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EFFECT OF pH AND TEMPERATURE ON CONFORMATIONAL CHANGES OF A HUMANIZED MONOCLONAL ANTIBODY

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**EFFECT OF pH AND TEMPERATURE ON CONFORMATIONAL
CHANGES OF A HUMANIZED MONOCLONAL ANTIBODY**

BY

SEJAL GANDHI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

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IN

PHARMACEUTICS

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2002

MASTER OF SCIENCE THESIS

OF

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2002

ABSTRACT

Maintenance of the structural integrity of a therapeutic protein is essential for its efficacy in relation to physiological and pharmacological activity. Therefore, a major challenge confronting the pharmaceutical scientist working with protein formulation is the instability of the protein during processing, handling, storage, and use. Hence well-defined preformulation studies for proteins need to be performed to understand the stability issues relevant to their production, formulation, and use. Vast numbers of drugs available in the market are micromolecular drugs, which have molecular weight less than 1000 D. There is a good reason to believe that polypeptides and proteins offer different challenges from conventional molecules. Protein drugs are probably the wave of the future.

Fluorescence spectroscopy has found wide use in studying denaturation of proteins by monitoring its folding-unfolding pattern. Protein conformational changes are usually accompanied by changes in the fluorescence emission spectrum. Fluorescence spectroscopy is not a normal component of preformulation scan of new substances. However, it may have the potential for that purpose, particularly for polypeptides and proteins.

The conformational changes of a humanized monoclonal antibody have been characterized by intrinsic and extrinsic fluorescence measurements in aqueous solutions of pH 3 to 8 in the temperature range of 5 °C to 70 °C. 1-anilinonaphthalene 8-sulfonate (ANS) was employed as a polarity-sensitive extrinsic probe. ANS is a compound that fluoresces strongly in hydrophobic non-

aqueous environments, but is almost non-fluorescent in water. Because of these characteristics, it has proved to be a very useful probe of accessible protein interior.

Changes in the fluorescence spectrum of the protein are observed with increasing temperature. The intensity of the tryptophan peak decreased linearly with temperature. Additionally, the tryptophan emission peak displayed a red shift from 335 nm to about 345 nm over the temperature range studied. The onsets of temperature of transition for the protein were estimated at each pH. The protein at pH 6, 7 and 8 was characterized by an onset of transition of about 60 °C, which was higher than at pH 3 to 5. Since higher onset of transition temperature reflects greater thermal stability, the preformulation studies suggest the protein is likely to be most thermally stable in an aqueous solution formulated at pH 6, 7, and 8.

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Finally, I would like to dedicate my thesis to my adorable nephew, Saarth, and my lovely newborn niece, Esha.

PREFACE

This document has been prepared in the format of the manuscript plan in accordance to section 11-3 of the Graduate Manual at the University of Rhode Island.

The thesis contains two manuscripts. Manuscript I: Use Of Fluorescence Spectroscopy To Study Protein Structure discusses some of the potentials of fluorescence spectroscopy in studying protein structure. Manuscript II is titled Potential Of Fluorescence Spectroscopy As A Preformulation Tool in which the experimental details of monitoring the stability of a protein under different temperature and pH conditions using fluorescence spectroscopy are described.

I performed all the experimental data presented in this thesis except the Differential Scanning Colorimetry (DSC) data (page 45), which was performed by my colleague, Mr. Eric Faulkner, at Biogen, Inc., Cambridge, MA. The DSC data obtained by Mr. Faulkner is included in my thesis because it is supportive of the conclusions that I have reached from my spectrophotometric studies. A general summary of conclusions and bibliography for the entire thesis follows at the end.

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OBJECTIVES

The main objectives of this study have been listed below:

1. To develop methods for intrinsic and extrinsic fluorescence techniques of protein analysis.
2. To investigate the effect of pH and temperature on structural changes of a humanized monoclonal antibody (protein) characterized by intrinsic and extrinsic methods of fluorescence spectroscopy.
3. To determine the optimum pH at which the aqueous formulation of protein is most stable.
4. To correlate the onsets of temperature of transition estimated using intrinsic and extrinsic methods of fluorescence.

MANUSCRIPT 1

USE OF FLUORESCENCE SPECTROSCOPY TO STUDY PROTEIN STRUCTURE

ABSTRACT

Fluorescence spectroscopy plays a major role in protein folding studies. It is now one of the most useful techniques for the study of protein structure. A variety of fluorescence observables can be used to follow denaturation or renaturation of the protein native structure induced by changes in temperature, pH, or additions of solutes. Unfolding of proteins results in increased exposure of hydrophobic residues to aqueous environment revealed by fluorescence red shift. The emission maximum of tryptophan is markedly affected by solvent polarity. This manuscript discusses some of the potentials of fluorescence methods in studying protein structure. Characteristics of protein fluorescence and fluorescence emission parameters have been discussed.

1. PROTEIN STABILITY

Proteins are condensation products of amino acids condensed together by peptide bonds. Typically, proteins have four levels of structure: primary structure, secondary structure, tertiary structure, and quaternary structure. The complex hierarchy of a native protein may be disrupted by multiple possible destabilizing mechanisms (1,2). Changes at any level of the protein structure can impact the molecule's activity. Protein chains fold spontaneously into their native (biologically active) conformation, most often with the hydrophobic residues interior to the protein and the charged residues on the surface. Protein denaturation occurs when a polypeptide loses its higher level structure and often results in loss of biological activity.

Typically, the native structure exhibits only marginal stability that is easily upset by even subtle environmental changes in shear stress, temperature, pH, ionic strength, or a combination thereof. Denaturation is usually induced at gas-liquid, liquid-liquid, or container-liquid interfaces, although heat, pH variations, solvents, salts, and excipients may also contribute. A number of pharmaceutical-associated activities have been found to induce denaturation. Formulation activities and filling activities along with shipping of liquid formulation often result in significant exposure of proteins to gas-liquid interfaces.

Proteins are different from "traditional" low-molecular-weight drugs. The essential distinction between the approaches used to formulate and evaluate proteins, compared to the conventional low molecular weight drugs, lies in the

need to maintain several levels of protein structure and the unique chemical and physical properties. The integrity of primary, secondary, tertiary, and quaternary structure of proteins should be guaranteed during their shelf life. Rational design of formulations that optimize the native structure and the bioactivity of a protein is therefore of great importance.

2. IMPORTANCE OF PROTEIN PREFORMULATION STUDIES

Preformulation is an integral part of the entire development process. It encompasses the phase of formulation development in which the pharmaceutical scientist takes an initial “look” at the protein molecule to identify the conditions that are likely to be best suited for development of a formulation possessing long-term stability. Well-defined preformulation studies yield much knowledge of the inherent degradative pathways to which a particular protein molecule is susceptible. The identification of the degradation mechanisms allows rational design decisions during formulation development. Unfortunately, no one technique is able to provide information about various structural aspects of proteins. Therefore, protein formulation development must be supported by multiple analytical approaches to capture a complete profile of the chemical and physical stability of prototype formulations.

3. FLUORESCENCE SPECTROSCOPY

3.1. Introduction

Fluorescence spectroscopy is an extremely powerful analytical tool that has been used in areas ranging from biology to physics. The term “fluorescence” was coined by George Stokes in 1852. He determined that the emission from quinine was at a longer wavelength than the excitation. He was the first to propose the use of fluorescence as an analytical tool.

The availability and simplicity of basic data acquisition and analysis are important practical features behind the popularity of fluorescence as compared to other spectroscopic techniques. Fluorescence is a dynamic phenomenon and the lifetime of the excited state is sufficient for a variety of chemical and physical reactions to take place prior to emission. Fluorescence spectroscopy is essentially a probe technique sensing changes in the local environment of the fluorophore, which distinguishes it from generalized techniques, such as, calorimetry, far-ultraviolet circular dichroism (CD), and infrared (IR) spectroscopy.

3.2. Basic Principle

Fluorescence activity can be schematically described with the classical Jablonski diagram as shown in Figure 1. It was first proposed by Professor Alexander Jablonski in 1935 to describe absorption and emission of light.

Biomolecules containing nonbonded electrons can be excited by light in the ultraviolet or visible regions of the spectrum. When such a molecule is irradiated by light of an appropriate wavelength, an electron is excited from the singlet ground state (S^0) to the first excited singlet state (S^1), a process that is

called absorption (10^{-15} sec). Depending on the configuration of the molecule at the moment of excitation, an electron can populate any one of several different vibrational levels in the S^1 state. However, since the higher vibrational levels exhibit higher energies, electrons that initially reside in these levels quickly (10^{-12} sec) relax down to the lowest vibrational level of the S^1 state. This process is called internal conversion. Once the lowest vibrational level of the S^1 state is reached, one of several competing processes can ensue. These include: (1) decay to the S^0 state by emission of a photon (fluorescence); (2) decay to the S^0 state by nonradiative processes; and (3) “intersystem crossing” to the first excited triplet state (T^1), followed by decay back to the S^0 state (phosphorescence).

Thus, fluorescence emission is observed when an excited electron returns from the first excited state back to the ground state. As some energy is always lost by non-radiative processes, such as vibrational transitions, the energy of the emitted light is always less than that of the absorbed light. Hence the fluorescence emission invariably occurs at a longer, less energetic wavelength than absorption. This red shift in wavelength was first observed by Stokes (1852), and in his honor is referred to as the “Stokes’ shift”.

3.3. Fluorescence Parameters

3.3.1. Fluorescence Emission:

The most basic fluorescence experiments irradiate the sample with light at or close to its absorption maximum, and measure the resulting fluorescence as a function of emission wavelength. Fluorescence intensity measured as a function of

wavelength comprises the emission spectrum. The position of the emission spectrum reflects changes in energetics of the excited and ground state between excitation and emission of a photon. The position and shape of an emission spectrum will not change with excitation wavelength. Since the time taken for the electron to relax down to the lowest vibrational level in the excited state is short compared to the total time spent in the excited state, the lowest level will always be reached before fluorescence occurs. Therefore, the resulting spectra will depend on the probabilities of relaxing into the ground state only. Fluorescence spectra are generally mirror images of their absorbance spectra. Any fine structure observed in absorption spectra reflects the vibrational energy levels of the excited state. An emission spectrum reflects the distribution of electrons in the vibrational levels of the ground state.

3.3.2. Fluorescence Lifetime

Fluorescence lifetime is the average time the fluorophore spends in the excited state. Lifetimes are generally of the order of 10^{-8} sec and require specialized equipment in order to be measured. Since the functionally important fluctuations that occur in biological systems and fluorescence lifetimes occur on a similar time scale, measurement of these lifetimes is a useful probe of the dynamics in these systems.

3.3.3. Fluorescence Anisotropy or Polarization

Fluorescence anisotropy is the degree to which fluorescence emission is depolarized relative to polarized exciting light. If the fluorophore is excited by polarized light, then only molecules whose transition moment is in the correct orientation will be excited. The extent of depolarization depends on the movement of the fluorophore during fluorescence lifetime. The measurement of such parameters can therefore be used to measure the degree of rotational freedom of the fluorophore on the lifetime time scale. Experimental determinations of anisotropy fall into two general categories: steady-state and dynamic, depending on the instrumentation employed. In steady-state measurements one determines the time-averaged polarization of fluorophore, while in dynamic measurements the change in polarization is monitored over the duration of the fluorescence lifetime. Both, the theoretical and technical aspects of fluorescence anisotropy have been extensively reviewed (3-6).

3.3.4. Fluorescence Quenching

Fluorescence quenching is the means by which some compounds have the ability to decrease the fluorescence of some fluorophores (7). There are two kinds of quenching: static, which involves a complex formation between the quencher and the fluorophore, and dynamic quenching, which requires a collision between the two substances. In each case, contact between the fluorophore and quencher during the lifetime of the excited state causes the fluorophore to return to the ground state, transferring its energy to the quencher without fluorescence occurring. The result is a decrease in fluorescence intensity and lifetime. Since

contact between fluorophore and quencher is required, the ability to quench a particular fluorescent group is indicative of the accessibility and dynamics of the system under study.

3.3.5. Energy Transfer

Energy transfer is the effect when an electronically excited fluorophore (donor) can transfer its energy to another fluorophore (acceptor), which then emits the energy as fluorescence (8). The amount of energy transfer depends on the extent of overlap of the donor's fluorescence and the acceptor's absorption spectra. The efficiency of the process also depends on the relative orientation of the donor's and acceptor's transition dipoles, and the distance they are apart. The major application of energy transfer is therefore as a measure of distance on the atomic scale between fluorescent groups.

4. PROTEIN FLUORESCENCE

4.1 Intrinsic Fluorescence

The fluorescence of proteins originates from phenylalanine, tyrosine, and tryptophan residues. They all absorb light in the UV region of the spectrum and emit fluorescence between 250 and 400 nm. Emission spectra of the three amino acids are shown in Figure 2. In proteins containing all three aromatic amino acids, fluorescence is usually dominated by the contribution of the tryptophan residues, because both, their absorbance at the wavelength of excitation and their quantum yield of emission are considerably greater than the respective values for tyrosine

and phenylalanine (Table 1). Phenylalanine exhibits the weakest fluorescence (quantum yield of 0.02) of the three and the lowest extinction coefficient. Tyrosine is more fluorescent than phenylalanine, but still emits relatively weakly compared to tryptophan. Emission of both tyrosine and tryptophan residues is observed when protein fluorescence is excited near the absorbance maximum around 280 nm. Excitation at or above 295 nm gives emission spectrum of tryptophan almost exclusively. Tryptophan residues are particularly valuable intrinsic probes since the indole ring is very sensitive to its environment and since there are often only a few tryptophan residues in a protein. In water, tryptophan's emission spectrum is typically centered between 348 to 350 nm and has a quantum yield of 0.23. This maximum shifts toward shorter wavelengths as the polarity of the solvent decreases. The fluorescence quantum yield of tryptophan residues in proteins reflects its position and interactions with neighboring residues.

The various environments of the fluorophores of a folded protein and the unique stereochemistry of the polypeptide chain affect the fluorophores in many ways which can be used to characterize and to follow changes in the folded conformation in solution (9,10).

Fluorescence spectroscopy is widely used in studying the denaturation of proteins by monitoring its folding-unfolding pattern. Unfolding of proteins is usually accompanied by a red-shifted emission maximum, depolarization of tryptophan fluorescence, enhanced accessibility to quenchers such as iodide, and changes in fluorescence lifetimes. It is used primarily for the assessment of tertiary structure of proteins.

Both, shifts in wavelength and changes in intensity are generally observed upon unfolding in proteins containing tryptophan (10). In native proteins, the tryptophan is deeply buried in the hydrophobic core. As the protein unfolds, the tryptophan gets exposed to the aqueous solvent and the intensity of fluorescence emission decreases. This is accompanied by a red shift. A blue shift signifies a deeply buried or shielded tryptophan residue. Changes in protein conformation, such as unfolding, thus very often lead to large changes in the fluorescence emission.

4.2 Extrinsic Fluorescence

Frequently, the natural fluorescence properties of macromolecules are not adequate for the desired experiments. In these cases, fluorophores foreign to the system under study but displaying improved spectral properties are chosen. They can be either covalently or noncovalently bound to the protein. The fluorescence signal reflects polarity of the molecules surrounding the polarity-sensitive probe. If the dye has a specific binding site on a macromolecule, it is possible to assess the polarity of the site. The dye is thus a useful probe of the degree of exposure of hydrophobic sites as the structure of a protein is perturbed (11,12)

The selection of the dye generally depends on application. In cases where, sensitivity is critical, such as ligand binding, the visible-absorbing fluorescein dyes with high molar absorptivity and quantum yields are frequently used. Dansyl chloride is widely used to label proteins where polarization measurements are anticipated. 1-Anilino-8-naphthalene sulfonic acid (1,8-ANS or ANS), 2-p-

toluidinylnaphthalene-6-sulfonic acid (2,6-TNS or TNS), and similar derivatives are frequently used as non-covalent labels for proteins and membranes. ANS is essentially nonfluorescent in water, but are highly fluorescent when dissolved in nonpolar solvents or when bound to macromolecules (13). It binds only to small hydrophobic pockets on a protein's surface and, hence, generally does not bind to native proteins. This therefore provides a useful method of following protein folding.

