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LITERATURE SURVEY

Fluorescence Immunoassay

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Keyphrases □ Fluorescence immunoassay—overview of methods currently in use, homogeneous and heterogeneous methods compared to radioimmunoassay and chromatographic determinations □ Immunoglobulins—role in pharmaceutical analysis, fluorescence immunoassays of drug–carrier protein conjugate □ Homogeneous fluorescence immunoassays—reactant-labeled fluorescence, quenching and enhancement immunoassays, fluorescence polarization, time-resolved fluorescence immunoassays, role in pharmaceutical analysis □ Drug–carrier protein conjugate—overview of fluorescence immunoassay methods currently in use

The recent advances made in the area of immunoassays of biologically active compounds have had a significant impact on the health care community. Highly specific and sensitive immunological procedures are available that provide picomole sensitivity on a routine basis (1). In most of these assays, a radioactive label is used to monitor the competitive binding between a labeled and unlabeled ligand for receptor sites on specific antibodies (2, 3). The analysis of this competitive binding allows the construction of a standard curve representing the antibody-bound radioactivity as a function of the unlabeled ligand concentration. This approach gives the analyst the capacity to quantitate, quite specifically, the ligand (analyte) of interest as well as the ability to detect it at far lower concentrations than is possible by other chemical methods due to the extremely sensitive methods available for the detection of radioactivity.

The major limitations of radioimmunoassay result from the biological hazards of radioactivity, the difficulties associated with various licensing requirements, the safe disposal of wastes, and the expense and inconvenience incurred by the short radioactive lifetimes of the labels (4, 5).

A rather recent development in the area of immunoassays provides a major alternative to the use of radiolabels. Fluorescent probes are used in place of the radiolabels, and their spectral characteristics are monitored as a function of the immunoreactions between the ligand and the antibody. In the foreseeable future, this new immunological technique may possibly be comparable in both selectivity and sensitivity to current radioimmunoassay methods.

In this overview of fluorescence immunoassay, the methods currently in use will be evaluated and likely future developments will be discussed.

NATURE OF IMMUNE RESPONSE AND ITS ROLE IN PHARMACEUTICAL ANALYSIS

The ability of an organism to synthesize specialized proteins (antibodies) directed against an invasive foreign material forms the basis of the immune response. Antibodies are a group of structurally related proteins known as the immunoglobulins (Ig). There are five major classes of immunoglobulins: IgG, IgM, IgA, IgD, and IgE. The predominant immunoglobulin in serum, IgG, is usually associated with the majority of antigen binding activity in the highly diluted solutions used in sensitive immunoassays (6). The IgG class has an average molecular weight of 150,000–160,000 daltons. The structure of IgG proteins is characterized by four polypeptide chains, two of which are heavy or H-chains (50,000 daltons) and two of which are light or L-chains (20,000 daltons). Each immunoglobulin molecule contains two binding sites at the terminal regions of the H-chains. These binding sites contain variable amino acid sequences and appear to be responsible for the binding specificity common to antibodies (7).

The immunoglobulins are produced by the B-lymphocytes. The process of the maturation and development of these lymphocytes into cells capable of antibody production is not understood fully (8).

The ability of a molecule to elicit an immunoresponse appears to depend on its size. Molecules with molecular weights below 10,000 daltons usually are not immunogenic. Most proteins, large polymeric carbohydrates, and lipopolysaccharides are immunogenic. The antibodies developed in response to these molecules recognize and bind to only a small section of the antigen. Antibody specificity usually involves no more than six or seven amino acid residues of a large protein-like structure (9).

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The investigations performed by Landsteiner (10) determined that an immunoresponse can be elicited from an organism to small molecules (haptens) coupled to proteins or polypeptides; antibodies to the hapten–protein conjugate, capable of recognizing part of the hapten even when it was not coupled to the carrier protein, could be generated. Drugs and their metabolites normally are not immunogenic unless they are first coupled covalently to macromolecules.

