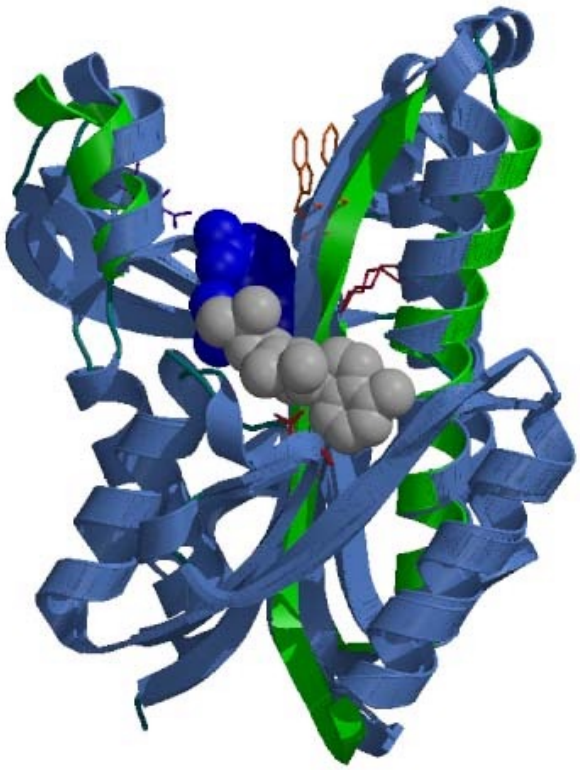
Spectroscopic Analysis of the Interaction between Dihydropteridine Reductase (DHPR) its Cofactor and Folic Acid (FA)



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Chapter 1

Spectroscopic Analysis of the Interaction between Dihydropteridine Reductase (DHPR) its Cofactor and Folic Acid (FA)

Preface

My work centered on exploring the interaction between the enzyme dihydropteridine reductase (DHPR), cofactor NADH, and ligands, which in my case was folic acid (FA). DHPR is an enzyme which is important in a biochemical pathway that recycles a substance called tetrahydrobiopterin. Tetrahydrobiopterin is important in the conversion of precursors of neurotransmitter like serotin. Deficiency in DHPR leads to phenylketonuria disease (PKU).

Enzyme and inhibitor interactions were observed both in the presence and the absent of the cofactor. The third chapter of my thesis will include a discussion about the principles behind the UV-VIS, IR and fluorescence spectroscopy. In the fourth chapter, I'll talk about the characteristics and properties of the protein and inhibitors studied. Chapter 5 will focus on the result we obtained from our UV-VIS and fluorescence work. Chapter 6 will be devoted to the IR studies. In both chapters 6 and 7, critical analysis of the data and the conclusion drawn from the results will be presented. The last chapter will include the calculated simulations for folic acid. This will support and explain the results presented in chapters 6 and 7.

Chapter 2

Introduction

The overall goal of our research is to understand the interplay between protein structure, inhibitor, and the cofactor. The enzyme Dihydropteridine reductase (DHPR) was studied in a model system. DHPR is needed for the normal cycling of tetrahydrobiopterin, which is a very important cofactor in the brain. If we don't have tetrahydrobiopterin then we won't have neurotransmitters such as serotonin. Serotonin is essential for the brain functioning. In humans, DHPR deficiency causes a neurological disorder known as phenylketonuria (PKU). PKU is a genetic disorder that is characterized by an inability of the body to utilize the essential amino acid, phenylalanine. Amino acids are the building blocks for body proteins. Essential amino acids can only be obtained from the food we eat as our body does not normally produce them. In classic PKU, the enzyme phenylalanine hydroxylase is completely deficient, which breaks down phenylalanine. This enzyme normally converts phenylalanine to another amino acid, tyrosine. Without this enzyme, phenylalanine and its breakdown chemicals from other enzyme routes, accumulate in the blood and body tissues and result in PKU.

Fluorescence spectroscopy plays an important role in investigating the interaction between proteins (enzymes), and small molecules, such as ligands and cofactors. We compared the DHPR with inhibitor and with both inhibitor and cofactor with the help of fluorescence. Data showed that a change in fluorescence intensity and fluorescence

peaks results when the cofactors and/or inhibitors were complex with DHPR. Infrared spectroscopy was used to identify the IR bands in the ligand folic acid. Calculated simulations for folic acid and its protonated form were obtained from the Gaussian03 program in order to compare the experimental data of folic acid and see which bonds are interacting when the ligand is within the protein.

Chapter 3

Theory behind spectroscopy

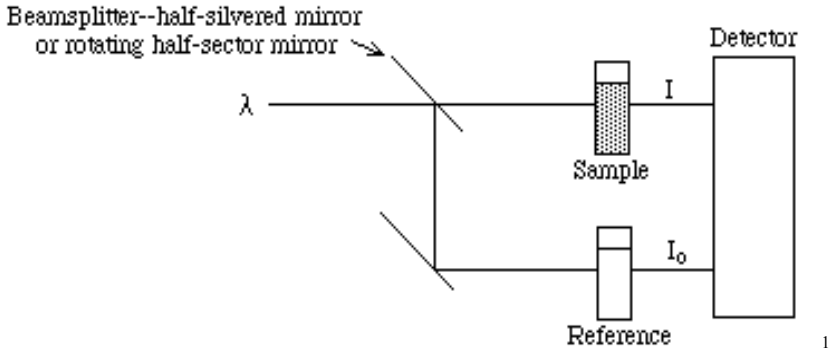
Spectroscopy is a type of analysis done by shining the light on the molecule (sample) to learn more about it and to see what is inside. In spectroscopy, chemists measure the absorbance, amount of light passes and absorbed by the sample. By knowing what wavelengths of lights are absorbed by the sample, we can learn more about that sample. On the other hand, if we shine the light at the wrong wavelength of light to study about the specific characteristic of a molecule then we will never learn anything about that molecule because different characteristics of molecules absorb energies at different wavelengths of lights. That's why there is a need for different forms of spectroscopy such as, ultraviolet spectroscopy (UV), fluorescence spectroscopy, and infrared spectroscopy (IR). These different forms of spectroscopy investigate specific characteristics of a sample. For example, IR spectroscopy is used to examine how the molecules in a sample vibrate, while ultraviolet spectroscopy (UV) is used to investigate how certain chemical bonds in a molecule are arranged.

UV-Vis spectroscopy

UV range is from 200 to 400 nm and visible (VIS) range is 400 to 800 nm. UV spectroscopy is useful to investigate electronic structure of unsaturated molecules and to determine the amount of conjugation in a molecule. In our research, we use it to see the absorption peak for the compound so that we can excite the compound at the absorption peak in fluorescence. Absorption of ultraviolet or visible light by a molecule depends on

electron transitions between molecular orbital energy levels. Absorption spectroscopy is mostly based on electronic transitions of non-bonding (n) or bonding (π) to the anti bonding (π*) excited state because these transitions fall in the region of 200-700nm. For example, C=C is a π to π* transition and C=O is a n to π* transition. When an electron goes from a higher to a lower energy state, a photon of definite wavelength and frequency is emitted. However, a photon is absorbed when electromagnetic radiation in the UV-Vis range passes through a molecule containing multiple bonds. As a result, in folic acid, photon is absorbed due to its double and carbonyl bond. Therefore, amount of energy absorbed depend on the compound structure. Molar absorptivity (ε) measures how strongly the species absorb light at a given wavelength. My ligand folic acid has a molar absorptivity of $7.95 \times 10^{3} M^{-1} cm^{-1}$. Every molecule has a characteristic electronic spectrum depending on its characteristic ΔEs. There is a general rule that describes the effect of double bonds conjugation on the energy absorbed by the π system which is the greater the number of conjugated multiple bonds in a compound, the longer the wavelength of the light that the compound will absorb. Folic acid has seven conjugated bonds from the rings and thus has an absorption peak at 363 nm. Schematic diagram of UV-VIS spectrometer is shown in figure one.

Figure #1



[1]

The function of the UV instrument is relatively straightforward. It has the source, the sample and the detector. A beam of light from a UV light source is separated into its component wavelengths by a diffraction grating or prism. Each monochromatic (single wavelength) beam is split into two equal intensity beams. One beam is the sample beam where we place the cuvette with our sample and the other beam is the reference beam where we place the cuvette with our solvent only. Detectors then measure the intensities of these light beams.

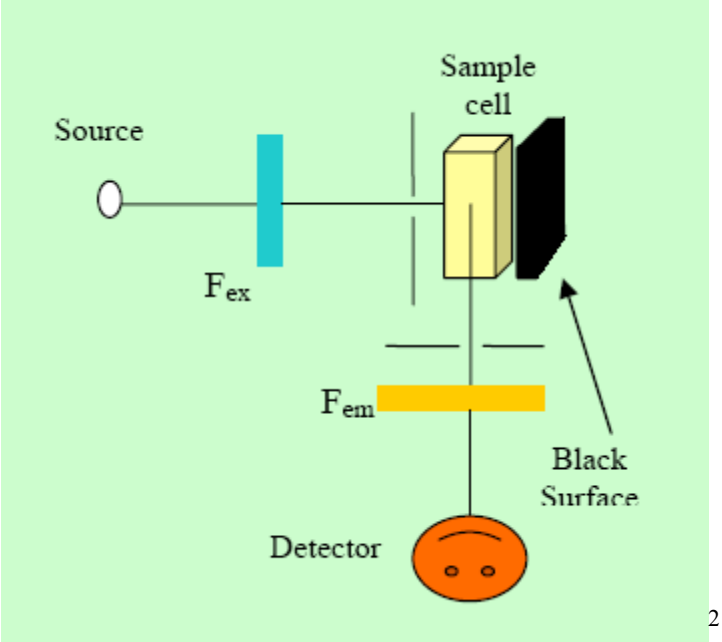
Fluorescence spectroscopy

Fluorescence spectroscopy is a type of electromagnetic spectroscopy used for analyzing fluorescent spectra. It is primarily concerned with electronic states and vibrational states. In fluorescence spectroscopy, the electron is first excited by absorbing a photon of light from its ground electronic state to one of the various vibrational states in the excited electronic state. Collisions with other molecules cause the excited molecule to lose vibrational energy until it reaches the lowest vibrational state of the excited

[1] Spectroscopy – UV/Vis. http://www.sci.sdsu.edu/TFrey/Bio750/UV-VisSpectroscopy.html

electronic state. The molecule then drops down to one of the various vibrational levels of the ground electronic state again, emitting a photon in the process. As molecules drop down into any of the vibrational levels of this ground state, the photons will have different energies, and frequencies. Therefore, by analyzing the different frequencies of light emitted in fluorescent spectroscopy, the structure of these different vibrational levels can be determined.

When electrons go from the excited state to the ground state, there is a loss of vibrational energy. As a result, the emission spectrum is shifted to longer wavelengths than the excitation spectrum. Emission and excitation spectra are obtained from fluorescence spectroscopy. Emission spectrum is wavelengths emitted by fluorophore after it has been excited by a photon of light. Fluorophore is the specific region of a molecule, which is capable of exhibiting fluorescence. The fluorescence emission spectrum is plotted as relative intensity as a function of wavelength and it is positioned in longer wavelengths than the excitation spectrum. Excitation spectra are generated by scanning through the absorption spectrum of a molecule while monitoring the emission at a single peak. In a similar manner to the absorption spectrum, the excitation is broadened due to vibrational and rotational relaxation of excited molecules. Absorption and excitation spectra are distinct but they often overlap. Schematic diagram of a fluorometer is shown in figure two.



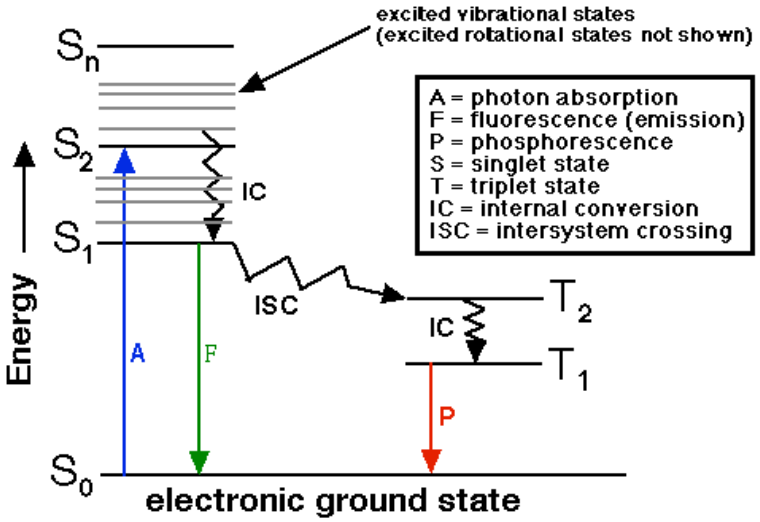
[2]

Fluorescence instrument has the source, the sample and the detector as in UV/VIS. First, the light would pass through an excitation filter and then it will be absorbed by the sample. After the excitation, fluorescence will be emitted. Then, light would pass through the emission filter and the amount of light pass through can be measured by the detector.

[2] Fluorometers. http://www.iugaza.edu/users/abdellatif/Instrumental%20Analysis/Lectures/Lectures%2021-/L30.pdf

Figure #3

Jablonski diagram[3]



Jablonski diagram is shown in figure three and it basically shows the electronic states and the transitions between them in a molecule. The states are arranged vertically by energy and grouped horizontally by the spin multiplicity. Straight arrows indicate the radiative transitions while the squiggly arrows indicate the nonradiative transitions. When the fluorophore absorbs energy, it is excited to a higher vibrational energy level in the first singlet excited state (S_1) before it goes to the lowest energy level and this process is known as absorption. Internal conversion takes place when there is a transition between energy states of the same spin state ($S_2 \rightarrow S_1$). On the other hand, intersystem crossing transition takes place between different spin states ($S_1 \rightarrow T_2$). Process of photon emission between states of the same spin state is known as fluorescence ($S_1 \rightarrow S_0$) and the photo emission between different spin state is knows as phosphorescence ($T_1 \rightarrow S_0$).

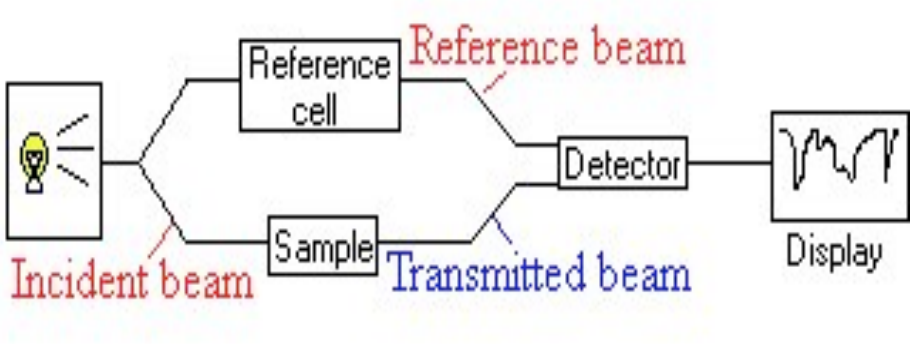
[3] Jablonski Diagram. Research\Jablonski Diagram.htm.

