

EXPERIMENT 4

IRON IN BIOLOGY: IRON CONTENT IN FERRITIN, THE IRON STORAGE PROTEIN

Purpose:

- 1) To illustrate the use of spectrophotometry in trace analysis of iron.
- 2) To understand how iron is stored in living cells.

Pre-Lab Preparation:

- 1) Read Appendix C (The Use of the Spectrophotometer), Appendix E (Graphing Experimental Data), and Appendix G (Linear Regression Computer Program).
- 2) The Pre-Lab Assignment for Experiment 4 on the course homepage.

I. INTRODUCTION

A. Ferritin Protein

Iron is an essential element for all living organisms. Although iron is also the most abundant transition metal in the earth's crust, its chemical properties hinder its availability to plants and animals. Iron is readily oxidized to Fe(III) by exposure to the atmosphere. Fe(III) is not normally soluble at pH 7, because it precipitates as iron hydroxide. The solubility product of $\text{Fe}(\text{OH})_3$ is 10^{-39} , which means that at physiological pH 7, the $[\text{Fe}(\text{III})]$ is 10^{-18} M. In most organisms, the intracellular concentration of iron is between 10^{-5} and 10^{-8} M. Thus animals and plants need mechanisms to both solubilize and take up Fe(III) from their environment, and to store Fe(III) in a soluble form for later use.

In this experiment, you will examine the chemical properties of ferritin, one of the most common iron-storage proteins in plants and animals. The ferritin protein resembles a spherical shell. It is made up of 24 protein subunits (Figure 1). When the protein shell is empty and contains no iron, it is referred to as apoferritin and has a molecular weight of 474,000. This spherical space can be filled up with as many as 4500 iron atoms, which is equivalent to an iron concentration of ~ 0.25 M. Iron is stored within the soluble ferritin protein as an Fe(III) oxo-hydroxide structure that contains some phosphate and is similar to rust. The overall stoichiometry of the iron core is $[\text{FeO}(\text{OH})]_8[\text{FeO}(\text{H}_2\text{PO}_4)]_8$, which has a formula weight of 879.7 g/mole or 97.7 g/mole of iron.

There are also engineering and material science applications of the ferritin proteins. They have been used to prepare highly uniform metal oxide particles that have interesting electronic and structural properties. These particles have been used in the nanoscale engineering of semiconductors and new composite materials.

Iron is added and removed from the ferritin core through channels that are formed where the 24 protein subunits join together (Figures 1 and 2). There are two types of channels, 3-fold and 4-fold. See the computer tutorial for a more detailed description of the channels and the structure of ferritin.

In the first part of this experiment you will use dilute acid to break apart the ferritin protein and liberate the enclosed iron. The Fe(III) will be reduced using dihydroxyfumarate (DHF) so the iron released can be quantified by spectrophotometry of the Fe(II) complexed with a ligand, ferrozine²⁻ (Figure 3). In the second part of this experiment, you will use dihydroxyfumarate (Figure 4) as a reducing agent to remove iron from the intact protein, via the protein channels. This reduction process mimics the biochemistry of iron transport out of ferritin that occurs when, for example, a new hemoglobin molecule has to be synthesized.

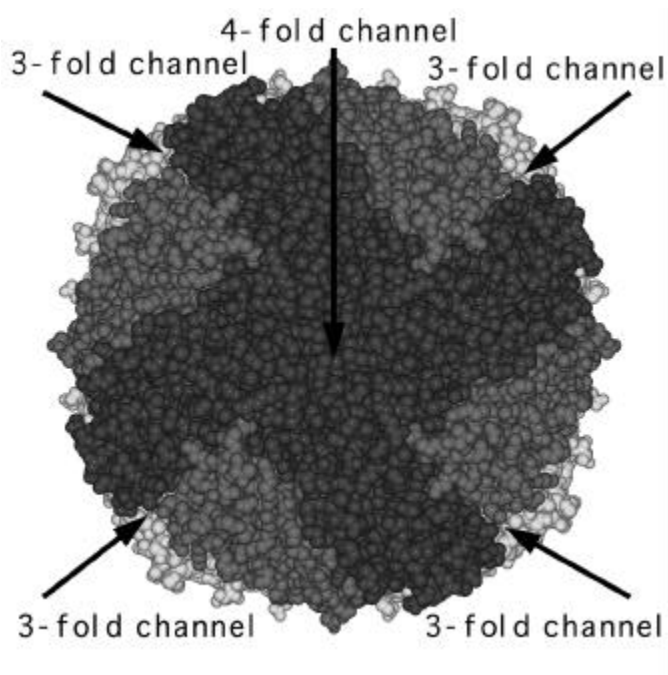


Figure 1.

