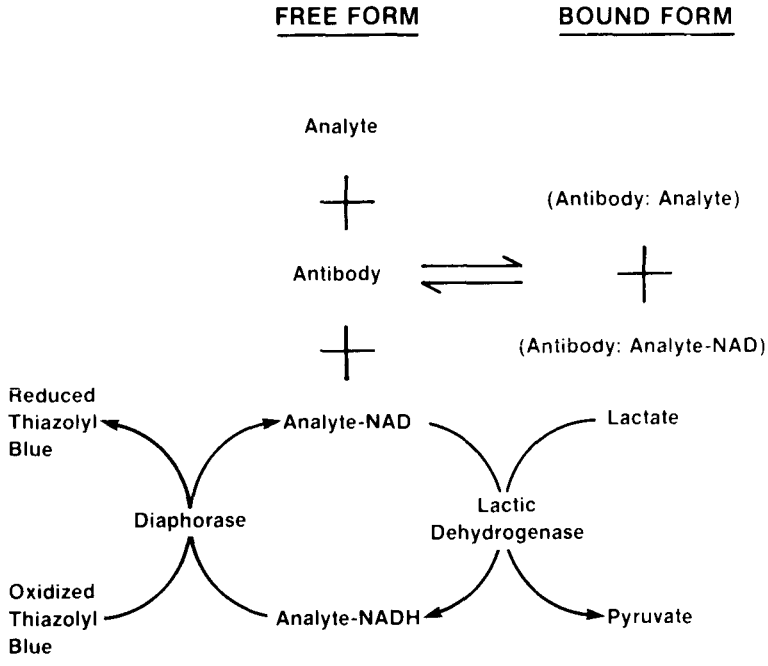

Fig. 1. Schematic illustration of a homogeneous CPB assay monitored by cofactor conjugates.

conjugates which are active in the cycling scheme given in Fig. 1 (*7*). The activity of the cofactor conjugate could be inhibited by the addition of a specific binding protein, namely avidin or antibody to DNP. Thus, as illustrated in Fig. 1, a homogeneous assay was assembled in which the analyte–NAD conjugate is distributed between the bound and free forms in accord with the principles of CPB analysis. The level of conjugate in the free form is, of course, dictated by the amount of analyte in the specimen and can be determined by the activity it expresses in the cycling reaction given in Fig. 1. Again since the bound and free forms of the conjugate express significantly different levels of activity, one form can be measured in the presence of the other, thus eliminating the need to separate the two forms prior to measurement. A similar assay using a cyclic system based on malic and alcohol dehydrogenase-catalyzed reactions and malic enzyme in an indicator reaction has been developed for the quantitation of estriol (*8*).

The cofactor scheme is based on a label that has a dual function, namely the monitoring of the CPB reaction and the introduction of an amplification step. Each time the anlayte–cofactor conjugate is cycled, a stoichiometric amount of product is produced, and the amount of product accumulates as the assay proceeds. If the cycling scheme is efficient and the product stable, considerable amplification may be achieved by this mechanism. Both NAD– and ATP–analyte conjugates have also been used to monitor homogeneous specific binding reactions in which the indicator reactions were bioluminescent (*9*, *10*).

