AN INVESTIGATION INTO THE ANALYTICAL UTILITY OF CHANGES IN POLARIZATION ACCOMPANYING ANALYTICAL DERIVATIZATION REACTIONS (FLUORESCENCE)

CHRISTOPHER P. HALLEN
University of New Hampshire, Durham

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AN INVESTIGATION INTO THE ANALYTICAL UTILITY
OF CHANGES IN POLARIZATION ACCOMPANYING
ANALYTICAL DERIVATIZATION REACTIONS

BY

Christopher P. Hallen
A.B., Assumption College, 1980

A DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Doctor of Philosophy
in
Chemistry

December, 1986
This dissertation has been examined and approved.

W. Rudolf Seitz
Dissertation director, W. Rudolf Seitz
Professor of Chemistry

Christopher F. Bauer
Assistant Professor of Chemistry

Miyoshi Ikawa
Professor of Biochemistry

Paul R. Jones
Professor of Chemistry

James H. Weber
Professor of Chemistry

11/20/86
Date
This Dissertation is Dedicated to my Parents

P. Edward and Blanche E. Hallen

Whose Love and Support Made This All Possible
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ABSTRACT

AN INVESTIGATION INTO THE ANALYTICAL UTILITY
OF CHANGES IN POLARIZATION ACCOMPANYING
ANALYTICAL DERIVATIZATION REACTIONS

by

Christopher P. Hallen

University of New Hampshire, December, 1986

Derivatization reactions between amines, alcohols and carbonyl groups with various reagents were studied. The goal of the work discussed in this dissertation was to generate basic data to know whether and in what context changes in fluorescence polarization are useful for following analytical derivatization reactions.

Reactions between primary amines and fluorescein isothiocyanate were initially studied. Changes in polarization were observed upon conjugation for many of the amines primarily due to changes in fluorescence lifetime. This reaction was also studied in varying amounts of glycerol to judge the effect of glycerol on the reaction rate. Optimum percentages of glycerol were determined. Rate data of reactions of fluorescein isothiocyanate with aniline and p-chloroaniline are reported.

Reactions between amines and alcohols with dichlorotriazinyl fluorescein were studied next. Minimal changes in polarization were observed upon conjugation because the
lifetimes were very similar to that of the reagent. Glycerol was a problem as it reacts rapidly with the reagent. In a set of kinetics experiments with polyethyleneimine, changes in polarization were observed but could not be distinguished from randomness brought about by the experimental conditions.

Dansyl chloride was more difficult to work with than either of the fluorescein compounds due to its sensitivity to environment. This was illustrated by dramatic changes in lifetime as the amount of glycerol in the solution was varied for Perrin plots.

The final study involved dansyl hydrazine and reactions with carbonyls. Polarizations again changed upon conjugation due to lifetime changes. Spectral shifts were also noted upon conjugation to two carbonyl compounds. Rate data for reactions with anisaldehyde are reported. A different TLC solvent system is suggested for separation of conjugate and free dansyl hydrazine.
CHAPTER I

FLUORESCENCE POLARIZATION AND DERIVATIZATION REACTIONS

Introduction

Because of the high sensitivity of fluorescence methods of analysis, reactions have been developed to derivatize nonfluorescent analytes to make them more detectable. Ideally, these reactions should be fast, quantitative and yield a product with significantly different fluorescence characteristics than the original reagent (1). There are few reactions that meet the latter stipulation, i.e., the fluorescence of the product is usually similar to that of the reagent. This means the derivatization reactions must be followed by an often lengthy separation step to purify the fluorophor-analyte conjugate before the final analysis.

Under certain conditions, the technique of fluorescence polarization should be able to determine the fluorophor-labelled analyte without a separation step. Fluorescence is depolarized by molecular rotation during the lifetime of the excited state. Since the product of a derivatization reaction is larger than the reagent, it will rotate less rapidly in solution and therefore not depolarize fluorescence to the same extent as the free fluorophor. In addition, the fluorescence lifetime of the fluorophor-
labelled analyte may differ from that of the free fluorophor. A change in lifetime will also change the polarization by allowing more or less time for depolarization to occur. Any change in polarization because of lifetime or slower rotation should allow the analyte to be measured without a separation step (2).

**Derivatization Reactions**

Fluorescence is a more sensitive method of chromatographic detection than ultraviolet absorption. However, it is limited in scope because few molecules have intrinsic fluorescence. Fluorescence derivatization reactions address this limitation by converting non- or weakly luminescent analyte molecules into highly fluorescent products (Figure 1-1). The reaction modifies the fluorophor in several ways. The derivative is larger and less mobile in solution. Since fluorescence is a molecular property, conjugation to an analyte may cause changes in luminescence excitation and emission spectra or in the excited state fluorescence lifetime.

Generally desirable properties for luminescence derivatization include 1) a fast, quantitative reaction that is simple to perform and specific for the analyte of interest; 2) ability to distinguish product fluorescence from fluorescence from side reactions or excess reagent, or,
Figure 1-1: *Fluorescence Derivatization Reaction Between a Fluorophor (F) and an Analyte (A) to Form a Fluorescent Conjugate.*
if this is not possible, to use a simple separation step to remove unreacted fluorophor; 3) high fluorescence efficiency of product for maximum sensitivity; 4) excitation and emission wavelengths greater than 330 nm so that less expensive glass optics can be used; 5) a derivative that is stable and does not undergo rapid decomposition. Many derivatization reactions allow for most of these requirements (1,3-9).

Fluorescent derivatives are useful for detection of species separated by thin layer chromatography (TLC) where the reaction can be carried out before or after the separation. In this case, the speed of the reaction is not crucial. In addition, reagent fluorescence is not a critical parameter as long as the conjugate is separated from the free reagent during the chromatographic steps. Post-separation derivatization requires that reagents not fluoresce but that conjugates do so they can be detected. A complete discussion of TLC with many examples is found in Stahl's book (10).

Fluorescence detectors are gaining in popularity as indicated by the increasing number of papers in the literature (11). To be useful, any excess fluorescent reagent must not be detectable under the same conditions as the labelled analyte.

Fluorescence detection is also widely used with liquid chromatography. As with TLC, the derivatization reaction can be performed before or after separation. Derivative
formation prior to separation can complicate the separation if the solution has multiple components. Each component could be bound to reagent therefore becoming more similar hindering the separation. The separation can get more complex if the reagent can bind at more than one site per analyte. An advantage of precolumn derivatization is that more time is available for reactions to occur. This is desirable as long as the derivatives can be adequately separated. Post column derivatization reactions are performed by introducing the necessary reagents to the column effluent in a specially designed reaction detector. Some loss of chromatographic resolution results due to the increased volume of the cell and mixing as the reagents are added. Advantages of post column reaction are that artifact formation is usually not a problem and that the derivatization reaction does not have to go to completion or be well-defined, provided it is reproducible. However, post column derivatization reactions must be fast to minimize the extent of band-broadening prior to detection. The actual time available varies from under thirty seconds to twenty minutes depending on the design of post column reactor (7,9).

The ideal eluent for a separation is not necessarily the ideal solvent system for running the derivatization reaction. To overcome this problem, post-column reactors have been designed to allow for phase separation where immiscible solvents are needed.
Fluorescence polarization could be used as a detection method for liquid chromatography where excess reagent is present as long as a polarization change exhibited upon conjugation is large enough to distinguish the reagent from the conjugate.

Other uses of derivatization reactions include analysis of single analytes without separation as long as the polarization changes upon binding. It is possible to estimate the ratio of bound:free fluorophor from measured polarizations using

\[
\frac{F_b}{F_f} = \frac{Q_f}{Q_b} \cdot \frac{P - P_f}{P_b - P}
\]

where the ratio of bound to free fluorophor \((F_b/F_f)\) is related to the quantum efficiency ratio \((Q_f/Q_b)\) and the polarizations of the free fluorophor \((P_f)\), bound fluorophor \((P_b)\) and the observed polarization of the reaction mixture \((P)\). Examples of single analyte detection include protein and enzyme assays as well as immunochemical detection (7,9).

Fluorescence Polarization: Background Information

Fluorescence polarization (12-13) is based upon theories put forth by Perrin (14-15) and Valvila and Levschin (16) in the 1920's. It was not until 1952, however, that applications of fluorescence polarization to biochemical problems were developed by Gregorio Weber (17-18). Weber's theory is summarized in a book edited by
Hercules (19).

A molecule will absorb radiation if it has a transition moment coinciding with a component of the excitation light electric vector. When plane-polarized light of the proper frequency is used, fluorophor molecules oriented with the plane of polarization of the excitation radiation will be most efficiently excited. If the transition moment of the fluorophor is not parallel to the electric vector, the probability of absorption is lessened. The probability of absorption is related to the degree of overlap between vectors of the molecule and excitation radiation. When the excitation vector is vertical, the probability of excitation goes down as the fluorophor's transition moment moves by some angle \( \theta \) relative to vertical (Figure 1-2). The probability of absorption is given by:

\[
\varepsilon = \varepsilon_\parallel \cos^2 \theta
\]  

(1-2)

where \( \varepsilon \) is the observed molar absorptivity and \( \varepsilon_\parallel \) is the absorptivity of a species perfectly aligned with the excitation vector. The larger the angle between vectors, the lower the probability of excitation, ultimately reaching a limit where \( \theta = 90^\circ \) and no light is absorbed.

Emitted light will also tend to be polarized in the plane of the transition moment. Again using Figure 1-2, \( I_v \), the intensity emitted parallel to the plane of the excitation radiation is

\[
I_v \propto \cos^2 \theta
\]  

(1-3)

where \( \theta \) would now be the angle of analyzer or emission
Figure 1-2: Angle $\theta$ Between Excitation Beam Vector (→) and the Fluorophor's Transition Moment (→→→)
polarizer relative to the excitation/emission vector. \( I_h \), the intensity emitted perpendicular to the plane of incident radiation, is

\[
I_h \propto \sin^2 \Theta \sin^2 \phi
\]  

(1-4)

where \( \phi \) is the azimuthal angle (Figure 1-3).

Experimentally, a fluorophor sample is excited with plane polarized light. Emission is observed at right angles to the excitation beam and resolved by an analyzer into intensities of light polarized parallel and perpendicular with respect to the excitation radiation. The degree of polarization \( (P) \) is calculated from these intensities:

\[
P = \frac{I_v - I_h}{I_v + I_h}
\]

(1-5)

Polarization is simply the difference in intensities normalized to the total intensity. The intrinsic polarization, \( P_0 \), is the maximum value for any fluorophor. It reflects the orientation of the transition moment for the excitation process relative to the transition moment for emission in the absence of depolarizing processes.

The depolarization of fluorescence causes the value of \( P \) to be less than expected. Depolarization in solution occurs as the fluorophor rotates during the excited state lifetime. In low viscosity solvents this happens at any concentration. Furthermore, excited state energy migration or energy transfer, which occurs at high fluorophor concentrations, also depolarizes fluorescence. Energy transfer is not a problem for this research work due to the
Figure 1-3: Effect of Fluorophor Rotation in Sample Chamber of Fluorometer.

S - source
P - excitation polarizer
A - absorption moment of fluorophor
θ - angle between excitation vector and absorption moment of fluorophor
ϕ - angle between emission vector of fluorophor and direction of emission beam.
P_a - analyzer oriented vertically (I_v)
P_h - analyzer oriented horizontally (I_h)
D - detector
low concentrations used. Experimental conditions and the resulting effect of polarizations are summarized in Table 1-1 (13,19). The amount of rotational depolarization of the fluorophor in solution is dependent upon several factors including solution temperature and viscosity, fluorescence lifetime, molecular size and shape. Perrin's theory derives an equation to relate these variables:

\[
\left( \frac{1}{P} - \frac{1}{3} \right) = \left( \frac{1}{P_0} - \frac{1}{3} \right) \cdot \left( 1 + \frac{3\tau}{\rho} \right) \tag{1-6}
\]

where \( P \) is the observed polarization, \( P_0 \) is the intrinsic polarization, \( \rho \) is the rotational relaxation time and \( \tau \) is the excited state fluorescence lifetime. Where \( 3\frac{\tau}{\rho} \) approaches 0, \( P \) approaches \( P_0 \). The larger this term becomes, the smaller the observed polarization.

For a spherical molecule, \( \rho \) is related to the molar volume and to viscosity by

\[
\rho = \frac{3V\eta}{RT} \tag{1-7}
\]

where \( R \) is the gas constant, \( T \) is absolute temperature, \( \eta \) is the solution viscosity and \( V \) is the molecular volume of the spherical fluorophor. Combining the last two equations yields

\[
\left( \frac{1}{P} - \frac{1}{3} \right) = \left( \frac{1}{P_0} - \frac{1}{3} \right) \left( 1 + \frac{RT\tau}{\eta V} \right) \tag{1-8}
\]

To obtain values for these numbers, a Perrin Plot can be
<table>
<thead>
<tr>
<th>Condition</th>
<th>Observed Polarization</th>
<th>Molecular Properties Responsible</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Dilute solution in very viscous solvents</td>
<td>Maximum possible $</td>
<td>P_o</td>
</tr>
<tr>
<td>2. Concentrated solutions in very viscous solutions</td>
<td>$0 &lt;</td>
<td>P</td>
</tr>
<tr>
<td>3. Dilute solutions in solvents of low viscosity</td>
<td>$0 &lt;</td>
<td>P</td>
</tr>
</tbody>
</table>
drawn from equation 1-8. The axes of a Perrin plot are \( T\tau/\eta \) vs \( 1/P \). The intercept is \( 1/P_0 \) and the slope is \( R/V \). If the molecule is nonspherical, the Perrin plot will be curved. This is caused by different rotation rates around different axes of the molecule.

Derivatization reactions can influence polarization in two ways. It has been demonstrated (17,20) that when a fluorophor molecule binds to another species of sufficient size, the unit as a whole will rotate more slowly in solution than the lone fluorophor due to the increase in molecular volume. In addition, the fluorophor-analyte conjugate will often have a different excited state fluorescence lifetime than the lone fluorophor due to changes in the electronic environment. As can be seen in equation 1-8, both volume and lifetime can affect the observed polarization but may work in opposite directions. The net result is that the conjugate may not depolarize fluorescence to the same extent as the free fluorophor. An important and easily controlled experimental parameter is the viscosity of the solution. In nonviscous media, unless the analyte molecule is exceptionally large, brownian motion will cause almost complete depolarization of the conjugate. In order to have a measurable polarization, one needs to use a medium where only partial rotational depolarization takes place. The viscosity becomes a very important parameter when choosing a reaction medium.

If a derivatization reaction involves a change in
polarization, then it is possible to determine the extent of the reaction by measuring polarization. There are several advantages to using fluorescence polarization to follow a reaction. Since a separation step is not needed, error due to perturbing an equilibrium is eliminated. The sensitivity of fluorescence allows measurements over a large range of concentrations. Of course, the disadvantage of fluorescence polarization is that a net change in polarization must occur upon reaction for the method to be of any use. The reaction must also yield a fluorophor-analyte conjugate. Another disadvantage is that polarization will have a lower detection limit than normal fluorescence because of the decreased throughput of the polarizers and the fact that the polarization involves measuring a relatively small difference between two intensities.

Fluorescence Polarization Spectrum

In order to obtain information from the polarization of fluorescence, it is necessary to select excitation and emission wavelengths that yield a sufficient polarization. The intrinsic polarization depends upon the angle between excitation and emission transition moments (β). The Jablonski equation (eqn 1-9; 19) relates $P_o$ to the angle $\beta$.

$$P_o = \frac{3 \cos^2 \beta - 1}{\cos^2 \beta + 3} \quad (1-9)$$

between transition moments. The limits occur when $\beta = 0^\circ$ and $90^\circ$ where $P_o$ has values of $1/2$ and $-1/3$ respectively. As the
excitation wavelength is changed, $\beta$ changes. This means that $P_0$ will also change because different transitions of the fluorophor are being excited. Depolarization mechanisms can be minimized by using dilute solutions ($\sim 10^{-6}$ M) at very high viscosity such as 99+% glycerol. Using these experimental conditions, the polarization observed as a function of wavelength constitutes the fluorescence polarization spectrum of the fluorophor. In general, the spectrum will contain a negative polarization region at lower wavelengths which corresponds to $S_0 \rightarrow S_2$ transitions and a region of high polarization at longer wavelengths due to $S_0 \rightarrow S_1$ transitions. The differences in the two polarization regions indicate that the two absorption transition moments are at a large angle relative to each other. A complete discussion of these phenomena is given by Gregorio Weber (19).
Goals of this Project

The goal of the work discussed in this dissertation was to generate basic data to determine whether and in what context changes in fluorescence polarization would be useful for observing analytical derivatization reactions. In addition, it would be desirable to have an idea of how fast some of the derivatization reactions are. This information is not present in the literature. The effect of media will also be studied since glycerol must be added in order to observe polarization changes. The fluorescent reagents studied were Fluorescein Isothiocyanate (FITC), Dichlorotriazinylamino Fluorescein (DTAF), Dansyl Chloride (DANSC) and Dansyl Hydrazine (DANSNH). The evaluation procedure involved Perrin plots and kinetic measurements for the reagent by itself and coupled to various analytes including amines, phenols and hydroxy and carbonyl compounds.
CHAPTER II

MATERIALS AND METHODS

Instrumentation

The instrument used to measure fluorescence polarization was an SLM 8000/8000S Photon Counting Spectrofluorometer (SLM-AMINCO Instruments, Inc. Urbana, IL.). The spectrofluorometer, which is illustrated in Figure 2-1, is a modular instrument. Excitation of the sample is accomplished via a 450 watt Xenon lamp (LH-450). The excitation wavelength is selected by a dual concave holographic grating monochromator (MC640). The sample is contained in the OP450 optical module. The latter also houses the excitation polarizer (Glan-Thompson calcite prism), two analyzers (Polaroid HNP B polarizers) and emission filter holders that support 50 x 50 mm filters. Detection is via photomultiplier tubes with variable independent voltage supplies for each of two channels A and B. The photomultiplier tube housings snap fit onto the optical module. The sample compartment for solution measurements contains a cuvette holder with built-in magnetic stirrer and temperature control. For obtaining emission spectra, an MC320 motorized single, concave holographic grating monochromator may be placed on the optical module on the channel A side. Data acquisition is
Figure 2-1: Schematic of the SLM 8000 / 8000S Spectrofluorometer.

- S: shutters
- B/S: beam splitter
- F: filter holders
- P: excitation polarizer
- L: focusing lenses
- A: analyzers
controlled electronically by an SPC 822/823 Electronics Unit. The SLM electronics control voltage to the PMT's, data acquisition and display as well as allow for background correction. RS-232 output ports allow for computer and plotter interfacing. Monochromators are controlled from an SMC-220 Monochromator Controller which sends signals to the stepper motors of the individual monochromators. Hard copies of the spectra are obtained from an Hewlett-Packard plotter (HP7470A) interfaced to either the SLM 8000S or to a computer.

Data collection and manipulation were controlled by a MINC-11 with RX-02 processors (Digital Equipment Corporation). This computer uses 8 inch floppy diskettes and a BASIC operating system. Data was obtained from the SLM 8000S from a serial digital output and converted by an onboard processor. Control programs were written by the researcher as necessary. The major data collection programs are presented in the Appendices.

Other instrumentation includes an Orion Digital Ionanalyzer/501 with glass combination electrode for pH measurement. A Torbal Torsion balance (Model EA-1) was used for all mass measurements requiring 4 decimal place accuracy. For special cases, a Mettler H51AR analytical balance with 5 place accuracy was used. In early work, fluorescence excitation and emission spectra were obtained on a Perkin Elmer MPF-44 Spectrofluorometer on loan from USGS. For UV-VIS measurements, a Bausch and Lomb 200 UV
Spectrometer was used. Data were analyzed on the MINC or the University DEC-10 mainframe computer.

Techniques

Preparation of Solutions

All chemicals used in this research were reagent grade or better. The water for all aqueous solutions was doubly deionized by passing house-deionized water through a mixed bed ion exchange resin (Ultrapure, Fisher) and then distilled in an all glass still (Megapure, Corning).

Glycerol (Fisher or Aldrich) solutions (v/v) for Perrin plots were prepared by determining the volume of anhydrous glycerol needed and converting to mass by using the density 1.2613 g/mL. The mass was then determined with a triple beam balance. Many methods of glycerol transfer were attempted before finally deciding on the use of 10 ml plastic syringes. The syringe minimized air contact and gave some control with respect to the amount of glycerol added. Water was added to yield the proper volume percentage. To prevent further water absorption by the glycerol, these solutions were not prepared on days of moderate to high humidity and were used promptly after preparation.

Buffer solutions were prepared by adding concentrated NaOH or HCl to the buffer solution until the desired pH was obtained. The solution was brought to volume and then the pH rechecked.
All glassware or plasticware was cleaned by soaking in a labcleaner (Micro, International Products) or 10% HNO₃ (Fisher) for 24 hours. If special cleanliness was required or if the previous cleaners were insufficient, glassware was soaked in Nochromix (Godax Laboratories) for 2 hours or in 1:1 concentrated nitric:sulfuric acids. All cleaning was completed with triple rinsing in deionized water and a single rinse in distilled water.

Fluorescence Spectra

In early work, spectra were obtained with the Perkin Elmer scanning fluorometer, on loan from the USGS. When this instrument was returned, the original SLM was upgraded to have motorized monochromators so it could scan spectra. The emission monochromator was placed on the channel A side if emission spectra were desired. The monochromator severely limited light throughput so the gain was set very high. As a result, dark current increased lowering the signal to noise level. Fluorescence spectra were obtained by finding manually the maximum intensities. These were compared to literature values where possible. The fluorescence was scanned over a limited range with both polarizers oriented vertically. The spectra were collected directly by the MINC for plotting in a standard format using the HP7470A plotter. The program is illustrated in the Appendix.
Polarization Spectra

Polarization spectra were obtained by preparing a 1.00 x 10^{-6} M solution of the fluorophor or fluorophor-conjugate under study and adding it to glycerol to prepare a final solution containing 99+% glycerol by volume. Glycerol was miscible with all solvents used to dissolve the fluorophors. Solutions were mixed for 15 seconds on a Vortex Genie (Fisher). This was shown to be a sufficient length of time to insure complete mixing by measuring fluorescence emission for several aliquots of the solution. A sample was then added to a clean quartz cuvette which was placed in the instrument. To eliminate the need to correct for dark current, the emission monochromator was removed and wavelength selection was performed via matching longpass filters near the maximum wavelength of the sample. The effect of polarizer/analyzer orientation is illustrated in Figure 2-2. When the polarizer is oriented vertically (Figure 2-2a), channels A and B respond to vertical and horizontal emission respectively. Turning the polarizer horizontally (Figure 2-2b) causes both channels to view light perpendicularly with respect to the excitation radiation and therefore both channels should see the same intensity. These intensities will vary depending upon relative gains to the individual detectors. Polarization values were determined at 5 or 10 nm intervals of excitation wavelength by 1) making sure the highest intensity signal for either channel and polarizer setting was close to 90000
Figure 2-2: Vertical and Horizontal Emission is Dependent Upon Polarizer / Analyzer Orientation.
cps, the highest signal level recommended by SLM, Inc. and that 2) channel A and B signals were roughly equal when the excitation polarizer was set to horizontal. In order to collect two channels at a time, the readings were collected by the operator and entered directly into the MINC for storage and analysis. Dark currents were also collected at each wavelength and subtracted from all readings (see program in Appendix). Polarization values were calculated using equation 2-1 where

\[
P = \frac{A_v - B_v \times (A_h/B_h)}{A_v + B_v \times (A_h/B_h)}
\]  

(2-1)

\(A_v\) is channel A with the excitation polarizer vertical, i.e. parallel emission, \(B_v\) is channel B with the excitation polarizer vertical, i.e. perpendicular emission. Likewise \(A_h\) and \(B_h\) indicate channels A and B, respectively, with the excitation polarizer horizontal. As noted earlier, the latter readings should be the same and are used to adjust the perpendicular intensity for any differences between gain or response of photomultiplier tubes.

