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THE ACQUISITION AND ANALYSIS OF TIME-RESOLVED FLUORESCENCE DATA FROM COMPLEX SYSTEMS: APPLICATIONS TO PROBLEMS OF PHOTOCHEMICAL, BIOPHYSICAL, AND ENVIRONMENTAL INTEREST

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University
1998

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ABSTRACT

Time-resolved fluorescence data, measured by the Time-Correlated Single-Photon Counting (TCSPC) method are analyzed and interpreted to provide insight into problems of photochemical, biophysical, and environmental interest. The results of these experiments are combined with data from other spectroscopic and computational methods to investigate problems as diverse as the photochemistry of dialkyldiazirines, the photophysics of psoralens, energy-transfer processes in photosynthetic reaction centers, and the fluorescence quenching of polyaromatic hydrocarbon compounds (PAHs) in environmental matrices.

Some challenges involved in the interpretation of TCSPC data are discussed, and particular attention is given to the problem of extracting chemically meaningful information from the fluorescence intensity decays by appropriate methods of data analysis.

We have conducted an extensive investigation of the excited states and photochemistry of the important class of carbene precursors known as dialkyldiazirines. This work represents the first recorded lifetime measurements for dialkyldiazirines. Decays have been obtained for 3,3-dimethyldiazirine, cyclohexyldiazirine, and adamantyldiazirine in a variety of solvents, over a wide range of temperatures (77 K - 320 K), at several excitation wavelengths. The fluorescence decays are single-exponential for all three dialkyldiazirines. The fluorescence lifetimes at ambient temperature were measured to be 0.04 ns for 3,3-dimethyldiazirine, 0.08 ns for cyclohexyldiazirine, and 0.24 ns for adamantyldiazirine. Arrhenius treatment of the fluorescence lifetime data indicates that the rate-limiting barriers for activated processes in the dialkyldiazirine excited states are between 2.8 - 3.5 kcal/mol.
The fluorescence lifetime of adamantyldiazirine was unaffected by deuteration of the solvent, solvent polarity, or excitation energy. The steady-state absorption and fluorescence emission spectra of the dialkyldiazirines in a variety of solvents are reported and discussed, as are the infrared and Raman spectra of adamantyldiazirine. These spectra are interpreted with the help of ab initio (RHF/6-31G* and CIS/6-31G*), density functional (B3LYP/6-31G*), and semiempirical (PM3) calculations. The fluorescence quantum yield of adamantyldiazirine at ambient temperature was calculated to be ~ 0.0012. Analysis of these data in the light of previous research suggests that little or no intermolecular chemistry is attributable to photoexcited dialkyldiazirines in solution at ambient temperature. Rather, fluorescence competes with one or more intramolecular photochemical processes. These conclusions are supported by time-resolved infrared experiments for adamantyldiazirine that show the growth of a band at 2042 cm⁻¹ corresponding to the formation of 2-diazaadamantane concurrent with the depletion of the diazirine N=N stretching mode at 1574 cm⁻¹.

We next describe the development of a methodology to measure the time-resolved fluorescence from photosystem II reaction centers (PS II-RCs) isolated from spinach leaves and Chlamydomonas reinhardtii algae. We identify some critical issues of data quality and discuss how they may be systematically optimized for specific chemical systems. We describe the use of a computer program to generate synthetic TCSPC data of a desired data precision (CPC), data resolution (number of channels), and time scale. This technique is used to test the ability of a particular data analysis algorithm to recover reproducible and reliable parameters from data under these specified conditions. We present and discuss some preliminary PS II-RC data, and analyze these data using sums of up to four exponentials and by lifetime distribution analysis using the Exponential Series Method (ESM). We have identified the presence of a long-lived (~ 29 ns) component in the fluorescence decay as diagnostic of a healthy reaction center preparation, as this fluorescence represents the delayed emission of P680 following recombination of the photogenerated radical pair species. We argue that the
complexity and heterogeneity of the PS II-RCs makes it more likely that these decays should be described by continuous distributions of lifetimes, rather than by discrete components. We suggest that ESM analyses provide an excellent model-independent representation of TCSPC data and propose using the results of such analyses to design appropriate global target analyses to recover kinetic parameters from multidimensional TCSPC data surfaces. To our knowledge, this work represents the first analysis of TCSPC data for PS II-RCs using unparameterized distributions of lifetimes.

We found that the integrity of PS II-RCs could be preserved by deoxygenating the samples, by minimizing their exposure to the laser, by keeping the temperature near 4 °C, and by providing continuous stirring. A precision of 20K – 50K CPC, data resolution of at least 2048 channels, and a time base of 50 – 75 ns are recommended for optimum recovery of the underlying distributions of amplitudes and lifetimes for these samples.

With respect to psoralen photophysics, we have succeeded in obtaining TCSPC data for four psoralen derivatives and two related compounds (visnagin and khellin) that are of higher precision (10K CPC) and resolution (512 channels) than those reported in the literature. We have obtained these data for a large matrix of solvents and emission wavelengths, and have analyzed the data in three different ways. By using the pre-exponential weighted mean lifetime in conjunction with sum-of-exponential and ESM lifetime distribution analyses, we have arrived at the first interpretation of the fluorescence decay data that can be successfully harmonized with the so-called “proximity effect.”

All of the psoralen ESM lifetime distributions revealed multi-mode behavior. Our data reveal the expected increases in the pre-exponential weighted mean lifetime with solvent polarity, as well as with emission wavelength. The latter observation is based on the assumption that polar solvents will reorient around the photoexcited psoralen in its $S_1$ state, lowering its energy relative to both $S_2$ and $S_0$. The stabilization relative to $S_2$ reduces the impact of the proximity effect and causes the increase in the fluorescence lifetime, while the stabilization
relative to $S_0$ lowers the energy gap between these two states and red-shifts the emission. Consequently, emission from the red edge of the fluorescence spectrum represents a preferential observation of emission from solvent-equilibrated species.

Finally, we present an analysis of TCSPC data obtained for aqueous pyrene in the presence of various humic substances. Two different methods were employed to analyze the data. Our original approach involved a "filtering" of the data in the time domain. The complex, short-lived fluorescence decay of the humic materials was excluded from the analysis by fitting only the "tails" of the fluorescence decay curves. The longer-lived pyrene decays could then be adequately described by a single exponential with a lifetime of ~ 194 ns using this "fitting-window" approach. We also analyzed these data using ESM fluorescence lifetime distributions. This permitted pyrene's fluorescence lifetime and the lifetime distributions of the humic materials to be recovered simultaneously. The pyrene lifetimes thus obtained were identical to those determined by the "fitting-window" approach. The humic substances themselves possess a broad distribution of lifetimes with 2 - 3 modes. Roughly 25% of the steady-state fluorescence intensity of these mixtures may be attributed to subnanosecond lifetime components, while the majority of the remainder have lifetimes between 2 - 9 ns. Less than 10% of the intensity was due to longer-lived species.

We have established that the fluorescence quenching of pyrene in these matrices is static in nature, being caused by ground-state interactions between pyrene and the humic materials. We have also identified dynamic quenching of pyrene's fluorescence by molecular oxygen. We therefore caution against the use of fluorescence quenching data to measure partition coefficients for these systems unless the samples are first purged of oxygen. Finally, we have observed a slight reduction in pyrene's fluorescence lifetime in oxic solutions that is proportional to the concentration of humic substances. We suggest that this may be due to additional dynamic quenching of pyrene by molecular oxygen due to enhanced concentrations of both pyrene and oxygen within the matrix of humic materials.
Dedicated to my Lord and Savior Jesus Christ and to Nancy, my precious bride.
ACKNOWLEDGMENTS

"I said to the Lord, 'You are my Lord; apart from you I have no good thing.'"
Psalm 16:2, NIV

"Whatever you do, work at it with all your heart, as working for the Lord, not for men, since you know that you will receive an inheritance from the Lord as a reward. It is the Lord Christ you are serving." Colossians 3:23–24, NIV

"A wife of noble character who can find? She is worth far more than rubies. ... Her husband is respected at the city gate, where he takes his seat among the elders of the land. ... She watches over the affairs of her household and does not eat the bread of idleness. ... Give her the reward she has earned, and let her works bring her praise at the city gate." Proverbs 31:10, 23, 27, 31, NIV

All praise and honor to my Lord Jesus Christ, who has provided motivation, strength, and endurance throughout the preparation of this dissertation. Without Him, everything would be "meaningless, a chasing after the wind." I am grateful for His provision of my wife Nancy, who has worked and prayed much harder than I have to bring this moment to pass.

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Summed regions: < 0.09 ns, 0.09 - 0.2 ns, 0.2 - 0.9 ns, 0.9 - 2.1 ns, > 2.1 ns. Fluorescence monitored at 500 nm

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Figure 5.65: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for khellin in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.45 ns, 0.45 - 0.8 ns, 0.8 - 2.0 ns, > 2.0 ns

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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>5-MOP</td>
<td>5-methoxypsoralen</td>
</tr>
<tr>
<td>8-MOP</td>
<td>8-methoxypsoralen</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom ($10^{-10}$ m)</td>
</tr>
<tr>
<td>$\Delta G_{cs}$</td>
<td>free energy of charge separation</td>
</tr>
<tr>
<td>$\phi_{\text{carbene}}$</td>
<td>quantum yield for carbene formation</td>
</tr>
<tr>
<td>$\phi_{\text{diaz}}$</td>
<td>quantum yield for diazo formation</td>
</tr>
<tr>
<td>$\Phi_F$</td>
<td>fluorescence quantum yield</td>
</tr>
<tr>
<td>$\mu$s</td>
<td>microsecond ($10^{-6}$ s)</td>
</tr>
<tr>
<td>ADA</td>
<td>ammonium dihydrogen arsenate</td>
</tr>
<tr>
<td>ALHA</td>
<td>Aldrich Humic Acid</td>
</tr>
<tr>
<td>Br-MOP</td>
<td>5-bromo-8-methoxypsoralen</td>
</tr>
<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>CCD</td>
<td>charge-coupled device</td>
</tr>
<tr>
<td>CFD</td>
<td>constant fraction discriminator</td>
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<tr>
<td>Chl</td>
<td>chlorophyll</td>
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<tr>
<td>CIS</td>
<td>configuration interaction singles</td>
</tr>
<tr>
<td>cm$^{-1}$</td>
<td>wavenumbers</td>
</tr>
<tr>
<td>CPC</td>
<td>counts in peak channel</td>
</tr>
<tr>
<td>CVI</td>
<td>C for virtual instrumentation</td>
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<tr>
<td>DCE</td>
<td>discrete component equivalents</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPB</td>
<td>diphenylbutadiene</td>
</tr>
<tr>
<td>ESM</td>
<td>exponential series method</td>
</tr>
<tr>
<td>eV</td>
<td>electron volt</td>
</tr>
<tr>
<td>fc</td>
<td>frozen core</td>
</tr>
<tr>
<td>FQ</td>
<td>fluorescence quenching</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width at half-maximum</td>
</tr>
<tr>
<td>FWTM</td>
<td>full width at tenth-maximum</td>
</tr>
<tr>
<td>GHz</td>
<td>gigahertz ($10^9$ Hz)</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HOMO</td>
<td>highest occupied molecular orbital</td>
</tr>
<tr>
<td>HS</td>
<td>humic substance</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz ($s^{-1}$)</td>
</tr>
<tr>
<td>IR</td>
<td>infrared</td>
</tr>
<tr>
<td>IRF</td>
<td>instrument response function</td>
</tr>
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</table>
K  kelvin
Kₐ  partition coefficient
kcal  kilocalorie
kD  kilodalton
L  liter
LDA  lifetime distribution analysis
LFFA  Lake Fryxell Fulvic Acid
LUMO  lowest unoccupied molecular orbital
M  molar
MCA  multichannel analyzer
MCP-PMT  microchannel plate photomultiplier tube
MC-SCF  multi-configuration self-consistent field
MCT  mercury-cadmium-telluride
MEM  maximum entropy method
mg  milligram
MHz  megahertz (10⁶ Hz)
 mL  milliliter
mm  millimeter
mol  mole
mV  millivolt
mW  milliwatt
Nd:YAG  Neodymium:Yttrium Aluminum Garnet
nm  nanometer (10⁻⁹ m)
NOC  nonpolar organic compound
ns  nanosecond (10⁻⁹ s)
OOP  out-of-plane
PAH  polyaromatic hydrocarbon
PBS  phosphate buffer solution
Pheo  pheophytin
PMT  photomultiplier tube
ps  picosecond (10⁻¹² s)
PS I  photosystem I
PS II  photosystem II
PS II-RC  photosystem II reaction center
PSO  psoralen
R6G  Rhodamine 6G
RC  reaction center
RP  radical pair
S₀  singlet ground state
S₁, S₂  first and second excited singlet states
SRFA  Suwannee River Fulvic Acid
SRHA  Suwannee River Humic Acid
TAG  time-to-amplitude converter

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TCSPC</td>
<td>time-correlated single-photon counting</td>
</tr>
<tr>
<td>TRIR</td>
<td>time-resolved infrared</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UV/VIS</td>
<td>ultraviolet-visible</td>
</tr>
<tr>
<td>W</td>
<td>watt</td>
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CHAPTER 1

INTRODUCTION

Time-Resolved Fluorescence Spectroscopy

Time-resolved fluorescence spectroscopy has become an ubiquitous research tool in many branches of science. Its popularity stems from its inherently high sensitivity relative to other techniques (e.g., absorption spectroscopies) and its convenient time-scale. Specifically, the mean time between the absorption of a photon and subsequent fluorescent emission is on the order of picoseconds or nanoseconds for most molecules. This quantity, termed the fluorescence lifetime, may be measured by several instrumental methods. Fluorescence lifetimes not only contain information about the photophysical properties of the molecule under investigation (i.e., the fluorophore), they also serve to report on aspects of the microenvironment experienced by the fluorophore as well as providing insight into any photochemical processes that compete with fluorescence to depopulate the first excited singlet ($S_1$) state. The time scales of many of these processes (e.g., solvation, photodegradation, energy transfer, electron transfer) are often similar to those of fluorescence, so changes in the kinetics of these processes (which might be difficult or impossible to measure directly) are reflected in corresponding changes in the measured fluorescence lifetimes.

Fluorescence lifetimes may be studied in the time domain or in the frequency domain. In the time domain, data are typically collected by recording the intensity decay of
a fluorophore's fluorescence emission subsequent to pulsed excitation. These data map out
a straightforward plot of intensity vs. time. Frequency domain measurements employ
sinusoidally-modulated light sources and obtain fluorescence lifetimes indirectly by
measuring the phase angle and demodulation factor between the absorbed and emitted
radiation. The fluorescence lifetimes are subsequently calculated from these measured
values.¹

The choice of the time or frequency domain for fluorescence measurements often
boils down to personal preference and available instrumentation. The relative merits of the
two approaches continue to be the source of considerable debate,²,³ yet several types of
instrumentation are available commercially for both time- and frequency-domain
measurements, and both methods can claim broad acceptance. It seems likely that the
techniques will coexist for the foreseeable future.

In this dissertation, time-resolved fluorescence data, measured in the time domain
are analyzed and interpreted to provide insight into problems of photochemical, biophysical,
and environmental interest. The results of these experiments are combined with data from
other spectroscopies and computational methods to investigate problems as diverse as the
photochemistry of diazirines, the photophysics of psoralens, energy-transfer processes in
photosynthetic reaction centers, and the fluorescence quenching of polyaromatic
hydrocarbon compounds (PAHs) in environmental matrices.

**Time-Correlated Single-Photon Counting**

Our time-resolved fluorescence measurements were carried out using the Time-
Correlated Single-Photon Counting (TCSPC) technique. This method has been widely used
since the 1970s, and descriptions of the technique abound in the literature.¹⁻¹² Consequently,
only a very brief introduction will be given here. The specific instrumentation employed in our experiments will be discussed in detail in the next chapter.

The TCSPC approach requires a source of pulsed excitation. Flashlamps have traditionally filled this role, but they have been replaced by laser systems in many modern instruments. The ideal pulse would have zero duration, and the ultimate time resolution of a given instrument is often largely determined by the pulse width of the excitation source. This does not hold true for instruments based on ultrafast laser systems, as the femtosecond and picosecond widths of their pulses are submerged beneath the transit-time spreads of the photomultiplier and detection electronics. A modern TCSPC system that has been carefully optimized is capable of routinely measuring lifetimes on the order of 10 – 20 ps, and even shorter lifetimes have been reported in the literature.13-22

The excitation pulse train is split into two optical paths, one arm of which is directed to the sample while the other terminates at a fast photodiode. The arrival of the pulse at the photodiode initiates a timer in the detection electronics that is subsequently halted by the arrival of the first fluorescence photon at the photomultiplier tube. The time interval between the arrival of these two pulses (corrected for differences in the two optical pathlengths) corresponds to the time between absorption and emission in the sample. This time interval is digitized and registered in the appropriate bin of a histogram of decay times. As this process is repeated thousands and millions of times, the histogram acquires the shape of the fluorophore's time-domain fluorescence decay.
Analysis of TCSPC Data

Fundamental Challenges in the Analysis of TCSPC Data

One of the most challenging aspects of time-resolved fluorescence spectroscopy is the analysis of the experimental data. For frequency-domain measurements, the data usually take the form of plots of phase angle and demodulation factor vs. the modulation frequency of the excitation source, while time-domain measurements (such as TCSPC) simply represent the fluorescence intensity as a function of time. Extracting meaningful chemical information from these raw data is occasionally straightforward. More often, however, a reliable interpretation of these data represents a considerable analytical challenge.

Obstacles to be faced in interpreting TCSPC data include (1) accounting for distortion in the observed decay curve introduced by the finite response time of the instrument; (2) identifying artifacts, interferences, or noise in the experimental data and making the appropriate adjustments or corrections; and (3) obtaining accurate and chemically meaningful kinetic parameters from the fluorescence intensity decays by appropriate methods of data analysis.

The distortion of fluorescence decay curves due to the finite width of the instrument response function is usually only of significant consequence when measuring lifetimes for systems near the frontier of the capabilities of the instrument (c. 10 ps – 5 ns). In any event, these distortions should be minimized by optimization of various experimental parameters. As long as the response function of the instrument is known and is stable over time, it can usually be accounted for in a straightforward fashion in the data analysis. The most widely used procedure involves convolution of the instrument response function (IRF) with a trial decay law. The agreement with the experimental data is tested by some statistical parameter such as the reduced chi-square ($\chi^2_r$) and the process is repeated in an iterative manner until the correspondence of the decay law and the data is judged to be satisfactory.
The presence of artifacts or interferences in the TCSPC data is a more serious concern. Even subtle effects of electrical noise, nonlinear responses, radio-frequency interferences, optical reflections, excitation source instabilities, and similar problems with the instrumentation can have profound consequences on the form of the data. It is usually not possible to account for these distortions with mathematical corrections, so these problems must be identified and rectified. This process is often quite tedious and difficult.

The third obstacle is primarily an issue of information content. In scientific research, it is generally not the fluorescence decay curves themselves that are of interest. Rather, the ultimate goal is the extraction of meaningful kinetic parameters for the underlying chemical system. This is accomplished by using an appropriate mathematical analysis of the decay curves. The challenge here lies in assessing exactly what qualifies as an “appropriate” mathematical analysis. There are at least three critical questions that must be answered in order to address this issue: (1) What is the appropriate equation or set of equations to which the data ought to be fit? (2) Do the fluorescence decays contain a sufficient amount of information to solve for the variable parameters in the equation(s) of interest? and (3) Are the data of sufficient quality (e.g., signal-to-noise ratio, resolution, etc.) to permit successful recovery of these parameters?

Sadly, when one surveys the references to time-resolved fluorescence spectroscopy in the literature, it seems that many researchers have failed to address these questions adequately. As a consequence, their conclusions are of dubious value. Kinetic parameters extracted from improper models or from data of low resolution or inadequate information content are often meaningless.

General Considerations in the Analysis of TCSPC Data

In the previous section it was noted that the distortion of TCSPC data due to the finite width of the instrument response function can be accounted for by deconvolution
techniques, assuming that the form of the instrument response function is stable and known.

Over the years, several techniques have been employed to deconvolute the instrument response function from the experimental data to recover the underlying decay law. These have included least-squares iterative reconvolution algorithms, the method of moments, Laplace transforms, Fourier transforms, the method of modulating functions, and phase plane methods, among others.\textsuperscript{4,10,26} While all of these methods have been used with success to analyze single-exponential decays that were relatively free of artifacts, the least-squares iterative reconvolution approach has been found to be more successful in the recovery of complex decay laws from data containing appreciable noise or distortion.\textsuperscript{10,26}

Regardless of the method, the deconvolution process is an ill-conditioned problem for noisy data.\textsuperscript{1,27-29} Practically speaking, this means that the recovered parameters will, in general, not be unique. In practice this usually does not present serious problems, provided that proper precautions are taken to ensure that no serious instabilities are introduced by the ill-conditioned nature of the data analysis process. Exceptions have been noted in the literature, however, and it seems likely that the problem has gone undetected in some investigations.\textsuperscript{29} Symptoms of instability might include the inability to recover similar kinetic parameters from repeated measurements of the same sample or the recovery of qualitatively different parameters when different optimization algorithms are employed to fit the data.\textsuperscript{29}

Attempts to interpret TCSPC decays beyond the limits justified by their information content are more likely to cause problems than instabilities in the analysis algorithms.\textsuperscript{30} When data are fit to multi-parameter equations, there is a considerable probability that the recovered equation will bear little or no resemblance to the underlying kinetic model. Instead, the equation will simply represent a mathematical parameterization of the data that essentially amounts to drawing a line through the data points. Once again the unsuspecting scientist might be led to believe that he has uncovered a complex kinetic model when the data are not adequate to distinguish the proposed model from a set of other feasible solutions. This set of
feasible solutions can be very large indeed. This pitfall may also be avoided by applying various checks and balances, some of which will be discussed later in this dissertation. Nevertheless, one must always stop short of claiming to have “proven” a particular complex kinetic model. The strongest statement that can be made is that the data have been shown to be more consistent with a proposed scheme and set of kinetic parameters than with other models explicitly considered as alternatives. In general, a more complex kinetic model will require more open-mindedness and humility.

Common Models Applied to the Analysis of TCSPC Data

The time-resolved fluorescence intensity of a single fluorophore in a homogeneous environment may, in general, be modeled as a single-exponential decay:

\[ I(t) = I_0 e^{-t/\tau} \]

In this equation, \( I(t) \) represents the fluorescence intensity at time \( t \), \( \alpha \) represents the amplitude and \( \tau \) is the fluorescence lifetime. This lifetime is a function of the intrinsic photophysical properties of the fluorophore, its photochemistry, and its environment. This straightforward situation is encountered perhaps more often than one might suppose, all things considered. Much more likely however, especially in chemical systems encountered in the “real world,” is the observation of a more complex time-resolved fluorescence response that cannot be described as a single-exponential decay. There are several factors that might contribute to this observed complexity including, but by no means limited to: (1) the presence of multiple fluorophores, (2) fluorescence from multiple conformations of a single fluorophore that cannot equilibrate during the lifetime of the excited state, (3) quenching phenomena, or (4) fluorescence from heterogeneous microenvironments.
Under these circumstances, one of five approaches is generally adopted to model the data:

1. **The data may be fit to a discrete sum of several (2 – 6) exponentials:**

   \[ I(t) = \sum_{i=1}^{n} a_i e^{-\lambda_i t} \]

   In the equation above, the sum is over \( n \) discrete exponentials, each with a characteristic amplitude \( (a_i) \) and lifetime \( (\lambda_i) \).

2. **The data may be fit to a sum of parameterized continuous distributions of exponentials.**

   In this case it is necessary to postulate a shape for the lifetime distributions (e.g., Gaussian, Lorentzian) in lifetime space.

3. **The data may be analyzed using an unparameterized continuous distribution of exponentials.**

   This approach involves no *a priori* assumptions about the shapes of the lifetime distributions. An example of this is the so-called Exponential Series Method (ESM). In this case a continuous distribution of lifetimes is *approximated* by a sum of many (c. 50 – 300) exponentials. The functional form of this model is identical to the discrete sum-of-exponentials described previously, except for the much larger value of \( n \). One key difference in this approach, however, is that the lifetime values are held constant during the fitting process, and are generally equally spaced on a logarithmic axis. Only the relative amplitudes are adjusted during the optimization process.

4. **If quenching processes are involved, the data may be fit directly to a quenching equation.**

   In this case the particular equation employed will be justified by the type of quenching believed to be present in the particular chemical system.
The data may be modeled using global simultaneous analysis of multiple fluorescence experiments using discrete or distributed parameters. The last two approaches are qualitatively different from the first three methods. Approaches 1 – 3 have the goal of obtaining an accurate, unbiased (model-independent) representation of a single decay curve in terms of amplitudes and lifetimes. These experiments can estimate the dimensionality of the system under investigation and expose general trends. The results of such analyses permit the development of hypothetical kinetic models. The last two techniques have a completely different goal. When one performs a global analysis or fits experimental data to a specific quenching equation, the goal is to determine physical mechanisms that will accurately predict how the amplitudes and lifetimes will change as a function of specific experimental parameters.

In general, a successful global analysis for a particular chemical system is preceded by successful model-independent analyses of one or more of the first three types listed above. The methods should not be viewed as competitive with one another. Rather, the first three approaches provide an unbiased, model-independent representation of the data that provides a rational framework for the design of subsequent global (model-testing) or quenching analyses.

When independent experiments or theory suggest a particular model, it is sometimes possible to proceed directly to an appropriate quenching (common) or global (less common) analysis. Of course, many systems do not require such complex analysis. In addition to the single-exponential case discussed previously, data described by two or three lifetime components or by a unimodal distribution of lifetimes often give rise to straightforward data analyses.

We note in closing this section that it is in general not possible to select an appropriate model for TCSPC data solely on the basis of a statistically acceptable fit to the data. In other words, the fact that one obtains a favorable value of the reduced chi-
square, observes random residuals, and a flat autocorrelation function does not mean that the data have been fit to a model that accurately represents the kinetics of the chemical system. In fact, for low-precision data, an acceptable value of the reduced chi-square can usually be obtained for a 2 - 4 exponential fit regardless of the "true" decay law. As the precision of the data (measured by the counts in the peak channel, or CPC) increases, it is often possible to reduce the size of the set of feasible solutions, but the selection of the model ultimately chosen to represent the data should always be guided by theoretical considerations and supported by additional, independent experiments whenever possible.

Quality Issues in TCSPC Data

When an instrument is optimized and the data are analyzed according to an appropriate model, problems may still arise. Many of these relate to the quality of the data. Data must be collected to a sufficiently high level of precision to provide an adequate signal-to-noise ratio for meaningful analysis. TCSPC data are governed by Poisson statistics, so the standard deviation for each datum (MCA channel) is given by the square root of the number of counts in that channel. Consequently as data are collected to higher values of CPC, the precision of the recovered parameters and the ability to discriminate between kinetic models will improve. While successful analyses of single-exponential decays often require less than 10,000 CPC, modeling data with complex decay laws may require 20,000 - 500,000 CPC.

In a similar way, the successful analysis of TCSPC data will require the range and resolution of the data to be optimized for the particular system under investigation. The full-scale range of the time-to-amplitude converter (TAC) is generally adjustable. Setting the time window too wide will compress the data into a fraction of the available data channels and resolution will be lost. Choosing a time window that is too narrow will cause information to be lost on long-lived components of the fluorescence decay, and analyses of such data will yield inaccurate values for the amplitudes and/or lifetimes of long-lived components. For a
given component, the time window will be optimized if its intensity decay is distributed across
the full range of the TAC. By its very nature, this condition requires that only one component
in a complex decay may be rigorously optimized for a given experiment. For multi-
component decays, an acceptable compromise range must be selected. Once a full-scale time
range has been chosen, it is usually also an option to select the resolution of the data, as
defined as the number of MCA channels. Collecting data into more channels improves the
resolution of lifetimes that are either closely-spaced or highly dissimilar. Unfortunately, there
is an inherent trade-off between the resolution of the data and its precision for a fixed
acquisition time. For example, doubling the resolution of the MCA from 512 to 1024
channels also doubles the data acquisition time required to obtain an equivalent data
precision.

Lifetime Distribution Analysis

Motivation for Lifetime Distribution Analysis

When faced with a nonexponential fluorescence decay curve, thoughtful
consideration must be given to the subsequent analysis of the data. It is absolutely crucial for
prior knowledge about the specific chemical system under investigation to be incorporated
into the analysis at this stage. The most natural option would seem to be to fit the data to a
discrete sum of 2 – 6 exponentials, adding additional components until an acceptable fit is
obtained. This is indeed the most common approach taken in the literature. As mentioned
previously, caution is necessary here as experience indicates that a 3 – 4 exponential fit will
provide statistically acceptable fits to nearly any set of TCSPC data. Whether the lifetimes
and amplitudes correspond to any parameters of real chemical significance is another question
entirely.
For some systems, there are good reasons to assign meaning to the parameters obtained from discrete-component analyses. The kinetics of excimers or exciplexes in homogenous solutions or of a single fluorescence probe partitioned into two discrete, homogeneous microenvironments should give rise to double-exponential fluorescence decays under most circumstances. Of course, mixtures of non-interacting fluorophores in homogeneous solutions will also give rise to a decay that possesses the same number of exponentials as there are components in the mixture.

If a system is known to undergo energy transfer, participate in excited-state reactions, form complexes, or experience collisional quenching of its fluorescence during its excited-state lifetime, other models need to be considered in analyzing the data. A discrete sum of exponential terms might provide meaningful insight under certain circumstances, but other equations, specific to the kinetic model of interest, might have to be considered. Lakowicz provides an excellent discussion of various quenching phenomena and appropriate equations used in their analysis in his *Principles of Fluorescence Spectroscopy*.¹

Similarly, some systems might possess fluorophores that are known to exist in a well-defined continuous distribution of states, microenvironments, etc., that would affect their fluorescence lifetimes. If these states, microenvironments, etc., are unable to interconvert and equilibrate during the lifetime of the excited state, the data might be best modeled as a parameterized distribution of exponential decays. Either Gaussian or Lorentzian lineshapes might be appropriate, depending on the nature of the distributed parameter.

Often, however, the prior knowledge of a system’s kinetics will be insufficient to suggest a particular mathematical model for the fluorescence analysis. This will often be the case for very complex samples and matrices, such as those encountered in biological and environmental contexts. Or the system of interest might be known to possess a complex, heterogeneous mixture of fluorophores or experience some other distributed parameter for
which no a priori form may be assigned to the distribution. For these systems, preliminary investigations might be best served by fitting the data to a model-independent parameterization of the data that imposes minimal bias on the structure of the set of recovered parameters. An approach that has gained popularity in recent years for such systems is the technique of Lifetime Distribution Analysis (LDA). Two specific methods of LDA that differ only in their optimization algorithm are the Maximum Entropy Method (MEM) and the Exponential Series Method (ESM). In both of these LDA methods the data are fit to a fixed set of lifetimes that are generally equally spaced on a logarithmic scale. Only the amplitudes of the lifetimes are varied in the optimization process. If a sufficiently large (c. 50 – 300) number of lifetimes are included in the analysis set, the recovered array of amplitudes will trace out a discrete approximation to the underlying continuous distribution of lifetime components that is presumed to exist in the sample.

Some specific systems that have been analyzed using LDA or have been recommended for such analysis include molecules absorbed on surfaces, fluorophores subject to multiple-conformation quenching phenomena, fluorescence from intrinsic or extrinsic probes localized in heterogeneous polymer matrices, bilayers, Langmuir-Blodgett films, micelles, cyclodextrins, biological macromolecules, membranes, or cells, molecular clusters in supersonic beams or supercritical fluids, and complex environmental matrices such as humic materials and coal liquids. For a relatively recent review of many of these applications, see the chapter by Ware in Photochemistry in Organized and Constrained Media. For microheterogeneous systems, distributed systems, complex mixtures, etc., lifetime distributions may represent a more logical starting point for analysis than discrete sums of exponentials. If the underlying kinetics of the system under investigation are due to distributions, fitting the data to a sum of a few (2 – 6) discrete exponential terms will, in general, result in chemically meaningless parameters.
Approaches to Lifetime Distribution Analysis

In the literature, lifetime distribution analysis is used as an "umbrella" term for fitting data to both parameterized and unparameterized lifetime distributions. This is somewhat unfortunate, as the philosophy and approach of the two types of lifetime distributions are so different. Parameterized distributions are most suited for systems that are known to possess a distributed parameter that is expected to give rise to a lifetime distribution of a known functional form. As such, this type of lifetime distribution analysis is more model-dependent than the unparameterized approaches to lifetime distribution analysis.

Parameterized distribution functions have been especially popular among scientists collecting time-resolved fluorescence data in the frequency domain. For example, Gilmore et al. have recently published work based on lifetime distribution analysis of photosystem II (PS II) Chlorophyll a fluorescence in thylakoids and leaves of barley wild-type and chlorina f104 and f2 mutants. Each data set was fit to a bimodal parameterized Lorentzian distribution model. Such fits involved five independent fitting parameters: the lifetime centers and widths of the two Lorentzian modes and the relative fractional contribution of each mode. Note that the relative fraction is essentially a ratio, and thus constitutes a single parameter. A similar study by Gilardi et al. concluded that although Trp48 in wild-type Pseudomonas aeruginosa Azurin displayed a single-exponential decay, the fluorescence responses of two mutants (apo-Ile7Ser and apo-Phe110Ser) were best described by Lorentzian distributions of lifetimes. These results were interpreted to be consistent with a loosening of the matrix around the indole side chain of Trp48 in the mutants. Several other examples exist in the literature, and the early work of Alcala et al. should be consulted for detailed descriptions of the relevant mathematics and critical variables in the resolvability of these parameterized distributions.
The analysis of fluorescence data using unparameterized distributions is quite different. No a priori form is assumed for the shapes of the distributions, and the data are usually analyzed using many (c. 50 – 300) independent fitting parameters. Such techniques permit asymmetrical distributions to be recovered with success, and provide a nearly bias-free representation of the data due to the very fluid nature of the parameter space employed. It should be noted that this fact is neither "better" nor "worse" than the other methods used to fit time-resolved fluorescence data. We stress again that the science of the system under investigation must guide the data analysis. When there are good reasons to expect parameterized distributions or discrete sums of a few exponentials, these should be employed instead of unparameterized distributions, as the fluid nature of the parameter space could be disadvantageous in the recovery of unique, chemically meaningful parameters. On the other hand, if the underlying chemistry is largely unknown or if there are good reasons to expect complex or skewed lifetime distributions, an unparameterized distribution might help to guard against correlations between fitting parameters, in which an incorrect parameterization of the system would cause fitting parameters to "compensate" for other parameters' deficiencies in unpredictable and chemically meaningless ways. Consequently, unparameterized lifetime distributions may often serve as precursors to simpler, parameterized (discrete, distributed, global) analyses. In this case the unparameterized distribution may be used to estimate the number, type, and symmetry of modes to be included in the parameterized description of the data and to expose basic trends in multidimensional data surfaces.

Historically, two techniques have been extensively employed to analyze unparameterized lifetime distributions. These are the Maximum Entropy Method (MEM) and the Exponential Series Method (ESM). Recently a third approach, the so-called Gakamsky method has been heralded as possessing certain advantages over the two traditional techniques, but it seems too early to evaluate these claims. The fundamental
fitting equation for the MEM and ESM techniques was discussed earlier, and detailed discussions may be found in the work of Livesey and Brochon (MEM), Rhodes and Fox (ESM), and in several papers by Ware et al. (MEM and ESM). The MEM approach has been adopted by many groups to analyze both time- and frequency-domain fluorescence data. The ESM method, in contrast, appears to have been used primarily to analyze data in the time-domain. Siemiarczuk et al. have determined that MEM and ESM are virtually identical in their ability to resolve lifetime distributions, but the ESM algorithm arrives at the solutions approximately five times faster. TCSPC data in this dissertation have been analyzed using the ESM approach with a program generously provided by Dr. Timothy Rhodes. Details of the algorithm are available in his recent paper discussing the application of ESM lifetime distributions to phenanthrene solvent-solute interactions in supercritical carbon dioxide.

Global Analysis

The ultimate goal of any analysis of time-resolved fluorescence data is to uncover details of the underlying chemical phenomena. This information is often not available directly from the amplitudes and lifetimes or distributions of these parameters obtained from model-independent data analysis, especially for complex systems. Once the dimensionality of a particular fluorescent system has been estimated, and the critical experimental variables have been identified, so-called "global" or "target" analyses are often performed on the data to test specific kinetic models.

A global analysis is performed when there is reason to believe that certain parameters may be mathematically linked across individual sets of data on a multidimensional data surface. This linking of parameters could correspond to a simple equality (e.g., of lifetimes) or could represent a complex mathematical relationship. The advantages of global
analyses over multiple single-curve analyses include enhanced parameter recovery and model
discrimination. These advantages are achieved because the chi-square hypersurface is
"steeper" and better defined in global analyses. Global analyses are also expected to lead
to superior recovery of closely-spaced lifetime components under certain conditions. While
statistically acceptable fits are almost always obtained when TCSPC data are analyzed with
discrete sums of exponentials or lifetime distributions, this is not the case for global analyses.
This is because global analysis, as a model-testing approach, has a parameter space that is
intrinsically limited by the parameterization of the specific model put forward as a
hypothetical description of the underlying chemical system. Statistically acceptable fits will
be obtained only when the entire data surface may be represented in an internally consistent
fashion by the model under test. As the data are fit directly to the rate constants, activation
energies, and other chemical properties of interest, global analyses guards against unjustified
interpretation of trends in abstracted parameters (i.e., amplitudes and lifetimes). Global
analyses are especially powerful because they are not limited to time-resolved fluorescence
data. The results of many dissimilar experiments may be analyzed in conjunction with the
time-resolved fluorescence data, as long as a suitable mathematical linkage exists between
relevant fitting parameters in the data sets. Excellent general discussions of global analysis
may be found in the work of Beechem et al. and Holzwarth. Additionally, Janssens
and Boens have published systematic studies of the data quality required for global analyses
of TCSPC data.

Overview of Research

The research presented in this dissertation represents investigations of diverse
chemical systems of photochemical, biophysical, and environmental interest. While
numerous experimental and computational methodologies have been employed to study the
chemistry of these systems, the analysis of time-resolved fluorescence data collected on a TCSPC instrument is the common theme linking these otherwise dissimilar investigations.

In Chapter 2, all of the relevant experimental and computational methods used in this dissertation research are described and discussed.

Chapter 3 represents an extensive, multi-pronged investigation of the excited states and photochemistry of the important class of carbene precursors known as dialkylidiazirines. In this chapter, time-resolved fluorescence data collected under a variety of experimental conditions (e.g., solvent, excitation wavelength, emission wavelength, temperature) are analyzed and combined with steady-state absorption, fluorescence, infrared, and Raman spectra; time-resolved infrared spectra; and semiemirical, density functional, and ab initio calculations to gain insight into the initial events in the excited-state chemistry of several dialkylidiazirines. This work represents the first recorded lifetime measurements for dialkylidiazirines. Any previous attempts to measure their time-resolved fluorescence were presumably limited by some combination of their very low quantum yields, very short lifetimes, and photochemical instability. As the fluorescence decays of dialkylidiazirines were found to be well-described by single exponentials, the analysis of the fluorescence data was straightforward for these systems.

Chapter 4 explores some of the critical variables in the analysis of TCSPC data for complex systems. A systematic approach to the determination of threshold values of data precision, data resolution, time base, etc. required to recover reliable fluorescence lifetime distributions for specific systems is presented. Particular emphasis is given to a discussion of the value of generating and analyzing synthetic TCSPC data to gauge the reliability of recovered parameters. The computer program that we have developed to generate these synthetic fluorescence decays is described. The complex fluorescence decays of photosystem II reaction centers, isolated from spinach leaves and Chlamydomonas reinhardtii algae are analyzed using four-exponential decays and ESM lifetime distributions. The critical variables
in the acquisition and analysis of TCSPC data for these systems are identified using the
approach developed in this chapter, and the potential applications of the ESM method of
lifetime distribution analysis for PS II reaction centers are discussed.

In Chapter 5 the time-resolved fluorescence of a second class of organic molecules,
the psoralens, are investigated. TCSPC data for four psoralen derivatives and two related
compounds are collected in various solvents at several wavelengths, and a three-pronged
analysis method is used to interpret the data. A combination of fitting these data to sums of
2 – 4 exponentials, ESM lifetime distributions, and a preexponential-weighted mean lifetime
provides the first interpretation of psoralen time-resolved fluorescence that is consistent with
the so-called proximity effect, an established aspect of the photophysics of these important
antiviral agents.

In Chapter 6, the fluorescence quenching of pyrene in the presence of humic
substances is analyzed. Humic materials are ubiquitous in the environment, and have been
implicated in the solubility enhancement of PAH pollutants in natural waters. As the humic
materials fluoresce strongly across the emission spectrum of pyrene, the measurement of
pyrene’s fluorescence lifetime in these complex matrices requires a creative analytical
approach. Our solution was to “filter” the TCSPC data in the time domain by collecting very
high-precision data and analyzing only the “tails” of the pyrene fluorescence decays. As the
fluorescence of the humic materials is negligible after several nanoseconds, an effective
isolation of the long-lived pyrene decay may be obtained. Our research investigates the
effects of oxygen on the observed quenching of pyrene’s fluorescence in the presence of
humic materials. This chapter also responds to feedback received from other scientists
concerning our initial publication on this topic.56-58

We conclude with Chapter 7, a summary of the contributions of this dissertation
research and an outline of some future directions for each project.
References


CHAPTER 2

EXPERIMENTAL AND COMPUTATIONAL METHODS

Fluorescence Lifetime Measurements

Instrumentation

Fluorescence lifetimes were determined using a Time-Correlated Single-Photon Counting (TCSPC) instrument. A diagram of this instrument is provided in Figure 2.1. For experiments conducted at The Ohio State University, the pulse train of a synchronously-pumped cavity-dumped dye laser (Coherent 700 Series; Rhodamine 6-G or DCM) was directed to a beam splitter. A portion of the pulse train was routed to a fast photodiode that registered a "start" signal on a Time-to-Amplitude Converter (TAC; Tennelec TC-864) after passing through a Constant Fraction Discriminator (CFD; Tennelec TC-455). This established the time position of each pulse with great precision. The "start" pulses initiated the charging of a capacitor in the TAC. The remainder of the pulse train was frequency-doubled (when necessary) in an appropriate non-linear crystal (Inrad, RDA or ADA) to generate UV excitation within an absorption band of the sample. Fluorescence was detected at 90° after having passed through a polarization analyzer (oriented at 54.7° relative to the polarization of the laser excitation), a depolarizer, and a subtractive-double monochromator (American Holographic DB-10S). The detector was a Microchannel Plate Photomultiplier Tube (MCP-PMT; Hamamatsu R-2809U-07). The signals arising from single photons were amplified (Minicircuit ZHL-42) and passed through a second CFD. The CFD output
pulses then served as the “stop” signals for the capacitor in the TAC. The TAC capacitance was transferred to a Multi-Channel Analyzer (MCA; Tennelec PCA-II), where the data were binned and presented as a histogram. This histogram served as a representation of the fluorescence decay.

When the Minicircuit ZHL-42 amplifier failed, it was replaced with a GHz preamplifier (EG&G Ortec 9306). Additional modifications to the instrument included replacing one of the CFDs with a pico-timing discriminator (EG&G Ortec 9307) and running the experiment in the so-called “reverse” configuration. In “reverse” mode, fluorescence photons arriving at the photomultiplier supply the “start” pulses, and the photodiode pulses serve as the “stop” signals. This configuration minimizes the TAC dead-time, since signals are processed only when a fluorescence photon is observed. This is in contrast to the “forward” configuration, in which > 99% of the observed “start” pulses have no “stop” pulse associated with them.

The decays collected at Wright-Patterson AFB were obtained using a mode-locked Ti:sapphire laser system (Spectra-Physics 3960C-L1S), pumped by an argon ion laser (Spectra-Physics 2080A-25). The 82 MHz output of the Ti:sapphire (1.3 W, 742 nm, 3 ps) was passed through an acousto-optic pulse-pickoff (Spectra-Physics 3980-5C) to yield a pulse train at 4 MHz (~ 55 mW). The laser output was monitored on a 350 MHz oscilloscope (Tektronix 2467) to measure the pulse suppression ratio. By adjusting the pulse-picker, pulse suppression ratios > 100 were routinely obtained. After frequency-doubling, 3 – 5 mW of 371 nm radiation was available for excitation, while the residual 742 nm light was directed to the photodiode. The experiment was run in reverse mode, with pico-timing discriminators (EG&G Ortec 9307) in both channels. The MCP-PMT was a Hamamatsu R-3809U-01, the preamplifier was an EG&G Ortec 9306, and the MCA was a Tennelec / Oxford PCA-Multiport with PCA-III software. The collection optics and other electronic components were identical to those used at The Ohio State University.
The TCSPC systems were capable of resolving fluorescence lifetimes on the order of 10 ps using a data analysis technique involving convolution of the instrument response function with trial decay functions. Iterative comparison with the experimental data provided a set of amplitudes and lifetimes corresponding to the best-fit to the observed fluorescence decay. The excellent signal-to-noise ratio of the TCSPC technique makes it useful even for weak fluorophores. Consequently, it was an ideal probe to interrogate diazirine excited states. Another advantage of the TCSPC approach is the ability to monitor the fluorescence decay directly during data collection. This allows various artifacts that are ubiquitous to fluorescence lifetime measurements to be detected and corrected in real time.

This proved to be especially important for the photosystem II samples in which it was often possible to assess the health of a particular reaction center preparation by eye after only a few minutes of data collection. It also allowed the decays for pyrene in the presence of humic substances to be “filtered” in the time domain in a very straightforward fashion. A blank was prepared for each humic substance studied. The fluorescence response in the absence of pyrene was collected and a channel noted for which the emission of the humic materials had decayed to baseline, and this channel was selected as the “start” channel for subsequent data sets in which pyrene was present.

Data Analysis

Sums of up to four discrete exponential decay components were fit using software supplied by Dr. James N. Demas and Seth Snyder while the Exponential Series Method (ESM) was employed for lifetime distribution analyses, using a program supplied by Dr. Timothy Rhodes.

In order to characterize the confidence limits of various fits to the data, synthetic data were generated using a software package written by Dr. Alex Siemiarczuk. Simulated TCSPC data could be prepared as a sum of up to sixty discrete exponential components. The
decay was convolved with a simulated instrument response of specified width, and random Poisson noise was superimposed on the data. The program permitted the number of channels, counts in the peak channel (CPC), and the full scale TAC width to be specified for each file. In the later stages of this work, simulations were carried out using a program written by the author in LabWindows/CVI. This new program allowed decays of up to 8192 channels to be constructed of up to 400 exponential terms, and allowed the user to select a synthesized or experimentally determined instrument response function for the convolutions. Flexibility in the choice of convolution routines was also incorporated and the user was given the ability to toggle the inclusion of Poisson noise and/or dark counts. The source code for this program is included in Appendix A.

Two additional computer programs were written to facilitate data analysis. One, coded in Visual C++, is an MS-DOS utility for the batch conversion of data files among various ASCII formats for TCSPC data. Functionality is also included for converting the x-axis from channels to time units, given an experimental calibration file. The second utility, written in LabWindows/CVI, parses parameter files generated by Demas and Snyder’s SPC analysis program into tab-delimited files suitable for direct import into a spreadsheet. These programs comprise Appendices B and C, respectively.

Transient Infrared Spectroscopy

We monitored the nanosecond – millisecond kinetics of adamantylidiazirine using Time-Resolved Infrared (TRIR) spectroscopy. The techniques currently available to measure infrared spectra over this time window may be divided into experiments that employ infrared lasers and those that rely on conventional infrared sources. The laser-based techniques have typically employed carbon monoxide, carbon dioxide, or diode lasers as
sources of the probe radiation. While these lasers provide excellent power, they do so over a very limited spectral range. Therefore, while they are suited to some chemical systems, our requirements were better met by a technique based on a conventional infrared source.

The transient infrared methods may be further divided into step scan and dispersive methods. Step scan methods benefit from the multiplex advantage, in which data for the entire spectrum is collected simultaneously. On the other hand, they suffer from the Fourier disadvantage, in that noise at a single data point manifests itself throughout the entire spectrum. Furthermore, the fact that the entire spectrum is collected simultaneously is a hindrance when one merely wants to monitor the transient kinetics at a single wavelength. Under such circumstances, one would prefer to sit at a single wavelength and observe the growth or decay of a particular spectral feature directly. Dispersive methods possess similar sensitivity and time resolution to the step-scan technique, yet they permit this type of single-wavelength data acquisition. Our investigations of adamantylidiazirine involved the initial collection of a few time-resolved spectra, but the choice of a dispersive technique enabled us to follow up these initial experiments with a more thorough monitoring of changes at particular wavelengths.

Our TRIR measurements were carried out in the laboratory of Dr. John P. Toscano at the Johns Hopkins University. The instrument is patterned after the design of Yuzawa et al. This technique provides access to the entire mid-IR spectrum (4000 – 800 cm⁻¹) with high sensitivity and ~ 30 ns time resolution. The instrument was capable of 4 cm⁻¹ spectral resolution, although our experiments were conducted using 16 cm⁻¹ resolution. Excitation pulses were supplied by the third harmonic (355 nm; 200 Hz; 150 mW) of a Continuum HPO-300 diode-pumped Nd:YAG laser. The excitation beam was crossed with the broadband output of a MoSi₂ infrared source (JASCO) and the changes in infrared intensity were observed on a MCT photovoltaic IR detector (Kolmar Technologies, KMPV11-1-J1). The resulting signal was amplified and digitized with a Tektronix TDS520A oscilloscope. Spectra
were collected point-by-point with a JASCO TRIR-1000 spectrometer operating in
dispersive mode with a 4 cm⁻¹ step size. In order to obtain an adequate signal-to-noise ratio,
several thousand laser shots were averaged at each infrared frequency. A flowing sample cell
was used (~ 20 mL reservoir) since the diazirine samples were efficiently photolyzed by the
355 nm laser. The flow cell had an optical pathlength of 0.5 mm. The adamantyl-diazirine
solutions were prepared in n-pentane and were ~ 0.5 M.

Steady-State Spectroscopies

UV/VIS Absorption Spectra

Absorption spectra were collected on a Perkin-Elmer Lambda 20 UV/VIS
spectrometer (accuracy 0.30 nm), a Milton-Roy Spectronic-3000 array spectrophotometer
(accuracy 0.35 nm), or (in a few cases) on a Hewlett-Packard HP8452A diode array
spectrophotometer (accuracy 2.0 nm). When sufficiently structured spectra were available,
the vibronic bands were resolved by fitting the experimental data with PeakFit™. A
representative fit to the data is shown in Figure 2.2.

Fluorescence Emission Spectra

Steady-state fluorescence excitation and emission spectra were measured on a
SPEX Fluorolog-2. The excitation source was a 450 W Xenon lamp (SPEX 1907-OFR),
passed through a single-grating monochromator (SPEX 1681B Minimate; 1200 groove / mm; 3.77 nm / mm). Fluorescence was detected in a T-box sampling module (SPEX 1692T)
at 90° relative to the excitation through a double-grating monochromator (SPEX 1680B
Spectramate; 1200 groove / mm; 1.70 nm / mm) with a SPEX 1911F detector. The slit widths
were invariably 1.25 mm, so the excitation bandpass was 4.7 nm and the emission bandpass was 2.1 nm. Rough quantum yield calculations were performed according to standard methods: 

$$\frac{\phi_{F, \text{sample}}}{\phi_{F, \text{reference}}} = \left( \frac{N_{\text{sample}}}{N_{\text{reference}}} \right) \times \left( \frac{F_{\text{sample}}}{F_{\text{reference}}} \right) \times \left( \frac{A_{\text{reference}}}{A_{\text{sample}}} \right)$$

In this equation, $\phi_F$ is the fluorescence quantum yield, $n$ is the refractive index of the solvent, $F$ is the integrated area of the fluorescence emission spectrum (Simpson's Rule), and $A$ is the average absorbance across the excitation bandwidth. No corrections were made for the average detector response or for the wavelength-dependence of the solvent refractive indices. The fluorescence yield ($\phi_F = 0.44$) of diphenylbutadiene (DPB) in cyclohexane was used as a standard.

Infrared Spectra

The infrared spectrum of adamantylidiazirine was measured in a 275 mg KBr pellet (10 mg adamantylidiazirine) with a Bruker Equinox-55 infrared microscope, model #A590, operating in reflection mode. The spectrum was obtained from 512 scans with the instrument set at 0.5 cm$^{-1}$ resolution, although the specifications of the instrument only guaranteed 4 cm$^{-1}$ resolution in reflection mode.

Raman Spectra

Raman spectra were collected for adamantylidiazirine with excitation at 457 nm, 488 nm, 514.5 nm, and 785 nm. Measurements were performed on solid samples, as well as on solutions prepared in cyclohexane, carbon tetrachloride, methylene chloride, and methanol.

The 785 nm experiments were performed using an SDL 8350 laser system (~55 mW) and a Chromex Raman-1 imaging spectrograph with a Photometrics SDS-9000 CCD detector. A 1200 lines/mm grating was used (optimized at 750 nm), so the spectral
resolution was ~ 4 cm⁻¹. The spectra at 457 nm, 488 nm, and 514.5 nm utilized the appropriate line from an argon-ion laser system (Coherent Innova 70–4) and a Dilor Raman microprobe instrument. The gratings used were 1200 lines/mm and 1800 lines/mm. Spectra of solids were collected through a 10× or 50× microscope objective, while solutions were run in the more conventional “macro” mode.

Frequency calibrations were made using naphthalene, 4-acetaminophenol (Tylenol), or cyclohexane as standards. An intensity correction for the wavelength dependence of the detector response was made for the data collected at 785 nm and 514.5 nm using the method of Ray et al.⁹

Semiempirical, Ab Initio, Molecular Mechanics, and Density Functional Calculations

Ab initio and density functional calculations¹⁰⁻¹⁴ were performed using Gaussian 94™,¹⁵ while semiempirical calculations were run either with Gaussian 94™ or with HyperChem™, release 4.5. Molecular mechanics calculations for the pyrene-humic substance complex in Chapter 6 were carried out in HyperChem™ using MM+.

The PM3 semiempirical method was used in Restricted Hartree-Fock calculations. Configuration interaction was included for eight occupied and eight unoccupied orbitals. The ground-state optimized geometry was used in the calculation of S₀ and S₁. The gradient of the reference configuration was < 0.009 kcal / mol / Å.

In Gaussian 94™, the frozen-core (fc) approximation was used for all correlated geometries and the basis set employed was the standard 6-31G*. Excited state calculations utilized the Configuration Interaction with Single Excitations (CIS) method within the frozen-core approximation.¹⁶⁻²⁰
References


Figure 2.1: Schematic of TCSPC instrument at The Ohio State University
Figure 2.2: Vibronic structure (Gaussian lineshapes) of the absorption spectrum of adamantyldiazirine in n-pentane
CHAPTER 3

DIALKYLDIAZIRINE PHOTOCHEMISTRY

Introduction

Dialkyldiazirines

The chemistry of carbene intermediates has been of considerable interest to organic chemists since their involvement in the alkaline hydrolysis of chloroform was demonstrated by Hine et al. in the middle of this century.¹ These highly-reactive species are now widely used in synthesis and may be generated by photolyzing appropriate diazirines. Molecular nitrogen is released upon photoexcitation, and a transient carbene is created. However, it is often difficult to distinguish between the chemistry of the carbene and the excited state of its diazirine precursor.²³ Indeed, there are several possible decay pathways from the excited state of a diazirine. Some of these processes are illustrated in Figure 3.1. These include, but are not necessarily limited to, fluorescence, internal conversion, intersystem crossing, carbon-nitrogen bond scission followed by intramolecular rearrangement processes (e.g., to form a diazo compound or, coupled with nitrogen loss, alkene formation via 1,2-hydrogen migration), intermolecular reactions (e.g., with solvent or other diazirines), as well as simple nitrogen extrusion to generate the carbene intermediate. The picture is further complicated by the fact that many of the possible products of these reactions are also accessible via the carbene. These complexities have made a comprehensive understanding of diazirine photochemistry an elusive goal.
Diazirines are weakly absorbing chromophores, and often exhibit weak fluorescence. The fluorescence lifetime of a diazirine should therefore serve as an indirect probe of other excited-state processes. Any enhancement of a competing process should be manifested as a decrease in the diazirine fluorescence lifetime, and any retardation of an alternate decay pathway should result in an increase in the lifetime.

Our research included experimental and computational investigation of several dialkyldiazirines, as well as the parent diazirine (CH₃N₂). The structures of these diazirines, as well as the structures of many of the intermediates and products considered in their photochemistry, are provided in Figure 3.2.

**Adamantyl Diazirine**

Adamantyl Diazirine (6) has attracted the attention of several investigators. This is due, in part, to the fact that adamantyl Diazirine is easy to synthesize, and is a stable, crystalline solid that is easy to handle. The carbene anticipated from the fragmentation of the diazirine (adamantylidene, 8) must surmount substantial activation barriers to intramolecular rearrangement of carbon or hydrogen, owing to the highly strained nature of the products of such reactions. As a result, the carbene is expected to have a long lifetime and useful intermolecular chemistry. Bayley and Knowles have used adamantyl Diazirine in membrane-labeling studies and Brinker et al. have analyzed the photolysis products in the cavities of β-cyclodextrins and various zeolites. Recently, Bonneau and Liu have made an important contribution to the understanding of adamantyl Diazirine’s photochemistry. They have measured the quantum yields of formation for the transient species 2-diazoadamantane (7) (φ_diazo ~ 0.5) and adamantylidene (φ_carbene ~ 0.5) in isooctane solutions. Previous studies had only analyzed the stable photoproducts. These stable photoproducts vary with solvent. Some of the most commonly observed are adamantanone (9), 2,4-dehydroadamantane (10), solvent insertion products (e.g., 11), and adamantanone azine (13). We have focused our
interest primarily on the structure, kinetics, and chemistry of the photoexcited diazirine and subsequently generated transient species. Adamantylidiazirine, as per adamantylidene, will resist hydrogen and carbon migrations due to its rigid architecture. For this reason, it is an attractive candidate for spectroscopic analysis.

Other Dialkyldiazirines

Once fluorescence investigations had been performed successfully on adamantylidiazirine, similar measurements were made for cyclohexyldiazirine (4) and 3,3-dimethyldiazirine (2). These compounds presented additional experimental challenges. Cyclohexyldiazirine is a liquid at room temperature, while 3,3-dimethyldiazirine is a gas. Solutions of these compounds were therefore prepared immediately prior to measuring the lifetimes. Solutions of adamantylidiazirine, in contrast, could be reliably prepared in advance and stored in a dark freezer. The fluorescence quantum yields of cyclohexyldiazirine and 3,3-dimethyldiazirine are lower than that of adamantylidiazirine, and their lifetimes are shorter. Only after successfully acquiring decays for the relatively long-lived adamantylidiazirine could the weak, short-lived decays of the other dialkyldiazirines be confidently distinguished from fluorescent impurities or experimental artifacts.

Experimental

Sample Preparation

The diazirines were prepared according to literature processes. All of the solvents employed were obtained from Burdick and Jackson or Aldrich (Freon-113 and methylcyclohexane) and were used without further purification. None of the solvents
exhibited significant fluorescence, and interference from solvent Raman bands was avoided in the measurement of fluorescence lifetimes by careful selection of the excitation and monitoring wavelengths.

Fluorescence Lifetime Measurements and Data Analysis

The diazirine fluorescence decays were collected using the TCSPC methods described in Chapter 2, using excitation wavelengths between 330 – 371 nm. The decays were typically collected to a precision of $10^4$ counts in the peak channel (CPC). Some decays were collected to $10^5$ CPC once the nature of the fluorescence kinetics had been determined to be single exponential. Identical lifetimes were obtained within experimental uncertainty for single exponential decays regardless of whether the data were collected to $10^5$ or $10^4$ CPC. This conclusion was verified for real and simulated TCSPC data.

When we analyzed the diazirine decays using ESM lifetime distributions, the “distributions” that were recovered corresponded to a weighted average of the two lifetimes in the ESM distribution that bracketed the lifetime obtained upon least-squares fitting to a single exponential using the Demas & Snyder program. Consequently, we may confidently state that the fluorescence decays of these dialkyldiazirines are discrete exponentials and not distributions of lifetimes.

The fluorescence lifetime of adamantyldiazirine in $n$-pentane at ambient temperature was also determined using an ISS K2-002 digital, multi-frequency, phase and modulation fluorimeter. This experiment was conducted in collaboration with Dr. Martin J. vandeVen of ISS. The excitation was centered at 354 nm using the output of a 300 W Xe arc lamp running at 18 W. The excitation slit widths were 2 mm, and a 3-74 emission filter was employed to block the excitation light from reaching the PMT. The cross-correlation frequency of the experiment was 80 Hz. Data collection required 2–10 turret rotations (sample/reference) for
each measurement. The analysis at each frequency was performed until the standard deviation of the phase was 0.2° and the standard deviation of the demodulation was 0.004. Ten excitation frequencies were employed, from 20 – 230 MHz.

The time-resolved infrared (TRIR) experiments, the computational methods, and the various steady-state spectroscopies employed in this investigation have been described in Chapter 2.

Results

Absorption and Fluorescence Emission Spectra

The absorption and fluorescence emission spectra of adamantyldiazirine exhibit a well-correlated mirror image relationship (Figure 3.3). In polar solvents, both spectra experience a red-shift, and a loss of vibrational structure. Among polar solvents, the absorption spectra in those solvents that are capable of forming hydrogen bonds with the nitrogen lone-pairs in adamantyldiazirine’s ground state are blue-shifted relative to spectra taken in solvents of similar polarity that are not capable of forming hydrogen bonds. (Figure 3.4) For example, the absorption maximum was 372 – 373 nm in n-pentane (ε ~ 245) and similar hydrocarbons, 375 – 376 nm in methanol and homologous alcohols, and 377 nm in acetonitrile. The peaks that we observed in n-pentane correspond precisely with those reported by Bayley and Knowles and Morgan. No solvent isotope effect was observed in either the absorption or emission spectra in cyclohexane/cyclohexane-d₁₂, and we observed no changes in the shape of the fluorescence emission spectra when the excitation wavelength was scanned across the absorption band from 330 nm – 370 nm. (Figure 3.5) Important spectral features are summarized in Table 3.1. The average fluorescence quantum yield was calculated to be 0.0012 ± 0.0004, and did not vary systematically with solvent. The
fluorescence intensity increased at low temperatures. As can be seen in Figure 3.6, when the
temperature was lowered to 79 K the emission intensity in methylcyclohexane increased 25-
fold relative to ambient temperature. Arrhenius treatment of the fluorescence intensities in
this solvent gave an average activation energy of 3.6 ± 1.0 kcal/mol. This activation energy
corresponds to the rate-limiting barrier in the S₁* state of adamantyl diazirine for any thermally
activated processes that compete with fluorescence. We had previously reported a value of
2.9 kcal/mol.¹¹ The recalculation reflects our decision to limit the data to the same
temperature regime investigated in the measurement of fluorescence lifetimes, as well as the
decision to average the activation energies obtained at the peaks of five separate vibronic
bands in the emission spectrum in which the activation energy at each peak was itself an
average activation energy over a 1.5 nm region at the peak (three data points). The Arrhenius
plots for the five different vibronic bands are provided in Figure 3.7. The reported
uncertainty corresponds to twice the standard deviation of the calculated activation energies.

The absorption spectra of cyclohexyldiazirine and cyclohexyldiazirine-d₄ exhibit
structure similar to that found in adamantyl diazirine (Figure 3.8) but the fluorescence
spectrum lacks any vibronic structure. The photolysis product(s) of cyclohexyldiazirine do
not appear to absorb between 300 – 400 nm, but the spectrum < 270 nm displayed significant
changes after being exposed to the laser used in the TCSPC experiments. (Figure 3.9, Table
3.2)

Vibrational Spectra

The infrared spectrum of adamantyl diazirine was obtained in a KBr pellet. (Figure
3.10) Some of the more prominent bands were found at 1570 cm⁻¹, 1508 cm⁻¹, 1467 cm⁻¹,
1450 cm⁻¹, 1350 cm⁻¹, 1132 cm⁻¹, 1080 cm⁻¹, 1067 cm⁻¹, and 996 cm⁻¹. Morgan reported a
band centered at 1575 cm⁻¹ in 3-methylpentane¹⁸, Bonneau and Liu¹⁶ observed the same peak
in isooctane at 1574 cm\(^{-1}\), and the solution spectrum in CHCl\(_3\) has been reported to exhibit peaks at 1540 cm\(^{-1}\) (with shoulders) and 1450 cm\(^{-1}\).\(^{11}\) In CCl\(_4\), the strongest band was found at 1550 cm\(^{-1}\).\(^{19}\)

We also obtained the infrared spectrum of a solution of adamantyldiazirine in \(n\)-pentane after it had been subjected to extensive photolysis at 355 nm. As may be seen in Figure 3.11, prominent absorptions occur at 1644.5 cm\(^{-1}\), 1450 cm\(^{-1}\), 1317.4 cm\(^{-1}\), 1075.5 cm\(^{-1}\), 951.5 cm\(^{-1}\), 879.6 cm\(^{-1}\), and 800.5 cm\(^{-1}\).

To our knowledge, the Raman spectrum of adamantyldiazirine has not previously been published. As mentioned in the experimental section of this work, we have obtained spectra of the solid and of solutions prepared in cyclohexane, methanol, carbon tetrachloride, and methylene chloride. Excitation was at 457 nm, 488 nm, 514.5 nm, or 785 nm. A representative spectrum is shown in Figure 3.12 for solid adamantyldiazirine excited at 488 nm. The labeled peak positions correspond to the best-fit parameters obtained using Lorentzian line shapes.

Some of the more prominent bands in the Raman spectrum were found at 1573 cm\(^{-1}\), 1434 cm\(^{-1}\), 1251 cm\(^{-1}\), 1225 cm\(^{-1}\), 1160 cm\(^{-1}\), 1131 cm\(^{-1}\), 1093 cm\(^{-1}\), 1066 cm\(^{-1}\), 946 cm\(^{-1}\), 844 cm\(^{-1}\), 774 cm\(^{-1}\), 700 cm\(^{-1}\), 558 cm\(^{-1}\), and 335 cm\(^{-1}\).

