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Immunoassays with Time-Resolved Fluorescence Spectroscopy: Principles and Applications

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This review deals with the application of time-resolved fluorescence spectroscopy in the field of immunoassay. The advantages of time-resolved fluorescence over conventional fluorometry are discussed and it is explained why the fluorescent lanthanide chelates, and especially the Eu^{3+} chelates, are the labels of choice in such applications. A brief description of commercially available time-resolved fluorometers is given. The principles of phase-resolved fluorometry are described. The relative merits and disadvantages of the two time-resolved assay principles currently available commercially are critically discussed. General assay designs by using either Eu^{3+} or a new Eu^{3+} chelate as the label are also discussed in some detail. A personal view of future trends in the field of immunoassay concludes this review.

KEY WORDS: non-isotropic immunoassay; europium; time-resolved fluorescence; fluorescent labels.

Radioimmunoassay (RIA) was introduced about 27 years ago by Yalow and Berson (1). At the same time, Ekins described a competitive binding assay using a naturally occurring binding protein (2). This technique has contributed significantly to the advancement of science because it offered the possibility of assaying a variety of molecules with exceptional sensitivity and specificity. Specificity and strong binding are intrinsic abilities of the antibodies, which are necessary for their function as defence molecules. These same characteristics give specificity and sensitivity to immunoassays. The sensitivity of the technique arises also from the use of radioisotopes as the detection system. Current state of the art competitive and noncompetitive radioisotopically based immunoassays are capable of measuring in the subpicomolar range, the limit being $\sim 10^7$ molecules/mL, according to Ekins (3).

Immunoassay techniques were used traditionally for the assay of polypeptide, thyroid and steroid hormones in human serum, contributing most to studies in endocrinology. Increasingly, immunoassays have come to contribute to other areas of clinical biochemistry. A variety of metabolites, vitamins, drugs, viral antigens, antibodies, and tumor markers can now be measured. Moreover, immunoassays are being used in veterinary medicine, agriculture, the food industry, environmental health, and forensic science (4).

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The ^{125}I radioactive nuclide is used almost exclusively as the tracer in radioimmunoassay because of attractive properties described in the literature (5). Immunoassay is a general analytical technique that relies upon the reaction of an antigen with a specific antibody, under competitive or noncompetitive conditions. To monitor the reaction, the label does not have to be a radioactive nuclide. In the last 15 years, many alternative detection systems have been explored, because radioactive labels have some serious disadvantages (Table 1).

The selection of an alternative label for immunoassay is based mainly on sensitivity of detection and freedom from the matrix effects of biological samples containing the analyte. Alternative labels have applicability for assays that do not demand exceptional sensitivity. Fluorescence and enzyme immunoassays not having a separation step (homogenous immunoassays), have almost completely eliminated isotopic techniques from the field of therapeutic drug monitoring. Indeed, certain techniques (e.g., fluorescence polarization immunoassay) are suitable only for small molecules like drugs and do not work at all for the assay of macromolecules.

Alternatives with potential for sensitivities equivalent to radioactive labels include enzyme labels, luminescent labels, and fluorescent labels. Comparative sensitivities for thyrotropin assay by various techniques have been published by Kaihola *et al.* (6). A summary of recent findings is shown in Table 2.

Specific activity of labels

The relative specific activity (defined as the number of detectable events per labelled molecule per unit time) of several commonly used labels is shown in Table 3 (3). In the case of ^{125}I radioactivity, only a minute fraction of the total events generated by the ^{125}I label are available for use during the 1-min counting time usually employed. Most of the potentially detectable events are lost during the storage of the label and are never utilized for assay purposes. A chemiluminescent label will produce at best one detectable event per labelled molecule during the measurement in the cuvette. An enzyme label can produce many detectable "events" per enzyme molecule depending on the turnover number of the enzyme used. A fluorescent label can also produce

TABLE 1
Advantages and Disadvantages of Radioactive Labels

Advantages	Disadvantages
1. Very high sensitivity	1. Potential health hazard
2. Freedom from environmental interference (pH, temp. ionic strength, etc.)	2. Require licensing 3. Special disposal 4. Limited shelf-life 5. Expensive γ-counters
3. Precise measurement of radioactivity	6. Automation difficult
4. No background signal from samples or reagents	

many detectable events per labelled molecule because one molecule can cycle many times through the excited and ground state during a short measurement period. These apparent advantages in specific activity of non-isotopic labels are, however, offset by background, matrix, quenching, light scattering, or other effects that limit the signal-to-noise ratio.

Enzyme immunoassay (11–16) and luminescence immunoassay (17–20) have been extensively reviewed previously. In this review, we will concentrate on fluoroimmunoassays and immunofluorometric assays using time-resolved detection.

Conventional fluorescence vs. time-resolved fluorescence in immunoassays

Several fluors have been used in immunoassays (21,22) but fluorescein is often found in kits. In the following discussion, we will identify some limitations of conventional fluorescence immunoassays and show why the use of europium chelates and the time-resolved principle is theoretically superior.

With close to 100% isotopic abundance, ^{125}I must be present in an amount of about 2 fmol per tube to yield 10,000 disintegrations in 1 min. A detection precision of 1% results. For a fluorescein label to be measured with the same precision in 1 mL would require 0.5 to 20 pmol of fluorescein (23). Fluorescein labelled tracers have been applied successfully for the assay of analytes

TABLE 2
Sensitivity of Serum Thyrotropin Assays

Type of Assay	Sensitivity milli-int. units/L	Reference
TR-FIA	0.02	7
ELIA	0.04	7
EIA	0.1	8
IRMA	0.02–0.1	9
IRMA	0.07–0.25	10
RIA	0.7	10

Note: TR-FIA, time-resolved fluorescence immunoassay (DELFIA™); ELIA, enhanced luminescence immunoassay (Amerlite™); EIA, enzyme immunoassay (Abbott); IRMA, immunoradiometric assay (five different kits in Ref. 9 and six different kits in Ref. 10); RIA, radioimmunoassay (Diagnostic Products Corp.). All assays, except RIA, are noncompetitive "sandwich" type and use either monoclonal or polyclonal antibodies.

TABLE 3
General Indication of Relative Specific Activities of Commonly Used Labels

Specific activity of ^{125}I
One detectable event per second per 7.5×10^6 labelled molecules

Specific activity of enzyme label
Determined by enzyme "amplification factor" and detectability of reaction product

Specific activity of chemiluminescent label
One detectable event per labelled molecule

Specific activity of fluorescent label
Many detectable events per labelled molecule

Note the low specific activity of ^{125}I. Note also that an enzyme label, by "amplifying" the number of detectable events (e.g., using a radioactive substrate) may greatly enhance the effective specific activity of the label and hence the sensitivity of noncompetitive assay systems.

