Principles of Fluorescence Spectroscopy

Joseph R. Lakowicz

Principles of Fluorescence Spectroscopy

Joseph R. Lakowicz

University of Maryland School of Medicine
Baltimore, Maryland

Plenum Press • New York and London

Library of Congress Cataloging in Publication Data

Lakowicz, Joseph R.
Principles of fluorescence spectroscopy.

1. Fluorescence spectroscopy. I. Title.
QD96.F56L34 1983 574.19'285 83-6280
ISBN 0-306-41285-3

A Division of Plenum Publishing Corporation
233 Spring Street, New York, N.Y. 10013

Printed in the United States of America

Principles of Fluorescence Spectroscopy

Preface

Fluorescence methods are being used increasingly in biochemical, medical, and chemical research. This is because of the inherent sensitivity of this technique, and the favorable time scale of the phenomenon of fluorescence. Fluorescence emission occurs about 10^{-8} sec (10 nsec) after light absorption. During this period of time a wide range of molecular processes can occur, and these can effect the spectral characteristics of the fluorescent compound. This combination of sensitivity and a favorable time scale allows fluorescence methods to be generally useful for studies of proteins and membranes and their interactions with other macromolecules.

This book describes the fundamental aspects of fluorescence, and the biochemical applications of this methodology. Each chapter starts with the theoretical basis of each phenomenon of fluorescence, followed by examples which illustrate the use of the phenomenon in the study of biochemical problems. The book contains numerous figures. It is felt that such graphical presentations contribute to pleasurable reading and increased understanding. Separate chapters are devoted to fluorescence polarization, lifetimes, quenching, energy transfer, solvent effects, and excited state reactions. To enhance the usefulness of this work as a textbook, problems are included which illustrate the concepts described in each chapter. Furthermore, a separate chapter is devoted to the instrumentation used in fluorescence spectroscopy. This chapter will be especially valuable for those performing or contemplating fluorescence measurements. Such measurements are easily compromised by failure to consider a number of simple principles.

Insofar as is possible the presentation has been kept simple, with the minimum use of theory and mathematics. Where extensive equations are used, a good deal of text is included to explain the origin and meaning of each expression. The contents of the book should be valuable both for the person who is considering the use of fluorescence methods, and for the experienced researcher who desires further background.

The author is indebted to many individuals for their assistance and encouragement. Special thanks are owed to a gifted and dedicated librarian, Mrs. Jean Van Grasstic. The author also thanks Drs. Carlota Sumbilla, Richard Thompson, and Susan Keating for their comments and suggestions for improvement of the text. And finally, the author thanks the American Heart Association, the National Institute of Health, and the National Science Foundation, for research support which maintained interest in this subject. Of course, the tolerance of my family was invaluable.

Joseph R. Lakowicz

Contents

1. Introduction to Fluorescence

1.1. Jablonski Diagram 3
1.2. Characteristics of Fluorescence Emission 5
1.2.1. Stokes' Shift 5
1.2.2. Invariance of the Emission Spectrum with Excitation Wavelength 6
1.2.3. Mirror Image Rule 7
1.3. Fluorescence Lifetimes and Quantum Yields 9
1.4. Fluorescence Anisotropy 11
1.5. Time Scale of Molecular Processes in Solution 12
1.6. Fluorophores 14
1.6.1. Intrinsic or Natural Fluorophores 14
1.6.2. Extrinsic Fluorophores 15

2. Instrumentation for Fluorescence Spectroscopy

2.1. Excitation and Emission Spectra 19
2.1.1. An Ideal Spectrofluorometer 22
2.1.2. Distortions in Excitation and Emission Spectra 23
2.2. Light Sources 23
2.3. Monochromators 26
2.4. Optical Filters 32
2.5. Photomultiplier Tubes 34
2.6. Photon-Counting versus Analog Detection of Fluorescence 37
2.7. Corrected Fluorescence Spectra 39
2.7.1. Corrected Excitation Spectra 39
2.7.2. Corrected Emission Spectra 40
2.8. Effects of Sample Geometry 43
2.9. Absorption of Light and Deviations from the Beer–Lambert Law 47
2.9.1. Absorption of Light 47
2.9.2. Deviations from Beer's Law 48