The human and bovine serum albumins and, recently, some synthetic polypeptides have been used as carriers for drugs and other small molecules. However, the population of antibodies directed toward the hapten–carrier protein conjugate is not homogeneous. Rather, some of the antibodies produced may recognize portions of the protein carrier and some recognize the hapten itself. Only those antibodies directed toward the hapten will be useful in immunoassay, and it is the heterogeneity of the isolated antibodies that makes it difficult to describe the immunoreactions quantitatively in terms of simple competitive equilibria.

To synthesize a drug–carrier protein conjugate, the drug must have at least one functional group available for attachment to the carrier reactive sites under suitable chemical conditions. The coupling of the drug to the carrier must not cause significant structural alterations or denature the carrier itself. Low molecular weight substances have commonly been conjugated to proteins through carboxyl, amino, and hydroxyl groups (11). A detailed review of the procedures and applications of various conjugation procedures was published by Beiser *et al.* (12). Selected methods and applications of pharmaceutical interest will be discussed briefly.

The selection of a conjugation procedure for the coupling of a hapten with a protein is determined by the available functional groups on the hapten, the stability and solubility of the hapten, and the mode of attachment to the protein (13). The most common sites of conjugation on a protein molecule are the amino groups of the *N*-terminal and lysine residues, the carboxyl groups of the *C*-terminal and aspartic and glutamic acid residues, the phenolic functions of tyrosine, and the secondary NH group of histidine. The sulfhydryl groups of cysteine also have been used for conjugation (13).

An example of a conjugative procedure used to couple a hapten with a carboxyl group to a protein carrier is provided by the production of the D-lysergic acid–poly(L-lysine) conjugate. The agent used to initiate the reaction was a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (14, 15). The D-lysergic acid was bonded covalently by its carboxyl function to the ϵ-amino group of poly(L-lysine) (mol. wt. 95,000). The resulting condensation resulted in amide bond formation to give a poly(L-lysine)–lysergamide conjugate (16).

Haptens with aromatic amino groups may be conjugated to proteins by a relatively simple diazotization procedure. The reaction involves conversion of the aromatic amine to a diazonium salt that couples to the aromatic and other side chains of a protein carrier (17, 18). Nitrous acid reacts with the aromatic amine to form the diazonium salt; at alkaline pH, the diazonium salt couples to the tyrosine, histidine, and tryptophan residues of the carrier through the azo group (19). An important pharmaceutical application of this procedure is the conjugation of chloramphenicol to bovine γ-globulin (20). In these experiments, the nitro group of the chloramphenicol was reduced to an amino group prior to conjugation.

Assuming that a suitable procedure for conjugation can be found, it should be possible to obtain specific antibodies for any biologically active compound. However, there is a certain amount of cross-reactivity of the hapten-directed antibodies with molecules of structure similar to that of the hapten. This problem is not significant in most instances, but it should be considered in any immunoassay. Another difficulty that may arise is the binding of the active compound by nonantibody proteins in the test serum, urinary, or cerebrospinal fluid solution. Again, this problem may not be significant, but the possibility of interference should be recognized.

FLUORESCENT PROBES

The fluorogenic molecules used to label covalently the ligands (drugs) and, in some instances, the antibodies in a fluorescence immunoassay fall into the category of fluorescent probes (21, 22). Fluorescent probes are small molecules whose fluorescent properties are altered subsequent to interactions with proteins or other macromolecules (23). The proper selection of a fluorescent label and the technique employed for its conjugation to the ligand are critical to the sensitivity and selectivity obtained in an immunoassay.