Infrared spectroscopy

Infrared spectroscopy deals with the infrared region of the electromagnetic spectrum. It is used to identify a compound and to investigate the composition of a sample. The infrared portion of the electromagnetic spectrum is divided into three regions which are near, mid and far infrared. The far-infrared lies in the region of 400-10 cm^{-1}, mid-infrared is from 4000-400 cm^{-1} and the near-infrared is from 14000-4000 cm^{-1} . Infrared spectroscopy works because chemical bonds have specific frequencies at which they vibrate corresponding to energy levels. Vibrational frequencies depend upon the strengths and geometry of the chemical bonds. The energy of a vibration is measured by its amplitude so the higher the vibrational energy, the larger the amplitude of the motion.

Only the bond that exhibits the change in dipole moment when it vibrates would be seen in IR. For instance, asymmetric stretches will not be seen in IR since there will be no change in dipole moment. In order to measure a sample, a beam of infrared light is passed through the sample, and the amount of energy absorbed at each wavelength is recorded. This can be done by using a Fourier transform instrument to measure all wavelengths at once. From this, a transmittance or absorbance spectrum would be plotted, which shows at which wavelengths the sample absorbs the IR, and allows an interpretation of which bonds are present. Schematic illustration of FT-IR is presented in figure four.

Figure #4



[4]

A Fourier Transform Infrared (FTIR) spectrometer obtains infrared spectra by collecting sample signal with an interferometer. It measures the frequencies of infrared at the same time. An FTIR spectrometer obtains the interferogram, carries out the Fourier Transform function, and displays the spectrum.

[4] Infrared Spectroscopy. http://sis.bris.ac.uk/~sd9319/spec/IR.htm

Chapter 4

Characteristics and properties of the protein and inhibitors studied

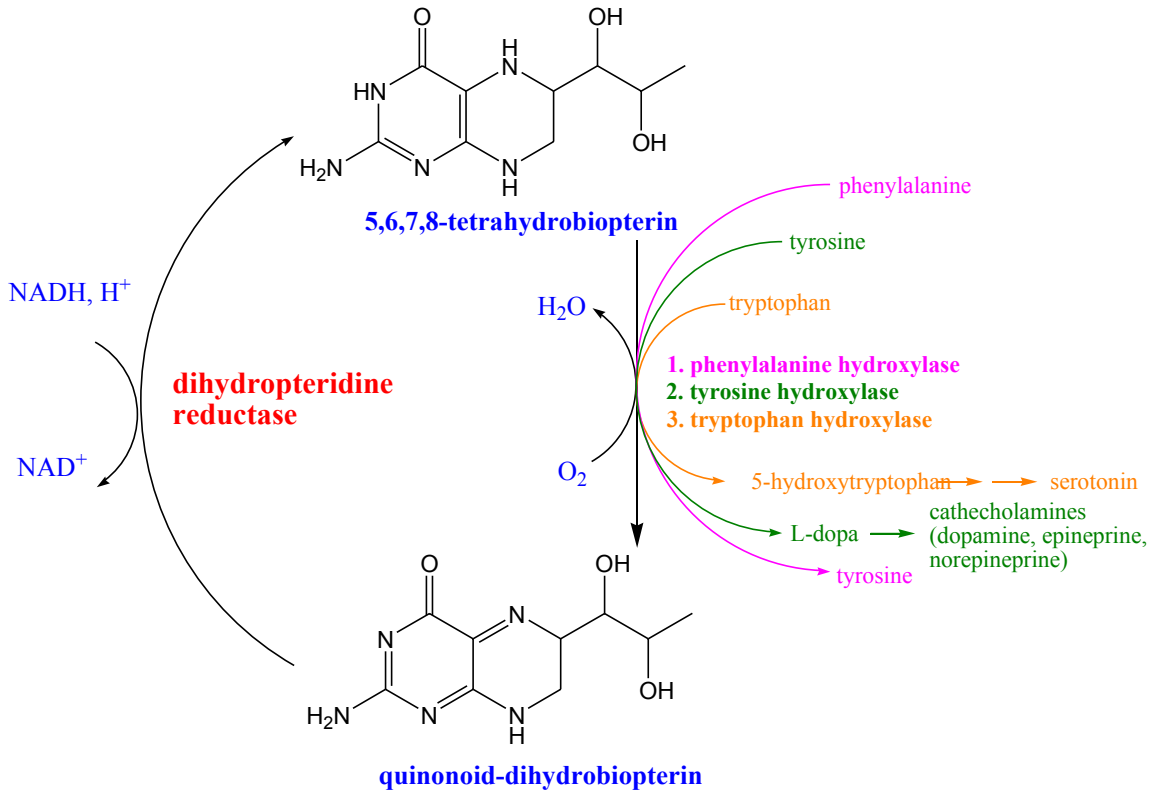
Dihydropteridine reductase (DHPR) is an enzyme involved in the recycling of tetrahydrobiopterin, which is the cofactor of the aromatic amino acid hydroxylases. Quite number of studies has been done on DHPR. The studies show that monomeric DHPR has a Km value of 7.8×10^{-5}M and the dimeric DHPR has a km value of 1.1×10^{-5} M. Studies also show the molecular weights of the monomeric and the dimeric enzymes are 26,000 and 52,000 respectively. [5]

We are currently working on the protein extraction from rat liver. The intention of this project was to extract the protein dyhydropteridine reductase (DHPR) so that it can be used in our research to govern the interaction within protein, ligand, and cofactor. Centrifugation, extraction, and dialysis methods were used mainly throughout this project. After dialysis, UV peak at 280 nm showed the indication of protein peak. However, this project is not completed yet.

[5] Higher Education Commission. http://www.hec.gov.pk/htmls/thesis/thesis_detail.asp?op=72

Figure #5

Dihydropteridine Reductase (DHPR)

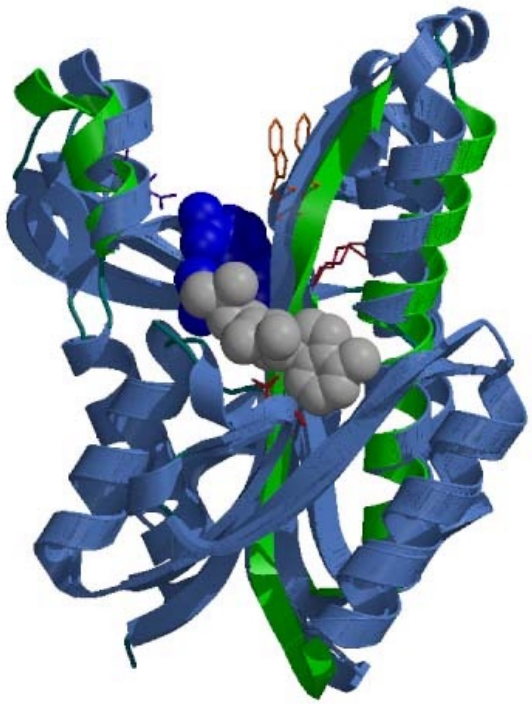


Dihydropteridine Reductase (DHPR) is shown in figure five and this cycle is showing us that why the dihydropteridine reductase (DHPR) enzyme is important. As you can see, DHPR recycles Tetrahydrobiopterin which is the important cofactor in the brain. Tetrahydrobiopterin is essential in the conversion of aromatic amino acids to precursors of neurotransmitters like serotin, which is important for the brain function. It is also essential in the conversion of phenylalanine to tyrosine by the enzyme

phenylalanine hydroxylase. Therefore, defect in the DHPR enzyme or the lack of phenylalanine hydroxylase causes phenylketonuria (PKU).

Figure #6

Crystal Structure of DHPR

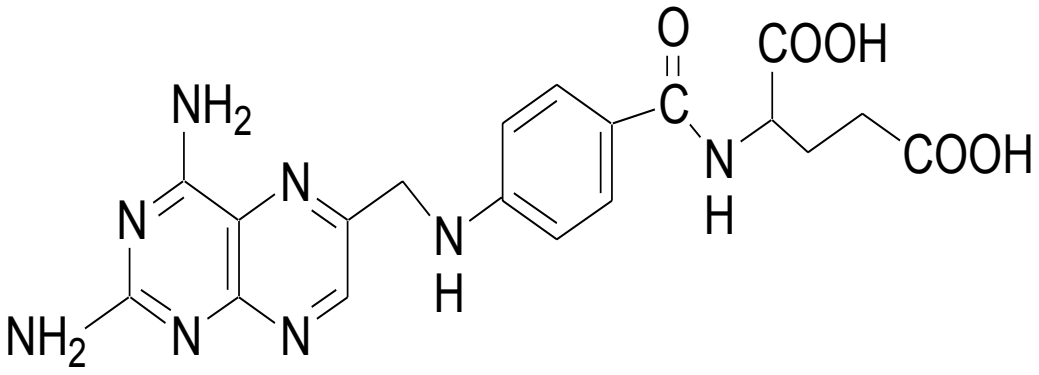


Apo form of x-ray crystal structure of the DHPR, which is superimposed to that of the NADH bound-form, is shown above. The blue ribbon represents the enzyme DHPR by itself while the one in green are the domains that shift upon NADH binding. This shift upon NADH binding triggered us to study the interaction between the protein structure DHPR, cofactor NADH, and the ligand folic acid. However, the

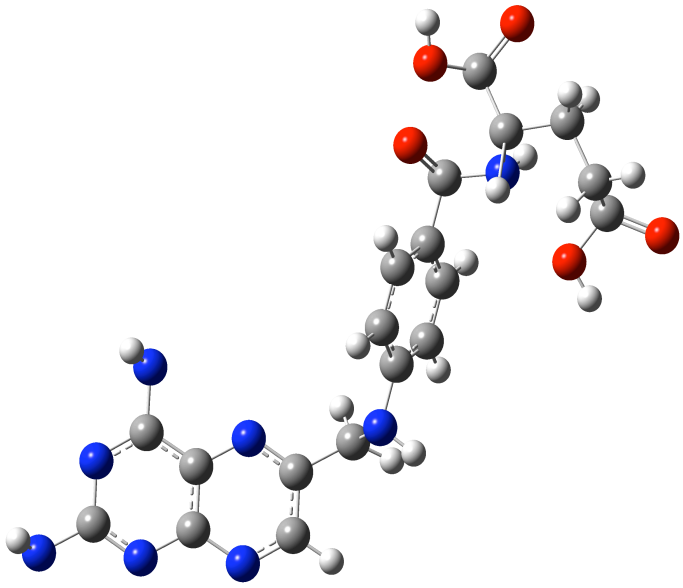
Crystal structure of the substrate bound enzyme has not been solved. Therefore, it would be our long-term goal to find the crystal structure of the ternary system involving enzyme/cofactor and the substrate.

Figure #7

Ligand Folic Acid (FA)



(a)

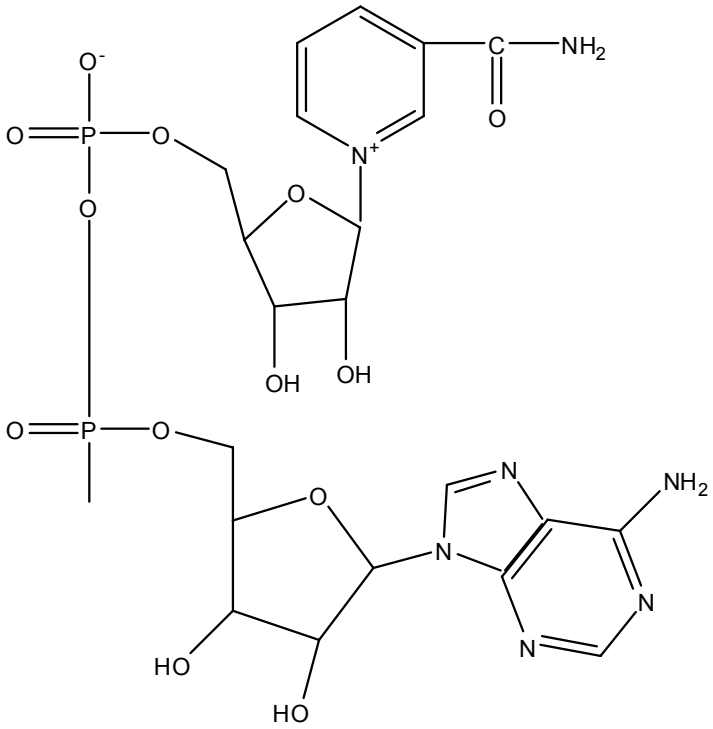


(b)

Structure of folic (a) acid and its 3D form (b) is shown on figure seven. As you can see, it has carboxylic and amine functional groups as well with two fused and one separate benzene rings. Folic acid resembles similar structure as the substrate dihydrobiopterin and thus would be important to study folic acid. Folic acid is involved in the formation of new cells and therefore essential for the normal growth and development of the fetus. Folic acid is active as a dietary component, but must be metabolized to the reduced dihydrofolate and tetrahydrofolate forms for biological activity. A deficiency of folic acid impairs DNA synthesis and cell division.

Figure #8

Structure of the cofactor NADH



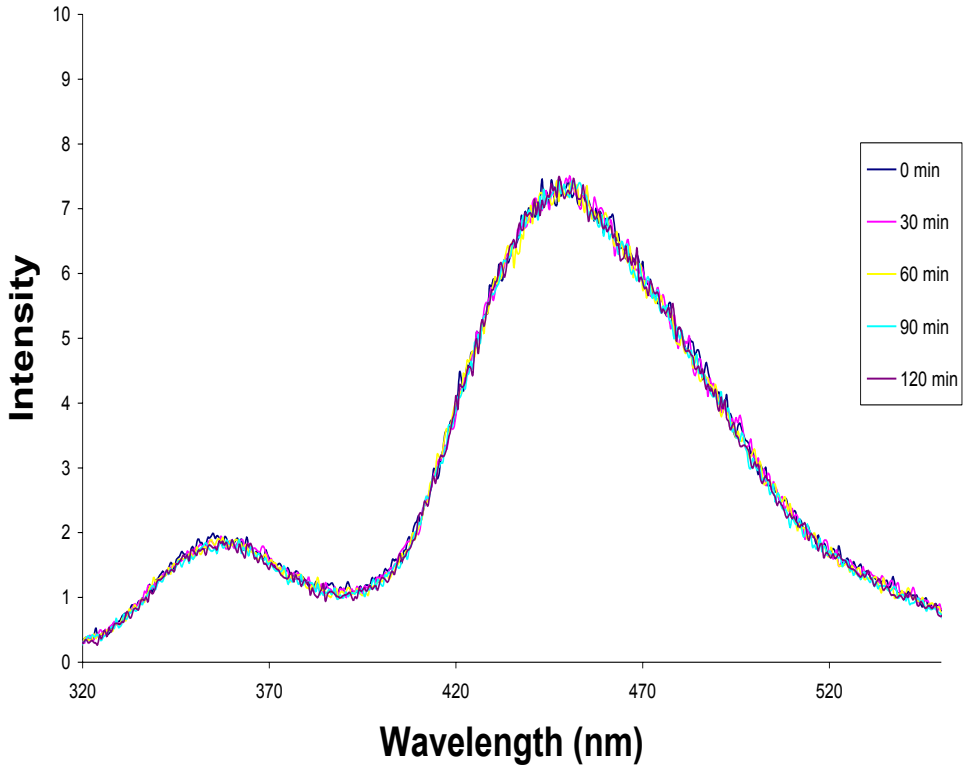
Beta-nicotinamide adenine dinucleotide (NADH) is shown in figure eighteen. NADH is an important cofactor that helps enzymes in the work they do throughout the body. Each of the body's cells contains an energy powerhouse called the mitochondria. A series of reactions called the energy electron transfer chain that takes place in the mitochondria to produce cellular energy in the form of ATP. NADH plays a key role in this process. It also participates in the production of L-dopa, which the body turns into the important neurotransmitter dopamine.