3. Measurement of Fluorescence Lifetimes

3.1. Pulse Lifetime Measurements 52
3.2. Phase and Modulation Measurements of Fluorescence Lifetimes 53
3.3. Pulse Lifetime Measurements and Instrumentation 56
3.3.1. Flash Lamps 59
3.3.2. Pulse-Sampling Method 62
3.3.3. Single-Photon-Counting Method 64
3.4. Analysis of Time-Resolved Decays of Fluorescence Intensity . . 65
3.4.1. Least-Squares Analysis of Time-Resolved Decays . . . 67
3.4.2. Method of Laplace Transformation 70
3.5. Time-Resolved Emission Spectra 71
3.6. Phase Shift and Demodulation Measurements of Fluorescence Lifetimes 75
3.6.1. Light Modulators 76
3.6.2. Cross-Correlation Detection 78
3.7. Analysis of Phase and Modulation Data 79
3.7.1. Analysis of Phase-Modulation Data by Simulation . . . 80
3.7.2. Direct Calculation of α_i and τ_i 81
3.7.3. Analysis of Variable Frequency Phase and Modulation Data 81
3.7.4. Effects of Ground-State Heterogeneity on the Apparent Phase and Modulation Lifetimes 82
3.8. Color Effects in Photomultiplier Tubes 86
3.8.1. Correction of Color and/or Geometric Effects in Lifetime Measurements 87
3.9. Evaluation of the Performance of Lifetime Instruments 89
Problems 93

4. Phase-Sensitive Detection of Fluorescence

4.1. Theory of Phase-Sensitive Detection of Fluorescence 96
4.2. Analysis of Heterogeneous Fluorescence by Phase-Sensitive Detection 100
4.3. Prospectus on the Resolution of Heterogeneous Fluorescence by PSDF 106
Problems 109

5. Fluorescence Polarization

5.1. Definitions of Polarization and Anisotropy 112
5.2. Theory for Polarization in Dilute Vitrified Solution 115
5.3. Polarization Spectra of Fluorophores 120
5.4. Measurement of Fluorescence Anisotropies 125
5.4.1. L-Format or Single-Channel Method 126
5.4.2. T-Format or Two-Channel Method 128
5.4.3. Comparison of the T-Format and the L-Format Methods 130

5.4.4. Alignment of Polarizers 130
5.4.5. Elimination of Polarization Effects on Fluorescence Intensity and Lifetime Measurements; Magic Angle Polarizer Conditions 131
5.5. Extrinsic Causes of Fluorescence Depolarization 132
5.6. Effects of Rotational Diffusion on Fluorescence Anisotropies; The Perrin Equation 134
5.7. Biochemical Applications of Anisotropy Measurements 139
5.7.1. Estimation of the Microviscosities of Cell Membranes . . 139
5.7.2. Rotational Diffusion of Proteins 142
5.7.3. Measurement of Association Reactions 145
5.7.4. Denaturation of DNA 150
Problems 151

6. Time-Dependent Decays of Fluorescence Anisotropy

6.1. Theory of Time-Resolved Decays of Anisotropy 156
6.1.1. Nonsymmetric Fluorophores 159
6.1.2. Hindered Fluorophores 160
6.1.3. Segmental Mobility of a Biopolymer-Bound Fluorophore 161
6.1.4. Time-Resolved Anisotropies for a Mixture of Fluorophores 162
6.2. Biochemical Applications of Time-Resolved Anisotropy Measurements 164
6.2.1. Lipid Bilayers Labeled with Diphenylhexatriene (DPH) . 164
6.2.2. Segmental Mobility of an Antibody Molecule 167
6.2.3. Depolarization of the *Y*-base on Yeast Phenylalanine tRNA ($tRNA^{phe}$) 169
6.3. Differential Polarized Phase Fluorometry 171
6.3.1. Measurement of Differential Phase Angles 171
6.3.2. Theory of Differential Polarized Phase Fluorometry . . . 171
6.3.3. Effects of Anisotropic Rotations on $\tan \Delta$ 176
6.3.4. Effects of Hindered Rotations on Differential Tangents 176
6.4. Lifetime-Resolved Measurements of Fluorescence Anisotropy . 179
Problems 183