The approximately spherical exterior of the ferritin protein is formed by 24 cylindrical protein chains packed together, as shown to the left. The arrows point to channels through which molecules can enter and leave the ferritin protein. The 3-fold (hydrophilic) channels are largely hidden in this view. A better view of the channels and the structure as a whole can be observed in the computer module.

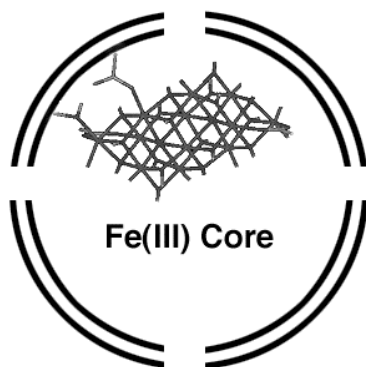


Figure 2.

A cross-section of the protein is shown to the left. The breaks in the double lines represent the protein channels. The geometric pattern inside the protein represents the microcrystalline mineral structure of $[\text{FeO}(\text{OH})]_8[\text{FeO}(\text{H}_2\text{PO}_4)]$ that forms when Fe(II) enters the protein sphere, is oxidized by oxygen to Fe(III), and reacts with water and phosphate.

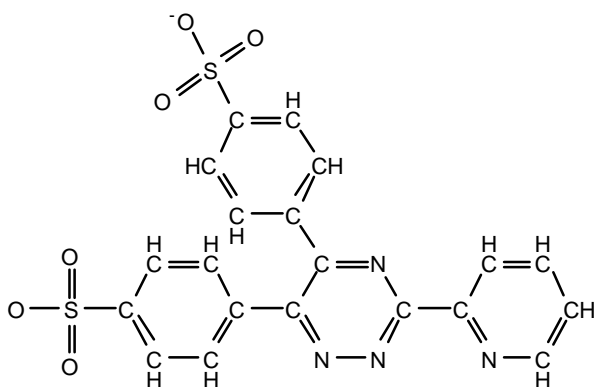


Figure 3. Ferrozine²⁻, an organic ligand that binds Fe(II) to form a purple complex.

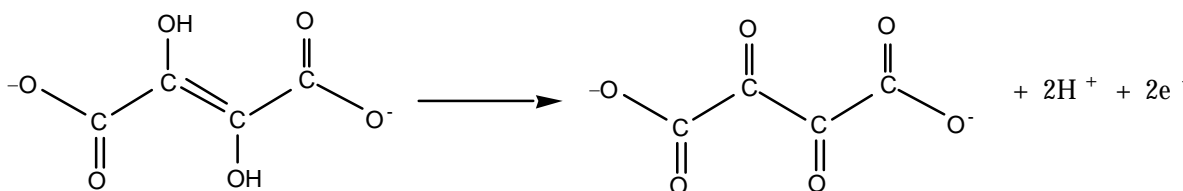


Figure 4.

The oxidation half reaction for dihydroxyfumarate.

B. Analysis of Ferritin

Each pair of students will quantify the amount of iron present in the ferritin protein by trace analysis of the iron using a spectrophotometer. In this lab you will be given a known amount of ferritin in solution (i.e., you will know its concentration in mg/ml of ferritin). It is reasonable to assume that all of the iron is released from the protein by acid treatment. You will break apart the protein and quantify the amount of iron released using the chromophoric complexing agent, ferrozine²⁻. You will determine the number of moles of iron that were present per mole of apoferritin (ferritin without any bound iron). From that figure, you can determine the number of iron atoms per molecule of ferritin and the percentage of iron binding sites that were occupied in your sample of ferritin (see section I.E. for data analysis).