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in the nanomolar range and above but because of detection limits, the picomolar range is not accessible. By contrast, certain lanthanide complexes provide potentially very high detection sensitivity compared with the conventional fluorescent probes, and may even surpass radioisotopes (24).

The problems of conventional fluorometry as applied to immunoassays have been reviewed (21,22). They include separation of fluorescence emission from excitation; Rayleigh and Raman scattering; background fluorescence from cuvettes, optics, and sample; nonspecific binding of the reagents; and fluorescence quenching. One advantage of a lanthanide complex (e.g., an adduct of Eu^{3+} with hydrolyzed 4,7-bis (chlorosulfophenyl)-1, 10-phenanthroline-2,9-dicarboxylic acid) (BCPDA-Eu^{3+}) is evident from its excitation and emission spectra (Figure 1), shown with the spectra of fluorescein and human serum for comparison.

The autofluorescence of serum, which is primarily due to albumin bound bilirubin, overlaps significantly with the emission spectrum of fluorescein and contributes to a high background signal. In the case of the BCPDA-Eu^{3+} complex, there is an exceptionally large Stokes shift (290 nm as compared to ~28 nm for fluorescein), making separation of excitation and emission wavelength very easy. Fluorescence quenching is also reduced and serum autofluorescence effects disappear. The emission band width of the BCPDA-Eu^{3+} complex is narrow (<10 nm at 50% emission) allowing use of narrow bandpass emission filters without loss of energy.

A characteristic feature of europium complexes is that the fluorescence emitted is long lived as compared to classical fluorescent probes (Table 4). By delaying measurement of fluorescence after a flash excitation of the sample, background short lived fluorescence due to serum, solvents, cuvettes, and reagents is excluded. The only background signal observed is due to nonspecific binding of the tracer. In the following section some useful Eu^{3+} complexes and their spectroscopic properties will be described.

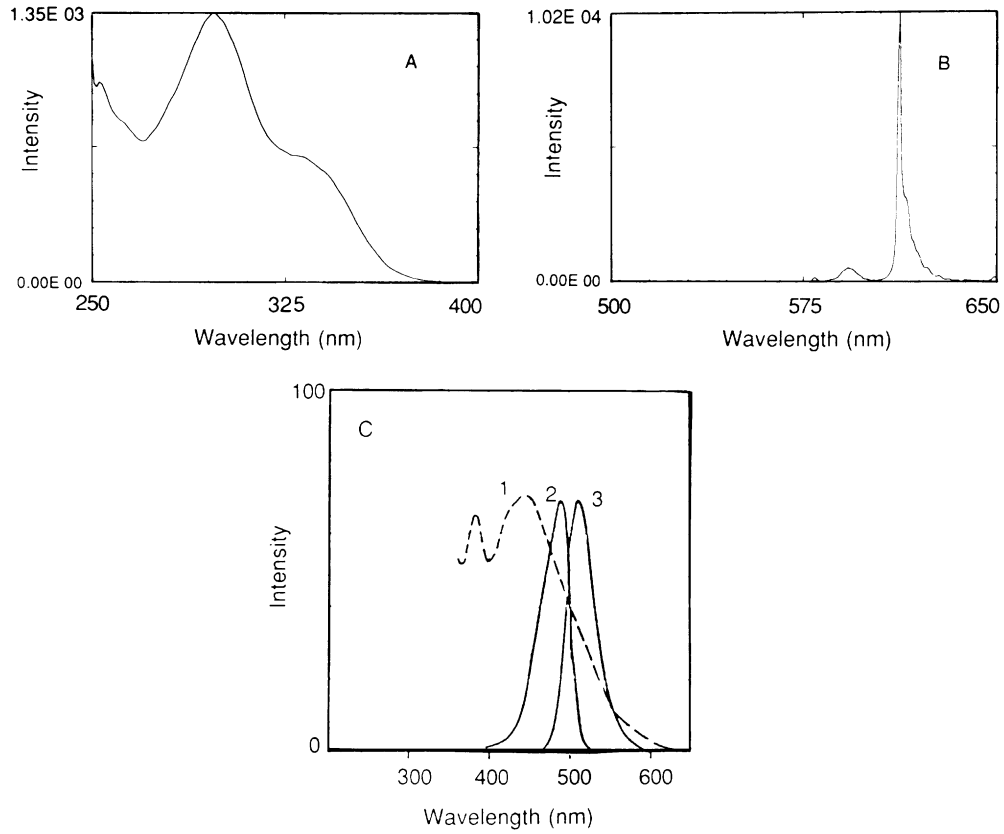

Figure 1—Excitation (A) and emission (B) spectrum of the hydrolyzed 4,7-bis (chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid-Eu^{3+} complex (BCPDA-Eu^{3+}) in solution. Emission spectrum of serum autofluorescence after excitation at 340 nm (C1) and excitation (C2) and emission (C3) spectrum of fluorescein in solution.

Europium complexes and spectroscopic properties

The ground state outer electron configuration of the europium atom is $4f^76s^2$. It loses three electrons to form the stable Eu^{3+} ion with a configuration of $4f^6$. Eu^{3+} forms relatively weak complexes with halides, halates, phosphates, sulfate and nitrate (25,26). Eu^{3+} in aqueous solution is surrounded by an inner hydration sphere of eight to nine water molecules. These water molecules can be excluded by a number of inorganic or organic ligands thereby affecting the fluorescence properties of the complexes formed. Eu^{3+} precipitates at alkaline pH due to formation of insoluble hydroxides.

Various polycarboxylic acids such as ethylenediaminetetraacetic acid (EDTA), ethylene bis (oxyethylenenitrilo) tetraacetic acid (EGTA), and diethylenetrinitrilopentaacetic acid (DTPA) complex with Eu^{3+}. These complexes are very stable, with stability constants in the range of 10^{16} to 10^{22} $Lmol^{-1}$, for the 1:1 metal-chelate adducts (27), and are useful for carrying Eu^{3+} in a noncovalent manner when the polycarboxylic acids are linked covalently to antibodies. Eu^{3+} can be dissociated completely from such ligands by lowering the pH because the concentration of the complexing anionic species is diminished. The Eu^{3+}-polycarboxylic acid complexes are, however, not very fluorescent.

