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Speaker

PHOSPHORESCENCE AT EXTREMELY LOW CONCENTRATIONS

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The persistence of phosphorescence is just lately emerging as a major spectroscopic tool. In particular, the extended lifetime of this form of luminescence brings into view a spectrum freed from shorter lifespan phosphorescence and fluorescence. And with abundant materials to display this lingering emission, as well as the sensitivity of phosphorescence to perturbations in molecular activity and environment, we are not faced with a shortage of laboratory applications. As might be expected from a phenomenon discovered long ago, implementation in research awaited an effective measurement technique; with its advent — a pulsed-lamp phosphorimeter (1) — phosphorescence is probing a multitude of spectrofluorometric questions.

The SPEX Phosphorimeter combines a flash lamp with signal-gating circuitry that inserts a variable delay between the excitation and sampling periods in such a way that spectral features can be conveniently isolated according to their lifetimes. In addition, since the peak intensity of the pulsed lamp is so much greater than the conventional 450 W lamp, sampling times can be extremely short to further sharpen time discrimination. Millisecond (or lower) sampling times offer yet another bonus; photomultiplier dark counts contribute only minutely to the signal so excellent detectivity and accuracy are attained with inexpensive PMTs. And since data accumulates only when the excitation source is off, stray light is never seen at all.

With these attributes, then, it is not surprising that phosphorimetry inherits the problems of extremely weak concentrations—trace analyses where the lower limit of detection is established by the smallest bit of phosphor that can be identified— or phosphorescing ions (especially europium and terbium fluorescent tags) probing biological systems with typical relevant concentrations of 10^{-5}M.

Phosphorescence is attributable to a change in electron spin multiplicity as an excited molecule or atom reverts to the ground state. This can be the result of a decay from a triplet (T) to a singlet state (S), for instance. A quick insight into the factors influencing the intensity of such transitions can be gained from this simplified equation (2):

$$\Phi_p = \Phi_{ST} \frac{k_p}{k_p + k_q (Q)} \qquad [1]$$

Here Φ_p is the quantum yield for phosphorescence and Φ_{ST} is the quantum yield for intersystem crossing from an excited S to an excited T state. Since the excitation of the particular molecule directly into an excited T state involves the coupling of the electron spin with the orbital angular momentum (spin-orbit interaction) and in most systems this coupling is feeble, the primary source to populate the triplet state is through a higher S state that has already been excited. The greater the probability of this intersystem crossing, the higher the phosphorescence yield. The radiative constant, k_p, reflects the strength of the coupling between the excited state and the ground state into which it decays, while k_q summarizes all contributions from "quenching" whereby the constituents of the sample (or the matrix a phosphor inhabits) tend to depopulate the excited state through non-radiative transitions. It is a function of the concentration of the quenching species, Q. (An additional term can be introduced to account for unimolecular, non-radiative processes such an internal conversion.)

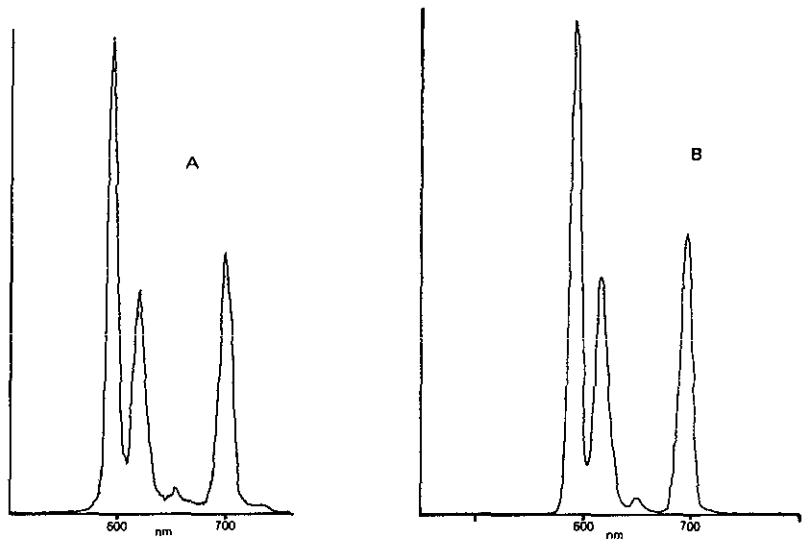


Fig 1 Europium spectra in H_2O (A) and D_2O with a 30 μsec delay between excitation and sampling. Though the features appear identical, the intensity of B is actually 40 times that of A.

