Stability Studies of Recombinant Hamster Polymorphic Arylamine *N*-Acetyltransferase

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Stability Studies of Recombinant Hamster Polymorphic Arylamine N-Acetyltransferase

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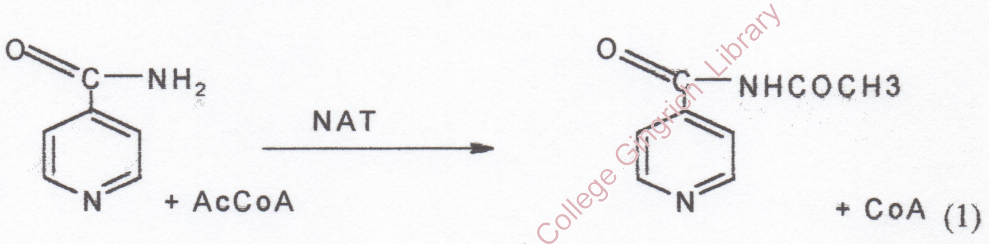
Abstract

The overall goal of this project is to investigate the difference in stability between recombinant hamster polymorphic (rNAT2) and monomorphic (rNAT1) arylamine N-acetyltransferase isozymes. Using fluorescence spectroscopy the equilibrium unfolding of rNAT2 was investigated using urea as a denaturant. Urea-induced unfolding of rNAT2 was studied by excitation at 280 and 295 nm. Excitation at 280 nm was done to study the contribution of tryptophan and tyrosine to the overall fluorescence of rNAT2. Excitation at 295 nm was also done, to just study the contribution of tryptophan residues to the emission spectra. The process produced four groups of spectra within each experiment that suggest the presence of an intermediate during the unfolding transition. Initial analysis of data using relative fluorescence versus urea concentration yielded considerable amounts of scatter. Analysis using average emission wavelength reduced the scatter, allowing for further conformation of the presence of an intermediate during the unfolding process. Results of the equilibrium unfolding experiments suggest that unfolding of rNAT2 does not occur via a simple two-state transition. Initial circular dichroism studies were conducted in the far UV region (190 - 250 nm).

Introduction

Biological Importance of Arylamine N-Acetyltransferases

The enzyme, acetyl coenzyme A: arylamine N-acetyltransferase (NAT) (EC 2.3.1.5) became a target of interest in 1952 when the ability to acetylate arylamines was discovered to be the first hereditary trait that affects the human drug response (Hanna, 1994). There are several roles that N-acetyltransferases play in the human body including metabolic disposition, detoxification, and bioactivation (toxification) of a diverse group of drugs, carcinogens, and other xenobiotics (Hanna, 1994). Arylamine N-acetyltransferase specifically plays a role as a catalyst in the principal detoxification pathways for most primary arylamines, hydrazines, and hydrazides. The enzyme catalyzes the metabolic conversion of a primary arylamine to an arylamide in an acetyl coenzyme A-dependent reaction as shown in reaction 1 (Hanna, 1996):



Reaction 1: Metabolic acetylation of *p*-aminobenzoic acid catalyzed by NAT

Humans are exposed to primary arylamines through a variety of experiences with environmental and dietary compounds. A major source of exposure to primary arylamines is through therapeutic agents, such as sulfonamide antibacterials. Exposure also occurs from tobacco smoke, consuming well-cooked meat and various occupational settings (Hanna, 1996). Primary arylamines are often not directly toxic to the human

body following ingestion or exposure, but unwanted effects can be attributed to one or more reactive metabolites. Usually the toxicity of arylamines is determined by a set of competing biochemical reactions, which may result in the bioactivation (toxification) or detoxification of the primary arylamine. Metabolic N-acetylation is primarily a detoxification process in mammalian tissues (Hanna, 1996).

Interindividual genetic differences in the regulation of the NAT loci has been implicated as an important factor in determining phenotypic variability in acetylation capacity (Hanna, 1994). An individual's varying capacity to acetylate arylamines, appears to be the link between NAT and certain cancers. Epidemiological studies have revealed that bladder cancer patients are typically slow acetylators while those affected by colorectal carcinoma are rapid acetylators (Hanna, 1994). Point mutations, which destabilize the polymorphic NAT proteins, appear to be responsible for the human slow acetylator phenotype (Hanna, 1994). Also, postmenopausal women who smoke have a greater risk for breast cancer, if they have a decreased capacity to detoxify carcinogenic aromatic amines via acetylation by the polymorphic NAT (Sticha, 1997).

N-acetyltransferases are cytosolic proteins with molecular masses of approximately 33kDa. NAT activity is highest in the liver, with various levels of activity in most other mammalian tissues. Research has been done on a variety of species with human, rabbit, mouse and hamster being studied in detail. Two cytosolic NAT isozymes have been found to be present in hamsters, as well as in other mammalian species (Hanna, 1994). The amino acid sequences of approximately 15 arylamine NATs have been determined, with mammalian NATs exhibiting between 65-85% sequence homology (Bergstrom, 1995). The two isozymes, which differ in their substrate

specificity, are regulated by two NAT loci (*NAT1* and *NAT2*). In each species, expression of the isozymes is under independent regulation by either a monomorphic or polymorphic gene. Large-scale purification of the recombinant monomorphic and polymorphic NATs from hamster, rNAT1 and rNAT2, respectively has made possible more extensive research on their structure and function. The hamster rNAT1 and rNAT2 are designated rNAT1-9 and rNAT2-70D, respectively (Sticha *et al.* 1998; Sticha *et al.*, 1997). Bioactivation and inhibition studies have shown the rNATs to be catalytically and functionally identical to the native hamster NATs. During studies involving the two recombinant isozymes, observations by Sticha and co-workers (1997) led to the discovery that rNAT2 remains active for longer periods of time than rNAT1. This may be attributed to rNAT2 possessing a greater thermodynamic stability than rNAT1. Equilibrium unfolding experiments can be conducted to examine a protein's stability, and would allow for comparison of the thermodynamic stabilities of rNAT1 and rNAT2.

Fluorescence Studies of Proteins

Fluorescence spectroscopy can be a valuable tool in studying conformational changes within a protein. This technique takes advantage of the intrinsic fluorescence resulting from the three aromatic amino acids present in proteins, phenylalanine, tyrosine and tryptophan. During the fluorescence process electromagnetic radiation is absorbed by these amino acids to put them in the excited state. The excited molecules return to the ground state through emitted electromagnetic radiation or fluoresence. Fluorescence emission is sensitive to changes in the molecular environment allowing the study of conformational changes within proteins. Tryptophan and tyrosine, each of which have a greater quantum yield then phenylalanine, are particularly useful in the study of protein

conformational changes with fluorescence. Comparing the three using the sensitivity parameter, phenylalanine has a value of four while tyrosine and tryptophan are 200 and 730, respectively (Schmid, 1997). Examination of the tryptophan residues can be done selectively by exciting at 295 nm, where its molar absorptivity is much greater than tyrosine. Excitation at 280 nm allows one to observe the contributions by both the tryosine and tryptophan residues.

The intrinsic fluorescence of the protein resulting from tryptophan and tyrosinse is monitored in its native and its unfolded state. Various means may be used to induce an unfolding transition, thus altering the tertiary structure of the protein. Some methods for inducing unfolding or denaturation of a protein include the use of chemical denaturants, such as urea and guandine hydrochloride, increasing temperature, pH and pressure. Significant shifts in fluorescence wavelength and intensity are generally observed during unfolding. Upon denaturation, the environment of the fluorescent amino acids within the protein changes. Initially, in a protein's folded state contains the fluorescent residues are generally located within the hydrophobic core, and as the protein unfolds the residues become more exposed. Thus, one might expect the increased mobility of the sidechains to lead to a decrease in fluorescence. A combination of effects contributes to the overall fluorescence of the protein. Residue absorbance is influenced by the microenvironment in which it is located. Often an increase in fluorescence can also be observed as a protein unfolds. Therefore both increases and decreases in fluorescence can be observed after the unfolding transition. During denaturation experiments, the emission maximum typically shifts to 350nm in the unfolded state corresponding to the fluorescence maximum of tryptophan. Other factors, such as solvent conditions and temperature, contribute to the

observed fluorescence spectrum emission. As temperature increases a decrease in fluorescence intensity usually results. Chemical denaturants, like urea, produce a significant, concentration dependent effect. A combination of these factors contributes to the overall measurement of fluorescence for the respective protein and provides information pertaining to changes in conformation.