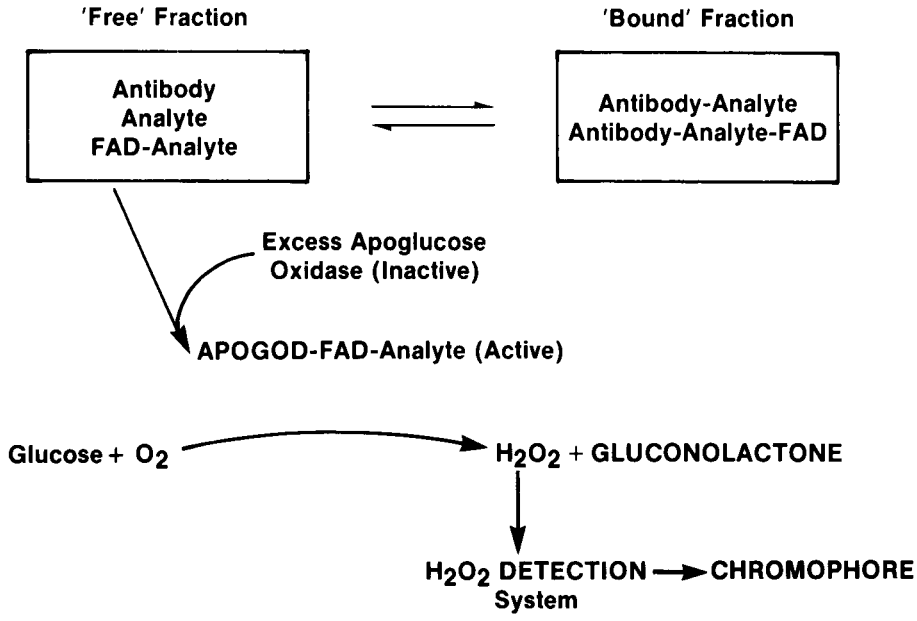

Fig. 2. Schematic illustration of the principles of the Prosthetic Group Labeled Immunoassay.

Prosthetic Group Labels

Prosthetic groups, such as flavin adenine dinucleotide (FAD), can also serve as labels in homogeneous immunoassays, as illustrated in Fig. 2. The analyte and a constant amount of analyte–FAD conjugate are allowed to compete for a limited number of antibody binding sites. At equilibrium the level of free conjugate is controlled by the amount of analyte in the assay mixture and can be determined through the addition of apoglucose oxidase (APOGOD) to the assay mixture. The apo-enzyme combines with the free, but not the antibody-bound form of the conjugate to generate glucose oxidase in proportion to the amount of free conjugate in the mixture. The level of enzyme thus generated can be determined by any one of a number of standard assays. Since a catalyst, the enzyme, is generated in the procedure an amplification mechanism is also built into this assay. This procedure has been used to assay theophylline (*11*) and human IgG (*12*).

Enzyme Modulator Labels

Substances capable of modulating enzyme activity, such as inhibitors, can serve as labels in homogeneous immunoassay. The first example of this type of label was reported in 1979 (*13*). Here acetazolamide, which is an inhibitor of carbonic anhydrase, was covalently bound to biotin. The resulting conjugate was a potent inhibitor of the enzyme. However, in the presence of avidin, the specific binding protein for biotin, the conjugate was incapable of inhibiting the enzyme. Thus, it was possible to assemble a CPB assay for biotin in which the level of free inhibitor–analyte conjugate was detectable through the degree of inhibition of enzyme activity observed when carbonic anhydrase was included in the assay mix-

ture. Recently, other assays employing inhibitor labels have been reported for various drugs, steroids, hormones (*14*), and DNP-lysine (*15*).

Fluorescence Methods

In the past 5 years, numerous applications of fluorescence methods for monitoring immunoassays have been reported. Whereas absorption measurements are restricted to the determination of band position and absorbancy, fluorescence measurements include band position, intensity, polarization, and lifetime. Recent improvements in the design of instruments for measuring fluoresence spectra, lifetimes, and polarization have done much to permit diversified application of fluorescence techniques to immunoassays and may well carry fluorescence methods to a position of prime importance in clinical diagnostics. With the exception of radiochemical techniques, fluorescence spectroscopy offers the clinical laboratory one of the most sensitive detection methods available.

Recent advancements in the design of fluorophores that exhibit specific properties desired for a particular kind of immunoassay have also advanced fluorescence measurements in clinical applications. These fluorescent compounds are of two kinds, the fluorescent probe labels and the fluorogenic enzyme substrate labels.

Fluorescent probe labels are ideally suited for monitoring homogeneous immunoassays because fluoresence is a good indicator of the properties of the immediate environment of the label. When a fluorophore is labeled to an analyte, binding of the fluorophore-labeled analyte to antibody may modulate some parameter of the fluorescent label. Immunoassay procedures based on fluorescence quenching or enhancement, electronic energy transfer, polarization, and fluctuation can thus distinghuish the antibody-bound labeled analyte from the free labeled analyte and alleviate the need for a separation step.