Quenching and Uranium at the ppb Level

The effects of quenching can be appreciated from Fig 1. Here A is the spectrum of a sample of $EuCl_3 \cdot H_2O$, while B is that of $EuCl_3 \cdot D_2O$, both excited at 395 nm which is in the absorption band of europium. These spectra were obtained on the SPEX FLUOROLOG spectrofluorometer with the 1934 Pulsed-lamp Phosphorimeter inserting a 30 μsec delay between each flash and the sampling which followed. Each point represents the average intensity resulting from 10 flashes.

Though the spectra appear virtually identical, the emission of the deuterized sample is actually 40 times more intense. The

mechanism responsible for this enhancement involves the high-frequency O-H vibrations in the ambient medium of the aqueous europium which provide a radiationless path to the ground state before a photon can be emitted. When deuterium is substituted for hydrogen in the solutions, the more massive isotope reduces the energy of these vibrations, making this path of relaxation more difficult by requiring more steps to bridge the gap between states. Thus, the phosphor is granted more time to phosphoresce. A lifetime determinination, calculated from a plot of intensity as a function of delay time, confirmed this interpretation, showing that the isotopic substitution extends the lifetime of the excited state from 1.25×10^{-4} to 3.45×10^{-3} seconds.

The energy gap between terbium excited and ground states is somewhat wider than that of europium and we would expect the non-radiative vibrational path to be less effective here and, consequently, the deuterium enhancement smaller. This is evidenced by an increase in terbium intensity and lifetime of only tenfold (4.3×10^{-4} to 4.45×10^{-3} seconds) going from H_2O to D_2O.

Despite all this, the replacement of hydrogen by deuterium does not alter the symmetry of an ion's environment and only those changes associated with the closer vibrational spacing of the system occur, so the spectral integrity is preserved and the quantum yield increased by limiting k_q in Eq 1 without altering the structure of the emission—a principal consideration for possible laser applications (3). And the consequences of this form of enhancement are readily expanded to trace analysis.

The detection of uranium whether in explorations for new supplies or in monitoring plant effluents and the environment, is often limited by interference from the natural fluorescence of organic constituents of the sample which compete with the uranium emission. But a pulsed-lamp phosphorimeter readily segregates the uranium emissions from these sources of background.

Uranium salts respond to uv excitation with a characteristic green phosphorescence as shown in Fig 2 which is the spectrum of $UO_2(NO_3)_2 \cdot H_2O$ at a concentration of 100 ppb taken with the Phosphorimeter, and the FLUOROLOG's slits set to 5 nm. When the slits were opened to maximum bandpass (about 60 nm) concentrations down to 10 ppb were immediately and unambiguosly identified.

In the routine analysis of uranium salts, it is customary to add some type of enhancer to extend the limits of detection. One of these is FLURAN, a proprietary reagent from Scintrex. Yet we have discovered that phosphoric acid offers a similar, though somewhat subdued, enhancement. The mechanism of this reaction is essentially the same as that in the Eu-Tb study above which reduces the non-radiative paths to the ground state

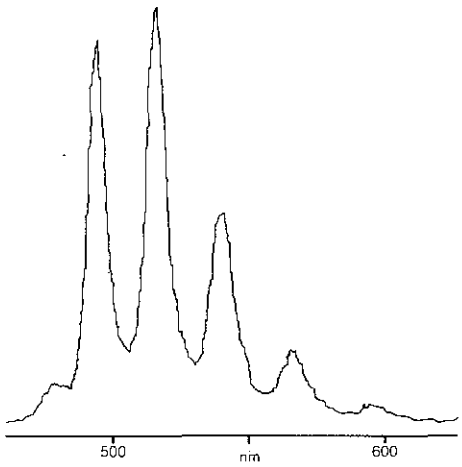


Fig 2 Phosphorescence spectrum of uranium at the 100 ppb level taken on the FLUOROLOG with the 1934 Phosphorimeter. Slit width was set to 5 nm.

without altering the spectra.