Circular Dichroism (CD) Studies of Proteins

Equilibrium fluorescence studies of proteins can only provide information reflecting changes in the protein's tertiary structure; CD spectroscopy can be used to gain insight into changes in both tertiary and secondary structure. CD spectra may also be used to estimate the amount of specific types of secondary structures present in a particular protein. Thus, CD spectra allow for a greater understanding of protein structure, and may be used to monitor changes in both secondary and tertiary structure.

Circular dichroism measures the difference between the absorption of left- and right-handed circularly polarized light by a protein's optically active (chiral) centers. The three aromatic acids in proteins, tryptophan, tyrosine and phenylalanine have finite CD signals and readily absorb in the near-UV region (250-290nm). A loss in tertiary structure commensurate with denaturation is monitored by the decrease in the near-UV signal as compared to the native protein (Bondensen *et al.*, 2001). In the far-UV region (180-250nm), the protein's secondary structure is readily studied because peptide bonds absorb in this region. The far-UV spectrum can be further used in estimating the protein's secondary structure, because α-helices and β-sheets absorb uniquely to produce CD peaks at different wavelengths. Using CD to study the unfolding process induced by chemical denaturants or heat, can be followed by CD spectroscopy. Further comparison

of rNAT1 and rNAT2 stability can be accomplished by comparing CD denaturation profiles.

Measuring Conformational Stability of Protein

Measuring the conformational stability of a protein involves calculating the equilibrium constant and the free energy change (ΔG) for the reaction, which describes the equilibrium between folded and unfolded protein as seen in reaction 2:

$$\text{Folded (F)} \leftrightarrow \text{Unfolded (U)} \qquad (2)$$

Equilibrium unfolding experiments using a chemical denaturant, such as urea, provide denaturation curves that can be analyzed to determine the stability of a protein. Such curves are prepared by following the changes at a chosen emission wavelength, where the folded and unfolded states differ significantly. Denaturation curves are split into three different regions: (1) the pre-transition region, which represents the folded protein, (2) the transition region where the unfolding occurs and (3) the post-transition region, which represents the unfolded protein. Many small proteins appear to unfold by a two-state transition and analysis assumes that under equilibrium conditions any intermediates are not significant enough to be considered in the analysis. While a two state mechanism does not necessarily mean that there are no intermediates, non-adherence to a two state transition may be indicative of folding intermediates. The midpoint of the denaturation curve can be used to characterize the protein's conformational stability. Other studies have revealed that some proteins do not undergo a simple two-state mechanism and yield information about transient or stable intermediates. Creating fluorescence profiles may contribute to an understanding of the intermediates that form during the unfolding

transition. These studies provide valuable information about protein folding and relative stability.

Materials and Methods

Materials

Competent non-K-12 derived *E. coli* strain TOPP3 cells transformed with pPH70D were a gift from the Carstairs R. Wagner laboratory. SnakeSkin™ dialysis tubing, ColorMeRanger™ Unstained Protein Molecular Weight Marker Mix, GelCode® Blue Stain Reagent, and Coomassie® Plus Protein Assay Reagent were purchased from Pierce Chemical Company. Ampicillin, Dnase1, dithiothretol (DTT), *p*-aminobenzoic acid (PABA), acetyl coenzyme A (AcCoA), sodium nitrite, ammonium sulfamate, *N*-1-napthylethylenediame dihydrochloride, lysozyme, trichloroacetic acid, and human thrombin were all purchased from Sigma-Aldrich. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from GibcoRCL UltraPure. Urea was purchased from USB. Tetracycline was purchased from Nutritional Biochemicals Coporation. Resin AG501-X8 and SDS were purchased from BioRad. DEAE-52 Pre-Swollen was purchased from Whatman. Sephadex G-75 was purchased from Pharmacia Fine Chemicals. All other reagents were ACS reagent grade and were purchased from Fisher or Sigma-Aldrich.

Isolation and Purification of rNAT2

Expression of the fusion protein rNAT2-F-D-T-70D. Transformed TOPP3/pPH70D cell stocks stored at -80°C were thawed and 1.0 µL was used to inoculate 10 mL of LB glucose (0.4%)/ampicillin (100 µg/ml) medium with tetracycline (50 µg/mL) contained in a capped culture tube (18 x 150mm). The inoculated cultures were grown aerobically overnight on an orbital shaker at 37°C. Additional cell stock solutions were prepared by mixing 0.3 mL if the overnight culture with 0.9 mL of 25% glycerol in a 1.5 mL centrifuge tube. The stock solutions were frozen in a dry ice/ethanol bath and stored at -80°C. Fresh LB glucose/ampicillin medium (1.0 L) contained in a

loosely capped 2.0 L flask was inoculated with an entire overnight culture (10 mL). The cultures were grown aerobically as described previously. Following incubation the density of the culture was monitored by measuring the absorbance at 600nm. When the absorbance at 600 nm was approximately 0.4, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM. After an additional 2.5 hr incubation, the cells were harvested by centrifugation at 5000 x g for 15 minutes at 4°C. The supernatant was decanted and the cell pellets were frozen and then transferred to a conical tube (50mL) and stored at -80°C.

TOPP3/pPH70D bacterial cell lysis. TOPP3/pPH70D bacterial cell pellets, retrieved from the 1.0 L of TOPP3/pPH70D culture were thawed using warm water. The cells were then suspended in 12.5 mL of degassed lysis buffer A (50 mM Tris, pH 8.0; 5 mM EDTA; 1.0 mg lysozyme/mL; 50 μg NaN_3/mL) per gram of cells. The mixture was then transferred to a 250 mL centrifuge bottle, and incubated at room temperature for 5-10 minutes. Next 1.25 mL of lysis buffer B (1.5 M NaCl; 0.1 M $CaCl_2$; 0.1 M $MgCl_2$; 20 μg Dnase1/mL; 1 mM PMSF) per gram of cells was added and the mixture was incubated at room temperature for another 5-10 minutes. Finally the volume of the lysed cells was measured and dithiothretol (DTT) was added to a final concentration of 5 mM. The mixture was layered with argon and spun at 25,000 x g for 30 minutes at 4°C. The supernatant was transferred to a 50 mL conical tube, layered with argon and stored on ice. At this step 0.1 mL was saved for protein and activity assays.

DEAE (diethylaminoethyl) anion exchange chromatography. The supernatant was transferred to SnakeSkin™ 10,000 MWCO dialysis tubing and dialyzed against 2.0 L of degassed and potassium phosphate buffer (PE; 20 mM, pH 7.4; 1mM EDTA, 1 mM

DTT), which had also been purged with argon. While dialyzing the buffer was gently stirred while being purged with argon through a fritted glass tube at 4°C for 1 hour. The buffer was changed to fresh 2.0 L of PE every hour, for a total of three hours. Then the dialysate was transferred to a 100 mL graduated cylinder and layered with argon. The dialysate was then assayed for protein concentration and activity. The sample was loaded onto a 2.5 x ~40 cm DEAE column, which was prepared following manufacturer's instructions and equilibrated with degassed and purged PE buffer. The column was loaded using a flow adapter at a flow rate of 0.5 mL/min, followed by a 50 mL wash with degassed and purged PE buffer. Ten-milliliter fractions were collected. The wash was then followed by a 800 mL, 0-0.5 M KCl gradient in PE buffer. The eluent absorbance was monitored at 280 nm and the peak fractions were assayed for enzyme activity. The highest activity peak fractions were pooled. A 0.1 mL sample was saved to assay. The pooled fractions were then concentrated to at least 1 mg/mL using Centriplus 30 ultrafiltration units. Glycerol was added to give a final concentration of10% and the solution quick frozen in a dry ice/ethanol bath and stored at -80°C.

Thrombin cleavage of partially purified fusion protein rNAT2. The protein solution was thawed in a room temperature water bath and dialyzed as previously described against degassed and purged thrombin cleavage buffer (50 mM Tris, pH 8.0; 0.1 M NaCl; 2.5 mM $CaCl_2$; 1 mM DTT) for three hours. Five units of human thrombin per mg of protein were added to the dialyzed fusion protein. The solution was mixed, layered with argon, and allowed to incubate overnight at 4°C. A 100 µL sample of the dialysate was saved for gel electrophoresis.