Spectra at all four of the excitation wavelengths are presented for solid samples of adamantyldiazirine and carbon tetrachloride solutions in Figures 3.13 – 3.14. For the most part, these spectra exhibit only minor differences. However, the bands marked with an asterisk (*) in the solid spectra (1207 cm\(^{-1}\) and 1617 cm\(^{-1}\)) appeared only under short-wavelength excitation and their relative intensities increased during data collection. These bands were not observed in any of the solution spectra, presumably because diffusion removed the photoproducts from the optical path.
Time-Resolved Infrared Spectroscopy

Time-resolved infrared spectroscopy was employed to monitor the growth and decay of species relevant to adamantyldiazirine's photochemistry in the microsecond regime. The regions of interest (2100 cm⁻¹ – 2000 cm⁻¹ and 1700 cm⁻¹ – 1548 cm⁻¹) were scanned first, with a 10 μsec time window. This revealed the growth of absorption features at 2042 cm⁻¹ and 1664 cm⁻¹ and a depletion feature centered at 1576 cm⁻¹. The latter two features may be seen in Figure 3.15. Once this survey was completed, the next step was to obtain higher-quality kinetic traces at the wavelengths of interest. The biphasic growth of the 2042 cm⁻¹ feature (59 ns and 2.6 μsec) can be seen in Figure 3.16, and the persistence of this feature over a 200 μsec window is apparent from Figure 3.17. The feature at 1664 cm⁻¹ also grows in with a rise time of 1.3 – 1.8 μsec and persists over the monitored time scale (Figure 3.18) although its intensity is somewhat weaker. The band at 1576 cm⁻¹, in contrast, exhibits a biphasic decay with lifetimes of 67 ns and 1.75 μsec. The decay of the intensity from 1596 cm⁻¹ – 1560 cm⁻¹ is plotted in Figure 3.19.

Fluorescence Lifetime Measurements

This work contains the first direct measurements of dialkylidiazirine fluorescence lifetimes. The data were consistently well-described by a single exponential or a sum of two exponentials. There was no discernable solvent dependence of the fluorescence lifetime of adamantyldiazirine at ambient temperature. The primary decay component fit to a lifetime of 0.24 ± 0.01 ns with no systematic deviation across the range of solvents studied (Table 3.1). A typical fluorescence decay, a two-exponential fit to the data, and a plot of the weighted residuals are provided in Figure 3.20. Additional representative decays in various solvents may be found in Figure 3.21. The ambient temperature lifetime was later confirmed
using an ISS K2-002 digital, multi-frequency, phase and modulation fluorimeter. (Figure 3.22) In this case, the fluorescence lifetime in n-pentane was measured to be 0.243 ± 0.007 ns.

The TCSPC method was also used to measure the fluorescence lifetimes of cyclohexyldiazirine (4), cyclohexyldiazirine-d₄ (5), 3,3-dimethyldiazirine (2), and 3,3-dimethyldiazirine-d₈ (3) (Table 3.3). Within the precision of our data, deuteration of the carbon atoms adjacent to the diazirine center did not appear to affect the fluorescence lifetime of either molecule. For cyclohexyldiazirine in n-pentane at 293 K the fluorescence lifetime was ~84 ps and for 3,3-dimethyldiazirine in Freon-113 at 283 K the fluorescence lifetime was ~40 ps.

The fluorescence lifetime of adamantyldiazirine did not exhibit a solvent isotope effect in cyclohexane/cyclohexane-d₁₂ and the fluorescence lifetime was unaffected by excitation energy. Lifetimes measured with 330 nm excitation were identical to those obtained with 371 nm excitation.

The fluorescence lifetime data (τᵣ) can be related to the natural lifetime of adamantyldiazirine (τₒ) via the fluorescence quantum yield (Φᵣ):

\[ τₒ = \frac{τᵣ}{Φᵣ} \]

Using this expression, we estimated the natural lifetime of adamantyldiazirine to be 206 ± 49 ns at ambient temperature. These data are included in Table 3.1.

A long-lived (0.6 – 3.2 ns) component was present as a minor contribution to the adamantyldiazirine fluorescence decay in every solvent (5 – 31%) when the data were collected to 10⁴ CPC, although the decays were monoexponential in methylcyclohexane between 173 K and 230 K. Monoexponential decays were also obtained in n-pentane at all
temperatures when the data collection was stopped at $10^3$ CPC. We attribute the long-lived component to the fluorescence of one or more photoproducts, for reasons that will be addressed later.

As was true for the fluorescence intensity, decreasing the temperature increased the fluorescence lifetimes of the dialkyldiazirines. In methylcyclohexane the fluorescence lifetime of adamantyldiazirine increased to 7.57 ns at 78 K (Figure 3.23). In $n$-propanol, the lifetime was 8.82 ns at 78 K. Arrhenius treatments of the fluorescence lifetimes in $n$-pentane, methylcyclohexane, and $n$-propanol resulted in activation energies of $3.45 \pm 0.08$ kcal/mol, with preexponential factors of $1.2 \pm 0.7 \times 10^{12}$ s$^{-1}$. As was the case with the Arrhenius parameters determined from the fluorescence intensity data, these values differ from those we reported previously$^{21}$ since they represent a recalculation based on fewer data points in each solvent in order to reflect the same temperature regime investigated for the other dialkyldiazirines. They also incorporate recent data for adamantyldiazirine in $n$-pentane. The reported uncertainties correspond to twice the standard deviation of the parameters obtained from linear regressions to data collected in the three solvents. Arrhenius plots were also prepared for cyclohexyldiazirine and cyclohexyldiazirine-$d_4$ in $n$-pentane and for 3,3-dimethylidiazirine and 3,3-dimethylidiazirine-$d_4$ in Freon-113. These appear in Figure 3.24, and the data used to construct the plots as well as the activation energies and pre-exponential factors obtained from the plots, are tabulated in Table 3.3. Representative decays of these dialkyldiazirines at various temperatures are provided in Figures 3.25 – 3.26.

**Semiempirical, Ab Initio, and Density Functional Calculations**

The PM3 method was used to calculate properties of the ground state and first excited singlet state of adamantyldiazirine. Semiempirical calculations carried out with the PM3 method have been shown to predict successfully a number of experimental observables, including molecular geometries, ionization potentials, and dipole moments. Seeger *et al.*
have established that vibrational calculations using the PM3 method often provide frequencies and force constants that agree well with experimental values and with those obtained from \textit{ab initio} calculations that require much more intensive computational resources.\(^{24}\)

To test the appropriateness of the PM3 method for diazirines, we performed a geometry optimization on the ground state of diazirine (1, $\text{H}_2\text{CN}_2$) and compared the results with those obtained from the \textit{ab initio} (6-31G*) calculations of other research groups.\(^{4,25,36}\) (Table 3.4)

Our PM3 calculation yielded a nitrogen-nitrogen bond length of 1.226 Å, a carbon-nitrogen bond length of 1.492 Å, and an N–N–C bond angle of 65.7°. These values are in excellent agreement with the corresponding \textit{ab initio} values of 1.22 Å, 1.50 Å, and 66.0° obtained by Yamamoto \textit{et al.}\(^4\) Both methods yield geometries that correspond well with the experimentally-determined bond lengths of 1.228 Å and 1.482 Å obtained from Pierce and Dobyn's analysis of diazirine's microwave spectrum.\(^{27}\) Encouraged by this result, we performed a similar PM3 calculation on adamantyl diazirine (6). We found a 1.228 Å nitrogen-nitrogen bond, a 1.496 Å carbon-nitrogen bond, and an N–N–C bond angle of 65.8°. The R–C–R angle at the diazirine carbon was calculated to be 112.5° in adamantyl diazirine, which is nearly identical to the 113.1° angle found for the H–C–H angle in diazirine 1 (PM3). These results suggest that the adamantyl cage has little or no effect on the geometry of the diazirine moiety. When the PM3 method was used to calculate the properties of the lowest excited singlet state of 6 (at the ground-state optimized geometry), an expansion of the dipole moment from 2.5 D in $S_0$ to 3.8 D in $S_1^*$ was indicated, consistent with the dipole moment expansion of 1.5 D obtained from Lombardi \textit{et al.}'s analysis of the Stark spectrum of difluorodiazirine.\(^{28}\)

We also performed \textit{ab initio} (RHF/6-31G* and MP2/6-31G*) and density functional (B3LYP/6-31G*) calculations on adamantyl diazirine using the \textit{Gaussian 94}™
software package. These results are also summarized in Table 3.4. The calculations were facilitated by the fact that adamantyldiazirine possesses $C_{2v}$ symmetry. At the RHF/6-31G* level the N=N bond was found to be 1.203 Å, the C–N bonds were 1.441 Å, the N–N–C bond angle was 65.3°, and the R–C–R angle at the diazirine carbon atom was 112.8°. An inspection of some of the significant molecular orbitals of ground-state adamantyldiazirine calculated at this level of theory (Figure 3.27) confirms that the highest-occupied molecular orbital ($\phi_{HOMO}$, HOMO) is nonbonding and antisymmetrical with respect to the nitrogen lone-pairs ($n_\pi$), while the lowest-unoccupied molecular orbital (LUMO) is antibonding, with $\pi^*$ symmetry. With correction for electron correlation at the second-order Møller-Plesset perturbation (MP2) level, the N=N bond was 1.269 Å, the C–N bonds were 1.479 Å, the N–N–C bond angle was 64.6°, and the R–C–R angle was found to be 114.1°. The B3LYP/6-31G* calculation yielded an N=N distance of 1.237 Å, C–N bond lengths of 1.477 Å, a 65.2° N–N–C bond angle, and a 113.3° R–C–R bond angle.

Using the frozen core approximation, a CIS(fc)/6-31G* // MP2(fc)/6-31G* calculation was performed to examine the vertical transitions to the first four excited singlet states of 6. In this context the double slash (//) has its usual meaning to indicate that the calculation was carried out at the MP2(fc)/6-31G* optimized geometry of the ground state. The ground state (singlet) has $A_1$ symmetry and a calculated dipole moment of 2.62 D along the z-axis, perpendicular to the N=N bond. The first four excited singlet states were calculated to be of B$_1$, A$_2$, B$_2$, and A$_g$ symmetries, respectively. Single-photon transitions to the A$_g$ states are forbidden under $C_{2v}$ symmetry, so only transitions to the first ($f = 0.0016$, x-polarized) and third ($f = 0.0421$, y-polarized) excited singlet states were predicted to have nonzero oscillator strengths. These vertical transitions were predicted to appear at 3.64 eV (341 nm) and 7.40 eV (168 nm). The calculation for $S_1^*$ agrees well with the experimental absorption spectrum of adamantyldiazirine, measured in n-pentane. Its origin appears at 3.32 eV (373 nm).
Interestingly, each of the first four vertical transitions terminates in the same \( \pi^* \) unoccupied molecular orbital \( (\phi_{d^*}, \text{LUMO}) \), but the dominant CI excitations for each transition (Table 3.5) correspond to excitations from different combinations of occupied molecular orbitals. These differences are illustrated in the charge density difference plots provided in Figure 3.28, where the solid lines correspond to charge accumulation in the excited state and dashed lines correspond to charge depletion from the ground state. Figure 3.28 (top) shows that the first two vertical excited states accumulate charge density in a similar spatial region \( (\pi^*) \) but Figure 3.28 (bottom) shows that the two states deplete charge density from very different areas. In the transition to \( S_1^* (1B_1) \), a considerable amount of electron density is taken from the lone-pair region in "front" of the nitrogens on the diazirine ring. Additional electron density is drawn from the entire adamantyl cage. The transition to \( S_2^* (1A_2) \), in contrast, depletes the lone-pair region to the "sides" of the nitrogens in the diazirine ring and takes very little electron density from the far side of the adamantyl cage. The transitions to \( S_3^* \) and \( S_4^* \) (not shown) are less easily summarized, but they appear qualitatively similar to the transition to \( S_1^* \) in that they draw more from the "front" of the nitrogens than from the "sides."

Attempts to optimize the geometries of the \( S_1^* (1B_1) \) state did not locate a minimum within \( C_{2v} \) symmetry. Instead, the optimum \( C_{2v} \) geometry corresponded to a transition state. Similarly, the \( S_2^* \) state, which is \( 1A_2 \) under \( C_{2v} \) symmetry, also yielded a transition state rather than an energy minimum in its optimized \( C_{2v} \) geometry. Our calculations showed that the state ordering and energies are extremely sensitive to the geometry changes that lead to these transition states. The CIS(fc)/6-31G* optimized geometries are given in Table 3.6.

To explore the reaction surface further, we nudged both of the transition states \( (1B_1 \) and \( 1A_2) \) along the normal coordinates of their imaginary vibrational frequencies and optimized their geometries under the resulting \( C_1 \) symmetry. The mirror plane containing the diazirine ring was retained in both cases, but the other mirror plane was broken. In \( C_1 \), both
states possessed \( A'' \) symmetry and converged on the same minimum geometry (Table 3.6, Figure 3.29). Mixing of these two states (forbidden by symmetry in \( C_{2v} \)) was made possible when the symmetry was reduced to \( C_2 \). The implications for adamantyl diazirine's photochemistry are discussed below.

Our calculated ground-state geometries seem quite reasonable in comparison with published results for smaller diazirines. They agree well with experimental studies of difluorodiazirine by Lombardi et al.,\textsuperscript{24} semiempirical calculations (SINDO1) for diazirine (1) and 3,3-dimethyldiazirine (2) by Müller-Remmers and Jug,\textsuperscript{29} and for diazirine (1) by the \textit{ab initio} calculations of Yamamoto et al., Devaquet et al., and Boldyrev et al.\textsuperscript{4,25,26} Pictures of the frontier molecular orbitals of adamantyl diazirine (6), calculated semiempirically with the AM1 method, have been published by Morgan.\textsuperscript{14} We note that the orbitals look very similar to those discussed here.

To assist with the interpretation of the absorption, fluorescence, and infrared spectra, we performed semiempirical (PM3), \textit{ab initio} (RHF/6-31G*), and density functional (B3LYP/6-31G*) vibrational analyses on the \( 1A_1 \) (\( C_{2v} \)), \( 1B_1 \) (\( C_{2v} \)), \( 1A_2 \) (\( C_{2v} \)), and \( 1A'' \) (\( C_2 \)) states of adamantyl diazirine with \textit{Gaussian 94}™. The 72 ground-state fundamental vibrations and their symmetry assignments under \( C_{2v} \) are \( 22a_1 \) \( (v_1-v_{22}) \), \( 14a_2 \) \( (v_{23}-v_{36}) \), \( 18b_1 \) \( (v_{37}-v_{53}) \), and \( 18b_2 \) \( (v_{55}-v_{79}) \). These are tabulated in Tables 3.7 - 3.8 and presented graphically in Figures 3.30 - 3.32. Synthetic infrared and Raman spectra generated from the RHF/6-31G* intensities and scaled frequencies are provided in Figure 3.33. Our calculations indicate that the \( \text{N} = \text{N} \) stretching mode \( (v_{17}) \) is unique among adamantyl diazirine’s \( a \) modes in that it experiences a major frequency reduction upon excitation to \( S_1^* \). Its RHF/6-31G* frequency in the \( C_2 \)-optimized geometry of \( S_1^* \) is 260 cm\(^{-1} \) lower than that of the \( C_{2v} \)-optimized ground state. Most of the other modes shift by only a few wavenumbers.
Discussion

The absorption, fluorescence, and vibrational spectra of diazirines provide information about their ground- and excited-state structures. We begin our discussion by examining the vibrational assignments for the ground and excited states of adamantyl diazirine (6). We then move to a discussion of the time-resolved infrared data, and outline the microsecond-regime photochemistry of adamantyl diazirine. We compare our experimental vibrational spectra with semiempirical, \textit{ab initio}, and density functional calculations to analyze the geometries, fundamental vibrations, and electron density distributions of the species and electronic states under consideration. We then discuss the fluorescence lifetime data for each of the dialkyl diazirines investigated. The fluorescence lifetime serves as a probe of the dynamics of the excited state, and its temperature- and solvent-dependence provide indirect access to the rates of the competing excited-state decay processes. We discuss the implications of our data with regard to the relative contributions of inter- and intramolecular processes in the excited state photochemistry of dialkyl diazirines.

Vibrational Assignments

We were able to assign normal modes to most of the bands in the infrared (Figure 3.10) and Raman (Figure 3.12) spectra of solid adamantyl diazirine for the frequencies $< 1700$ cm$^{-1}$. This was achieved by comparing the spectra against each other and referencing the PM3, RHF/6-31G*, and B3LYP/6-31G* calculated frequencies and the RHF/6-31G* calculated infrared and Raman intensities. We were also guided by the vibrational assignments for diazirine (1) and 3,3-dimethyldiazirine (2) tabulated by other research groups.$^{10,31}$ These assignments, and the experimental and computational data used to obtain them, are tabulated in Tables 3.7 - 3.8. These modes are presented graphically in Figures 3.30 - 3.32.
It is clear that the strong band observed in the infrared spectrum at 1570 cm\(^{-1}\) (1573 cm\(^{-1}\) Raman) can be assigned to \(v_{177}\), a mode that is best described as an N=N stretch, although it also includes a contribution from an R–C–R scissoring motion on the diazirine carbon. This vibration has \(a_1\) symmetry under \(C_{\infty v}\). Calculations at the PM3, B3LYP/6-31G*, and RHF/6-31G* levels overestimated the frequency of this vibration by at least 135 cm\(^{-1}\). We performed similar calculations for diazirine (1), 3-methyldiazirine, 3,3-dimethyldiazirine (2), and 3,3-difluorodiazirine. In each case, the calculated N=N stretching frequency was higher than the reported experimental values. \(^{30-33}\) In order to obtain satisfactory agreement with experimental values for these smaller diazirines, vibrational analyses at the MP2/6-31G* level were required. We expect that an MP2/6-31G* vibrational analysis would also successfully model \(v_{17}\) for adamantylidiazirine, but we were unable to perform such a large calculation with our current resources. We estimate that about 16 GB of disk space would have been required. While the MP2/6-31G* vibrational analysis proved to be unwieldy, we were able to optimize the geometry of adamantylidiazirine at the MP2/6-31G* level. For smaller diazirines, the MP2/6-31G* frequency of the N=N stretch exhibited a nearly linear inverse dependence on the calculated MP2/6-31G* N=N bond length (Figure 3.34). If this simple correlation can be extended to adamantylidiazirine, we would expect the 1.269 Å calculated N=N bond length (Table 3.4) to correspond to an MP2/6-31G* stretching frequency of \(\sim 1563\) cm\(^{-1}\), in excellent agreement with the 1570 cm\(^{-1}\) band observed in the infrared spectrum.

Beyond \(v_{177}\), assigning the vibrations between 1320 cm\(^{-1}\) – 1570 cm\(^{-1}\) becomes more difficult. There are fourteen normal modes with calculated frequencies that could be considered for the eight infrared and four Raman bands that we observed. Of these bands, only two (1467 cm\(^{-1}\) infrared/1469 cm\(^{-1}\) Raman and 1442 cm\(^{-1}\) infrared/1441 cm\(^{-1}\) Raman) appear in both spectra. Qualitatively, each of these bands may be viewed as a deformation of the adamantyl cage structure. Some of the modes also couple to a diazirine ring stretch to some extent. As discussed above, these frequencies are probably not well-described by PM3,
RHF/6-31G*, or B3LYP/6-31G* calculations, owing to the mixing of these modes with $\nu_{17}$. Consequently, our assignments for these bands should be viewed as somewhat tentative. We suggest that the 1508 cm$^{-1}$ band in the infrared spectrum arises from $\nu_{16}(a_i)$ while the bands at 1467 cm$^{-1}$ (1469 cm$^{-1}$ Raman) and 1450 cm$^{-1}$ are attributable to $\nu_{50}(b_j)$ and $\nu_{69}(b_j)$. An infrared band appears at 1442 cm$^{-1}$ as a low-energy shoulder to the 1450 cm$^{-1}$ band. A similar band appears at 1441 cm$^{-1}$ as a high-energy shoulder to the 1434 cm$^{-1}$ band in the Raman spectrum. Only $\nu_{15}(a_i)$ is expected to possess significant infrared and Raman intensity in this region, so we assign it to this vibration. The Raman band at 1434 cm$^{-1}$ does not appear in the infrared spectrum, so it is assigned to $\nu_{34}(a_2)$, which is infrared-forbidden. Strong infrared bands appear at 1373 cm$^{-1}$ and 1350 cm$^{-1}$ and are attributed to $\nu_{14}(a_i)$ and $\nu_{13}(a_i)$, respectively on the basis of the RHF/6-31G* infrared intensities of these modes. The weak Raman band at 1355 cm$^{-1}$ is difficult to assign. It might also be due to $\nu_{13}(a_i)$, or it could be $\nu_{33}(a_2)$—either assignment would be consistent with the calculated Raman intensity. And we cannot completely rule out $\nu_{68}(b_j)$, although this band is predicted to have negligible intensity in both spectra. The infrared band at 1337 cm$^{-1}$ is attributed to $\nu_{49}(b_j)$ since it is weak or absent in the Raman spectrum. We excluded $\nu_{67}(b_j)$ and $\nu_{33}(a_2)$ from consideration for this band since both are predicted to be absent from the infrared spectrum. The $\nu_{67}(b_j)$ mode is predicted to have negligible intensity, while $\nu_{33}(a_2)$ is symmetry-forbidden. This leaves only the infrared band at 1325 cm$^{-1}$ unassigned. We suggest that this is $\nu_{48}(b_j)$ since $\nu_{67}(b_j)$ has negligible intensity and $\nu_{32}(a_2)$ is symmetry-forbidden, according to the RHF/6-31G* calculations.

Things become a little bit clearer once we reach the 1311 cm$^{-1}$ band in the infrared spectrum. There is a corresponding band at 1312 cm$^{-1}$ in the Raman spectrum, so this vibration is probably due to $\nu_{12}(a_i)$. Another band appears in both spectra at 1296 cm$^{-1}$ (1297 cm$^{-1}$ Raman). Based on the calculated intensities, we suggest that this is $\nu_{66}(b_j)$. As $\nu_{47}(b_j)$ is also anticipated to have infrared intensity, we speculate that it might also contribute to this
peak in the infrared spectrum. There is a strong Raman band at 1251 cm$^{-1}$, with no infrared equivalent. We believe that this arises from the infrared-forbidden $\nu_{31} (a_2)$ mode. A peak at 1239 cm$^{-1}$ is present in both spectra, although its Raman intensity is weak. This is probably $\nu_{46} (b_1)$. Strong Raman bands appear at 1225 cm$^{-1}$ and 1160 cm$^{-1}$. The corresponding bands in the infrared are either weak or nonexistent, so these are assigned to $\nu_{11} (a_1)$ and $\nu_{30} (a_2)$, respectively.

We are more confident in our assignments of the bands in the low-energy regions of the infrared and Raman spectra. In general, the calculations give excellent predictions for the frequencies of these bands, and there are numerous peaks that appear in both the Raman and infrared spectra that serve to anchor our assignments. The first of these appears at 1132 cm$^{-1}$ in the infrared spectrum and at 1131 cm$^{-1}$ in the Raman. This is assigned to $\nu_{10} (a_1)$, which is predicted to be one of the strongest Raman bands in the spectrum of adamantyldiazirine. The RHF/6-31G* calculations also attribute significant infrared intensity to this band. A weak band appears at 1109 cm$^{-1}$ in the infrared and 1107 cm$^{-1}$ in the Raman. We attribute this to $\nu_{45} (b_1)$. A stronger peak is found at 1093 cm$^{-1}$ in both spectra. This vibration is assigned to $\nu_9 (a_1)$. There is a strong infrared absorption at 1080 cm$^{-1}$, with a very small corresponding peak at the same wavenumber in the Raman spectrum. We identify this as $\nu_{44} (b_1)$. The band at 1067 cm$^{-1}$ (1066 cm$^{-1}$ Raman) is strong in both spectra and is assigned to $\nu_{63} (a_2)$. A weak band found at 1030 cm$^{-1}$ in the infrared spectrum and at 1032 cm$^{-1}$ in the Raman is tentatively assigned as $\nu_8 (a_1)$. This band is paired with one of similar intensity at 1027 cm$^{-1}$ in the Raman spectrum. This latter vibration is assigned as $\nu_{62} (b_2)$. The intense band observed at 996 cm$^{-1}$ in both spectra is assigned to $\nu_{43} (b_1)$. The band at 955 cm$^{-1}$ in both spectra is somewhat weaker, and probably corresponds to $\nu_{61} (b_2)$. A strong band is found at 946 cm$^{-1}$ in both spectra and we assign it to $\nu_7 (a_1)$. There is a band at 883 cm$^{-1}$ in the infrared spectrum that might also be faintly visible in the Raman spectrum. We attribute this to $\nu_{42} (b_1)$. The strong band at 871 cm$^{-1}$ in the infrared (872 cm$^{-1}$ Raman)
is identified as $v_{50} (b_2)$. There is also a band at 844 cm$^{-1}$ that appears in both spectra and it is assigned as $v_6 (a_1)$. The band at 835 cm$^{-1}$ is relatively strong in the infrared but weak in the Raman spectrum. Hence, it is assigned as $v_{41} (b_1)$. The band at 796 cm$^{-1}$ appears only in the infrared spectrum, where it is relatively strong. Consequently, we assign it to $v_{59} (b_2)$. On the other hand, the vibration at 774 cm$^{-1}$ is the most intense band in the Raman spectrum, yet is very weak in the infrared at 773 cm$^{-1}$. This peak is assigned to $v_5 (a_1)$. There is another very strong Raman band at 700 cm$^{-1}$, but the infrared spectrum is silent in this region. This band is clearly $v_4 (a_1)$. The last strong band in the infrared spectrum occurs at 663 cm$^{-1}$, and there is a corresponding, albeit weak band in the Raman spectrum at 664 cm$^{-1}$. This logically corresponds to $v_{88} (b_2)$. There is also a weak infrared absorption at 636 cm$^{-1}$ and a similarly weak peak in the Raman spectrum at 634 cm$^{-1}$. These bands are assigned to $v_{40} (b_1)$.

Even without the benefit of an infrared spectrum, the low-energy region of the Raman spectrum may be assigned. The B3LYP/6-31G* frequencies and RHF/6-31G* calculated Raman intensities are in excellent agreement with the measured spectrum. The peaks at 558 cm$^{-1}$, 524 cm$^{-1}$, and 469 cm$^{-1}$ are assigned to $v_3 (a_1), v_{57} (b_2), and v_{25} (a_2)$. The calculated frequencies and predicted intensities match up very well here. The same is true of the bands at 440 cm$^{-1}$ and 396 cm$^{-1}$, which can be assigned as $v_2 (a_1)$ and $v_{38} (b_1)$. The final two bands in the Raman spectrum, at 335 cm$^{-1}$ and 260 cm$^{-1}$ are also in excellent agreement with the predicted frequencies and relative intensities for $v_1 (a_1)$ and $v_{37} (b_1)$.

Bonneau and Liu found a band at 1646.2 cm$^{-1}$ for adamantanone azine (13) in isoctane, and they noted the appearance of this band in photolyzed solutions of adamantylidiazirine in the same solvent. Consequently, we may confidently assign the 1645 cm$^{-1}$ band in the infrared spectrum of adamantylidiazirine’s photolysis products in $n$-pentane to this species.
As mentioned previously, the Raman spectra of solid adamantyldiazirine obtained with 457 nm and 488 nm excitation exhibited bands at 1207 cm\(^{-1}\) and 1617 cm\(^{-1}\) that grew in to the spectrum during data collection (Figure 3.35) These bands are certainly attributable to a photoproduct, the most probable candidate again being adamantanone azine (13). The infrared spectrum of the photolysis product(s) in solutions of \(n\)-pentane possessed a peak at 1201 cm\(^{-1}\), which might correspond to the 1207 cm\(^{-1}\) vibration observed in the Raman spectrum of the solid. There are no bands in the infrared spectrum near 1617 cm\(^{-1}\); the nearest band is the strong peak at 1645 cm\(^{-1}\). This band is itself absent from the Raman spectra of solid adamantyldiazirine. These observations support the identification of the photoproduct as adamantanone azine, since semiempirical calculations of the normal modes of this molecule predict an infrared-forbidden \(\text{Ad} = \text{N} = \text{N} = \text{Ad}\) vibration that is red-shifted by 53 cm\(^{-1}\) relative to another \(\text{Ad} = \text{N} = \text{N} = \text{Ad}\) band that is predicted to be the strongest band in adamantanone azine’s infrared spectrum. As may be seen from Figure 3.11, the 1645 cm\(^{-1}\) band is clearly the most intense band in the infrared spectrum of the photoproduct(s).

The mirror image relationship between the absorption and emission spectra (Figure 3.3) of adamantyldiazirine proves that the fluorescent species is adamantyldiazirine rather than an impurity, and its structure is consistent with the molecule’s rigid geometry. It confirms a nonzero oscillator strength for the \(S_1^* - S_0\) absorption, and indicates that the vibrational spacings in \(S_1^*\) are similar to those of \(S_0\). In \(n\)-pentane, where the vibrational structure is well-resolved, our data seem to justify a preliminary assignment of several bands (Table 3.9). The most prominent fluorescence bands appear at 374 nm and 395 nm, respectively. There is another sharp band between them at 389 nm, although it is less intense than the band at 395 nm. A broader band at 378 nm appears as a shoulder to the 374 nm band. The mirror image bands in the absorption spectrum occur at 373 nm (most intense), 369 nm (shoulder), 359 nm (sharp), and 354 nm (sharp, strong). A satisfying interpretation may be
obtained by taking the most intense band in each spectrum as 0–0 and using the spacings to
the next three bands as vibronic origins. We have labeled these vibronic origin bands as \( \alpha_i \),
\( \beta_i \), and \( \gamma_i \) in the absorption spectrum and as \( \alpha_i' \), \( \beta_i' \), and \( \gamma_i' \) in the fluorescence emission
spectrum. In the fluorescence spectrum these spacings correspond to 1460 cm\(^{-1}\) (\( \alpha_1 \)), 1033
cm\(^{-1}\) (\( \beta_1 \)), and 334 cm\(^{-1}\) (\( \gamma_1 \)). There are several ground-state normal modes of
adamantydiazirine with calculated frequencies very close to these values. In fact, only a few
of these modes need to be considered, as the vibrational structure of the absorption band must
involve totally symmetric (\( a_i \)) vibrations.\(^{34} \) Therefore, using the CIS(fc)/6-31G* scaled
frequencies, the most likely possibilities are \( v_1 \) (319 cm\(^{-1}\)), \( v_6 \) (1002 cm\(^{-1}\)), \( v_{14} \) (1384 cm\(^{-1}\)),
and \( v_{15} \) (1458 cm\(^{-1}\)). From the vibrational spectra, we know that adamantydiazirine has bands
at 335 cm\(^{-1}\), 1030/1032 cm\(^{-1}\), 1441/1442 cm\(^{-1}\), 1450 cm\(^{-1}\), and 1467/1469 cm\(^{-1}\). The vibronic
spacings in the absorption spectrum are quite similar to their counterparts in the emission
spectrum: 1429 cm\(^{-1}\) (\( \alpha_1 \)), 1008 cm\(^{-1}\) (\( \beta_1 \)), and 289 cm\(^{-1}\) (\( \gamma_1 \)). This mirror image relationship
is consistent with the results of our CIS(fc)/6-31G* calculations (Table 3.5) that predict very
slight changes in the molecular geometry upon excitation to \( S_1 \). The lack of a Stokes shift
and the mirror image between the spectra also supports the conclusion that \( S_1 \) is 1B, rather
than 1A, states should have zero oscillator strength under \( C_{2v} \). The scaled CIS(fc)/
6-31G* frequencies for the 1B, state are \( v_1 \) (318 cm\(^{-1}\)), \( v_6 \) (1001 cm\(^{-1}\)), \( v_{14} \) (1388 cm\(^{-1}\)),
and \( v_{15} \) (1458 cm\(^{-1}\)). These values are essentially unchanged relative to their ground-state
counterparts.

We note that there is some uncertainty in these assignments, arising from the width
of the vibronic bands in the solution-phase absorption and emission spectra at room
temperature and the relatively low resolution of these spectra. (Data points in the absorption
spectra were spaced by 24 – 40 cm\(^{-1}\) and points in the emission spectra were spaced by 40 –
80 cm\(^{-1}\)). Nevertheless, our assignments are supported by the remaining peaks in the
absorption and fluorescence spectra. For example, the absorbance band at 354 nm (\( \alpha_1, 15_1 \))

initiates a series of harmonics, spaced by ~ 1429 cm⁻¹ that appear at 337 nm (α₂, 15₀°) and 321 nm (α₃, 15₀°). The mirror image bands in the fluorescence spectrum, spaced by ~ 1460 cm⁻¹, appear at 395 nm (α₁', 15₀°) and 421 nm (α₂', 15₀°). This last band is rather weak and is largely submerged beneath the fluorescence envelope, so it is not surprising that its frequency is a somewhat poor match. The vibronic origin at 359 nm (β₁', 8₈₀°) initiates a progression of bands in ν₁₂ that appear at 342 nm (β₂', 8₀₁₅₀°) and 326 nm (β₃', 8₀₁₅₀°). The corresponding bands in the fluorescence spectrum appear at 389 nm (β₁', 8₀₈₀°) and 414 nm (β₂', 8₀₁₅₀°). Again, the uncertainty in the position of this last band is considerable. Finally, the vibronic origin at 369 nm (γ₁', 1₄₀°) also initiates a progression in ν₁₂ to 348 nm (γ₂', 1₀₁₅₀°) and 331 nm (γ₃', 1₀₁₅₀°). In this case, there is some uncertainty in the position of all three bands. The mirror image bands appear at 378 nm (γ₁', 1₄₀°) and 400 nm (γ₂', 1₀₁₅₀°) in the fluorescence spectrum.

Vibrational frequencies tend to decrease slightly in excited states relative to their corresponding ground state frequencies. Our assignments and CIS(fc)/6-31G* calculations reflect this. As mentioned previously, only the N=N stretching mode is predicted to experience a significant shift in frequency (Δν = -260 cm⁻¹) upon excitation to S₁*. We note that if the N=N stretch (~ 1570 cm⁻¹ in the ground state infrared spectrum) contributes to the vibronic structure in the absorption and emission spectra, it does so only weakly, as none of the stronger bands in the absorption or emission spectra exhibit spacings of this magnitude. This was also observed for diazirine (1, H₂CN₂). On the other hand, when Lombardi et al. investigated 3,3-difluorodiazirine (F₂CN₂), they observed a long progression in the N=N stretch. The difference in behavior between diazirine and 3,3-difluorodiazirine was explained by appealing to a Mulliken population analysis that indicated that the n orbital in ground state diazirine contains 50% more density on the carbon atom than the same orbital in 3,3-difluorodiazirine. This is a consequence of the electron-withdrawing nature of fluorine relative to hydrogen. Carbon–nitrogen bonding is
therefore more important in the n orbital of diazirine than in the corresponding orbital in 3,3-difluorodiazirine. This causes the subsequent $^1(n-\pi^*)$ excitation in diazirine to be weighted more toward a lengthening of a C–N bond rather than a lengthening of the N=N bond. As alkyl groups tend to be electron-donating, our observation of a progression in a C–N mode ($v_{15}$) rather than the N=N mode ($v_{17}$) in adamantyldiazirine is consistent with their interpretation. Our *ab initio* calculations predict a significant deformation of the diazirine ring in $S_1^*$ as the molecule moves from $C_{2v}$ to $C_s$ symmetry (Figure 3.29). This deformation is accompanied by a large reduction in the frequency of the N=N stretch. The reduced symmetry allows the N=N stretch to mix with several of the CH$_2$ scissoring modes of the adamantyl cage. This also helps to explain the absence of the N=N stretch from the vibronic structure. Under these conditions it is reasonable to expect a relatively small Frank-Condon overlap for this mode, giving rise to weak vibronic coupling. All of these results are consistent with our CIS(fc)/6-31G* calculation of the vertical transition to $S_1^*$ (1B$_2$). This excitation involves promotion of an electron primarily from an n orbital ($\phi_{20}$) into an antibonding $\pi^*$ orbital ($\phi_{17}$). This weakens the bond between the nitrogens, reducing the force constant of the vibration. The frequency reduction that results from the weaker force constant makes this mode roughly isoenergetic with CH$_2$ scissoring vibrations in the adamantyl cage, and these modes mix under $C_s$ symmetry.

We note that these results are consistent with those found for F$_2$CN$_2$ by Lombardi *et al.*, Simmons *et al.*, and Sieber *et al.*

**Time-Resolved Infrared Spectroscopy**

The transient infrared data provided insight into the photochemical fate of adamantyldiazirine in the microsecond regime. As mentioned previously, we observed a biphasic decay in the infrared absorption of adamantyldiazirine in n-pentane between 1560 – 1596 cm$^{-1}$. An instantaneous decay (given the time resolution of the experiment) was
followed by a much slower decay with a lifetime of \( \sim 1.75 \) µsec. Coincident with this bleaching, a new absorption feature grew in at 1664 cm\(^{-1}\) with a rise time of 1.3 – 1.8 µsec. A biphasic growth was also observed at 2042 cm\(^{-1}\). An instantaneous rise was followed by a slower growth with a 2.6 µsec rise time.

The band centered at 1576 cm\(^{-1}\) obviously corresponds to the N=N stretch (\( \nu_{\text{N=N}} \)) of adamantyldiazirine (6). The band at 2042 cm\(^{-1}\) is similarly straightforward to assign as the diazo band of 2-diazaadamantane (7). The logical assignment for the band at 1664 cm\(^{-1}\) would be the known photoproduct, adamantanone azine (13). This last conclusion is jeopardized, however, by the observation of the azine band at 1650 cm\(^{-1}\) in the post-photolysis steady-state spectrum measured on the same instrument.

One possible interpretation that is consistent with the TRIR data involves the formation of an intermediate, perhaps an excited state complex (exciplex), within 1.3 – 1.8 µsec that possesses an absorption feature at 1664 cm\(^{-1}\). This complex then either proceeds to the ultimate azine product (13), or falls apart to reform the diazo compound (corresponding to the 2.6 µsec growth at 2042 cm\(^{-1}\)). Just such an intermediate has recently been proposed.\(^{36}\)

As will be discussed below, 2-diazaadamantane (7) and adamantylidene (8) are formed in a 50:50 ratio on a timescale that is instantaneous relative to the that of the TRIR experiment. Consequently, a complex of either of these species with a ground-state molecule of adamantyldiazirine (6) would appear to be the most likely candidate(s) for the exciplex that is formed. In an effort to consider as many options as possible, we performed PM3 vibrational calculations on adamantyldiazirine (6), 2-diazaadamantane (7), adamantylidene (8), adamantene (12), adamantanone azine (13), and two possible exciplex structures (14 and 15). As mentioned previously, the PM3 method is very successful at predicting \textit{differences} in vibrational frequencies, even when its ability to predict the
absolute frequencies is poor or unknown. Consequently, we sought to identify the species that would exhibit a vibration with a frequency approximately 14 cm\(^{-1}\) higher in frequency than that of adamantanone azine, the major ultimate photoproduct. A scaled PM3 frequency of 1686 cm\(^{-1}\) was obtained for the infrared-allowed Ad=N=N=Ad stretch of adamantanone azine. Of the other molecules and species considered, only complex 15 possessed a PM3 vibrational frequency within 100 cm\(^{-1}\) of the azine band. The calculated (PM3) frequency for the appropriate band of 15 is 1737 cm\(^{-1}\) (scaled), corresponding to a blue shift of 51 cm\(^{-1}\) relative to adamantanone azine.

We therefore suggest that a species with structure 15 (or a similar structure) is formed in 1.3 – 1.8 \(\mu\)sec, probably from adamantylidene (8) and a molecule of adamantylidiazirine (6) in its ground state. This exciplex then either rearranges to form the adamantanone azine (13) or falls apart to regenerate the starting materials. It is also possible that some of the complex rapidly decomposes to form adamantylidene (8) + 2-diazoadamantane (7). This would explain the 2.6 \(\mu\)sec growth of the 2-diazoadamantane absorption feature at 2042 cm\(^{-1}\).

Excitations, Excited States, and Immediate Photochemistry

The observed red shifts in the steady-state absorption and fluorescence spectra with increasing solvent polarity indicate that the excited state of the diazirine is more polar than the ground state and that the excited state \((S_1^*)\) interacts more strongly than the ground state \((S_0)\) with more polar solvents. This is consistent with the results of our \textit{ab initio} and semiempirical calculations that indicate an increase in dipole moment upon excitation that can be attributed to a localization of electron density on the diazirine nitrogens in \(S_1^*\). This interpretation also explains the red-shift of the spectra in CH\(_3\)CN relative to solvents of similar polarity that are capable of forming hydrogen bonds with the nitrogen lone pairs in \(S_0\). Hydrogen bonding solvents would be expected to stabilize the ground state more than the \(^1(n-\pi^*)\) excited state,
so the energy gap between the states is expected to be larger under these circumstances. It is not surprising that this is a weak effect, however, as Haselbach et al. found that the two electrons in the \( n \) antisymmetric "lone pair" molecular orbital of 3,3-dimethyldiazirine are only 56% localized on the two nitrogen atoms.\(^{37}\)

We believe that the fluorescence of dialkyl diazirines originates from their lowest excited singlet states, \( S_1^* \), since neither the structure of adamantyl diazirine's fluorescence emission spectrum nor its fluorescence lifetime displayed a dependence upon excitation wavelength. The observed mirror image relationship between the absorption and fluorescence spectrum lends further support to this conclusion.

Our data appear to be largely consistent with the current theoretical understanding of the reactivity of diazirines and alkyl-substituted diazirines. The general scheme, initially developed at the semiempirical level by Müller-Remmers and Jug\(^{29}\) has recently been mapped out in more detail and extended with \textit{ab initio} MC-SCF methods by Yamamoto et al.\(^4\) Specifically, excitation at the red edge of the absorption spectrum creates an \(^1(n - \pi^*)\) state that can, in principle, decay by a variety of pathways. For diazirine (1, \( H_2CN_3 \)) three nonradiative decay processes have been identified.\(^4\) The most straightforward of these involves isomerization to diazomethane \textit{via} a conical intersection at a bent-in-plane (bip), diazomethane-like diradicaloid structure. A very slight (0.55 kcal/mol) barrier must be surpassed to initiate this reaction from the \( S_1^* \) minimum. A second pathway arises from vibrational leakage from the first pathway, across a bifurcation point on the \( S_1^* \) surface, along a bent-out-of-plane (bop) pathway to a second conical intersection at a ring-opened diazirine diradicaloid structure. Movement through this funnel leads to the formation of a carbene and molecular nitrogen. A third possibility involves thermal population of \( S_2^* \) from the \( S_1^* \) surface. The \( S_0 \) product surface is then accessed \textit{via} the same conical intersection employed by the second pathway, to yield nitrogen and singlet carbene. Intersystem crossing to a triplet
state is not expected to contribute significantly to the depopulation of $S_1$, and this has been confirmed experimentally by Modarelli et al. for 3-methyldiazirine and 3,3-dimethyldiazirine (2). Seburg and McMahon reached the same conclusion for 3-methyldiazirine.

We note that diazirine's $S_2$ state has been assigned as $(n-\sigma^*)$ by Yamamoto et al., but our CIS(fc)/6-31G* calculations on adamantyl diazirine (6) indicate that transitions to each of its first four excited singlet states terminate primarily in the LUMO, which has $\pi^*$ character. This was noted previously in our discussion of the charge density difference plots in Figure 3.28. We believe that the first few excited states of adamantyl diazirine should be adequately defined at this level of theory. We have calculated the first six CIS vertical excited states at the MP2(fc)/6-31G* geometry for diazirine (1) using several basis sets. The energies of these states are tabulated in Appendix D. We found that the excited state ordering is identical for the first three excited states for all four basis sets employed (6-31G*, 6-31+G*, 6-311++G**, and 6-31+G*R) and the energies of these states change very little as the basis set is improved (Table 3.10). These results suggest that the spatial distribution of the first three excited states should be reasonably well-described at the 6-31G* level. The CIS(fc)/6-31G* state orderings for the first four excited states of adamantyl diazirine (6) are identical to those calculated at the CIS(fc)/6-31G* level for diazirine (1), and the energies of these states are also quite similar. Therefore, we would not expect our results for adamantyl diazirine to be significantly improved with a larger basis set.

The extent to which adamantyl diazirine's photochemistry resembles that of diazirine is unclear. Most alkyl- and dialkyl- diazirines possess $\alpha$-hydrogens that have been postulated to migrate either concurrent with or subsequent to nitrogen extrusion to generate alkenes. The $\alpha$-hydrogens of adamantyl diazirine are not expected to undergo these rearrangements, as the rigid adamantyl cage strongly inhibits the formation of internal double bonds. With these rearrangement pathways blocked, the options available to a photoexcited molecule of adamantyl diazirine are nearly identical to those open to diazirine itself: internal
conversion, fluorescence, intersystem crossing, carbene formation, and rearrangement to form a diazo compound. Adamantyldiazirine has also been shown to undergo an \textit{intramolecular} insertion reaction under certain conditions (usually at temperatures > 200 °C) to form the highly strained 2,4-dehydroadamantane (10),\textsuperscript{13,17-19} but to our knowledge this product has never been observed to account for more than ~ 7% of the product distribution in photochemical experiments conducted at ambient temperature in any of the solvents employed in our investigations.\textsuperscript{13,18}

Since our experiments were carried out in solution, \textit{intermolecular} chemistry was also a possibility. While the possible decay pathways are therefore quite similar to those in diazirine, there are enough differences between the two species to anticipate that the topologies of the reaction surfaces in the excited state might be somewhat different, leading to dissimilar partitioning among the possible decay pathways. For example, the electron-donating nature of the adamantyl cage could stabilize pathways such as carbene formation. On the other hand, reaction pathways that involve dramatic changes in the molecular geometry might be more hindered in adamantyldiazirine than in the more flexible diazirine. Consequently, it would be unwise to expect the reaction surface mapped out by Yamamoto \textit{et al.}\textsuperscript{4} to apply equally well to adamantyldiazirine. Recent experiments conducted by Bonneau and Liu\textsuperscript{16} in isooctane solutions of adamantyldiazirine suggest that the photochemistry partitions in approximately equal ratios to form 2-diazoadamantane and adamantylidene.

The increases in fluorescence intensity and fluorescence decay time observed at low temperatures for each of the diazirines studied confirms that fluorescence competes with at least one activated chemical process in the diazirines' $S_1^*$ states. Arrhenius treatments of these data yielded activation energies of 2.8 – 3.7 kcal/mol, depending on the diazirine (Table 3.3). This energy corresponds to the lowest (\textit{i.e.}, rate-limiting) barrier on the excited state ($S_1^*$) surface. There was good agreement between activation parameters obtained from the
steady-state fluorescence intensities and those derived from the fluorescence lifetimes. The calculations of Yamamoto et al. found a rate-limiting barrier of 0.55 kcal/mol for the isomerization of diazirine to diazomethane. While they were unable to locate a transition state along a pathway leading to carbene, they were able to establish a maximum barrier for this process of 4 kcal/mol. Müller-Remmers and Jug also predicted a barrier of a few kcal/mol for the analogous process in dimethyldiazirine, so our range of 3.4 – 3.7 kcal/mol for adamantyldiazirine seems quite reasonable. It confirms our intuitive sense that the photochemistry ought to be somewhat more hindered in adamantyldiazirine relative to diazirine due to the rigid molecular architecture, but not prohibitively so, as the diazirine moiety is identical in both systems.

Our CIS(fc)/6-31G* explorations of the excited state surfaces of adamantyldiazirine lend further support to these conclusions. Neither $S_1^*$ nor $S_2^*$ exhibited minima under $C_{2v}$ symmetry. Both of the states become A" symmetric under $C_\pi$ when the mirror plane containing the diazirine ring is retained. Consequently, mixing of the states (which was symmetry-forbidden in $C_{2v}$) is possible under $C_\pi$. Both states converge to the same optimum geometry under these circumstances. We obtained identical results for diazirine (1, H$_2$CN$_2$) at the CIS(fc)/6-31G*, CIS(fc)/6-31+G*, and the CIS(fc)/6-311++G** levels of theory. In every case (for diazirine and adamantyldiazirine) the optimized $C_\pi$ structure possesses a lengthened (1.65 Å – 1.66 Å) and a shortened (1.37 Å – 1.39 Å) carbon–nitrogen bond. Our data do not map out a complete photochemical reaction surface for adamantyldiazirine, but the optimized $C_\pi$ structure is consistent with the formation of 2-diazoadamantane (7) via a bent-out-of-plane mechanism. This seems to support the conclusions drawn from the experimental work of Bonneau and Liu. We note, however, that our results are at variance with the MC-SCF (6-31G*) calculations of Yamamoto et al. in which the $S_1^*$ and $S_2^*$ states
of diazirine (1) possessed minima under C₂ᵥ symmetry and the photochemical conversion to
diazomethane was described as a surface-crossing mechanism via a conical intersection along
an asymmetric (C₁) bent-out-of-plane pathway.

Within the precision of our fluorescence lifetime measurements, we did not observe
an isotope effect for hydrogens at positions alpha (α) to the diazirine carbon in 3,3-
dimethyl Diazirine (2), 3,3-dimethyl Diazirine-d₄ (3), cyclohexyl Diazirine (4), or
cyclohexyl Diazirine-d₄ (5). If hydrogen migrations from α-positions proceeded at rates that
were competitive with that of fluorescence or the rate-limiting excited-state processes, we
would have expected to observe fluorescence lifetimes for the deuterated species that were
consistently longer than their counterparts under identical experimental conditions.
Therefore, our data do not support the theory that hydrogen migrations occur in concert with
carbene formation in the excited states of dialkyl Diazirines. It should be noted, however, that
the measured fluorescence lifetimes for the deuterated species were generally somewhat
longer than their counterparts, but this trend was not observed with sufficient strength or
consistency to suggest this hydrogen-migration pathway as an alternative to diazo- or
carbene-formation from the diazirine excited state.

Since neither the fluorescence lifetime nor the steady-state fluorescence spectrum of
adamantyl Diazirine exhibited a solvent isotope effect, the abstraction of hydrogen atoms from
the solvent by the diazirine excited state also does not appear to be a process that competes
with fluorescence to any significant extent. In fact, the lack of any solvent-dependence on the
room temperature fluorescence lifetime of adamantyl Diazirine suggests that the solvent does
not play a crucial role in determining the rates of any activated processes that compete with
fluorescence to depopulate the S₁* state. We conclude that any solvent-insertion processes
that do occur¹³ are attributable to chemistry of subsequent intermediates (e.g.,
adamantylidene (8) or 2-diazoadamantane (7)), and not that of the diazirine excited state. The
lack of a solvent-dependence or noticeable oxygen-dependence for the fluorescence lifetime

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also seems to be inconsistent with the hypothesis that exciplexes of certain solvents or triplet oxygen ($^3\text{O}_2$) with the excited state of adamantylidiazirine might be responsible for some of the observed photochemistry.\textsuperscript{13}

### Photoproducts

The ultimate products of adamantylidiazirine's photolysis in several solvents have been analyzed by many groups.\textsuperscript{14} Depending on the experimental conditions, adamantanone azine (13), adamantanone (9), 2,4-dehydroadamantane (10), and assorted solvent-insertion products (11) are formed in various product ratios. Bonneau and Liu observed the characteristic infrared absorption of adamantanone azine (13) at 1646.2 cm$^{-1}$ in photolyzed isooctane solutions of adamantylidiazirine.\textsuperscript{16} We also observed a strong band at 1645 cm$^{-1}$ in the FT-IR spectrum of photolyzed solutions of adamantylidiazirine prepared in $n$-pentane.

The UV/VIS absorption spectrum of cyclohexylidiazirine in $n$-pentane (Figure 3.9) exhibits several new bands < 270 nm after photolysis at 355 nm. The spectrum at longer wavelengths is unchanged, other than the expected reduction in absolute intensity due to the photolysis of the diazirine. The peak maxima are tabulated in Table 3.2. The strongest of these new absorption features is at 219 nm. It is not clear whether these bands arise from the diazo form of cyclohexylidiazirine, the azine photoproduct, or another photogenerated species.

In the Results section of this chapter we mentioned that a second decay component was often observed in the fluorescence of adamantylidiazirine. We attribute the minor component of the fluorescence decays to luminescent photoproducts. None of the solvents exhibited significant fluorescence, and the relative contribution of the long-lived decay component seemed to depend only upon the amount of time a given sample had spent in the laser beam. While we are unable to discount completely the possibility that this minor component had its origin in relevant photophysical processes of adamantylidiazirine, it would
be difficult to explain the observed reproducibility of the major (0.24 ns) component and the variability of the minor component simultaneously. For example, the minor component had a lifetime of 0.57 ns in n-pentane, but in n-hexane the lifetime was 1.0 ns. Similarly, the minor component was responsible for 31%, 9%, and 18% of the decay in methanol, n-propanol, and n-butanol, respectively. The collection times for these samples (a very rough measure of the extent of photolysis) were 4761, 1800, and 3458 seconds. Photoproduct fluorescence was also indicated in steady-state emission spectra measured in Freon-113. (Figure 3.35) A broad, featureless fluorescence with a maximum at 416 nm replaced the more structured spectrum due to adamantylidiazirine (emission maximum at 398 nm) as the sample was subjected to 600 intense pulses of 351 nm radiation from an XeF excimer laser.

Additional support for attributing the long-lived component to photoproduct fluorescence comes from the emission wavelength dependence of the fluorescence lifetimes (Table 3.11). In Freon-113, there were negligible changes in the relative contributions of the two decay components as the emission wavelength was varied near the maximum for adamantylidiazirine (390 nm – 400 nm) but when the decay was monitored at 420 nm (near the emission maximum of the photoproduct(s)) the relative contribution of the long-lived species increased from 6% to 18%. When the sample was subjected to extensive 350 nm photolysis, the contribution of the long-lived component reached 69%, even when monitored at 390 nm. Among the photoproducts that have been observed previously, adamantanone (9) and adamantanone azine (13) possess chromophores that would make them possible sources of the long-lived fluorescence. It is possible, however, that the species responsible for the fluorescence is formed in microscopic quantities and has gone undetected in previous analyses, owing to the extreme sensitivity of fluorescence techniques relative to other methods such as gas chromatography.
Conclusions and Future Directions

The fluorescence lifetimes of 3,3-dimethyldiazirine (2), 3,3-dimethyldiazirine-d₆ (3), cyclohexyldiazirine (4), cyclohexyldiazirine-d₄ (5), and adamantyldiazirine (6) were measured using the time-correlated single-photon counting technique and found to be on the order of 40 ps for 2 and 3, 84 ps for 4 and 5, and 240 ps for 6 at ambient temperature. To our knowledge, these are the first such measurements to be reported in the literature for diazirines. Our Arrhenius treatment of the temperature dependence of the fluorescence provides experimental support for the calculations of previous groups that predicted a barrier to carbene and/or diazo formation on the S₁* surface of diazirines.¹²⁻³⁻⁵⁻⁷⁻¹¹²⁻¹³⁻¹⁷⁻²⁶ For adamantyldiazirine, this value was determined to be between 3.4 - 3.7 kcal/mol. For cyclohexyldiazirine the activation energy was 2.81 - 2.86 kcal/mol, and for 3,3-dimethyldiazirine it was 3.19 - 3.26 kcal/mol. The high activation energy for adamantyldiazirine is reasonable, given the constraints imposed on rearrangement by the adamantyl cage. It is less obvious why the activation energy for cyclohexyldiazirine would be smaller than that for 3,3-dimethyldiazirine. One possible explanation would be that the cyclohexane ring preferentially aligns the active orbitals in a favorable position for the rearrangement reaction(s).

Our CIS calculations did not find energy minima for the S₁* and S₂* states under Cᵥ symmetry for diazirine or adamantyldiazirine. Previous calculations at the MC-SCF/6-31G* level found a shallow minimum for the S₁* state under Cᵥ.⁴ Furthermore, while our calculations are consistent with photoisomerization of diazirines to diazo compounds, we have found a potential intermediate at a Cᵥ geometry (retaining the mirror plane containing the diazirine ring) that corresponds to an energy minimum on both the S₁* (1Bᵥ) and S₂* (1Aᵥ) surfaces. Our data therefore allow for a simple mixing between these states, without appealing to the S₁*/S₂* conical intersection indicated in the MC-SCF calculations.⁴
There are at least two possible reasons for the different structures and mechanism found by the two computational methods. The CIS method ignores excitations above singles, so it might not treat higher excited states (e.g., $S_2^*$) as adequately as does the MC-SCF approach. On the other hand, the success of the MC-SCF method depends on the choice of the "active space" under consideration. For example, the calculations of Yamamoto et al. employed an active space of six electrons in six active orbitals. Our CIS calculations reveal that a significant contribution to the $S_1^*$ state involves an excitation from $\phi_{4s}$ (HOMO – 3), as well as non-negligible contributions from molecular orbitals of even lower energy (e.g., $\phi_{3s}$). These orbitals were not included in the active space of the MC-SCF approach. The success of the CIS approach in the calculation of $^1(n-\pi^*)$ excitations in formaldehyde, acetaldehyde, acetone, formamide, and benzaldehyde is well-documented. We are therefore inclined to trust the CIS treatment of the $S_1^*$ state of diazirine and adamantylidiazirine. Nevertheless, we concede that the MC-SCF approach possesses certain advantages in the calculation of higher excited states. We also note that our calculations involved discrete geometry optimizations, while Yamamoto et al. were able to explore significant regions of the relevant potential energy surfaces. More calculations will probably be required to resolve the apparent discrepancies.

The fact that we did not observe a solvent-dependence on the fluorescence lifetime of adamantylidiazirine lends further support to the hypothesis that the primary nonradiative decay pathways in the excited state are intramolecular processes such as carbene and/or diazo formation, rather than intermolecular processes such as solvent insertion. Such intermolecular processes would be expected to exhibit some solvent dependence. While some of the ultimate products of the photolysis of adamantylidiazirine arise from intermolecular chemistry, our data suggest that these are generated from one or more
intermediates generated subsequent to depopulation of the diazirine excited state, such as adamantylidene or 2-diazoadamantan. In this we agree with the conclusions of Bonneau and Liu.16

We observed a biphasic depletion of the infrared absorption corresponding to the N=N stretch of adamantylidiazirine, as well as the growth of two new infrared absorption features that were assigned to 2-diazoadamantan (7) and an intermediate/exciplex that probably arises from the interaction of photogenerated adamantylidene (8) with ground-state adamantylidiazirine. The structure of this transient species was suggested to resemble 15. The growth of the band attributed to the complex was instantaneous on the time scale of the TRIR experiment, but the growth of the 2-diazoadamantan band was biphasic. We suggested that the slow growth superimposed on the instantaneous rise might be due to the dissociation of the previously-mentioned excited state complex (15) into 2-diazoadamantan and adamantylidene.

Our analysis of the absorption and fluorescence emission spectra in a variety of solvents has demonstrated that adamantylidiazirine possesses a larger dipole moment in S₁* than in S₀, a conclusion that is confirmed by our semiempirical and ab initio calculations. We were also able to assign most of the vibrations of adamantylidiazirine < 1700 cm⁻¹ using the infrared absorption and Raman spectra of adamantylidiazirine in conjunction with semiempirical, density functional, and ab initio calculations.

While semiempirical methods and RHF/6-31G* calculations appear to be adequate for the determination of diazirine geometries, we found that vibrational analyses at the MP2(fc)/6-31G* level or higher were required to predict accurately the absolute frequencies of vibrational modes involving the diazirine ring moiety. While electron correlation was required to obtain the absolute frequencies of these modes, the semiempirical, density functional, and Hartree-Fock methods appeared to be adequate to assign the normal modes
and evaluate the general nature of the molecular vibrations. The less rigorous calculations also seemed to be capable of calculating the absolute frequencies of modes that did not significantly involve the diazirine ring.

Our data suggest that the N=N stretch experiences a significant reduction in force constant and frequency of vibration upon photoexcitation. In this we concur with earlier investigations of difluorodiazirine by Lombardi et al.24 On the other hand, the vibrational modes localized in the adamantyl cage are relatively unaffected by the excitation.
References


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<th>solvent</th>
<th>absorption bands (nm)*</th>
<th>fluorescence bands (nm)*</th>
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<th>$\phi_f$</th>
<th>$\tau_0$ (ns)</th>
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<td>more structured spectra than methanol</td>
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<td>very broad, featureless spectra</td>
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<td>0.0009</td>
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*Peaks were assigned based on a fit to a minimum number of Gaussian bands required to yield nonsystematic residuals with the PeakFit software package. The peak responsible for the global minimum in each spectrum is tabulated in italics. Only peaks deemed to be unambiguous contributors to the observed spectrum have been reported. *Excitation was at 335 nm. Solvents with C-H bonds exhibited a strong Raman band near 371 nm. To account for this, a spectrum of the pure solvent was collected and the Raman peaks were assigned with PeakFit. The position and width parameter of these peaks were then held constant in the subsequent fits for the diazirine solutions, although the amplitudes were allowed to vary. The Raman bands are not tabulated. *Only the lifetime of the major (>80%) component has been tabulated. See text for explanation.

**Table 3.1:** Solvent dependence of absorption spectrum, fluorescence emission spectrum, fluorescence lifetime, fluorescence quantum yield, and natural lifetime for adamantyl diazirine at ambient temperature
Peaks were assigned based on a fit to a minimum number of Gaussian bands required to yield nonsystematic residuals with the PeakFit software package. The peak responsible for the global maximum of each spectrum is tabulated in italics.

**Table 3.2:** Absorption bands (Gaussian bandshapes) for n-pentane solutions of cyclohexyldiazirine at ambient temperature before and after photolysis
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<thead>
<tr>
<th>Temperature (K)</th>
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Table 3.3: Temperature dependence and Arrhenius parameters for dialkyldiazirine fluorescence lifetimes
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Table 3.3 (continued): Temperature dependence and Arrhenius parameters for dialkyldiazirine fluorescence lifetimes
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Table 3.4: Calculated and experimental ground state geometries for diazirines
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<th>orbitals (coefficients)</th>
<th>μ of final state&lt;sup&gt;c&lt;/sup&gt;</th>
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<sup>a</sup>Transition energies are in eV.  <sup>b</sup>Transition wavelengths are in nanometers.  <sup>c</sup>Dipole moments are in debye.

Table 3.5: CIS (fc) / 6-31G* vertical transitions for adamantylidiazirine at the MP2 / 6-31G* ground-state optimized geometry
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*aDistances are in angstroms. *Angles are in degrees. *Number of imaginary vibrational frequencies. *$\Delta E$ is the difference in energy (eV) between the ground and excited states, without ZPE correction.

Table 3.6: CIS (fc) / 6-31G* optimized structures and energies for ground and excited singlet states of adamantyldiazirine
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Table 3.7: Experimental and calculated vibrational frequencies for the ground electronic state of adamantyldiazine. RHF / 6-31G* scaling factor = 0.89. Observed infrared and Raman bands are tabulated in accordance with normal mode assignments discussed in the text. Modes sorted by symmetry.
| C<sub>n</sub> | RHF/6-31G* PM3 B3LYP RHF/6-31G* Observed RHF/6-31G* Observed |
|-----------|-------------|-------------|-------------|-------------|-------------|
|           | Mode        | Scaled v (cm<sup>-1</sup>) v (cm<sup>-1</sup>) v (cm<sup>-1</sup>) IR Intensity | IR v (cm<sup>-1</sup>) | Raman Intensity | Raman v (cm<sup>-1</sup>) |
| 37        | 249         | 265         | 258         | 0.01203     | 0.00128     | 260          |
| 38        | 385         | 412         | 399         | 0.03183     | 0.00346     | 396          |
| 39        | 425         | 456         | 446         | 0.01036     | 0.00001     |              |
| 40        | 619         | 679         | 651         | 0.000026    | 636         | 0.00306      | 634          |
| 41        | 802         | 891         | 842         | 0.01416     | 835         | 0.00760      | 835          |
| 42        | 872         | 928         | 908         | 0.00247     | 883         | 0.00207      |              |
| 43        | 968         | 1002        | 1011        | 0.02030     | 996         | 0.01825      | 996          |
| 44        | 1063        | 1054        | 1101        | 0.07127     | 1080        | 0.00068      | 1080         |
| 45        | 1090        | 1122        | 1133        | 0.00112     | 1109        | 0.00540      | 1107         |
| 46        | 1218        | 1216        | 1264        | 0.01105     | 1239        | 0.00092      | 1239         |
| 47        | 1298        | 1296        | 1332        | 0.00880     |              | 0.00444      |              |
| 48        | 1330        | 1325        | 1364        | 0.00420     | 1325        | 0.00095      |              |
| 49        | 1344        | 1352        | 1390        | 0.00570     | 1337        | 0.00010      |              |
| 50        | 1465        | 1409        | 1520        | 0.06048     | 1467        | 0.00023      | 1469         |
| 51        | 2847        | 2906        | 3035        | 0.35564     |              | 0.11723      |              |
| 52        | 2869        | 2968        | 3062        | 0.09311     |              | 0.07203      |              |
| 53        | 2877        | 2970        | 3064        | 0.61506     |              | 0.11808      |              |
| 54        | 2882        | 3043        | 3075        | 0.65949     |              | 0.35019      |              |

Table 3.7 (continued): Experimental and calculated vibrational frequencies for the ground electronic state of adamantylazide. RHF / 6-31G* scaling factor = 0.89. Observed infrared and Raman bands are tabulated in accordance with normal mode assignments discussed in the text. Modes sorted by symmetry.
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Table 3.8: Experimental and calculated vibrational frequencies for the ground electronic state of adamantyl diazin. RHF/6-31G* scaling factor = 0.89. Observed infrared and Raman bands are tabulated in accordance with normal mode assignments discussed in the text. Modes sorted by RHF/6-31G* frequency.
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Table 3.8 (continued): Experimental and calculated vibrational frequencies for the ground electronic state of adamantyldiazirine. RHF / 6-31G* scaling factor = 0.89. Observed infrared and Raman bands are tabulated in accordance with normal mode assignments discussed in the text. Modes sorted by RHF / 6-31G* frequency
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Table 3.9: Transition frequencies and assignments for adamantyldiazirine in n-pentane
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Table 3.10: CIS vertical excited singlet state energies (eV) calculated using various basis sets for diazirine (1) at the MP2 (fc) / 6-31G* optimized geometry
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**Table 3.11**: Emission wavelength dependence of adamantyldiazirine's fluorescence lifetime in Freon-113
Figure 3.1: Some possible decay processes for dialkyldiazirine excited states
Figure 3.2: Some of the diazirines and species derived from diazirines that were investigated in this work.
Figure 3.3: Absorption and fluorescence emission spectra of adamantyl diazirine in (a) n-pentane and (b) methanol.
Figure 3.4: Absorption spectra of adamantyldiazirine in (a) n-hexane, (b) n-butanol, and (c) acetonitrile
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Solvent: n-hexane

$\chi^2_r = 1.2172$

$\tau_1 = 0.245 \text{ ns (93.4\%)}$

$\tau_2 = 1.051 \text{ ns (6.6\%)}$
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Introduction

The time-resolved fluorescence data for dialkyldiazirines may be described by a simple, single-exponential decay law, as discussed in the previous chapter. Specifically, the fluorescence intensity at any time \( t \) is simply:

\[
I(t) = I_0 e^{-t/\tau}
\]

In the previous chapter, it was shown that the fluorescence lifetimes recovered from single-exponential fits to TCSPC data could be directly related to aspects of dialkyldiazirine photophysics and photochemistry such as activation energies. The fluorescence of these samples followed a single-exponential decay law presumably because the dialkyldiazirines were the only fluorophores of consequence present in solution and their fluorescence originated from a homogeneous solvent environment. We noted further that complexity was introduced in the form of a second decay component when the solutions were exposed to the laser beam for extended periods of time. This too could be attributed in a direct way to the appearance of a new fluorophore in a homogeneous environment.