Fig 3A is the spectrum of such a phosphoric acid-enhanced solution containing only 1.7 ppb uranium. Since the slits were so wide (to permit maximum throughput), the individual peaks are no longer resolved, though the position of the emission brands it as originating from uranium. The lower feature is background from the solvent which is subtracted away by the Spex SC-31 Programmable Data Handler in Fig 3B.

In practice, when many samples are regularly processed, it is not necessary to run complete scans to obtain accurate determinations. The peak maximum in 3B represents some 50,000 counts accumulated during a 10 second integration time. To gauge the uranium concentration, it is sufficient to record that point and one to either side of it and, therefore, only 30 seconds of operating time are actually consumed.

1.7 ppb is hardly the lower limit of detection with this system. The signal-to-noise ratio in Fig 3B, for instance, is about 25:1 and if a ratio of 10:1 is considered adequate to identify a uranium peak, the 0.2 ppb level is already achieved. Furthermore, with refinements in the technique, such as an increase in the integration time or the substitution of FLURAN for phosphoric acid, the parts per trillion level emerges as accessible.

Modifications of Transition Moments in Europium

As noted, the sensitivity of phosphorescing ions to changes in their local environment can be applied to exploring the structure of complex molecules. Usually, alterations in the spectra when a phosphor is introduced into a system are due to shifts in the energy levels (which would appear as a displacement of the peaks) or to a modification of transition moments that, in turn, is reflected in the peak intensities. In europium, the 4f orbitals responsible for phosphorescence are well shielded by the overlying $5s^2$ and $5p^6$ shells, so the position of the emission is relatively immune to outside perturbations. However, the coupling between the excited and ground states (k_p in Eq 1) may be markedly affected and the consequent alteration in transition moments is indicated by an enhancement of the emission, specifically those transitions from the excited $5D_0$ to the ground state 7F multiplet.

The magnitude of these modifications can be appreciated by referring to Fig 4 and Table 1 in which europium was correlated to a number of different substances. The Eu (III) ion emission spectrum in water (A) excited at 395 nm (the center of the europium absorption band) serves as a convenient standard for comparison with other ligand species. In solutions of EDTA (B) and DPA (C), a dramatic enhancement of the emission as a whole is recognized. A closer examination of the features reveals, however,

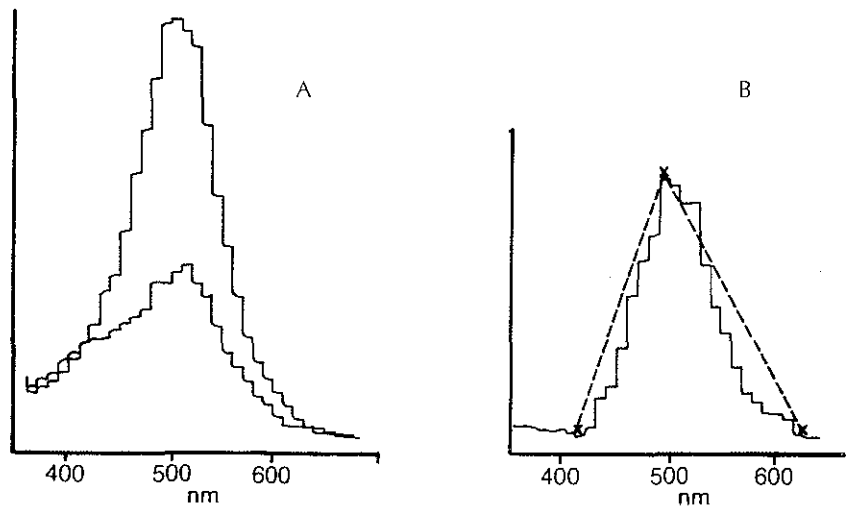


Fig 3 Phosphorescence of uranium (enhanced with phosphoric acid) at a concentration of 1.7 ppb. The FLUORLOG's slits were opened to 60 nm and the individual peaks are no longer resolved. The inset in A is the solvent background which is subtracted out in B. 3B also indicates how uranium concentration may be gauged with only three points.