On the other hand, when a fluorophore is labeled to an antibody, binding of the labeled antibody to the analyte may not affect fluorescence. The fluorophore-labeled antibody, however, can diffuse and bind to a surface which has previously been coated with the analyte. The use of total internal reflection technique to differentially excite the fluorophore on the immunologically active surface would permit a distinction between the fluorophore-labeled antibody that is surface bound from nonsurface bound material.

Fluorogenic enzyme substrate labels can also be used in homogeneous immunoassays to monitor enzyme activity when a change in fluorescence occurs during the cleavage of a specific bond linking a fluorophore to the rest of the molecule. Ideally, the intact substrate labels should be nonfluorescent, while the free fluorophores, which are liberated by enzyme action, should be highly fluorescent. Since the fluorogenic enzyme substrate offers greater sensitivity than the chromogenic substrates, only minute quantities of enzyme are required.

Fluorescent Probe Labels

Fluorescence Quenching or Enhancement Some fluorescent dyes are sensitive to the microenvironment. When a fluorophore is labeled to an analyte, the fluorescence intensity of the probe in the analyte–fluorophore conjugate can undergo fluo-

rescence quenching or enhancement, as a result of noncovalent interaction of the analyte–fluorophore with the analyte-specific antibody. Thus, Shaw et al. (*16*) observed that the fluorescence of a fluorescein–gentamicin conjugate is partially quenched upon binding to gentamicin-specific antiserum. Labeled gentamicin and unlabeled gentamicin from a serum sample were allowed to compete for an appropriate amount of antiserum and the extent of fluorescence quenching was related to the gentamicin content in the sample.

On the other hand, Voss and Kardost (*17*) described fluorescence enhancement of 1-naphthylamine-5-sulfonic acid in the fluorophore–nucleotide conjugate after nucleotide specific antibodies were allowed to bind to the conjugate. The degree of fluorescence enhancement was quantitatively related to the antinucleotide antibody activity in biological fluids.

Labeling of the fluorophore with analyte having heavy atoms can produce quenching of fluorophore fluorescence. Smith (*18*) reported the fluorescence quenching of fluorescein in the fluorescein–thyroxine conjugate. This quenching presumably resulted from the intramolecular interaction of the fluorophore with thyroxine, which contains four iodine atoms. Heavy atom quenching is a consequence of enhanced rate of intersystem crossing to the triplet state that competes with fluorescence decay. Binding of thyroxine-specific antiserum to the fluorescein–thyroxine conjugate, however, gave relief of quenching. This enhancement effect was utilized in a fluoroimmunoassay for thyroxine in which the enhancement was progressively reversed in proportion to increasing thyroxine concentration.

Binding of the fluorophore to antifluorophore antibody can also alter the fluorophore's fluorescence properties. Lopatin and Voss (*19*) found that antibody against fluorescein caused up to 90% quenching of fluorescence upon binding. This phenomenon was exploited in the fluorescence protection immunoassay (*20*) for human IgG in which fluorescein-labeled IgG and IgG from a clinical sample were allowed to compete for a limiting number of binding sites on anti-IgG. Antifluorescein was then added. Because of steric hindrance, only the free fluorescein-labeled IgG could combine with antifluorescein, producing fluorescence quenching. The fluorescein-labeled IgG that was bound by anti-IgG antibody was sterically protected from the antifluorescein, and thus fluorescence from this species was not quenched. In a competitive binding reaction, as the IgG level increased, the amount of free fluorescein-labeled IgG increased. Thus an increasing amount of free fluorescein-labeled IgG was available for reaction with antifluorescein producing an increasing degree of quenching.