Chapter 5

Results from UV-VIS and Fluorescence

One of our objectives is to see whether our ligand folic acid is stable at various temperatures with respect to change in time because if our ligand is not stable then it would be hard for us to govern the protein-ligand interaction. Therefore, we ran the folic acid with a pH of 7.40 at 4, 25 and 44^{0}C for two hours and observed the folic acid to be stable (Figure 9a, b and c).

Figure #9a



The fluorescence spectrum of 50μM Folic Acid that was excited at 280 nm and ran at 4 ^{0}C displays two fixed peaks at 360 nm and 450 nm regardless of increase in time (Figure #9a). It demonstrates the stability of ligand folic acid at 4 ^{0}C since there are no changes in the intensity levels or in the peak positions.

Figure #9b

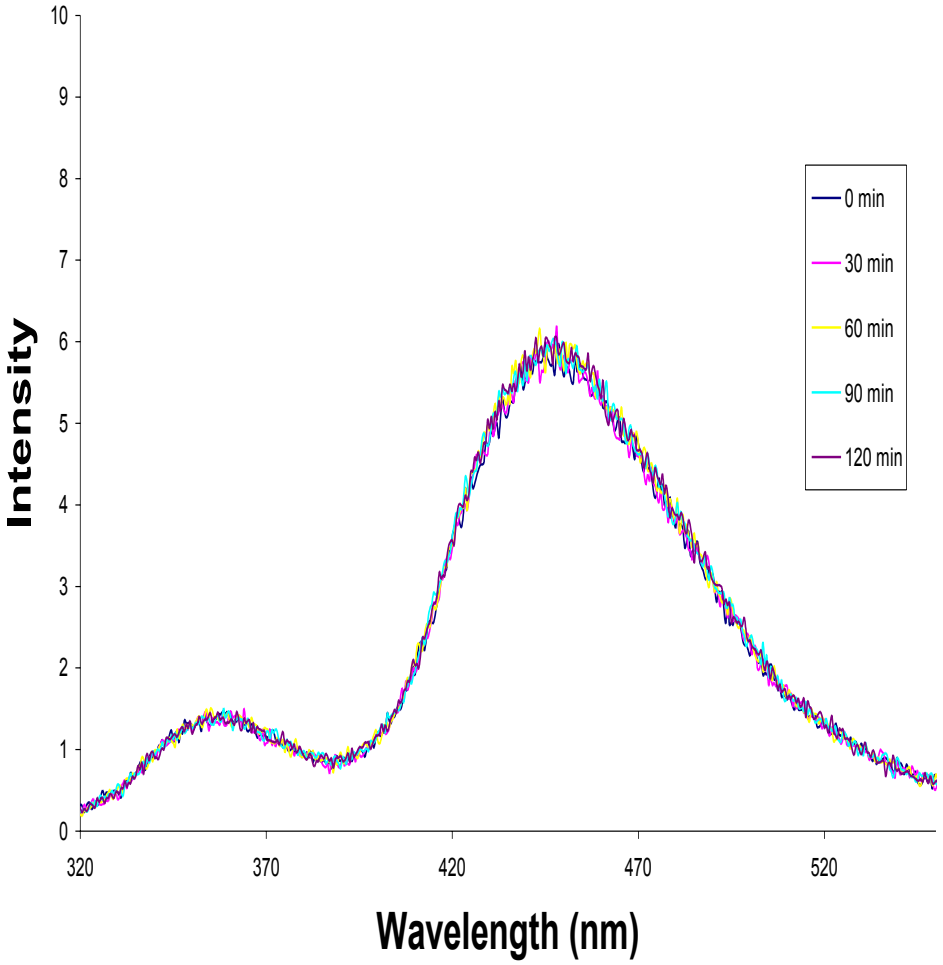


Figure nine (b) shows the fluorescence spectrum of 50μM Folic Acid which was excited at 280 nm and ran at 25 ^{0}C. It displays two fixed peaks at 360 nm and 450 nm regardless of increase in time and thus the folic acid is stable at 25 ^{0}C because there are no changes in the intensity levels or in the peak positions.

Figure #9c

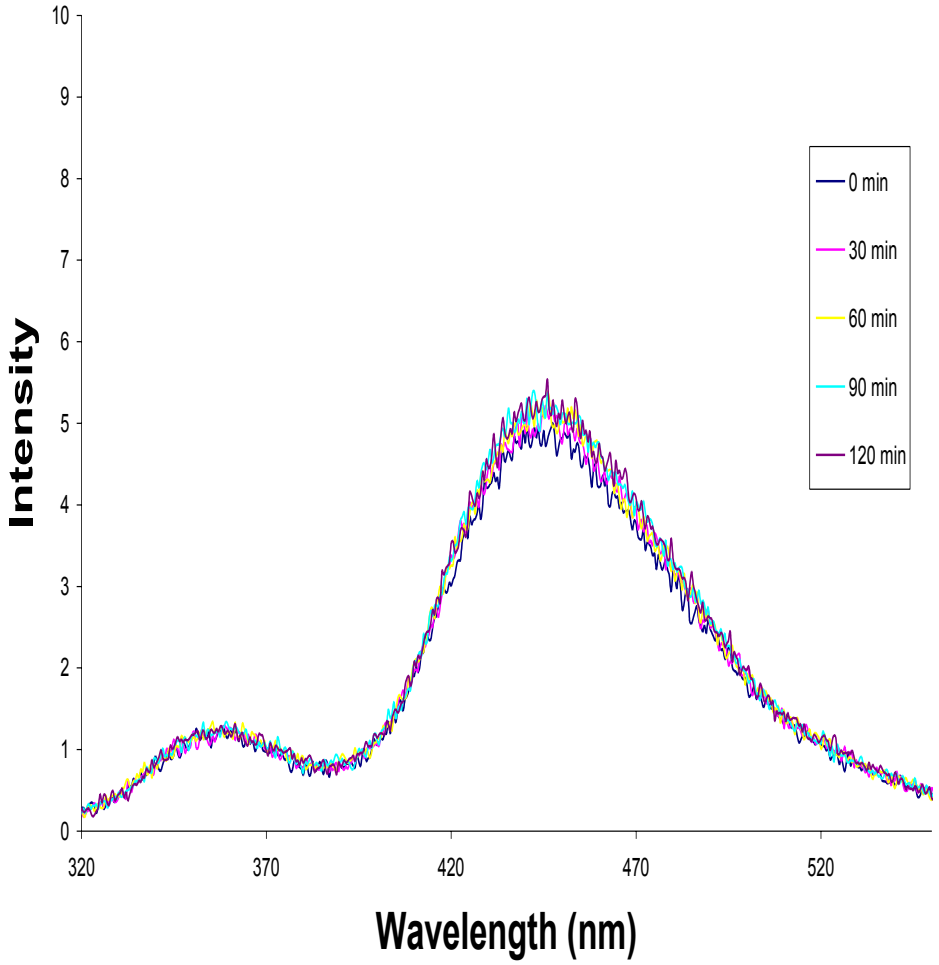


Figure nine (c) illustrates the fluorescence spectrum of 50μM Folic Acid which was excited at 280 nm and ran at 44 ^{0}C. It also displays two fixed peaks at 360 nm and 450 nm with no change in the intensity levels regardless of change in time. Therefore, ligand folic acid is also stable at 44 ^{0}C.

UV for folic acid

Absorbance of folic acid (3μM), binary (3μM folic acid + 0.7μM DHPR), and ternary (3μM folic acid + 0.7μM DHPR + 3μM NADH) system with the pH of 7.40 were ran in UV and peaks were observed at 280 and 360 nm. These UV spectra were presented in figure 10 a, b and c respectively.

Figure #10a

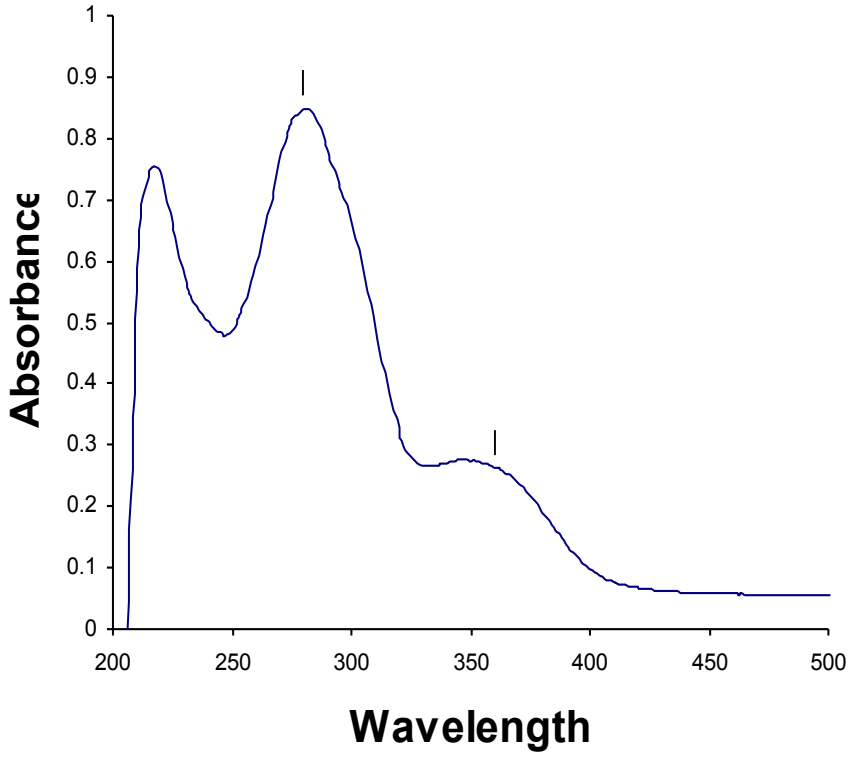
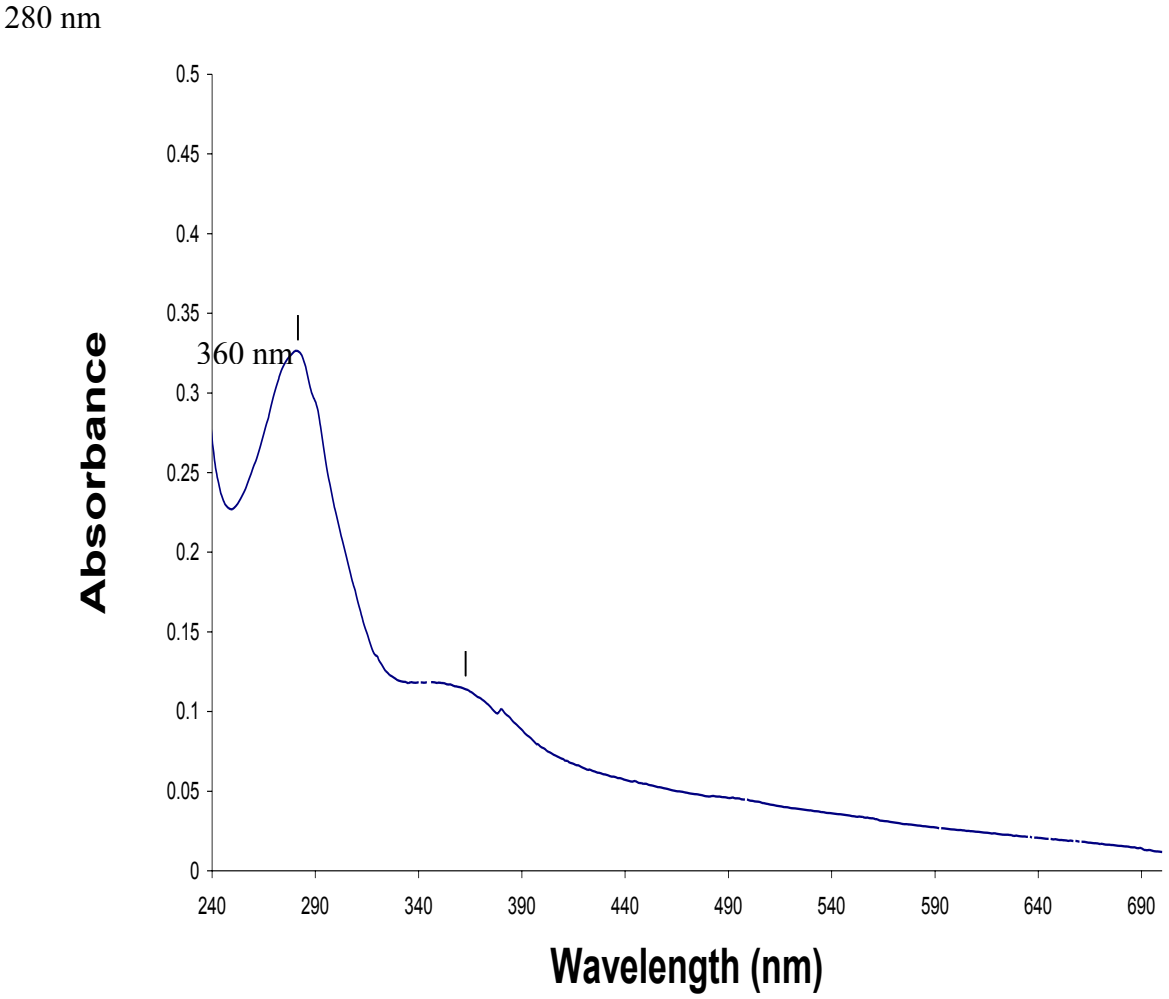


Figure ten (a) shows the peaks of 280nm and 360nm with the absorbance of 0.87 and 0.30 respectively.