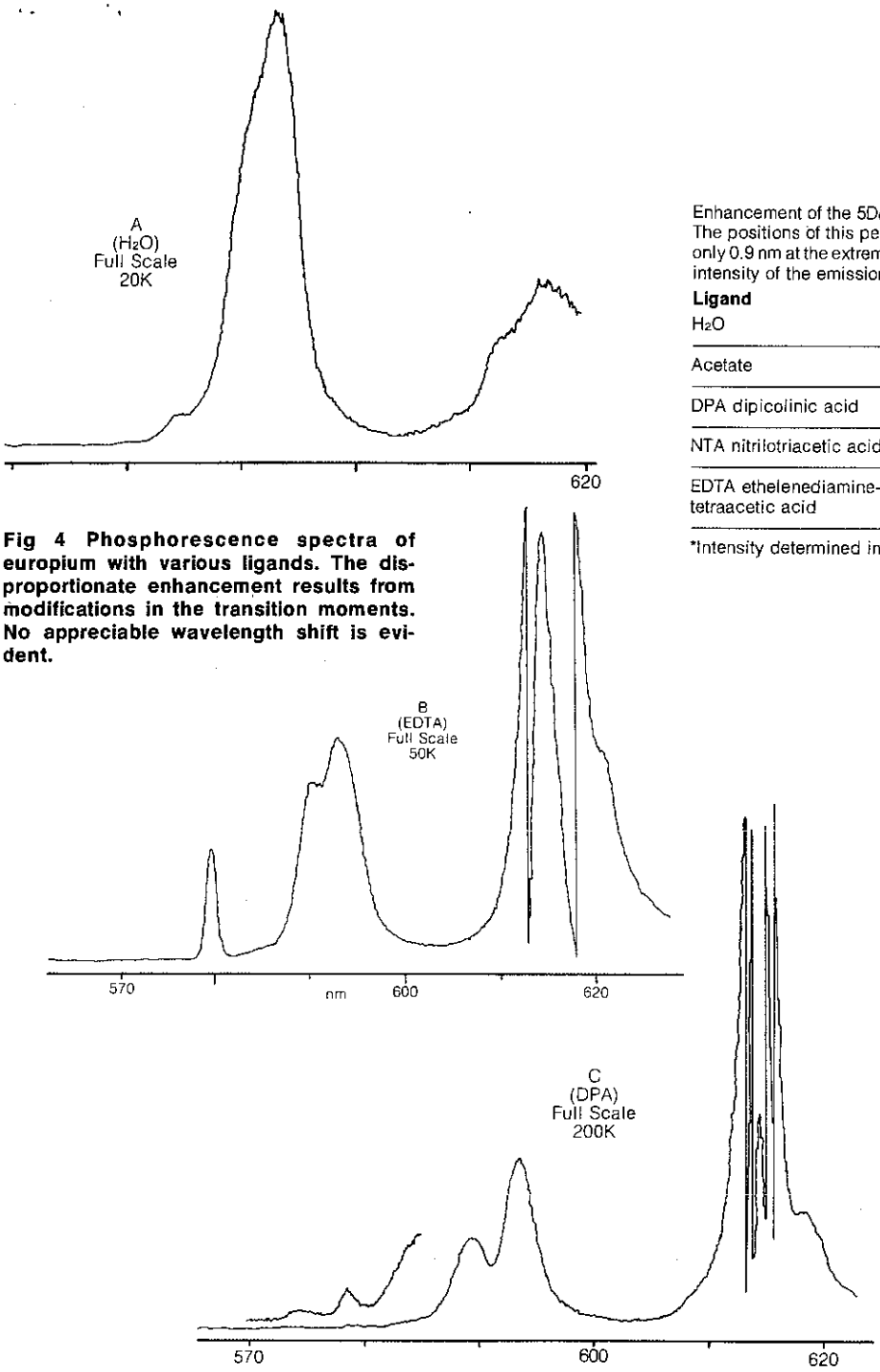


Fig 4 Phosphorescence spectra of europium with various ligands. The disproportionate enhancement results from modifications in the transition moments. No appreciable wavelength shift is evident.

Table I

Enhancement of the $5D_0 \rightarrow 7F_0$ emission of Eu(III) as a function of Ligand species. The positions of this peak are also tabulated. Though the wavelength shifted by only 0.9 nm at the extreme, transition moment modifications drastically altered the intensity of the emission.