C. Kinetic Study of Ferritin

A distinguishing kinetic feature of enzyme-catalyzed reactions is that these reactions often show saturation, which means that above a certain concentration, the addition of more substrate does not increase the rate. This saturation is not generally seen for simple chemical reactions. Nearly all enzyme-catalyzed reactions show a first-order dependence of rate on substrate concentration at very low concentrations, but instead of increasing indefinitely as the concentration increases, the rate approaches a limit at which there is no dependence of rate on concentration and the reaction becomes zero-order with respect to the substrate. This behavior is illustrated in Figure 5. This saturation is generally observed when the substrate is in large excess (10:1 or greater) over the enzyme. Under these conditions, the substrate molecules compete for a limited number of binding sites

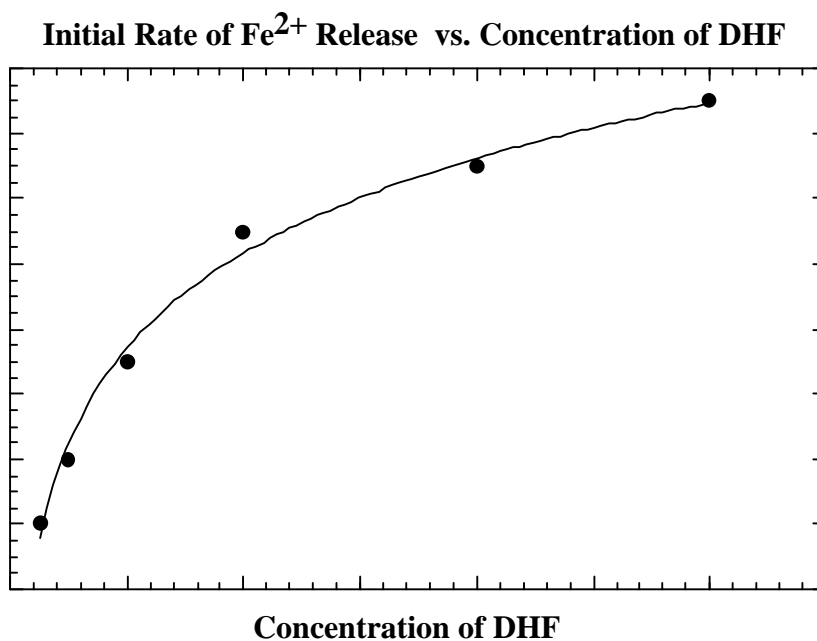


Figure 5.

Example of a saturation plot for release of iron from ferritin in the presence of dihydroxyfumarate.

on the enzyme; thus, at very high substrate concentrations, all the available binding sites are occupied, and the rate of reaction no longer increases with increasing substrate concentration.

In this experiment you will study dependence of rate of Fe^{2+} release on DHF concentration. Although saturation of the rate of iron release occurs at increasing concentrations of the reducing agent, dihydroxyfumarate (DHF), you will conduct your experiments at DHF concentrations that fall in the initial linear portion of the curve in Figure 5. Ferritin is not, strictly speaking, an enzyme, but it does share some properties with enzymes. (You will be studying the rates of enzymes in the computer exercise for Chem 152, Experiment 6.) When enzyme-catalyzed reactions are studied under conditions where the rate is dependent on substrate concentration, it is possible to learn a great deal about the binding mechanism, including how tightly substrates are bound to the enzyme, the actual rate of transformation, and how tightly substrate analogs (inhibitors) are bound to the enzyme. Detailed understanding of an enzyme mechanism is very important in the design of enzyme inhibitors, which can act as therapeutic drugs. You will not examine the mechanism of ferritin iron-release at that level of detail, but it is important to learn what distinguishes an enzyme-catalyzed reaction from simple chemical reactions.

You will determine the amount of iron released over time by intact ferritin in the presence of the reducing agent. You will use the same analysis used for determining the total iron present, but in the procedure you will not digest the protein with acid, so the protein remains intact. The reductant enters the protein through the channels, and the iron leaves through the channels of the intact ferritin protein. In this part of the experiment, the ferritin is functioning in a similar manner to its role in a biological system. The amount of iron released over time will be graphed for each DHF concentration, and the initial rate of release will be determined from the slope of the graph. (The initial rate equals the slope.)