In quantitative immunoassays using fluorescent labels, the fluorochrome should possess several basic chemical and spectroscopic properties. The labeled ligand should have a relatively high water solubility because immunoassays are usually carried out in the aqueous environment of biological samples (*i.e.*, serum, urine, and cerebrospinal fluid). The presence of certain potentially reactive functionalities on the fluorochrome facilitates its conjugation to the ligand. Derivatives of the fluorochromes containing reactive groups such as acid chlorides, isothiocyanates, and diazonium salts can be used for the conjugation of the label to the ligand. A major concern in labeling a ligand with a fluorescent probe is the alteration of the specificity of the ligand for its antibody. The site of conjugation should provide maximum exposure of the functional groups necessary for antibody recognition. The stability and shelflife are also important.

The spectral characteristics of greatest importance in the selection of a fluorochrome are the molar absorptivity at the selected excitation wavelength, the quantum yield of fluorescence of the labeled species, the spectral regions of absorption and emission of radiation, and the Stokes shift. Ideally, the fluorescent label should have a high molar absorptivity in the visible region of the electromagnetic spectrum, which is well removed from the excitation spectra of proteins and other endogenous interferences normally present in biological fluids. The emission wavelength should also lie well in the visible region with Stokes shift (displacement of the fluorescence maximum from the longest wavelength absorption maximum) of at least 50 nm. A high quantum yield of fluorescence in the antibody-bound or free labeled ligand is also desirable.

Due to the rather stringent requirements placed on fluorescent labels used for monitoring immunoreactions,

only a few substances have given satisfactory results. The labels most commonly used are fluorescein isothiocyanate and several rhodamine dyes (usually tetramethylrhodamine isothiocyanate). Fluorescein isothiocyanate has a relatively high quantum yield and can be conjugated to drugs and other ligands under fairly mild conditions (24). Gentamicin, as the free base, was conjugated with fluorescein isothiocyanate at alkaline pH, forming fluoresceinthiocarbamyl gentamicin. The reaction mixture was purified on a Sephadex column, with unreacted gentamicin being well separated from the fluorescein-labeled gentamicin. Polarization and quenching immunoassays (*vide infra*) were performed with the conjugate, and the results were comparable in sensitivity to bioassay and radioimmunoassay (25, 26).

Recent work in ligand labeling with fluorescent metal chelates has opened a new area of research in fluorescent probes (27). The chelates of rare earth metals have unique emission characteristics in that, upon excitation of aromatic portions of the ligand of the lanthanide complex, the energy of excitation is efficiently transferred to the lanthanide ion. This causes f–f transitions, which produce narrow, almost line-like emission bands with fairly long decay times (1 μsec–1 msec). In addition, the rare earth chelates possess large quantum yields in combination with very large Stokes shifts. The excitation region of these chelates is fairly broad and allows excellent sensitivity and selectivity, enabling the use of fairly wide bandwidths for excitation and narrow bandwidths for emission. With the proper combination of a rare earth chelate and fluorometric technique, sensitive immunoassays can be developed that avoid interferences commonly encountered in the immunoassay.

The development of new fluorescent probes for fluorescent immunoassay, having greater environmental sensitivity and larger Stokes shifts than observed in the compounds in use currently, should provide a fertile area for research.

HOMOGENEOUS FLUORESCENCE IMMUNOASSAY

Fluorescence immunoassays can be divided into two broad classes, homogeneous and heterogeneous. The homogeneous technique differs from the heterogeneous primarily in that fluorometric quantitation of the ligand can be performed without separating the free ligand from the antibody-bound labeled ligand. The elimination of the need to separate bound and free ligands, normally present in other immunoassays (*e.g.*, radioimmunoassay) represents a major advantage of fluorescence immunoassay and provides the opportunity for a simple, fast, and reliable quantitation. This procedure can be performed because of the different microenvironments in which the free and bound ligands reside and their effects on the spectral characteristics of the label.

