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Principles of luminescence spectroscopy. Luminescent determination of clinically and agriculturally important samples

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Abstract - The paper describes the basic principles of fluorescence spectroscopy, especially as applied to enzyme reactions. The utilization of front surface fluorescence for analysis of enzyme reactions and the future of this technology are discussed. The application of chemiluminescence spectroscopy for the assay of enzymes and metabolites, like cholesterol, triglycerides, the isoenzymes LDH and CPK etc. are discussed.

1. INTRODUCTORY REMARKS

1.1. History of luminescence

Luminescence is one of the oldest and most established analytical techniques, having been first observed by Monardes in 1565 from an extract of Ligirium nephiticiem. Sir David Brewster noted the red emission from chlorophyll in 1833, and Sir G.G. Stokes described the mechanism of the absorption and emission process in 1852. Stokes also named fluoresence after the mineral fluorspar (latin fluo = to flow + spar = a rock), which exhibits a blue-white fluorescence.

Phosphorescence dates back to the early 1500s, being so named after the Greek word for 'light bearing'. In fact the element phosphorus was named from this same Greek word in 1669 since it was found to produce a bright light in a dark room.

1.2. Light and its interaction with matter

Light is a form of electromagnetic radiation, the propagation of which is regarded as a wave phenomenon. Light is characterized by a wavelength λ and a frequency ν interrelated by the equation

$$\nu = \frac{c}{\lambda}$$

where c is the velocity of light, 3×10^{10} cm/sec.

When light impinges upon matter, two things can happen: it can pass through the matter with no absorption taking place, or it can be absorbed either entirely or in part. In the latter case energy is transferred to the molcecule in the absorption process.

Absorption of energy must occur in integral units, called quanta. The quanta-energy relationship can be expressed by the equation

$$E = h\nu = \frac{hc}{\lambda}$$

where E is the energy, h is Planck's constant (6.62×10^{-27} erg-sec). Note that energy E is inversely related to wavelength λ.

Every molecule possesses a series of closely spaced energy levels and can go from a lower to a higher energy level by the absorption of a discrete quantum of light equal in energy to the difference between the two energy states (Fig. 1). Only a few molecules are raised to this higher excited state and hence capable of exhibiting luminescence. Between each main electron state are the various vibration levels of the molecule. In Fig. 1, which illustrates the various potential energy levels of a diatomic molecule, are indicated the various vibrational levels, represented as O, 1, 2, 3, and 4, of each curve. The ground state is indicated by G, the first excited singlet electronic state by S*, and the first excited triplet by T*. Differences in the singlet and triplet are differences in the spin of the electron, S. All electrons have a spin S equal to $\pm 1/2$. The arrow designations often used to denote spin ($\uparrow\downarrow$) arise from the right-hand rule. If one curls the fingers of the right hand in the direction of the spin, the thumb points in the direction of the arrow, and vice versa. The spin, S, can be denoted as +1/2 or -1/2.

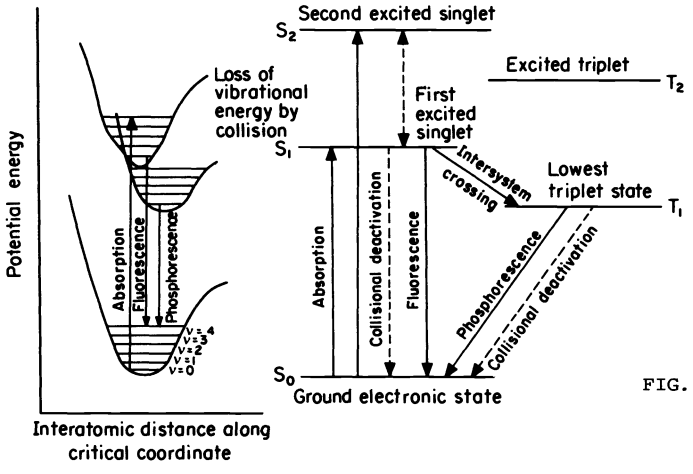


FIG. 1. Schematic energy-level diagram for a diatomic molecule.

A normal polyatomic molecule in the ground state G usually has an even number of electrons with paired spins. Thus there is one electron with $S = +1/2$ and one with $S = -1/2$. Multiplicity is a term used to express the orbital angular momentum of a given state and is related to the spin by the equation

$$M = 2S + 1.$$

Thus when all electrons are paired, $S = 0$ $(+ 1/2 - 1/2 = 0)$, and the multiplicity equals 1. This is called a singlet electronic state. When the spin of a single electron is reversed, the molecule finds itself with two unpaired electrons and $S = 1$ $(+ 1/2 + 1/2 = 1)$ and the multiplicity is 3 $(2(1) + 1 = 3)$. This electronic state is called a triplet.

Photons in the ultraviolet and visible regions of the electromagnetic spectrum have energies of 35 to 145 kcal/mole and promote electronic transitions. More energetic photons cause photodecomposition, rather than electronic transitions. Less energetic photons have only enough energy to cause a vibrational or rotational transition. The less energetic vibrational transitions are often superimposed on the electronic transition and are observed as fine structure.

When a quantum of light impinges on a molecule, it is absorbed in about 10^{-15} sec, and a transition to a higher electronic state takes place (Fig. 1). This absorption of radiation is highly specific, and radiation of a particular energy is absorbed only by a characteristic structure. The electron is raised to an upper excited singlet state, S_1, S_2, etc. These ground-to-singlet transitions are responsible for the visible and ultraviolet-absorption spectra observed for molecules. The absorption transitions usually originate in the lowest vibrational level of the ground electronic state.

During the time the molecule can spend in the excited state, 10^{-4} sec, some energy in excess of the lowest vibrational energy level is rapidly dissipated. The lowest vibrational level ($\nu = 0$) of the excited singlet state S is attained. If all the excess energy is not further dissipated by collisions with other molecules, the electron returns to the ground electronic state, with the emission of energy. This phenomenon is called fluorescence. Because some energy is lost in the brief period before emission can occur, the emitted energy (fluorescence) is of longer wavelength than the energy that was absorbed.

The phenomenon of phosphorescence involves an intersystem crossing, or transition, from the singlet to the triplet state. A triplet state results when the spin of one electron changes so that the spins are the same, or unpaired. The transition from the ground state to the triplet excited state is a forbidden (highly improbable) transition. Internal conversion from the singlet to the triplet (electronic-spin reversal) is more probable since the energy of the lowest vibrational level of T* is lower than that of S*. Molecules in T* can then return to the ground state G directly, since a return via S* could result only by acquiring energy from the environment (this sometimes occurs and is called delayed fluorescence, as we shall see). Transition times of 10^{-4} to 10 sec are observed in phosphoresence. Hence a characteristic feature of phosphorescence is an afterglow, that is, emission that continues after the exciting source is removed. Because of the relatively long lifetime of the triplet state, molecules in this state are much more susceptible to radiationless deactivation processes, and only substances dissolved in a rigid medium phosphoresce.

2. TYPES OF LUMINESCENCE

The various types of luminescence can be classified according to the means by which energy is supplied to excite the luminescent molecule.

When molecules are excited by interaction with photons of electromagnetic radiation, the form of luminescence is called photoluminescence. If the release of electromagnetic energy is immediate or from the singlet state, the process is called fluorescence, whereas phosphorescence is a delayed release of energy from the triplet state. Some molecules exhibit a delayed fluorescence that might incorrectly be assumed to be phosphorescence. This results from two inter-system crossings, first from the singlet to the triplet, then from the triplet to the singlet.

If the excitation energy is obtained from the chemical energy of reaction, the process is chemiluminescence. In bioluminescence the electromagnetic energy is released by organisms.

Triboluminescence (Greek tribo, to rub) is produced as a release of energy when certain crystals, such as sugar, are broken. The energy stored on crystal formation is released in the breaking of the crystal.

Other types of luminescence - cathodoluminescence resulting from a release of energy produced by exposure to cathode rays, or thermoluminescence which occurs when a material existing in high vibrational energy levels emits energy at a temperature below red heat, after being exposed to small amounts of thermal energy - are much less commonly encountered.

3. TYPES OF FLUORESCENCE AND EMISSION PROCESSES

The fluorescence normally observed in solutions is called Stokes fluorescence. This is the re-emission of less energetic photons, which have a longer wavelength (lower frequency) than the absorbed photons.

If thermal energy is added to an excited state or a compound has many highly populated vibrational energy levels, emission at shorter wavelengths than those of absorption occurs. This is anti-Stokes fluorescence, often observed in dilute gases at high temperatures. A common example is the green emission from copper-activated cadmium sulfide excited by red light.