5. CONCLUSIONS

In order for any spectroscopic method to be used to monitor a conformational transition of a macromolecule, there must be a discernable difference in some signal between the two or more macroscopic states. Fluorescence spectroscopy is very useful for such studies because fluorescence signals are extremely sensitive to the microenvironment of a fluorophore. Changes in protein conformation can lead to changes in emission maximum or quantum yield. Fluorescence is a very convenient and adaptable method for various sample chambers, types of preparations, or experimental designs. A broad range of protein concentrations can be studied (nanomolar to millimolar), which can minimize the amount of sample needed. This is especially advantageous in preformulation studies when only a modest amount of protein is available for performing various stability studies. Fluorescence is a multi-dimensional method and enables measurements to be made as a function of intensity, wavelength, time (i.e., decay time of the excited state or reaction time for a conformational transition),

polarization angle, or solution conditions (i.e., a range of salt concentration, pH, temperature, pressure, etc.)

It is a rapid monitoring method, which makes it possible to have a rapid throughput in data collection. This is particularly useful in the preformulation stage to obtain quick information about the compatibility of different excipients with the protein under investigation. The emission signal is measured above a low background level hence high sensitivity. The sensitivity of fluorescence techniques is as much as 1000 times more sensitive than absorption spectroscopy. Because it is a non-invasive technique, fluorescence does not interfere with the sample. The excitation light levels required to generate a fluorescence signal are low, reducing the effects of photo-bleaching, and living tissue can be investigated with no adverse effects on its natural physiological behavior.

Fluorescence measurements are usually more selective than absorption spectrophotometry measurements. For example, although tyrosine and tryptophan absorb at similar wavelengths, the latter emits with a maximum near 350 nm while the emission maximum of tyrosine is at 303 nm. By appropriate choice of emission wavelengths, it is possible to obtain the absorption spectrum of either of these chromophores in the presence of the other.

The richness of information content, together with the sensitivity of the measurements and the existence of intrinsic fluorescing groups and the ability to introduce extrinsic groups, has made fluorescence spectroscopy a method of particular importance in studies with proteins.

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TABLE 1: ABSORBANCE AND FLUORESCENCE PROPERTIES OF AROMATIC AMINO ACIDS

Compound	Absorbance λ_{\max} (nm)	Fluorescence λ_{\max} (nm)	Quantum Yield
Tryptophan	280	348	0.2
Tyrosine	274	303	0.14
Phenylalanine	257	282	0.04

FIGURE 1: JABLONSKI DIAGRAM OF ENERGY

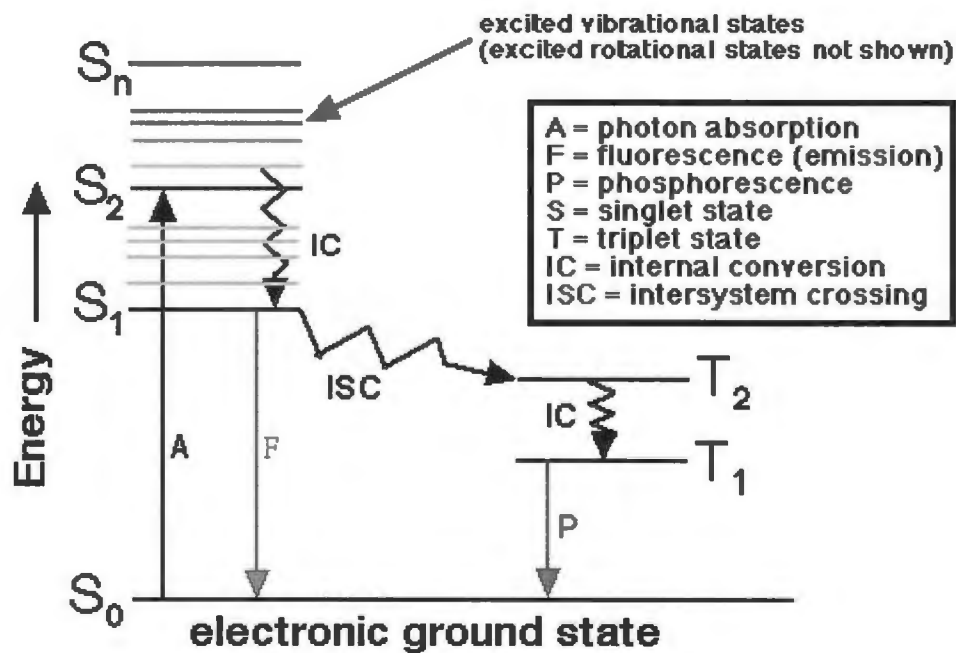
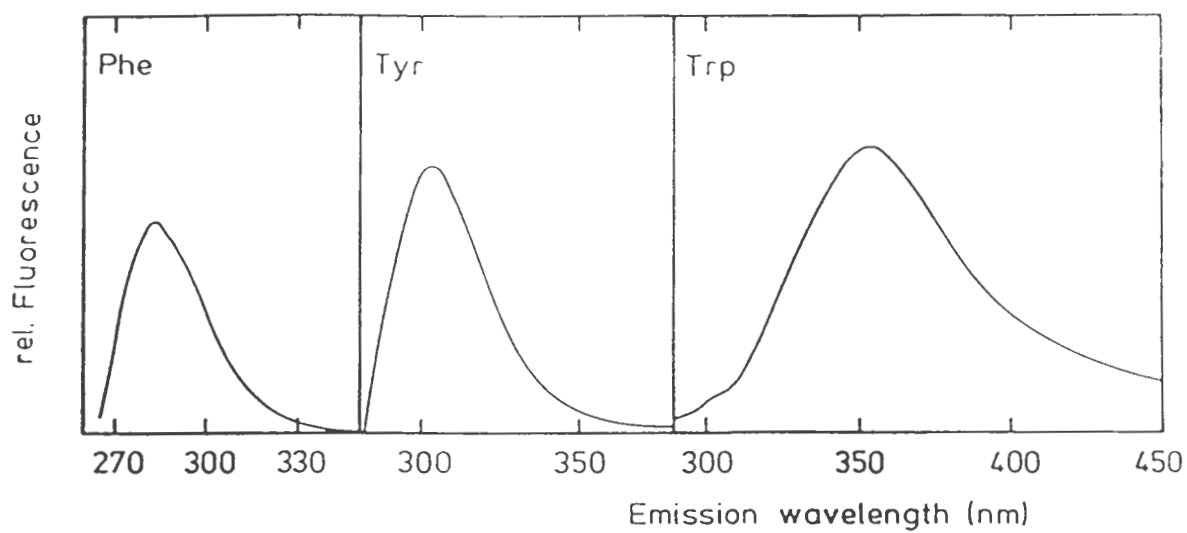


FIGURE 2: FLUORESCENCE SPECTRA OF THE AROMATIC AMINO ACIDS



MANUSCRIPT II

POTENTIAL OF FLUORESCENCE SPECTROSCOPY AS A PREFORMULATION ANALYTICAL TOOL

ABSTRACT

The conformational changes of a humanized monoclonal antibody (Mab) have been characterized by intrinsic and extrinsic methods of fluorescence spectroscopy. The intrinsic and extrinsic fluorescence emission spectra were obtained from 5 °C to 70 °C in 20 mM citrate buffers in the pH range of 3 to 8 at 5 °C intervals. Changes in the fluorescence spectrum of the protein were observed with increasing temperature. The tryptophan emission peak displayed a red shift from 335 nm to about 345 nm over the temperature range studied. This may indicate that even at 70 °C, the protein is not extensively unfolded, in which case a peak position of about 355 nm would be expected. 1-anilinonaphthalene 8-sulfonic acid (ANS) was used as a hydrophobic probe for extrinsic studies. The extrinsic intensity reflects the polarity of the molecules surrounding the hydrophobic probe ANS. The binding of the dye to protein at pH 3, as reflected by emission intensity, is relatively higher as compared to that at higher pH values. A possible explanation for the above result would be that at low pH, the dye might bind to protein by electrostatic interaction in addition to hydrophobic interaction. In addition, the protein may be unfolded at pH 3. The onsets of temperature of transition were

estimated at each pH. Higher temperature of transition indicates greater thermal stability. The protein exhibited an onset of temperature of transition of about 60 °C at pH 6, 7, and 8 suggesting that the protein would be most stable in an aqueous solution formulated at these pH values. At pH 3 to 5, an early onset of transition of about 35 °C was observed. The results reported using fluorescence spectroscopy are supported by the DSC data. This clearly indicates the potential of fluorescence spectroscopy for protein drugs as a routine preformulation tool.

1. INTRODUCTION

Monoclonal antibodies (Mabs) are immunoglobulins, usually of the IgG class, consisting of two identical heavy and two identical light chains, which are covalently linked by disulphide bonds and folded into compact globular regions. Each domain in an immunoglobulin has a similar structure of two beta sheets packed tightly against each other in a compressed antiparallel barrel. They consist of six short regions (of about 10 amino acids) of highly variable amino acid composition forming the antigen-binding site. The hinge region allows a fair amount of rotational flexibility relative to the rest of the molecule. Comprehensive reviews about structure and function of immunoglobulins have been given by Jeske and Capra (1) and by Edmundson and Ely (2). The structure, folding, and stability of immunoglobulins and their fragments are well characterized (3-7)

Mabs belong to the new generation of biotechnological products that are becoming increasingly important in pharmaceutical sciences. An important aspect of pharmaceutical Mab preparations is their physico-chemical stability upon storage (8). Protein precipitation can be either reversible or irreversible and can be induced by low or high ionic strength, heating, extremes of pH, freezing and thawing, and mechanical forces (9). Structural changes in proteins that lead to exposure of hydrophobic sites often result in loss of biological activity. Most protein solutions are not thermally stable above room temperature for prolonged periods of time; therefore accelerated studies are typically conducted.

The protein of interest in this study is a humanized monoclonal antibody of molecular weight of about 150 kD. Characterization of drug molecule is a very

important step at the preformulation phase of product development. Every protein has unique characteristics, some of which may cause difficulty in designing stable formulations. Preformulation studies can provide great insight into specific solution conditions that confer the greatest stability to a given protein. Simply avoiding extremes in pH can drastically reduce the rate of deamidation (8,10). The melting temperature of a protein provides one measure of the physical stability of the molecule. Melting temperature (T_m) is defined as the temperature at which equal amounts of native and denatured protein exist in equilibrium. Conditions producing increases in the T_m (or, more precisely, the Gibbs free energy of denaturation) for a particular protein provide for greater resistance to thermal denaturation and thus greater physical stability (11).

Proteins are naturally fluorescent. Hence, protein fluorescence is ideal for probing many structural aspects of proteins including folding and conformations. Protein chains fold spontaneously into their native conformation, most often with the hydrophobic residues interior to the protein and the charged residues on the surface. Typically, the native structure exhibits only marginal stability that is easily upset by even subtle environmental changes in shear stress, temperature, pH, ionic strength, or a combination thereof. Changes in protein conformation, such as unfolding, very often lead to large changes in the fluorescence emission.

In the present study, the conformational changes of the humanized Mab were investigated as a function of pH and temperature and thus generated the preformulation data. The optimum pH at which the protein formulation is likely to be most stable was determined by estimating the temperature of onset of transition

at each pH. The structural changes were probed using intrinsic and extrinsic methods of fluorescence spectroscopy.

Fluorescence can often be used to follow changes in protein conformation during the process of denaturation, particularly when the protein contains tryptophan residues that are buried in the native structure but become exposed to water during unfolding. With progressive exposure of tryptophan residues to water, which acts as a moderately effective quencher, the emission intensity typically decreases, although the extent of intensity change can vary considerably, depending on the local environment of the tryptophan residues in the native structure. In addition, as the tryptophan residues are shifted from the protein interior of low dielectric to an aqueous environment of high dielectric, there is a red shift in the fluorescence emission maximum, typically from 335 nm to 354 nm (12). In general, the changes in tryptophan emission intensity and the emission wavelength occur synchronously during the denaturation process (13-16)

1-anilinonaphthalene 8-sulfonic acid (ANS) was used as the fluorescent hydrophobic probe. While ANS is only weakly fluorescent in aqueous solutions, its fluorescence is greatly enhanced when it is buried in the protein matrix and screened from water quenching (17,18). It has frequently been used as a probe to reveal the presence of partially folded intermediates due to the presence of increased exposure of contiguous hydrophobic surface area (19-21). It was initially applied to the investigation of the hydrophobicity of native proteins (17). Stryer has established that interaction of ANS with the solvent-exposed hydrophobic clusters of apomyoglobin and apohemoglobin results in considerable increase of

ANS fluorescence intensity. These intensity changes were accompanied by a significant blue shift of the fluorescence spectrum of this probe.

The preformulation studies provided essential data needed for optimum drug formulation development. Protein stability was closely related to the changes in temperature and pH conditions. The temperature dependence of the denaturation rate changed at around transition temperature (T_m). These results illustrate the importance of preformulation and stability characterization of protein pharmaceuticals in support of both process and formulation development.

2. MATERIALS AND EQUIPMENT

2.1. Materials

The humanized monoclonal antibody, an immunoglobulin of the subtype IgG was obtained from Biogen, Inc.

20 mM citrate buffers of pH 3, 4, 5, 6, 7, and 8 were obtained from Biogen, Inc.

The fluorescent hydrophobic probe, 1-anilinonaphthalene 8-sulfonate (ANS), Lot # PE0121, was obtained from Spectrum.

Ethanol supplied by Spectrum, Catalog # ET 107

Pierce Slider-A-Lyzer® cassette (10,000 MWCO Catalog No. 66425)

0.22 μm Millipore Millex-Gv filter, Catalog # SLGV025LS

3 ml sterile disposable syringe, Becton Dickinson™, Catalog # 309585

Needle 20 G 1 Precision Glide, Becton Dickinson™, Catalog # 305175

2.2. Equipment

Fluoromax-2 spectrofluorometer connected to a PC equipped with Datamax software, manufactured by JY Horiba, ID 1343, S/N 6340

Diode Array UV-visible spectrophotometer: Hewlett Packard Model 8452A, ID 2212, S/N 8452AX

3. DEVELOPMENT OF METHOD

3.1. Preparation of the protein sample

The protein sample was dialyzed in the buffer medium of appropriate pH for 24 hours with four buffer exchanges at about 4 °C. The concentration of the dialyzate was measured by UV/Vis spectrophotometer at 280 nm. Appropriate dilutions were made to get the required concentrations.

3.2. Fluorescence measurements

3.2.1. Intrinsic Fluorescence

The intrinsic fluorescence spectrum of tryptophan was monitored, using an excitation wavelength of 295 nm to avoid excitation of tyrosine.

To obtain an emission spectrum for a protein, the excitation wavelength should be offset from the emission start scan by 15-20 nm. Monitoring the emission at excitation wavelength gives information about light scattering of the protein, and hence useful in studying protein aggregation. The initial objective of this work was to study protein conformational changes as well as aggregation at different pH and temperature conditions. Hence the protein emission was monitored from 280-400 nm. Since the light scattering data obtained was difficult to interpret, it has not been included in the thesis.

The scan rate was optimized to give the best S/N ratio. By increasing the scanning time, the signal is averaged longer, resulting in a better S/N ratio. To select the optimum scan rate for analysis, the protein was scanned at 1 nm/0.25 sec, 1 nm/0.5 sec, 1 nm/sec, and 1 nm/2 sec (data not shown). The scan rate of 1 nm/sec gave the highest S/N ratio.

The excitation and emission slits were set to 4.25 nm band pass equivalent to 1 nm. The protein concentration employed was 0.05 mg/ml. Fluorescence measurements were made using teflon-stoppered quartz cuvettes with magnetic stir bars.

3.2.2. Extrinsic Fluorescence

It is necessary to determine the appropriate ANS: protein ratio for the extrinsic fluorescence experiments. 1, 3, 5, 7.5 and 10 μ L of 10 mM ANS solution

in ethanol were spiked in 1 ml of 0.5 mg/ml protein solutions. The fluorescence spectrum was then monitored at 25 °C, using an excitation wavelength of 372 nm. As seen from Figure 1, an increase in fluorescence intensity was observed with increase in ANS concentrations. 7 μM ANS concentration was found to be optimum so that the ANS: protein ratio was 20:1.

Protein samples (0.5 mg/ml) were excited at 380 nm and emission was monitored from 400-600 nm with scan rate of 1 nm/sec. The emission spectrum of ANS in appropriate buffer was used as a blank.