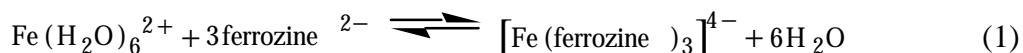
D. Trace Analysis

Some metal ions form intensely colored complexes with organic molecules. (Some of the complexes you synthesized in Experiment 3 of Chem 151 were this type of complex.) The intense color allows trace analysis to be done on these ions in solution using light absorption measurements. In this experiment such a technique is employed for the trace analysis of iron in aqueous solutions.

The ferrous ion, Fe(II) , becomes highly absorbing to visible light when it forms a complex with the organic chelator, ferrozine²⁻. This very stable complex, $[\text{Fe(ferrozine)}_3]^{4-}$, is dark purple

and can be spectrophotometrically detected even at very low concentrations (e.g., 10^{-7} M). This provides a rapid and convenient analytical technique for determining ferrous ion concentration, as described in more detail below.

Since the formation equilibrium constant, K_f , is approximately 10^{20} , we can assume that Fe(II) quantitatively converts into $[\text{Fe}(\text{ferrozine})_3]^{4-}$ in the presence of ferrozine²⁻ because the products are favored over the reactants (see Eqs. 1 and 2).



$$K_f = \frac{[\text{Fe}(\text{ferrozine})_3]^{4-}}{[\text{Fe}(\text{H}_2\text{O})_6^{2+}][\text{ferrozine}^{2-}]^3} \approx 10^{20} \quad (2)$$

E. Data Analysis

1. Calculation of Total Iron Present and Iron Released Over Time

The concentration of Fe(II) in the 25 ml volumetric flask from the ferritin digestion is calculated by using Eq. 3, which was derived in the Introduction of Experiment 3 in Chem 151. The pathlength, b , is equal to 1 cm for this experiment and ϵ is $2.79 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ for the ferrozine-iron complex at λ_{max} .

$$\left(\begin{array}{c} \text{molar concentration of} \\ \text{Fe(II) in the 25 ml flask} \end{array} \right) = \left(\frac{A}{\epsilon b} \right) \quad (3)$$

The mmoles of Fe(II) in the 25 ml volumetric flask is calculated using Eq. 4.

$$\left(\begin{array}{c} \text{mmoles of Fe(II)} \\ \text{in the 25 ml volumetric flask} \end{array} \right) = \left(\begin{array}{c} \text{molar concentration of} \\ \text{Fe(II) in the 25 ml flask} \end{array} \right) \cdot (25.00 \text{ ml}) \quad (4)$$

As mentioned earlier in the Introduction, the mineral core of ferritin is made up of $[\text{FeO}(\text{OH})]_8[\text{FeO}(\text{H}_2\text{PO}_4)]$, which has a formula weight of 879.7 g/mole. Since there are 9 atoms of iron per molecule of core, the core weight is divided by 9, giving 97.70 grams of core for every mole of iron present. So the weight of the iron core in milligrams can be calculated using Eq. 5.

$$(\text{weight of iron core}) = (\text{mmoles of Fe(II)}) \cdot (97.70 \text{ g/ mole}) \quad (5)$$

The total weight of ferritin in milligrams is the weight of ferritin you place in the 25 ml volumetric flask and this quantity can be calculated using Eq. 6.

$$(\text{total weight of ferritin}) = \left(\frac{\text{concentration of stock ferritin solution}}{\text{in mg/ml}} \right) \cdot \left(\frac{\text{volume of stock ferritin solution}}{\text{added in ml}} \right) \quad (6)$$

The weight of apoferritin (ferritin without the mineral core) in milligrams can be calculated using Eq. 7. Using this value and the molecular weight of apoferritin of 474,000 g/mole, the mmoles of apoferritin can be calculated using Eq. 8.

$$(\text{weight of apoferritin}) = (\text{total weight of ferritin}) - (\text{weight of iron core}) \quad (7)$$

$$(\text{mmoles of apoferritin}) = \left(\frac{\text{weight of apoferritin}}{474,000 \text{ g/mole}} \right) \quad (8)$$

The number of atoms of Fe(II) contained in each ferritin molecule is equal to the number of mmoles of Fe(II) per mmole of apoferritin (because Avogadro's number cancels out). The mmoles of apoferritin is equal to the value calculated in Eq. 8, and the mmoles of Fe(II) was calculated in Eq. 4, so the number of Fe(II) atoms per ferritin can be calculated using Eq. 9.