DEAE anion exchange chromatography. To separate rNAT2 from FLAG-L54F DHFR and other contaminating proteins, the digest was dialyzed against 2.0 L of PE buffer as described previously. The dialysate was loaded on a 2.5 x ~ 40 cm DEAE column equilibrated with degassed and purged PE at a flow rate of 0.5 mL/min. Ten-mL fractions were collected. The column was eluted with a 50 mL wash of degassed and purged PE followed by a 600 mL, 0-0.3 M KCl gradient in PE buffer. The fractions were monitored by their absorbance at 280 and 260nm. Selected fractions were assayed for activity and the peak activity fractions were pooled. The protein concentration and activity of the pool was then measured. The pool was concentrated to at least 3.5mL using Centriplus 10 units. The retentate was adjusted to10% solution with glycerol and quick frozen in a 15 mL conical tube using a dry ice/ethanol bath and stored at -80°C.

Gel filtration chromatography. To remove any high molecular weight contaminants the concentrated protein solution was applied to a gel filtration column. The protein was thawed and applied to a Sephadex G-75 (superfine) column (1.5 x ~65 cm), which had been equilibrated with degassed and purged PE. The sample was loaded at a flow rate of 0.5 mL/min while collecting 2.5 mL fractions. The asbsorbances at 280 and 260nm were read and the activity of the peak fractions assayed. The appropriate fractions were pooled and the protein concentration and NAT activity determined. The pool was then concentrated to ~ 0.3mg/mL and then the solution were adjusted to 10% with glycerol to stabilize the protein activity. The protein was then aliquoted out in 0.1 mg samples, quick-frozen and stored at -80°C.

SDS PAGE electrophoresis.

Protein samples were analyzed on precast 12% SDS-PAGE gels according to the method of Laemmli (Laemmli, 1970). The ColorMeRanger™ Unstained Protein Molecular Weight Marker Mix was used as standards. Prior to loading the protein samples were prepared by mixing 1 part sample with two parts sample buffer (2.9mL deionized water, 1.0mL of 0.5M Tris-HCl, pH 6.8, 2.0mL glycerol, 1.6mL 10% (w/v) SDS, 0.4mL β-mercaptoethanol and 0.1mL 1.0% bromophenol blue) and boiled for 60 seconds. The protein bands were visualized by staining the gels with GelCode® Blue Stain Reagent according to the manufacturer's instructions. The following amounts of protein were run on the gel: 10.0 μg of E. coli lysate, 5.0 μg of first DEAE column pool, 5.0 μg of thrombin digest, 2.0 μg of second DEAE column pool and 2.0 μg of G75 column pool.

AcCoA/p-Aminobenzoic acid (PABA) Assay for NAT Activity.

The assay spectrophotometrically measures the amount of free amine remaining after termination of the reaction. The amount of acetylated amine was determined by comparison to blank samples, which contained no enzyme or AcCoA, but only free amine. The enzyme activity, expressed as μmol of amine acetylated/mg of protein/min, was determined by the following equation: [(absorbance of control (free amine only) – absorbance of sample)/absorbance of control] x [(initial concentration of free amine x volume of incubation)/(mg of protein in the incubation x time of incubation)].

The assay was performed as described previously (Hein, 1997; Weber 1971). Incubation tubes (1.5 mL), containing enzyme (protein concentrations in a final volume of 0.1667 mL were 3 μg of protein extract/mL or 0.5μg of pure rNAT2 protein/mL) and

sufficient tetrasodium pyrophosphate buffer (50 mM, pH 7.0; 1 mM DTT) to bring the volume to 0.1 mL. When determining the activity of chromatography fractions, 5 µL of each fraction was used in the assay. In addition to enzyme and buffer, 16.7 µL of AcCoA (6 mM solution in deionized water) was added. The control incubation mixtures contained 16.7 µL of deionized water instead of AcCoA and buffer instead of enzyme. Reactions initiated by addition of 50 µL of PABA (0.333 mM) were carried out for 2 minutes at 37°C in a heat block and terminated by the addition of 333 µL of cold 5% trichloroacetic acid in deionized water. The tubes were then centrifuged at 12000 rpm for 20 minutes at 4°C in a microfuge, this step will remove any precipitated proteins. The supernatants were transferred to disposable 1.5mL cuvettes and the reaction rate determined by diazotization of the unacetylated PABA. The supernatant was treated by sequentially adding 50 µL of 0.05% sodium nitrite, 50 µL of 0.5% ammonium sulfamate and 250 µL of 0.05% *N*-1-naphthylethylenediamine dihydrochloride at two minute time intervals. The absorbance was read at 540 nm against deionized water

Determination of Protein Concentration.

Protein concentrations were determined by the method of Bradford (Bradford, 1976) using BSA as the standard. The Bradford assay was performed using Coomassie® Plus Protein Assay Reagent following the manufacturer instructions for the microscale assay.

rNAT2 sample preparation.

Aliquots of rNAT2, which are stored in 10% glycerol at -80°C, were thawed, transferred to a Slide-A-Lyzer 10,000 MWCO cassette, and dialyzed against 1L of PE

buffer for three hours, changing the buffer every hour. Protein concentration and activity assays were then performed on the dialyzed protein.

Reagent preparation.

The PE buffer and 9M urea in PE stock solutions were prepared fresh on the day of the experiment. Urea solutions were treated with BioRad AG501-X8 Resin for a minimum of 30 minutes. The solutions were degassed for 15 minutes and filtered through 0.2 μm syringe filters. The refractive index of the urea stock solution and of the buffer were measured using an Abbe refractometer at 25°C. These values were used to calculate ΔN, which equals the refractive index of urea – the refractive index of PE. The concentration of urea was determined using equation 3 (Pace and Scholtz, 1997):

$$117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3 \qquad (3)$$

The calculated concentration of urea should be within 10% of the expected value (9M) to proceed with sample preparation.

Spectrofluorometric Analysis

Equilibrium unfolding as a function of urea concentration was monitored using a SPEX Fluoromax Spectrofluorometer. The excitation wavelength was 280 nm or 295 nm (2.0-nm bandpass). The emission spectrum was monitored over a range of 400 – 300 nm (6.0-nm bandpass) at 1.0 nm increments and 0.15 sec integration time as an average of 16 scans. The measurements were made using a 5mm x 10 mm fluorescence cuvette. The samples were equilibrated overnight at 10°C prior to making the measurements. A blank containing no protein was prepared at each concentration of urea. The protein concentration used for the fluorescence studies was 9 μg/mL.

Fitting of Equilibrium Data

The data was fit to equation 4 (Pace and Scholtz, 1997) by a non-linear least squares analysis using KaleidaGraph (Synergy Software).

$$y = \{(Y_f + m_f[D]) + (Y_u + m_u[D]) \times \exp[m \times ([D]-[D]_{1/2})/RT]\}/(1 + \exp[m \times ([D] - [D]_{1/2})/RT]) \quad (4)$$

This equation is used in the linear extrapolation method for analysis of denaturation curves **(Figure 1).** The transition region is characterized by m, which measures its steepness and $[D]_{1/2}$ the midpoint of the transition represents the denaturant concentration when the protein is half folded and unfolded. The parameters, Y_F and Y_U and m_F and m_U represent the intercepts and slopes, respectively, of the pre- and post-transition regions. The fraction unfolded (Fu) for any point in the transition region can then be calculated using equation 5. The term Y_F is calculated over the entire concentration range from the equation for the pre-transition region, and Y_U is calculated from the equation from the post-transition region.

$$Fu = (Y_F-Y)/(Y_F-Y_U) \quad (5)$$

The equilibrium unfolding constant (K) for the unfolding process can be calculated using equation 6.

$$K = Fu/(Fy) = Fu/(1-Fu) \quad (6)$$

The free energy change, ΔG, can then be calculated using equation **7,** where R is the ideal gas constant and T the absolute temperature.

$$\Delta G = RT\ln K \quad (7)$$

The transition region is analyzed by equation 8.

$$\Delta G = \Delta G(H_2O) - m[D] \quad (8)$$

The slope (m) of this plot, expresses the dependence of the transition region on the concentration of the denaturant [D]. The y-intercept, creates a value known as $\Delta G(H_2O)$, measure of the conformational stability of a protein at zero denaturant concentration. The term $[D]_{1/2}$ which represents the midpoint of the transition is determined from equation 9.

$$[D]_{1/2} = \Delta G(H_2O)/m \quad (9)$$

The three values m, $\Delta G(H_2O)$ and $[D]_{1/2}$ can be used to describe not only a protein's denaturation curve but serve as a means to measure the conformational stability of a given protein, and can be used as a basis of comparison.