The intrinsic and extrinsic fluorescence emission spectra were obtained from 5 °C to 70 °C in 20 mM citrate buffers in the pH range 3 to 8 at 5 °C intervals with incubation at each temperature for 5 min prior to data acquisition. The temperature was maintained by a water bath connected to the fluorometer, which was pre programmed to increase temperature from 5 °C to 70 °C. The sample was continuously stirred using a magnetic stirrer during the incubation period. The spectra were collected for each temperature interval. Each spectrum was replicated twice, and the wavelength emission maxima were determined for all spectra using polynomial linear regression of third order. The onset of the temperatures of thermal transition for the protein was estimated from the intrinsic and the extrinsic fluorescence data by extrapolation of the intensity curves at each pH.

4. RESULTS AND DISCUSSION

4.1. Intrinsic Fluorescence

4.1.1. Effect of Accelerated Temperature Ramping on Intrinsic Intensity

4.1.1.1. Intrinsic Emission Spectra (Raw Data)

An emission spectrum is obtained by plotting fluorescence intensity versus wavelength. Figures 2 (a-f) show the emission spectra of the protein from 5 °C to 70 °C at 10 °C intervals at each pH. A transition at about 40 °C, characterized by an increase in intensity, is observed at pH 3 and 4. At higher pH values, changes in intensities were observed between 50 °C and 60 °C indicating the onset of transition.

4.1.1.2. Effect of pH on Onset of Transition Temperature

Changes in the fluorescence spectrum of the protein are observed with increasing temperature. The fluorescence emission of tryptophan is highly sensitive to the polarity of its environment, thus providing a useful tool for probing conformational changes in proteins. The effect of temperature on protein tertiary structure was examined by monitoring changes in intrinsic fluorescence intensity as a function of temperature.

The effect of accelerated temperature ramping from 5 °C to 70 °C on the emission intensity of tryptophan at 336 nm at each pH is as shown in Figure 3. The intensity measurements were made at 336 nm since maximum intensity was observed at that wavelength.

The fluorescence intensity displayed a linear decrease with temperature, with transition observed at the onset of temperature of thermal transition (T_o) at each pH. Up until 35 °C, the intensity of the tryptophan at 336 nm decreased linearly with temperature at all pH values. At pH 6, 7, and 8, the onset of transition was observed at about 60 °C at which temperature an increase in intensity was observed. Fluorescence is a radiative process involving transfer of electron from first excited triplet state to ground state. As the temperature increases, the rate of emission of the photon by other non-radiative processes such as internal conversion increases, resulting in a decrease in the fluorescence emission intensity. Further, tryptophan may undergo photo-degradation to non-fluorescent kynurenine, which again results in a decrease in the fluorescence emission intensity with increase in temperature.

Since the protein under study has multiple domains, more than one transition was observed at pH 3, 4, and 5. At pH 3, it displayed an earliest onset of transition at about 35 °C. On decreasing the pH from neutrality to approximately to 3.0, proteins become maximally positively charged, since the pKs of most carboxylic groups of amino acid residues are ≥ 3 . The resulting intramolecular repulsion between the positively charged groups is the driving force of acid unfolding of proteins as they fail to overcome the interactions such as hydrophobic forces, salt bridges, and metal ion-protein interactions that favor folding. Hence at pH 3, the protein may exist already in unfolded state. The protein at pH 3 unfolds exposing the hydrophobic residues to the aqueous solvent until about 35 °C, as indicated by a decrease in the intrinsic fluorescence emission intensity.

As in pH 3, the protein exhibited multiple transitions at pH 4 and 5. The onsets of temperature of thermal transition at each pH were estimated by extrapolation of intrinsic intensities are as shown in Table 1.

4.1.1.3. Effect of pH on Intensity at Extreme and Room Temperature Conditions

Figure 4 shows the intrinsic tryptophan intensity at 5 °C, 25 °C, and 70 °C at 336 nm with respect to pH. At pH 5, 6, 7, and 8, the protein displayed a decrease in intensity as the temperature was increased from 5 °C to room temperature (25 °C) and further to 70 °C. At lower pH values of 3 and 4, the protein showed a decrease in intensity as the temperature was increased to 25 °C. This was followed by an increase in emission intensity as the temperature was raised to 70 °C. This may suggest the formation of an intermediate partially unfolded state referred to as molten globule state. This state is considered to consist of an overall fold similar to that of the native state in which the secondary structure is somewhat changed and the tertiary structure largely disrupted (22).

4.1.2. Effect of Accelerated Temperature Ramping on Intrinsic Peak Wavelength

The polarity of the environment of tryptophan as reflected in fluorescence emission maximum (λ_{max}) also changes with the temperature of the protein solution. A common transition, characterized by an increase in the wavelength of the emission maximum from approximately 335 to 345 nm, was observed during thermal perturbation of the protein in the pH range studied (Figure 5). This

increase in emission maximum position presumably reflects some increase in exposure of one or more buried tryptophan residues at higher temperatures. A fully exposed tryptophan in an aqueous buffer is expected to have λ_{max} well over 350 nm (23,24). This indicates that thermal treatment does not completely unfold the protein and even at 70 °C, the tryptophan though exposed, is somewhat protected from the aqueous environment.

The emission intensity decreased linearly with temperature. This was not accompanied by a red shift initially as shown in Figure 6. The protein exhibited a blue shift as the temperature was increased from 5 °C to 25 °C, followed by a red shift on exposure to higher temperatures. The possible explanation for this behavior could be that the unfolding is only partial, and thus the tryptophans remain in a protein-like interior. This partially unfolded state is the molten globule state as suggested earlier. The further unfolding that occurs at much higher temperature finally exposes the tryptophans to the high dielectric of an aqueous medium, causing the expected red shift in their emission.

4.2. Extrinsic Fluorescence

4.2.1. Effect of pH on Emission Intensity at Room Temperature (25 °C)

Binding experiments with the polarity-sensitive dye 1-anilinonaphthalene 8-sulfonate (ANS) were performed in order to investigate the relative hydrophobicity of the accessible protein surface of the different conformational states as a function of temperature and pH. ANS is essentially nonfluorescent in aqueous solutions but becomes appreciably fluorescent in nonpolar environments. In addition, the

fluorescence emission maximum of the dye shifts to lower wavelengths in nonpolar environment. Thus, the dye is a useful probe of the degree of exposure of hydrophobic sites as the structure of a protein is perturbed.

Figure 7 shows the emission spectra of protein at 25 °C at each pH. The peak intensities obtained from Figure 7 were then plotted as a function of pH as shown in Figure 8.

The extrinsic fluorescence intensity reflects the polarity of the molecules surrounding the hydrophobic probe ANS. ANS is accompanied by an increase in its fluorescence intensity as well as by a blue shift of the fluorescence emission in non-aqueous environments. The emission intensity at room temperature decreased sharply as the pH was increased as seen from Figures 7 and 8. The extrinsic intensity at higher pH values is much smaller as compared to that at pH 3. This indicates that ANS is most shielded from aqueous solvent and therefore, extensively bound to the hydrophobic pockets on protein at pH 3.

The protein increases the fluorescence intensity of ANS about 32 folds at pH 3 (480 nm, 25 °C). The extrinsic intensity of the protein at pH 3 (480 nm, 25 °C) is 70-fold higher than the intensity at pH 8 suggesting the dye is extensively bound to the protein at low pH as compared to that at higher pH values.

The wavelength of the emission maximum of ANS bound to protein was approximately 480 nm. Hence, the extrinsic intensities at different pH values have been compared at 480 nm.

4.2.2. Effect of Accelerated Temperature Ramping on Extrinsic Emission Intensity

4.2.2.1. Extrinsic Emission Spectra (Raw Data)

The emission spectra of ANS bound to protein from 5 °C to 70 °C at 10 °C intervals at each pH is as shown in Figures 9 (a-f). The spectra obtained at pH 6, 7, and 8 are comparable, with the onset of transition observed at about 60 °C. At pH 3, the spectrum was markedly different. The changes in intensity with increase in temperature were minimum at pH 3 in comparison to the changes observed at higher pH values.

4.2.2.2. Effect of pH on Onset of Transition Temperature

The effect of temperature on intensity of ANS bound to protein in the pH range studied is shown in Figure 10. The intensities are normalized by dividing the intensity at each temperature by the intensity at 5 °C at that pH at 480 nm.

Intensity changes were monitored at 480 nm. At pH 6, 7, and 8, increase in extrinsic intensity was observed at about 60 °C. The increase in extrinsic intensities at higher temperatures indicates exposure of hydrophobic residues to aqueous solvent at higher temperatures and hence reflects greater thermal stability of the protein.

At pH 3, there was not an appreciable change in intensity observed with increase in temperature from 5 °C to 70 °C. An increase in binding of dye to the protein is indicated by an increase in ANS intensity between 35 and 55 °C at pH 4. The intensity at pH 5 displayed an increase at 50°C.

The onset of the temperature of thermal transition estimated from extrapolation of the extrinsic intensity curves are summarized in Table 2.

4.2.2.3. Effect of pH on ANS binding

The emission intensities measured at 480 nm are normalized by dividing the extrinsic intensity at each temperature by the intensity at 5 °C at pH 3 as in Figure 11.

The fluorescence emission intensity of ANS bound to protein at pH 3 is appreciably greater than that displayed at pH 8 in the temperature range studied. This indicates that some hydrophobic residues that were accessible to ANS at pH 3 became inaccessible at higher pH. The weak fluorescence emission intensity of the ANS-protein complex at pH 6, 7, and 8 suggests low dye binding.

ANS (Figure 12) consists of a solubilizing sulfonate SO_3^- group and a nearly water-insoluble anilinonaphthalene moiety. Recent works on ANS fluorescence suggest that the enhancement of ANS fluorescence upon binding to proteins results from the protection of the naphthalene moiety from water molecules (25).

ANS is a charged hydrophobic dye (21) having negative sulfonate ion at $\text{pH} \leq 3.6$ (26) and can interact both by hydrophobic as well as electrostatic interactions depending on the pH studied. Since the protein has a positive charge at low pH, the dye may bind to the protein by electrostatic interaction in addition to hydrophobic interaction. This explains the greater ANS-protein binding as reflected by increased emission intensity exhibited by the ANS-protein complex at

pH 3 (Figure 12). In addition, since the protein is unfolded at low pH, the hydrophobic residues are exposed to the aqueous solution, thereby, allowing greater ANS binding and hence higher emission intensity.

4.2.2.3. Comparison of Intensities at Extreme Temperature and Room Temperature Conditions

The emission spectra of ANS bound to protein at extreme temperature conditions of 5 °C and 70 °C are as shown in Figure 13. The spectrum obtained at 5 °C is identical to that obtained at room temperature (25 °C). This may indicate equivalent thermal stability at 5 °C and 25 °C in the pH range studied.

Figure 14 compares the peak intensities of the protein at extreme temperatures and room temperature at different pH values. The intensities of ANS bound to protein at 5 °C and 25 °C are nearly the same. This suggests that the extent of exposure of hydrophobic residues for binding to ANS at 5 °C is approximately the same as that at room temperature. At 70 °C, the protein unfolds exposing the hydrophobic clefts to aqueous solvent. This results in enhancement of ANS-protein binding as reflected by increase in emission intensity.

The intensity of protein at pH 3 is greatest in the temperature range studied (Figure 14). At pH 6, 7, and 8, the protein displayed a small increase in intensity, as the temperature was raised from 5 °C to 70 °C. In comparison, there was a significant increase in intensity observed at pH 4 and 5. This suggests that the protein is unfolded to a greater extent at low pH, which may reflect lower thermal

stability of protein. As indicated earlier, the protein at pH 3 may already be in unfolded state at 5 °C. Hence, ANS may be bound to most of the exposed hydrophobic residues of protein at 5 °C. Therefore, subjecting the protein to higher temperatures does not result in further exposure of free unbound hydrophobic residues for binding to ANS. The protein, thus, does not show an appreciable change in intensity with increase in temperature at pH 3.

4.3 Correlation Between Fluorescence Data and DSC Data

As seen from Tables 1 and 2, the onset of thermal transition temperatures estimated from intrinsic fluorescence data correlate with those estimated from extrinsic fluorescence data. The protein exhibited an onset of transition at about 60 °C in aqueous solutions of pH 6, 7, and 8 as demonstrated by using both, intrinsic and extrinsic fluorescence methods of analysis. This indicates greater thermal stability of the protein in aqueous solutions of pH 6, 7, and 8. Since the dye may bind to protein by electrostatic interaction in addition to hydrophobic interaction at low pH, the correlation of the onsets of transition temperature between intrinsic and extrinsic fluorescence data was found to be better at higher pH values.

The results obtained from the fluorescence studies correlate with those obtained from DSC data (Table 3). The DSC studies for the estimation of onsets of temperature of transition for the protein under study, were performed by Mr. Eric Faulkner, at Biogen, Inc., MA.

5. CONCLUSIONS

Drug product development of proteins is a challenge for pharmaceutical teams. Dealing with biopharmaceuticals in terms of preformulation is specific, due to the different nature of drugs encountered. This study demonstrates the valuable information that fluorescence spectroscopy, as an analytical tool, can provide about protein stability at preformulation stage when very little is known about the molecule.

The protein at pH 6, 7 and 8 is characterized by an onset of transition of about 60 °C. Since higher onset of transition temperature reflects greater thermal stability, the protein is likely to be thermally stable in an aqueous solution formulated at pH 6, 7, and 8. At pH 3 to 5, the protein exhibited early onsets of transition indicating lower thermal stability.

The correlation of the onsets of temperature of transition estimated using DSC and fluorescence techniques of analysis indicates the usefulness of fluorescence spectroscopy in monitoring the conformational changes of the protein under different pH and temperature conditions.

6. ACKNOWLEDGEMENTS

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**TABLE 1: ONSET OF TRANSITION TEMPERATURES ESTIMATED
USING INTRINSIC FLUORESCENCE**

pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
33 °C	41 °C	56 °C	62 °C	61 °C	63 °C
54 °C	51 °C	63 °C	-	-	-
65 °C	64 °C	-	-	-	-

**TABLE 2: ONSET OF TRANSITION TEMPERATURES ESTIMATED
USING EXTRINSIC FLUORESCENCE**

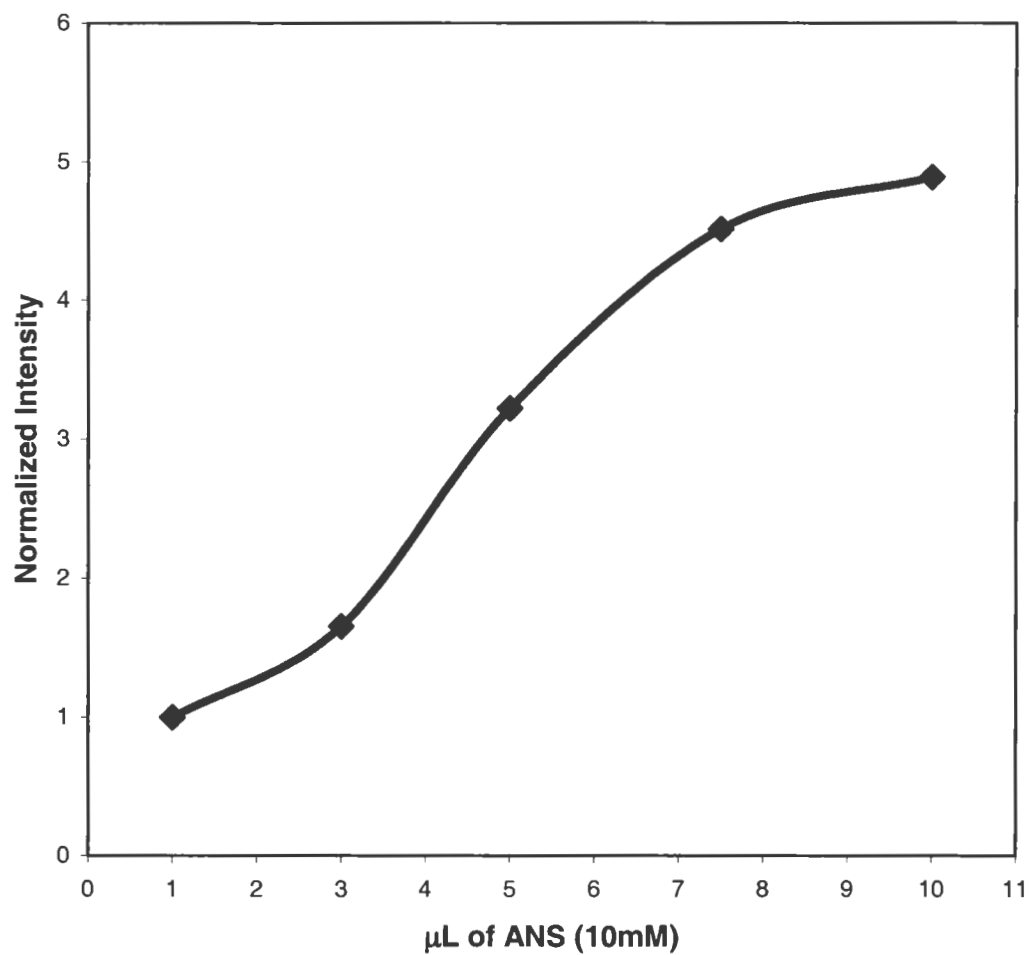
pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
48 °C	37 °C	53 °C	62 °C	62 °C	62 °C
57 °C	51 °C	58 °C	-	-	-
-	63 °C	-	-	-	-