**Perrin Plots**

Perrin plots were obtained by varying viscosity instead of temperature. Using wavelengths chosen from the polarization spectrum, solutions containing the same concentration of fluorescent species were compared in solutions containing 35, 58, 65 and 70% glycerol by weight.
The maximum percentage of glycerol was held to 70% for convenience. Above 75%, the flow of a glycerol solution is very slow and absorption of atmospheric water can be a problem (21). For convenience, bulk solutions of glycerol-water were prepared such that 9.90 ml of the glycerol solution and 0.100 ml of fluorescent species would yield the desired viscosity for the resulting solution. Literature values for viscosity of glycerol-water mixtures (21,22) were used since the percentage of other solvents always was <1%. Linear Perrin plots were generally observed indicating these viscosities to be appropriate for this research work. By plotting $1/P$ vs $T/n$, polarizations of the fluorophor and its derivatives can be compared. As long as the concentrations of fluorophor and conjugate are the same, a comparison of relative intensities will yield fluorescent lifetimes of the derivatives ($\tau_1$). The derivative lifetime is related to the lifetime of the original fluorophor ($\tau_0$) using equation 3-2 by knowing the total intensities $I_0$ and $I_1$

$$\frac{\tau_0}{\tau_1} = \frac{I_0}{I_1}$$  \hspace{1cm} (3-2)

$I_1$ of the reagent and derivative respectively. Total intensities are calculated from the vertical intensity

$$I = I_v + 2 I_h$$  \hspace{1cm} (3-3)

plus two times the horizontal intensities. For $\tau_0$ values, literature values were used.
CHAPTER III

FLUORESCIN ISOTHIOCYANATE

Introduction

Fluorescein Isothiocyanate (FITC) (Figure 3-1), has been widely used in clinical labs as a sensitive reagent for amino acids (23-27) and proteins (28-39). Much of the work to date involves the formation of a conjugate followed by separation by electrophoresis, elution, thin layer or high performance liquid chromatography to remove excess FITC from the conjugate in order to quantitate the derivative. FITC is advantageous for many reasons, including sensitivity of detection and selectivity. FITC reacts with amines and is not subject to interference from side reactions. For the experimenter, FITC is convenient to use because the reaction will go to completion in aqueous media under favorable pH conditions that will not lead to the decomposition of the analyte.

Martin and Lindqvist (40) have shown that the charge on fluorescein depends on pH (Figure 3-2). The highest molar absorptivity has been found for the dianion which forms in basic solutions (Table 3-1). They also measured quantum efficiencies for the various species. It is expected that the values for FITC will be similar.

H. Elmgren (41) measured the fluorescent lifetimes of
Figure 3-1: Structure and Fluorescence Data for 3',6'-dihydroxy-6-isothiocyanato-spiro(isobenzofuran-1(3H),9'-[9H]xanthen)-3-one; commonly Fluorescein Isothiocyanate.
Figure 3-2: Effect of pH on Electronic Structure of Fluorescein Isothiocyanate. Very acidic pHs, cation (I); mildly acidic pHs, neutral molecule (II); neutral pHs, monoanion (III); basic pHs, dianion (IV).
Table 3-1: Effect of pH on Fluorescence and Absorbance Characteristics of Fluorescein

<table>
<thead>
<tr>
<th>fluorescein species</th>
<th>excitation wavelength (nm)</th>
<th>molar absorptivity (M⁻¹ cm⁻¹)</th>
<th>quantum yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>cation</td>
<td>437</td>
<td>55000</td>
<td>.9 - 1</td>
</tr>
<tr>
<td>neutral</td>
<td>437</td>
<td>16000</td>
<td>.2 - .25</td>
</tr>
<tr>
<td>monoanion</td>
<td>437</td>
<td>30000</td>
<td>.25 - .35</td>
</tr>
<tr>
<td></td>
<td>475</td>
<td>31000</td>
<td>.25 - .35</td>
</tr>
<tr>
<td>dianion</td>
<td>491</td>
<td>88000</td>
<td>.93</td>
</tr>
</tbody>
</table>

1. Data from M. Martin and L. Lindqvist (40).
FITC as a function of pH. He found lifetimes of 3.18 ns for the cation, 3.44 ns for the neutral species, 3.91 ns for the monoanion and 4.65 ns for the dianion.

The high molar absorptivity and quantum efficiency of the dianionic species help to make FITC highly desirable for fluorescence labeling. Proteins and other reactants that may denature in acidic or strongly basic environments can be derivatized and detected at pHs where FITC emission is optimum.

Reactions of FITC with Amines

Maeda, Kawauchi and coworkers (24,25) have used FITC to study end terminal amino groups of proteins by reaction to form a fluorescein thiocarbonyl species. They describe the reaction sequence as shown in Figure 3-3. The reaction goes rapidly to completion in aqueous media. F. Sokol et al. (31) found a pH dependence for the reaction between FITC and γ-globulin. They reported that the amount of FITC bound to the protein was highly dependent upon the pH of the reaction mixture. They postulated a mechanism where electrons on the amine group attack the carbon of the thiocyanate group. The mechanism is depicted in Figure 3-4. Sokol also pointed out that the first step is slow in acidic media because the amino group is protonated. Until the proton is lost, (reaction 1 of mechanism), the nitrogen can not nucleophilically attack the thiocyanate carbon. This depends upon the pKa of the amine and the pH of the solution.
Figure 3-3: Reaction Sequence Between Fluorescein Isothiocyanate (IV) and Primary Amines (VI) to become Fluorescein Thiocarbamylate (VII).
Figure 3-4: Reaction Mechanism for Thiocarbamylation
Reaction Between Fluorescein Isothiocyanate and Amines. See text for references.
Zahradnik (42,43) published a similar mechanism for reactions of isothiocyanates with hydroxide ions and amines. He proved polarographically that the reactions are bimolecular additions where the isothiocyanate group behaves as an electrophile. It was demonstrated that the initial step of the reaction is based on a polar intermediate resonance structure formed as shown in reaction 2 of the mechanism. Zahradnik confirmed this (44) by calculating electron densities around the NCS group for phenyl-, 1-naphthyl and 2-naphthyl isocyanates. Nitrogen had 1.619 electrons, sulfur 1.613 electrons and carbon 0.716 electrons which supported the electrophilic intermediate. Since the work by Maeda, Kawauchi et al. (23-27), these reactions have become common for many biomedical analyses.

**Kinetics of the Thiocarbamylation Reaction**

There have been few kinetic studies of FITC reactions with proteins in the literature. For the most part, researchers allow the reaction to proceed for 10-24 hours depending upon the reaction conditions used. Many of these procedures are based on work of Como and Kaplan (29) who used an 18 hour reaction time to label antibodies with FITC. In an interesting set of experiments, McKinney, Spillane and Pearse (33) labeled bovine serum albumin and γ-globulin with FITC under a variety of reaction conditions. They found the rate of reaction increased as pH increases reporting that the reaction occurs very rapidly at pH's 9
and above. For example, they added 25 µg of dye per mg protein at pH 9 and room temperature. By the time they adjusted the pH of the reaction mixture and separated the conjugate from the free dye, 15 µg of dye were bound per mg protein. Also indicated in the same paper was that the ratio of µg FITC bound: mg protein was only slightly higher when the reaction time was 21 hours instead of 2 hours. There is an effective upper limit to the increase in reaction rate with pH. Kawauchi et al. (26) report that FITC degrades to aminofluorescein at pHs above 10.

F. Sokol et al. (31) studied the kinetics of the reaction between FITC and γ-globulin. They discovered no difference in the efficiency of binding between reactions stopped at 3 hours and 18 hours. T. Tokumura (39) also studied the γ-globulin reaction and observed that the highest probability for binding is at the first of eight amino sites. Total binding of eight FITC molecules onto one globulin takes a week but after one hour an average of 0.5 sites is coupled to fluorescein.

Andersson et al. (45) studied the binding of FITC to bovine serum albumin (BSA). This system is relatively simple since few binding sites are available. 750 µM FITC was reacted with 75 µM BSA at pH 8. They saw rapid conjugation of FITC to the first site with 1:1 conjugation finished in an hour. After one site is filled, however, they report that other sites become partially blocked and it takes over 10 hours for two more fluorophor molecules to bind.
Maeda and coworkers (25) studied the kinetics of the fluorescein-thiocarbamylation reaction using insulin and neocarzinostatin. For both species an excess of FITC was used in the reaction. After gel filtration to remove free FITC, a plot of absorbance at 490 nm vs. reaction time leveled off to constant absorbance at 2 hours for insulin and 1.5 hours for neocarzinostatin at pH's 9 and 9.5, respectively. Maeda noted that amino groups other than the α-amino terminus became active at pH's greater than 9.5. He also noted, as did McKinney (33), that the reaction proceeds more slowly as pH is lowered. In another report, Kawauchi et al. (26) studied the rate of alanine binding to excess FITC. Using absorbance vs. time plots it was shown that the reaction reached completion in less than an hour at pH 9.

Other groups have measured rate constants for reactions between aryl isothiocyanates and various amines. Part of the work done for this dissertation was to try to relate these studies to our conditions.

Rao and Ventkataraghavan (46) determined the effects of substituents on reactions between para-substituted phenyl isothiocyanates with aniline. They found that the addition reaction was second order and that the rate constants for a substituted phenyl isothiocyanate ($k$) and an unsubstituted phenyl isothiocyanate ($k_0$) are related through the Hammett substituent value ($\sigma$) of the group;

$$\log \frac{k}{k_0} = 0.071 + 2.133\sigma \quad (3-1)$$
Similarly, substituted anilines were reacted with phenyl isothiocyanates to yield a second linear relationship

\[
\frac{k}{k_0} = 0.017 - 4.443\sigma
\]

(3-2)

where \(k\) is now the rate constant for substituted aniline and \(k_0\) for unsubstituted aniline. Both equations were obtained at 40°C. The rate increased as the electron donating power of the para-substituent increased. Rao and Venkataraghavan's results are tabulated in Table 3-2 along with some results from Akiyama et al. (47) who ran similar reactions between phenyl isothiocyanate and substituted anilines. Both sets of results show reasonable agreement.

Other kinetic studies were performed by Drobnica and Augustine (48,49). In their first report, they reacted twenty different aryl isothiocyanates with glycine at pH 9.80 following the reaction spectrophotometrically. They determined rate constants which varied from 0.078 ± 0.015 to 2.72 L mole\(^{-1}\) sec\(^{-1}\) with most values falling between 0.18 and 0.33 M\(^{-1}\) sec\(^{-1}\). As with Rao's work, they also found a linear relationship between log rate constant and the Hammett \(\sigma\)-values. Their rate constants are several orders of magnitude higher than others reported.
Table 3-2: Rate Constants from the Literature for Reactions Between Phenyl Isothiocyanate and Substituted Anilines

<table>
<thead>
<tr>
<th>Substituent</th>
<th>Hammett $\sigma$-value</th>
<th>rate constant (M$^{-1}$ sec$^{-1}$)</th>
<th>temperature ($^\circ$C)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Br</td>
<td>.232</td>
<td>$6.3 \times 10^{-5}$</td>
<td>40$^o$</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$9.5 \times 10^{-5}$</td>
<td>54.5$^o$</td>
<td>46</td>
</tr>
<tr>
<td>p-Cl</td>
<td>.232</td>
<td>$1.68 \times 10^{-5}$</td>
<td>20$^o$</td>
<td>47</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>$8.40 \times 10^{-5}$</td>
<td>20$^o$</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$3.53 \times 10^{-4}$</td>
<td>30$^o$</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$6.25 \times 10^{-4}$</td>
<td>40$^o$</td>
<td>46</td>
</tr>
<tr>
<td>p-CH$_3$</td>
<td>-.170</td>
<td>$4.62 \times 10^{-4}$</td>
<td>20$^o$</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.45 \times 10^{-3}$</td>
<td>30$^o$</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$3.29 \times 10^{-3}$</td>
<td>40$^o$</td>
<td>46</td>
</tr>
</tbody>
</table>
Determination of Rate Constants

The reaction between amines and FITC is second order. The rate law is therefore,

\[
\frac{dx}{dt} = k \left[ a-x \right] \left[ b-x \right] \quad (3-3)
\]

where \(a\) is the concentration of amine, \(b\) is the concentration of the FITC, and \(x\) is the amount of limiting reagent reacted. The derivative of the rate law (50) is

\[
t = \frac{1}{k} \cdot \frac{2.303}{(a-b)} \log \frac{b(a-x)}{a(b-x)} \quad (3-4)
\]

The rate constant can be calculated from individual reaction completion times or by plotting the derivative equation where the slope is \(1/k\).

Experimental

Fluorescence and polarization spectra were obtained as described in Chapter II. A \(1.00 \times 10^{-7}\) M FITC solution in 0.1M bicarbonate buffer was used to obtain the fluorescence spectra. For the latter, a \(1.00 \times 10^{-6}\) M FITC solution in 99.9% glycerol was used with emission wavelengths selected via longpass filters with cutoffs of 518 nm.
Reaction with 1-Amino-2-naphthol-4-sulfonic Acid

The reaction system was a modification of a protein labeling experiment previously used by this research group (51). In order to force the reaction to completion, excess amine relative to fluorophor was used. The conjugate was formed by mixing 10 mL of the chosen amine with 10 mL of 0.85% sodium chloride (Baker), 3.00 mL of 0.5 M sodium carbonate (Baker) buffer (pH 9), 5.0 mL of acetone (Fisher) and 1.5 mL of fluorescein isothiocyante (Sigma) solution dissolved in HPLC grade or better acetone (Fisher). The first reaction tried was between 0.026 M 1-amino-2-naphthol-4-sulfonic acid (ANSA - Eastman Kodak) and 0.0097 M FITC. The mixture was stirred for two hours at room temperature in the dark. This was due to the light sensitivity of ANSA. By keeping ANSA in large excess, it was hoped to force all the FITC into the thiocarbonyl form. Reaction completion was checked by thin layer chromatography as used by Kawauchi (26). Kawauchi found that free FITC migrates with the solvent front while the conjugated form lags behind. Baker-flex silica-gel TLC plates (Fisher) were dried for 4 hours at 110° C and stored in a desiccator over CaCl₂. A benzene:ethyl acetate:acetic acid (5:1:1, v:v:v) solution was used to develop the plates. No fluorescence emission was observed from the solvent front which indicated the reaction had gone to completion. The FTC-ANSA complex appeared as a yellow patch under UV light. Due to severe band spreading, no Rf values could be obtained.
Perrin plots were obtained by adding 0.050 ml of FITC or FTC-ANSA to solutions of varying percentages of glycerol and water. In addition, acetone alone was added to glycerol to do a blank correction. The spectrofluorometer was set up using the FITC solutions after a quick check illustrated that its fluorescence yield was higher than that of the FTC-ANSA. All values were collected by averaging ten 1 second data acquisitions per channel and polarizer settings.

Effect of pH and Viscosity

The above reaction was used to test the system for interferences from glycerol and pH effects. Glycerol solutions of three different viscosities were prepared. FITC was reacted with amino naphthol sulfonic acid, polyphenylalanine and albumin. A tenfold excess of reactants with respect to FITC were used. In addition, the reaction between FITC and amino naphthol sulphonic acid was run using buffers from pH's 7-10 to observe the effect of pH.

Effect of Amine Size on Polarization

The next step was to confirm a basic premise of the work, i.e. that changes in the size of the analyte would change the polarization. A group of five amines DL-alanine (Eastman Kodak), 1-naphthylamine hydrochloride (Eastman Kodak), 1,4-phenylene diamine dihydrochloride (Eastman Kodak), 1-amino-2-naphthol-4-sulfonic acid (Fisher) and
adenosine triphosphate (Sigma) were chosen because of the molecular weight spread they encompassed. The diamine was chosen to compare with 1-naphthylamine since the two species are approximately the same size. In order to judge size effects, three larger "amines" were purchased from Sigma Chemical Co., polyphenyl-alanine, lysozyme and bovine serum albumin. The entire group had a MW range up to 65400 grams mole⁻¹.

Using the same conditions as before, 10.00 mL of 0.0260 M amine solution were added to 18.0 mL of a solution of salt, carbonate buffer and acetone in the same amounts as previously. Finally 1.00 mL of 0.0100 M FITC was added. Eppendorf pipets were used to measure smaller volumes. Acid washed class A pipets were used for the larger volumes. The reaction vessels, 125 mL Erlenmeyer flasks, were covered with parafilm and a cork and placed in the dark with stirring for two hours at room temperature. Final conjugate concentrations were 3.33 x 10⁻⁴ M assuming the reaction ran to completion. The validity of this assumption was confirmed by TLC and dialysis. TLC of the samples all illustrated no fluorescence at the solvent front indicating no free FITC remained. Some of the mixture was diluted 1:100 with HPLC grade acetone. Then 0.0500 mL of this dilution was added to a 10 mL class A volumetric flask which was filled to the mark with glycerol-water solution to yield final concentrations of 1.67 x 10⁻⁸ M FITC or FTC-A at three different viscosities.
Reaction of FITC with Bovine Serum Albumin

Two similar reactions were run. The first used the same experimental conditions as in the previous study, i.e., 10 mL of 0.85% NaCl solution, 3 mL of 0.5M carbonate buffer and 6 mL of acetone were added to 10 mL of albumin solution contained in a flask on a magnetic stirrer. One mL of FITC solution was added and the mixture stirred. An aliquot was placed into the lightpath of the spectrofluorometer which had been set up using a FITC blank made up of all materials except albumin for which water was substituted. Intensity data was obtained for each channel and polarizer setting. The aliquot was then returned to the reaction vessel. Aliquots were sampled every five minutes for a period of twenty minutes and then every ten minutes until two hours had passed.

In the second sample, glycerol was added. Glycerol should make it easier to follow the kinetics by changing polarizations to a greater degree. Because isothiocyanates also react with hydroxyls, a blank reaction was run to confirm that FITC would not react rapidly with glycerol. Nine mL of glycerol were obtained by weighing into a flask. Ten mL of salt solution and 10 mL of the albumin solution that was made up with additional buffer to match earlier conditions, were added to the reaction vessel. FITC was then added and an aliquot placed into a ground glass stoppered quartz cuvette. In order to minimize exposure of the
glycerol to moisture, the reaction was run in the cuvette. The sample was stirred using a microstirbar and the magnetic stirrer built into the SLM sample compartment.

Reaction of FITC with small monoamines

The rates of reactions between FITC and small amines were measured in glycerol/water solutions. Glycerol solutions were prepared of NaCl solution, acetone and glycerol to equal the amounts from previous reactions. A 12.5 mL aliquot was added to 1.5 mL of amine then 2.9 mL of the latter mixture was placed into a cuvette and 0.1 mL of FITC was added by pipet with shaking. The reaction was allowed to proceed for 30, 60 or 90 minutes depending on the concentration of amine and FITC used. Concentrations of amines were $3.00 \times 10^{-6}$ M, $6.00 \times 10^{-7}$ M and $3.00 \times 10^{-7}$ M. The amines were in tenfold excess over the FITC concentration. Intensity values were collected using the MINC computer and stored directly on floppy diskettes. Prior to taking readings for the sample, blanks of glycerol, FITC and amine were run to confirm that the background signal from the supporting reagents was not excessive.

Kinetics with Anilines

Rao and Venkataghvan used p-bromo, p-methyl and p-methoxy anilines to establish their rate law comparison (equation 3-2). None of these species was available in-house
but p-chloroanilene, which has the same Hammett value as p-bromo \( (\sigma = 0.232) \) was available. Aniline and p-chloroanilene were reacted using the same methodology as used for the smaller monoamines.

After data collection using the MINC, plots were drawn. Polarization and intensities were plotted versus reaction run time. In most cases a distinct change in slope was observed. This change in slope was taken as the endpoint of the reaction \((25, 26, 31, 48, 49)\) and the time taken for calculation of rate constants. In addition, plots of \( \ln(P_{\text{inf}} - P) \) vs. reaction time and Fraction of FITC free vs. reaction time were drawn.

**Results and Discussion**

Fluorescence spectra of \(1.00 \times 10^{-7}\) M FITC are illustrated in Figure 3-5. These spectra are not corrected for instrumental distortions. The polarization spectrum of \(1.00 \times 10^{-6}\) M FITC in 99.9% glycerol is plotted in Figure 3-6. Both are consistent with literature spectra.

**Reaction with 1-Amino-2-naphthol-4-sulphonic Acid**

Polarizations and fluorescence lifetimes (Table 3-3) were calculated from the intensity data. These are plotted as Perrin plots (Figure 3-7). The temperature was 31° C when the intensity data were collected. The Perrin equation holds
Figure 3-5: Uncorrected Fluorescence Excitation (---) and Emission (-----) Spectra of Fluorescein Isothiocyanate.
Figure 3-6: Excitation Polarization Spectrum of Fluorescein Isothiocyanate in 99.9% glycerol.
Table 3-3: Calculated Values of Polarizations and Fluorescent Lifetimes of FITC and FTC-ANSA

<table>
<thead>
<tr>
<th>Viscosity</th>
<th>Polarizations</th>
<th>Lifetimes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FITC</td>
<td>FTC-ANSA</td>
</tr>
<tr>
<td>.0297 P</td>
<td>.0484</td>
<td>.0786</td>
</tr>
<tr>
<td>.0821 P</td>
<td>.108</td>
<td>.141</td>
</tr>
<tr>
<td>.111 P</td>
<td>.128</td>
<td>.159</td>
</tr>
<tr>
<td>.151 P</td>
<td>.159</td>
<td>.203</td>
</tr>
<tr>
<td>.225 P</td>
<td>.196</td>
<td>.229</td>
</tr>
</tbody>
</table>
Figure 3-7 Perrin Plots of FITC (o) and FTC-ANSA (x)
for FITC as indicated by the linearity of the line. The FTC-ANSA conjugate has a slightly nonlinear response. Nonlinearity indicates the molecular species is not spherical. The lifetime of the free FITC is given as 4.65 nanoseconds (41). From this value, the lifetime of the FTC-ANSA conjugate is calculated to be 1.39 ns based on intensity. This value is an average of the lifetimes in Table 3-3 since there was no trend indicating the viscosity was affecting the lifetimes of either the free FITC or the conjugate. The change in polarization upon conjugation is attributed to changes in lifetime rather than to changes in the degree of rotational depolarization due to increased mass.