$$\left(\frac{\# \text{ of Fe(II) atoms}}{\text{ferritin molecule}} \right) = \left(\frac{\text{mmoles of Fe(II)}}{\text{mmoles of apoferritin}} \right) \quad (9)$$

As stated in the Introduction, each ferritin molecule can hold 4500 atoms of iron. To calculate the percent of sites occupied, use Eq. 10.

$$(\% \text{ of the sites occupied}) = \left(\frac{\# \text{ of Fe(II) atoms / ferritin molecule}}{4500} \right) \cdot 100 \quad (10)$$

2. Calculation of Iron Released by Dihydroxyfumarate-A Time Course

The concentration of reductant used is calculated using Eq. 12, where the concentration and volume of the stock solution are known.

$$\left(\frac{\text{concentration of}}{\text{DHF}} \right) = \left(\frac{\text{concentration of stock}}{\text{DHF solution}} \right) \cdot \left(\frac{\text{volume of DHF stock solution}}{25.00 \text{ ml}} \right) \quad (12)$$

The mmoles of Fe(II) released by dihydroxyfumarate is calculated using Eqs. 3 and 4 where A is the absorbance reading after 12 minutes.

To calculate the percent of Fe(II) released by the reductant during the time period, use Eq. 11 where mmoles of Fe(II) released is calculated from line 3 of the Report. The total mmoles of Fe(II) was calculated using Eqs. 3 and 4 and the absorbance reading from part II.C. of this experiment. The factor of 10 is included because the original stock solution was ten times more concentrated in part II.B. than part II.A.

$$\% \text{ of total Fe(II)} = \left(\frac{\text{mmoles of Fe(II) released}}{\text{mmole of total Fe(II)} \cdot 10} \right) \cdot 100 \quad (11)$$

You will obtain the initial rate of iron release by calculating the concentration of Fe(II) for each time period using Eq. 3 and then graphing the concentration of Fe(II) vs. time elapsed. The slope of this straight line gives the initial rate of release because the slope is the change in concentration with time. Make sure that the time is in units of minutes so all of the rates are reported in the same units. Although the graph should go through the origin, **do not** include (0,0) as part of your graph.

3. Calculation of the Concentration of an Iron Unknown

The concentration of Fe(II) in the 25 ml volumetric flask is calculated using Eq. 1 where the value of b is 1.00 cm and the ϵ is the literature value ($2.79 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). Once you have the concentration of the solution in the 25 ml volumetric flask, the concentration of the original unknown solution is obtained using Eq. 13. The final factor in Eq. 13 multiplies the concentration by the diluted volume then divides by the original volume transforming the concentration back to the original concentration.

$$\left(\text{Concentration of Fe(II)} \right)_{\text{in the original solution}} = \left(\text{Concentration of Fe(II)} \right)_{\text{in the 25 ml volumetric flask}} \cdot \left(\frac{25 \text{ ml}}{0.5 \text{ ml}} \right) \quad (13)$$

II. PROCEDURE

A. Determination of Total Iron in Ferritin (students work in pairs)

Your TA will have placed the following items at your bench:

- (a) eight 25 ml volumetric flasks with stoppers
- (b) eight cuvettes with caps

At the end of the lab period, you should wash these items and check with your TA, who will record the successful completion of these tasks in his or her gradebook.

Here you will break open the protein with acid, freeing the iron core. The iron will then be complexed by ferrozine²⁻, so you can determine its concentration. Place 1.00 ml of the stock ferritin solution in a clean (but may be distilled-water-wet) 25 ml volumetric flask. This ferritin solution is obtained from the Brinkmann dispenser labeled 0.3 mg / ml. Using your graduated cylinder, now add the following:

- 2 ml 2 M H₂SO₄
- 2 ml dihydroxyfumarate

Wait 30 minutes for the shell to be stripped from the molecule, then add:

- 4 ml 2.5 M NaOAc (sodium acetate)
- 2 ml ferrozine²⁻

Wait another 30 minutes, and then dilute to the 25 ml mark with distilled water (using a clean Beral pipet to add the last few mls), stopper and mix.