Ligand	Position, nm	Enhancement
H_2O	578	1*
Acetate	577.7	200
DPA dipicolinic acid	578.4	300
NTA nitrilotriacetic acid	578.6	2000
EDTA ethelenediamine-tetraacetic acid	578.5	5000

*Intensity determined in a separate experiment

that the enhancement is not equally proportioned throughout the spectra. The $5D_0 \rightarrow 7F_0$ transition, at about 578 nm, that is too weak to be discerned in the aqueous solution, is distinct when europium is chelated by EDTA, which is a hexadentate ligand. With the DPA bidentate ligand, another emission surfaces at 573 nm, as seen in the insert. Further distinctions between the spectra are apparent in the transitions at 590 nm ($5D_0 \rightarrow 7F_1$) and 614 nm ($5D_0 \rightarrow 7F_2$).

From the table it is evident that the energy levels remain substantially undisturbed for the reference peak ($5D_0 \rightarrow 7F_0$) shifted only 0.9 nm at the extreme. Rather, the alteration in transition moments coupling states of different multiplicity allows the phosphorescence to compete more effectively with other mechanisms that might depopulate the phosphorescence state before it can emit. This can be contrasted with the study of quenching in which the non-radiative path itself was limited, and the emission character unaffected. There, the lifetime of the europium increased on the same order of magnitude as the enhancement. In the present case, the lifetimes of individual transitions were modified disproportionately, even though they arose from the same excited $5D_0$ state. The spectral features were therefore altered as well. Also, though the enhancement with EDTA was a factor of 5000, the lifetime of this emission was increased by less than tenfold.

Energy Transfer to Phosphorescence Probes

Finally, we treat the manner in which the excited state responsible for phosphorescence can be preferentially populated. Normally, when studying a specific ion, we would consider only the mechanisms through which intersystem crossing allows that ion to transfer from an excited state incapable of phosphorescence to one which can emit. However, when ions probe biological or chemical systems, an alternative route to populate these excited states materializes. If a molecule possesses its own phosphorescing region that happens to overlap the ion's excited states, it can serve as a pump to populate these ion states. Thus, absorption of a photon by the molecule raised it to an S state that then crosses to a T that can readily transfer its energy to the ion as follows:

$$S_0(\text{molecule}) \text{ - } \rightarrow S_1(\text{molecule}) \text{ - } \rightarrow T_1(\text{molecule}) \text{ - } \rightarrow \text{Ion excited level}$$

(Of course, since the excited S states of the molecules are so densely populated, fluorescence interference would swamp the phosphorescence were it not for the phosphorimeter).

To see how strong such a reaction can be, note the spectrum in Fig 5 where energy transfer from the strong absorption band of bipyridyl oxide, through the triplet state of the chelate, populates the multiple responsible for europium phosphorescence (4). Transitions too weak to be observed in spectra like Fig 1 now appear. This is even more striking in the inset where the scale has been expanded. Transitions from the $5D_1$ to the 7F multiplet, rarely reported, occur at 520 (F_0), 532 (F_1), and 555 nm (F_2). On the other hand, the mean lifetime has increased to only 8×10^{-4} seconds, less than a fifth that of the same ion in the deuterated environment. This tends to confirm that radiationless transitions are still at work and that energy transfer, not a reduction of quenching, is responsible for the enhancement. And even though lifetime measurements confirm a modification of transition moments, the excitation wavelength corresponded to the absorption band of the chelate and not the ion itself.

Energy transfer is emphatically illustrated in Fig 6, the fluorescence spectrum of bipyridyl oxide. Note the bite the europium $7F_0 \rightarrow 5D_2$ transition has taken out of the emission at 464 nm. The inset is the excitation spectrum of that transition. The positions correspond exactly.