Effect of pH and Viscosity on Rate

FTC-ANSA was prepared in a series of buffers between pHs 6.8 and 10.2. TLC indicated that all reactions had gone to completion during the two hour experiment. Only one fluorescent spot was visible on any TLC plate indicating side reactions were minimal. The data, Table 3-4, show increasing intensity as pH increases. This trend is supported by Martin and Lindqvist (40) who reported the monoanionic form of fluorescein occurs at neutral pH. This form is excited at shorter wavelength than the dianion and has a shorter fluorescence lifetime, lower molar absorptivity and lower quantum efficiency. The net result should be a lowering of intensity as pH is lowered. A
Table 3-4: Effect of pH on the Fluorescence of FTC-ANSA

<table>
<thead>
<tr>
<th>pH</th>
<th>$I_v$</th>
<th>$I_H$</th>
<th>$I_T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.85</td>
<td>25.3</td>
<td>19.4</td>
<td>64.0</td>
</tr>
<tr>
<td>7.52</td>
<td>33.8</td>
<td>26.4</td>
<td>86.6</td>
</tr>
<tr>
<td>9.24</td>
<td>54.5</td>
<td>43.6</td>
<td>141.6</td>
</tr>
<tr>
<td>9.41</td>
<td>55.7</td>
<td>44.8</td>
<td>145.4</td>
</tr>
<tr>
<td>9.81</td>
<td>55.8</td>
<td>44.9</td>
<td>145.6</td>
</tr>
<tr>
<td>10.06</td>
<td>54.7</td>
<td>44.2</td>
<td>143.1</td>
</tr>
</tbody>
</table>

1. $I_H$ is corrected for differences in PMT response.
2. $I_T$ is calculated from $I_v + 2I_H$. 
similar trend was observed for the FITC blank.

Aminonaphtholsulfonic acid, polyphenylalanine and albumin were reacted with FITC in solutions of three different viscosities to see if viscosity would have any effect upon thiocarbamylation kinetics. In each case there was a change in polarization with respect to the FITC blank for each trial. However, the time required for the reaction to go to completion was not affected by viscosity.

**Effect of Amine Size on Polarization**

Polarization results are summarized in Table 3-5. In seven of eight cases there was a noticeable polarization change. In the eighth case, adenosine triphosphate, the molecular rotation due to the increased mass of ATP seems to have been negated by lifetime considerations. Using intensity data, fluorescence lifetime values were calculated for all the derivatives. As anticipated, there is a difference in lifetime for the FITC-ATP complex as well as most of the complexes. Lifetimes are tabulated in Table 3-6. Lifetimes were calculated from that of FITC. Intensities indicate lifetimes, with the exception of FTC-albumin, were not affected by viscosity, therefore, lifetimes are averages for all viscosities sampled and include the standard deviation. The lifetime of the FTC-albumin conjugate changed from 2.41 ns in 35.1% aqueous glycerol to 2.01 ns in 65.4% glycerol.

The observed changes in polarization are primarily due to changes in lifetime rather than volume. This makes sense
Table 3-5: Effect of Analyte Size on Polarizations in Solutions of 3 Different Viscosities

<table>
<thead>
<tr>
<th>Analyte</th>
<th>FTC-A conjugate</th>
<th>Polarizations at viscosities (poise)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MW</td>
<td>.0297</td>
</tr>
<tr>
<td>free FITC</td>
<td>0</td>
<td>.0482</td>
</tr>
<tr>
<td>alanine</td>
<td>89</td>
<td>.0635</td>
</tr>
<tr>
<td>1-naphthylamine hydrochloride</td>
<td>180</td>
<td>.0602</td>
</tr>
<tr>
<td>1,4-phenylenediamine dihydrochloride</td>
<td>181</td>
<td>.108</td>
</tr>
<tr>
<td>1-amino-2-naphthol-4-sulfonic acid</td>
<td>239</td>
<td>.0620</td>
</tr>
<tr>
<td>adenosine triphosphate</td>
<td>551</td>
<td>.0489</td>
</tr>
<tr>
<td>polyphenylalanine</td>
<td>3000</td>
<td>.0567</td>
</tr>
<tr>
<td>lysozyme</td>
<td>13930</td>
<td>.244</td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>65400</td>
<td>.366</td>
</tr>
</tbody>
</table>
Table 3-6: Fluorescence Lifetimes of Thiocarbamyl Conjugates Calculated from Intensity Data

<table>
<thead>
<tr>
<th>FTC-A conjugate</th>
<th>lifetime (±Sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>free FITC</td>
<td>4.65 ± 1</td>
</tr>
<tr>
<td>alanine</td>
<td>1.89 ± .09</td>
</tr>
<tr>
<td>l-naphthylamine</td>
<td>3.02 ± .27</td>
</tr>
<tr>
<td>1,4-phenylenediamine</td>
<td>1.61 ± .18</td>
</tr>
<tr>
<td>l-amino-2-naphthol-4-sulfonic acid</td>
<td>1.39 ± .14</td>
</tr>
<tr>
<td>adenosine triphosphate</td>
<td>2.09 ± .16</td>
</tr>
<tr>
<td>polyphenylalanine</td>
<td>2.89 ± .11</td>
</tr>
<tr>
<td>lysozyme</td>
<td>4.61 ± .31</td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>ca. 2.26 ± 2</td>
</tr>
</tbody>
</table>

1. H. Elmgren (41).
2. Lifetime of FTC-albumin varied with viscosity (see text)
since polarization should vary with volume as a function of the cube root of mass. With small amines or species that can appear smaller by wrapping around the fluorophor, the change in polarization with volume would not be significant. Thus the polarization technique can be utilized to detect small amines as well as the larger amines where size effects will be important.

The polarization difference between the mono and diamines of similar size can also be explained by the lifetime. The lifetime of the diamine is significantly different from that of the monoamine. The lifetime of the diamine conjugate could be affected by the presence of a small amount of difluorescein derivative. Such a species has precedence in the literature (52) where two N,N-diisopropylamino isothiocyanates were bound to hydrazine. The difluorophor species suggested herein would be affected by energy transfer depolarization and concentration quenching because of the close proximity of the two fluorescein moieties leading to a shorter fluorescent lifetime as observed here.

The Perrin plots from the data are nonlinear indicating that the species are nonspherical. Molecular volumes, therefore, were calculated only for the larger species to illustrate the magnitude of the volumes. These lifetime corrected volumes are summarized in Table 3-7. The data illustrate that the proteins lysozyme and albumin have a molecular volume much larger than fluorescein alone.
Table 3-7: Molecular Volumes of Nonspherical Thiocarbamyl Species at Viscosity .0297 poise and 27° C

<table>
<thead>
<tr>
<th>species</th>
<th>volume (L·mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>free FITC</td>
<td>0.37</td>
</tr>
<tr>
<td>FTC-lysozyme</td>
<td>3.4</td>
</tr>
<tr>
<td>FTC-albumin</td>
<td>5.1</td>
</tr>
</tbody>
</table>
supporting the view that only larger species would depolarize fluorescence by volume.

Reaction of FITC with Bovine Serum Albumin

The addition of glycerol did not change the observed rate of the reaction between FITC and albumin. For both mixtures tested, an experiment with 35.1% glycerol and an experiment without glycerol present, plots of polarization with reaction time (Figure 3-8) show a change in slope around the sixty minute mark. Initially it was believed that the change was due to a second binding site being activated. After many trials with smaller monoamines, it was concluded that this is an indication that the reaction has reached completion. TLC of the reaction mixtures indicated no unbound FITC remained after the two hour reaction period. Using a reaction time of 60 minutes and assuming 95% of the FITC had reacted yielded a second order rate constant of $1.79 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$ at 21 °C.

The presence of glycerol led to increased polarization changes without affecting the reaction rate. The changes in polarization were 0.037 with 35.1% glycerol compared to 0.015 without glycerol.

In experiments run several months later, the reaction between FITC and albumin, again in 35.1% glycerol, seemed to go much faster although the temperature was only 26° C. The initial intensity of the reaction mixture in the cuvette after addition of fluorophor and shaking was much lower than
Figure 3-8  Polarization Changes as a Function of Time for Reaction Between FITC and Albumin
the intensity of the blank solution. In addition, the intensities held fairly constant over the two hour period of experimentation (Table 3-8). Polarizations also changed drastically between the blank and the reaction mixture. The polarization of the blank was 0.188 compared to an initial polarization of 0.210 for the reaction mixture. Polarizations held within 1% for the remainder of the experiment. Earlier work in this project showed that the lifetime of the FTC-albumin conjugate was less than the free fluorophor and that the polarization values of the conjugate were larger. It could be assumed that the reaction had gone to completion almost immediately which is more consistent with results of reactions between FITC and smaller amines. Consequently, a rate constant could not be calculated.

Reaction of FITC with Small Monoamines

The reaction between glycine and FITC seemed to be complete by the time the first intensity values were measured. As with the latter albumin experiments, the intensities of the reaction mixtures were lower than those of the FITC blank (Table 3-9). There was no change in slope for any of the experimental plots. Polarizations stayed fairly constant varying between 0.126 and 0.135 over the entire time span for the reaction. The change in polarization could be attributed to temperature effects. As the temperature increases, the viscosity decreases leading to greater rotational depolarization.
<table>
<thead>
<tr>
<th></th>
<th>$I^V$</th>
<th>$I^N$</th>
<th>$I^T$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC blank solution</td>
<td>62.8</td>
<td>42.9</td>
<td>148.6</td>
<td>.188</td>
</tr>
<tr>
<td>Initial readings at start of reaction</td>
<td>37.3</td>
<td>24.3</td>
<td>85.9</td>
<td>.210</td>
</tr>
<tr>
<td>Final readings 122 minutes after the reaction start</td>
<td>37.5</td>
<td>24.5</td>
<td>86.5</td>
<td>.210</td>
</tr>
</tbody>
</table>
Table 3-9: Experimental Values from a Kinetics Trial Between FITC and Glycine

<table>
<thead>
<tr>
<th></th>
<th>IV</th>
<th>II</th>
<th>IT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC blank solution</td>
<td>51.9</td>
<td>41.0</td>
<td>134.0</td>
<td>.117</td>
</tr>
<tr>
<td>Initial readings at</td>
<td>22.3</td>
<td>17.1</td>
<td>56.6</td>
<td>.131</td>
</tr>
<tr>
<td>start of reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final readings 30</td>
<td>26.5</td>
<td>20.6</td>
<td>67.7</td>
<td>.126</td>
</tr>
<tr>
<td>minutes after the</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction start</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
It would not be inconsistent to have the lifetime of glycine/FITC conjugate be smaller than that of free FITC resulting in lower intensities. Alanine, another small amino acid, had a lifetime of 1.89 ns. A short lifetime would support the evidence of a fast reaction as indicated by the change in intensity. There was no way to check the results by TLC because the low concentrations would not be visible on the plate.

A fast reaction between glycine and FITC is consistent with studies on the substituent effects on rates. Drobnica and Augustin (48,49) reported rate constants for arylisothiocyanates with glycine that were several orders of magnitude higher than the rate constants with other analytes.

Rate of Aniline/FITC Reaction

The rate of the aniline/FITC reaction was studied so that the rate in glycerol/water mixtures could be related to rates measured by Rao and Ventkataraghavan (46) and by Akiyama et al. (47) in other studies.

Table 3-10 shows the aniline and FITC concentrations for various experiments as well as the times required for the reactions to go to completion. Temperatures were held at 21.5 °C ± 1°C. The best fit line for the derivative of the second order rate law (Figure 3-9), assuming 95% of the FITC has reacted to form conjugate, is \( Y = 5.5 \times 10^{-6} X + 0.053 \) (\( r^2 = .995 \)) where the slope is \( 1/k \). The calculated rate
Table 3-10: Initial Concentrations of aniline and FITC with Graphically Determined Reaction Completion Times

<table>
<thead>
<tr>
<th>[aniline]</th>
<th>[FITC]</th>
<th>minutes (± Sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3.00 \times 10^{-6}$</td>
<td>$3.00 \times 10^{-7}$</td>
<td>$5.51 \pm .40$</td>
</tr>
<tr>
<td>$6.00 \times 10^{-7}$</td>
<td>$6.00 \times 10^{-8}$</td>
<td>$30.6 \pm 2.6$</td>
</tr>
<tr>
<td>$3.00 \times 10^{-7}$</td>
<td>$3.00 \times 10^{-8}$</td>
<td>$58.4 \pm 1.9$</td>
</tr>
</tbody>
</table>
Figure 3-9 Derivative of Second Order Rate Law (equation 3-4) for the FITC / Aniline Reaction where 
a is concentration of amine, b is the 
concentration of fluorophor and x is 95% of 
limiting reagent
constant is $1.8 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ or $3.0 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$. This is a much larger rate constant than found by either Rao and Ventkataraghavan or Akiyama et al. in their reactions between phenyl isothiocyanates and substituted anilines. This seems to indicate that the fluorescein moiety is a very good electron withdrawing group. Resonance structures can be written that have the carbonyl group pull electron density from the pi-system and from the phenyl ring to which the isothiocyanate group is bound. This would pull electron density from the isothiocyanate nitrogen which would help the reaction. Induction by the rich pi-system in the para position would be negligible. Rao and Ventkataraghavan indicate that an electron withdrawing $R$ group will assist the reaction towards completion, assigning the resonance structure given in Figure 3-10.

The results of the reaction between $p$-chloroaniline and FITC appear in Table 3-11. The best fit line, $Y = 3.2 \times 10^{-6} X + 8.5 \ (r^2=.989)$ is illustrated in Figure 3-11. The second order rate constant is calculated to be $3.1 \times 10^5 \text{ M}^{-1} \text{ min}^{-1}$ or $5.2 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$. Rao and Ventkataraghavan's $p$-bromoaniline reacted an order of magnitude slower than unsubstituted aniline. The $p$-chloroaniline used in this work reacted slightly faster than the unsubstituted aniline. Obviously the FITC system has other effects that the phenyl isothiocyanate system does not have.
Figure 3-10  Resonance Structure Suggested by Rao and Ventkataraghavan (46) showing stabilization of the isothiocyanate moiety by an electron withdrawing R-group
Table 3-11: Initial Concentrations of p-chloroaniline and FITC with Graphically Determined Reaction Completion Times

<table>
<thead>
<tr>
<th>[p-chloroaniline]</th>
<th>[FITC]</th>
<th>minutes (± Sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.00 x 10^{-6}</td>
<td>3.00 x 10^{-7}</td>
<td>13.4 ± 2.9</td>
</tr>
<tr>
<td>6.00 x 10^{-7}</td>
<td>6.00 x 10^{-8}</td>
<td>23.1 ± 2.9</td>
</tr>
<tr>
<td>3.00 x 10^{-7}</td>
<td>3.00 x 10^{-8}</td>
<td>44.2 ± 3.0</td>
</tr>
</tbody>
</table>
Figure 3-11 Derivative of Second Order Rate Law (equation 3-4) for the FITC / p-Chloroaniline Reaction
where a is the concentration of amine, b is the concentration of fluorophor and x is 95% of the limiting reagent.

\[
\frac{2.303}{a-b} \log \frac{b(a-x)}{a(b-x)} \quad (x \times 10^{-6})
\]
Conclusion

Fluorescein isothiocyanate reacted rapidly and quantitatively with a series of amine containing compounds as judged by changes in fluorescence polarization and confirmed by TLC.

Initially, polarization was expected to be useful only for larger species where the molecular volume would be large enough to depolarize fluorescence. As shown in this part of the thesis, changes in fluorescence lifetime upon conjugation allowed the thiocarbamylation reaction to be followed by fluorescence polarization even for small amines. This increases the effectiveness of the technique.

The reactions with aliphatic amines went so quickly even at the $10^{-7}$ M level that the rate constants could not be obtained. The "slow" reaction between the weak base aniline and FITC yielded a rate constant of $1.8 \times 10^5$ M$^{-1}$ min$^{-1}$ at 21.5°C. Other reactions seem to go even faster and are essentially over by the time the sample can be shaken and placed into the fluorometer. In some cases, during the first collection of data in a sequence, the intensities dropped during the collection of points and were rock steady from the next sequence 30 seconds later to the end of the experiment. This confirms the very quick reaction. Rapid reactions would allow polarization to be considered as a post-column derivatization detector for liquid chromatography.
The presence of glycerol in the reaction vessel did not interfere with the kinetics of the reactions. This made the experiments much easier since glycerol could be added with the reactants instead of placing aliquots of reaction solution into glycerol for analysis. Glycerol is needed to slow rotational depolarization and allow polarizations to be measured. A medium containing 35.1% glycerol was found to be satisfactory for determining polarizations. At this level, glycerol mixes readily with other solvents.
CHAPTER IV

DICHLOROTRIAZINYLANOFLUORESCEIN

Introduction

The reactivity of di- and trichloro-triazines has led to their widespread use in the dye industry to immobilize dyes on fabric. Fluorescein is noted for its high fluorescence efficiency and long emission wavelength that reduce the effects of background fluorescence from proteins. To improve the labeling efficiency of fluorescein to alcohols, a triazinyl chloride moiety was added to fluorescein by Barskii (54). The new compound, dichlorotriazinylaminofluorescein (DTAF - Figure 4-1) was found to have a molar extinction coefficient and fluorescence properties similar to another popular fluorescein compound, fluorescein isothiocyanate. The reaction between 5-aminofluorescein and cyanuric chloride is fast and economical with a good yield (55). The major advantages of the triazinyl derivative over the isothiocyanate are not just reaction efficiency and the ability to derivatize alcohols but also the stability and purity of DTAF with respect to FITC especially as the isothiocyanate moiety decomposes over months while the triazinyl chloride moiety is stable for years (56).
Excitation wavelength: 492 nm
Emission wavelength: 520 nm
Molar absorptivity: 82000 M$^{-1}$·cm$^{-1}$
Quantum yield: 0.6 - 0.8
Fluorescence lifetime: 4.65 ns

Figure 4-1: Structure and Fluorescence Characterizations of 5-[(4,6)-dichloro-1,3,5-triazin-2-yl)amino]-3',6'-dihydroxy-spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one, hydrochloride; dichlorotriazinylaminofluorescein;
Reactions of cyanuric chloride

Cyanuric chloride or 2,4,6-trichloro-1,3,5-triazine has been shown to react rapidly with ammonia (57), amines (58), phenols, carboxylic acids, esters, amides and Grignard reagents. A review of reactions has been written by Smolin and Rapoport (59).

Early work by Fries (58,60), Diels (57) and Serier (61) described the reaction between cyanuric chloride and various amines. All showed that individual chlorides could be replaced depending upon the temperature. Diels (57) illustrated this by adding methylamine to cyanuric chloride in a freezing bath followed by recrystallization and then adding a mole of ethylamine at a slightly elevated temperature. The third chloride remained unreacted.

Banks (62,63) reacted 2-chloro-4,6-diamino-s-triazine with aniline showing the reaction to be fast and quantitative in forming a compound with analgesic properties. He suggested a mechanism for the reaction noting that addition of acid increased the reactivity. Banks et al. then prepared several substituted s-triazine compounds using elevated temperatures to complete the replacement of the third chloride from triazine. Cuthbertson and Moffatt (65) also illustrated the effect of temperature on the reaction as they stepwise prepared a series of amine derivatives by using 0 °C, room temperature and over 100 °C to remove the three chlorides.

A group from American Cyanamid published several papers
(66-74) describing reactions between cyanuric chloride and several species illustrating the reactivity of s-triazine and the effect of temperature on replacement of chloride ions. Thurston et al. (66) prepared a series of monoamino-dichloro- and diamino-monochloro-s-triazines. The reactions went to completion in less than an hour. To prepare monoamines, temperatures were maintained around 0 °C. Diamines were prepared by allowing the reaction, which is exothermic, to warm up to room temperature. Dudley et al. (68) prepared trialkoxy, dialkoxy-monoamine and monoalkoxy-diamine derivatives of s-triazine in good yield illustrating the reactivity towards alcohols. The reaction with alcohols took longer than the reaction with amines. Schaefer et al. (69) prepared aryloxy derivatives again using temperature to control the amount of substitution taking place.

Reactions of DTAF

DTAF has been used for fluorescence immunoassay of many biologically active species in the last decade. The compound was first synthesized by Barskii (54) whose procedure was modified by Blakeslee and Baines (55) and then by Keith et al. (78).

The fluorophor was suggested (55,75-79) to have the same reactivity as monosubstituted cyanuric chloride. The reaction between DTAF and amine or alkoxy containing species is considered to be bimolecular with one mole of reagent and one mole of reactant combining (Figure 4-2). The general
Figure 4-2: Reaction Between Dichlorotriazinylamino Fluorescein (I) and Amines or Alcohols to Form Fluorescent Conjugates.
consensus is that only one species binds per DTAF at room temperature and that the reaction occurs rapidly at pH 9 (55,75-79).

Blakeslee and Baines (55) first used DTAF to modify rabbit IgG for immunofluorescence assay. They report a rapid reaction between DTAF and IgG with 70 percent of the fluorophor bound in an hour resulting in 2.4 moles DTAF per mole protein. The optimum conditions were found to be pH 9 for one hour with a 5:1 excess of fluorophor. In a second study, Blakeslee (56) reports that DTAF hydrolyzes rapidly in pH 9.0 borate buffer with 12.5 percent hydrolysis occurring in a one hour reaction period.