While you are waiting the 30 minutes, prepare a blank to use in the spectrophotometer. To prepare the blank mix:

- one drop of 2 M H₂SO₄ using a Beral pipet
- 2 ml of the dihydroxyfumarate
- 2 ml of 2.5 M NaOAc
- 2 ml of the ferrozine²⁻ solution

Wait 30 minutes, then dilute to 25 ml with distilled water. You may do one of the kinetic analysis runs in II.B. while you are waiting.

After waiting 30 minutes, pour an aliquot of the solution from the volumetric flask containing the ferritin into a cuvette. Using the blank (prepared above) in position #1 of the spectrophotometer, obtain the absorbance for your ferritin solution. The Genesys 5 should be in the ABS/%T/CONC program (See Appendix C.5.b.) and set for a wavelength of 561 nm. Be sure the ∇ markers on the cuvettes are always facing to the right and to AUTO ZERO before reading the absorbance. Write the absorbance in your lab notebook. The concentration of iron can be calculated using Eq. 1.

Save the blank prepared in this section to use as the blank in II.C..

B. Determination of Iron Released by Dihydroxyfumarate — A Time Course (students work in pairs)

In this procedure, iron is released through the channels of intact ferritin molecules by reducing the iron in the protein core from Fe(III) to Fe(II). The Fe(II) released from the core is trapped in the highly colored ferrozine²⁻ complex. This part of the experiment measures initial rate dependence of the reduction of iron on the concentration of the reducing agent in the reactions. You will do the time course study with two different reducing-agent concentrations.

To prepare the blank for this section place the following in a 25 ml volumetric flask:

- 2 ml 2.5 M NaOAc
- 2 ml ferrozine²⁻
- 0.5 ml dihydroxyfumarate (Add volumes using your Mohr pipet.)

Dilute to the mark with distilled water and mark the flask as “blank”. Be sure to record the concentration of the stock DHF in your notebook.

Place the following in a clean (but may be distilled water wet) 25 ml volumetric flask:

- 2 ml 2.5 M NaOAc
- 2 ml ferrozine²⁻
- 9 ml distilled water
- 0.5 ml dihydroxyfumarate

Mix these solutions well. **Begin timing as you now add 1.00 ml** of the 3 mg/ml stock ferritin from the properly labeled Brinkmann dispenser to the reaction flask (not the blank). Carefully dilute to the 25 ml mark. Stopper, mix thoroughly, transfer to a cuvette and as soon as possible, obtain an absorbance reading. The time of this absorbance reading is the time elapsed since mixing. Use the blank prepared in this section in position #1, the same λ_{max} and the ABS/%T/CONC program as above. Don't forget to face the ∇ mark on the cuvette to the right and to AUTO ZERO first. Write down the absorbance and the total time elapsed from the addition of ferritin in your lab notebook. Take an absorbance reading every two minutes for 12 minutes. Other students will be using the same Genesys at the same time, so be sure to note which position contains your sample.

When you have completed the first kinetic run with 0.5 ml of DHF solution, go back to the start of II.B. and repeat the procedure using 2.0 ml of DHF.

The concentration of Fe(II) is calculated using Eq. 3 (I.E. of this experiment). Although the graph should go through the origin, **do not** include (0,0) as part of your graph. For detailed instructions on graphing with Kaleidagraph see Appendix G.

C. Determination of an Unknown Iron Concentration (students work alone)

Each student will be given a vial containing an unknown solution of Fe(II) prepared from ferrous ammonium sulfate ($\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$). Be sure to write your unknown number in your notebook. Pipet 0.5 ml of the unknown solution into each of three clean (may be wet with distilled water) 25 ml volumetric flasks. Use a graduated cylinder to add to each of these flasks:

2 ml of dihydroxyfumarate

2 ml of the ferrozine²⁻ solution

Mix the contents of each flask thoroughly. **Do not dilute** to the mark until after you have allowed them stand for 10 minutes. Use your Beral pipet to add the last few drops of water so you do not overshoot the mark.

Using the blank solution you prepared in II.A., the same ABS/%T/CONC program, and the same λ_{max} , obtain absorbance readings of these solutions. Calculate the concentration of the solution in the 25 ml volumetric flask and in the original unknown solution.

