FLUORESCENCE AND ITS MEASUREMENT

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As the measurement of fluorescence of catecholamines does not differ materially from the measurement of fluorescence of other materials, I shall present a few general remarks about the occurrence of fluorescence and follow with a description of some of the problems of its measurement.

Fluorescence involves absorption of incident radiation with the production of an electronically excited state. This excited state consists of a change in the electronic orbital to a higher energy condition; it normally has a lifetime of the order of 10^{-8} sec. This lifetime is required for the normal process of fluorescence wherein the return of the excited state to the ground state is accompanied by the emission of radiation. The emitted radiation is ordinarily somewhat longer in wavelength than the incident radiation due to the loss of energy during the conversion to the excited state and back again.

During the time that the molecule is in the excited state, other processes compete for the energy. If the energy is dissipated in less than the normal lifetime of the excited state fluorescent emission will not be produced. One of the processes available is thermal deactivation by gradual decay of the excited state to lower and lower levels until all of the energy is dissipated thermally without fluorescent radiation. The energy may also be utilized in the form of photochemical energy to cause dissociation of a bond, or by production of ionization, or by causing the excitation of another structure—this second structure's excited state decaying either because of any of the competing processes or by fluorescence of the newly excited structure. This latter process, in which the energy of an excited state of one structure is donated to a second structure with consequent radiation from the second structure, accounts for some of the large differences in energy occasionally observed between exciting and emitting light. For the excited state to persist long enough to favor the fluorescence process, the excited state must be insulated from thermal vibrational systems and bond structures susceptible to dissociation and easily ionized structures, as well as from other absorbing structures capable of becoming excited at the expense of the original excited structure.

This insulation or the opposite, coupling with other molecules, is dependent upon local molecular environment, so that the viscosity and dielectric constant of the solvent, as well as hydrogen bonding, dimer formation and the presence of foreign molecules influence the fluorescence process. The more highly excited a structure is, the more reactions it is capable of activating so that the number of possible susceptible structures is likely to be greater than in the case of a less excited structure. For a bond dissociation to occur, the energy of the excited state must be greater than the energy of the bond structure. There is a minimum energy of the excited state required for the disruption of a bond of particular energy. This minimum energy is represented as the wavelength of light required to pro-

duce an excited state, so that a material absorbing in the far ultraviolet may be incapable of fluorescing because of the presence of a susceptible bond, while the same bond may be present in a material absorbing in the near ultraviolet without destroying its ability to fluoresce.

The presence or absence of susceptible bonds, the influence of the dielectric constant of the solution, the change in coupling between the solute molecules, and the ions and the various interionic and intermolecular forces make the prediction of fluorescence exceedingly difficult. It is frequently easier to explain the phenomenon after the fact rather than to predict it.

Consideration of these theoretical aspects leads to the thought that one might well convert nonfluorescent materials to fluorescent materials or increase the sensitivity of materials already fluorescent to improve the sensitivity of their determination. It may also be possible to reduce the interference of materials which cannot be separated by other means. From the theoretical considerations we would conclude that it would be easier to quench the fluorescence of a short wave absorber than a long wave absorber. It might require only a minor chemical treatment to shift the absorption band into a lower energy region to reduce the influence of the quenching effect of side chains or other easily dissociated bonds. Thermal dissipative processes are in general inhibited by lowering the temperature or increasing the viscosity of the solvent to minimize the number of collisions and therefore reducing the likelihood of degradation by vibrational steps, the extreme of this process being the observation of fluorescence in the frozen state. The frozen state, however, favors an additional conversion to the phosphorescent state (3) which we will not consider here except to say that in the hands of Dr. Keirs (2), the observation of the phosphorescence of materials frozen in a mixture of alcohol, ether and isopentane at the temperature of liquid nitrogen shows some very interesting possibilities in relation to characterization and determination of fluorescent materials.