Resonance fluoresence is the re-emission of photons possessing the same energy as the absorbed photons. This type of fluorescence is never observed in solution because of solvent interactions, but it does occur in gases and crystals. It is also the basis of atomic fluorescence. Atomic fluorescence spectroscopy is an excellent technique for the assay of many elements and will be discussed.

If an electron is excited by an absorbed photon of energy to a higher vibration level with no electronic transition, energy is entirely conserved and a photon of the same energy is re-emitted within 10^{-15} sec as the electron returns to its original state. The emitted light has the same wavelength as the exciting light since the absorbed and emitted photons are of the same energy. The emitted light is referred to as Rayleigh scattering and occurs at all wavelengths. Its intensity, however, varies as the fourth power of the wavelength, so its effect can be minimized by working at longer wavelengths. It is a problem when the intensity of fluorescence is low in comparison with the exciting radiation and when the absorption and fluorescence spectra of a substance are close together.

Another form of scattering emission related to Rayleigh scattering is the Raman effect. Raman scatter appears in fluorescence spectra at higher and lower wavelengths (the former being more common) than the Rayleigh-scatter peak, and these Raman bands are satellites of the Rayleigh-scatter peak with a constant frequency difference from the exciting radiation. These bands are due to vibrational energy being added to, or subtracted from, this excitation photon. The Raman bands are much weaker than the Rayleigh-scatter peak but become significant when high-intensity sources are used. The relationship between the fluorescence band, Rayleigh scatter, and Raman scatter is shown in Fig. 2.

4. EXCITATION SPECTRUM

Any fluorescent molecule has two characteristic spectra: the excitation spectrum (the relative efficiency of different wavelengths of exciting radiation to cause fluorescence) and the emission spectrum (the relative intensity of radiation emitted at various wavelengths).

The shape of the exitation spectrum should be identical with that of the absorption spectrum of the molecule and independent of the wavelength at which fluorescence is measured. This is seldom the case, however, the differences being due to instrumental artifacts. Examination of the excitation spectrum indicates the positions of the

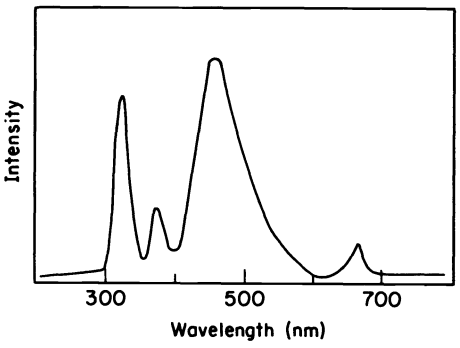


FIG. 2

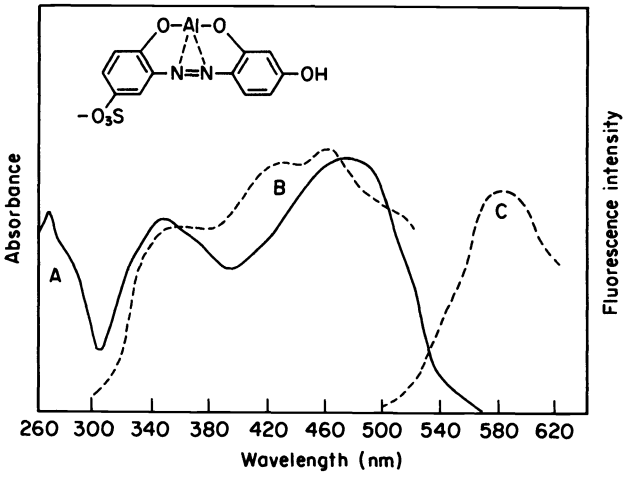


FIG. 3

FIG. 2. Fluorescence spectra of quinine sulfate in 0.1 N sulfuric acid (λ_{ex} = 320 nm). Peaks 320 nm, Rayleigh scatter; 360 nm, Raman scatter of water; 450 nm, quinine fluorescence; 640 nm second-order Rayleigh scatter; 720 nm, second-order Raman scatter.

FIG. 3. Absorption and fluorescence spectra of the aluminum complex with acid Alizarin Garnet R (0.008%): curve A, the absorption spectrum; curve B, the fluorescence-excitation spectrum; curve C, the fluorescence-emission spectrum.

absorption spectrum that give rise to fluorescence emission; for example, the excitation spectrum of the aluminum chelate of acid Alizarin Garnet R (Fig. 3) indicates peaks at 350, 430 and 470 nm. The absorption spectrum (run on a spectrophotometer) exhibits peaks at 270, 350 and 480 nm. The two spectra do not agree because (a) photomultiplier sensitivity changes, (b) the bandwidth of the monochromator changes, and (c) the slits remain constant in fluorescence. To obtain the true, or 'corrected'. spectra of the compound the apparent excitation curve would have to be corrected for these factors and then the absorption spectrum should be obtained.

A general rule of thumb is that the longest wavelength peak in the excitation spectrum is chosen for excitation of the sample. This minimizes possible decomposition caused by the shorter wavelength, higher energy, radiation.

5. EMISSION SPECTRUM

The emission, or fluorescence, spectrum of a compound results from the re-emission of radiation absorbed by that molecule. The quantum efficiency and the shape of the emission spectrum are independent of the wavelength of the exciting radiation. If the exciting radiation is at a wavelength that differs from the wavelength of the absorption peak, less radiant energy will be absorbed and hence less will be emitted. The emission spectrum of the aluminum-acid Alizarin Garnet R complex indicates a fluorescence peak at 580 nm (curve C, Fig. 3).

Each absorption band to the first electronic state will have a corresponding emission, or fluorescence, band. These two bands, or spectra, will be approximately mirror images of each other. In fact this mirror-image principle is useful in distinguishing whether an absorption band is another vibrational band in the first excited state or a higher electronic level. Fluorescence peaks other than the mirror image of the absorption spectrum indicate scatter or the presence of impurities. Rayleigh and Tyndall scatter can be observed in the emission spectrum at the same wavelength as the excitation wavelength and also at twice this value (second-order grating effect). In very dilute solutions one may also observe Raman scatter. The wider the fluorescence band, the more complex and less symmetrical the compound.

Figure 4 shows the absorption and emission spectra of anthracene and quinine. Four major absorption peaks are observed in the anthracene spectrum; all correspond to transitions from S_0 to S_1* but denote transitions to different vibrational levels. Four major emission peaks, each a mirror image of the peaks in the absorption spectrum, are likewise observed. For quinine two excitation peaks are observed, one at 250 nm corresponding to an $S_0 \rightarrow S_2$* transition. Only one emission peak, corresponding to the $S_0 \rightarrow S_1$ transition is observed.

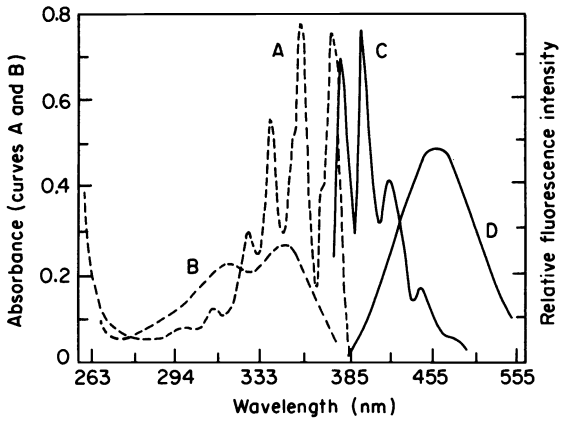


FIG. 4. Absorption and fluorescence spectra of anthracene (in ethanol) and quinine (in 0.1 N sulfuric acid): curve A, anthracene absorption; curve B, quinine absorption; curve C, anthracene fluorescence; curve D, quinine fluorescence.

The fact that some compounds possess several excitation and/or emission peaks is of analytical usefulness. If two compounds have overlapping excitation bands, as in the case of anthracene and quinine, both could be excited together and then differentiated by their emission spectra. Quinine could be measured at a λ_{em} of 450 nm, whereas anthracene could be monitored at a λ_{em} of 400 nm. Similarly, if two compounds emit radiation at the same wavelength, they can still be measured together in the same solution if they have different, non-overlapping, excitation peaks. This, in fact, is one of the major advantages that fluorescence spectroscopy has over absorption spectroscopy.

Any portion of the spectrum where absorption occurs can produce fluorescence since emission almost always takes place from the lowest vibrational level or the state to which the molecule is originally excited. The fluorescence peak will be at the same wavelength regardless of the excitation wavelength; however, the intensity of the fluorescence will vary with the relative strength of the absorption (or the sum total of all the absorptions).