Fluorescence Electronic Energy Transfer The theory of resonance transfer of electronic excitation energy between donor and acceptor molecules of suitable spectroscopic properties was first presented by Forster (*21*). According to his theory, the rate constant for singlet electronic energy transfer from an excited donor to a chromophore acceptor which may or may not be fluorescent is proportional to r^{-6}, where r is the distance between the molecules involved. Forster proposed that singlet–singlet transfer occurs by a resonance interaction of the dipole pair between the energy donor and acceptor chromophores. In his quantitative treatment,

the distance R_0, the critical distance at which transfer efficiency is 50%, is related to the quantum yield of energy donor, spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor, and the orientation factor K^2.

The Forster energy transfer theory explains the phenomena of (a) quenching of donor fluorescence, and (b) sensitized fluorescence of acceptor. The quenching of the emission of a donor species by an acceptor is a measure of energy transfer from the donor to the acceptor molecule. Alternatively, in the process of sensitized fluorescence, emission at a longer wavelength characteristic of the acceptor molecule can occur and is a result of energy trasnsfer since the acceptor molecule does not absorb the incident energy. The energy exchanges in both cases are found to be sensitive to and indicative of the prevailing distances and orientation of the chromophore pair.

Using the energy transfer between the donor–acceptor pair in the analyte-antibody complex, a fluorescent excitation transfer immunoassay has been described (*22*). In this method as illustrated in Fig. 3, the analyte is coupled covalently to the energy donor, fluorescein, and an antibody to the analyte is labeled with the energy acceptor, rhodamine. When the labeled antibody is bound to the labeled analyte, the energy donor and the energy acceptor are brought in close proximity to each other and rhodamine quenches the fluorescence of fluorescein. Thus in a competitive binding assay, the degree of quenching, or sensitized fluorescence that occurs is a measure of energy transfer and is related to analyte concentration.

Rotational Diffusion: Fluorescence Polarization As illustrated in Fig. 4, suppose a fluorescent solution is placed at coordinate O and is illuminated by a beam of polarized light whose electric vector is in the OZ direction. Suppose the fluorescent light emitted along OY is observed. The intensity of two linearly polarized components, one parallel to OZ, $I_{\parallel}$, and the other along OX, $I_{\perp}$, *can be determined by orienting a polarizer so as to first transmit one component, then the other. The fluorescence polarization, p ,* is given by (*23*)

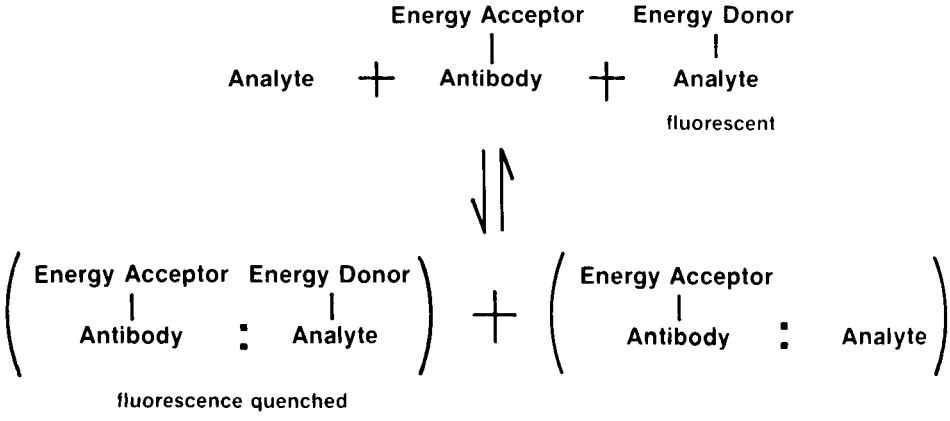

Fig. 3. Principle of the excitation transfer immunoassay.