7. Effects of Solvents on Fluorescence Emission Spectra

7.1. Stokes Shifts and Solvent Relaxation 189
7.2. General Solvent Effects on Fluorescence Spectra: The Lippert Equation 190
7.3. Derivation of the Lippert Equation 194
7.4. Specific Solvent Effects 201
7.5. Biochemical Applications of Solvent Effects 208
7.5.1. Localization of Membrane-Bound Fluorophores 208
7.5.2. Localization of Protein-Bound Fluorophores 211
Problems 215

8. Mechanisms and Dynamics of Solvent Relaxation

8.1. Effects of Solvent Relaxation on Steady State Emission Spectra . 219
8.2. Theories of Time-Dependent Solvent Relaxation 225
8.2.1. Continuous Model for Solvent Relaxation 226
8.2.2. Two-State Model for Solvent Relaxation 228
8.2.3. Experimental Distinction between Continuous and Two-State Relaxation 229
8.3. Phase-Modulation Studies of Solvent Relaxation 230
8.3.1. Proof of an Excited State Process from Phase and Modulation Data 232
8.3.2. Resolution of Individual Spectra from Phase-Modulation Data 233
8.3.3. Differentiation of Two-State versus Continuous Relaxation by Phase Fluorometry 235
8.4. Time-Resolved Measurements of Solvent Relaxation 236
8.5. Lifetime-Resolved Measurements of Solvent Relaxation 242
8.6. Analysis of Solvent Relaxation by Phase-Sensitive Detection of Fluorescence 244
8.7. Analysis of Spectral Relaxation in Model Membranes and Proteins 247
8.7.1. Time-Resolved Emission Spectra of Labeled Membranes 247
8.7.2. Phase-Modulation Measurements of Spectral Relaxation in Membranes 250
8.7.3. Lifetime-Resolved Emission Spectra of Labeled Membranes 251
8.7.4. Spectral Relaxation in Labeled Proteins 252
Problems 255

9. Quenching of Fluorescence

9.1. Quenchers of Fluorescence 258
9.2. Theory of Collisional Quenching 260
9.2.1. Derivation of the Stern–Volmer Equation 261
9.2.2. Interpretation of the Bimolecular Quenching Constants . 262
9.3. Theory of Static Quenching 264
9.4. Combined Dynamic and Static Quenching 266
9.5. Examples of Static and Dynamic Quenching 167
9.6. Deviations from the Stern–Volmer Equation; Quenching Sphere of Action 271
9.7. Origin of the Smoluchowski Equation 274
9.8. Biochemical Applications of Quenching 275
9.8.1. Effects of Steric Factors in Quenching: The Ethidium Bromide–DNA Complex 275
9.8.2. Charge Effects in Quenching 276

9.8.3. Permeation of Charged and Neutral Quenchers into Proteins . . . 278
9.8.4. Surface Accessibility of Tryptophan Residues in Proteins by Iodide Quenching of Fluorescence . . . 279
9.8.5. Surface Accessibility of Membrane-Bound Proteins by Picolinium Derivatives . . . 284
9.8.6. Localization of Membrane-Bound Fluorophores by Quenching . . . 285
9.8.7. Oxygen Quenching of Membrane-Bound Fluorophores . 287
9.8.8. Quenching of Membrane-Bound Fluorophores as a Method to Determine the Diffusion and Partition Coefficients of Quenchers . . . 288
9.8.9. Effects of Viscosity on the Quenching Efficiency . . . 291
9.8.10. Exchange of Cytochrome b_5 between Membranes . . . 292
Problems . . . 297