A physical constant that is characteristic of luminescent molecules is the difference between the wavelengths of the excitation and emission maxima. This constant is called the Stokes shift and indicates the energy dissipated during the lifetime of the excited state before return to the ground state:

$$\text{Stokes shift} = 10^7 \frac{1}{\lambda_{ex}} - \frac{1}{\lambda_{em}},$$

where λ_{ex} and λ_{em} are the corrected maximum wavelength for excitation and emission, and are expressed in nanometers. The Stokes shift is of interest to analytical chemists since the emission wavelength can be greatly shifted by varying the form of the molecule being excited. The fluorescence-maximum shift of 5-hydroxyindole from 330 nm at pH 7 to 550 nm in strong acid occurs with no change in the excitation peak (295 nm) and is due to excited-state protonation.

6. QUANTUM YIELD

Every molecule possesses a characteristic property that is described by a number called the quantum yield, or quantum efficiency, Φ. This is the ratio of the total energy emitted per quantum of energy absorbed:

$$\Phi = \frac{\text{number of quanta emitted}}{\text{number of quanta absorbed}} = \text{quantum yield}$$

The higher the value of Φ, the greater the fluorescence of a compound. A non-fluorescent molecule is one whose quantum efficiency is zero or so close to zero that the fluorescence is not measureable. All energy absorbed by such a molecule is rapidly lost by collisional deactivation.

7. RELATION BETWEEN FLUORESCENCE INTENSITY AND CONCENTRATION

The basic equation defining the relationship of fluorescence to concentration is

$$F = \Phi I_o (1 - e^{-\varepsilon bc}),$$

where Φ is the quantum efficiency, I_o is the incident radiant power, ε is the molecular absorptivity, b is the path length of the cell, and c is the molar concentration.

The basic fluorescence intensity-concentration equation indicates that there are three major factors other than concentration that affect the fluorescence intensity:

1. The quantum efficiency Φ. The greater the value of Φ, the greater will be the fluorescence, as already discussed.

2. The intensity of incident radiation, I_0. Theoretically, the more intense source will yield the greater fluorescence. In actual practice a very intense source can cause photodecomposition of the sample. Hence one compromises on a source of moderate intensity (i.e. a mercury or xenon lamp is used).

3. The molar absorptivity of the compound ε. In order to emit radiation a molecule must first absorb radiation. Hence, the higher the molar absorptivity, the better will be the fluorescence intensity of the compound. It is for this reason that saturated non-aromatic compounds are nonfluorescent.

For very dilute solutions the equation reduces to one comparable to Beer's law in spectrophotometry,

$$F = K \Phi I_0 \varepsilon b c.$$

Thus a plot of fluorescence versus concentration should be linear at low concentrations and reach a maximum at higher concentrations (Fig. 5). At high concentrations quenching becomes so great that the fluorescence intensity decreases (inner-cell effect). The linearity of fluorescence as a function of concentration holds over a very wide range of concentration (Fig. 5). Measurements down to 10^{-5} µg/ml are feasible, and linearity extends up to 100 µg/ml or higher.

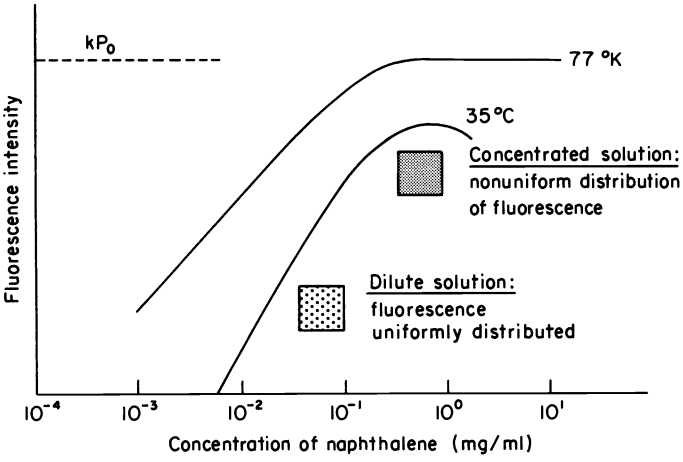


FIG. 5. Dependence of fluorescence on the concentration of fluorophor and temperature.

Generally a linear response will be obtained until the concentration of the fluorescent species is large enough to absorb significant amounts of exciting light. For a linear response to be obtained the solutions must absorb less than 5% of the exciting radiation. At higher concentrations light scattering as well as the inner-cell effect become important. In the concentration regions where fluorescence is proportional to concentration, fluorescence is measured in the absence of significant radiation. In this region the energy available for excitation is uniformly distributed through the solution.

8. INTRODUCTION TO EXPERIMENTATION

The fundamental principles of fluorescence measurements are illustrated by Fig. 6, a simplified schematic representation of a filter fluorometer. The desired narrow band of wavelengths of exciting radiation is selected by a filter (called the primary filter) placed between the radiation source and the sample. The wavelength of fluorescence radiant energy to be measured is selected by a second optical filter (called the secondary filter) placed between the sample and a photodetector located at a 90° angle from the incident optical path. The output of the photodetector, a current that is proportional to the intensity of the fluorescent energy, is amplified to give a reading on a meter or a recorder. In a spectrofluorometer the filters are replaced by prism or grating monochromators, and an x-y recorder is used to display the excitation and emission spectra.

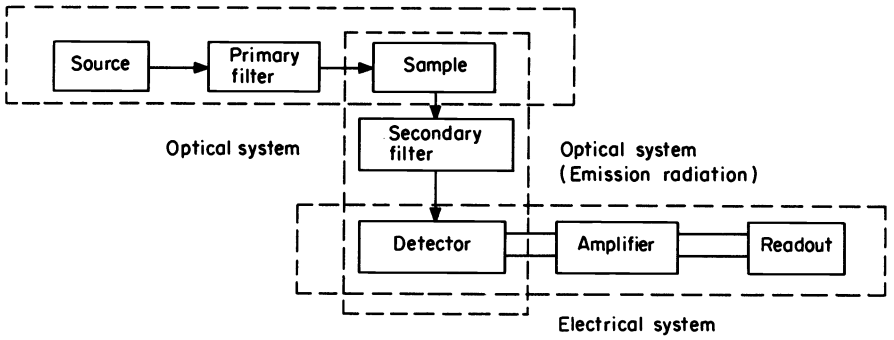


FIG. 6. Schematic diagram of the optical components of a typical filter fluorometer.

The geometry and nature of the fluorometric measurement accounts for its excellent sensitivity, which surpasses absorption methods by three to four orders of magnitude. The fluorometer is capable of measuring low concentrations of substances with good reliability, whereas the spectrophotometer loses accuracy as the signal from the sample approaches that of the reference.

Because of the 180^{o} geometry of the spectrophotometer, the detector constantly views the source, which is very intense. This results in a large electronic signal. In fluorometry it is not necessary to measure small differences between the sample and blank; rather, the detector senses only the sample fluorescence since it is placed at a right angle. Hence the background is zero, and the sample reading can be adjusted to 100% by controlling the intensity of the source by varying the monochromator slits or by controlling the detector amplification. Thus the accuracy is almost completely independent of concentration.

9. PRACTICAL CONSIDERATIONS

9.1. Advantages of fluorescence

Molecular emission (fluorescence and phosphorescence) is a particularly important analytical technique because of its extreme sensitivity and good specififity. Fluorometric methods can detect concentrations of substances as low as one part in ten billion, a sensitivity of 1000 times greater than that of most spectrophotometric methods. The main reason for this increased sensitivity is that in fluorescence the emitted radiation is measured directly and can be increased or decreased by altering the intensity of the exciting radiant energy. An increase in signal over a zero background signal is measured in fluorometric methods. In spectrophotometric methods the analogous quantity, absorbed radiation, is measured indirectly as the difference between the incident and the transmitted beams. This small decrease in the intensity of a very large signal is measured in spectrophotometry with a correspondingly large loss in sensitivity.

The specificity of fluorescence is the result of two main factors: (a) there are fewer fluorescent compounds than absorbing ones because all fluorescent compounds must necessarily absorb radiation, but not all compounds that absorb radiation emit; (b) two wavelengths are used in fluorometry, but only one in spectrophotometry. Two compounds that absorb radiation at the same wavelength will probably not emit at the same wavelength. The difference between the excitation and emission peaks ranges from 10 to 280 nm (emission at the same wavelength as excitation is scatter, as we have seen).

Materials that possess native fluorescence, those that can be converted to fluorescent compounds (fluorophors), and those that extinguish the fluorescence of other compounds can all be determined by fluorometry.