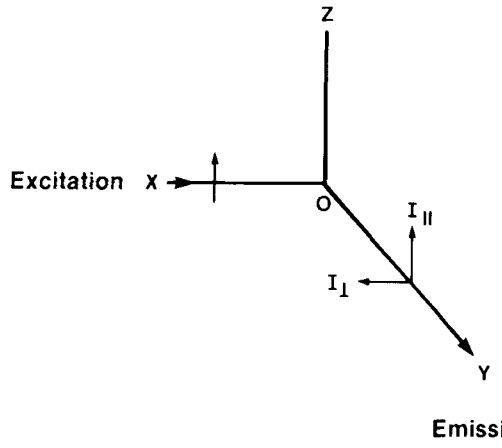

Fig. 4. Schematic illustration of the principles of fluorescence polarization with solution placed at 0.

$$p = \frac{(I_{11} - I_{\perp})}{(I_{11} + I\perp)}$$

The orientation of the polarizer can be changed manually or electronically (*24*) by applying an electric field to a liquid crystal.

Measurement of polarization of fluorescence has been used to monitor homogeneous competitive binding reactions. The degree of polarization of a fluorescent solution can be correlated to the rotational Brownian movement of the fluorescent labels. For the simple case of rigid spherical molecules in solution, the rotational relaxation time, defined as the time necessary for the average emission oscillator to rotate through an angle equal to arc cos (e^{-1}), is related to parameters of both the fluorescent labels and the solvent by

$$\rho = \frac{3\eta V}{RT}$$

where T and η are the temperature and viscosity of the solution, respectively, V is the apparent spherical volume of the fluorescent label, and R is the gas constant. From Perrin (*25*), the fluorescence emitted at right angles to the direction of excitation by linearly polarized light will have the observed polarization, p, given by

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

where τ is the lifetime of the excited state of fluorescence and p_0 is the maximum possible polarization. Polarization of fluoresence can therefore be affected by any change in rotational restriction, size, or conformation of the fluorescent labels, as well as by changes in temperature or solvent viscosity. Fluorescence polarization is also sensitive to changes in molecular size as a result of binding of other molecules

to the fluorophore. When a small fluorophore–analyte conjugate of low polarization binds to an antibody, the fluorescence polarization increases because the increased size of the complex results in a restriction of rotational motion of the label. In CPB assays the ratio of free-to-bound labeled analyte is governed by the level of analyte in the unknown. Fluorescence polarization can provide a direct evaluation of the relative amounts of free and bound labeled analyte present in solution without a separation. Fluorescence polarization immunoassays were described as early as in 1965 (*26*). More recent clinical applications include determinations for therapeutic drugs (*27–29*), cortisol (*30*), and insulin (*31*).

In a fluorescence polarization immunoasssay an analyte is labeled with a fluorophore. Reaction between the fluorophore–analyte conjugate and antibody to form a complex results in an increase in the effective size of the conjugate. This interaction, in turn, causes a retardation in Brownian rotational motion that is manifested by an increase in the polarization of fluorescence. In the presence of unlabeled analyte, a smaller percentage of the labeled analyte is bound to the antibody resulting, in a lower degree of polarization than that observed in the absence of unlabeled analyte. Hence, in an immunoassay as the level of unlabeled analyte increases, the level of fluorescence polarization decreases.

Lateral Diffusion (1) Fluorescence Correlation (Fluctuation). The fluorescence correlation immunoassay (*32, 33*) is based on (a) the number of fluctuations of particles within a prescribed volume in solution caused by random Brownian motion and (b) diffusion of particles in which the diffusion coefficient is inversely related to particle size. Fluorescence correlation analysis was originally used to study translational diffusion of fluorescent particles across the exciting laser beam by measuring spontaneous concentration fluctuations (*34*). The fluorescence of the particles is used to monitor particle concentrations. To improve sampling statistics, the sampling volume is periodically scanned through the sample solution. The autocorrelation function of the recorded intensity, which is a mathematical transform of the fluorescence, is calculated on line by a computer at a time equal to the scanning period. As illustrated in Fig. 5, transformation of the concentration fluctuations via the autocorrelation function eliminates most unwanted noise sources. Since these unwanted noise sources are not periodic in the sampling time, they appear only in autocorrelation function equal to zero. Only the desired concentration fluctuation noise is periodic and thus contributes to the autocorrelation function.

