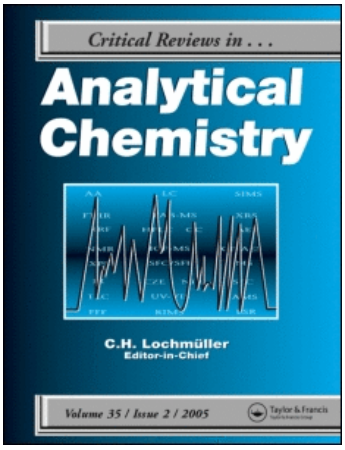

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TIME-RESOLVED FLUORESCENCE OF LANTHANIDE PROBES AND APPLICATIONS IN BIOTECHNOLOGY

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I. INTRODUCTION

A. Definition of Time-Resolved Fluorometry

The relaxation of excited molecular states is often followed by the emission of light. Molecular excited states are related to the orbital, vibrational, and rotational levels of the orbital electrons. In addition, the electron spin determines the singlet and triplet states. In the singlet state the spin of an electron is paired, whereas in the triplet state the spin is not paired. When a molecule is excited to an upper singlet energy level, such as S_2 (Figure 1), it can rapidly (10^{-10} sec) go to the lowest excited state S_1 without emission of light. From S_1 the molecule may go to any of the rotational and vibrational levels of the ground electronic state S_0. This happens either by fluorescence emission or by nonradiative internal processes, referred to as intersystem crossing. From T_1 the molecule can return to S_0 by nonradiative processes or by a radiative process called phosphorescence. Under suitable conditions S_1 and T_1 can also transfer their excitation energy to other molecules.

The important processes of fluorescence excitation and emission are characterized by the fluorescence spectrum, quantum yield, fluorescence decay time, and polarization. The fluorescence spectrum represents the intensity of fluorescence at different wavelengths. The more complex and less symmetrical the compound, the wider the fluorescence emission spectrum. The difference between the wavelengths of the excitation and emission maxima is called the Stokes shift. The extent of the Stokes shift indicates the energy dissipated during the lifetime of the excited state before it returns to the ground state. The fluorescence quantum yield is the fraction of excited molecules that emit light. Quenching is the reduction of fluorescence by a competing deactivating process from interaction with another substance present in the system. The effect of quenching can be stronger for fluorescent substances with a long decay time. The fluorescence decay time τ is defined by the equation

$$I(t) = I_0 e^{-t/\tau} \quad (1)$$

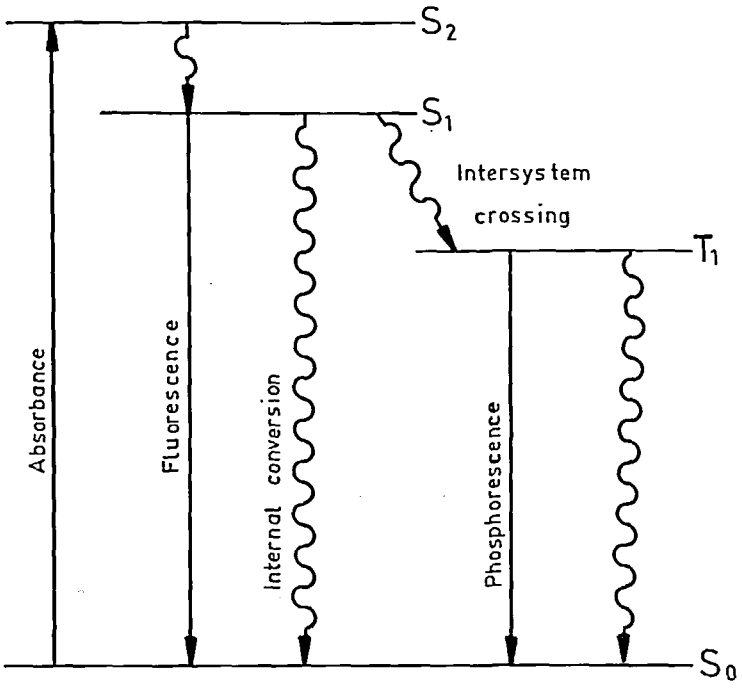

FIGURE 1. The electronic energy levels and transitions in a fluorescent organic molecule. S_0 = ground-state singlet level, $S_{1\text{-}2}$ = excited singlet levels, $T_{1\text{-}2}$ = triplet levels, → = radiative energy transfer, ⇝ = nonradiative energy transfer.

which describes the decay of fluorescence intensity after an instantaneous pulse of excitation light. The excited state decay time of a molecule at S_1 may range from a few tenths to several hundred nanoseconds, depending on the molecular structure. T_1 has a comparatively long decay time ranging from some hundred microseconds to several seconds, depending both on the molecular structure and on the temperature. Certain inorganic substances, particularly the lanthanides, have a relatively long fluorescence decay time ranging from 1 μsec to 1 msec, which is here called "ion fluorescence."

The registration of fluorescence emission spectra at different decay times from the excitation pulse is called time-resolved fluorescence spectroscopy. This technique has widely been used in the study of the conformation and dynamics of molecules.[1] The fluorescence spectrum, quantum yield, and decay time are dependent on the molecular structure. They are also sensitive to the chemical environment, and for that reason fluorescence spectroscopy is a useful tool in the study of the interactions between macromolecules and their molecular environment.[2] Time-resolved fluorescence spectra are composed of different types of emissions. During the excitation pulse Rayleigh and Raman scattering and extremely short decay fluorescence components are dominating. Later, these spectra consist of fluorescence emissions with different, longer decay times. Time-resolved fluorometry naturally makes it possible to distinguish between short and long decay components, in particular, a long decay emission can be separated from the background signal, which most often consists of short decay fluorescence and scattering.[3,4]

In relation to the instrumentation the field can be divided into three main categories:

1.	Phosphorescence	decay time 1 msec to 100 sec
2.	"Microsecond" fluorescence	decay time 1 μsec to 1 msec
3.	"Nanosecond" fluorescence	decay time 1 nsec to 1 μsec

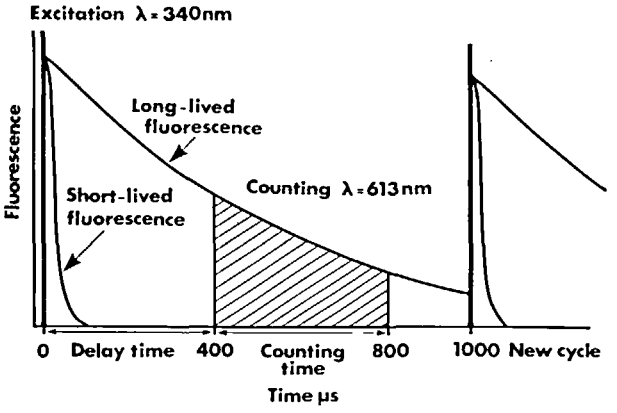

FIGURE 2. Schematic presentation of operation of a pulsed source time-resolved fluorometer and phosphorimeter.

The principles used to measure phosphorescence, long decay fluorescence, and nanosecond fluorescence are basically the same (Figure 2). However, the instrument technology applied to each category differs significantly.

Traditional phosphoroscopy is based on the use of rotating discs or cylinders as mechanical choppers exposing the sample to the excitation light source and the photodetector at different times. Delay times and exposure times are adjusted by varying the rotation speed and the width of the window.[5]

Microsecond fluorescence requires a much faster function than is possible using mechanically moving components. Xenon flash tubes have been found applicable, the flash time being typically 1 to 10 μsec. A photomultiplier tube can be used as detector and this is often equipped with electronic gating through certain of its electrodes. The need for gating is dependent on the intensity of the flash pulse. With low intensity flashes a photomultiplier tube gating is not necessary and satisfactory.[6]

Nanosecond fluorescence analysis requires a pulsed light source with pulses of less than 1 nsec duration. Two alternatives have been widely used: gas-filled spark light sources and lasers.

Typically, the nanosecond time-resolved fluorometers use very low excitation intensity and monitor only one photon per excitation pulse (here called the "monophoton fluorometric system"). In this case the resolution time of the photodetector should be shorter than the decay time. The excitation intensity is low enough to allow detection of an average less than one photon per pulse. Decay time studies and time-resolved spectroscopy are normally based on the registration of the time of arrival of emitted single photons. The histogram of a large number of such events represents the actual decay curve. The precise registration of a decay curve or a complete time-resolved emission spectrum takes a long time; this is because the maximum repetition frequency of a pulsed light source is limited (e.g., 1000 pulses per second). Consequently, the monophoton systems are not useful for routine high-throughput analysis.

Microsecond fluorometry and phosphorimetry are "multiphoton fluorometric systems" and therefore can provide short counting times.

B. Definition of Applications

The subject of this review is microsecond fluorometry and related fluorescent probes used for labeling organic and biochemical compounds. In time-resolved fluorometry the fluorescence emission can be distinguished into prompt fluorescence and long decay fluorescence

(Figure 2). The most important sources of the background of biochemical and biological samples are the "autofluorescence" and the scattering. The light emitted from these sources appears simultaneously with the excitation as the fluorescence decay time of bioorganic substances is very short. Time-resolved fluorometry efficiently discriminates the background from the long decay fluorescence of the probe, because measurement does not occur until a certain time has elapsed form the moment of excitation. Thus, it offers an almost ideal way of measuring long decay ion fluorescence and considerably improves the sensitivity compared with the conventional measurement methods with short decay time organic fluorescent probes.

Certain aspects of nanosecond fluorometry are also discussed, as this technique can be regarded as a useful analytical tool in routine applications in the future. The particular applications can be called "biospecific assays" based upon a specific reaction between the analyte and the reactant. The fluorescent label coupled to the analyte, or to the reactant, provides the means of reaching a very high detection sensitivity.

The reactants to be labeled are typically antibodies or antigens and the related assays, called immunoassays, are an important part of the routine workload in every clinical biochemistry laboratory today. The antigen (the analyte) or the antibody itself can be attached to a label and, respectively, an assay of this type is called a competitive binding immunoassay or a noncompetitive immunometric assay.

Radioimmunoassays (RIA, IRMA) are the most widely used immunoassays. Although extremely sensitive and quite precise, these assays have several drawbacks. Radioactivity may be a health hazard and special attention to handling of reagents, training of staff, and storage of waste is required. The useful lifetime of a kit is limited by the half-life of the isotope and, consequently, by the efficiency of kit distribution after manufacture. Measuring radioactivity often involves long counting times, which often means that certain delays in producing results are encountered.

For these and other reasons, alternative analytical methods have been developed. Among the alternative labels developed as a substitute for radiotracers are enzymes, luminescent compounds, metals,[7,8] and fluorescent probes.

Another important group of assays is based on the use of DNA probes as the specific binding reactant. Today only phosphorus-32 can be regarded as a satisfactory label owing to its sensitivity and practicability. The short half-life, high cost, and radiation hazard are major disadvantages in the use of these labels and, consequently, suitable nonisotopic alternatives are urgently required.

Within the scope of this review the following general requirements are suggested for an assay with fluorescent labels applicable to biospecific assays:

- Fast measurement, high throughput
- Sample size: 10 to 100 $\mu\ell$, reaction volume 0.1 to 1 mℓ
- Precision 1 to 10%
- Sensitivity 1 ng to 1 pg/mℓ
- Reduction of the "autofluorescence" and scattering
- Applicability to haptens, polypeptides, antibodies, viruses, DNA, etc.

Microsecond fluorescence with time-resolved detection is a potential choice, because it fulfills all the requirements defined above. It is a multiphoton fluorometric system and the excitation pulse repetition rate can be made reasonably high (up to 1000 Hz) which allows the samples to be measured with satisfactory precision in a counting time of 1 sec. Fluorescence decay times ranging from 100 to 1000 μsec are the most optimal and the electronics required can be made using conventional digital counting circuits and components. This naturally makes the instrumentation more simple and less expensive.

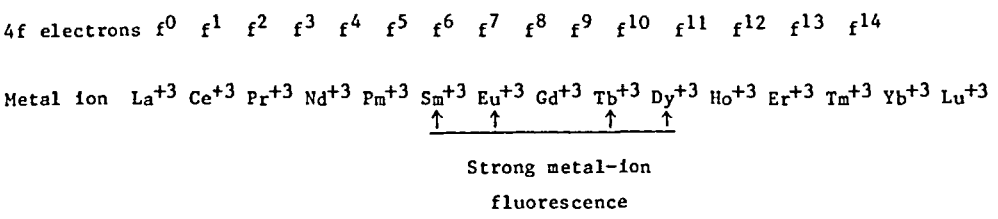

FIGURE 3. Lanthanides and structure of the 4f electronic shell.

Lanthanides and their chelates are a special group of compounds which display ion fluorescence with decay times on the order of 50 to 1000 μsec. This makes these compounds very useful for time-resolved microsecond fluorometry. In comparison to the best organic fluorescent compounds the lanthanide chelates can provide comparable, or better overall sensitivity when used in time-resolved fluorescence detection.[9] This is the case despite the lower quantum yield, which is around one tenth or less than that of the best organic fluorescent compounds. The low quantum yield stems largely from the wide Stokes shift and the long decay time of the fluorescent signal.

II. FLUORESCENCE OF LANTHANIDES AND LANTHANIDE CHELATES

A. Physical Characteristics

The 15 lanthanide elements shown in Figure 3 usually display a characteristic tervalence which determines the majority of their chemical properties. The lanthanide ions differ quite significantly by the possession of an unfilled 4f electronic shell which can hold up to 14 electrons. The 4f electronic configuration plays an important role in the radiative transition of these elements, particularly when they are complexed with organic ligands.

The fluorescence of inorganic salts of the lanthanides is weak because the absorption of the ion itself is very low. The fluorescence can be dramatically enhanced when the metal ion M^{3+} is bonded to appropriate organic ligands.

Weissman[10] concluded that the excitation light is absorbed by the organic part of the lanthanide complex and emitted as the line spectrum of the lanthanide ion. He thus postulated an internal energy transfer from the organic ligand to the central lanthanide ion and observed that the efficiency of the energy transfer depends on the type of ligand attached to the lanthanide ion. The complete mechanism published by Crosby et al.[11] is illustrated in Figure 4.

The first step involves the energy absorption by the organic part of the complex (ligand) leading to its excitation from the ground singlet state S_0. This excitation brings the molecule to one of the vibrational multiples of the excited state S_1. The molecule rapidly loses its excess vibrational energy through some nonradiative deactivation process and falls to the lowest level of S_1. At this stage there are two alternative routes available. The molecule can either deactivate by radiative transition from S_1 to S_0 (prompt ligand fluorescence) or undergo transition to one of the triplet states T. From the triplet state the molecule can return to the ground state by means of a spin-forbidden transition ($T \rightarrow S_0$) (molecular phosphorescence) or the excitation can be transferred to the central lanthanide ion. The latter is a special feature of the lanthanide chelates, which has no parallel in either the fluorescence or the phosphorescence of organic molecules. Essentially, this process consists of a transfer of energy from the triplet state of the ligand to some appropriate 4f energy level of the lanthanide ion. When receiving energy from the triplet states the ion comes to the resonance state and can undergo a radiative transition resulting in the characteristic line emission of the ion (ion fluorescence). Not all excited energy levels of the lanthanide ions, however, have this ability. Those that do, and are therefore responsible for the metal-ion fluorescence, are generally designated as "resonance levels".

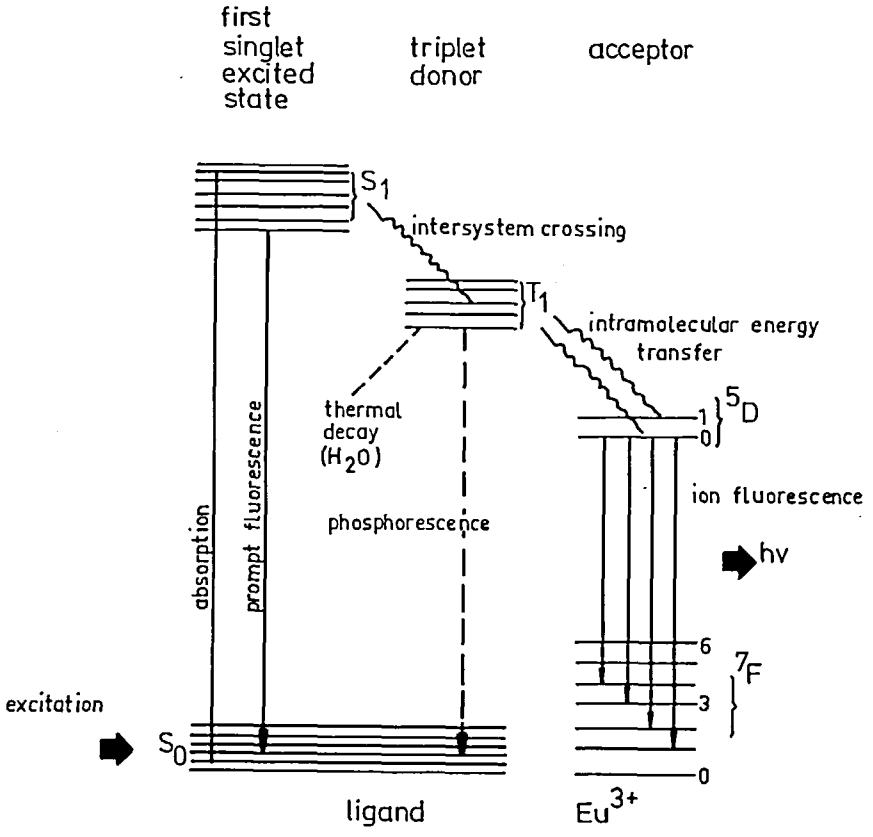

FIGURE 4. Schematic diagram of the radiative processes of the chelate leading to Eu metal ion fluorescence.