9.2. Limitations of fluorescence

The principle disadvantage of fluorescence as an analytical tool is its serious dependence on the environment (temperature, pH, ionic strength, etc.)

9.2.1. Photochemical decomposition

The ultraviolet bright light used for exitation may cause photochemical changes in, or destruction of, the fluorescent compound, giving a gradual decrease in the intensity reading. In a practical sense one can take three measures to avoid photochemical

decomposition: (a) always use the longest wavelength radiation for excitation; (b) measure the fluoresence of the sample immediately after excitation - not allow the exciting radiation to strike the sample for long periods; (c) protect photochemically unstable standard solutions, such as quinine sulfate, from sunlight and ultraviolet laboratory lights by storing in a black bottle.

9.2.2. Quenching

Quenching, the reduction of fluorescence by a competing deactivating process resulting from the specific interaction between a fluorophor and another substance present in the system, is also frequently a problem.

The general mechanism for the quenching process can be denoted as follows:

$$M + h\nu \rightarrow M^* \quad \text{(light absorption)}$$

$$M^* \rightarrow M + h\nu \quad \text{(fluorescence emission)}$$

$$M^* + Q \rightarrow Q^* + M \quad \text{(quenching)}$$

$$Q^* \rightarrow Q + \text{energy}.$$

Four common types of quenching are observed in luminescence processes: temperature, oxygen, concentration, and impurity quenching. One of the most notorious quenchers is dissolved oxygen, which causes a reduction in fluorescence intensity and a complete destruction of phosphorescent intensity. Small amounts of iodide and nitrogen oxides are very effective quenchers and interfere.

Small amounts of highly absorbing substances like dichromate interfere by robbing the fluorescent species of the light available for excitation. For this reason most workers prefer not to wash their cuvettes with dichromate cleaning solution.

a. Temperature quenching. As the temperature is increased, the fluorescence decreases. The degree of temperature dependence varies from compound to compound. Tryptophan, quinine, and indoleacetic acid are compounds whose fluorescence varies greatly with temperature.

Temperature effects on luminescence are a type of excited-state quenching by encounter. The fluorescence changes are nearly those of molecular activity with temperature, which suggests that increasing temperature increases molecular motion and collisions, and hence robs the molecule of energy.

The change in fluorescence is normally 1% per $1^{\circ}C$; however, in some compounds, such as tryptophan or Rhodamine B, it can be as high as 5%.

b. Concentration quenching. Absorption causes many problems during a fluorometric assay, just as fluorescence causes a problem when the absorbance of a solution is measured.

In order for fluorescence to be observed absorption must occur. As we have already seen, the fluorescence intensity is proportional to the molar absorptivity: the more highly absorbing the substance, the greater its fluorescence. But when the absorption is too large, no light can pass through to cause excitation. Thus, at low concentrations, when the absorbance is less than about 0.05, there is a linear relationship between fluorescence and concentration (Fig. 7). At intermediate concentrations the light is not evenly

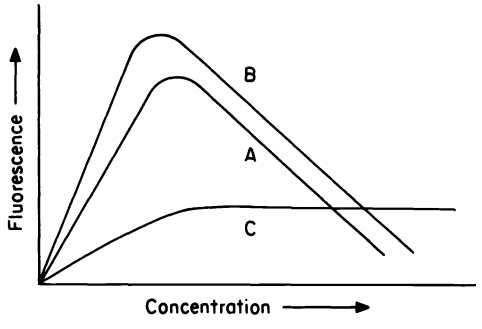


FIG. 7. Relationship between fluorescence and concentration.

A: End-on detector
B: Right angle detector
C: Surface detector

distributed along the path of light. The portion of the solution nearest the light source absorbs so much radiation that less and less is available for the rest of the solution. As a result, considerable excitation occurs at the front of the solution (Fig. 8), but less and less occurs throughout the rest of the cell. This type of concentration quenching causes a fluorescence loss that is called the inner-cell effect.

When fluorescence is measured at the surface (Fig. 8), the fluorescence increases linearly and then levels off as predicted by Eq. 7. This is explained by the fact that even in concentrated solutions only the surface is observed, and hence quenching is not important. However, in solutions, quenching does become important; hence the fluorescence decreases at high concentrations.

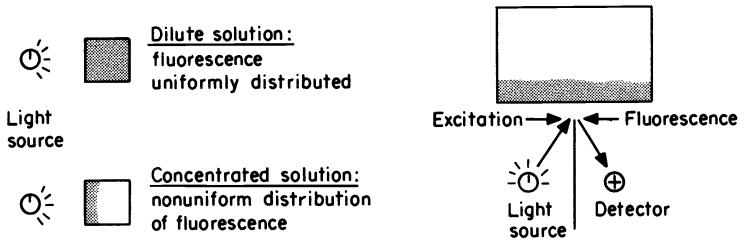


FIG. 8. Effect of concentration on fluorescence

Hence an unknown sample should always be tested for concentration quenching. Consider curve A in Fig. 7. There are two possible concentrations for each relative fluorescence value, one on each side of the maximum. To know which is correct, the analyst must dilute his sample and read again. If the fluorescence increases, then the previous reading was on the negative slope, and an incorrect value was obtained. In this case the solution should be diluted and read again. If the fluorescence decreases, then a correct value is obtained. All fluorescence methods should incorporate a linear curve with checks for concentrations quenching.

10. STRUCTURAL EFFECTS

10.1. General considerations

Fluorescence phenomena are not sensitive to the finer details of molecular structure; fluorescence is not generally useful as a 'fingerprinting' technique. Of the huge number of known organic and inorganic compounds, only a small fraction exhibits intense luminescence. In order to understand how molecular structure affects fluorescence, one must realize that fluorescence always competes with a variety of other processes. When a molecule is promoted to an electronically excited state, it may divest itself of its excess energy in a number of different ways the principal decay process being (a) fluorescence, (b) nonradiative decay (internal conversion of intersystem crossing) and (c) photochemical reaction. Which of these three processes dominates depends entirely on their relative rates. Thus, for fluorescence to dominate, one desires that the rate constant for radiative transitions be large relative to those for nonradiative decay or photodecomposition.

In general, therefore, strongly fluorescent molecules possess the following characteristics:

a. The spin-allowed electronic absorption transition of lowest energy is very intense (i.e. has a large ε_{max}). The intensity of absorption is directly proportional to the rate constant for the radiative transition. Because fluorescence is simply the reverse of absorption, it follows that, the more probable the absorption transition, the more probable (hence more rapid) will be the reverse (fluorescence) transition. Therefore, in order to predict whether or not a given molecule will fluoresce, an examination of its absorption spectrum is of considerable assistance.

b. The energy of the lowest spin allowed absorption transition should be reasonably low. The greater the energy of excitation, the more probable the occurrence of photodissociation.

c. The electron that is promoted to a higher level in the absorption transition should be located in an orbital not strongly involved in bonding. Otherwise, bond dissociation may accompany excitation, and fluorescence is unlikely to be observed.

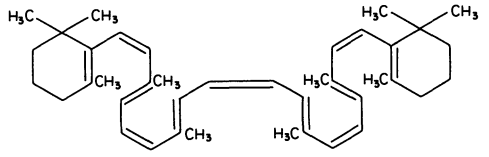
d. The molecule should not contain structural features or functional groups that enhance the rates of radiationless transitions. Although the theory of nonradiative processes is still in the process of development, we observe that certain structural features greatly increase the rates of radiationless processes and therefore adversely affect fluorescence intensities.

10.2. Factors of structure

10.2.1. π electron effect

On the basis of these few simple considerations we can easily understand why aromatic hydrocarbons are usually very intensely fluorescent. In these systems π electrons, which are less strongly held than σ electrons, can be promoted to π* antibonding orbitals by absorption of electromagnetic radiation of fairly low energy without extensive disruption of bonding. Furthermore, π → π* transitions in most aromatic hydrocarbons are strongly allowed (ε_{max} 10^4). The combination of these two factors singifies that aromatic compounds possessing low-lying (π, π*) singlet states usually fluoresce strongly.

In saturated hydrocarbons there are no π-bonding or nonbonding electrons; thus all electronic transitions involve σ-bonding electrons. We expect transitions involving σ electrons to occur at very high energies and, in addition, to significantly disrupt bonding in the molecule. In fact saturated hydrocarbons do fluoresce, though the fluorescence is very weak (fluorescence quantum efficiencies are on the order of 10^{-3}) and occurs in the 140- to 170-nm region (vacuum ultraviolet). In aliphatic carbonyl compounds n → π* transitions can occur, and these compounds consequently can exhibit fluorescence in the 'normal' ultraviolet or even the visible region, though the quantum efficiences are likewise very small. Some nonaromatic, but highly conjugated, compounds, such as β-carotene (1), vitamin A(2), and vitamin A aldehyde (3), are fluorescent, due to the occurence of π → π* transitions. In general, however, the vast majority of intensely fluorescing organic compounds are aromatic, and it is with such systems that we shall be mainly concerned.