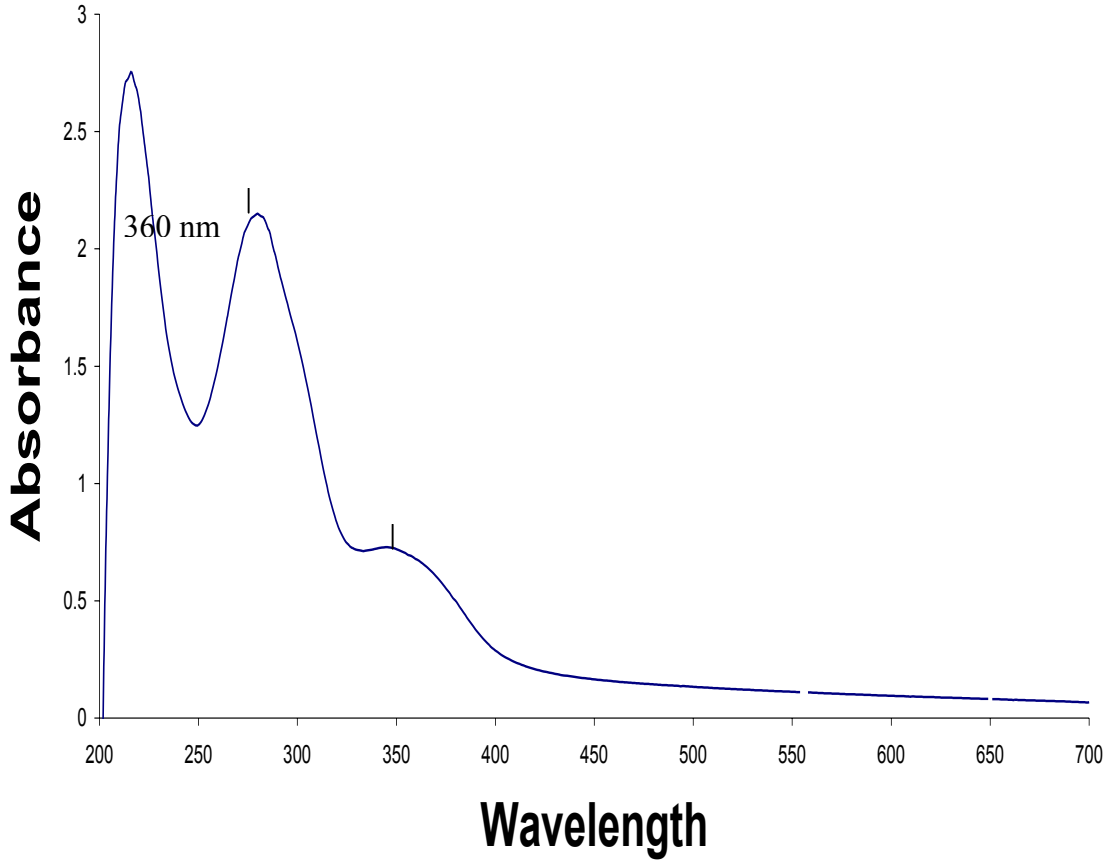
Figure #10 b



Peaks were revealed at 280 nm and 360 nm with the absorbance of 0.33 and 0.14 respectively (Figure #10 b). The absorbance of folic acid is lower when it's in binary than when it is by it self.

Figure #10 c

280 nm



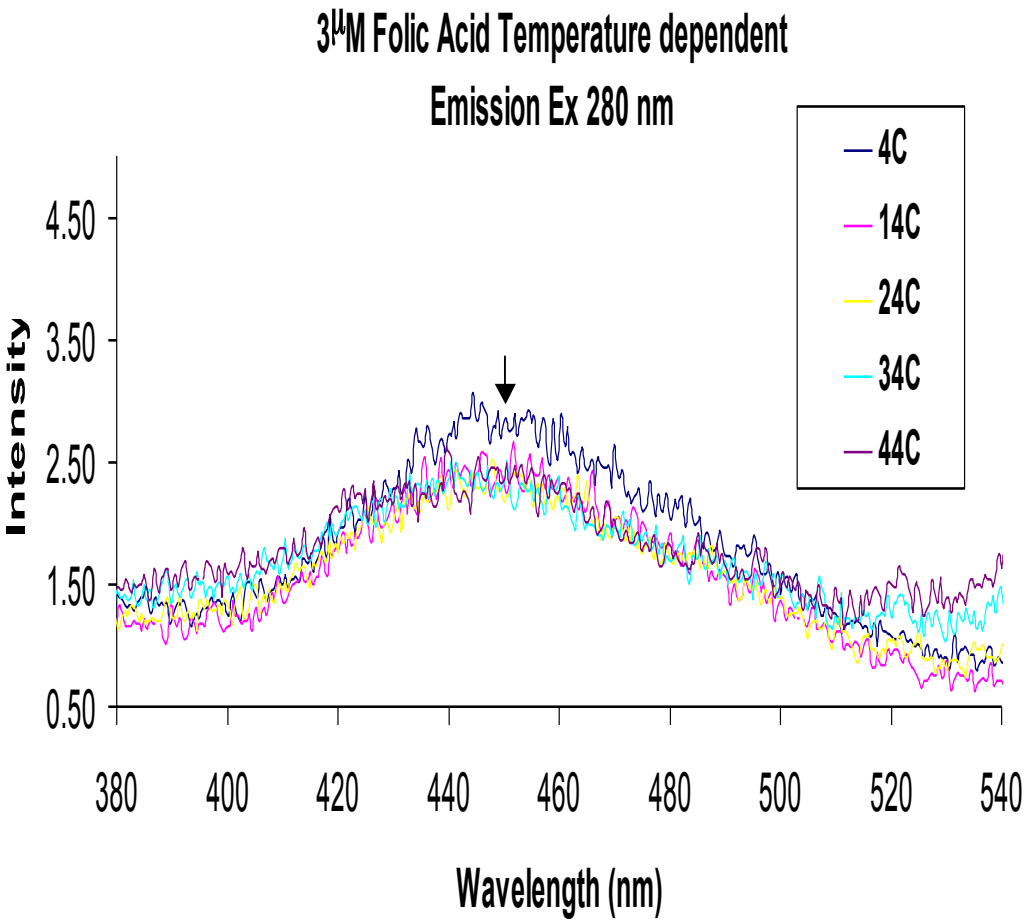
Peaks were observed at 280 nm and 360 nm with the absorbance of 0.25 and 0.85 respectively (Figure #10 c). The absorbance of folic acid is higher when it's in ternary than when it is by it self and in binary.

445 nm

Folic acid in fluorescence

Folic acid (3μM), binary (3μM folic acid + 0.7μM DHPR), and ternary (3μM folic acid + 0.7μM DHPR + 3μM NADH) system with the pH of 7.40 were excited at 280nm, 340nm and 360nm under the temperature dependent in fluorescence. These spectra were presented in 11 a, b, c, d, e, f, g, h, and i respectively. Then, Folic acid (3μM), binary (3μM folic acid + 0.7μM DHPR), and ternary (3μM folic acid + 0.7μM DHPR + 3μM NADH) system with the pH of 7.40 were emitted at 345 nm and 445nm and exhibited the peak at 280nm, 345 nm and 360 nm respectively. These excitation spectra are shown in figure 12a, b, c, d, e, and f. In all of the spectra, observed the decrease in intensity when the temperature was raised.

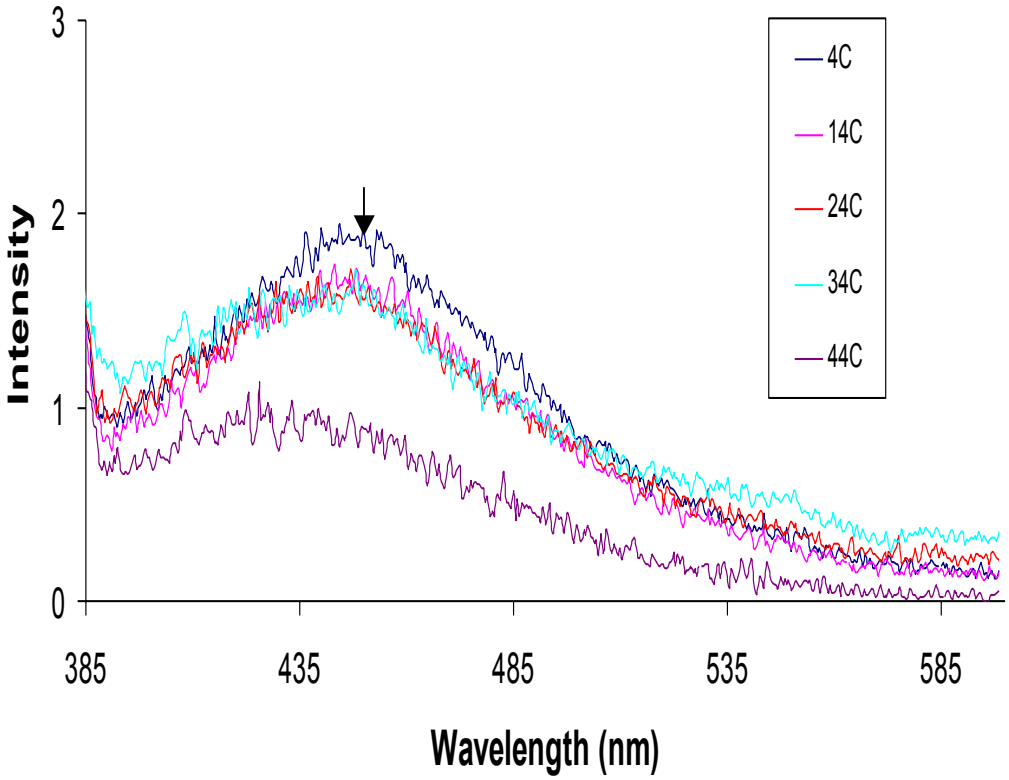
Figure #11a



445 nm

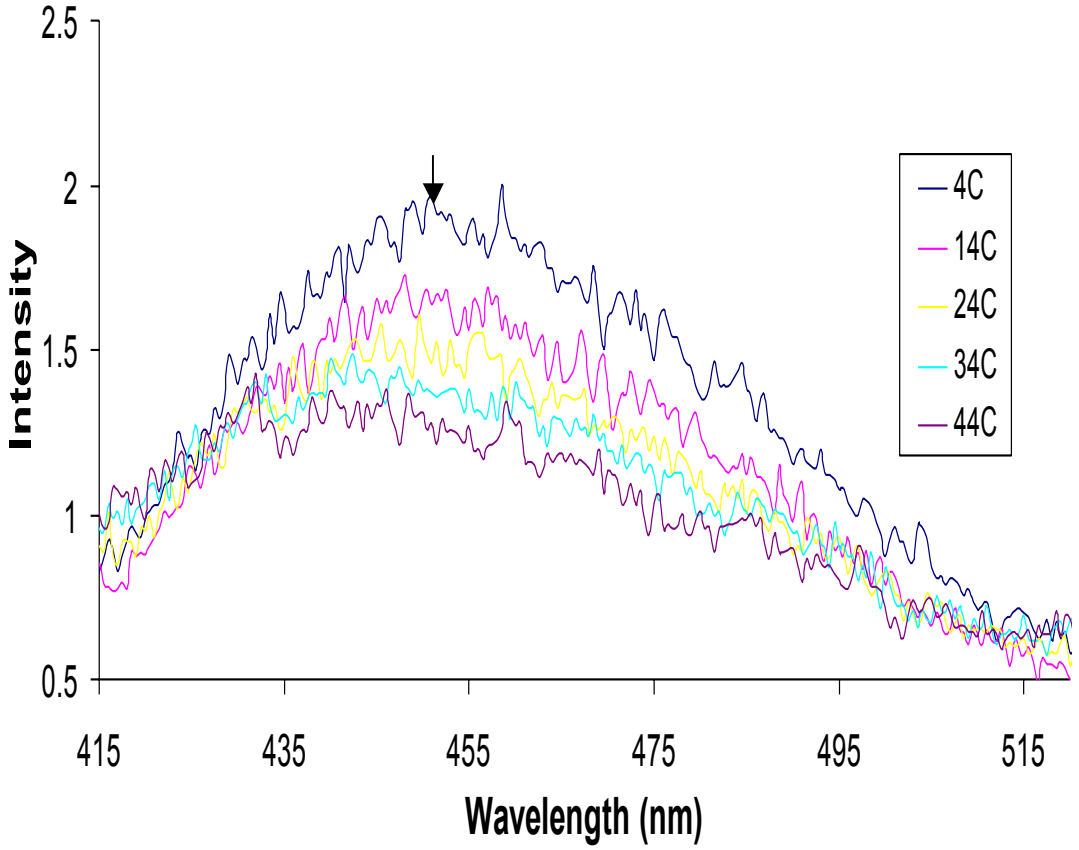
Figure 11 (a) illustrates the temperature dependent emission at 280nm for 3μM folic acid. Peak was observed at 445 nm with the intensity of 2.95. Decrease in intensity was observed with the increase in temperature.

Figure #11b



Folic acid (3μM) was excited at 340 nm under various temperatures (4, 14, 24, 34 and 44 ^{0}C) and the peak was observed at 445 nm with the intensity of 1.95 (Figure #11b). Intensity level was decreased when the temperature was increased.

Figure #11c



Folic acid (3μM) was excited at 360 nm under various temperatures (4, 14, 24, 34 and 44 ^{0}C) and the peak was observed at 445 nm with the intensity of 1.95 (Figure #11c). Intensity level was decreased when the temperature was increased.

345 nm

Figure #11 d

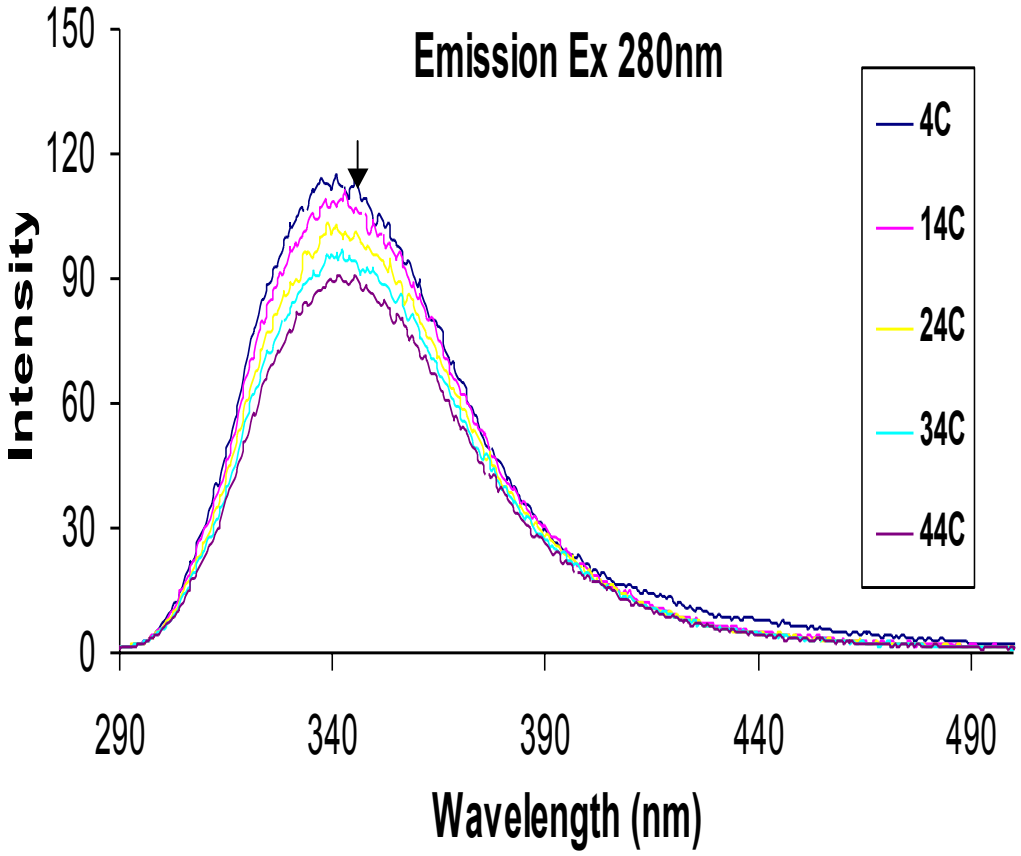


Figure eleven (d) illustrates the temperature dependent emission at 280nm for binary folic acid (3μM folic acid +0.7μM DHPR). Peak was observed at 445 nm with the intensity of 110. Decrease in intensity was observed with the increase in temperature.