As we discussed in Chapter 1, the fluorescence of complex, heterogeneous systems rarely conforms to such straightforward analysis. In general, heterogeneity on a molecular level will give rise to heterogeneity in the observed fluorescence decay. Multiple
fluorophores, distributed microenvironments, various chemical gradients, constrained media, and conformational heterogeneity are some of the myriad factors that might affect the form of time-resolved fluorescence data. For such systems, more sophisticated analytical methods must be applied to recover useful information. In this chapter we present preliminary results related to the acquisition and analysis of TCSPC data for photosystem II reaction centers (PS II-RCs) isolated from spinach leaves and *Chlamydomonas reinhardtii* algae. These complex systems are a classic example of the impact of chemical heterogeneity. As may be seen clearly in a representative TCSPC decay curve for PS II-RCs, their fluorescence may certainly not be adequately described as a single exponential decay (Figure 4.1). Since the interpretation of these data has long been recognized as essential in comprehending the kinetics and biophysics of these important systems, there is strong motivation to develop appropriate methods of data analysis.

**Photosystem II Reaction Centers**

The absorption of solar energy by plant pigments and the subsequent conversion of this energy into a stable separation of charge is a key process in photosynthesis. The reaction sequence that accomplishes this charge separation has a remarkably high quantum yield, approaching or exceeding 90%. This enables photosynthetic organisms to harvest the sun's energy with high efficiency. Understanding these processes has been and continues to be the goal of extensive research. Not only are the scientific questions intrinsically fascinating, the possibility of some day emulating the process to collect solar energy for human benefit holds significant economic and social appeal.

The fundamental processes in the conversion of solar energy into chemical energy occur in photosynthetic reaction center (RC) complexes. Photosynthetic organisms can be classified as either oxygenic or non-oxygenic. Non-oxygenic organisms such as purple bacteria contain a single type of reaction center, while oxygenic organisms such as
cyanobacteria and photosynthetic eukaryotes possess two distinct types of reaction centers (photosystem II and photosystem I) linked in series. Of these reaction centers, the photosystem II (PS II) reaction complex is unique in possessing a sufficiently large redox potential to drive the oxidation of water. Specifically, the active chlorophyll(s) in PS II are 400 mV more oxidizing than free chlorophyll (Chl'/Chl) in solution. In contrast, the special pair of chlorophylls in the bacterial RC and the active pigments of PS I are more reducing than Chl'/Chl. The chemical origin of this difference in redox potentials is not well understood.

Since 1987 it has been possible to isolate PS II-RC complexes. These isolated RCs retain the ability to separate charge, but quinone reduction, tyrosine oxidation, and subsequent processes are blocked. The structure of the PS II-RC has not yet been determined to the same level of detail as that of the purple bacteria RC. The two structures are believed to possess a significant amount of homology, but there are some differences, many of which are undoubtedly photochemically significant. Structural differences are likely to factor heavily into the variation in redox potentials discussed previously, for example. Another difference that has plagued spectroscopic investigations of PS II-RC is the spectral congestion of its pigments. The pigments of bacterial RC can be assigned to relatively well-resolved spectral positions, but the spectra of PS II-RC pigments exhibit significant overlap. This complicates the interpretation of experimental data. Kinetic processes in PS II-RCs, as measured by various spectroscopic techniques, possess highly nonexponential kinetics and exhibit considerable excitation and emission wavelength dependence. The assignment of rate constants and the chemical origins of measured lifetimes remains a topic of considerable debate, despite several years of investigation.

The PS II-RC consists of two transmembrane proteins of 32 kD and 31 kD that constitute the so-called D1/D2 heterodimer. The complex also contains two smaller proteins that neither bind pigments nor participate in the photochemistry: a cytochrome b559 with a single heme and two protein subunits (9 kD) and a psb I protein (4.8 kD). The pigment
composition of PS II-RC includes 4 – 6 Chl $a$ molecules, two Pheophytins (Mg-less Chlorophylls), and at least one carotenoid. The primary electron donor in PS II-RC is called P680. It is believed to consist of a pair of excitonically coupled Chls, although some evidence still points to a monomer structure. Together with the two Pheophytins (Pheo$_1$ and Pheo$_2$) and two accessory Chls (Chl$_{acc1}$ and Chl$_{acc2}$), P680 forms a reaction center core that resembles the bacterial RC. The accessory Chls are ~ 30 Å away from P680. This PS II-RC core also binds two antenna Chls (Chl$_{ant1}$ and Chl$_{ant2}$) on its periphery. There are no pigments analogous to these antenna Chls in the bacterial RC. Additional Chls may also be bound to a small fraction of PS II-RCs isolated for particular samples. Three spectrally different “pools” of these pigments have been identified, and are believed to account for much of the observed variation in pigment content of PS II-RC preparations: Chl$_{ant1}$, Chl$_{ant2}$, Chl$_{ant3}$. As mentioned in the previous paragraph, this heterogeneity in pigment composition has frustrated attempts to resolve the photophysics of the initial energy transfer processes. One current interpretation, advanced by Holzwarth et al., is that P680 is preferentially excited, followed by ultrafast (< 1 ps) equilibration with adjacent pigments. This equilibrated state then undergoes charge separation with a lifetime of ~ 3 ps. If excitation occurs within antenna Chls, 20 – 60 ps are required to transfer the energy to P680 before the remainder of the sequence can be initiated.

Once energy has been transferred from the various pigments to P680, charge is separated and a radical pair (or sequence of radical pairs) is formed. The kinetics of this formation and any subsequent relaxation processes are also unsolved issues. Different models have been tested, including the presence of a single radical pair (RP), a static distribution of free energies for charge separation ($\Delta G_{cs}$) values for the formation of RPs, and a continuous-relaxation model in which an initially formed RP gradually relaxes via coupling interactions with the protein matrix. These models were recently tested using Förster theory to calculate rate constants for pairwise energy transfer. The approach used to
test these models was based largely on a global target analysis of a large matrix of time-
resolved fluorescence data. This recent example highlights the prominent role given to time-
resolved fluorescence data in studies of PS II-RCs.  

The Role of Site-Directed Mutagenesis

The technique of site-directed mutagenesis has been widely used in the study of bacterial RCs and has recently been applied to studies of PS I. Its potential utility for investigations of PS II-RCs has also been discussed for some time. Site-directed mutagenesis involves selective genetic manipulation of specific amino acid residues in a protein complex of interest. It is generally employed in order to elucidate structure-function relationships by monitoring changes (or lack thereof) in experimental observables upon mutation of residues that have been postulated to play functional roles in a given biophysical process.

Until 1994, the only successful studies of the primary photochemistry of PS II-RCs had been performed on preparations isolated from higher plants such as pea seedlings and spinach leaves. While convenient, these systems are not readily suitable for genetic manipulation. The successful isolation of PS II-RCs from *Chlamydomonas reinhardtii* has recently been achieved. In contrast to higher plants, *Chlamydomonas* is an ideal organism for site-directed mutagenesis. As a single-celled alga, cultures can be grown rapidly. This permits adequate quantities of samples developed with specific mutations to be prepared in a reasonable amount of time. Specific point mutations of the D1 and D2 polypeptides of *Chlamydomonas reinhardtii* PS II-RC is anticipated to be a fruitful approach to obtaining further insight into the initial events in photosynthesis.
Our Objectives

The research presented in this chapter represents preliminary results in a collaborative effort with Dr. Richard T. Sayre, a professor in the Departments of Plant Biology and Biochemistry. The overall objective is to characterize the protein-chromophore and steric interactions that contribute to the remarkably high midpoint potential of P680. Specifically, we hope to assess the extent of homology in chromophore organization between PS II-RC and purple bacteria RC. We are also seeking insight into the regulation of charge transfer processes and redox potentials by the reaction center. By using structural models of the PS II complex, Sayre has predicted some of the amino acid residues that may be responsible for positioning the chlorophylls of the RC complex and he has targeted these sites for specific mutations. For example, strategic mutations could help resolve the question of whether P680 is a monomer or a dimer. Other mutations could locate the binding sites of Chl$_{sec1}$ and Chl$_{sec2}$. Once a stable, reproducible reaction center preparation has been developed these mutants will be studied using various spectroscopic techniques.

While this chapter clearly represents a work in progress, we have succeeded in analyzing *Chlamydomonas* time-resolved fluorescence decays. Specific contributions that will be addressed in this chapter include: (1) a discussion of critical variables in the acquisition and analysis of TCSPC data for these systems, (2) comparison of the time-resolved fluorescence of wild-type (unmutated) *Chlamydomonas* PS II-RCs with data measured on RCs isolated from spinach leaves, and (3) the development of a TCSPC diagnostic to assess the health of a particular reaction center preparation.

Current Approaches to the Analysis of TCSPC Data for PS II Reaction Centers

Trends in the analysis of time-resolved fluorescence data for photosynthetic systems have followed—or perhaps driven—developments in data analysis methods over the years.
Early studies attempted to fit the data to a minimum number of exponential terms. An effort was then made to assign meaning to the parameters thus obtained, with little success. Rarely could correlations be found with experimental parameters, and the acquisition of higher quality data would often result in poorer fits, necessitating the inclusion of additional exponential terms. As the parameters of discrete sum-of-exponential fits are highly correlated with one another, the values of the parameters previously recovered would inevitably change when new parameters were added.

As the theory of TCSPC data analysis developed, and as the ability to perform complex calculations became more commonplace, global analysis techniques were developed and employed to link lifetimes among multiple data sets, collected at various excitation wavelengths, emission wavelengths, and temperatures. These analyses were considerably more successful in identifying reproducible lifetime components and in identifying trends in the data, but it was still often difficult or impossible to correlate the recovered parameters with the underlying chemistry.

Other groups were following a somewhat different approach. Considering the complex, heterogeneous nature of the photosynthetic reaction centers, they attempted to fit their data to parameterized distributions of lifetimes. These groups also experienced some success, but this approach suffers from a fundamental limitation. In order to parameterize the fitting equation properly, it is necessary to know the number and functional form of the lifetime distribution(s) that are required. To some extent this proved to be self-defeating, as it was necessary to know part of the answer before the question could be properly framed. Nevertheless, this approach was helpful in bringing to light the theoretical justifications for using distributions of lifetimes, rather than sums of a few discrete components, to describe these highly heterogeneous systems. Distributed systems are more naturally described by distributed parameters.
In the past few years Holzwarth et al. have begun to employ global target analysis in the analysis of time-resolved fluorescence data from photosynthetic systems.\textsuperscript{43,17} Recall that in global analyses multiple TCSPC data sets are analyzed simultaneously by linking certain parameters (e.g., lifetimes) across data sets. Global target analysis goes one step further. Global target analysis fits the data directly to a proposed kinetic model, bypassing the intermediate step of obtaining a set of amplitudes and lifetimes that might not possess any direct correspondence to meaningful chemical properties of the system. The fitting parameters in this case are the fundamental chemical properties of interest: rate constants, activation energies, \textit{etc.} The primary advantage of global target analysis is that statistically acceptable fits to the data are generally obtained \textit{only} when a hypothesized kinetic model is consistent with the experimental data. Other methods of analysis typically result in acceptable values of the reduced chi-square regardless of the validity of the model, as long as they are permitted a sufficiently fluid parameter space. In addition to the excellent work of Holzwarth's group, Beechem \textit{et al.} have effectively promoted the global target analysis approach to the interpretation of TCSPC data.\textsuperscript{23-27}

As successful as these early forays into global target analysis have been, the approach cannot be immediately applied to new systems. The approach is inherently model-dependent. A scientist must propose rational kinetic hypotheses, design appropriate sets of experiments to acquire the data, and perform a separate global target analysis on each proposed kinetic scheme. Each aspect of this process can be quite lengthy. In the very recent paper by Konermann \textit{et al.}, a typical global target analysis of a PS II-RC fluorescence data matrix required approximately two weeks on a DEC 3000–800 computer!\textsuperscript{44} Hence, a successful global target analysis will require preliminary foundational experiments, analyzed by more mundane "empirical" methods. The results of these initial experiments might then suggest reasonable kinetic models to be submitted for further testing. Global target analysis represents (for now) the pinnacle of TCSPC data analysis.
The Appropriateness of ESM Lifetime Distribution Analysis for PS II Reaction Centers

As we mentioned in Chapter 1, unparameterized lifetime distributions are often valuable in the analysis of complex, heterogeneous fluorescence decays. This is especially true in the early stages of an investigation, when very little is understood about the origins of the various lifetime components. Using a technique such as the Exponential Series Method (ESM), it is possible to obtain an essentially unbiased description of the time signature of a fluorescence decay curve. Given the inherent complexity of the energy transfer processes in photosynthetic reaction centers, it is unlikely that these recovered distributions will correspond directly to meaningful chemical information. Nevertheless, an ESM (or MEM) fit to the data can reveal the minimum number of components (or modes) required to fit the data acceptably, and the shapes and widths of the recovered distributions can suggest functional forms for future analyses using parameterized distributions and/or discrete exponentials. By fitting preliminary data to ESM lifetime distributions and interpreting these data in concert with other spectroscopic studies, rational models can then be developed for testing by global target analysis.

Experimental and Computational Methods

We investigated PS II-RCs isolated from wild-type *Chlamydomonas reinhardtii* algae and spinach leaves. The preparations were carried out in the Sayre laboratory, and the details of the relevant procedures are provided as Appendix E.

A sample absorption spectrum of a spinach photosystem II reaction center complex is provided in Figure 4.2. The following bands are clearly present: cytochrome b559 (417.5 nm), Chl Soret (435.5 nm), Pheo Q₉ (543 nm), Chl Q₇ (675.5 nm).
Time-Correlated Single-Photon counting measurements were carried out using the instrumentation described in Chapter 2. Experiments were conducted using excitation pulses from 600 – 678 nm generated by picosecond R6G or DCM dye lasers at repetition rates of 0.5 – 4 MHz. The absorbance of the sample at the excitation wavelength was usually between 0.1 – 1.0, and the monitoring bandwidth was generally 5 nm. A typical instrument response function had a FWHM of ~ 75 ps and a FWTM of ~ 235 psec.

In order to protect the samples from photodegradation, the average power at the sample was kept low (< 10 mW). Additionally, the samples were maintained near 4 °C and oxygen was excluded from the samples either by the addition of glucose oxidase/catalase or (in more recent experiments) by bubbling with argon. Samples were also magnetically stirred and frequently replaced with fresh solutions. The solutions were allowed to reach thermal equilibrium in the dark prior to data collection, and the samples were removed from the beam immediately after each experiment.

Data were initially analyzed using the SPC software supplied by Demas and Snyder. Eventually, ESM lifetime distribution analyses were performed using the software generously provided by Dr. Timothy Rhodes. In order to test the critical variables in TCSPC data for PS II-RCs, we developed an additional program in LabWindows/CVI to generate synthetic data when supplied with an input distribution of lifetimes and parameters to specify the quality of the data such as CPC, time base, and the number of channels. The source code for this program is provided as Appendix A.

Identification of Critical Variables

In Chapter 1 we mentioned that there are three essential issues to be considered before TCSPC data of complex systems may be confidently interpreted on the basis of recovered fitting parameters. These include (1) addressing the distortion imposed on the data
by the finite time response of the system, (2) identifying and eliminating or otherwise accounting for distortions of the data caused by chemical or instrumental artifacts, and (3) ensuring that the data are of sufficient quality (in terms of information content) to permit recovery of meaningful parameters. In this section we discuss our systematic investigation of some of these issues. While the specific samples considered here are PS II-RCs, the approach is quite general and should be applied to any complex system on a case-by-case basis.

Distortion Due to Instrument Response

Regardless of the functional form adopted by a particular system for the fluorescence intensity as a function of time, the observed fluorescence decay curve does not directly represent \( I(t) \). Rather, the observed signal, \( e(t) \), corresponds to a convolution of the finite response function of the instrument, \( L(t) \), with the fluorescence decay:

\[
e(t) = \int_0^t L(t - t')I(t)dt'
\]

As TCSPC data are not continuous, but rather represent a histogram comprised of \( n \) discrete bins of width \( \Delta t \), this equation is more appropriately represented as:

\[
e(t) = (\Delta t)\sum_{i=0}^{t} L(t - i\Delta t)I(i\Delta t)
\]

As we have mentioned in Chapter 1, the method of deconvolution that has proven to be the most reliable is the method of iterative reconvolution. This approach convolves a measured IRF with a trial decay law and adjusts the fitting parameters until the agreement between the data and the fitting equation is optimized. Careful consideration of the convolution equation for discrete data reveals a subtlety in the analysis of complex TCSPC decays that seems to have gone unnoticed (or at least unemphasized) in the literature. Empirically, one tends to encounter difficulty obtaining excellent chi-square values when data
are analyzed over an entire decay curve. Presumably this is due to problems associated with quantization of the data into discrete channels or the potentially disproportionate effects of minor temporal instabilities on the rising edge of a fluorescence decay curve. Hence, it is common to fit TCSPC data from the peak channel forward, and occasionally from a few channels further into the decay. Provided that the data have been collected to adequate precision (CPC), this will have absolutely no effect on the analysis of single-exponential decays. As a zeroth-order kinetic process, the recovered lifetime will be the same regardless of the fitting window that is analyzed.

What has not been emphasized in the literature is that this does not hold true for complex fluorescence decays, especially when one or more of the components of interest is very short-lived. If the entire decay curve is not included in the analysis, an intrinsic bias will be introduced into the fitting procedure that could artificially emphasize the longer-lived components since the first few channels of the decay contain proportionately more information about the shorter-lived components.

We observed this problem when we fit experimental data from PS II-RCs to ESM lifetime distributions with fits performed from the peak channel. Slightly different lifetime distributions were recovered when the data were analyzed from the base of the rise of the fluorescence signal. This is evident in Figures 4.3–4.4. The differences are more apparent when the weighted residuals of a similar pair of fits are examined (Figure 4.5). Significant variations are observed at early times, especially in the first 200 ps.

A similar problem manifested itself in the generation of synthetic data. Different algorithms treat the convolution integral in somewhat different ways. These methods use various methods of approximation to represent the convolution integral. Specifically, the method of numerical integration employed by each method can lead to subtle differences in the convolutions. The literature is also somewhat silent on this point, although Demas has a good discussion of it in the sixth chapter of *Excited State Lifetime Measurements*. Here
again the problem is more severe for complex fluorescence decay curves. When synthetic
data were generated for single-exponential decays, the form of the decay was essentially
identical regardless of the convolution algorithm employed. On the other hand, when data
generated from complex lifetime distributions were synthesized, the shapes of the generated
decay curves were often visibly different.

How can these complexities be addressed? We carried out the following test
sequence: (1) fit an experimental PS II-RC TCSPC data file to an ESM lifetime distribution,
(2) use this distribution as the input distribution for a synthetic decay curve using a different
convolution routine than the one employed by the fitting program, (3) resubmit the synthetic
decay curve for ESM analysis. We found acceptable agreement between the ESM
distributions obtained from the synthetic data and the original data only when the fits were
conducted over the entire fluorescence decay curves. When fits were attempted from the
peak channels, the recovered distributions were often visibly different.

We believe these complexities merit further consideration. As a precautionary
measure, we chose to perform all of our ESM analyses over the entire fluorescence decay.
While this generally resulted in slightly higher values of the reduced chi-square than
equivalent fits conducted from the peak channel, the fits are more reproducible and probably
more reliable using this approach.

Distortion Due to Instrumental or Chemical Artifacts

In Chapter 1 we mentioned a number of instrumental considerations that must be
addressed to eliminate artifacts from the observed fluorescence decay curves. Such concerns
have been extensively addressed by others, and the methods of identifying and correcting
these optical and electrical artifacts are the same, regardless of the nature of the sample.

Of particular concern for photosynthetic systems is the issue of sample integrity.
The PS II-RCs were often observed to degrade during exposure to the laser beam, especially
under aerobic conditions. This photodegradation is largely attributable to singlet oxygen generation. As mentioned in the experimental section of this chapter, the problem of photodegradation could be minimized by deoxygenation of the samples. Maintaining the temperature near 4 °C and shielding the sample from the laser beam between experiments were also helpful in protecting the sample from damage. Continuous stirring of the solution removed photolyzed and/or unrelaxed reaction centers from the optical path. Despite all of these precautions, we found it prudent to replace the samples regularly. Quick checks of sample integrity were performed by comparing decay curves throughout a set of experiments. Damage was indicated by a loss of the longest-lived lifetime component(s) of PS II-RCs.

Issues of Data Quality

The importance of assessing the information content of TCSPC data was also discussed in Chapter 1. If meaningful information is to be extracted from time-resolved fluorescence data for a system as complex as a photosynthetic reaction center, it is first necessary to determine the optimum experimental conditions for data collection. If the data are not of sufficient quality, any discussion of the relative merits of applying various kinetic models is futile.

Role of Synthetic Data. It is not possible to specify a general level of TCSPC data quality that will suffice for all chemical systems. Each sample is different, so the optimum levels of precision, resolution, width of the time window, etc. will vary. These values may also depend on the width and shape of the instrumental response function, as well as the form of the underlying lifetime distribution. It has long been recognized that generating synthetic decays of various data quality and submitting these decays to analyses identical to those used for the experimental data is an excellent way to ensure that the data are of sufficient quality. “Sufficient” in this context generally refers to the ability to recover parameters in a reproducible fashion with uncertainties that are appropriate to the goals of the analysis.
The generation of synthetic data is more reliable for TCSPC experiments than for some other spectroscopic techniques since the data are governed by straightforward Poisson statistics.

We have written a program in LabWindows/CVI that generates synthetic data of the required quality using any one of four different convolution algorithms. The instrumental response function can be an actual IRF from an experiment (more common) or synthesized as a sum of Gaussians (less common). Variable input parameters include the data precision (CPC), data resolution (number of channels), time base (TAC range), and the desired set of amplitudes and lifetimes. Dark counts may also be added if desired. Poisson noise is generally superimposed on the data, although there is an option to disable this feature.

**Data Precision.** There has long been a rule of thumb for the acquisition of TCSPC data that says that experiments should be conducted until 10,000 counts have been collected in the peak channel (CPC). As seems to be the case for many issues of TCSPC data analysis, this principle must be discarded for complex decay curves. Ten thousand counts are usually sufficient for single-exponential decays—indeed, successful parameter recovery is often possible with 1000 counts or less. Multi-exponential decays are another matter entirely. We have already alluded to the fact that acceptable values of the reduced chi-squared can usually be obtained with fits to 2-4 exponentials when low-precision data are collected, regardless of the complexity of the underlying decay law. If higher precision data are collected for the same sample, the chi-square value will increase steadily as the inadequacy of the simple kinetic model is exposed.

We used the following methodology to test the CPC-dependence of photosystem II TCSPC data: (1) several experimental PS II-RC data files were fit to a sum of four exponentials, (2) synthetic data files were generated with similar lifetimes and relative contributions at various levels of data precision (CPC), (3) four-exponential fits were conducted on the synthetic data, and the recovered parameters were compared with the input parameters. The results are tabulated in Table 4.1 and presented graphically in Figure 4.6.
Clearly, these data suggest that by collecting data to higher CPC, the kinetic parameters can be extracted with increasingly higher levels of accuracy. If a 5% error is deemed to be acceptable, and if PS II-RC samples are truly described by four exponentials, then 10K CPC will permit successful recovery of all of the parameters except one. Only τ₁ is poorly resolved at this level of data precision. A consideration of the input parameters reveals why this is the case. The 400 ps lifetime makes only a minor (4.48%) contribution to the decay, and its intensity is difficult to isolate from those of the 40 ps and 4 ns components because its lifetime is intermediate between these two values. If the data precision is improved to 25K CPC, all of the parameters may be determined to better than 2.2% accuracy.

It is important to keep in mind that the values above apply only if the kinetics of photosynthetic samples are truly represented by a four-exponential decay law. If the kinetics are more complex, a successful chi-square might be obtained for a four-exponential fit at 10K CPC, but the recovered parameters might not correspond to any chemically meaningful properties of the sample. As there are good reasons to believe that the fluorescence kinetics of PS II-RCs might be significantly more complex than a four-exponential decay, we also tested the level of data precision required under the assumption that the fluorescence decay was better described by a complex continuous distribution of lifetimes. Once again, experimental (50K CPC) data were analyzed, but this time the model was an ESM lifetime distribution with 100-components, logarithmically spaced from 0.010 – 150 ns. Additional components were included at 0.001 ns and 1500 ns to account for any scatter and/or dark counts. The lifetime distribution obtained from the ESM fit was used as the input distribution for synthetic data, generated at various levels of data precision. These data are plotted in Figure 4.7. ESM analyses were then performed on the synthetic data, and the recovered distributions are tabulated with the input distribution in Figure 4.8. While the essential form of the distribution is recovered at 10K CPC, only at data precisions 50K CPC do the recovered distributions truly begin to resemble the input distribution. The agreement
continues to improve as data precision is increased, but a value of 20K – 50K CPC seems to be sufficiently precise to permit recovery of the majority of the features of the input distribution. These values represent a higher level of precision than the traditional rule of thumb, yet are still within the realm of the practical. Collecting data to > 100K CPC would present logistical challenges. This is especially true for PS II-RCs, which are subject to photodegradation. Consequently, we acquired data for our most recent experiments to a precision of 20K – 50K CPC.

Data Resolution. The second aspect of data quality that we investigated was the resolution of the data, as measured by the number of bins in the histogram. From an instrumental point of view, this corresponds to the number of channels in the multichannel analyzer. The more channels that are employed, the more the data will resemble a continuous function, and fewer errors will be introduced by the digital nature of the time axis. There is a straightforward tradeoff, however, between data resolution and time of analysis. If the number of channels are doubled, all other factors being equal, the time required to achieve a particular level of data precision (CPC) will also be doubled.

We applied a test method analogous to that employed for the CPC-dependence to assess the impact of data resolution on the ability to recover parameters for a four-exponential fit. Several synthetic decays (20K CPC) were generated with the same lifetimes and relative amplitudes used in the previous test. Decays were synthesized using 256, 512, and 1024 channels. The fitting program crashed when we tried to fit the 256-channel data to a sum of exponentials, presumably because of the ill-conditioned nature of the problem at this low resolution. The recovered parameters for the 512 and 1024 channel decays are included in Table 4.1 and the results are presented graphically in Figure 4.9. The improvement in
parameter recovery upon moving from 512 to 1024 channels is not dramatic, but it is significant. It would have been interesting to measure the impact of collecting data into 2048 channels, but the SPC program does not permit the analysis of files with more than 1024 points.

Most of our early photosystem experiments were conducted at a resolution of 1024 channels. In our most recent experiments we used resolutions of 1024 and 2048 channels. We found that when the data were analyzed using ESM lifetime distributions, the fits were significantly more reproducible and reliable when the data were collected across 2048 channels. While our TCSPC instrument has the capability of analyzing 4096 or 8192 channels, we believe the benefits of the additional resolution would be more than offset by the increased time required for data acquisition. Perhaps of equal importance is the fact that the time required to fit the data increases significantly as the number of channels are increased.

**Time Base.** Of the three experimentally-determined aspects of TCSPC data tested in this section, the time base is the least straightforward. While it is fairly clear that increasing the data precision or data resolution will result in superior parameter recovery, the time base can only be rigorously optimized for one lifetime component at a time. This means that for systems described by a sum of exponentials or by a continuous distribution of lifetimes, there will always be tradeoffs. Optimizing the experiment’s ability to measure a short-lived component will increase the magnitude of the uncertainties in the lifetimes and fractions of any long-lived components and vice versa. One can usually not determine *a priori* what the “optimum” time base will be for a particular sample.

As Gakamsky *et al.* have noted,37 for any given time base there exists a \( \tau = \tau_{\text{max}} \) beyond which the component’s decay will be indistinguishable from a constant background, and there exists a \( \tau = \tau_{\text{min}} \) for which components with lifetimes shorter than \( \tau_{\text{min}} \) will be indistinguishable from scatter.
Once again we turned to the generation of synthetic data to determine an optimal time base for TCSPC investigations of PS II-RCs. Several 20K CPC, 1024-channel decays were synthesized using time bases of 3 - 192 ns. The input parameters corresponded to the same four-exponential decay used for the other tests. The results are again included in Table 4.1 and presented in Figure 4.10. As expected, no single time base simultaneously optimizes the recovery of all four components. A time base of at least 24 ns is required to obtain even a reasonable estimate of the 40 ns lifetime. In fact, as a consequence of the correlation among the fitting parameters, the relative contributions of all four the components are poorly resolved for time bases < 24 ns. While the 192 ns time base is nearly ideal for the recovery of the 40 ns lifetime, all of the information about the 40 ps and 400 ps lifetimes is compressed into the first few channels of the decay curve, and the ability to resolve these two components accurately begins to deteriorate. Our results suggest that any time base between 24 - 96 ns should permit acceptable recovery of the majority of the fitting parameters. The choice for a specific experiment will be further influenced by the component(s) of greatest interest for the particular investigation. For our initial experiments we were specifically interested in the long-lived component(s) of PS II-RC fluorescence, so we selected time bases between 53 - 75 ns, although we occasionally measured decays with a much shorter time base to obtain more accurate information about the short-lifetime regime of the lifetime distribution.

Evaluation of Critical Variables

Optimizing the quality of TCSPC data is a nontrivial exercise and is a complex function of the properties of the particular chemical system under investigation and the specific goals of the experiments. There will inevitably need to be compromises made between data quality and speed of analysis, as well as in the ability to resolve the short-lifetime region or the long-lifetime region of a particular decay curve.
This last compromise is avoided to a considerable extent when the data can be analyzed globally, as experiments can be conducted at several time bases and fitting parameters linked across the entire data surface. As complex fluorescence decays often contain contributions from components that span several decades in lifetime space, a more satisfying solution would address the underlying problem more directly. The TCSPC instrument, in its traditional configuration, possesses a linear time axis. For complex decays, a logarithmic time axis would be more convenient, as it would permit accurate simultaneous measurement of a broad distribution of lifetimes. Some commercial time-resolved fluorescence instruments have been introduced recently that possess this very capability. This option will probably become more prevalent in the next generation of time-resolved fluorescence instrumentation.

In the meantime, the generation of synthetic data and applying a testing method similar to the one conducted above appears to be the best way to optimize the time base of a TCSPC instrument for a particular application. As mentioned previously, we made measurements using time bases of 53 - 75 ns, and currently prefer values at the high end of this range. We have also decided to collect PS II-RC data into 2048 channels, as we believe this gives us sufficient resolution to resolve the short-lived components of the lifetime distribution without slowing down data acquisition and analysis to an impractical extent. We have found 20K CPC to be sufficient, in general, to recover complex ESM lifetime distributions from PS II-RCs, although we prefer to collect 50K CPC whenever possible. For four-exponential fits, 10K CPC appears to be adequate.

The only similar systematic investigation of TCSPC data quality issues that we discovered in the literature was a study performed by Janssens et al. to test the effect of some of these parameters on the global analysis of time-resolved fluorescence data for mixtures of several discrete-lifetime components. They concluded that for a fixed number of total fluorescence counts in a data surface (across multiple data files), the superior approach was
to collect a few files with excellent precision (CPC) rather than to collect a larger number of lower-precision files. Similarly, they suggested that it was more important to optimize the data precision than the data resolution for the systems that they investigated. From the perspective of global analysis, they concluded that the advantages of global analysis would be best realized if the decays in the data surface were strategically selected to maximize the differentiation in the relative amplitudes of the lifetime components as a function of time base, excitation/emission wavelengths, temperature, etc. Our studies were addressing somewhat different questions on a completely different chemical system, but our conclusions appear to be in qualitative agreement with theirs.

Results for Spinach and Chlamydomonas reinhardtii Reaction Centers

In this section we present the preliminary results of our time-resolved fluorescence investigations of PS II-RCs. Our primary goals in this stage of the project were to develop reliable, reproducible sample preparations for the Chlamydomonas reinhardtii reaction centers and to develop appropriate methods for the acquisition and analysis of TCSPC data for PS II-RCs. The first objective has proven to be more of a challenge than we might have hoped, and the Sayre group is continuing to approach the problem in new ways. While we believe we have had one or two adequate PS II-RC samples from Chlamydomonas, the majority of our experiments on these reaction centers have indicated a loss of photochemical activity. The analysis of spinach PS II-RCs has been conducted in order to assess the instrument’s performance on a more rugged, well-established preparation. We believe that we have largely obtained our second goal of developing an appropriate methodology for measuring time-resolved fluorescence from PS II-RCs. We also believe that we have made a significant contribution to the analysis of TCSPC data for these samples by fitting our data to ESM lifetime distributions.
We do not believe that extensive chemical information will be available from these data until reliable preparation methods have been achieved and global analysis routines can be invoked to analyze multidimensional data surfaces for these complex samples. Consequently, in this section we will present only the empirical results of our sum-of-exponential fits and ESM lifetime distribution analyses. As we mentioned previously, there is little reason to believe that these lifetime components correspond directly to any meaningful kinetic parameters in the reaction center. We will therefore not attempt to interpret the decay components, other than to compare them with the results of other research groups. We see no reason to doubt that the data for our spinach samples and the healthiest of our *Chlamydomonas* samples are consistent with the kinetic models thus far advanced in the literature. We expect that our innovation in fitting PS II-RC time-resolved fluorescence data to unparameterized lifetime distributions will ultimately prove to be a powerful tool to be applied to the improvement of these kinetic models.

Representative TCSPC decays for "good" preparations of spinach and *Chlamydomonas reinhardtii* PS II-RCs are presented along with a decay for a damaged (inactive) *Chlamydomonas* PS II-RC in Figure 4.11. The qualities of the reaction center preparations were independently verified in other experiments. A cursory inspection of the decay curves reveals that the healthy reaction centers contain a significant (> 15%) contribution from one or more long-lived components. The damaged reaction centers lack this long-lived component. We found this observation to be quite general for all of the reaction center preparations that we studied. Four-exponential fits for several PS II-RC preparations are provided in Tables 4.2 - 4.4. Most of the *Chlamydomonas* PS II-RCs contain little or no contribution from a lifetime longer than 10 ns or so, while all of the spinach PS II-RC preparations contain this feature. This long-lived component is generally acknowledged in the literature to arise from P680 fluorescence subsequent to recombination of the charge-separated state. When this component is not present, it suggests that the PS II-
RC is incapable of separating charge, and is therefore inactive. We found that this quick
diagnostic was generally in excellent agreement with other techniques used to assess sample
integrity.

Of all of the *Chlamydomonas* PS II-RCs studied, only one possessed a significant
fraction of this long-lived component. This sample was prepared on 2/19/94. Subsequent
preparations occasionally contained a long-lived contribution, but it never accounted for
more than 10% of the intensity. In contrast, between 16 – 18% of the 2/19 preparation’s
fluorescence intensity could be attributed to a 27 – 29 ns lifetime. Our best spinach sample
(11/3/95) contained a 40 – 46% contribution from a lifetime component between 29 – 32 ns.

Comparing the parameters from four-component fits to our best preparations of
spinach and *Chlamydomonas* PS II-RCs, we see that the lifetime components are nearly
identical, with the differences in the samples’ time-resolved fluorescence signatures
originating primarily in the relative fractions of the four components. Using the first entry in
Table 4.4 for the 2/19/94 *Chlamydomonas* preparation and the first entry in Table 4.2 for the
11/3/95 spinach preparation, we see that both samples are dominated by a component with a
lifetime near 5 ns. This component is known from the literature\(^\text{2,13}\) to correspond to uncoupled
(free) chlorophyll. We have confirmed this assignment, as we obtained a single-exponential
lifetime of 5.62 ± 0.05 ns for chlorophyll in aqueous solution (Figure 4.12). We have already
referred to the ~ 29 ns component that accounts for 43% of the spinach PS II-RC decay and
17% of the *Chlamydomonas* decay. This is attributed to P680 fluorescence subsequent to
charge recombination. Both decays also contain a very short (< 75 ps) component that is
responsible for less than 3% of the intensity, and an intermediate-lifetime component of 0.76
ns (spinach, 5.5%) or 1.5 ns (*Chlamydomonas*, 6.8%). These latter two components are
related to the initial energy transfer and radical-pair relaxation processes. These four-
exponential fits are similar to those obtained by Giorgi *et al.* for PS II-RCs isolated from pea
seedlings (*Pisum sativum*) and *Chlamydomonas*, although the 280 ns time base used in their
experiments caused the two short-lived components to be recovered as a single 1.2 ns component, while the long-lived component was resolved into a 14 − 17 ns component and a 51 − 53 ns component. They concluded (and we agree) that the time-resolved fluorescence decays of *Chlamydomonas* PS II-RCs are sufficiently similar to those of higher plants to justify extending the conclusions of future research based on site-directed mutagenesis of *Chlamydomonas* to apply to higher plants.

Nevertheless, the fact that our recovered lifetimes were different than those obtained by Giorgi et al. suggests that the fluorescence of PS II-RCs may not be adequately described by four exponentials. We have already argued that for a system as complex, heterogeneous, and distributed as a photosynthetic reaction center, a continuous distribution of lifetimes is a more logical initial model than discrete lifetime components. When we fit our PS II-RC data to ESM lifetime distributions, we confirmed that the data are well-described by these distributions, and that the recovered distributions are sufficiently complex to suggest that no fewer than five components ought to be used in any attempt to analyze PS II-RC fluorescence. Indeed, these data seem to be better described by lifetime distributions than by discrete components, as we and others had suspected. Fit results and weighted residuals for four-exponential and ESM fits to the same TCSPC data file are compared in Figure 4.13. Some representative fits to 100-component distributions are provided in Figure 4.14. By summing the fractional contributions of each lifetime component in a given mode, we obtained Discrete Component Equivalents (DCEs) for these distributions (Figure 4.15). The two distributions selected for presentation exhibit some significant differences. For example, the long-lived component for the spinach sample is distributed between 10 − 20 ns, in contrast to the 30 − 40 ns distribution for the *Chlamydomonas* sample. Other major differences include the fractional contributions of the 44 − 88 ps component, and the locations of the intermediate-lifetime components. The *Chlamydomonas* sample exhibits small peaks between 200 − 300 ps and 500 − 600 ps, and a large distribution of lifetimes between 2 − 3 ns.
In contrast, the spinach sample has some intensity between 100 – 200 ps. Its other feature is a broad distribution spanning 0.5 – 3.0 ns. Both samples possess the mode attributable to free chlorophyll, which ranges from 3 – 7 ns.

We were unable to compare spinach and *Chlamydomonas* decays collected under identical conditions. Unfortunately, we were never in possession of healthy RC preparations for these two systems at the same time. Consequently, we were not sure to what extent some of the differences in the recovered distributions might have been due to differences in data quality or other factors. In the absence of a direct comparison, we devised the following test: (1) independent ESM analyses were performed for a *Chlamydomonas* PS II-RC decay and a spinach PS II-RC decay, (2) the recovered distribution for spinach was used as the input distribution to generate a synthetic decay curve of identical data quality (*i.e.*, same IRF, CPC, time base, resolution) as the experimental *Chlamydomonas* data, and (3) the synthetic “spinach” data was fit to a lifetime distribution. As may be seen from Figures 4.16 – 4.17, the distribution recovered from the synthetic “spinach” decay continues to resemble the distribution obtained from the experimental spinach data rather than the *Chlamydomonas* data. This suggests that if these differences in lifetime distributions are real, we would have been able to resolve them under the experimental conditions used to acquire the 2/19 *Chlamydomonas* sample.

To get an estimate of the reproducibility and reliability of the ESM distributions, we analyzed two independent 10K CPC decay files for the 2/19 preparation of *Chlamydomonas*. We then generated a third file by summing the counts in all three of the decay files collected for this particular sample at these particular experimental conditions and performed an ESM analysis on this “cumulative” file. As may be seen in Figures 4.18 – 4.19, the fits are very reproducible. All of the recovered amplitudes and lifetimes are similar to one another, with the values for the “cumulative” file intermediate between those for the individual decays, as expected.
Finally, we provide a preview of the types of analyses that we hope to perform in the future. Lifetime distributions and DCEs are shown for the excitation wavelength- and temperature-dependence of ESM distributions for the 6/24 preparation of spinach PS II-RC. The excitation wavelength study was performed on the 6/24-2 sample, while the variable temperature investigation was performed on a fresh sample, 6/24-3. The results are presented in Figures 4.20 – 4.23. These data clearly exhibit some “spectra” and temperature-dependence. It is, in general, unnecessary to analyze for trends in these data since the fractions and lifetimes *per se* do not correspond directly to any meaningful kinetic parameters. What we do see in these data is the potential for future global analyses and/or global target analyses. Similar data surfaces could be globally analyzed in terms of parameterized distributions of lifetimes, or fit directly to specific kinetic models.

We close this section by presenting an ESM distribution for a *Chlamydomonas* PS II-RC decay curve that was collected on a much shorter (~ 2.5 ns) time base (Figures 4.24 – 4.25). As mentioned in previous sections, the lifetime distributions in the short-lifetime regime should be better described using this time base. Unfortunately, the longest-lived components cannot be resolved from the component due to free chlorophyll on this time scale. While this presents a problem for single-decay analysis, it is an opportunity for global analysis. By collecting multiple decay curves across several different time scales and by performing experiments at various temperatures, excitation wavelengths, emission wavelengths, etc., a multidimensional data surface may be fit globally to parameterized distributions. Alternately, a specific kinetic hypothesis may be tested by fitting the data surface directly to the rate constants, activation energies, etc. that are of interest. In global analysis the limitations of optimizing the experiment to recover a particular decay component may be overcome.
Preliminary Conclusions

In this chapter we have outlined an approach for investigating the structure-function relationships of photosystem II reaction centers using time-resolved fluorescence spectroscopy. By making strategic mutations in the reaction center proteins of *Chlamydomonas reinhardtii* algae, we expect to be able to observe and interpret changes in the time-resolved fluorescence decays.

While the Sayre group is still working to develop a reliable method of isolating active *Chlamydomonas* PS II-RCs, we have succeeded in developing an effective methodology for acquiring TCSPC data for these systems. Furthermore, we have applied the Exponential Series Method of lifetime distribution analysis to these samples for the first time. We believe that unparameterized distributions of lifetimes, rather than discrete exponential components, represent a better starting point in the analysis of fluorescence from this very heterogeneous, complex, distributed biochemical system.

We have presented preliminary data for several preparations of *Chlamydomonas* PS II-RCs and have demonstrated our ability to distinguish active from damaged reaction centers on the basis of the presence or absence of a significant (> 15%), long-lived (~ 29 ns) fluorescence decay component that corresponds to P680 charge-recombination fluorescence. We have also measured the time-resolved fluorescence of several PS II-RCs isolated from spinach leaves, and compared the recovered lifetimes and lifetime distributions with those that were obtained for *Chlamydomonas*. We found four-exponential fits to these two RC samples to be similar enough to suggest essential homology in the kinetics of the two systems, although there were enough differences in the ESM lifetime distributions recovered from these two systems to suggest that this issue ought to be investigated further before proceeding on to studies of *Chlamydomonas* mutants.
Our recovered lifetime components appeared to be in reasonable agreement with results published in the literature, although we believe four-exponential fits are insufficient to describe these TCSPC data and suggest that future work be conducted with ESM lifetime distributions. We consistently observed 5 – 6 discrete modes in the lifetime distributions recovered from PS II-RCs, so future global analyses could be conducted using 5 – 6 discrete components or parameterized distributions.

Our systematic investigation of TCSPC data quality for these systems leads us to recommend a threshold data precision of 20K CPC and a data resolution of at least 2048 channels. If only one time base is to be analyzed, a 24 – 96 ns time scale will permit satisfactory recovery of most of the lifetime components. Ultimately, data from several time scales should be combined in global analyses for optimum recovery of the lifetime distributions.

This chapter has summarized several of the issues that must be considered when attempting an analysis of complex, biological samples using Time-Correlated Single-Photon counting. The data must be acquired in a manner that does not damage the sample, and analyzed according to a model that is appropriate to the system. Furthermore, the information content of the data—as measured by its precision, resolution, and time base—must be adequate to recover the desired kinetic parameters. While these considerations have caused progress on this project to advance more slowly than one might hope, the precautions are necessary if the ultimate conclusions are to be believed.
References


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### Table 4.1: Effect of (a) data precision (CPC), (b) data resolution (# of MCA channels), and (c) time base on the relative errors in recovered parameters for four-exponential fits to synthetic TCSPC data. Each table entry represents an average over five synthetic decays. The values, standard deviations, and errors relative to the “true” (input) values are tabulated for each parameter. The input lifetimes and fractions are similar to those obtained for four-exponential fits to PS II-RC data.

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<th>f₁</th>
<th>Relative Error (f₁)</th>
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Table 4.1: Effect of (a) data precision (CPC), (b) data resolution (# of MCA channels), and (c) time base on the relative errors in recovered parameters for four-exponential fits to synthetic TCSPC data. Each table entry represents an average over five synthetic decays. The values, standard deviations, and errors relative to the “true” (input) values are tabulated for each parameter. The input lifetimes and fractions are similar to those obtained for four-exponential fits to PS II-RC data.

159
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<th>Sample</th>
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<th>(\lambda_{\text{em}}) (nm)</th>
<th>(\chi^2)</th>
<th>(\tau_1) (ns)</th>
<th>(\tau_2) (ns)</th>
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<th>(\tau_4) (ns)</th>
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<th>(f_2)</th>
<th>(f_3)</th>
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**Table 4.2:** Sum-of-exponential fits to spinach PS II-RC fluorescence decays
Table 4.3: Sum-of-exponential fits to Chlamydomonas reinhardtii PS II-RC fluorescence decays. Most recent preparations

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<th>A1, (mm)</th>
<th>A2, (mm)</th>
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<th>X2, (ms)</th>
<th>X3, (ms)</th>
<th>X4, (ms)</th>
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<th>f2</th>
<th>f3</th>
<th>f4</th>
<th>f5</th>
<th>f6</th>
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<td>2.0%</td>
<td>4.235</td>
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Table 4.3: Sum-of-exponential fits to Chlamydomonas reinhardtii PS II-RC fluorescence decays. Most recent preparations

161
| Sample            | CPC Channels | Time Base (ns) | λ_ex (nm) | λ_em (nm) | x² | τ1 (ns) | τ2 (ns) | τ3 (ns) | τ4 (ns) | f1   | f2   | f3   | f4   | τ_m (ns) | μ > (ns)
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<td></td>
</tr>
<tr>
<td>6/7/94</td>
<td>10K</td>
<td>1024</td>
<td>75</td>
<td>634</td>
<td>685</td>
<td>1.08</td>
<td>5.17</td>
<td>10.20</td>
<td>0.780</td>
<td>0.046</td>
<td>80.5%</td>
<td>14.5%</td>
<td>3.5%</td>
<td>1.5%</td>
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<td>1024</td>
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<td>687</td>
<td>1.32</td>
<td>2.51</td>
<td>5.63</td>
<td>0.257</td>
<td>0.046</td>
<td>21.5%</td>
<td>75.0%</td>
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<td>8.58</td>
<td>0.714</td>
<td>0.052</td>
<td>78.1%</td>
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<td>1.14</td>
<td>5.32</td>
<td>8.95</td>
<td>0.902</td>
<td>0.054</td>
<td>84.3%</td>
<td>10.2%</td>
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<tr>
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<td>0.052</td>
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<tr>
<td>6/7/94</td>
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<td>1.16</td>
<td>5.32</td>
<td>12.00</td>
<td>0.892</td>
<td>0.056</td>
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<td>10.0%</td>
<td>3.9%</td>
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<tr>
<td>6/7/94</td>
<td>70K</td>
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<td>634</td>
<td>707</td>
<td>1.17</td>
<td>5.13</td>
<td>11.40</td>
<td>0.870</td>
<td>0.063</td>
<td>84.6%</td>
<td>10.3%</td>
<td>3.6%</td>
<td>1.5%</td>
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<td>707</td>
<td>1.16</td>
<td>5.20</td>
<td>10.60</td>
<td>0.707</td>
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<td>13.1%</td>
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Table 4.4: Sum-of-exponential fits to Chlamydomonas reinhardtii PS II-RC fluorescence decays. Early preparations.
Figure 4.1: Form of a typical PS II-RC fluorescence decay. Note the logarithmic y-axis. Data from Spinach PS II-RC (6/24-2 prep), 50K CPC, 71.5 ns time base, 2048 channels, excitation ~ 673 nm, emission ~ 683 nm
Figure 4.2: A typical PS II-RC absorption spectrum. The following bands are clearly present: cytochrome b559 (417.5 nm), Chl Soret (435.5 nm), Pheo Q_s (543 nm), Chl Q_y (675.5 nm)
Figure 4.3: Effect of fitting range on 100-component ESM fluorescence lifetime distributions for Spinach PS II-RCs: (a) fit from base of rise, components logarithmically spaced from 0.010 ns to 150 ns and (b) fit from peak channel, components logarithmically spaced from 0.010 ns to 110 ns. Both fits were to the same data file: 6/24-3 prep, 20K CPC, 71.5 ns time base, 2048 channels, excitation ~ 664 nm, emission ~ 683 nm. Additional components at 0.001 ns and 1100 or 1500 ns to account for scatter and/or dark counts.
Figure 4.4: Effect of fitting range on “Discrete Component Equivalents” for ESM fluorescence lifetime distributions for Spinach PS II-RCs (6/24-3 prep). Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.9 ns, 0.9 - 3.0 ns, 3 - 9 ns, 9 - 20 ns, > 20 ns
Figure 4.5: Weighted residuals for fits from (a) the peak channel and (b) the base of the rise for 100-component ESM fluorescence lifetime distributions for Spinach PS II-RCs. Only the first two nanoseconds are shown. Both fits were to the same data file: 6/24-3 prep, 20K CPC, 71.5 ns time base, 2048 channels, excitation ~ 664 nm, emission ~ 683 nm. INSET: Differences in the weighted residuals over the first 1.2 nanoseconds.
Figure 4.6: Data-precision (CPC) dependence of the relative errors in recovered fractions and lifetimes for four-exponential fits to synthetic data. Input parameters: 0.040 ns (5.97%), 0.400 ns (4.48%), 4.000 ns (59.70%), and 40 ns (29.85%). Data synthesized with 1024 MCA channels on a 96 ns time base.
Figure 4.7: Synthetic decay curves at various levels of data precision (CPC). Data generated using an ESM lifetime distribution for experimental data from Spinach PS II-RC (6/24-2 prep, 50K CPC, 71.5 ns time base, 2048 channels, excitation ~ 664 nm, emission ~ 683 nm). Solid lines correspond to experimental data and IRF, symbols correspond to synthetic data.
Figure 4.8: ESM fluorescence lifetime distributions (100 components) for simulated data of variable precision (CPC): (a) input distribution, (b) 10K, (c) 20K, (d) 50K, (e) 100K, (f) 1000K. The input distribution was obtained from an ESM fit (100 components) to a Spinach PS II-RC (6/24-2 prep, 50K CPC, 71.5 ns time base, 2048 channels, excitation ~ 664 nm, emission ~ 683 nm). Lifetime components for all ESM fits were logarithmically spaced from 0.010 ns - 150 ns with additional components at 0.001 ns and 1500 ns to account for scatter and/or dark counts.
Figure 4.9: Data-resolution (# of MCA channels) dependence of the relative errors in recovered fractions and lifetimes for four-exponential fits to synthetic data. Input parameters: 0.040 ns (5.97%), 0.400 ns (4.48%), 4.000 ns (59.70%), and 40 ns (29.85%). Data synthesized to 20K CPC on a 75 ns time base.
Figure 4.10: Time base dependence of the relative errors in recovered fractions and lifetimes for four-exponential fits to synthetic data. Input parameters: 0.040 ns (5.97%), 0.400 ns (4.48%), 4.000 ns (59.70%), and 40 ns (29.85%). Data synthesized to 20K CPC with 1024 MCA channels.
Figure 4.11: TCSPC decays for various preparations of PS II-RCs: (a) Spinach 11/3/95 prep, (b) Chlamydomonas 2/19/94 prep, and (c) Chlamydomonas 5/9/94 prep. All decays normalized to 10K CPC.
Figure 4.12: Fluorescence decay for aqueous chlorophyll. The decay is a single exponential with a lifetime of 5.62 ± 0.05 ns
Figure 4.13: Comparison of fitting methods for PS II-RCs isolated from Chlamydomonas reinhardtii. The same TCSPC data file (10K CPC) was analyzed using (a) a sum of 4 exponentials with 5 dark counts, and (b) an ESM lifetime distribution comprised of 100 exponentials, logarithmically spaced between 0.010 ns - 150 ns. The ESM fit included components at 0.001 ns and 1500 ns to account for scatter and/or dark counts.
Figure 4.14: ESM fluorescence lifetime distributions (100 components) for PS II-RCs: (a) Spinach 6/24-2 prep, (b) Chlamydomonas 2/19 prep. Spinach data: 50K CPC, 2048 channels, 71.5 ns time base, excitation ~ 668 nm, emission ~ 683 nm. Chlamydomonas data: 30K CPC, 1024 channels, 53 ns time base, excitation ~ 612 nm, emission ~ 687 nm. Lifetime components for ESM fits were logarithmically spaced from 0.010 ns - 150 ns with additional components at 0.001 ns and 1500 ns to account for scatter and/or dark counts.
Figure 4.15: “Discrete Component Equivalents” for ESM fluorescence lifetime distributions for PS II-RCs; (a) Chlamydomonas 2/19 prep, (b) Spinach 6/24-2 prep. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.13 ns, 0.13 - 0.4 ns, 0.4 - 1.6 ns, 1.6 - 3.1 ns, 3.1 - 10 ns, > 10 ns
Figure 4.16: ESM fluorescence lifetime distributions (100 components) for experimental and simulated data from PS II-RCs: (a) Chlamydomonas 2/19 prep, (b) Spinach 6/24-2 prep, (c) Synthetic data generated using ESM fit to (b) using the IRF and experimental conditions of (a). Chlamydomonas data: 10K CPC, 1024 channels, 53 ns time base, excitation ~ 612 nm, emission ~ 687 nm. Spinach data: 50K CPC, 2048 channels, 71.5 ns time base, excitation ~ 664 nm, emission ~ 683 nm. Synthetic data: 10K CPC, 1024 channels, 53 ns time base. Lifetime components for ESM fits were logarithmically spaced from 0.010 ns - 150 ns with additional components at 0.001 ns and 1500 ns to account for scatter and/or dark counts.
Figure 4.17: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions of experimental and synthetic PS II-RC data: (a) Chlamydomonas 2/19 prep, (b) Spinach 6/24-2 prep, and (c) Synthetic data generated using (b) as the input distribution and the experimental conditions of (a). Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.2 ns, 0.2 - 0.3 ns, 0.3 - 0.8 ns, 0.8 - 3.0 ns, 3 - 10 ns, > 10 ns
Figure 4.18: Reproducibility of 100-component ESM fluorescence lifetime distributions for Chlamydomonas PS II-RCs: (a) fit to file #1 (10K CPC), (b) fit to file #2 (10K CPC), (c) fit to sum of file #1, file #2, and file #3 (10K CPC, not shown). All fits were for the 2/19 prep, 53 ns time base, 1024 channels, excitation ~ 612 nm, emission ~ 678 nm. Lifetime components were logarithmically spaced from 0.010 ns to 150 ns with additional components at 0.001 ns and 1500 ns to account for scatter and/or dark counts.
Figure 4.19: Reproducibility of "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for Chlamydomonas PS II-RCs (2/19 prep). Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.2 ns, 0.2 - 0.3 ns, 0.3 - 0.8 ns, 0.8 - 3.8 ns, 3.8 - 10 ns, > 10 ns
Figure 4.20: Excitation wavelength dependence of ESM fluorescence lifetime distributions (100 components) for Spinach PS II-RCs (6/24-2 prep, 50K CPC, 71.5 ns time base, 2048 channels, emission ~ 683 nm): (a) 664 nm, (b) 669 nm, (c) 673 nm, and (d) 678 nm. The 678 nm data were 20K CPC. Lifetime components for ESM fits were logarithmically spaced from 0.010 ns - 150 ns with additional components at 0.001 ns and 1500 ns to account for scatter and/or dark counts.
Figure 4.21: Excitation wavelength dependence of "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for Spinach PS II-RC (6/24-2 prep). Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.02 ns, 0.02 - 0.15 ns, 0.15 - 0.30 ns, 0.30 - 2.8 ns, 2.8 - 7.0 ns, 7.0 - 30 ns, > 30 ns
Figure 4.22: Temperature dependence of ESM fluorescence lifetime distributions (100 components) for Spinach PS II-RCs (6/24-3 prep, 20K CPC, 71.5 ns time base, 2048 channels, excitation ~ 664 nm, emission ~ 683 nm): (a) 263 K, (b) 269 K, (c) 280 K, and (d) 290 K. Lifetime components for ESM fits were logarithmically spaced from 0.010 ns - 150 ns with additional components at 0.001 ns and 1500 ns to account for scatter and/or dark counts.
Figure 4.23: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for various temperatures of Spinach PS II-RC (6/24-3 prep). Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.9 ns, 0.9 - 3.0 ns, 3 - 9 ns, 9 - 20 ns, > 20 ns.
Figure 4.24: Short time base ESM fluorescence lifetime distribution (100 components) for Chlamydomonas PS II-RC (2/19 prep, 10K CPC, 2.5 ns time base, 512 channels, excitation ~ 612 nm, emission ~ 687 nm). Lifetime components for ESM fit were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 4.25: "Discrete Component Equivalents" for short time base Chlamydomonas PS II-RC (2/19 prep) ESM fluorescence lifetime distribution. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.5 ns, 0.5 - 2.0 ns, 2.0 - 9.0 ns, > 9 ns.
CHAPTER 5

ANALYSIS OF TIME-RESOLVED FLUORESCENCE DATA FOR PSORALENS AND RELATED COMPOUNDS

Introduction

Molecules containing a coumarin fused in a linear fashion to a furan ring are known as psoralens. A related class of compounds, the angelicins (or isopsoralens), possess an angular fusion of the same two ring systems. Taken together, these molecules are classified as furocoumarins. Many of these tricyclic molecules are natural products found in plants. They are specifically prevalent in the Umbelliferae, Rutacea, and Leguminosae families and are believed to function as natural insecticides in many of these plants. In all, several dozen psoralens have been isolated and characterized from natural sources. Several of these, as well as some analogs, have been synthesized. The structure of the parent psoralen (PSO) is provided in Figure 5.1, along with the numbering scheme used for derivative compounds. Some of the more common psoralens, such as 8-methoxypsoralen (8-MOP) are commercially available.

Psoralens are of interest due to their phototherapeutic value. For example, 8-MOP has been used in folk medicine for thousands of years to treat various dermatological problems, and has been used clinically since 1970 in the photochemotherapy of vitiligo and psoriasis (PUVA treatment). It is one of the most potent skin sensitizers known. Psoralens penetrate most biological structures and are generally nontoxic in the absence of actinic radiation (c. 320 – 400 nm). Psoralens have been shown to intercalate between base pairs in
double-stranded DNA with association constants of approximately $10^4$. Since psoralens absorb light in a region of the spectrum that is largely transparent to the nucleic acids that comprise DNA, applications have been developed to initiate selectively photochemistry at these intercalation sites. Specifically, each psoralen absorbs two ultraviolet photons and effects a $C_2$-cycloaddition reaction between the 3,4 and 4',5' double bonds of the psoralen and the 5,6 double bonds of adjacent pyrimidine bases. This reaction is depicted in Figure 5.2. The bifunctional adduct thus formed serves to cross-link the DNA, and this technique has been used to probe DNA structure. More recently, psoralens have come under investigation as possible antiviral agents that could protect the blood supply from pathogens such as HIV and the hepatitis virus. In theory, donated blood could be sterilized by the addition of a suitable psoralen derivative, followed by irradiation with ultraviolet light prior to transfusion. This approach, in principle, would selectively target the viruses, as neither red blood cells nor platelets contain genomic nucleic acids.

Recently, Platz et al. have determined that brominated psoralens (bromopsoralens) sensitize viral inactivation to an even greater extent than their non-halogenated analogs. They postulate that the excited singlet state of a bromopsoralen can abstract an electron from a nearby guanine residue to generate a DNA-bound, psoralen-centered radical. This radical species subsequently induces DNA strand cleavage. Additionally, the residual guanine radical cation produced by this process is itself believed to stimulate singlet oxygen formation, whose presence leads to additional DNA damage.

The Proximity Effect

The photophysics and photochemistry of these important molecules are fascinating, yet poorly understood. Further insight is necessary in order to comprehend adequately their photosensitizing and antiviral mechanisms. One of the factors complicating the interpretation of psoralens' photochemical and photophysical properties is the fact that, as aromatic
carbonyl compounds, psoralens tend to exhibit the phenomenon known as the "proximity effect," or more formally, pseudo Jahn-Teller distortion. This effect arises due to the small spacings between these molecules' lowest energy $^1\pi\pi^*$ and $^1\pi\pi^*$ states. The proximity effect is essentially a vibronic interaction between these two states that induces potential energy distortions and geometric displacements along vibrationally active out-of-plane (OOP) bending modes, providing an efficient, radiationless decay pathway to the ground state (internal conversion). Consequently, these molecules tend to have low quantum yields of fluorescence and short fluorescence lifetimes. As will be seen shortly, this is certainly the case for psoralens.

Under the influence of the proximity effect, the efficiency of the $S_1 - S_0$ internal conversion becomes a strong function of the $S_1 (\pi\pi^*) - S_2 (\pi\pi^*)$ energy gap. The proximity effect also enhances the rates of other radiationless transitions, such as intersystem crossing to $T_1$, but does so to a lesser extent because the effect is in direct proportion to the magnitude of the energy gap separating the two states. Since the $S_1 - S_0$ gap is generally significantly larger than the $S_1 - T_1$ spacing, internal conversion tends to be accelerated to a greater degree than intersystem crossing. One consequence of this is that the branching ratio between internal conversion and intersystem crossing becomes very dependent upon the $S_1 (\pi\pi^*) - S_2 (\pi\pi^*)$ spacing.

The photophysical properties of a molecule are, in general, perturbed by the presence of a solvent. For molecules that exhibit the proximity effect, the implications of solvation are even more significant. The ability of a solvent to stabilize an excited state depends on the polarity of the excited state and of the solvent medium. The more polar state of $S_1$ and $S_2$ will be preferentially stabilized by polar solvents, resulting in a change in the magnitude of the energy gap between these states. In some cases this solvation might even serve to invert the $\pi\pi^* - \pi\pi^*$ state ordering. In general, $\pi\pi^*$ states are more polar than $\pi\pi^*$ states, so psoralens would be expected to possess larger $S_1 - S_2$ energy gaps in polar solvents.
Using Fluorescence to Study the Proximity Effect

If polar solvents increase the $S_1 - S_2$ energy gap in psoralens, the fluorescence emission properties should provide insight into these changes. An increase in the energy gap would decrease the vibronic coupling between the $S_1$ and $S_2$ states, and the quantum yield of fluorescence would increase as a result. If solvent reorientation occurs on a time scale similar to the fluorescence lifetime of the psoralen, one might expect to see an emission wavelength dependence in the fluorescence decay. Emission from the solvated molecule would be red-shifted relative to the "free" fluorophore, and the fluorescence lifetime would be expected to be somewhat longer at these wavelengths since the larger $S_1 - S_2$ energy gap should serve to retard processes such as internal conversion and intersystem crossing that compete with fluorescence to depopulate the excited state.

Results of Previous Investigations

A consistent understanding of the fluorescence properties of psoralens has not yet been achieved. Sasaki et al. reported an increase in 8-MOP's maximum emission wavelength and fluorescence intensity with increasing solvent polarity, consistent with the proximity effect. Later, Menger et al. determined the quantum yields for fluorescence and phosphorescence of 8-MOP at 77 K, and found them to be 0.019 and 0.13, respectively. These values are quite small, consistent with the enhancement of internal conversion that one would expect from the proximity effect. This work was followed by a study by Matsumoto and Isobe that systematically investigated the effects of solvents on the absorption and fluorescence emission spectra of several psoralens. Their data agreed with previous results, and showed that electron-donating substituents (such as alkoxy groups) red-shifted the fluorescence emission spectra, presumably due to stabilization of the $\pi\pi^*$ state. The fluorescence yield was also observed to increase in hydroxylic solvents, and this is probably
attributable to two factors: a blue-shift in the nπ* state caused by ground-state hydrogen bonding between the psoralen and solvent, and a red-shift in the ππ* state due to solvent stabilization of this state. These two factors both serve to increase the energy gap between S1 and S2, and therefore reduce the proximity effect. The quantum yield of fluorescence for 8-MOP in ethanol at ambient temperature was later determined by two different groups and found to be 0.00136 or 0.0020.10 These values agree well with the earlier measurements of Matsumoto and Isobe for 8-MOP in methanol. They found Φf = 0.0012 in this solvent, Φf = 0.0002 in acetonitrile, and the quantum yield in cyclohexane was too small to measure. These quantum yield data are also consistent with the predictions of the proximity effect. Paik and Shim found the correlations between solvent polarity and the fluorescence emission maxima and quantum yields to be sufficiently reliable to use these properties as probes of the local polarity of the microenvironment experienced by psoralens associated with various micelles.11

The situation becomes much less straightforward when one turns to discussion of the time-resolved fluorescence decays of 8-MOP and similar psoralens. Several groups have reported attempts to analyze the fluorescence decays of these molecules, yet no consensus has been reached and most groups confess to having little or no insight into the nature of the observed decays or trends in these data. As early as 1979, Menger et al. reported the fluorescence lifetime of 8-MOP in neutral, aqueous solution measured by two independent techniques. They reported a value of 2.6 ± 0.6 ns using a nitrogen laser/oscilloscope apparatus, and a lifetime of 1.6 ± 0.6 ns using a TCSPC instrument. The form of the fluorescence decay was not published, however, and no details were given concerning the measurement and analysis of the data.

A more thorough investigation was published by Lai et al. in 1982, and TCSPC decays for 8-MOP in ethanol at several temperatures and wavelengths were presented.6 Lifetimes at 298 K were also reported for 8-MOP in acetonitrile (< 0.10 ns), and
trifluoroethanol (2.30 ns). The fluorescence lifetime could not be measured under these conditions in 3-methylpentane, as the intensity was too weak to be detected. In ethanol, these authors found the fluorescence decay to be exponential at high temperatures (e.g., 298 K) and long wavelengths (e.g., 540 nm) but the decays became non-exponential upon cooling or when the fluorescence was monitored at shorter wavelengths (e.g., 410 nm). Lai et al. reported two-exponential fits to these data, although they did not report any values of reduced chi-square or other statistical parameters by which the adequacy of the fits could be gauged. The lifetime at 297 K, monitored at 540 nm, was reported to be 0.39 ns, increasing to 3.22 ns at 233 K. An additional, short-lived (c. 0.70 – 1.15 ns) component was required to fit data collected at lower temperatures, although the fitted lifetimes showed no clear trends with respect to their temperature-dependence. The wavelength-dependence was not explicitly tabulated, although visual inspection of the published figure suggests that a short-lived component grew in importance relative to a long-lived (3.22 ns) component as the monitoring wavelength was blue shifted from 540 nm to 410 nm at 233 K. It is very important to note that these TCSPC data were collected to only ~ 1000 CPC across less than 256 channels. Such decays lack the necessary precision to distinguish a two-exponential decay from more complex behavior. Furthermore, no indication was given of the TCSPC instrument’s time resolution.