In the aqueous environment, the free labeled antigen experiences strong polarizing forces and perhaps hydrogen bonding forces at acidic or basic functional groups as a result of interactions with water molecules. These forces are exerted to different degrees in the ground and excited states of the labeled antigen because these states have different dipole moments. Moreover, certain functional groups on the fluorophore are free to rotate subsequent to the fluorescent transition in water. On the other hand, in the hydrophobic environment of an antibody binding site, the dielectric strength is low, and rotation of functional groups on the fluorophore is severely restricted. The solvation and restricted rotational freedom of the antibody-bound labeled antigen usually cause this species to fluoresce at shorter wavelengths than the free labeled antigen because the relative stabilization of the excited state of the latter, by strong electrostatic and electromeric interactions, is much greater than in the bound labeled antigen. In addition, the weak solvation and restricted rotational freedom of the antibody-bound probe also cause the bound probe to fluoresce more intensely than the free probe because the bound probe is somewhat shielded from internal and external conversions, which compete with fluorescence for deactivation of the excited state.

If the fluorescent emission spectrum of the bound labeled ligand is shifted sufficiently from that of the free labeled ligand, the resulting spectroscopic measurements can be used for quantitation without a separation step. In essence, a spectroscopic separation rather than a chemical separation is employed and contributes to the simplicity and speed of analysis.

Despite their speed and simplicity, homogeneous assays may not always provide the sensitivity needed in certain solutions due to the endogenous background fluoresence of the proteinaceous material present. The Rayleigh and Raman scattering caused by the high protein content in solution and the intrinsic fluorescence from certain amino acid residues are major obstacles to a sensitive assay.

The three major types of homogeneous fluorescence immunoassay are quenching and enhancement, fluorescence polarization, and reactant-labeled fluorescence immunoassay. Each technique will be discussed briefly and evaluated for its sensitivity and reliability.

Reactant-Labeled Fluorescence Immunoassay—An interesting variation of homogeneous immunoassay in which neither the labeled ligand *per se* nor the antibody-labeled ligand complex is fluorescent is seen in reactant-labeled fluorescence immunoassay. The technique is based on the quantitation of the fluorescent product resulting from the enzymatic cleavage of the unbound labeled ligand. The fluorescent probe labeling the ligand exists in nonfluorescent form as long as it is linked covalently to the ligand through the bond that will be cleaved by enzymatic action (usually hydrolysis). If the labeled ligand is complexed by specific antibodies prior to hydrolysis, it cannot act as a substrate for the enzyme. The complex prevents any reaction between the enzyme and labeled ligand and thereby inhibits fluorescence from excited labels (28, 29). The unavailability of the conjugated substrates (drug and label) to the enzyme is reversed by the presence of free unlabeled ligand (in the unknown solution), which then competes with the labeled conjugate for the antibody binding sites. The labeled ligand thus displaced from its antibody binding site can be cleaved by the enzyme, liberating the fluorescent form of the label. The extent of fluorescence is proportional to the free ligand or analyte concentration.

A reactant-labeled fluorescence immunoassay was applied to the analysis of gentamicin in human serum (30). Gentamicin is a widely used antibiotic but, in systemic therapy, the range between therapeutic and potentially toxic serum levels is narrow (31). Thus, a fast, reliable, and

sensitive procedure was developed based on reactant-labeled fluorescence. The assay uses umbelliferyl β-galactoside as a label for gentamicin and the enzyme β-galactosidase to obtain fluorescent products. Gentamicin is conjugated to β-galactosylumbelliferone to form a nonfluorescent substrate. Hydrolysis of the uncomplexed β-galactosylumbelliferone–gentamicin conjugate is catalyzed by the enzyme β-galactosidase to form a fluorescent product, the free 7-hydroxycoumarinate anion. However, hydrolysis is inhibited if the gentamicin conjugate is bound with specific antigentamicin antibodies since steric hindrance from the antibody prevents the enzyme from acting on the substrate. The extent of fluorescence is proportional to the gentamicin concentration, and the sensitivity is comparable to that of radioimmunoassay.