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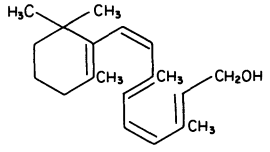
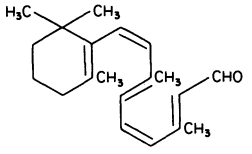
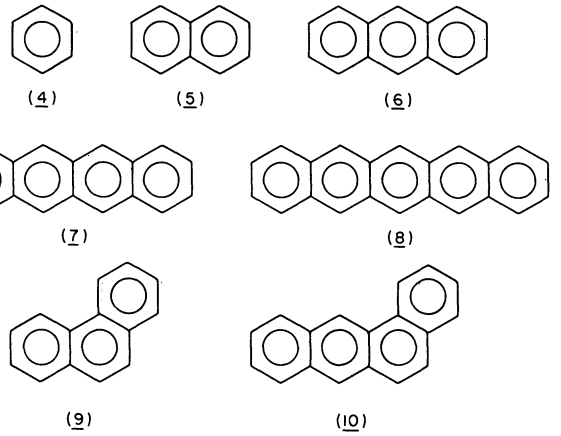


(2)



(3)



(4) (5) (6) (7) (8) (9) (10)

Most unsubstituted aromatic compounds exhibit an intense fluorescence in the ultraviolet or visible region. As the degree of conjugation increases, the intensity of fluorescence often increases and a bathochromic shift (shift to longer wavelengths) is observed (Table I). Thus benzene (4) and naphthalene (5) fluoresce in the ultraviolet, anthracene (6) in the blue, tetracene (7) in the green, and pentacene (8) in the red. For a given number of aromatic rings it is nearly always observed that linear ring systems fluoresce at longer wavelength than nonlinear systems. Thus λ_{em} is at 400 nm for anthracene (6) and at 350 nm for phenanthrene (9); similarly, λ_{em} is at 480 for tetracene (7) and at 380 nm for benz|a|-anthracene (10).

Table I. Luminescence of condensed linear aromatics in EPA[a] glass at 77 K

Compound	Φ_F	λ_{ex} (nm)	λ_{em} (nm)
Benzene[b]	0.11	205	278
Naphthalene[b]	0.29	286	321
Anthracene[c]	0.46	365	400
Tetracene[d]	0.60	390	480
Pentacene[e]	0.52	580	640

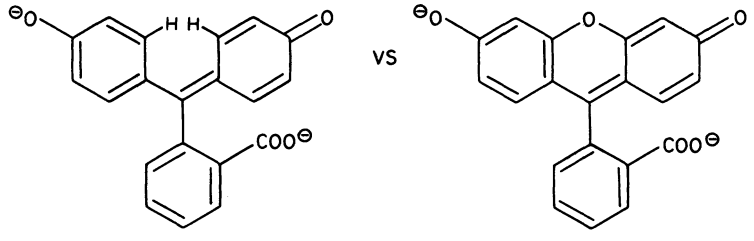
[a] A mixture of diethyl ether, isopentane, and ethanol, 5:5:2 (v/v/v).
[b] Fluoresces in the ultraviolet. [c] Fluoresces in the blue.
[d] Fluoresces in the green. [e] Fluoresces in the red.

10.2.2. Substituents

A simple generalization is that ortho-para-directing substituents often enhance fluorescence, whereas meta-directing groups repress it. Many of the common meta-directing substituents possess low-lying (n, π^*) singlets. Electron withdrawing groups (e.g. $-NO_2$) repress fluorescence. Electron donating groups (CN-, Cl-) enchance the fluorescence.

10.2.3. Rigid co-planarity

Rigid co-planarity is also a requirement of good fluorescence. In a comparison of phenophalen (11) vs fluorescein (12), the latter fluoresces strongly, because of rigid planatary, the former does not because it is free to rotate and dissipate its energy in solution.



(11) (12)

11. ENVIRONMENTAL EFFECTS

Environmental effects are of importance in fluorescence also: The nature of the solvent, pH, heavy atoms, oxygen, and temperature.

11.1. Solvent

In the case of solute-solvent pairs in which neither is appreciably popular, a red shift from the vapor spectrum is still observed. This shift is caused by a dispersive interaction resulting from the fact that electron transitions produce changes in the electron densities of solute molecules. Even if both solvent and solute are nonpolar in the initial and final states, the occurrence of an electronic transition in a solute requires a finite transition dipole which polarizes the surrounding solvent shell. This 'polarization shift' governs the magnitude of the red shift observed in going from gas-phase to solution spectra.

The fluorescence intensities of aromatic compounds can also be affected by electrostatic solvent effects. These effects are generally insignificant if both solute and solvent are nonpolar. For polar solute-solvent pairs electrostatic intensity perturbations are minor relative to those produced by specific short-range interactions (complex formation, hydrogen bonding, etc).

11.2. pH

Most hydroxy substituent aromatic compounds fluoresce better at high pH while the -OH group emerges to an electron donating substituent. The sole exception is phenol, which fluoresces better at pH 1 than 13.

11.3. Temperature

The higher the temperature, the lower the fluorescence. This is due to increased molecular collision resulting in dissipation of energy.

11.4. Heavy atoms

These promote an intersystem crossing from the singlet to the triplet: Phosphorescence is enhanced, fluorescence reduced. Diiodo-fluorescein is phosphorescent, chlorofluorescein is fluorescent.

11.5. O_2

The presence of O_2 effects a quenching of fluorescence, but it has the most effect on phosphorescence. This is the main reason solutions must be reduced to a low temperature or frozen in the solid state before phosphorescence is observed. The effect on fluorescence is minimal, and generally, judging O_2 is not necessary.

12. ASSAY OF ENZYMES IN CLINICAL AND AGRICULTURAL CHEMISTRY

12.1. Cellulase

Guilbault and Heyn (1) tested several fluorogenic substrates for cellulase: fluorescein dibutyrate, 1- and 2-naphthyl acetate, indoxyl acetate, and resorufin butyrate. The latter is cleaved by cellulase to the highly fluorescent resorufin (λ_{ex} 540, λ_{em} 560 nm). By the use of this substrate, from 0.00010 to 0.060 units of cellulase can be determined in only 1 min with an accuracy and precision of 1.5%. This compares most favourably with other methods which take 1 to 5 h and have a precision and accuracy of only 5 to 10%.

12.2. Lipase

Guilbault and Kramer (2,3) described a rapid and simple method for the determination of lipase based on its catalysis of the hydrolysis of the non-fluorescent dibutyryl ester of fluorescein:

Dibutyrylfluorescein ⟶ fluorescein

This reaction can be monitored by measuring the rate at which the highly fluorescent fluorescein is produced with time. The concentration of enzyme can then be calculated from linear calibration plots of ΔF/min versus enzyme concentration.

In a thorough study of fluorometric substrates for lipase Guilbault and Sadar (4) evaluated 12 different compounds from the aspect of stability, spontaneous hydrolysis, enzymatic hydrolysis, Michaelis constant for the enzyme-substrate complex, and total fluorescence of the final product. Optimum conditions of analysis were found for all substrates, and the lowest detectable enzyme concentration was found for each substrate. From all aspects, 4-methylumbelliferone heptanoate was found to be the best substrate for pig-pancreas lipase, and 4-methylumbelliferone octanoate was best for fungal lipase. As little as 1×10^{-5} unit could be determined by a direct reaction-rate method, with an accuracy and precision of about 1.5%.

Guilbault and Hieserman (5) prepared several new fluorometric substrates for the assay of lipase. A study of six N-methylindoxyl esters as substrates for lipase indicated N-methylindoxyl myristate to be best. By using this ester, from 0.0002 to 4.0 unit/ml of pig **pancreas can be determined, in the presence of several other esterases, with an accuracy and precision of about 1.5%. Analysis is performed by a direct initial-reaction-rate method in 2 to 3 min.**

12.3. Nucleases

A fluorometric assay for nucleases has been suggested by Stevens (6), who synthesized sulfonyl chlorides of fluorescent compounds and showed that they reacted with the amino groups of deoxyribonuclease to yield nondialysable fluorescent derivatives. Stevens pointed out that should such a fluorescent derivative be acted on by deoxyribonuclease, it will yield dialyzable fluorescent products as a measure of the nuclease activity.