A different class of Eu^{3+} chelates are the β-diketones. Many investigators have studied β-diketone-Eu^{3+} or Tb^{3+} complexes that are usually very fluorescent (28–31). The maximum number of ligands per metal ion is three. The β-diketones have two oxygen atoms available for coordination so that a total of six oxygens are coordinated around the metal ion. The Eu^{3+} ion, however, prefers eight to nine oxygen molecules in its coordination sphere, the balance usually being made available by coordinated water molecules. An "insulating sheath" can be created around an Eu^{3+} ion, if a

TABLE 4
Lifetimes of Some Fluorescent Probes Used in FIA

Probe	τ, ns
Fluorescein (FITC, DTAF)	4.5
Rhodamines	
RBITC	3
RB 200 SC	1
DANS	14
ANS	16
Fluorescamine	7
N-(3-Pyrene)-maleimide	100
Eu-(ß-NTA)$_3$	500,000

Note: FITC, Fluorescein isothiocyanate; DTAF, dichlorotriazinylaminofluorescein; DANS, dansyl chloride; ANS, anilinonaphthalenesulfonic acid; RBITC, rhodamine B isothiocyanate; RB 200 SC, lissamine rhodamine B sulfonyl chloride; NTA, naphthoyltrifluoroacetone.

synergistic agent having an oxygen atom available for coordination can be found, thereby excluding water. Trioctylphosphine oxide (TOPO) has a lone oxygen atom suitable for this purpose and a Eu(β-diketone)$_3$ (TOPO)$_2$ complex can be formed. The exclusion of H_2O from the coordination sphere of Eu^{3+} is desirable because the quantum efficiency of fluorescence is higher.

Europium itself will fluoresce when excited by an intense radiation but this property is not very useful because the quantum yield is low. When Eu^{3+} is suitably complexed, it can form highly fluorescent complexes that can be used to measure Eu^{3+} down to 10^{-13} mol/L (32). An interesting characteristic of the fluorescent Eu^{3+} complexes is that they absorb radiation at the wavelength characteristic of the ligand and emit radiation with the wavelength characteristic of Eu^{3+}. This is now known to be due to energy transfer from the ligand to the metal ion (31,33,34), (Figure 2). Energy is absorbed by the organic ligand leading to an excited state as the electrons migrate from the ground state singlet to the excited state singlet. The excitation may lead to any vibrational multiplet of the excited state S_1. The molecule rapidly returns to the lowest energy level in S_1 by a nonradiative process. At this stage, there are two possibilities. Either the molecule can return from S_1 to S_0 by a radiative transition (ligand fluorescence) or a nonradiative transition, or it can go over to one of the triplet states, T_1. From the triplet level, the molecule can go to the ground state S_0 by a $T_1 \rightarrow S_0$ radiative (molecular phosphorescence) or a nonradiative process. The energy can also be transferred to the metal ion, which may be excited, and subsequently emit characteristic radiation (ion-fluorescence, $d \rightarrow S_0$). For these series of events to be completed successfully, the following requirements must be fulfilled: (a) nonradiative deactivating transitions and

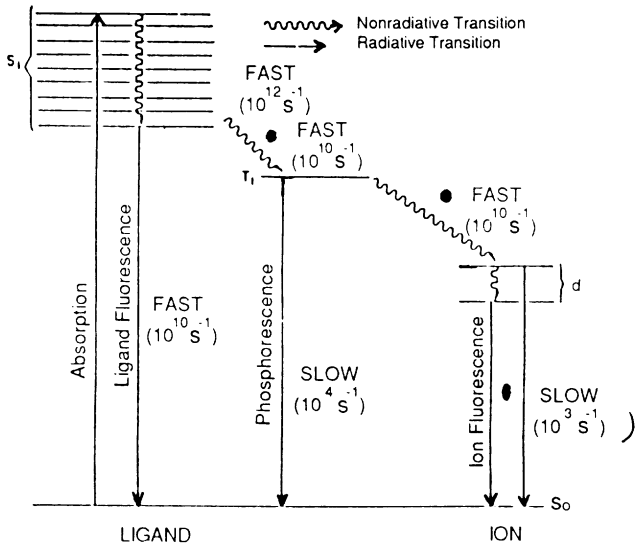

Figure 2—The energy transfer mechanism. For details see text. The non-radiative transitions from $S_1 \rightarrow S_0$, $T_1 \rightarrow S_0$ and $d \rightarrow S_0$ may occur but they are not shown. In parenthesis are the approximate rate constant values according to Kleinerman. The value of $10^4 s^{-1}$ is for an uncomplexed ligand transition.

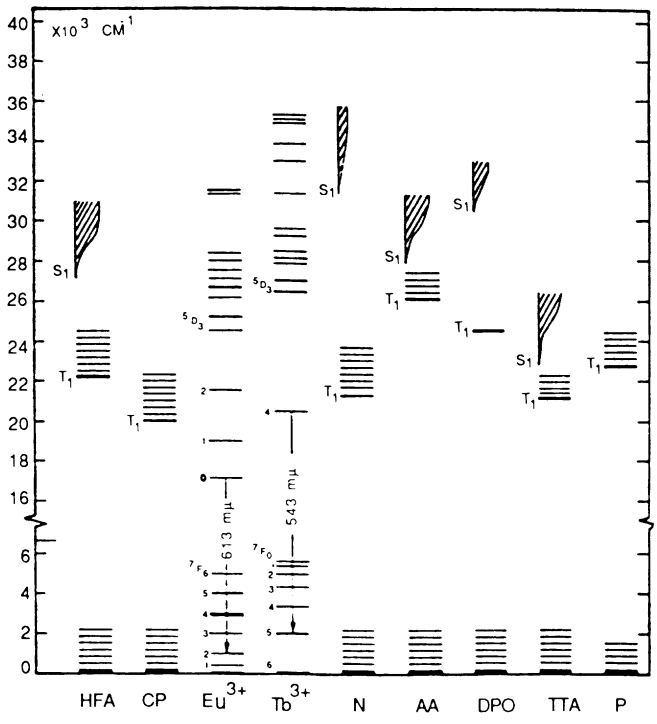

Figure 3—Energy levels of some organic compounds and Eu^{3+} and Tb^{3+}. HFA: hexafluoroacetyl-acetonate; CP: *cis*-piperylene; N: naphthalene; AA: acetylacetonate; DPO: diphenylene oxide; TTA: thenoyltrifluoroacetonate; P: perylene. Reprinted with permission. See Ref. 34.

$S_1 \rightarrow S_0$ and $T_1 \rightarrow S_0$ radiative transitions should be minimal (b) the energy of the ion-resonance level should be close to, and preferably just below, that of the triplet state level of the ligand. In that case, the probability of the triplet to resonance level transition ($T_1 \rightarrow d$) is high, and (c) the radiationless transitions of the excited metal ion should be low. Kleinerman (33) has proposed that except for the $T_1 \rightarrow d$ transition, it is possible to have intersystem crossing via the ligand excited singlet state without the participation of the triplet state ($S_1 \rightarrow d$). This may happen if the rate constant of energy transfer from the singlet excited state of the ligand to the triplet state is relatively small. The preferred pathway depends on the nature of ligand participating.