Quenching and Enhancement Immunoassays—Fluorescent quenching methods are based on the observed reduction in fluorescence intensity of a labeled ligand upon complexation by the antibody. The exact mechanism has not been established, but it is generally agreed that it involves alteration of the electronic structure and perhaps of the vibrational composition of the electronic states of the labeled antigen upon binding by the antibody. Alteration in the electronic distribution of the bound labeled ligand can enhance the probability of radiationless deactivation of the first excited singlet state. A possible explanation might be the vibrational coupling of the bound labeled ligand with the normal vibrational modes of the protein, a process that could enhance the rate of internal conversion to the ground state. If the extent of quenching of the bound labeled ligand is great enough, the competitive binding of unlabeled ligand for antibody binding sites can be quantitated by measuring the fluorescence of the displaced labeled ligand.

Serum gentamicin levels were studied using a quenching fluoroimmunoassay (32). With fluorescein-labeled gentamicin as the labeled conjugate, the degree of quenching of its fluorescence emission upon binding by an antibody was related to the concentration of unlabeled gentamicin present, using a standard curve.

A novel homogeneous quenching assay was developed for morphine that employs a fluorescein-labeled antigen and a quencher-labeled antibody (33). The mechanism of quenching is associated with energy transfer from the fluorescent donor (fluorescein) to the fluorescent acceptor (rhodamine) or quencher. The emission of the fluorescein-labeled antigen is quenched by the rhodamine-labeled antibody upon complexation. The effectiveness of energy transfer quenching is dependent on the overlap of the fluorescence and absorption spectra of the donor and acceptor, respectively. The efficiency of energy transfer is also dependent on the distance between the bound labeled antigen and the energy acceptor (quencher) in the antibody complex. The quenching can be prevented by competitive binding with unlabeled antigen, which forms the basis for the analysis of the latter. The sensitivity obtained for the morphine assay was in the nanomolar range, but unusually high purity requirements for antibodies and background interference, as well as the difficulty and tedium of labeling of both antigen and antibody, limit routine applications.

Fluorescent enhancement assays are similar to those of the quenching techniques in that there is a change in fluorescent intensity upon antibody binding. The difference is that there is an increase in the quantum yield of ligand fluorescence upon binding. This phenomenon was reported for the binding between fluorescein-labeled thyroxine and its antibodies (34). In this case, the proposed mechanism is a decrease in the thyroxine-induced spin-orbital coupling between the singlet and triplet states of fluorescein. This decrease is a result of the alteration of the separation between the lowest excited singlet states of fluorescein and thyroxine, induced by the low polarity of the antibody binding site (35). The sensitivity is less than that of typical radioimmunoassay procedures.

Fluorescence Polarization—Fluorescence polarization provides valuable information on the interactions of antigens or haptens with antibodies. The use of fluorescence polarization in the study of macromolecules was initiated by Weber in 1952 (36) and has become a major tool in structural investigations of the binding of small molecules to proteins.

The physical principle underlying fluorescence polarization immunoassay involves the selective elimination of light waves whose electric vectors do not all lie in a single plane. This is accomplished by passing the exciting light through a polarizing filter. The resulting polarized radiation selectively excites those molecules whose absorption transition moments have a significant component in the plane of the electrical vector of the exciting beam (37). As a result, molecules excited with polarized light emit radiation that is polarized in the same direction as the exciting light, to a degree that is inversely related to the amount of Brownian rotation occurring during the interval between absorption and emission of light (38). In other words, the photoselected molecules originally excited by polarized light and having fairly small volumes (*i.e.*, free labeled antigen) have a rotational relaxation time that is much shorter than their fluorescence decay time and thus become completely randomized before fluorescing. Therefore, little polarized fluorescence is displayed. However, photoselected molecules having very large volumes, such as antibody proteins and their complexes, rotate at a rate comparable to or slower than the rate at which they fluoresce. Consequently, randomization of the fluorescent transition moments does not occur in the large molecules, and substantial fluorescence polarization is observed.