TABLE 3: ONSET OF TRANSITION TEMPERATURES USING DSC

pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
32 °C	40 °C	53 °C	65 °C	65.5 °C	65.5 °C
51 °C	63 °C	65 °C	-	-	-
-	-	-	-	-	-

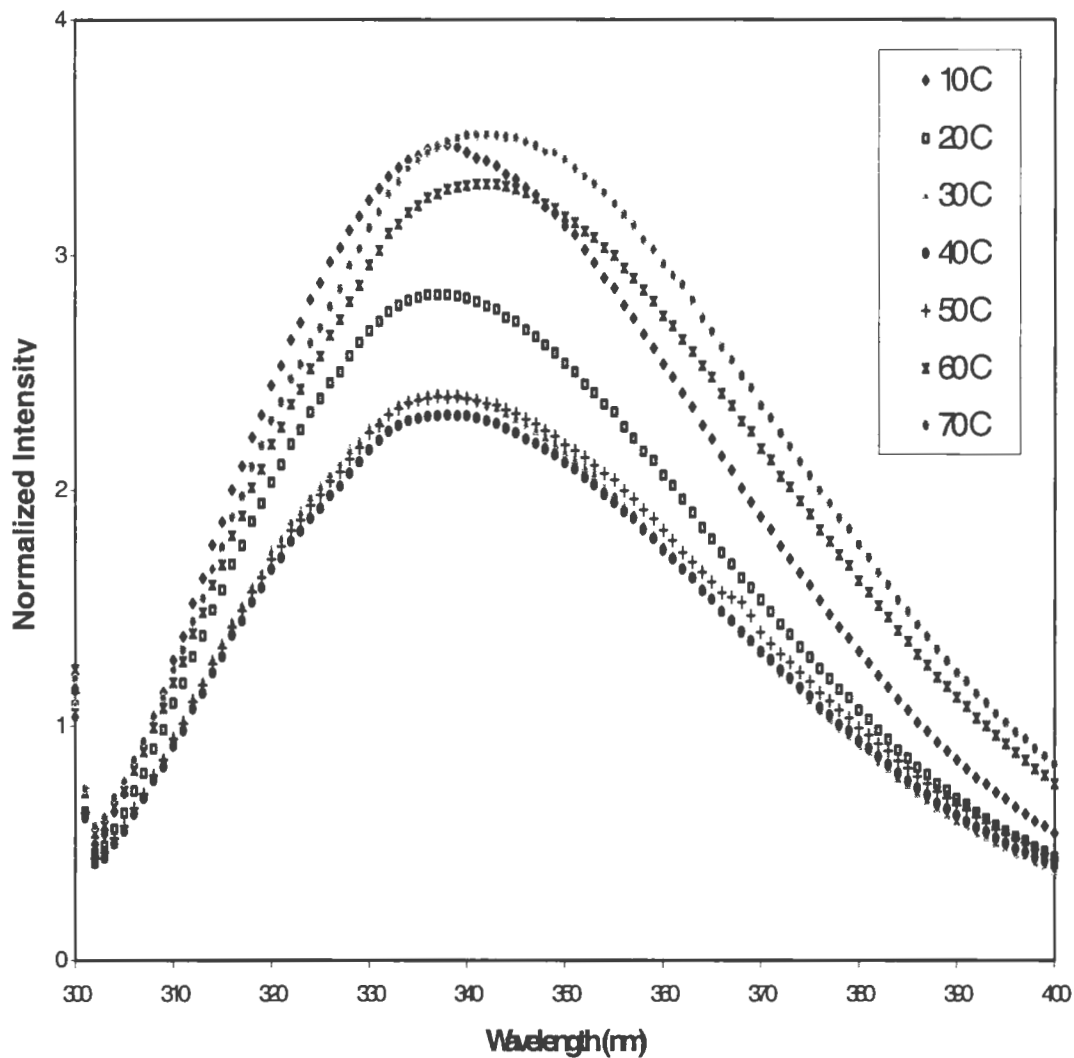
(Data generated by Mr. Faulkner and included with permission)

FIGURE 1: ANS TITRATION



The intensities are normalized by dividing the intensity at each ANS concentration by the intensity at ANS concentration of 1 μL

FIGURE 2 (a): INTRINSIC EMISSION SPECTRA AT pH 3



The intensities in Figures 2 (a-f) are normalized by dividing the intensity at each wavelength by the intensity at 300 nm at: a) pH 3, b) pH 4, c) pH 5, d) pH 6, e) pH 7, f) pH 8