The number of quanta available in the light source is limited, whereas the number of quanta capable of being absorbed by the solution is so many times greater it would seem that a more intense exciting light would greatly increase the sensitivity of the fluorescence determinations. There is a physical limitation, however, imposed by the fact that other processes also scatter light. The local fluctuations in the refractive index of the solution due to molecular vibrations produce Raleigh scattering of the incident light in the same manner that the molecules of the air scatter a small quantity of light to produce the blue sky. This is a consequence of the structure of the solvent and cannot be eliminated. Scattering by suspended particles or micellar structure may also be very difficult to minimize. We find that high intensities pose an instrumental problem in the requirement of the reduction of scattered light in the monochromator, and every monochromator or filter will pass some unselected light. Another form of scattering from the solution is produced by the Raman effect where incident light is shifted in wavelength in the scattering process just as in true fluorescence. In Raman scattering, however, when the exciting wavelength is changed the emission also shifts in the same direction so that a constant energy difference between incident and emitted light

is maintained. The bonded OH Raman difference of 3400 cm^{-1} has frequently been observed.

The general guide lines that we have just put down in regard to structure and fluorescence can be summarized in simple but incomplete fashion by generalizing that those structures absorbing in the highly energetic or far ultraviolet region are more likely to be quenched or to encounter internal conversion mechanisms to dissipate the absorbed energy and prevent its appearance as fluorescence. Those materials that absorb in the near visible or visible region have less energy available for dissociative processes.

If we wish to characterize or to make an estimate of the concentration of a material that is adequately fluorescent, we are faced with the selection of a proper instrument. An ideal fluorometer is one in which a source will adequately excite the structure to emit and a measuring device will measure the emitted fluorescence to the exclusion of the exciting light. If it were possible to have ideal geometry so that the incident radiation passed through the solution without being scattered, and if we were able to look at right angles or along any axis that did not include direct incident radiation, we should be able to observe the fluorescence emission without contamination with incident radiation. As we customarily deal with high intensity sources and high sensitivity detectors, it is obvious that even minute amounts of scattered radiation will swamp our detecting device and render our determinations difficult. The practical approach is to filter the incident radiation highly, make use of the most favorable geometry to select emitted radiation to the exclusion of incident radiation, and to filter the fluorescence radiation selectively before it reaches the detector. With these considerations in mind, let us consider the light source. The ideal source should have a high output of radiation capable of exciting the fluorescent material in question. Any light it puts out above this wavelength is of nuisance value since this is the energy that heats the lamp house, which when scattered and passed by the secondary filtering system reaches our detector to be indicated along with the true fluorescence. Herein lies the value of excitation with a mercury lamp, as a large fraction of the radiation from a mercury lamp is distributed in the region which is effective in exciting fluorescence. The few lines it has in the visible region are easily removed as they are fairly well spaced and can be effectively filtered out. The line spectrum of emission of a mercury lamp, however, limits us to a few regions of the spectrum for excitation and reduces the possibility of at least one of the advantages of spectrophotofluorimetry (1).

If we have continuous or near continuous radiation for excitation we can, by observing the fluorescence of the material, make a close estimate of the absorption curve of this material even though the total quantity is inadequate for effective absorption spectrophotometry. I have said "approximate absorption" spectrum, since the spectrophotofluorometers currently available have not performed well enough in the low wavelength ultraviolet region to allow an accurate determination of absorption spectra without recourse to elaborate correction schemes to correct for instrumental errors and the falling off of the emission of the source in the far ultraviolet.

We have selected a xenon arc as the most practical source since it offers the very nearly continuous spectrum extending well down into the ultraviolet characteristic of arcs. This source, save for a few xenon lines, has a spectral output characteristic of incandescent bodies. The amount of radiation in the visible and ultraviolet is only a very small portion of the total output if we include the infrared portion of the spectrum. Only a few percent of the total energy of the emission lie below 250 mμ. As the infrared portion does not affect our detector system, most of it can be disregarded. A small difference in the arc temperature makes a large difference in the amount of ultraviolet emitted so that the color temperature, which is determined by the current through the system, is a determinant not only of the intensity but of the shape of the curve of the ultraviolet emission.

We have tried to stabilize these lamps by various approaches but have found that even if we do stabilize the current to the lamps, fluctuations in the arc path inside the lamps produce almost as much variation as the electrical variation, so that we are left essentially with selection of lamps as an effective way of reducing the variation of the ultraviolet output.