CD Spectroscopy.

A Jasco Model J810 CD spectrometer was used to measure the CD spectrum. In the far UV readings a 0.1 cm cell was used, and a 1.0 cm cell was used for the near UV readings. The protein concentration was maintained at 0.1mg/ml in both regions of the spectrum. The scan start for far UV was 260-190nm and 250-310 for near UV. Bandwith was 1nm with a data pitch of 1nm, a total of 10 scans were accumulated, the sensitivity level was standard, the response time was 1.0sec, the speed 20nm/min and the temperature was 25°C.

Results

rNAT2 Isolation and Purification

A total of four rNAT2 preparations were conducted during the course of these studies. Protein concentration and enzyme activity was monitored at each stage of the isolation procedure. Results from a typical preparation are shown in Table 1. Overall, the procedure yielded 2.1 – 4.2 mg of rNAT2 per preparation with a specific activity that ranged from 65.2-76.2 μmol/min/mg.

Table 1: Purification of rNAT2-70D

Purification Step	Total protein (mg)	Specific Activity (μmol/min/mg)	Total Activity (μmol/min)	% Recovery	Purification Fold
Lysate	194	3.83	743	100	1.00
DEAE 1	43.1	20.4	879	118	5.32
DEAE 2	5.67	45.8	260	35.0	12.0
Sephadex G-75	3.30	65.2	215	28.9	17.0

Urea-Induced Equilibrium Unfolding Experiments

The urea-induced equilibrium unfolding transition of rNAT2 was followed by fluorescence spectroscopy at two different excitation wavelengths. Initial studies of urea-induced unfolding followed by fluorescence had considerable scatter. Subsequently, the temperature was monitored externally using a temperature probe and the cell chamber was flushed with nitrogen gas in an effort to eliminate condensation.

Excitation at 280 nm.

Emission spectra of rNAT2 were recorded by exciting at 280 nm (**Figure 2**). By exciting at 280 nm the contributions of both tyrosine and tryptophan can be observed in the overall fluorescence of rNAT2. Four different groups of spectra with similar character resulted from this experiment **(Figure 3)**. The first group of samples, with urea concentration 0-2.52 M, showed similar behavior because their peak maxima was approximately 335 nm, where the protein maximally absorbs in its native state. In the next group, in the 3.03-3.52 M urea concentration range, there is a red shift in the maxima, to ~ 340 nm. The third group of spectra, urea concentration 3.79-6.80 M, has more of a red shift to ~ 345 nm, with a noticeable decrease in fluorescence intensity. In the last group of spectra, urea concentration 7.06-8.07 M, the maxima occur at ~ 355 nm but the intensity increases as the urea concentration increases. In **Figure 4**, the relative fluorescence at 335nm, the wavelength where the maximum change occurs, is plotted

versus the concentration of denaturant. The overlapping effects occurring within these four groups of spectra lead to considerate scatter. Complete analysis of the equilibrium curve over the entire urea concentration range including the folded and unfolded baselines could not be done using **equation 4**. Average emission wavelength analysis was then applied to the same data set. The intensity weighted average of all the wavelengths scanned was calculated and plotted as the average emission wavelength versus the concentration of urea **(Figure 5).** The folded baseline is in the 0-2.52 M urea concentration range and the unfolded in 7.56-8.07 M. From 3.27-6.06 M there is the presence of a thermodynamically stable intermediate of rNAT2, which corresponds to the third region in the emission spectra (**Figure 3 and Figure 5**).

Excitation at 295 nm.

Another equilibrium unfolding study of rNAT2 was conducted under the same conditions as the first experiment, in an effort to analyze the contributions of just tryptophan residues to the overall fluorescence of rNAT2. Since there are a large amount of aromatic residues in rNAT2 studying the contributions of just tryptophan may simplify the studies of the unfolding process, and allow for better comparisons of stability.

Emission spectra of rNAT2 were recorded by exciting the protein at 295 nm (**Figure 6**). The emission spectra of rNAT2 were collected into four groups with similar character, as in the first experiment **(Figure 7)**. The first group of samples, with urea concentration 0-2.5 M, the maxima occur at 335 nm, again at the place where the native rNAT2 absorbs maximally. The second group of spectra, urea concentration 3-6.5 M, show an increase in intensity compared to the first group with a red shift to ~ 340 nm. The next group, with urea concentrations 6.75 and 7.0 M, showed only a slight red shift

in maxima, to ~ 345 nm, but there was a decrease in intensity compared to the second group. The last group of spectra, at urea concentrations of 7.5 and 8.0 M showed a slight decrease in fluorescence intensity, but there was a further red shift to ~ 355 nm. **Figure 8** shows the relative fluorescence at 335 nm as a function of urea concentration. Again, the relative fluorescence data had considerable scatter, like in the first experiment, due to the fluctuations in the intensity of the spectra groups (**Figure 8**). The analysis using the average emission wavelength showed similar results to the experiment conducted with excitation at 280 nm **(Figure 9)**. There is evidence of a folded and unfolded baseline, and also a thermodynamically stable intermediate throughout the unfolding transition. The folded baseline is in the 0-2.0 M urea concentration range. The unfolded baseline is from 7.5-8.0 M urea concentration range. The presence of a thermodynamically stable intermediate is seen from urea concentration 3.5-6.0 M (**Figure 9**).

Early studies using circular dichroism (CD) were also conducted, and produced data on how rNAT2 will interact with circularly polarized light in the near- and far-UV regions. The far-UV spectrum of rNAT2 (**Figure 10**) will be quite useful in future studies of rNAT2 involving CD.

Discussion

RNAT2 Purification

The purification steps yielded lower total protein amounts throughout the preparation than those published (Sticha *et al.*, 1997). Overall this resulted in a lower enzyme yield (**Table 1**). Alterations was made in changing the selection of transformed TOPP3/pPH70D by growing the cells on LB agar to insure the selection of active cells. This did not increase the overall yield of the protein. Personal communications with

collaborators at University of Minnesota have been promising. There has been the development of a new line of transformed *E. coli* cells that have allowed them to achieve a greater yield of rNAT2. Use of this new cell line in future experiments conducted at Albright College will help in eliminating the yield problem associated with the current isolation and purification procedure.

Urea-Induced Equilibrium Unfolding Studies

The initial and ultimate goal of this project has been to investigate the differences in stability between rNAT1 and rNAT2 and to produce an experimental design that allows for the accumulation of consistent data and results. Equilibrium unfolding studies were conducted to determine and compare the stability of the two isozymes. To this date only studies on rNAT2 have been completed using fluorescence spectroscopy and circular dichroism (CD). Using urea to induce an unfolding transition of rNAT2 and studying the fluorescence of rNAT2 contributed by aromatic residues, tryptophan and tyrosine, the protein's conformational stability can be determined.

Often scatter produced by experimental conditions and error plagued initial unfolding studies. In an effort to eliminate scatter alterations to the experimental method and conditions were made. To eliminate scatter due to condensation because of the operating temperature of 10°C, nitrogen gas was used to flush the cell chamber. The type of cuvette used was changed as well. During initial studies a 5 mm x 5 mm quartz cuvette was used. This was held by a plastic holder, which does not fully contact the cell block. In subsequent experiments, a 5 mm x 10 mm. This allows the cuvette to have direct contact with the cellblock and to transfer the temperature to the samples in a more efficient manner. This prevents the solution from sitting at a higher temperature, and

allows for more accurate results. Lastly another change was the method used to monitor the temperature. Optimal results were obtained when the temperature was monitored by an external probe. This allows for the direct observation of the temperature of individual samples, which is crucial in maintaining consistency throughout the experiment. Due to the sensitive nature of fluorescence, the more consistent and reproducible the experimental method, the better the results that can be achieved.