Each alternative route leading to ion fluorescence meets strong competition from parallel nonradiative deactivating transitions which have to be minimized for efficient ion fluorescence. Thus, in order to obtain a good, long decay fluorescent label, ligand fluorescence ($S_1 \rightarrow S_0$) and phosphorescence ($T \rightarrow S_0$) have to be minimized.

Thus, in relation to the choice of a suitable lanthanide ion, two important factors have to be considered:[12,13]

1. The energy of the resonance level should be close to, but somewhat lower than, that of the triplet level, so that the probability of the triplet-to-resonance level transition is high.
2. The probability of the nonradiative deactivation of the resonance level should be small compared with that of the radiative transition.

On the basis of these facts, Whan and Crosby[14,15] have classified the lanthanide ions into three groups:

1. The first group consists of La^{3+}, Gd^{3+}, and Lu^{3+} ions. The chelates of these do not exhibit ion fluorescence, but exhibit very strong molecular fluorescence and phosphorescence. La^{3+} has a completely empty and Lu^{3+} a completely filled 4f shell and neither of them has a suitable level which can receive energy from the triplet state. For Gd^{3+} ($4f^7$) the lowest resonance level lies far above the triplet level of any of the

known ligands. In this group the entire emitted energy appears as molecular fluorescence or phosphorescence.

2. The second group consists of Pr^{3+}, Nd^{3+}, Ho^{3+}, Er^{3+}, Tm^{3+}, and Yb^{3+}. The chelates of these show weak ion fluorescence as well as weak molecular fluorescence and phosphorescence. The weak molecular fluorescence is indicative of efficient singlet-to-triplet energy transfer. At the same time weak phosphorescence and ion fluorescence indicate efficient quenching of this energy via nonradiative transitions. This is due to the fact that these ions have closely spaced energy levels which make the nonradiative transitions easy.
3. The third group consists of Sm^{3+}, Eu^{3+}, Tb^{3+}, and Dy^{3+}. The chelates of this group show strong ion fluorescence with weak molecular fluorescence and phosphorescence. These characteristics indicate (1) an efficient intramolecular triplet-to-resonance level energy transfer and (2), a less pronounced nonradiative deactivation in the lanthanide ion. Characteristic (1) is attributed to the fact that in these ions the resonance level energy is close to that of the triplet level, whereas characteristic (2) is attributed to the fact that the energy levels are not so closely spaced in the region of interest.

Having received energy from one of the ligands to which it is bound, the metal ion has moved up to its lowest triplet state from where it can decay to a lower energy state by the emission of a photon. The point of relaxation can be any of the several sublevels of the ground electronic state of the metal ion. The emission spectrum of a rare-earth ion therefore consists of a series of distinct frequencies, each corresponding to the transition from one resonance level to one specific sublevel of the ground state. Usually, for each metal ion one or two of the ground-state sublevels are favored over all others. Thus, in the emission spectrum there is usually a band which considerably exceeds all others in intensity (see Figures 5 and 6). Many chelates of Eu^{3+} and Tb^{3+}, including those of benzoylacetonates and dibenzoylmethides, are especially bright emitters.

To exhibit optimal fluorescent characteristics these chelates require an anhydrous environment.[16] The quantum efficiency of e.g., the Eu-(4,4,4-trifluoro-1-[2-thienyl-1,3-butanedionato]) chelate ($Eu[TTA]_3$) at 614-nm peak in alcohol is 0.18.[17] Dy^{3+} and Sm^{3+} are weaker emitters, but obviously useful in certain applications. These four ions are useful as fluorescent labels, because of decreased internal quenching and not because of improved energy transfer. The principal quenching mechanism is the deactivation of the ion via vibronic coupling to the ligands and hence to the surrounding solvent.[14]

Fluorescence studies, therefore, have been confined mainly to the chelates of Eu^{3+}, Tb^{3+}, Dy^{3+}, and Sm^{3+} of which the Eu- and Tb-chelates are the most extensively studied and used in practical applications.[13]

The fluorescence of $Eu(TTA)_3$ and other β-diketone chelates in various solutions was reported by Halverson et al.[17,18] The authors introduced the concept of an "insulating sheath" in which a "synergic agent" forms complexes with the chelate. The synergic agents act as a shield, protecting the chelate from external interactions and efficiently reducing nonradiative energy degradation of the ion fluorescence. Trioctylphosphine oxide (TOPO), tributylphosphate, and dihexylsulfoxide are examples of effective synergic agents.

The decay times of the fluorescent compounds of Eu^{3+}, Tb^{3+}, Dy^{3+}, and Sm^{3+} vary between 10 and 1000 μsec and are dependent on the chemical environment and especially on the solvent. The composition of the chelate affects the decay time as well as the quantum efficiency. Generally, the longer the decay time, the more intense the fluorescence. Decay times of Eu-chelates vary between 50 and 1000 μsec, those of Tb-chelates between 400 and 1600 μsec[19], Dy-chelates in the order of 10 μsec, and Sm-chelates from 10 to 50 μsec.[13] The insulating sheath as proposed by Halverson[17] also lengthens the decay time.

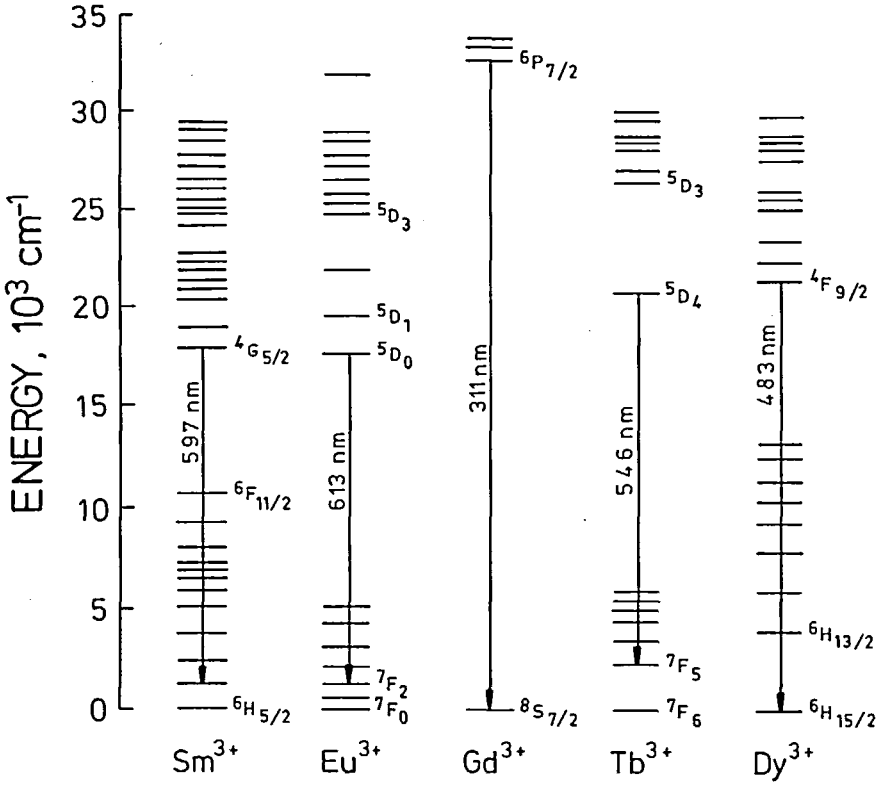

FIGURE 5. Principal emission lines of certain lanthanide ions.

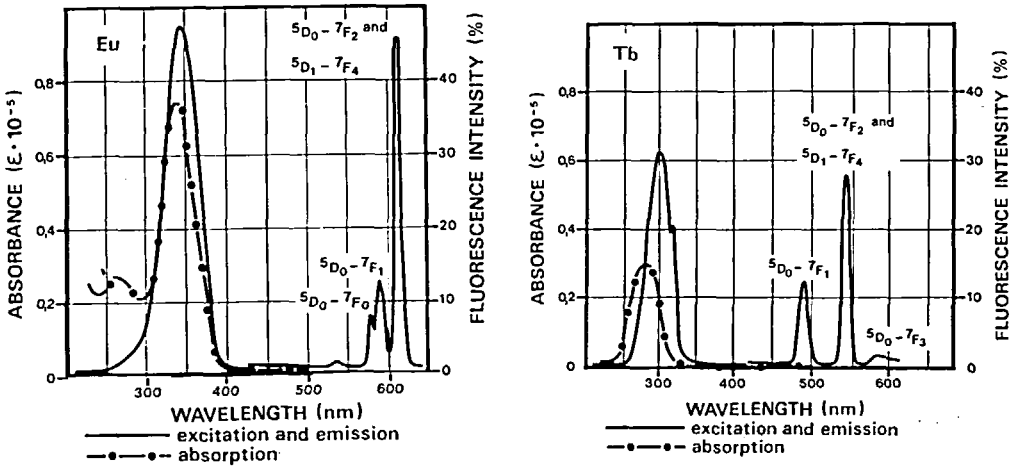

FIGURE 6. The excitation and emission spectra of Eu^{3+} and Tb^{3+}. The metal ion emits energy as narrow-band emission. The excitation band is typically broad and the Stokes shift more than 200 nm.

B. Fluorescent Lanthanide Chelates

The intensity of the lanthanide ion fluorescence is mainly controlled by two important factors: first, the ability of the triplet state of the ligand to receive energy from the excited singlet state and to transfer it to the resonance level of the ion; second, the extent of nonradiative deactivation (quenching) from the excited state of the lanthanide chelate. These

factors are influenced by the nature of the ion, the ligand, the ion-ligand bond, loosely attached adduct molecules, and the solvent. The influence of these different factors on fluorescence properties has been reviewed by Sinha.[13] The effect of the organic ligand on the intramolecular energy transfer in europium and terbium chelates has been studied by Filipescu et al.[16]

A large number of different lanthanide chelates have been investigated in order to explain the effect of the ligand on the intensity of the ion fluorescence. The central metal ion has usually been europium or terbium and the most commonly used ligands have had the basic structure of β-diketone. The chelates which have been studied can be classified into the following general structures:

1. Tris chelates
2. Tetrakis chelates
3. Mixed ligand complexes
4. Complexes with neutral donors
5. Others, including, e.g., phthalate, picrate, and salicylate complexes

The most extensive studies have been carried out using various tris-β-diketone chelates employing either gadolinium,[20] europium, or terbium[16] as the central ion. The aim has been to find a correlation between the structure of the ligand, the position of the triplet level, and the intensity of the fluorescence. Although some correlation was found between the structure of the ligand and the position of the triplet level, no systematic trends were observed between these two parameters and the variation in the fluorescence intensity. In spite of this, certain qualitative conclusions regarding the energy transfer process can be drawn.[13,16]

1. The efficiency of the energy transfer from the triplet state to the ion is controlled by the nature of the ligand-metal bond. The energy transfer is more efficient through covalent bonding than it is through ionic bonding.
2. Substituents to a phenyl ring in the ligand consisting of electron-donating groups such as hydroxy, methoxy, and methyl groups increase the fluorescence intensity. The opposite effect is seen when an electron-withdrawing nitro group is attached in the meta or para position.
3. The fluorescence intensity is increased by fluorine substitution in the ligand. The energy transfer to the metal ion increases as the electronegativity of the fluorinated group makes the europium-oxygen bond of a more covalent nature.
4. Increased conjugation, for example, by aromatic substituents or by replacing phenyl by naphtyl groups results in enhanced ionic fluorescence.
5. The ligand affects the radiative transition probability of the electron in the metal ion from the resonance to a lower level. This has been observed since the ratio of the intensities of two different emission lines originating from the same resonance level is strongly dependent on the nature of the organic ligand moiety.
6. β-Diketone ligands substituted both with aromatic and aliphatic substituents, for example, benzoyltrifluoroacetone, show a marked increase in ion fluorescence.

Thus it is evident that the overall ligand-ion energy transfer governs the absorption and emission properties of the ion. As the process is also influenced by the resonance level of the lanthanide ion, the correct ligand-ion combination has to be found for optimal fluorescence intensity. Although no strict correlation exists between ligand structure and fluorescence intensity, the qualitative conclusions stated above have been validated when the conditions for sensitive time-resolved fluorescence measurement were optimized.[21,22]

The fluorescent properties of β-diketone ligand structures have been investigated in great detail and, more recently, the fluorescent properties of a number of other aromatic complexes with lanthanide ions have been studied.[23] Only a semiquantitative registration of fluorescence was carried out and the intensity was classified as weak, medium, or strong. However, the energy transfer properties of a great number of aromatic lanthanide complexes were measured. The aromatic compounds were classified into 11 groups, based on their structure, as follows: acetylene derivatives, five-membered heterocyclic rings, benzoic acid derivatives, biphenyl derivatives, pyridine derivatives, pyrimidine and pyrazine derivatives, di- and tripyridyl derivatives, quinoline derivatives, phenanthroline derivatives, cytostatic aza-uracil derivatives, cytostatic purine derivatives, and organic phosphonimido derivatives. In each group either europium or terbium complexes were found which were characterized as strongly fluorescent. Although the compounds as such do not form strong chelates, their energy transfer properties in relation to ligand structure should be studied in more detail.

III. EVOLUTION OF TIME-RESOLVED FLUORESCENCE TECHNIQUES FOR BIOSPECIFIC ASSAYS

The key elements of time-resolved fluorescence techniques for biospecific assays are (1) a time-resolved fluorometer and (2) a long decay time fluorescent label and its conjugation with biomolecules. Each of these two elements had been developed separately and the possibility of combining them became obvious in the middle of the 1970s. Since the introduction of time-resolved fluoroimmunoassay (TR-FIA) for practical routine use at the beginning of the 1980s, the question of the original contributions in this particular field has been discussed in the scientific literature. It can be seen from the comments of various authors that the scientific evolution of this special technology has not yet been reviewed sufficiently in the literature. Therefore, it is worth studying how the key elements and the complete concept have been created.

A. Time-Resolved Fluorometry

Research with fluorescent and phosphorescent material has a long history. In 1942 Weissman[10] used a rotating disc phosphoroscope which comprised of a high pressure mercury arc light source, a quartz monochromator, and a photocell with galvanometer. Certain parameters characteristic of the fluorescence emission from europium were registered using europium salicylaldehyde and europium chloride hexahydrate. These parameters were excitation and emission wavelengths, quantum yield, and decay time.

Time-resolved phosphorimetry was used for chemical analysis by Keirs et al. in 1957.[24] They showed that a binary mixture of structurally similar compounds with decay times of 6 and 8 msec could be resolved by choice of the delay time between termination of the excitation radiation and activation of the photodetector. The phosphorimeter was equipped with a rotating disc shutter.

When using traditional instrumentation for long decay ion fluorescence and phosphorescence, the signal has been recorded with intermittent excitation of the sample. In order to discriminate between prompt and long decay emission, the exciting radiation has been periodically interrupted by a mechanical shutter and the emitted radiation has been viewed only during the dark period. Mechanical phosphorimeters suffer from certain disadvantages, in particular: the speed and versatility of these instruments in limited. Optimization of the optical geometry is not easy. In addition, it is extremely inconvenient to alter the decay time and counting time in a mechanical phosphorimeter; for this reason the time-resolved spectra cannot be recorded using mechanical choppers.

Later, the development was directed towards using flash lamps and electronic gating of the photodetector or associated counting electronics. In this approach the switch-off time

(the tail) of the flash lamp and the saturation problems of the photodetector are critical. It was realized that a combination of a fast mechanical chopper and flash lamps with a gated detector might solve these problems and provide the highest separation of the specific and unspecific fluorescence.

Halverson et al., in 1964,[17] used an oxygen flash lamp for measuring decay times of a larger number of europium chelates.