In Figure 3, the energy levels of some organic molecules and of Eu^{3+} and Tb^{3+} are shown. The predominant radiative transition of excited Eu^{3+} after an energy transfer from a triplet state of the ligand is $^5D_0 \rightarrow {}^7F_2$ with an emission wavelength of 613 nm. For Tb^{3+}, the $^5D_4 \rightarrow {}^7F_5$ transition is prominent, with an emission wavelength 543 nm.

The fluorescence lifetime of the Eu^{3+} complexes is 10 to 1000 μs compared to nanoseconds for most conventional fluorescent probes (Table 4). The lifetime depends on temperature and the solvent present. The long lifetime is mainly due to the $^5D_0$ state of Eu^{3+} (and the $^5D_4$ state of Tb^{3+}). The transitions of the $S_1 \rightarrow S_0$ type (molecular fluorescence or radiationless) and those of the $S_1 \rightarrow T_1$ and $T_1 \rightarrow d$ types are several orders of magnitude faster than the radiative transitions of the

excited rare earth ions. The $T_1 \rightarrow S_0$ transition (ligand phosphorescence or radiationless) may also be slow but this transition does not usually occur appreciably because of the rapid energy migration from the long lived T_1 level to the *d* levels of the ion.

Instrumentation for time-resolved fluorescence

Time-resolved fluorometers have all the usual components of a conventional fluorometer plus a system for time-gated measurements of only a portion of the total emission cycle. During the rest of the emission cycle, the photomultiplier is inactive and unwanted events (e.g., short-lived fluorescence) are undetected. The time-gating has to be optimized for the chemical system used. Figure 4 shows a typical sequence of events.

The excitation light is usually a flash of very short duration in comparison to the fluorescence lifetime of the chemical system. In the "ARCUS" fluorometer (24,35), commercially available from LKB, Turku, Finland, the excitation light pulse duration is ≤ 1 μs. The flash tube used is a bulb-type xenon lamp. A special stabilization circuit allows for a fixed integrated photon emission from the lamp. The flash lamp is activated about 1,000 times per second. Overall, the sequence of events on the ARCUS is as follows: excitation light flash duration, ≤ 1 μs; delay time during which any short-lived fluorescence is decayed, 400 μs; measuring time with photomultiplier active, 400 μs; recovery time before the next cycle, 200 μs. The full cycle is 1 ms and the measuring time per well is 1 s (~1000 flashes). The excitation band is isolated with an interference filter. The time-resolving operation of the ARCUS is maintained by the action of a pulse generator that activates the excitation source and the timing of the emission counter.

In the CyberFluor 615 gated fluorometer and some other instruments described in the literature for research purposes (36,37), the excitation source is a laser. This light source has high output power (photon flux); monochromaticity; good spatial definition; pulse widths of the order of nanoseconds; and excellent between pulse stability. Kuo *et al.* (36) used a helium-cadmium ion laser interrupted at 500 Hz by a light chopper, with interference filters to isolate the specific delayed fluorescence. The CyberFluor 615 analyzer uses a nitrogen laser. The gated system characteristics are as follows: laser emission wavelength 337.1 nm; laser pulse duration 3 to 4 ns; repetition rate 20 pulses/s; delay time 200 μs; measurement time 400 μs; recovery time after completion of measurement to next flash 49.4 ms; cycle time 50 ms; measurement time/well 1 s (20 flashes); usable fluorescence measurements/well is 16.

The CyberFluor instrument differs from the ARCUS and other instruments reported in that it measures fluorescence from a solid phase and not in solution, requiring a special optical system (Figure 5). The ARCUS and CyberFluor 615 analyzers measure samples in an

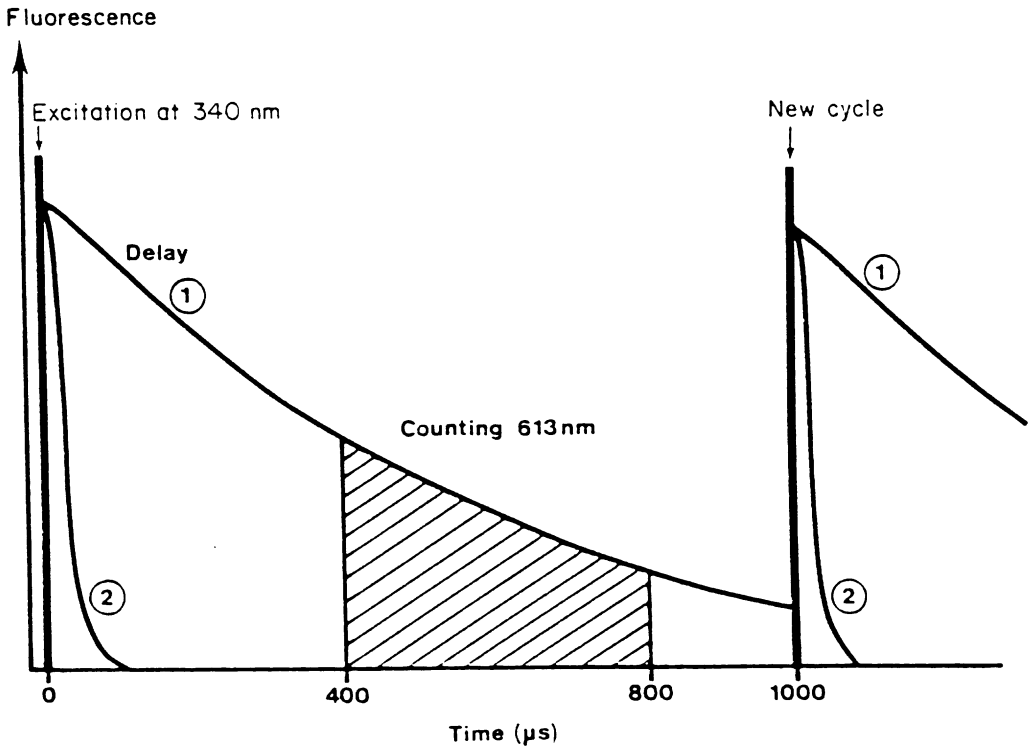

Figure 4—Measurement principle of time-resolved fluorescence. The cycle time is 1 ms and pulsed excitation less than 1 μs occurs at the beginning of each cycle. The delay time after the pulsed excitation is 400 μs and the actual counting time within the cycle has the same duration. The total measurement time per cuvette is 1 s. Curve 1 represents the fluorescence of the europium chelate and curve 2 the background fluorescence (actual decay time less than 1 μs). Reprinted by permission of John Wiley & Sons, Ltd. See Ref. 51.