FIGURE 2 (b): INTRINSIC EMISSION SPECTRA AT pH 4

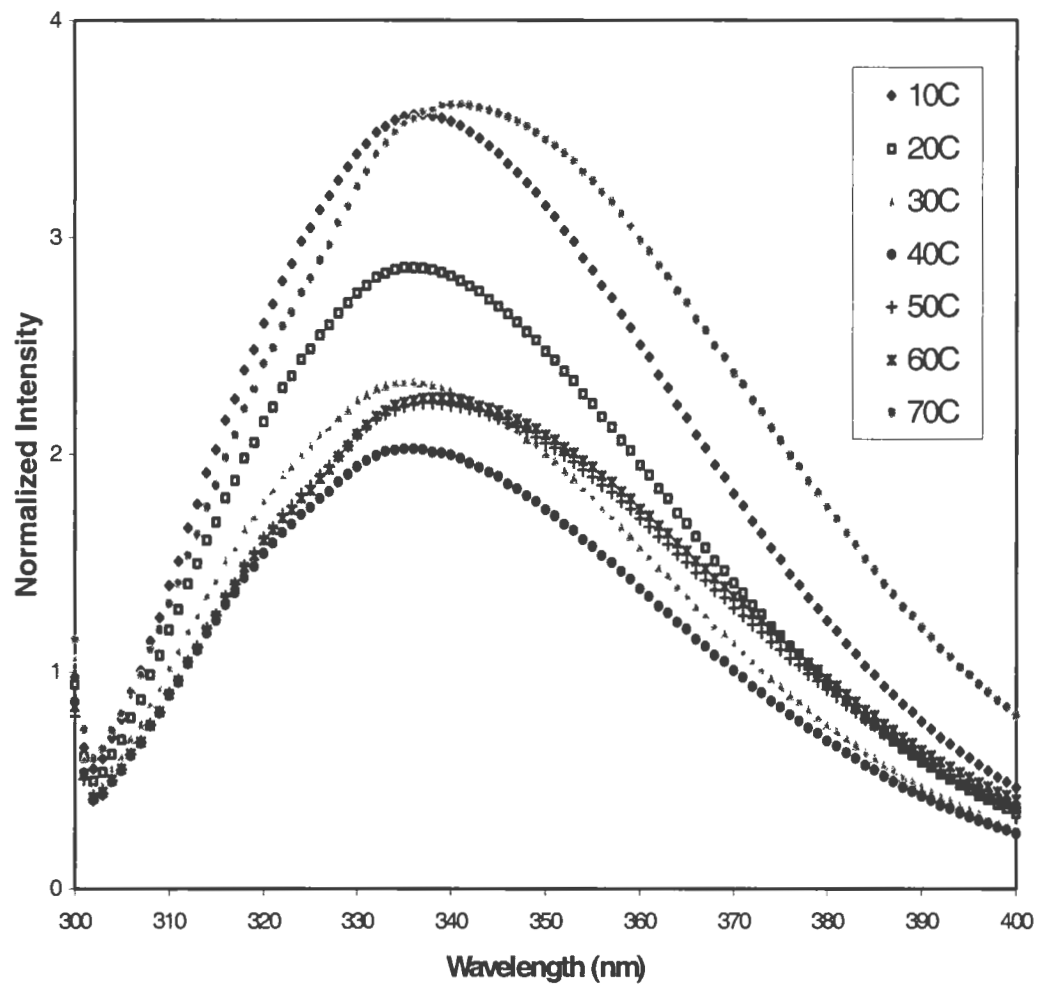


FIGURE 2 (c): INTRINSIC EMISSION SPECTRA AT pH 5

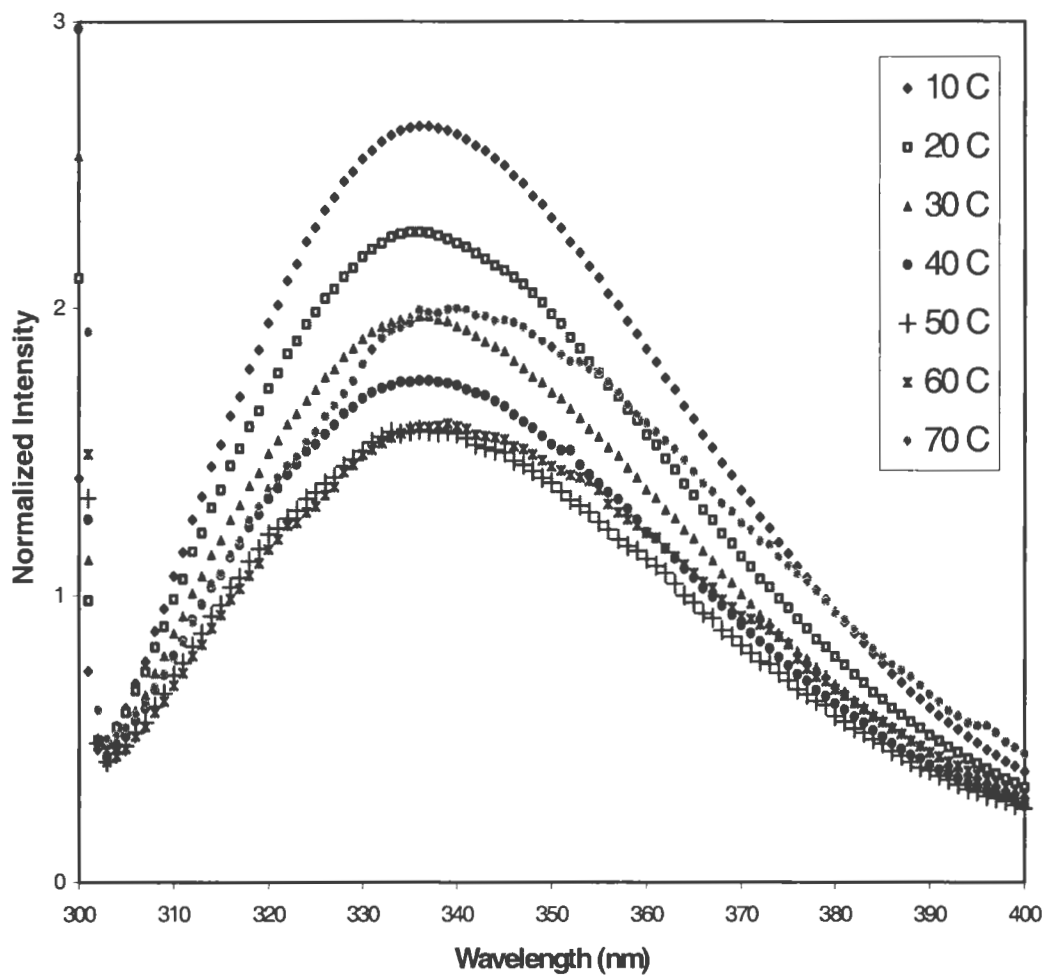


FIGURE 2 (d): INTRINSIC EMISSION SPECTRA AT pH 6

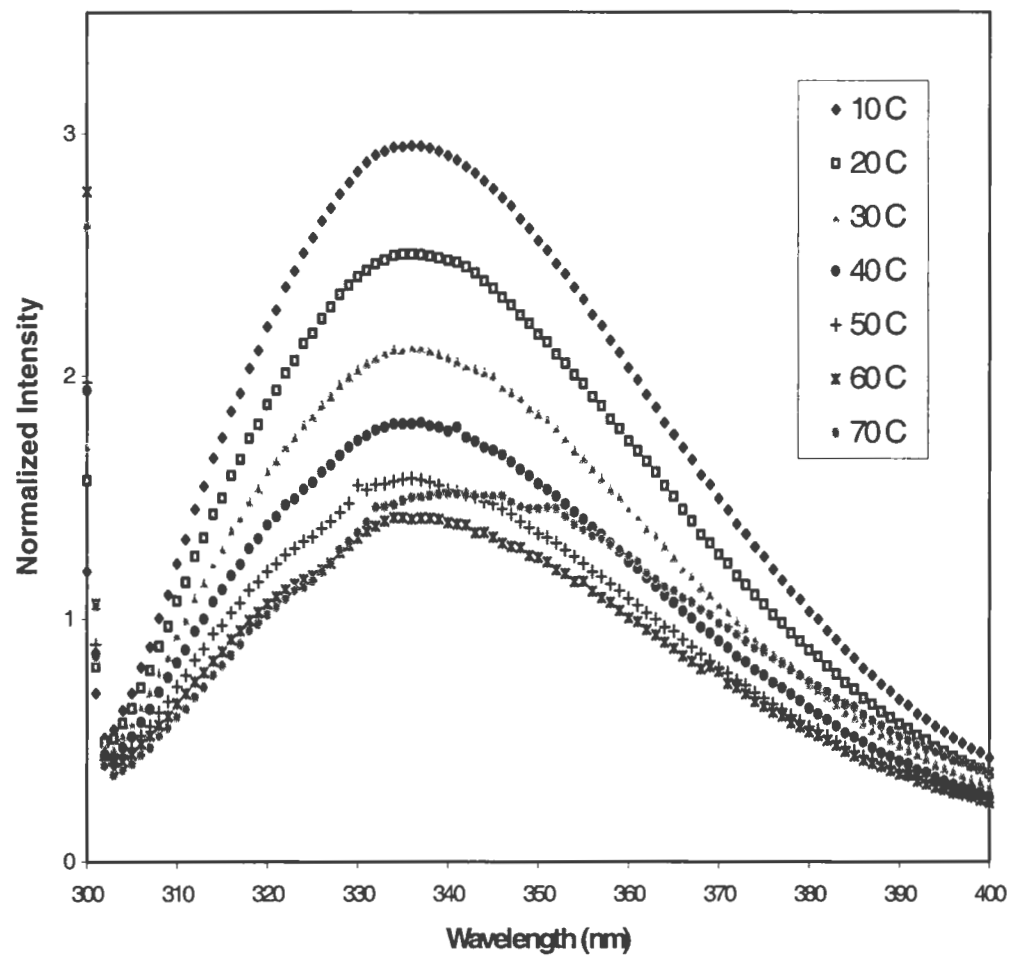


FIGURE 2 (e): INTRINSIC EMISSION SPECTRA AT pH 7

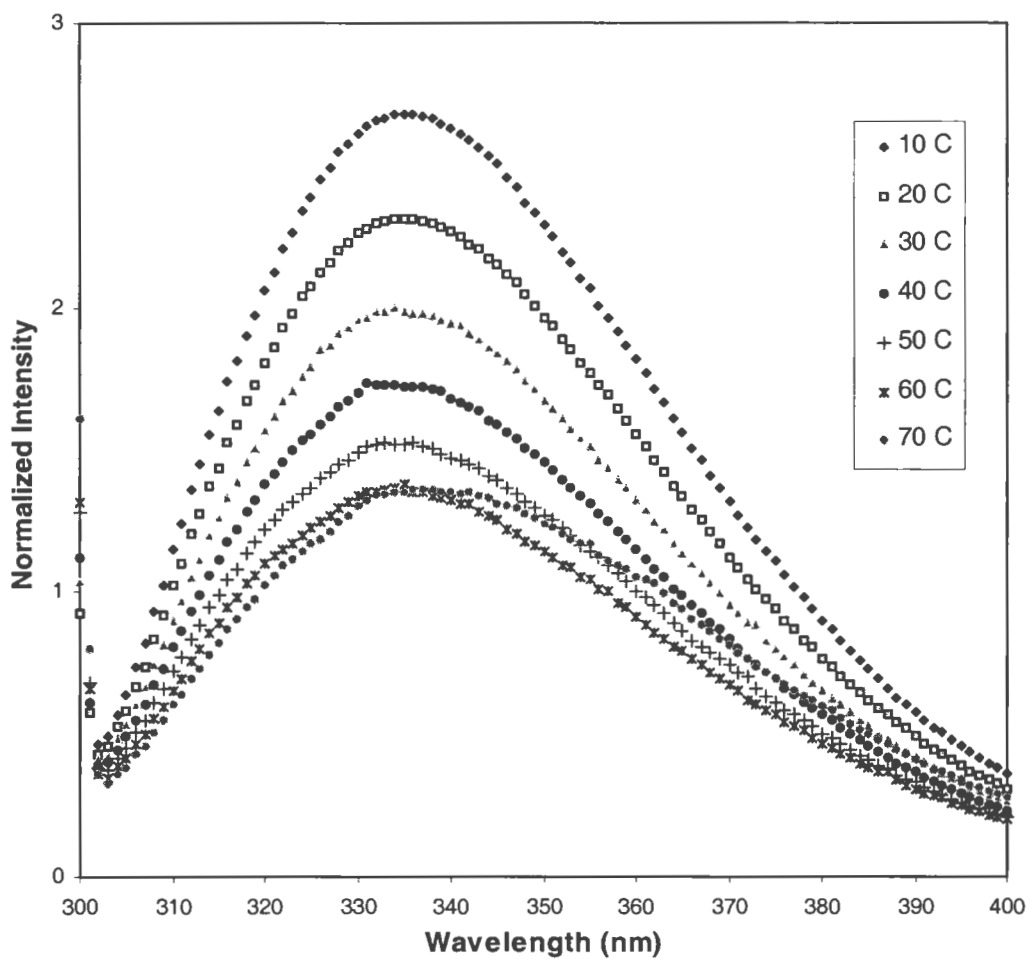


FIGURE 2 (f): INTRINSIC EMISSION SPECTRA AT pH 8

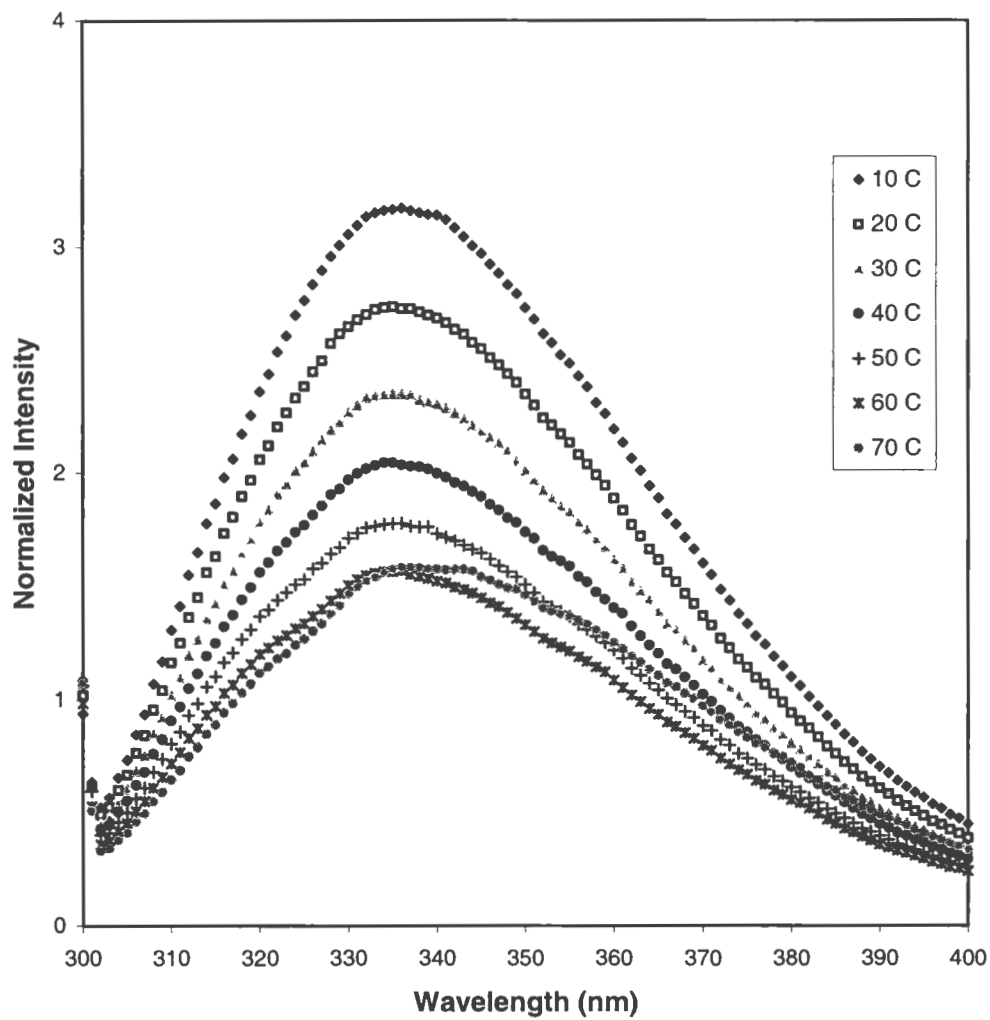
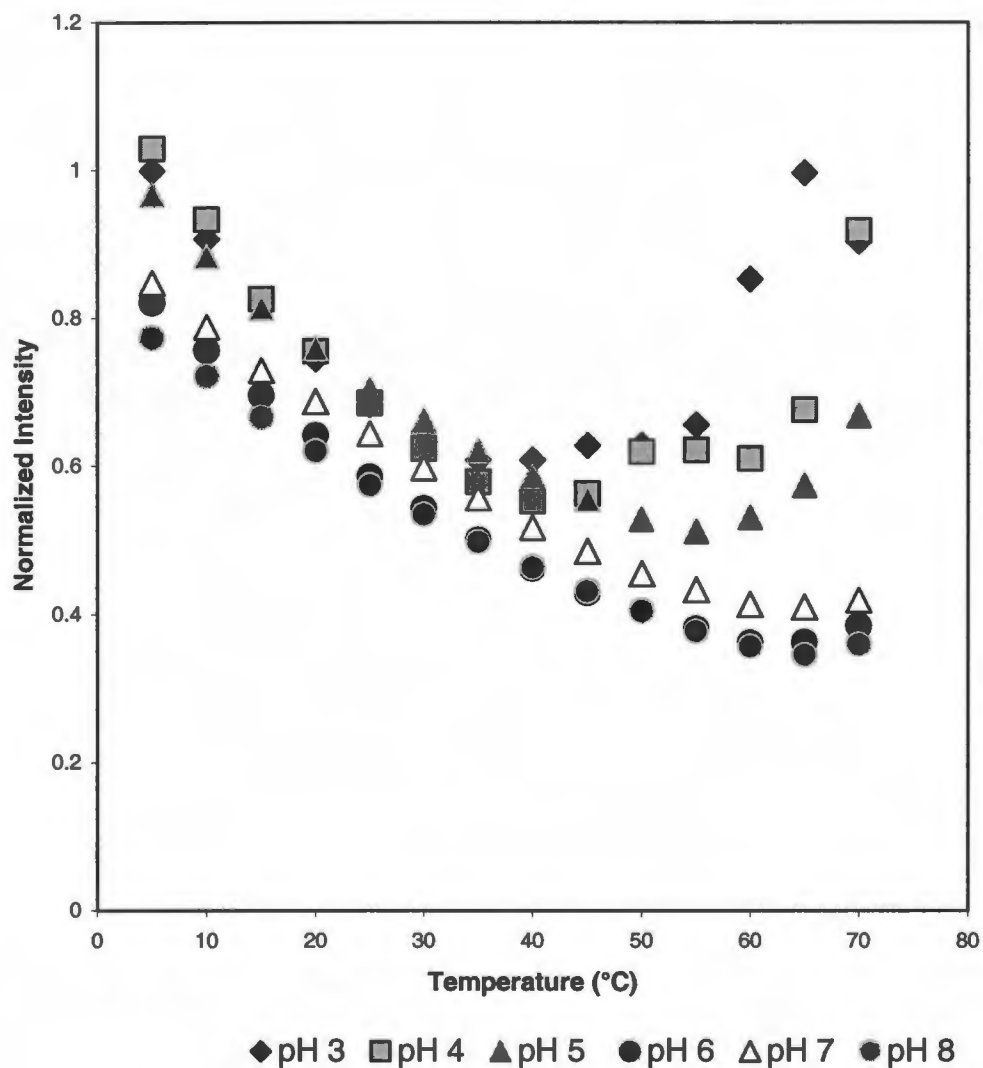
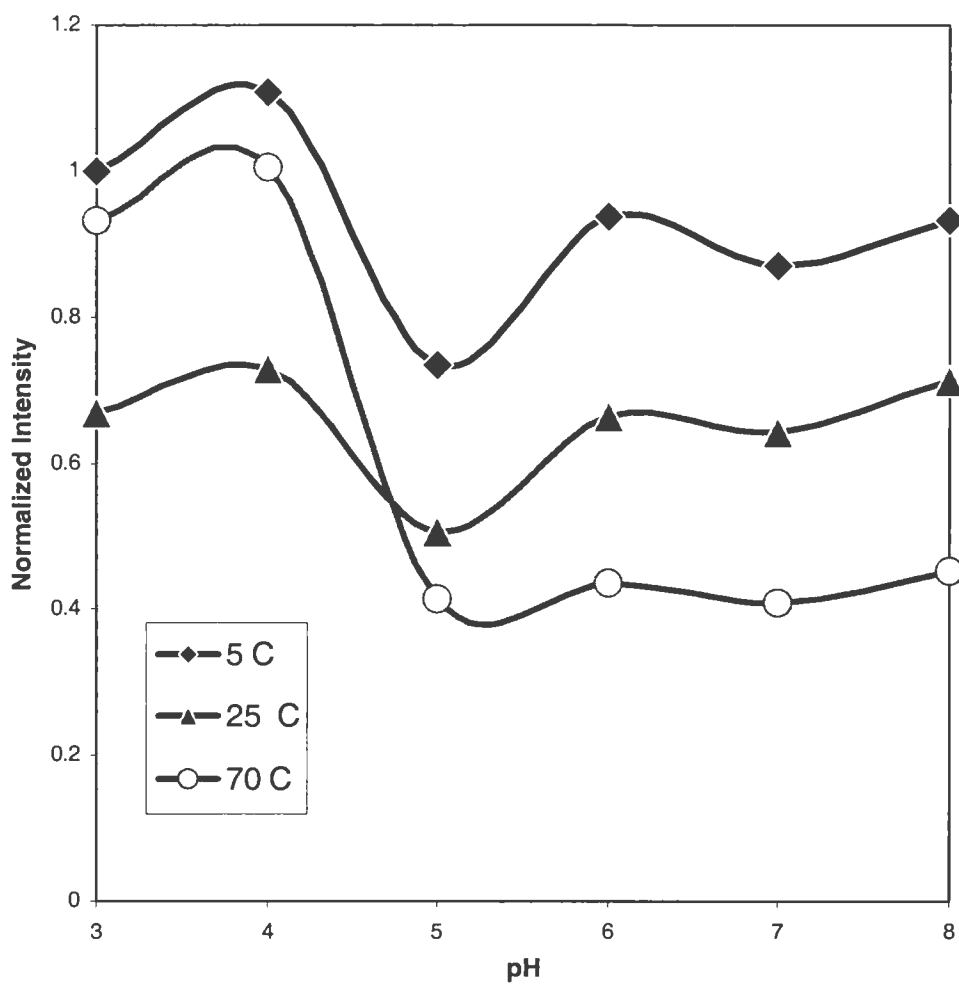


FIGURE 3: EFFECT OF ACCELERATED TEMPERATURE RAMPING ON INTRINSIC INTENSITY (336 nm)



The emission intensities measured at 336 nm are normalized by dividing the intrinsic intensity at each temperature by the intensity at 5 °C at pH 3

FIGURE 4: EFFECT OF pH ON INTRINSIC INTENSITY AT EXTREME AND ROOM TEMPERATURE (25 °C) CONDITIONS



The intensities measured at 336 nm are normalized by dividing each intensity by the intensity at 5 °C at pH 3