A remarkable contribution to the development of phosphorimetric and time-resolved fluorometric instrumentation was made by the Department of Chemistry, University of Florida. In 1966 Prof. Winefordner and co-workers proposed the use of an electronic gate which switches the photomultiplier tube only during the dark period.[25] The same authors discussed the variability and optimization of the decay time and counting time of a time-resolved phosphorimeter in more detail and derived mathematical expressions for this purpose.[5] In another article, in 1969, the authors verified the improvement in sensitivity of the measurement of many organic molecules with long decay times. They reported the detection limits of many drugs to be of the order of 0.1 ng/mℓ, mainly due to rejection of interferences from prompt fluorescence and incident scattered light. The dark current of the photomultiplier tube was also reduced to a fraction using this gated system. The authors also suggested the use of single photon counting as an additional potential improvement of the signal-to-noise ratio.[3]

In 1957, Hendee et al. designed a time-resolved spectrometer with improved sensitivity in which the photomultiplier was gated by pulsing the high voltage.[26] Peterson et al. studied, in 1963, the fluorescence of the Tb^{3+} ion with a time-resolved fluorometer.[6] The instrument incorporated a xenon-flash tube with a repetition rate of 40 flashes per second. A small amount of hydrogen was added in order to quench the tail of the flash. The system was equipped with a grating monochromator and with a gated photomultiplier detector. The signal from the photomultiplier tube was integrated over many flashes and so the precision and the signal-to-noise ratio were improved despite the somewhat low flash intensity. The authors also found that the time-resolved excitation technique was most useful in removing short-lived "lattice" fluorescence. The time resolution of this fluorometric system was 2.5 μsec.

Parker described, in 1969, a phosphorimetric microscope with two synchronously driven mechanical choppers; he reported significant reduction of the blank due to solvent impurities and Raman scattering.[27]

The development of nanosecond flash lamps made it possible to measure the decay of nanosecond fluorescence directly. Bennett[28] and Ware et al.[29] designed instrumentation for a nanosecond time-resolved fluorometry. The first commercial nanosecond fluorometric system, the TRW Model 31 B, was largely based on the light source designed by Mackey et al.[30]

In 1974 Lytle and Kelsey[31] designed a system consisting of a cavity-dumped continuous wave argon-ion laser and a fast photomultiplier tube connected to a sampling oscilloscope. The system was operated as a time-resolved fluorometer and it allowed the display of the fluorescence spectrum of certain fluorescent compounds without interference from scatter and Raman lines. The authors saw the potential of this method in the analysis of macromolecular samples where scattered radiation is a severe problem. According to the authors, the detection limit could be improved by the factor of 10^2 to 10^3 using single photon counting, and a level of sensitivity of 10^{-12} M could be reached.

The references above are not comprehensive and only the papers of most importance have been cited. It can be seen that the technology for both microsecond and nanosecond time-resolved fluorometry was created as early as the beginning of the 1970s. The reason why commercial time-resolved fluorometers for routine application were not introduced until the beginning of the 1980s is that the whole analytical problem was not associated with instrument

technology itself. The main obstacle was the lack of applications and suitable long-decay fluorescent probes.

The practical long decay fluorescent probes for routine applications became available much later in the 1980s and made possible the introduction of commercial time-resolved fluorometers, as discussed in more detail in Section IV.

B. Long-Decay Time-Fluorescent Labels and Their Conjugation with Biomolecules

It has been known since the early 1930s that the salts of certain lanthanides around gadolinium are fluorescent. The reaction of lanthanide salts with nucleic acids was discussed in a number of publications during the 1930s and the 1940s. A lanthanum-containing reagent was employed for the fixation of nucleic acid structures in UV microscopy.[32]

It was discovered by Weissman, in 1942, that β-diketone complexes of tervalent europium, terbium, and samarium exhibit unusual luminescence properties when excited by near UV light. The compounds emit visible radiation characteristic of the lanthanide ions. Intra-4f electronic transitions within the lanthanide ions occur whenever the excitation is carried out in the intense ligand absorption bands. These ligand bands belong to $\pi - \pi^*$ couplings and are characteristic of the coordinated ligands surrounding the central chelated ions. Weissman realized that the energy was being pumped into the electronic system of the organic complex characterized by the π-electronic levels of the ligands and was, subsequently, migrating to the central chelated lanthanide ion, from where the characteristic fluorescence of the ion occurred. He designated this process as the "intramolecular energy transfer."[10]

Stern et al. studied, in 1953, high-polymer preparations of DNA with a fluorescent europium probe[33] and reviewed the interaction of lanthanide compounds with substances of biological interest.[34] The fluorescent spectra and the decay times were investigated in the 1950s and reported by, for example, Dieke et al.[35] In 1961 to 1962 the intramolecular energy transfer in lanthanide chelates was studied by Crosby[36,37] and Filipescu.[16] Halverson et al. published their principle of "insulating sheath" in 1964[17] and Ohnesorge made a comprehensive article on the fluorescence of metal chelate compounds in 1960.[38] Crosby also published an extensive review on luminescent organic complexes of lanthanides in 1966.[15] The fluorometric determination of europium with benzoyl trifluoroacetone was described by Shigematsu et al. in 1969.[39] In 1971 Fisher and Winefordner[40] optimized the fluorometric assay of europium and terbium as hexafluoroacetylacetone-trioctylphosphine oxide complexes.

In 1969 Scaff et al.[41] showed that $Eu(TTA)_3$ can be firmly bound within microorganisms and that it emits fluorescence in the 630-nm region. $Eu(TTA)_3$ was used by Anderson et al. in 1971[42] and in 1975[43] in the microscopy of soil propagules and their photomicrographic counting.

Eu^{3+} and Tb^{3+} were used as fluorescent complexes with *Escherichia coli* tRNA by Kayne et al. in 1974.[44] and with DNA and chromation by Yonuschot et al. in 1975.[45] Because the fluorescence was quenched by water, the DNA-Tb precipitate was dried before measurement. In 1975 Wolfson et al.[46] used Eu^{3+} as a fluorescent probe in the investigation of the tRNA structure. The decay time was recorded using a Xenon flash-lamp fluorometer with a memory oscilloscope.

Leif et al.[47] discussed, in 1975, the possibility of using lanthanide chelates which are complexed with antibodies for detecting multiple cellular antigens fluorometrically. As a complex suitable for labeling in biochemistry and cytology, Leif suggested the use of europium with chelating complexes of the type $Eu(\beta\text{-diketonate})_3$ and Eu(1,10-phenanthroline-x). He assumed that this complex might have following properties:

- A high-intensity narrow-line fluorescence
- A suitable fluorescence half-life

- A reactive site facilitating easy binding to selected groups of biological substrates
- Sufficient solubility in the desired aqueous solvent mixture

He also assumed that the complex would have high thermodynamic stability with chemical inertness under the desired conditions of solvent, pH, temperature, and concentration. In addition, Leif assumed that the complex would offer the following advantages:

1. A high intensity fluorescence. The β-diketonate and 1,10-phenanthroline ligands possess electronic energy levels appropriate for absorption of exciting radiation and for transfer of intramolecular energy to the emission levels of the central Eu^{3+} ion.
2. Effective coupling of the labeling complex to the substrate. The presence of a single substrate-binding group on a sterically exposed site of the ligand would favor such a coupling.
3. Greater stability of the Eu^{3+} ion complex. The caging effect of the four planar and rigid ligands should confer this greater stability. This is also expected to prevent dissociation and bleaching of the Eu^{3+} ion under experimental conditions while in addition, minimizing the fluorescence quenching caused by water.

Leif was actually one of the very first authors who suggested a complete concept of chemistry for time-resolved fluoroimmunoassay. The design of suitable instrumentation with pulsed light excitation and time-resolved detection was already available at that time if needed. It was later found that the concept developed by Leif was inadequate, mainly because of the difficulty in developing a stable and quench-resistant chelate system for use in an aqueous environment. For this reason Leif's concept did not lead to any practical system at the time.

In the field of nanosecond fluorometry long decay pyrene butyric acid conjugated with human IgM was measured by Knopp et al. in 1969.[178] They used a time-resolved fluorometer equipped with a free running spark lamp and a fast photomultiplier. The signal and its decay were registered using an oscilloscope. Yguerabide et al. studied, in 1970[48] and in 1972,[2] the formation of an antibody-antigen complex with fluorescent ϵ-dansyl-L-lysine using a nanosecond time-resolved fluorometric system. Weltman et al.[49] studied, in 1973, some applications of *N*-(3-pyrene) maleimide which forms fluorescent adducts with organic compounds and proteins and emits fluorescence with a decay of 100 nsec. The IgM macroglobulin was of special interest in this work.

Finally, the first paper describing a complete time-resolved nanosecond fluorometric system for a biospecific assay was published by Sacchi et al.[50] The system was made for a microscope; it included a pulsed nitrogen laser, a fast photomultiplier for recording the decay in nanoseconds, and a sampling oscilloscope. A blood sample was stained with Pararosaliline SO_2 for the measurement of DNA. "The noise such as background fluorescence and autofluorescence can be discriminated on the basis of different temporal behavior," the authors wrote. They also claimed that the overall sensitivity could be increased by several orders of magnitude and that the system had the potential to measure extremely low concentrations of fluorescent material. Sacchi's system was not made for routine assays, but it obviously included all the essential elements of a time-resolved fluorescence assay.

Wieder (1978)[51] published a paper on background rejection in fluorescence immunoassay and also obtained a patent on the same subject.[52] The claims of this patent are obvious from the contributions by Leif[47] and Sacchi[50] but Wieder did not refer to these earlier papers. He proposed the use of $Eu(TTA)_3$ as a long decay fluorescent probe. From many earlier and later works it has become evident that this method was not a viable possibility because of severe problems with quenching. Another problem is that these β-diketones bind europium with very low thermodynamic stability. In a stable labeling system the dissociation constant

should be at least 10^{10}, but that of the Eu^{3+} complexes with β-diketones is in the order of 10^3 to 10^6 only, which is not sufficient. Europium chelates with β-diketones will dissociate even in optimal conditions and the fluorescence will be extinguished at micromolar and lower concentrations,[16,53-56] The corresponding German patent by Wieder[57] introduces a functional group into one of the β-diketones chelated to europium. It was supposed that the $Eu(TTA)_3$ could be modified to $Eu(TTA)_2$ ($TTA\text{-}CH_2\text{-}NH_2$) and the amino group would then be converted to an isothiocyanate and the chelate conjugated with an antibody using standard methods. The coupling, however, does not eliminate the quenching caused by water and the stability of the described coupling is far too low for a practical fluorescent antibody conjugate.

Fluoroimmunoassays, including the possibility of time-resolved fluorometry, were reviewed by Soini et al. in 1979.[58] In this work references were also made to certain seminar reports made in 1974 and in 1975, where the basic concept of time-resolved fluoroimmunoassay using lanthanide chelates was discussed for the first time.

The central problem of a practical time-resolved fluoroimmunoassay until the late 1970s was the availability of useful long-decay fluorescent probes. It was understood that organic fluorescent probes and an applicable nanosecond fluorometric system would lead to very expensive instrumentation. Due to the limited speed of photomultiplier tubes and associated electronics, the maximum photon counting rate is quite low and substantially dependent on the repetition rate of the excitation light source. In monophoton fluorometric systems the maximum single photon counting rate is always lower than the light source repetition frequency. In the multiphoton fluorometric system for fluorescent labels having decay times between 10 and 200 nsec, the maximum rate of photon counting is naturally dependent on the time resolution of the single photon counter. With satisfactory linearity limited by the dead time of the counting system, the maximum counts per flash would be on the order of ten counts. Using light sources with a repetition frequency between 100 and 1000, a maximum count rate between 1000 and 10,000 counts per second can be obtained, but that is not satisfactory for the dynamic range required.

Lanthanide chelates remained the only potential alternative for long decay time fluorescent probes and the design concept of suitable instrumentation was also clear from many publications in the 1960s. The central problem was the stability and quenching of the lanthanide chelate. To be able to keep the ion or chelate firmly on the immunoreagent or some other macromolecule at low concentrations in the vicinity of competing chelating compounds or heavy metals, a very strong chelating agent was required. The quenching effect of water was a problem, particularly since europium has a strong tendency to fill its coordination sphere up to coordination number 9 with water molecules.

Frank and Sundberg[59] understood the basic prerequisites for stable labeling conditions with lanthanide chelates and developed a conjugation method which was supposed to be stable in an aqueous environment. The fluorescent lanthanide chelate was incorporated covalently with polymeric beads derived from loaded latex. The particles were strongly fluorescent in water, but obviously some other problems were involved, because no further applications of this method for a practical immunoassay have been found in the literature.

Finally, a viable solution for a time-resolved fluoroimmunoassay employing lanthanide chelates was found by the research team at the Wallac Biochemical Laboratory, Turku, Finland,[60] in cooperation with the Department of Molecular Endocrinology, the Middlesex Hospital, Middlesex, England.[21,61] The associated instrumentation was developed by Soini and Kojola.[9] The procedure, which now has been found sufficiently reliable and robust for clinical routine use, was named "DELFIA" Dissociation-Enhanced Lanthanide Fluoroimmunoassay. The problems discussed above were solved by using a stable Eu-EDTA chelate and later an Eu-DTPA chelate[62] for labeling the antibody with a virtually nonfluorescent label. In 1971 to 1977 the work of Poluektov and Tishchenko[63,64] and especially the work of Sundberg and Meares et al.[65-68] was of great value to this progress.[69]

Table 1
TYPICAL RATINGS FOR GAS-FILLED FLASH, SPARK, AND CHOPPED DISCHARGE LAMPS

	Pulse duration	Repetition frequency (kHz)	Typical total electric power	Optical output pulse energy (J)
Xenon flash	1 μsec—1 msec	<1	10—100 W	10^{-6}
Spark caps	1—5 nsec	<10	10 W	$<10^{-12}$
Chopped discharge lamps	>100 μsec	<1	0.5—5 kW	$>10^{-3}$

After the immunoreaction and separation the Eu-ion was dissociated at low pH with a solution called "Enhancement Solution", which contains a suitable β-diketone and certain synergistic additives forming a stable, highly fluorescent micellar structure in water. The synergistic agents were based on the model presented by Halverson et al. in 1964.[17] The DELFIA method and related applications have recently been described in a number of review articles[70-81] and will be discussed in more detail later in Section V.

Diethylenetriaminepentaacetic acid (DTPA) has been successfully used for stable complexes of lanthanides and proteins.[19,62,82] An immunoassay using a terbium-transferrin complex as the fluorescent probe has been proposed[83] and some new fluorescent chelates have been patented.[84-86] An article on metal chelates as probes of biological systems has been published[87] and a time-resolved fluoroimmunoassay using phenobarbital[88] and a phosphoroimmunoassay using tetraiodofluorescein as label have been proposed.[89]

IV. INSTRUMENTATION

A. Pulsed Light Source

Light sources which emit short duration pulses can be divided into the following categories:

- Flash tubes
- Mechanically or electronically chopped discharge lamps
- Spark gaps
- Pulsed lasers

The most important parameters in the selection of the pulsed light source for time-resolved fluorometric systems are the pulse duration and the intensity at the desired wavelength. Other important parameters are repetition rate, afterglow, and size of the plasma (see Table 1).

1. Gas-Filled Discharge Lamps

Gas-filled flash tubes are a traditional solution for microsecond fluorometry and phosphorimetry; they produce light pulses of micro-to-millisecond duration. The pulse duration of a flash tube is usually limited by the time constant of the discharge circuit. The range of commercially available tubes with different power and flash durations is large and an optimal tube can be found for every application. Typical small Xenon flash tubes used in time-resolved fluorometry provide 10 μJ photon emission per pulse over the whole spectrum, 1 μsec pulse duration, and 50 kW max peak power at a 1-kHz repetition rate. The tubes can be specified for shortest possible afterglow and the manufacturer optimizes the choice of the glass envelope and gas-filling mixture accordingly.

A small plasma cap distance of the flash tube electrodes ensures good focus and high excitation intensity. The emission spectrum of the flash tube is specific to the filling-gas

mixture. Xenon has a wide continuous spectrum and is particularly useful in time-resolved fluorometry of lanthanides as well as in phosphorimetry.[90] The lifetime of flash tubes is limited and dependent on the peak power used and number of pulses (typically 10^9 pulses for half intensity). The pulsed sources are not necessarily driven continuously, since a limited number of light pulses is used for each measurement. Each pulse may excite a relatively small number of molecules (e.g., 1000) which will produce emitted photons (multiphoton fluorometric system). In systems where Xenon flash tubes are used it is typical to trigger the flash tube 1000 times for each measurement. Consequently, the lifetime of the flash tube permits the measurement of a million samples.

An alternative cylindrical flash lamp has been reported, having point electrodes with a 2-cm gap and a mercury-argon gas mixture filling. The lamp produced pulses of 60 μsec duration, 1 kHz repetition rate, and 1 μJ photon emission intensity over the whole spectrum.[30]

Continuously operated discharge lamps can be pulsed using a mechanical[24,91] or an electronic chopper device, and, thus, only a small part of the total emission is in effective use. Continuously operated discharge lamps require very high electric power (0.5 to 5 kW), whereas the flash tubes can operate with a very small duty cycle and small power dissipation. A pulsed discharge lamp system is expensive and clumsy in relation to its performance and hardly competitive against flash tube systems anymore. Mechanical choppers have a limited speed (at its best 10 to 100 μsec on/off time). Electronic choppers work normally with polarized light only and have a limited on/off contrast which is not satisfactory for high sensitivity time-resolved fluorometry.