The advantages of energy transfer with trivalent lanthanide ions such as Eu (III) and Tb (III) are intimately tied to their atomic properties. Their effective radii are very close to that of Ca^{2+} ions and both lanthanides lack strong geometrical preferences in binding groups. Calcium, on the other hand, is a basic structrual and functional entity in most proteins, but it is spectroscopically silent. All these factors suggest that the lanthanides might replace Ca^{2+} ions in protein molecules without the structural modifications that would otherwise nullify their effectiveness

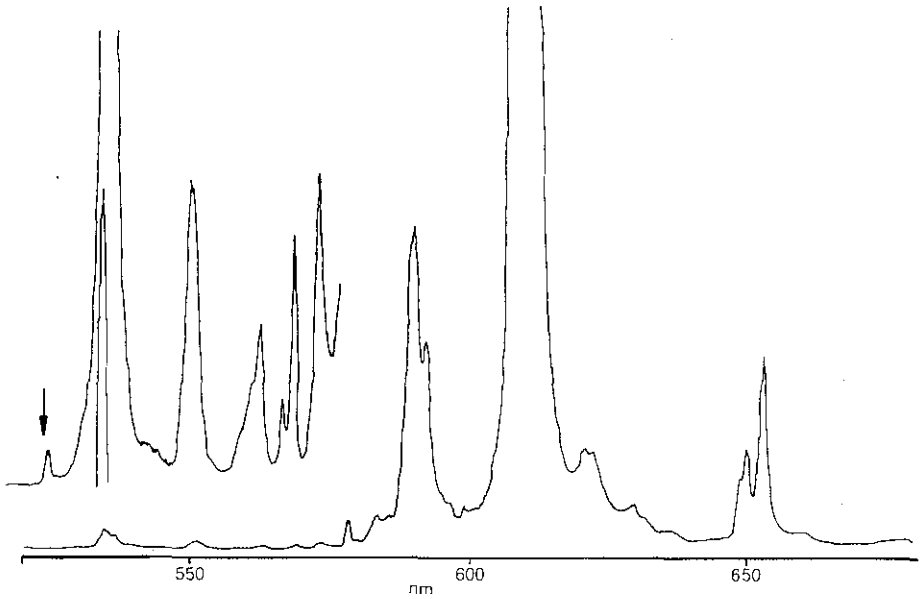


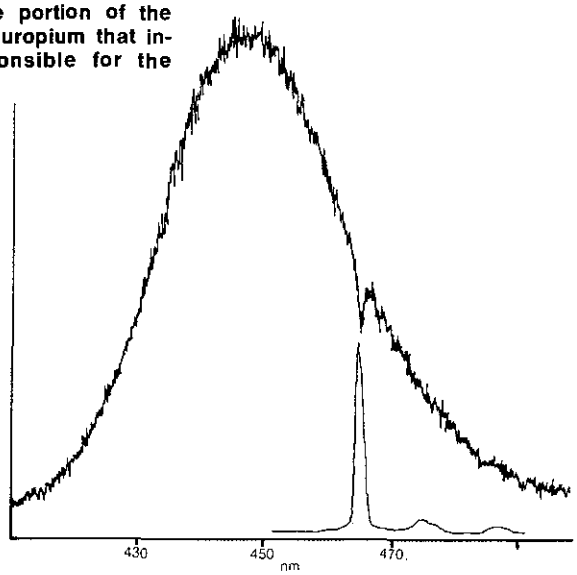
Fig 5 Phosphorescence spectrum of europium chelated by bipyridyl oxide. Excitation was into the absorption band of the ligand (270 nm) that, in turn, preferentially populates the europium excited states. Note the fine structure revealed in the inset, especially the $5D_1 \rightarrow 7F_0$ transition at 520 nm, that is rarely reported.

(5). And the resulting phosphorescence emission, enhanced by energy transfer, will provide the information necessary to map the conformations of such molecules at the rarified concentrations peculiar to biological systems.

Trypsin, for instance, is a calcium-binding protein known to exhibit phosphorescence over a region from 400 to 500 nm which effectively envelopes the avilable electron states of terbium. Furthermore, when terbium ions are introduced into a solution of trypsin, the ions should migrate to the calcium binding sites where the enhancement of the phosphorescence emission should continue until all the sites are occupied and the intensity reaches a maximum.

Fig 7 is a spectrum of terbium chelated in this manner by trypsin with the intensity enhanced by a factor of 10^4 over the unchelated terbium. Lifetime determinations confirm energy transfer as responsible for the increase. Also, excitation was at 295 nm, in the absorption band of the trypsin.