12.4. Peptidase

Smith and Hill (7) utilized naphthylamine-containing peptides to demonstrate aminopeptidase activity by fluorescence microscopy. Subsequently Greenburg (8) reported the use of phenylalanyl-β-naphthylamide for the fluorometric assay of aminopeptidase activity in solution. By carrying out the reaction in a 0.1-ml volume at pH 8.0 (tris buffer) it was possible to measure as little as 10-12 mole of aminopeptidase by following the appearance of napthylamine (λ_{ex} 335, λ_{em} 410 nm). Subsequently Roth (9) utilized L-leucyl-β-naphthylamide to assay leucine aminopeptidase in kidney extracts, serum, urine and duodenal juice.

Oxytocinase, an enzyme that cleaves oxytocin at the cystinyl-tyrosine bond, also splits cystine-di-β-naphthylamide to yield free naphthylamine. Roth (9) adapted this reaction to the fluorometric assay of oxytocinase in the serum of pregnant women.

12.5. Proteases

Riedel and Wünsch (10) introduced benzolarginine-β - naphthylamide as a substrate for trypsin assay. In their studies the naphthylamine formed as a result of tryptic activity as assayed colorimetrically. More recently Roth (11) used the same substrate to develop a highly sensitive fluorometric procedure for trypsin assay. Assay of trypsin activities equivalent to 1 μg of the crystalline enzyme is relatively simple and can be carried out kinetically using incubation times of 1 to 5 min. The method can be used to measure much smaller amounts of enzyme. Fluorescence is measured at a λ_{ex} of 338 and a λ_{em} of 410 nm.

Trypsin can be assayed by its esterolytic activity with the substrate p-tosylarginine methyl ester hydrochloride (TAME). The methanol that is released can be assayed by a variety of procedures. Sardesai and Provido (12,13) determined the enzymatically formed methanol by a fluorometric procedure that involves oxidation to formaldehyde and condensation of the latter with acetylacetone and ammonia to a fluorophor. The procedure can be used to determine as little as 100 ng of trypsin and has been applied to measure trypsin activity in serum. Although the substrate is not specific for trypsin and can also be hydrolyzed by plasmin and thrombin, it can be made specific for trypsin by carrying out the assay with and without trypsin inhibitor.

A fluorometric method for the determination of trypsin by using α-benzoyl-L-arginine-β-naphthylamide (BANA) as substrate was described by Uete, Asahara, and Tsuchikura (14). The method is reported to be simpler and more sensitive than the Bratton-Marshall reaction.

The specific chrymotrypsin substrates N-acetyl-L-tryptophan ethyl ester (ATrEE) and N-acetyltyrosine ethyl ester (ATEE) are fluorescent, as are the products of chymotryptic digestion, N-acetyltryptophan and N-acetyltyrosine.

Bielski and Freed (15) noted that in both cases fluorescence increased on de-esterification. They utilized this finding to develop a sensitive procedure for chymotrypsin assay. One limitation of the method is that chymotrypsin itself is fluorescent at these wavelengths because of its aromatic amino acids. This is no problem as long as the concentration of enzyme is two orders of magnitude less than that of the substrate. Under such conditions there is an almost threefold increase in the fluorescence of ATrEE on hydrolysis and an almost fivefold increase in the fluorescence of ATEE. Because it fluoresces more intensely with excitation and emission at longer wavelengths, ATrEE is preferred.

When proteins are used as substrates of proteolytic enzymes, it is possible to measure enzyme activity by following the appearance of amino acids in the supernate after precipitation with trichloroacetic acid. Guroff (16) followed proteolytic activity with protein substrates by fluorometric assay of the increase in trichloroaceticacid-soluble tyrosine (free and peptide) during incubation. The method he used involved condensation with nitrosonaphthol.

Lüscher and Käser-Glasszmann (17) have developed a method for the measurement of fibrinolysin with the fluorescent dye Lissamine Rhodamine B 200, which is chemically bound to fibrinogen and is liberated on lysis of the fibrin clot. The fluorescent fibrinogen is prepared by treating the protein with the sulfonyl chloride of the dye. To measure fibrinolytic activity of a tissue sample the dye-labelled protein is incubated with it and other components required for clot formation. The fluorescence liberated in the fluid on clot lysis is proportional to fibrinolytic activity (18).

Fluorescein-labelled fibrin has been used as a substrate for the fluorometric assay of fibrinolysin. Strassle (19) used a similar method for the assay of fibrinolytic activity.

Gray et al (20) have shown that the 4-methylumbelliferone esters of benzoylglycine, benzyloxcarglycine and benzoncarbonylcitruline are good substrate for proteases.

13. BIOLUMINESCENT DETERMINATION OF CLINICALLY IMPORTANT SERUM SAMPLES

Bioluminescence is the subdivision of chemiluminescence, found in a biological system, and can be defined as the light accompanying a chemical reaction.

A widely used bioluminescent reaction is the pyridine nucleotide linked reaction; which is represented by the following:

$$NADH + FMN \xrightarrow{\text{Dehydrogenase}} NADH^{+} + FMNH_2 \qquad (1)$$

$$FMNH_2 + R\text{-}\overset{O}{\overset{\|}{C}}\text{-}H + O_2 \xrightarrow{\text{Luciferase}} h\nu + RCOOH + H_2O + FMN \qquad (2)$$

The light produced is related to the substrate concentration which is related to the production of NADH in prior steps. This method is extremely sensitive, selective, gives good linear response and requires simple instrumentation at low cost with ease of operation. The main disadvantage would be with the enzyme stability in solution which can be improved via enzyme immobilization. Currently we are investigating the following systems:

1. Glycerol & Triglyceride

$$\text{Glycerol} + NAD^+ \xrightarrow[(EC1.1.1.6)]{GDH} DHA + NADH + H^+ \quad (3)$$

$$NADH + FMN \xrightarrow{(NADH:\ FMN\ Oxidoreductase)} NAD^+ + FMNH_2 \quad (4)$$

$$FMNH_2 + \text{Decanal} + O_2 \xrightarrow[(Photobacterium\ Fischeri)]{Luciferase} h\nu + FMN + H_2O + RCOOH \quad (5)$$

For the analysis of triglycerides, the same procedure for assay of the glycerol is followed, after the hydrolysis of triglyceride to Glycerol:

$$\text{Triglyceride} + \xrightarrow[(E\ C.3.1.1.3)]{Lipase} \text{Glycerol} + \text{Free Fatty Acid} \quad (6)$$

2. Lactate Dehydrogenase

The total lactate dehydrogenase can be easily assayed as follows:

$$\text{Lactate} + NAD^+ \xrightarrow[(E\ C.1.1.1.27)]{LDH} NADH + \text{pyruvate} + H^+ \quad (7)$$

$$NADH + FMN \longrightarrow FMNH_2 + NAD^+ \quad (8)$$

$$FMNH_2 + \text{R-C-H} + O_2 \xrightarrow{Luciferase} h\nu + RCOOH + FMN + H_2O \quad (9)$$

The determination of the isoenzyme, LDH-1, is achieved following prior immunochemical separation (using the Isomune-LD kit from Roche Diagnostics).

3. Cholesterol

For the analysis of cholesterol, we have utilized more than one procedure:

a. $$\text{Cholesterol} \xrightarrow[\text{Dehydrogenase}]{\text{Hydroxysteroid}} \Delta^4 \text{ Cholesterone} + NADH \quad (10)$$

$$NADH + FMN \xrightarrow{\text{Dehydrogenase}} FMNH_2 + NAD^+ \quad (11)$$

$$FMNH_2 + RCOH + O_2 \xrightarrow{Luciferase} h\nu + FMN + RCOOH + H_2O \quad (12)$$

b. $$\text{Cholesterol} \xrightarrow[NAD^+]{CDH} \Delta^4 \text{ cholesterone} + NADH \quad (13)$$

$$NADH^+ \text{ .....} \longrightarrow h\nu + \text{ .....} \quad (14)$$

c. $\text{Cholesterol} + \text{COD} \longrightarrow \Delta^4 \text{ cholesterone} + H_2O_2$ (15)

$H_2O_2 + \text{EtOH} \xrightarrow{\text{Catalase}} \text{acetaldehyde}$ (16)

$\text{Acetaldehyde} \xrightarrow{\text{ALDDH}} \text{acetate} + \text{NADH} + H^+$ (17)

$\text{NADH} + \text{FMN} \longrightarrow FMNH_2 + NAD^+$ (18)

$FMNH_2 + \text{RCOH} + O_2 \xrightarrow{\text{Luciferase}} h\nu + \text{RCOOH} + \text{FMN} + H_2O$ (19)

d. $\text{Cholesterol} \xrightarrow{\text{COD}} \Delta^4 \text{ cholesterorone} + H_2O_2$ (20)

$H_2O_2 + \text{FMN} + \text{RCOH} \xrightarrow[\text{photobacterium phosphonium}]{\text{Luciferase}} h\nu$ (21)

For assay of cholesterol ester, first hydrolysis must be effected, then the same procedure is used:

$\text{Cholesterol ester} \xrightarrow{\text{cholesterolesterase}} \text{cholesterol} + \text{fatty acid}$ (22)

GDH = Glycerol Dehydrogenase
DHA = Dihydroxy Acetone
NAD = Nictoinamide Adenine Dinucleotide
FMN = Flavin Mononucleotide
NADH = Reduced NAD
$FMNH_2$ = Reduced FMN
$RCOH^2$ = Decanal
CDH = Cholesterol Dehydrogenase, 'could be isolated from nocardia'
COD = Cholesterol Oxidase
ALDDH = Aldehydedehydrogenase

Data on analysis using these systems will be described.