Later studies of psoralen and 8-MOP fluorescence decays in ethanol and water at room temperature performed by Andreoni et al. suffer from similar limitations of data quality and ambiguities in methodology.¹²⁻¹⁴ Their 8-MOP data required three-exponential fits in ethanol (0.4 ns, 71%; 1.5 ns, 23%; 7 ns, 6%) and two-exponential fits in water (1 ns, 97%; 4 ns, 3%). It should be noted that they reported the percent contributions as being “relative initial amplitudes” without further explanation, so it is not clear whether these values refer to true fractions of the steady-state fluorescence intensity as given by $\alpha_i \tau_i / \sum \alpha_i \tau_i$. Again, only 200 channels of data were collected and the FWHM of the instrumental response function was
reported to be 240 ps, so the data are of insufficient quality to resolve additional components, especially short-lived components. As far as interpreting their results, Andreoni et al. could only say that "the multiexponential fluorescence decay of linear furocoumarins seems to be related both to the structure of the molecule and to the solvent environment. On the basis of the reported results, however, a simple explanation for this behaviour is rather difficult."^{13}

Despite these uncertainties, Andreoni et al. suggested that the multi-exponential decay might be due to independent fluorescence emission from the furan and coumarin moieties of the molecule,^{12} although this seems unlikely to us. Furthermore, they noticed no emission wavelength dependence on the amplitudes and lifetimes recovered for 8-MOP in ethanol,^{13} a conclusion that cannot be reconciled with the work of Lai et al. discussed previously.^{6} We have no reason to question their observation that the concentration of 8-MOP did not affect the recovered amplitudes and lifetimes.^{14} This fact, combined with their report that the Beer-Lambert law was obeyed across the entire concentration range, was interpreted to indicate that aggregation was not a significant issue with respect to the observed fluorescence behavior. A study of 5-methoxypsoralen (5-MOP) by Sae Melo and coworkers in 1988 concluded that the presence of dissolved oxygen reduced the quantum yield and lifetime of fluorescence by only 2% or so.^{16} This suggests that acceptable fluorescence decays of psoralen solutions may be measured in air-equilibrated solutions.

Current Understanding

In summary, trends in the steady-state fluorescence data for psoralens appear to be adequately described by the proximity effect and straightforward solvent polarity considerations. Observed changes in the fluorescence quantum yields can be satisfactorily explained in terms of perturbations of the relative energies of the \( S_1 (\pi\pi^*) \) and \( S_2 (\pi\pi^*) \) states. The proximity effect results in accelerated internal conversion induced by vibronic interactions between closely-spaced states. Therefore, any factor that increases the spacing
between these two states reduces the impact of the proximity effect and increases the quantum yield of fluorescence. Fluorescence is generally emitted from a molecule's lowest excited singlet state. Since the $S_1 (\pi\pi^*)$ states of psoralens are more polar than their $S_0$ counterparts, polar solvents will stabilize the $S_1$ states to a greater extent than $S_0$ states, and the observed fluorescence will exhibit maxima at longer wavelengths in more polar solvents as a consequence of these reductions in the $S_1 - S_0$ energy gaps.

On the other hand, the fluorescence lifetime data have thus far defied a coherent interpretation. While it seems likely that neither dissolved molecular oxygen, nor sample aggregation is responsible, psoralens tend to exhibit highly non-exponential fluorescence decays under most conditions. Trends have proved to be difficult to interpret, especially given the relatively low precision of the published TCSPC data. Our goals were therefore (1) to measure high-precision TCPSC fluorescence decays for several psoralens in various solvents and at several wavelengths and (2) to attempt to analyze these data in a physically meaningful or (at least) a statistically acceptable fashion. The ultimate goal, of course, is a unified understanding of the photophysics and photochemistry of psoralens that will explain their phototherapeutic activity and facilitate the intelligent design of useful derivatives.

Experimental

Instrumentation and Samples

Time-Correlated Single-Photon Counting (TCSPC) measurements were carried out using the instrument described in Chapter 2. Excitation was at approximately 300 nm, using the frequency-doubled output of an R6G dye laser, and the fluorescence decays were monitored at 400, 450, 500, 550, or 580 nm with emission bandwidths of 2 – 5 nm. The absorbance at the excitation wavelength was kept between 0.1 – 1.0 for all solutions, and was
typically less than 0.25. The repetition rate of the laser system was 1 MHz, and the FWHM of the instrumental response function was approximately 80 ps. Data were collected into 512 channels to a precision of at least 10,000 CPC. The full scale time base was typically 16 – 20 ns, although many experiments were confirmed using a time base of ~ 3 ns full scale (data not shown).

In addition to 8-MOP, three other psoralens and two related compounds were investigated. The psoralens included an analog of 8-MOP, brominated at the 5 position. We will refer to this molecule as Br-MOP. The other psoralen analogs replace the methyl group in 8-MOP and Br-MOP with a quaternary ammonium substituent designed to enhance aqueous solubility. These two analogs will be referenced as 8c and 8b, respectively. The two related compounds, visnagin and khellin, are technically not psoralens although their structures are quite similar. There is a report in the literature that visnagin might be less mutagenic than psoralens such as 8-MOP or 5-MOP.17

Visnagin, khellin, and 8-MOP were obtained from commercial sources, while the other three molecules were synthesized in the Platz laboratory. The structures of all of these molecules are provided in Figure 5.3. Decays were collected for 8-MOP, visnagin, and khellin in n-pentane, acetonitrile, methanol, and water. Since Br-MOP was not sufficiently soluble in aqueous solution, decays were measured in n-pentane, acetonitrile, and methanol. Solutions of 8b and 8c prepared in methanol, water, and PBS buffer solution. All of the TCSPC data were obtained at ambient temperature.

Data Analysis

With the possible exception of visnagin in methanol, all of these samples displayed non-exponential decay kinetics in all solvents at all wavelengths. When the data were fit to a discrete sum of exponentials, at least three components (and usually four) were required to obtain acceptable (< 1.4) values of the reduced chi-square statistic. The data were
independently analyzed using the ESM method of lifetime distribution analysis. In this case the data were fit to a sum of 100 exponentials, logarithmically spaced between 0.010 – 10 ns, with the first and last lifetimes set to 0.001 and 100 ns, respectively, to allow for contributions of scatter or dark counts. Data for visnagin in methanol were analyzed using lifetimes spaced between 0.010 – 40 ns to improve the resolution of its longer lifetime components. These results were then summarized by visually inspecting the distributions and assigning each mode to a “bin” and summing up the fractional contributions of each mode to the steady-state fluorescence intensity. In this way, the results of the ESM lifetime distribution analyses could be compared with the fits to sums of exponentials in a straightforward fashion. Hence, we have chosen to call these results “Discrete Component Equivalents,” or DCEs. In most cases the analyses gave similar results, suggesting that a sum of four discrete exponentials might be an adequate model to describe these data. There is no a priori reason to believe that the ESM distributions would have contained four or fewer modes if this were not the case.

In analyzing these data, it was also helpful to calculate the pre-exponential weighted mean lifetime as defined by Carraway, Demas, and DeGraff.

\[
\tau_m = \frac{\Sigma \alpha_i \tau_i}{\Sigma \alpha_i}
\]

This value of the “average lifetime” is useful for evaluating trends for an entire decay. As long as a statistically acceptable fit is used to calculate \( \tau_m \), its value may be accurately calculated, regardless of whether the decay model employed bears any resemblance to the “true” decay model.

Results

The fluorescence decay kinetics of psoralens and related compounds are quite complex. As may be seen from Figure 5.4, the fluorescence of 8-MOP is highly non-exponential and strongly solvent-dependent. It is also obvious from Figures 5.5–5.8 that the
form of the decay is also dependent upon the monitoring wavelength. Figures 5.7 and Figures 5.9 – 5.12 testify to the fact that even when the fluorescence is monitored at the same wavelength, in the same solvent, the form of the decay is unique to each species.

It is easy to see why previous investigators have struggled to interpret the time-resolved fluorescence of these systems. Especially when the data are analyzed using the traditional sum-of-exponentials approach, one wonders whether the parameters recovered correspond in any meaningful way with the underlying photophysics. The possibility that the fit simply represents trade-offs between correlated fitting parameters and that the recovered decay law is simply a meaningless “curve-fit” to the data is difficult to discount. While our results must still be viewed as a preliminary attempt to sort through the complexity of time-resolved psoralen fluorescence, we believe that the additional data-fitting flexibility provided by lifetime distribution analysis makes genuine trends in the fits both more apparent and more believable. As will be seen in the discussion that follows, the results of lifetime distribution analyses often gave DCEs that were in excellent agreement with fits to three or four discrete components. This strengthens our confidence in both approaches, as we are not aware of an a priori reason, given the differences in the fitting algorithms, to expect the two methods to converge on similar results unless the results represent genuine models to the data. The following sections explain the data that is presented graphically in Figures 5.4 – 5.85. The individual figures will usually not be referenced from within the text.

8-MOP

To our knowledge, 8-methoxypsoralen (8-MOP) is the only molecule that we investigated for which the results of fluorescence lifetime measurements have been previously reported. Its decay has been analyzed using 1 – 3 exponential terms. We observed non-exponential behavior for 8-MOP’s fluorescence under all of the experimental conditions explored in this investigation. The data consistently required at least three or four
components for an adequate fit, although we were able to obtain a satisfactory fit to two exponentials for one data file (8-MOP in acetonitrile, $\lambda_{em} = 450$ nm, $\chi^2 = 1.24$). Two of the aqueous samples (400 nm and 450 nm) and one sample prepared in n-pentane (550 nm) might be better described by five or six components, as evidenced by their lifetime distributions. Acceptable values of the reduced chi-square were obtained for four-exponential fits to these data, however. The results of the sum-of-exponential fits for 8-MOP (as well as the other molecules and ions studied in this chapter) and the associated pre-exponential weighted mean lifetimes calculated from these fits are tabulated in Table 5.1.

The decay in n-pentane is dominated (up to 82%) by a $3.7 - 5.7$ ns component at 400 nm and 450 nm, with a substantial secondary contribution (up to 36%) from a component with a lifetime between $0.9 - 2.8$ ns. These components are still present at longer wavelengths (500 nm and 550 nm), but a short-lived component gains importance. This component has a lifetime of less than 60 ps, yet accounts for up to 43% of the overall steady-state intensity at 550 nm. Three-component fits suffice to describe the decay at all wavelengths, although the fit to the 550 nm data is described much better by fits to four or more components, as judged by the value of the reduced chi-square. This conclusion is supported by the fits to lifetime distributions that suggest that a component between $0.23 - 0.41$ ns is also present at all wavelengths, and its relative importance increases with increasing wavelength. This component accounts for 18% of the intensity at 550 nm. The four-exponential fit to the 550 nm data agrees with this conclusion, and attributes 23% of the decay to an 0.23 ns component. The pre-exponential weighted mean lifetime summarizes all of these observations, as the value of $\tau_M$ decreases steadily from 1.4 ns at 400 nm to 0.04 ns at 550 nm. Despite the complexity of the decay, it is clear that the lifetimes and fractional contributions of the long-lived components decrease at the expense of increased fractional contributions of shorter lived components at longer emission wavelengths.
8-MOP's fluorescence decay in acetonitrile is more easily summarized. The data are adequately described by three components, regardless of the emission wavelength or fitting method employed. A short-lived component with a lifetime between 30 – 50 ps is responsible for the majority (58 – 61%) of the intensity and a long-lived component (4.0 – 6.5 ns) accounts for most of the remainder (33 – 41%). A minor component (1 – 9%) with an intermediate lifetime (0.86 – 0.96 ns) gains importance at longer wavelengths, yet it is very weak or absent at 450 nm. The fractional intensity of this intermediate component comes at the expense of the long-lived component. Nevertheless, $\tau_m$ is essentially constant (0.058 – 0.062 ns) at all wavelengths.

The decay in methanol is also relatively straightforward. In this case the intensity is dominated (60 – 79%) by a component with a lifetime of approximately 0.38 ns. Adequate description of the remainder of the intensity, however, requires three additional components. The longest lived of these (6.5 – 10 ns) is the most important at 450 nm and 500 nm, but the 0.9 – 1.8 ns component eclipses its contribution at 550 nm. The remainder (3.0 – 6.5%) of the intensity is due to a short-lived (~30 ps) component at all wavelengths. In all of this, the value of $\tau_m$ remains relatively constant, between 0.23 – 0.27 ns. A four-exponential fit to 8-MOP's decay in methanol, monitored at 550 nm, is compared with an ESM distribution (100 exponentials) for the same file in Figure 5.86. The value of the reduced chi-square and the plot of the weighted residuals are quite comparable, and this observation is quite general for most of the psoralen decays.

While at least four components are required to describe 8-MOP's fluorescence behavior in aqueous solutions, the 0.8 – 1.3 ns component is clearly the most important, accounting for 31 – 91% of the total steady-state intensity. The fractional contribution of this component increases steadily with the emission wavelength. A long-lived (3.7 – 6.6 ns) component accounts for up to 30% of the decay at 400 nm, but gives rise to only 3 – 4% of the intensity between 550 – 580 nm. Similarly, a short-lived component (0.13 – 0.47 ns) is
responsible for up to 23% of the intensity at 400 nm, but is reduced to a 3 – 4% contribution between 550 – 580 nm. The fourth component is very short-lived (< 65 ps) and is also important only at 400 nm, where it accounts for up to 15% of the intensity. At other wavelengths its contribution is less than 6%. In summary, 8-MOP’s decay in water is characterized by an increasing fractional contribution of the 0.8 – 1.3 ns component at the expense of the other three components as the emission wavelength is increased from 400 – 580 nm. The value of $\tau_m$ is 0.12 ns at 400 nm and reaches a maximum of 0.88 ns at 550 nm.

Upon assessing the solvent-dependence of 8-MOP’s fluorescence, it is clear that the lifetime of the major component increases with the solvent’s polarity and/or hydrogen-bonding capability in acetonitrile, methanol, and water. This is also reflected in the values of $\tau_m$ (averaged for all wavelengths) for these solvents: acetonitrile (0.06 ns), methanol (0.25 ns), and water (0.36 ns). The decays in $n$-pentane are complex, and do not conform to this trend. Indeed, the pre-exponential weighted mean lifetime is longest in $n$-pentane, being approximately 0.38 ns.

**Br-MOP**

Br-MOP is structurally identical to 8-MOP, except for the fact that it is brominated at the 5 position. This one change was enough to prevent it from being soluble in water, and to induce some significant differences in its fluorescence decay properties, especially in $n$-pentane.

Three exponentials are required to fit Br-MOP’s fluorescence decays in $n$-pentane at all wavelengths. At 550 nm, four components give a significantly improved fit. In every case, the dominant component is very short-lived (15 – 34 ps) and comprises 49 – 64% of the steady-state intensity. At 400 nm a long lived (1.3 – 3.2 ns) component contributes up to 34% of the intensity, but the importance of this component trails off quickly as the emission wavelength is increased. By 550 nm it accounts for only about 1% of the total decay. Of
increasing importance at longer wavelengths is a component with a lifetime between 0.13 – 0.33 ns. While responsible for less than 5% of the decay at 400 nm, this component accounts for up to 38% of the intensity at 550 nm. The fourth component, with an intermediate lifetime of 0.54 – 0.89 ns, is responsible for the remainder. It should be noted that there is some discrepancy between the three-component fits to these data and the DCEs obtained from lifetime distribution analysis. While the data are statistically well-described by three-component analyses, the fitting program appears to suffer from a correlation of parameters with the low-intensity, long-lifetime components. On the other hand, the parameters for the short-lived, high-intensity components are in reasonable agreement with the DCEs taken from the lifetime distributions. The value of $\tau_m$ is essentially constant between 25 – 39 ps for all of the $n$-pentane solutions.

In acetonitrile, the Br–MOP decays were again adequately described by three-component fits. In this solvent, the dominant component (64 – 87%) had a lifetime between 77 – 110 ps. The lifetime of this component may increase slightly with increasing wavelength. A component between 0.15 and 0.40 ns becomes increasingly more important in terms of fractional contribution and shifts to longer lifetime as the wavelength increases. This latter observation is true of the three-component fits and the DCEs, but the trend in fractional contribution is reversed for the three-component fits. This anomaly is probably due to the width of the underlying lifetime distributions (Figure 5.13). Fitting these data to discrete components seems to force the parameters to adopt artifactual values, due to the highly correlated nature of these parameters. The third component (1.2 – 3.2 ns) is responsible for about 5% of each decay, and its lifetime increases with wavelength. The value of $\tau_m$ tracks the behavior of the major component, increasing from 97 ps to 112 ps from 450 nm – 550 nm.

Four exponentials are required to describe Br–MOP’s fluorescence in methanol, although here again a full 58 – 78% of the intensity is due to one component. This component has a lifetime between 0.25 – 0.50 ns, with a peak intensity near 0.35 ns. This value does not
appear to have an associated wavelength dependence. A long-lived component (5 – 10 ns) accounts for 9 – 16% of the decay. Its contribution decreases with increasing wavelength. A lifetime with an intermediate value (1.0 – 2.8 ns) is also present to a similar extent (3 – 16%), but also becomes less important as the wavelength increases. A short-lived component contributes 9 – 13% of the total intensity, and its importance also decreases with increasing wavelength. Its lifetime is difficult to pinpoint, however. At 450 nm (where it is the most important) the lifetime distribution divides it into two modes: one at extremely short lifetime (10 – 15 ps) and another at ~ 110 ps. The distributions at the other two wavelengths smear this intensity out across the region spanning these two extremes. Perhaps predictably, the three-component fit yields a “compromise” lifetime between 44 – 57 ps. While Br–MOP’s fluorescence behavior in methanol is complex, it can be summarized by noting that the dominant component at ~ 0.35 ns gains intensity as a function of wavelength at the expense of the other three components. This has the effect of imposing a slight growth on the value of $\tau_M$, which increases from 0.23 ns at 450 nm to 0.26 ns at 550 nm.

The solvent-dependence of Br–MOP’s time-resolved fluorescence is similar to that of 8–MOP, the exception being the behavior in n-pentane. Once again, the lifetime of the major component increases with solvent polarity. The wavelength-averaged value of $\tau_M$ goes from 0.028 ns in n-pentane, to 0.105 ns in acetonitrile, to 0.248 ns in methanol.

8b

The structure referred to as 8b in this work is structurally identical to Br–MOP, except for the fact that the methoxy group at the 8 position of Br–MOP has been replaced with a longer alkoxy substituent that is terminated by a quaternary ammonium moiety that gives the species a positive charge. Consequently, 8b is less soluble in nonpolar solvents, but more soluble in water and PBS buffer. As such, it would be a more promising candidate for antiviral applications in biological matrices than Br–MOP.
In methanol, four components are required to fit 8b's fluorescence decays. Between 58 - 80% of its intensity in this solvent arises from a component with a lifetime between 0.2 - 0.3 ns. The fractional contribution of this lifetime increases with wavelength. A long-lived component (4 - 10 ns) accounts for up to 20% of the decay at 450 nm, but its significance diminishes to less than 6% at 550 nm. On the other hand, a short-lived component (25 - 140 ps) accounts for 12 - 17% of the intensity at all three wavelengths and its lifetime increases with wavelength. The remainder of the fluorescence (2 - 8%) arises from a component with a lifetime between 0.9 - 2.0 ns. Once again, the value of $\tau_M$ tracks the behavior of the major component. At 450 nm, $\tau_M = 0.12$ ns but at 550 nm $\tau_M$ has increased to 0.21 ns.

In aqueous solutions, an adequate fit to the fluorescence of 8b also requires at least four components. Between 29 - 81% of the decay arises from a component with a lifetime of 0.6 - 1.1 ns. The fractional contribution of this component increases significantly going from 450 nm to 500 nm and remains constant at 550 nm. Of secondary importance is a long-lived (5.0 - 6.6 ns) component that is actually of similar intensity (32%) to the major component at 450 nm, but accounts for less than 10% of the decay at higher wavelengths. A third component (0.18 - 0.35 ns) gives rise to 10 - 19% of the fluorescence. While there is no clear trend in its fractional contribution, its lifetime clearly decreases with increasing wavelength. A fourth component has a very short lifetime (0.01 - 0.08 ns) and accounts for less than 2% of the decay. The exception is for the 450 nm data, in which this component contributes up to 10% of the intensity. A fifth component also appears in the 450 nm lifetime distribution between 1 - 2 ns. Once again we observe a trend toward higher $\tau_M$ at longer wavelengths.

As might be expected, the fluorescence behavior in PBS buffer is very similar to that in aqueous solution. Once again, an 0.5 - 1.3 ns component accounts for 31 - 69% of the decay, and this fraction increases with wavelength. The long-lived 5 - 8 ns component is also present to the extent of up to 29% at 450 nm, but its importance again diminishes to as low
as 12% at 550 nm. The third component (0.16 – 0.37 ns) is also duplicated, accounting for somewhere between 11 – 29% of the total decay, depending on the fitting methodology. The short-lived component is somewhat more important in PBS. It has a lifetime between 15 – 45 ps and accounts for 5 – 9% of the decay. A component near 2 ns again comprises up to 28% of the 450 nm decay, yet contributes only 15% to the 500 nm decay and is completely absent from the 550 nm decay. As was the case for the aqueous solutions, the fluorescence of 8b in PBS buffer can be summarized as an increase in the fractional contribution of the major component at the expense of the other components. Once again, the trend is toward larger values of \( \tau_M \) at long wavelength.

Although the range of polarities explored for solutions of 8b is smaller than for 8-MOP or Br-MOP, we once again observed an increase in the lifetime of the major component upon going from methanol to water or PBS.

8c

The structure that we have termed 8c is identical to 8b except for lacking the bromine substituent at the 5 position of the psoralen ring system. It is therefore analogous to 8-MOP, but more water-soluble. Its fluorescence decay characteristics are similar to the other psoralens.

In methanol, at least four components are necessary for an acceptable fit to the data. The dominant (52 – 66%) component has a lifetime of 0.26 – 0.30 ns. A long-lived (6 – 11 ns) component accounts for up to 27% of the decay at 450 nm, but this fraction decreases with wavelength to about 7% at 550 nm. The intensity is directed primarily toward a component with a lifetime between 0.9 – 1.5 ns that contributes as little as 7% at 450 nm, but up to 23% at 550 nm. The fourth component in methanol has a short lifetime (26 – 180 ps). The two fitting methodologies disagree on whether the fractional contribution of this component increases or decreases with wavelength, but both techniques indicate an increase in the
lifetime of this component with wavelength. In the lifetime distributions, the lifetimes span 50 - 180 ps, but in the discrete exponential approach the fitted lifetime is 26 - 71 ps. 8c in methanol follows the trend for psoralens in showing an increase in $\tau_m$ from 0.15 ns at 450 nm to 0.28 ns at 550 nm.

Aqueous solutions of 8c require four-component fits at 450 nm, but three components suffice at 500 nm or 550 nm. A component with a lifetime of approximately 1.2 ns dominates the decay, accounting for 66 - 92% of the intensity, with the fraction increasing as a function of wavelength. This increase in intensity comes at the expense of the long-lived component (3.7 - 7.0 ns). The contribution of this component diminishes from up to 18% at 450 nm to as low as 5% at 450 nm. With respect to short lifetimes, a component found in the 450 nm lifetime distribution (60 - 80 ps) and in the 500 nm lifetime distribution (~ 120 ps) coalesces in the 550 nm distribution with another component, found between 200 - 300 ps in the 450 nm distribution and between 300 - 400 ps in the 500 nm distribution. The union of these two components has a lifetime between 100 - 200 ps. This result is mirrored in the fit to discrete lifetimes, in which the 500 nm and 550 nm data can be fit to either three or four exponentials with success, and the three-component fit corresponds to a compromise between the two shortest lifetimes from the four-exponential fit. It is likely that this observation stems not from a change in the photophysical properties of 8c, but from a loss of information about these (probably distinct) components at long wavelength since their aggregate contribution to the overall intensity is reduced from roughly 18% at 450 nm to as low as 5% at 550 nm. None of these complexities change the familiar trend in $\tau_m$, which increases from 0.32 ns at 450 nm to 0.91 ns at 550 nm.

The data for 8c in PBS were acceptably described by three exponentials, but four distinct modes are evident in the lifetime distributions for these decays. As was true for the aqueous samples, the dominant component has a lifetime of 0.9 - 1.7 ns and its fractional contribution increases from 79% at 450 nm to 91% at 550 nm. In this case, both methods of
data analysis suggest that the lifetime of this component decreases with increasing wavelength. The intensity increase again comes at the expense of a long-lived (2.3 – 4.0 ns) component for which the contribution decreases from approximately 9% to roughly 2% with increasing wavelength. A shorter-lived component (0.15 – 0.30 ns) also contributes less as the emission wavelength is increased, being reduced from a value as high as 12% at 450 nm to as low as 5% at 550 nm. The remainder (~2%) of the decay is due to short lived (~30 ps) contributions. This last component is absent from the three-exponential fits. It is likely that these fits artificially shorten the lifetime of the 0.15 – 0.30 ns component to compensate for this. This is yet another example of lifetime distribution analysis providing greater flexibility in parameter space for complex fluorescence decays. As was true for every other psoralen, $\tau_m$ increased with increasing wavelength, from 0.70 ns to 0.87 ns.

In terms of solvent-dependence, 8e is similar to the other psoralens studied. Its major component has a longer lifetime in the more polar, aqueous environment than in methanol. Similarly, the $\tau_m$ averaged over all of the wavelengths studied, increases with solvent polarity.

Khellin

Khellin is a molecule that, while technically not a psoralen derivative, possesses a very similar ring structure. It differs from visnagin only in the number of methoxy substituents. Khellin has two such substituents, oriented para- to one another across the central aromatic ring. Visnagin has one methoxy group, located on the same side of the ring system as the carbonyl group (Figure 5.3).

As was true for the psoralens, khellin's fluorescence in n-pentane is best described by at least four components, or modes. At short wavelengths (400 nm and 450 nm) the decay is dominated by a very short-lived (20 – 30 ps) component that comprises up to 66% of the total intensity. As the monitoring wavelength is increased, however, the lifetime of this
component increases somewhat (up to ~ 50 ps) and its relative contribution is eclipsed by two longer-lived components. The first of these (0.16–0.28 ns) is present at 400 nm and accounts for about 18% of the decay. Its importance increases with wavelength, however, and reaches a maximum of nearly 50% at 500 nm before falling off somewhat at 550 nm. The second, somewhat longer-lived (0.7–1.7 ns) component accounts for less than 7% of the 400 nm decay, but increases in importance monotonically to a nearly 38% contribution at 550 nm. The fourth component has a lifetime of 1.9–6.3 ns and is responsible for 10–23% of the decay. There is no clear trend for the lifetime or fractional contribution of this component.

In summary, khellin’s behavior in n-pentane essentially amounts to a decrease in the importance of a short-lived component and a concurrent increase in the importance of two longer-lived components as the emission wavelength increases. As one might expect, this results in $\tau_M$ increasing from 0.018 ns to 0.159 ns.

In acetonitrile, khellin’s fluorescence can be successfully fit to only two exponentials. The majority (80–89%) of the intensity is due to a 1.4–1.5 ns component, whose lifetime and relative contribution increase slightly with increasing wavelength. The remainder of the decay is due to an 0.5–0.8 ns component that also experiences a slight increase in its lifetime with increasing wavelength, but whose relative contribution (obviously) decreases somewhat with increasing wavelength. The net effect on $\tau_M$ is an increase from 1.17 ns to 1.30 ns from 450 nm to 550 nm.

In methanol, the vast majority of khellin’s fluorescence decay can be discussed in terms of three components. At 450 nm, a long-lived (6–7 ns) component accounts for up to 75% of the intensity, but the contribution of this component decreases drastically to 13% at 550 nm. The lifetime of this component remains constant, however. To compensate for the reduction in relative intensity experienced by the long-lived component, an 0.28–0.31 ns component increases in importance from 14% to 81% of the steady-state intensity. The only other significant component is short-lived (15–70 ps) and its relative contribution decreases.
with wavelength, although its lifetime seems to increase. The effects observed for khellin in methanol appear to have opposing effects on $\tau_{\text{eff}}$, as its value displays no clear trend for this system.

The fluorescence decay of khellin in water is difficult to summarize. The lifetime distributions are quite broad and exhibit up to six distinct modes. Nevertheless, values of the reduced chi-square are quite good for four-exponential fits to these data, so it is not clear upon which paradigm the discussion should be based. If some minor features in the lifetime distributions are overlooked, one can construct DCEs that reasonably approximate the results of the four-exponential fits, and some trends may be discussed. Specifically, the two most important components are long-lived. A $1.2 - 2.0$ ns component comprises up to 50% of the decay at 450 nm, but this is reduced to $\sim 27\%$ at 550 nm. An even longer ($4.3 - 6.5$ ns) component accounts for almost 30% of the 450 nm decay, but its importance is also diminished to $\sim 16\%$ in the 550 nm decay. The two shorter-lived components experience concomitant increases in relative intensity. The most important of these has a lifetime of $0.1 - 0.7$ ns and contributes 16% of the intensity at 450 nm, increasing to 30% at 550 nm. The short-lifetime component has a lifetime of $10 - 70$ ps, and increases from less than 7% intensity at 450 nm to up to 32% intensity at 550 nm. As one might expect, the decreasing importance of the long-lived components and the increasing importance of the short-lived components causes $\tau_{\text{M}}$ to decrease with decreasing wavelength.

There are no obvious trends to discuss for the solvent-dependence of khellin’s time-resolved fluorescence.
Visnagin

As mentioned previously, visnagin is nearly identical to khellin. The only difference in their structure being an additional methoxy substituent on the central aromatic ring of khellin. One would therefore expect visnagin’s fluorescence behavior to resemble that of khellin. For the most part, this is what we observed.

In n-pentane, visnagin’s fluorescence decays are extremely complex, requiring at least four components to obtain a satisfactory fit. A component with a lifetime of 0.10–0.33 ns accounts for 45–49% of the decay from 450 nm – 550 nm, although it only contributes 19% at 400 nm. The lifetime of this component also increases with wavelength. A long-lived (2.4–3.5 ns) component is responsible for 38% of the decay at 400 nm, but the importance of this mode is reduced to less than 10% at longer wavelengths. The remainder of the intensity is nearly equally divided between a short-lifetime (18–80 ps) component and an intermediate-lifetime (0.5–1.2 ns) component. The relative fractions of these two components seem to exchange intensity with one another, with the short-lived component going through a maximum near 450 nm. The lifetime of the short-lived component increases monotonically with wavelength while the 0.5–1.2 ns lifetime decreases with wavelength. The pre-exponential weighted mean lifetime is 0.068 ns at 400 nm, goes through a minimum of 0.059 ns at 450 nm, and then increases to 0.16 ns at 550 nm.

Visnagin’s fluorescence is similarly complex in acetonitrile, a fact that is somewhat surprising given the fact that khellin’s fluorescence was adequately described by two exponentials in this solvent. In visnagin’s case, at least three, and probably four exponentials are required to obtain an acceptable value of the reduced chi-square statistic. The dominant component is long-lived, with a lifetime between 6–8 ns. The relative fraction of this component increases from 24% at 450 nm to 57% at 550 nm. A component at 3.3 ns appears only in the 450 nm distribution, but accounts for 25% of that decay. It seems likely that this is an artifact of a poor ESM fit to these data, and that this component should actually be
merged with the 1.4 – 2.2 ns component that is found for all three wavelengths. If these two components are thus binned together, the contribution of this component would be observed to decrease from 54% at 450 nm to 25% at 550 nm. The remainder of the decay can be attributed to an 0.38 – 0.50 ns component that accounts for 15 – 18% of the intensity and a 25 – 73 ps component that contributes 2 – 5% of the intensity. Both of these components increase in their relative importance with increasing wavelength, although this conclusion should be stated tentatively as the effects are slight. The lifetime of the short-lived component clearly increases with wavelength, however. The overall effect on $\tau_M$ is a net increase from 1.00 ns at 450 nm to 1.47 ns at 550 nm.

Of all of the systems studied in this chapter, visnagin in methanol stands out in its simplicity. The decays are well-described as a sum of two exponentials, and greater than 95% of the intensity is due to a single component with a lifetime between 4.7 – 8.6 ns. The remainder of the decay can be accounted for by a component with a lifetime between 0.7 – 3.0 ns. If a third component is present, it is even shorter-lived (0.1 – 0.3 ns) and contributes less than 1% of the steady-state intensity. The fluorescence behavior of visnagin appears to be largely wavelength-independent from 450 nm – 550 nm.

Three components are required to describe visnagin’s fluorescence in aqueous solution. A 1.9 – 2.8 ns component dominates, and accounts for 56 – 71% of the decay. A long-lived (4.9 – 7.0 ns) component also makes a significant (26 – 39%) contribution to the steady-state intensity. The vast majority of the remainder of the intensity can be ascribed to an 0.35 – 0.41 ns component that is responsible for 3 – 5% of the decay. While the relative intensities of these components exhibit no clear wavelength-dependence, the lifetime of the major component decreases with increasing wavelength. This has the effect of lowering $\tau_M$ as a function of wavelength.
In general, the pre-exponential weighted mean lifetime (averaged over all wavelengths) increases with solvent polarity for visnagin. The values for $n$-pentane (0.096), acetonitrile (1.296), and water (2.057) follow a straightforward trend. Methanol solutions do not follow this pattern, however, as their wavelength-averaged $\tau_m = 5.4$ ns. The reason for this is not immediately apparent.

**Discussion**

We have successfully achieved the two goals set forth in the introduction to this chapter. We have measured high-precision TCSPC fluorescence decays for four psoralen derivatives and two related compounds and we have analyzed these data in three different ways to interpret these data in a fashion that is both statistically acceptable and reliable. Fitting the data to discrete sums of exponentials, ESM lifetime distributions, and calculating the pre-exponential weighted mean lifetime has been shown to be a robust suite of analysis techniques. The tools are complementary, as the weaknesses of any individual approach are compensated by the strengths of the others. Consequently, an interpretation that is consistent with all three results gains credibility, even when the experimental data are quite complex.

The ESM lifetime distributions provide the flexibility in parameter space necessary to permit the data to be described as a sum of many exponential components or to approximate continuous distributions of lifetimes. This prevents some of the errors in correlation of fitting parameters introduced by fitting the data to fewer exponentials than required by the photophysics of the system under investigation. On the other hand, fitting the data to a discrete sum of exponentials establishes the minimum number of lifetime components required to give a statistically acceptable fit to the data. This guards against over-zealous interpretation of ESM lifetime distributions, beyond the limitations imposed by the quality and nature of the experimental data. In other words, if the data are adequately
described by a sum of several exponentials, one should exercise caution in attributing significance to a lifetime distribution that “resolves” a significantly larger number of modes. Finally, the pre-exponential weighted mean lifetime possesses the inherent strengths and weaknesses of possessing a single value for a given decay. It can cut through the complexity to reveal overall trends in a series of non-exponential decay curves, yet it lacks the high information content of the other two approaches. Taken together, the three tools comprise a formidable data-fitting arsenal.

Following the discussion of data quality presented in Chapter 4, we conclude that future experiments would probably benefit from data precision of greater than 20,000 CPC and data resolution of at least 1024 channels, considering the complex nature of the lifetime distributions observed for these samples. Nevertheless, the data reported here are significantly more precise and of higher resolution than those published previously. More importantly, the fits to these data were reproducible, in general, and exhibited consistent trends. While we would expect higher quality data to “clean up” these observations, we are largely confident in the analysis presented here.

We have measured and analyzed the time-resolved fluorescence for a large matrix of samples, wavelengths, and solvents in this chapter. Nevertheless, our results permit several statements to be made of a general nature. First, and most importantly, we have finally harmonized the time-resolved fluorescence data of psoralens with the predictions made by the proximity effect. The proximity effect anticipates an increase in the observed fluorescence lifetime with increasing solvent polarity due to preferential stabilization of the $S_1 (\pi\pi^*)$ state relative to the less polar $S_2 (\pi\pi^*)$ state in these solvents. This has the effect of increasing the energy gap between $S_1$ and $S_2$, and therefore reducing the importance of the proximity effect. The rates of processes competing with fluorescence to depopulate $S_1$ (e.g. internal conversion and intersystem crossing) are reduced and the expected consequence is an increase in the observed fluorescence lifetime. We observed just such an increase in the fluorescence
lifetimes of all four of the psoralen derivatives that we investigated (8-MOP, Br-MOP, 8b, and 8c) as measured either by \( \tau_m \) or by the lifetime of the component that accounted for the majority of the steady-state intensity as a function of solvent polarity, consistent with the proximity effect (Figures 5.87 – 5.88). The lone exception to this trend was for 8-MOP in \( n \)-pentane. Given 8-MOP’s low quantum yield of fluorescence and low solubility in this solvent, it is possible that this one sample might have been subject to interference from a long-lived fluorescent impurity. It is also possible that the data are accurate, and that an as-yet-unexplained factor accounts for the anomaly. In any event, the rest of the data are unified in their agreement with the predictions of the proximity effect for psoralen derivatives.

Secondly, for all of the samples based on a psoralen ring system, the pre-exponential weighted mean lifetime increased as a function of emission wavelength in acetonitrile, methanol, water, and PBS buffer (Figures 5.89 – 5.92). This is also consistent with the predictions of the proximity effect, if one assumes that the long-wavelength fluorescence emission originates from a more solvent-equilibrated population of fluorophores. The process of solvation in a polar solvent should stabilize the \( S_1 \) state relative to both \( S_2 \) and \( S_0 \). The stabilization relative to \( S_2 \) reduces the importance of the proximity effect (discussed previously) and increases the observed fluorescence lifetime. The stabilization relative to \( S_0 \) reduces the energy gap between these two states, which serves to red-shift the fluorescence under these conditions.

As solvation is an extremely rapid process at room temperature, it is especially intriguing that for all six of the systems studied, in all solvents, the lifetimes of any short-lived (< 100 ps) components were observed to increase (or at least not decrease) at longer emission wavelengths. This is also consistent with a preferential observation of solvent-equilibrated fluorescence at longer wavelengths.
The data for khellin and visnagin, while largely consistent with one another, displayed behavior that was in general incompatible with and more complex than that of the psoralens. Apparently there is enough structural dissimilarity between the ring systems of these molecules to distinguish their photophysical behavior from that of psoralens. For example, while the pre-exponential mean lifetime for these compounds increased as a function of wavelength in n-pentane and acetonitrile, the effect on $\tau_M$ was ambiguous in methanol and its value actually decreased as a function of emission wavelength in aqueous solutions (Figures 5.93–5.94).

Finally, we note that our pre-exponential weighted mean lifetimes for 8-MOP are in reasonable agreement with the values of the fluorescence lifetime published previously for this molecule. A lifetime of less than 0.10 ns was reported for 8-MOP in acetonitrile. Our wavelength-averaged value of $\tau_M$ for 8-MOP in acetonitrile was 0.061 ns. We did not find any references to 8-MOP's fluorescence lifetime in methanol, but the literature value for its lifetime in ethanol is 0.39 ns. Our wavelength-averaged $\tau_M$ for 8-MOP in methanol was 0.253 ns. Finally, lifetimes of 2.6 ± 0.6 ns and 1.6 ± 0.6 ns have been reported for aqueous 8-MOP. Our value of 0.356 ns for the $\tau_M$ of 8-MOP in water initially appears to be at variance with these values, but the component responsible for 31–91% of the steady-state intensity in these solutions had a lifetime of 0.8–1.3 ns. Given the low-resolution of those early measurements, we suspect that they might not have adequately assessed the impact of the short-lifetime components in aqueous 8-MOP's fluorescence decay.

There is much in the time-resolved fluorescence data of psoralens and related compounds that remains unexplained. Nevertheless, this work represents both the first recorded measurements of fluorescence lifetimes for these compounds in hydrocarbon solvents and an improvement in the quality of the time-resolved fluorescence data for psoralens in acetonitrile, methanol, and water. Furthermore, by simultaneously analyzing the data using the traditional sum-of-exponentials approach in conjunction with ESM lifetime
distributions and a calculation of the pre-exponential weighted mean lifetime, we have been able to analyze these data in a way that permits an interpretation of the time-resolved data that is consistent with the proximity effect. Previous studies, limited as they were by data resolution and precision as well as by correlations among fitting parameters in sum-of-exponentials analysis, had not been able to arrive at an interpretation that demonstrated this level of agreement with theory. Our work should open a door to future research, in which the time-resolved fluorescence of psoralens and related compounds may be analyzed to gain additional insight into their photophysical properties as well as an understanding of the photochemistry that gives rise to their phototherapeutic and antiviral activities.
References


SampI»
8b

Solvent x«,(nm )
tiM
mcflumol
450
1.07 0.230
SCO Î.4Ô
550
1.16 0.256

tz W
4.960
0.057
0.074

T,(m#
0.(06
2619
6.626

,* (n # f.
f» T«(W
1.472 5 8 ^ 19.6% 14.5% 7.6% 0.122
6 ^ '7 2 9 % 125% 7.5% 6.0% 0.189
1.754 79.5% 11.7% 5.3% 3.5% 0.213

8b

PB8

450
500
550

1.13 1.348 6.264 0.346 0.028 39.1% 28.9% 23.5% 8.5% 0.247
1.13 1039 a S K i 6.W 0.024 420% âl6 % 19.3% 5.2% 0.291
l:lT 0.907' Ô.281 5.779 O^CW 60.5% 224% 124% 4 8% 0J29\

8b

waltr

450
500
550

1.16 1.118 6.263
1.11 0.776 0.222
1.20 0.786 0.212

8c

methanol

450
500
550

1.03 0.262 9 .5 a 0.026 1.146 521% 27.1% 124%
1.05 0 . ^ 0.971 8.812 0:040 65:5% 147% 11.0%
1.01 0.274 1.012 10.662; Ô.071 60.3% 224% 8.9%

450
500
550

1.18 1.258 0.152 4.056 0.000 78.6% 120%
1.09 1.199 0.195 3.758 0.000 67.3% 7.3%
0.97 1.141 2301 0.147 0.000 87.0% 7.8%

8c

0.295 0.025 41.8% 324% 18.6%
6 .2 3 (ï()13 80.9% ÎO.7% 7.3%
6.206 0.020 7&4% 120% 8.8%

7.2% 0.256
12% 0.411
1.8% 0.403
8.4%
8.8%
8.3%

0.149
0.206
0.277

9.4% 0.0%
5.4% 0.0%
5.2% 0.0%

0.6
0.8
0.871

8c

water

450
500
æo

1.06 1.272 6.507 0.198 0.022 66.1% 16.2% 13.4% 4.3% 0.318
1.02 1.196 0.211 5.337 0.017 83.4% 7.6% 7.5% 1.5% 0.516
1.24 i:i3 1 0 .m Ô.Ô5 3.651 922% 11.2% 8.2% 4.7% 0.908

8-MOP

acalonIMIa

450
500
550

1.08 0.036
1.15 0.036
1.19 0 037

5.027 1.824 0.000 58.2% 34.9% 6.9%
5.7%
5.271 0 .9 3 0.000 5 9 .» 33.0% 7.8%

4.942 0.862 0.000 60.6% 33:8%

0.0% 0.062
0.0% 0.058
0.0% 0.062

8-MOP

mcflianoi

450
500
550

1.17 0.386 9.087 1.799 0.028 60.3% 26.3% 6.8% 6.5% 0.251
1.20 Ô.3TO 8 . ^ 1.060 0.026 74.3% 124% 7.7% 5.6% 0.234
1.05 0.378 0.949 9.732 0.031 726% 13.6% 8.9% 4.9% 0.274

8-MOP

n-pentene

400
450
500
550

1.19
1.20
1.09
1 44

4.232
3.886
3.737
0.055

2103
1.467
0.044
4.263

0.063
0.049
0.765
0.567

0.000
0.000
0.000
0.000

0.0%
0.0%
0.0%
0.0%

1.448
0.727
0.257
0.120

8-MOP

water

400
450
500
550
580

1.18
1.04
1.18
1.10
1.21

1.350 6.565
1.077 4.758
1.019 0 .4 3
0.969 0.239
0.972 0.019

0.226
0.291
4.189
3.957
3.724

0.020 39.2% 28.2% 18.7% 13.9%
0 .0 3 727% 11.8% 9.9% 5.7%
0.017 83.4% 8.1% 5.1% 3.4%
O.tDOO 922% 4.9% 3.0% 0.0%
0J241 87.4% 5.7% 4.0% 3.0%

0 .1 3
0.273
0.328
0.875
0.243

Br-MOP

acetonitrile

450
500
550

1.39 0.077
1.21 0.069
1.25 0.CH6

Br-MOP

methanol

450
500
550

1.01 0.365 8.203
1.17 0.343 5.410

Î.12 b.l^

77.9%
81.7%
727%
43.1%

19.5% 26%
13.0% 5.3%
15.5% 11.8%
322% 24.7%

0.152 1.578 0.000 636% 31.4%
0.275 2158 0.000 830% 124%
0.476 3.231 0.000 ^ 2 % 11.1%

OJOS4

5.0%
4.6%
5.7%

0.0% 0.097
0.0% 0.102
0.0% 0.112

2685 0.047 59.0% 15.0% 139% 120%
0 044 i.046 727% 135% 7.5% 6.3%
6.788 1.114 77.0% 8.9% 7.8% 6.2%

0.234
0.254
0.257

Note: fi, fj. and represent Ihe factional contribution (am/Som) of each component to the steady-state intensity.
TMis the pre-mponential weighted mean IHet'me (Zcoxi/Sca).

Table 5.1: Sum-of-exponential fits to psoralen fluorescence decays
219


<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>λ_{ex} (nm)</th>
<th>x^2</th>
<th>τ1 (ns)</th>
<th>τ2 (ns)</th>
<th>τ3 (ns)</th>
<th>τ4 (ns)</th>
<th>f1</th>
<th>f2</th>
<th>f3</th>
<th>f4</th>
<th>τM (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br-MOP</td>
<td>n-pentane</td>
<td>400</td>
<td>1.07</td>
<td>0.022</td>
<td>1.875</td>
<td>7.581</td>
<td>0.000</td>
<td>55.9%</td>
<td>35.9%</td>
<td>9.2%</td>
<td>0.0%</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td>450</td>
<td>1.17</td>
<td>0.015</td>
<td>0.330</td>
<td>1.741</td>
<td>0.000</td>
<td>56.8%</td>
<td>30.9%</td>
<td>12.3%</td>
<td>0.0%</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>1.00</td>
<td>0.018</td>
<td>0.305</td>
<td>2.152</td>
<td>0.000</td>
<td>64.3%</td>
<td>28.5%</td>
<td>7.1%</td>
<td>0.0%</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td></td>
<td>550</td>
<td>1.40</td>
<td>0.020</td>
<td>0.238</td>
<td>1.308</td>
<td>0.000</td>
<td>59.3%</td>
<td>33.5%</td>
<td>7.2%</td>
<td>0.0%</td>
<td>0.032</td>
</tr>
<tr>
<td>khellin</td>
<td>acetonitrile</td>
<td>450</td>
<td>1.20</td>
<td>1.419</td>
<td>0.591</td>
<td>0.000</td>
<td>65.0%</td>
<td>15.0%</td>
<td>0.0%</td>
<td>1.0%</td>
<td>0.0%</td>
<td>1.173</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>1.04</td>
<td>1.461</td>
<td>0.640</td>
<td>0.000</td>
<td>86.2%</td>
<td>13.8%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>1.242</td>
</tr>
<tr>
<td></td>
<td></td>
<td>550</td>
<td>1.38</td>
<td>1.548</td>
<td>0.804</td>
<td>0.000</td>
<td>79.7%</td>
<td>20.3%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>0.0%</td>
<td>1.303</td>
</tr>
<tr>
<td>khellin</td>
<td>methanol</td>
<td>450</td>
<td>1.20</td>
<td>0.012</td>
<td>2.392</td>
<td>0.537</td>
<td>0.000</td>
<td>84.4%</td>
<td>20.4%</td>
<td>13.2%</td>
<td>0.0%</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>1.07</td>
<td>0.301</td>
<td>6.242</td>
<td>0.043</td>
<td>0.000</td>
<td>57.8%</td>
<td>30.5%</td>
<td>11.7%</td>
<td>0.0%</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td></td>
<td>550</td>
<td>1.08</td>
<td>0.305</td>
<td>6.458</td>
<td>0.019</td>
<td>0.000</td>
<td>81.1%</td>
<td>13.1%</td>
<td>5.8%</td>
<td>0.0%</td>
<td>0.019</td>
</tr>
<tr>
<td>khellin</td>
<td>n-pentane</td>
<td>450</td>
<td>1.11</td>
<td>0.012</td>
<td>2.392</td>
<td>0.367</td>
<td>0.000</td>
<td>66.4%</td>
<td>29.4%</td>
<td>12.4%</td>
<td>0.0%</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>450</td>
<td>1.15</td>
<td>0.018</td>
<td>0.225</td>
<td>1.744</td>
<td>0.000</td>
<td>47.2%</td>
<td>30.4%</td>
<td>25.3%</td>
<td>5.5%</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>1.21</td>
<td>0.276</td>
<td>0.031</td>
<td>1.081</td>
<td>0.000</td>
<td>46.7</td>
<td>24.5</td>
<td>22.1</td>
<td>4.5%</td>
<td>0.100</td>
</tr>
<tr>
<td>khellin</td>
<td>water</td>
<td>450</td>
<td>1.11</td>
<td>1.633</td>
<td>5.181</td>
<td>0.308</td>
<td>0.019</td>
<td>84.4%</td>
<td>20.4%</td>
<td>13.2%</td>
<td>0.0%</td>
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Note: f1, f2, f3, and f4 represent the fractional contribution (a0t/Σa0t) of each component to the steady-state intensity. τM is the pre-exponential weighted mean lifetime (Σa0t/Σa).

Table 5.1 (Continued): Sum-of-exponential fits to psoralen fluorescence decays
Figure 5.1: Structure of psoralen (PSO) and the numbering scheme used to locate substituents of psoralen derivatives
Figure 5.2: Consecutive cycloaddition reactions of 8-MOP to two uracil derivatives. Figure reproduced from The Spectrum (1995) 8:11-20
Figure 5.3: Structures of the psoralens and related molecules investigated in this work.
Figure 5.4: TCSPC fluorescence decays monitored at 500 nm for 8-MOP in various solvents: (a) n-pentane, (b) acetonitrile, (c) methanol, (d) water
Figure 5.5: TCSPC fluorescence decays for 8-MOP in n-pentane. Emission monitored at: (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm
Figure 5.6: TCSPC fluorescence decays for 8-MOP in acetonitrile. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.7: TCSPC fluorescence decays for 8-MOP in methanol. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.8: TCSPC fluorescence decays for 8-MOP in water. Emission monitored at: (a) 400 nm, (b) 450 nm, (c) 500 nm, (d) 550 nm, (e) 580 nm.
Figure 5.9: TCSPC fluorescence decays for 8c in methanol. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.10: TCSPC fluorescence decays for 8b in methanol. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.11: TCSPC fluorescence decays for visnagin in methanol. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.12: TCSPC fluorescence decays for khellin in methanol. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.13: ESM fluorescence lifetime distributions (100 components) for Br-MOP in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.14: ESM fluorescence lifetime distributions (100 components) for 8-MOP in (a) n-pentane, (b) acetonitrile, (c) methanol, and (d) water. Fluorescence monitored at 500 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.15: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8-MOP in (a) n-pentane, (b) acetonitrile, (c) methanol, and (d) water. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.09 ns, 0.09 - 0.9 ns, 0.9 - 1.5 ns, > 1.5 ns. Fluorescence monitored at 500 nm.
Figure 5.16: ESM fluorescence lifetime distributions (100 components) for 8-MOP in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.17: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8-MOP in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.09 ns, 0.09 - 0.2 ns, 0.2 - 0.5 ns, 0.5 - 3.0 ns, > 3.0 ns
Figure 5.18: ESM fluorescence lifetime distributions (100 components) for 8-MOP in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
**Figure 5.19:** "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8-MOP in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 2.0 ns, > 2.0 ns
Figure 5.20: ESM fluorescence lifetime distributions (100 components) for 8-MOP in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.21: “Discrete Component Equivalents” for ESM fluorescence lifetime distributions for 8-MOP in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.7 ns, 0.7 - 2.0 ns, > 2.0 ns.
Figure 5.22: ESM fluorescence lifetime distributions (100 components) for 8-MOP in water. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, (d) 550 nm, and (e) 580 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.23: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8-MOP in water. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, (d) 550 nm, and (e) 580 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.7 ns, 0.7 - 2.0 ns, > 2.0 ns.
Figure 5.24: TCSPC fluorescence decays monitored at 500 nm for Br-MOP in various solvents: (a) n-pentane, (b) acetonitrile, (c) methanol
Figure 5.25: ESM fluorescence lifetime distributions (100 components) for Br-MOP in (a) n-pentane, (b) acetonitrile, and (c) methanol. Fluorescence monitored at 500 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.26: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for Br-MOP in (a) n-pentane, (b) acetonitrile, and (c) methanol. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.2 ns, 0.2 - 1.0 ns, > 1.0 ns. Fluorescence monitored at 500 nm.
Figure 5.27: TCSPC fluorescence decays for Br-MOP in n-pentane. Emission monitored at: (a) 400 nm (b) 450 nm, (c) 500 nm, (d) 550 nm
Figure 5.28: ESM fluorescence lifetime distributions (100 components) for Br-MOP in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.29: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for Br-MOP in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.4 ns, 0.4 - 1.5 ns, > 1.5 ns
Figure 5.30: TCSPC fluorescence decays for Br-MOP in acetonitrile. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.31: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for Br-MOP in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.03 ns, 0.03 - 0.15 ns, 0.15 - 0.6 ns, 0.6 - 3.0 ns, > 3.0 ns.
Figure 5.32: TCSPC fluorescence decays for Br-MOP in methanol. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.33: ESM fluorescence lifetime distributions (100 components) for Br-MOP in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.34: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for Br-MOP in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.2 ns, 0.2 - 1.0 ns, 1.0 - 4.0 ns, > 4.0 ns
Figure 5.35: TCSPC fluorescence decays monitored at 500 nm for 8b in various solvents: (a) methanol, (b) water, (c) PBS
Figure 5.36: ESM fluorescence lifetime distributions (100 components) for 8b in (a) methanol, (b) water, and (c) PBS. Fluorescence monitored at 500 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.37: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8b in (a) methanol, (b) water, and (c) PBS. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.4 ns, 0.4 - 2.0 ns, > 2.0 ns. Fluorescence monitored at 500 nm.
Figure 5.38: ESM fluorescence lifetime distributions (100 components) for 8b in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.39: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8b in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.2 ns, 0.2 - 0.8 ns, 0.8 - 3.0 ns, > 3.0 ns
Figure 5.40: TCSPC fluorescence decays for 8b in water. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.41: ESM fluorescence lifetime distributions (100 components) for 8b in water. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.42: “Discrete Component Equivalents” for ESM fluorescence lifetime distributions for 8b in water. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.5 ns, 0.5 - 1.1 ns, 1.1 - 2.0 ns, > 2.0 ns.
Figure 5.43: TCSPC fluorescence decays for 8b in PBS. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.44: ESM fluorescence lifetime distributions (100 components) for 8b in PBS. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.45: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8b in PBS. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.3 ns, 0.3 - 1.1 ns, 1.1 - 4.0 ns, > 4.0 ns.
Figure 5.46: TCSPC fluorescence decays monitored at 500 nm for 8c in various solvents: (a) methanol, (b) water, (c) PBS
Figure 5.47: ESM fluorescence lifetime distributions (100 components) for 8c in (a) methanol, (b) water, and (c) PBS. Fluorescence monitored at 500 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.48: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8c in (a) methanol, (b) water, and (c) PBS. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 1.0 ns, 1.0 - 2.0 ns, > 2.0 ns. Fluorescence monitored at 500 nm.
Figure 5.49: ESM fluorescence lifetime distributions (100 components) for 8c in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.50: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8c in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.05 ns, 0.05 - 0.2 ns, 0.2 - 0.8 ns, 0.8 - 2.0 ns, > 2.0 ns
Figure 5.51: TCSPC fluorescence decays for 8c in water. Emission monitored at: (a) 450 nm, (b) 500 nm, and (c) 550 nm.
Figure 5.52: ESM fluorescence lifetime distributions (100 components) for 8c in water. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.53: “Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8c in water. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.05 ns, 0.05 - 0.5 ns, 0.5 - 3.0 ns, > 3.0 ns
Figure 5.54: TCSPC fluorescence decays for 8c in PBS. Emission monitored at: (a) 450 nm, (b) 500 nm, and (c) 550 nm.
Figure 5.55: ESM fluorescence lifetime distributions (100 components) for 8c in PBS. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.56: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for 8c in PBS. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.5 ns, 0.5 - 3.0 ns, > 3.0 ns.
Figure 5.57: TCSPC fluorescence decays monitored at 500 nm for khellin in various solvents: (a) n-pentane, (b) acetonitrile, (c) methanol, (d) water
Figure 5.58: ESM fluorescence lifetime distributions (100 components) for khellin in (a) n-pentane, (b) acetonitrile, (c) methanol, and (d) water. Fluorescence monitored at 500 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.

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Figure 5.59: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for khellin in (a) n-pentane, (b) acetonitrile, (c) methanol, and (d) water. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.09 ns, 0.09 - 0.2 ns, 0.2 - 0.9 ns, 0.9 - 2.1 ns, > 2.1 ns. Fluorescence monitored at 500 nm.
Figure 5.60: TCSPC fluorescence decays for khellin in n-pentane. Emission monitored at: (a) 400 nm, (b) 450 nm, (c) 500 nm, (d) 550 nm
Figure 5.61: ESM fluorescence lifetime distributions (100 components) for khellin in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.62: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for khellin in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.07 ns, 0.07 - 0.45 ns, 0.45 - 1.7 ns, 1.7 - 9.0 ns, > 9.0 ns
Figure 5.63: TCSPC fluorescence decays for khellin in acetonitrile. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.64: ESM fluorescence lifetime distributions (100 components) for khellin in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.65: “Discrete Component Equivalents” for ESM fluorescence lifetime distributions for khellin in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.45 ns, 0.45 - 0.8 ns, 0.8 - 2.0 ns, > 2.0 ns
Figure 5.66: ESM fluorescence lifetime distributions (100 components) for khellin in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.67: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for khellin in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.09 ns, 0.09 - 0.4 ns, 0.4 - 5.0 ns, > 5.0 ns
Figure 5.68: TCSPC fluorescence decays for khellin in water. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.69: ESM fluorescence lifetime distributions (100 components) for khellin in water. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.70: “Discrete Component Equivalents” for ESM fluorescence lifetime distributions for khellin in water. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.09 ns, 0.09 - 0.9 ns, 0.9 - 3.0 ns, > 3.0 ns
Figure 5.71: TCSPC fluorescence decays monitored at 500 nm for visnagin in various solvents: (a) n-pentane, (b) acetonitrile, (c) methanol, (d) water
Figure 5.72: ESM fluorescence lifetime distributions (100 components) for visnagin in (a) n-pentane, (b) acetonitrile, (c) methanol, and (d) water. Fluorescence monitored at 500 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts. Lifetime components in methanol were logarithmically spaced from 0.010 ns - 40 ns with additional components at 0.001 ns and 400 ns to account for scatter and/or dark counts.
Figure 5.73: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for visnagin in (a) n-pentane, (b) acetonitrile, (c) methanol, and (d) water. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.5 ns, 0.5 - 3.0 ns, > 3.0 ns. Fluorescence monitored at 500 nm.
Figure 5.74: TCSPC fluorescence decays for visnagin in n-pentane. Emission monitored at: (a) 400 nm, (b) 450 nm, (c) 500 nm, (d) 550 nm
Figure 5.75: ESM fluorescence lifetime distributions (100 components) for visnagin in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.76: “Discrete Component Equivalents” for ESM fluorescence lifetime distributions for visnagin in n-pentane. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 0.4 ns, 0.4 - 1.5 ns, > 1.5 ns
Figure 5.77: TCSPC fluorescence decays for visnagin in acetonitrile. Emission monitored at: (a) 450 nm, (b) 500 nm, (c) 550 nm
Figure 5.78: ESM fluorescence lifetime distributions (100 components) for visnagin in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.79: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for visnagin in acetonitrile. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.1 ns, 0.1 - 1.0 ns, 1.0 - 3.0 ns, 3.0 - 4.0 ns, > 4.0 ns
Figure 5.80: ESM fluorescence lifetime distributions (100 components) for visnagin in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 40 ns with additional components at 0.001 ns and 400 ns to account for scatter and/or dark counts.
Figure 5.81: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for visnagin in methanol. Emission monitored at (a) 450 nm, (b) 500 nm, and (c) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 3.0 ns, 3 - 10 ns, > 10 ns
Figure 5.82: TCSPC fluorescence decays for visnagin in water. Emission monitored at: (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm
Figure 5.83: ESM fluorescence lifetime distributions (100 components) for visnagin in water. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.84: "Discrete Component Equivalents" for ESM fluorescence lifetime distributions for visnagin in water. Emission monitored at (a) 400 nm, (b) 450 nm, (c) 500 nm, and (d) 550 nm. Each DCE is labeled by its region of significant fractional intensity and corresponds to a summation of the fractional contributions across a subset of the ESM distribution. Summed regions: < 0.3 ns, 0.3 - 1.0 ns, 1.0 - 4.0 ns, > 4.0 ns
Figure 5.85: ESM fluorescence lifetime distributions (100 components) for all of the psoralens and related compounds studied in this work. Emission monitored in methanol at 500 nm for (a) 8b, (b) 8c, (c) 8-MOP, (d) Br-MOP, (e) khellin, and (f) visnagin. Lifetime components were logarithmically spaced from 0.010 ns - 10 ns with additional components at 0.001 ns and 100 ns to account for scatter and/or dark counts. Lifetime components for visnagin were logarithmically spaced from 0.010 ns - 40 ns with additional components at 0.001 ns and 400 ns to account for scatter and/or dark counts.
Figure 5.86: Comparison of fitting methods for 8-MOP in methanol. Fluorescence monitored at 550 nm. The same TCSPC data file (10K CPC) was analyzed using (a) a sum of 4 exponentials, and (b) an ESM lifetime distribution comprised of 100 exponentials, logarithmically spaced between 0.010 ns - 10 ns. The ESM fit included components at 0.001 ns and 100 ns to account for scatter and/or dark counts.
Figure 5.87: Pre-exponential weighted mean lifetime ($\tau_\text{w}$) as a function of solvent polarity for (a) 8-MOP, (b) Br-MOP, (c) visnagin, and (d) kheillin at three emission wavelengths. Solvent polarities are represented by Dimroth/Reichardt's $E_r(30)$ values (Reichardt, C. (1988) *Solvents and Solvent Effects in Organic Chemistry*, 2nd Ed., VCH, Weinheim). The $E_r(30)$ values were 0.009 for $n$-pentane, 0.076 for acetonitrile, 0.160 for methanol, and 0.545 for water.
Figure 5.88: Lifetime of the major decay component as a function of solvent polarity for (a) 8-MOP, (b) Br-MOP, (c) visnagin, and (d) khellin at three emission wavelengths. Solvent polarities are represented by Dimroth/Reichardt's $E_{30}$ values (Reichardt, C. (1988) Solvents and Solvent Effects in Organic Chemistry, 2nd Ed., VCH, Weinheim). The $E_{30}$ values were 0.009 for n-pentane, 0.076 for acetonitrile, 0.160 for methanol, and 0.545 for water.
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Figure 5.90: Pre-exponential weighted mean lifetime ($\tau_\omega$) as a function of emission wavelength for Br-MOP in (a) $n$-pentane, (b) acetonitrile, and (c) methanol.
Figure 5.91: Pre-exponential weighted mean lifetime ($\tau_w$) as a function of emission wavelength for 8b in (a) PBS, (b) methanol, and (c) water
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Figure 5.93: Pre-exponential weighted mean lifetime ($\tau_m$) as a function of emission wavelength for khellin in (a) $n$-pentane, (b) acetonitrile, (c) methanol, and (d) water
Figure 5.94: Pre-exponential weighted mean lifetime ($\tau_m$) as a function of emission wavelength for visnagin in (a) $n$-pentane, (b) acetonitrile, (c) methanol, and (d) water
CHAPTER 6

THE FLUORESCENCE QUENCHING OF PYRENE BY HUMIC SUBSTANCES

Introduction

The scientific community is still in the early stages of understanding the impact of anthropogenic activity on the environment. One of the more pressing issues in this crucial area of research is the quantitative analysis of the fate and behavior of nonpolar organic compound (NOC) pollutants in natural waters. Of particular concern are the class of contaminants known as polycyclic aromatic hydrocarbons (PAHs). These compounds are produced as byproducts of incomplete combustion, and are also found in areas contaminated by oil spills, creosote, or coal tar. Many PAHs are known to be carcinogenic or otherwise toxic, others are under investigation.¹

Dissolved humic substances have been shown to play an important role in the facilitated transport of NOCs in the environment. As a consequence, many groups have sought to develop accurate techniques to measure partition coefficients (K_{oc}) between NOC pollutants and humic substances sampled from subsurface and surface waters and sedimentary porefluids.²⁻⁹ The value of K_{oc} simply represents the ratio of pollutant concentration bound to humic material relative to its concentration in solution. While the extent to which dissolved organic materials such as humic and fulvic acids affect these environmental processes, and the exact mechanisms by which they do so are just beginning to be understood, the fact that these substances do increase the effective aqueous solubility of
NOCs is beyond question. It is very unlikely that the humic materials significantly affect the solvation power of water *per se*, so the *apparent* solubility enhancements are probably attributable to hydrophobic interactions between the NOCs and the humic materials, or portions thereof. These hydrophobic interactions may include a combination of van der Waals forces and entropic effects. In any event, the solubility enhancement method, based on this phenomenon, has become one of the more popular techniques employed to measure the partition coefficients between NOCs and humic materials. This method is generally reliable and is relatively well-understood, but it is time- and labor-intensive and requires copious quantities of humic substances (up to 5 mmol/L as organic carbon). Furthermore, it inherently involves a chemical separation step, making the method subject to errors that might be introduced by incomplete separations. It may also be insensitive to substances that possess low liquid or subcooled liquid solubilities (mole fraction < 10⁻³).²

Fluorescence quenching (FQ) has recently emerged as an alternative approach in the measurement of $K_\infty$ values for fluorescent nonpolar organic compounds.²⁷,¹⁰⁻¹³ This technique correlates an observed reduction in fluorescence intensity for an NOC with the aqueous concentration of a particular humic material. The reduction in fluorescence intensity thus observed is presumed to be due to ground-state associations (binding) between the humic substances and the NOC that render the NOC nonfluorescent. The fluorescence intensity is measured at several concentrations of humic material and the value of $K_\infty$ is obtained from the slope of a Stern-Volmer plot prepared from these data. This method has several advantages over current alternatives: (1) it does not require an initial chemical separation step; (2) it is a ratiometric technique, so the exact NOC concentrations need not be measured; (3) it is less time-consuming; and (4) requires less sample.² However, the quenching mechanisms are not well understood, having been the subject of considerable debate.²⁴,⁶,¹⁰⁻¹⁴ Even more troubling is the fact that some studies have found nonlinear Stern-Volmer plots, and the $K_\infty$ values
obtained for polyaromatic hydrocarbon (PAH) compounds by the FQ technique have often been larger than those measured using other methods such as reverse-phase chromatography or dialysis.\textsuperscript{14}

The accuracy of the FQ technique rests on the validity of the assumption that the observed quenching is due to ground-state interactions between the fluorophore and the quenching species. This condition is known as static quenching. Anomalous quenching behavior can arise from a variety of sources, some of which would threaten the applicability of the FQ technique under certain circumstances. For example, deviations from linearity in Stern-Volmer plots could suggest the presence of multiple fluorophores, multiple quenchers, or heterogeneous microenvironments. Or these deviations could reflect the presence of quenching mechanisms in addition to static quenching. These might include dynamic (collisional) quenching, distance-dependent quenching, or transient quenching. If NOC fluorescence were quenched by one or more of these processes in addition to the ground-state interactions with humic substances, the values for $K_\infty$ measured by the FQ technique would not be accurate, and their disparity from $K_\infty$ values obtained by the solubility enhancement method and other techniques might be rationalized.