In the competitive correlation immunoassay, antibodies to analyte are covalently attached to beads (Ab_b) and fluorophore-labeled analyte (Ag*) is allowed to compete with unlabeled analyte (Ag) in a sample, for the antibody sites on the beads. To distinguish the large, slowly diffusing bead, Ag* : Ab_b from the small rapidly diffusing Ag*, the fluctuations in fluorescence intensity that occur for a given small volume element in the solution, excited with a laser source are measured and the temporal autocorrelation function of the fluctuations in the fluorescent intensity is calculated.

Nicoli (*33*) has shown that the peak height of this autocorrelation function is dominated entirely by the bead fluorescence. Thus, the fluorescence correlation technique is insensitive to small, fast-diffusing Ag* and allows the elimination of separation steps in the immunoassay.

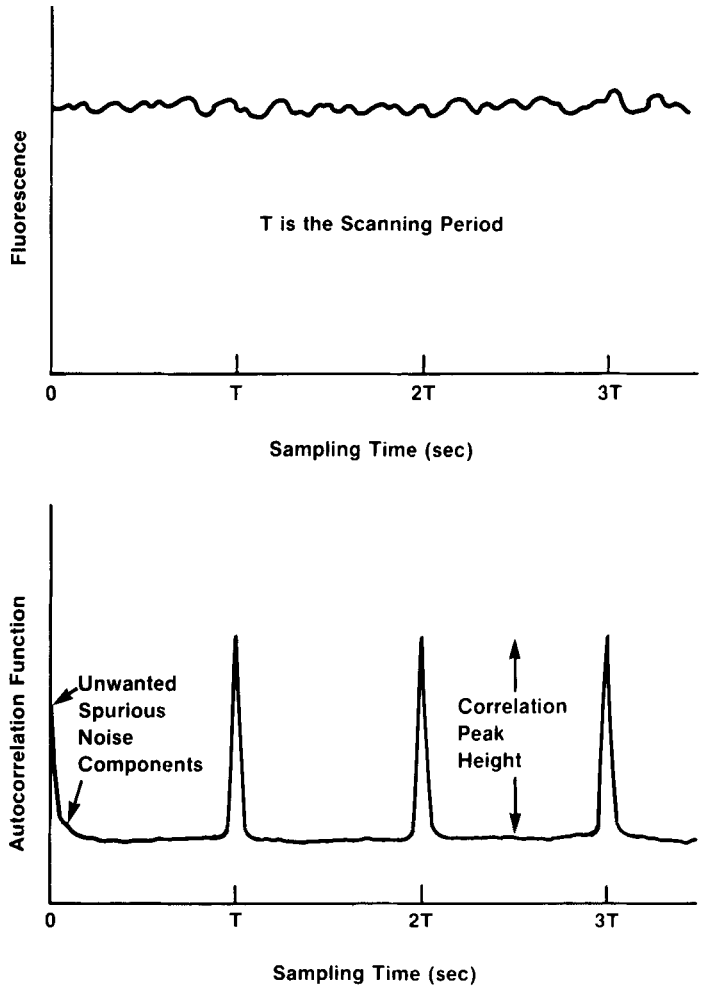

Fig. 5. Schematic illustration of concentration fluctuations monitored by fluorescence and the mathematical transformation into the autocorrelation function.

The fluorescence correlation technqiue has also been applied to a sandwich immunoassay (*32*) in which the antibody is labeled with the fluorophore (Ab*) and the sandwich complex on the bead, Ab* : Ag : Ab_b is distinguished from the fluorophore-labeled antibody, Ab* by the fluorescence correlation technique.