M.E. Jolley et al. (75) monitored DTAF-labelled aminoglycosides in a fluorescence polarization immunoassay (FPIA). A tenfold excess of aqueous aminoglycoside was added to a methanol solution of DTAF adjusted to pH 9.0. The mixture was allowed to sit for one hour at room temperature before hydrolyzed and conjugated fluorophors were separated by chromatography. They reported good agreement between FPIA and radioimmunoassay methods for aminoglycosides. In two patents, Wang, Stroupe and Jolley (76,77) described conditions for conjugation of forty antibiotics and drugs with DTAF followed by chromatography and analysis by FPIA. They report the reaction will go to completion for ligands with molecular weights between 50 and 4000 amu and at any pH between 3 and 12 although smaller ligands and pH between 6 and 9 are optimal.
Gualandnis et al. (79) labeled lectins with DTAF using 0.1 M carbonate buffer (pH 9.0) with stirring at 4 °C for four hours. They used an excess of lectin (20 µg DTAF/mg) but did not report how much labeling was achieved. Hiebert et al. (81) labeled muramyldipeptide with DTAF to study distribution of the peptide in the body. The reaction ran for 6 hours at 45 °C in a mixture of aqueous 0.4 M sodium carbonate and acetone. The conjugate was then separated from excess reagent by gel filtration resulting in a net 47 percent yield. Many groups (78,80,82) have reacted DTAF with tubulin or microtubules in vitro. The reaction is allowed to run for 5-10 minutes at 37 °C followed by centrifugation and separation by polyacrylamide gel electrophoresis.

DeBelder and Granath (83) prepared crystals of dichlorotriazinylaminofluorescein-labeled dextrans by adding solid reagent to a solution of dextran. A pH-stat was used to keep the pH at 10. After one hour, the dextran conjugate was precipitated from the aqueous solution by adding ethanol. The crystals were then dissolved in water and recrystallized by adding ethanol.

**Experimental**

DTAF (Research Organics) was dissolved in HPLC grade acetone (Fisher) to make a 1.00 x 10^{-4} M solution. 0.100 mL was added to 9.90 mL of glycerol for a polarization
spectrum. As a check of purity, TLC was performed with chloroform:methanol:acetic acid (66:33:1,v/v/v) (55). Only two spots were visible on the plate. The first was the major reagent spot and the second very faint spot was the hydrolyzed DTAF. To verify this hypothesis, DTAF was added to a solution of 0.5 M sodium hydroxide (Baker) and boiled for twenty minutes to completely hydrolyze the triazinyl chlorides (56) followed by TLC.

Preparation of derivatives

A series of amine and hydroxyl containing species were reacted with DTAF. 5.00 mL of 9.00 x 10^{-6} M DTAF in acetone was added to 5.00 mL of 9.00 x 10^{-5} M solutions of each of the following: glycine, aniline, p-chloroaniline, 17,900 MW dextran and 153,000 MW dextran dissolved in 0.1 M borate buffer and left for an hour with stirring in the dark. 5 \mu L of concentrated HCl was added to additional samples of aniline and 17,900 MW dextran to see if the reaction was assisted by acid catalysis as suggested by Banks (62,63). At the end of an hour, a TLC plate was spotted with solution and developed in chloroform: methanol: acetic acid. In addition, a 1.00 mL aliquot of the reaction mixture was added to 14 mL of aqueous glycerol to yield solutions of 35 to 76% glycerol by volume to obtain Perrin plots. Intensity data were taken with an excitation wavelength of 480 nm and emission resolved using 518 nm longpass filters.
Kinetics of DTAF Reaction

Aniline and p-chloroaniline were reacted with DTAF in the same concentrations as used in the FITC study. Both 35 and 70% glycerol by volume were utilized to increase the viscosity. In addition, attempts were made to study reactions between dextrans and DTAF.

After glycerol was suspected of being an interferent, alternative methods of including glycerol were investigated. When polarizations still did not change, dialysis was used to determine whether conjugation occurred.

Results and Discussion

Free DTAF had an Rf of 0.97 in the developer used. The hydrolyzed product, prepared by the method of Blakeslee (56) has an Rf of 0.76. The faint spot appearing on the original TLC plate had an Rf of 0.75 illustrating that the reagent is contaminated by a small amount of hydrolyzed compound. Uncorrected excitation and emission spectra of hydrolyzed and pure DTAF compounds were identical. The excitation and emission spectra of pure $1.00 \times 10^{-7}$ M DTAF in bicarbonate buffer are shown in Figure 4-3. The polarization spectrum is in Figure 4-4. Both spectra are similar to those of FITC showing that the triazinyl moiety does not affect the fluorescence properties of the fluorescein moiety.
Figure 4-3: Uncorrected Fluorescence Excitation and Emission Spectra of $1.00 \times 10^{-7}$ M Dichlorotriazinylamino Fluorescein in 0.10 M Bicarbonate Buffer.
Figure 4-4: Fluorescence Excitation Polarization Spectrum of 1.00 x 10^-6 M Dichlorotriazinylamino fluorescein in 99.9% Glycerol by Volume.
Preparation of Derivatives

Thin layer chromatography showed that all free reagent was reacted in each of the samples. The TLC of the dextran mixtures had many spots including hydrolyzed reagent. Polarizations did not change for any species (Table 4-1). This lack of polarization change with conjugation stems from the lack of change in fluorescence lifetime upon conjugation (Table 4-2). Fluorescence lifetimes of even the larger species did not change with conjugation. The fluorescein moiety is located away from the reaction center. The s-triazine ring is probably not electronically conjugated with the fluorescein pi electron system because we see no spectral change due to an increased pi system. This is illustrated by the shape and wavelengths of spectra of fluorescein, FITC and DTAF. The reaction site is even further removed and does not affect the lifetime of the fluorescein moiety. The failure to observe a change in polarization for DTAF/dextran conjugates is presumably because of independent rotation of DTAF relative to dextran resulting in the same degree of rotational depolarization for bound DTAF as free DTAF.

Effect of HCl on Reaction Rate

The rate of reagent hydrolysis clearly increases in the presence of hydrochloric acid. TLC data for the aniline/DTAF reaction mixture showed no free reagent was present in the
Table 4-1: Polarization Values for DTAF and Conjugates in Various Percentages of Glycerol.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Polarizations at each % Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35 %</td>
</tr>
<tr>
<td>DTAF</td>
<td>.0803</td>
</tr>
<tr>
<td>DTAF-aniline</td>
<td>.0783</td>
</tr>
<tr>
<td>DTAF-p-chloro aniline</td>
<td>.0772</td>
</tr>
<tr>
<td>DTAF-glycine</td>
<td>.0772</td>
</tr>
<tr>
<td>DTAF-17,900 MW dextran</td>
<td>.0765</td>
</tr>
<tr>
<td>DTAF-153,000 MW dextran</td>
<td>.0829</td>
</tr>
</tbody>
</table>
Table 4-2: Intensity Data for DTAF and Conjugates at 0.0297 Poise and 21 °C.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$I_v$</th>
<th>$I_m$</th>
<th>$I_t$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTAF</td>
<td>94.1</td>
<td>80.1</td>
<td>254.3</td>
</tr>
<tr>
<td>DTAF-aniline</td>
<td>90.7</td>
<td>77.6</td>
<td>245.8</td>
</tr>
<tr>
<td>DTAF-p-chloro aniline</td>
<td>85.7</td>
<td>73.4</td>
<td>232.6</td>
</tr>
<tr>
<td>DTAF-glycine</td>
<td>89.1</td>
<td>76.3</td>
<td>241.7</td>
</tr>
<tr>
<td>DTAF-17,900 MW dextran</td>
<td>92.0</td>
<td>78.9</td>
<td>249.9</td>
</tr>
<tr>
<td>DTAF-153,000 MW dextran</td>
<td>87.1</td>
<td>73.7</td>
<td>234.5</td>
</tr>
</tbody>
</table>
acid-catalyzed mixture but a large amount of the hydrolyzed species was present. Conversely, the uncatalyzed mixture had very little hydrolyzed reagent present. A reaction blank consisting of DTAF alone in borate buffer also had very little hydrolysis product present showing that DTAF is fairly stable in water under basic conditions agreeing with literature results (55,75). No free DTAF was found in an acid catalyzed blank mixture. These results also agree with literature reports on cyanuric chloride (62) that indicate hydrolysis is much faster in acidic media.

The polarization values after adding acid-catalyzed samples to glycerol were similar to values without HCl present (Table 4-3). No change in polarization was observed upon conjugation.

Effect of Glycerol on Reaction

Glycerol was used at 35.1 and 70.1% by volume in several kinetics experiments between aniline, p-chloroaniline and 17,900 MW dextran with DTAF. A change of slope at the 4 minute mark occurred for each experiment independent of concentrations. A reaction blank with aqueous glycerol and DTAF in buffer was run and had an identical change in slope (Figure 4-5). It is apparent that glycerol, a triolcohol, reacts fairly efficiently with DTAF. Any change in polarization from conjugate formation with analyte could be hidden by the glycerol reacting with DTAF.

In order to eliminate glycerol from the reaction vessel
Table 4-3: Polarization Values for DTAF and Conjugates in Various Percentages of Glycerol For Acid Catalyzed Reaction Samples.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Polarizations at each % Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35 %</td>
</tr>
<tr>
<td>DTAF</td>
<td>.0756</td>
</tr>
<tr>
<td>DTAF-aniline</td>
<td>.0738</td>
</tr>
<tr>
<td>DTAF-17,900 MW dextran</td>
<td>.0729</td>
</tr>
</tbody>
</table>
Figure 4-5: Change of Polarization with Time for DTAF Blank in 70.1% Glycerol. Change in Slope is Attributed to Reaction Between DTAF and Glycerol.
during kinetics measurements, a precision experiment was run to determine whether unmatched plastic cuvettes (Spectrocell) would increase error relative to rinsing and refilling a quartz cuvette. Data and results appear in Table 4-4. The results indicate that either method can offer similar precision. The plastic cuvettes were used for the kinetics experiments. In addition, the amount of waste solutions should be kept in mind. Rinsing the quartz cuvette generates roughly four times the waste. From this experiment, small changes in polarization would be hidden by the noise associated with the plastic cuvettes or rinsing quartz cuvettes.

Kinetics of the Reaction

Unfortunately, after removing glycerol from the reaction vessel the observed changes in polarization were too small to be resolved from the cuvette-to-cuvette variations. If the DTAF is rotating independently with respect to the analyte bound to it, only a small change in polarization would be expected.
Table 4-4: Comparison of Precision Between Plastic Cuvettes and Rinsing a Quartz Cuvette for Kinetics Trials

**Plastic Cuvettes:**

<table>
<thead>
<tr>
<th>Iv</th>
<th>Ih</th>
<th>It</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>73.6</td>
<td>46.6</td>
<td>166.9</td>
<td>0.225</td>
</tr>
<tr>
<td>74.9</td>
<td>47.0</td>
<td>168.9</td>
<td>0.229</td>
</tr>
<tr>
<td>72.7</td>
<td>46.3</td>
<td>165.2</td>
<td>0.222</td>
</tr>
<tr>
<td>69.7</td>
<td>45.9</td>
<td>161.5</td>
<td>0.206</td>
</tr>
<tr>
<td>76.2</td>
<td>48.2</td>
<td>172.5</td>
<td>0.225</td>
</tr>
</tbody>
</table>

mean: 73.4, Sd: 2.5

**Quartz Cuvette:**

<table>
<thead>
<tr>
<th>Iv</th>
<th>Ih</th>
<th>It</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>69.4</td>
<td>42.1</td>
<td>153.7</td>
<td>0.244</td>
</tr>
<tr>
<td>70.1</td>
<td>42.1</td>
<td>154.3</td>
<td>0.249</td>
</tr>
<tr>
<td>73.0</td>
<td>43.9</td>
<td>160.8</td>
<td>0.250</td>
</tr>
<tr>
<td>70.6</td>
<td>42.7</td>
<td>156.0</td>
<td>0.246</td>
</tr>
<tr>
<td>63.9</td>
<td>38.2</td>
<td>140.2</td>
<td>0.252</td>
</tr>
</tbody>
</table>

mean: 69.4, Sd: 3.4
Conclusions

Dichlorotrizinylaminofluorescein reacts with amines and hydroxyls but does not show a change in polarization. The reaction, therefore, can not be followed by fluorescence polarization. The probable reason for this is that the reaction site is set away from the fluorescein moiety and not coupled in any way electronically and the analyte, once bound, does not stop rotational depolarization.

During the study it was apparent that DTAF and glycerol react. It became necessary to add glycerol after the reaction of DTAF and analytes has reached its completion. The simplest method was found to be the use of plastic cuvettes instead of continual rinsing and refilling of a quartz cuvette.
CHAPTER V

DANSYL CHLORIDE

Introduction

One of the most widely used derivatizing agents for the analysis of proteins and amino acids (84, 85) is dansyl chloride (DANS - 5-dimethylaminonaphthalene-1-sulfonyl chloride - Figure 5-1). It was originally introduced by Gregorio Weber (18) as a reagent to prepare fluorescent conjugates of proteins for fluorescence polarization studies. Dansyl chloride has also been widely used to determine N-terminal amino acids by reaction followed by cleavage of the then labeled amino acid. Another characteristic of dansyl derivatives is their sensitivity to environment. Spectral shifts and fluorescence lifetime changes occur as solvents of different polarity are used (86). In many cases, spectral shifts also occur when dansyl is conjugated to an analyte. The literature contains many examples of the advantages and disadvantages of dansyl chloride as a derivatizing agent.

Dansyl Chloride/Analyte Reactions

Dansyl chloride, like other aromatic sulfonyl chlorides (84) reacts rapidly with primary and secondary amines at slightly alkaline pHs. The reaction is illustrated in Figure
Excitation wavelength 360 nm (EtOH)
Emission wavelength 520 nm (EtOH)
Molar absorptivity 3700 M\(^{-1}\cdot\text{cm}^{-1}\) (EtOH)
Quantum yield 0.5 (EtOH)
Fluorescence lifetime 12.9 ns (EtOH)
Sensitive to Environment

Figure 5-1: Structure and Fluorescence Characterizations of 5-dimethylaminonaphthalene-1-sulfonyl chloride; dansyl chloride;
5-2. Higher pHs are required for reactions with phenols and imidazoles. The reactivity is dependent on the relative basicity of the analyte species (84). The optimum pH for the reaction is between pHs 7 and 9.5 (84,87,88). The lower limit is determined by protonation of the amine or phenol to be dansylated. When the amine or phenol is protonated, the rate of dansylation reaction is slowed. The upper limit is set by the hydrolysis of the reagent (87,88,91). Gros and Labouesse (87) report that the rate of hydrolysis is constant up to pH 9 after which the rate increases very rapidly. At pHs 10 and above, hydrolysis destroys most of the reagent (Figure 5-3a). This side reaction limits the ability of dansyl chloride to react with many phenols.

Another important decomposition reaction (84,88,89,91) is common for amino acids or other species with more than one reactive site when there is a large excess of dansyl chloride. The dansyl will conjugate at both sites, then the didansylated amino acid will break apart to form an aldehyde, carbon monoxide, dansyl sulfonic acid and dansyl amide (Figure 5-3b). This latter reaction is favored by sterically hindered amino acids. Neadle and Pratt (89), using conditions that produced less than 1% dansyl amide due to decomposition of many amino acids, found 15% dansyl amide for leucine and norleucine and 45% dansyl amide for $\alpha$-amino-isobutyric acid. They found an increase in decomposition rate for species where there is a free hydrogen on the amido nitrogen and an electron donating alkyl group on the C2
Figure 5-2: Reaction Between Dansyl Chloride (I) and Amines to Form a Fluorescent Conjugate.
Figure 5-3:  

a) Decomposition of Dansyl Chloride by Hydrolysis to Dansyl Sulphonic Acid (II) at Alkaline pH's;

b) Decomposition of Didansylated α-Amino Acids.
Boyle (90) found another side reaction when using dansyl chloride in dimethylsulfoxide. The solvent reacted with reagent to form dansyl sulfonic acid and chlorodimethyl sulfite. No other solvent problems have been reported in the literature.

Dansyl Chloride as a Derivatizing Agent

As mentioned earlier, G. Weber (18) used dansyl chloride to study ovalbumin and bovine serum albumin by fluorescence polarization. Weber dissolved the dye in acetone then added it to the protein dissolved in phosphate or carbonate buffer with stirring at 0-3°C. The reaction was judged to be complete when the turbid suspension that formed upon addition of the two solutions cleared, usually 5 to 12 hours. The conjugate was then purified by dialysis against 0.15 M K$_2$SO$_4$ or 0.2 M KCl until no fluorescence was observed in the outer liquid, generally a period of two days. He found 50-60% conjugation had occurred. Weber also reported no change in lifetime occurs between pH 1.5 and 14 for the stable conjugate.

Chen (94) also studied the interaction of albumin and dansyl chloride. His labeling procedure was similar to Weber's. Albumin was dissolved in bicarbonate buffer and dansyl chloride in acetone. The reaction was allowed to proceed for 15 hours with an excess of dansyl chloride relative to albumin. Chen found that both lifetime and
quantum yield dropped until four moles of dansyl were conjugated per mole of albumin.

In 1974, Kinoshita et al. (95,96) reported the use of cycloheptaamylose to increase the solubility of dansyl chloride in water making the reaction with albumin run faster yielding an increased number of dansyl molecules per mole of protein. Another advantage of the cycloheptaamylose-dansyl chloride complex is a decrease in moisture sensitivity making it easier to handle than free dansyl chloride.

Dudich, Nezlin and Franck (97) recently studied binding of dansyl to various immunoglobulins. They measured lifetimes of IgG conjugates to be 7.2 to 7.5 ns. The human serum albumin conjugate had a lifetime of 17.7 ns.

Sikorski and Daczyriska (99) prepared a thin layer of solid dansyl chloride in the bottom of test tubes by evaporation of an acetone solution. A 1-mL sample of 10-20 mg/mL protein in 0.1 M phosphate buffer was added to the tube and it was shaken for 1-3 hours at 4 or 22° C. The reaction was stopped by removing the solution. They found the reaction rate to increase as the pH was raised from 6 to 8. A greater degree of labeling was found for albumin at the warmer temperature.

Hartley and Massey (92) first prepared labeled amino acids using reaction times of 16 to 96 hours in length depending on the amino acid. Little was done on characterizing the fluorescence of dansylated amino acids
until Chen (93) studied many of them, finding quantum efficiencies to be different for many of the conjugated amino acids as well as large shifts in emission wavelength and quantum yield depending on the solvent used. Table 5-1 illustrates Chen's results for dansyl-\textsuperscript{DL}-tryptophan in a variety of solvents.

Gros and Labouesse (87) studied the kinetics of glycine dansylation after first determining the rate of hydrolysis of dansyl chloride. They found that hydrolysis was fairly slow below pH 9.5 but the rate became rapid as pH rose above 9.5. The glycine reaction was found to have a rate constant of 3.2 M\textsuperscript{−1} \cdot \text{sec}^{-1} at 20° C. Gros and Labouesse recommend standard conditions for labeling peptides and amino acids. Equal volumes of 0.2 - 0.02 mM analyte in aqueous 0.1M bicarbonate buffer are added to 10 mM dansyl chloride in acetone. The reaction is run at room temperature taking 6 to 30 minutes for 95% dansylation to occur.

Spivak et al. (91) dissolved amino acids in potassium bicarbonate buffer and added an aliquot to an acetone solution of dansyl chloride. They reported reaction times should be kept short to preserve the dansyl amino acid or it will decompose to dansyl sulfonic acid. A reaction time of 40 minutes was shown to be sufficient to quantitate amino acids.

Baya et al. (100) reported on HPLC determination of amino acids using precolumn derivatization. A 10-20 fold excess of dansyl chloride is added to amino acid samples in
Table 5-1: Effect of Solvent on the Emission Wavelength and Quantum Yield of Dansyl-DL-tryptophan in Ethanol 0.01 M Tris buffer (1:1) at pH 7.0.¹

<table>
<thead>
<tr>
<th>solvent</th>
<th>dielectric constant (debyes)</th>
<th>quantum yield</th>
<th>emission wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td>78.5</td>
<td>0.068</td>
<td>578</td>
</tr>
<tr>
<td>methanol</td>
<td>31.2</td>
<td>0.37</td>
<td>533</td>
</tr>
<tr>
<td>ethanol</td>
<td>25.8</td>
<td>0.50</td>
<td>529</td>
</tr>
<tr>
<td>n-butanol</td>
<td>19.2</td>
<td>0.50</td>
<td>519</td>
</tr>
<tr>
<td>dimethylformamide</td>
<td>36.7</td>
<td>0.59</td>
<td>517</td>
</tr>
<tr>
<td>acetone</td>
<td>21.5</td>
<td>0.35</td>
<td>513</td>
</tr>
<tr>
<td>ethyl acetate</td>
<td>6.11</td>
<td>0.54</td>
<td>510</td>
</tr>
<tr>
<td>glycerol</td>
<td>42.5</td>
<td>0.18</td>
<td>553</td>
</tr>
<tr>
<td>ethylene glycol</td>
<td>37</td>
<td>0.36</td>
<td>543</td>
</tr>
<tr>
<td>propylene glycol</td>
<td>32</td>
<td>0.37</td>
<td>538</td>
</tr>
</tbody>
</table>

¹ excitation wavelength 335 nm.
titrisol buffer. The reaction goes to completion in less than one hour using $10^{-8}$ M amino acids. At pH 9.0, hydrolysis was not reported to be a problem when the reagent was present in 10-fold excess.

Tapuhi et al. (88) also studied amino acids by HPLC. They found that the amount of conjugate that formed was dependent upon the ratio of DANS-C to amino acid. A 10:1 ratio gave satisfactory results without much decomposition by didansyl amino acid formation. Many reactions went to maximum yield in less than 30 minutes including proline (6 minutes) and tryptophan (7 minutes). In addition, the effect of UV irradiation on 5 dansylated amino acids was studied. Upon irradiation of the samples, intensities of the DANS-AA samples were reduced and the amount of DANS-NH$_2$ formed increased. For most of the samples, 86% of the intensity remained after one hour of irradiation and 64% after 3 hours. DANS-methionine dropped to 63% and 11% for one and three hours of irradiation, respectively. The authors recommend keeping the samples and the reaction away from light.

Other amine-containing species have been studied by chromatography after derivatization with dansyl chloride. Frei and Lawrence (101-106) labeled carbamates at 45° using conditions similar to the recommended conditions of Gros and Labrouesse (87). The carbamates were first hydrolyzed to methyamine and substituted phenols before labeling with DANS-C was carried out. Due to increased temperatures,
hydrolysis of the reagent was also important. They report the coupling reaction proceeds faster than hydrolysis of carbamate or of reagent. The carbamates were then quantitated by TLC (101-103), HPLC (104) or fluorimetry (105-106).

Nachtmann, Spitzy and Frei (107) studied alkaloids and adrenaline by conjugation to dansyl. The drugs were dissolved in buffer; then a solution of dansyl chloride dissolved in acetone was added. The mixture was stirred in the dark at various elevated temperatures for 20 minutes. The amount of water was held under 60% to reduce the rate of hydrolysis. Wavelength shifts were found to depend upon the drug bound to dansyl. All derivatives were resolved by TLC with a solvent system consisting of 3:1 benzene:methanol (v/v).