Dynamic quenching, in particular, can usually be reliably distinguished from static quenching on the basis of Stern-Volmer plots prepared with fluorescence lifetime data. Static quenching involves a ground-state interaction that renders a fluorophore completely nonfluorescent. Consequently, while the fluorescence intensity is reduced by such quenching, the lifetime of any residual fluorescence should be unaffected, as it originates from the unassociated portion of the fluorophore population. Dynamic (collisional) quenching, in contrast, arises from collisions of photoexcited fluorophores with quenching species. These collisions add an additional nonradiative decay pathway to the excited state of the fluorophore, reducing the average amount of time spent in the excited state, and
consequently, the fluorescence lifetime. If collisional quenching occurs, one expects to observe a decrease in the measured fluorescence lifetime as the quencher concentration is increased.

Our original goals for this project were therefore (1) to compare results obtained with the fluorescence quenching technique with those obtained from the more traditional solubility enhancement method and (2) to test the possibility of dynamic (collisional) quenching as an explanation for any differences in the recovered $K_{\infty}$ values by measuring the effect of humic substances on NOC fluorescence lifetimes. Pyrene was chosen as the analyte, as it is representative of many polycyclic aromatic hydrocarbons. It is highly fluorescent, and readily available. Additional advantages of PAH compounds include the fact that they are nonpolar, do not possess functional groups, and do not easily form ions in solution. This minimizes the number of quenching/binding mechanisms that have to be considered in order to explain the results.

Humic substances are complex, heterogeneous mixtures of polyfunctional, highly aromatic naturally-occurring organic compounds that are ubiquitous in the environment. Although several theories have been advanced regarding the microscopic nature of the interactions of NOCs with humic substances, one of the most common views involves a partitioning of the NOCs into hydrophobic regions formed by self-organization processes in aqueous humic substances, similar to those observed in micelles. The pseudomicellar regions thus formed are believed to provide a larger, looser environment than true micelles and their sizes and binding properties are believed to depend strongly on properties of the bulk solution such as ionic strength and pH. These ground-state binding/partitioning interactions between hydrophobic portions of humic substances and NOCs are presumed to be the source of the apparent enhancement of the NOCs' aqueous solubilities, as well as the primary means by which the fluorescence intensity of NOCs are reduced in the presence of humic materials. That such an explanation is plausible is demonstrated in Figure 6.1. This AMBER geometry-
optimized structure represents the interactions of pyrene with a typical humic acid fragment. A two-dimensional representation of the complex is provided in Figure 6.2, which is derived from Figure 6 in Puchalski et al. The optimized geometry clearly places the pyrene molecule in a hydrophobic "pocket" that consists of individual acid moieties linked via hydrogen bonds. It is important to stress, however, that the heterogeneous nature of these humic substances and the complex matrices of naturally-occurring aquatic humic materials limit this figure to the illustration of a possibility. Nevertheless, one can easily visualize hydrophobic interactions making strong contributions to the binding of PAHs, regardless of the other forces involved. That π–π interactions between PAHs and aromatic moieties of humic substances are an important component of these partitioning forces has been shown by Gauthier et al., who demonstrated a strong correlation between measured $K_{oc}$ values and three independent measures of aromaticity in various humic materials. This correlation was later independently confirmed.

We examined pyrene's interactions with four different types of humic substances. These included fulvic and humic acids from the Suwannee River in Georgia (SRFA and SRHA), fulvic acids from Lake Fryxell (LFFA) in Antarctica, and a reference standard of humic acids obtained from Aldrich (ALHA). Some of the physical and chemical properties of these humic substances have been tabulated and discussed. In general, humic materials derived from the decomposition products of higher plants (e.g., SRFA and SRHA) tend to be larger than those derived from microbial sources (phytoplankton and bacteria), such as LFFA. The humic acids tend to be somewhat larger and more polydisperse with respect to their molecular weights than their fulvic acid counterparts. For example, the number-averaged molecular weights of SRFA and SRHA are 1390 and 1810, respectively. The fulvic acids were found to contain less than 28% aromatics (13% for LFFA), while the aromaticity of humic acids exceeded 40%. Fulvic acids, however, are more acidic.
During the course of our investigations, it became clear that dissolved oxygen was affecting pyrene's fluorescence. This led to our third experimental objective: to investigate the role of oxygen on the observed quenching processes.

**Experimental Methods**

Aqueous solutions of pyrene and the various humic substances investigated were obtained from the laboratory of Karlin M. Danielsen and Yu-Ping Chin. Their methods of isolation, solution preparation, and characterization have been published.\textsuperscript{14-16} Measurements of partition coefficients, $K_{ac}$, employing the solubility enhancement method and steady-state fluorescence quenching were also carried out in their laboratory.

Lifetime measurements were conducted using the TCSPC instrument in our laboratory. The instrument has been described in Chapter 2. The dye laser (Rhodamine-6G) provided a pulse train at 566 nm. This was converted to a wavelength suitable for exciting pyrene fluorescence by generating the second harmonic at 283 nm using a temperature-tuned ADA crystal from INRAD. The emission monochromator was centered at 373 nm, near the fluorescence maximum of pyrene, and the bandwidth employed was either 2 or 5 nm. The polarization rotator and scrambler were not used in the collection optics, as they would have needlessly reduced the fluorescence intensity at the PMT. Any artifacts introduced by rotational reorientation are irrelevant on the time scale of these experiments ($\sim 1.58$ ns/channel; $\sim 750$ ns full scale). Measurements were made at ambient temperature and therefore were subject to thermal fluctuations throughout the course of the experiments. Such fluctuations should have been minor, however, as the laboratory was climate-controlled.

When anoxic conditions were desired, the samples were bubbled with argon for $\sim 3$ minutes. The custom-built cuvettes were sealed with ground glass joints wrapped in Teflon
tape. A "headspace" column of argon extended for several centimeters above the solutions, which served to inhibit oxygen diffusion from the atmosphere. Consequently, the samples remained anoxic for several hours at a time.

Data Analysis

While pyrene exhibits a straightforward single-exponential decay in aqueous solutions, data analysis was complicated by the presence of intense, multi-exponential fluorescence interference originating from the fulvic and humic acids (Figure 6.3). The complexity of these samples' fluorescence decays, arising from the inherent heterogeneity of the acids themselves, are well-known. In fact, McGown et al. selected aqueous solutions of humic materials as prototypical solutions for total lifetime distribution analysis for this very reason.17 Fortunately, the fluorescence lifetimes of humic materials are much shorter than those of pyrene. Consequently, we were able to fit each pyrene decay to a single exponential (plus a constant background) by simply restricting the fitting window to the portion of the decay where the fluorescence contribution of the humic substances was insignificant. This was achieved by collecting a decay for each humic substance alone, and noting a channel (time position) at which fluorescence had decayed to near-background intensity. We then collected decays for pyrene in the presence of various concentrations of the humic substances. To achieve an adequate signal-to-noise ratio, we collected the fluorescence to a level of ~ 10^4 counts in our first fitting channel (typically ~ 115 ns into the decay). Under these conditions, the humic substances often exhibited intensities of > 10^6 CPC. The data were distributed across 512 channels, each of which was ~ 1.58 ns wide. Fits were performed using PeakFit™ (Jandel Scientific).
The validity of the "fitting-window" approach was questioned by Green and Blough. They criticized the technique based on the fact that approximately 59% of pyrene's fluorescence photons are emitted prior to 115 ns, where we began our fits. They suggested that our results were heavily influenced by low-quality (in terms of signal-to-noise ratio) data near the tail of the TCSPC decays. They appear to have overlooked the fact that we collected \(10^4\) fluorescence counts in the first fitting channel precisely to avoid the uncertainties of low-quality data. Furthermore, our data analyses were weighted according to Poisson statistics, so the "tails" of the fluorescence decays received their proper statistical weight. We therefore stand by our conviction that this is a valid approach for the analysis of TCSPC data, and we believe that Figure 6.4 illustrates this fact. From this figure, we note that (1) the data are obviously linear (on a logarithmic scale) across the fitting window, corresponding to a single-exponential decay in this region; (2) the signal-to-noise ratio at the beginning of the fitting window is quite high—statistically equivalent to \(10^4\) CPC; and (3) neither the convolution of the data with the instrument response function, nor fluorescence from humic substances will have any effect on fluorescence lifetimes analyzed from 115 ns forward.

It should also be noted that it was not necessary to use an iterative convolution technique to fit these data, as the aqueous fluorescence lifetime of pyrene (> 120 ns) is several orders of magnitude longer than the FWHM of the instrument response function. This was particularly important at the time these experiments were performed, as we did not yet have the ESM lifetime distribution analysis software which would have permitted us to analyze complex decays via iterative convolution. Consequently, we would have been limited to a maximum of four exponential terms in the analysis of TCSPC data using Demas and Snyder's SPC program. It would have been futile to analyze these data using such a program, since the humic substances themselves could not be adequately described by four-exponential fits. As
mentioned above, this problem was circumvented by filtering the data in the time domain by restricting the data analysis to a time window in which the fluorescence from the humic substances was negligible.

Results

As this work was a collaborative effort, only the results of the fluorescence lifetime measurements will be reported in this section. Results of the other aspects of the investigations are reviewed in the discussion, and have been published.\textsuperscript{14-16}

When experiments were conducted in the absence of dissolved oxygen, the fluorescence lifetime of pyrene was found to be 194 ± 5 ns, regardless of the presence or concentration of humic substances (Figure 6.5 and Table 6.1). When no attempt was made to deoxygenate the samples, we observed a significant decrease in pyrene's fluorescence lifetime in aqueous solutions (141 ± 8 ns). Furthermore, under oxic conditions, pyrene's fluorescence lifetime was shortened by approximately 10 ns in the presence of humic substances. These results are illustrated in Figure 6.6. The exact change in pyrene's fluorescence lifetime depended on the identity and concentration of humic substance. These data are plotted in Figure 6.7 and tabulated in Table 6.1. There also seems to have been a slight decrease in pyrene's lifetime in proportion to the concentration of humic material. These changes, despite their consistency, are within the range of experimental error, so they must be viewed with some skepticism.

The fluorescence lifetime of pyrene was the same for a given decay curve regardless of the method used to fit the data. For example, the fluorescence lifetimes of pyrene in the presence of 3.6 and 11.7 mg C/L SRFA (deoxygenated solutions) were determined by the “fitting window” technique to be 193.5 ns and 194.3 ns, respectively. When the ESM lifetime distribution method was used to fit these same data (over the entire fluorescence decays), two
adjacent lifetime components (184.5 ns and 198.1 ns) accounted for 92% and 77% of the total fluorescence intensity of these samples. This may be clearly seen in Figure 6.8. Taking a weighted average of these two components resulted in pyrene lifetimes of 194.3 ns and 193.7 ns. Both methods of data analysis give reliable, reproducible results for these measurements.

It should be noted that the absolute values of the reduced chi-square for ESM fits to these data were, in general, very poor ($\chi_r^2 > 100$). Visual inspection of the weighted residuals (Figure 6.9) reveals that the first few channels of the decays were poorly described by the set of 100 lifetimes used to construct the ESM distribution. This was not surprising, since the "real" width of the instrument response function is less than 100 ps FWHM. Our data were collected with a time resolution of 1.58 ns/channel, so the ESM convolutions were performed using an instrument response function that was more than an order of magnitude wider than its actual value, despite the fact that it was confined to a single channel. This artifact, due to the inherent quantization of the IRF under such conditions, is well-known. The effect on the reduced chi-square value is exacerbated by the fact that the lifetime distributions of humic substances possess significant contributions from subnanosecond components. This increases the statistical weight of these early channels in the determination of the reduced chi-square. Nevertheless, these artifacts do not adversely affect the recovered values of longer-lived components, and the weighted residuals are small and randomly distributed over the entire decay after the first few channels.

These experimental conditions were optimized to determine the fluorescence lifetime of pyrene. Our data suggest that accurate determinations of the lifetime distributions of the humic materials themselves would have required a time base < 100 ns and (preferably) more than 512 channels. Nevertheless, we were able to obtain approximate lifetime distributions for deoxygenated solutions of the humic materials in the absence of pyrene by analyzing our data with the ESM technique. As can be seen from Figures 6.10 - 6.11, there is considerable similarity among the lifetime distributions of the different humic materials,
although the humic acids might be slightly more biased toward long-lifetime components than their fulvic acid counterparts. For ALHA, approximately 22% of the fluorescence originated from species with lifetimes shorter than 2 ns. The majority of the fluorescence emission (~ 58%) could be described by lifetime components between 2 – 9 ns in duration. The remainder of the emission (~ 20%) could be attributed to components with lifetimes between 9 – 50 ns, with longer-lived species comprising < 0.3% of the total intensity. The distribution for SRHA was similar, with nearly 21% of the intensity arising from lifetime components < 2 ns. The fraction attributable to 2 – 9 ns components (70%) was even higher than that for ALHA, while the 9 – 50 ns components accounted for only 9% of the intensity. Components longer than 50 ns again comprised < 0.3% of the total. The majority (~ 57%) of SRFA’s fluorescence could also be attributed to 2 – 9 ns components, but in this case nearly 33% of the intensity was due to lifetime components shorter than 2 ns. Conversely, only 10% of the SRFA decay could be assigned to lifetimes between 9 – 50 ns. Once again, lifetimes longer than 50 ns contributed less than 0.2% of the total intensity.

Discussion

Danielsen et al. measured the binding of pyrene by aquatic humic substances using both the fluorescence quenching and the solubility enhancement methods. The solubility enhancement data were fit to the following equation, developed by Chiou et al.:*9

\[
\frac{S_w^*}{S_w} = 1 + K_{as} [\text{HS}]
\]

The apparent aqueous solubility of pyrene in the presence of the humic substance is given by \(S_w^*\), while its solubility in the absence of humic materials is given by \(S_w\). The concentration of humic substances, expressed as kilograms of organic carbon per liter, is represented by \([\text{HS}]\). The partition coefficients were therefore obtained from the slopes of the lines that resulted from plotting these data at different concentrations of humic substance. In
an analogous manner, the fluorescence quenching data were fit to the steady-state version of the Stern-Volmer equation:

\[ \frac{F_0}{F} = 1 + K_{\infty}[HS] \]

In this equation, \( F_0 \) and \( F \) represent, respectively, the observed fluorescence intensities in the absence and presence of the humic substance of interest. A linear plot (with the same slope as the solubility enhancement method) is expected if (1) the fluorescence quenching arises from ground-state (static) interactions between the humic substance and pyrene and (2) this quenching is complete; i.e., the pyrene thus bound is rendered nonfluorescent. The \( K_{\infty} \) values recovered by Danielsen et al. using the FQ method were 1.6 – 2.7 times higher than those determined with the solubility enhancement method. They interpreted these differences to be indicative of the presence of additional (non-static) fluorescence quenching that resulted in steeper Stern-Volmer plots and, consequently, higher recovered values of \( K_{\infty} \). In this interpretation the accuracy of the \( K_{\infty} \) values obtained by solubility enhancement were explicitly presumed to be correct. In light of the previously-mentioned concerns inherent in the solubility enhancement method, this presumption might need to be reexamined. Furthermore, the fact that the two techniques yielded \( K_{\infty} \) values that differed by (roughly) a factor of two should be placed in perspective. Literature values for the \( K_{\infty} \) values isolated from identical environmental sources often differ by more than an order of magnitude and are strongly dependent upon factors such as the solution pH, ionic strength, and the concentration of bivalent cations. Additionally, there is considerable variation in the sampling techniques employed, as well as the details of the experimental methodology. For example, some of the fluorescence quenching studies explicitly mentioned efforts to remove or otherwise control the concentration of dissolved oxygen (a known dynamic quencher) while others made no mention of such efforts. It is also important to keep in mind that the humic substances themselves are inherently inhomogeneous—a variation in composition
(e.g., aromaticity) could introduce differences between measurements.\textsuperscript{3} In view of these and other complicating factors, a two-fold variation in the recovered $K_{\text{on}}$ values does not seem quite as significant.

While Danielsen \textit{et al.} also appealed to nonlinearity in some of their Stern-Volmer plots as evidence for the presence of additional quenching mechanisms, such behavior was observed only for very high concentrations of dissolved humic materials ($> 3 \times 10^{-5}$ kg/L). Furthermore, these experiments were performed without controlling the concentration of dissolved oxygen.\textsuperscript{14}

The possibility of dynamic fluorescence quenching due to the humic materials themselves is contraindicated by our observation that the fluorescence lifetime of pyrene is unchanged in the presence of humic materials, regardless of their identity or concentration.

The work of Chen \textit{et al.} argues against the possibility that some fluorophores might be somehow bound to humic substances while remaining fluorescent.\textsuperscript{14} They observed no change in the rotational correlation times of napropamide or 1-naphthol in the presence of humic acid as measured by time-resolved fluorescence anisotropy. One would have expected to have observed increases in the rotational correlation times if these fluorophores had attached themselves to humic substances and continued to emit luminescence.

That pyrene's fluorescence is collisionally quenched by dissolved oxygen is apparent from our fluorescence lifetime measurements. We found its decay time to be $\sim 194$ ns in anoxic samples, but on the order of $141$ ns when the solution was exposed to the atmosphere.

While the invariance of pyrene's fluorescence lifetime in the presence of humic substances was not surprising, and the oxygen-dependence of the fluorescence lifetime of free pyrene is well-known, the data in \texttt{DB03104.JNB} are more difficult to explain. We observed an 8 -- 10 ns decrease in pyrene's fluorescence lifetime in oxic solutions in the presence of humic materials relative to similar solutions that lacked humic substances. Furthermore, this
effect seems to have become more pronounced as the concentration of humic material was increased, although the effects were small and the trends somewhat ambiguous, especially for ALHA.

In our publication with Danielsen et al., we interpreted these observations to be suggestive of a synergistic quenching mechanism that involved both the humic materials and dissolved oxygen. Specifically, we speculated that the local concentration of dissolved oxygen, itself a nonpolar organic molecule, might be somewhat enhanced in the vicinity of the nonpolar “pockets” formed by dissolved humic materials. If there exists a population of pyrene molecules that are not bound by humic substances (in the sense that would give rise to static quenching) and yet are somehow localized in the vicinity of the humic materials (as opposed to the bulk aqueous phase), these molecules might be subject to an increased local concentration of dissolved oxygen, that could give rise to additional dynamic quenching of pyrene’s fluorescence by oxygen. A similar suggestion had been advanced previously by Morra and coworkers. We noted that the increased solubility of oxygen in organic solvents relative to water (e.g., a factor of thirty in 2-propanol) supported this possibility. Under this scheme, the observed reductions in pyrene’s fluorescence lifetime are not directly due to the humic materials. Instead, they represent an enhancement of dynamic quenching due to oxygen.

While this theory was received with some skepticism, we believe that some of the critiques leveled against it were either invalid or not relevant to the particular experimental conditions. Nevertheless, there is much in the position of Green and Blough that we are grateful for and with which we now concur. A portion of our response has been published, but a more detailed response to their specific concerns relevant to our fluorescence lifetime measurements and their interpretations follow.
Green and Blough cited (from the literature) four lines of evidence that the quenching of fluorescence due to NOCs in the presence of humic materials was primarily static in nature. These included the following experimental observations: (1) substantial quenching is observed at very low PAH concentrations; (2) as the HS:PAH ratio is increased, the fluorescence intensity asymptotically approaches zero; (3) little or no increase in quenching is detected with increasing temperature; and (4) fluorescence lifetimes of PAHs are constant in the presence and absence of HA. The latter two observations cited the work of Chen et al.

We note first of all that these studies were carried out on different NOCs with much shorter fluorescence lifetimes. Chen et al. reported lifetimes of 3.3 ns for napropamide, 8.1 ns for 1-naphthol, and 44 ns for fluoranthene. Not only are these substances chemically distinct from pyrene, but their short lifetimes make them intrinsically less sensitive to dynamic quenching. We also note, without disagreeing with their conclusion, that the temperature range that was investigated was rather small (283 K – 313 K). We agree with Green and Blough that these observations support the conclusion that pyrene is not dynamically quenched by humic substances. But we reached the same conclusion based on our observation of a constant pyrene lifetime under anoxic conditions, regardless of the presence or concentration of humic substances! What Green and Blough seem to have missed is that nothing in these observations rules out a more elaborate quenching mechanism. The "sphere of action" mechanism of Morra et al. remains a possibility, as does our suggestion of synergistic effects based on increased local oxygen concentrations.

Green and Blough go on to note that, because we only analyzed data beyond 115 ns, we were limiting our observations to unbound pyrene. If the presence of humic substances significantly shortened pyrene's fluorescence lifetime, its fluorescence might have been hidden beneath the background fluorescence due to humic substances. This is a valid observation, although it does presume a very large difference in lifetimes between the
"bound" and "free" forms of pyrene, if they exist. The "fitting-window" approach to data analysis is inherently blind to this possibility, but an ESM lifetime distribution analysis performed on high-resolution TCSPC data might be able to distinguish the presence of pyrene fluorescence in the presence of multi-exponential interference from humic substances. This possibility could also be tested by monitoring changes in pyrene's fluorescence intensity and lifetime simultaneously. If, after subtracting the background fluorescence due to humic substances, the contribution due to long-lived pyrene could not account for the remainder of the intensity, it would suggest the presence of a short-lived population of fluorescent pyrene. We have already addressed their general critiques of the "fitting-window" approach, and will not repeat the discussion here.

Green and Blough also assert that our maximum concentration of HS (~ 45 mg C/L, corresponding to ~ 30 μM) could quench only ~ 5% of the pyrene fluorescence, assuming diffusion-controlled kinetics. Here again, they seem to have misread our paper to assume that we were supporting dynamic quenching due to humic substances. The concentration of dissolved oxygen in aerated solutions at ambient temperature is almost a factor of ten higher than the concentration of humic substances, and could certainly quench a significant fraction of pyrene's fluorescence. They go on to imply that we had suggested that the presence of dissolved humic materials somehow increased the bulk aqueous solubility of oxygen to an appreciable extent. They then show that such low concentrations of humic materials would have very little effect on the bulk solution. While this is an interesting sidelight, we never suggested this possibility so it has no relevance to a critique of our paper.

Nevertheless, Green and Blough do note four assumptions implied in our suggested "synergistic quenching" mechanism. These are (1) that (in the absence of oxygen) pyrene within the HS matrix has the same lifetime as pyrene in the bulk solution, (2) a significant number of molecules within the HS matrix must be fluorescent and available for quenching, (3) oxygen within the matrix must out-compete the matrix itself in quenching fluorescence,
(4) kinetics must remain single-exponential despite unusual gradients in quencher concentration. They claim that (2) is contraindicated by previous studies that showed almost complete quenching of fluorescence at high concentrations of HS, and that the others lack sufficient experimental evidence to justify such a complex quenching mechanism. We agree that these assumptions seem to be implicit in the proposed mechanism and that more evidence would be necessary to assert this “synergistic quenching” mechanism as fact. However, we are not yet willing to rule it out completely. We note further that assumption (2) does not seem to exclude the possibility that the matrix itself could completely quench fluorescence at sufficiently high concentrations of HS.

We do agree that more work should be done to confirm or refute this hypothesis. Specifically, one would want to measure the steady-state fluorescence intensities and fluorescence lifetimes simultaneously under both oxic and anoxic conditions. Our fluorescence lifetime measurements lacked independent measurements of the fluorescence intensity, and Danielsen et al. did not duplicate their steady-state Stern-Volmer plots under anoxic conditions. At the current time, we can make no statements regarding the extent of quenching due to this novel mechanism, if it exists.

Furthermore, since our original publication of these results, alternative explanations have occurred to us or have been suggested to us by others.19 The large standard deviations that we observed for the oxic samples of pyrene alone (± 8 ns) might be due, at least in part, to fluctuations in ambient laboratory temperature. This would change not only the fluorescence lifetime of pyrene, but its aqueous solubility as well. However, if the pyrene-only solutions were not permitted the same opportunity to equilibrate with the atmosphere as were the solutions containing humic materials, it would provide a trivial explanation for the observation of a longer fluorescence lifetime of aqueous pyrene in the absence of humic materials in oxic solutions.
The expected value of pyrene's fluorescence lifetime in the presence of dissolved oxygen can be calculated using our value for its lifetime in the absence of oxygen in conjunction with the fluorescence lifetime version of the Stern-Volmer equation:

$$\frac{\tau_d}{\tau} = 1 + k_q \tau_0 [O_2]$$

Here, $\tau_0$ is the fluorescence lifetime of aqueous pyrene in the absence of oxygen (194 ns), $\tau$ is the expected fluorescence lifetime in the presence of oxygen, $k_q$ is the second-order diffusion-controlled Stern-Volmer quenching constant, and $[O_2]$ is the molar concentration of dissolved oxygen. For aqueous solutions at 25 °C, $k_q$ should be $\sim 1 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$, and $[O_2]$ should be $\sim 266 \mu\text{M}$. This calculation yields an expected value for $\tau$ of $\sim 130$ ns, in excellent agreement with our measurements of pyrene's fluorescence lifetime in the presence of humic materials and dissolved oxygen. (Table 6.1) Furthermore, while our average value for pyrene's fluorescence lifetime in oxic aqueous solutions in the absence of humic materials was 141 ns, inspection of the fits to individual decay curves revealed that the measured lifetime decreased almost monotonically throughout the course of the experiments (a time period of $\sim 36$ hours). Early pyrene-only decays had lifetimes of $\sim 149$ ns, while those collected near the end of the experiments had fitted lifetimes of approximately 132 ns. Since this latter value is in excellent agreement with the measured pyrene lifetimes in oxic aqueous solutions containing low concentrations of humic materials, and since it is very close to the calculated value of $\tau$ for pyrene that is subject to dynamic quenching due to dissolved oxygen under these conditions, we now prefer this trivial explanation for the apparent $8 - 10$ ns decrease in pyrene's fluorescence lifetime in the presence of dissolved humic substances. The pyrene-only solutions seem to have been given insufficient time to equilibrate with the atmosphere prior to measuring the fluorescence decays. This trivial explanation explains both the large standard deviation for measurements of pyrene's fluorescence lifetime in oxic solutions in the
absence of humic substances as well as the poor agreement between our mean value for pyrene's lifetime under these conditions (141 ns) and the expected value as calculated by the Stern-Volmer equation (130 ns).

Nevertheless, the weaker effect on the lifetime of pyrene, observed as the concentration of humic materials was increased, might still be attributable to "local enhancements" of the concentrations of pyrene and dissolved oxygen in the vicinity of the humic materials. Attributing this weaker effect to the dissolved oxygen concentration addresses the concerns raised by Green and Blough\textsuperscript{17} concerning the ability of humic substances to effect the equivalent of an ~ 10\% increase in dissolved oxygen concentration in hydrophobic microenvironments in and around humic materials. This effect, if present, is at least an order of magnitude less effective than was originally suggested, and should therefore require a proportionally smaller increase in the local dissolved oxygen concentration.

An alternative explanation for the observed reduction in pyrene's fluorescence lifetime as the concentrations of humic materials were increased would be the formation of aggregates of humic materials that do not associate efficiently with pyrene. These could force pyrene into solution where it would be more accessible to oxygen. We do not favor this interpretation, however, as it would be difficult to harmonize with the increase in static quenching of pyrene that is observed as the concentrations of humic substances are increased.

The presence of dissolved oxygen might introduce complexities into the interpretation of PAH fluorescence quenching data in addition to those previously mentioned. The photochemical generation of singlet oxygen and/or various radical species could initiate chemistry that directly or indirectly affects PAH fluorescence. Our initial concerns in this regard\textsuperscript{16} have subsequently been supported by the work of Tiller and Jones.\textsuperscript{1} They noted that the steady-state fluorescence intensities of oxic aqueous solutions of several PAHs, including pyrene, decreased over time as they were exposed to ultraviolet radiation. Engebretson and von Wandruszka observed the photodegradation of pyrene at the rate of ~ 15\% per hour.
under similar conditions. We can neither confirm nor deny the presence of this phenomenon for our experiments, as we were unable to monitor the steady-state fluorescence intensity in concert with the fluorescence lifetime measurements. We do not believe, however, that such processes contributed to the observed reductions in fluorescence lifetime that we observed throughout the course of the experiments, since the introduction of a fresh aqueous solution of pyrene did not interrupt the steady trend toward shorter lifetimes as time progressed.

Conclusions and Future Directions

The use of fluorescent probe molecules is a double-edged sword. The fact that a probe molecule's fluorescence properties critically depend on its microenvironment makes it simultaneously useful in measuring changes in the target properties yet vulnerable to artifacts or unanticipated changes in the microenvironment of the fluorophore. Our experiments have conclusively demonstrated the need to deoxygenate samples prior to attempting to measure values of $K_{\infty}$ for the interaction of PAHs with humic substances via the fluorescence quenching method. Failure to do so can lead to overestimation of the $K_{\infty}$ values relative to those obtained by other approaches, such as the solubility enhancement method. This is due to additional quenching of probe fluorescence due to collisions of dissolved molecular oxygen with photoexcited probe molecules that render them nonfluorescent. The errors introduced by this additional quenching do not appear to be large, however, relative to the effects of static quenching as measured by the Stern-Volmer equation. The values of $K_{\infty}$ obtained by the FQ method in the presence of dissolved oxygen were 1 – 3 times as large as those measured by the solubility enhancement method. This variation is small relative to determinations of $K_{\infty}$ in different laboratories for supposedly identical samples. In this case, $K_{\infty}$ values have been
reported that differ by several orders of magnitude. This undoubtedly stems from the fact that all methods currently used to measure partition coefficients are subject to their own particular artifacts and difficulties.

The fact that we observed a constant lifetime of $194 \pm 5$ ns for anoxic aqueous solutions of pyrene, regardless of the presence or concentration of four different humic substances, is conclusive evidence that these materials do not contribute to the dynamic quenching of pyrene’s fluorescence. On the other hand, we have had to revise our initial interpretation of the apparent enhanced dynamic quenching due to oxygen in the presence of humic substances. We now suspect that our pyrene-only solutions for these experiments were not given sufficient opportunity to equilibrate with the atmosphere prior to measuring the fluorescence lifetimes. Consequently, we obtained a wide range of lifetimes for pyrene under oxic conditions and the values thus obtained decreased steadily throughout the course of the experiment. After roughly 36 hours of equilibration, the measured lifetime values had approached the values calculated using the fluorescence lifetime form of the Stern-Volmer equation, and were roughly equal to the measured fluorescence lifetimes for pyrene in the presence of humic materials.

Nevertheless, our data continue to suggest a synergistic quenching of pyrene’s fluorescence in the presence of oxygen and humic materials. Measured fluorescence lifetimes decrease steadily in oxic solutions as the concentrations of humic substances are increased. This effect, while consistent, is quite small and approaches the reasonable error limits of the lifetime measurements. Furthermore, we lack critical supporting data (such as simultaneous steady-state fluorescence emission intensity measurements) that would tend to confirm or refute this theory.

We have found many of the criticisms of Green and Blough to be valid and helpful, yet we have responded to several aspects of their comments that we believe to be unresolved, inconclusive, inaccurate, or irrelevant to the ideas advanced in our original publication.
Where appropriate, we have softened or retracted statements and conclusions. We agree, in
genral, with their assertion that these ideas should be subjected to additional testing before
receiving widespread acceptance.

Our data were analyzed by filtering the data in the time domain to avoid interferences
from fluorescence due to humic substances. We have shown this method to be valid in
general, but it is inherently blind to processes that might significantly shorten the lifetime of,
without extinguishing completely, the fluorescence of our probe molecule. On the other
hand, our preliminary application of the ESM method of lifetime distribution analysis to these
data suggest that the technique is capable of recovering accurate lifetime values for the long-
lived probe molecule in the presence of highly heterogeneous fluorescence decays due to the
humic materials. Our results for anoxic aqueous samples of the humic substances alone
suggest that the lifetime distributions of fulvic acids might contain a higher fraction of short
lived (< 2 ns) components than their humic acid counterparts. In future experiments,
fluorescence decays should be collected with a higher time resolution (fewer ns/channel; more
channels; shorter time base) and the data should be analyzed using lifetime distributions to
monitor the changes (if any) in the short-lifetime regime of the lifetime distributions. These
systems would be ideal for a fluorescence lifetime system that was capable of collecting data
on a logarithmic time axis, as the lifetimes of interest are several decades apart.
References


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Table 6.1: Pyrene fluorescence lifetimes ("fitting-window" method) measured in the (a) presence and (b) absence of oxygen. Reported standard deviations reflect the larger of (a) the standard deviation of replicate measurements, or (b) twice the value of the standard deviation of the fit.
Figure 6.1: AMBER optimized geometry of the pyrene-humic acid complex from Figure 6.2. The pyrene molecule (center) occupies a hydrophobic “pocket” formed by the humic acids.
Figure 6.2: Schematic, two-dimensional representation of pyrene’s interactions with a dissolved humic acid. Figure adapted from Environ. Sci. Technol. (1992) 26:1787-1792
Figure 6.3: TCSPC fluorescence decays monitored at 373 nm for various dissolved humic substances in the absence of oxygen: (a) LFFA, (b) SRFA, (c) SRHA, (d) ALHA
Figure 6.4: Illustration of the “fitting-window” method of measuring pyrene fluorescence lifetimes. TCSPC fluorescence decays for LFFA in the (a) absence and (b) presence of pyrene. Emission monitored at 373 nm in the absence of oxygen.
Figure 6.5: Fluorescence lifetimes for deoxygenated pyrene solutions in the presence of various dissolved humic substances. Error bars represent the larger of (a) the standard deviation of replicate measurements or (b) twice the value of the statistical uncertainty in the fit to the TCSPC data.
Figure 6.6: ESM fluorescence lifetime distributions (100 components) for (a) ALHA (deoxygenated), (b) pyrene in the presence of dissolved oxygen, (c) pyrene (deoxygenated), and (d) pyrene in the presence of 5.3 mg Carbon/L ALHA (deoxygenated). Emission was monitored at 373 nm. Lifetime components were logarithmically spaced from 0.500 ns - 500 ns with additional components at 0.050 ns and 5000 ns to account for scatter and/or dark counts. The fit to (c) represents a single exponential fit with the “fitting-window” technique, rather than an ESM lifetime distribution.
Figure 6.7: Fluorescence lifetimes for pyrene solutions in the presence of oxygen and dissolved humic materials. Error bars represent the larger of (a) the standard deviation of replicate measurements or (b) twice the value of the statistical uncertainty in the fit to the TCSPC data.
Figure 6.7 (Continued): Fluorescence lifetimes for pyrene solutions in the presence of oxygen and dissolved humic materials. Error bars represent the larger of (a) the standard deviation of replicate measurements or (b) twice the value of the statistical uncertainty in the fit to the TCSPC data.
Figure 6.8: ESM fluorescence lifetime distributions (100 components) for (a) pyrene in the presence of 3.6 mg Carbon/L SRFA, (b) pyrene in the presence of 11.7 mg Carbon/L SRFA, and (c) SRFA in the absence of pyrene. All solutions were deoxygenated and emission was monitored at 373 nm. Lifetime components were logarithmically spaced from 0.500 ns – 500 ns with additional components at 0.050 ns and 5000 ns to account for scatter and/or dark counts.
Figure 6.9: Typical weighted residuals obtained from ESM lifetime distribution analyses of TCSPC decays for solutions containing aqueous humic substances. Note the break in the y-axis. The plotted residuals are for the 100 component fit to a deoxygenated solution of Suwannee River Fulvic Acids from Figure 41. $c_r^2 = 336$
Figure 6.10: ESM fluorescence lifetime distributions (100 components) for deoxygenated aqueous solutions of (a) Aldrich Humic Acids (ALHA) and (b) Suwannee River Humic Acids (SRHA). Emission monitored at 373 nm. Lifetime components were logarithmically spaced from 0.500 ns – 500 ns with additional components at 0.050 ns and 5000 ns to account for scatter and/or dark counts (not shown).
Figure 6.11: ESM fluorescence lifetime distributions (100 components) for deoxygenated aqueous solutions of (a) Suwannee River Fulvic Acids (SRFA) and (b) Suwannee River Humic Acids (SRHA). Emission monitored at 373 nm. Lifetime components were logarithmically spaced from 0.500 ns – 500 ns with additional components at 0.050 ns and 5000 ns to account for scatter and/or dark counts (not shown)
CHAPTER 7

ASSESSMENT AND FUTURE DIRECTIONS

Overview

The acquisition and analysis of time-resolved fluorescence data is occasionally straightforward. There are systems, such as the aqueous chlorophyll sample from Chapter 4, that exhibit a simple single-exponential decay with a unique lifetime (Figure 4.12). Other samples, such as laser dyes, can withstand extended exposure to laser excitation without degradation. But these systems are the exception rather than the rule, especially when it comes to problems of photochemical, biophysical, and environmental interest. These systems are often photolabile, and usually exhibit a considerable amount of heterogeneity: in composition, chemical potential, microenvironment experienced by the fluorescent “solute,” etc. Other processes such as energy transfer, excimer/exciplex formation, electron/proton transfer, or other excited state reactions might compete with fluorescence to depopulate the excited state. It is rare indeed that one of these systems may be analyzed in a routine manner. In this dissertation we have highlighted several of the crucial issues that must be addressed when measuring the time-resolved fluorescence of complex systems. We have also outlined some of the strategies employed to analyze these data, and discussed the strengths and weaknesses of each approach. In general, every complex chemical system will require a unique strategy, and data from other spectroscopies, analytical methods, and computational techniques will need to be incorporated into the analysis to interpret the results. We have
investigated four distinct complex chemical systems in this work. In the sections that follow, we will review the methods employed to acquire and analyze these data, discuss the significance of the scientific conclusions, and outline some directions for future research.

Dialkyldiazirine Photochemistry

Dialkyldiazirines are photochemical precursors to carbenes, divalent organic species that are of considerable synthetic utility in organic chemistry. The photolability of these compounds, combined with the weakly-absorbing nature of the diazirine ring system chromophore, has often hindered their investigation. In Chapter 3 we employed a combination of spectroscopic (steady-state absorption, fluorescence, Raman, and infrared; time-resolved fluorescence and infrared) methods in conjunction with high-level calculations (semiempirical, \textit{ab initio}, and density functional) to investigate the photochemistry of several dialkyldiazirines.

Our work represents the first direct measurements of fluorescence lifetimes for dialkyldiazirines, as well as the first time-resolved study of their infrared spectra. We obtained steady-state infrared and Raman data for adamantyl diazirine, and have analyzed these spectra in conjunction with the vibronic spacings in the absorption and fluorescence spectra to assign several of its ground- and excited-state vibrations. We were guided in our interpretation by vibrational analyses of adamantyl diazirine, performed at various levels of theory.

Our fluorescence lifetime data show that dialkyldiazirines decay with a single, short lifetime. At room temperature, we measured lifetimes of 0.24 ns for adamantyl diazirine, 0.08 ns for cyclohexyl diazirine, and <0.04 ns for dimethyl diazirine. By preparing Arrhenius plots of the temperature-dependence of our fluorescence data, we were able to calculate activation barriers for the rate-limiting photochemical processes that compete with fluorescence to depopulate dialkyldiazirine $S_1$ states. These barriers were found to be approximately 3.4 kcal/
mol for adamantyldiazirine, 2.8 kcal/mol for cyclohexyldiazirine, and 3.2 kcal/mol for dimethyldiazirine. The fluorescence lifetimes were not observed to change as a function of solvent or excitation wavelength.

Our high-level *ab initio* calculations for adamantyldiazirine assigned the first four excited singlet states as possessing $B_1$, $A_2$, $B_2$, and $A_2$ symmetries, respectively. By examining the character of the excitations that give rise to these states, we found that all four transitions transferred electron density to the $\pi^*$ molecular orbital localized on the diazirine nitrogen atoms. Previous calculations, carried out at lower levels of theory had identified the transition from $S_0 - S_1$ as $n-\pi^*$, but had assigned the $S_0 - S_2$ transition as $n-\sigma^*$.

Our calculations also suggested that the $S_1$ and $S_2$ states of adamantyldiazirine do not possess energy minima under $C_2v$ symmetry. Rather, they converge to a structure of $C_1$ symmetry that breaks the symmetry perpendicular to the plane of the diazirine ring by shortening one of the C-N bonds while lengthening the other. This permits mixing of the $S_1$ and $S_2$ states (symmetry-forbidden in $C_2v$) and suggests a photochemical route to 2-diazoadamantane and/or adamantylidene that does not require the $S_1/S_2$ conical intersection suggested by Yamamoto *et al.*

As the fluorescence lifetime of adamantyldiazirine did not change with solvent, we concluded that the rate-limiting processes that compete with fluorescence are probably *intramolecular* rather than *intermolecular* events. Our data are consistent with the rearrangement discussed in the previous paragraph that ultimately forms a mixture of 2-diazoadamantane and adamantylidene, in an approximately 50:50 ratio. Our time-resolved infrared measurements were also consistent with this interpretation, as we observed a biphasic depletion of the N=N stretch mode in conjunction with the appearance of a new band at 2042 cm$^{-1}$ corresponding to the diazo band of 2-diazoadamantane.
Our future plans for investigating the photochemistry of dialkyl-diazirines include measuring the picosecond and/or femtosecond transient absorption of these species, as well as their resonance Raman, and time-resolved resonance Raman spectra. These experiments would provide additional insight into the ultrafast dynamics of these molecules, and, in the case of the Raman experiments, provide mode-specific information on the rearrangement process. These experiments will be supplemented by additional computations, perhaps including the calculation of potential energy surfaces for some of the smaller dialkyl-diazirines.

**Time-Resolved Fluorescence of Photosystem II Reaction Centers**

An understanding of the structure-function relationships in the energy- and electron-transfer processes of photosystem II reaction centers would not only provide insight into one of the most fascinating aspects of biophysics, but it could also be extremely helpful for the development of solar-energy devices that might eventually emulate this behavior. The time-resolved fluorescence of these reaction centers contain a wealth of information about these processes, but obtaining these data and extracting the relevant information is a formidable challenge.

We have identified several critical variables in the acquisition and analysis of TCSPC data for these complex systems, and have succeeded in obtaining decay curves for several preparations of PS II-RCs isolated from wild-type *Chlamydomonas reinhardtii* algae and spinach leaves. We have identified the presence of a long-lived (~29 ns) component in the fluorescence decay as diagnostic of a healthy reaction center preparation, as this fluorescence represents the delayed emission of P680 following recombination of the photogenerated radical pair species. To our knowledge, we are also the first to analyze PS II-RC fluorescence with unparameterized lifetime distribution analysis. We believe this represents a more logical
starting point than discrete component analysis in the analysis of these systems, based on the complexity and intrinsically heterogeneous nature of the protein environment.

We found that the integrity of PS II-RCs could be preserved by deoxygenating the samples, minimizing their exposure to the laser, keeping the temperature near 4 °C, and providing continuous stirring. We developed a software package to assist in the evaluation of the necessary precision, resolution, and time base for PS II-RC time-resolved fluorescence data. We concluded that if the data were adequately described by four-exponential fits, data of 10K CPC precision would be sufficient. However, if we are correct in our assessment that ESM lifetime distributions are more suitable for the analysis of these data, a precision of 20K - 50K CPC seems more appropriate. Under these conditions we have also determined that a data resolution of at least 2048 channels, and a time base of 50 - 75 ns, provide optimum recovery of the underlying lifetimes and relative contributions.

Our initial ESM analyses of TCSPC data for reaction centers isolated from spinach leaves and *Chlamydomonas reinhardii* algae suggest that at least five components are required to describe adequately the fluorescence decay of these samples. As there are good theoretical reasons to expect continuous distributions of states, microenvironments, and energy-transfer distances in these reaction centers, we have concluded that these data are more appropriately modeled as lifetime distributions than as discrete components.

Unfortunately, our investigations were hindered by difficulties in obtaining PS II-RC preparations of reproducible quality. When these become available, we hope to measure complete TCSPC data matrices (*i.e.*, temperature, excitation/emission wavelength, etc.) for wild-type spinach and *Chlamydomonas reinhardii* reaction centers. Lifetime distribution analyses using the unparameterized ESM approach will be used to determine the number of discrete components or parameterized distributions appropriate for inclusion in subsequent global analyses of entire reaction surfaces. These global analyses could first be performed to recover fluorescence amplitudes and lifetimes, but the ultimate goal would be to perform a
global target analysis that fit the data directly to the kinetic parameters of interest such as rate constants, activation energies, etc. As global target analysis involves testing specific kinetic models, these data should be supplemented by the results of other steady-state and time-resolved spectroscopies. Specifically, the absorption and emission spectra, as well as the transient absorption and Raman spectra could eventually be incorporated into the analysis.

The development of the kinetic models will be facilitated by the strategic mutation of the reaction centers in *Chlamydomonas reinhardtii*. The Sayre group has already targeted specific residues for mutation, and have succeeded in growing some of the more promising mutant species. Once a stable preparation procedure has been identified, the path will be clear for future research.

**Time-Resolved Fluorescence of Psoralens and Related Compounds**

Psoralens are phototherapeutic agents that have long been used to treat a variety of dermatological conditions. Recently they have been the study of considerable additional investigation on account of their antiviral activity. The Platz group has been involved in studies of several psoralen derivatives that might eventually be used to sterilize units of donated blood from deadly viruses, such as HIV. Understanding the photophysics and photochemistry of these species will be critical to the strategic development of analogs that are less toxic and more water-soluble than the psoralens currently used, yet possess equal or greater antiviral activity.

Much of the photophysics of psoralens has been explained by the so-called proximity effect, or pseudo-Jahn-Teller distortion. This vibronic interaction between closely-spaced $S_1$ ($\pi\pi^*$) and $S_2$ ($n\pi^*$) states results in enhanced radiationless decay via internal conversion. This effect is expected to be more pronounced in nonpolar solvents, as polar solvents would be expected to preferentially stabilize the $\pi\pi^*$ state relative to the $n\pi^*$ state, increasing the
energy gap between them and reducing the impact of the proximity effect. Experimentally, this should correspond to an increase in the observed fluorescence lifetimes of psoralens in polar solvents.

While various experimental data had been cited by previous groups to demonstrate the presence of the proximity effect in psoralens, the time-resolved fluorescence data had eluded a consistent interpretation. The observed decays were quite complex, and required at least three exponential terms to obtain statistically acceptable fits. These data were generally of low resolution and low precision, however, and no attempt was made to interpret the various lifetime components.

We have succeeded in obtaining TCSPC data for four psoralen derivatives and two related compounds (visnagin and khellin) that are of higher data precision (10K CPC) and resolution (512 channels) than those reported in the literature. We have obtained these data for a large matrix of solvents and emission wavelengths, and have analyzed the data in three different ways. By using the preexponential weighted mean lifetime, in conjunction with sum-of-exponential and ESM lifetime distribution analyses, we have arrived at an interpretation of the data that can be harmonized with the proximity effect.

The ESM lifetime distributions revealed multi-mode behavior for all of the psoralens in every solvent. The form of the distributions varied considerably with solvent, and the data for most of the solvents also exhibited an emission wavelength dependence. We believe that the discrete component analyses have established the minimum number of components required to adequately describe each psoralen in a given solvent. The ESM lifetime distributions, on the other hand, have a more fluid parameter space that reduces the correlations among fitting parameters that might be experienced in discrete component analyses. Finally, the preexponential weighted mean lifetime provides a single lifetime value that can be correlated with the emission wavelength or a solvent property. We believe that these three approaches, used in concert, comprise a powerful data-fitting toolbox.
Our data reveal the expected increases in the preexponential weighted mean lifetime with solvent polarity, as well as with emission wavelength. This latter observation is based on the assumption that polar solvents will reorient around the photoexcited psoralen in its $S_1$ state, lowering its energy relative to both $S_2$ and $S_0$. The stabilization relative to $S_2$ reduces the impact of the proximity effect and causes the increase in the fluorescence lifetime, while the stabilization relative to $S_0$ lowers the energy gap between these two states and red-shifts the emission. Consequently, emission from the red edge of the fluorescence spectrum represents a preferential observation of emission from solvent-equilibrated species.

This work represents an essential first step in a full-scale investigation of the photophysics and photochemistry of psoralens. To our knowledge, no high-level calculations have been reported for psoralen derivatives in the literature. There is every reason to believe that such calculations would be at least as useful in understanding these systems as they were in our investigation of dialkyldiazirine photophysics. By investigating the effects of specific substituents on the relative energies of various states of these psoralens, it should be possible to select or synthesize analogs with known $S_0 - S_1$ and $S_1 - S_2$ energy gaps.

Also, given the framework for interpreting the time-resolved fluorescence data developed in Chapter 5, systematic studies of the solvent polarity, emission wavelength, and pH dependence of the TCSPC data of some psoralen derivatives should be initiated. Specifically, global analyses of the multidimensional data surfaces should be attempted using data obtained in a solvent of tunable polarity (e.g., water-dioxane mixtures) and adjustable pH, monitoring the fluorescence at several wavelengths across the emission spectrum. The proper number of lifetime components to include in the global analysis of the data surface should be determined by a combination of discrete-component and ESM lifetime distribution analysis as outlined in Chapter 5.
The global analyses of these data will also require a systematic collection of steady-state absorption and emission spectra for each point in the data matrix. When these data are combined with the results of the *ab initio* or density functional calculations, it should be possible to correlate the observed fluorescence decay components with specific states and/or chemical events of the psoralens. Of course the acquisition of transient absorption and/or transient Raman data for these systems would also provide useful insight into their photophysics and photochemistry. This understanding should be of great value in the development of antiviral agents based on the psoralen ring system.

**Fluorescence Quenching of Pyrene by Humic Substances**

Humic substances are complex, heterogeneous mixtures of polyfunctional, highly aromatic organic compounds found in a wide variety of natural environments. They have been implicated in the solubility enhancement and facilitated transport of nonpolar organic compounds (NOCs) in surface and subsurface waters and sedimentary porefluids. Of particular interest are the class of organic molecules known as polycyclic aromatic hydrocarbons (PAHs). PAHs are formed as byproducts of incomplete combustion, and many have been implicated as being carcinogenic or otherwise toxic.

The quenching of PAH fluorescence by humic substances is a popular method used to measure partition constants for PAHs in humic substance matrices. This technique is based upon the assumption that the quenching process is static in nature. In other words, it assumes that a ground-state association occurs between PAH and humic substance that renders the PAH nonfluorescent. If some or all of the quenching was due to collisions between photoexcited PAH molecules and the humic substances (dynamic quenching), the recovered partition coefficients would be inaccurate.
Fluorescence lifetime measurements are often used to distinguish static from dynamic quenching. While static quenching reduces fluorescence intensity, it does not affect fluorescence lifetimes. Dynamic quenching, on the other hand, affects both the intensity and the lifetime of fluorescence since collisions compete with fluorescence emission to depopulate excited states.

The measurement of fluorescence lifetimes for PAHs in humic substance matrices is complicated by the fluorescence of the humic materials themselves. These substances possess complex lifetime distributions that reflect their chemical heterogeneity. Consequently, it is difficult or impossible to fit PAH fluorescence data to a simple sum-of-exponentials, even if the lifetimes are quite different.

We collected fluorescence decays for aqueous solutions of pyrene in the presence of humic substances isolated from four distinct environmental sources. We found that we were able to fit the data successfully in two different ways. Our original approach involved a “filtering” of the TCSPC data in the time domain. The lifetime distributions of humic materials are dominated by short (2 – 9 ns) components, and the fluorescence due to these substances is completely negligible after about 100 ns. Pyrene, in contrast, has a room temperature lifetime of 194 ± 5 ns in deoxygenated aqueous solutions, and retains considerable fluorescence intensity after 100 ns. By collecting TCSPC data of sufficiently high-precision, we were able to fit only the “tails” of the fluorescence decay curves, thus completely avoiding the fluorescence of the humic substances. We typically collected data to 10K CPC in our first fitting channel, ~115 ns into the decay. This usually corresponded to a precision of greater than 10^6 CPC at time zero. The pyrene decays could be adequately described by single exponentials using this “fitting-window” method.
We recently re-analyzed our data using ESM fluorescence lifetime distributions. We found this approach to be effective in recovering the complex lifetime distributions of the humic materials and the discrete lifetime of pyrene simultaneously. The pyrene lifetimes obtained using this approach were identical to those obtained using the "fitting-window" approach. The humic substances themselves were found to possess a broad distribution of lifetimes with 2 – 3 modes. Roughly 25% of the steady-state fluorescence intensity of these mixtures may be attributed to subnanosecond lifetime components, while the majority of the remainder have lifetimes between 2 – 9 ns. Less than 10% of the intensity was due to longer-lived species.

We observed significant quenching of pyrene's fluorescence in aqueous solutions due to dissolved molecular oxygen. The fluorescence lifetime of pyrene at room temperature was reduced to 141 ± 8 ns in oxic solutions, relative to its value of 194 ± 5 ns in anoxic solutions. As references to the fluorescence quenching technique in the literature made no mention of this effect, we suspect that many measurements of partition coefficients might have been rendered inaccurate by this artifact. Future attempts to measure partition coefficients by the fluorescence quenching method should certainly be performed on anoxic samples.

We did not observe a change in pyrene's fluorescence lifetime in deoxygenated solutions of humic materials. We therefore conclude that the quenching of pyrene's fluorescence by these materials is static in nature, as required by the fluorescence quenching technique. However, we did notice a slight decrease in pyrene's fluorescence lifetime as a function of humic substance concentration for the oxic samples. This led us to suggest that the local concentrations of oxygen and pyrene might be enhanced within or near the dissolved humic substances, and that the dynamic quenching of pyrene's fluorescence due to oxygen might be enhanced as a result. This effect, if present, is weaker than we originally reported, but is still suggested by the data.
These experiments revealed the necessity of deoxygenating any solutions used to measure partition coefficients between PAHs and humic substances by the fluorescence quenching technique. We also think we might have identified a "synergistic quenching" mechanism in oxic aqueous solutions of pyrene in the presence of humic substances due to the increased local concentrations of these nonpolar species in or near the humic matrix.

This last conclusion must be held tentatively, and should be supported or refuted by additional experiments. Specifically, steady-state fluorescence measurements should be performed in conjunction with the time-resolved measurements. Unfortunately, at the time these experiments were conducted, we did not have access to a steady-state fluorimeter on-site. Collecting the steady-state spectrum would allow the changes in intensities and lifetimes to be monitored simultaneously. Future experiments should also rigorously control the oxygen content of the aerated solutions via some combination of temperature control and bubbling oxygen into the cuvettes. The cuvettes should then be sealed to prevent equilibration with the atmosphere.

Conclusion

We have studied a wide variety of chemical systems using the Time-Correlated Single-Photon Counting approach for the measurement of time-resolved fluorescence. We have analyzed the data from these systems in several creative and innovative ways, on a case-by-case basis. While the traditional sum-of-exponentials approach was occasionally acceptable, the data interpretation for other systems either required or was enhanced by fitting the data to unparameterized distributions of lifetimes using the Exponential Series Method. For some systems, filtering the data in the time domain or calculating a preexponential weighted mean lifetime facilitated the interpretation. Information from other spectroscopies and computational chemistry was incorporated into the analyses as appropriate. Throughout
this dissertation we have discussed several of the most important issues in acquiring and analyzing TCSPC data for complex systems of photochemical, biophysical, and environmental interest. These samples are often photolabile and/or highly heterogeneous. If naive, oversimplified, or erroneous interpretations of the time-resolved data are to be avoided, each system must be approached carefully. The chemistry of each system must be considered at every stage: in designing appropriate experiments, controlling the conditions in the laboratory, and in determining the best model(s) for analyzing the data.

We believe that the research presented in this document represents a significant contribution to the understanding of several important chemical systems, and provides new paradigms for the analysis of TCSPC data. We are hopeful that it will also lay a foundation for future research.
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SYNTHESIZE: A WINDOWS95 COMPUTER PROGRAM TO: (1) GENERATE SYNTHETIC TCSPC DATA AND (2) CONVERT TCSPC DATAT FILES (WRITTEN IN LABWINDOWS/CVI V4.01)

The project consists of the following files:
CV14702.C
CV14702.H
CV14702.UIR
RANLIB.C
RANLIB.H
LINPACK.H
COM.C

CV14702.C

----------
#include <ansic.h>
#include <analysis.h>
#include <formatio.h>
#include <string.h>
#include <math.h>
#include <userint.h>
#include "cv14702.h"
#include "ranlib.h"

#define PATH_SIZE 260
#define LINE_SIZE 260
#define FILE_SIZE 260
#define EXT_SIZE 5
#define CONVOLVED_ARRAY_SIZE 16384
#define MAX_ARRAY_SIZE 8192
// #define MAX_ARRAY_SIZE 256
#define SMALL_ARRAY_SIZE 260
#define MAX_EXPONENTIALS 400
// #define MAX_EXPONENTIALS 101
#define GAUSSIANS 8 // Number of Gaussians to define simulated IRF
#define DEFAULT_PATH "e:\\jsb\\db\\Part 1"
#define COMMA_EXTENSION ".unc"
#define IRF_EXTENSION ".irf"
#define SPACE_EXTENSION ".dt2"
#define PCA_EXTENSION ".asc"
#define SIMULATION_EXTENSION ".fit"
#define FIT_EXTENSION ".txt"
/* min and max macros */
#ifndef max
#define max(a,b) (((a) > (b)) ? (a) : (b))
#endif
#ifndef min
#define min(a,b) (((a) < (b)) ? (a) : (b))
#endif

struct dataStruct {
    double x[MAX_ARRAY_SIZE];
    double y[MAX_ARRAY_SIZE];
    double spc[MAX_ARRAY_SIZE];
    double irf[MAX_ARRAY_SIZE];
    double sigma[MAX_ARRAY_SIZE];
    int xStart, xStop;
};

struct smallDataStruct {
    double x[SMA L L ARRAY_SIZE];
    double y[SMA L L ARRAY_SIZE];
};

struct headerStruct {
    char fileName[F I L E SIZE + 1], spectrumId[L I NE SIZE + 1];
    int numberOfChannels, peakChannel;
    unsigned int elapsedTime;
    double peakCounts;
    double totalCounts;
};

struct simulationStruct {
    int numberOfChannels, numberOfExponential s, darkCounts, irfSource, convolutionType,
    noise;
    unsigned int countsPeakChannel;
    double TACIncrement, TACRange;
    double tau[MAX_EXPONENTIALS];
    double amp[MAX_EXPONENTIALS];
    double fraction[MAX_EXPONENTIALS];
};

struct regressionStruct {
    double R_Square;
    double slope;
    double intercept;
};

struct pointStruct {
    double x;
    double y;
};

struct gaussianStruct {
    double amp[GAUSSIANS];
    double center[GAUSSIANS];
    double width[GAUSSIANS];
};

static struct dataStruct data;
// static struct calData;
static struct smallDataStruct points, calDataZoom;
static struct pointStruct cursor1, cursor2;
static struct headerStruct header;
static struct simulationStruct sim;
static structure gaussianStruct gauss;
static char currXUnitName[260];
static char buffer[LINe_SIZE + 2];
static char fileName[PATH_SIZE + 1];
static char commaFile[PATH_SIZE + 1];
static char irffile[PATH_SIZE + 1];
static char spaceFile[PATH_SIZE + 1];
static char headerFileName[PATH_SIZE + 1];
static char **fileList;

static int currYAxis = 1;
static int currXUnit = 1;
static int numberOfFiles = 0;
static int numberOfNewFiles;
static int fileIndex;
static int fileSelErr;
static int headerStatus = 0;
static int panelHandle;
static int fileHandle;
static int commaFileHandle;
static int irffileHandle;
static int spaceFileHandle;
static int headerFileHandle;
static int reverseMode;
static int textStyleIndex = 0;
static int textErr;
static int dataPlot = 0;
static int virginDataPlot = 1;
static int virginCalibrationPlot = 1;

static double nspPerChannel = 1.0;
static double activeYData[MAX_ARRAY_SIZE + 1];
static double Y[MAX_ARRAY_SIZE][MAX_EXPONENTIALS];
// static double H[MAX_ARRAY_SIZE][MAX_EXPONENTIALS]; // Temporary
static double convolved[CONVOLVED_ARRAY_SIZE];
static double cl[MAX_ARRAY_SIZE]; // Temporary array for convolutions.

void SetupTextbox (void);
int Convolute (void);
void conv (int, double[], int, double[], double[]);

void main ()
{
    panelHandle = LoadPanel (0, "cv14702.ui", PANEL);
    DisplayPanel (panelHandle);
    SetupTextbox ();
    RunUserInterface ();
}

/* FUNCTION: conv.c - convolution of x[n] with h[n], resulting in y[n]
   Downloaded from the internet.
*/

void conv(M, h, L, x, y)
double *h, *x, *y;
int M, L;

int n, m;
for (n = 0; n < L+M; n++)
    for (y[n] = 0, m = max(0, n-L+1); m <= min(n, M); m++)
        y[n] += h[m] * x[n-m];

/* FUNCTION: ConvoluteMartin

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• Convolve the instrumental response function with a sum of exponentials.