445 nm

Figure #11e

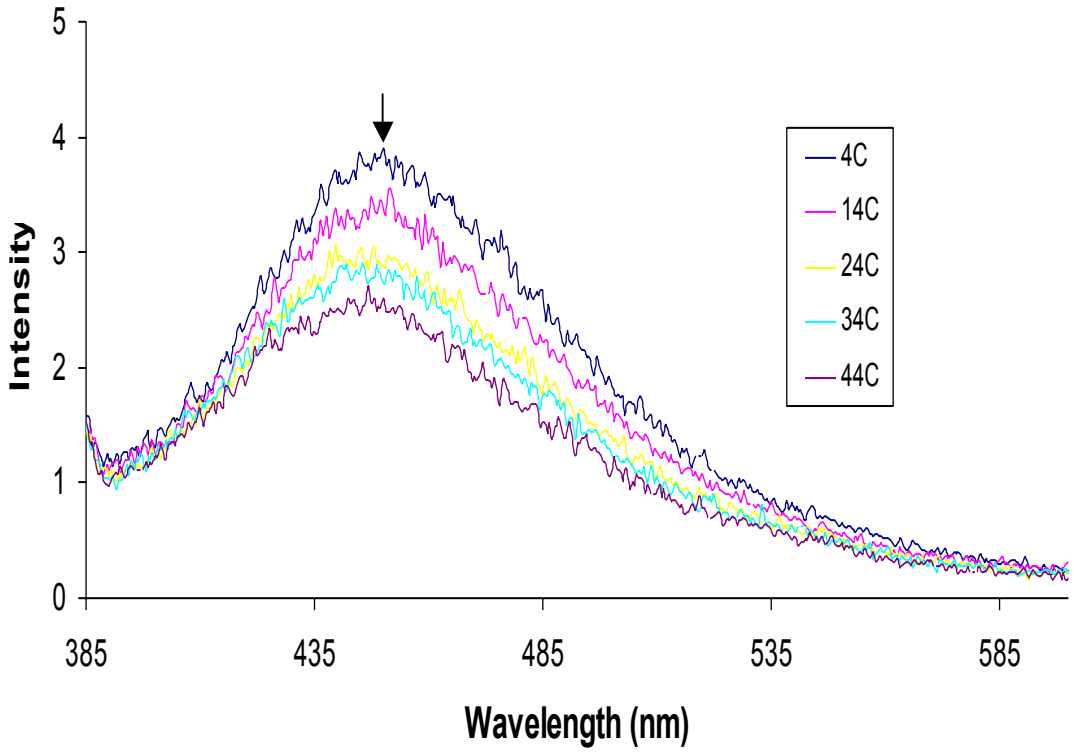


Figure eleven (e) shows the temperature dependent emission at 340nm for binary folic acid (3μM folic acid +0.7μM DHPR). Peak was observed at 445 nm with the intensity of 3.99. Decrease in intensity was observed with the increase in temperature.

445 nm

Figure #11 f

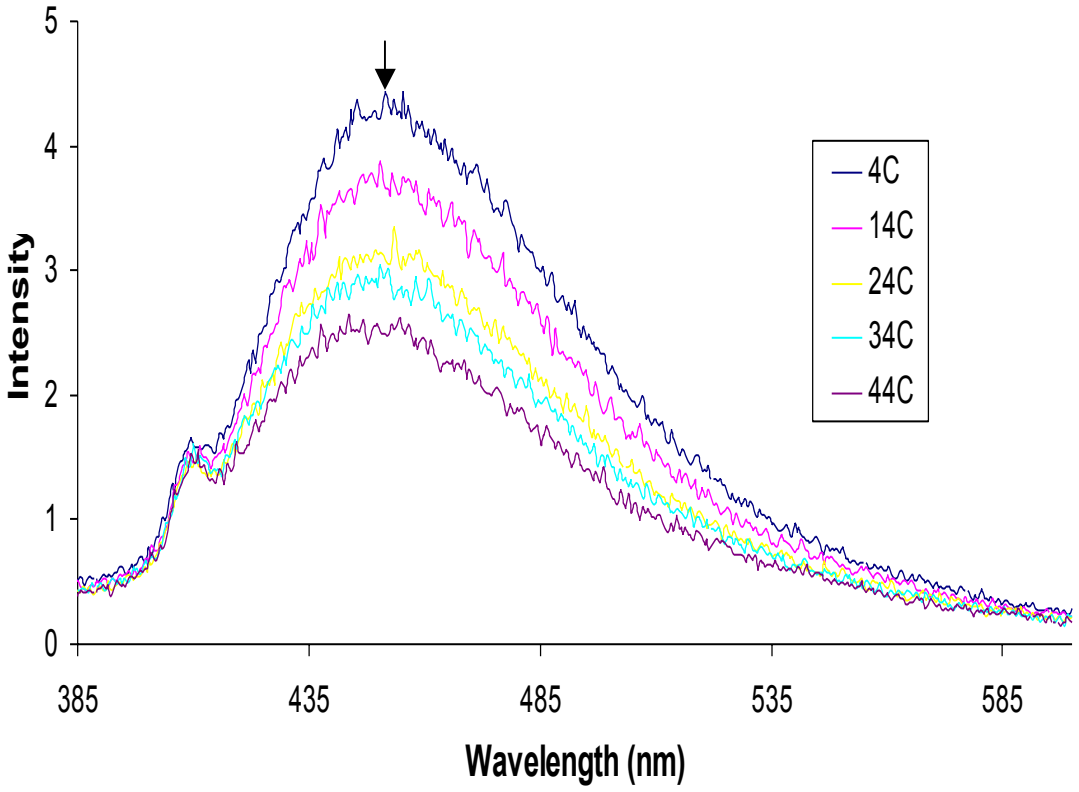


Figure eleven (f) illustrates the temperature dependent emission at 360 nm for binary folic acid (3μM folic acid +0.7μM DHPR). Peak was observed at 445 nm with the intensity of 4.35. Decrease in intensity was observed with the increase in temperature.

345 nm

Figure #11g

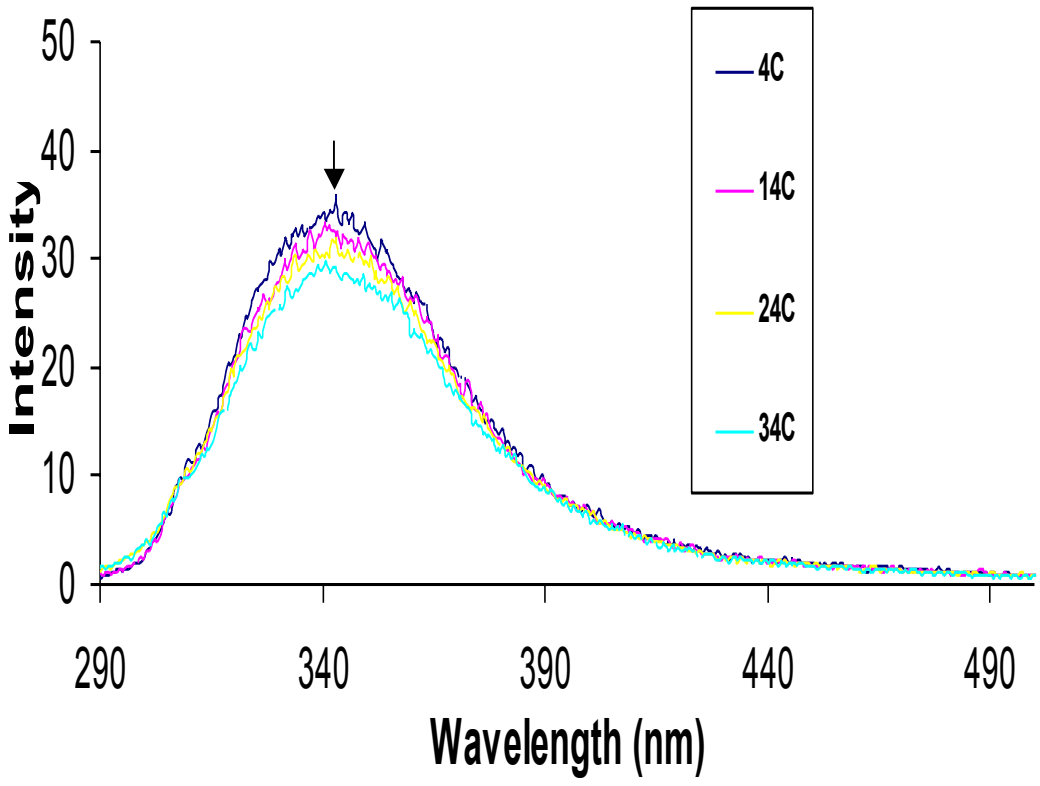
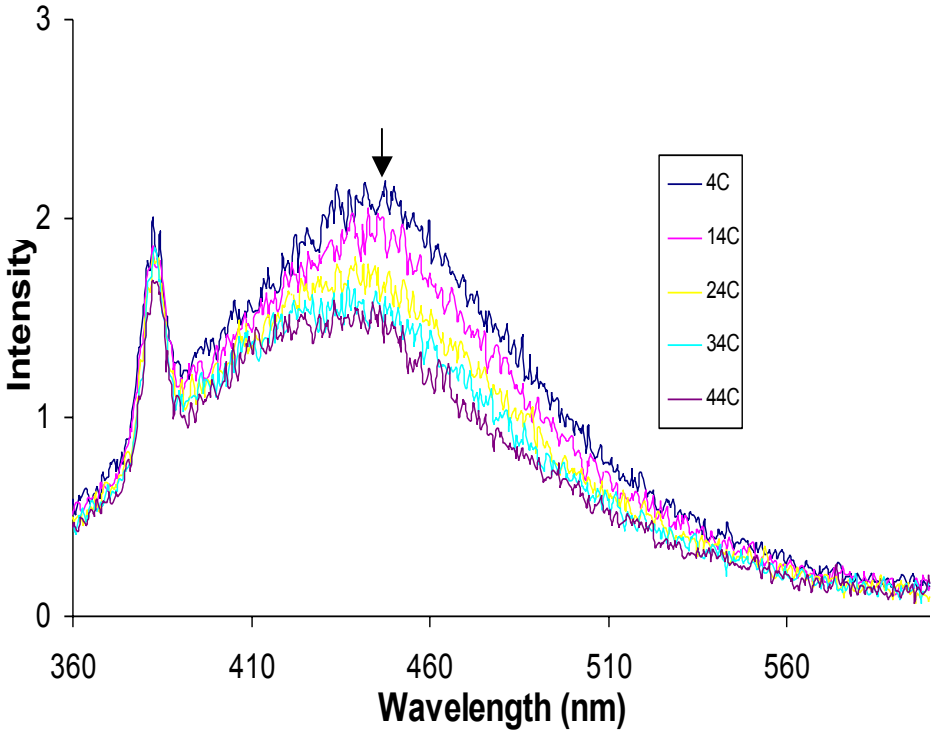


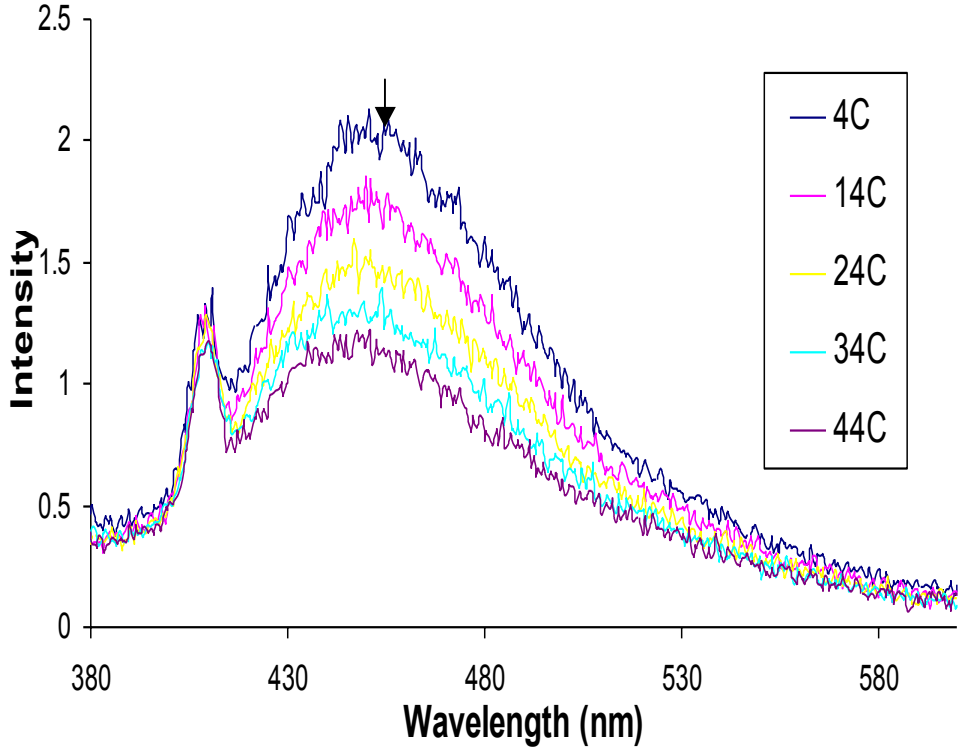
Figure eleven (g) shows the temperature dependent emission at 280nm for ternary folic acid (3μM folic acid +0.7μM DHPR + 3μM NADH). Peak was observed at 345 nm with the intensity of 34. Decrease in intensity was observed with the increase in temperature.

Figure #11 h



Ternary folic acid (3μM folic acid +0.7μM DHPR + 3μM NADH) was excited at 340 nm under various temperatures (4, 14, 24, 34 and 44 ^{0}C) and the peak was observed at 445 nm with the intensity of 2.15 (Figure #11 h). Intensity level was decreased when the temperature was increased.

Figure #11 i



Ternary folic acid (3μM folic acid +0.7μM DHPR + 3μM NADH) was excited at 360 nm under various temperatures (4, 14, 24, 34 and 44 ^{0}C) and the peak was observed at 445 nm with the intensity of 2.15 (Figure #11 i). Intensity level was decreased when the temperature was increased.