The spark gap is the traditional low-intensity pulsed light source for nanosecond time-resolved fluorometry. The pulse duration is 1 to 5 nsec and the repetition rate is 1 to 10 kHz. These lamps normally require continuous gas flow and an external gas supply. The photon emission intensity is very low, less than 1 pJ per pulse over the whole spectrum, and spark gaps are useful only in the monophoton fluorometric systems.[92]

2. Pulsed Lasers

Pulsed lasers for the 300- to 500-nm range have currently replaced spark caps in modern nanosecond fluorescence spectroscopy. There are four general methods of pulsing lasers on a submicrosecond time scale (see Table 2):

1. Lasers with pulsed excitation
2. Lasers with G-switching
3. Mode locked lasers
4. Cavity dumped lasers

Pulsed nitrogen lasers (337 nm) have often been used as an excitation source in time-resolved fluorometry and phosphorimetry.[93] Yamada et al.[94,95] designed a multiphoton fluorometric system consisting of a nitrogen laser and a gated single photon counter. The laser operated at 15 to 25 Hz and produced 0.5 mJ pulses of 10 nsec duration. A very high detection sensitivity was obtained for the $Eu(TTA)_3$ chelate, reaching up to 2×10^{-15} mol/ℓ. Due to the low pulse repetition rate of the laser, the counting times for reasonable statistical precision were, of necessity, greater than 10 sec.

A general problem is the difficulty of producing pulses of the desired duration (less than 1 nsec) and particular repetition rate (selectable up to 10 MHz). The rate may be either so low that the signal averaging requires too long a time (methods 1 and 2) or the rate is so high that any reasonably long-lived decay will be interrupted by the next excitation pulse (method 3).

Pulsed lasers which offer flexibility in terms of repetition rate and pulse duration are based on method 4 or, more often (currently), on a combination of mode locking, cavity dumping, and optical switching.

Table 2
GENERAL METHODS FOR PULSING LASERS

	Pulse duration	Repetition frequency	Typical total electric power (kW)	Optical pulse peak power or energy	
Lasers with pulsed excitation					
Flash lamp-excited dye lasers	10 nsec—10 μsec	<100 Hz	<1		
Electrically excited nitrogen lasers	1—10 nsec	<100 Hz	<0.5	200 kW	10^{-4} J
Nitrogen laser-excited dye lasers	1—10 nsec	<100 Hz	<0.5		
Lasers with Q-switching					
Rotating mirrors	>10 μsec	<1 kHz			
Saturable or bleachable absorbers	—	—	<10	200 kW	10^{-3} J
Electro-optical shutter (Kerr cell)	>1 nsec	~10 kHz			
Lasers with mode locking					
Acousto-optic modulation of the laser intensity at a frequency equal to the mode frequency spacing	<1 nsec	~100 MHz	1—10	200 W	10^{-9} J
Lasers with cavity dumping					
Acousto-optic grating used for diffracting the light from the cavity	<10 nsec	1 Hz—10 MHz	1—10	1 kW	10^{-8} J

a. Cavity Dumping

Certain continuously operated lasers, e.g., the argon ion laser, can be equipped with a cavity dumper. In an ordinary laser one mirror is made totally reflective while the other mirror is semireflective, and its transmission-reflectance ratio is optimized for good resonance gain and sufficient laser beam output. Using an appropriate optical switch or some other controllable reflective element in the resonance cavity, the laser action can be kept at the maximum with totally reflective or transmissive optical elements. The radiation is then in the form of a standing wave within the resonator. With an appropriate optical element, the whole beam is reflected out of the cavity, and the radiation exits from the cavity within the duration of one or several round-trip transit times and a short intense laser pulse is obtained. After this so-called cavity dumping the resonator gain is zero, and no lasing will occur until the resonator is reset to its initial condition.

In practice, the pulsing effect can be made possible using a Bragg cell where a piezoelectric transducer generates an acoustic wave across the laser beam and diffracts the light from the cavity. The output peak power is very great because it contains nearly all of the intracavity power. The switching time is limited to 5 to 10 μsec by the acoustic transit time across the laser path. The maximum repetition rate depends on the length of the time necessary for regenerating the intracavity power, which varies from 100 nsec to 10μsec. Consequently, the useful repetition rate of a cavity-dumped laser system is from single shot to 0.1 to 10 MHz.

Lytle et al.[31] reported the use of cavity-dumped Argon ion laser as an excitation source in time-resolved fluorometry as early as 1974. Their system was flexible with adjustable pulse duration and frequency (1 Hz to 10 MHz) and particularly applicable to time-resolved fluorometry and phosphorimetry for decay times from 0.5 nsec up. The system efficiently separated Rayleigh scattering and Raman bands from the fluorescence spectra and, consequently, the sensitivity was improved drastically. The disadvantage of the cavity-dumping method is the duration of the laser pulse, which is not satisfactory in all time-resolved studies with typical fast decay organic fluorescent material. For lanthanides the pulse duration of the cavity-dumped Argon ion laser system is perfect. The 364-nm emission line is somewhat aside from the excitation maximum and the energy pulse quite low, 10^{-8}J, but enough for monophoton fluorometric systems.

b. Mode Locking

Mode locking is a method capable of producing extremely short duration laser pulses. Mode locking is based on a specific phase relationship between the discrete frequency components of the laser emission, called longitudinal "modes" of the laser. The modes are separated by a constant frequency interval, which is inversely related to the round-trip time for light in the cavity. In the absence of any control, the modes have a random phase resulting in a periodic fluctuation of the output intensity of the laser.

Organizing the modes to have a common phase can be achieved by modulation of the laser intensity, with radiation having a frequency equal to the mode spacing. An example of cavity configuration for a modulated mode-locked CW argon ion laser is shown in Figure 7. A Bragg cell driven with 44 MHz through a piezoelectric transducer is used as a modulator which diffracts the light from a 1.7-m-long cavity twice per cycle. The output of the laser is a continuous train of pulses with a duration of 0.2 nsec spaced 11.4 nsec apart.

c. Combined Laser Systems

In many time-resolved fluorometric applications the short period between the mode-locked laser pulses can result in an overlap of temporal information. Therefore, reduction of the typical high pulse rate of 40 to 150 MHz is necessary for the analysis of fluorescent material with decay times longer than 10 nsec. The reduction of the pulse rate down to a desired

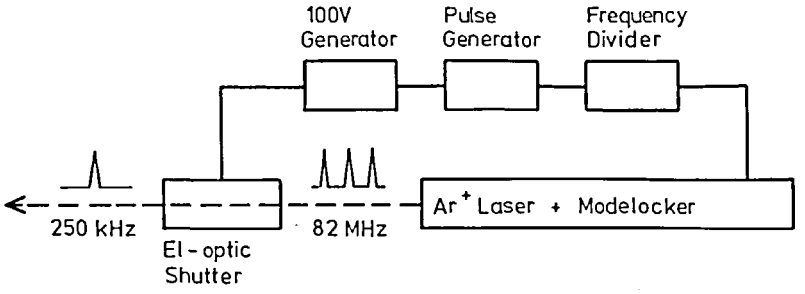

FIGURE 7. Block scheme of mode-locked argon ion laser with pulse frequency reduction.

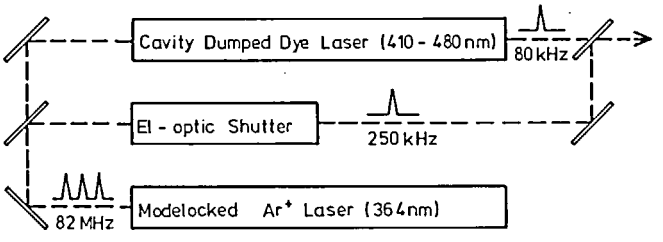

FIGURE 8. Block diagram of combined mode-locked argon ion laser and cavity-dumped dye laser.

frequency can be accomplished with an electro-optical shutter, e.g., the Pockel's cell, which is electronically synchronized with the mode-locker frequency using a frequency divider and a pulse generator with adjustable pulse width and delay. A large variety of selectable frequencies is then available.

Schneckenburger et al. built a laser system consisting of a mode-locked Argon ion laser with an 82-MHz pulse rate and he reduced the repetition rate with a low-voltage Pockel's cell down to 250 kHz. The system shown in Figure 7 was used for fluorescence studies with 4-methylumbelliferone (MU) (exc. 364 nm) having a decay time of 3.0 ± 0.5 nsec. The lowest detection level of MU was reduced from about 10^{-13} mol (obtained using classical fluorometry) to 10^{-16} mol.[96]

The same author has later reported results with a modified laser system consisting of a mode-locked Argon ion laser (364 nm) and a cavity-dumped Dye laser (410 to 480 nm) with an electro-optical shutter for reducing the high pulse rate down to 250 kHz (see Figure 8).[97]

Cavity-dumped and mode-locked laser systems are expensive (~100,000 USD) and require special care for reliable operation. Mode locking requires very stable mechanical design and installation.

An Argon ion laser tube requires a long warm-up and the lifetime of it is limited to 5000 hr. Consequently, the total working time becomes long despite a limited amount of analyses. The spare plasma tube is expensive. These are the facts which limit the use of pulsed lasers in analytical systems and also demotivate the development of related nanosecond fluorometric techniques for routine applications at the moment. In the field of laser technology new innovations can, however, appear unexpectedly and change the situation drastically at any time.

d. Semiconductors

It has been proposed that semiconductor lasers would be used as pulsed excitation light

source in time-resolved fluorometry. The variety of suitable emission wavelengths is very limited, but GaA1As laser diode for 800 nm emission has been claimed to be useful with lanthanide chelates of Nd^{3+} and Yb^{3+}. The advantages of using semiconductors as pulsed light sources are the simplicity of the associated electronics and the low power dissipation.[98]

3. Discussion

As a summary we can draw the conclusion that low-cost pulsed flash lamps are a practical choice for use in routine time-resolved fluorometers for lanthanide chelates. The technology of pulsed laser systems is also mature and available, but much more expensive. The parameters of a laser system can be optimized for each particular application and, in addition, certain configurations offer flexibility of pulse rate and duration. Pulsed laser systems also provide the possibility to exploit a large variety of organic fluorescent probes with fast decay. It is, however, obvious that the separation of a specific fluorescence signal from the background fluorescence, one having a decay time of 20 nsec and the other 5 nsec, is not possible with the same efficiency as when using long decay time fluorescent labels such as lanthanide chelates, with a decay time of 50 to 1000 μsec. On the other hand, organic fluorescent probes may have a much better quantum yield which compensates the difference, but it is not likely that nanosecond systems for organic fluorescent labels would be any more sensitive than the current lanthanide chelate methodology. In microanalytical studies, however, the easy focusing and the high point intensity of the laser offer outstanding advantages.[50,97] The potential of light-emitting semiconductors is very much dependent on the usefulness of the near IR fluorescent lanthanide labels Nd^{3+} and Yb^{3+} in the assay environment.

B. Detector and Instrumental Background

In time-resolved fluorometry the fast photomultiplier tube is the only practical single-photon detector available today. Good single-photon resolution is also an advantage in counting photons from long decay fluorescent probes, such as lanthanide chelates. A single-photon counting system is less sensitive to instability caused by high voltage and temperature fluctuations. Naturally, nanosecond fluorometry requires the extreme speed and resolution of single-photon counting.

The excitation light pulse is always sensed by the detector despite the 90° angle optical configuration and monochromators or interference filter (IF-filters) used to separate the emission light from the excitation light. Leakage of the excitation pulse to the detector can go through different ways:

- Scattering and leakage of the secondary IF-filters
- Prompt fluorescence of the sample or the cuvette
- Leakage of the primary IF-filter and scattering

There are five sources of instrumental background noise and each originates from one or more of the above-mentioned routes. These sources will be discussed in more detail and are the following:

- Recovery of the photomultiplier tube after the excitation pulse shock
- Afterglow of the pulsed light source
- Afterpulsing of the photomultiplier tube
- Thermal noise
- Long decay fluorescence or phosphorescence of the optical components including the cuvette and solvent

1. Recovery of the Photomultiplier Tube

The leakage of the IF-filters always causes saturation of the photon counting electronics. However, the current of the photomultiplier tube is not generally saturated during the excitation pulse. Saturation is, however, possible with samples having a strong component of prompt fluorescence (e.g., serum) in the multiphoton fluorometric systems. The recovery of the photomultiplier tube depends on the degree of saturation and takes a certain period of time. If pulse counting is activated during this period, extra pulses will be counted during the time taken for the tube to reach its normal operation level.

Electronic gating may relieve the saturation and recovery process,[3,4,26,90,91] but the gating system does not help if the afterglow of the pulsed light source dominates the background. Gating of the first dynode does not completely eliminate signals caused by the excitation light during the gate-off period. Long-lived excited states in the vicinity of the photocathode, including released free photoelectrons from the photocathode, will be activated when the tube is switched on again and a strong pulse appears simultaneously at the output of the tube.

2. Afterglow

As discussed in Section IV.A, the principal pulse duration of the pulsed light source is very short in lasers and about 1 μsec in bulb-type Xenon flash tubes. In the latter case, light is still emitted long after the electric current in the flash tube has reached zero, but in a pulsed laser the afterglow exposure of the sample is so small that it is not measurable in practice. The strength and duration of the flash tube afterglow is related in a complex manner to several factors. These factors include the total energy of discharge, the length of the plasma cap, the type of the capacitor, and the composition of the fill gas. In bulb-type Xenon flash tubes, the afterglow decays below the detection level in 50 μsec and the delayed part of the emission is dominated by the red end of the emission spectrum.

3. Afterpulsing

Another effect which may contribute to time-resolved fluorometric detection is afterpulsing. Afterpulsing may be caused by light feedback from the area of the anode, or possibly certain dynodes, to the photocathode. It is characterized by a delay of 40 to 50 nsec. A second type of afterpulse can be the result of ionization of gas in the region between the cathode and first dynode and usually ranges from 200 nsec to over 1 μsec after the excitation. When the ion strikes the photocathode, several secondary electrons may be emitted. Present photomultiplier processing techniques are designed to minimize the problem of afterpulsing, but from the experimental studies we have gathered that there are other additional mechanisms which produce afterpulsing as a result of strong excitation. Therefore, it is difficult to define exactly whether the most important component of the background is afterpulsing rather than the flash lamp afterglow.

Afterpulsing may be a big problem in multiphoton nanosecond fluorometry, but in monophoton nanosecond fluorometry the effect of the weak excitation pulse to the photomultiplier is minimal. In microsecond fluorometry the delay times are normally significantly longer than the afterpulsing delay times. In our opinion, the afterglow of the pulsed light source is a more important reason for the recovery of the photomultiplier tube and for long decay background.

4. Thermal Noise

Thermal electronic noise is the fourth important source of the instrumental background signal and is dependent on the choice of the photomultiplier tube and particularly on the type of the photocathode. The emission wavelength of the fluorescent label determines the optimization of the signal-to-noise ratio. Europium, with its principal emission peak at 614

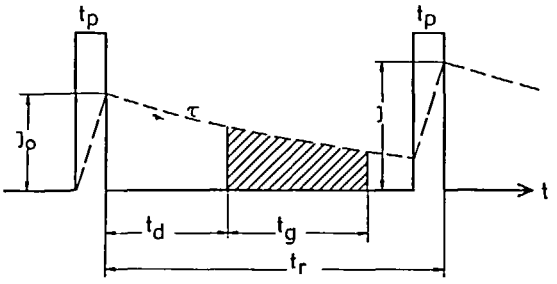

FIGURE 9. Schematic presentation of operation of the time-resolved fluorometric system.

nm, is a case where it is difficult to choose between bialkali and multialkali cathodes. At 614 nm the quantum efficiency of small bialkali cathodes is 2 to 3% and multialkali cathodes 8 to 10%, but these have 10 to 200 and 500 to 2000 cps noise rates, respectively. These noise rates will be reduced by the duty cycle of the time gating. Despite the very low quantum efficiency, a bialkali cathode is often a better choice, especially when detection of the terbium emission at 560 nm is required. Another aspect is the temperature dependence of the thermal noise which increases dramatically at a certain temperature. For bialkali tubes this temperature is 35 to 50°C, and for multialkali (red sensitive) much lower, 25 to 35°C.

In the case of near infrared-emitting labels, Nd^{3+} and Yb^{3+}, the thermal noise of the red sensitive photon detector plays a very important role. There is an obvious need to cool down the photomultiplier tube, unless the intensity level of the excitation and emission can be kept so high that one can go to analog signal mode in the photomultiplier or use a semiconductor photodiode detector.

5. Background from Optical Components

In low fluorometric studies the fluorescence and phosphorescence of optical components, including the flash tube glass envelop, lenses, reflectors, filters, and cuvette play an important role. This background may be very harmful in time-resolved fluorometers if the emission from optical components has a long decay time. Selection of material for these optical components is very important, because normal optical glass and even plastics contain impurities, including heavy metals, which act as fluorophors and emit fluorescence and phosphorescence with different decay times. The use of unselected standard optical material always leads to poor results.