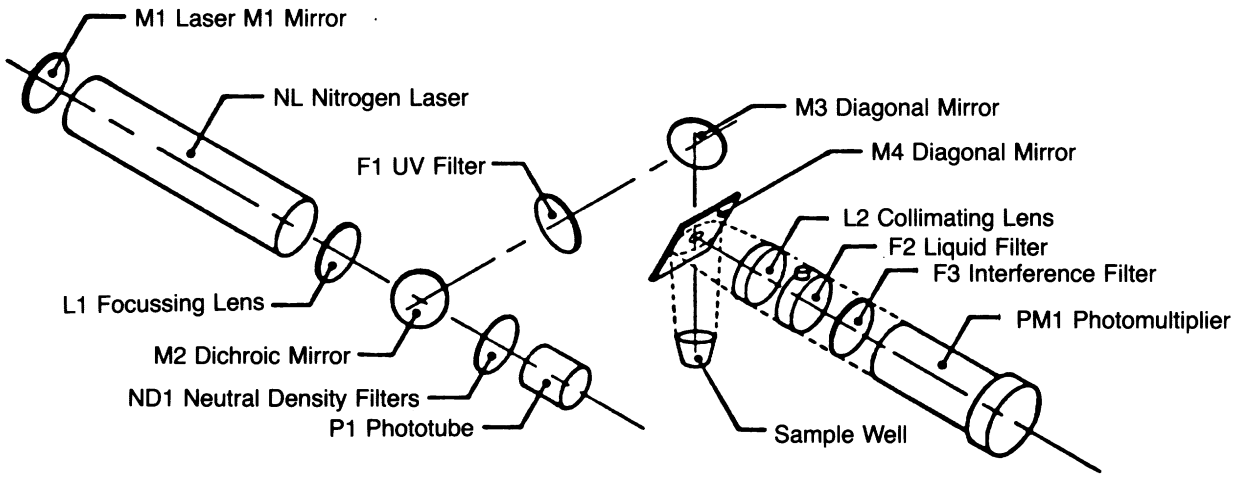

Figure 5—The optical system of the CyberFluor 615 time-resolved fluorometer/analyzer. Phototube P1 is used to monitor the output power level of the laser. The sample well is a white microtitration well. The beam reaches the well through a hole in the diagonal mirror M4 which also reflects fluorescence light to the direction of the photomultiplier.

automated fashion at speeds of 2000 to 3000 samples/h. A more detailed description of the ARCUS is given in the literature (24,35).

Fluorescence lifetime

Every fluorescent molecule is characterised by its own lifetime. How fast the fluorescence decays after excitation can range from nanoseconds to milliseconds. There are various ways of measuring the fluorescence lifetime. One way is by the pulse technique (38). The sample of interest is illuminated with an intense, brief pulse of light and the intensity of the resulting fluorescence is monitored as a function of time. This procedure is identical to the one used for measuring the fluorescence signal in a "time-resolved" fashion for an immunoassay.

The fluorescence decay curve follows first-order kinetics and can be described by the equation:

$$I(t) = I(0) \cdot e^{-k.t} \quad [1]$$

where $I(0)$ and $I(t)$ are the fluorescence intensities at time 0 and t, respectively and k is a rate-constant. If we define the fluorescence lifetime τ as the time required for the excited state population to be reduced to $^1/e$ of $I(0)$, then Equation [1] can be rewritten as follows:

$$I(t) = I(0) \cdot e^{-t/\tau} \quad [2]$$

because when $I(\tau) = I(0)e^{-1}$, $\tau = k^{-1}$. The lifetime so defined is the reciprocal of the rate-constant. Taking the logarithms of Equation [2], we obtain:

$$\ln I(t) = \ln I(0) - {}^{t}/\tau \quad [3]$$

The plot of $\ln I(t) = f(t)$ is a straight line with a slope of τ^{-1} (Figure 6). This plot is not linear when the system analyzed has more than one fluorescent compound exhibiting different lifetimes but there are ways of calculating individual species lifetime from such cumulative experimental data (38).

The fluorescence lifetimes of many conventional fluorescent probes fall in the nanosecond time range. It has to be kept in mind that measuring fluorescence lifetimes with the pulsed excitation technique may be difficult if the lifetimes are very short because the exciting pulse must have a duration substantially less than the lifetime of the fluor. The detection and signal processing electronics must also have a very fast response.

Nanosecond and subnanosecond lifetime measurements can be best undertaken with a different technique called phase-resolved fluorometry (PRF). This method was described in 1927 by Gaviola (39). It involves the use of continuous, sinusoidally modulated excitation combined with phase-sensitive detection. The lifetime resolution with this principle is in the range of 1 to 100 picoseconds.

In the phase modulation approach, the sample is excited with light having a time-dependent intensity $E(t)$ of the form:

$$E(t) = A(1 + m_{ex}\sin \omega t) \quad [4]$$

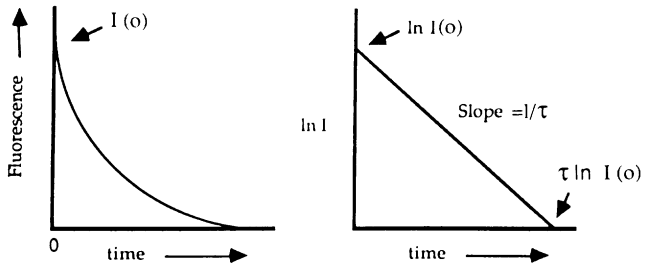

Figure 6—Left panel: fluorescence decay as a function of time after an instant excitation at time zero. With the plot of the right panel, the fluorescence lifetime τ can be calculated. For details see text.

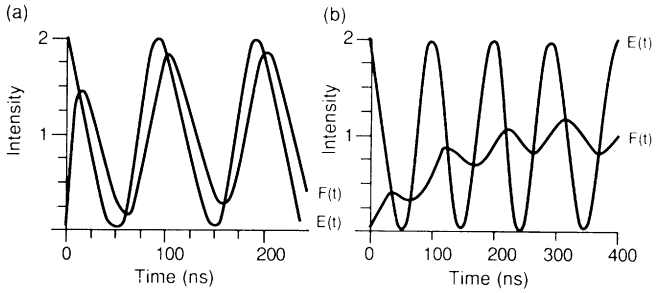

Figure 7—Transient response of luminescent samples to a sinusoidal excitation starting at t = 0. Modulation frequency = 10 MHz. (a) τ = 10 ns; (b) τ = 100 ns. For more details see text. Reprinted with permission from the American Chemical Society. See Ref. 39.

where A is the constant component of the exciting beam, m_{ex} is the ratio of the amplitude of the sinusoidal modulation to the constant component, and ω is the angular modulation frequency ($\omega = 2\pi f$ where f is the linear modulation frequency in hertz).