Clean your cuvettes and volumetric flasks, and show your TA that all are present and clean so that you are checked off in their gradebook.

DATA GUIDE

This page summarizes the minimum key data that must be recorded in your lab notebook. All other observations and data also should be recorded in your notebook. The stapled blue copies of the notebook pages should be submitted to your TA at the end of each laboratory period.

II.A. Determination of Total Iron in Ferritin

1. The absorbance of the solution and the wavelength for the measurement.

II.B. Determination of Iron Released by Dihydroxyfumarate: A Time Course

2. Concentration of the stock dihydroxyfumarate.
3. The time of the ferritin addition.
4. The time / absorbance readings every two minutes for 12 minutes.

II.C. Determination of an Unknown Iron Concentration

5. The number of your unknown.
6. The absorbance readings for these solutions.

Report
(3 sides)

EXPERIMENT 4
Iron in Biology: The Iron Content in Ferritin,
the Iron Storage Protein

Submit your stapled Report to the RED locker before the deadline given in the introduction and course outline.

Name _____
(Last) (First)

TA (print) _____ Chem ID # _____

Partner _____

II.A. Determination of Total Iron in Ferritin

1. What is the concentration of Fe(II) in the 25 ml volumetric flask from the ferritin digestion?
_____ M
2. How many mmoles of Fe(II) are present in the 25 ml volumetric flask?
_____ mmoles
3. What is the weight of the iron core in the ferritin? _____ mg
4. What is the weight of the apoferritin? _____ mg
5. How many mmoles of apoferritin were present? _____ mmoles
6. How many atoms of Fe(II) were present per molecule of ferritin? _____
7. If ferritin can hold 4500 atoms per molecule, what percentage of Fe(II) binding sites were occupied? _____
8. Show your calculation for II.A.4, II.A.5, and II.A.6 below.
(Copy the calculations from your notebook.)

II.B. Determination of Iron Released by Dihydroxyfumarate-A Time Course

	Run 1 0.5 ml DHF	Run 2 2.0 ml DHF
1. Concentration of dihydroxyfumarate in the 25 ml volumetric flask, M:		
2. Absorbance at λ_{\max} after 12 minutes: (or after your last reading)		
3. Mmoles of Fe(II) released by dihydroxyfumarate:		
4. % of total Fe(II) per protein molecule:		

5. Show your calculation for % of total Fe(II) for Run 1 below:
(Copy the calculation from your notebook.)

6. Plot the concentration of Fe(II) released vs. time in minutes for each run. Fit the data to a straight line using linear regression. Report the slope (initial rate in $\text{M}\cdot\text{min}^{-1}$) for each run. Although the graph should go through the origin, **do not** include (0,0) as part of your graph. Partners can work together on this graph, but each student must generate a graph, and is responsible for the graph turned in with the report. Clearly label the concentration of DHF used and your Chem ID number. Staple the graphs to the report for grading.

7. What is the initial rate of iron release for each of the concentrations of dihydroxyfumarate used?

Run 1: _____ (0.5 ml of DHF) Run 2: _____ (2.0 ml DHF)

II.C. Determination of an Unknown Iron Concentration

Unknown number: _____

	Flask 1	Flask 2	Flask 3
1. Concentration of Fe(II) in the 25 ml volumetric flask, M:			
2. Concentration of Fe(II) in the original solution, M:			
3. Average concentration of Fe(II) in the original solution, M:			

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for

Experiment 4
Pre-Lab Assignment

Name _____

Chem ID _____

TA _____

- Staple this page to the front of your written Pre-lab Assignment for this experiment.
- Submit this assignment to the GREEN slotted locker outside McMillen 206 by 5 minutes after the hour at the start of the lab period in which this experiment is to be performed. It is strongly advised to submit this assignment early (hours or days) to avoid any chance of being late since a late Pre-lab Assignment will receive no credit.

COVER PAGE

for

Experiment 4
Tutorial Assignment

Name _____

Chem ID _____

TA _____

- Staple this page to the front of your written Tutorial Assignment for this experiment.
- Submit this assignment to the GREEN slotted locker outside McMillen 206 before the deadline given in the course outline (pages 4 and 6 of the lab manual). It is strongly advised to submit this assignment early (hours or days) to avoid any chance of being late and receiving a late penalty.