• Adapted from Thomas Martin's SIMSPC.C program.

```c
int ConvoluteMartin (void)
{
    int t, tp, i, respmax, respmin;
    double resparea = 0.0, temp, dt;

    for (i = 0; i < sim.numberofChannels; i++) {
        resparea += data.irf[i];
    }
    resparea = sim.countsPeakChannel / resparea;

    for (t = 0; t < sim.numberofChannels; t++) {
        data.y[t] = 0.0;
        for (i = 0; i < sim.numberofExponentials; i++) {
            temp = 0.0;
            for (tp = 0; tp < sim.numberofChannels && tp <= t; tp++) {
                temp -= data.irf[tp] * exp((tp - t) * sim.TAC Increment / sim.tau[i]);
            }
            data.y[t] = data.y[t] += temp * sim.amp[i];
        }
        data.y[t] = data.y[t] * resparea;
        data.y[t] = data.y[t] * sim.countsPeakChannel;
    }
    return 1;
}
```

/* FUNCTION: ConvoluteTCSPC  
* Convolve the instrumental response function with a sum of exponentials.  
* Translated from the FORTRAN code of O'Connor & Phillips, pp. 192-196.  
*/

```c
int ConvoluteTCSPC (void)
{
    double fact;
    // double cl[MAX_ARRAY_SIZE];  // For intermediate results. Now defined globally.
    double a[(2 * MAX_EXPONENTIALS)];  // Array of fitting parameters

    int nchan;
    int i;
    int j1, j2;
    fact = sim.TAC Increment;  // ns / channel
    nchan = sim.numberofChannels;
    ic = sim.numberofExponentials;

    for (j = 0; j < ic; j++) {
        j1 = 2 * j;
        j2 = j1 + 1;
        a[j1] = sim.amp[j];
        a[j2] = sim.tau[j];
    }

    /* The convolution integral is calculated using the trapezoidal rule. */
    for (i = 0; i < nchan; i++) {
        data.y[i] = 0.0;
    }
    c1[0] = 0.0;
    for (j = 0; j < ic; j++) {
        j1 = 2 * j;
```
\[ j_2 = j_1 + 1; \]

for \( i = 1; i < \text{nchan}; i++ \) {
    \[ c[i] = \exp(-\text{fact} / a[j_2]) \times c[i - 1] \]
    \[ - a[j_1] \times \text{fact} \times 0.5 \times (\text{data.irf}[i - 1] \times \exp(-\text{fact} / a[j_2]) + \text{data.irf}[i]); \]
}

\[ \text{data.y}[i] = \text{data.y}[i] + c[i]; \]
}\]

/* Check that data points are not < 0 */
for \( i = 0; i < \text{nchan}; i++ \) {
    if (\text{data.y}[i] < 0.0) {
        \text{data.y}[i] = 0.0;
    }
}
return 0;
\]

/* FUNCTION: FitConvolveESM
ConvolveESM for fitting algorithm. */
double FitConvolveESM (double x, double coef[], int ncoef)
{
    int i, j;
    double fit;
    i = (int) x;

    /* This is the only calculation that must be performed on each iteration. */
    for \( j = 0; j < ncoef; j++ \) {
        fit = coef[j] \times Y[i][j];
    }
    return fit;
}

/* FUNCTION: ConvolveESM
Generates the data matrix from the current array of amplitudes and the Y matrix.
This approach adapted from FITIT.EXE by Timothy Rhodes. */
int ConvolveESM (void)
{
    int i, j;

    /* This is the only calculation that must be performed on each iteration. */
    for \( i = 0; i < \text{sim.numberOfChannels}; i++ \) {
        for \( j = 0; j < \text{sim.numberOfExponentials}; j++ \) {
            \text{data.y}[i] += \text{sim.amp}[j] \times Y[i][j];
        }
    }

    return 0;
}

/* FUNCTION: GenerateYMatrix
Convolve the instrumental response function with a sum of exponentials.
Uses code translated from the FORTRAN of O'Connor & Phillips, pp. 192-196. */
int GenerateYMatrix (void)
{
    int i, j;
}
for (i = 0; i < sim.numberofChannels; i++) { // Initialize data array.
data.y[i] = 0.0;
}

for (j = 0; j < sim.numberofExponentials; j++) { // Loop through exponentials.
  /* The convolution integral is calculated using the trapezoidal rule. */
  for (i = 0; i < sim.numberofChannels; i++) {
    Y[i][j] = 0.0; ...... // Initialize exponential array.
    cl[0] = 0.0;
    for (i = 1; i < sim.numberofChannels; i++) {
      cl[i] = exp(-sim.TACIncrement/sim.tau[j])*cl[i-1]
               + sim.TACIncrement*0.5*(data.irf[i-1]*exp(-sim.TACIncrement/
               sim.tau[j])+data.irf[i]);
      Y[i][j] = Y[i][j] + cl[i];
    }
  }
  return 0;
}

/* FUNCTION: GenerateHMatrix */
* Convolve the instrumental response function with a sum of exponentials.
*/

/* int GenerateHMatrix (void) */
{
  int i, j, m;
  /* Generate synthetic decay */

  /* for (m = 0; m < (sim.numberofExponentials + 1); m++) { // Loop through exponentials.
    i = m - 1;
    /* The convolution integral is calculated using the trapezoidal rule. */
    /* for (i = 0; i < sim.numberofChannels; i++) { */
      H[i][m] = 0.0; ...... // Initialize exponential array.
    } 
    cl[0] = 0.0;
    if (m == 0) {
      for (i = 0; i < sim.numberofChannels; i++) {
        H[i][m] = 1;
      }
    } else {
      for (i = 1; i < sim.numberofChannels; i++) {
        cl[i] = exp(-sim.TACIncrement/sim.tau[j])*cl[i-1]
                + sim.TACIncrement*0.5*(data.irf[i-1]*exp(-sim.TACIncrement/
                sim.tau[j])+data.irf[i]);
        H[i][m] = H[i][m] + cl[i];
      }
    }
  }
  return 0;
}

/* FUNCTION: FitOneDataFile */

int FitOneDataFile (char* fileName)
{
    static int saveFile, dataPlot, done, intBuffer, peakChannel, fileCloseErr, scanErr,
    ticErr;
    static int i = 1;
    static int j = 1;
    static int k = 1;
    static int bufferSize = sizeof(buffer);
    static double realBuffer, norm, meanSquaredError;

double arrayIndex[MAX_ARRAY_SIZE];
char stringBuffer[LINE_SIZE + 1];
char distributionFile[LINE_SIZE + 1];

FillBytes (buffer, 0, bufferSize - 1, 0);   // Fill Bytes with 0s
FillBytes (spaceFile, 0, PATH_SIZE + 1, 0);  // Fill Bytes with 0s

/* Read in file for decay. */
fileHandle = OpenFile (fileName, VAL_READ_ONLY, VAL_TRUNCATE,_
                          VAL_ASCII);

done = 1;
for (i = 0; done != -2; i++) {
    done = ReadLine (fileHandle, buffer, (bufferSize - 1));
    Scan (buffer, "$%f%\n", &data.x[i], stringBuffer, &data.spc[i]);
    header.numberOfChannels = i;
}

fileCloseErr = CloseFile (fileHandle);

DeleteGraphPlot (panelHandle, PANEL_DATA_GRAPH, -1, VAL_IMMEDIATE_DRAW);

    /* Find the channel with the maximum # of counts & its index. */
MaxMinID (data.spc, header.numberOfChannels, &header.peakCounts,
                   &header.peakChannel, &realBuffer, &intBuffer);   // MaxMinID

    /* For the area under the decay, we are only interested in channels
       from the peakChannel forward. Therefore, take a subset and sum the elements. */
SubsetID (data.spc, header.numberOfChannels, header.peakChannel,
               (header.numberOfChannels - header.peakChannel),
                   activeYData, header.totals.Counts);
SumID (activeYData, (header.numberOfChannels - header.peakChannel),
            &header.totals.Counts);

    /* Read in file for IRF. */
FileSelectPopup ("e:\jsb\db\Part 1", "*.dt2", "", "Choose Space-Delimited IRF File",
                          VAL_OK_BUTTON, 0, 0, 1, 1, irfFile);
    fileHandle = OpenFile (irfFile, VAL_READ_ONLY, VAL_TRUNCATE,
                          VAL_ASCII);

done = 1;
for (i = 0; done != -2; i++) {
    done = ReadLine (fileHandle, buffer, (bufferSize - 1));
    Scan (buffer, "$%f%\n", &data.x[i], stringBuffer, &data.irf[i]);
    sim.numberOfChannels = i;
}

fileCloseErr = CloseFile (fileHandle);

    // Insert test here to check if sim.numberOfChannels = header.numberOfChannels
sim.TACIncrement = (data.x[1] - data.x[0]);

sim.TACRange = sim.numberOfChannels * sim.TACIncrement;

dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x, data.spc,
                   header.numberOfChannels, VAL_DOUBLE, VAL_DOUBLE,
                   VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_RED);

dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x,
                   data.irf, header.numberOfChannels,
                   VAL_DOUBLE, VAL_DOUBLE, VAL_THIN_LINE,
                   VAL_NO_POINT, VAL_SOLID, 1, VAL_BLUE);

SetCtrlVal (panelHandle, PANEL_NUMERIC_NO_OF_CHAN,
            header.numberOfChannels);

SetCtrlVal (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS,
            header.totalCounts);

SetCtrlVal (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, header.peakCounts);

SetCtrlVal (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL,
            header.peakChannel);

SetCtrlAttribute (panelHandle, PANEL_STRING_FILENAME, ATTR_DIMMED, 1);

SetCtrlAttribute (panelHandle, PANEL_STRING_SPECTRUM_ID, ATTR_DIMMED, 1);

SetCtrlAttribute (panelHandle, PANEL_NUMERIC_NO_OF_CHAN, ATTR_DIMMED, 0);

SetCtrlAttribute (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS, ATTR_DIMMED, 0);

SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, ATTR_DIMMED, 0);

SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL, ATTR_DIMMED, 0);

SetCtrlAttribute (panelHandle, PANEL_NUMERIC_COLLECT_TIME, ATTR_DIMMED, 1);

SetCtrlVal (panelHandle, PANEL_NUMERIC_TAC_RANGE, sim.TACRange);

SetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, header.numberOfChannels);

SetCtrlVal (panelHandle, PANEL_NUMERIC_INCREMENT, sim.TACIncrement);

SetCtrlVal (panelHandle, PANEL_NUMERIC_CPC, (int) header.peakCounts);

/* dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, sim.tau, sim.fraction,
                     sim.numberOfExponentials, VAL_DOUBLE, VAL_DOUBLE,
                     VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1,
                     VAL_BLUE);

SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_XMAP_MODE,
                  VAL_LINEAR);

SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_YMAP_MODE,
                  VAL_LOG);

*/

MaxMinID (data.irf, header.numberOfChannels, &norm,
          &peakChannel, &realBuffer, &intBuffer);

/* Find the IRF channel with the maximum # of counts & its index. */

for (i = 0; i < header numberOfChannels; i++) {
   data.irf[i] = data.irf[i] / norm;
   data.spc[i] = data.spc[i] * header.peakCounts;
}

/* Read in a FITIT.EXE distribution to get the set of lifetimes. */

FillBytes (buffer, 0, bufferSize - 1, 0);
FillBytes (commaFile, 0, PATH_SIZE + 1, 0);
FileSelectPopup ("e:\\jdb\\Part 1", ".txt", ",", "Choose Distribution File Containing Lifetimes", VAL_OK_BUTTON, 0, 0, 1, 1, distributionFile);

fileHandle = OpenFile (distributionFile, VAL_READ_ONLY, VAL_TRUNCATE,
                       VAL_ASCII);

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/* Read and discard first two lines. Read the third line and get the # of
  exponentials. */
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
Scan (buffer, "\%d", &sim.nNumberOfExponentials);
if (sim.nNumberOfExponentials < 1 || sim.nNumberOfExponentials > MAX_EXPONENTIALS) {
  MessagePopup ("Invalid Distribution File",
               "Number of Exponentials Must be 1 - 400");
  return 1;
}
/* Read the lifetimes & amplitudes for the sum of exponentials. */
for (j = 0; j < sim.nNumberOfExponentials; j++) {
  scanErr = ScanFile (fileHandle, "\%f[\%f[\%f]n", &sim.tau[j], &sim.amp[j]);
}
fileCloseErr = CloseFile (fileHandle);
/* Reset all of the amplitudes to 1 / numberOfExponentials */
/* for (j = 0; j < sim.nNumberOfExponentials; j++) {
  sim.amp[j] = 1 / sim.nNumberOfExponentials;
} */
/* Normalize the ESM Amplitudes */
SumId (sim.amp, sim.nNumberOfExponentials, &norm);
for (j = 0; j < sim.nNumberOfExponentials; j++) {
  sim.amp[j] = sim.amp[j] / norm;
}
GenerateYMatrix();
//GenerateHMatrix();
/* for (i = 0; i < sim.nNumberOfChannels; i++) {*/
  //arrayIndex[i] = (double) i;
/* } */
/* NonLinearFit (arrayIndex, data.spc, data.y, sim.nNumberOfChannels,
  *FitConvolveESM, sim.amp, sim.nNumberOfExponentials,
  &meanSquaredError); */
/* for (i = 0; i < sim.nNumberOfChannels; i++) {
  if (data.spc[i] > 0) {
    data.sigma[i] = 1 / sqrt(data.spc[i]);
  } else {
    data.sigma[i] = 1;
  }
}
fitErr = GenLSFit (Y, sim.nNumberOfChannels, sim.nNumberOfExponentials,
  data.spc, NULL, 0, data.y, sim.amp, NULL,
  &meanSquaredError);
/* for (i = 0; i < sim.nNumberOfChannels; i++) {*/
  FmtFile (commaFileHandle, "\%f, \%f\n", data.x[i], data.y[i]);
/* } */
dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x, data.y,
  sim.nNumberOfChannels, VAL_DOUBLE, VAL_DOUBLE,
  VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_GREEN);
SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_YMAP_MODE,
  VAL_LOG);
SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_XMAP_MODE, VAL_LINEAR);

return 0;
}

/* FUNCTION: SetupTextbox
 * Sets up column titles in a textbox that will display file summary information
 * in the same format used in the header file to be generated.
 */

void SetupTextbox ()
{
    textErr = InsertTextBoxLine (panelHandle, PANEL_TEXTBOX_HEADER_INFO,
                                  ....... textLineIndex, "Total | Peak | Peak | Collect |");
    textLineIndex++;
    textErr = InsertTextBoxLine (panelHandle, PANEL_TEXTBOX_HEADER_INFO,
                                  ....... textLineIndex, "Counts | Counts | Chan | Time(s) | Decay ID");
    textLineIndex++;
    textErr = InsertTextBoxLine (panelHandle, PANEL_TEXTBOX_HEADER_INFO,
                                  ....... textLineIndex, "FILENAME | CHAN |");
    textLineIndex++;
}

int Quit (int panel, int control, int event,
           void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
    case EVENT_COMMIT:
        QuitUserInterface (0);
        break;
    }
    return 0;
}

/* FUNCTION: SelectFiles
 * Uses a multi-file select popup to allow the user to generate a list of
 * data files to process. If the function is run > 1 time before files are
 * processed, new files are appended to the end of the current queue.
 */

int SelectFiles (int panel, int control, int event,
                 void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
    switch (event) {
    case EVENT_COMMIT:
        /* Reinitialize index & offset each time the function is called. */
        index = 0;
        offset = 0;

        /* If SelectFiles has been run > 1 time, add new files to end of list. */
        offset = numberOfFiles;
        if (offset != 0) {
            index = index + offset;
        }
        /* Select the files and store them in filelist */
fileSelErr = MultiFileSelectPopup (DEFAULT_PATH, "*.*", "*.asc, *.unc",
    "Select Files to Process", 0, 0, 1,
    &numberOfNewFiles, &fileList);
/* Total # of files = # of new files + # of previously selected files. */
    numberOfFiles += numberOfNewFiles;
/* This rather messy loop adds each new filename to the list of pending files */
    for (loopIndex = 0; loopIndex < numberOfNewFiles; loopIndex++) {
        listErr = InsertListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
            index, fileList[loopIndex],
            index++; /* Increment index */
            ... ...
        }
    } break;
}
return 0;

/* FUNCTION: doOnePcaToComma */
* Reads and tabulates information from PCA-II header rows. Reads first 2 columns *
* from PCA-II file and places them into an output file. Discards ROI column. */
int doOnePcaToComma (char* fileName)
{
    static int saveFile;
    static int dataPlot;
    static int fcloseErr;
    static int intBuffer;
    static int extensionIndex, slashIndex;
    static int i = 1;
    static int m = 1;
    static int bufferSize = sizeof(buffer);
    double realBuffer;
    char stringBuffer[L_INE_SIZE + 1];
    char headerString[L_INE_SIZE + 1];
    fillBytes (header.spectrumId, 0, LINE_SIZE + 1, 0);
    fillBytes (header.fileName, 0, FILE_SIZE + 1, 0);
    fillBytes (headerString, 0, LINE_SIZE + 1, 0);
    fillBytes (buffer, 0, bufferSize - 1, 0);
    fillBytes (commaFile, 0, PATH_SIZE + 1, 0);
    fileHandle = OpenFile (fileName, VAL_READ_ONLY, VAL_TRUNCATE,
        VAL_ASCII);
    if (headerStatus != 0) headerFileHandle = OpenFile (headerFileName, VAL_WRITE_ONLY,
        VAL_APPEND, VAL_ASCII);
    if (headerStatus == 2) {
        WriteLine (headerFileHandle, " I # of I Total I Peak I Peak ! Collect !", -1);
WriteLine (headerFileHandle, "Filename | Chan | Counts | Counts | Chan |
Time(s) | Decay ID", -1);
WriteLine (headerFileHandle,
"================================================================================================================================================", -1);
  headerStatus = 1;
}

ReadLine (fileHandle, buffer, LINE_SIZE + 1);
Scan (buffer, "%37c[dl%39c[d]", &header.elapsedTime); /* discard date, time, & "Elt:" */
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%4c%124c", buffer, header.spectrumId);

/* Don’t display default message */
m = CompareBytes (header.spectrumId, 0,
  "No spectrum identifier defined", 0, 30, 1);
if (m == 0) FillBytes (header.spectrumId, 0, LINE_SIZE - 1, 0);
ReadLine (fileHandle, buffer, (bufferSize - 1));

/* discard "Memory Size: INTEGER Chls Conversion Gain: " */
Scan (buffer, "%37c%124c", stringBuffer, &intBuffer, stringBuffer,
&header.numberOfChannels, stringBuffer);
ReadLine (fileHandle, buffer, (bufferSize - 1));
ReadLine (fileHandle, buffer, (bufferSize - 1));
for (i = 0; i < header.numberOfChannels; i++) {
  ReadLine (fileHandle, buffer, (bufferSize - 1));
  Scan (buffer, "%f%lx%4f", &data.x[i], &data.y[i]);
}

fcloseErr = CloseFile (fileHandle);

/* Reverse the order of Y data if experiment was run in reverse mode. */
if (reverseMode == 1) {
  Reverse (data.y, header.numberOfChannels, data.y);
}

/* Find the channel with the maximum # of counts & its index. */
MaxMinID (data.y, header.numberOfChannels, &header.peakCounts,
  &header.peakChannel, &realBuffer, &intBuffer);

/* For the area under the decay, we are only interested in channels from the peakChannel forward. Therefore, take a subset and sum the elements. */
SubsetID (data.y, header.numberOfChannels, header.peakChannel,
  (header.numberOfChannels - header.peakChannel) ,
  activeYData);
SumID (activeYData, (header.numberOfChannels - header.peakChannel),
  &header.totalCounts);

/* For our table, we are not interested in the file extension or the path. The actual fileName will be between the right-most backslash and a period. */
slashIndex = FindPattern (fileName, 0, -1, "\\", 0, 1);
extensionIndex = FindPattern (fileName, 0, -1, ".", 0, 0);

/* Copy the appropriate subset of the full path into the header structure. */
CopyString (header.fileName, 0, fileName, (slashIndex + 1),
  (extensionIndex - slashIndex - 1));
DeleteGraphPlot (panelHandle, PANEL_DATA_GRAPH, -1, VAL_IMMEDIATE_DRAW);

CopyString (commaFile, 0, fileName, 0, -1);
extensionIndex = FindPattern (commaFile, 0, -1, ".", 0, 0);
CopyBytes (commaFile, extensionIndex, COMMA_EXTENSION, 0, EXT_SIZE);
commaFileHandle = OpenFile (commaFile, VAL_WRITE_ONLY, VAL_TRUNCATE,
...... VAL_ASCII);

for (i = 0; i < header.numberOfChannels; i++) {
    FmtFile (commaFileHandle, "%f, %f
", data.x[i], data.y[i]);
}

fileCloseErr = CloseFile (commaFileHandle);

dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x, data.y,
header.numberOfChannels, VAL_DOUBLE, VAL_DOUBLE,
VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_RED);

SetCtrlVal (panelHandle, PANEL_STRING_FILENAME, header.fileName);
SetCtrlVal (panelHandle, PANEL_STRING_SPECTRUM_ID, header.spectrumId);
SetCtrlVal (panelHandle, PANEL_NUMERIC_NO_OF_CHAN,
header.numberOfChannels);
SetCtrlVal (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS,
header.totalCounts);
SetCtrlVal (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, header.peakCounts);
SetCtrlVal (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL,
header.peakChannel);
SetCtrlVal (panelHandle, PANEL_NUMERIC_COLLECT_TIME,
header.elapsedTime);

SetCtrlAttribute (panelHandle, PANEL_STRING_FILENAME, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_STRING_SPECTRUM_ID, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_NO_OF_CHAN, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_COLLECT_TIME, ATTR_DIMMED, 0);

Fmt (headerString, "%s %w8 ! %w5 %w9p %f [w9p] %i[w5p] %s",
header.fileName, header.numberOfChannels, header.totalCounts,
header.peakCounts,
header.peakChannel, header.elapsedTime, header.spectrumId);

textErr = InsertTextBoxLine (panelHandle, PANEL_TEXTBOX_HEADER_INFO,
...... textLineIndex, headerString);
textLineIndex++;

/** Write header information to file. */
if (headerStatus != 0) {
    WriteLine (headerFileHandle, headerString, -1);
    fileCloseErr = CloseFile (headerFileHandle);
}

return 0;
}

/* FUNCTION: doOneSimulationToComma
 * Reads and tabulates information from simulation data header rows. Reads 3 columns
 * from file and creates 2 comma-delimited output files (IRF & Decay).
 */
int doOneSimulationToComma (char* fileName)
{
    static int saveFile;
static int dataPlot;
static int fileCloseErr;
static int intBuffer;
static int extensionIndex, slashIndex;
static int i = 1;
static int m = 1;
static int bufferSize = sizeof(buffer);
double realBuffer;
char stringBuffer[LINE_SIZE + 1];
char headerString[LINE_SIZE + 1];

FillBytes (header.spectrumId, 0, LINE_SIZE + 1, 0);
FillBytes (header.fileName, 0, FILE_SIZE + 1, 0);
FillBytes (headerString, 0, LINE_SIZE + 1, 0);
FillBytes (buffer, 0, bufferSize - 1, 0);
FillBytes (commaFile, 0, PATH_SIZE + 1, 0);

if (headerStatus != 0) headerFileHandle = OpenFile (headerFileName, VAL_WRITE_ONLY, VAL_TRUNCATE, VAL_APPEND, VAL_ASCII);

if (headerStatus == 2) {
    WriteLine (headerFileHandle, " # of Total Peak Peak |
    Collect !", -1);
    WriteLine (headerFileHandle, "Filename | Chan | Counts | Counts | Chan |
    Time(s) | TAC Increment(ns/chann)", -1);
    WriteLine (headerFileHandle, "-" * (header.numberOfChannels + 1));
}

ReadLine (fileHandle, buffer, LINE_SIZE + 1);
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
ReadLine (fileHandle, buffer, LINE_SIZE + 1);

Scan (buffer, "%c%d%i%u%29c", &header.numberOfChannels, header.spectrumId);

for (i = 0; i < header.numberOfChannels; i++) {
    ReadLine (fileHandle, buffer, (bufferSize - 1));
    Scan (buffer, "%f%f%f%f", &data.x[i], &data.y[i], &data.cf[i], &data.y[i]);
}

fileCloseErr = CloseFile (fileHandle);

/* Reverse the order of Y data if experiment was run in reverse mode. */
if (reverseMode == 1) {
    Reverse (data.y, header.numberOfChannels, data.y);
}

/* Find the channel with the maximum # of counts & its index. */
MaxMin1D (data.y, header.numberOfChannels, &header.peakCounts,
    &header.peakChannel, &realBuffer, &intBuffer);

/* For the area under the decay, we are only interested in channels
    from the peakChannel forward. Therefore, take a subset and sum the elements.
    */
Subset1D (data.y, header.numberOfChannels, header.peakChannel,
    (header.numberOfChannels - header.peakChannel),
    activeYData);
SumID (activeYData, (header.numberOfChannels - header.peakChannel),
       header.totalCounts);

/* For our table, we are not interested in the file extension or the path.
   The actual file name will be between the right-most backslash and a period. */
slashIndex = FindPattern (fileName, 0, -1, "\\", 0, 1);
extensionIndex = FindPattern (fileName, 0, -1, ".", 0, 0);

/* Copy the appropriate subset of the full path into the header structure. */
CopyString (header.fileName, 0, fileName, (slashIndex + 1),
      (extensionIndex - slashIndex - 1));

DeleteGraphPlot (panelHandle, PANEL_DATA_GRAPH, -1, VAL_IMMEDIATE_DRAW);

CopyString (commaFile, 0, fileName, 0, -1);
extensionIndex = FindPattern (commaFile, 0, -1, ",", 0, 0);
CopyBytes (commaFile, extensionIndex, COMMA_EXTENSION, 0, EXT_SIZE);
commaFileHandle = OpenFile (commaFile, VAL_WRITEONLY, VAL_TRUNCATE,
                      ...... VAL_ASCII);

for (i = 0; i < header.numberOfChannels; i++) {
    FmtFile (commaFileHandle, "%f, %f\n", data.x[i], data.y[i]);
}

fileCloseErr = CloseFile (commaFileHandle);

dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x, data.y,
            header.numberOfChannels, VAL_DOUBLE, VAL_DOUBLE,
            VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_RED);

SetCtrlVal (panelHandle, PANEL_STRING_FILENAME, header.fileName);
SetCtrlVal (panelHandle, PANEL_STRING_SPECTRUM_ID, header.spectrumId);
SetCtrlVal (panelHandle, PANEL_NUMERIC_NO_OF_CHAN,
        header.numberOfChannels);
SetCtrlVal (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS,
        header.totalCounts);
SetCtrlVal (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, header.peakCounts);
SetCtrlVal (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL,
        header.peakChannel);
SetCtrlVal (panelHandle, PANEL_NUMERIC_COLLECT_TIME,
        header.elapsedTime);

SetCtrlAttribute (panelHandle, PANEL_STRING_FILENAME, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_STRING_SPECTRUM_ID, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_NO_OF_CHAN, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_COLLECT_TIME, ATTR_DIMMED, 0);

Fmt (headerString, "%s[w8] %s[w5p] %s[w9p0] %s[w9p] %s[w5p] %s[w9p] %s",
      header.fileName, header.numberOfChannels, header.totalCounts,
      header.peakCounts,
      header.peakChannel, header.elapsedTime, header.spectrumId);
textErr = InsertTextBoxLine (panelHandle, PANEL_TEXTBOX_HEADER_INFO,
                               ...... textLineIndex, headerString);
textLineIndex++;

/* Write header information to file. */
if (headerStatus !=0) {
    WriteLine (headerFileHandle, headerString, -1);
fileCloseErr = CloseFile (headerFileHandle);
}

/* Save IRF data as a separate comma-delimited file. */
CopyString (irfFile, 0, fileName, 0, -1);
extensionIndex = FindPattern (irfFile, 0, -1, ".", 0, 0);
CopyBytes (irfFile, extensionIndex, IRF_EXTENSION, 0, EXT_SIZE);
irfFileHandle = OpenFile (irfFile, VAL_WRITE_ONLY, VAL_TRUNCATE,
.... VAL_ASCII);
for (i = 0; i < header.numberOfLines; i++) {
   FmtFile (irfFileHandle, "%T , %f\n", data.x[i], data.irf[i]);
}
fileCloseErr = CloseFile (irfFileHandle);
return 0;

/* FUNCTION: AddPoissonNoiseToOneFittedFile
* Reads the generated ESM fit from FITIT.EXE and adds Poisson noise.
* Creates a comma-delimited output file using the current value of nsPerChannel.
*/
int AddPoissonNoiseToOneFittedFile (char* fileName)
{
    static int scanErr, timeCalErr, seedErr, saveFile, dataPlot, fileCloseErr, intBuffer;
    static int extensionIndex, slashIndex;
    static int i = 1;
    static int m = 1;
    static int bufferSize = sizeof(buffer);

    static long is1 = 1; // from ranlib.c
    static long is2 = 1; // from ranlib.c

    static unsigned long intYData = 1;
    double realBuffer;

    char stringBuffer[L LINE_SIZE + 1];
    char headerString[L LINE_SIZE + 1];
    char test; // from ranlib.c

    FillBytes (buffer, 0, bufferSize - 1, 0);
    FillBytes (commaFile, 0, PATH_SIZE + 1, 0);

    fileHandle = OpenFile (fileName, VAL_READONLY, VAL_TRUNCATE,
VAL_ASCII);

    /* Read and discard first two lines. Read the third line and get the # of channels. */
    ReadLine (fileHandle, buffer, LINE_SIZE + 1);
    ReadLine (fileHandle, buffer, LINE_SIZE + 1);
    Scan (buffer, "%I[u]", header.numberOfLines);

    /* Read the data for the ESM best-fit equation. */
    for (i = 0; i < header.numberOfLines; i++) {
        scanErr = ScanFile (fileHandle, "%f[x]\%f[x]\n", &data.x[i], &data.y[i]);
    }

    fileCloseErr = CloseFile (fileHandle);
    DeleteGraphPlot (panelHandle, PANEL_DATA_GRAPH, -1, VAL_IMMEDIATE_DRAW);
    CopyString (commaFile, 0, fileName, 0, -1);
}
extensionIndex = FindPattern (commaFile, 0, -1, ".", 0, 0);
CopyBytes (commaFile, extensionIndex, COMMA_EXTENSION, 0, EXT_SIZE);
commaFileHandle = OpenFile (commaFile, VAL_WRITE_ONLY, VAL_TRUNCATE,
...... VAL_ASCII);

dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x, data.y,
header.numberOfChannels, VAL_DOUBLE, VAL_DOUBLE,
VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_BLUE);

/* Prompt for time calibration in ns / channel. */
timeCalErr = PromptPopup (fileName,
"Enter Time Calibration in ns / channel.\n", buffer, bufferSize - 1);
Scan (buffer, "%f", &sim.TACIncrement);

/* Prompt for phrase that will be used to seed the random number generator. 
Use different phrases to generate different sets of random numbers. */
seedErr = PromptPopup ("Random Number Seed Generator",
"Enter phrase to seed random number generator.\n", buffer, bufferSize - 1);
phrtsd(buffer, &isl, &is2); // Generates seeds from phrase. From ranlib.c
setall(is1, is2); // I do not know the purpose (if any) of this function.

/* Add noise to the data and save in a comma-delimited file. */
for (i = 0; i < header.numberOfChannels; i++) {
    data.x[i] = data.x[i]*nsPerChannel; // Convert channel number to time in ns.
    intYData = (unsigned long) data.y[i]; // Convert double to unsigned long int.
    if (intYData != 0) { // Cannot generate Poisson noise on zero counts!
        intYData = ignpol(intYData); // Add Poisson noise. From ranlib.c
        data.y[i] = intYData;
    }
}

/* Output to comma-delimited file, convert counts to unsigned long int. */
FmtFile (commaFileHandle, "%f, %ln", data.x[i], (unsigned long) intYData);

fileCloseErr = CloseFile (commaFileHandle);
DeleteGraphPlot (panelHandle, PANEL_DATA_GRAPH, -1, VAL_IMMEDIATE_DRAW);
dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x, data.y,
header.numberOfChannels, VAL_DOUBLE, VAL_DOUBLE,
VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_RED);
return 0;

FUNCTION: SynthesizeOneDataFile
* Reads a distribution of exponentials in the form generated by FITIT.EXE.
* Synthesizes a TCPSC data file according to the current parameters for
  Counts in Peak Channel (CPC), # of Channels, Time Base, Dark Counts and
  convolves it with an instrumental response function. Poisson noise is added
  and the file is saved as a comma-delimited (Time, Counts) file. nsPerChannel.
*/

int SynthesizeOneDataFile (char* fileName)
{
static int scanErr, multErr, timeCalErr, seedErr, buildErr, saveFile, dataPlot;
static int fileCloseErr, intBuffer, slashIndex, extensionIndex, peakChannel, done,
offset;
static int i = 1;

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static int j = 1;
static int k = 1;
static int m = 1;
static int bufferSize = sizeof(buffer);

static long is1 = 1; // from ranlib.c
static long is2 = 1; // from ranlib.c

static unsigned long intYData = 1;
static double realBuffer, norm;

char stringBuffer[LINER_SIZE +1];
char headerString[LINER_SIZE +1];
char test; // from ranlib.c

/* Sum of Gaussians from Experimental IRF */
/* (Currently CV01803.DT2) */
gauss.amp[0] = 6044.59664;
   gauss.center[0] = -0.0073797;
   gauss.width[0] = 0.01260564;
gauss.amp[1] = 3379.97550;
   gauss.center[1] = 3.0026e-06;
   gauss.width[1] = 0.00935079;
   gauss.center[2] = 0.01101850;
   gauss.width[2] = 0.01311009;
gauss.amp[3] = 717.151778;
   gauss.center[3] = 0.02793337;
   gauss.width[3] = 0.0201741;
gauss.amp[4] = 308.766919;
   gauss.center[4] = 0.07298302;
   gauss.width[4] = 0.03498277;
gauss.amp[5] = 207.191256;
   gauss.center[5] = 0.17846651;
   gauss.width[5] = 0.0409437;
gauss.amp[6] = 64.4378161;
   gauss.center[6] = 0.21990441;
   gauss.width[6] = 0.02129296;
   gauss.center[7] = 0.27503357;
   gauss.width[7] = 0.10995785;

/* Get the simulation parameters from the control panel. */
GetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, &sim.numberOfChannels);
GetCtrlVal (panelHandle, PANEL_NUMERIC_CPC, &sim.countsPeakChannel);
GetCtrlVal (panelHandle, PANEL_NUMERIC_DARK_CTS, &sim.darkCounts);
GetCtrlVal (panelHandle, PANEL_NUMERIC_INCREMENT, &sim.TACIncrement);
GetCtrlVal (panelHandle, PANEL_NUMERIC_TAC_RANGE, &sim.TACRange);
GetCtrlVal (panelHandle, PANEL_SWITCH_IRF, &sim.irqSource);
GetCtrlVal (panelHandle, PANEL_SWITCH_NOISE, &sim.noise);

FillBytes (buffer, 0, bufferSize - 1, 0);
FillBytes (commaFile, 0, PATH_SIZE + 1, 0);

fileHandle = OpenFile (fileName, VAL_READ_ONLY, VAL_TRUNCATE,
   VAL_ASCII);

/* Read and discard first two lines. Read the third line and get the # of
   exponentials. */
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
ReadLine (fileHandle, buffer, LINE_SIZE + 1);
Scan (buffer, "%%lu", &sim.numberOfExponentials);
if (sim.numbosExponentials < 1 || sim.numbosExponentials > MAX_EXPONENTIALS) {
    MessagePopup("Invalid Distribution File", "Number of Exponentials Must be 1 - 400");
    return 1;
}

/* Read the lifetimes & amplitudes for the sum of exponentials. */
for (i = 0; i < sim.numbosExponentials; i++) {
    scanErr = ScanFile (fileHandle, "%f%n", &sim.tau[i], &sim.amp[i]);
}

fileCloseErr = CloseFile (fileHandle);
DeleteGraphPlot (panelHandle, PANEL_DATA_GRAPH, -1, VAL_IMMEDIATE_DRAW);

CopyString (commaFile, 0, fileName, 0, -1);
extensionIndex = FindPattern (commaFile, 0, -1, ".", 0, 0);
CopyBytes (commaFile, extensionIndex, COMMA_EXTENSION, 0, EXT_SIZE);
commaFileHandle = OpenFile (commaFile, VAL_WRITE_ONLY, VAL_TRUNCATE, 

/* Test to check whether first & last amplitudes are dark & scatter. */
if (sim.numbosExponentials > 1) {
    multErr = MulID (sim.tau, sim.amp, sim.numbosExponentials, sim.fraction);
    if (sim.tau[0] < sim.tau[1]) {
        sim.fraction[0] = 0; /* Remove scatter component. */
    }
    if (sim.tau[sim.numbosExponentials - 1] < sim.tau[sim.numbosExponentials - 1]) {
        sim.fraction[sim.numbosExponentials - 1] = 0; /* Remove dark count component. */
    }
}

dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, sim.tau, sim.fraction, sim.numbosExponentials, VAL_DOUBLE, VAL_DOUBLE, VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_BLUE);
SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_YMAP_MODE, VAL_LINEAR);
SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_XMAP_MODE, VAL_LOG);
}

if (sim.irfSource == 0) {
    /* Read IRF from file */
    FileSelectPopup ("e:\\sb\\db\\Part 1", ".dt2", ",", "Choose Space-Delimited IRF File", 

        VAL_OK_BUTTON, 0, 0, 1, 1, irffile);
    fileHandle = OpenFile (irffile, VAL_READ_ONLY, VAL_TRUNCATE, 
        VAL_ASCII);
    done = 1;
    for (i = 0; done != -2; i++) {
        done = ReadLine (fileHandle, buffer, (bufferSize - 1));
        Scan (buffer, "%f", &data.x[i], stringBuffer, &data.irf[i]);
        sim.numbosChannels = i;
    }
    sim.TACIncrement = (data.x[1] - data.x[0]);
    sim.TACRange = sim.numbosChannels * sim.TACIncrement;
    fileCloseErr = CloseFile (fileHandle);
SetCtrlVal (panelHandle, PANEL_NUMERIC_TAC_RANGE, sim.TACRange);
SetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, sim.numbosChannels);
```c
SetCtrlVal (panelHandle, PANEL_NUMERIC_INCREMENT, sim.TACIncrement);
SetCtrlVal (panelHandle, PANEL_NUMERIC_CPC, sim.countsPeakChannel);
SetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, sim.numberOfChannels);
}
else {
    // Generate synthetic IRF
    for (i = 0; i < sim.numberOfChannels; i++) {
        data.x[i] = i * sim.TACIncrement;
        data.irf[i] = 0;
        for (j = 0; j < (GAUSSIANS - 1); j++) {
            data.irf[i] = data.irf[i] + gauss.amp[j] * exp(-0.5 * ((data.x[i] -
                gauss.center[j]) / gauss.width[j]));
        }
    }

    /* Find the channel with the maximum # of counts & its index. */
    MaxMinLD (data.irf, sim.numberOfChannels, &norm, 
              &peakChannel, &realBuffer, &intBuffer);

    /* Normalize the IRF */
    for (i = 0; i < sim.numberOfChannels; i++) {
        data.irf[i] = data.irf[i] / norm;
        data.irf[i] = data.irf[i] * sim.countsPeakChannel;
    }

    /* Normalize the ESM Amplitudes */
    SumLD (sim.amp, sim.numberOfExponentials, &norm);
    for (i = 0; i < sim.numberOfExponentials; i++) {
        sim.amp[i] = sim.amp[i] / norm;
    }

    switch (sim.convolutionType) {
    case 0:
        .......
        // ESM / O'Connor & Phillips
        GenerateMatrix();
        ConvolveESM();
        break;
    case 1:
        .......
        // No Convolution
        /* Generate synthetic decay */
        for (i = 0; i < sim.numberOfChannels; i--) {
            data.y[i] = 0;
            for (j = 0; j < sim.numberOfExponentials; j++) {
                if (data.x[i] >= 0) {
                    data.y[i] = data.y[i] + sim.amp[j] * exp(-data.x[i] / sim.tau[j]);
                }
            }
        }
        break;
    case 2:
        .......
        // conv.c - same as Convolve()
        /* Generate synthetic decay */
        for (i = 0; i < sim.numberOfChannels; i++) {
            data.y[i] = 0;
            for (j = 0; j < sim.numberOfExponentials; j++) {
                if (data.x[i] >= 0) {
                    data.y[i] = data.y[i] + sim.amp[j] * exp(-data.x[i] / sim.tau[j]);
                }
            }
        }
```

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conv (sim.numberofChannels, data.irf, sim.numberofChannels, data.y, convolved);
for (i = 0; i < sim.numberofChannels; i++) {
    data.y[i] = convolved[i];
}
break;

// O'Connor & Phillips
ConvolutedCSPC ();
break;

// Thomas Martin's GOMER.C
ConvolutedMartin ();
break;

// LW/CVI Convolve() function
/* Generate synthetic decay */
for (i = 0; i < sim.numberofChannels; i++) {
    data.y[i] = 0;
    for (j = 0; j < sim.numberofExponentials; j++) {
        if (data.x[i] > 0) {
            data.y[i] = data.y[i] + sim.amp[j] * exp(-data.x[i] / sim.tau[j]);
        }
    }
    Convolve (data.y, sim.numberofChannels, data.irf, sim.numberofChannels, convolved);
    for (i = 0; i < sim.numberofChannels; i++) {
        data.y[i] = convolved[i];
    }
    break;
}
default:
    break;

/* Check that data points are not < 0 */
for (i = 0; i < sim.numberofChannels; i++) {
    if (data.y[i] < 0.0) {
        data.y[i] = 0.0;
    }
}

/* Find the channel with the maximum # of counts & its index. */
MaxMin1D (data.y, sim.numberofChannels, sim.worm, speakChannel, &realBuffer, &intBuffer);

/* Normalize the decay and add dark counts. */
for (i = 0; i < sim.numberofChannels; i++) {
    data.y[i] = data.y[i] / norm;
    data.y[i] = data.y[i] * sim.countsPeakChannel;
    data.y[i] = data.y[i] + sim.darkCounts;
}
if (sim.noise) {       /* Add noise. */
    /* Prompt for phrase that will be used to seed the random number generator.
       Use different phrases to generate different sets of random numbers. */
    FillBytes (buffer, 0, bufferSize - 1, 0);
seedErr = PromptPopup ("Random Number Seed Generator",
"Enter phrase to seed random number generator.",
buffer, bufferSize - 1);
phrtsd(buffer,&is1,is2); // Generates seeds from phrase. From ranlib.c
setall(&is1,is2); // I do not know the purpose (if any) of this function.

From ranlib.c

/* Add noise to the data and save in a comma-delimited file. */
for (i = 0; i < sim.numberofChannels; i++) {
    intYData = (unsigned long) data.y[i]; // Convert double to unsigned long
    if (intYData != 0) { // Cannot generate Poisson noise on zero counts!
        intYData = ignpoi(intYData); // Add Poisson noise. From ranlib.c
        data.y[i] = intYData;
    }
    /* Output to comma-delimited file, convert counts to unsigned long int. */
    FmtFile (commaFileHandle, "%f, %i
", data.x[i], (unsigned long) intYData);
} else {
    .......
    // Do not add noise.
    for (i = 0; i < sim.numberofChannels; i++) {
        intYData = (unsigned long) data.y[i]; // Convert double to unsigned long
        data.y[i] = intYData;
        /* Output to comma-delimited file, convert counts to unsigned long int. */
        FmtFile (commaFileHandle, "%f, %i
", data.x[i], (unsigned long) intYData);
    }
}

fileCloseErr = CloseFile (commaFileHandle);
DeleteGraphPlot (panelHandle, PANEL_DATA_GRAPH, -1, VAL_IMMEDIATE_DRAW);
dataPlot = PlotXY (panelHandle, PANEL_DATA_GRAPH, data.x, data.y,
sim.numberofChannels, VAL_DOUBLE, VAL_DOUBLE,
VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLI, 1, VAL_RED);
SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_XMAP_MODE,
VAL_LINEAR);
SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_YMAP_MODE,
VAL_LINEAR);

return 0;

/* FUNCTION: doOneCommaToSpace */
* Reads and tabulates information from PCA-II header rows. Reads 2 columns
* from file with data pairs in (channel, counts) format and places them into
* an output file in (channel counts) format. Eventually this function will
* perform an x-conversion on the channel number using the current calibration
* equation to yield data in (time counts) format.
*/

int doOneCommaToSpace (char* fileNName)
{
    static int saveFile;
    static int dataPlot;
    static int fileCloseErr;
    static int intBuffer;
    static int extensionIndex, slashIndex;
static int i = 0;
static int m = 1;
static int done;
static int bufferSize = sizeof(buffer);
double realBuffer;
char stringBuffer[LIN_SIZE + 1];
char headerString[LIN_SIZE + 1];

FillBytes (header.fileName, 0, FILE_SIZE + 1, 0);
FillBytes (buffer, 0, bufferSize - 1, 0);
FillBytes (spaceFile, 0, PATH_SIZE + 1, 0);

fileHandle = OpenFile (fileName, VAL_READ ONLY, VAL_TRUNCATE, VAL_ASCII);

done = 1;
for (i = 0; done != -2; i++) {
    done = ReadLine (fileHandle, buffer, (bufferSize - 1));
    Scan (buffer, "\%f%3c%f", &data.x[i], stringBuffer, &data.y[i]);
    header.numberOfChannels = i;
}

fileCloseErr = CloseFile (fileHandle);

/* Find the channel with the maximum # of counts & its index. */
MaxMinID (data.y, header.numberOfChannels, &header.peakCounts,
        header.peakChannel, &realBuffer, &intBuffer);

/* For the area under the decay, we are only interested in channels from the peakChannel forward. Therefore, take a subset and sum the elements. */

SubsetID (data.y, header.numberOfChannels, header.peakChannel,
        (header.numberOfChannels - header.peakChannel),
        activeYData);

SumID (activeYData, (header.numberOfChannels - header.peakChannel),
        header.totalCounts);

/* For our table, we are not interested in the file extension or the path. The actual fileName will be between the right-most backslash and a period. */
slashIndex = FindPattern (fileName, 0, -1, "\\", 0, 1);
extensionIndex = FindPattern (fileName, 0, -1, ".", 0, 0);

/* Copy the appropriate subset of the full path into the header structure. */
CopyString (header.fileName, 0, fileName, (slashIndex + 1),
           (extensionIndex - slashIndex - 1));

DeleteGraphPlot (panelHandle, PANEL_DATA GRAPH, -1, VAL_IMMEDIATE DRAW);

dataPlot = PlotXY (panelHandle, PANEL_DATA GRAPH, data.x, data.y,
                   header.numberOfChannels, VAL_DOUBLE, VAL_DOUBLE,
                   VAL_THIN_LINE, VAL_NO_POINT, VAL_SOLID, 1, VAL_RED);

SetCtrVal (panelHandle, PANEL_STRING_FILENAME, header.fileName);
SetCtrVal (panelHandle, PANEL_NUMERIC_NO_OF_CHAN,
           header.numberOfChannels);
SetCtrVal (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS,
           header.totalCounts);
SetCtrVal (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, header.peakCounts);
SetCtrVal (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL,
           header.peakChannel);
SetCtrlAttribute (panelHandle, PANEL_STRING_FILENAME, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_STRING_SPECTRUM_ID, ATTR_DIMMED, 1);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_NO_OF_CHAN, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_TOTAL_COUNTS, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_COUNTS, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_PEAK_CHANNEL, ATTR_DIMMED, 0);
SetCtrlAttribute (panelHandle, PANEL_NUMERIC_COLLECT_TIME, ATTR_DIMMED, 1);
saveFile = ConfirmPopup ("Confirmation", "Has this file converted properly?");
if (saveFile == 1) {
    CopyString (spaceFile, 0, fileName, 0, -1);
    extensionIndex = FindPattern (spaceFile, 0, -1, ".", 0, 0);
    CopyBytes (spaceFile, extensionIndex, SPACE_EXTENSION, 0, EXT_SIZE);
    spaceFileHandle = OpenFile (spaceFile, VAL_WRITE_ONLY, VAL_TRUNCATE,
        ...... VAL_FSYNC);
    for (i = 0; i < header.numberChannels; i++) {
        fmtFile (spaceFileHandle, "%f \n", data.x[i], data.y[i]);
    }
    fileCloseErr = CloseFile (spaceFileHandle);
} else {
    MessagePopup ("Conversion Aborted", "This file was NOT converted.");
}
return 0;

FUNCTION: SimulationToComma
* Loop through the files in the pending list, read their paths from the list,
* and convert one simulated data file -> two comma-delimited files.
*
int SimulationToComma (int panel, int control, int event,
    void *callbackData, int eventData1, int eventData2) {
    switch (event) {
        static int getFileErr, checkErr;
        static int pendingErr;
        static int properFileType, confirmNoHeader;
        static int extensionIndex;
        static int goAhead;
        case EVENT_COMMIT:
            if (headerStatus == 0) {
                confirmNoHeader = ConfirmPopup ("No Header File Selected!",
                    "Do you wish to continue without one?");
                if (confirmNoHeader == 0) {
                    MessagePopup ("Conversion Aborted", "Select Header File and
                    Begin Again.");
                    return 0;
                }
            }
            for (fileIndex = 0; fileIndex < numberOfFiles; fileIndex++) {
                getFileErr = GetValueFromIndex (panelHandle,
                    PANEL_LISTBOX_PENDING_FILES, .......
                fileName);
                checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
                    ...... fileIndex, 1);
                extensionIndex = FindPattern (fileName, 0, -1, ".", 0, 0);
properFileType = CompareStrings (fieldName, extensionIndex, 

SIMULATION_EXTENSION, 0, 0);
        if (properFileType == 0) {
            doOneSimulationToComma (fieldName);
        } else {
            goAhead = ConfirmPopup ("Possible File Type Mismatch", 

            "This file does not have the expected extension. Continue anyway?");
            if (goAhead == 1) doOneSimulationToComma (fieldName);
        }
        checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES, 

            fileName, 0);
        }
        for (fileIndex = (numberOfFiles - 1); fileIndex >= 0; fileIndex--)
            DeleteListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES, 

                fileName, 

                1);
        
        numberOfFiles = 0;
        break;
    }
    return 0;
}

int ESMFits (int panel, int control, int event,
    void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
        static int getFileErr, checkErr;
        static int pendingErr;
        static int properFileType, confirmNoHeader;
        static int extensionIndex;
        static int goAhead;
        
            case EVENT_COMMIT:
            for (fileIndex = 0; fileIndex < numberOfFiles; fileIndex++) {
                getFileErr = GetValueFromIndex (panelHandle, 

                    PANEL_LISTBOX_PENDING_FILES, 

                        fileName);
                checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES, 

                    fileName, 1);
                extensionIndex = FindPattern (fileName, 0, -1, ".", 0, 0);
                properFileType = CompareStrings (fieldName, extensionIndex, 

                    SPACE_EXTENSION, 0, 0);
                if (properFileType == 0) {
                    FitOneDataFile (fieldName);
                } else {
                    goAhead = ConfirmPopup ("Possible File Type Mismatch",

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..... "This file does not have the expected extension. Continue anyway?";
   if (goAhead == 1) FitOneDataFile (fileName);
   }
   checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
   .... fileIndex, 0);
   }
   for (fileIndex = (numberOfFiles - 1); fileIndex >= 0; fileIndex--) {
     DeleteListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
     fileIndex,
     .... 1);
   }
   numberofFiles = 0;
   break;
   return 0;
   }
   
   /* FUNCTION: AddNoiseToFittedFiles
   * Loop through the files in the pending list, read their paths from the list,
   * and add noise to fitted files from FITIT.EXE, creating comma-delimited files.
   */
   int AddNoiseToFittedFiles (int panel, int control, int event,
   void *callbackData, int eventData1, int eventData2)
   {
     switch (event) {
       static int getFileErr, checkErr;
       static int pendingErr;
       static int properFileType, confirmNoHeader;
       static int extensionIndex;
       static int goAhead;
       
       case EVENT_COMMIT:
       for (fileIndex = 0; fileIndex < numberofFiles; fileIndex++) {
         getFileErr = GetValueFromIndex (panelHandle,
         .... PANEL_LISTBOX_PENDING_FILES,
         .... fileName);
         checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
         .... fileIndex, 1);
         extensionIndex = FindPattern (fileName, 0, -1, ".", 0, 0);
         properFileType = CompareStrings (fileName, extensionIndex,
         .... FIT_EXTENSION, 0, 0);
         if (properFileType == 0) {
           AddPoissonNoiseToOneFittedFile (fileName);
         } else {
           goAhead = ConfirmPopup ("Possible File Type Mismatch",
           .... "This file does not have the expected extension. Continue anyway?");
           if (goAhead == 1) AddPoissonNoiseToOneFittedFile 
           (fileName);
         }
         checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
         .... fileIndex, 0);
         }
       for (fileIndex = (numberofFiles - 1); fileIndex >= 0; fileIndex--) {
       
       }
int SynthesizeData (int panel, int control, int event, 
    void *callbackData, int eventData1, int eventData2) 
{ 
    switch (event) 
    { 
        static int getFErr, checkErr;
        static int pendingErr;
        static int properFileType, confirmNoHeader;
        static int extensionIndex;
        static int goAhead;

        case EVENT_COMMIT: 
            for (fileIndex = 0; fileIndex < numberOfFiles; fileIndex++) 
            { 
                getFErr = GetValueFromIndex (panelHandle, 
                    PANEL_LISTBOX_PENDING_FILES, 
                    .......
                    fileName); 
                checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES, 
                    .......
                    fileIndex, 
                    .......
                    fileName); 
                extensionIndex = FindPattern (fileName, 0, -1, 
                    ".", 0, 0); 
                properFileType = CompareStrings (fileName, extensionIndex, 
                    .......
                IF_EXTENSION, 0, 0); 
                if (properFileType == 0) 
                { 
                    SynthesizeOneDataFile (fileName); 
                } 
                else 
                { 
                    goAhead = ConfirmPopup ("Possible File Type Mismatch", 
                    .......
                    "This file 
                    does not have the expected extension. Continue anyway?"); 
                    if (goAhead == 1) 
                        SynthesizeOneDataFile (fileName); 
                } 
                checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES, 
                    .......
                    fileIndex, 0); 
            } 
            for (fileIndex = (numberOfFiles - 1); fileIndex >= 0; fileIndex--) 
            { 
                DeleteListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES, 
                    .......
                    fileIndex, 
                    .......
                    1); 
            } 

            numberOfFiles = 0;

        }
break;
}
return 0;
}

/* FUNCTION: PcaToComma
 * Loop through the files in the pending list, read their paths from the list,
 * and convert one file PCA-II -> comma-delimited on each in turn.
 */
int PcaToComma (int panel, int control, int event,
 void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
    static int getFileErr, checkErr;
    static int pendingErr;
    static int properFileType, confirmNoHeader;
    static int extensionIndex;
    static int goAhead;
    case EVENT_COMMIT:
        if (headerStatus == 0) {
            confirmNoHeader = ConfirmPopup("No Header File Selected!",
                  "Do you wish to continue without
      one?");
            if (confirmNoHeader == 0) {
                MessagePopup("Conversion Aborted", "Select Header File and
            Begin Again.");
                return 0;
            }
        }
        for (fileIndex = 0; fileIndex < numberOfFiles; fileIndex++) {
            getFileErr = GetValueFromIndex (panelHandle, PANEL_LISTBOX_PENDING_FILES,

                  fileName);
            checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
                  fileIndex, 1);
            extensionIndex = FindPattern (fileName, 0, ".", 0, 0);
            properFileType = CompareStrings (fileName, extensionIndex,

                  PCA_EXTENSION, 0, 0);
            if (properFileType == 0) {
                doOnePcaToComma (fileName);
                }
            else {
                goAhead = ConfirmPopup("Possible File Type Mismatch",
                  "This file
does not have the expected extension. Continue anyway?");
                if (goAhead == 1) doOnePcaToComma (fileName);

            }
            checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,

                  fileIndex, 0);
        }
        for (fileIndex = (numberOfFiles - 1); fileIndex >= 0; fileIndex--) {
            DeleteListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,

                  fileIndex,

                  1);
    }}
int CommaToSpace (int panel, int control, int event, 
    void *callbackData, int eventData1, int eventData2) 
{
    switch (event) {
        static int getErr, checkErr;
        static int pendingErr;
        static int properFileType;
        static int extensionIndex;
        static int goAhead;
        case EVENT_COMMIT:
            for (fileIndex = 0; fileIndex < numberOfFiles; fileIndex++) {
                getErr = GetValueFromIndex (panelHandle, ?????
...................... fileIndex, 
...................... fileName);
                checkErr = CheckList {panelHandle, PANEL_LISTBOX_PENDING_FILES, 
...................... fileIndex, 1};
                extensionIndex = FindPattern (fileName, 0, -1, ",", 0, 0);
                properFileType = CompareStrings (fileName, extensionIndex, 
...................... COMMA_EXTENSION, 0, 0);
                if (properFileType == 0) {
                    doOneCommaToSpace {fileName};
                } else {
                    goAhead = ConfirmPopup ("Possible File Type Mismatch", 
...................... "This file 
...................... does not have the expected extension. Continue anyway?""1);
                    if (goAhead == 1) doOneCommaToSpace {fileName};
                }
                checkErr = CheckList {panelHandle, PANEL_LISTBOX_PENDING_FILES, 
...................... fileIndex, 0};
            }
            for (fileIndex = (numberOfFiles - 1); fileIndex >= 0; fileIndex--) {
                DeleteListItem {panelHandle, PANEL_LISTBOX_PENDING_FILES, 
...................... fileIndex, 
...................... 1};
            }
            numberOfFiles = 0;
            break;
        }
    return 0;
}

/* FUNCTION: SetConfiguration */
* Reads the TCSPC experimental configuration (forward or reverse)
  * from the appropriate panel switch.
*/

int SetConfiguration (int panel, int control, int event,
  void *callbackData, int eventData1, int eventData2)
{
  switch (event) {
    case EVENT_COMMIT:
      GetCtrlVal (panelHandle, PANEL_SWITCH_CONFIGURATION, &reverseMode);
      break;
  }
  return 0;
}

/* FUNCTION: SelectHeaderFile
 * Allows the creation or selection of a header file to store data file summary
 * information. If an existing file is chosen, new information is appended to
 * the previous file. Otherwise, a new file is created.
 */

int SelectHeaderFile (int panel, int control, int event,
  void *callbackData, int eventData1, int eventData2)
{
  static cnar headerFileDisplay[FILE_SIZE + 1];
  static int extensionIndex, slashIndex;

  switch (event) {
    case EVENT_COMMIT:
      headerStatus = FileSelectPopup ("e:\jsb\db\Part 1", "*.hdr", "", "Choose
      or Create Header File",
      .....  VAL_OK_BUTTON, 0, 0, 1, 1,
      headerFileName);
      if (headerStatus != 0) {
        slashIndex = findPattern (headerFileName, 0, -1, "\", 0, 1);
        extensionIndex = findPattern (headerFileName, 0, -1, ".", 0, 0);
        /* Copy the appropriate subset of the full path for display
           purposes. */
        CopyString (headerFileDisplay, 0, headerFileName, (slashIndex + 1),
          (extensionIndex - slashIndex - 1));
        SetCtrlVal (panelHandle, PANEL_STRING_HDR_FILENAME,
          headerFileDisplay);
        SetCtrlAttribute (panelHandle, PANEL_STRING_HDR_FILENAME,
          ATTR_DIMMED, 0);
        } 
      break;
  }
  return 0;
}

/* FUNCTION: ChooseScale
 * Reads the value from the appropriate switch to toggle between a linear
 * or logarithmic y-axis for the data graph.
 */

int ChooseScale (int panel, int control, int event,
  void *callbackData, int eventData1, int eventData2)
{
  switch (event) {
    static int getYScaleErr;
    static int yScaleErr;
    case EVENT_COMMIT:
getYScaleErr = GetCtrlVal (panelHandle, PANEL_SWITCH_CHCX, &curvYAxis);
yScaleErr = SetCtrlAttribute (panelHandle, PANEL_DATA_GRAPH, ATTR_YMAP_MODE, curvYAxis);
break;
}
return 0;
}

int CVICALLBACK setTACIncrement (int panel, int control, int event,
void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
        case EVENT_COMMIT:
            GetCtrlVal (panelHandle, PANEL_NUMERIC_INCREMENT, &sim.TAICrement);
            GetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, &sim.numberOfChannels);
            sim.TACRange = sim.TAICrement * sim.numberofChannels;
            SetCtrlVal (panelHandle, PANEL_NUMERIC_TAC_RANGE, sim.TACRange);
            SetCtrlVal (panelHandle, PANEL_NUMERIC_CPC, sim.countsPeakChannel);
            SetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, sim.numberofChannels);
            break;
    }
    return 0;
}

int CVICALLBACK setTACRange (int panel, int control, int event,
void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
        case EVENT_COMMIT:
            GetCtrlVal (panelHandle, PANEL_NUMERIC_TAC_RANGE, &sim.TACRange);
            GetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, &sim.numberofChannels);
            sim.TAICrement = sim.TACRange / sim.numberofChannels;
            SetCtrlVal (panelHandle, PANEL_NUMERIC_INCREMENT, sim.TAICrement);
            SetCtrlVal (panelHandle, PANEL_NUMERIC_CPC, sim.countsPeakChannel);
            SetCtrlVal (panelHandle, PANEL_NUMERIC_CHANNELS, sim.numberofChannels);
            break;
    }
    return 0;
}

int CVICALLBACK SetConvolutionType (int panel, int control, int event,
void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
        case EVENT_COMMIT:
            GetCtrlVal (panelHandle, PANEL_RING_CONV_TYPE, &sim.convolutionType);
            break;
    }
    return 0;
}

int CVICALLBACK SetNoise (int panel, int control, int event,
void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
        case EVENT_COMMIT:
            GetCtrlVal (panelHandle, PANEL_SWITCH_NOISE, &sim.noise);
            break;
    }
    return 0;
}
#include <userint.h>

#ifdef __cplusplus
extern "C" {
#endif

/* Panels and Controls: */
#define PANEL 1
#define PANEL_BUTTON_COMMA_TO_SPACE 2 /* callback function: CommaToSpace */
#define PANEL_BUTTON_PCA_TO_COMMA 3 /* callback function: PcaToComma */
#define PANEL_BUTTON_FILES_SELECT 4 /* callback function: SelectFiles */
#define PANEL.Quit 5 /* callback function: Quit */
#define PANEL.TEXTBOX_HEADER_INFO 6
#define PANEL_LISTBOX_PENDING_FILES 7
#define PANEL_SWITCH_NOISE 8 /* callback function: SetNoise */
#define PANEL_SWITCH_IRF 9
#define PANEL_SWITCH_CONFIGURATION 10 /* callback function: SetConfiguration */
#define PANEL_NUMERIC_COLLECT_TIME 11
#define PANEL_NUMERIC_PEAK_CHANNEL 12
#define PANEL_NUMERIC_PEAK_COUNTS 13
#define PANEL_SWITCH_CHOOSE_SCALE 14 /* callback function: ChooseScale */
#define PANEL_NUMERIC_TOTAL_COUNTS 15
#define PANEL_NUMERIC_NO_OF_CHAN 16
#define PANEL_STRING_HDR_FILENAME 17
#define PANEL_STRING_FILENAME 18
#define PANEL_STRING_SPECTRUM_ID 19
#define PANEL.BUTTON_SELECT_HEADER 20 /* callback function: SelectHeaderFile */
#define PANEL.BUTTON_SYNTHESIZE 22 /* callback function: SynthesizeData */
#define PANEL.BUTTON_FIT_TO_COMMA 23 /* callback function: AddNoiseToFittedFiles */
#define PANEL.BUTTON_SIM_TO_COMMA 24 /* callback function: SimulationToComma */
#define PANEL_NUMERIC_INCREMENT 25
#define PANEL_NUMERIC_CHANNELS 26
#define PANEL_NUMERIC_CPC 27
#define PANEL_NUMERIC_DARKCTS 28
#define PANEL_NUMERIC_TAC_RANGE 29
#define PANEL_COMMANDBUTTON_RANGE 30 /* callback function: setTACRange */
#define PANEL_COMMANDBUTTON_INC 31 /* callback function: setTACIncrement */
#define PANEL_RING_CONV_TYPE 32 /* callback function: SetConvolutionType */
#define PANEL_BUTTON_ESM_FIT 33 /* callback function: ESMFits */

#ifdef __cplusplus
}
#endif

/ * Menu Bars, Menus, and Menu Items: */
/* (no menu bars in the resource file) */

/* Callback Prototypes: */
int CVICALLBACK AddNoiseToFittedFiles(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK ChooseScale(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK CommaToSpace(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK EMFits(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK PcaToComma(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK Quit(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK SelectFiles(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK SelectHeaderFile(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK SetConfiguration(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK SetConvolutionType(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK SetNoise(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK setTACIncrement(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK setTACRange(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK SimulationToComma(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
int CVICALLBACK SynthesizeData(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);

#ifdef __cplusplus

;
#endif
THE FOLLOWING FILES WERE OBTAINED FROM THE INTERNET

RANLIB.C

---

#include "ranlib.h"
#include <stdio.h>
#include <stdlib.h>
#include <math.h>

#define ABS(x) ((x) >= 0 ? (x) : -(x))
#define min(a,b) ((a) <= (b) ? (a) : (b))
#define max(a,b) ((a) >= (b) ? (a) : (b))

//ftntstop(char*);

float genbet(float aa, float bb)
{
    float genbet(float aa, float bb)
    GenErate BETA random deviate
    Function
    Returns a single random deviate from the beta distribution with
    parameters A and B. The density of the beta is
    x^ ( a-1 ) * (1-x)^( b-1 ) / B( a,b ) for 0 < x < 1
    Arguments
    aa -> First parameter of the beta distribution
    bb -> Second parameter of the beta distribution

    Method
    R. C. H. Cheng
    Generating Beta Variate with Nonintegral Shape Parameters
    (Algorithms BB and BC)

    if (qsame) goto S2G;
    if((aa < 0.0 || bb < 0.0)) goto S10;
    puts("AA or BB < 0 in GENBET - Abort!",stderr);
    printf(stderr,"AA: %16.6E BB %16.6E\n",aa,bb);
    exit(1);
    S10:
    olda = aa;
    oldb = bb;
    S20:
    if((min(aa,bb) > 1.0)) goto S100;
    Algorithim BB
    Initialize
    if(qsame) goto S30;
    a = min(aa,bb);
    b = max(aa,bb);
    alpha = a+b;
beta = sqrt((alpha-2.0)/(2.0*a*b-alpha));
gamma = a+1.0/beta;

S30:
S40:
  ul = ranf();
  /*
   * Step 1
   */
  u2 = ranf();
  v = beta*log(ul/(1.0-ul));
  if(!(v > expmax)) goto S50;
  w = infnty;
  goto S60;
S50:
  w = a*exp(v);
S60:
  z = pow(u1,2.0)*u2;
  r = gamma*v-1.3862944;
  s = a+r-w;
  /*
   * Step 2
   */
  if(s+2.609438 >= 5.0*z) goto S70;
  /*
   * Step 3
   */
  t = log(z);
  if(s > t) goto S70;
  /*
   * Step 4
   */
  if(r-alpha*log(alpha/(b-w)) < t) goto S40;
S70:
  /*
   * Step 5
   */
  if(!(aa == a)) goto S80;
  genbet = w/(b-w);
  goto S90;
S80:
  genbet = b/(b+w);
S90:
  goto S130;
S100:
  /* Algorithm BC
   * Initialize
   */
  if(qsame) goto S110;
  a = max(aa,bb);
  b = min(aa,bb);
  alpha = a+b;
  beta = 1.0/b;
  delta = 1.0+a-b;
  k1 = delta*(1.38889E-2+4.16667E-2*b)/(a*beta-0.777778);
  k2 = 0.25-(0.5-0.25/delta)*b;
S110:
S120:
  ul = ranf();
  /*
   * Step 1
   */
  u2 = ranf();
  if(ul >= 0.5) goto S130;
  /*
Step 2

\[ y = ul \cdot u2; \]
\[ z = ul \cdot y; \]
\[ \text{if}(0.25 \cdot u2 + z - y \geq k1) \text{ goto S120}; \]
\[ \text{goto S170}; \]
S130:

/*

Step 3

\[ z = \text{pow}(ul, 2.0) \cdot u2; \]
\[ \text{if}(! (z \leq 0.25)) \text{ goto S160}; \]
\[ v = \text{beta} \cdot \log(ul/(1.0 - ul)); \]
\[ \text{if}(! (v > \text{expmax})) \text{ goto S140}; \]
\[ w = \text{infty}; \]
\[ \text{goto S150}; \]
S140:
\[ w = a \cdot \exp(v); \]
S150:
\[ \text{goto S200}; \]
S160:
\[ \text{if}(z \geq k2) \text{ goto S120}; \]
S170:

/*

Step 4

Step 5

\[ v = \text{beta} \cdot \log(ul/(1.0 - ul)); \]
\[ \text{if}(! (v > \text{expmax})) \text{ goto S180}; \]
\[ w = \text{infty}; \]
\[ \text{goto S190}; \]
S180:
\[ w = a \cdot \exp(v); \]
S190:
\[ \text{if}(\alpha \cdot (\log(\alpha/(b+w)) + v) - 1.3862944 < \log(z)) \text{ goto S120}; \]
S200:

/*

Step 6

\[ \text{if}(! (a == aa)) \text{ goto S210}; \]
\[ \text{genbet} = w/(b+w); \]
\[ \text{goto S220}; \]
S210:
\[ \text{genbet} = b/(b-w); \]
S220:
S223:
return genbet;

*undef expmax
*undef infty
*/

float genchi(float df)
/*

******************************************************************************
float genchi(float df)
Generate random value of CHI square variable
Function
Generates random deviate from the distribution of a chisquare
with DF degrees of freedom random variable.
Arguments
df -> Degrees of freedom of the chisquare
(Must be positive)
Method
Uses relation between chisquare and gamma.
*******************************************************************************/

402
static float genchi;
if(!(df <= 0.01)) goto S10;
fputs("DF <= 0 in GENCHI - ABORT",stderr);
fprintf(stderr,"Value of DF: %.16.6E\n",df);
exit(1);
S10:
genchi = 2.0*gend(1.0,df/2.0);
return genchi;

float genexp(float av)
/*
 * static float genexp;
 *
genexp = expo()*av;
return genexp;

float genf(float dfn,float dfd)
/*
 * static float genf,xden,xnum;
 */
if(!(dfn <= 0.0 || dfd <= 0.0)) goto S10;
fputs("Degrees of freedom nonpositive in GENG - abort!",stderr);
fprintf(stderr,"DFN value: %.16.6E DFD value: %.16.6E\n",dfn,dfd);
exit(1);
S10:

GENerate EXPonential random deviate
Function
Generates a single random deviate from an exponential
distribution with mean AV.

Arguments
AV -> The mean of the exponential distribution from which
a random deviate is to be generated.

Method
Renames SEXPO from TOMS as slightly modified by BWB to use RANF
instead of SUNIF.
For details see:
Ahrens, J.H. and Dieter, U.
Computer Methods for Sampling From the
Exponential and Normal Distributions.

GENerate random deviate from the F distribution
Function
Generates a random deviate from the F (variance ratio)
distribution with DFN degrees of freedom in the numerator
and DFD degrees of freedom in the denominator.

Arguments
DFN -> Numerator degrees of freedom
(Must be positive)
DFD -> Denominator degrees of freedom
(Must be positive)

Method
Directly generates ratio of chi-square variates

GENerate random deviate from the F distribution
Function
Generates a random deviate from the F (variance ratio)
distribution with DFN degrees of freedom in the numerator
and DFD degrees of freedom in the denominator.