Time-resolved fluorometry is an extremely sensitive method and very low concentrations can be detected with a high signal-to-noise ratio. Consequently, the method sets high requirements on the purity of the solvent. In time-resolved fluorometry of lanthanides an appropriate chelating system is always used. Free chelating agents are highly reactive with any heavy metal originating from laboratory glass or environmental dust and the background is easily raised as a result of improper handling or poor laboratory practice.

C. Signal Analysis

1. Growth of Signal to Steady-State Level

The operation of the time-resolved fluorometric system is shown schematically in Figure 9. The light source is pulsed, having a width of t_p. The signal has a maximum instantaneous intensity I_p at the end of the excitation pulse. The excited states of the fluorescent label decay with time constant τ and after a suitable delay time, t_d, the signal from the detector is sampled for a certain period of gate time, t_g. The excitation is repeated at repetition frequency f and the time interval between the pulses $t_r = 1/f$. The fluorescence signal will be integrated during the gate time t_g over a definite number of measurement cycles (n).

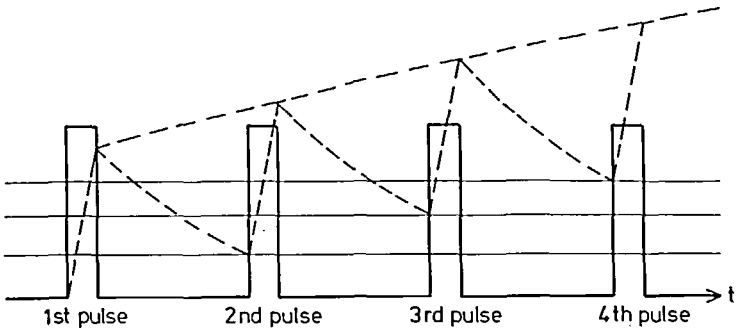

FIGURE 10. Illustrative presentation of the excitation process with a pulse train. The pulse width and interval in this figure are not in real scale, but the excitation pulses are supposed to be very short and the pulse interval t_r shorter than the decay time τ. The fluorescence signal level will attain the steady-state level within a low number of pulses.

Instrumentation which is available and applicable for time-resolved fluorometric studies with lanthanide chelates should be equipped with a pulsed light source producing very short light pulses in comparison with the actual decay times. Normally, the time interval, t_r, between the pulses is at least two orders of magnitude longer than the excitation pulse, t_p. For this reason the pulse duration can be assumed $t_p = 0$ in any mathematical model without any significant error.

The absorption of the energy from the excitation pulse is instantaneous, but the rise time of the fluorescence emission depends on the actual energy-transfer mechanism from the intermediate energy levels to the final energy level. In the Eu-dibenzoylmethide chelate, for example, the fluorescence rise time of the 613-nm line of the Eu^{3+} ion after one short excitation pulse is in the order of 1 μsec and its has been assumed that the energy is first transferred to a higher intermediate level and its relaxation time to reach the emitting level is on the order of 1 μsec.[12,99] Secondly, due to the long decay time of the final emission level, the relaxation is not complete when the next excitation pulse is transmitted and, consequently, the emission intensity increases during the initial part of the excitation pulse train. In the mathematical model below, the possible lifetime of intermediate energy levels has been omitted.

The total number of excitation pulses, n, for one measurement is high (e.g., 1000) and it can be assumed that the pulse-to-pulse steady-state fluorescence emission (Figure 10) will be attained during a low number of excitation pulses.

The instantaneous intensity of the fluorescence signal, I_p, of single photon counts as function of time, t, is given by the exponential function

$$I_p = I_0 \exp(-t/\tau) \tag{2}$$

where τ is the decay time constant of the fluorescent label. The initial signal intensity I_0 after the first short excitation pulse depends on the light source intensity, optical conditions, absorption of the sample, and the quantum yield of the fluorescent label. The subsequent excitation pulses increase the excitation of the sample until it reaches a steady-state level as illustrated in Figure 10. The instantaneous intensity of the fluorescence signal at the moment of t after n excitation pulses is given by

$$\mu_{II} \approx \frac{e\,a^2}{\hbar}\frac{W}{V_0}\eta\frac{\bar{z}}{z}$$

$$I_{pn} = I_0 \exp\left(-\frac{t}{\tau}\right)\left[1 + \exp\left(-\frac{t_r}{\tau}\right) + \exp\left(-\frac{2t_r}{\tau}\right) + \exp\left(-\frac{(n-1)\,t_r}{\tau}\right)\right] \quad (3)$$

which can be written

$$I_{pn} = I_0 \exp\left(-\frac{t}{\tau}\right)\left[\frac{1 - \exp\left(-\frac{nt_r}{\tau}\right)}{1 - \exp\left(-\frac{t_r}{\tau}\right)}\right] \quad (4)$$

The steady state will be attained when $n \rightarrow \infty$.

$$I_{p,\infty} = I_0 \frac{\exp\left(-\frac{t}{\tau}\right)}{1 - \exp\left(-\frac{t_r}{\tau}\right)} \quad (5)$$

At the moment $t = 0$ after n pulses the instantaneous intensity is

$$I_{p,\infty} = I_0 \frac{1}{1 - \exp\left(-\frac{t_r}{\tau}\right)} \quad (6)$$

From this equation it can be seen that the level of the steady-state signal intensity is dependent on the ratio between the pulse interval and decay time as follows:

for $t_r/\tau = 1$ $\quad I_{p,\infty} = 1.58\ I_0$

for $t_r/\tau = 1/5$ $\quad I_{p,\infty} = 5.52\ I_0$

for $t_r/\tau = 1/10$ $\quad I_{p,\infty} = 10.51\ I_0$

The signal can be strengthened with a higher pulse repetition rate. This, however, leads to the need for a pulsed light source with a very short pulse duration a decay afterglow that is low and fast. A long decay time phosphorescent background source, even in a very low concentration in the sample itself or in the optics, may become significant with high excitation pulse rates. Optimization of the pulse repetition rate for the actual decay time should be a compromise between the afterglow and long decay time background. In time-resolved fluorometers for lanthanide chelates[9,19] equipped with a Xenon flash lamp the optimal delay time, t_d, has been 100 to 400 μsec. With shorter delay times the signal-to-background ratio decreases. It is not yet quite clear to us whether it is the afterglow or the long decay background fluorescence which is more important in this respect. Studies using a pulsed laser would obviously clarify this problem, because the lasers are substantially free of the afterglow.

2. Total Photon Counts

Barnes and Winefordner have produced an article on the optimization of time-resolved

phosphorimetry[100] which is also applicable to time-resolved fluorometry as well. The article is based on the earlier contributions of the same research group,[5,25] and mathematical expressions have been derived for the time-resolved signal analysis in a pulsed light source system. The model covers the exponential growth and decay of emission during and after the excitation pulse including the case, where the pulse interval is of the same duration or shorter than the decay time. The model gives the optimum delay time and window time for different decay times.

The number of emitted photons per flash cycle is the integral of the steady-state intensity (Equation 5) during the gate time:

$$N_p = \int_{t_d}^{t_d+t_g} I_{p\infty}\, dt \tag{7}$$

$$N_p = \frac{I_0}{1 - \exp\left(-\frac{t_r}{\tau}\right)} \int_{t_d}^{t_d+t_g} \exp\left(-\frac{t}{\tau}\right) dt \tag{8}$$

$$N_p = \frac{I_0\, \tau \exp\left(-\frac{t_d}{\tau}\right)}{1 - \exp\left(-\frac{t_r}{\tau}\right)} \left[1 - \exp\left(-\frac{t_g}{\tau}\right)\right] \tag{9}$$

With the steady-state signal strength the number of single photons integrated over a period of n excitation pulses (neglecting the error caused by the increase of the signal at the beginning of the period) is

$$N = I_0 n \tau \frac{\exp\left(-\frac{t_d}{\tau}\right)}{1 - \exp\left(-\frac{t_r}{\tau}\right)} \left[1 - \exp\left(-\frac{t_g}{\tau}\right)\right] \tag{10}$$

In this model, it has been assumed that the excitation light source intensity remains constant. This is not the case; in practice, the intensity of the flash lamp or the laser varies from pulse to pulse and the integrated intensity over a period of 1 sec or longer may also be unstable. Using a reference detector with a signal integrator in the system discussed above, a feedback loop can be arranged which controls the length of the pulse train (number of pulses n), so that the pulsing (with a constant frequency f) is interrupted when a preset total integrated signal level has been reached in the reference detector. In other words, if in Equation 10 I_0 will change as a result of instability, n will be compensated so that the product $I_0 \cdot n$ remains constant. The integrated photon emission from the light source is stabilized by this method with a precision of $\pm\frac{1}{n} \cdot 100\%$.[9]

This stabilization method has many advantages. First of all, the system is simple; the pulsed light source and its power supply can be made without any stabilization circuit and less-expensive components with lower stability can be used. The temperature dependence of the system can be minimized by a single temperature compensation element. The pulsed light source is operated only during the measurement, thus ensuring a long lifetime. The possible fatigue of the pulsed light source will be automatically compensated without any effect on the total signal, only the measurement time needed for one sample varies.

3. Sensitivity

The number of emitted photons for one flash obtained from a fluorescent sample is

$$N_E = 2.3 \cdot \epsilon \cdot d \cdot n_0 \cdot g_1 \cdot Q \cdot k \text{ photons} \quad (11)$$

where ϵ = molar absorptivity of the supposed lanthanide chelate = 150,000 $\ell mol^{-1} cm^{-1}$; d = path length of cuvette = 1 cm; n_0 = photon intensity from a typical Xenon flash lamp in the position of the sample cuvette = $7.0 \cdot 10^{11}$ photon/flash; g_1 = factor dependent on the transmission and reflections of the cuvette = 0.74; Q = quantum yield of the supposed lanthanide chelate = 0.06 at the main emission peak; and k = concentration of the sample mol/ℓ.

The numerical values above are valid for the instrument reported elsewhere.[9] The number of emitted photons for one flash in this example is

$$N_E = 1 \cdot 10^{16} \cdot k \text{ photons} \quad (12)$$

The number of counts for one flash from the single photon detector is given in the following formula:

$$N_p = \cdot g_3 \cdot g_4 \cdot g_5 \cdot g_6 \cdot q \cdot N_E \quad (13)$$

where g_3 = fraction of pulses passed through the single photon pulse discriminator = 0.2; g_4 = transmission of the sample solution, cuvette and optical parts at the emission wavelength including reflection losses = 0.67; g_5 = geometrical photon collection efficiency = 0.28; g_6 = transmission of the secondary filter = 0.25; and q = quantum efficiency of the photocathode at the emission wavelength = 0.08. The numerical value of N_p in our example is

$$N_p = 8.0 \cdot 10^{12} \cdot k \text{ photons} \quad (14)$$

The number of counts is the integral of the intensity

$$N_p = \int_0^\infty I_p dt \quad (15)$$

and from Equation 2

$$N_p = \int I_0 \exp(-t/\tau) = \tau I_0 \quad (16)$$

and

$$I_0 = \frac{1}{\tau} N_p \quad (17)$$

I_0 gives the required instantaneous counting speed of single photons at the end of the flash:

$$I_0 = 8.0 \cdot 10^{12} \cdot \tau^{-1} \cdot k \text{ photons} \quad (18)$$

For a sample with 800 μsec decay time and different concentrations the required counting speed is

$$k = 10^{-12} \text{ mol}/\ell \qquad I_0 = 10 \text{ kHz}$$

$$k = 10^{-9} \text{ mol}/\ell \qquad I_0 = 10 \text{ MHz}$$

For a satisfactory pulse pair resolution and counting linearity, the theoretical maximum counting speed of the electronics should be on the order of 50 to 100 MHz.

For one measurement consisting of n = 1000 excitation pulses with 1 kHz repetition frequency (t_r = 1 msec), t_d = 400 μsec delay time, and t_g = 500 μsec gate time, the total number of counts can be calculated combining Equation 10, which gives in our example N = 0.32 I_0, and Equation 18. The total number of counts is then

$$N = 3.2 \cdot 10^{15} \cdot k \text{ counts} \tag{19}$$

The typical instrumental background in our example[9] is 100 counts for the same counting time as above (~1 sec). Assuming that the background counts are free of other variation than pure Poisson statistics, the potential and theoretical sensitivity of this instrument on the basis of three standard deviations is on the order of 10^{-14} mol/ℓ. This sensitivity is better than the sensitivity obtainable with a high specific activity ^{125}I label with a long counting time.[9]

D. Instrumentation for Time-Resolved Fluorometry of Lanthanides

1. Instrument for In Vitro Studies

Instrumentation applicable to time-resolved fluorometry of lanthanides with pulsed light source and gated detector has been described in various publications since 1957.[4,9,26,90,93,95] Three commercial instruments are available today; Perkin-Elmer Micro Filter Fluorometer LS-2, Perkin-Elmer Luminescence Spectrometer Model LS 5, and LKB-Wallac Time-Resolved Fluorometer Model 1230 "Arcus".

The Perkin-Elmer LS-2 is a filter fluorometer with individual excitation filters and scanning emission filter. The LS-2 is equipped with a flow cell and a peristaltic pump.

The Perkin-Elmer LS-5 is a rationing spectrometer for research use equipped with separate excitation and emission scanning monochromators and digital displays of wavelengths and signal intensity. The pulsed light source is a Xenon flash lamp. The excitation wavelength covers the range from 220 to 720 nm and the emission wavelength from 250 to 800 nm, both with a selectable slit from 2.5 to 20 nm. The sample holder and sample changer are operated manually.

Both the LS-2 and LS-5 can be used in "integrated luminescence mode" and in "phosphorescence mode", i.e., time-resolved mode.

The LKB-Wallac Time-Resolved Fluorometer model 1230 "Arcus" is an automatic bench top, large sample capacity fluorometer intended for routine applications and especially for "DELFIA" immunoassays. The Arcus is a filter fluorometer. The excitation light source is a pulsed Xenon flash. The principal design of the fluorometric system and the signal analysis are as described elsewhere.[9] Figure 11 shows the optical and electronic schematics of the Arcus. It has space for 30 sample racks of microtitration strips or 12-mm plastic tubes. The sample capacity therefore is 360 microtitration wells or 300 plastic tubes. The excitation light beam is focused onto the sample through the side of the cuvette and the generated fluorescence is measured through the bottom of the cuvette. An automatic light shutter protects the single photon counting detector when the lid of the sample magazine is open.

A comparison of the sensitivities of the fluorometers referred to above has been made in our laboratory.[101] A dilution series of a β-diketone chelate of europium has been measured in

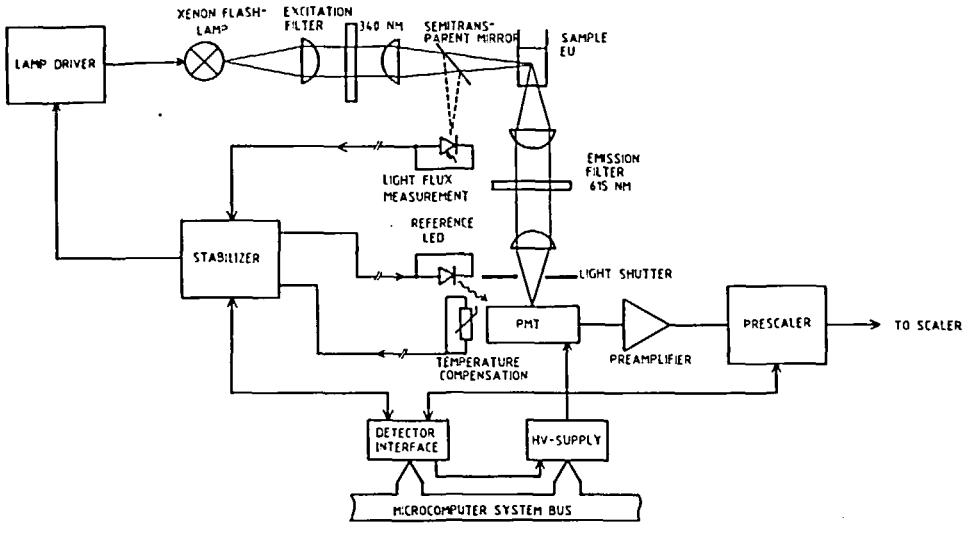

FIGURE 11. Optical and electronic block scheme of a time-resolved fluorometer.

1. "Integral luminescence mode" with LS-5
2. "Phosphorescence mode" with LS-5
3. With Arcus (time-resolved mode only)

The detection limit for this particular sample was in the order of 10^{-9} mol/ℓ in case 1, 10^{-12} mol/ℓ in case 2, and 10^{-14} mol/ℓ in case 3.