Upon the binding of an antigen to an antibody, there is a reduction or a restriction in the rotational Brownian motion as well as an increase in the overall size of the fluorescent label. This result causes considerable polarization of the fluorescence along or perpendicular to the optical axis of the excitation polarizer, depending on whether the fluorescence transition moment of the molecule is oriented closer to 0 or 90° to the transition moment associated with the absorption band excited. The case where the transition moments for excitation and fluorescence are parallel (or nearly so) will be discussed first.

If a second polarizing film (emission polarizer) is placed between the fluorescing sample and the photodetector of the fluorometer with its optical axis perpendicular to that of the polarizing film between the lamp and the sample, the highly polarized fluorescence from the antibody-labeled ligand is filtered to a much greater extent than is the unpolarized fluorescence from the same concentration of

free labeled ligand excited under the same conditions. If the optical axes of both polarizers are parallel and the excitation and emission moments of the fluorophore are parallel (or nearly so), the emission polarizer passes relatively more radiation from the bound labeled ligand than from the free labeled ligand to the detector, because the polarized emission is concentrated along the optical axis of each polarizer while the unpolarized emission is dispersed over all angles to the optical axis of the emission polarizer and some, therefore, is filtered. Regardless of the orientation of the optical axis of the second polarizer with respect to the first, the fluorescence intensity registered by the detector, ideally, should be the same for unpolarized fluorescence (*i.e.*, that of the free labeled ligand). In the case of parallel absorption and fluorescence transition moments, the intensity of the polarized fluorescence (that from the bound labeled ligand) measured when the optical axes of the polarizers are parallel ($F_{\parallel}$) should be greater than when the optical axes of the polarizers are perpendicular ($F_{\perp}$). In the case of perpendicular absorption and fluorescence transition moments, $F_{\parallel} = F_{\perp}$ also for the free labeled ligand (unpolarized fluorescence) and $F_{\parallel} < F_{\perp}$ for the bound labeled ligand (polarized fluorescence).

The degree of polarization may be defined as:

$$p = \frac{F_{\parallel} - F_{\perp}}{F_{\parallel} + F_{\perp}} \quad \text{(Eq. 1)}$$

Ideally, at any given excitation wavelength, p should be a property of a pure species and be invariant with respect to concentration. For a free labeled ligand, $F_{\parallel} = F_{\perp}$ so that $p = 0$ at all excitation wavelengths. For a bound labeled ligand, it is possible to have $+\frac{1}{2} > p > -\frac{1}{3}$, depending on the excitation wavelength. However, if a system is contrived that originally contains all antibody-bound labeled ligand and to which the free unlabeled ligand is added so that some labeled ligand is displaced, the relative increase in unpolarized fluorescence and decrease in polarized fluorescence from the solution cause a net decrease in p, as calculated from its operational definition in Eq. 1. If all of the labeled ligand is ultimately displaced from the antibody complex, p falls to zero. The fact that, depending on the extent of binding of the labeled ligand, the degree of polarization varies between some nonzero value and zero permits the construction of a calibration curve of the degree of fluorescence polarization *versus* the concentration of unlabeled ligand and permits execution of a homogeneous immunoassay.

The use of polarized fluorescence for the quantitation of several antigen–antibody reactions was reported (39, 40). Two assays of particular interest were those applied to the measurement of serum levels of gentamicin and phenytoin (41, 42). Fluorescein-labeled ligands were used in both cases, and the polarization measurements correlated well with other instrumental and bioassay techniques.

The use of fluorescence polarization as a routine method for drug level determinations has been limited not only by the expense of the instrumentation but also by the energy losses due to the polarizing films and the background interferences that result in reduced sensitivity. In addition, the nonlinear polarization response as a function of concentration, combined with the nonlinear relationship in competitive binding between antigen and antibody, severely limits the dynamic range of the procedure (5).