10. Energy Transfer

10.1. Theory of Energy Transfer for a Donor–Acceptor Pair . . . 305
10.2. Distance Measurements by Energy Transfer . . . 312
10.3. Association Reactions of Macromolecules Revealed by Energy Transfer . . . 316
10.3.1. Self-Association of ATPase Molecules in Lipid Vesicles 316
10.3.2. Dimerization of Gramicidin in Lipid Bilayers . . . 318
10.3.3. Binding of Hemoglobin to Red Blood Cell Membranes 320
10.3.4. Lateral Organization of Membranes . . . 321
10.4. Static and Dynamic Mobility of Macromolecules Revealed by Time-Resolved Decays of Donor Fluorescence . . . 324
10.5. Energy Transfer in the Rapid Diffusion Limit . . . 331
10.6. Energy Transfer in Solution . . . 333
10.7. Analysis of the Transfer Efficiency in the Presence of Nondipolar Quenching Mechanisms . . . 335
Problems . . . 337

11. Protein Fluorescence

11.1. Spectral Properties of the Aromatic Amino Acids . . . 342
11.1.1. Excitation Polarization Spectra of Tyrosine and Tryptophan . . . 344
11.1.2. Effects of Solvent on the Emission Spectra of Tryptophan Derivatives . . . 345
11.2. General Characteristics of Protein Fluorescence . . . 347
11.2.1. Tyrosine and Tryptophan Emission . . . 347
11.2.2. Energy Transfer from Tyrosine to Tryptophan . . . 350
11.3. Factors Affecting the Emission Spectra of Proteins . . . 354
11.3.1. Three-Dimensional Structure . . . 354

11.3.2. Association of Proteins with Substrates and Other Macromolecules 357
11.3.3. Possible Tyrosinate Emission in Proteins 359
11.4. Fluorescence Lifetimes of Proteins 363
11.5. Resolution of Individual Tryptophan Residues in Proteins from Time-Resolved Decays of Fluorescence 368
11.6. Dynamics of Proteins Revealed by Fluorescence Methods . . 370
11.6.1. Dynamics of Proteins as Revealed by Quenching of Protein Fluorescence 371
11.6.2. Time-Dependent Decays of Fluorescence Anisotropy . 375
11.6.3. Wavelength-Dependent Lifetimes of Tryptophan Fluorescence 379
Problems 381

12. Spectral Characteristics of Systems Which Undergo a Reversible Two-State Reaction
12.1. Reversible Two-State Reaction 386
12.2. Time-Resolved Decays of Fluorescence for a Reversible Two-State Reaction 388
12.2.1. Resolution of the Individual Emission Spectra from Time-Resolved Decays 391
12.2.2. Differential-Wavelength Deconvolution of Time-Resolved Decays 392
12.2.3. Excited State Dissociation of 2-Naphthol; An Example of a Reversible Excited State Reaction 394
12.3. Analysis of Excited-State Reactions by Phase-Modulation Fluorometry 399
12.3.1. Phase Angles and Demodulation Factors for Spectra Which Overlap 404
12.3.2. Resolution of Unrelaxed and Relaxed Emission Spectra from $\phi(\bar{\nu})$ and $m(\bar{\nu})$ 405
12.4. Model Calculations for Phase-Modulation Fluorometry 407
12.5. The Excited State Protonation of Acridine 416
12.6. Phase-Modulation Studies of Excited State Reactions . . . .
12.6.1. Resolution of the Emission Spectrum of Anthracene and its Exciplex with Diethylaniline 420
12.6.2. Measurement of ϕ_F and ϕ_R for 2-Naphthol by Phase-Sensitive Detection of Fluorescence 420
12.6.3. Detailed Analysis of Exciplex Formation by Phase-Modulation Fluorometry 424
Problems 429