2. *Microscopy*

The very first report on phosphorimeter microscopy was published by Parker[27] in 1969. The paper describes how a conventional spectrophosphorimeter with synchronously driven mechanical choppers can be combined with a microscope to give an instrument capable of measuring the fluorescence or phosphorescence spectra of specimens with sizes down to 1 μm. The author discusses factors affecting the sensitivity of the analyzing system and reports that the microscope was capable of distinguishing between long-lived emission and prompt emission. He reports that in this system the choppers worked as a 100% efficient filter for the exciting light and a completely black field was obtained in the absence of a phosphorescent specimen even without primary or secondary filter. This indicates that the system worked on a reasonably high light level and the long-lived emission from the optics and from the object glass was not interfering. Consequently, the system sensitivity would have been better with higher excitation intensity and photon counting sensitivity.

Sacchi et al.[50] combined a tunable pulsed dye laser and a microscope. The system allowed for the first time the nanosecond fluorescence decay to be measured directly in the cell. Background fluorescence and autofluorescence were reduced significantly and the overall sensitivity was increased by the time-resolved detection.

Schneckenburger[97] used a mode-locked Argon ion laser in combination with a cavity-dumped Dye laser for the same purpose. A time-resolved microscope having high sensitivity for lanthanide chelates and reduced autofluorescence and background scattering as principal features has not yet been developed or published.

V. TIME-RESOLVED FLUOROIMMUNOASSAY

A. Dissociation-Enhanced Lanthanide Fluoroimmunoassay "DELFIA"

In order to apply lanthanides as labels in immunoassays, essentially two requirements must be fulfilled. First, the lanthanide has to be bound to one of the immunoreactive components in a kinetically stable form. Second, the lanthanide should form a fluorescent complex with a ligand and thus allow quantitative measurement based on time-resolved fluorometry. Although these requirements in principle can be combined in the same chelating structure, their separation brings about certain benefits in the design of an immunoanalytical system. The "DELFIA" is based on a separation of these two functions.[81] The lanthanide is bound to one of the immunocomponents in an essentially nonfluorescent form. The immunoassay is carried out as a noncompetitive or competitive reaction in a solid-phase system. After the immunoreaction has been completed, part of the labeled component (antigen or antibody) is bound to the solid phase and the separation of the free and bound fraction is accomplished by extensive washing of the solid phase surface. In the final step in the assay the lanthanide ion is dissociated from the immunocomponent on the solid phase into a solution where a highly fluorescent lanthanide chelate is formed. The lanthanide concentration in the solution is measured with high sensitivity using time-resolved fluorometry.

The first requirement, binding of the lanthanide, for example, europium ions, to an immunoreactive component is accomplished with derivatives of strongly chelating compounds such as EDTA and DTPA containing a suitable functional group for covalent coupling. A variety of chelating compounds have been synthesized for the purpose, but it has been difficult to combine good chelating properties with the excitation energy absorption and transfer characteristics required for the europium fluorescence. Some of the chelating compounds tested, which also are satisfactory as ligands, do not form chelates stable enough. The stability of the chelate is a prerequisite for sensitive immunoassays which are based on separation.

The second requirement, the formation of strongly fluorescent ligand-lanthanide complex, which allows a sensitive detection of the lanthanide, is achieved by adding an excess of a suitable ligand into a detergent solution containing a synergistic agent.[61,81] Under optimal conditions europium concentrations down to 10^{-14} mol/ℓ can be measured in solution. The choice of the appropriate ligand is dependent on the lanthanide intended to be measured. Consequently, the composition of the so-called enhancement solution varies with the lanthanide and a compromise has to be accepted if more than one lanthanide is to be measured in the same solution.[22] The formation of a fluorescent ligand-lanthanide complex is extremely slow in the presence of strongly chelating agents such as the polycarboxylic acid-based complexones which bind the lanthanide label to the immunocomponent with a very high binding constant.[54] The disadvantage is, however, circumvented in the immunoassays based on the DELFIA principle. The stability of a lanthanide chelate is in practice governed by the conditional constant, which is strongly dependent on pH. If the pH is lowered the stability of the chelate complex decreases and the lanthanide is readily dissociated into solution. The conditional stability is utilized as the label is dissociated from the immunocomponent on a solid phase, after the immunoreaction has been completed by adding an enhancement solution having a low pH. The fluorescent lanthanide ligand complex is formed in solution and the concentration of the label is measured with time-resolved fluorometry. The detailed composition and function of the enhancement solution has been previously described.[21,81]

The advantage of the DELFIA is that it allows the lanthanide label to be measured in solution under very favorable conditions both in regard to chemical and instrumental factors and, thus, a high sensitivity is achieved. The fluorescence intensity also reaches a constant level after the enhancement which makes even repeated reading feasible any time after the enhancement has been completed. The addition of the enhancement solution constitutes,

however, a certain disadvantage, as it requires an additional step in the assay and as it can be contaminated if not handled properly. The labeled immunocomponents are also essentially nonfluorescent, which implies that they cannot be directly quantitated. This limits their utility, for example, in assays requiring a direct fluorometric quantitation on a solid phase.

The dissociation-enhanced lanthanide fluoroimmunoassay has already proven its usefulness in a number of differently designed solid-phase assays. In noncompetitive immunometric assays both the high sensitivity and the wide dynamic range are beneficial in a number of applications. The competitive assays have been optimized to cover the clinically relevant range and thus they do not display the potential sensitivity of the label technology. In addition, a number of virus antigen and serological assays have been developed. A more detailed description of the potential of DELFIA is given in Section VI.

B. Immunoassays with Directly Fluorescent Probes

A directly fluorescent lanthanide label requires that both the fluorescent and the chelating properties are incorporated into one ligand-lanthanide complex. When such a complex is bound to an immunoreactive component completely new assay designs based on time-resolved fluorometry become real. An ideal requirement for the lanthanide chelate would be that it has a very high stability constant, it is water soluble, it has a suitable excitation spectrum with a high absorbance, efficient energy transfer to the chelated lanthanide ions, and it has to contain a functional group for covalent coupling to an immunoreactive component. It has, however, been difficult to combine these requirements in the same molecular structure, as those lanthanide chelates which are stable at high dilution are usually poorly fluorescent, and those which are highly fluorescent dissociate at high dilution.

Some EDTA analogs which form stable chelates with lanthanide ions and, in addition, contain a para-substituted phenyl group for binding to biological molecules exhibit a measurable fluorescence in the presence of europium and terbium ions.[102] Although the stability of the chelates is acceptable, their utility is limited by the relatively low sensitivity, because of the inefficient transfer of the absorbed energy to the chelated lanthanide ion from the aromatic ring adjacent to the EDTA.[37,102] In this system terbium gives a higher fluorescence intensity when compared to europium, because terbium ions are excited when the binding sites of proteins have nearby aromatic side chain chromophores from which energy transfer may occur.[103] A 50% probability of energy transfer occurs at a donor-to-acceptor distance of 5 to 10 Å.

Alternatively, lanthanides have been suggested to be bound to immunocomponents using chelators with good absorption and energy-transfer properties, but a low stability constant for the lanthanide chelate complex.[51] Although the fluorescent properties of different β-diketone chelates are excellent,[51,61] the poor stability[54] seriously limits their use in any immunoassay system.

In most of the reported immunoassays employing directly fluorescent lanthanide probes and time-resolved detection, terbium complexes have been used. Terbium ions have been bound to the immunocomponent via a bifunctional chelating agent, as in the measurement of human serum albumin[19] and immunoglobulin G.[104] Also, specific ion binding sites on proteins such as transferrin have been utilized in an assay for gentamicin.[83] In all these assays the detection sensitivity of the label is limited by the efficiency of the transfer of the excitation energy. Thus, the labeling concept is suitable only for the assay of analytes for which high sensitivity is not required.

Another alternative to directly produce fluorescent lanthanide probes and to decrease the quenching of the fluorescence by water is to incorporate lanthanide chelates into the hydrophobic phase of a loadable polymeric latex.[56] A number of different energy-absorbing ligands can be used as chelating agents within the latex particles, but β-diketones were preferred because of their high energy-transfer efficiency. In this technique up to 7.5% by weight of

the polymeric latex particle can consist of the fluorescent rare earth chelate which gives a detection level of 10^{-14} to 10^{-15} mol/ℓ. The latex particle-based label technology has, however, not yet proven to be useful in practical immunoassays, as no reports on its performance characteristics in different applications are presently available.

C. Homogenous Immunoassays

A homogenous immunoassay is defined as an assay that does not require any separation.[105] This eliminates one source of error and makes the whole procedure very simple to perform. The separation of the free- and bound-labeled component in the assay is avoided, because the properties of the label are in some way affected by the immunoreaction. Either the physical properties of the label change or the degree of immunoreaction influences a biological activity that can be quantitated. In most homogenous immunoassays a labeled antigen ligand is allowed to react with a specific antibody in the presence of an unknown amount of the ligand intended to be quantitated. When the antibody binds the labeled antigen the signal from the label is modified. In the assay the degree of modification of the signal can be directly related to the concentration of the antigen in the sample.

Several homogenous fluorescence-based immunoassays have been developed; in these the biospecific affinity reaction in some way or other affects the physical signal of the label and a change in the signal level is registered. Such a change in the fluorescence signal level caused by the immunoreaction can, for example, be based on fluorescent polarization, quenching, enhancement, energy transfer, or use of fluorogenic enzyme substrates.[106-108]

Experience in the use of time-resolved fluorometry for homogenous immunoassays is still relatively limited. A homogenous fluoroimmunoassay for phenobarbital employing a fluorescein-labeled antigen has recently been reported.[88] The signal-to-background ratio in the assay was improved by phase-resolved fluorescence measurements. The ratio of free and antibody fraction of the antigen is obtained from the difference in fluorescence lifetimes of the two species and the difference in the fluorescence intensity. The lifetimes of the free and bound antigen were 4.04 and 3.94 nsec, respectively. Matrix interference was minimized by a 3000-fold dilution of the sample.

Apparently, no reports have yet been published on homogenous immunoassays employing lanthanide labels and time-resolved fluorometry. It has, however, been proven that the ligand field surrounding the lanthanide ion affects both the intensity and the lifetime of the fluorescence.[16,36,37,81,109] Therefore, it can be expected that fluorescent lanthanide chelates, when used as labels in homogenous immunoassays, will change their physical properties during the immunoreaction. In a homogenous assay the immunocomponent labeled with the lanthanide chelate could be bound to the specific antibody, which changes the chemical environment around the label. When the ligand field around the lanthanide ion changes the effect on the energy absorption, transfer and emission properties are foreseen. This will influence the intensity and/or the lifetime of the lanthanide ion fluorescence. In our own laboratory the above assumption has been proven to be correct, as europium chelates have been used as labels in homogenous immunoassays.[110]

Although the lanthanide chelates and time-resolved fluorometry can be used for homogenous immunoassays, the assay is limited by the same factors as the competitive assays, in general.[70] Consequently, the two major advantages of the lanthanide chelate labels, the high specific activity and the extremely wide measurement range, are not actually utilized in this assay concept. In spite of this, the properties of the labels make them suitable for simple homogenous assays in which sensitivity is not the major requirement.

VI. APPLICATION OF TIME-RESOLVED FLUOROIMMUNOASSAY

Since the introduction of the immunoassay based on the DELFIA concept, a great variety

Table 3
NONCOMPETITIVE IMMUNOMETRIC ASSAYS BASED ON THE DELFIA PRINCIPLE

Analyte	Ref.
Rabbit IgG	61, 158
hCG	60, 81, 159
hTSH	77, 130, 160—165
AFP	166
Phospholipase A_2	167, 168
Insulin	169
C-reactive protein	170
LH	77, 171
Myelin basic protein	172
Ferritin[a]	
FSH[a]	
Prolactin[a]	
IgE[a]	
CEA[a]	

[a] Developed in our laboratory; results are to be published.

of assays have been developed. In principle, the technique is applicable generally and assay procedures have successfully been worked out for essentially all analytes intended to be measured with the novel system. This section reviews the already-published time-resolved fluoroimmunoassay procedures, which are based on the basic work carried out in our own laboratory and on other reported assays employing the same principle.

A. Protein and Peptide Hormone Assays

The specific activity of the lanthanide labels is at least 10^6-fold higher than that of commonly used radioisotopes.[9] In addition, the dynamic range for lanthanide measurements is extremely wide.[78] The labels as such thus fulfill the requirements often set as prerequisites for the successful introduction of a new nonisotopic technique. To exploit the advantage of the high specific activity of the europium chelates, the first assays were carried out using a noncompetitive immunometric assay design.[70,75] In the assay system based on labeled antibodies the full sensitivity potential of the technique is utilized. Table 3 lists the reported noncompetitive immunometric assays of proteins and peptide hormones that are based on the dissociation-enhanced time-resolved fluoroimmunoassay principle.

Most of the noncompetitive assays listed in Table 3 have been performed using monoclonal antibodies (except C-reactive protein and phospholipase A_2) and employing polystyrene microtitration strip wells as the solid phase. Monoclonal antibodies have been preferred when available, as they are clearly advantageous in noncompetitive-labeled antibody-based immunometric methods.[111] Several of the assays are carried out with a one-step procedure, which implies that all the reagents and the specimen are introduced at the same time to the microtitration strip well. The practical performance of the assays becomes quite simple as the number of incubations is reduced to a minimum. The separation step in the assay is carried out very simply by efficient washing of the wells, which decreases the error in the determination of the nonspecific binding. The efficiency of the separation step as such becomes important, because a large excess of labeled antibody is used; this increases the relative error in the estimation of nonspecific binding if washing is inadequate. Flow charts

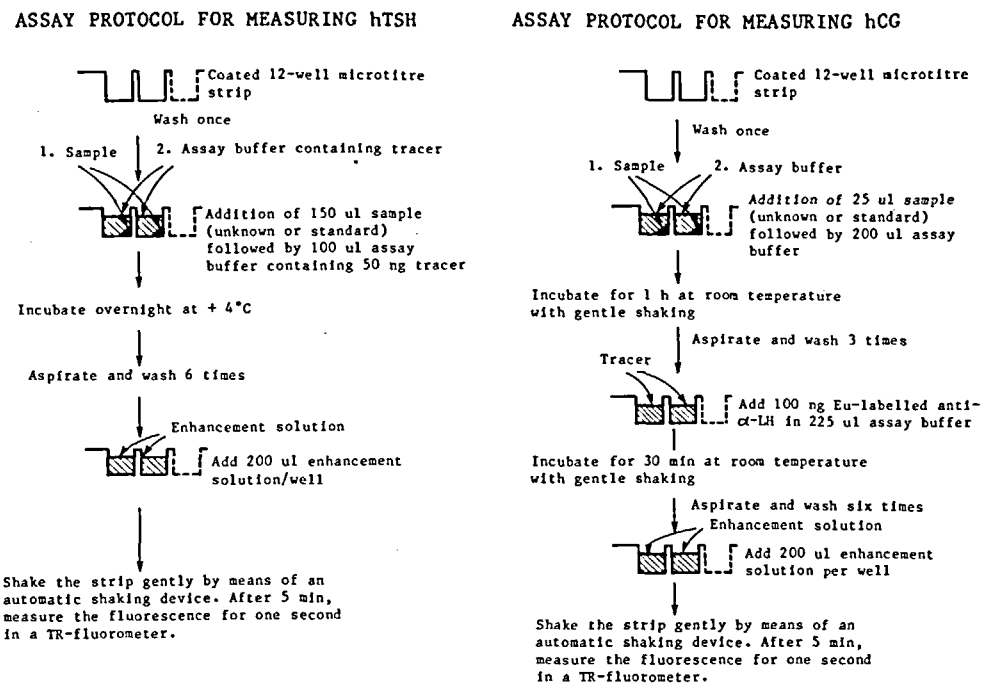

FIGURE 12. Procedure for the one-step assay of hTSH and the two-step assay of hCG.

for two typical assay protocols (hTSH and hCG) are shown in Figure 12. All other non-competitive and competitive analyte protocols are essentially similar to those presented in the flow chart.

The major benefits of the time-resolved fluorometry in the noncompetitive immunometric assays are sensitivity and a wide dynamic range. For example, in assays of serum thyroid stimulating hormone (hTSH), where the determination of low and normal hormone levels contributes to an improved clinical discrimination of patients with thyroid disease,[112,113] these properties are important. Figure 13 shows a standard curve from an hTSH assay in which the time-resolved fluorescence signal (cps) is depicted against the concentration (μIU/mℓ). The standard curve is linear up to at least 324 μIU/mℓ;[77] although the highest standard in the figure is only 25 μIU/mℓ. The sensitivity of the assay is 0.003 μIU/mℓ, when calculated as the dose corresponding to three times the standard deviation of the zero standard. The assay exhibits a coefficient of variation (CV) below 10% from 0.025 to 324 μIU/mℓ hTSH. The performance characteristics of the assay are, thus, excellent and there is at present no IRMA or nonisotopic hTSH assay which exceeds the time-resolved fluorescence-based technique in sensitivity and range. Hyperthyroid, euthyroid, and hypothyroid serum hTSH levels can be covered in a one-step procedure.