(2) Total Internal Reflection Fluorescence. When an antibody is labeled with a fluorophore, binding of the antibody to an analyte may not affect the fluorescence intenstiy of the label. However, the free fluorophore-labeled antibody can be distinguished from the analyte-bound fraction by virtue of lateral diffusion of the labeled antibody and total internal reflection fluorescence spectroscopy (*35*). Here, a fixed amount of analyte is first adsorbed onto a quartz surface. This immunologically active surface is then placed in contact with a sample solution containing analyte and a fixed amount of fluorophore-labeled antibody. The labeled antibody distributes itself between the free and surface-bound analyte. The

distribution is controlled by the level of analyte in the specimen. Excitation laser light is then introduced into the optically transparent quartz surface where it suffers total reflection. The penetrating light (the evanescent wave) only excites the fluorophore-labeled antibody that is in contact with the reflecting surface and not the labeled antibody in solution. This preferential excitation of the fluorophore-labeled antibody that is bound to the immunological reactive surface therefore affords an indication of the distribution of label between the solution and surface. Thus, in an assay, as analyte concentration increases, less labeled antibody will be available to react at the surface.

Fluorogenic Enzyme Substrate Labels

Homogeneous immunoassays have also been monitored by enzymic hydrolysis of a fluorogenic enzyme substrate that is covalently coupled to the analyte (*36–38*). The intact substrate–analyte conjugate must be nonfluorescent under conditions of the assay. However, after enzymic hydrolysis, it must be highly fluorescent. Binding of the analyte-specific antibody to the substrate–analyte conjugate renders the conjugate inactive as an enzymic substrate. Competitive binding reactions are set up with a constant amount of substrate–analyte conjugate and a limiting amount of analyte-specific antibody. Analyte and substrate–analyte conjugate compete for antibody binding sites. Any substrate–analyte that is not antibody-bound is hydrolyzed by the enzyme giving fluorescence intensity that is related to the analyte concentration.

Three different fluorogenic substrate–analyte conjugates have been described. The first conjugate (*36*) is composed of an analyte (biotin) that is coupled directly to a dye (umbelliferone) through an ester bond. Hydrolysis of the nonfluorescent ester with an esterase yields a fluorescent product.

The second type of conjugate (*37*) uses an enzyme substrate (a galactosyl umbelliferone derivative) coupled to analytes such as haptens, e.g., theophylline, or proteins such as IgG (*39, 40*). This fluorogenic enzyme substrate does not possess any inherent fluorescence background at the optimal excitation and emission wavelengths used in the assay (*41*) and represents the ideal case for uncomplicated detection of fluorescent products from the enzymic reaction.

This substrate labeled fluorescent immunoassay approach has been used for the measurement of various therapeutic drugs (antibiotics, antiasthmatics, antiepileptics, and antiarrhythmics) and specific proteins (IgG, IgM, and IgA). This procedure has also been incorporated into a paper strip format (*42*) to produce assays that eliminate reagent manipulation and require only a single sample dilution. Thus they represent an appreciable improvement in convenience over conventional wet chemistry procedures.

The third conjugate (*38*), as illustrated in Fig. 6, is based on a fluorophore–quencher–analyte combination. Here, the fluorogenic enzyme substrate molecule contains a fluorescer–quencher pair, separated by a chain containing a bond that can be hydrolyzed by an enzyme. In a competitive protein-binding assay, the fluorogenic enzyme substrate is covalently labeled with an analyte on the quencher portion of the molecule. Enzyme hydrolysis of the labeled substrate