After making the necessary changes to the experimental method, the equilibrium unfolding studies were conducted on rNAT2 by exciting at two different wavelengths, 280 and 295 nm. The first experiment with excitation at 280 nm created emission spectra of rNAT2, exciting both the tryptophan and tyrosine residues (**Figure 3**). The four groups of spectra illustrated in **Figure 5** show the different transitions that rNAT2 is undergoing throughout the unfolding process. The initial (blue) group shows the folded or native state of rNAT2 before the presence of urea really begins to affect the native folded conformation. As the urea concentrations increase rNAT2 begins to undergo the unfolding transition. This is evident due to the red shift in spectra maxim and the decrease in overall fluorescent intensity of rNAT2. Once the protein reaches an unfolded state (red group) the fluorescence intensity begins to increase from the transition state. As a protein unfolds it becomes more random in structure, and the intensity between the black and green groups increases during the transition. This increase could be attributed to the change in the microenvironment of the residues. The subsequent increase in urea concentration induces the intensity to drop and a slight shift in maxima, which would correspond to the intermediate state that rNAT2, is believed to achieve during the unfolding process. The decrease in fluorescence would imply that a more mobile

structure is achieved, and the microenvironment of the aromatic residues has changed in this part of the transition. The final (red) group shows another increase in intensity, which would imply an unfolded state.

Excitation at 295 nm

The second experiment conducted, which had rNAT2 excited at 295 nm instead of 280 nm showed different results, but still confirmed the presence of an intermediate structure during rNAT2's unfolding pathway. By exciting at 295 nm the intent was to only concentrate on how tryptophan contributes to the overall fluorescence of the protein, with the desire to simplify the emission spectra results. Four groups of emission spectra were also observed with this experiment, but yielded different results from the first experiment (**Figure 7**). The first (blue) group represents the folded state of rNAT2, and upon transition to a more unfolded state (red group) there is a more random structure involved initially. The transition from the folded to intermediate shows an initial increase in intensity, which would imply changes in the residues' microenvironment. That would correlate with a transition between the unfolded and intermediate structures. As the urea concentrations increase the intensity drops. The transition to the unfolded state shows an increase in intensity. Since these residues are at different positions along the length of the chain, the observed results are the net effect felt by these multiple residues. There are a large amount of aromatic residues in rNAT2, which is a major complication in analyzing the fluorescence results. Though exciting at 295 nm has eliminated tyrosine signals from the overall fluorescence of rNAT2, it still has a considerable amount of fluorescence.

Since the unfolding transition of rNAT2 was assumed initially to be a simple two-state transition with no accumulating intermediates, data was analyzed using relative fluorescence at 335 nm. By analyzing the data in this particular manner, the data is concentrated on what happens throughout the unfolding process where rNAT2 absorbs maximally. This type of analysis proved to be difficult and didn't allow for good interpretations of the unfolding process. Since monitoring rNAT2 at one wavelength doesn't account for the multiple processes that may change in the unfolding process, the results produced a large amount of scatter as seen in **Figures 4** and **8**. Subsequently the data was analyzed using average emission wavelength, which helped to confirm that rNAT2 does not undergo a simple two-state transition while unfolding.

The average emission wavelength produced a better measure of the shift or change in the shape of emission spectra. Overall, the benefit of analyzing the data by this particular method, is that there is less influence by noise on the fluorescence data. The results from analysis using this method in **Figures 5** and **9** showed that rNAT2 transitions from the folded state to a stable intermediate and then transitions again to the fully unfolded state. This observation coincides with the rise and fall seen in intensity with the emission spectra in **Figures 3** and **7**. Overall this analysis has helped in understanding the considerable scatter in relative fluorescence data.

Circular Dichroism Studies of rNAT2

In an effort to find other methods of analyzing stability differences between rNAT1 and rNAT2, initial scans of rNAT2 using circular dichroism (CD) have been conducted. The initial scans involved looking at rNAT2 in the far-UV (190-250 nm) in

its native state. By taking this approach results may be simplified making analysis of stability differences between rNAT1 and rNAT2 less complicated.

Future Experiments

Since the initial goal of this experiment was to ultimately compare the stabilities between rNAT1 and rNAT2, the simplest method would be preferred. Using fluorescence spectroscopy has proven to be difficult because of its sensitive nature, and the presence of an intermediate creates complicated emission spectra. In initial studies done by Frieda Texter using thermal denaturation monitored by CD results showed a simple two-state transition that occurred. This would be useful in future stability studies, because CD can be used in comparing stability of rNAT1 and rNAT2. On the other hand fluorescence spectroscopy can be used in mechanistic studies of these particular isozymes, due to its sensitive nature and conformation of a stable intermediate forming during the unfolding process.

Finally the development of a finite and reproducible stability study for rNAT2 will allow for experimentation on rNAT1 to begin. So that one might begin to pursue the differences for these differences.

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APPENDIX OF FIGURES

Figure 1 – Fluorescence Intensity versus Urea Concentration (M)
The protein being studied was RNase T1. Readings were taken at 25°C in 30mM Mops buffer at pH 7.0. From Pace and Scholtz, 1997