The resulting time-dependent emission $F(t)$ will be demodulated and phase shifted to an extent determined by the fluorescence lifetime of the species:

$$F(t) = A' (1 + m_{ex}\, m \sin(\omega t - \phi)) \quad [5]$$

where A' is the constant component of the fluorescence emission, ϕ is the phase shift of the species, and m is the demodulation factor:

$$m = \cos \phi \quad [6]$$

The demodulation factor can also be expressed as the ratio of the degree of sinusoidal modulation of the fluorescent species to the degree of sinusoidal modulation of a scattering solution. The phase shift and demodulation effects are shown in Figure 7 for two fluorescent molecules having lifetimes of 10 ns and 100 ns.

The fluorescence lifetime τ can be calculated from the phase shift of the species:

$$\tau = \omega^{-1} \tan \phi \quad [7]$$

or from the demodulation:

$$\tau = \omega^{-1} (m^{-2} - 1)^{1/2} \quad [8]$$

In practice, the phase shift and demodulation of a species are measured relative to either a scattering solution (τ = 0) or a reference fluorophore of known lifetime.

If a molecule has a fluorescence lifetime that changes upon binding to a macromolecule, then it may be possible to measure in a homogenous solution the free fluor in the presence of the bound even if the spectral characteristics of the free and bound form are exactly the same (39). This approach has been successfully applied to a homogeneous assay for phenobarbital (40), using fluorescence lifetime differences of the order of pico-

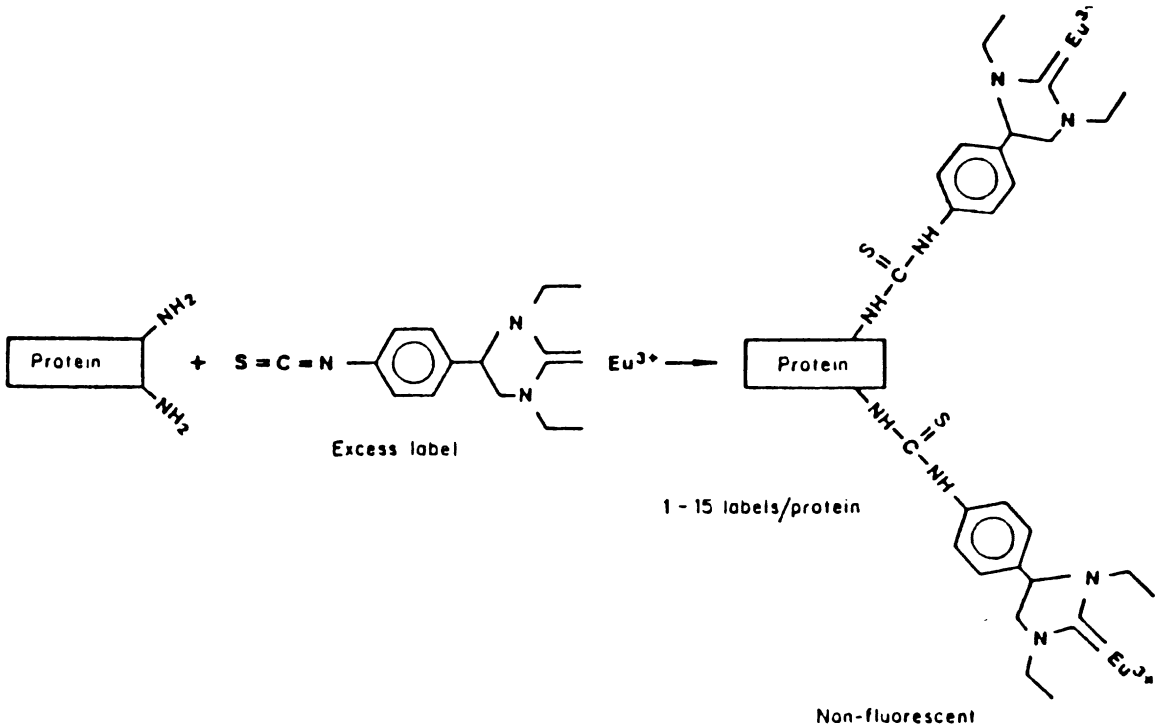

Figure 8—The principle for labelling of protein with isothiocyanatophenyl-EDTA-Eu. The protein is reacted with a 60-fold molar excess of the label at pH ~9.3 overnight at +4°C. The labelled protein is separated from excess reagent by gel filtration on a Sepharose 6B column. Conjugation yield is obtained by measuring the europium fluorescence of the labelled protein in comparison with europium standards. Reprinted by permission of John Wiley & Sons, Ltd. See Ref. 51.

seconds. Other immunoassays based on the same principle have been described for albumin (41) and lactoferrin (42).

Two time-resolved fluoroimmunoassay systems are currently commercially available.

The LKB system

Soini, Hemmila, and their colleagues (43–54), and Wieder (55), were the first to initiate systematic studies for the use of europium as a tracer in immunoassays in the mid 1970s. The LKB system is a general analytical method using Eu^{3+} linked to antibody. It is suitable for the immunoassay of large and small molecules, polypeptides (56–60), steroid, and thyroid hormones, drugs, antigens, and antibodies related to infectious agents (61), tumor markers, etc. The LKB tracer can be prepared as shown in Figure 8. The bridge molecule is isothiocyanatephenyl-EDTA which reacts with the free amino groups of the antibody molecule and is incorporated at a usual yield of 5 to 15 molecules per molecule of IgG. The LKB tracer can be used in both competitive and noncompetitive immunoassays in the usual manner. In competitive immunoassays, the antibody is labelled and the antigen immobilized. These immunoassays require separation of free antibody from antibody bound to a solid-phase. Polystyrene microtiter wells are used as solid-phase with noncovalent adsorbtion of antigen, or, in the case of noncompetitive assays, of antibody. The quantitation of Eu^{3+} is performed by dissociating it at pH 3.2 and complexing it with the diketone 2-naphtoyltrifluoroacetone and the synergistic agent trioctylphosphine oxide, in the presence of Triton X-100. The postulated fluorescent product is shown in Figure 9. The system can detect as little as 10^{-13} mol/L Eu^{3+}. A typical "sandwich" type noncompetitive immunoassay with the LKB system is shown in Figure 10.

The LKB assays, although highly sensitive, have some limitations. (a) Direct quantitation of tracer on the solid-phase is not possible because the EDTA-Eu^{3+} complex does not fluoresce strongly. Europium has to be dissociated and measured in solution in the presence of a different chelate which gives higher fluorescence, adding an extra step to the procedure. (b) The LKB enhancement solution containing the Eu^{3+} chelates is vulnerable to Eu^{3+} contamination from the environment and its background fluorescence increases with time even with extremely careful handling. The relatively high Eu^{3+} concentration in serum (10^{-8} mol/L) (62) should not interfere because it is washed out before the final measurement, but nonspecific binding of serum Eu^{3+} to the microtiter well can contribute to the final fluorescence measurement. Dissociation of Eu^{3+} from the antibody by competition with other serum cations does not seem to be a problem.