TIME-RESOLVED FLUORESCENCE IMMUNOASSAY

All of the fluorometric techniques considered so far have been based on the measurement of the intensity of fluorescence produced under "steady-state" conditions. Steady-state fluorometry is derived from the excitation of the sample with a continuous temporal output of exciting radiation. The lamps and their power supplies used in conventional fluorometers are sources of continuous radiation. After a short initial excitation of the sample, a steady state is established in which the rate of excitation of the analyte is equal to the sum of the rates of all processes deactivating the lowest excited singlet state (fluorescence, internal conversion, and intersystem crossing). When the steady state is established, the observed fluorescence intensity becomes time invariant and produces the temporally constant signal which is measured by the photodetector.

However, with the development of modern electrooptics, it has become possible to excite a potentially fluorescent sample with a thyratron-pulsed flash lamp that emits its radiation in bursts of 2–10-nsec duration with ~0.2 msec between pulses. A fluorescent sample excited with such a pulsed source does not fluoresce continuously. Rather, its fluorescence intensity, excited by a single pulse, decays exponentially until the next pulse again excites the sample. The pulsed source acts much as does a mechanical chopper in phosphorimetry. The fluorescence from the sample excited by the pulsed source can be represented, after detection, as a function of time on a fast sampling oscilloscope or on an x–y plotter used with a multichannel pulse analyzer.

The former approach is called stroboscopic fluorometry, and the latter is called time-correlated single-photon counting. In either case, fluorescences with decay times much longer than the lamp pulse characteristics can be treated in the same way that radioactive decay curves are analyzed. A semilogarithmic plot of fluorescence intensity against time yields a straight line (or a series of overlapping lines if several fluorophores have comparable but not identical decay times) whose slope is proportional to the decay time and whose vertical axis intercept can be compared with that of a standard solution of the fluorophore for quantitative analysis. However, if the lamp pulse time and the decay time of the fluorophore are comparable, the lamp characteristics must be subtracted from the observed signal to obtain the fluorophore's decay characteristics. A computer is usually used to solve a deconvolution integral representing the composite temporal characteristics of the lamp and the fluorophore output (43–45).

The pulsed-source (time-resolved) method effects spectroscopic separation of the emissions of several fluorescing species by taking advantage of differences in their decay times rather than their fluorescence intensities. Thus, several overlapping fluorescences, such as those of free and antibody-bound ligand, can be quantitated simultaneously. Although the stroboscopic approach is useful when a single species has a decay time much longer than other species in the solution, it does not give good results when several species in solution have fairly close (within an order of magnitude) decay times. In this regard, time-correlated single-photon counting is indispensable. Time-resolved fluorometry is also extremely useful for the elimination of interferences due to Rayleigh and Raman

scatter. Since these processes occur on a time scale of 10^{-14}–10^{-13} sec, they are faster than the lamp pulse time and are not represented in the signal that reaches the detector.

Time-resolved fluorometry has not reached its full potential. Its routine use in fluorescence immunoassay is inhibited by the high cost and complexity of the instrumentation, but this situation could change in the foreseeable future.

HETEROGENEOUS ASSAYS

Most research and development of fluorescence immunoassays have been concentrated in the area of homogeneous assay. However, a major limitation in the application of homogeneous fluoroimmunoassay to the therapeutic monitoring of drug concentrations in human serum is the presence of endogenous interferences. This limitation usually takes the form of a loss of fluorometric sensitivity due to the presence of a high background emission from endogenous proteins and other species and a small signal from the analyte. Notwithstanding the speed and simplicity of the homogeneous assays, it is occasionally desirable to circumvent the problem of background emission by physically separating the antibody–ligand complex from other species in the sample before fluorometric quantitation. This approach forms the basis of heterogeneous immunoassay.

The separation of the antibody-bound–ligand complex from free ligands and other fluorescing species present in solution can be accomplished by various methods that are already in use in radioimmunoassay. These methods are based on the chemical or immunological differences between the free ligand and the antibody–ligand complex. The charge, size, solubility, and surface configuration (immunological specificity) are among the determinants used to separate the free and bound forms (1).