Appendix: Answers to Problems 433
References 469
Index 489

1

Introduction to Fluorescence

Luminescence is the emission of photons from electronically excited states. Luminescence is divided into two types, depending upon the nature of the ground and the excited states. In a singlet excited state, the electron in the higher-energy orbital has the opposite spin orientation as the second electron in the lower orbital. These two electrons are said to be paired. In a triplet state these electrons are unpaired, that is, their spins have the same orientation. Return to the ground state from an excited singlet state does not require an electron to change its spin orientation. A change in spin orientation is needed for a triplet state to return to the singlet ground state. Fluorescence is the emission which results from the return to the lower orbital of the paired electron. Such transitions are quantum mechanically "allowed" and the emissive rates are typically near 10^8 sec^{-1}. These high emissive rates result in fluorescence lifetimes near 10^{-8} sec or 10 nsec. The lifetime is the average period of time a fluorophore remains in the excited state. Phosphorescence is the emission which results from transition between states of different multiplicity, generally a triplet excited state returning to a singlet ground state. Such transitions are not allowed and the emissive rates are slow. Typical phosphorescent lifetimes range from milliseconds to seconds, depending primarily upon the importance of deactivation processes other than emission. Throughout this book we will be concerned primarily with the more rapid phenomenon of fluorescence.

Substances which display significant fluorescence generally possess delocalized electrons formally present in conjugated double bonds. Some typical fluorescent substances (fluorophores) are shown in Figure 1.1. One widely encountered fluorophore is quinine, which is present in tonic water. If one observes a glass of tonic which is exposed to sunlight, a faint blue glow is frequently visible. This glow is most apparent when the glass is observed at a right angle relative to the direction of the sunlight, and when the dielectric constant is decreased by additives. The quinine, which is present in the tonic, is excited by the ultraviolet light from the sun. Upon

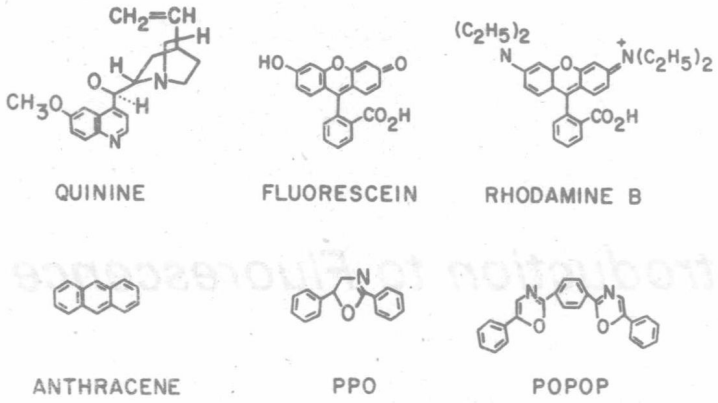

Figure 1.1. Structures of typical fluorescent substances.

return to the ground state the quinine emits blue light with a wavelength near 450 nm. Additional fluorophores are also frequently encountered. The green or red–orange glow sometimes seen in antifreeze is probably due to trace quantities of fluorescein or rhodamine, respectively (Figure 1.1). Polynuclear aromatic hydrocarbons, such as anthracene and perylene, are also fluorescent, and may be partially responsible for the blue fluorescence frequently seen from gasoline. And finally, compounds such as PPO and POPOP are used in scintillation cocktails and are thus frequently encountered in biochemical research. These compounds are highly fluorescent. Numerous additional examples could be presented. Instead of listing them here, examples will appear throughout the book, with reference to the useful properties of the individual fluorophores. In contrast to aromatic organic molecules, atoms are generally nonfluorescent in condensed phases. One notable exception is the group of elements commonly known as the lanthanides.[1] The fluorescence from europium and terbium ions results from electronic transitions between *f* orbitals. These are shielded from the solvent by higher filled orbitals.

Fluorescence spectral data are generally presented as emission spectra. A fluorescence emission spectrum is a plot of the fluorescence intensity versus wavelength (in nanometers) or wave numbers (in cm^{-1}). Two typical fluorescence emission spectra are shown in Figure 1.2. Emission spectra vary widely and are dependent upon the chemical structure of the fluorophore and the solvent in which it is dissolved. The spectra of some compounds, such a perylene, show significant structure due to the individual vibrational energy levels of the ground and excited states. Other compounds, such as quinine, show spectra which are devoid of vibrational structure.