Fig 6 Fluorescence spectrum of bipyridyl oxide showing how energy transfer to europium depletes the chelate's excited states. The inset is the portion of the excitation spectrum of europium that includes the band responsible for the $7F_0 \rightarrow 5D_2$ transition.



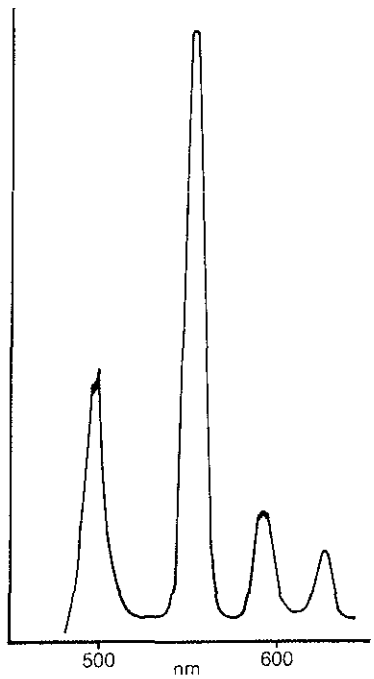


Fig 7 Phosphorescence spectrum of terbium chelated by trypsin. The enhancement is about 10^4 over the unchelated terbium.

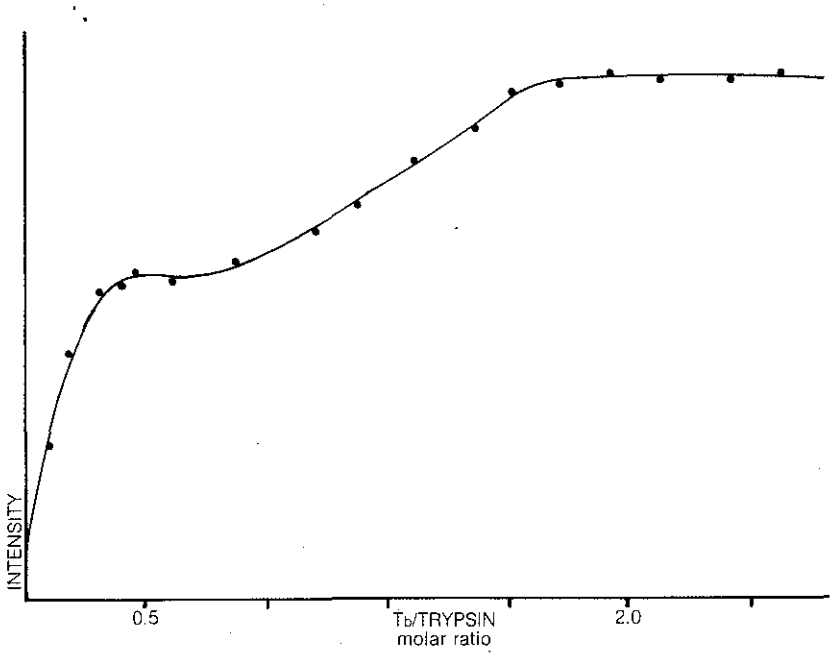


Fig 8 Titration curve tracing the enhancement of phosphorescence as a function of terbium concentration in a solution of trypsin. The final plateau indicates two binding sites for calcium.

Titration of a 10^{-5} M solutions of trypsin in a Tris buffer with 50 μl aliquots of a 10^{-5} M solution of $TbCl_3$ led to the curve in Fig 8. The intensity of the emission increased until the terbium concentration was double that of the trypsin molecules. At this point, additional terbium ions could no longer locate positions on the molecules at which to chelate and participate in energy transfer. So we conclude that trypsin possesses two binding sites for calcium.

Viewed over its entirety, the irregular shape of the curve is indicative of a system governed by multiple equilibrium constants that reflect the preferential nature of the binding sites. The intensity of the emission rises sharply until a mild plateau is approached when just one terbium ion has been added for every two molecules. The binding then continues with decelerated enhancement until saturation is achieved at two atoms per molecule. So there are two equilibrium constants for the system as we have prepared it.

To further justify the selection of terbium as a probe in this experiment, the procedure was repeated with europium. No concentration-related enhancement could be detected.