Figure #12a

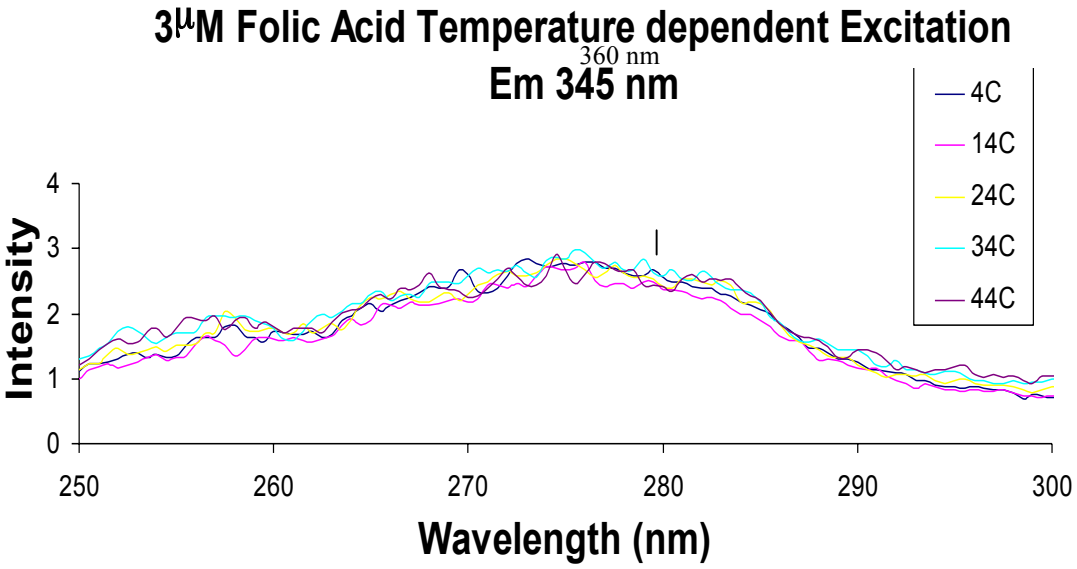
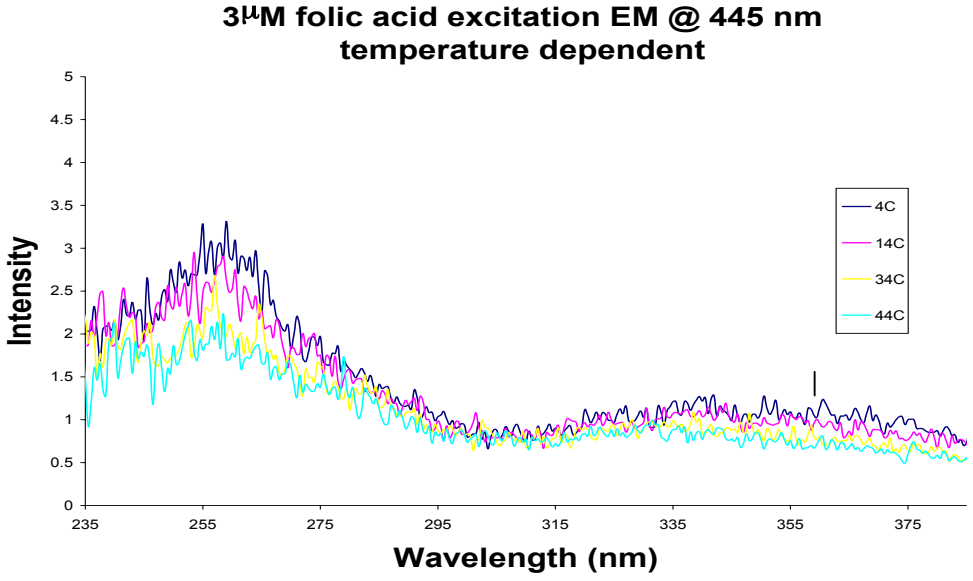
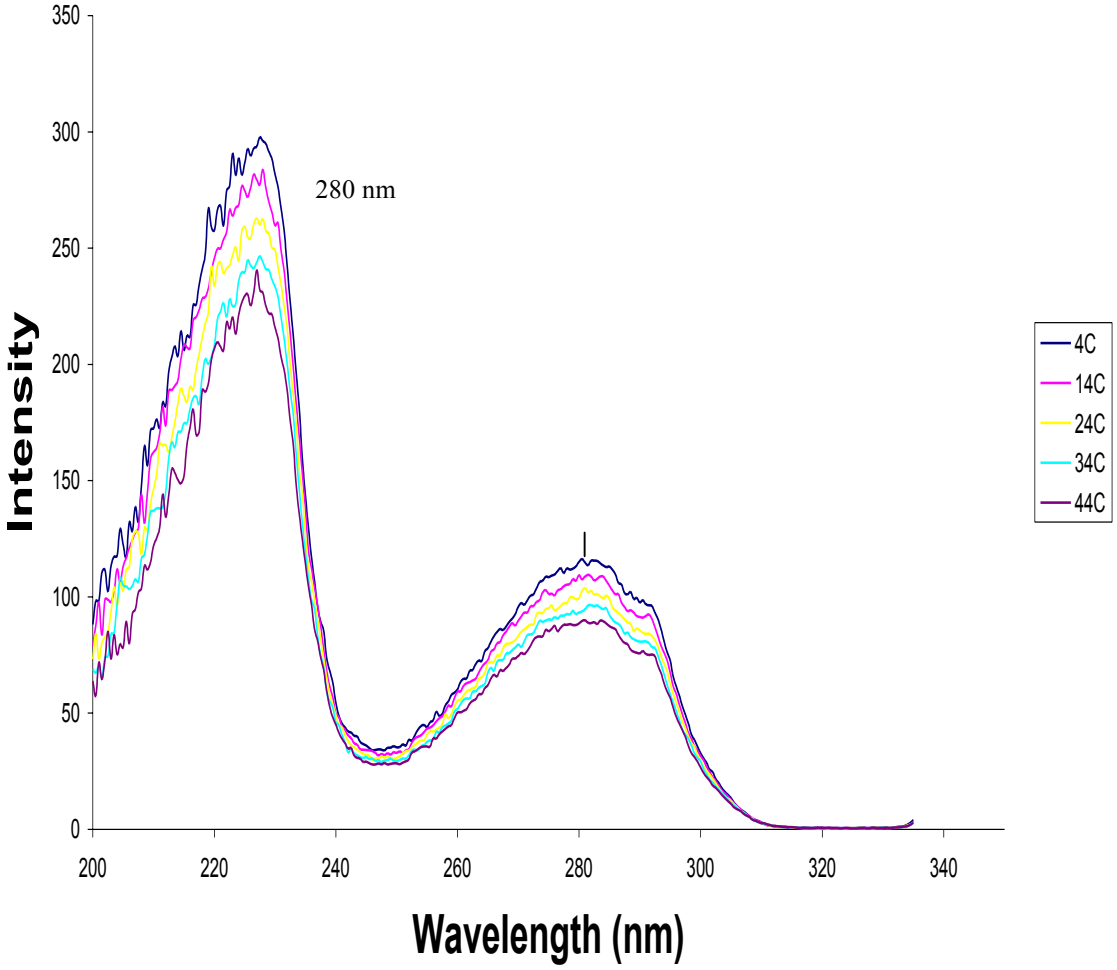


Figure #12 b



Folic acid (3μM) was emitted at 345 nm (Figure #12a) and at 445 nm (Figure #12 b) and has a peak at 280 nm and at 360 nm respectively.

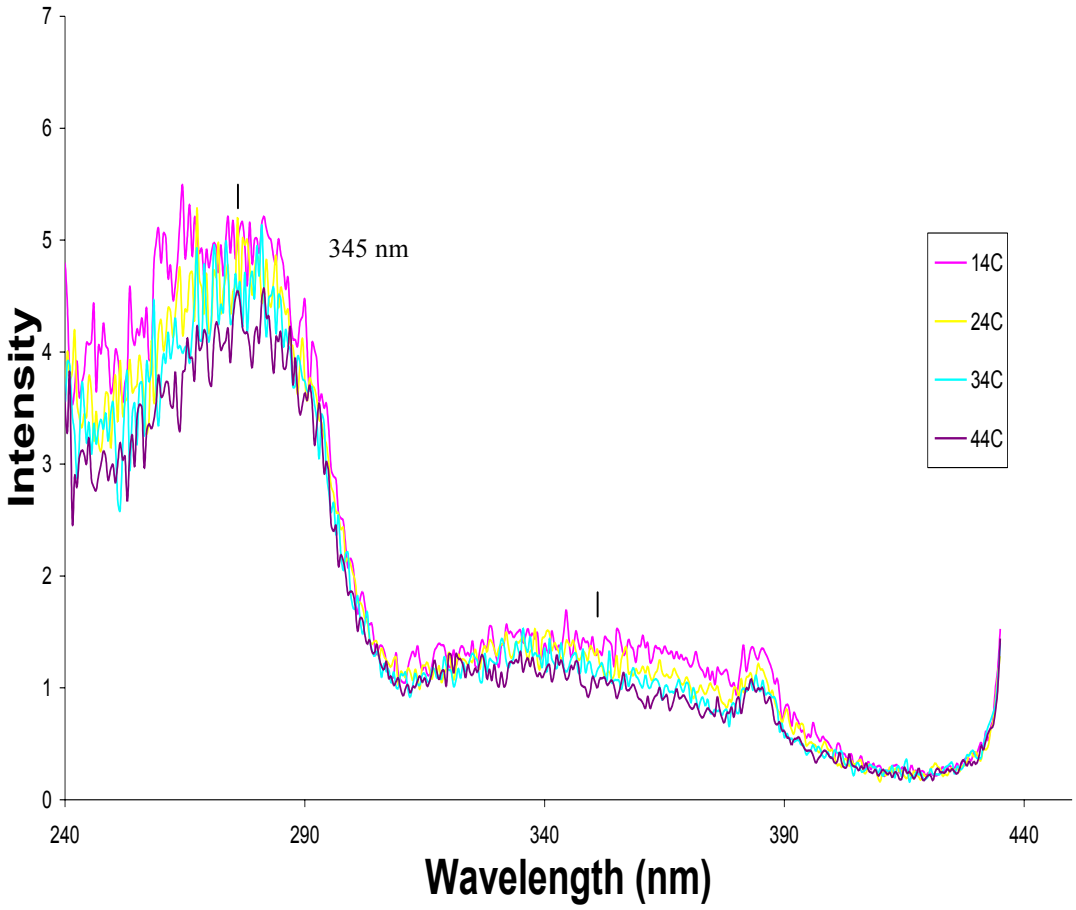
Figure #12 c



Binary folic acid (3μM folic acid +0.7μM DHPR) temperature dependent was emitted at 345 nm and reveals a peak at 280 nm.

Figure #12 d

280 nm



Binary folic acid (3μM folic acid +0.7μM DHPR) temperature dependent was emitted at 445 nm and reveals peaks at 280 nm and 345 nm.

345 nm

Figure #12 e

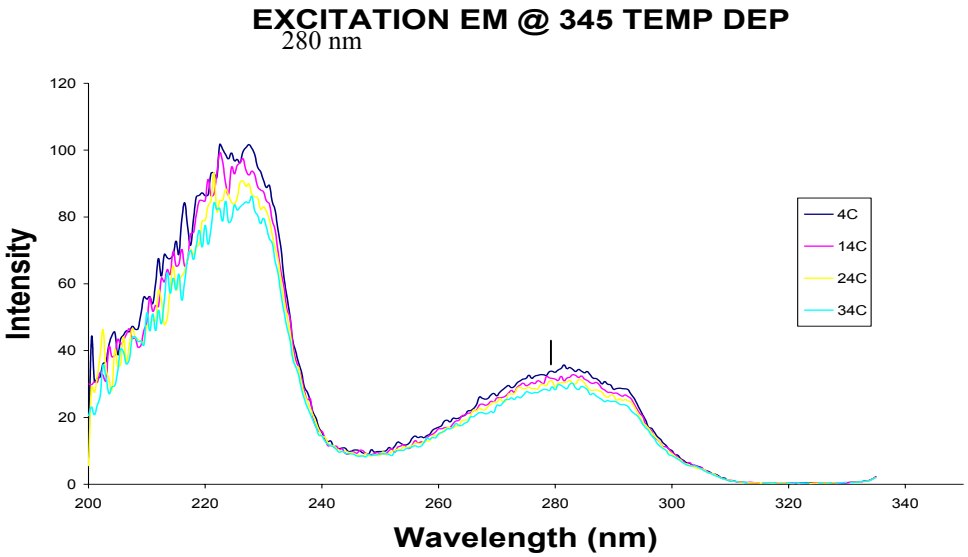
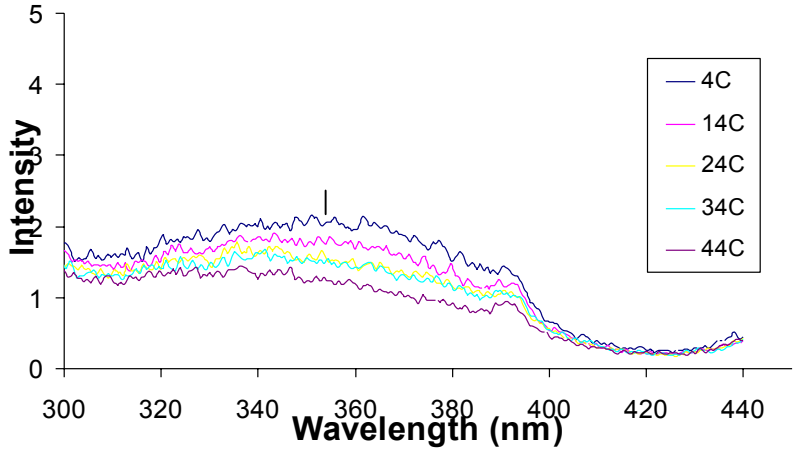


Figure #12 f



Ternary folic acid (3μM folic acid +0.7μM DHPR + 3μM NADH) was emitted at 345 nm (Figure #12 e) and at 445 nm (Figure #12 f) and has a peak at 280 nm and at 345 nm respectively.

Chapter 6

Folic acid in IR

Various concentrations of folic acid (100, 50, 25, 10, 5 and 1mM) with the pH of 7.40 were prepared and ran in IR in order to identify their IR bands. Then, calculated simulations were performed to folic acid using Gaussian 03 program to compare and contrast with the experimental result. Folic acid was protonated with the help of Gaussian 03 program and then subtracted from calculated simulation of folic acid to observe the changes in IR bands. These IR spectra are shown in figure 13a, b, c, d, e, f, g, and h.