FIGURE 5: EFFECT OF ACCELERATED TEMPERATURE RAMPING ON INTRINSIC PEAK WAVELENGTH

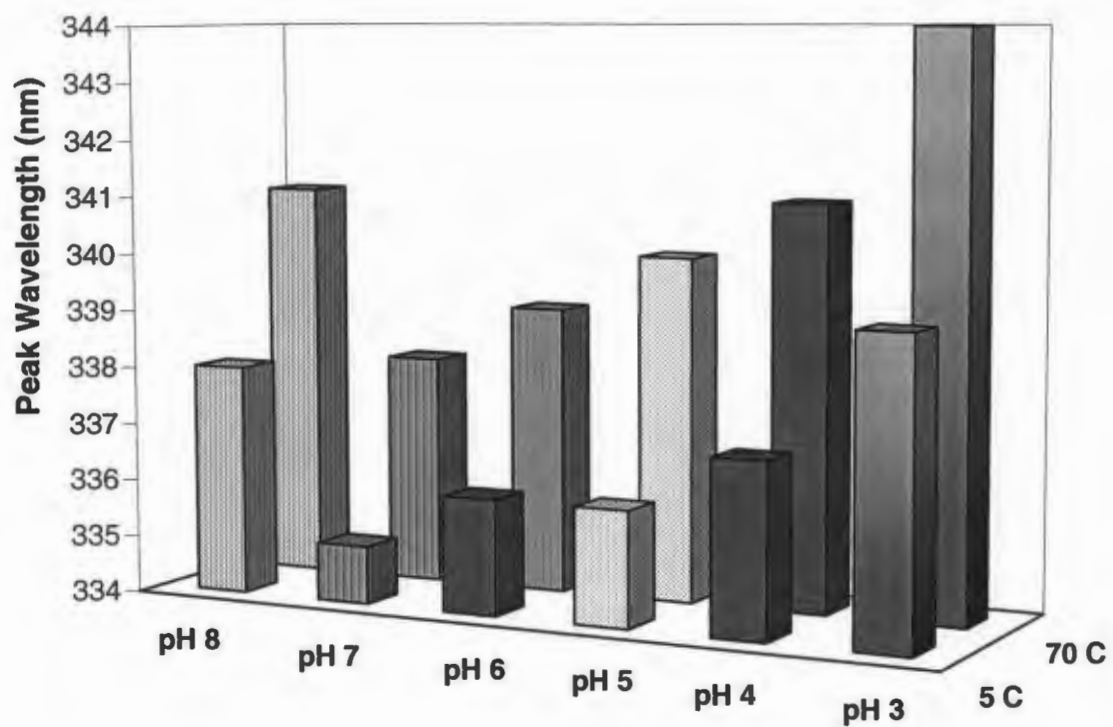


FIGURE 6: INTRINSIC PEAK WAVELENGTH AT EXTREME AND ROOM TEMPERATURE (25 °C) CONDITIONS

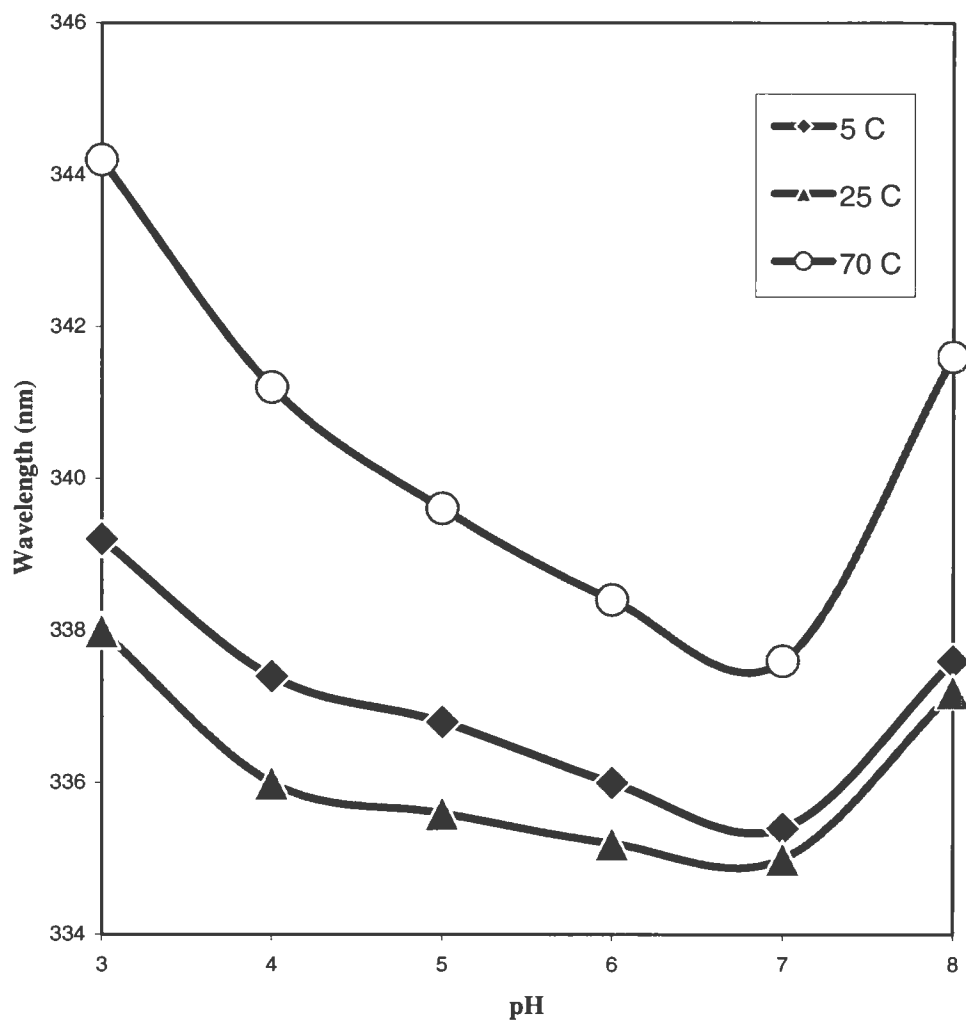
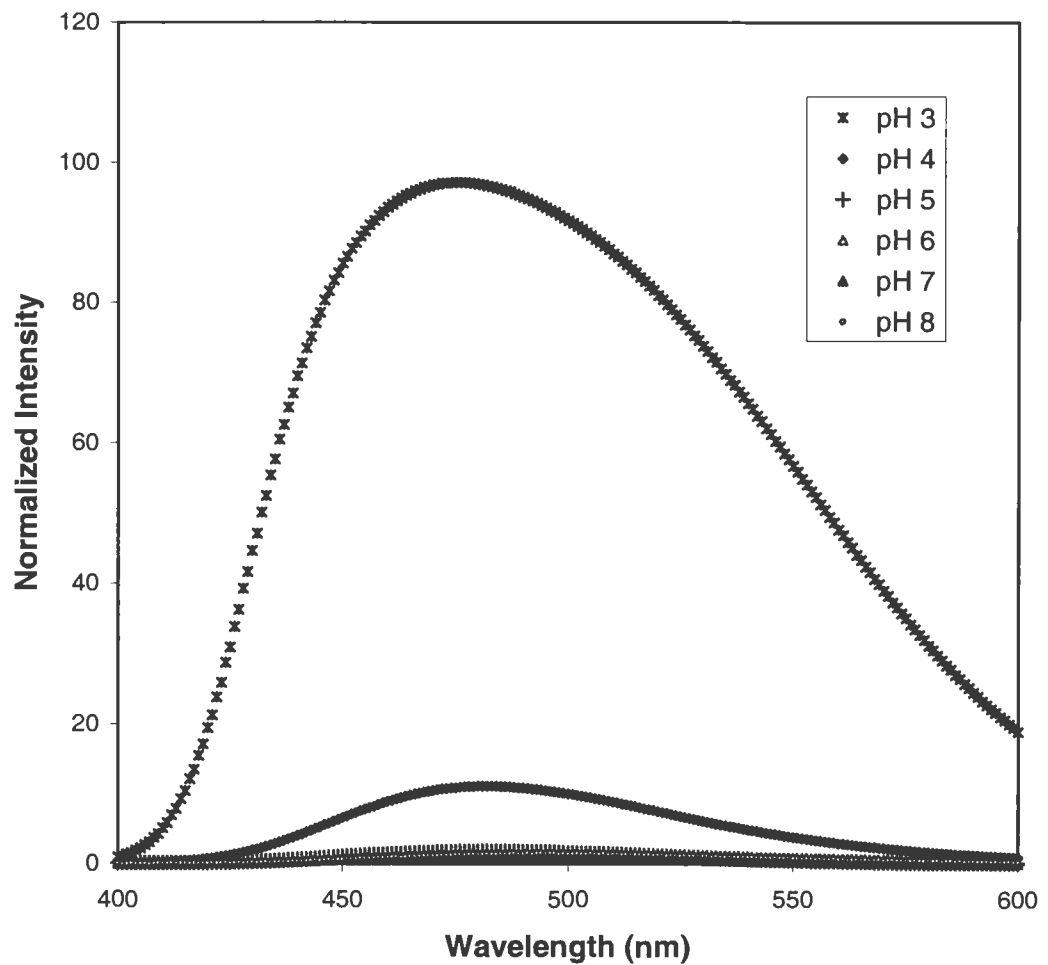
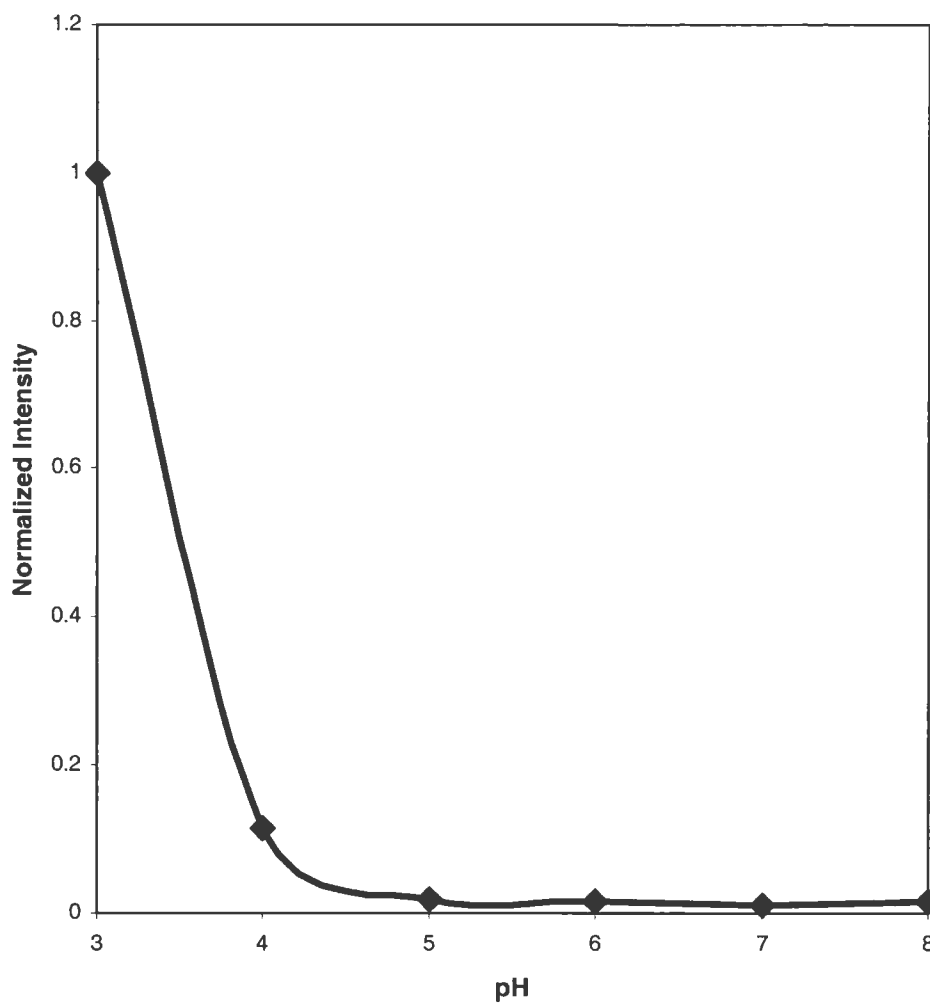


FIGURE 7: EXTRINSIC EMISSION SPECTRA AT ROOM TEMPERATURE (25 °C)



The extrinsic intensities are normalized by dividing the intensity at each wavelength by the intensity at 400 nm at 5 °C at pH 3

FIGURE 8: EFFECT OF pH ON EXTRINSIC PEAK INTENSITIES AT ROOM TEMPERATURE (25 °C)



The intensities are normalized by dividing the peak intensity at each pH by the peak intensity at pH 3 at 25 °C

FIGURE 9 (a): EXTRINSIC EMISSION SPECTRA AT pH 3

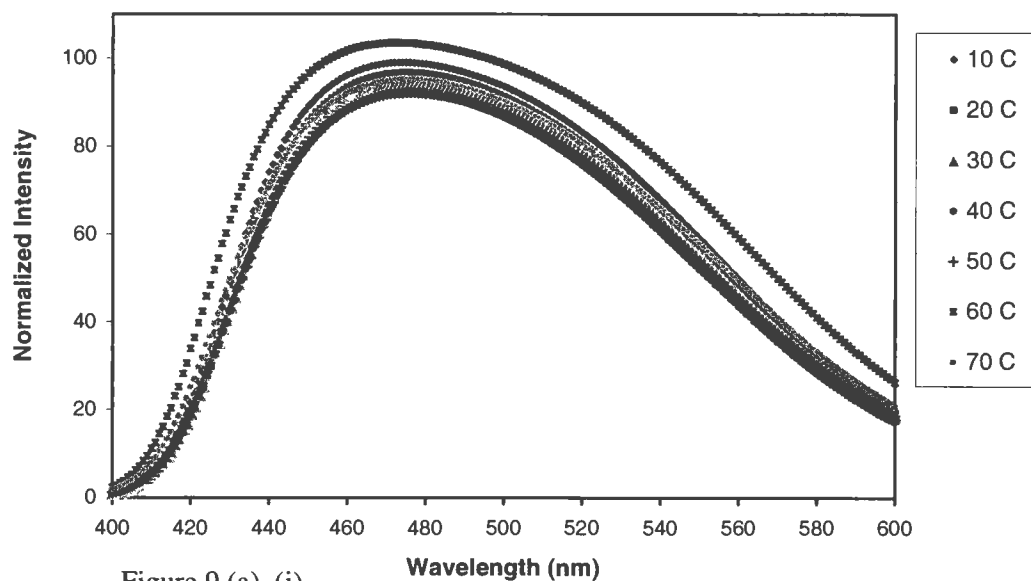


Figure 9 (a) (i)

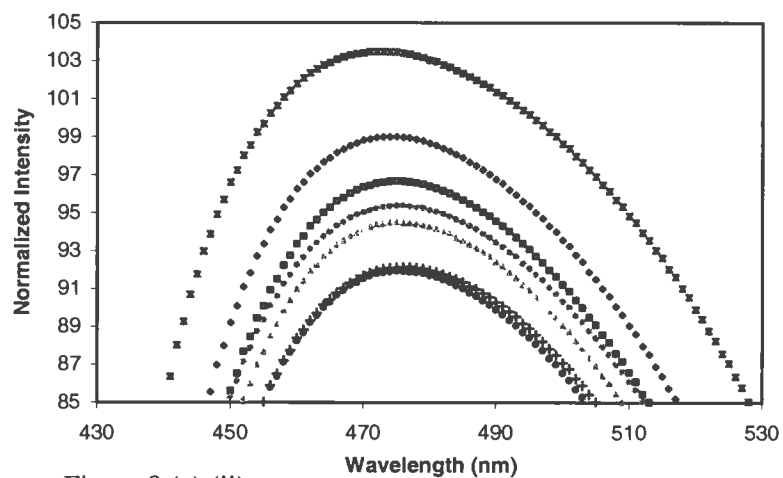


Figure 9 (a) (ii)

The intensities in Figures 9 (a-f) are normalized by dividing the intensity at each wavelength by the intensity at 5 °C at 400 nm at: a) pH 3, b) pH 4, c) pH 5, d) pH 6, e) pH 7, f) pH 8

The graph in Figure 9 (a) (ii) is a magnified section of the graph in Figure 9 (a) (i)