Systems regulating the output of xenon arcs in the visible region may have rather good performance characteristics for regions of the spectrum in the visible and long ultraviolet range, but it is extremely difficult to prevent the changes in the region below 250 mμ. At the present time the best we can say about this region is that it is not very dependable, and the technical details of adequate stabilization in this region have not been completely worked out. As this region has only recently become accessible to filter instruments by the development of ultraviolet filters, of which only a very few are in use, this problem is essentially limited to the problem of spectrophotofluorometers used for absorption or excitation spectra in the short wave ultraviolet region.

Since the problem of standardizing this end of the spectrum has not been solved it is essential, in reporting data, to give instrumental details. The use of the fluorometer to obtain absorption spectra may also be in error whenever there is more than one band of excitation. Because of the different quantum efficiencies of the excitation bands, the quenching factor is likely to vary for the different energies of the excited states and absorption spectra determined by fluorescence emission may be in error. With all of these disadvantages and limitations, we still can compare materials for identity and, in many cases, make adequate quantitative determinations for present purposes.

The structures of the spectra of fluorescence emission and activation are likely to be distorted when the concentration of the solution is excessive. A general rule when plotting curves with a spectrophotofluorometer is to work at the highest dilution capable of giving a satisfactory curve and to recheck at a higher dilution. The latter should always give a curve of the same shape if the system is free from concentration effects that produce absorption scattering or quenching.

If we utilize very high dilutions and the scattered light peak begins to overlap the fluorescence, and if such problems as the fluorescence of the cuvette are en-

countered, the curve will again be distorted. The optimum range for obtaining satisfactory curves can be attained only by understanding the problems involved in the use of a particular instrument. Very careful attention to the details in the instrument instruction manual and a knowledge of the limitations of the cuvettes as to ultraviolet transmission, fluorescence and scattering will frequently clarify the problem.

Mention should be made of the problem of overlapping orders in the use of grating monochromators. Here again, an understanding of the type of spectrum that a grating produces will suggest the type of error to be expected. The essential point is that a diffraction grating at a given setting will pass light of a particular wavelength and all of its harmonics. For example, the wavelengths 200, 400 and 800 mμ can all come through a grating monochromator when it is set for 800 mμ, so that they cannot be distinguished without an additional filter to separate the light at the entrance or exit of the monochromator. This is an extreme case where all three are in the region of interest. Ordinarily we are concerned mostly with such values as 300 and 600 mμ, where a fluorescence emission seems to have two peaks—one at 300 and a smaller one at 600. We can identify the true peak by insertion of a reddish filter, and if the red peak does not go through a red filter, it is obviously an artifact. The use of one or two simple filters will usually immediately clarify this type of problem.

A fluorometer requires an intense light source and an optical system of very high light gathering capacity with high efficiency in separating wavelengths of light. High efficiency narrow band filters offer considerable promise for the construction of special purpose fluorometers. Once the wavelengths of activation and emission are established and the conditions of the determination are set up, one needs only a single wavelength source and a single wavelength isolated for emission to make determinations. Filters currently available have high transmission and offer the possibility of very high aperture so that for sensitivity one could probably design a filter instrument or modify existing filter instruments to function better than a spectrophotofluorometer for one particular method. But I would like to point out again the advantages of a scanning system which will plot the entire curve which may indicate the presence of interferences. Such interference may be due to other fluorescent materials overlapping the one under examination or may be due to excessive scattering. Some solutions produce sufficient scattered light to add significantly to the apparent fluorescence. This problem would not be distinguishable from true fluorescence in a straight filter instrument unless an adequate blank could be obtained.

The implications of the theory of fluorescence indicate that there is a great deal to be learned by application of fluorescence to the study of the molecular interaction and energy transfer mechanisms that are now attracting a great deal of attention.

The instrumentation currently available was designed for past problems so that one must always exercise caution in accepting data in the new field without a very careful appraisal of instrument error. A high-performance instrument

operating over the full range of effective fluorescence will probably not be made until the full range of its usefulness becomes more established.

REFERENCES

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