14. SEMISOLID SURFACE FLUORESCENCE

14.1 General

Fluorescent procedures are several orders of magnitude more sensitive than colorimetric methods and thus have replaced colorimetric assays in numerous instances. Further advantages are greater selectivity (since two wavelengths are used) and an accuracy independent of region of measurement.

Previous fluorometric methods, although an improvement over other methods of determining enzyme activity, have not eliminated all the problems associated with enzymatic analyses. Fluorometric analysis depends on the production of a fluorescent compound as a result of enzymatic activity between a substrate and enzyme. The rate of production of the fluorescent compound is related to both enzyme and substrate concentration. This rate can be quantitatively measured by exciting the fluorescent compound as it is produced and by recording the quantity of fluorescence emitted per unit time with a fluorometer.

Fluorometrically measuring enzyme activities or enzymatic reactions is usually done by wet chemical methods that rely on the reaction of a substrate solution with an enzyme solution. Unfortunately, wet chemicals methods involved the preparation of costly substrates, cofactors, coenzymes, and enzyme solutions. For example, when determing the presence and concentration of an enzyme, a substrate must be accurately measured and dissolved in a large amount of buffer solution, usually about 100 ml, to prepare a stock solution. The enzymatic reaction usually is carried out by measuring a certain volume of stock solution into an optical cuvette, adding a measured amount of enzyme solution to the substrate solution, and recording the change in absorbance emitted from the resultant solutions. When determining the concentration of a specific substrate kinetically in an enzyme-catalysed reaction, the procedure is even more cumbersome and costly. A relatively large amounts of expensive enzyme, usually 0.1 ml of stock solution, is needed to make the reaction proceed at a conveniently measurable rate. These enzyme solutions must be prepared fresh daily. This standard wet chemical method requires considerable technician time and relatively large quantities of expensive substrates or enzymes.

Rietz and Guilbault (21) developed solid-surface fluorometric methods using a 'reagentless' system for the assay of enzymes, substrates, activators and inhibitors. An attachment to an Aminco filter fluorometer has been adapted to accept, instead of a glass cuvette, a metal slide (a cell) that is painted black to reduce the background. A silicone rubber pad is placed on the slide. All the reagents for a quantitative assay are placed in the form of a solid reactant film on the surface of the pad. The sample of the fluid containing the substance to be assayed is then added to the pad. The change in fluorescence with time is measured and equated to the concentration of the substance determined.

These reagent pads are simple to prepare; hundreds could be conveniently manufactured at one time. They are stable for months or longer when stored under specified conditions. There is no need for the cumbersome, time-consuming preparation of reagents when performing an analysis, since essentially all the reagents for a quantitative assay are already present on the pad. If samples are hard to obtain, the pad method could be a great advantage, since only 3-25 µl of sample is required.

14.2. ASSAY OF IMPORTANT SUBSTRATES WITH IMMOBILIZED ENZYMES

14.2.a. Urea Nitrogen in Serum. The determination of serum urea nitrogen is presently the most popular screening test for the evaluation of kidney function. Elevated urea nitrogen values are found in patients with acute glomerulonephritis, chronic nephritis, polycystic kidney, nephrosclerosis and tubular necrosis.

A fluorometric method for the determination of urea in serum was developed using a coupled enzyme system (22).

$$\text{urea} \xrightarrow{\text{urease}} 2NH_4^+ \qquad (23)$$

$$NADH + 2NH_4^+ + \alpha\text{-ketoglutarate} \xrightarrow{\text{glutamate dehydrogenase}} NAD + \text{glutamic acid} \qquad (24)$$

The rate of disappearance of NADH fluorescence (λ_{ex} = 365 nm; λ_{em} = 460 nm) was proportional to the content of urea in serum. Reaction conditions and reagent concentrations were similar to that found using the spectrophotometric reagent kit by Calbiochem, but less NADH was used. The rate of decrease in the fluorescence of NADH, triggered by the addition of urea, was measured. The system worked well for assay of serum urea. The calibration plot is linear up to 25 mg of urea/dl. The method affords a rapid, simple, and inexpensive means for urea assay, the results of which correlate well with the automatic diacetyl monoxime method (correlation coefficient, 0.998).

14.2.b. Creatine in Urine. Creatine tolerance tests, which measure the ability of an individual to retain a test dose of creatine, are of great diagnostic value in indicating extensive muscle destruction and disease of the kidney. The following reaction scheme was investigated to develop a sensitive fluorometric method, and later on, a fluorometric pad procedure for determining creatine in biological samples (23).

$$\text{Creatine} + ATP \xrightarrow{CK} \text{creatine phosphate} + ADP \qquad (25)$$

$$\text{phosphoenolpyruvate (PEP)} + ADP \xrightarrow{PK} \text{pyruvate} + ATP \qquad (26)$$

$$\text{pyruvate} + NADH \xrightarrow{LDH} \text{lactate} + NAD \qquad (27)$$

The rate of fluorescence change of NADH, $\Delta F/min$, was proportional to the amount of creatine in the sample.

This scheme was successfully applied to urine creatine. The content of creatine in urine is about 10 times that in serum. Fluorogenic substances in urine interfere but can be easily removed by activated carbon. There was little or no loss of creatine as determined by a spectrophotometric method using 10 ml of urine treated with 0.5 g of activated carbon followed by filtration. The overall reaction occurred at pH 9 with Tris buffer. A linear calibration curve was obtained for 0-20 and 0-100 mg/dl creatine. The 0-20 mg/dl curve covered the normal range of urine creatine. The amounts of NADH, ATP, PEP, Mg, buffer mixture, and LDH-PK mixture were optimized. Two-fifths of the amount of NADH, as in the spectrophotometric method, gave a reasonable slope change, $\Delta F/min$, and about 3 min of linearity of the slope was obtained.

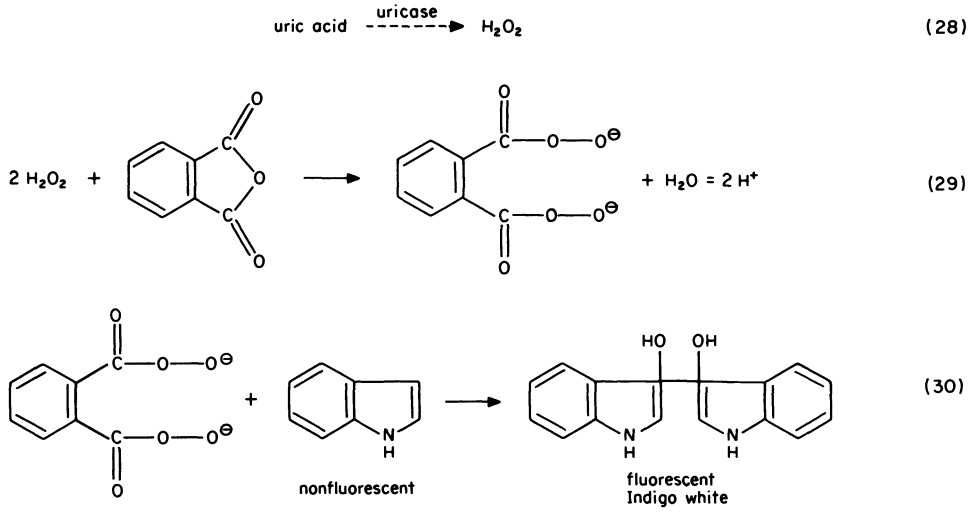
The complexity of this solution mixture made its preparation difficult; for many laboratories it would be impractical. A silicone pad method in which all the reagents were lyophilized or immobilized would be an ideal answer to this problem. However,

the crystalline lyophilized reagent is stable only for about 48 hr under refrigeration. Attempts are now being made to add some stabilizer to the mixture, such as mannitol, gum, or ammonium sulfate, and/or to lyophilized the reagents separately on a pad.