FIGURE 9 (b): EXTRINSIC EMISSION SPECTRA AT pH 4

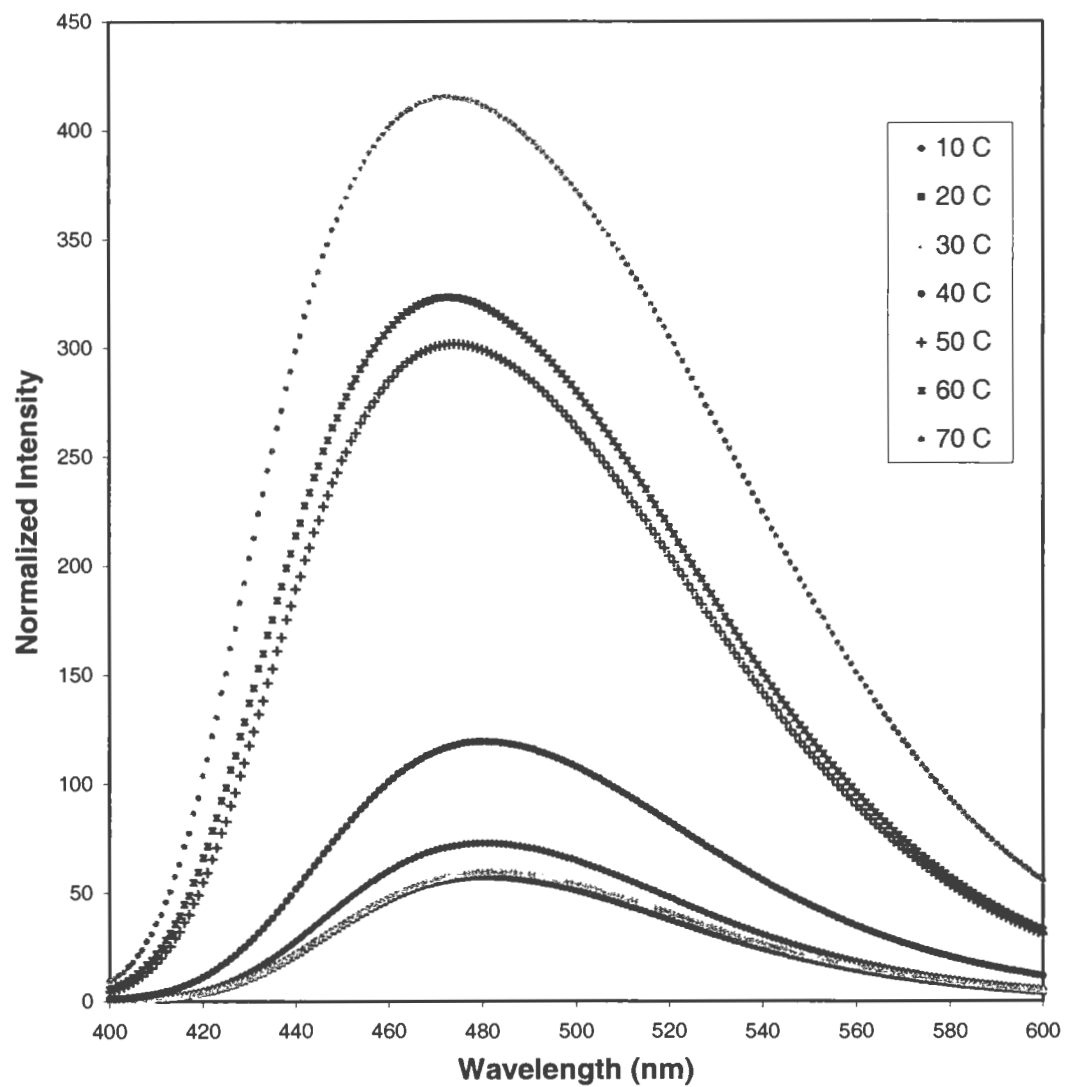


FIGURE 9 (c): EXTRINSIC EMISSION SPECTRA AT pH 5

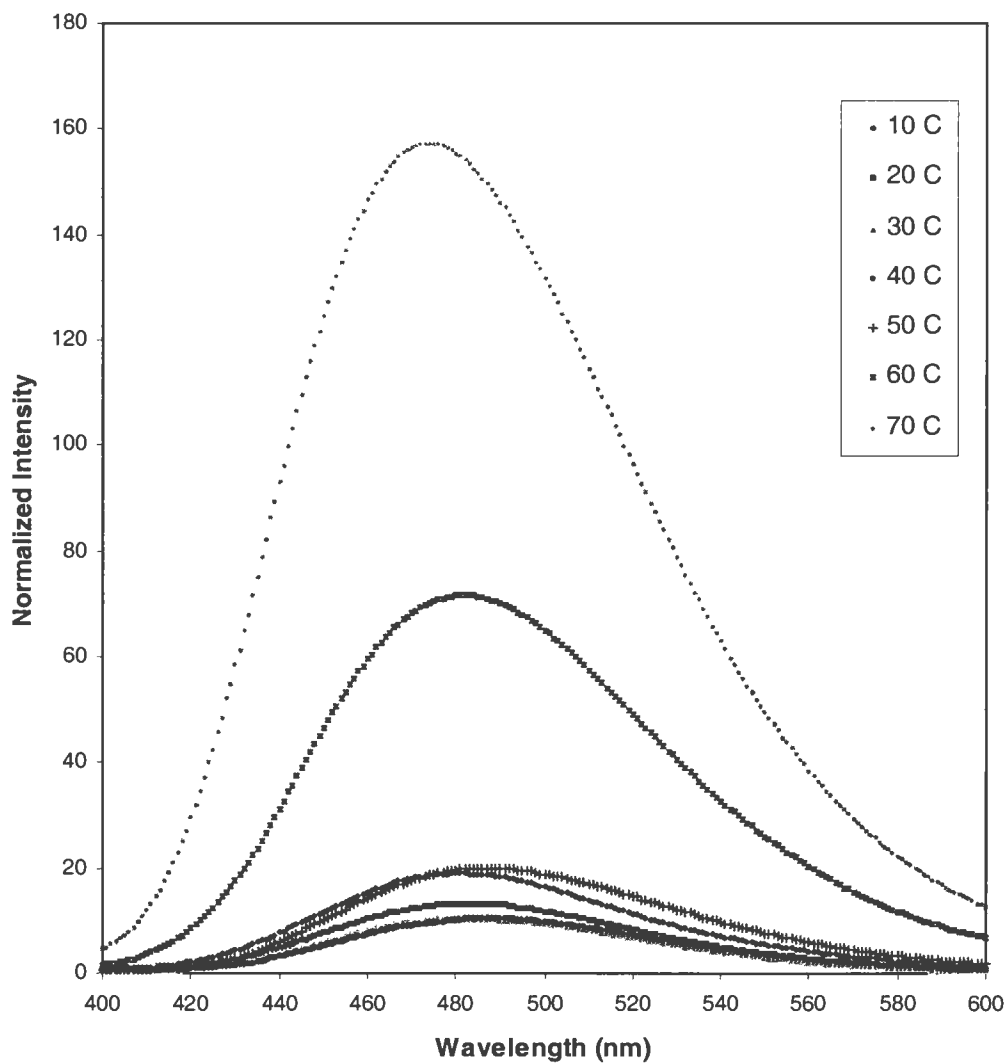


FIGURE 9 (d): EXTRINSIC EMISSION SPECTRA AT pH 6

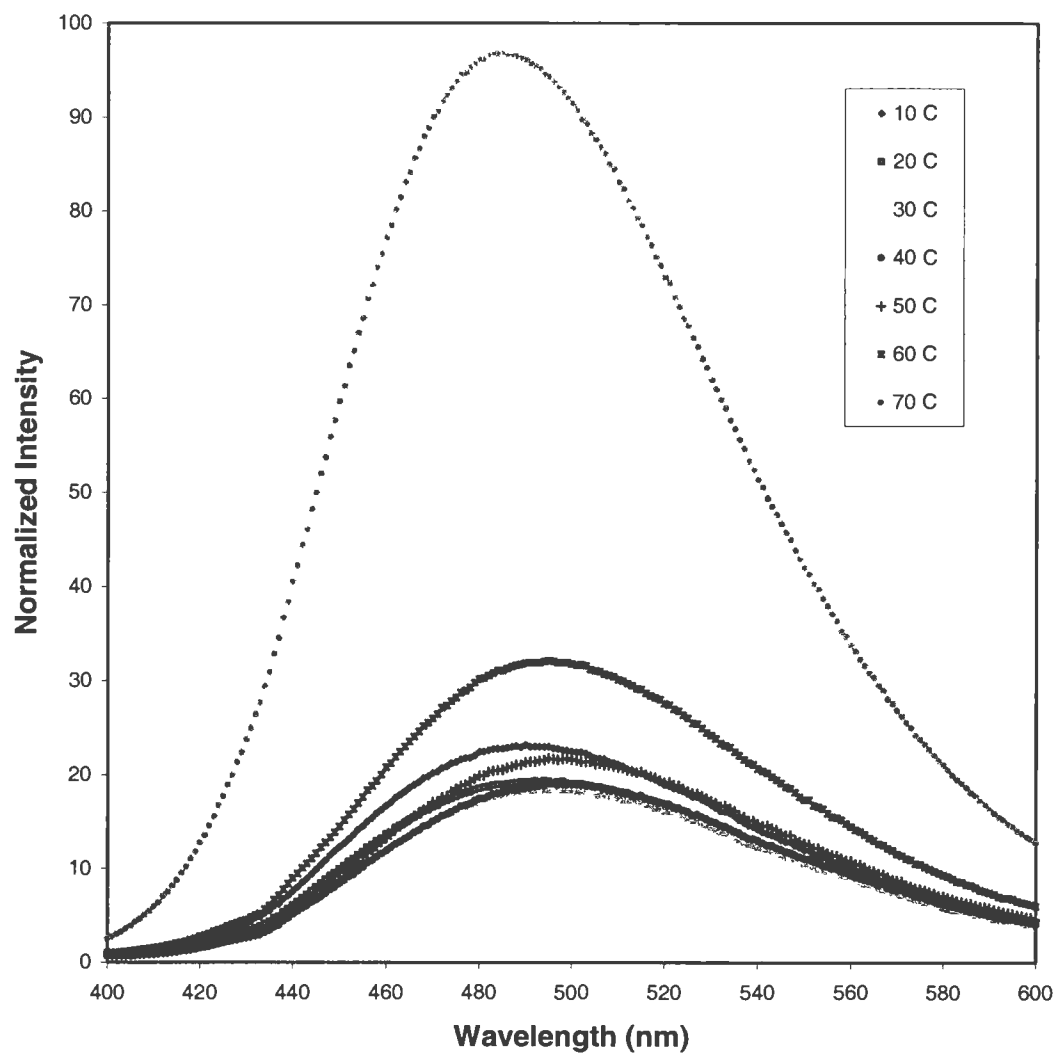


FIGURE 9 (e): EXTRINSIC EMISSION SPECTRA AT pH 7

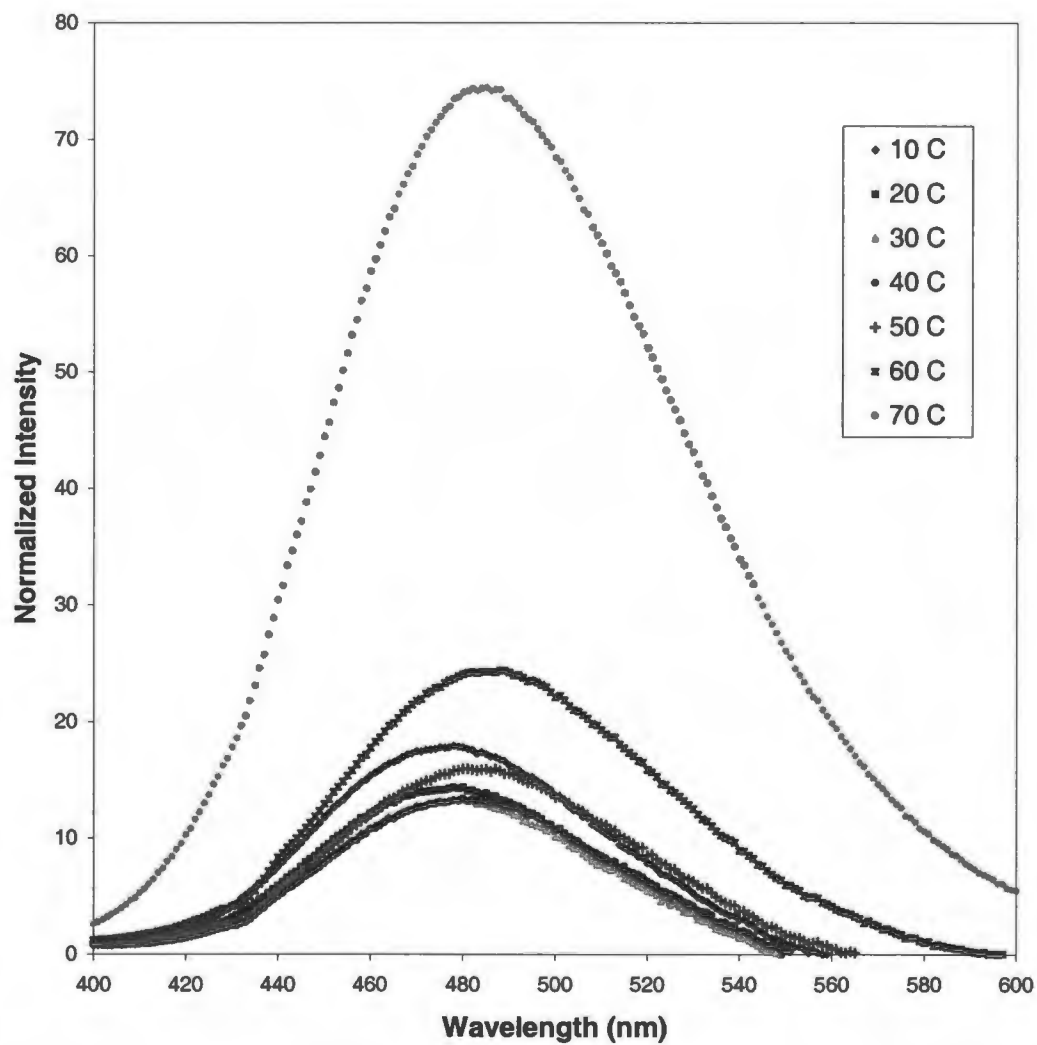


FIGURE 9 (f): EXTRINSIC EMISSION SPECTRA AT pH 8

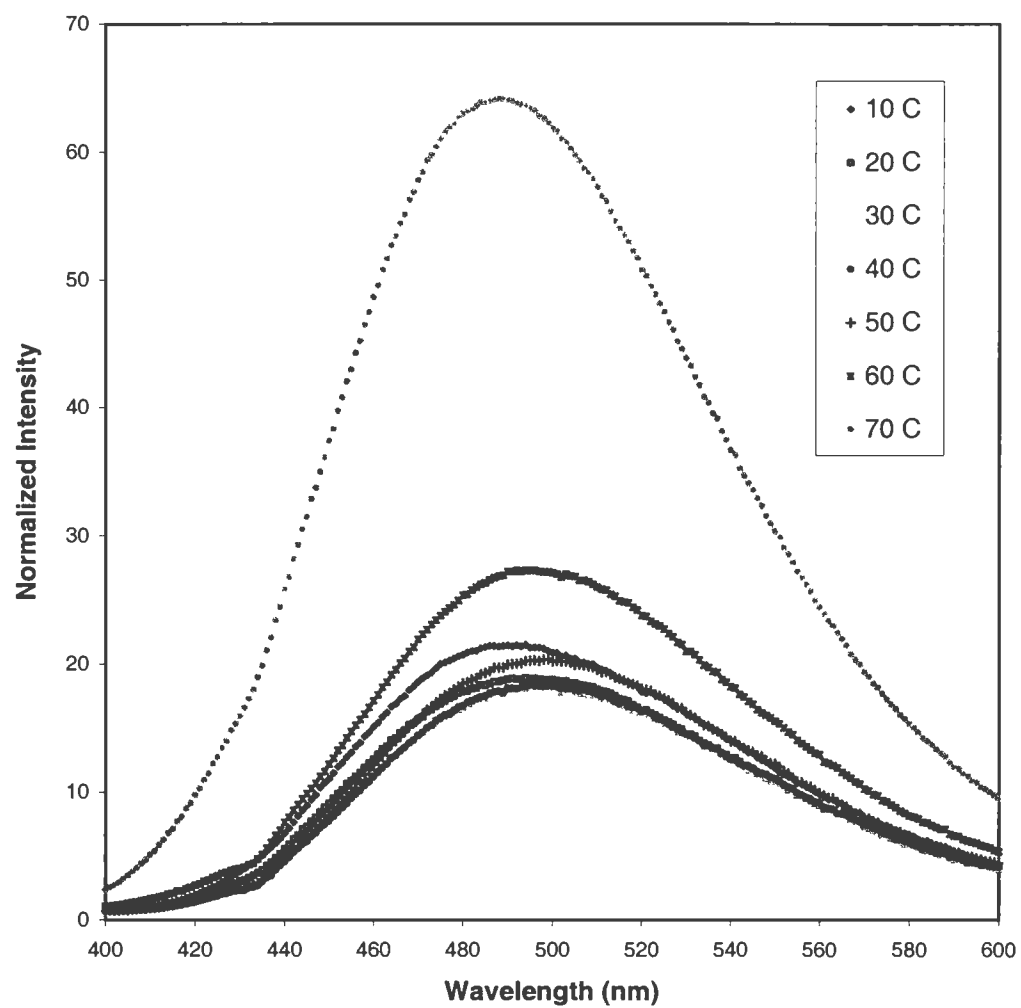
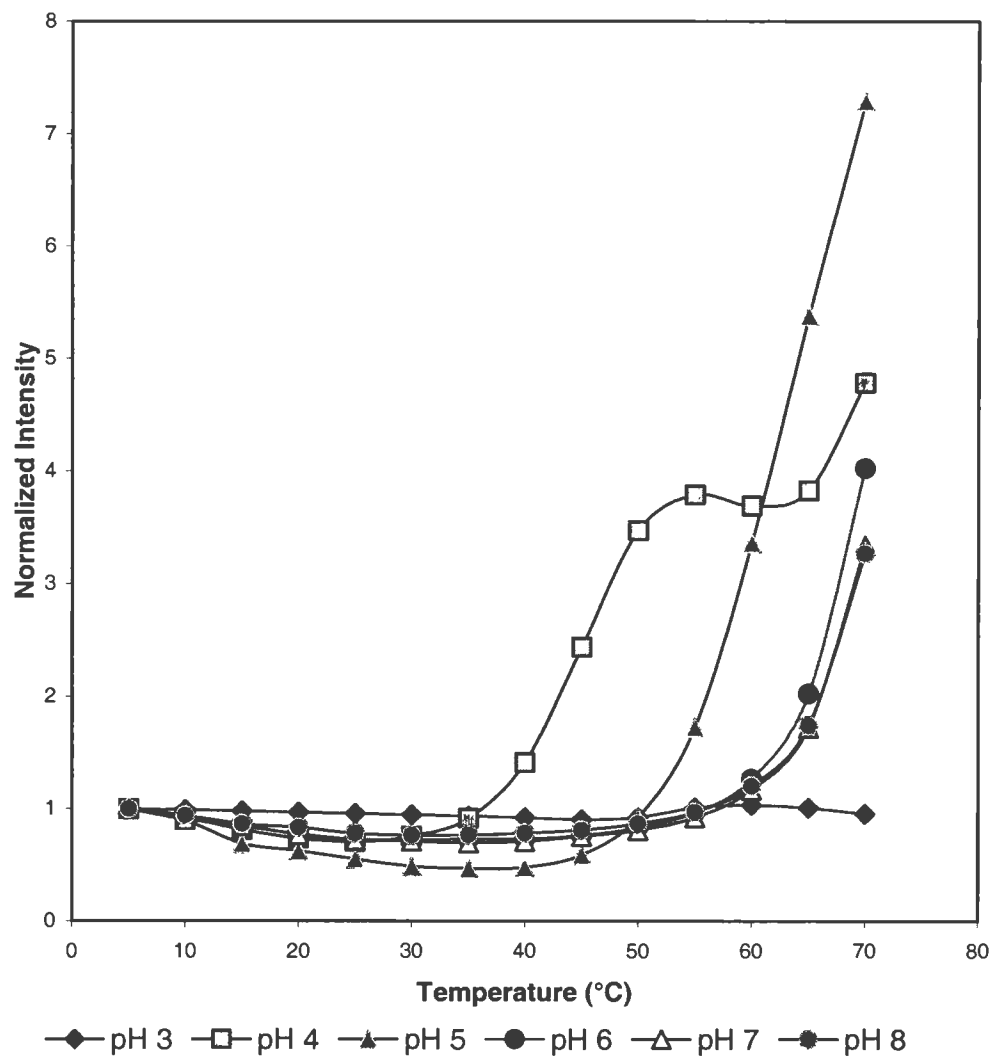
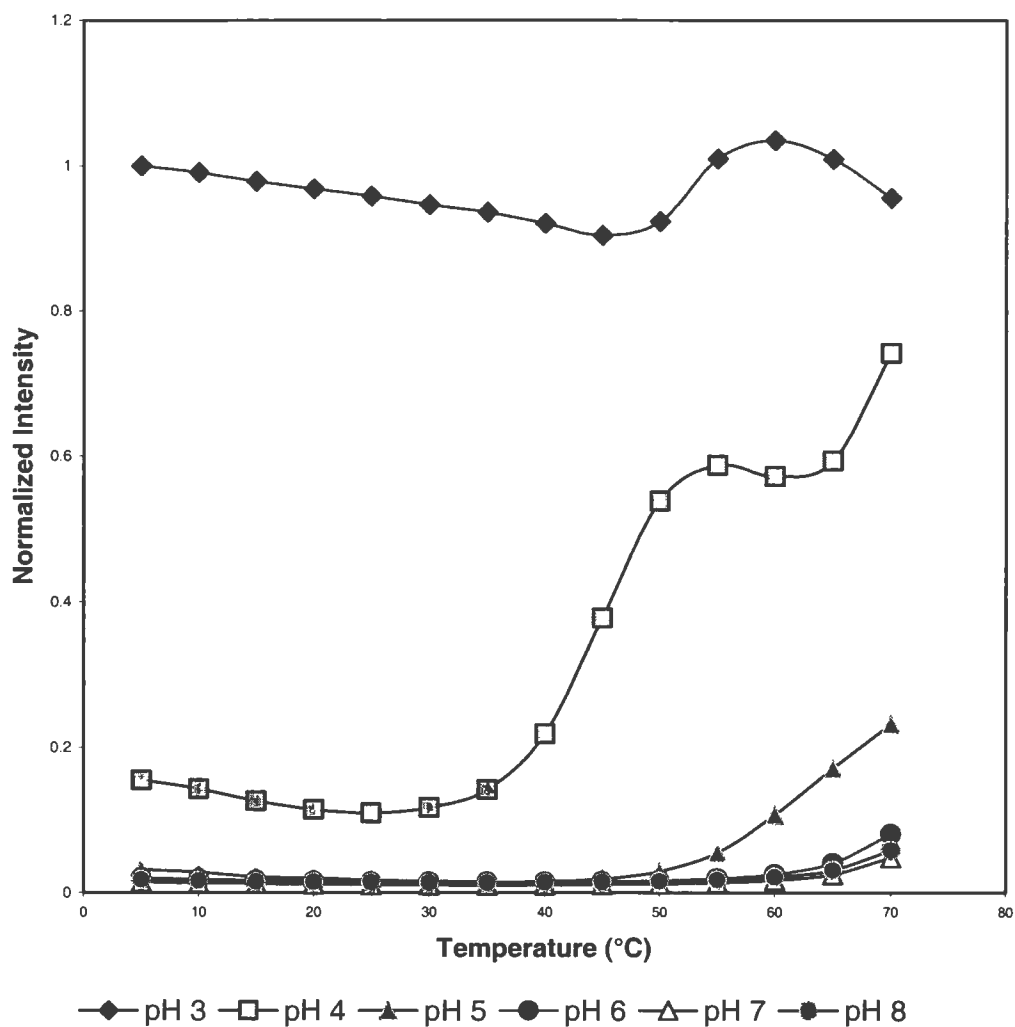