Figure #13a

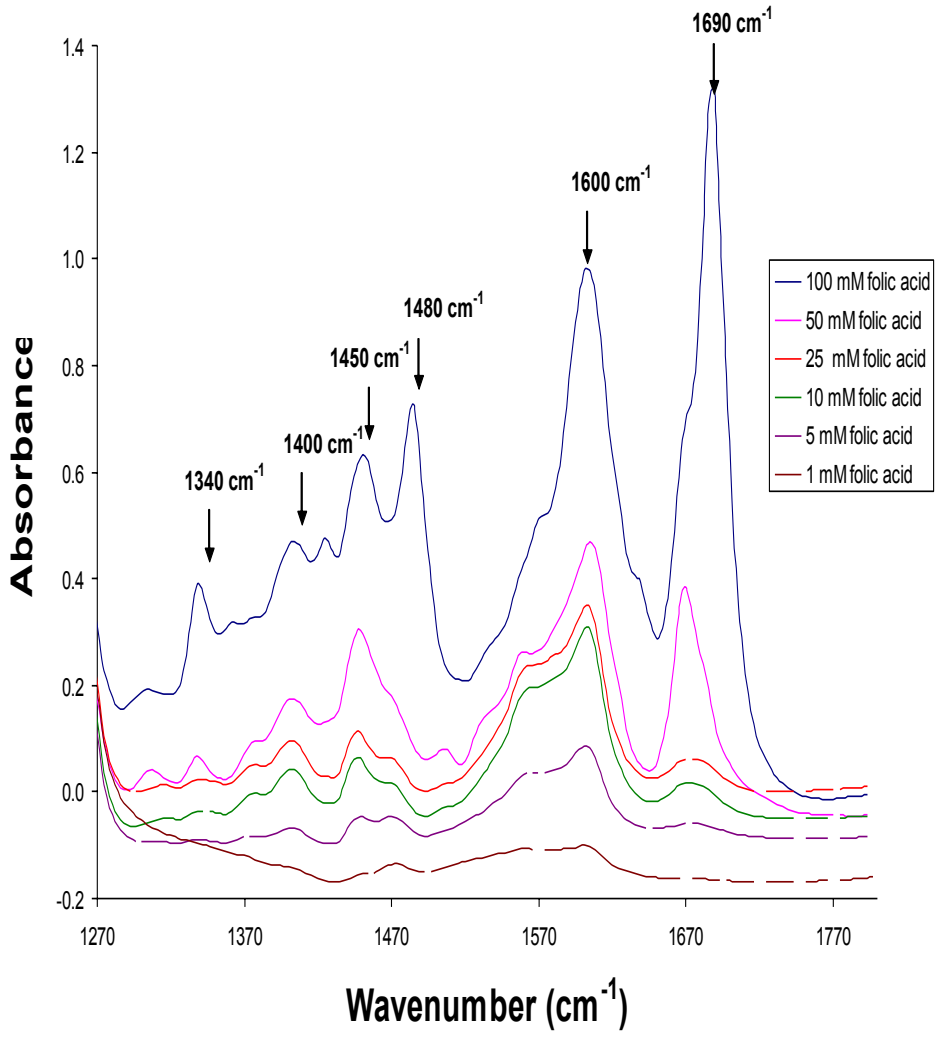
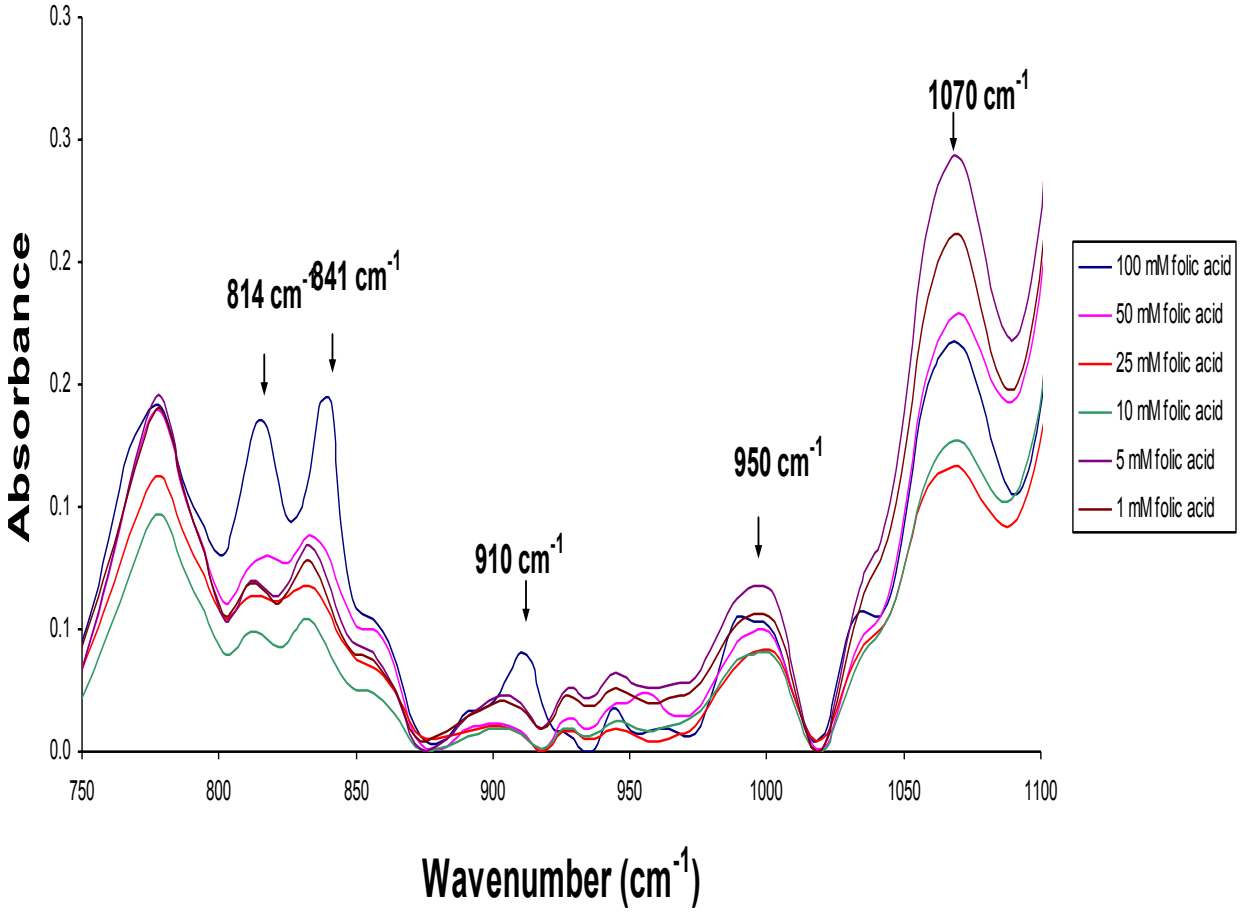


Figure #13b



Various concentrations of folic acid were run in infrared and IR peaks were identified. Observed a higher IR signal for higher concentration of folic acid. Regions of 1800-1200 cm^{-1} and 1100-750 cm^{-1} are shown in figures 13(a) and (b) respectively.

950 cm^{-1} 980 cm^{-1}

Calculated simulations for folic acid

Figure #13c

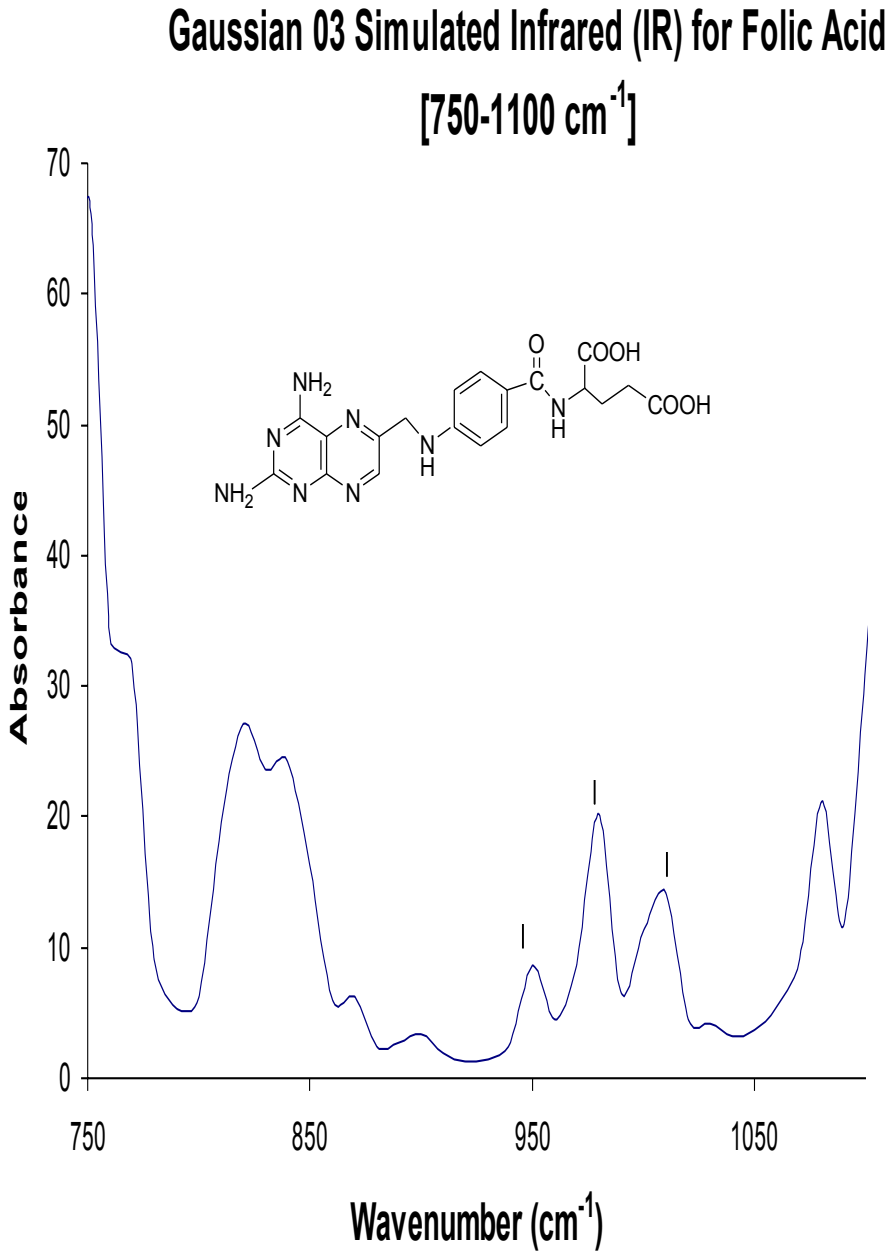
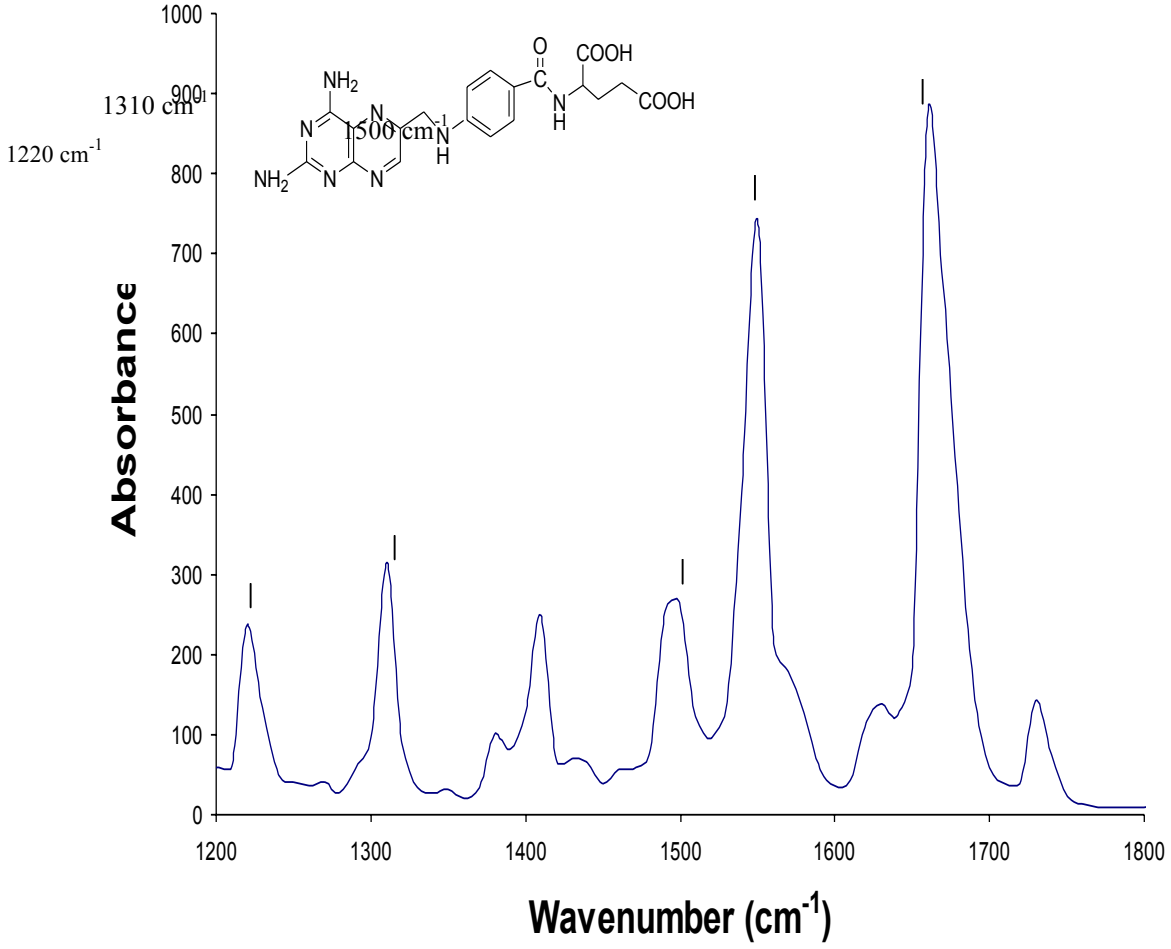