**Experimental**

Dansyl chloride was obtained from Sigma Chemical Co. (St.Louis, MO) and used without further purification. A 0.0100 M stock solution was prepared by weighing 0.0270 grams of dansyl chloride into a 10 mL volumetric flask and diluting with spectrophotometric grade acetone. The solutions were stored in a freezer when not in use. Using step dilutions, a 1.00 x 10^{-6} M DANS solution in 99.9% glycerol by volume was prepared. A polarization spectrum was
collected as described in chapter II. Emission wavelengths were selected using longpass filters with 50% transmittance at 442 and 447 nm respectively for channels A and B.

Dansylated Amino Acids

Dansylated aspartic acid, glycine, phenylalanine and tyrosine were purchased from Sigma and used without further purification. Stock solutions (1 x 10^-3 M) were prepared for each compound in absolute ethanol. For fluorescence spectra, 1.00 x 10^-5 M solutions were also prepared in absolute ethanol. Vertical intensity data were collected using the MINC. Perrin plots were constructed from data obtained by adding 0.100 mL of the latter solutions into class A volumetric flasks and filling with glycerol solutions. Final volume percentages of glycerol were 35 to 70%. To collect the data, fluorescence was excited at 400 nm. Emission was resolved by longpass filters with 50% transmittance at 442 and 447 nm for channels A and B respectively. Ten intensity values were averaged for each channel and polarizer setting.

Reaction Between Polyethyleneimine and Dansyl Chloride

Polyethyleneimine had been purchased from Aldrich as a 50% aqueous solution. The density was determined by carefully filling a tared 10 mL volumetric flask with the 50% solution and weighing. The density was found to be 1.076 g/mL. When preparing solutions, a 0-100 μL positive
displacement pipet (Gordon-Keeble) was used to measure the volume of polyethylene imine required as calculated from the density assuming a molecular weight of 55000 g·mol.

A 2.00 x 10⁻² M solution of polyethyleneimine was prepared in 0.1 M sodium bicarbonate buffer. A 20.0 mL aliquot was placed in a 125 mL Erlenmeyer flask fitted with a ground glass stopper, then 20.0 mL of 2.00 x 10⁻³ M dansyl chloride in ethanol was added. Parafilm was used to seal the flask which was placed on a magnetic stirrer in a darkroom for 90 minutes. The mixture was then placed into 12-14,000 MW cutoff dialysis tubing (Spectrapor) and dialyzed in 0.1 M bicarbonate buffer for 48 hours. A calibration curve was constructed using solutions of dansyl chloride in bicarbonate buffer. These were used to measure the concentration of dansyl in the dialysate on the Spectronic 200 UV spectrophotometer.

Glycerol solutions were then used to construct Perrin plots of the conjugate and free reagent using excitation at 356 nm and emission resolved by 493 nm longpass filters.

**Kinetic study:** Solutions of polyethyleneimine and dansyl chloride having concentrations of 1.00 x 10⁻⁵, 5.00 x 10⁻⁶, and 1.00 x 10⁻⁶ M were prepared. The polyethyleneimine solutions were made with 30% glycerol in bicarbonate buffer or in the buffer alone. For each kinetics trial, 2.50 mL of polyethyleneimine was placed into a cuvette and 0.250 mL of dansyl chloride was added with shaking. The reaction was allowed to proceed for 45-75 minutes depending on the
Results and Discussion

The uncorrected fluorescence excitation and emission spectra of 1.00 x 10^{-5} M DANS in absolute ethanol (Figure 5-4) and polarization spectrum of 1.00 x 10^{-6} M DANS in 99.9% glycerol (Figure 5-5) are similar to those in the literature.

Dansylated Amino Acids

The fluorescence spectra obtained for the dansylated amino acids in ethanol showed slight shifts in wavelengths compared to dansyl chloride. The excitation/emission wavelengths are in Table 5-2.

Polarization data collected to construct Perrin plots are listed in Table 5-3. Polarizations are similar for all species. After looking at the total intensities (Table 5-4), this is not surprising. The intensities are almost identical indicating lifetimes are similar for these conjugates with the possible exception of dansyl tyrosine which had intensities consistently lower than all the other species. Without any change in lifetime for analytes of approximately the same size, a change in polarization is not expected. All conjugates show increased total intensity as the percentage of glycerol increases. This illustrates the effect of
Figure 5-4: Uncorrected Fluorescence Excitation and Emission Spectra of $1.00 \times 10^{-5}$ M Dansyl Chloride in Absolute Ethanol. Excitation: 360 nm; Emission: 516 nm.
Figure 5-5: Fluorescence Excitation Polarization Spectrum of 1.00 x 10^-6 M Dansyl Chloride in 99.9% Glycerol.
Table 5-2: Excitation and Emission Wavelengths of Dansyl Chloride and four Dansyl Amino Acids. All Solutions are $1.00 \times 10^{-5}$ M in Absolute Ethanol

<table>
<thead>
<tr>
<th>compound</th>
<th>excitation</th>
<th>emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>dansyl chloride</td>
<td>360 nm</td>
<td>516 nm</td>
</tr>
<tr>
<td>dansyl aspartic acid</td>
<td>344 nm</td>
<td>506 nm</td>
</tr>
<tr>
<td>dansyl glycine</td>
<td>350 nm</td>
<td>504 nm</td>
</tr>
<tr>
<td>dansyl phenylalanine</td>
<td>348 nm</td>
<td>504 nm</td>
</tr>
<tr>
<td>dansyl tyrosine</td>
<td>335 nm</td>
<td>496 nm</td>
</tr>
</tbody>
</table>
Table 5-3: Polarization of Dansyl Chloride and four Dansyl Amino Acids in Various Percentages of Glycerol. All Solutions are $1.00 \times 10^{-7}$ M.

<table>
<thead>
<tr>
<th>compound</th>
<th>Polarization at each % glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35.1%</td>
</tr>
<tr>
<td>DANS-chloride</td>
<td>.159</td>
</tr>
<tr>
<td>DANS-aspartic acid</td>
<td>.203</td>
</tr>
<tr>
<td>DANS-glycine</td>
<td>.189</td>
</tr>
<tr>
<td>DANS-phenylalanine</td>
<td>.157</td>
</tr>
<tr>
<td>DANS-tyrosine</td>
<td>.191</td>
</tr>
</tbody>
</table>
### Table 5-4: Total Intensities of Dansyl Chloride and four Dansyl Amino Acids in Various Percentages of Glycerol. All Solutions are 1.00 x 10^{-7} M.

<table>
<thead>
<tr>
<th>compound</th>
<th>Total Intensities at each % glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35.1%</td>
</tr>
<tr>
<td>DANS-chloride</td>
<td>43.0</td>
</tr>
<tr>
<td>DANS-aspartic acid</td>
<td>43.0</td>
</tr>
<tr>
<td>DANS-glycine</td>
<td>42.3</td>
</tr>
<tr>
<td>DANS-phenylalanine</td>
<td>46.8</td>
</tr>
<tr>
<td>DANS-tyrosine</td>
<td>41.9</td>
</tr>
</tbody>
</table>
solvent composition on the lifetime or quantum yield of the
dansyl moiety (86) and is probably due to a slower rate of
internal conversion caused by increased viscosity. Lifetime
corrected Perrin plots are shown in Figures 5-6 and 5-7 for
the dansylated amino acids. For comparison, dansyl chloride
appears in both figures.

Reaction Between Polyethyleneimine and Dansyl Chloride

The reaction between 0.0400 mmol of polyethyleneimine
and dansyl chloride proceeded to an endpoint. After
exhaustive dialysis, the inner buffer fluoresced under UV
irradiation indicating conjugation had occurred. All
retentate was combined and the total volume measured. An
aliquot was quantitated against dansyl chloride standards in
bicarbonate buffer (Table 5-5). The molar absorptivity by
linear regression for dansyl in 0.1 M bicarbonate buffer is
3460 M⁻¹•cm⁻¹. The concentration of dansyl in the dialysate
is 2.12 x 10⁻⁵ M corresponding to 0.0239 mmol of dansyl
chloride. The inner buffer, therefore, has 0.0161 mmol of
conjugate formed corresponding to 40% reaction. The volume
of inner buffer was increased to 75.0 mL to yield a solution
2.15 x 10⁻⁴ M in conjugate. An equivalent concentration of
reagent was prepared for a Perrin plot. Data appear in
Tables 5-6 and 5-7 and Figure 5-8. The data illustrate many
things. First, the difference in intensities for free
reagent relative to the conjugate indicates that a large
change in lifetime occurs upon conjugation. Using Weber's
Figure 5-6: Lifetime Corrected Perrin Plots of Dansyl Chloride (*), Dansyl Aspartic Acid (o) and Dansyl Glycine (x).
Figure 5-7: Lifetime Corrected Perrin Plots of Dansyl Chloride (*), Dansyl Phenylalanine (o) and Dansyl Tyrosine (x).
Table 5-5: Calibration Curve for Dansyl Chloride in 0.1 M Bicarbonate Buffer. Molar Absorptivities Calculated by Beers Law.

<table>
<thead>
<tr>
<th>[DANSCL]</th>
<th>Absorbance</th>
<th>Molar Absorptivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.50 x 10^{-4}</td>
<td>0.858</td>
<td>3430 M^{-1}</td>
</tr>
<tr>
<td>2.00 x 10^{-4}</td>
<td>0.708</td>
<td>3540 M^{-1}</td>
</tr>
<tr>
<td>1.00 x 10^{-4}</td>
<td>0.349</td>
<td>3490 M^{-1}</td>
</tr>
<tr>
<td>5.00 x 10^{-5}</td>
<td>0.172</td>
<td>3440 M^{-1}</td>
</tr>
<tr>
<td>retentate</td>
<td>0.076</td>
<td>-</td>
</tr>
</tbody>
</table>

calibration curve: \( Y = 3462 \times X + 0.00245 \)
Table 5-6: Total Intensities for $2.15 \times 10^{-6}$ M Dansyl Chloride and Dansyl Polyethyleneimine.

<table>
<thead>
<tr>
<th>Viscosity (poise)</th>
<th>Total Intensity</th>
<th>Lifetime conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DANS</td>
<td>PEI</td>
</tr>
<tr>
<td>0.0821</td>
<td>68.3</td>
<td>184.6</td>
</tr>
<tr>
<td>1.112</td>
<td>75.3</td>
<td>193.4</td>
</tr>
<tr>
<td>1.1507</td>
<td>81.8</td>
<td>222.5</td>
</tr>
<tr>
<td>1.2248</td>
<td>90.1</td>
<td>179.3</td>
</tr>
<tr>
<td>Viscosity (poise)</td>
<td>Polarization DANS-C</td>
<td>Polarization DANS-PEI</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>.0821</td>
<td>0.0426</td>
<td>0.258</td>
</tr>
<tr>
<td>.1112</td>
<td>0.0524</td>
<td>0.269</td>
</tr>
<tr>
<td>.1507</td>
<td>0.0626</td>
<td>0.282</td>
</tr>
<tr>
<td>.2248</td>
<td>0.0809</td>
<td>0.302</td>
</tr>
</tbody>
</table>
Figure 5-8: Lifetime Corrected Perrin Plots of Dansyl Chloride (*) and Dansyl Polyethyleneimine (o).
value of 14 ns for the lifetime of dansyl chloride in glycerol (18), the calculated lifetime of the conjugate is 40 ± 4 ns. The lifetime corrected volume calculated using the Perrin equation is 25.7 L·mole⁻¹ using a Po value of 0.363 at 356 nm.

Kinetic study: With the large polarization obtained for the Perrin plot for the conjugate, it was hoped that the reaction could be followed by polarization. The results obtained from the kinetics experiment, however, are purely qualitative.

For all experiments where glycerol was present, a change in slope occurred at the 5-8 minute mark after a rapid increase in polarization and intensity. The intensities and polarizations continued to rise throughout the experiments without a second change in slope. This indicates that the reaction was continuing but at a relatively slow rate and probably at several different sites on the polyethyleneimine. The experiments where glycerol was not present had the increasing intensities but no changes in slope. The rationale for this is that conjugation leads to a less polar environment so the conjugate fluoresces more efficiently. Dialysis of the samples indicated some conjugate was present after the experiment but the amount could not be quantitated.
Conclusion

Dansyl chloride is more difficult to work with than either of the fluorescein compounds because of its sensitivity to environment. In addition, the reactions appear to go to completion more slowly than the fluorescein species. DANS-Cl also has a poorer molar absorptivity and quantum efficiency than fluorescein.

Lifetime changes are not an important factor for the smaller analytes studied. Of the dansyl amino acids studied, only one, dansyl tyrosine, had a significant change in lifetime upon conjugation. The lifetime of reagent and conjugates is very dependent upon the amount of glycerol present.

The dansyl conjugate of polyethyleneimine was formed. The lifetime of the conjugate was 40 ns in aqueous glycerol. During the kinetic experiments, it was apparent that the kinetics were complex and that more than one site reacted.
CHAPTER VI

DANSYL HYDRAZONE

Introduction

The utility and reactivity of dansyl chloride prompted R. Chayan et al. (109) to have a hydrazine derivative of dansyl prepared as a reagent for carbonyl compounds. The new compound, dansyl hydrazine (DANSH - Figure 6-1), has since been used to sensitively determine many carbonyl containing species. It shares many of dansyl chloride's advantages and disadvantages. Both react rapidly and selectively under mild conditions but both also degrade under ultraviolet light and are subject to several side reactions.

Reactions of Hydrazines and Carbonyl Compounds

There are many examples of reactions between carbonyls and hydrazines or other amines (110). The accepted mechanism for the reaction between hydrazines and carbonyl compounds to form imines or Schiff bases (110-114) has two steps (Figure 6-2). The first step is addition as the nitrogen lone pair attacks the carbonyl carbon while the carbonyl oxygen adds a proton from the nitrogen. The next step is dehydration of the carbinolamine intermediate to form the hydrazone. Depending upon the reaction conditions used, either step may be rate determining (111-114). R. Moscovici
**Figure 6-1:** Structure and Fluorescence Data for 5-dimethylaminonaphthalene sulfonyl hydrazine; Dansyl Hydrazine.
Figure 6-2: Reaction Between Dansyl Hydrazine (I) and a Carbonyl Containing Species to form a Dansyl Hydrazone (III) via a Carbonilimine Intermediate (II).
et al. (114) report that under acidic conditions, the first step is the slow step, presumably due to protonation of the hydrazinyl nitrogen. Under neutral or basic conditions, dehydration is the slow step. This was illustrated by the authors using phenylhydrazine with three different carboxaldehydes. Moscovici et al. also calculated rate constants for the reaction as a function of pH. The maximum rate was found in highly acidic media. At pH 1, the rate constant was $10^4 \text{ M}^{-1} \text{ min}^{-1}$. The constant dropped steadily to a plateau between pH's 5 and 6 ($k \approx 10^1 \text{ M}^{-1} \text{ min}^{-1}$) finally dropping down to the slowest rate at pH's greater than 8 ($k = 10^{-1} \text{ M}^{-1} \text{ min}^{-1}$). The rate constant at the plateau varied depending on the aldehyde used. The reactions were run in 20% aqueous ethanol at 25°C. Aldehyde was the limiting reagent with concentrations of $5 \times 10^{-5}$ M. Based on the curve shape, Moscovici and coworkers determined that the rate determining step in acidic media was acid catalyzed attack of the nucleophile. In neutral or basic media, the rate determining step was acid catalyzed dehydration of the carbinolimine intermediate (114).

**Reactions of Dansyl Hydrazines**

Dansyl hydrazine has been widely used in the past decade as a carbonyl specific label for forming many conjugates prior to chromatography. Recent applications include conjugates of sugars (116) and steroids (117) separated by TLC, gel electrophoretic separation of
glycoprotein conjugates (118) and HPLC separations of sugars (119,120), steroids (121-123), glycoconjugates (124), bile acids (125-126) and pesticides (104). The common aspects of the derivatizations are the use of ethanol to make a stock dansyl hydrazine solution and the use of slightly acidic conditions for the reactions. Most researchers also used elevated temperatures.

Chronologically, the earliest reactions involving DANSH were reported by Chayan et al. (109). They reacted dansyl hydrazine with ketone-containing steroids using the same conditions as used by Smith and Fuell (115) for a reaction between ketosteroids and isonicotinic acid hydrazide. Conditions for the reaction depend upon the reactivity of the ketone. Chayan and coworkers found that conjugated ketones will react at room temperature but 17-ketosteroids require significantly higher temperatures. They added equal volumes of slightly acidic ethanol and DANSH dissolved in ethanol to dry steroid. For the conjugated steroids, cortisol and testosterone, the mixture was left for 75 minutes in the dark at room temperature, then separated by thin layer chromatography on Kieselgel G plates using ethanol:chloroform (5:95) developer. The spot was recovered by scraping and dissolving in absolute ethanol followed by centrifugation and fluorometric analysis. A linear relation was obtained for 2-20 µg steroid in the sample. The 17-keto steroid was heated in a boiling water bath for ten minutes followed by extraction and subsequent chromatography and
fluorometric analysis as for the other sample. In order to stabilize the fluorescence of the dansyl group, triethanolamine was added to the Kieselgel slurry used to prepare the TLC plates. Chayan et al. also note the applicability of the technique to simple aldehydes and ketones, dependent upon a suitable system to separate the derivatives from each other and from excess reagent.

R.W. Frei and J.F. Lawrence (104) used elevated temperatures and glacial acetic acid to form dansyl hydrazone derivatives of hexanal and 3-hydroxy-2-butanone for analysis by HPLC after evaporation and dissolution in benzene. They report that the reagents all come out with the dead volume or slightly after. P. Weber and L. Hof (124) oxidized glycoproteins to form carbonyl groups and then reacted them with dansyl hydrazine in acetate buffer at room temperature for a variety of reaction times. They reported that the reaction goes to completion within two hours. A.E. Eckhardt et al. (118) separated glycoproteins by gel electrophoresis and then oxidized and stained them in situ. The staining with dansyl hydrazine in slightly acidified DMSO took 2 hours in a 60°C water bath. G.Avigad (116) formed hydrazones of 18 reducing sugars in solutions acidified with trichloroacetic acid at 80°C for ten minutes. Reasonable separation was achieved using 5 different TLC developing solutions. In general Avigad found good reaction efficiency for aldoses but relatively poor yields for the hydrazones of 2-amino-2-deoxy aldose and for the ketoses
α-fructose and L-sorbose even upon heating to 100°C. W.F. Alpenfels (119) followed Avigad's procedure for labelling various sugars prior to HPLC separation as did K. Mopper and L. Johnson (120).

W. Funk et al. (117) reacted cortisol with dansyl hydrazine at room temperature in the dark for 30 minutes in ethanol with a trace of trichloroacetic acid added. After evaporating under nitrogen, the residue was redissolved in ethanol and subjected to HPTLC. A preservative was added to stabilize fluorescence. T. Kawasaki et al. (121-123) labelled a variety of steroids using various experimental conditions and reagents. They reported sensitive determinations of each dansyl derivative during subsequent HPLC analysis.

**Experimental**

Dansyl hydrazine was purchased from Molecular Probes (Eugene, OR). A 0.0100 M stock solution was prepared in absolute ethanol. To test compound purity, a sample was spotted on a TLC plate and developed using chloroform: tertiary butanol: acetic acid (5:4:1). All TLC plates were oven dried for 2-4 hours at 110°C prior to use. Avigad (116) has used this system to separate dansyl hydrazine and dansyl hydroxide, a common side reaction product. Bright fluorescence was emitted from the solvent front under UV irradiation. This is where Avigad indicated pure dansyl
hydrazine would be. Nothing was visible near Rf 0.3 where the hydroxide form was reported to be. A second spot of emission showed very dimly at the point of spotting. Both spots were scraped off the plate, dissolved in ethanol and centrifuged. Fluorescence and polarization spectra were obtained as described in chapter II. Due to the low fluorescence efficiency of dansyl hydrazine, a 1.00 x 10^{-6} M solution in ethanol was used for the fluorescence spectra and a 1.00 x 10^{-5} M solution in 99.1% glycerol was used for the polarization spectrum.

**Reaction of Dansyl Hydrazine with carbonyl compounds**

The labeling conditions used for this project were similar to those of Chayan et al. (109). Acidified ethanol was prepared by adding 0.325 mL conc. HCl to 500 mL of absolute ethanol. A group of four different carbonyl containing compounds was found around the department including an aldehyde (anisaldehyde), a ketone (acetonaphthone), an ester (benzyl acetate) and a carboxylic acid (lauric acid) (Figure 6-3). 0.100 M stock solutions of each of the compounds were prepared using absolute ethanol. Each sample was diluted 1:10 with the acidified ethanol. Ten milliliters of the latter solution were placed in a 125 mL Erlenmeyer flask followed by 10 mL of stock DANSH solution. A TLC plate was spotted with sample, then the flasks were covered and left on magnetic stirrers in a photographic darkroom for a period of two hours. At the end of two
Figure 6-3: Structures of the Carbonyl Containing Compounds used for this Project.
hours, another TLC plate was spotted with each sample. The TLC plates were developed in chloroform: t-butanol: acetic acid as used earlier. Also at the end of two hours, 0.100 mL samples were placed into 10 mL volumetrics which were filled to the mark with glycerol solutions. Final percentage glycerol was 55.8, 60.6, 65.4 and 70.4 percent. A sample of reagent was also treated similarly and added to the glycerol solutions. Perrin plots were obtained as described earlier.

**Energy Transfer**

In an effort to illustrate that the reaction had indeed occurred, fluorescence spectra were obtained from each of the reaction mixtures after diluting 0.025 mL of reaction mixture in a 10 mL volumetric flask using a 1:1 mixture of acidic ethanol and absolute ethanol made to mimic the solvents already in the reaction mixture. Absorption measurements were taken initially in order to find the dilution required to yield an absorbance of roughly 0.1. In addition, absorption spectra were obtained for solutions diluted .05 : 10 with the same solvent mixture.

**Kinetics Study**

Anisaldehyde was chosen for the kinetic study in order to use spectral changes accompanying conjugation to assist in the rate determination. The reaction was run in 65.5% glycerol to boost the viscosity and increase polarization
changes that might occur. This viscosity was based on the Perrin plots obtained with dansyl derivatives.

Solutions of $3 \times 10^{-4}$ M anisaldehyde and $3 \times 10^{-5}$ M DANSH were prepared from stock solutions. The former was diluted using acidic ethanol, the latter using absolute ethanol. After further dilution to eliminate inner filter effects, fluorescence spectra of both solutions illustrated that the anisaldehyde fluorescence was too weak to interfere.