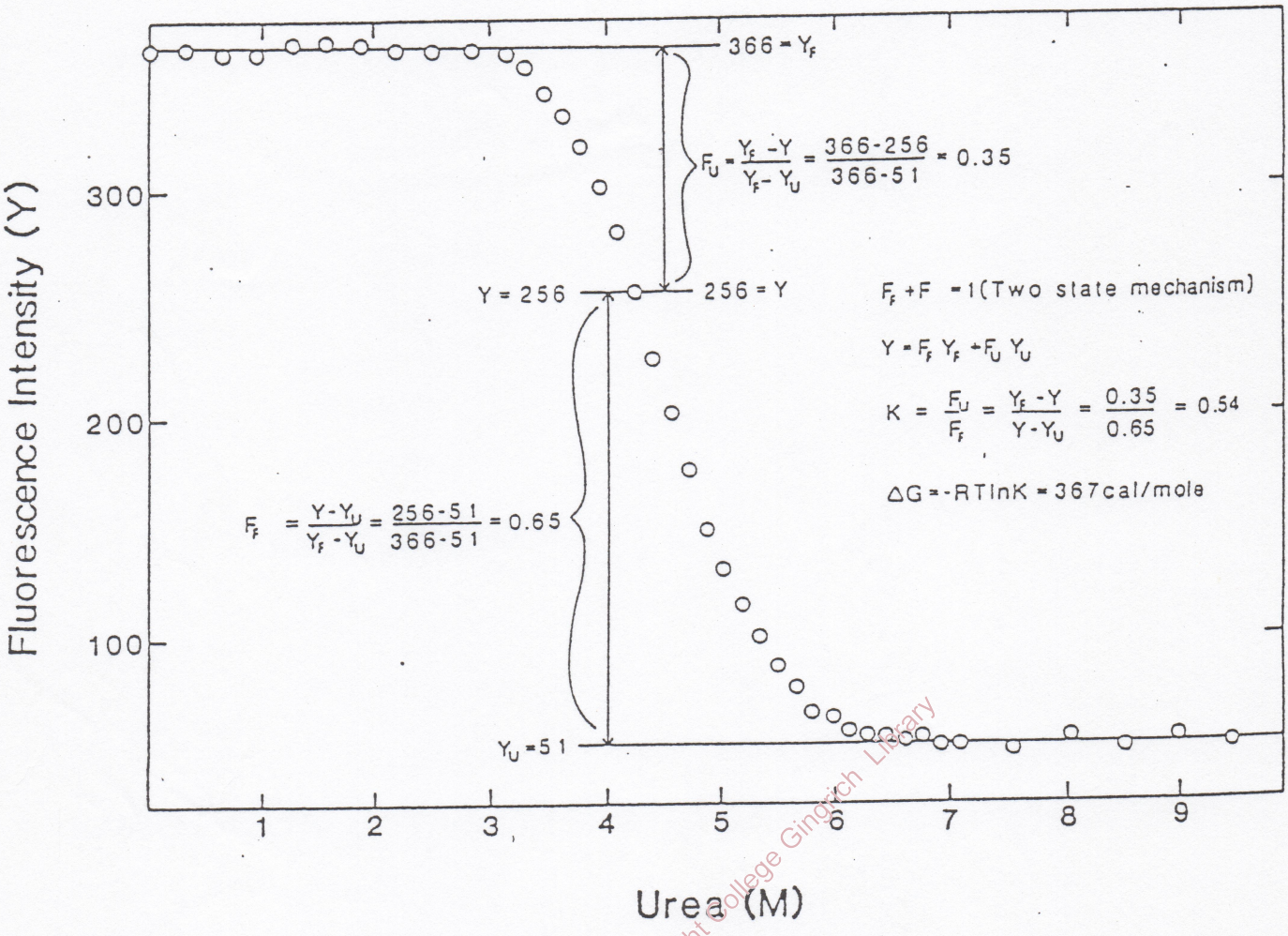

Figure 2: Corrected rNAT2 Samples Excitation at 280 nm

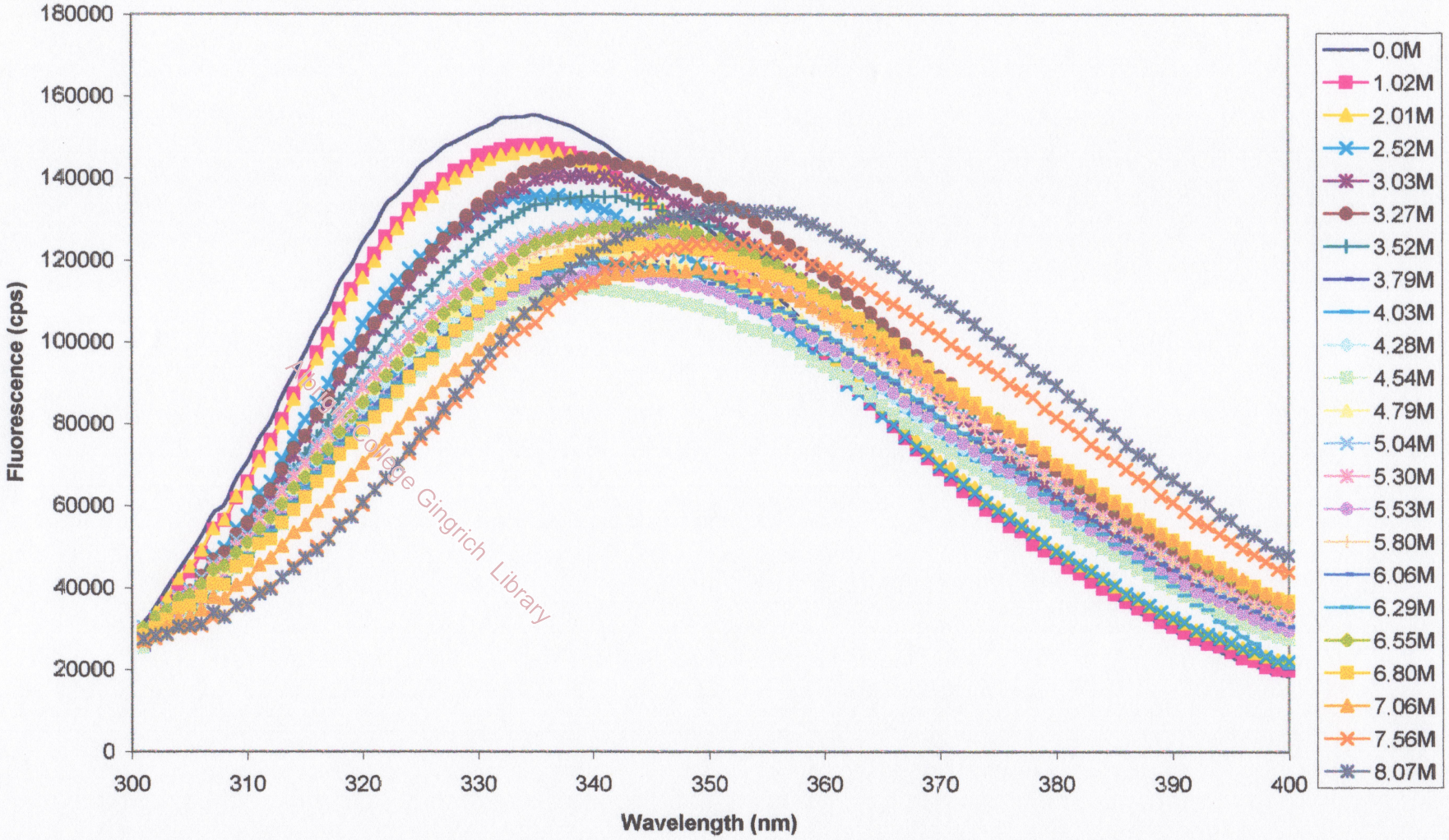

Figure 3: Corrected rNAT2 Samples Excitation at 280 nm

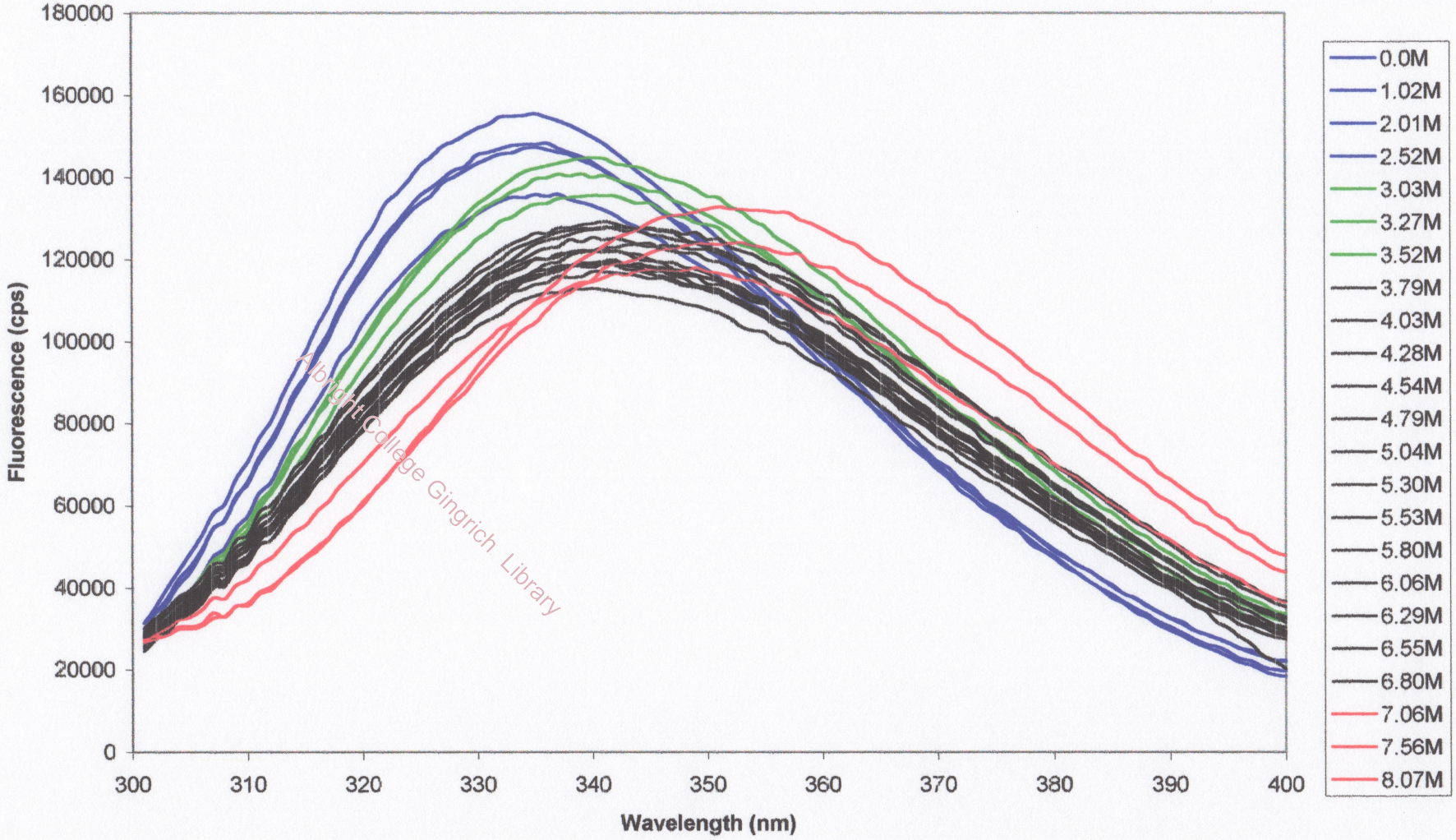

Figure 4: Relative Fluorescence at 335nm versus Urea Concentration
Excitation at 280 nm