Arguments
DFN -> Numerator degrees of freedom
(Must be positive)
DFD -> Denominator degrees of freedom
(Must be positive)

Method
Directly generates ratio of chi-square variates
\[ xnum = \text{genchi}(\text{dfn})/\text{dfn}; \]

\[
\text{GENF} = \left( \frac{\text{GENCHI}(\text{DFN})}{\text{DFN}} \right) / \left( \frac{\text{GENCHI}(\text{DFD})}{\text{DFD}} \right)
\]

\[ xden = \text{genchi}(\text{dfd})/\text{dfd}; \]

if(!xden <= 9.99999999998E-39*xnum) goto S20;

fputs(" GENF - generated numbers would cause overflow", stderr);

fprintf(stderr," Numerator \%16.6E Denominator \%16.6E\n", xnum, xden);

fputs(" GENF returning 1.0E38", stderr);

gnf = 1.0E38;

goto S30;

S20:

gnf = xnum/xden;

S30:

return gnf;

float gengam(float a, float r)
/*
-----------------------------------------------
float gengam(float a, float r)
GENerates random deviates from GAMma distribution
Function
Generates random deviates from the gamma distribution whose
density is
\[ (a^r)/\Gamma(r) \times x^{r-1} \times \exp(-a\times x) \]
Arguments
a -> Location parameter of Gamma distribution
r -> Shape parameter of Gamma distribution
Method
Renames SGAMMA from TOMS as slightly modified by BWB to use RANF
instead of SUNIF.
For details see:
(Case R >= 1.0)
Ahrens, J.H. and Dieter, U.
Generating Gamma Variates by a
Modified Rejection Technique.
Algorithm GD
(Case 0.0 <= R <= 1.0)
Ahrens, J.H. and Dieter, U.
Computer Methods for Sampling from Gamma,
Beta, Poisson and Binomial Distributions.
Computing, 12 (1974), 223-246/
Adapted algorithm GS.
-----------------------------------------------*/
[
static float gengam;

gengam = sgamma(r);

gengam /= a;

return gengam;
]

void genmn(float *parm, float *x, float *work)
/*
-----------------------------------------------
void genmn(float *parm, float *x, float *work)
GENerate Multivariate Normal random deviate
Arguments
parm -> Parameters needed to generate multivariate normal
deviates (MEANV and Cholesky decomposition of
COVM). Set by a previous call to SETGMN.
1 : 1 - size of deviate, P
2 : P + 1 - mean vector
P+2 : P*(P+3)/2 + 1 - upper half of cholesky
-----------------------------------------------*/

404
decomposition of cov matrix

\[ X \leftarrow \text{Vector deviate generated.} \]
work \leftarrow \text{Scratch array \hspace{1cm} Method}
1) \text{Generate P independent standard normal deviates - } E_1 \sim \mathcal{N}(0,1)
2) \text{Using Cholesky decomposition find } A \text{ s.t. } \text{trans}(A)^*A = \text{COVM}
3) \text{trans}(A)E + \text{MEANV} \sim \mathcal{N}(\text{MEANV}, \text{COVM})

\[
\begin{align*}
&\text{\texttt{*/}} \\
&\text{static long i, icount, j, p, D1, D2, D3, D4;} \\
&\text{static float ae;} \\
&p = (\text{long}) (*\text{parm}); \\
&\text{\texttt{*/}} \\
&\text{Generate P independent normal deviates - WORK \sim \mathcal{N}(0,1)} \\
&\text{\texttt{*/}} \\
&\text{for}(i=1; i<p; i++) \{ \text{\texttt{work} + i-1} = \text{snorm}(); \} \\
&\text{\texttt{*/}} \\
&\text{\texttt{PARM} \leftarrow (P+2) \leftarrow \text{trans}(A) = \text{Cholesky decomposition of the desired covariance matrix.}} \\
&\text{\texttt{trans}(A)(1,1) = PARM(P+2)} \\
&\text{\texttt{trans}(A)(2,1) = PARM(P+3)} \\
&\text{\texttt{trans}(A)(2,2) = PARM(P+2+P)} \\
&\text{\texttt{trans}(A)(3,1) = PARM(P+4)} \\
&\text{\texttt{trans}(A)(3,2) = PARM(P+3+P)} \\
&\text{\texttt{trans}(A)(3,3) = PARM(P+2+P)} \\
&\text{\texttt{trans}(A)\leftarrow \text{WORK} + \text{MEANV} \sim \mathcal{N}(\text{MEANV}, \text{COVM})} \\
&\text{icount = 0;} \\
&\text{ae = 0.0;} \\
&\text{for}(j=1, D1=1, D2=(1-j+D1)/D1; D2>0; D2-=, j+=D1) \{ } \\
&\text{icount += \{\}-1; } \\
&\text{ae += (*\text{parm} + (j-1)*p-icount)*\{\text{work} + j-1\}); } \\
&\text{\{x+i-1 \} = ae+\{\text{parm}+i\}; } \\
&\text{\} } \\
&\text{\} } \\
\text{\texttt{void genmul( long n, float *p, long ncat, long *ix)}} \\
&\text{\texttt{*/}} \\
\text{\texttt{-----------------------------}} \\
\text{\texttt{void genmul(int n, float *p, int ncat, int *ix)}} \\
\text{\texttt{GENERate an observation from the MULTinomial distribution}} \\
\text{\texttt{Arguments}} \\
\text{\texttt{N \rightarrow Number of events that will be classified into one of}} \\
\text{\texttt{the categories 1..NCAT}} \\
\text{\texttt{P \rightarrow Vector of probabilities. \ P(i) is the probability that}} \\
\text{\texttt{an event will be classified into category i. Thus, \ P(i)}} \\
\text{\texttt{must be \ [0,1]. Only the first NCAT-1 \ P(i) must be defined}} \\
\text{\texttt{since \ P(\text{NCAT}) is 1.0 minus the sum of the first}} \\
\text{\texttt{NCAT-1 \ P(i).}} \\
\text{\texttt{NCAT \rightarrow Number of categories. Length of P and IX.}} \\
\text{\texttt{IX \leftarrow Observation from multinomial distribution. All IX(i)}} \\
\text{\texttt{will be nonnegative and their sum will be N.}} \\
\text{\texttt{Method}} \\
\text{\texttt{Algorithm from page 559 of}} \\
\text{\texttt{Devroye, Luc}} \\
\text{\texttt{Non-Uniform Random Variate Generation. Springer-Verlag,}} \\
\text{\texttt{New York, 1986.}} \\
\text{\texttt{-----------------------------}}
static float prob, ptot, sum;
static long i, icat, ntot;
if(n < 0) ftntop("N < 0 in GENMUL");
if(ncat <= 1) ftntop("NCAT <= 1 in GENMUL");
ptot = 0.0F;
for(i=0; i<ncat-1; i++) {
    if(* (p+i)  < 0.0F) ftntop("Some P(i)  < 0 in GENMUL");
    if(* (p+i) > 1.0F) ftntop("Some P(i)  > 1 in GENMUL");
    ptot += *(p+i);
}
if(ptot > 0.99999F) ftntop("Sum of P(i)  > 1 in GENMUL");

/* Initialize variables */
ntot = n;
sum = 1.0F;
for(i=0; i<ncat; i++) ix[i] = 0;
/* Generate the observation */
for(icat=0; icat<ncat-1; icat++) {
    prob = *(p+icat)/sum;
    *(ix+icat) = lgnbin(ntot,prob);
    ntot -= *(ix+icat);
    if(ntot <= 0) return;
    sum -= *(p+icat);
}
ix+ncat-1 = ntot;
/* Finished */
return;
}

float gennch(float df, float xnonc)
/
**************************************************************************
float gennch(float df, float xnonc)

Generate random value of Noncentral CHIsquare variable
Function
Generates random deviate from the distribution of a noncentral
chi-square with DF degrees of freedom and noncentrality parameter
xnonc.
Arguments
DF -> Degrees of freedom of the chi-square
    (Must be > 1.0)
XNONC -> Noncentrality parameter of the chi-square
    (Must be >= 0.0)
Method
Uses fact that noncentral chi-square is the sum of a chi-square
deviate with DF-1 degrees of freedom plus the square of a normal
deviate with mean XNONC and standard deviation 1.
**************************************************************************
/*
{ static float gennch;

if(!((df <= 1.0 || xnonc < 0.0))) goto S10;
fpnts("DF <= 1 or XNONC < 0 in Gennch - ABORT", stderr);
fprintf(stderr,"Value of DF: %16.6E Value of XNONC%16.6E\n", df, xnonc);
exit(1);
S10:
    gennch = gennchi(df-1.0)+pow(gennor(sqrt(xnonc),1.0),2.0);
    return gennch;
float gennf(float dfn, float dfd, float xnonc)
/*
-------------------------
float gennf(float dfn, float dfd, float xnonc)
GENERate random deviate from the Noncentral F distribution
Function
Generates a random deviate from the noncentral F (variance ratio)
distribution with dfn degrees of freedom in the numerator, and dfd
degrees of freedom in the denominator, and noncentrality parameter
XNONC.
Arguments
dfn -> Numerator degrees of freedom
(Must be >= 1.0)
dfd -> Denominator degrees of freedom
(Must be positive)
xnonc -> Noncentrality parameter
(Must be nonnegative)
Method
Directly generates ratio of noncentral numerator chisquare variate
to central denominator chisquare variate.
*/
{
static float gennf, xden, xnum;
static long qcond;
qcond = dfn <= 1.0 || dfd <= 0.0 || xnonc < 0.0;
if (!qcond) goto S10;
puts("In GENNF - Either (1) Numerator DF <= 1.0 or", stderr);
puts("(2) Denominator DF < 0.0 or ", stderr);
printf(stderr,
"DFN value: %16.6E DFD value: %16.6E XNONC value: %16.6E
", dfn, dfd, xnonc);
exit(1);
S10:
xnum = gennch(dfn, xnonc)/dfn;
/*
GENNF = ( GENNCH(DFN, XNONC) / DFN ) / ( GENCH(DFD) / DFD )
*/
xden = gench(dfd)/dfd;
if (!xden <= 9.99999999999E-39*xnum) goto S20;
puts("GENNF - generated numbers would cause overflow", stderr);
printf(stderr,
"Denominator %16.6E Numerator %16.6E
", xnum, xden);
puts("GENNF returning 1.0E38", stderr);
gennf = 1.0E38;
goto S30;
S20:
gennf = xnum/xden;
S30:
return gennf;
}
float gennor(float av, float sd)
/*
-------------------------
float gennor(float av, float sd)
GENERate random deviate from a NORMAL distribution
Function
Generates a single random deviate from a normal distribution
with mean, AV, and standard deviation, SD.
Arguments
av -> Mean of the normal distribution.
sd -> Standard deviation of the normal distribution.
Method
407
Renames SNORM from TOMS as slightly modified by BWB to use RANF instead of SUNIF.
For details see:
Ahrens, J.H. and Dieter, U.
Extensions of Forsythe’s Method for Random Sampling from the Normal Distribution.

```c

static float gennor;

    gennor = sd*snorm()+av;
    return gennor;
}

float genunf(float low, float high)

    Generates a real uniformly distributed between LOW and HIGH.
    Arguments
    low -> Low bound (exclusive) on real value to be generated
    high -> High bound (exclusive) on real value to be generated

    
```
Arguments

gset -> 0 Get
1 Set

q <- Number of the current random number generator (1..32)

```c
#define numg 32L
static long curntg = 1;
if(getset == 0) *g = curntg;
else {
    if(*g < 0 || *g > numg) {
        puts(" Generator number out of range in GSCGN",stderr);
        exit(0);
    }
    curntg = *g;
}
#endif numg

void gsrgs(long getset,long *qvalue)
{ /*
void gsrgs(long getset,long *qvalue)
Get/Set Random Generators Set
Gets or sets whether random generators set (initialized).
Initially (data statement) state is not Set
If getset is 1 state is set to qvalue
If getset is 0 state returned in qvalue
*/
static long qinit = 0;
if(getset == 0) *qvalue = qinit;
else qinit = *qvalue;
}

void gssst(long getset,long *qset)
{ /*
void gssst(long getset,long *qset)
Get or Set whether Seed is Set
Initialize to Seed not Set
If getset is 1 sets state to Seed Set
If getset is 0 returns T in qset if Seed Set
Else returns F in qset
*/
static long qstate = 0;
if(getset != 0) qstate = 1;
else *qset = qstate;
}
long ignbin(long n, float pp)
{ /*
long ignbin(long n, float pp)
GENERate BINomial random deviate
Function
Generates a single random deviate from a binomial
distribution whose number of trials is N and whose
probability of an event in each trial is P.
Arguments
n -> The number of trials in the binomial distribution
    from which a random deviate is to be generated.
p -> The probability of an event in each trial of the

```
binomial distribution from which a random deviate
is to be generated.

ignbin <- A random deviate yielding the number of events
from N independent trials, each of which has
a probability of event P.

Method

This is algorithm BTPE from:

Kacitvichyanukul, V. and Schmeiser, B. W.

Binomial Random Variate Generation.

Communications of the ACM, 31, 2

(February, 1988) 216.

---------------------------------------------------------------------

SUBROUTINE BTPEC(N,PP,ISEED,JX)
BINOMIAL RANDOM VARIATE GENERATOR
MEAN .LT. 30 - INVERSE CDF
MEAN .GE. 30 - ALGORITHM BTPE: ACCEPTANCE-REJECTION VIA
FOUR REGION COMPOSITION. THE FOUR REGIONS ARE A TRIANGLE
(SYMMETRIC IN THE CENTER), A PAIR OF PARALLELOGRAMS (ABOVE
THE TRIANGLE), AND EXPONENTIAL LEFT AND RIGHT TAILS.
BTPE REFERS TO BINOMIAL-TRIANGLE-PARALLELOGRAM-EXPONENTIAL.
BTPEC REFERS TO BTPE AND "COMBINED." THIS BTPE IS THE
RESEARCH AND BTPEC IS THE IMPLEMENTATION OF A COMPLETE
USABLE ALGORITHM.
REFERENCE: Voratas Kacitvichyanukul and Bruce Schmeiser,
"BINOMIAL RANDOM VARIATE GENERATION,"
COMMUNICATIONS OF THE ACM, FORTHCOMING
LAST REVISED: MAY 1985, JULY 1987
REQUIRED SUBPROGRAM: RAND() - A UNIFORM (0,1) RANDOM NUMBER
GENERATOR
ARGUMENTS
N : NUMBER OF BERNOULLI TRIALS (INPUT)
PP : PROBABILITY OF SUCCESS IN EACH TRIAL (INPUT)
ISEED: RANDOM NUMBER SEED (INPUT AND OUTPUT)
JX : RANDOMLY GENERATED OBSERVATION (OUTPUT)
VARIABLES
PSAVE: VALUE OF PP FROM THE LAST CALL TO BTPEC
NSAVE: VALUE OF N FROM THE LAST CALL TO BTPEC
XNP: VALUE OF THE MEAN FROM THE LAST CALL TO BTPEC
P: PROBABILITY USED IN THE GENERATION PHASE OF BTPEC
FM: TEMPORARY VARIABLE EQUAL TO XNP + P
M: INTEGER VALUE OF THE CURRENT MODE
FM: FLOATING POINT VALUE OF THE CURRENT MODE
XP: TEMPORARY VARIABLE USED IN SETUP AND SQUEEZING STEPS
P1: AREA OF THE TRIANGLE
C: HEIGHT OF THE PARALLELOGRAMS
M: CENTER OF THE TRIANGLE
XR: RIGHT END OF THE TRIANGLE
AL: TEMPORARY VARIABLE
XLL: RATE FOR THE LEFT EXPONENTIAL TAIL
XLR: RATE FOR THE RIGHT EXPONENTIAL TAIL
P2: AREA OF THE PARALLELOGRAMS
P3: AREA OF THE LEFT EXPONENTIAL TAIL
P4: AREA OF THE RIGHT EXPONENTIAL TAIL
U: A U(0,P4) RANDOM VARIATE USED FIRST TO SELECT ONE OF THE
FOUR REGIONS AND THEN CONDITIONALLY TO GENERATE A VALUE
FROM THE REGION
V: A U(0,1) RANDOM NUMBER USED TO GENERATE THE RANDOM VALUE
FROM REGION 1 OR TRANSFORMED INTO THE VARIATE TO ACCEPT OR
REJECT THE CANDIDATE VALUE
IX: ABSOLUTE VALUE OF (IX-M)

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F: THE HEIGHT OF THE SCALED DENSITY FUNCTION USED IN THE
ACCEPT/REJECT DECISION WHEN BOTH M AND IX ARE SMALL
ALSO USED IN THE INVERSE TRANSFORMATION
R: THE RATIO P/Q
G: CONSTANT USED IN CALCULATION OF PROBABILITY
MP: MODE PLUS ONE, THE LOWER INDEX FOR EXPLICIT CALCULATION
OF F WHEN IX IS GREATER THAN M
IX1: CANDIDATE VALUE PLUS ONE, THE LOWER INDEX FOR EXPLICIT
CALCULATION OF F WHEN IX IS LESS THAN M
I: INDEX FOR EXPLICIT CALCULATION OF F FOR BTEP
AMAXP: MAXIMUM ERROR OF THE LOGARITHM OF NORMAL BOUND
YNORM: LOGARITHM OF NORMAL BOUND
ALV: NATURAL LOGARITHM OF THE ACCEPT/REJECT VARIATE V
IX, IX1, W, Z2, F2, AND WC ARE TEMPORARY VARIABLES TO BE
USED IN THE FINAL ACCEPT/REJECT TEST
QN: PROBABILITY OF NO SUCCESS IN N TRIALS

REMARK
IX AND IX COULD LOGICALLY BE THE SAME VARIABLE, WHICH WOULD
SAVE A MEMORY POSITION AND A LINE OF CODE. HOWEVER, SOME
COMPILERS (E.G., CDC MNF) OPTIMIZE BETTER WHEN THE ARGUMENTS
ARE NOT INVOLVED.
ISEED NEEDS TO BE DOUBLE PRECISION IF THE IMSL ROUTINE
GGUBFS IS USED TO GENERATE UNIFORM RANDOM NUMBER, OTHERWISE
TYPE OF ISEED SHOULD BE DICTATED BY THE UNIFORM GENERATOR

 static float psave = -1.0;
 static long nsave = -1;
 static long igmbin, i, ix, ix1, k, m, mp, TI;
 static float al, aiv, amaxp, c, f, f1, f2, ffm, fmp, pl, p2, p3, p4, q, qn, z, vl, w, w2, x, xl,
 x2, xl1, xl1r, cm, xnp, xnpq, xc, ynorm, z, z2;

 if (pp != psave) goto S10;
 if (n != nsave) goto S20;
 if (xnp < 30.0) goto S140;
 goto S30;

 S10:
 
 S20:

 S140:

 S30:

 S50:

 xnp = n*p;
 nsave = n;
 if (xnp < 30.0) goto S140;
 ffm = xnp+p;
 m = ffm;
 tm = m;
 xnpq = xnp*q;
 pl = (long) (2.195*sqrt(xnpq)-4.6*q)+0.5;
 xl = xm-pl;
 xr = xm+pl;
 c = 0.134+20.5/(15.3+fm);
 al = (ffm-xl)/(ffm-xl*p);
 xll = al*(1.0+0.5*al);
 al = (xl*ffm)/(xl*r);
 xlr = al*(1.0+0.5*al);
 p2 = pi*(1.0+c+c);
 p3 = p2-c/xll;
 p4 = p3+c/xlr;
S30:
/*
****GENERATE VARIATE
*/
   u = ranf()*p4;
   v = ranf();
/*
TRIANGULAR REGION
*/
   if(u > p1) goto S40;
   ix = x[m-p1]*v+u;
   goto S170;
S40:
/*
PARALLELOGRAM REGION
*/
   if(u > p2) goto S50;
   x = x[l]+(u-p1)/c;
   v = v+c+1.0-ABS(xm-x)/pl;
   if(v > 1.0 || v <= 0.0) goto S30;
   ix = x;
   goto S70;
S50:
/*
LEFT TAIL
*/
   if(u > p3) goto S60;
   ix = x[l]-log(v)/x[l];
   if(ix < 0) goto S30;
   v *= (u-p2)*x[l];
   goto S70;
S60:
/*
RIGHT TAIL
*/
   ix = x[r]-log(v)/x[r];
   if(ix > n) goto S30;
   v *= (u-p3)*x[r];
S70:
/*
****DETERMINE APPROPRIATE WAY TO PERFORM ACCEPT/REJECT TEST
*/
   k = ABS(ix-m);
   if(k > 20 & & k < xnpq/2-1) goto S30;
   /*
   EXPLICIT EVALUATION
   */
   f = 1.0;
   r = p/q;
   q = (n+1)*r;
   T1 = m-ix;
   if(T1 < 0) goto S80;
   else if(T1 == 0) goto S120;
   else goto S100;
S80:
   mp = m+1;
   for(i=mp; i<=ix; i++) f *= (q/i-r);
   goto S120;
S100:
   ixl = ix+1;
   for(i=ixl; i<=m; i++) f /= (q/i-r);
S120:
   if(v <= f) goto S170;
   goto S30;
S130:
SQUEEZING USING UPPER AND LOWER BOUNDS ON ALOG( f(X) )

alpha = k/x*n*p*q * (k * (k / 3.0 + 0.625) + 0.166666666666666)
ynorm = -(k/k) / (2.0 * x*n*p*q)
\[ \text{if (alv < ynorm - amaxp) goto S170;} \]
\[ \text{if (alv > ynorm + amaxp) goto S30;} \]

STIRLING'S FORMULA TO MACHINE ACCURACY FOR THE FINAL ACCEPTANCE/REJECTION TEST

\[ \text{if (alv <= x*m*log(f1/x1) + (n-m-0.5)*log(z/w) + (i-x-m)*log(w*p/(x1*q)) + (13860.0 - (462.0 - (132.0 - (99.0 - 140.0) / x2) / x2) / x1) / 166320.0) getoto S170;} \]
goto S30;

/*
 INVERSE CDF LOGIC FOR MEAN LESS THAN 30
*/

qn = pow(q, (double)n);
r = p/q;
g = c*(n+1);

if(u < f) goto S170;
if(i > 110) goto S1b0;
u -= f;
i += 1;
f = (g/i - r);
goto S160;

if(psave > 0.5) ix = n-ix;
ignbin = ix;
return ignbin;
;
long ignmbn(long n, float p)

----------------------------------------------------------------------------------------------

long ignmbn(long n, float p)
GENerate Negative BiNomial random deviate Function
Generates a single random deviate from a negative binomial distribution.

Arguments
N -> The number of trials in the negative binomial distribution from which a random deviate is to be generated.
P -> The probability of an event.

Method
Algorithm from page 480 of
Devroye, Luc


*****************************************************************************
/

static long ignnbn;
static float y,a,r;
/*
  . Executable Statements ..
/
*

Check Arguments
*/
if(n < 0) ftntstop("N < 0 in IGNNBN");
if(p <= 0.0F) ftntstop("P <= 0 in IGNNBN");
if(p >= 1.0F) ftntstop("P >= 1 in IGNNBN");
/*
Generate Y, a random gamma (n, (1-p)/p) variable
*/
r = (float)n;
a = p/(1.0F-p);
y = gengam(a,r);
/*
Generate a random Poisson(y) variable
*/
ignnbn = ignpoi(y);
return ignnbn;
}
long ignpoi(float mu)
/*
*****************************************************************************
	long ignpoi(float mu)
	GENerate POisson random deviate
	Function
Generates a single random deviate from a Poisson
distribution with mean AV.

Arguments
av <- The mean of the Poisson distribution from which
a random deviate is to be generated.
genexp <- The random deviate.

Method
Renames KPOIS from TOMS as slightly modified by BWB to use RANF
instead of SUNIF.
For details see:
Ahrens, J.H. and Dieter, U.
Computer Generation of Poisson Deviates
From Modified Normal Distributions.
ACM Trans. Math. Software, 8, 2
(June 1982),163-179
*****************************************************************************

POISSON DISTRIBUTION

*****************************************************************************

FOR DETAILS SEE:

AHRENS, J.H. AND DIETER, U.

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INTEGER FUNCTION IGNPOI(IR, MU)
INPUT: IR=CURRENT STATE OF BASIC RANDOM NUMBER GENERATOR
       MU=MEAN MU OF THE POISSON DISTRIBUTION
OUTPUT: IGNPOI=SAMPLE FROM THE POISSON-(MU)-DISTRIBUTION
MUPREV=PREVIOUS MU, MU0LD=MU AT LAST EXECUTION OF STEP P OR B.
TABLES: COEFFICIENTS AQ-A7 FOR STEP F. FACTORIALS FACT.
COEFFICIENTS A(K) - FOR PX = FK*V*VSUM(A(K)*V**K)-DEL.
SEPARATION OF CASES A AND B

♦

(extern float fsign(float num, float sign);
static float a0 = -0.5;
static float a1 = 0.3333333;
static float a2 = -0.2500068;
static float a3 = 0.2000118;
static float a4 = -0.1661269;
static float a5 = 0.1421878;
static float a6 = -0.1384794;
static float a7 = 0.1250066;
static float mu0ld = 0.0;
static float muprev = 0.0;
static float fact[10] = {1.0, 1.0, 2.0, 6.0, 24.0, 120.0, 720.0, 5040.0, 40320.0, 362880.0};
static long ignpoi, j, kflag, i, m;
static float b1, b2, c, c0, cl, c2, c3, del, difmuk, e, fk, fx, fy, g, omega, p0, px, py, q, s,
t, u, v, x, xx, pp[35];

/*
CASE A. (RECALCULATION OF S, D, L IF MU HAS CHANGED)
*/
if((mu == muprev) goto S10;
if((mu < 10.0) goto S120;
/*
MUPREV = MU;
S = SQRT(MU);
I = 6.0*MU*MU;
*/

THE POISSON PROBABILITIES PK EXCEED THE DISCRETE NORMAL
PROBABILITIES FK WHENEVER K >= M(MU). L=FIX(MU-1.1484)
IS AN UPPER BOUND TO M(MU) FOR ALL MU >= 10.

/*
l = (long) (mu-1.1484);
S10:
*/
STEP N. NORMAL SAMPLE - SNORM(IR) FOR STANDARD NORMAL DEVIATE
/*
q = mu+s*snorm();
if((q < 0.0) goto S20;
ignpoi = (long) (q);
*/
STEP I. IMMEDIATE ACCEPTANCE IF IGNPOI IS LARGE ENOUGH
/*
if(ignpoi >= 1) return ignpoi;
*/
STEP S. SQUEEZE ACCEPTANCE - SUNIF(IR) FOR (0,1)-SAMPLE U
/*
fk = (float)ignpoi;
difmuk = mu-fk;
*/

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u = ranf();
if(d^u >= difmuk* difmuk * difmuk) return ignpoi;

S20:
/*
STEP P. PREPARATIONS FOR STEPS Q AND H.
(RECALCULATIONS OF PARAMETERS IF NECESSARY)
.3969423=(2*PI)**(-.5) .416667E-1=1./24. .1428571=1./7.
THE QUANTITIES B1, B2, C3, C2, C1, C0 ARE FOR THE HERMITE
APPROXIMATIONS TO THE DISCRETE NORMAL PROBABILITIES FK.
C=.1069/MU GUARANTEES MAJORIZATION BY THE 'HAT'-FUNCTION.
*/
if(mu == muold) goto S30;
muold = mu;
omega = 0.3969423/s;
b1 = 4.166667E-2/mu;
b2 = 0.3*b1*b1;
c3 = 0.1428571*b1*b2;
c2 = b2-15.0*c3;
c1 = b1-6.0*b2+45.0*c3;
c0 = 1.0-b1+3.0*b2-15.0*c3;
c = 0.1069/mu;
S30:
if(g < 0.01 goto S50;

/*
'SUBROUTINE' F IS CALLED (KFLAG=0 FOR CORRECT RETURN)
*/
kflag = 0;
goto S70;

S40:
/*
STEP Q. QUOTIENT ACCEPTANCE (RARE CASE)
*/
if(fy-u*fy <= py*exp(px-fx)) return ignpoi;
S50:
/*
STEP E. EXPONENTIAL SAMPLE - SEXPO(IR) FOR STANDARD EXPONENTIAL
DEViate E AND SAMPLE T FROM THE LAPLACE 'HAT'
(IF T <= -.6744 THEN PK < FK FOR ALL MU >= 10.)
*/
a = sexpo();
u = ranf();
u = (u-1.0);
t = 1.8*sign(a,u);
if(t <= -.6744) goto S50;
ignpoi = (long) (mu+s*t);
fk = (float) ignpoi;
difmuk = mu-fk;
/*
'SUBROUTINE' F IS CALLED (KFLAG=1 FOR CORRECT RETURN)
*/
kflag = 1;
goto S70;

S60:
/*
STEP H. HAT ACCEPTANCE (E IS REPEATED ON REJECTION)
*/
if(c*fabs(u) > py*exp(px+e)-fy*exp(fx+e)) goto S50;
return ignpoi;
S70:
/*
STEP F. 'SUBROUTINE' F. CALCULATION OF PX, PY, FX, FY.
CASE IGNPOI .LT. 10 USES FACTORIALS FROM TABLE FACT
*/
if(ignpoi >= 10) goto S80;
px = -mu;

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\[ p_y = \text{pow}(\mu, (\text{double})\text{ignpoi}) \times (\text{fact} + \text{ignpoi}) \]

goto S110;

S60:
/ * CASE IGNPOI .GE. 10 USES POLYNOMIAL APPROXIMATION A0-A7 FOR ACCURACY WHEN ADVISABLE .8333333E-1 = 1./12. .3999423 = (2×PI)**(-.5)*/

de1 = 8.333333E-2/fk;
de1 = (4.8×del×del×del);
\[ v = \text{difmuk}/fk; \]
if(abs(v) <= 0.25) goto S90;
\[ px = \text{fk}×\log(1.0+v)-\text{difmuk}-\text{del}; \]
goto S100;
S90:
\[ px = \text{fk}×v×v×((((((a7×v+a6)×v+a5)×v+a4)×v+a3)×v+a2)×v+a1)×v+a0)-\text{del}; \]
S100:
\[ p_y = 0.3999423/\text{sqrt}(\text{fk}); \]
S110:
\[ x = (x_d-\text{difmuk})/s; \]
\[ xx = x×x; \]
\[ fx = -0.5×xx; \]
\[ fy = \text{omega}×((((c3×xx+c2)×xx+c1)×xx+c0); \]
if(kflag <= 0) goto S40;
goto S60;
S120:
/ * CASE 2. (START NEW TABLE AND CALCULATE P0 IF NECESSARY)*/
muprev = 0.0;
if(\mu = \text{muold}) goto S130;
muold = \mu;
m = \text{max}(1L, (\text{long}) \{\mu\});
l = 0;
p = \text{exp}(-\mu);
q = p0 = p;
S130:
/ * STEP U. UNIFORM SAMPLE FOR INVERSION METHOD */
\[ u = \text{ranf}(); \]
\text{ignpoi} = 0;
if(u <= p0) return \text{ignpoi};
/ * STEP T. TABLE COMPARISON UNTIL THE END PP(L) OF THE PP-TABLE OF CUMULATIVE POISSON PROBABILITIES (0.458=PP(9) FOR MU=10)*/
if(1 <= 0) goto S150;
j = 1;
if(\mu > 0.458) j = \text{min}(1L, m);
for(k = j; k <= i; k++) {
if(u <= *(pp+k-1)) goto S180;
}
if(1 >= 35) goto S130;
S150:
/ * STEP C. CREATION OF NEW POISSON PROBABILITIES P AND THEIR CUMULATIVES Q=PP(K)*/
l = 1;
for(k = 1; k <= 35; k++) {
p = p×\mu/(float)k;
q += p;
*(pp+k-1) = q;
if(u <= q) goto S170;

l = 35;
goto S130;

S170:
l = k;
S180:
ignpoi = k;
return ignpoi;

long ignuin(long low, long high)
/
***********************************************************************
long ignuin(long low, long high)
Generate Uniform Integer
Function
Generates an integer uniformly distributed between LOW and HIGH.
Arguments
low -> Low bound (inclusive) on integer value to be generated
high -> High bound (inclusive) on integer value to be generated
Note
If (HIGH-LOW) > 2,147,483,561 prints error message on * unit and
stops the program.
***********************************************************************
IGNUNI generates integers between 1 and 2147483562
MAXNUM is 1 less than maximum generable value
*/
#endif define maxnum 2147483561L
static long ignuin, ign, maxnow, range, ranpl;

if(!((low > high)) goto S10;
    fputs(" low > high in ignuin - ABORT",stderr);
    exit(1);

S10:
    range = high-low;
    if(!((range > maxnum)) goto S20;
    fputs(" high - low too large in ignuin - ABORT",stderr);
    exit(1);

S20:
    if(!((low == high)) goto S30;
    ignuin = low;
    return ignuin;

S30:
    /*
     Number to be generated should be in range 0..RANGE
     Set MAXNOW so that the number of integers in 0..MAXNOW is an
     integral multiple of the number in 0..RANGE
    */
    ranpl = range+1;
    maxnow = maxnum/ranpl*ranpl;
S40:
    ign = ignlgi()-1;
    if(!((ign <= maxnow)) goto S50;
    ignuin = low+ign%ranpl;
    return ignuin;
S50:
    goto S40;
#endif undef maxnum
#endif undef err1
#endif undef err2

long lенноб( char *str )
{" Returns the length of str ignoring trailing blanks but not
other white space.
"
{
long l, i_nb;

for (i=0, i_nb= -IL; *(str+i); ++)
  if (!(*(str+i) != ' ')) i_nb = i;
return (i_nb-1);
}

long mlttnod(long a, long s, long m)
/*

long mlttnod(long a, long s, long m)
Returns (A*S) MOD M
This is a transcription from Pascal to Fortran of routine
MULTMod_Decompos from the paper
I’Ecuycr, P. and Cote, S. “Implementing a Random Number Package
with Splitting Facilities.” ACM Transactions on Mathematical
Arguments
a, s, m ->
*/
#define n 32768L
static long mlttnod,a0,al,k,p,qh,rh;
/*
H = 2**(b-2)/2 where b = 32 because we are using a 32 bit
machine. On a different machine recompute H
*/
if(!(a <= 0 || a >= m || s <= 0 || s >= m)) goto 310;
锻ps(" a, m, s out of order in mlttnod - A B O R T!",stderr);
 flirtf(stderr, " a = %12ld s = %12ld m = %12ld",a,s,m);
锻ps(" mlttnod requires: 0 < a < m; 0 < s < m",stderr);
exit(1);
$10: if((a < h)) goto 320;
a0 = a;
p = 0;
goto $120;
$20:
al = a/h;
x0 = a-h*al;
qh = m/h;
rh = m-h*qh;
if(!(al >= h)) goto 350;
al -= h;
k = s/qh;
p = n*(s-k*qh)-k*rh;
$50:
if(! (p < 0)) goto 40;
p = m;
goto $30;
$40:
goto $60;
$50:
p = 0;
$60:
/*
P = (A2*S*H)MOD M
*/
if(!(al != 0)) goto 90;
q = m/a1;  
k = s/q;  
p = (k*(m-a1*q));  
if(p > 0) p -= m;  
p += (a1*(s-k*q));  
S70:  
  if(!(p < 0)) goto S80;  
p += m;  
goto S70;  
S90:  
S80:  
k = p/qh;  
  
  p = (h*(p-k*qh)-k*rh);  
S100:  
  if(!(p < 0)) goto S110;  
p -= m;  
goto S100;  
S120:  
S110:  
  if(!(a0 != 0)) goto S150;  
  
  p = ((A2*H + A1)*S)MOD M  
  
p = h*(p-k*qh)-k*rh;  
S100:  
  if(!(p < 0)) goto S110;  
p -= m;  
goto S100;  
S120:  
S110:  
  if(!(a0 != 0)) goto S150;  
  
  p = ((A2*H + A1)*H*S)MOD M  
  
  q = m/a0;  
k = s/q;  
p = (k*(m-a0*q));  
if(p > 0) p -= m;  
p += (a0*(s-k*q));  
S130:  
  if(!(p < 0)) goto S140;  
p += m;  
goto S130;  
S150:  
S140:  
  mltmod = p;  
  return mltmod;  

void phrtsd(char* phrase, long *seed1, long *seed2)  
/*  
PHrase To SeeDs  

Function  

Uses a phrase (character string) to generate two seeds for the RGN  
random number generator.  
Arguments  
phrase -> Phrase to be used for random number generation  
seed1 <- First seed for generator  
seed2 <- Second seed for generator  

Note  

Trailing blanks are eliminated before the seeds are generated.  
Generated seed values will fall in the range 1..2^30  
(1..1,073,741,824)  */
\{ 
static char table[] =
"abcdefghijklmnopqrstuvwxyzABCDEFGHIJKLMNOPQRSTUVWXYZ0123456789\n!#$%^&*()_+[]:\"<>?,./; 

long ix;
static long twop30 = 1073741824L;
static long shift[5] = {
 1L, 64L, 4096L, 262144L, 16777216L 
};
static long i, ichr, j, lphr, values[5];
extern long lennob(char *str);

*seed1 = 1234567890L;
*seed2 = 1234567891L;
lphr = lennob(phrase);
if(lphr < 1) return;
for(i=0; i<=lphr-1; i++) {
  for(ix=0; table[ix]; ix++) if(*(phrase+i) == table[ix]) break;
  if (!table[ix]) ix = 0;
  ichr = ix % 64;
  if(ichr == 0) ichr = 63;
  for(j=1; j<5; j++) {
    *(values+j-1) = ichr;
    if(*(values+j-1) < 1) *(values+j-1) += 63;
  }
  for(j=1; j<5; j++) {
    *seed1 = (*seed1**((shift+j-1)**(values+j-1))) % twop30;
    /* The original expression for *seed2 downloaded from the web was
"seed2 = (*seed2**(shift+j-1)**(values+6-j-1)) % twop30;
I modified it because it was causing pointer arithmetic errors.
I have no idea what consequence, if any, this has on the random
numbers generated by this routine. JSB 8/8/97
*/
    *seed2 = (*seed2**(shift+j-1)**(values+j-1)) % twop30;
  }
}
#undef twop30

float ranf(void) 
\/*
  \******************************************************************************
  \* float ranf(void) \*
  \* \******************************************************************************
  \*
  RANDOM number generator as a Function
  \* Returns a random floating point number from a uniform distribution
  \* over 0 - 1 (endpoints of this interval are not returned) using the
  \* current generator
  \* This is a transcription from Pascal to Fortran of routine
  \* Uniform_01 from the paper
  \* L'Ecuyer, P. and Cote, S. "Implementing a Random Number Package
  \* With Splitting Facilities." ACM Transactions on Mathematical
  \******************************************************************************
  
  */
  static float ranf;
  /*
  \* 4.656613057E-10 is 1/M1. M1 is set in a data statement in IGNLG1
  \* and is currently 2147483563. If M1 changes, change this also.
  */
  ranf = ignlg1() * 4.656613057E-10;
\}
return ranf;
}

void setgmn(float *meanv, float *covm, long p, float *parm)
{/*
void setgmn(float *meanv, float *covm, long p, float *parm)

SET Generate Multivariate Normal random deviate

Function

Places P, MEANV, and the Cholesky factorization of COVM

in GENMN.

Arguments

meanv -> Mean vector of multivariate normal distribution.
covm <-> (Input) Covariance matrix of the multivariate

normal distribution

(Output) Destroyed on output

p -> Dimension of the normal, or length of MEANV.

parm <- Array of parameters needed to generate multivariate norma

deviates (P, MEANV and Cholesky decomposition of

COVM).

1 : 1 - P

2 : P + 1 - MEANV

P+2 : P*(P+3)/2 + 1 - Cholesky decomposition of COVM

Needed dimension is (p*(p+3)/2 + 1)
*/
{
extern void spofa(float *a, long lda, long n, long *info);
static long T1;
static long i, icount, info, j, D2, D3, D4, D5;
T1 = p*(p+3)/2 + 1;
/*
* TEST THE INPUT
*/
if(! (p <= 0)) goto S10;
puts("P nonpositive in SETGMN",stderr);
fprintf(stderr,"Value of P: $12.1d\n",p);
exit(1);
S10:
'*parm = p;
/*
* PUT P AND MEANV INTO PARM
*/
for (i = 2, D2 = 1, D3 = (p + 1 - i + 02) / D2; D3 > 0; D3--, i += D2) {*param+i-1) = *(meanv+i-2);
/*
* Cholesky decomposition to find A s.t. trans(A)*A = COVM
*/
spofa(covm, p, p, &info);
if (! (info != 0)) goto S30;
puts(" COVM not positive definite in SETGMN", stderr);
exit(1);
S30:
icount = p-1;
/*
* PUT UPPER HALF OF A, WHICH IS NOW THE CHOLESKY FACTOR, INTO PARM

COVM(1,1) = PARM(P+2)

COVM(1,2) = PARM(P+3)

COVM(1,P) = PARM(2P+1)

COVM(2,2) = PARM(2P+2) ...
*/
for (i = 1, D4 = 1, D5 = (p - 1 + D4)/D4; D5 > 0; D5--, i += D4) {
for (j = i - 1, j < p; j++) {
icount += 1;
'*parm+icount-1) = *(covm+i-1+j*p);
}

}
float sexpo(void)
/*
   *************************************************/

(STANDARD-) EXPONENTIAL DISTRIBUTION

 *************************************************/

FOR DETAILS SEE:

AHRENS, J.H. AND DIETER, U.
COMPUTER METHODS FOR SAMPLING FROM THE
EXPONENTIAL AND NORMAL DISTRIBUTIONS.
COMM. ACM, 15,10 (OCT. 1972), 873 - 882.

ALL STATEMENT NUMBERS CORRESPOND TO THE STEPS OF ALGORITHM
'SA' IN THE ABOVE PAPER (SLIGHTLY MODIFIED IMPLEMENTATION)

Modified by Barry W. Brown, Feb 3, 1988 to use RANF instead of
SUNIF. The argument IR thus goes away.

*************************************************/

Q(N) = SUM(ALOG(2.0)*-K/K!)  K=1,...,N, THE HIGHEST N
(HERE 8) IS DETERMINED BY Q(N)=1.0 WITHIN STANDARD PRECISION
*/

static float q[8] = {
  0.6931472,0.9333737,0.9988778,0.9984959,0.9998293,0.9998333,0.9999986,1.0
};
static long i;
static float sexpo,a,u,ustar,umin;
static float q1 = q;
a = 0.0;
  u = ranf();
goto S30;
S20:
a += q1;
S30:
  u += u;
  if(u <= 1.0) goto S20;
  u = 1.0;
  if(u > q1) goto S60;
  sexpo = a+u;
  return sexpo;
S60:
i = 1;
  ustar = ranf();
  umin = ustar;
S70:
  ustar = ranf();
  if(ustar < umin) umin = ustar;
  i += 1;
  if(u > *(q+i-1)) goto S70;
  sexpo = a+umin*q1;
  return sexpo;
}
float sgamma(float a)
/*
   *************************************************/
PARAMETER $A \geq 1.0$

FOR DETAILS SEE:
AHRENS, J.H. AND DIETER, U.
GENERATING GAMMA VARIATES BY A MODIFIED REJECTION TECHNIQUE.
COMM. ACM, 25,1 (JAN. 1982), 47 - 54.

STEP NUMBERS CORRESPOND TO ALGORITHM 'GD' IN THE ABOVE PAPER
(STRAIGHTFORWARD IMPLEMENTATION)
Modified by Barry W. Brown, Feb 3, 1988 to use RANF instead of SUNIF. The argument IR thus goes away.

PARAMETER $0.0 < A < 1.0$

FOR DETAILS SEE:
AHRENS, J.H. AND DIETER, U.
COMPUTER METHODS FOR SAMPLING FROM GAMMA, BETA, POISSON AND BINOMIAL DISTRIBUTIONS.

(INPUT: $A$ = PARAMETER (MEAN) OF THE STANDARD GAMMA DISTRIBUTION
OUTPUT: SGAMMA = SAMPLE FROM THE GAMMA-(A)-DISTRIBUTION
COEFFICIENTS $Q(K)$ - FOR $Q0 = \sum(Q(K) \cdot A^{-K})$
COEFFICIENTS $A(K)$ - FOR $Q = Q0 + (T \cdot T / 2) \cdot \sum(A(K) \cdot V^{**K})$
COEFFICIENTS $E(K)$ - FOR $EXP(Q) - 1 = 1 + \sum(E(K) \cdot Q^{**K})$
PREVIOUS A PRE-SET TO ZERO - AA IS A', AAA IS A''
SQRT32 IS THE SQUARE ROOT OF 32 = 5.656885449492380

```c
extern float fsign( float num, float sign);
static float q1 = 4.1666666E-2;
static float q2 = 2.0831485E-2;
static float q3 = 8.011911E-3;
static float q4 = 1.44121E-3;
static float q5 = -7.3908E-5;
static float q6 = 2.45E-4;
static float q7 = 2.424E-4;
static float a1 = 0.33333333;
static float a2 = -0.250003;
static float a3 = 0.2000062;
static float a4 = -0.1662921;
static float a5 = 0.1423657;
static float a6 = -0.1367177;
static float a7 = 0.1233795;
static float a1 = 1.0;
```
```
static float e2 = 0.4999897;
static float e3 = 0.166825;
static float e4 = 4.07753E-2;
static float e5 = 1.0293E-2;
static float aa = 0.0;
static float aha = 0.0;
static float sqrt32 = 5.656854;

if (a == aha) goto S10;
if (a < 1.0) goto S120;

/*
STEP 1: RECALCULATIONS OF S2, S, D IF A HAS CHANGED
*/

aa = a;
s2 = s-0.5;
s = sqrt(s2);
d = sqrt32-12.0*s;

S10:
/*
STEP 2: T=STANDARD NORMAL DEVIATE,
X=(S,1/2)-NORMAL DEVIATE.
IMMEDIATE ACCEPTANCE (I)
*/
t = snorm();
x = s+0.5*t;
sgamma = x*x;
if (t >= 0.0) return sgamma;

/*
STEP 3: U=0.1 -UNIFORM SAMPLE. SQUEEZE ACCEPTANCE (S)
*/
u = ranf();
if (u <= t*t*t) return sgamma;

/*
STEP 4: RECALCULATIONS OF Q0, B, SI, C IF NECESSARY
*/
if (a == aha) goto S40;
aha = a;
r = 1.0/a;
q0 = ((((q7-r-q6)*r+q5)*r+q4)*r+q3)*r+q2)*r+q1)*r;

/* APPROXIMATION DEPENDING ON SIZE OF PARAMETER A
THE CONSTANTS IN THE EXPRESSIONS FOR B, SI AND C WERE ESTABLISHED BY NUMERICAL EXPERIMENTS */
if (a <= 3.686) goto S30;
if (a <= 13.022) goto S20;

/* CASE 3: A .GT. 13.022 */

/* CASE 2: 3.686 .LT. A .LE. 13.022 */

/* CASE 1: A .LE. 3.686 */
```

425
\[ b = 0.463s + 0.178s^2; \]
\[ s_1 = 1.235; \]
\[ c = 0.195/s - 7.9E-2 - 1.6E-1s; \]

**STEP 5: NO QUOTIENT TEST IF X NOT POSITIVE**

```plaintext
/*
if (x <= 0.0) goto S70;
*/
```

**STEP 6: CALCULATION OF V AND QUOTIENT Q**

```plaintext
/*
v = t/(s-s);
if (fabs(v) <= 0.25) goto S50;
q = q0 - 0.25*t*t - (s2 + s2) * log(1.0 + v);
goto S60;
S50:
q = q0 + 0.5*t*t*(((a7+v+a6)*v+a5)*v+a4)*v+a3)*v+a2)*v+a1)*v;
S60:
*/
```

**STEP 7: QUOTIENT ACCEPTANCE (Q)**

```plaintext
/*
if (log(1.0 - u) <= q) return sgamma;
*/
```

**STEP 8: E=STANDARD EXPONENTIAL DEVIATE**

```plaintext
e = sexp();
u = ranf();
u = (u-1.0);
t = b*sign(s1*e, u);
*/
```

**STEP 9: REJECTION IF T LT. TAU(l) = -.71874483771719**

```plaintext
/*
if (t < -0.7187449) goto S70;
*/
```

**STEP 10: CALCULATION OF V AND QUOTIENT Q**

```plaintext
/*
v = t/(s-s);
if (fabs(v) <= 0.25) goto S80;
q = q0 - 0.25*t*t - (s2 + s2) * log(1.0 + v);
goto S90;
S80:
q = q0 + 0.5*t*t*(((a7+v+a6)*v+a5)*v+a4)*v+a3)*v+a2)*v+a1)*v;
S90:
*/
```

**STEP 11: HAT ACCEPTANCE (H) (IF Q NOT POSITIVE GO TO STEP 9)**

```plaintext
/*
if (q <= 0.0) goto S70;
if (q <= 0.5) goto S100;
w = exp(q)*e-1.0;
goto S110;
S100:
w = (((e5+e4)*q+e3)*q+e2)*q+e1)*q;
S110:
*/
```

**IF T IS REJECTED, SAMPLE AGAIN AT STEP 8**

```plaintext
/*
if (c*fabs(u) > w*exp(e-0.5*t*t)) goto S70;
x = s*0.5*t;
sgamma = x*x;
return sgamma;
S120:
*/
```
ALTERNATE METHOD FOR PARAMETERS A BELOW 1 (.3678794 = exp(-1.))

```
\* 
\* a = 0.0;
\* b = 1.0+0.3678794*a;
\* S130:
\* p = b*ranf();
\* if(p >= 1.0) goto S140;
\* sgamma = exp(log(p)/ a);
\* if(sexp0() < sgamma) goto S130;
\* return sgamma;
\* S140:
\* sgamma = -log((b-p)/ a);
\* if(sexp0() < (1.0-a)*log(sgamma)) goto S130;
\* return sgamma;
\*/
```

STANDARD- NORMAL DISTRIBUTION

FOR DETAILS SEE:

AHRENS, J. H. AND DIETER, U.
EXTENSIONS OF FORSYTHE'S METHOD FOR RANDOM
SAMPLING FROM THE NORMAL DISTRIBUTION.
MATH. COMPUT., 27, 124 (OCT. 1973), 927 - 937.

ALL STATEMENT NUMBERS CORRESPOND TO THE STEPS OF ALGORITHM 'FL'
(M=5) IN THE ABOVE PAPER (SLIGHTLY MODIFIED IMPLEMENTATION)

Modified by Barry W. Brown, Feb 3, 1988 to use RANF instead of
SUNIF. The argument IR thus goes away.

THE DEFINITIONS OF THE CONSTANTS A(K), D(K), T(K) AND
H(K) ARE ACCORDING TO THE ABOVEMENTIONED ARTICLE

```
static float a[32] = {
0.0, 0.3, 0.176096, -2.7, 0.81241E-2, 0.11777, 0.1573107, 0.1970991, 0.2372021, 0.2776904,
0.3186394, 0.36013, 0.4022501, 0.4450965, 0.4887764, 0.5334097, 0.5791322,
0.626099, 0.6744898, 0.724514, 0.7764218, 0.8305109, 0.8871466, 0.9467818,
1.0099, 1.077516, 1.150349, 1.229859, 1.318011, 1.417797, 1.534121, 1.67594,
1.862732, 1.153875
};
static float d[31] = {
0.0, 0.0, 0.0, 0.0, 0.0, 0.263843, 0.2425085, 0.2255674, 0.2116342, 0.1999243,
0.1899108, 0.1812252, 0.1736014, 0.1668419, 0.1607967, 0.1553497, 0.1504094,
0.1459026, 0.14177, 0.1379632, 0.1344418, 0.1311722, 0.128126, 0.1252791,
0.1226109, 0.1201036, 0.1177417, 0.1155119, 0.1134023, 0.1114027, 0.1095039
};
static float t[31] = {
7.633828E-4, 2.306878E-3, 3.860618E-3, 5.438454E-3, 7.0507E-3, 8.708936E-3,
1.042357E-2, 1.220953E-2, 1.408125E-2, 1.605579E-2, 1.81529E-2, 2.039573E-2,
2.261177E-2, 2.543072E-2, 2.830296E-2, 3.146822E-2, 3.499233E-2, 3.895483E-2,
4.345878E-2, 4.840335E-2, 5.468334E-2, 6.184222E-2, 7.047983E-2, 8.113195E-2,
9.462444E-2, 0.1123001, 0.136498, 0.1716886, 0.2276241, 0.330498, 0.5847031
};
static float h[31] = {
427
```
static long i;
static float snorm, u, s, ustar, aa, w, y, t;

\[ u = \text{ranf}(); \]
\[ s = 0.0; \]
\[ \text{if} (u > 0.5) s = 1.0; \]
\[ u = (u-s) \]
\[ i = \text{(long)} (u); \]
\[ \text{if}(i == 32) i = 31; \]
\[ \text{if}(i == 0) \text{goto S100;} \]

```
  START CENTER
  ustar = u-(float)i;
  aa = *(a+i-1);
  S40:  \text{if}(ustar <= *(t+i-1)) \text{goto S60;}
        \text{w} = (ustar-(t+i-1))*(h+i-1);
  S50:  \text{ */ }
        \text{EXIT (BOTH CASES)}
        \text{y} = aa+w;
        \text{snorm = y;}
        \text{if}(s == 1.0) \text{snorm = -y;}
        \text{return snorm;}
  S60:  \text{ */ }
        \text{CENTER CONTINUED}
        u = \text{ranf}();
        w = u*(aa-i-aa);
        tt = (0.5*w+aa)*w;
        \text{goto S80;}
  S70:  \text{tt} = u;
        \text{ustar = ranf();}
  S80:  \text{if}(ustar > tt) \text{goto S50;}
        u = \text{ranf}();
        \text{if}(ustar >= u) \text{goto S70;}
        \text{ustar = ranf();}
        \text{goto S40;}
  S100: \text{ */ }
        \text{START TAIL}
        i = 6;
        aa = *(a+31);
        \text{goto S120;}
  S110: \text{aa += *(d+i-1);}
        i += 1;
  S120: \text{u += u;}
        \text{if}(u <= 1.0) \text{goto S110;}
        u -= 1.0;
  S140: \text{w = u**(d+i-1);}
```
\[ tt = (0.5 \times w + sa) \times w; \]

goto S160;

S150:
\[ tt = u; \]

S160:
\[ u\text{star} = \text{ranf}(); \]
\[ \text{if}(u\text{star} > tt) \text{ goto } S50; \]
\[ u = \text{ranf}(); \]
\[ \text{if}(u\text{star} >= u) \text{ goto } S150; \]
\[ u = \text{ranf}(); \]
\[ \text{goto } S140; \]

float fsign(float num, float sign)
/* Transfers sign of argument sign to argument num */
{
if (\ ((sign>0.0f && num<0.0f) \mid (sign<0.0f && num>0.0f) )
    \return -num;
else \return num;
}

FTNSTOP:
Prints msg to standard error and then exits
***********************************************
void ftnstop(char *msg)
/* msg = error message */
{
 if (msg != NULL) fprintf(stderr, "%s\n", msg);
 exit(0);
}
/* Prototypes for all user accessible RANLIB routines */

extern void advnst(long k);
extern float genbet(float aa, float bb);
extern float genchi(float df);
extern float genexp(float av);
extern float genf(float dfn, float dfd);
extern float gengam(float a, float r);
extern void genmm(float *parm, float *x, float *work);
extern void genmul(long n, float *p, long ncat, long *ix);
extern float gennch(float df, float xnonc);
extern float gennf(float dfn, float dfd, float xnonc);
extern float gennor(float av, float sd);
extern void gennrm(long *larray, int larray);
extern float gennunf(float low, float high);
extern void getsd(long *iseed1, long *iseed2);
extern void gscgn(long getset, long *g);
extern long ignbin(long n, float pp);
extern long ignbnn(long n, float p);
extern long ignlgi(void);
extern long ignpoi(float mu);
extern long ignuin(long low, long high);
extern void initgn(long isdtype);
extern long multmod(long a, long s, long m);
extern void phrtd(char * phrase, long * seed1, long * seed2);
extern float ranf(void);
extern void setall(long iseed1, long iseed2);
extern void setant(long qvalue);
extern void setgmn(float *meanv, float *covm, long p, float *parm);
extern void setsd(long iseed1, long iseed2);
extern float sexpo(void);
extern float sgamma(float a);
extern float snorm(void);
#include <math.h>
float sdot(long n, float *sx, long incx, float *sy, long incy)
{
    static long i,ix,iy,m, mpl;
    static float sdot, stemp;
    stemp = sdot = 0.0;
    if(n <= 0) return sdot;
    if(incx == 1 && incy == 1) goto S20;
    ix = iy = 1;
    if(incx < 0) ix = (-n+1)*incx+1;
    if(incy < 0) iy = (-n+1)*incy+1;
    for(i=1; i<=n; i++) {
        stemp += (*(sx+ix-1) * *(sy+iy-1));
        ix *= incx;
        iy += incy;
    }
    sdot = stemp;
    return sdot;
}

void sfsa(float *a, long lda, long n, long *info)
/*
 SPOFA FACTORS A REAL SYMMETRIC POSITIVE DEFINITE MATRIX.
 SPOFA IS USUALLY CALLED BY SPOCO, BUT IT CAN BE CALLED
 DIRECTLY WITH A SAVING IN TIME IF RCOND IS NOT NEEDED.
 (TIME FOR SPOCO) = (1 + 18/N) * (TIME FOR SPOFA).
 ON ENTRY
 A REAL(LDA, N)
 THE SYMMETRIC MATRIX TO BE FACTORED. ONLY THE
 DIAGONAL AND UPPER TRIANGLE ARE USED.
 LDA INTEGER
 THE LEADING DIMENSION OF THE ARRAY A.
 N INTEGER
 THE ORDER OF THE MATRIX A.
 ON RETURN
 A AN UPPER TRIANGULAR MATRIX R SO THAT A = TRANS(R)*R
 WHERE TRANS(R) IS THE TRANSPOSE.
 THE STRICT LOWER TRIANGLE IS UNALTERED.
 INFO INTEGER
 = 0 FOR NORMAL RETURN.
 = K SIGNALS AN ERROR CONDITION. THE LEADING MINOR
 OF ORDER K IS NOT POSITIVE DEFINITE.
 LINPACK. THIS VERSION DATED 08/14/78.
 CLEVE MOLER, UNIVERSITY OF NEW MEXICO, ARGONNE NATIONAL LAB.
 SUBROUTINES AND FUNCTIONS
 BLAS SDOT
 FORTRAN SQRT
 INTERNAL VARIABLES
*/
extern float sdot(long n, float *sx, long incx, float *sy, longincy);
static long i, jml, k;
static float t, s;

BEGIN BLOCK WITH ...EXITS TO 40
/*
for(j=1; j<=n; j++) {
  *info = j;
  s = 0.0;
  jml = j-1;
  if(jml < 1) goto S20;
  for(k=0; k<jml; k++) {
    t = *(a+k+(j-l)*lda) - sdot(k, *(a-k*lda), IL, *(a+(j-l)*lda), IL);
    t /= *(a+k*lda);
    *(a+k+(j-l)*lda) = t;
    s += (t*t);
  }
S20:
  s = *(a+j-1+(j-1)*lda)-s;
  /*
  ......EXIT
  */
  if(s <= 0.0) goto S40;
  *(a+j-1+(j-1)*lda) = sqrt(s);
  *info = 0;
S40:
  return;
}
COM.C

#include "ranlib.h"
#include <stdio.h>
#include <stdlib.h>

void advnst(long k) {
    /*
    void advnst(long k)
    ADV-a-N-ce ST-ate
    Advances the state of the current generator by 2^K values and
    resets the initial seed to that value.
    This is a transcription from Pascal to Fortran of routine
    Advance_State from the paper
    L'Ecuyer, P. and Cote, S. "Implementing a Random Number Package
    with Splitting Facilities." ACM Transactions on Mathematical
    Arguments
    k -> The generator is advanced by 2^K values
    */
    
    #define numg 32L
    extern void gset(long getset,long *qvalue);
    extern void gsgn(long getset,long *q);
    extern long Xm1,Xm2,Xa1,Xa2,Xcg1[],Xcg2[];
    static long q,1,ib1,ib2;
    static long qsgn;
    
    gset(0L,qsgn);
    if(qsgn) goto S10;
    fputs(" ADVNST called before random generator initialized - ABORT",stderr);
    exit(1);

    S10:
    gsgn(0L,q);
    ic1 = Xa1;
    ib2 = Xa2;
    ib1 = mltmod(ib1,ib1,Xm1);
    ib2 = mltmod(ib2,ib2,Xm2);
    }
    setsd(mltmod(ib1,*(Xcg1+q-1),Xm1),mltmod(ib2,*(Xcg2+q-1),Xm2));
    
    NOW, IB1 = A1**K AND IB2 = A2**K
    */
    #undef numg
}

void getsd(long *iseed1,long *iseed2) {
    /*
    GET SeeD
    Returns the value of two integer seeds of the current generator
    This is a transcription from Pascal to Fortran of routine
    Get_State from the paper
    L'Ecuyer, P. and Cote, S. "Implementing a Random Number Package
    with Splitting Facilities." ACM Transactions on Mathematical
    Arguments
    iseed1 <- First integer seed of generator G
    */
```c
#define numg 32L
extern void gsrgs(long getset, long *qvalue);
extern void gsrgn(long getset, long *g);
extern long Xcgl[1], Xcg2[1];
static long g;
static long qrgnin;
/
* Abort unless random number generator initialized */
gsrgs(OL, &qrgnin);
if(!qrgnin) goto S10;
printf(stderr, "GETSD called before random number generator initialized — abort!\n");
exit(0);
S10:
gsrgn(OL, &g);
  *iseed1 = *(Xcgl+g-1);
  *iseed2 = *(Xcg2+g-1);
#undef numg
long lgnlgi(void)
/
******************************************************************************

long lgnlgi(void)

Generate Large Integer

Returns a random integer following a uniform distribution over (1, 2147483562) using the current generator.
This is a transcription from Pascal to Fortran of routine
Random from the paper
L’Ecuyer, P. and Cote, S. “Implementing a Random Number Package
With Splitting Facilities.” ACM Transactions on Mathematical

******************************************************************************
/
#define numg 32L
extern void gsrgs(long getset, long *qvalue);
extern void gsrsst(long getset, long *qset);
extern void gsrgn(long getset, long *g);
extern void irgcm(void);
extern long Xml, Xm2, Xa1, Xa2, Xcgl[1], Xcg2[1];
extern long Xqanti[1];
static long lgnlgi, curntg, k, s1, s2, z;
static long qqsd, qrgnin;
/
* IF THE RANDOM NUMBER PACKAGE HAS NOT BEEN INITIALIZED YET, DO SO.
IT CAN BE INITIALIZED IN ONE OF TWO WAYS: 1) THE FIRST CALL TO
THIS ROUTINE 2) A CALL TO SETALL. */
gsrgs(0L, &qrgnin);
if(!qrgnin) irgcm();
gsrsst(0, &qqsd);
if(!qqsd) setall(1234567890L, 1234567890L);
/
* Get Current Generator */
gsrgn(0L, &curntg);
s1 = *(Xcgl+curntg-1);
s2 = *(Xcg2+curntg-1);
k = s1/53668L;
s1 = Xa1*(s1-k*53668L)-k*12211;
```

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if(s1 < 0) s1 += Xm1;
k = s2/52774L;
s2 = Xa2*(s2-k*52774L)-k*3791;
if(s2 < 0) s2 += Xm2;
*(Xcg1+curntg-1) = s1;
*(Xcg2+curntg-1) = s2;
z = s1-s2;
if(z < 1) z += Xm1-1;
if(*Xqanti+curntg-1) z = Xm1-z;
ignlgi = z;
return ignlgi;

void initgn(long isdtype)
{
    INIT-generate current G-e-N-erator
    Reinitializes the state of the current generator
    This is a transcription from Pascal to Fortran of routine
    Init-Generator from the paper
    L’Ecuyer, P. and Cote, S. “Implementing a Random Number Package
    with Splitting Facilities.” ACM Transactions on Mathematical
    Arguments
    isdtype => The state to which the generator is to be set
    isdtype = -1 => sets the seeds to their initial value
    isdtype = 0 => sets the seeds to the first value of
    the current block
    isdtype = 1 => sets the seeds to the first value of
    the next block

    do nothing
    /*
    #define numg 32L
    extern void qgrgs(long getset,long *qvalue);
    extern void qscgn(long getset,long *q);
    extern long Xm1,Xm2,Xa1w,Xa2w,Xig1[],Xig2[],Xlg1[],Xlg2[],Xcgl[],Xcg2[];
    static long g;
    static long qrgnin;
    */
    Abort unless random number generator initialized
    /*
    qgrgs(0L,&qrgnin);
    if(qrgnin) goto S10;
    fprintf(stderr,“%s\n”,
        “INITGN called before random number generator initialized - abort!”);
    exit(1);
    S10:
    qscgn(0L,&g);
    if(-1 != isdtype) goto S20;
    *(Xig1+g-1) = *(Xig1+g-1);
    *(Xig2+g-1) = *(Xig2+g-1);
    goto S50;
    S20:
    if(0 != isdtype) goto S30;
    goto S50;
    S30:
    /*
    do nothing
    */
    if(1 != isdtype) goto S40;
    *(Xlg1+g-1) = mitmod(Xa1w,*:(Xlg1+g-1),Xm1);
    *(Xlg2+g-1) = mitmod(Xa2w,*:(Xlg2+g-1),Xm2);
    goto S50;
S40:
    printf(stderr,"%s\n","isdtype not in range in INITGN");
    exit(1);
S50:
    *(Xcg1+q-1) = *(Xlg1+q-1);
    *(Xcg2+q-1) = *(Xlg2+q-1);
    #undef numg

void incgcm(void)  
/*
  * Initialize Random number Generator Common
  * Function
  * Initializes common area for random number generator. This saves the
  * nuisance of a BLOCK DATA routine and the difficulty of
  * assuring that the routine is loaded with the other routines.
  */
{
    #define numg 32L
    extern void gsrgs(long getset, long *qvalue);
    extern long Xml,Xm2,Xa1,Xa2,Xa1w,Xa2w,Xa1vw,Xa2vw;
    extern long Xqanti[];
    static long TI;
    static long li;
    /*
     * V=20; W=30;
     * ALW = MOD(A1**(2*W),M1)     A2W = MOD(A2**(2*W),M2)
     * ALVW = MOD(A1**(2**(V+W)),M1) A2VW = MOD(A2**(2**(V+W)),M2)
     * If V or W is changed ALW, A2W, ALVW, and A2VW need to be recomputed.
     * An efficient way to precompute a**(2**j) MOD m is to start with
     * a and square it j times modulo m using the function MLTMOD.
     */
    Xml = 2147483563L;
    Xm2 = 2147483399L;
    Xa1 = 40014L;
    Xa2 = 40692L;
    Xa1w = 1033780774L;
    Xa2w = 1494757890L;
    Xa1vw = 2082007225L;
    Xa2vw = 784306273L;
    for(i=0; i<numg; i++) *(Xqanti+i) = 0;
    TI = 1;
    /*
     * Tell the world that common has been initialized
     */
    gsrgs(1L,$TI);
    #undef numg
}
void setall(long iseed1,long iseed2)  
/*
  * sets all random number generators
  * Sets the initial seed of generator 1 to iseed1 and iseed2. The
  * initial seeds of the other generators are set accordingly, and
  * all generators states are set to these seeds.
  * This is a transcription from Pascal to Fortran of routine
  * Set Initial Seed from the paper
  * L'Ecuuyer, P. and Cote, S. “Implementing a Random Number Package
  * with Splitting Facilities.” ACM Transactions on Mathematical
  * Arguments
  * iseed1 -> First of two integer seeds
  */

Iseed$2 \rightarrow \text{Second of two integer seeds}

\[
\begin{align*}
\text{\#define numg 32L} \\
\text{extern void gsrcs(long getset, long *qvalue);} \\
\text{extern void gsst(long getset, long *qset);} \\
\text{extern void gscqn(long getset, long *q);} \\
\text{extern long Xm1,Xm2,Xa1w,Xa2w,Xig1[],Xig2[];} \\
\text{static long T1;} \\
\text{static long g,ocgn;} \\
\text{static long qrqin;} \\
\text{T1 = 1;} \\
\end{align*}
\]