A 0.01 mL aliquot of anisaldehyde was added to 0.01 mL of acidic ethanol and 2.97 mL of glycerol solution in a cuvette. The reaction started when 0.01 mL of DANSH solution was added to the cuvette. Data were collected for 45 minutes using the MINC to control data collection. During the experiment 35 sets of intensity data points were collected. Between each data point collection, the cuvette was inverted to insure mixing of reactants and the excitation beam was shuttered to stop the degradation of the dansyl group which occurs with UV light. Before beginning the kinetics experiment, currents and voltages to the PMTs were set using a dansyl hydrazine blank prepared in the same manner as the reaction mixture except with acidic ethanol instead of anisaldehyde solution. Intensity data for blank solutions of dansyl hydrazine, glycerol and anisaldehyde were stored in the data file. Excitation was at 311 nm for one trial and at 353 nm for a second. Emission was resolved by a pair of 518 nm longpass filters. Concentrations of reactants in the
Cuvette at the start of the reaction were $1.00 \times 10^{-6}$ M aldehyde and $1.00 \times 10^{-7}$ M dansyl hydrazine.

In addition, two other experiments with initial concentrations $3.00 \times 10^{-6}$ M aldehyde/ $3.00 \times 10^{-7}$ M dansyl hydrazine and $3.00 \times 10^{-7}$ M aldehyde/ $3.00 \times 10^{-8}$ M dansyl hydrazine were run. For these experiments, the excitation wavelength was 353 nm.

**TLC Developer Determination**

Complete separation of the derivative and reagent using literature conditions were not successful. The sample used for testing the solvent systems was a 1:1 mixture of stock DANSH solution and a 1 day old reaction mixture of anisaldehyde conjugate. Various combinations of chloroform:methanol:acetic acid (50:49:1, 66:33:1, 85:14:1) and hexane:tertiary butanol:acetic acid (25:74:1, 55:44:1, 85:14:1) were prepared to find a reasonable solvent strength that would separate the two materials. The best resolution was observed using the 55:44:1 mixture of hexane:t-butanol:acetic acid but overlap still occurred. Consultation with colleagues (127) indicated that solvent systems consisting of ethyl acetate and hexanes separated many hard to resolve species including derivatives and enantiomers. Four ethyl acetate:hexane mixtures were prepared, 0.5:1, 2:1, 3:1 and 5:1.
**Effect of Glycerol on Reaction**

After determining the best solvent system to separate the derivative from the reagent, two reactions were run between dansyl hydrazine and anisaldehyde to compare the results of the reaction in ethanol media and in ethanol/aqueous glycerol media. TLC plates were spotted and developed every 15 minutes during the first hour of the reaction. The nonglycerol sample was resolved successfully, however, the other samples were not adequately resolved from the glycerol even when two-dimensional chromatography was used (10,128). The solvents for two-dimensional TLC were water or methanol for the first separation to remove the glycerol from the dansyl compounds and then ethyl acetate: hexane to separate the derivative and reagent.

Excitation and emission spectra were obtained from diluted samples. Solutions were prepared with added glycerol to make sure any spectral changes were not due to solvent effects.

**Results and Discussion**

The fluorescence and polarization spectra for DANSH are illustrated in Figures 6-4 and 6-5. Excitation and emission peaks for the compound appear at 347 nm and 521 nm, respectively, in ethanol. These values agree with literature results. The two spots appearing on the TLC plate for the stock solution also have been observed previously (116,117).
Figure 6-4: Uncorrected Fluorescence Excitation (---) and Emission (----) Spectra of $1.00 \times 10^{-6}$ M Dansyl Hydrazine in Ethanol.
Figure 6-5: Excitation Polarization Spectrum of $1 \times 10^{-5}$ M Dansyl Hydrazine in 99.1% Glycerol.
After scraping and redissolution in ethanol, the spot which did not migrate up the plate did not fluoresce to a measurable extent. Accordingly, reagent purification was not undertaken.

**Affect of Glycerol on Reaction**

When glycerol was added to the reaction medium, TLC information could not be obtained because the spots were not resolved from the glycerol. Two dimensional chromatography eventually separated the free reagent and conjugate spots from the glycerol but not from each other. The separation from glycerol indicates there was no reaction between dansyl hydrazine and glycerol.

Emission spectra indicated that the products of the reaction between anisaldehyde and dansyl hydrazine were identical when the reaction was run in an ethanol or ethanol/aqueous glycerol media. Data appear in Table 6-1. The emission wavelength shift with respect to the reagent is the same in various media indicating that the conjugate has formed.

Because glycerol did not react with DANSH, 65.4% glycerol was added to the solution for the kinetics trial to increase the polarization change without worrying about any glycerol/DANSH conjugate formation.
Table 6-1: Excitation/Emission spectral data illustrating the effect of glycerol on intensity (I) and wavelength of dansyl anisaldehyde conjugates.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Excitation (nm)</th>
<th>I</th>
<th>Emission (nm)</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>one(^1).</td>
<td>354</td>
<td>20.9</td>
<td>518</td>
<td>20.4</td>
</tr>
<tr>
<td></td>
<td>356</td>
<td>19.9</td>
<td>516</td>
<td>19.9</td>
</tr>
<tr>
<td></td>
<td>358</td>
<td>19.1</td>
<td>534</td>
<td>19.1</td>
</tr>
<tr>
<td>two(^2).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no glycerol added</td>
<td>356</td>
<td>19.9</td>
<td>516</td>
<td>19.9</td>
</tr>
<tr>
<td>with glycerol added</td>
<td>358</td>
<td>19.1</td>
<td>534</td>
<td>19.1</td>
</tr>
<tr>
<td>Dansyl hydrazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no glycerol added</td>
<td>354</td>
<td>17.7</td>
<td>552</td>
<td>16.8</td>
</tr>
<tr>
<td>with glycerol added</td>
<td>352</td>
<td>17.4</td>
<td>556</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td>364</td>
<td>17.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. reaction solvents: aqueous ethanol with 65.4% glycerol (v/v) present.
2. reaction solvent: ethanol.
Reaction of Dansyl Hydrazine with Carbonyl Compounds

Only two spots appeared on the TLC plates both before and after the reaction using Avigad's conditions (116). The first spot was the completely retained spot which again did not fluoresce after redissolution. The other spot had approximately the same retention distance on the plate under UV irradiation as pure dansyl hydrazine.

These results can be explained in two ways: 1) Very little product formed resulting in a spot too dim to be visualized or 2) product was not being separated from reagent. The former explanation would require that less than one percent of the DANSH reacted (as determined by spotting and developing various concentrations of DANSH).

Results of experiments in glycerol appear in Tables 6-2 and 6-3. One sample, acetonaphthone, precipitated upon addition of the glycerol solution. The evidence indicates that some reaction occurred because of polarization and intensity changes. The Perrin plots obtained are shown in Figure 6-6. There was no way to determine the extent of the reaction so lifetimes could not be calculated, however, the large change in polarization for the anisaldehyde conjugates indicate that there is a change in lifetime. The dansyl chloride study using dansyl amino acids did not have a change in lifetime for most of the conjugates. It is possible, however, that there could be a slight change in lifetime for DANSH conjugates because a different linkage is created. A change in lifetime would explain the changes in
Table 6-2: Experimental Intensities of Dansyl Conjugates in Various Percentages of Glycerol

<table>
<thead>
<tr>
<th>Dansyl Conjugate</th>
<th>Total Intensity at each % glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55.8%</td>
</tr>
<tr>
<td>.. hydrazine</td>
<td>32.7</td>
</tr>
<tr>
<td>.. anisaldehyde</td>
<td>25.6</td>
</tr>
<tr>
<td>.. benzyl acetate</td>
<td>33.1</td>
</tr>
<tr>
<td>.. lauric acid</td>
<td>32.6</td>
</tr>
<tr>
<td>.. acetonaphthone</td>
<td>-2</td>
</tr>
</tbody>
</table>

1. excitation: 350 nm; emission: longpass 493 nm filter
2. acetonaphthone solutions precipitated upon addition of glycerol.
<table>
<thead>
<tr>
<th>Dansyl Conjugate</th>
<th>Polarizations at each % glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>55.8%</td>
</tr>
<tr>
<td>hydrazine</td>
<td>0.0514</td>
</tr>
<tr>
<td>anisaldehyde</td>
<td>0.0797</td>
</tr>
<tr>
<td>benzyl acetate</td>
<td>0.0484</td>
</tr>
<tr>
<td>lauric acid</td>
<td>0.0518</td>
</tr>
<tr>
<td>acetonaphthone</td>
<td></td>
</tr>
</tbody>
</table>

1. excitation: 350 nm; emission: longpass 493 nm filter
2. Acetonaphthone solutions precipitated upon addition of glycerol.
Figure 6-6: Perrin Plots of Dansyl Hydrazine (x), Dansyl Anisaldehyde conjugate (o), Dansyl Benzyl Acetate conjugate (*) and Dansyl Lauric Acid conjugate (.).
polarizations observed for these samples.

Energy Transfer

Absorption spectra were obtained for each of the dansyl hydrazine derivatives. The concentrations of each species after dilution with 1:1 acidic ethanol: ethanol were $2.5 \times 10^{-5}$ M. All of the samples showed strong absorbance around 220 nm. This is due to the naphthalene moiety ($\epsilon=133,000$ in alcohol). There were a variety of other wavelength bands depending upon the compound. Of interest were the bands around 290 nm and the differences in absorbance for the band ca 220 nm (Table 6-4). It should be noted from the data that two conjugates, anisaldehyde and acetonaphthone, have much greater absorbances ca 290 nm than the original reagent or the other derivatives. Both of these species have a benzene ring adjacent to the carbonyl group conjugated to dansyl. It is believed that the presence of the benzene ring affects the absorption spectrum.

To test this hypothesis, excitation spectra were obtained for all the samples (Figure 6-7). The two derivatives in question had two distinct excitation wavelengths compared to a single excitation wavelength for the reagent and other derivatives. This is supportive evidence that absorption occurs followed by energy transfer to the dansyl moiety which would lead to an increased signal size.
Table 6-4: Absorbance Readings around 220 and 290 nm for Dansyl Hydrazine and four Dansyl Hydrazones.

<table>
<thead>
<tr>
<th></th>
<th>A ca 220 nm</th>
<th>A ca 290 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>dansyl hydrazine</td>
<td>219</td>
<td>288</td>
</tr>
<tr>
<td>DNS-anisaldehyde</td>
<td>220</td>
<td>280</td>
</tr>
<tr>
<td>DNS-benzyl acetate</td>
<td>220</td>
<td>286</td>
</tr>
<tr>
<td>DNS-lauric acid</td>
<td>225</td>
<td>285</td>
</tr>
<tr>
<td>DNS-acetonaphthone</td>
<td>225</td>
<td>288</td>
</tr>
</tbody>
</table>

1. all solutions are $2.5 \times 10^{-5}$ M in 1:1 acidic ethanol : ethanol.
Figure 6-7: Excitation Spectra of Dansyl Hydrazine (1) and Dansylated Conjugates of Acetonaphthone (2), Anisaldehyde (3), Benzyl Acetate (4) and Lauric Acid (5).
**TLC Solvent Determination**

The best separation of dansyl-anisaldehyde conjugate from free reagent was achieved using a 2:1 ethyl acetate:hexanes solvent system. *R*<sub>f</sub> values of 0.70 for dansyl hydrazine and 0.85 for the dansyl-anisaldehyde conjugate were measured. Only one spot was observed in the reaction mixture alone indicating the reaction had reached completion.

**Kinetics Study**

When anisaldehyde was mixed with dansyl hydrazine, intensities increased rapidly relative to the intensity for DANSH. Initial and final intensity values for a representative experiment are shown in Table 6-5. Polarizations also changed, dropping from an original reading of 0.1 before rising to a fairly stable value. The trend is represented in Figure 6-8. If the conjugate lifetime is longer than that of free DANSH, as intensity data indicates, a decrease in polarization is expected. Excitation spectra taken after the reaction was complete showed greater intensity for the anisaldehyde-conjugate than for the free reagent blank indicating that a reaction had occurred.

A reaction between $1.00 \times 10^{-4}$ M dansyl hydrazine and $1.00 \times 10^{-3}$ M anisaldehyde was followed by TLC. Judging by the *R*<sub>f</sub> values and the number of spots on the plates, the
Table 6-5: Experimental Results from a Kinetics Trial Between 3.00 x 10^{-6} M anisaldehyde and 3.00 x 10^{-7} M Dansyl Hydrazine

<table>
<thead>
<tr>
<th></th>
<th>IV</th>
<th>IH</th>
<th>IT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DANSH blank solution</td>
<td>29.7</td>
<td>24.2</td>
<td>78.1</td>
<td>.101</td>
</tr>
<tr>
<td>Initial readings at</td>
<td>36.5</td>
<td>30.3</td>
<td>97.1</td>
<td>.0925</td>
</tr>
<tr>
<td>start of reaction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final readings 15</td>
<td>37.7</td>
<td>31.0</td>
<td>99.6</td>
<td>.0975</td>
</tr>
<tr>
<td>minutes after the</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>reaction start</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 6-8: Polarization Changes during a Kinetics Experiment between $3.00 \times 10^{-6}$ M anisaldehyde and $3.00 \times 10^{-7}$ M Dansyl Hydrazine at $21^\circ$C.
reaction reached at least 99% completion between 15 and 30 minutes after addition of reagent. This range of completion times is consistent with values obtained by Mopper and Johnson (120) for hydrazone formation of sugars and by Kawasaki, Maeda and Tsuji (121) for dansylation of 17-oxysteroids.

Table 6-6 illustrates the reaction times at which changes in slope occurred for various initial concentrations of anisaldehyde and dansyl hydrazine for those reactions monitored by polarizations. The TLC data is inconsistent with the times appearing in Table 6-6 if the latter data are assumed to represent reaction completion as done for the FITC experiments. The more concentrated the reactants, the faster the reaction reaches 99% completion as long as no other factors affect the rate constant. Perhaps there is a rapid rise in the concentration of carbinolamine intermediate before final dehydration and this causes changes in intensities and polarizations. This could explain the dip in the polarization curve.

For an indication of the magnitude of the rate constant, the TLC data is used. The second order rate constant would fall between 330 M\(^{-1}\) min\(^{-1}\) and 170 M\(^{-1}\) min\(^{-1}\) using 15 and 30 minutes as the limits of reaction completion.
Table 6-6: Initial Concentrations of anisaldehyde and DANSH with Reaction Time at the Change in Slope

<table>
<thead>
<tr>
<th>[anisaldehyde]</th>
<th>[DANSH]</th>
<th>minutes ($\pm$ Sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3.00 \times 10^{-6}$</td>
<td>$3.00 \times 10^{-7}$</td>
<td>$4.53 \pm 0.53$</td>
</tr>
<tr>
<td>$1.00 \times 10^{-6}$</td>
<td>$1.00 \times 10^{-7}$</td>
<td>$6.90 \pm 0.82$</td>
</tr>
<tr>
<td>$3.00 \times 10^{-7}$</td>
<td>$3.00 \times 10^{-8}$</td>
<td>$31.4 \pm 2.8$</td>
</tr>
</tbody>
</table>

1. slope change of experimental plots: I vs time, P vs time;
Conclusions

Dansyl Hydrazine reacts with carbonyl compounds as evidenced by the rapid change in polarization and intensities during the kinetics study. The reaction may take awhile to reach completion as shown by the TLC study. Reaction completion took between 15 and 30 minutes for 1.04 x 10^-2 M anisaldehyde and 1.04 x 10^-3 M DANS H indicating a second order rate constant between 330 M^{-1} min^{-1} and 170 M^{-1} min^{-1}. The presence of glycerol does not affect the rate of the reaction nor does the glycerol interfere in the reaction. Glycerol is needed to increase the polarization changes observed upon conjugation. Even with 65.4% glycerol, the polarizations are small at the wavelengths used.

Energy transfer may occur when the conjugate has a carbonyl group located alpha to an aromatic ring as observed for acetonaphthone and anisaldehyde. These spectral changes can be turned to the advantage of the researcher when following reactions although blue shifts of wavelength will be accompanied by a smaller intrinsic polarization as illustrated by the polarization spectrum.

The TLC developing solution of 2:1 ethyl acetate and hexane performed much better for separating the conjugates formed in this project than literature solvent systems that have been used to separate dansyl sugar derivatives.

Dansyl Hydrazine is limited by its low fluorescence efficiency which makes detection of low concentrations difficult.
CHAPTER VII

CONCLUSION

Polarization changes may be used to resolve derivatized analyte from unreacted fluorophor in the absence of spectral changes provided the analytical reaction is accompanied by a change in lifetime or size which affects the extent of rotational depolarization. Reagents that showed measurable polarization changes were fluorescein isothiocyanate and dansyl hydrazine. Studies with model analytes showed that both lifetime and size changes were responsible for polarization changes. These two fluorophors indicate that fluorescence polarization can be used to monitor derivatization reactions. The reaction conditions needed for fluorescein isothiocyanate are pH 9 and 35% glycerol to stop rotational depolarization. Signal intensities are readily measurable even for $10^{-8}$ M samples because of the high molar absorptivity and fluorescence efficiency of fluorescein. Dansyl hydrazine reacts better in slightly acidic media and needs more glycerol for polarization changes to be observed; 65% glycerol by volume was sufficient.

Dansyl chloride reacted with polyethyleneimine yielding a conjugate with a lifetime of 40 ns. The reaction did not go to completion and yielded little kinetic information presumably because many different sites react. The fluorescence lifetime of reagent and all conjugates was
dependent upon the amount of glycerol present.

The biggest problem with fluorescence polarization was illustrated by dichlorotriazinyl fluorescein. For this compound, polarization did not change upon conjugation. One difficulty stemmed from the fact that lifetimes did not change significantly upon conjugation. In addition, analyte size did not influence polarization as expected. Independent rotation between the reagent with respect to analyte is a possibility. The last difficulty with DTAF was the reaction between reagent and glycerol.

Special care must be taken with glycerol. Although tightly capped and sealed with Parafilm, glycerol still absorbed water with time. This causes problems with viscosity control. Other problems encountered were a lack of consistency between suppliers. Polarization values and background fluorescence fluctuated depending upon the manufacturer of the glycerol. Aldrich's Gold Label glycerol seemed to give consistent results.

Between fluorescein isothiocyanate, dansyl chloride and dansyl hydrazine, amines and carbonyls are covered. It would be desirable to study other fluorophors to find a reagent to react with alcohols and other important analytes that would also exhibit a change in polarization with conjugation. A fluorescein derivative to react with these important analytes would allow fluorescein's fluorescence characteristics to be utilized.

Polarization will allow the use of efficient
fluorescent reagents such as fluorescein without a separation step in certain favorable cases. The need for glycerol, however, and the time required to obtain a complete reaction are limits to applicability.
Many computer programs were written to assist the research. The computer proved invaluable for collection and manipulation of the large volume of numbers involved in this research. The most useful programs have been illustrated here as examples. Many other programs which are specialized spinoffs of these programs are not included. These programs all operate individually instead of having one master menu program as favored by other programmers.

Program KINET3

This program was the workhorse for the research project. It collects data points during kinetics trials for storage and output. In order to fit into the computer's memory, this program had to be separated into three parts, NSETUP.BAS, NRUNIN.BAS and NOUTPT.BAS. NSETUP.BAS sets up a timing sequence for the data collection as well as records experimental conditions such as number of data points and ambient temperature. The second part, NRUNIN.BAS, controls the collection of data points from the SLM using conditions set in the first part. Three values per channel are collected and polarizations calculated from average intensities. The data are outputed directly to a printer in case of computer crash as well as into a data file. The third part, NOUTPT.BAS, prints hard copies of the data in tabular form and produces plots using the HP7470A plotter.
Program SPECTR.BAS

This program collects fluorescence excitation and emission spectra from the SLM for storage on disk as well as output to the plotter for in a standardized format. The program allows the user to set the wavelength ranges to scan and the wavelength step to use. These values, of course, must be the same as the values on the SMC-220. The program also allows some flexibility for axes and labelling on the hard copy.

Program MANPOL.BAS

This program will collect a polarization spectrum interactively for storage on disk and subsequent output to the plotter. It was found that the best polarization spectrum was obtained where horizontal intensities are balanced at each wavelength rather than scanning and calculating polarizations from the scans as suggested by the SLM manual (53). MANPOL.BAS enters data from the SLM, corrects for dark currents and outputs to the disk and printer. In addition, a data file is created that can be plotted out on the HP7470A using a generic plotting program such as PLOT.BAS. To use the program, the user will usually collect ten samples using the SLM integration electronics (SPC 822/823) and then enter the average to the computer at the prompt. This method allows both channels to be sampled at one time for a more efficient experiment. The SLM will
not allow two channels to be sampled at one time when in a scanning mode or if either peripheral port (printer or plotter) is in operation.