Some other groups have used a similar system to develop time-resolved immunoassays (37,63), successfully using Tb^{3+} and Sm^{3+} as well as Eu^{3+}. Kuo et al. (36) used a Tb^{3+}-EDTA complex bound to antigen in

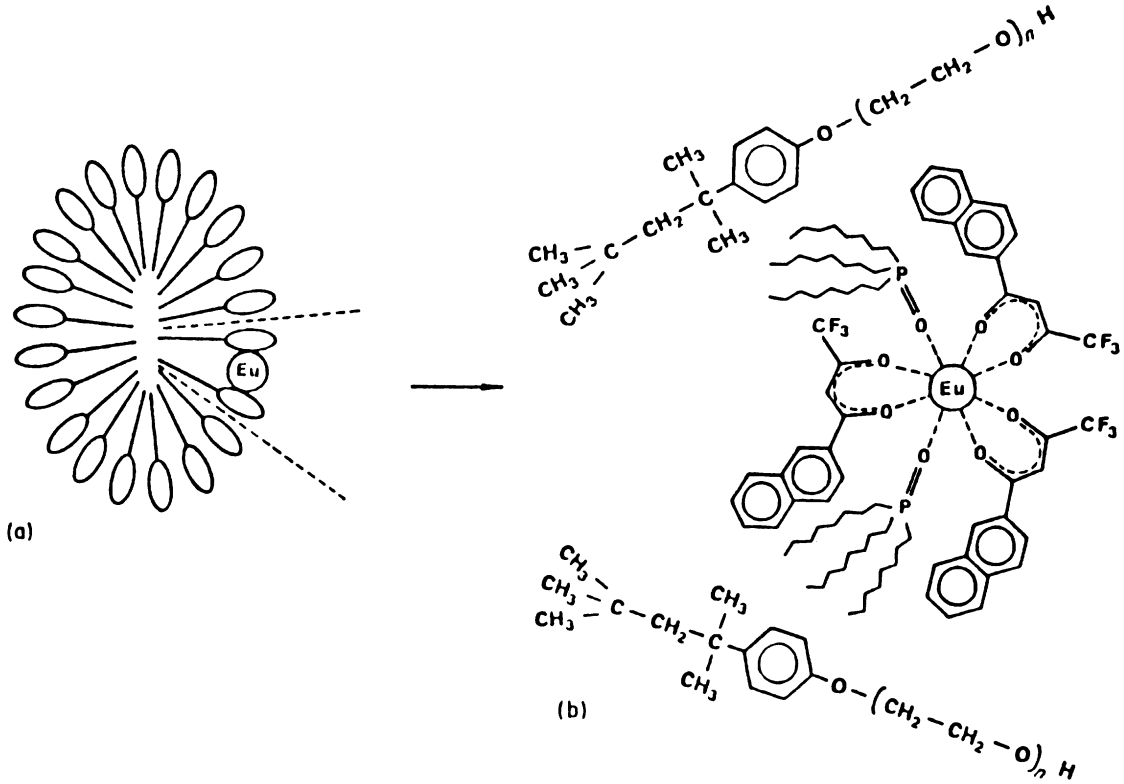

Figure 9—(a) A micelle consisting of Triton X-100 molecules with an association number of about 140, in which a fluorescent europium chelate is solubilized. (b) A hypothesized form of the europium chelate, consisting of an europium ion, three 2-napthoyltrifluoroacetone and two tri(*n*-octyl)phosphine oxide molecules are solubilized in between Triton X-100 structures. Reprinted by permission of John Wiley & Sons, Ltd. See Ref. 51.

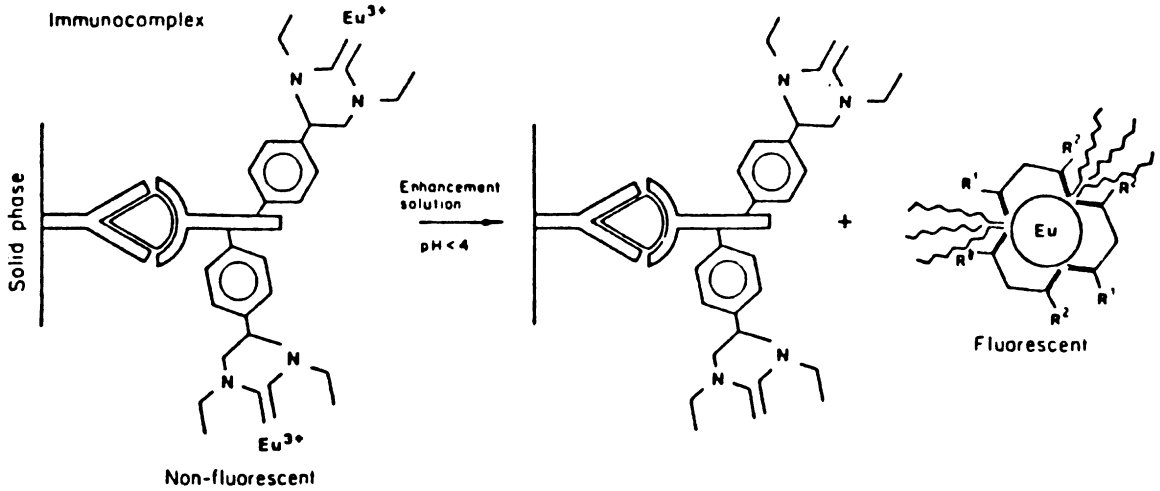

Figure 10—Principle for the europium release after the immunometric assay has been completed. As a final step before fluorescence measurement an enhancement solution is added consisting of acetone-potassium hydrogen phthalate, 100 mmol/L, pH 3.2, containing 2-naphthoyltrifluoroacetate, 15 μmol/L, tri(*n*-octyl)phosphine oxide, 50 μmol/L, and Triton X-100, 1 g/L. The europium ion dissociates from the labelled protein and forms a new fluorescent chelate in solution. Reprinted by permission of John Wiley & Sons, Ltd. See Ref. 51.

devising a competitive time-resolved fluorescence immunoassay of IgG. The fluorescence of free labelled antigen was measured in solution, thus obviating the need for dissociating Tb^{3+} from EDTA before measurement.

The CyberFluor system

Instead of using europium as label for time-resolved immunoassays, it is possible to employ a europium chelate using excess Eu^{3+} to form the complex. After the immunoreaction and all washings have been completed in a competitive or noncompetitive immunoassay, the fluorescence of the chelate-europium complex can be measured either on the solid-phase or in solution.