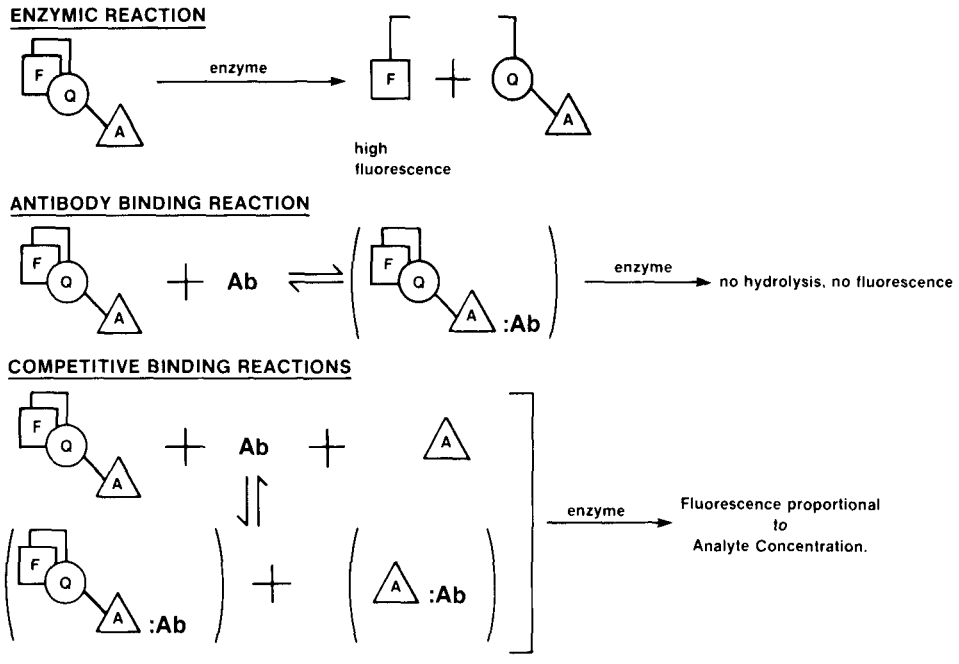

Fig. 6. Principle of the Substrate Labeled Fluorescent Immunoassay using an Intramolecularly Quenched Fluorogenic Substrate. F is the fluorescer, and Q is the quencher that is covalently attached to the analyte, A.

brings about relief of fluorescence quenching through release of the fluorescing component. When antibody to analyte binds to the labeled substrate, enzymic hydrolysis is inhibited. Finally, when competing analyte is added, the inhibition of enzymic hydrolysis is diminished and fluorescence is produced because of the release of the fluorescent label in proportion to analyte concentration.

Miscellaneous

Spin Labels

The homogeneous spin immunoassay, which utilizes a nitroxide radical as a paramagnetic label, has been developed for drugs of abuse such as morphine (*43*). The spin-labeled morphine, being a small molecule, tumbles rapidly in solution and gives rise to sharp electron spin resonance (ESR) peaks. When the spin-labeled morphine is bound to antibody, the free radical in the resulting complex becomes immobilized and results in a broadening of the ESR spectrum of the nitroxide. Since only the spin-labeled morphine that is not bound to antibody contributes to the ESR signal, the free form can be determined without the need to separate it from the antibody-bound form. In the presence of an increasing amount of unlabeled morphine, the antibody-bound spin-labeled morphine is progressively displaced, resulting in an increase in the concentration of labeled morphine. Thus in the competitive protein binding assay, the ESR signal increases in proportion to the analyte concentration.

Conclusion

In conclusion, we have described in this article different examples of labels that have been utilized in the development of novel, separation-free, homogeneous immunoassays for the determination of analytes having high and low molecular weight.

Many of the assays that rely on spectrophotometric methods are based on the use of enzyme, cofactor, prosthetic group, or enzyme modulator labels. One particularly interesting aspect about most of these labels is their ability to introduce an amplification mechanism into the assay.

Fluorescent methods have also been used to monitor homogeneous immunoassays. These fluorescent immunoassays have been developed only after specialized fluorescent probe labels and fluorogenic enzyme substrate labels became available. These labels exhibit interesting fluorescence properties, such as quenching and enhancement, energy transfer, polarization, and fluorescence fluctuation. Utilizing these different fluorescence properties of the labels various homogeneous immunoassays schemes have been developed.

Most of the spectrophotometric and fluorescent immunoassays are applicable to simple clinical instruments. These assays are, therefore, expected to exert significant impact on future clinical diagnostics. For the fluorescent immunoassays, there is no doubt that future advances will occur in the design of ingenious fluorescent probes and fluorogenic substrates. The use of novel fluorescent labels to develop new and innovative fluorescent immunoassays will continue, especially since the theoretical understandings of fluorophore–antibody interaction and fluorophore photo- and organic chemistry become more sophisticated and as experimental fluorescence techniques and instrumentation become further refined and improved.

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