Program PLOT.BAS

Written to be functionally very simple, this program will take data from either the terminal or a data file for output to the HP7470A plotter. Data entered from the terminal is also stored on disk for future use. Most of the plots in this dissertation were plotted using this program or one of its family of programs. The data files are set up to read in the number of data points, the individual data points followed by axes labels and titles.
- PROGRAM NSETUP.BAS -

10 DISPLAY_CLEAR \ ROLL_AREA(6,11) \ CHAR_MODE("BOLD")
20 PRINT ' KINET3.BAS'
30 CHAR_MODE("-BOLD")
40 PRINT \ PRINT \ PRINT '
   written by CPHallen'
50 PRINT '  on 28-March 1985'
60 BOX("BOLD",5,20,13,60) \ PAUSE(10) \ DISPLAY_CLEAR
70 PRINT ' Welcome to KINET3.BAS. This program has been
   written to'
80 PRINT ' help you collect data from the SLM Fluorescence
   Spectrometer,'
90 PRINT ' using the MINC 1103 computer.'
100 PRINT ' This part of KINET3.BAS is called NSETUP.BAS
   and is used to '
110 PRINT ' prepare the instrument for data collection.'
120 PRINT ' This program records fluorescence data in
   the following '
130 PRINT ' order: 1. channel A with exc. polarizer 9'
140 PRINT ' 2. channel B with ExPol 9'
150 PRINT ' 3. channel B with ExPol 0'
160 PRINT ' 4. channel A with ExPol 0'
170 PRINT ' Also recorded are time of data collection using
   the MINC '
180 PRINT ' internal clock and polarization which is calculat
   ed as follows: ' \ PRINT
190 PRINT ' [ A0 - BO( A9/B9 )] '
200 PRINT ' P = ------------------- ' 
210 PRINT ' [ A0 + BO( A9/B9 )] ' \ PRINT
220 PRINT \ PRINT ' When ready to continue, hit [cr] '; \ 
   INPUT Y$
230 DISPLAY_CLEAR
240 PRINT ' Time allowed between data collections in
   this program is up'
250 PRINT ' to the user. Recommended is 3-4 minutes between
   runs 1-3 where lots'
260 PRINT ' of cuvette shuffling occurs then fast runs (4-8)
   for initial'
270 PRINT ' kinetics followed by 25-30 seconds for runs to
   30 minutes (9-37).'
280 PRINT ' Be advised that the computer will not respond to
   external commands'
290 PRINT ' when running in the PAUSE mode so keep it reason
   able. Maximum allowed'
300 PRINT ' is 86000 seconds.'
310 GOSUB 890
320 GOTO 470
330 PRINT \ PRINT ' User can set up to six different
   time delays depending on'
340 PRINT ' ones individual purposes. Delays should be enter
   ed as seconds, so'
350 PRINT ' a 4 minute delay is 240 seconds.'
360 PRINT \ PRINT 'Enter time delay and first region, e.g. 
    for 100 second delay'
370 PRINT 'over the range of readings 1 through 3, you 
    should enter :'
380 PRINT '': 100,1,3'
390 PRINT 'Time delay and region one '; \ INPUT T0,N1,N2
400 PRINT 'Time delay and region two '; \ INPUT T1,N3,N4
410 PRINT 'Time delay and region three '; \ INPUT T2,N5,N6
420 PRINT 'Time delay and region four '; \ INPUT T3,N7,N8
430 PRINT 'Time delay and region five '; \ INPUT T4,N9,M1
440 PRINT 'Time delay from ';M1+1;' to end of run '; \ 
    INPUT T6
450 DISPLAY_CLEAR
460 PRINT 'Time delays are now:' \ PRINT \ GOTO 480
470 PRINT \ PRINT 'Default values have been chosen and are 
    as follows :'
480 PRINT \ PRINT 'From ';N1;' to ';N2;', a ';T0;' second 
    delay;'
490 PRINT 'From ';N3;' to ';N4;', a ';T1;' second delay;'
500 PRINT 'From ';N5;' to ';N6;', a ';T2;' second delay;'
510 PRINT 'From ';N7;' to ';N8;', a ';T3;' second delay;'
520 PRINT 'From ';N9;' to ';M1;', a ';T4;' second delay;'
530 PRINT 'From ';M1+1;' to finish, a ';T6;' second delay.'
540 PRINT \ PRINT
550 PRINT 'Is this satisfactory (Y)es or (N)o --- (a no 
    will require'
560 PRINT 'that yo redo all time delays and regions); [Y]; 
    INPUT Y$
570 IF Y$='N' THEN 330
580 IF Y$='NO' THEN 330
590 PRINT \ PRINT 'Enter todays temperature '; \ INPUT C
600 PRINT \ PRINT 'How many trials are to be collected '; 
    \ INPUT N
610 DISPLAY_CLEAR \ PRINT 'In order to rapidly aquire data 
    points, several parameters must be set'
620 PRINT 'Set the following on the SPC-822/823:'
630 PRINT ' Acquisition Time 10x.1'
640 PRINT 'Set the following on the SMC-220:'
650 PRINT ' Time Base 2x1'
660 PRINT ' Lower Limit (excitation) user chosen'
670 PRINT ' Upper Limit (excitation) LL + 1'
680 PRINT ' Monochromator Selection EX'
690 PRINT ' Wavelength Increment 1.0 nm'
700 PRINT ' Spectrum Acquisition Multiscan'
710 PRINT ' Select Number 1'
720 PRINT \ PRINT ' To collect a data point, push toggle 
    to START'
730 PRINT 'and a point will be collected.' \ PRINT
740 PRINT 'All systems are up and running. Good Luck!'
750 PRINT \ PRINT 'To continue experiment, run NRUNIN.BAS,'
760 PRINT 'which is part 2 in the KINET3.BAS program set.'
770 OPEN 'HOLD.DAT' FOR OUTPUT AS FILE #1
780 PRINT #1,T0,'';N1;'';N2
790 PRINT #1,T1;'';N3;'';N4
PROGRAM NRUNIN.BAS

10 .DIM A0(100),B0(100),A9(100),B9(100),P(100),T(100),T$(100)
20 PRINT "Welcome to KINET3.BAS. This program has been written to'
30 PRINT 'help you collect data from the SLM Fluorescence Spectrometer'
40 PRINT 'using the MINC 1103 computer.'
50 PRINT 'This part of KINET3.BAS is called NRUNIN.BAS and is the actual'
60 PRINT 'data collection part of the program. This part will also look over'
70 PRINT 'an existing data file. Data is signal averaged (each point taken'
80 PRINT 'three times). TURN ON LP!'""""""""""""""""
90 PRINT """"""""""""
100 PRINT 'Do you wish to access/make an OLD or NEW file [NEW]; \ LINPUT Y$'
110 IF Y$='NEW' OR IF Y$=' ' THEN 140
120 IF Y$='OLD' THEN 1560
130 GOTO 90
140 DISPLAY_CLEAR
150 PRINT 'Have you run NSETUP.BAS [Y]; \ INPUT Y$
160 IF Y$='Y' OR IF Y$='YES' THEN 220
170 IF Y$=' ' THEN 220
180 PRINT "YOU HAVE BEEN LOGGED OUT OF NRUNIN.BAS !'
190 PRINT 'YOU MUST RUN NSETUP.BAS BEFORE RUNNING NRUNIN .BAS !!!!'
200 GOTO 32767
220 OPEN 'HOLD.DAT' FOR INPUT AS FILE #1
230 INPUT #1,T0,N1,N2
240 INPUT #1,T1,N3,N4
250 INPUT #1,T2,N5,N6
260 INPUT #1,T3,N7,N8
270 INPUT #1,T4,N9,M1
280 INPUT #1,T6
290 INPUT #1,C,N,D1,D2

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800 PRINT #1,T2;',';N5;',';N6
810 PRINT #1,T3;',';N7;',';N8
820 PRINT #1,T4;',';N9;',';M1
830 PRINT #1,T6
840 PRINT #1,C;',';N;',';D1;',';D2
850 CLOSE #1
860 GOTO 32767
890 REM Loop to assign standard time values
900 N1=1 \ N2=3 \ N3=4 \ N4=6 \ N5=7 \ N6=40
910 N7=41 \ N8=45 \ N9=46 \ M1=50
920 T0=25 \ T1=0 \T2=78 \ T3=78 \ T4=78 \ T6=78
930 RETURN
32767 END
158

300 CLOSE #1
310 PRINT \ PRINT 'Enter DSK: FILNAM. EXT for data storage '; \ LINPUT FS
320 OPEN FS FOR OUTPUT AS FILE #1
330 PRINT #1,CLK$; ',';DAT$; ',';C; ',';N
340 OPEN 'LP:' FOR OUTPUT AS FILE #2
350 PRINT #2,'Raw data from trials from ';FS \ PRINT #2 \ PRINT #2 \ CLOSE #2
360 PRINT 'All systems are now up and running. Good Luck !!'
370 PRINT 'Remember, data is collected : A9, B9, B0 then A0'
380 PRINT 'When ready to continue, hit [cr] '; \ INPUT A$
390 FOR I=1 TO N
400 IF I>M1 THEN T5=T6 \ IF I=M1 THEN T5=T4 \ IF I=N8 THEN T5=T3
410 IF I<=N6 THEN T5=T2 \ IF I=N4 THEN T5=T1 \ IF I=N2 THEN T5=T0
420 T$(I)=CLK$ \ D$=T$(I)
430 IF I=4 THEN 450
440 GOTO 470
450 U$=T$(4) \ H1=VAL(SEG$(U$,1,2))*3600 \ M1=VAL(SEG$(U$,4,5))*60
460 S1=VAL(SEG$(U$,7,8)) \ Q1=(H1+M1+S1)/60
470 D3=0 \ D4=0 \ D5=0 \ D6=0 \ A1=0 \ A2=0 \ B1=0 \ B2=0 \ A3=0 \ A4=0 \ B3=0 \ B4=0
480 OPEN 'LP:' AS FILE #2
490 PRINT #2,'A0','B0',' A9','B9','for run number ';I;' at time ';T$(I) \ PRINT #2
500 FOR J=1 TO 3
510 PRINT 'Acquire a value for channel A, ExPol 9'
520 CIN(,,I$,8,1,60)
530 D3=VAL(SEG$(I$,1,6)+'E '+SEG$(I$,7,8))
540 A1=A1+D3 \ A3=A3+(D3*D3)
550 PRINT I$,C$,'value to list :';D3 \ PRINT
560 PRINT 'Acquire a value for channel B, ExPol 9'
570 CIN(,,I$,8,1,60)
580 D4=VAL(SEG$(I$,1,6)+'E '+SEG$(I$,7,8))
590 B1=B1+D4 \ B3=B3+(D4*D4)
600 PRINT I$,C$,'value to list :';D4 \ PRINT
610 PRINT 'Acquire a value for channel B, ExPol 0'
620 CIN(,,I$,8,1,60)
630 D5=VAL(SEG$(I$,1,6)+'E '+SEG$(I$,7,8))
640 B2=B2+D5 \ B4=B4+(D5*D5)
650 PRINT I$,C$,'value to list :';D5 \ PRINT
660 PRINT 'Acquire a value for channel A, ExPol 0'
670 CIN(,,I$,8,1,60)
680 D6=VAL(SEG$(I$,1,6)+'E '+SEG$(I$,7,8))
690 A2=A2+D6 \ A4=A4+(D6*D6)
700 PRINT I$,C$,'value to list :';D6 \ PRINT
710 PRINT #2,D6,D5,D3,D4
720 NEXT J
730 S1=SQR((A4-A2*A2/3)/2)
740 S2=SQR((B4-B2*B2/3)/2)
750 S3=SQR((A3-A1*A1/3)/2)
760 S4=SQR((B3-B1*B1/3)/2)
159

770 PRINT #2 \ PRINT #2,S1,S2,S3,S4 \ PRINT #2
780 CLOSE #2
790 A9(I)=A1/3 \ AO(I)=A2/3
800 B9(I)=B1/3 \ B0(I)=B2/3
810 P=(A9(I)/B9(I)) \ P(I)=((A0(I)-B0(I)*P)/(A0(I)+B0(I)*P))
820 H2=VAL(SEG$(D$,1,2))*3600 \ M2=VAL(SEG$(D$,4,5))*60
830 S2=VAL(SEG$(D$,7,8)) \ Q2=(H2+M2+S2)/60
840 IF I<4 THEN T(I)=0
850 IF I>4 THEN T(I)=Q2-Q1
860 DISPLAY_CLEAR \ PRINT 'Run number ;I; at time ;T(I); MINUTES.'
870 PRINT \ PRINT 'Polarization value is ... ;P(I)
880 PRINT \ PRINT 'Intensity data ...'
890 PRINT \ PRINT 'A ExPol 0','B ExPol 0','A ExPol 9','B ExPol 9'
900 PRINT AO(I),BO(I),A9(I),B9(I)
910 PRINT #1,A0(I);',';B0(I);',';A9(I);',';B9(I);',';P(I);','

920 IF I=N THEN 980
930 PRINT \ PRINT 'First run at ;U$; with this run at : ';
940 IF T5>5 THEN 960
950 PRINT \ PRINT 'Now in a ;T5; second pause.' \ PAUSE(T5 ) \ GOTO 980
960 PRINT \ PRINT 'Now in a ;T5-5; second pause.' \ PAUSE(
970 PRINT \ PRINT 'Five second warning !'; \ PAUSE(5)
980 NEXT I
990 CLOSE #1 \ PRINT \ PRINT 'Session completed at ';CLK$; on DAT$
1000 PRINT \ PRINT 'Do you wish to see a table of data collected:
1010 PRINT ' (1) Intensities, (2) Polarizations or (3) no
table.'
1020 PRINT 'Number of choice '; \ INPUT Y
1030 IF Y=1 THEN 1070
1040 IF Y=2 THEN 1170
1050 IF Y=3 THEB 1270
1060 GOTO 1000
1070 DISPLAY_CLEAR \ PRINT 'Intensity results at time ';CLK$
1080 PRINT #1,'A ExPol 0','B ExPol 0','A ExPol 9','B ExPol 9' \ J+
1090 FOR I= 1 TO N
1100 PRINT I,A0(I),BO(I),A9(I),B0(I) \ J=J+1 \ IF J<10 THEN
1110 PRINT \ PRINT 'When ready to continue, hit [cr]'; \ INPUT A$
1120 J=0 \ DISPLAY_CLEAR
1130 PRINT 'Intensity results at time ';CLK$
1140 PRINT #1,'A ExPol 0','B ExPol 0','A ExPol 9','B ExPol 9' \ PRINT
1150 NEXT I
1160 PRINT \ PRINT \ GOTO 1000
1170 DISPLAY_CLEAR \ PRINT '#','Polarization','Time Diff.',
Time Collected' \ J=0
1180 FOR I=1 TO N
1190 PRINT I,P(I),T(I),T$(I) \ J=J+1 \ IF J<10 THEN 1230
1200 PRINT 'When ready to continue, hit [cr] ' \ INPUT A$
1210 J=0 \ DISPLAY_CLEAR
1220 PRINT '#','Polarization','Time Diff.','Time Collected' \ PRINT
1230 NEXT I
1240 PRINT \ PRINT \ GOTO 1000
1250 GOTO 1000
1260 PRINT 'Time is now ';CLK$;'.' \ PRINT
1270 PRINT 'Do you wish to see a plot of:'
1280 PRINT '  (1) Polarization vs. Time inclusive'
1290 PRINT '  (2) Polarization vs. Time w/o init. values'
1300 PRINT '  (3) no plot'
1310 PRINT 'Number of choice '; \ input y
1320 IF Y=1  THEN 1360
1330 IF Y=2 THEN 1400
1340 IF Y=3 THEN 1420
1350 GOTO 1270
1360 DISPLAY_CLEAR
1370 REGION(,2) \ GRAPH(,,T(1),P(1),,,2) \ LABEL(,'TIME','POLARIZATION',2)
1380 PRINT 'When ready to continue, hit [cr] ' ; \ INPUT A$
1390 DISPLAY_CLEAR \ GOTO 1270
1400 DISPLAY_CLEAR
1410 REGION(,2) \ GRAPH(,,T(4),P(4),,,2) \ LABEL(,'TIME','POLARIZATION',2) \ GOTO 1380
1420 DISPLAY_CLEAR
1430 PRINT 'Do you wish to . . . '
1440 PRINT '  (1) Return to tables of I or P'
1450 PRINT '  (2) Return to plots'
1460 PRINT '  (3) Take more data (in a new file)'
1470 PRINT '  (4) End run.'
1480 PRINT 'Number of choice '; \ INPUT Y
1490 IF Y>4 THEN 1430
1500 ON Y GOTO 1000,1270,20,1540
1540 PRINT 'Have a pleasant day !'
1550 GOTO 32767
1560 PRINT 'Enter DSK:FILNAM.EXT to read from '; \ LINPUT F$
1570 OPEN F$ FOR INPUT AS FILE #1
1580 INPUT #1,C$,D$,C,N
1590 FOR I=1 TO N
1600 INPUT #1,A0(I),B0(I),A9(I),B9(I),P(I),T(I),T$(I)
1610 NEXT I
1620 CLOSE #1
1630 GOTO 1000
32767 END
10 DIM AO(100), B0(100), A9(100), B9(100), P(100), T(100), T$(100)
20 DIM X(100), Y(100), Z(100)
30 PRINT 'What is the DSK:FILNAM.EXT for the file to be output to?'
40 PRINT 'printer or plotter'; 
50 OPEN F$ FOR OUTPUT AS FILE #1
60 INPUT #1, C$, D$, C, N
70 FOR I=1 TO N
80 INPUT #1, A0(I), B0(I), A9(I), B9(I), P(I), T(I), T$(I)
90 X(I)=T(I)
100 NEXT I
110 CLOSE #1
120 DISPLAY CLEAR
130 PRINT 'Output information to: '
140 PRINT ' (1) Printer (TURN ON LP:)' 
150 PRINT ' (2) Plotter (CONNECT AND TURN ON)'
160 PRINT 'Any other response will stop program.'
170 INPUT A
180 IF A=1 THEN 210
190 IF A=2 THEN 600
200 STOP
210 OPEN 'LP:' FOR OUTPUT AS FILE #1
220 PRINT #1, 'File '; F$; ' was collected at '; C$; ' on '; D$;
230 PRINT #1, 'Temperature was '; C; ' and '; N; ' data points were collected.'
240 PRINT #1 
250 PRINT #1 
260 FOR I=1 TO N
270 PRINT #1, I, A0(I), B0(I), A9(I), B9(I), P(I), T(I), T$(I)
280 PRINT I, P(I) 
290 PAUSE(2)
300 NEXT I
310 CLOSE #1
320 FOR I=N-4 TO N \ F=F+P(I) \ NEXT I
330 IF I=1 THEN 210
340 P1=F/5 \ PRINT ' P inf = '; P1
350 P2=P(2) \ P3=P1-P2
360 FOR I=1 TO N
370 A0(I)=LOG(ABS(P(I)-P1))
380 IF I=2 THEN A9(I)=1 \ GOTO 400
390 A9(I)=(P1-P(I))/(P(I)-P2)
400 B0(I)=(P1-P(I))/P3
410 IF I<3 THEN 450
420 IF I>N+1 THEN 450
430 B9(I)=(P(I+1)-P(I))/(T(I+1)-T(I))
440 Z(I)=((T(I+1)-T(I))/2)+T(I)
450 NEXT I
460 OPEN 'LP:' FOR OUTPUT AS FILE #1
470 PRINT #1 
480 PRINT #1, 'Run Number', 'Polarization', 'Ln[P-Pinf]', 'Fr/'

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- PROGRAM NOUTPT.BAS -

- PROGRAM NOUTPT.BAS -
PRINT #1 \ PRINT #1
FOR I=1 TO N
IF I<3 THEN 550
IF I>N+1 THEN 550 .
PRINT #1,I,P(I),A0(I),A9(I),B0(I),B9(I),Z(I)
GOTO 560
PRINT #1,I,P(I),A0(I),A9(I),B0(I)
GOTO 560
PRINT I,A0(I)  \ PAUSE(I)
NEXT I
CLOSE #1
GOTO 50
DISPLAY CLEAR
PRINT 'It is possible to plot :
PRINT '  (1) Polarization vs. Time'
PRINT '  (2) A ExPol 0 vs. time'
PRINT '  (3) A ExPol 9 vs. time'
PRINT '  (4) B ExPol 0 vs. time'
PRINT '  (5) B ExPol 9 vs. time'
PRINT '  (6) LNfP - Pinf! vs. time'
PRINT '  (7) free/bound fluorophor vs time'
PRINT '  (8) fraction fluorophor free vs. time'
PRINT '  (9) End'
PRINT \ PRINT \ PRINT
PRINT 'What is the number of your choice '; INPUT D9
IF D9>9 THEN 600
ON D9 GOTO 750,810,870,930,990,1050,1050,1050,32767
FOR I=1 TO N
Y(I)=P(I)*100
L1$="Polarization  (x 100)"
T$=F$
GOTO 1360
FOR I=1 TO N
Y(I)=A0(I)/1000
L1$="Relative Intensity"
T$="A ExPol 0 from "+F$
GOTO 1360
FOR I=1 TO N
Y(I)=A9(I)/1000
L1$="Relative Intensity"
T$="A ExPol 9 from "+F$
GOTO 1360
FOR I=1 TO N
Y(I)=B0(I)/1000
L1$="Relative Intensity"
T$="B ExPol 0 from "+F$
GOTO 1360
FOR I=1 TO N
Y(I)=B9(I)/1000
NEXT I
1000
L1$="Relative Intensity"
T$="B ExPol 9 from "+F$
GOTO 1360
DISPLAY_CLEAR
PRINT 'You must choose a value for Pinf or Pb.'
PRINT 'For your convenience, a list of polarizations is provided.'
PRINT \ PRINT 'I','Polarization'
FOR I=1 TO N
PRINT I,P(I)
NEXT I
PRINT \ PRINT 'Enter value of Pinf or Pb to be used';
\ INPUT P1
ON D9 GOTO 750,810,870,930,990,1140,1200,1280,32767
FOR I=1 TO N
Y(I)=LOG(ABS(P(I)-P1))
NEXT I
L1$='Ln ! P(I) - Pinf !
T$=F$
GOTO 1360
P2=P(2)
FOR I=1 TO N
Y(I)=(P1-P(I))/(P(I)-P2)
NEXT I
L1$='Free / Bound Fluorophor'
T$=F$
GOTO 1360
P3=P1-P(2)
FOR I=1 TO N
Y(I)=(P1-P(I))/P3
NEXT I
L1$='Frac. Fluor. Free'
T$=F$
GOTO 1360
PRINT \ PRINT \ PRINT \ PRINT 'RUNNING A MAX/MIN DETERMINATION'
N1=X(4) \ N2=X(4) \ N3=Y(4) \ N4=Y(4)
FOR I=1 TO 5
K1=N1-X(I) \ L1=N2-X(I) \ K2=N3-Y(I) \ L2=N4-Y(I)
IF K1>0 THEN N1=X(I)
IF L1<0 THEN N2=X(I)
IF K2>0 THEN N3=Y(I)
IF L2<0 THEN N4=Y(I)
NEXT I
PRINT \ PRINT \ PRINT 'COMPUTATION COMPLETED'
PRINT \ PRINT "Values are:"
PRINT \ PRINT 'X',N1,N2
PRINT \ PRINT 'Y',N3,N4
PRINT "Enter X starting and finishing points "
INPUT X1,X2
PRINT \ PRINT "Enter Y starting and finishing points "
INPUT Y1,Y2
points