The other noncompetitive time-resolved fluorescence-based peptide and protein assays show essentially the same properties as those described for the hTSH assay. The clinical usefulness sets the limits for sensitivity, range, and speed. Consequently, the requirements vary from analyte to analyte. It is, however, obvious that lanthanide labels and time-resolved fluorometry, in combination with noncompetitive immunometric assays and monoclonal antibodies, will contribute to the development of assays with very high sensitivity, which are likely to bring new clinically important information.

B. Hapten Assays

In competitive immunoassays, either analyte or antibody labeled, the increase in the

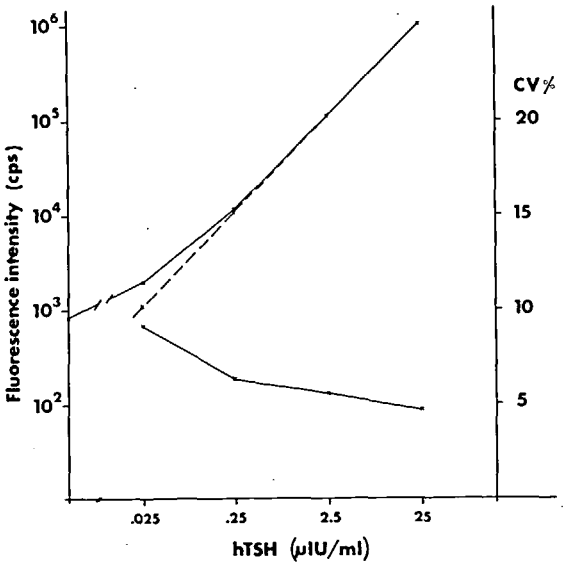

FIGURE 13. Dose-response curve and precision profile for a one-step hTSH assay. The test was carried out as outlined in Figure 1. A linear response is obtained after the background is subtracted (— — —).

specific activity of the label[70] does not essentially improve the assay sensitivity when compared to ^{125}I. The size of the haptens, on the other hand, renders the two-site immunometric assay impossible. Consequently, only competitive hapten assays have been designed using europium chelates as labels. The assays as such perform at least as well as corresponding radioimmunoassays.

Competitive assays are carried out either using labeled haptens or labeled antibodies and both assay designs yield maximal sensitivity when the antibody concentration approaches zero. When the time-resolved fluorescence technology is applied to competitive hapten assays antibodies are labeled with the europium chelate. The haptens are immobilized via a protein carrier to polystyrene microtitration strip wells. The procedure can be strictly defined as immunometric, because it is based on the labeling of a specific antibody instead of the antigen. The final result is obtained from the distribution of the labeled antibody between the endogenous hapten in the sample or the standard and the solid-phase-bound exogenous hapten. The assay principle is outlined in Figure 14. In the design of the assay the antibody immobilization is avoided as the solid phase is formed by a stable hapten protein conjugate. The problems associated with labeling of various hapten molecules are circumvented as essentially the same reaction conditions apply to the labeling of different antibody preparations. The practical performance of the assay is as simple as in any competitive immunoassay using labeled haptens. The assay design makes it also possible to utilize indirect procedures when, for example, Eu-labeled antispecies antibodies are available; this procedure, however, requires an additional incubation step.

The reported competitive hapten assays based on europium chelates as labels and time-resolved fluorometry are listed in Table 4. All the assays have been carried out by using the antibody as the labeled reactant. A typical standard curve for a thyroxine assay is shown in Figure 15. The sensitivity of the assay, when calculated as two standard deviations from the zero standard, was about 2 nmol/ℓ. The precision of the assay within the assay range was 5% or better. The performance characteristics of the thyroxine assay have been shown to meet all the criteria for a reliable and accurate test of thyroxine in serum.[114] The assay

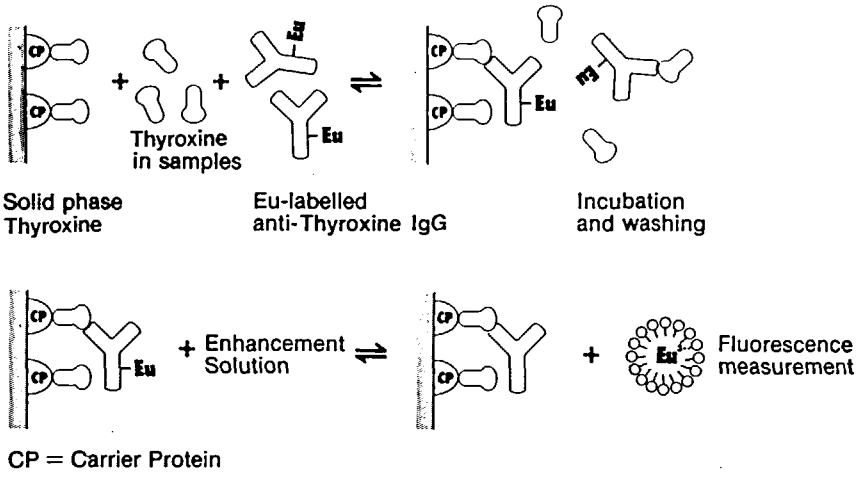

FIGURE 14. The principle of the competitive immunometric assay of thyroxine. A constant amount of protein-bound thyroxine is immobilized on the wall of microtitration strip wells and the antibody is labeled with europium. After the immunoreaction has been completed, the label is released into solution by addition of the enhancement solution.

Table 4
COMPETITIVE IMMUNOMETRIC ASSAYS BASED ON THE DELFIA PRINCIPLE

Analyte	Ref.
Cortisol	77, 173
Testosterone	77, 174, 175
Digoxin	176, 177
Thyroxine	114

has been optimized for the clinically important concentration range of the hormone and thus the potential sensitivity of the time-resolved fluorescence technique with europium labeling is not fully exploited. However, all the prerequisites exist for the development of highly sensitive hapten assays, the sensitivity of which is not restricted by the specific activity of the label, but governed by the common rules all competitive assays obey.

C. Serological Assays

In serological immunoassays labeled antispecies antibodies are widely used in various solid-phase indirect and direct assays to measure human serum antibody levels. The most commonly employed techniques are based on radioisotopes or enzymes as labels.[115,116] Because the europium chelates can be considered generally applicable as labels in immunoassays, they should also suit several serological applications. This has actually been proven in assays for rubella[117] and tetanus antibodies[118] and by labeling protein A with a europium chelate.[119]

In the rubella antibody assay, swine antihuman IgG was labeled with the europium chelate. The study showed that the test had a sensitivity and specificity comparable to RIA and other tests and an excellent overall correlation was found. The agreement between the tests was good, which suggests that the time-resolved fluorescence-based assay is a possible alternative to the procedures that are presently in use. A similar conclusion can be drawn from the

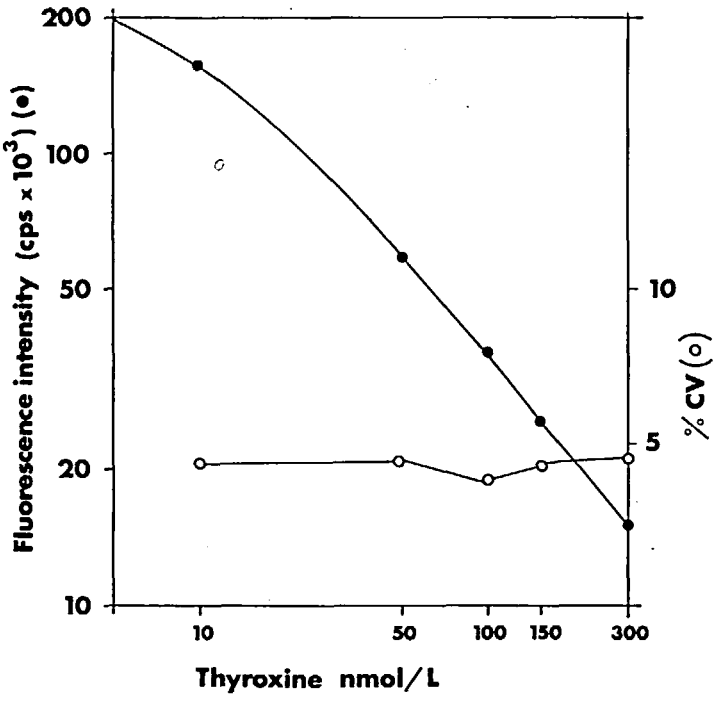

FIGURE 15. Dose-response curve (•) and precision profile (o) for the thyroxine assay. A 20-μℓ serum sample and 100-μl assay buffer containing 10 ng of the Eu-labeled antibody was added to the antigen-coated microtitration strip well. The incubation time was 2 hr at room temperature under gentle shaking. After washing and addition of the enhancement solution the time-resolved fluorescence was measured in an Arcus 1230 fluorometer.

assays developed for the tetanus antibodies. Both the direct and the indirect time-resolved immunoassay were more sensitive than the corresponding direct enzyme immunoassay. In addition, the wide dynamic range enabled the assay to be carried out in a single dilution, as the sensitivity of the measurement was 10- to 30-fold compared to the enzyme-based procedure. Although the sensitivity requirement in many of the possible serological applications is not very high, the sensitivity of the time-resolved assays in combination with the wide dynamic range is obviously advantageous.

D. Virus Antigen Assays

It can already be foreseen that antiviral drugs will be available in the near future for the treatment of viral diseases, which increases the importance of the availability of rapid viral diagnostic procedures. The lack of specific therapy for viral infections has actually delayed the development of rapid laboratory tests, but the situation is likely to change in the near future when the therapeutic possibilities improve. The diagnostic trend is to detect the presence of viral antigens directly in clinical specimens, which results in a diagnosis within an hour or less after the specimen has arrived at the laboratory. In addition, the aim is to detect early-occurring virus-specific IgM class antibodies. Traditional virus diagnostic procedures are being replaced by sensitive immunoassays, including radioimmunoassays and enzyme immunoassays.[120]

As the time-resolved fluorescence-based technology is generally applicable, its performance has also been tested in the assay of viral antigens in clinical specimens. Originally, the assays were laborious and complicated, because indirect assay procedures requiring three

different incubation steps were applied.[115,121] The tests carried out with europium chelates as labels showed, however, similar or better performance characteristics to the corresponding indirect radioimmunoassays and enzyme immunoassays. At the same time, it became obvious that the assays required simplification. The viral antigens are relatively large particles and thus contain several different or even similar epitopes. Therefore, it was self-evident that direct assays could be performed, but in addition it was demonstrated that the direct assay worked in a one-incubation procedure when the same antibody preparation was used, both immobilized on the solid phase and labeled with the europium chelate. The first successful one-incubation experiment based on the principle mentioned above was made in the measurement of the hepatitis-B surface antigen.[122] Monoclonal antibodies were used, and the catching and labeled antibodies were the same. The result of the one-incubation procedure was actually more sensitive than the conventional method with two incubations. The detection limit for a positive sample was 0.5 ng/mℓ if twice the signal level of the negative control is used as a limit. A decrease in the signal level is obtained at very high HBsAg concentrations due to the binding of the labeled antibody to the excess of the liquid phase antigen that is washed away. In spite of this, the one-incubation procedure still gives a positive-to-negative ratio of 7, with a specimen containing as many as 10^{13} HBsAg particles per milliliter (100 μg/mℓ).

The direct one-incubation assay has been worked out for the detection of rotavirus and adenovirus in stool specimens and for influenza A and adenovirus in nasopharyngeal aspirates.[120,123,124] All these assays were performed by using a polyclonal antibody preparation both on the solid phase and as the labeled reagent. The sensitivity of the assays has been at least as good as that obtained with corresponding indirect radioimmunoassay or enzyme immunoassay and the correlation between the assays is excellent. Moreover, the assays are easy to perform as they only require the addition of the specimen and the label, incubation and washing, addition of Enhancement Solution, and automatic reading in the fluorometer with time resolution.

Based on the present experience on the use of time-resolved fluorescence-based immunoassay in viral antigen determinations, it is obvious that it will be a viable alternative to the already-accepted immunoassays in the field. The high specific activity of the europium chelate used as the label in combination with good-quality monoclonal antibodies, which are becoming available for various viral antigens, will certainly be valuable in technically simple and sensitive assays. A certain limiting factor related to a more decentralized use of the technology in rapid viral diagnostics is the requirement for an instrument to read the final outcome of the test, because the specimens need only to be classified as either positive or negative.

E. Screening Assays from Dry Blood Spots

Several mass-screening programs for detecting inborn metabolic errors are already carried out from neonatal dried blood spot samples. These programs have become very important for diagnosing disorders which by early detection and treatment can be prevented. A typical example is the irreversible mental retardation caused by congenital hypothyroidism, which can be prevented by early treatment. Radioimmunoassays are generally used for the detection of the disorder by measuring either the thyroxine or the thyrotropin (hTSH) level in a blood sample from the infant.[126] A number of similar tests are presently being evaluated, including those for the screening of cystic fibrosis by measuring immunoreactive trypsin,[127] congenital adrenal hyperplasia by measuring 17α-hydroxiprogesterone,[125,128] and Down's syndrome by measuring α-fetoprotein.[129]

In all screening tests in which immunoassays are used for the measurement of the analyte concentration from dried blood spots, it is evident that a nonisotopic assay with good performance characteristics is advantageous. When hTSH measurements have been carried

out from dry blood spots using conventional radioimmunoassays or enzyme immunoassays,[33] long incubation times and large filter paper disc specimens have been used to improve the required sensitivity of the assay. This can be circumvented by applying the time-resolved fluorescence-based sensitive hTSH assay[130] to the blood spots. The whole assay is carried out in a one-incubation procedure in 2 hr by adding both the filter paper disc and the europium chelate-labeled monoclonal antibody preparation simultaneously into the microtitration strip well. Only one filter paper disc with a diameter of 3 mm (equivalent to about 2.7 μℓ of blood) is used per well. The sensitivity of the neonatal hTSH assay is 2 μIU/mℓ if the limit of sensitivity is defined as 3 × standard deviation of the 1-μIU/mℓ standard measurement value. The highest standard in the assay has been 100 μIU/mℓ and the intraassay precision — which to a great extent is dependent on the quality of the filter paper specimens — is typically better than 10% within the range. Also, the false-positive rate has been substantially lower than in the conventional screening test.[131] Because of the rapid assay procedure the screening results will be available sooner, which favors early diagnosis and treatment.

Quite obviously, the time-resolved fluoroimmunoassay-based screening tests will, in the future, represent a valuable alternative to the present testing procedures.

VII. LANTHANIDES AND NUCLEIC ACID LABELING

The understanding of the complexity of genomes in various types of cells has expanded dramatically with the development of recombinant DNA techniques and sensitive methods for analyzing the organization of specific genes. These techniques exploit the sensitivity and specificity that can be achieved by nucleic acid hybridization probes, which have become irreplaceable in molecular biology for the detection of specific, complementary nucleic acid sequences. Radioisotopes such as ^{32}P and ^{3}H are commonly used as labels for nucleic acid probes, but stability, detection sensitivity, and safety problems have stimulated the interest in the development of nonisotopic alternative labels.

The recent advances in the recombinant DNA technology have led to the possibility of using nucleic acid hybridization as a widespread method, not only in molecular biology, but also in fields such as diagnostic medicine. DNA probes are likely to to be used in certain unique areas of application such as genetic predisposition to disease, including inherited disease, virus detection, bacterial identification, and antibiotic sensitivity testing. It is, however, apparent that before the DNA probe-based technology can be generally applied in the clinical routine, the radiolabels have to be replaced by a nonisotopic detection system. Some alternative labels for hybridization probes have been developed based on, for example, biotinylated nucleotide analogs detected by streptavidin,[132,133] haptens bound to nucleotides for immunological detection,[134] and proteins cross-linked to the nucleic acid.[135] The final detection principle in all these alternative procedures is based on an enzyme-catalyzed reaction. So far, none of these procedures have been able to compete with radiolabeled probes in terms of assay simplicity and sensitivity. DNA probes represent a new alternative for future tests in routine diagnostics, but one of the prerequisites for the success of the technique is a simple and sensitive detection system.

The high specific activity of europium chelates, when measured by time-resolved fluorometry, provides a sensitivity which gives the label technology great potential as a nonisotopic alternative in the field of nucleic acid hybridization. The available europium chelate labels, which are essentially nonfluorescent before dissociation and enhancement[81] are, however, not suitable to be used in hybridization procedures such as Southern blotting, Northern blotting, and *in situ* hybridization. In these applications the primary label should preferably be fluorescent and the dissociation step must be avoided. In dot blot hybridizations, in which the denatured nucleic acid sample is immobilized on nitrocellulose or nylon mem-

branes, the label dissociation and fluorescence enhancement technique can be used, although it complicates the assay procedure.