Figure #13d

1550 cm-1

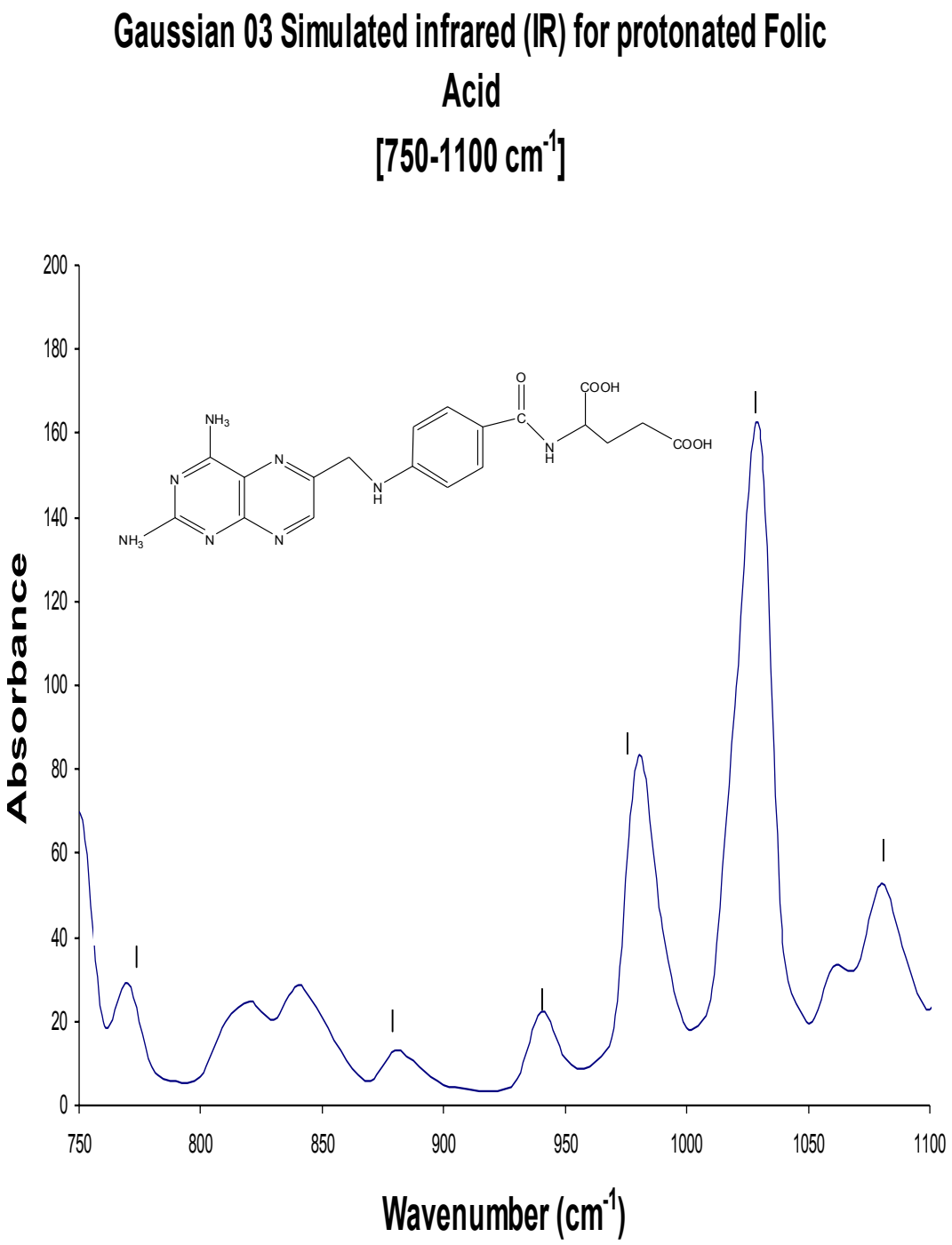
Gaussian 03 Simulated Infrared (IR) for Folic Acid
Region of [1200-1800 cm-1]



Calculated simulations for folic acid was run in Gaussian 03 and the IR spectra was obtained for the regions of 1800-1200 cm^{-1} and 1100-750 cm^{-1} were presented in figures (d) and (c) respectively.

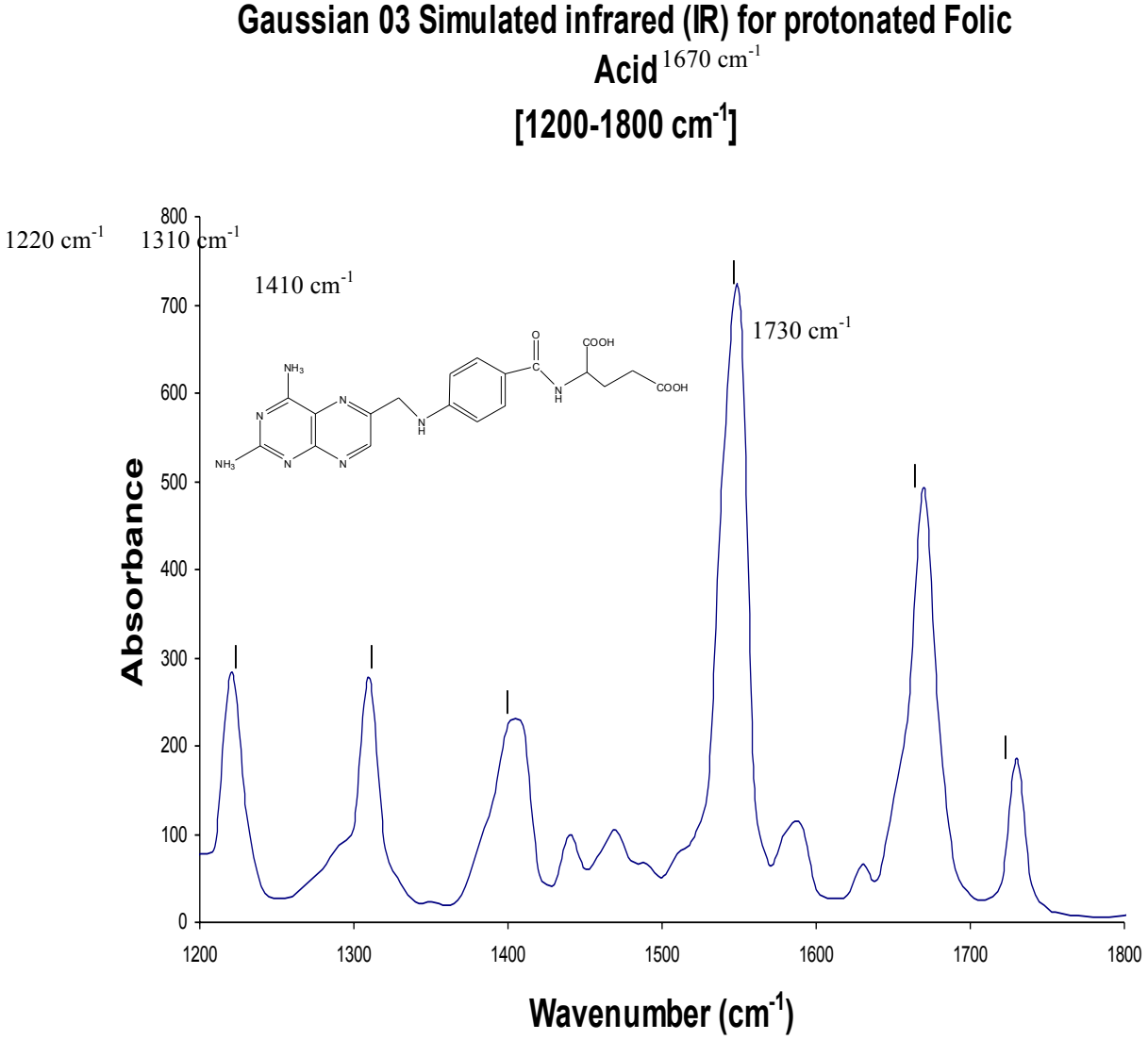
770 cm^{-1} 880 cm^{-1} 940 cm^{-1} 980 cm 1030 cm

Figure #13e



1550 cm^{-1}

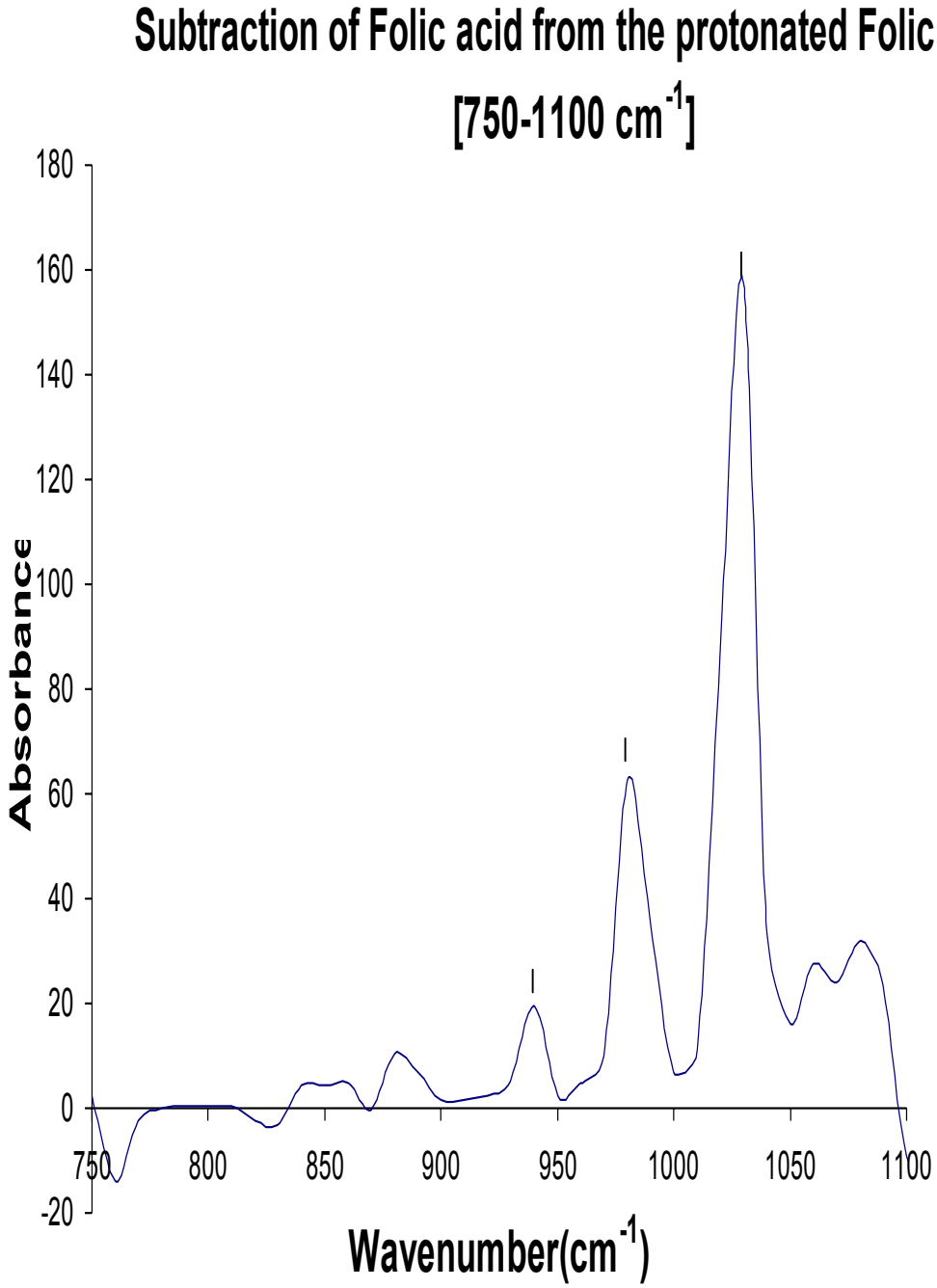
Figure #13f



Calculated simulations for protonated folic acid was run in Gaussian 03 and the IR spectra was obtained for the regions of 1800-1200 cm^{-1} (Figure #13f) and 1100-750 cm^{-1} (Figure #13e) and IR peaks were identified.

940 cm⁻¹ 980 cm⁻¹ 1030 cm⁻¹

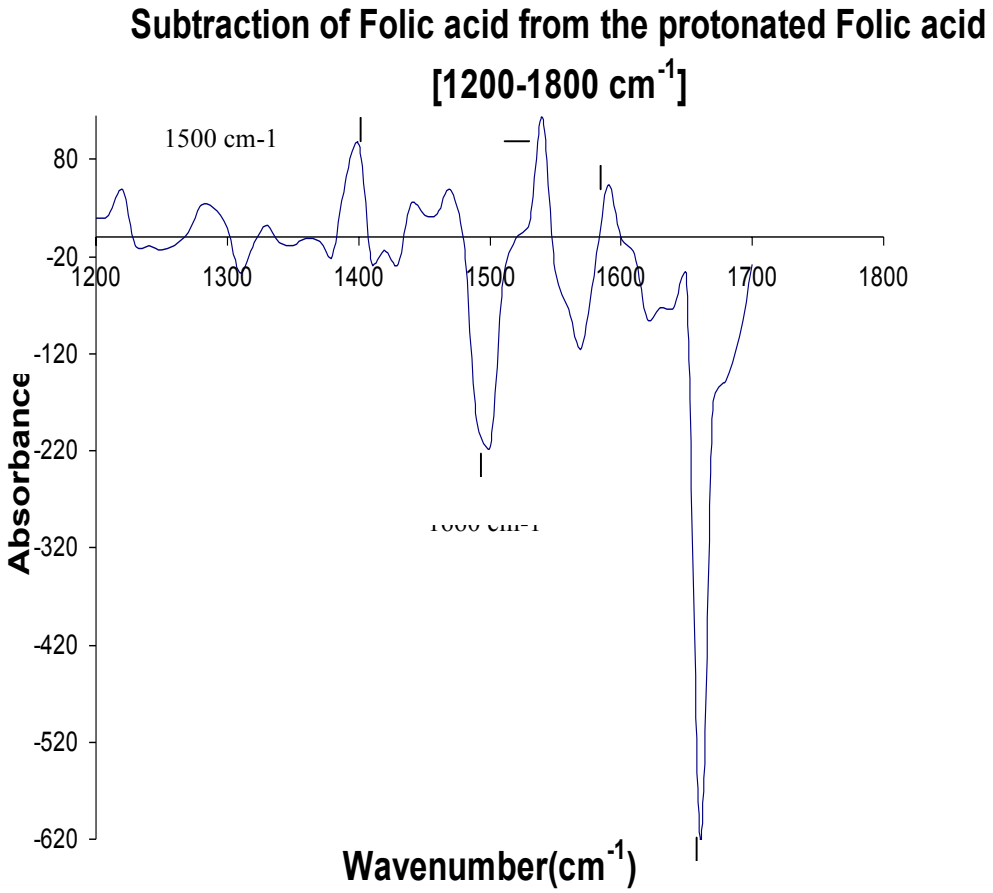
Figure# 13g



1400 cm-1

1540 cm-1 1590 cm-1

Figure #13h



Resultant (protonated – folic acid) calculated simulations for folic acid with the regions of 1800-1200 cm^{-1} and 1100-750 cm^{-1} and identified IR peaks were shown in figure 13 (h) and (g) respectively.

Chapter 7

Conclusions

Our main goal of this research is to study the interaction between protein structure Dihydropteridine Reductase, cofactor Nicotinamide Adenine Dinucleotide (NADH), and the inhibitor folic acid and thus we studied them using spectroscopy technique. Our excitation spectra revealed the peak at 280 nm and at 360nm as it was in the UV spectra. As a result, 280 nm and the 360 nm peaks are real because according to the theory if the UV peaks are seen in the excitation spectra then those peaks are real. Our fluorescence spectroscopy spectra show the intensity difference within folic acid (3μM) by it self, binary (3μM folic acid + 0.7μM DHPR), and ternary (3μM folic acid + 0.7μM DHPR + 3μM NADH) systems. These intensity changes exhibit the interaction of protein-ligand interaction. At the same time, we also observed the decrease in intensity level as the temperature was raised and indicating the inverse relationship with the fluorescence intensity and the temperature.

We ran the folic acid in IR to study the nature of the interaction. Various concentrations of folic acid were prepared and ran in IR as it was in figures 13a and b. Then, calculated simulations for folic acid was performed in Gaussian 03 to compare the IR data with the experimental data. Similar IR bands were observed in both calculated simulation and in experimental spectra. However, the minor differences in these IR bands are due to the fact that Gaussian calculations were done in vacuum while the experiments were carried out in tris HCl solvent. At the same time, folic acid structure was protonated in one and three position using Gaussian and subtracted from non-protonated folic acid to see which bonds are moving upon protein binding (figures 13 g and h).

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