1530 INPUT Y1,Y2
1540 PRINT \ PRINT "INITIALIZING THE PLOTTER"
1550 COUT(,"IN;SP1;PU;","",1)
1560 COUT(,"IP1500,2200,9300,6200;">","",1)
1570 COUT(,"SC"+STR$(X1)+","+STR$(X2)+"","+STR$(Y1)+"","+STR$(Y2)+";">","",1)
1590 PAUSE(2)
1600 PRINT \ PRINT "SETTING UP AXES"
1610 COUT(,"SI.15,.25;">","",1)
1620 COUT(,"TL1.5,0;">","",1)
1630 COUT(,"PA"+STR$(X1)+"","+STR$(Y1)+";">","",1)
1640 PAUSE(1)
1650 FOR X=X1 TO X2 STEP (X2-X1)/10
1660 COUT(,"PD;PA"+STR$(X)+"","+STR$(Y1)+"","PU;XT;PU">","",1)
1670 COUT(,"CP-.835,-1;">","",1)
1680 COUT(,"LB"+STR$(X)+CHR$(3),">","",1)
1690 COUT(,"PA"+STR$(X)+"","+STR$(Y1)+";">","",1)
1700 PAUSE(1.5)
1710 NEXT X
1720 COUT(,"TL1.0;">","",1)
1730 COUT(,"PA"+STR$(X1)+"","+STR$(Y1)+";">","",1)
1740 FOR Y=Y1 TO Y2 STEP (Y2-Y1)/5
1750 COUT(,"PD;PA"+STR$(X1)+"","+STR$(Y)+"","PU;YT;PU">","",1)
1760 COUT(,"CP-4.5,-.25;">","",1)
1770 COUT(,"LB"+STR$(Y)+CHR$(3),">","",1)
1780 COUT(,"PA"+STR$(X1)+"","+STR$(Y)+";">","",1)
1790 PAUSE(2)
1800 NEXT Y
1810 PAUSE(2)
1820 PRINT \ PRINT "PLOTTING THE DATA"
1830 COUT(,"PU;SP2;PU;">","",1)
1840 PAUSE(2)
1850 COUT(,"PA"+STR$(X(4))+"","+STR$(Y(4))+"","PD">","",1)
1860 FOR I=5 TO N
1870 COUT(,"PA"+STR$(X(I))+"","+STR$(Y(I))+";">","",1)
1880 PAUSE(1)
1890 NEXT I
1900 PRINT \ PRINT "PLOTTING ROUTINE COMPLETED"
1910 PAUSE(3)
1920 PRINT \ PRINT "LABELLING PLOT"
1930 COUT(,"SP1;PU;">","",1)
1940 COUT(,"DII,0;SI.20,.25;">","",1) \ PAUSE(1)
1950 COUT(,"PA"+STR$((X2-X1)/(2+X1)))+"","+STR$(Y1)+";">","",1)
1960 COUT(,"CP-9,-3;">","",1)
1970 PAUSE(2)
1980 COUT(,"LBReaction Run Time"+CHR$(13)+CHR$(3),">","",1)
1990 PAUSE(4)
2000 T=LEN(L1$)/2
2010 COUT(,"PA"+STR$(X1)+"","+STR$((Y2-Y1)/2+Y1)+";">","",1)
2020 COUT(,"DII,1;CP-"+STR$(T)+"",4;">","",1)
2030 PAUSE(2)
2040 COUT(,"LB"+L1$+CHR$(13)+CHR$(3),">","",1)
2050 PAUSE(4)
165

2060 T=LEN(T$)/2
2070 COUT(,"PA"+STR$((X2-X1)/2+X1)+"","+STR$(Y2)+"","",1)
   \ PAUSE(,5)
2080 COUT(,"DI1,0;CP"+STR$(T)+",3","",1)
2090 PAUSE(2)
2100 COUT(,"LB"+T$+CHR$(13)+CHR$(3),1)
2110 PAUSE(4)
2120 COUT(,"PA"+STR$(X2)+"","+STR$(Y2)+"","",1)
2130 T=LEN(D$)
2140 PAUSE(1) \ COUT(,"DI1,0;CP—"+STR$(T)+",6.5","",1)
2150 PAUSE(1) \ COUT(,"LB"+D$+CHR$(13)+CHR$(3),1)
2160 PAUSE(1) \ COUT(,"CP—8,—.75","",1)
2170 PAUSE(1) \ COUT(,"LBCHallen"+CHR$(13)+CHR$(3),1)
2180 COUT(,"SP0;","",1) \ GOTO 120
32767 END

- PROGRAM SPECTR.BAS -

10 DIM A(300),B(300),C(300),D(300),P(300)
20 PRINT "What is the DSK:FILNAM.EXT of the file to hold the
data \\
   \ INPUT F$
30 OPEN F$ FOR OUTPUT AS FILE #1 \ GOSUB 1600
40 PRINT 
50 PRINT "For the excitation spectrum : \\
   \ INPUT W1
60 PRINT "What is the wavelength to finish with \\
   \ INPUT W2
70 PRINT "Enter the wavelength step to use (.25, .5, \\
   \ INPUT W5
80 PRINT "For the emission spectrum : \\
90 PRINT "What is the wavelength to start with \\
   \ INPUT W6
100 PRINT "What is the wavelength to finish with \\
   \ INPUT W7
110 PRINT "What is the wavelength step to use " \\
   \ INPUT W0
120 PRINT #1,"EXCITATION"
130 PRINT #1,W1",";W2",";W5
140 PRINT "When ready to collect the first \\
   \ INPUT A$
150 W3=W1 \ W4=(W2-W1)/W5 \ V4=W4
160 FOR I=1 TO W4
170 CIN(I$,8,1,10)
180 C$=SEG$(I$,1,6)+'E '+SEG$(I$,7,8)
190 A(I)=VAL(C$) \ C(I)=W3
200 PRINT C(I),A(I) \ PRINT #1,C (I);",";A (I)
210 W3=W3+W5
220 NEXT I
230 PRINT #1,"EMISSION"
240 PRINT #1,W6",";W7",";W0
250 PRINT \ PRINT \ PRINT "When ready to collect the next spectrum, hit [cr]" \ INPUT A$
260 W3=W6 \ W4=(W7-W6)/W0
270 FOR I=1 TO W4
280 CIN(I,$,8,1,10)
290 CS=SEG$(I$,$,1,6)+'E'+SEG$(I$,$,7,8)
300 B(I)=VAL(C$) \ D(I)=W3
310 PRINT D(I),B(I) \ PRINT #1,D(I);",";B(I)
320 W3=W3+W0
330 NEXT I
340 CLOSE #1 \ DISPLAY_CLEAR
350 PRINT "Do you wish to have a hard copy of the spectra" \ INPUT Y$
360 IF Y$="YES" THEN 540
370 IF Y$="Y" THEN 540
380 PRINT \ PRINT ' Have a great day !!!' \ GOTO 32767
390 PRINT \ PRINT "Connect the MINC and the Plotter"
400 PRINT \ PRINT "RUNNING A MAX/MIN DETERMINATION"
410 N1=A(1)/1000 \ M1=A(1)/1000 \ N2=B(1)/1000 \ M2=B(1)/1000
420 FOR I=1 TO W4
430 A(I)=A(I)/1000 \ K=N1-A(I) \ L=M1-A(I)
440 IF K 0 THEN N1=A(I)
450 IF L 0 THEN M1=A(I)
460 NEXT I
470 FOR I=1 TO W4
480 B(I)=B(I)/1000 \ K=N2-B(I) \ L=M2-B(I)
490 IF K 0 THEN N2=B(I)
500 IF L 0 THEN M2=B(I)
510 NEXT I
520 PRINT \ PRINT \ PRINT "COMPUTATION COMPLETED"
530 PRINT \ PRINT "Values are: "
540 PRINT \ PRINT " EXCITATION","," \ PRINT " EMISSION"
550 PRINT \ PRINT " MINIMUM","MAXIMUM","MINIMUM","MAXIMUM"
560 PRINT \ PRINT " X ";W1,W2,W6,W7
570 PRINT \ PRINT " Y ";N1,M1,N2,M2
580 PRINT \ PRINT " Enter X starting and finishing points "; \ INPUT X1,X2
590 PRINT \ PRINT " Enter Y starting and finishing points "; \ INPUT Y1,Y2
600 PRINT \ PRINT " Enter number of tic marks on each axis - X,Y "; \ INPUT X3,Y3
610 PRINT \ PRINT \ PRINT " INITIALIZING THE PLOTTER"
620 COUT(,"IN;SPl;Pu; ",1)
630 COUT(,"IP500,2200,9300,6200");,1)
640 COUT(,"SC"+STR$(X1)+","+STR$(X2)+","+STR$(Y1)+","+STR$(Y2)+";",1)
650 PAUSE(2)
660 PRINT \ PRINT "SETTING UP AXES"
670 COUT(,"SI.30, .25");,1)
680 COUT(,"TL1.5,0");,1)
690 COUT(,"FA"+STR$(X1)+","+STR$(Y1)+";",1)
700 PAUSE 1
167

860 COUT("PD;PA"+STR$(X2)+","+STR$(Y1)+","+STR$(X2)+","+STR$(Y2)+","+STR$(X1)+","+STR$(Y1)+";PU;",1)
870 PAUSE(3)
880 X4=(X2-X1)/(X3-1) \ X=X1-X4
890 FOR X= 1 TO X3
900 X=X+X4
910 COUT("PA"+STR$(X)+","+STR$(Y1)+";XT;PU;",1)
920 COUT("CP-.835,-1;",1)
930 COUT("LB"+STR$(X)+CHR$(3),1)
940 PAUSE(1.5)
950 NEXT I
960 COUT("TL1.0;PA"+STR$(X1)+","+STR$(Y1)+";",1)
970 Y4=(Y2-Y1)/(Y3-1) \ Y=Y1-Y4
980 FOR I= 1 TO Y3
990 Y=Y+Y4
1000 COUT("PA"+STR$(X1)+","+STR$(Y)+";YT;",1)
1010 COUT("CP-3.25,-.25;",1)
1020 COUT("LB"+STR$(Y)+CHR$(3),1)
1030 PAUSE(1.5)
1040 NEXT I
1050 PRINT \ PRINT \ PRINT "PLOTTING THE DATA"
1060 COUT("PU;SP2;PU;",1)
1070 PAUSE(2)
1080 COUT("PA"+STR$(C(I))+","+STR$(A(I))+";PD;",1)
1090 FOR I=2 TO V4
1100 COUT("PA"+STR$(C(I))+","+STR$(A(I))+",",1)
1110 PAUSE(1.5)
1120 NEXT I
1130 PAUSE(4)
1140 COUT("PU;LT6;",1)
1150 PAUSE(2)
1160 COUT("PA"+STR$(D(I))+","+STR$(B(I))+";PD;",1)
1170 FOR I=2 TO W4
1180 COUT("PA"+STR$(D(I))+","+STR$(B(I))+",",1)
1190 PAUSE(.5)
1200 NEXT I
1210 COUT("PU;",1)
1220 PRINT \ PRINT \ PRINT "PLOTTING ROUTINE COMPLETED"
1230 PRINT \ PRINT \ PRINT "Do you wish to have axes labels and/or a title ( Y or N )"; INPUT Y$
1240 IF Y$="N" THEN 1520
1250 IF Y$="Y" THEN 1270
1260 GOTO 1230
1270 COUT("SP1;PU;",1)
1280 L$="Wavelength (nm)" \ T=LEN(L$)/2
1290 COUT("DI1,0;SI.3,.3;",1)
1300 PAUSE(2)
1310 COUT("PA"+STR$((X2-X1)/2+X1)+";"+STR$(Y1)+";",1)
1320 COUT("CP-"+STR$(T)+";",1)
1330 PAUSE(1)
1340 COUT("LB"+L$+CHR$(13)+CHR$(3),1)
1350 PAUSE(5)
1360 L$="Fluorescent Intensity" \ T=LEN(L$)/2
10 N=0 \ PRINT 'Enter DSK:FILNAM.EXT of file to hold the spectrum' \ LINPUT F$
20 OPEN F$ FOR OUTPUT AS FILE #2
30 DISPLAY_CLEAR
40 N=N+1
50 PRINT 'Enter excitation wavelength :'; \ INPUT W
60 PRINT 'Enter dark current, channel A :'; \ INPUT D1
70 PRINT 'Enter dark current, channel B :'; \ INPUT D2
80 PRINT \ PRINT
90 PRINT 'Enter signals as follows : A0,B0,A9,B9:'
100 PRINT 'A0:'; \ INPUT A1
110 PRINT 'B0:'; \ INPUT B1
120 PRINT 'A9:'; \ INPUT A2
130 PRINT 'B9:'; \ INPUT B2
140 PRINT \ PRINT \ PRINT
150 A9=A2-D1 \ A0=A1-D1
160 B9=B2-D2 \ B0=B1-D2

- PROGRAM MANPOL.BAS -

1370 COUT(,"DIO,1;",1)
1380 COUT(,"PA"+STR$(X1)+","+STR$((Y2-Y1)/2+Y1)+";",1)
1390 COUT(,"CP"+-STR$(T)+",4;",1)
1400 PAUSE(1)
1410 COUT(,"LB"+L$+CHR$(13)+CHR$(3),,1)
1420 PAUSE(5)
1430 PRINT \ PRINT \ PRINT "Enter title ... " \ LINPUT T$
1440 T=LEN(T$)/2
1450 COUT(,"D11,0;",1)
1460 PAUSE(2)
1470 COUT(,"PA"+STR$((X2-X1)/(2+X1))+","+STR$(Y2)+";",1)
1480 COUT(,"CP"+-STR$(T)+",3;",1)
1490 PAUSE(1)
1500 COUT(,"LB"+T$+CHR$(13)+CHR$(3),,1)
1510 PAUSE(5)
1520 COUT(,"SP0;",1) \ GOTO 32767
1600 REM SUBROUTINE FOR PROGRAM FIREUP
1610 DISPLAY_CLEAR
1620 PRINT CHR$(7)
1630 PRINT "IMPORTANT NOTE"
1640 PRINT \ PRINT \ PRINT "MAKE SURE THAT THE SLM IS CONNECTED TO THE"
1650 PRINT \ PRINT \ PRINT "MINC USING THE PROPER RS-232 PORT."
1660 PRINT \ PRINT \ PRINT "If the plotter is also plugged into the SLM, it must"
1670 PRINT \ PRINT "be turned on for the scanning feature to be enabled."
1680 PRINT \ PRINT \ PRINT \ PAUSE(2)
1690 RETURN
32767 END
P = (A₀ - B₀*A₉/B₉)/(A₀ + B₀*A₉/B₉)

PRINT 'POLARIZATION : ' ; P
PRINT 'Outputting to printer ... please sit by ...'
OPEN 'LP:' FOR OUTPUT AS FILE #1
PRINT #1, "Raw data : ", A1, B1, A2, B2, D1, D2
PRINT #1, "Corr data : " ; W, A₀, B₀, A₉, B₉, P
PRINT #1
PRINT #2, W; ', '; A₀; ', '; B₀; ', '; A₉; ', '; B₉; ', '; P
PRINT 'Do another wavelength [Y] '; INPUT N$
IF N$ = 'N' THEN 290
IF N$ = 'n' THEN 290
GOTO 30
CLOSE #1
PRINT #2, W; ', ';
CLOSE #2
PRINT 'What is the DSK:FILNAM.EXT to hold data for hard copy (PLOT.BAS) '; LINPUT Fl$
IF Fl$ = ' ' THEN 32767
OPEN Fl$ FOR OUTPUT AS FILE #3
OPEN F$ FOR INPUT AS FILE #2
PRINT #3, N
FOR I = 1 TO N
INPUT #2, W, A₀, B₀, A₉, B₉, P
PRINT #3, W; ', '; P
NEXT I
PRINT 'Enter title for the plot '; LINPUT T$
PRINT #3, 'EXCITATION WAVELENGTH (nm)'; ', '; 'POLARIZATION'; ', '; T$
CLOSE #2 \ CLOSE #3
32767 END

- PROGRAM PLOT.BAS -

DIM A(300), B(300), C(300), D(300), P(300)
Z9 = 0
PRINT 'Do you wish to plot a (N)ew or (E)xisting file [NE W] ' ; INPUT Y$
IF Y$ = 'E' THEN 80
IF Y$ = ' ' THEN 200
IF Y$ = 'N' THEN 200
GOTO 20
DISPLAY_CLEAR \ PRINT "What is the DSK:FILNAM.EXT of the file to view "; LINPUT Fl$
PRINT 'Is there a correction factor for the y-axis data Y or N ' ; INPUT A$
IF A$ = 'N' THEN A = 1 \ GO TO 120
PRINT 'Enter the correction factor ' ; \ INPUT A
OPEN Fl$ FOR INPUT AS FILE #2
 FOR I = 1 TO N
140 FOR I = 1 TO N
INPUT #2, C(I), A(I)
A(I) = A(I) * A
NEXT I
INPUT #2, X$, Y$, T$
CLOSE #2 \ GOTO 380
PRINT 'What is the DSK:FILNAM.EXT to be used for holding the data'; \ INPUT F$
OPEN F$ FOR OUTPUT AS FILE #1
PRINT 'How many data pairs are to be entered'; \ INPUT N
PRINT 'Enter '; N;' data pairs : X,Y'
PRINT \ PRINT #1, N
FOR I = 1 TO N
PRINT 'data point '; N; \ INPUT C(I), A(I)
PRINT #1, C(I); ','; A(I)
NEXT I
IF Z9 = 0 THEN 310
CLOSE #1 \ GOTO 880
PRINT \ PRINT \ PRINT 'Enter the following for the plot'
PRINT \ PRINT 'Entering nothing will mean nothing will be printed'
PRINT \ PRINT 'X-axis label '; \ LINPUT X$
PRINT \ PRINT 'Y-axis label '; \ LINPUT Y$
PRINT \ PRINT 'Title for plot '; \ LINPUT T$
PRINT #1, X$; ','; Y$; ','; T$
CLOSE #1
PRINT \ PRINT \ PRINT "Connect the MINC and the Plotter"
PRINT \ PRINT \ PRINT 'Also load pens - Left for axes and labels, Right for lines'; \ PAUSE(2)
PRINT \ PRINT \ PRINT "RUNNING A MAX/MIN DETERMINATION"
N1 = A(1) \ M1 = A(1) \ N2 = C(1) \ M2 = C(1)
FOR I = 1 TO N
K = N1 - A(I) \ L = M1 - A(I)
IF K 0 THEN N1 = A(I)
IF L 0 THEN M1 = A(I)
NEXT I
FOR I = 1 TO N
K = N2 - C(I) \ L = M2 - C(I)
IF K 0 THEN N2 = C(I)
IF L 0 THEN M2 = C(I)
NEXT I
PRINT \ PRINT \ PRINT "COMPUTATION COMPLETED"
PRINT \ PRINT "Values are: "
PRINT \ PRINT "Minimum", "Maximum"
PRINT \ PRINT "X", N2, M2
PRINT "Y", N1, M1
PRINT \ PRINT \ PRINT "Enter X starting and finishing points"; \ INPUT X1, X2
PRINT \ PRINT \ PRINT "Enter Y starting and finishing points"; \ INPUT Y1, Y2
PRINT \ PRINT \ PRINT "Enter number of tic marks on each axis - X,Y"; \ INPUT X3, Y3
PRINT \ PRINT \ PRINT "INITIALIZING THE PLOTTER"
COUT("IN;SP1;PU;", 1)
COUT("IP2000, 2200, 5500, 7000;", 1)
COUT("IP2000, 2200, 5500, 7000;", 1)
630  COUT(,"SC0","+STR$(Y3)+",0,"+STR$(X3)+";",1)
640  PAUSE(2)
650  PRINT \\ PRINT "SETTING UP AXES"
660  COUT(,"SI.30,.25;",1)
670  COUT(,"TL-1.5,0;",1)
680  PAUSE(2)
690  COUT(,"PU0,0;PD"+STR$(Y3)+",0,"+STR$(Y3)+","+STR$(X3)
       +","0,"+STR$(X3)+",0,0 P;",1)
700  PAUSE(4)
710  X4=(X2-X1)/(X3-1) 
    X=X1-X4
720  FOR X= 1 TO X3-1
730  X=X+X4 
    B=I*(X3/(X3-1))
740  COUT(,"PA"+STR$(Y3)+","+STR$(B)+";YT;P;",1)
750  COUT(,"DI0,1;CP-1,-1;",1)
760  COUT(,"LB"+STR$(X)+CHR$(3),,1)
770  PAUSE(1.5)
780  NEXT I
790  COUT(,"TL1.0;PA"+STR$(Y3)+",0;",1)
800  Y4=(Y2-Y1)/(Y3-1) 
    Y=Y1-Y4
810  FOR I= 1 TO Y3-1
820  Y=Y+Y4 
    Z=Y/100 
    B=I*(Y3/(Y3-1))
830  COUT(,"PA"+STR$(Y3-B)+",0;XT;P;",1)
840  COUT(,"CP-3.25,-.25;",1)
850  COUT(,"LB"+STR$(Z)+CHR$(3),,1)
860  PAUSE(1.5)
870  NEXT I
880  PRINT \\ PRINT \\ "PLOTTING THE DATA"
890  COUT(,"SC"+STR$(Y2)+"","+STR$(Y1)+","+STR$(X1)+","+STR$(X2)+";",1)
900  COUT(,"PU;SP2;P;",1)
910  PAUSE(2)
920  COUT(,"PA"+STR$(A(I))+","+STR$(C(I))+";PD;",1)
930  FOR I=2 TO N
940  COUT(,"PA"+STR$(A(I))+","+STR$(C(I))+";",1)
950  PAUSE(.5)
960  NEXT I
970  COUT(,"PU;",1)
980  PRINT \\ PRINT \\ "PLOTTING ROUTINE COMPLETED"
990  IF Z9=1 THEN 1270
1000  PRINT \\ PRINT "Do you wish to have (A)xes label
s, (T)itle or (B)oth on your plot"; 
     INPUT Q$
1010  IF Q$='A' THEN 1050
1020  IF Q$='B' THEN 1050
1030  IF Q$='T' THEN 1200
1040  GOTO 1000
1050  COUT(,"SP1;P;",1)
1060  T=LEN(X$)/2
1070  COUT(,"DI0,1;SI.3,.3;",1)
1080  PAUSE(2)
1090  COUT(,"PA"+STR$(Y1)+","+STR$((X2-X1)/2+X1)+";",1)
1100  COUT(,"CP-"+STR$(T)+",-2;",1)
1110  PAUSE(1)
1120  COUT(,"LB"+X$+CHR$(13)+CHR$(3),,1)
1130  PAUSE(5)
1140 T=LEN(Y$)/2
1150 COUT("DI-1,0;",1) \PAUSE(2)
1160 COUT("PA"+STR$(Y2-(Y2-Y1)/2)+","+STR$(X1)+";",1)
1170 COUT("CP-"+STR$(T)+","+3.8;",1) \PAUSE(1)
1180 COUT("LB"+Y$+CHR$(13)+CHR$(3),1)
1190 PAUSE(5) \ IF Q$='A' THEN 1270
1200 T=LEN(T$)/2
1210 COUT("DIO,1;",1)
1220 PAUSE(2)
1230 COUT("PA"+STR$(Y2)+","+STR$((X2-X1)/2+X1)+";",1)
1240 COUT("CP-"+STR$(T)+",2;",1) \PAUSE(1)
1250 COUT("LB"+T$+CHR$(13)+CHR$(3),l)
1260 PAUSE(5)
1270 COUT("SP0;",1)
1280 PRINT 'Do you wish to do another plot on the same axes [N]; " INPUT N$
1290 IF N$=' ' THEN 32767
1300 IF N$='N' THEN 32767
1310 IF N$='Y' THEN 1330
1320 GOTO 1280
1330 Z9=1 \ GOTO 30
32767 END


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