\text{TELL IGNLGI, THE ACTUAL NUMBER GENERATOR, THAT THIS ROUTINE \hspace{1cm} HAS BEEN CALLED.}

\[
\begin{align*}
\text{gsst}(1,&T1); \\
gscqn(0L,&ocgn); \\
\end{align*}
\]

\text{Initialize Common Block if Necessary}

\[
\begin{align*}
\text{gssrcs}(0L,&qrqin); \\
\text{if(qrqin) lnrgcm();} \\
\text{*Xig1 = Iseed1;} \\
\text{*Xig2 = Iseed2;} \\
\text{initgn(-LL);} \\
\text{for(q=2; q<numg; q++)} \\
\text{\{ g<<2, g=numg; g++ \} } \\
\text{gscqn(1L,&ocgn);} \\
\text{undef numg} \\
\end{align*}
\]

\text{void setant(long qvalue)}

\text{void setant(long qvalue)}

\text{SET ANTThetic}

\text{Sets whether the current generator produces antithetic values. If X is the value normally returned from a uniform \([0,1]\) random number generator then 1 - X is the antithetic value. If X is the value normally returned from a uniform \([0,N]\) random number generator then N - 1 - X is the antithetic value.}

\text{All generators are initialized to NOT generate antithetic values.}

\text{This is a transcription from Pascal to Fortran of routine \text{SetAntithetic from the paper}}

\text{L’Ecuyer, P. and Cote, S. “Implementing a Random Number Package \hspace{1cm} with Splitting Facilities.” ACM Transactions on Mathematical \hspace{1cm} Software, 17:98-111 (1991)}}

\text{Arguments}

\text{qvalue \rightarrow nonzero if generator G is to generating antithetic \hspace{1cm} values, otherwise zero}

\[
\begin{align*}
\text{\#define numg 32L} \\
\text{extern void gscqs(long getset, long *qvalue);} \\
\text{extern void gscqn(long getset, long *q);} \\
\text{extern long Xqanti[];} \\
\text{static long q;} \\
\text{static long qrqin;} \\
\end{align*}
\]
Abort unless random number generator initialized

```c
void setsd(long iseed1, long iseed2)
{
#define numg 32L
extern void gsrqs(long getset, long *qvalue);
extern void gscqn(long getset, long *qg);
extern long Xigl[], Xig2[];
static long g;
static long qrqnin;
/*
 * Abort unless random number generator initialized
 */
gsrqs(0L, &qrqnin);
if (qrqnin) goto S10;
fprintf(stderr, "%s"
, " SETSD called before random number generator initialized - abort!");
exit(1);
S10:
gscqn(0L, &q);
*(Xig1+g-1) = iseed1;
*(Xig2+g-1) = iseed2;
initgn(-1L);
#undef numg
}
long Xm1, Xm2, Xa1, Xa2, Xcg1[32], Xcg2[32], Xalw, Xalw, Xa2w, Xig1[32], Xig2[32], Xigl[32],
Xig2[32], Xalw, Xa2w;
long Xqanti[32];
```

This is a transcription from Pascal to Fortran of routine Set Seed from the paper L’Ecuyer, P. and Cote, S. “Implementing a Random Number Package with Splitting Facilities.” ACM Transactions on Mathematical Software, 17:98-111 (1991)

Arguments

iseed1 -> First integer seed
iseed2 -> Second integer seed
APPENDIX B

JSBPCA17: AN MS-DOS COMPUTER PROGRAM FOR THE CONVERSION AND CALIBRATION OF TCSPC DATA COLLECTED WITH PCA-II SOFTWARE (WRITTEN IN VISUAL C++ V1.0)

/* PCA FILE CONVERTER
* Created by Jeff Buterbaugh 8/25/94
* Takes raw ASCII data files from PCA-II software and converts them to files in a specified format. (Comma- or Space-delimited, Single column, etc.)
* Header file information is tabulated and stored in a separate file.
* This program also performs x-axis conversions, from channels -> time units.
*/

#include <iostream.h>
#include <iomap.h>
#include <stdio.h>
#include <stdlib.h>
#include <fstream.h>
#include <string.h>
#include <math.h>
#include <conio.h>
#include <direct.h>

enum boolean { false, true };  
enum filemode { input, outOver, outApp };  
const unsigned int LINE_SIZE = 128;  
const unsigned int PATH_SIZE = 64;  
const unsigned int FILE_SIZE = 8;  // DOS filename limit  
const unsigned int EXT_SIZE = 5;  // EXT - NULL  
const unsigned int MAX_ARRAY_SIZE = 2048;  // max # of data points - arbitrary  
const unsigned int SMALL_ARRAY_SIZE = 64;  // for smaller data structures  
const unsigned int CHANNEL_SIZE = 6;  // width of PCA-II Chn Column + NULL  
const double DEFAULT_THRESHOLD = 9500;  // min # of counts for calibration point  

struct headerStruct {  
char filename[FILE_SIZE + 1], spectrumId[LINE_SIZE + 1];  
int numberOfChannels, peakChannel;  
unsigned int elapsedTime;  
double peakCounts;  
double totalCounts;  
};  

struct dataStruct {  
double x[MAX_ARRAY_SIZE];  
double y[MAX_ARRAY_SIZE];  
};  

struct smallDataStruct {  
double x[SMALL_ARRAY_SIZE];  
};
double y[SMALL_ARRAY_SIZE];
int pointCount;

struct regressionStruct {
    double R_Square;
    double slope;
    double intercept;
};

// FUNCTION PROTOTYPES */
void displaySettings();
void displayHelpInfo();
void changeSettings();
void processPcaFiles();
void processCommaToSpace();
void processCommaToCounts();
void processTimeConversion();
void menu();

void useDefaults(char*);
void incrementFilename(char*);
void getDataStats(const dataStruct, headerStruct, int);
void getPotentialCalibrationPoints(const dataStruct, smallDataStruct, int);
void chooseCalPoints(smallDataStruct);

double sq(double);

/* GLOBAL DECLARATIONS */
dataStruct data;  // declared to accommodate huge array
smallDataStruct points;  // stores subset of data array
regressionStruct equation;  // x-conversion equation
fstream fhdr, fin, fout;  // header, input, and output files
char fullHeaderPath[PATH_SIZE + 1], fullInPath[PATH_SIZE + 1],
    fullOutPath[PATH_SIZE - 1];
char commaChanPath[PATH_SIZE + 1], commaTimePath[PATH_SIZE + 1],
    spacePath[PATH_SIZE + 1], headerPath[PATH_SIZE + 1], pcaPath[PATH_SIZE + 1];
char commaChanExt[EXT_SIZE + 1], commaTimeExt[EXT_SIZE + 1],
    spaceExt[EXT_SIZE + 1], headerExt[EXT_SIZE + 1], pcaExt[EXT_SIZE + 1];
char countsPath[PATH_SIZE + 1], countsExt[EXT_SIZE + 1];

boolean finished = false;

/* DEFAULTS: Under normal circumstances, read Defaults from an INI file */

char* inFfile = "C:\\jbpca.ini";  // location of INI file for defaults
char* defaultPcaPath = "C:\\data\\";  // extra slashes required for esc sequence
char* defaultPcaExt = ".asc";
char* defaultCommaChanPath = "C:\\data\";

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char* defaultCommaChanExt = ".unc";
char* defaultCommaTimePath = "C:\data\";
char* defaultCommaChanExt = ".dat";
char* defaultHeaderPath = "C:\data\";
char* defaultHeaderExt = ".hdr";
char* defaultCountsPath = "C:\data\";
char* defaultCountsExt = ".cts";
char* defaultSpacePath = "C:\data\";
char* defaultSpaceExt = ".dt2";

/* FUNCTION: sq
 * Returns the square of the argument.
 */
double sq(double x)
{
    return x * x;
}

/* FUNCTION: incrementFilename
 * Increments the final character in a DOS filename (<= 8 characters).
 * Designed for naming convention in which the final characters are digits
 * corresponding to a file number. Will work for files numbered 0 - 99.
 */
void incrementFilename(char* filename)
{
    int index = 0;
    while(filename[index] != '0') index++;
    if(filename[index - 1] == '9') {
        if(filename[index - 2] == '9') {
            filename[index - 1] = '0';
            if(filename[index - 2] != '0') {
                filename[index - 2] += 1;
            }
        }
    }
    else {
        filename[index - 1] += 1;
    }
}

/* FUNCTION: useDefaults
 * Sets parameters to their default values.
 */
void useDefaults(char* iniFile)
{
    ifstream fini;
    char buffer[LFILE_SIZE - 1];
    int test;

    if(openFile(iniFile, fini, input) == false) {
        cout << "INI file " << iniFile << " not available."
            " Using 'factory defaults'." << endl;
        strcpy(pcaPath, defaultPcaPath); // use default PCA path
        strcpy(commaChanPath, defaultCommaChanPath); // use default comma-delimited path
        strcpy(commaTimePath, defaultCommaTimePath); // use default time-converted path
        strcpy(countsPath, defaultCountsPath); // default single-column path
        strcpy(spacePath, defaultSpacePath); // use default space-delimited path
        strcpy(headerPath, defaultHeaderPath); // use default header path
        strcpy(pcaExt, defaultPcaExt); // use default PCA file extension
        strcpy(commaChanExt, defaultCommaChanExt); // default comma output extension
        strcpy(commaTimeExt, defaultCommaTimeExt); // default time-converted extension
        strcpy(countsExt, defaultCountsExt); // default single-column extension
        strcpy(spaceExt, defaultSpaceExt); // use default space-delimited extension
        strcpy(headerExt, defaultHeaderExt); // use default header extension
    }
    else {
    // INI file successfully opened
        do { // read lines until [Defaults] is found
            ......
fini.getline(buffer, LINE_SIZE + 1);
    test = strcmp(buffer, "[Defaults]");
    if (test != 0) {
        fini.getline(pcaPath, LINE_SIZE + 1);
        fini.getline(pcaExt, LINE_SIZE + 1);
        fini.getline(commaChanPath, LINE_SIZE + 1);
        fini.getline(commaChanExt, LINE_SIZE + 1);
        fini.getline(commaTimePath, LINE_SIZE + 1);
        fini.getline(commaTimeExt, LINE_SIZE + 1);
        fini.getline(headerPath, LINE_SIZE + 1);
        fini.getline(headerExt, LINE_SIZE + 1);
        fini.getline(countsPath, LINE_SIZE + 1);
        fini.getline(countsExt, LINE_SIZE + 1);
        fini.getline(spacePath, LINE_SIZE + 1);
        fini.getline(spaceExt, LINE_SIZE + 1);
    }
    fini.close();

    equation.R_Square = 1.0;  // initialize x-conversion equation
    equation.slope = 1.0;
    equation.intercept = 0.0; // to x(2) = x(1)
}

/* FUNCTION: displaySettings
 * Displays the current path & extension settings as well as the current
 * x-conversion equation.
 */
void displaySettings()
{
    cout << "Current Settings (Defaults in " << iniFile << ") : " << ")n"
    cout << "FILE FORMAT PATH EXTENSION
    cout << "[a] PCA-II file (Raw Data) : " << pcaPath << "\t[b] "
        << pcaExt << ")n"
    cout << "[c] (Channel, Counts) : " << commaChanPath << "\t[d] "
        << commaChanExt << "\n"
    cout << "[e] (Time, Counts) : " << commaTimePath << "\t[f] "
        << commaTimeExt << "\n"
    cout << "[g] Single-Column (Counts) : " << countsPath << "\t[h] "
        << countsExt << "\n"
    cout << "[i] Spaced (Time Counts) : " << spacePath << "\t[j] "
        << spaceExt << "\n"
    cout << "[k] Table of Header Info : " << headerPath << "\t[l] "
        << headerExt << "\n"
    cout << "[m] X-Conversion Equation : " << "x(2) = " << equation.slope
        << "x(1) ";
    /* Display of equation depends on the sign of the y-intercept. */
    if (equation.intercept >= 0) cout << ");
    cout << equation.intercept << "t"
        << "R-Squared = " << equation.R_Square << ")n"
    
    /* FUNCTION: changeSettings
 * Allow changes in input/output file paths and extensions from default values.
 */
void changeSettings()
{
    char choice;
    cin >> buffer[LINIE_SIZE + 1];
    displaySettings();
    cout << "Enter letter corresponding to the parameter 
"
do { 
    cin >> choice; 
    cingetline(buffer, LINE_SIZE + 1); 
} while(choice < 'a' || choice > 'm'); 

switch(choice) { 
    case 'a': 
        cout << "Enter new PCA-II path : " << endl; 
        gets(buffer); 
        strcpy(pcaPath, buffer); 
        break; 
    case 'b': 
        cout << "Enter new PCA-II extension : " << endl; 
        gets(buffer); 
        strcpy(pcaExt, buffer); 
        break; 
    case 'c': 
        cout << "Enter new Comma-Delimited path : " << endl; 
        gets(buffer); 
        strcpy(commaChanPath, buffer); 
        break; 
    case 'd': 
        cout << "Enter new Comma-Delimited extension : " << endl; 
        gets(buffer); 
        strcpy(commaChanExt, buffer); 
        break; 
    case 'e': 
        cout << "Enter new Time-Converted path : " << endl; 
        gets(buffer); 
        strcpy(commaTimePath, buffer); 
        break; 
    case 'f': 
        cout << "Enter new Time-Converted extension : " << endl; 
        gets(buffer); 
        strcpy(commaTimeExt, buffer); 
        break; 
    case 'g': 
        cout << "Enter new Single-Column path : " << endl; 
        gets(buffer); 
        strcpy(countsPath, buffer); 
        break; 
    case 'h': 
        cout << "Enter new Single-Column extension : " << endl; 
        gets(buffer); 
        strcpy(countsExt, buffer); 
        break; 
    case 'i': 
        cout << "Enter new Space-Delimited path : " << endl; 
        gets(buffer); 
        strcpy(spacePath, buffer); 
        break; 
    case 'j': 
        cout << "Enter new Space-Delimited extension : " << endl; 
        gets(buffer); 
        strcpy(spaceExt, buffer); 
        break; 
    case 'k': 
        cout << "Enter new Header path : " << endl; 
        gets(buffer); 
        strcpy(headerPath, buffer); 
        break; 
    case 'l': 
        cout << "Enter new Header extension : " << endl; 
        gets(buffer); 
}
strcpy(headerExt, buffer);
break;
case 'm':
cout << "Enter equation slope : ";
cin >> equation.slope;
cin.getLine(buffer, LINE_SIZE + 1);
cout << "Enter equation intercept : ";
cin >> equation.intercept;
brcak;
default:
brcak;
}

/* FUNCTION: openFile
* Opens a file in the specified mode and tests for success. Returns ok = true
* for a successful open; ok = false for a failure.
*/
bool openFile(char* fullPath, ifstream& currentFile, filemode currentMode)
{
    bool ok = true;
    switch(currentMode) {
        case input:
            currentFile.open(fullPath, ios::in | ios::nocreate);
brcak;
        case outFile:
            currentFile.open(fullPath, ios::out | ios::noreplace);
brcak;
        case outApp:
            currentFile.open(fullPath, ios::app);
brcak;
        default:
            ok = false;
brcak;
    }
    if(!currentFile) {
        ok = false;
cout << "Error opening file " << fullPath << endl;
        switch(currentMode) {
            case input:
                cout << "The file does not exist or could not be opened." << endl;
brcak;
            case outFile:
                cout << "The file already exists or could not be opened." << endl;
brcak;
            case outApp:
                cout << "The file could not be opened." << endl;
default:
                cout << "Something is awry with the code — please debug."
                    currentFile.close();
        }
        do {
            cout << "<ENTER> to continue..." << endl;
        } while (getchar() != '\n');
    } else ok = true; // currentFile opened
    return ok;
}

/* FUNCTION: setUpHeaderFile

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* Opens the header file for output (output mode), and writes
* column headings. The file is then closed until it is needed.
*/

```cpp
bool setUpHeaderFile(char* fullPath, ifstream& fhdr)
{
    char headerFile[FILE_SIZE + 1];
    char tempPath[PATH_SIZE + 1];
    bool ok = true;

    cout << "Header file path = " << header << "\t"
         << " Extension = " << headerExt << endl;
    cout << "Enter header filename :
    cin >> headerFile, FILE_SIZE + 1);

    strcpy(tempPath, headerPath);
    strcat(tempPath, headerFile);  // append filename to current path
    strcpy(fullHeaderPath, tempPath);  // copy the resulting path onto full path
    strcat(fullHeaderPath, headerExt);  // append the extension to the path

    ok = openFile(fullHeaderPath, fhdr, outputFile);
    if(ok == false) { return ok; }

    fhdr << "| Of | Total | Peak | Peak | Collect |\n"  << headerExt << endl;
    fhdr << "Filename | Chan | Counts | Counts | Chan | Time(s) | Decay ID |\n"  << headerExt << endl;
    fhdr << "\n" << headerExt << endl;
    fhdr.close();
    return ok;
}
```

```cpp
/* FUNCTION: commaToSpace
 * Converts a comma-delimited data file to a space delimited data-file.
 */

bool commaToSpace(char* filename)
{
    double x;
    double y;
    char ch;
    char tempPath[PATH_SIZE + 1];
    bool ok = true;

    strcpy(tempPath, commaTimePath);
    strcat(tempPath, filename);  // append filename to current comma path
    strcpy(fullInPath, tempPath);  // copy resulting path onto full path for input
    strcat(fullInPath, commaTimeExt);  // append the extension to the path

    /* Open comma-delimited file for input */
    ok = openFile(fullInPath, fin, input);
    if(ok == false) { return ok; }

    strcpy(tempPath, spacePath);
    strcat(tempPath, filename);
    strcpy(fullOutPath, tempPath);
    strcat(fullOutPath, spaceExt);

    ok = openFile(fullOutPath, fout, outputFile);  // open space-delim file for output
    if(ok == false) { return ok; }

    while(fin >> x) {
        for (int c = 1; c <= 3; c++) fin.get(ch);  // discard ", "
        fin >> y;
    }
```

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```cpp
function: commaToCounts
* Converts comma-delimited file (channels, counts) to a single-column (counts) file.*

```
double runningSum = 0;

/* Initialize values before beginning tests */
header.peakChannel = 0;
header.peakCounts = 0;

for (int k = 0; k <= maxChan; k++) { // loop through channels
    if (data.y[k] > header.peakCounts) { // test for new "leader"
        header.peakChannel = k;
        header.peakCounts = data.y[k];
        runningSum = 0; // reset lower limit of integral
    }
    runningSum += data.y[k];
}
header.totalCounts = runningSum; // total counts from peakChan -> end of decay

/* FUNCTION: getPotentialCalibrationPoints
  * Scans a data file for channels with counts >= a threshold value.
  * Channels that meet this criterion are then tested to see if they contain
  * more counts than the channels on either side. If so, it is a local maximum
  * and the channel is suggested as a calibration point for the x axis conversion.
  */

void getPotentialCalibrationPoints(const dataStruct& data,
                                  smallDataStruct points, int maxChan)
{
    points.pointCount = 0;
    double threshold = DEFAULT_THRESHOLD;
    int index = 0;
    char choice;
    char buffer[LINE_SIZE + 1];

cout << "Default threshold for calibration points = " << threshold << "\n";
cout << "Enter Y to accept or N to change : ";
d0 {
    cin >> choice;
    cin.getline(buffer, LINE_SIZE + 1);
    while((choice != 'Y') && (choice != 'y') &&
          (choice != 'N') && (choice != 'n'))
    {
        cout << "Enter new threshold : ";
        cin >> threshold;
    }
    for (int k = 0; k <= maxChan; k++) { // loop through channels
        /* Test each point to see if it meets the criteria. */
        if ((data.y[k] >= threshold) && (data.y[k] >= data.y[k-1])
            && (data.y[k] >= data.y[k+1])) {
            points.x[index] = k;
            points.y[index] = data.y[k];
            points.pointCount++;
            index++;
            if (points.pointCount >= SMALL_ARRAY_SIZE) return;
        }
    }
}

/* FUNCTION: chooseCalPoints
  * Takes the potential calibration points and displays them to the user.
  * The user then enters the array of points to be used for the actual
x-axis conversion. These points are then returned.

```cpp
void chooseCalPoints(smallDataStruct points)
{
    int index;
    char buffer[LINE_SIZE + 1];

    cout << "Suggested calibration points: " << "\n";
    cout << "Channel" " \t" << "Counts" " \n";
    for(index = 0; index < points.pointCount; index++)
    {
        cout << points.x[index] " \t";
        cout << points.y[index] " \n";
    }
    do
    {
        cout << "Enter number of calibration points: ";
        cin >> points.pointCount;
    } while(points.pointCount >= SMALL_ARRAY_SIZE);

    for(index = 0; index < points.pointCount; index++)
    {
        cout << "Enter channel for calibration point "
             "\t" << "\t" << "Enter corresponding time value for point "
             "\t" << "\t";
        cin >> points.x[index];
        cin >> points.y[index];
    }
    cout << "Actual calibration points: " "\n";
    cout << "Channel" " \t" << "Time Value" " \n";
    for(index = 0; index < points.pointCount; index++)
    {
        cout << points.x[index] " \t";
        cout << points.y[index] " \n";
    }
    cout << "<ENTER> to Continue" "\n";
    cin.getLine(buffer, LINE_SIZE + 1);
}

/* FUNCTION: calcRegression
* Takes a set of calibration points and fits them to a line.
* Returns a regressionStruct of the best fit.
*/

regressionStruct calcRegression(smallDataStruct points)
{
    regressionStruct bestfit;

double meanx, meany, sdevx, sdevy;
double sum = (double) points.pointCount;
double sumx = 0, sumy = 0;
double sumxx = 0, sumyy = 0, sumxy = 0;
for(int i = 0; i < points.pointCount; i++)
    {
        sumx += points.x[i];
        sumy += points.y[i];
        sumxx += sq(points.x[i]);
        sumyy += sq(points.y[i]);
        sumxy += points.x[i] * points.y[i];
    }

meanx = sumx / sum;
meany = sumy / sum;
sdevx = sqrt((sumxx - sq(sumx) / sum) / (sum - 1.0));
sdevy = sqrt((sumyy - sq(sumy) / sum) / (sum - 1.0));
bestFit.slope = (sumxy - meanx * meany * sum) /
\[ \text{bestFit.intercept} = \text{meany} - \text{bestFit.slope} \times \text{meanx}; \]
\[ \text{bestFit.R.Square} = \frac{\text{sdevx}}{\text{sdevy} \times \text{bestFit.slope}}; \]
\[ \text{return bestFit;} \]

/* FUNCTION: getXConversionEquation
 * Reads a comma-delimited file \( (\text{channel}, \text{counts}) \) into the data array.
 * Potential calibration points are identified with \text{getPotentialCalibrationPoints}
 * & the actual points for the x-axis conversion are selected with \text{getCalPoints}.
 * A linear regression is performed on the points using \text{calcRegression} and the
 * resulting regression structure is assigned to \text{equation}.
 */

\begin{verbatim}
boolean getXConversionEquation(dataStruct& data, smallDataStruct& points, regressionStruct& equation)
{
    char tempPath[PATH_SIZE + 1], filename[FILE_SIZE + 1];
    char ch;
    int index = 0;
    int maxChan = 0;

    boolean done = false;
    boolean ok = true;

    cout << "Current path = " << commaChanPath << \\
          "Extension = " << commaChanExt << endl;
    cout << "Enter calibration file name or X to exit : ";
    cin.getLine(filename, FILE_SIZE + 1);
    if(strcmp(filename, "X") == 0) {
        done = true;
    }
    else if(strcmp(filename, "x") == 0) {
        done = true;
    }
    else done = false;

    while(!done) {
        strcpy(tempPath, commaChanPath);
        strcat(tempPath, filename);
        strcpy(fullInPath, tempPath);
        strcat(fullInPath, commaChanExt);

        ok = openFile(fullInPath, fin, input);
        if(ok == false) { return ok; }

        while(fin >> data.x[index]) {
            for(int c = 1; c <= 3; c++) fin.get(ch); // discard ",",\n            fin >> data.y[index];\n            fin.get(ch); // discard newline character\n            index++;
        }
        fin.close();
        maxChan = index - 1; // negate effect of last index++
        getPotentialCalibrationPoints(data, points, maxChan);
        chooseCalPoints(points);
        equation = calcRegression(points);
        done = true;
    }
    return ok;
}
\end{verbatim}
from PCA-II file and places them into an output file. Discards ROI column.

```c
boolean pcaToComma(char* filename, dataStruct& data) {
    headerStruct header;
    double channel;
    double counts;
    char ch;
    char buffer[LINE_SIZE + 1];
    char tempPath[PATH_SIZE + 1];

    int index = 0;
    int maxChan;

    boolean ok = true;

    strcpy(tempPath, pcaPath);
    strcat(tempPath, filename);
    strcpy(fullInPath, tempPath);
    strcat(fullInPath, pcaExt);

    ok = openFile(fullInPath, fin, input);
    if(ok == false) { return ok; }

    strcpy(tempPath, commaChanPath);
    strcat(tempPath, filename);
    strcpy(fullOutPath, tempPath);
    strcat(fullOutPath, commaChanExt);

    ok = openFile(fullOutPath, fout, outOver);
    if(ok == false) { return ok; }

    strcpy(header.filename, filename);

    for (int i = 1; i <= 6; i++) fin >> buffer;    // discard date, time, & "Elt:"

    /* zap leading 0's to avoid octal interpretation of elapsed time */
    while ((fin.peek() == '0') || (fin.peek() == ' ')) fin.get(ch);
    fin.getLine(buffer, LINE_SIZE + 1);    // discard remainder of first header row
    fin >> buffer;    // discard "ID:"
    fin.get(ch);    // discard leading space
    fin.getLine(header.spectrumId, LINE_SIZE + 1);

    /* Don't display default message */
    int m = strcmp(header.spectrumId, "No spectrum identifier defined");
    if (m == 0) strcpy(header.spectrumId, "\0");
    fin >> buffer; fin >> buffer;    // discard "Memory Size:"

    /* zap leading 0's to avoid octal interpretation of number of channels */
    while (((fin.peek() == '0') || (fin.peek() == ' ')) fin.get(ch);
    fin >> header.numberOfChannels;
    for (int j = 1; j <= 3; j++) {
        fin.getLine(buffer, LINE_SIZE + 1);    // dump remainder
    }

    while (fin.getLine(buffer, CHANNEL_SIZE)) {
        fin >> counts;
        channel = index;
        data.x[index] = channel;
        data.y[index] = counts;
    }
}
```
/* Write to comma-delimited file */
fout << data.x[index] << " , " << data.y[index] << "\n";
fin.getline(buffer, LINE_SIZE);// discard ROI index++;
}

maxChan = (int)channel; // NOTE: channel wasn't incremented on final iteration
getD ataSt ats(data, header, maxChan);

/* Append header info for current file to header table */
ok = openFile(fullHeaderPath, fhdr, outApp);
if(ok == false) { return ok; }

fhdr << setiosflags(ios::fixed); // do not use scientific notation in table
fhdr << setprecision(0); // ignore fractional component
fhdr << setw(8) << header.filename;
fhdr << setw(7) << header.numberOfChannels;
fhdr << setw(13) << header.totalCounts;
fhdr << setw(12) << header.peakCounts;
fhdr << setw(6) << header.peakChannel;
fhdr << setw(10) << header.elapsedTime;
fhdr << " \n"; fhdr << header.spectrumId << "\n";

/* close streams */
fhdr.close();
fin.close();
fout.close();
cout << "PCA-II file " << fullInPath << " -> " << fullOutPath
<< " (Comma-delim)" << endl;
return ok;
}

/* FUNCTION: processPcaFiles */

void processPcaFiles()
{
char filename[FILE_SIZE + 1], nextFile[FILE_SIZE + 1];
boolean done = false;
strcpy(nextFile, "NBXXXYYZ");
strcpy(filename, nextFile);
cout << "\n";
cout << "Current PCA-II path = " << pcaPath << " Extension = " << pcaExt << endl;
cout << "Current comma-delim path = " << commaChanPath << " Extension = " << commaChanExt << endl;
cout << "Enter PCA-II filenames (<= 8 characters) to convert " << " to comma-delimited files." << endl;
cout << "Type X to exit at any time." << endl;
while(!done) {
    cout << "Next file (Default = " << nextFile << ": X = exit) : ":
    cin.getline(filename, FILE_SIZE + 1);
    if(strcmp(filename, "\0") == 0) {
        strcpy(filename, nextFile);
        if(pcaToComma(filename, data) == false) {
            done = true;
        }
    }
    else if(strcmp(filename, "X") == 0) {
        done = true;
    }
    else if(strcmp(filename, "x") == 0) {

done = true;
}
else {
    if (pcaToComma(filename, data) == false) {
        done = true;
    }
}
}
incrementFilename(filename);
strcpy(nextFile, filename);
}

/* FUNCTION: processCommaToSpace */

void processCommaToSpace()
{
    char filename[FILE_SIZE + 1], nextFile[FILE_SIZE + 1];
    boolean done = false;
    strcpy(nextFile, "NBXXXXYZ");
    strcpy(filename, nextFile);
    cout << "\n";
    cout << "Current comma-delimited path = " << commaTimePath << \"\t\n" << "Extension = " << commaTimeExt << endl;
    cout << "Current space-delimited path = " << spacePath << \"\t\n" << "Extension = " << spaceExt << endl;
    cout << "Enter comma-delimited filenames (<= 8 characters) to convert\n" << "to space-delimited files. Type X to exit at any time." << endl;
    while(!done) {
        cout << "Next file (Default = " << nextFile << "; X = exit) : " ;
        cin.getline(filename, FILE_SIZE + 1);
        if(strcmp(filename, "\0") == 0) {
            strcpy(filename, nextFile);
            if(commaToSpace(filename) == false) {
                done = true;
            }
        } else if(strcmp(filename, "X") == 0) {
            done = true;
        } else if(strcmp(filename, "X") == 0) {
            done = true;
        } else {
            if(commaToSpace(filename) == false) {
                done = true;
            }
        }
        incrementFilename(filename);
        strcpy(nextFile, filename);
    }
}

/* FUNCTION: processCommaToCounts */

void processCommaToCounts()
char filename[FILE_SIZE + 1], nextFile[FILE_SIZE + 1];
boolean done = false;

strcpy(nextFile, "NBXXXYZ");
strcpy(filename, nextFile);

cout << "\n";
cout << "Current comma-delimited path = " << commaChanPath << "\t"
<< " Extension = " << commaChanExt << endl;
cout << "Current single-column path = " << countsPath << "\t"
<< " Extension = " << countsExt << endl;
cout << "Enter comma-delimited filenames (<= 8 characters) to convert\n" << "to single-column files. Type X to exit at any time.\" << endl;

while(!done) {

cout << "Next file (Default = " << nextFile << "; X = exit) : ";
cin >> filename, FILE_SIZE + 1);

if(strcmp(filename, "\0") == 0) {
    strcpy(filename, nextFile);
    if(commaToCounts(filename) == false) {
        done = true;
    }
}
else if(strcmp(filename, "X") == 0) {
    done = true;
}
else if(strcmp(filename, "x") == 0) {
    done = true;
}
else {
    if(commaToCounts(filename) == false) {
        done = true;
    }
}

incrementFilename(filename);
strcpy(nextFile, filename);
}

/* FUNCTION: timeConvert
 * Uses current equation to batch convert x-axis of comma-delimited data files.
 * Output format is space-delimited.
 */

boolean timeConvert(char* filename, regressionStruct equation) {
    double x, new_x;
    double y;
    char ch;
    char tempPath[PATH_SIZE + 1];
    boolean ok = true;

    strcpy(tempPath, commaChanPath);
    strcat(tempPath, filename); // append filename to current comma path
    strcpy(fullInPath, tempPath); // copy resulting path onto full path for input
    strcat(fullInPath, commaChanExt); // append the extension to the path

    ok = openFile(fullInPath, fin, input); if(ok == false) { return ok; }
    strcpy(tempPath, spacePath);
    strcat(tempPath, filename);
    strcpy(fullOutPath, tempPath); // copy resulting path onto full path for output
    strcat(fullOutPath, spaceExt); // append the extension to the path

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ok = openFile(fullOutPath, fout, outOver); // open space-delim file for output
if(ok == false) { return ok; }

while(fin >> x) {
    for (int c = 1; c <= 3; c++) fin.get(ch); // discard "", "
    fin >> y;
    fin.get(ch); ...... // discard newline character
    new_x = equation.slope*x + equation.intercept;
    fout << new_x << " " << y << "\n"; // store as space-delimited points
}
fin.close();
fout.close();
cout << fullInPath << " \" " << fullOutPath << endl;
return ok;

FUNCTION: processTimeConversion

void processTimeConversion()
{
    char filename[FILE_SIZE + 1], nextFile[FILE_SIZE + 1];
    boolean done = false;
    strcpy(nextFile, "NBXXXYYZ");
    strcpy(filename, nextFile);
    cout << "\n";
    cout << "Enter comma-delimited files to convert with equation: " << "\n"
         << "x(2) = " << equation.slope << "x(1) ";
    if(equation.intercept >= 0) cout << "+ ";
    cout << equation.intercept << "\t"
         << "R-Squared = " << equation.R_Square << "\n"
         << "Files will be stored in space-delimited format" << endl;
    cout << "Current comma-delimited path = " << commaChanPath << "\t"
         << "Extension = " << commaChanExt << endl;
    cout << "Current space-delimited path = " << spacePath << "\t"
         << "Extension = " << spaceExt << endl;
    cout << "Type X to exit at any time." << "\n";

while(!done) {
    cout << "Next file (Default = " << nextFile << "; X = exit) : ";
    cin.getLine(filename, FILE_SIZE + 1);

    if(strcmp(filename, "\0") == 0) {
        strcpy(filename, nextFile);
        if(timeConvert(filename, equation) == false) {
            done = true;
        }
    }
    else if(strcmp(filename, "X") == 0) {
        done = true;
    }
    else if(strcmp(filename, "x") == 0) {
        done = true;
    }
    else {
        if(timeConvert(filename, equation) == false) {
            done = true;
        }
    }
}

incrementFileName(filename);
```c
strncpy(nextFile, filename);
}

/* FUNCTION: menu
 * Select program options.
 */
void menu()
{
    char choice;
    char buffer[L INE _S IZE + 1];
    cout << "\n";
    displaySettings();
    cout << "MAIN MENU:" << "\n";
    cout << "[1] Change path name, extension, or equation." << "\n";
        << "Space-Delimited (time cts) with current X-Conversion equation." << "\n";
    cout << "[5] Convert Comma-Delimited files [chan , cts] to "
        << "Single-Column (cts) VAX FORMAT" << "\n";
    cout << "[6] Convert Comma-Delimited files [time , cts] to "
        << "Space-Delimited (time cts)." << "\n";
    cout << "[7] Exit PCA FILE CONVERSION UTILITY" << "\n";
    cout << "[8] HELP for PCA FILE CONVERSION UTILITY" << "\n\n";
    do {
        cin >> choice;
        cin.getline(buffer, LINE_SIZE + 1);
        while(choice < '1' || choice > '8');

        switch(choice) {
            case '1':
                changeSettings();
                break;
            case '2':
                if(setUpHeaderFile(fullHeaderPath, hdr) == true) {
                    processPcafiles();
                }
                break;
            case '3':
                if(getXConversionEquation(data, points, equation) == false) {
                    cout << "Could not obtain X-Conversion Equation" << endl;
                }
                break;
            case '4':
                processTimeConversion();
                break;
            case '5':
                processCommaToCounts();
                break;
            case '6':
                processCommaToSpace();
                break;
            case '7':
                finished = true;
                break;
            case '8':
                displayHelpInfo();
                break;
            default:
                break;
        }
    }
} 455
```
void displayHelpInfo()
{
    cout << "Use of PCA FILE CONVERSION UTILITY is usually straightforward."
        << endl;
    cout << "If your objective is to convert raw data from the PCA-II" << endl;
    cout << "format (ASCII) into a form suitable for analysis by PeakFit or" << endl;
    cout << "Seth Snyder & Jim Demas' deconvolution program, choose menu" << endl;
    cout << "Selections 2, 3, and 4 in that order. Selection 2 will convert" << endl;
    cout << "the raw data to a comma-delimited format without converting the" << endl;
    cout << "X-axes into the time domain. It will also construct a summary" << endl;
    cout << "table from the header information present in each ASCII file." << endl;
    cout << "Selection 3 will use one of the files generated in the previous" << endl;
    cout << "step to calibrate the time axis. The program will search the" << endl;
    cout << "calibration file for local maxima that have counts >= a" << endl;
    cout << "threshold value specified by the user. These maxima will be" << endl;
    cout << "suggested as likely calibration points for the axis conversion." << endl;
    cout << "It is the user's responsibility to enter the actual channel-time" << endl;
    cout << "correlations to use in the conversion. An X-conversion equation" << endl;
    cout << "is computed by linear regression analysis of the calibration" << endl;
    cout << "points. The results are stored in memory. Selection 4 will then" << endl;
    cout << "convert selected files (comma-delimited) according to the current" << endl;
    cout << "X-conversion equation." << endl;
    cout << "To create files suitable for analysis with the VAX program," << endl;
    cout << "follow steps 2, 3, and 5 in an analogous manner." << endl;
    cout << "PRESS ANY KEY TO CONTINUE" << endl;

    while(!_kbhit())
        _getch();
}

/* MAIN */

main()
{
    useDefaults(iniFile);
    cout << "PCA FILE CONVERSION UTILITY PROGRAM - VERSION DATE 8/25/94" << "\n"
        << "Created for The Gustafson Group by Jeff Buterbaugh" << "\n"
        << "The Ohio State University Department of Chemistry" << "\n"
        << "140 W. 18th Ave. Columbus OH 43210" << "\n"
        << "buterbaugh.18osu.edu" << "\n"
        << "THIS UPDATE SHOULD CREATE VALID FILES FOR THE VAX PROGRAM **";
    cout << endl;
    cout << "** IT ALSO CONTAINS A HELP FILE ** ";
    cout << "PRESS ANY KEY TO CONTINUE" << endl;
    while(!_kbhit())
        _getch();

    While(!finished) menu();
    return 0;
}
APPENDIX C

PARAMETER: A WINDOWS 95 UTILITY TO PARSE PARAMETER FILES GENERATED BY DEMAS & SNYDER'S SPC.EXE INTO TAB-DELIMITED TABLES (WRITTEN IN LABWINDOWS/CVI 4.01)

The project consists of the following files:
DB06401.C
DB06401.H
DB06401.PRJ

DB06401.C
========

#include <analysis.h>
#include <ansi_c.h>
#include <formatio.h>
#include <userint.h>
#include "db06401.h"

#define DEFAULT_PATH "\jsb"
#define PARAMETER_EXTENSION ".prm"
#define PATH_SIZE 260
#define LINE_SIZE 260

#define MAX_EXPONENTIALS 4

static int panelHandle;
static int fileHandle;
static int outputFileHandle;
static int fileSelectErr;
static int fileCloseErr;
static int numberOfFiles, numberOfNewFiles;

static char **fileList;
static char fileName[PATH_SIZE + 1];
static char outputFileName[PATH_SIZE + 1];
static char buffer[LINE_SIZE + 2];
static char commaFile[PATH_SIZE + 1];

static double sortedFractions[MAX_EXPONENTIALS];

struct parameterStruct
{
    char decayFileName[PATH_SIZE + 1], irrFileName[PATH_SIZE + 1];
    int firstChan, lastChan;
    double tau[MAX_EXPONENTIALS];
    double amp[MAX_EXPONENTIALS];
    double fraction[MAX_EXPONENTIALS];

    457
double sigmaTau[MAX_EXPONENTIALS];
double sigmaAmp[MAX_EXPONENTIALS];
double darkCounts, timeShift;
double chisSquare, meanTau, averageTau, meanResidual, sigmaResidual;
double skewness, skewnessThreshold, kurtosis, kurtosisThreshold, durbinWatson;
};

static struct parameterStruct parameter;

/* FUNCTION: SelectFiles */
* Uses a multi-file select popup to allow the user to generate a list of *
* data files to process. If the function is run > 1 time before files are *
* processed, new files are appended to the list of the current queue. *
*/

int SelectFiles (int panel, int control, int event,
    void *callbackData, int eventData1, int eventData2)
{
    switch (event) {
        static int listErr, fileSelErr;
        static int index = 0;
        static int loopIndex = 0;
        static int offset = 0;
        case EVENT_COMMIT:
            /* Reinitialize index & offset each time the function is called. */
            index = 0;
            offset = 0;

            /* If SelectFiles has been run > 1 time, add new files to end of list. */
            offset = numberOfFiles;
            if (offset != 0) {
                index = index - offset;
            }

            /* Select the files and store them in fileList */
            fileSelErr = MultiFileSelectPopup (DEFAULT_PATH, "*.prm", "*.pr",
                "Select Files to Process", 0, 0, 1,
                &numberOfNewFiles, &fileList);

            /* Total # of files = # of new files + # of previously selected files. */
            numberOfFiles += numberOfNewFiles;

            /* This rather messy loop adds each new filename to the list of pending files
            at the position "index"
            */
            for (loopIndex = 0; loopIndex < numberOfNewFiles; loopIndex++) {
                listErr = InsertListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
                    fileList[loopIndex],
                    fileList[loopIndex],
                    index++); /* Increment index */
            }

            if (numberOfFiles > 0) {
                SetCtrlAttribute (panelHandle, PANEL_BUTTON_ANALYZE_FILES,
                    ATTR_DIMMED, 0);
        }
    }
}
FUNCTION: AnalyzeOneFile
* Reads and tabulates information from an SPC parameter file.
*
int AnalyzeOneFile (char* fileName)
{
    static int fileCloseErr;
    static int i, j, k, index;
    static int bufferSize = sizeof(buffer);
    static int sortedIndices[MAX_EXPONENTIAls];

    FillBytes (parameter.decayFileName, 0, PATH_SIZE + 1, 0);
    FillBytes (parameter.irfFileName, 0, PATH_SIZE + 1, 0);
    FillBytes (buffer, 0, bufferSize - 1, 0);
    FillBytes (outputFile, 0, PATH_SIZE + 1, 0);

    fileHandle = OpenFile (fileName, VAL_READ_ONLY, VAL_TRUNCATE,
                           VAL_ASCII);

    fileSelectErr = FileSelectPopup ("e:\jsb", "*.txt", "*.",
                                     .... "Select Tab-Delimited Output File",
                                     .... VAL_OK_BUTTON, 0, 0, 1,
                                    outputFile);

    outputFileHandle = OpenFile (outputFile, VAL_WRITE_ONLY,
                                 .... VAL_APPEND, VAL_ASCII);

    WriteLine (outputFileHandle, "Decay\tIRF\tChiSquare\tTau1\tTau2\tTau3\tTau4\t
Fraction\tFraction\tFraction\tFraction\tDark\tFitStart\tFitStop\tTimeShift\tMean\nTau1\tTau2\tMeanRes\tSigmaRes\tAmp1\tAmp2\tAmp3\tAmp4\tSA1\tSA2\tSA3\tSA4\t
ST1\tST2\tST3\tST4\tSkewness\tSkewness\tKurtosis\tKurtosis\tDurbinWatson", -1);

    while (!ReadLine (fileHandle, buffer, LINE_SIZE + 1)) { 
        // Look for end of file
        ReadLine (fileHandle, buffer, LINE_SIZE + 1);
        /* Get the decay and irf file names from the first line */
        j = 0; // current position in buffer
        while (!isspace(buffer[i])) {
            i++;
        }
        i -= 5;
        while (isalnum(buffer[j])) {
            j++;
            i--;
        }
        i++; // set index to first character in file name
        for (k = 0; k < j; k++) {
            parameter.decayFileName[k] = buffer[i];
            i++;
        }
        i += 13; // advance to irf filename
        k = 0;
        while (isalnum(buffer[i])) {
            parameter.irfFileName[k] = buffer[i];
            i++, k++;
        }
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%15c%1[u]%3c%1[u]", &parameter.firstChan,
&parameter.lastChan);
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%10c%1[x]", &parameter.chiSquare);
ReadLine (fileHandle, buffer, (bufferSize - 1));
i = 0;
while (buffer[1] != 68 && buffer[0] != 84) { // Check for "D" in Darks or "T" in Tshift
    Scan (buffer, "%7c%1[x]%4c%1[x]%8c%1[x]", &parameter.tau[i],
        &parameter.sigmaTau[i], &parameter.fraction[i]);
    ReadLine (fileHandle, buffer, (bufferSize - 1));
    Scan (buffer, "%7c%1[x]%4c%1[x]", &parameter.amp[i],
        &parameter.sigmaAmp[i]);
i++;
    ReadLine (fileHandle, buffer, (bufferSize - 1));
} if (buffer[1] == 68) {
    Scan (buffer, "%7c%1[x]", &parameter.darkCounts);
} else {
    Scan (buffer, "%7c%1[x]%23c%1[x]", &parameter.timeShift,
        &parameter.darkCounts);
}
ReadLine (fileHandle, buffer, (bufferSize - 1));
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%7c%1[x]%6c%1[x]", &parameter.meanTau,
&parameter.averagelTau);
ReadLine (fileHandle, buffer, (bufferSize - 1));
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%14c%1[x]", &parameter.meanResidual);
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%15c%1[x]", &parameter.sigmaResidual);
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%16c%1[x]%13c%1[x]", &parameter.skewness,
    &parameter.skewnessThreshold);
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%16c%1[x]%13c%1[x]", &parameter.kurtosis,
&parameter.kurtosisThreshold);
ReadLine (fileHandle, buffer, (bufferSize - 1));
Scan (buffer, "%21c%1[x]", &parameter.durbinWatson);
Sort (parameter.fraction, MAX_EXPONENTIALS, 1, sortedFractions);
i = 0;
j = 0;
for (i = 0; i < MAX_EXPONENTIALS; i++) {
    while (sortedFractions[i] != parameter.fraction[j]) {
        j++;
    }
    sortedIndices[i] = j;
    j = 0;
}
FmtFile (outputFileHandle, "%s\t%s\t%f\t",
    parameter.decayFileName, parameter.irfFileName,
    parameter.chiSquare);
for (i = 0; i < MAX_EXPONENTIALS; i++) {
    index = sortedIndices[i];
    FmtFile (outputFileHandle, "%f\t", parameter.tau[index]);
}
for (i = 0; i < MAX_EXPONENTIALS; i++) {
    index = sortedIndices[i];
    FmtFile (outputFileHandle, "%f\t", parameter.fraction[index]);
}
FmtFile (outputFileHandle, "%f\t%i\t%i\t%f\t",

460
parameter.darkCounts, parameter.firstChan, parameter.lastChan,  
parameter.timeShift);  
FmtFile (outputFileHandle, "%ftft", parameter.meanTau, parameter.averageTau);  
FmtFile (outputFileHandle, "%ftft", parameter.meanResidual,  
parameter.sigmaResidual);  
for (i = 0; i < MAX_EXPONENTIALS; i++) {  
    index = sortedIndices[i];  
    FmtFile (outputFileHandle, "%ft", parameter.amp[index]);  
}  
for (i = 0; i < MAX_EXPONENTIALS; i++) {  
    index = sortedIndices[i];  
    FmtFile (outputFileHandle, "%ft", parameter.sigmaAmp[index]);  
}  
for (i = 0; i < MAX_EXPONENTIALS; i++) {  
    index = sortedIndices[i];  
    FmtFile (outputFileHandle, "%ft", parameter.sigmaTau[index]);  
}  
FmtFile (outputFileHandle, "%ftftftftftftft",  
parameter.skewness, parameter.skewnessThreshold,  
parameter.kurtosis,  
parameter.kurtosisThreshold, parameter.durbinWatson);  
/* Reset all parameter values */  
for (i = 0; i < MAX_EXPONENTIALS; i++) {  
    parameter.tau[i] = 0;  
    parameter.amp[i] = 0;  
    parameter.amp[i] = 0;  
    parameter.sigmaAmp[i] = 0;  
    parameter.sigmaAmp[i] = 0;  
}  
parameter.firstChan = 0;  
parameter.lastChan = 0;  
parameter.darkCounts = 0;  
parameter.timeShift = 0;  
parameter.chiSquare = 0;  
parameter.meanTau = 0;  
parameter.averageTau = 0;  
parameter.meanResidual = 0;  
parameter.sigmaResidual = 0;  
parameter.skewness = 0;  
parameter.skewnessThreshold = 0;  
parameter.kurtosis = 0;  
parameter.kurtosisThreshold = 0;  
parameter.durbinWatson = 0;  
FillBytes (parameter.decayFileName, 0, PATH_SIZE + 1, 0);  
FillBytes (parameter.irffFileName, 0, PATH_SIZE + 1, 0);  
}  
fileCloseErr = CloseFile (outputFileHandle);  
return 0;  
}  
/* FUNCTION: AnalyzeFiles  
* Loop through the parameter files in the pending list, read their paths from the list,  
* and tabulate the relevant information in an easy-to-read format.  
*/  
int AnalyzeFiles (int panel, int control, int event,  
                  void *callbackData, int eventData1, int eventData2)  
{  
    switch (event) {  
    static int getFileErr, checkErr;  
    static int pendingErr;  
    static int properFileType;
static int extensionIndex;
        static int goAhead;
        static int fileIndex;
        
case EVENT_COMMIT:
            for (fileIndex = 0; fileIndex < numberOfFiles; fileIndex++) {
                err = GetValueFromIndex (panelHandle,
                PANEL_LISTBOX_PENDING_FILES,
                fileIndex,
                fileName);
                checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
                fileIndex, 1);
                extensionIndex = FindPattern (fileName, 0, ".", 0, 0);
                properFileType = CompareStrings (fileName, extensionIndex,
                PARAMETER_EXTENSION, 0, 0);
                if (properFileType == 0) {
                    AnalyzeOneFile (fileName);
                } else {
                    goAhead = ConfirmPopup ("Possible File Type Mismatch",
                    "This file does not have the expected extension. Continue anyway?");
                    if (goAhead == 1) AnalyzeOneFile (fileName);
                }
                checkErr = CheckListItem (panelHandle, PANEL_LISTBOX_PENDING_FILES,
                fileIndex, 0);
            }
            numberOfFiles = 0;
            break;
        }

int Quit (int panel, int control, int event,
        void *callbackData, int eventData1, int eventData2)
        {
        switch (event) {
        case EVENT_COMMIT:
            QuitUserInterface (0);
            break;
        }
        return 0;
        }

void main ()
        {
        panelHandle = LoadPanel (0, "db06401,uir", PANEL);
        DisplayPanel (panelHandle);
        RunUserInterface ();
        }
DB06401.H

#include <userint.h>

#ifdef __plusplus
    extern "C" {
#endif

    /* Panels and Controls: */

    #define PANEL 1
    #define PANEL_LISTBOX_PENDING/files 2
    #define PANEL_BUTTON_ANALYZE_FILES 3  /* callback function: AnalyzeFiles */
    #define PANEL_BUTTON_SELECT_FILES 4  /* callback function: SelectFiles */
    #define PANEL_BUTTON_QUIT 5  /* callback function: Quit */
    #define PANEL_TEXTMSG 6
    #define PANEL_TEXTMSG_2 7
    #define PANEL_TEXTMSG_3 8
    #define PANEL_TEXTMSG_4 9

    /* Menu Bars, Menus, and Menu Items: */

    /* (no menu bars in the resource file) */

    /* Callback Prototypes: */

    int CVICALLBACK AnalyzeFiles(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
    int CVICALLBACK Quit(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);
    int CVICALLBACK SelectFiles(int panel, int control, int event, void *callbackData, int eventData1, int eventData2);

#ifdef __cplusplus
    
#endif

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APPENDIX D

CALCULATED ENERGIES, GEOMETRIES, AND VIBRATIONAL FREQUENCIES FOR RELEVANT ELECTRONIC STATES OF ADAMANTYLDIAZIRINE

Adamantyldiazirine, C_{18}N_6 (1A') (PM3)
Energy at Zero Kelvin | -63.33626156 Hartree/Particle
Zero-Point Vibrational Energy | 0.228444 Hartree/Particle

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Frequencies: (cm\(^{-1}\))

| 126.8302 | 264.5559 | 291.5308 |
| 353.0343 | 354.7013 | 372.6817 |
| 412.4820 | 449.1990 | 454.2531 |
| 456.0089 | 553.7288 | 608.1565 |
| 678.9416 | 695.4029 | 777.2398 |
| 884.7326 | 889.7268 | 891.2421 |
| 913.0686 | 915.4324 | 928.0250 |
| 950.7799 | 958.7572 | 965.5683 |
| 970.1076 | 981.3264 | 1002.1240 |
| 1042.1346 | 1051.1185 | 1054.4219 |
| 1090.2535 | 1100.1015 | 1105.3437 |
| 1122.1300 | 1130.1195 | 1140.6493 |
| 1142.0197 | 1214.6424 | 1218.2854 |
| 1239.3718 | 1244.7704 | 1296.3505 |
| 1296.4252 | 1305.0808 | 1318.2071 |
| 1323.8764 | 1324.9972 | 1328.7390 |
| 1351.9096 | 1354.5692 | 1355.9445 |
| 1392.7342 | 1403.0069 | 1407.6319 |
| 1407.8881 | 1408.6348 | 1419.3326 |
| 2013.9577 | 2905.9880 | 2906.9569 |
| 2909.6055 | 2911.1871 | 2968.0803 |
| 2969.2250 | 2970.1945 | 2970.3621 |
| 2970.9976 | 3041.0593 | 3042.5508 |
| 3042.6716 | 3043.0258 | 3043.3739 |
### Adamantylidiazirine, C_{24} N_{8} S_{4} (1A_{u}) (B3LYP/6-31G*)

**Energy at Zero Kelvin**

\[ -498.942515716 \text{ Hartree/Particle} \]

**Zero-Point Vibrational Energy**

\[ 0.230163 \text{ Hartree/Particle} \]

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**Frequencies: (cm⁻¹)**

| 103.3020 | 258.2411 | 281.8915 |
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| 398.7984 | 440.0529 | 446.0210 |
| 474.0063 | 533.7522 | 567.9350 |
| 651.4623 | 677.8879 | 708.3273 |
| 779.4526 | 803.8625 | 841.6095 |
| 879.6070 | 879.8527 | 898.0217 |
| 907.8879 | 918.9477 | 958.1613 |
| 970.7894 | 1010.5373 | 1043.3527 |
| 1043.6257 | 1074.1327 | 1089.5107 |
| 1101.0985 | 1117.8408 | 1133.2411 |
| 1141.2387 | 1142.6451 | 1173.5699 |
| 1192.1127 | 1261.7881 | 1263.5392 |
| 1286.9191 | 1309.9048 | 1332.3853 |
| 1339.6636 | 1354.8787 | 1363.6925 |
| 1364.3657 | 1367.0037 | 1390.2886 |
| 1401.2475 | 1406.2180 | 1408.9053 |
| 1421.0005 | 1505.6669 | 1516.2454 |
| 1519.6874 | 1520.1650 | 1541.7446 |
| 1705.2169 | 3024.7106 | 3031.8646 |
| 1705.4133 | 3033.2721 | 3034.8884 |
| 3049.5565 | 3053.5087 | 3062.0152 |
| 3064.2766 | 3067.3249 | 3071.2676 |
Adamantylidiazine, C_{22}N_2 (1A_1) (RHF/6-31G*)

Energy at Zero Kelvin = -495.680681431 Hartree/Particle
Zero-Point Vibrational Energy = 0.247079 Hartree/Particle

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Energy at Zero Kelvin = -497.3118684 Hartree/Particle

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468
No Vibrational Analysis

Adamantrylidiazirine, C\textsubscript{7}N\textsubscript{2} S\textsubscript{3} (1B\textsubscript{1}) [Transition State] (CIS/6-31G*)

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Zero-Point Vibrational Energy = 0.243925 Hartree/Particle

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469
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Zero-Point Vibrational Energy = 0.241522 Hartree/Particle

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\end{align*}
\]
Adamantylidiazirine, C₈S₂(1A*) and S₂(2A*) (CIS/6-31G*)

Energy at Zero Kelvin = -495.652661125 Hartree/Particle
Zero-Point Vibrational Energy = 0.244934 Hartree/Particle

Center Atomic Coordinates (Angstroms)
Number Number X      Y      Z

Frequencies: (cm⁻¹)

104.4247    226.6060   289.4801
333.2442    337.2367   390.4686
424.7661    448.8568   455.9315
472.1790    475.2534   523.5056
638.9283    664.6337   765.6381
771.9100    831.9151  858.7924
859.9642    938.1738  975.5322
975.6903    978.2636 1029.3707
1036.4827   1083.3140 1120.6829
1124.6704   1145.2611 1158.9324
1187.8447   1210.5087 1219.0547
1223.8683   1240.3377 1277.3052
1279.6659   1362.6937 1385.9798
1397.0414   1413.3172 1454.0121
1467.3313   1477.7909 1481.4280
1485.5750   1486.8553 1512.9297
1518.7224   1526.5295 1529.4459
1544.2339   1631.9058 1638.4645
1646.3807   1647.5868 1666.6412
1671.5394   1794.5296 1805.4112
1720.3416   1737.8232 1780.2943
1727.5297   1729.9168 1732.0190
1724.9878   1724.1461 1725.3052
1725.7823   1728.4792 1734.6295
APPENDIX E

THE PREPARATION OF THYLAKOID, BBY, AND PS II REACTION CENTER PARTICLES FROM CHLAMYDOMONAS REINHARDTII

Thylakoids

Buffer 1: 0.35 M Sorbitol, (2 L/8 L culture)
20 mM HEPES, pH 7.5
2.0 mM MgCl₂

All procedures at 4 °C in the dark. You will need 50-100 mg of chlorophyll in cells (8 L, 4 day culture in TAP) to make a large enough prep for reaction center particles.

1. Pellet cells at 3,500 × G for 10 min at 4 °C or concentrate by filtration through the Pelicon device using the 0.11 μm filter.
2. Wash cells with 1/5 volume of Buffer 1 and repellet as above.
3. Resuspend cells in Buffer 1 to give 1.0 mg Chl/mL
4. Rupture cells using one of the following devices. The bionebulizer works best.
   B. Yeda Press Procedure: Rupture at 1,000 psi (for cell wall-less use 80 psi).
   C. Beadbeater Procedure. For the Beadbeater fill the 50 ml chamber half full with 0.5 mm zirconium beads. Add cells to fill chamber to the top. There should be no air. Fill collar with ice water mix. Blend at 20 sec intervals with a 30 sec cool down interval. Repeat 5 times for a total of two minutes blending. Filter through miracloth to remove beads and wash beads twice with 50 ml of Buffer 1.
   D. Bionebulizer Procedure: Assemble the Bionebulizer carefully so as not to crush the ceramic sphere against the orifice. Set the nitrogen pressure at 110 psi and adjust the flow rate to maximum. Place a small beaker of water at the inlet hose and adjust the dial on the top until no bubbles are forced through the water and/or no water is taken up through the tube. After adjustment turn the dial 6 notches counterclockwise so as to pull solution through the tube. Place a beaker under the outlet hose to collect broken cells. Suction cells through the device and collect Repeat once for a total of two passes through the nebulizer.
5. Pellet thylakoids at 40,000 × G (18,500 rpm in SS-34) for 20 min at 4 °C. Be sure to precool rotor and centrifuge to 4 °C.
6. Resuspend thylakoids in equal or greater volume of Buffer 1, homogenize and spin at 1,200xG for 30 sec in SS-34 (3,200 rpm) to pellet unbroken cells. Remove supernatant to new centrifuge tube and pellet thylakoids at 11,000xG for 12 min. (10,000 rpm in SS-34). Store at ≤ (-)80 °C at ≥ 1.0 mg Chl/ml.

Assay PS-II oxygen evolution: 0.3 M sorbitol, 20 mM HEPES, pH 7.5, 2 mM MgCl₂, 30 mM methylamine HCL, 2 mM KFeCN, 0.2 mM DMBQ (make 20 mM DMBQ stock in DMSO, to add last)
DENSITY GRADIENT THYLAKOIDS
(optional)

Step Gradient Thylakoid Buffers:

0.3 M sucrose (100 mls)
5.0 mM HEPES, pH 7.5
10 mM EDTA

Step Gradient Buffers: 1.8 M, 1.3 M, and 0.5 M sucrose in 5.0 mM HEPES, pH 7.5 (50 mls, each).

All procedures at 4°C in dark.
1) Resuspend thylakoids in 30 mls of 0.3 M sucrose, 5 mM HEPES pH 7.5 and 10 mM EDTA and pellet at 100,000xG for 15 min (36,000 rpm, Ti70) at 4°C.
2) Homogenize pellet in 40.0 mls of 1.8 M sucrose, 5 mM HEPES pH 7.5 and layer on bottom of 4 SW-28 tubes. Overlay with: 1) 20.0 mls of 1.3 M sucrose, 5 mM HEPES pH 7.5 and approx. 6.0 mls of 0.5 M sucrose, 5.0 mM HEPES pH 7.5. Spin gradients at 24,000 rpm (100,000xG) for 1 hour at 4°C.
3) Thylakoids band at 1.3 M/0.5 M sucrose interface. If thylakoids are the last step dilute with three volumes of 20 mM HEPES pH 7.5 and pellet at 100,000xG for 15 min otherwise dilute with Buffer 3 for BBY particles and pellet at appropriate G force. Store thylakoids in Buffer 2 at ≥ 1.0 mg Chl/ml.
BBY-TYPE PSII PARTICLES

Buffers:

3) 20 mM MES, pH 6.0 (200 mls)  T) 25% (w/v) Triton X-100 (25 mls)
15 mM NaCl
5.0 mM MgCl₂
5.0 mM EDTA
20 mM MES, pH 6.0
20 mM MES, pH 6.0
15 mM NaCl
5.0 mM MgCl₂
(Takes 30 min to dissolve Triton.)

4) 0.4 M sucrose (50 mls)  5) 2.0 M sucrose
15 mM NaCl
5.0 mM MgCl₂
20 mM MES, pH 6.0
20 mM MES, pH 6.0
5.0 mM MgCl₂
15 mM NaCl

All procedures and buffers at 4 °C in dark. Be sure and pre-cool rotors and centrifuges.

1. Remove starch from the thylakoids. Place 2.0 mls of Buffer 5 in the bottom of an SW-55 rotor tube and overlay to the top with the thylakoid prep. Spin at 100,000xG for 30 min at 4 °C. Remove supernatant and pellet at 11,000xG for 12 min. (10,000 rpm in SS-34).
2. Resuspend pellet in a large volume of Buffer 3 and homogenize. Pellet at 12,500xG for 5 min. (10,500 rpm in SS-34).
3. Resuspend pellet in Buffer 3 to give a Chl concentration ≥ 2.86 mg/ml. Add Buffer T dropwise (in dark cold room) to a beaker containing thylakoids with slow stir to give a Triton:Chl ratio of 25:1 and a final Chl concentration of 2.0 mg/ml. Stir in dark for 30 min at 4 °C.
4. Pellet PSII particles at 40,000xG at 4 °C (18,500 rpm in SS-34) for 20 min. Pour off supernatant and homogenize particle in equal volume of Buffer 3 and repellet as above. This removes excess Triton. Repeat the wash once more with Buffer 3.
5. Resuspend and homogenize BBY particles in Buffer 4 to give Chl concentration ≥ 1.0 mg/ml. Freeze in liquid N₂ and store at (-) 80 °C. From here go on to RC particle isolation.
6. Assay in Buffer 4 plus 1.0 mM potassium ferricyanide and 0.2 mM p-phenylbenzoquinone. Make 20 mM PBQ in DMSO and add last to reaction mixture.
7. BBY particles can be stored at > 1.0 mg Chl/ml in liquid nitrogen.
Reaction Center Particles

<table>
<thead>
<tr>
<th>Wash Buffer:</th>
<th>50 mM Tris-HCl, pH 7.2</th>
<th>Triton Buffer:</th>
<th>25% (w/v) Triton X-100</th>
</tr>
</thead>
<tbody>
<tr>
<td>(100 ml)</td>
<td></td>
<td>(40 ml)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Column Buffer:</th>
<th>25 mM Tris-HCl, pH 7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3 L)</td>
<td>0.2% Triton X-100</td>
</tr>
<tr>
<td></td>
<td>50 mM NaCl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BTS-200:</th>
<th>0.2 M sucrose</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.5 L)</td>
<td></td>
</tr>
</tbody>
</table>

| 20 mM BisTris, pH 6.5 |
| 20 mM MgCl₂         |
| 10 mM MgSO₄         |
| 5.0 mM CaCl₂        |
| 0.03 % (w/v) dodecyl maltoside |

All procedures at 4 °C and in dark.

1. Pellet BBY particles at 12,500xG (10,000 rpm in SS-34) for 10 min at 4 °C. Resuspend and homogenize with a minimal volume of Wash Buffer so that Chl concentration is ≥ 1.6 mg/ml.

2. Solubilize BBY particles at 1.0 mg Chl/ml with Triton Buffer (added dropwise) to give a final Triton concentration of 8% (w/v), equals Triton:Chl ratio of 80:1. Solubilize at 4 °C in the dark room with slow stirring for 1 hour.

3. Centrifuge at 100,000xG (32,000 rpm in Ti70) for 1 hour at 4 °C. Be sure to pre-cool Ti70 rotor and centrifuge.

4. Apply supernatant to a Q-Sepharose column (2.5 x 8 cm) equilibrated with Column Buffer. Wash with Column Buffer thoroughly until no more green color is eluted from column (Absorbance at 670 nm = 0.04). This will require at least 1.5 L of Column Buffer run at 2.0 ml/min for 12-24 hours. This must be done in complete darkness in cold room at 4 °C. Failure to remove all the color will result in RC particles contaminated with CP43, CP47 and LHC.

5. Wash column with ≥ 5 column volumes of BTS-200. Elute RC particles by application of a 200 ml gradient of 0-200 (10-210) mM MgSO₄ in BTS-200 (BTS-200 has 10 mM MgSO₄). Collect 3.0 ml fractions. The RC particles are extremely light sensitive and must be at 4 °C at all times. RC particle fractions are identified spectrophotometrically by the A₄15/434 peak ratio. Fractions enriched in the 415 nm peak (A₄15/434 ratio ≥ 1.15) are good RC particles. Pool fractions and concentrate by filtration in an Amicon device with a <100 kD cutoff filter under 70 psi nitrogen in dark at 4 °C. Exchange buffer to BTS-200 without MgSO₄. Store reaction centers at -80 °C or in liquid nitrogen in dark.

6. Light induced absorbance changes measured at 4 °C in 60 mM Tris-HCl, pH 8.5, 20% (v/v) glycerol at 4.8 uM Chl in the presence of 1.0 uM methyl viologen and 1-5 mg/ml dithionite.

7. Pigment composition can be determined using the following equations:

- Chla (uM) = (-1.709 A₄₂₀ + 11.979 A₋₂.₉₉₈ A - 5.708 A) X 0.893
- Chlb (uM) = (-0.171 A₄₃₃ - 0.23 A - 11.871 A + 13.248 A) X 0.907
- Car (uM) = (-0.43 A₄₅₈ - 0.251 A₄₃₈ - 4.376 A₄₄₂ - 13.216 A) X 0.957
- Pheo (uM) = (10.28 A - 8.38 A + 2.456 A - 1.233 A) X 0.893

BIBLIOGRAPHY


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