Conclusion

We have seen how a flexible, pulsed-lamp phosphorimeter, with a wide range of delay and sampling times, not only extends the limits of detection, but also correlates the magnitude of enhancement effects, and the alterations of lifetimes they produce, to expose the structure of the system. Needless to say, the types of persistent luminescence isolated by the instrument need not be restricted to phosphorescence. Delayed fluorescence, for example, resulting from thermal stiumulation of a molecule from an excited triplet back into an excited singlet (reverse intersystem crossing) where it radiates back to the ground state, is just as readily unmasked. Primarily observed in viscous and rigid media, this form of luminescence yields a spectrum identical to normal fluorescence, but it has an extra-long lifetime similar to phosphorescence (6).

Exploited to its full potential, the phosphorimeter welcomes the demands of industrial applications and opens the exploration of the microcosm to a new, wider perspective.

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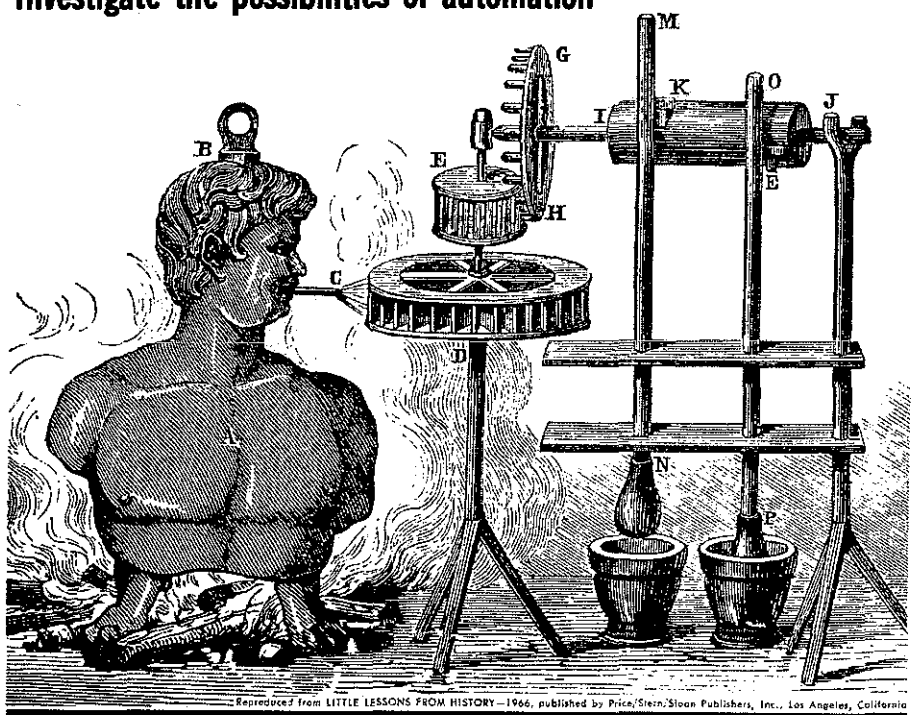

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Aluminum foil	2-mil piece	1 (b)	3 x 2	100-200
Candle wax	chunk	1.5	2	100-200
Chewing gum	chunk	1.5	2	100-200
Fish scales	10-mm flakes	1.5	2 x 2	200
Hair	dog clipping	0.5	2	200
Human vertebrae	7-mm pieces	2	2 x 2	200
Mouse skin	raw, ½ animal	2 (c)	3	200
Nylon (a)	3-mm beads	2	2 x 2 (e)	100-200
Permalloy 5	2-mm shot	2	3	30
Polyethylene	10-mil sheet	1	2	200
Rubber band	5-mm shearings	1.5	2 x 2	100
Rubber, oil-extended	5-mm shearings	1.5 (d)	2	25-50
Sheep wool	wad	0.5	2	200
Space food	stick	2	2	100
Steel wool	wad	0.5	2	100
Teflon	2-mil tape	3	2 x 2	100

(a) Three different nylons yielded similar results.
(b) 0.5 g of Tide detergent added.
(c) Equal weight of sodium sulfate as dehydrating agent
(d) Equal amount of sand added. Purpose: ethanol-toluene extraction.
(e) Two 2-min grinds with a one-min cooling period between.