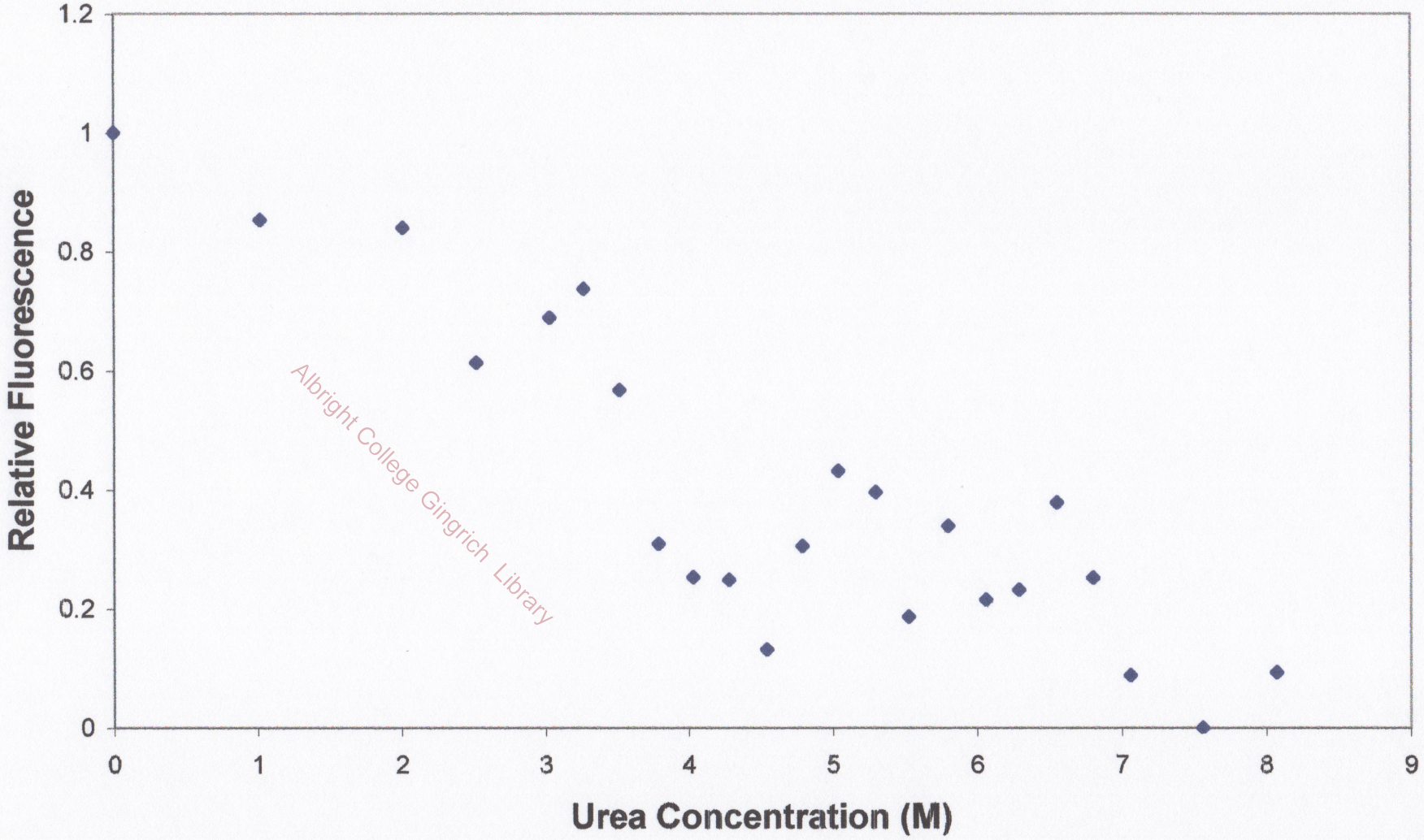

Figure 5 : Average Emission Wavelength Excitation at 280 nm

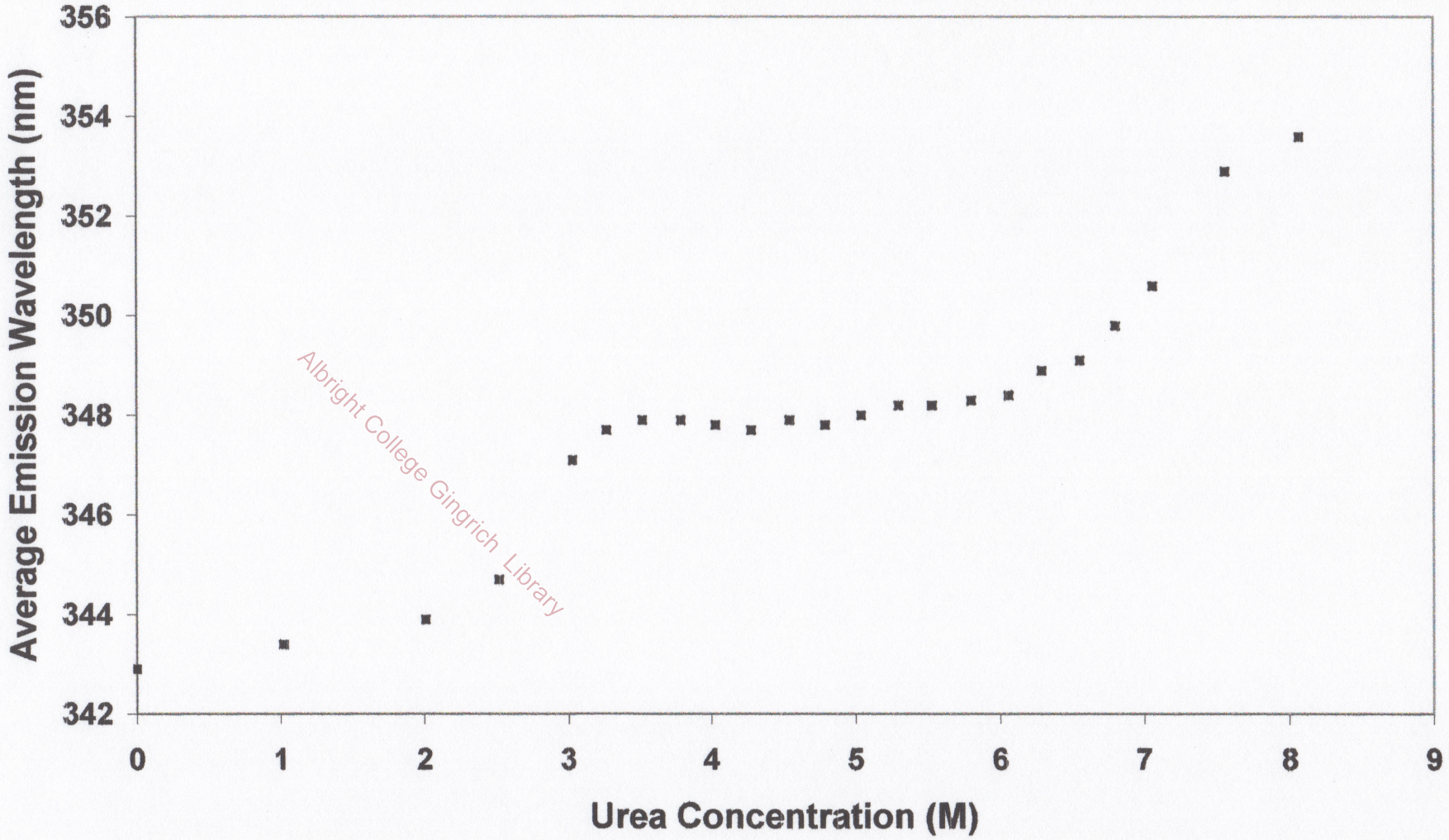

Figure 6: Corrected rNAT2 Samples Excitation at 295 nm