14.2.c. Uric Acid in Serum. Determination of serum uric acid is most helpful in the diagnosis of gout. Elevated levels of uric acid are found in patients with familial idiopathic hyperuricemia and in decreased renal function.

A direct reaction rate method was investigated for the sensitive determination of serum uric acid, based on a fluorometric Schoenemann reaction. The rate of formation of indigo white, $\Delta F/min$ (λ_{ex} = 395 nm, λ_{em} = 470 nm), is a measure of the uric acid content in the sample (24). Optimum conditions, such as pH, buffer system, and organic solvent of the hydrogen peroxide-phthalic anhydride, were studied. The optimum pH of the uricase reaction (pH 9.4) coincided with the optimum pH using phosphate buffer for the hydrogen peroxide-phthalic anhydride reaction (pH 9.1). Therefore, an overall pH of 9.4 was used. Phosphate buffer was found to be superior to Tris and borate buffers.

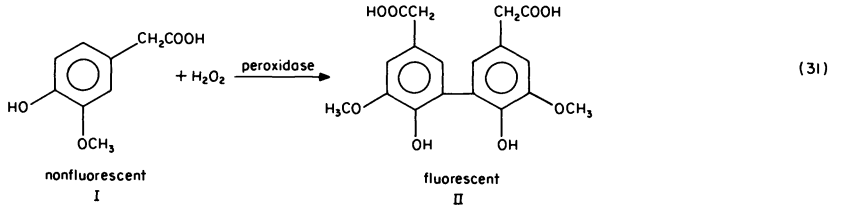
Acetone or methyl cellusolve both were good solvents for phthalic anhydride.



The rate of formation of hydrogen peroxide in the uricase-uric acid reaction was a function of uricase enzyme. Enough uricase enzyme was used so that in about 5 min the formation of hydrogen peroxide was completed, before the addition of the coupling fluorogenic reagents. The system works well and gives a linear response of 0-5 mg/dl of uric acid. Similar results were observed with the silicone pad method on which the enzyme and the buffer were placed.

Difficulties were encountered in serum samples, however. Protein had to be removed by either trichloracetic acid (TCA), acetone, or tungstate methods, and inhibition was still observed. Amino acids and ammonium hydroxide were found to inhibit the reaction so that lower values of uric acid were found.

Another fluorometric reaction system that holds great promise for a specific analysis of serum uric on silicone pads was developed (25). As shown, the method consists of oxidising uric acid with uricase to hydrogen peroxide which in turn oxidizes homovanillic acid (I) [reaction 31] to a highly fluorescent material (II) (λ_{ex} = 315 nm; λ_{em} = 425 nm). The rate of production of fluorescence is proportional to the amount of uric acid present.



A calibration curve was constructed from a series of standards and was linear from 0.1 to 14 mg/dl. The method is simple, precise and accurate. Comparison of the results obtained by this method with the standard enzymatic spectrophotometric method (disappearance of uric acid at 293 nm) gave a coefficient of variation of 0.93.

14.2.d. Cholesterol Assay. A method has been developed based on the following sequential enzymatic reactions.

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{cholesterol ester hydrolase}} \text{fatty acid} + \text{cholesterol} \quad (32)$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest-4-en-one} + H_2O_2 \quad (33)$$

$$H_2O_2 + \text{homovanillic acid} \xrightarrow{\text{peroxidase}} \text{fluorescent dimer} \quad (34)$$

The initial rate of fluorescent dimer formation is monitored and is proportional to the concentration of total cholesterol. A linear relationship was found in the range 0-400 mg/dl. Results had good precision and accuracy (26).

14.2e. Glucose Assay. Kiang et al. (27) proposed a simplifed assay of glucose using a solid-surface fluorescence method. Hexokinase and glucose 6-phosphate dehydrogenase were immobilised onto a silicone rubber pad and the NADH produced was measured. The pads may be used for 1 month after preparation and a good CV (3%) is obtained.

14.2f. Nitrate Ion Assay. In a prototype system that should be applicable to many other oxyanions, Kiang et al. (28) described a semisolid surface fluorescence (SSF) method for nitrate ion, using the NADH-dependent nitrate reductase from Chlorella vulgaris:

$$NO_3^- + NAD \xrightarrow{E} NADH + NO_2^- \quad (35)$$

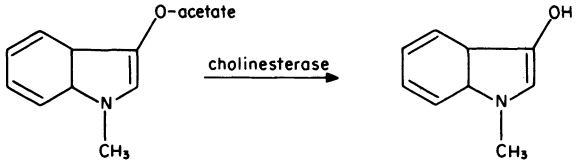
By placing the enzyme and NAD on a pad, the addition of NO_3^- effects conversion of NAD to NADH, which is measured fluorometrically. Good results were obtained. Attempts to extend this methodology to the assay of nitrite was only partially successful because of contamination of the nitrite reductase, isolated from Azotobacter chroococcum, with NADH reductase. The latter interferences in the assay by effecting a spontaneous reduction of NAD to NADH.

$$NO_2^- + 3\,NAD \xrightarrow[\text{reductase}]{\text{nitrite}} NH_3 + 3\,NADH \quad (36)$$

Both enzymes are highly specific, working only with nitrate or nitrite as substrate, respectively. The linear range was about 10^{-3} to 10^{-5} M substrate.

14.3. Assay of Enzymes with Immobilized substrates and/or Enzymes.

Simmerman and Guilbault (29) developed an SSF method for assay of cholinesterase, using immobilized N-methyl indoxyl acetate on a silicone rubber pad.



nonfluorescent N-methyl indoxyl acetate — fluorescent N-methyl indoxyl (37)

As little as 10^{-5} IU/liter of enzyme can be detected.

Guilbault and Vaughn (30) first developed an SSSF method for assay of acid and alkaline phosphatases in 1971. The substrate naphthol AS BI phosphate, a non-fluorescent compound, is used, which is cleaved by acid phosphatase at pH 5 or by a pH 8 to the highly fluorescent naphthol AS BI. The influence of substrate, drop volume, and shape of the drop on the background fluorescence was studied, as well as the effect of potential interferences such as bilirubin in blood.

Rietz and Guilbault (21) used 4-methylumbelliferone phosphate as substrate for the assay of acid and alkaline phosphatase. The highly fluorescent 4-methylumbelliferone is measured, the $\Delta F/\Delta t$ rate of formation being proportional to the concentration of this enzyme.

Rietz and Guilbault (31) developed an assay for γ-glutamyl transpeptidase using SSSF. The reagents N-γ-L-glutamyl-α-naphtylamide and glycylglycine are placed on the pad surface and the fluorescence is measured (λ_{ex} = 342 nm, λ_{em} = 445 nm):

$$\text{N-}\gamma\text{-L-glutamyl-}\alpha\text{-naphthylamide} + \text{glyglyc} \xrightarrow{\gamma\text{-GT}} \alpha\text{-naphthylamine} \qquad (38)$$

Only 10-50 µl of serum is required, and a direct assay is affected in 2-3 min. The range is 26-265 IU/liter with a precision of 2-3%.

Rietz and Guilbault (32) have subsequently described procedures for the assay of glutamate oxaloacetate transaminase and glutamate pyruvate transaminase using SSSF. Dade (Division of American Hospital Supply, Miami, Florida) tablets, containing all reagents necessary for a spectrophotometric assay, were dissolved in water, then 30 µl of the solution was added to the silicone rubber surface. After addition of 10 µl of serum, the fluorescence change due to NADH was measured and ΔF/min was plotted versus activity of GOT or GPT. The pads were stable for 3-4 days, and a linear range of 2.2-106 IU/liter was obtained. In this same report, a method was described for α-hydroxybutyrate dehydrogenase using SSSF. A layer of substrates palced on the pad was stable for up to 1 month.

Guilbault and Zimmerman (33) described an SSSF procedure for assay of LDH.

$$\text{lithium lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} \qquad (39)$$

In this method, 50 µl of 1 mM NAD, and 20 µl of 1 mM lithium lactate were placed onto a silicone rubber pad. Upon addition of 20 µl of sample (LDH), the fluorescence of NADH was produced and monitored. The linear range sas 160-820 IU/ml, with 3% CV. The pads prepared with immobilized lactate and NAD, were stable for 30 days, with a day-to-day variation of about 3%.

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