The ideal chelate for this type of immunoassay must meet the following criteria: (a) It should form very stable complexes with a rare-earth metal; (b) the complex must exhibit very strong fluorescence; (c) the excitation wavelength of the chelate should be close to 337.1 nm, the wavelength of a nitrogen laser; (d) the chelate should be easy to react covalently with proteins; (e) it should have minimum interaction with serum components.

The europium chelates of the diketone type do not fulfill criterion (a); the europium chelates of the polycarboxylic acid type (EDTA, etc.) fulfill criterion (a) but not (b). There are problems of aqueous solubility and stability with theonyltrifluoroacetone, a diketone chelate proposed by Wieder (42).

A new chelating agent, 4,7-bis(chlorosulfophenyl)-1,10phenanthroline-2,9dicarboxylic acid, BCPDA (Eurofluor-S™*) (Figure 11) meeting the above criteria is used as a tracer in the CyberFluor system (64). The molecule has two sulfonylchloride groups that can react covalently under mild conditions with available amino groups of protein molecules. The chelating site consists of two heteroaromatic nitrogens and two carboxyl groups.

Streptavidin labelled with BCPDA has been developed as a universal detection system. Streptavidin is available in pure form, it can be labelled with BCPDA easily and with high yield, and without loss of its biological activity. Both labelled streptavidin and the biotinylated antibodies used in the system are stable. A common detection reagent can be used for all competitive and noncompetitive assays with amplification of the response. Additionally, the nonspecific binding of labeled streptavidin is low. Typical assays with this system are shown in Figures 12 and 13. All reactions are carried out in white microtitration strip wells and fluorescence is measured on the solid-phase with a spe-

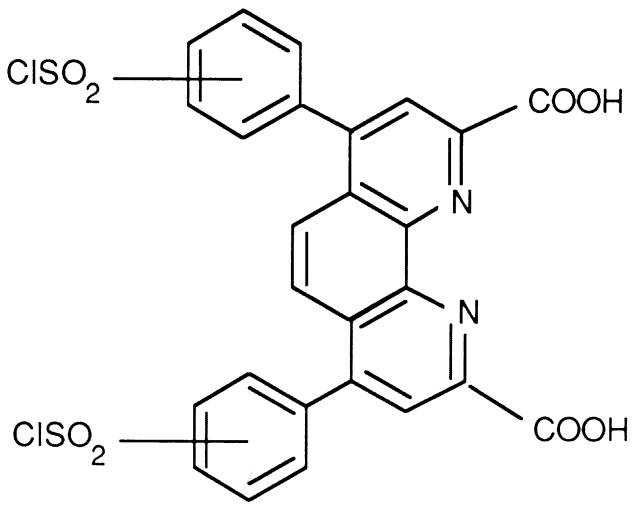

Figure 11—The structure of the Eu^{3+} chelate 4,7-bis-(chlorosulfophenyl)1,10phenanthroline-2,9-dicarboxylic acid. The sulfonylchloride groups are used for covalent bonding with available amino groups of streptavidin.

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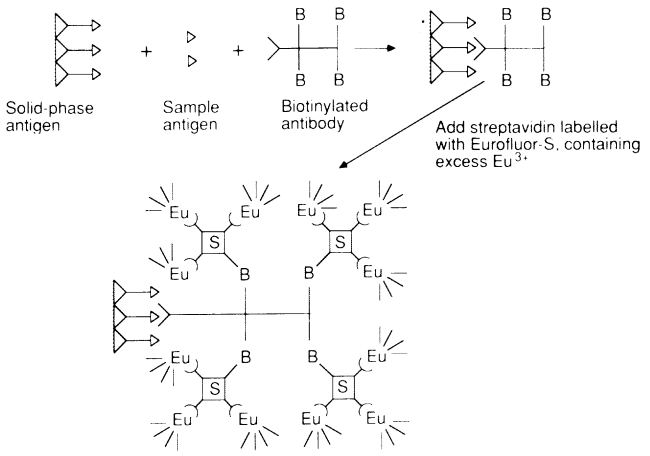

Figure 12—Schematic depiction of competitive immunoassay of a hapten with the CyberFluor system. B = biotin. S = streptavidin. The fluorescence of the final product is measured on the dry solid-phase.

cially designed instrument (Figure 5). This type of immunoassay lends itself to automation. Successful applications include the noncompetitive assay of alpha-fetoprotein (65) and choriogonadotropin (66) and the competitive assay of cortisol in serum (67).

Conclusion

During the next 5 to 10 years, nonisotopic immunoassay will continue to grow and isotopic immunoassay will steadily decline. The two techniques will coexist in the clinical laboratory for many years since isotopic labels have unique advantages, the instrumentation is now widely distributed, and the menu of isotopic immunoassay is very rich. Homogenous nonisotopic immunoassays are now preferred over isotopic immunoassays for assaying drugs, thyroid and some steroid hormones. These assays are suitable for antigens present in serum in the micro and nanomolar range.

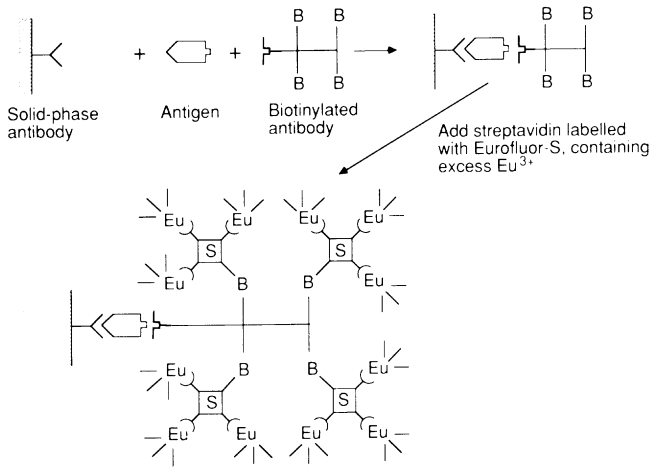

Figure 13—Non-competitive 'two-site' immunoassay of an antigen with the CyberFluor system. Other details as in Figure 12.

It is now clear that detection limit is not a restrictive factor in heterogenous nonisotopic immunoassay and there are many reports showing better detection limits for alternative immunoassays over isotopic immunoassays. The extent of vulnerability of alternative immunoassays to the matrix effects of biological samples remains to be established. In terms of automation, there are currently instruments in the market suitable for performing nonisotopic immunoassays in a fully automated fashion and with random access capability. Turnaround times vary between 30 and 120 min. The assay menu of these analyzers is still very limited and they are only suitable for high volume throughput (e.g., thyroid function tests). At the moment, the most promising alternative labels are enzymes, luminescent and fluorescent labels. Time-resolved fluorescence eliminates most of the limitations of conventional fluorescence and shows promise of becoming a prominent alternative immunoassay technique.

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