In principle, as in the case of immunoassay, the dot blot filter hybridizations can be classified in direct and indirect procedures. Usually, the direct assays are based on probes labeled with radioisotopes. The nonisotopic alternatives are still based on an indirect pro cedure, in which, for example, a slightly modified specific probe is recognized after hybridization by a labeled compound such as streptavidin or a specific antibody. The indirect filter hybridization assay has already been carried out using Eu-labeled antibodies and a DNA probe tagged with a hapten.[136] Adenovirus DNA was detected in a model system in which 5 to 7% of the bases in the probe were modified with 7-iodo-*N*-acetoxy-*N*-2-acetylaminofluorene. After the hybridization and antibody incubations the washed filters were transferred into an Enhancement Solution to dissociate the europium ion and to form a new highly fluorescent chelate in solution. In these experiments with 1.2 kb single-stranded probe the detection limit of Ad2 DNA was 5×10^5 molecules (20 pg DNA), which corresponds to 0.3 pg of actual target sequences. The sensitivity was improved tenfold compared to the corresponding enzyme-labeled procedure. Positive results have also been obtained in indirect hybridization using a biotinylated nucleic acid probe and streptavidin labeled with europium.[137]

Because of the complexity of the indirect hybridization assays direct assays should be preferred, and that is why the nucleic acid probe molecules have to be directly labeled with europium. Work on direct labeling has been initiated, but besides the labeling chemistry the stability of the europium chelates becomes critical because of the often quite demanding incubation conditions used in hybridizations.

Clearly, the results in the indirect hybridization assays in combination with a time-resolved fluorescence-based detection of the europium label have once again proven that the technology is a very good potential alternative to radioisotopes. A number of problems need, however, to be solved before direct assays can be performed with the new labels. This also applies, in general, to the DNA probe-based hybridization technology before it can be put into practical use in the clinical routine. The following main disadvantages are associated with the present assays: complex pretreatment of the specimen is required, the test procedure is complicated as it requires a long time and many manipulations, and the best assays are carried out with radiolabels because the nonisotopic detection systems need improvement. Despite this, the DNA probes are foreseen to have great potential in fields not covered by the already fast and relatively simple monoclonal antibody-based assay systems.

Furthermore, the time-resolved fluoroimmunoassay has been used to study DNA methylation by the use of Eu-labeled antibodies raised against protein-bound 5-methylcytidine.[138] The degree of methylation in human leukemic cell lines was followed, as it was implicated in the regulation of eukaryotic gene transcription, and the effects of drug treatment were uncertain. The developed procedure is a competitive solid phase immunoassay based on labeled antibodies, and the sample consists of a deoxynucleoside-containing supernatant prepared from highly polymerized DNA isolated from human leukemic cells. The performance characteristics of the assay were as good as those of a corresponding radioimmunoassay.

VIII. OTHER APPLICATIONS USING FLUORESCENT LANTHANIDES

There is an obvious interest in the use of lanthanides as nonisotopic labels in other fields besides immunoassays and nucleic acid hybridizations. Lanthanide labeling will, however, always change the molecular structure of the labeled compounds and, consequently, it is to be assumed that such compounds will undergo some change in their biochemical behavior.

It is evidently impossible that lanthanides could completely replace radiolabels such as ^3H, ^{14}C, and ^{32}P, because their usefulness in research is unchallengeable. In spite of this, other, new applications employing time-resolved fluorometric detection have been found.

In addition, ordinary lanthanide fluorescence has long been applied in the study of protein and nucleic acid conformation, as well as metal ion binding to proteins.

A. Protease Activity

Artificial substrates are commonly used in various enzyme activity determinations and the result is obtained by following the rate of change in either the substrate or the end product concentration by measuring their absorbance or fluorescence. An equivalent approach was made in the measurement of protease activity by using europium chelate-labeled casein as the substrate.[139] The enzyme activity was followed by measuring the release of labeled peptide fragments during hydrolysis. The labeled casein was immobilized on an insoluble matrix so that the released fragments could be separated from the substrate before measurement. After separation the europium content of the fragments was measured with time-resolved fluorescence using the Enhancement Solution. The sensitivity of the assay allowed the detection of α-chymotrypsin, trypsin, and subtilisin with detection levels of 2.5, 1.0, and 1.0 ng, respectively. A disadvantage of the procedure is the separation, sampling, and enhancement steps, which are required because the europium chelate bound to the substrate is not fluorescent. Once stable fluorescent chelates which can be coupled to substrates become available, the possibilities to apply the technology to enzyme activity determinations employing immobilized substrates will improve.

B. Binding to Phospholipids

One of the possible ways to study phospholipid membranes is the use of fluorochromes, which are capable of reflecting changes in the conformation and composition of the membrane structure by means of fluorescence intensity. Europium and terbium ion fluorescence, when measured in a time-resolved mode in the presence of different phospholipids, shows a difference in degree of enhancement depending on the phospholipid employed.[140] This means that the fluorescence signal strength from the lanthanides is modulated by the different phospholipids. In fact, the fluorescence intensity of europium in the presence of phospholipids is very low when compared to the optimal enhancement conditions in the immunoassays (at least a 10^6-fold increase in intensity is obtained in the normal Enhancement procedure). Acidic phospholipids gave a somewhat stronger enhancement than neutral phospholipids. The excitation energy absorption and transfer properties of the phospholipids are thus completely different from the chelators used as energy-absorbing ligands when high sensitivity is required. Lanthanide fluorescence can, nevertheless, be used to study cation binding to phospholipids and membrane structures, as the binding most likely affects the number of coordination centers occupied by water and, accordingly, the fluorescence intensity.

C. Assay of Target Cell Cytolysis

Since its introduction, the ^{51}Cr release test has been widely used for quantitative in vitro measurements of cell-mediated cytolysis.[141] The test is based on the fact that radioactive chromate diffuses through the cell membrane, remains in the cytoplasm, and is released only when the cell membrane is sufficiently damaged to allow the efflux of intracellular molecules. In principle, the target cells are labeled by ^{51}Cr, and as the effector cells kill the target cells the cytolysis is quantitated by following the release of chromium. The major effector cells with well-defined cytotoxic immune activity include monocytes, polymorphonuclear leukocytes, natural killer (NK) cells, and T lymphocytes. In particular, since its discovery more than 10 years ago, the NK-cell activity has been the aim of extensive research.

The chromium release test has, since its introduction, proven to be very reliable. The test is relatively easy to carry out and is very sensitive. The chromium does not change the morphology and characteristics of the target cells and its release from these cells is even and "smooth" with relatively low spontaneous release. In spite of this, several efforts have

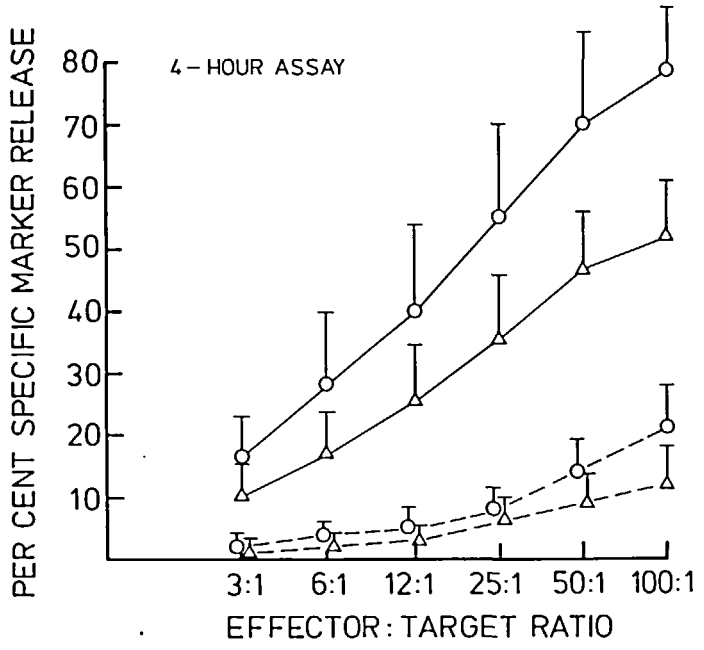

FIGURE 16. Percent specific marker release from K-562 and Raji target cells at different effector NK cells to target cell ratios. The targets were labeled either with Eu DTPA (○) or ^{51}Cr (△). The assay incubation time was 4 hr. Each value is the mean ± SD of ten assays.

been made to introduce new tests. The main reason for these efforts is the well-known disadvantage of the radioactivity used in the conventional assay.

For various reasons none of the nonisotopic alternatives has been able to replace the already established procedure.[142-145] A novel nonisotopic test for the measurement of cell-mediated cytolysis of target cells is based on the use of an europium chelate as the label.[146,147] The target cells are labeled with an essentially nonfluorescent Eu-DTPA chelate and, in order to make the cells permeable to the label, dextran sulfate treatment was employed. The cytolytic assay with the europium chelate-labeled target cells was carried out essentially as in the chromium release test. After the incubation the cells were pelleted by centrifugation. An aliquot from the supernatant was added to the Enhancement Solution to measure the amount of released europium by time-resolved fluorescence. The human erythroleukemic cell line K-562 and Raji cells derived from Burkitts's lymphoma were used as targets. A typical result is shown in Figure 16 where both the chromium and europium release is followed at different effector-to-target cell ratios using both cell lines and NK cells as effectors.

In the comparative experiments carried out with the chromium and europium release tests the significance, specificity, and high sensitivity of the new cytolysis assay was proven. Essentially identical results were obtained with the two release tests, but, in addition, the europium chelate-based assay provided some definite advantages. The high specific activity of the label and the amount of label that can be used per cell make the measurement of single-cell cytolysis conceivable. At the moment, however, in ordinary cytolysis tests the number of effector and target cells can be reduced if desired by making use of the europium label.

The lag phase for europium is shorter and the rate of release is almost twice as fast as chromium, which allows a shorter incubation time in the assay. In addition, the general

benefits of the nonisotopic time-resolved fluorescence-based technology should be considered. The use of europium chelates as labels for target cells offers a good alternative to ^{51}Cr for the measurement of cell-mediated cytolysis.

D. Conformation Studies and Metal Ion Binding

Various forms of interaction of lanthanides with substances of biological interest have already been used for a long time in the study of proteins and nucleic acids.[34] The binding of lanthanide ions to macromolecules provides a tool for experiments in which fluorescence is applied to investigate structure-function relationships. Until recently, most of these studies were carried out using ordinary fluorescence. Even here it has been possible to apply the fluorescent properties of the lanthanides, especially europium and terbium, in the investigation of the ion binding and structural properties of a number of proteins.[103,148-153] Similarly, europium and terbium have been found to form complexes with nucleic acids[33] and they have been used as fluorescent probes in the study of tRNA[44,46,154,155] and DNA[45] structures.

Fluorescent measurements showed that terbium binds in a stoichiometric manner to DNA until one terbium is present for each phosphate group.[45] The binding is sufficiently stable to make it possible to follow the completion of the reaction, but sufficiently loose to allow the terbium to be gently removed by chelating agents. The procedure was used to measure the availability of phosphate groups for binding in chromatin. The fluorescence was recorded after a solid, dry terbium-DNA precipitate was pressed into a pellet and measured in a solid-state front surface sample holder as it was emphasized that water quenches the fluorescence.

The interaction of lanthanides, when bound to tRNA, is followed by measuring the fluorescent enhancement caused mainly by the uncommon base, 4-thiouridine, which is present in tRNA. The number of lanthanide ions bound per tRNA molecule as well as their binding constant have been determined. In this context, also, lifetime measurement of europium fluorescence were used to support the assumptions which indicate that more than one type of binding site is involved.[46,154,155] Decay time measurements reveal at least two components, $\tau = 0.3$ msec and $\tau = 0.14$ msec, which lead to the conclusion that the most tightly bound europium has three water molecules coordinated to it, while the more weakly bound has six.

As mentioned previously, ordinary fluorescence has until recently been applied in the study of protein lanthanide interaction. The two lanthanides most commonly used are terbium and europium. The observations, however, indicate that the results obtained have to be interpreted with caution. The fluorescence of both lanthanides is sensitive to the number of water ligands in the primary hydration sphere and the fluorescence can be quenched due to energy transfer. In addition, protein aggregation and precipitation can occur. The sensitivity of the lanthanide fluorescence towards the ligand field around the ion has eventually been utilized by applying time-resolved fluorescence to the measurement of changes in decay constants. It was shown that fluorescence can be observed in quite dilute europium and terbium solutions using a pulsed dye laser excitation above 450 nm.[156] The direct excitation of metal ion levels eliminates the problems of protein absorption and photosensitivity. Simultaneously, the environmental sensitivity of the fluorescence decay constant was demonstrated. The method turned out to be a rather simple and accurate one for the determination of the number of water molecules coordinated to the lanthanide ion, which itself is coordinated to other ligands or bound to a protein. The technique has been further verified by examining the results obtained from structurally well-characterized crystalline solids and from corresponding data obtained on solutions.[149,157] Actually, when the difference in the observed reciprocal luminescence lifetimes for well-characterized crystalline hydrates of europium and terbium are plotted against the crystallographically determined numbers of water molecules in the first coordination spheres, a good linear correlation is obtained. Results on the protein parvalbumin and the enzyme thermolysin further prove the usefulness of the tech-

nique, as data for the lanthanide ion binding of these proteins have also been established by X-ray crystallography. Both techniques gave consistent results. The fluorescent decay constant measurement on europium and terbium, when bound at the calcium site 1 in thermolysin, suggests that about 1.5 water molecules are bound to the lanthanide ion under the conditions used. The corresponding value for parvalbumin was 1.2 to 1.3 water molecules.

These initial results suggest that the measurement of fluorescence decay times using the lanthanides europium and terbium as probes has considerable potential when the structure of macromolecules and their binding is investigated. Information is obtained in regard to the number of metal-coordinated water molecules and the characteristics of distinct binding sites in the macromolecule. The method is also well suited to establish the relationship between solution and solid-state structures. Furthermore, it is simple and applicable to dilute protein solutions.

Lanthanide ions are, therefore, considered as useful and sensitive fluorescent probes in the study of the structure of biological macromolecules.[149] The research applications do not always require time resolution in the fluorescence detection, but as shown in many cases, the measurement of the decay time of the emission provides additional information which contributes to the understanding of the structure and function of the complex compounds.

IX. SUMMARY

It can be concluded that the time-resolved fluorescence detection of lanthanides and their chelates has been applied in a wide variety of both routine and research applications. Among the routine applications the immunoassays, although challenged by a number of other non-isotopic alternatives, represent a technique which is anticipated to gain wide use for the measurement of numerous analytes of different origin. As the lanthanide labels have a very high specific activity, they are ideal for assays in which a high sensitivity is required. The properties of the labels are optimally utilized in noncompetitive immunometric assays based on monoclonal antibodies. Assuming that the immunochemical problems are solved, the assay sensitivities should approach the achievable theoretical maximum which is around 10^{-15} to 10^{-16} mol/ℓ, supposing that the affinity constant for the antibody is 10^{10} to 10^{11} ℓ/mol. Although the present clinical range for most of the analytes does not require the utmost sensitivity, it is evident that once it is achieved new research results will support the introduction of sensitive assays of new and already existing analytes. It has recently been demonstrated for analytes such as thyrotropin and α-fetoprotein that high sensitivity is a clinical prerequisite. In immunoassays time-resolved fluorometry is thus considered as one of the most promising alternatives available in the nonisotopic field.

The successful application of the new technology to immunoassays supports the opinion that it will be useful, also, in other future routine applications for which nonisotopic alternatives are to be desired. The acceptance of the DNA probe-based hybridization assays into the clinical routine is dependent partly on the possibilities to simplify the assay procedures, but also partly on the availability of a convenient and sensitive nonisotopic label. The assay requirements with regard to testing conditions are, however, often completely different from those in immunoassays. This sets stricter demands on the label which have to be fulfilled before direct assays with lanthanide-labeled probes can be carried out. It has, however, been proven in indirect hybridization assays that the necessary sensitivity can be reached, which indicates that in the future lanthanide chelates are likely to be used for labeling of nucleic acids.

The possible research applications of lanthanide chelates in combination with time-resolved fluorescence are numerous. Although the radioisotopes are impossible to replace in many of the fields, some of the applications mentioned in Section VIII are likely to be developed further into useful routine assays. The results obtained, especially for the measurement of

cell-mediated cytolysis using Eu-labeled target cells, indicate that the test can be of general use. Some of the older research reports also prove that the long decay time of lanthanide fluorescence was already applied in the research carried out on biochemical macromolecules before it was used to improve the detection sensitivity of fluorescent labels.

The future expectations in the field of lanthanide chelates and time-resolved fluorometry lie of course in a wide application of the present assay concepts, but also in the basic work carried out on the fluorescent and stability properties of the chelates. Obviously, the aim is to combine good energy absorbing and transfer properties of a ligand as well as chelate stability into the same molecular structure. When this is done such a lanthanide label will be available that makes new assay designs and applications possible. It is, however, impossible to predict when the two stated requirements will be fulfilled and if they will actually provide the sensitivity already achieved with the accessible europium and terbium chelates in solution. The results that have been obtained with the available technology in research and also in routine applications assure, on the other hand, that the basic research aimed to improve the technology continues.

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