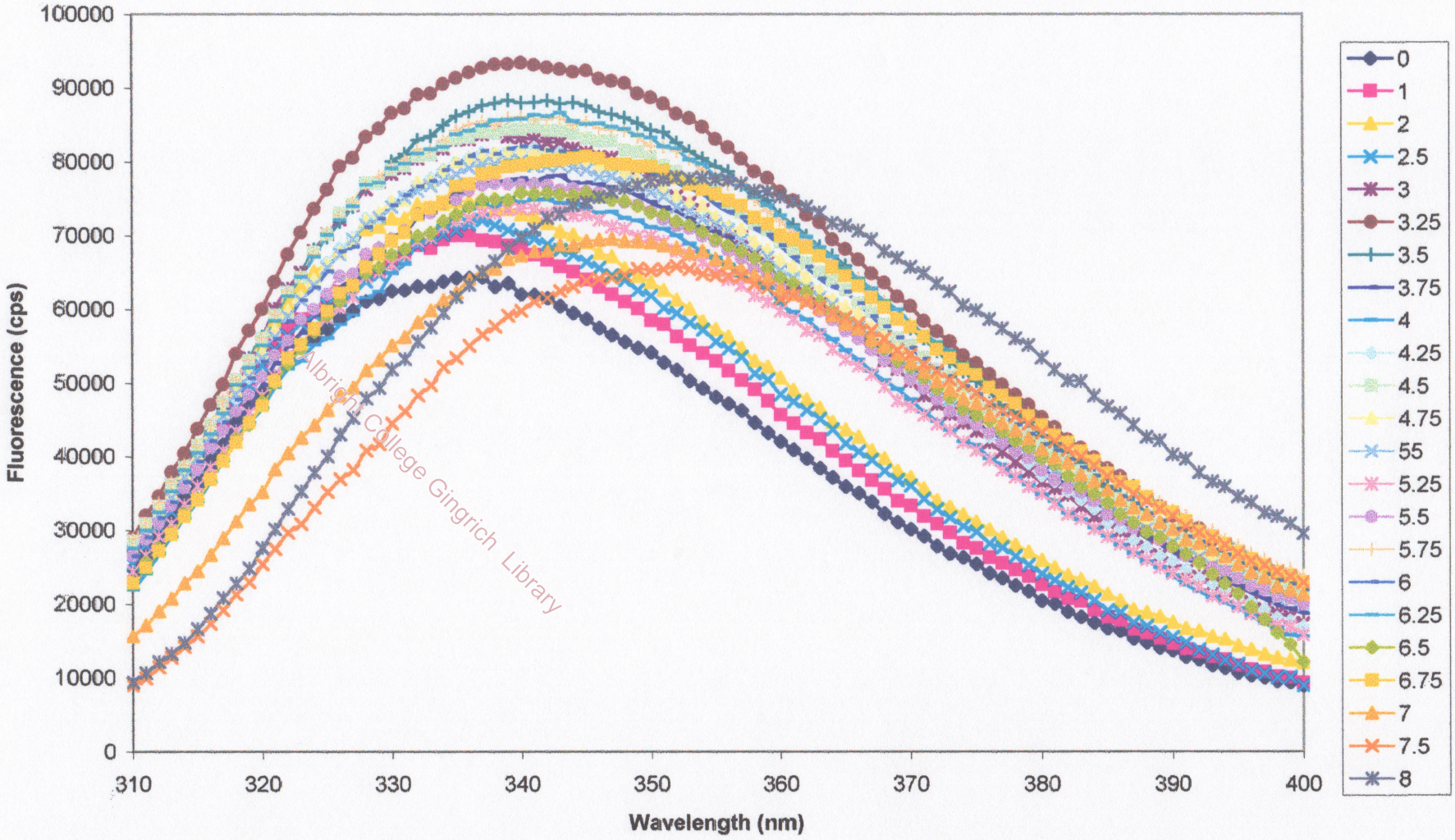

Figure 7: Corrected rNAT2 Samples Excitation at 295 nm

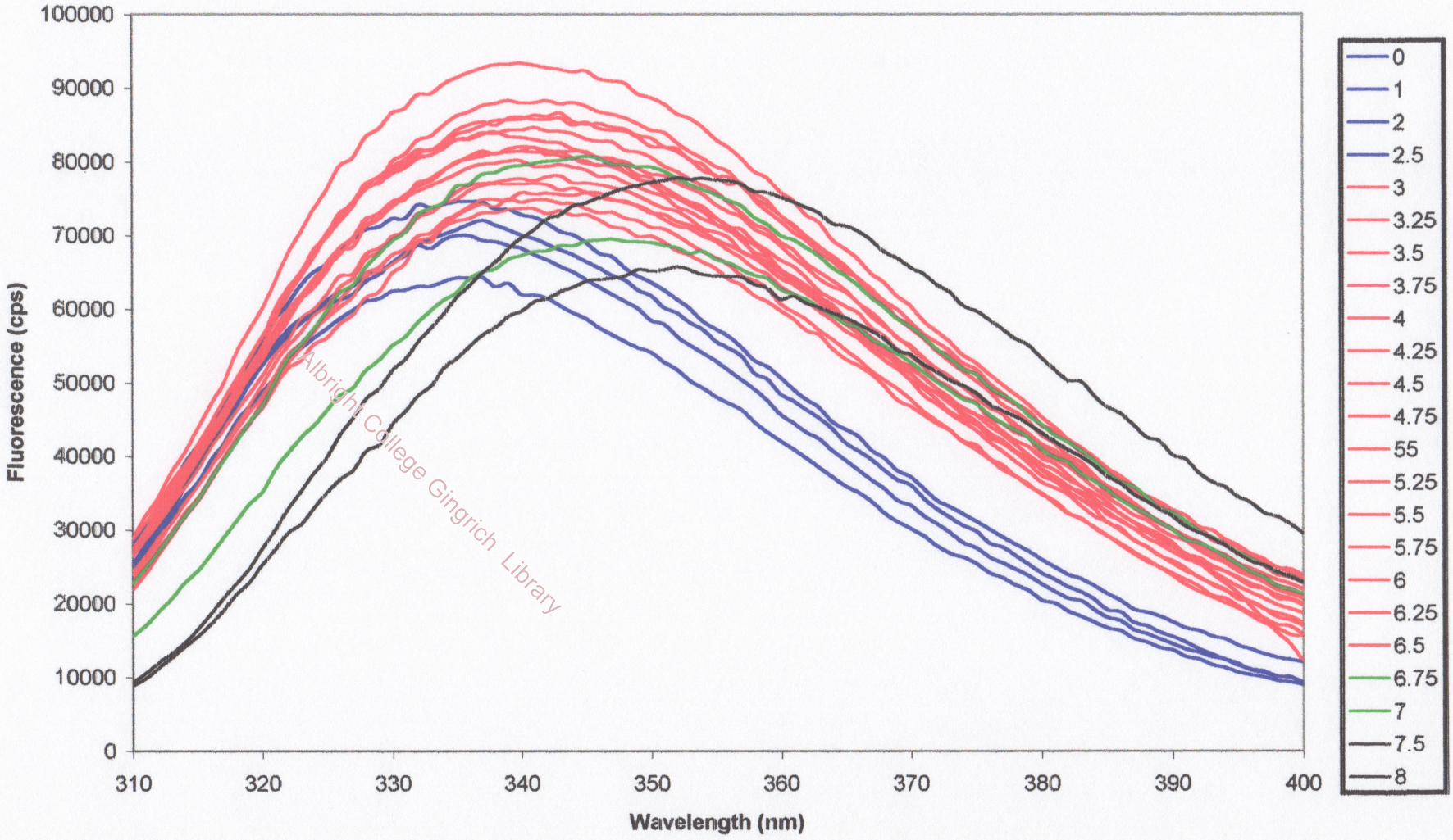

Figure 8: Relative Fluorescence at 335nm versus Urea Concentration
Excitation at 295 nm