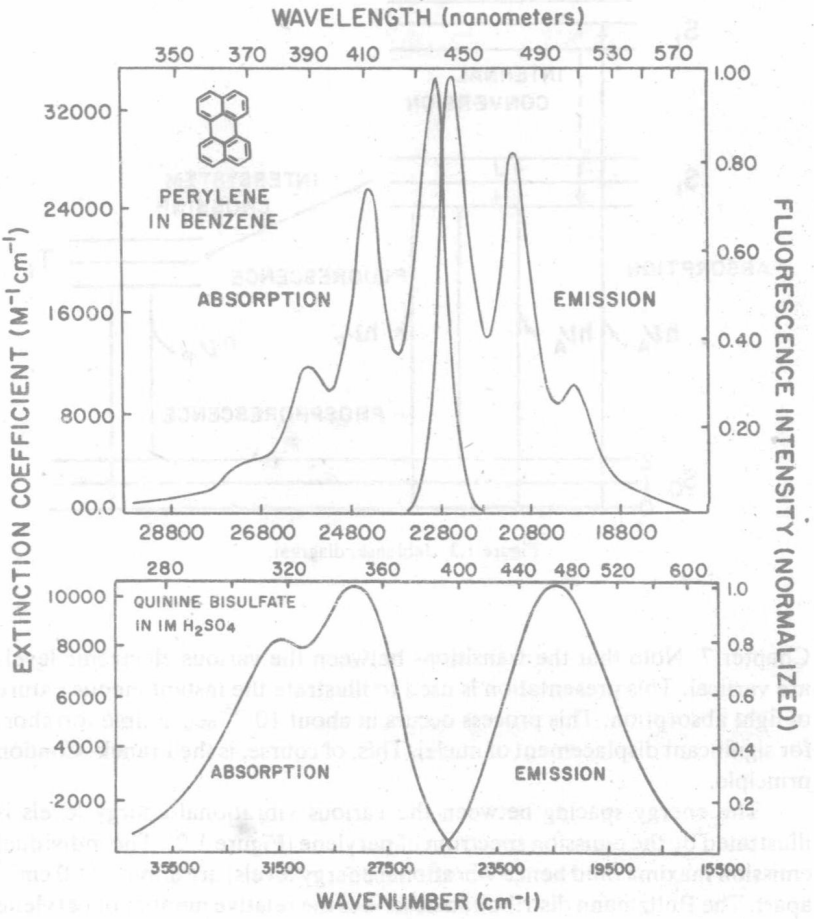

Figure 1.2. Absorption and fluorescence emission spectra of perylene and quinine. Emission spectra cannot be correctly presented on both the wavelength and wave number scales. The wave number presentation is correct in this instance. Wavelengths are shown for convenience. See Chapter 2. (From Ref. 2.)

1.1. Jablonski Diagram

The absorption and emission of light is nicely illustrated by the energy-level diagram suggested by A. Jablonski.[3] The ground, first, and second electronic states are depicted by S_0, S_1, and S_2, respectively (Figure 1.3). At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels, depicted by 0, 1, 2, etc. In this diagram we excluded solvent effects, which will be considered in more detail in