Some techniques that are most commonly encountered in heterogeneous methodology are gel permeation chromatography, chemical precipitation with inorganic salts or organic solvents, and double antibody methods (46, 47). Gel permeation or filtration chromatography enables the separation of free and bound forms of the fluorescent ligand due to the differences in molecular size between the micro- and macromolecular entities (48). The chemical precipitation methods used in radioimmunoassay have not been thoroughly investigated for use in fluoroimmunoassay. The procedures are based essentially on the differential precipitation of proteins. Antibodies can be precipitated with ethanol and dioxane or salted out with ammonium sulfate. Double antibody separation techniques are highly versatile and can be applied to almost any assay system. The separation of the ligand–antibody complex results from the precipitate that forms after a second antibody is introduced. The second antibody is directed against the antibodies of the primary ligand–antibody reaction and is produced in a different species of animal than the first. A good example is the heterogeneous assay of gentamicin. After the competitive binding between gentamicin and labeled gentamicin for sites on a rabbit antibody (directed against gentamicin) comes to equilibrium, separation is achieved by precipitation of the gentamicin–rabbit antibody and labeled gentamicin–rabbit antibody complexes with goat anti-rabbit immunoglobulins.

One promising separation (heterogeneous) technique involves the use of a solid phase support to which an antibody population is either adsorbed or bonded covalently (49). The solid material may be paper disks, the walls of test tubes, glass or plastic beads, or even Sephadex or Sepharose (50, 51).

Once the antibodies are immobilized on the solid support, labeled and unlabeled ligands are introduced and allowed to compete for available binding sites on the antibody. The bound labeled fraction then is separated from the labeled ligand remaining in solution by washing. The labeled ligand complexed to the antibody then can be measured directly, without removal from the solid phase, by a fluorometer with a front-surface fluorescence attachment (52). The fluorescent label also may be removed from the solid phase by a denaturant and measured in a conventional fluorometer (4).

A variation of the solid-phase methodology may involve a solid-phase ligand that competes with free ligand and labeled antibody. The solid-phase bound, labeled antibody then may be measured after washing to remove the unbound, labeled antibody.

The ability to reduce background interference commonly encountered in fluoroimmunoassays will make heterogeneous assays more attractive in the near future. The time and effort spent in immobilizing the antigen or antibody in this system, as well as the actual separation of the labeled and unlabeled species, will ultimately be rewarded with enhanced sensitivity. Advances in the technology of the solid phases and instrumentation used for heterogeneous immunoassay are likely. Already, specialized "immunofluorometers" designed for front-surface illumination are available commercially.

CONCLUSION

The application of fluorescence immunoassay to the detection and quantitation of biologically active compounds provides a major alternative to chromatographic and radiochemical methods of analysis. The elimination of potentially hazardous radioactive labels and the ability to measure, quite sensitively, serum levels of active agents without prior extraction and separation have propelled fluoroimmunoassay into the mainstream of modern analytical techniques. Further developments in fluorescent probes, antibody production, heterogeneous methodology, and fluorescence instrumentation will enhance the attractiveness of fluoroimmunoassay.

The development of novel fluorescent probes for use in immunoassays will be an important area of research. Probes having greater Stokes shifts, greater environmental sensitivities, and high quantum yields of fluorescence that are easily coupled to drugs may replace the limited number of probes now in routine use.

The introduction of new instrumentation and accompanying methodology will also broaden the scope of the applications and improve the sensitivity obtained in fluoroimmunoassay. A recent study reported the use of an argon-ion laser in the detection system of a high-pressure liquid chromatographic separation of free and bound fluorescent antigens (48). This technique employs gel per-

meation chromatography for the separation of fluorescein-labeled insulin, bound by specific antibodies, from the free fluorescent antigen. The detection limit is comparable to that obtained in radioimmunoassay. The laser as an excitation source will no doubt play a much greater role in fluoroimmunoassay (48).

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