FIGURE 10: EFFECT OF TEMPERATURE ON EXTRINSIC INTENSITY (480 nm)



The intensities are normalized by dividing the intensity at each temperature by the intensity at 5 °C at that pH at 480 nm

FIGURE 11: EFFECT OF pH ON EXTRINSIC INTENSITY (480 nm)



The emission intensities measured at 480 nm are normalized by dividing the extrinsic intensity at each temperature by the intensity at 5 °C at pH 3

FIGURE 12: STRUCTURE OF 1-ANILINONAPHTHALENE 8-SULFONIC ACID

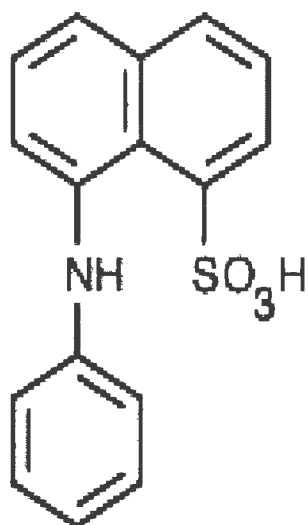
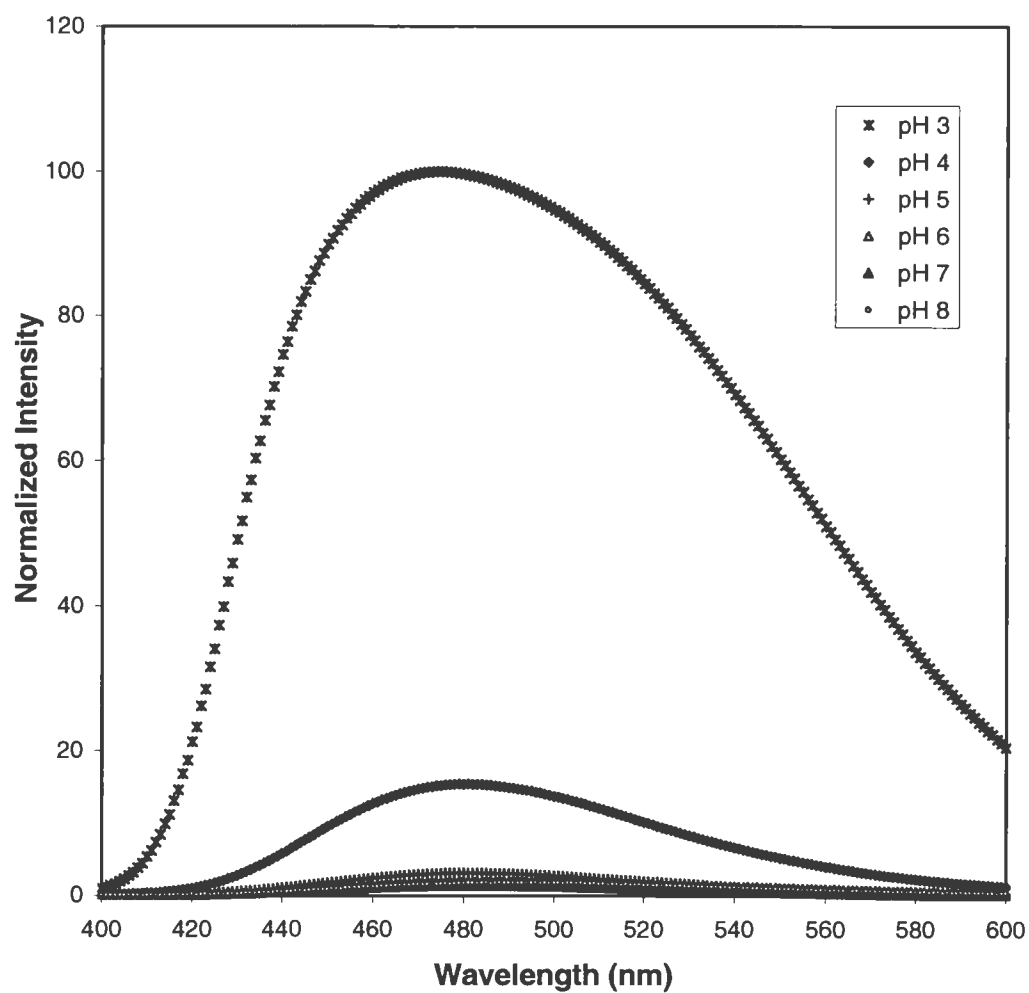


FIGURE 13 (a): EXTRINSIC EMISSION SPECTRA AT 5 °C



The extrinsic intensities are normalized by dividing the intensity at each wavelength by the intensity at 400 nm at pH 3 (a) at 5 °C and, (b) at 70 °C

FIGURE 13 (b): EXTRINSIC EMISSION SPECTRA AT 70 °C

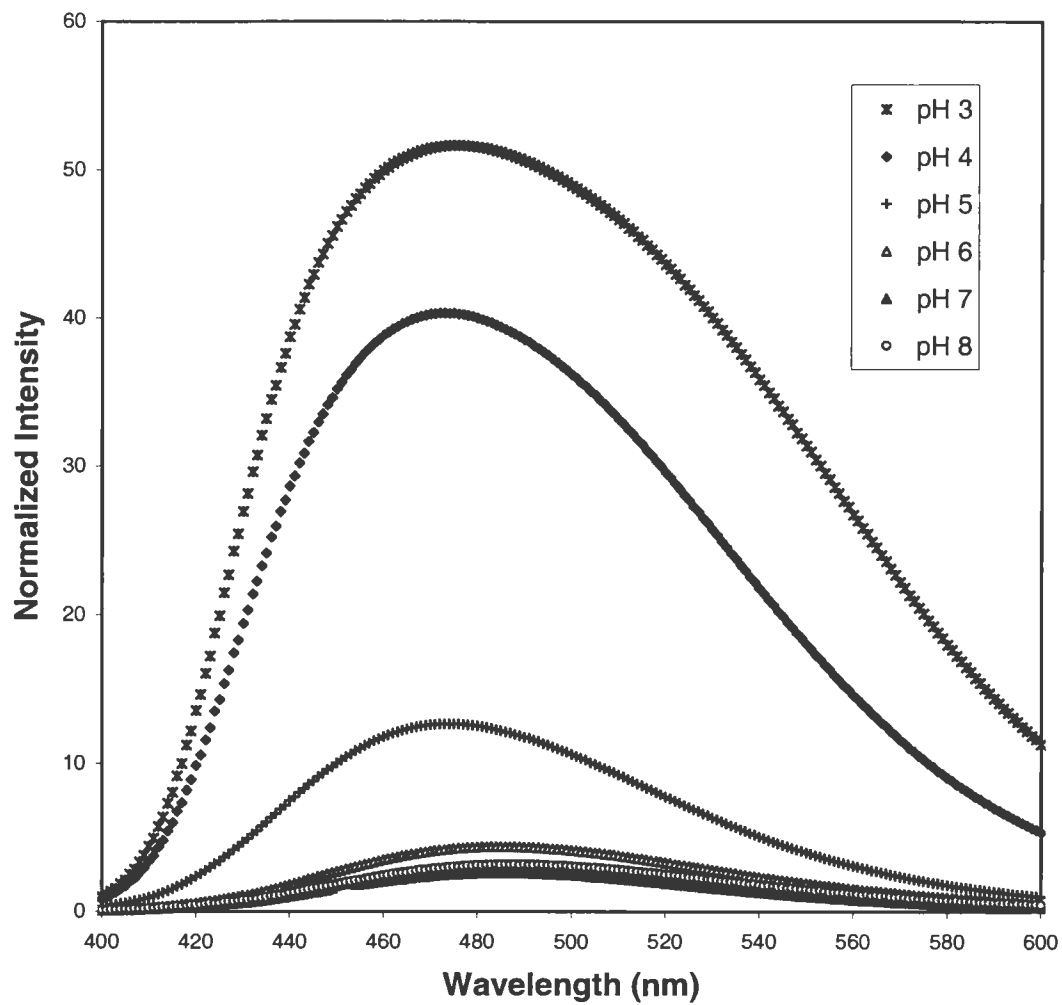
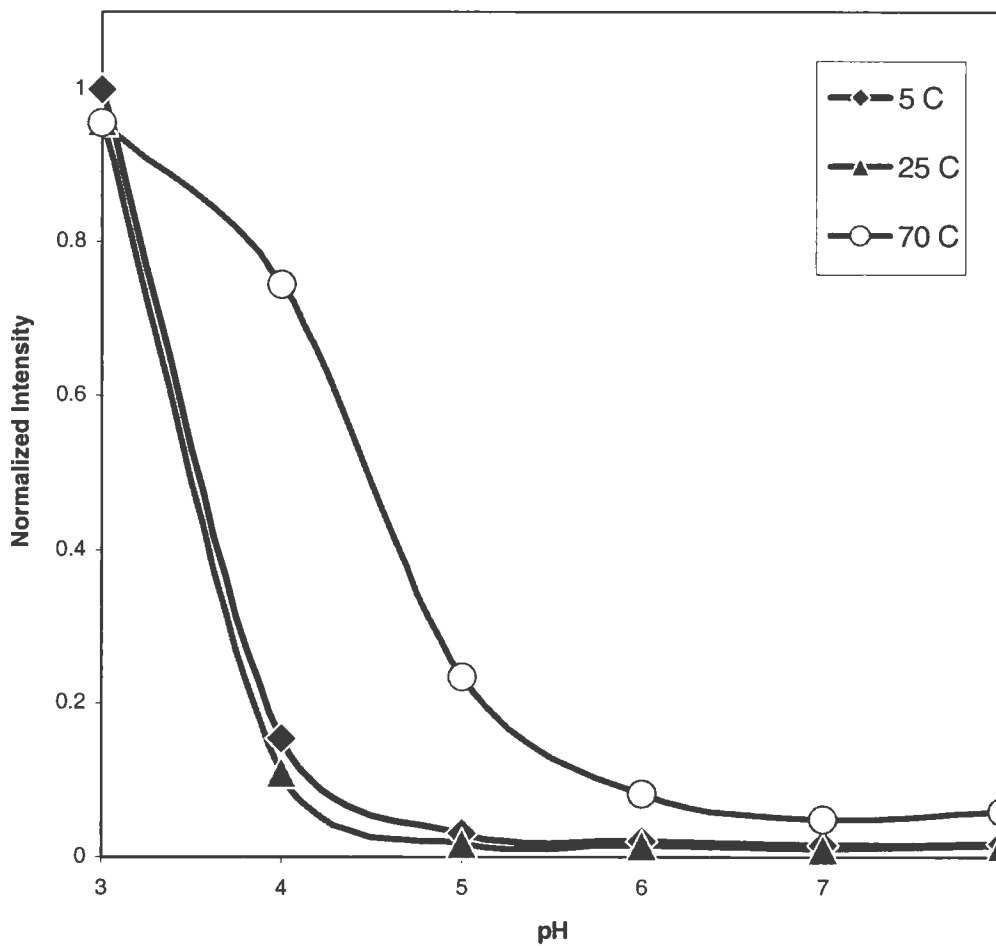


FIGURE 14: EXTRINSIC PEAK INTENSITIES AT EXTREME AND ROOM TEMPERATURE CONDITIONS



The intensities are normalized by dividing the peak intensities at each pH by the peak intensity at pH 3 at 5 °C

SUMMARY OF CONCLUSIONS

1. Changes in the fluorescence spectrum of the protein were observed with increasing temperature.
2. The fluorescence intensity displayed the expected linear decrease with temperature, with an increase observed near the onset of temperature of transition at each pH.
3. The red shifts in the intrinsic fluorescence emission at higher temperatures reflect some increase in exposure of one or more buried tryptophan residues to aqueous environment.
4. At low pH, the protein may exist already in unfolded state. Hence, increased temperature has no further significant effect on the peak intensity position.
5. The polarity-sensitive hydrophobic dye, ANS, may bind to protein by electrostatic interaction in addition to hydrophobic interaction at low pH. Therefore, the correlation of the onset of transition temperature between intrinsic and extrinsic fluorescence data was found to be better at higher pH values.
6. The spectroscopic data reported in this thesis is supported by the DSC data obtained from Mr. Eric Faulkner, at Biogen, Inc.

7. The onset of temperature of transitions (T_o) estimated using intrinsic and extrinsic methods of fluorescence technique correlate with those determined using DSC method of analysis.
8. The protein at pH 6, 7 and 8 is characterized by an onset of transition of about 60 °C. Since higher onset of transition temperature reflects greater thermal stability, the preformulation studies suggest the protein is most likely to be thermally stable in an aqueous solution formulated at pH 6, 7, and 8.
9. The data reported in this thesis clearly indicate that fluorescence spectroscopy for protein drugs has considerable potential as a routine preformulation tool and is likely to demonstrate substantial value in developing optimal stability.

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