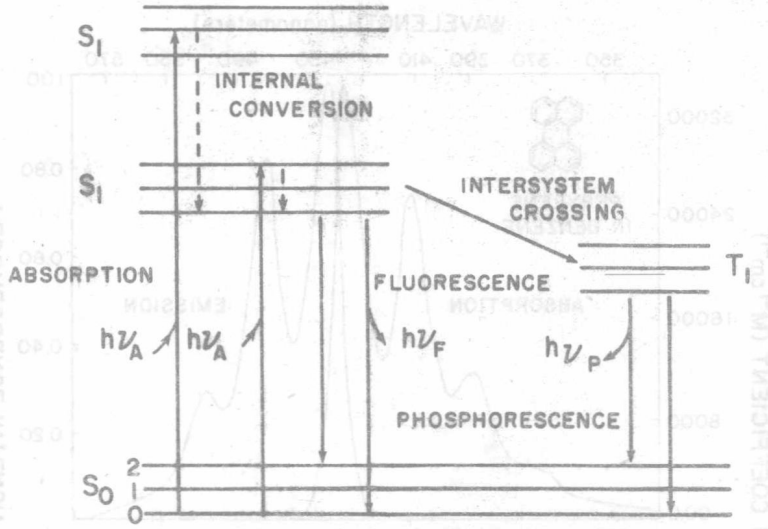

Figure 1.3. Jablonski diagram.

Chapter 7. Note that the transitions between the various electronic levels are vertical. This presentation is used to illustrate the instantaneous nature of light absorption. This process occurs in about 10^{-15} sec, a time too short for significant displacement of nuclei. This, of course, is the Franck–Condon principle.

The energy spacing between the various vibrational energy levels is illustrated by the emission spectrum of perylene (Figure 1.2). The individual emission maxima (and hence vibrational energy levels) are about 1500 cm^{-1} apart. The Boltzmann distribution describes the relative number of perylene molecules in the 0 and 1 vibrational states. The ratio (R) of molecules in each state is given by

$$R = e^{-\Delta E / kT} \tag{1.1}$$

where ΔE is the energy difference, k is the Boltzmann constant, and T is the temperature in degrees kelvin (K). Assuming room temperature of 300 K this ratio is about 0.01. Hence most molecules will be present in the lowest vibrational state, and light absorption results mainly from molecules in this energy level. Because of the larger energy difference between S_0 and S_1, essentially no fluorophores can populate S_1 as a result of thermal energy. It is interesting to note that even the small, thermally

induced population of molecules in the first excited vibrational state can be detected using absorption difference spectra at various temperatures.

Following light absorption, several processes usually occur. A fluorophore is usually excited to some higher vibrational level of either S_1 or S_2. With a few rare exceptions, molecules in condensed phases rapidly relax to the lowest vibrational level of S_1. This process is called internal conversion and generally occurs in 10^{-12} sec. Since fluorescence lifetimes are typically near 10^{-8} sec, internal conversion is generally complete prior to emission. Hence, fluorescence emission generally results from the thermally equilibrated excited state. As for absorption, the electronic transition down to the lowest electronic level also results in an excited vibrational state (Figure 1.3). This state will also reach thermal equilibrium in about 10^{-12} sec. An interesting consequence of these considerations is that the absorption spectrum of the molecule reflects the vibrational levels of the electronically excited states, and the emission spectrum reflects the vibrational levels of the ground electronic state. Generally, electronic excitation does not greatly alter the spacing of the vibrational energy levels. As a result, the vibrational structures seen in the absorption and the emission spectra are similar.

Molecules in the S_1 state can also undergo conversion to the first triplet state T_1. Emission from T_1 is termed phosphorescence, and generally is shifted to longer wavelengths (lower energy) relative to the fluorescence. Conversion of S_1 to T_1 is called intersystem crossing. Transition from T_1 to the ground state is forbidden, and as a result the rate constant for such emission is several orders of magnitude smaller than those of fluorescence. Although not indicated explicitly in Figure 1.3, a variety of other processes can influence the fluorescence emission. These factors include solvent effects, solvent relaxation, quenching, and a variety of excited state reactions. These will be considered in detail in later sections of this book.

1.2. Characteristics of Fluorescence Emission

The phenomenon of fluorescence displays a number of general characteristics. Exceptions are known, but these are infrequent. Generally, if any of the following characteristics are not displayed by a given fluorophore, one may infer some special behavior for this compound.

1.2.1. Stokes' Shift

Except for atoms in the vapor phase, one invariably observes a shift to lower wavelength (i.e., a loss of energy) of the emission relative to the absorption. This phenomenon was first observed by Stokes in 1852 in