THE KINETICS OF OXYGEN UPTAKE BY 6,7-DIMETHYLESTER PROTOPORPHYRIN IX IRON (II) IN SEVERAL SOLVENTS

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#### ABSTRACT

#### THE KINETICS OF OXYGEN UPTAKE BY 6,7-DIMETHYLESTER PROTOPORPHYRIN IX IRON(II) IN SEVERAL SOLVENTS

By

James R. Baker

The kinetics of the reaction of the complex 6,7dimethylester protoporphyrin IX iron(II) with oxygen has been investigated in benzene/pyridine and pyridine/imidazole solutions. Though the results of the reaction in the benzene/pyridine solution have been reported in a previous work<sup>1</sup>, substantial reason was found to justify a reexamination of the kinetics results. Also obtained in this work were the kinetics of this same reaction in a pyridine solution saturated with imidazole. It was previously reported<sup>2</sup> that the latter system yielded a product that was reversible in its reaction with oxygen. In order to describe the reaction more fully, kinetics data have been obtained.

In both systems a prominent visible absorption spectrum was obtained for the non-oxygenated 6,7-dimethylester protoporphyrin IX iron(II). As the complex was allowed to react with oxygen, the visible spectra showed marked alterations in their visible absorption and the intensities of the absorbances decreased as the reaction proceeded. By using absorbance as a measure of the concentration of the non-oxygenated complex, the decrease of the absorbance with time measures the rate of this reaction.

In reexamining the reaction of the iron(II) complex with oxygen in benzene/pyridine solution a new rate equation of a mixed first- and second-order character was found, while the previous results showed mainly first-order character for the reaction. These results suggested a new mechanism should be proposed. The kinetics of this same reaction in a pyridine solution saturated with imidazole also fit a mixed-order rate equation. Unfortunately, not enough data were obtained and not enough is known about the reaction to propose a reaction mechanism. It was, however, verified by a visible spectral study that this was, indeed, a reversible oxygen carrying system.

- 1. I. A. Cohen and W. S. Caughey, Biochem., 7, 636 (1968).
- A. H. Corwin and S. D. Bruck, <u>J. Amer. Chem. Soc.</u>, <u>80</u>, 4736 (1958).

#### THE KINETICS OF OXYGEN UPTAKE BY

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## IN SEVERAL SOLVENTS

By

James R. Baker

## A THESIS

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To my Mother and Father

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#### INTRODUCTION

In recent years a considerable amount of interest has been expressed in the study of the reversible oxygen carrying ability of hemoglobin.<sup>1</sup> Of course, hemoglobin was long ago identified as a component of blood and as the prime oxygen carrier in most animal systems. Heme (protoporphyrin IX iron(II)) is the actual oxygen carrying unit of hemoglobin and can combine reversibly with oxygen and has been the subject of considerable study for many years<sup>2</sup>,<sup>3</sup>,4,5. Throughout these studies, however, the actual oxygen to iron interaction and the detailed mechanism of how oxygen can enter through the globin for reaction with the iron heme has not yet been established.

Hemoglobin<sup>6,7,8</sup> consists of four protein subunits each containing a heme group surrounded by the polypeptide globin that consists of four subunits ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ , and  $\beta_2$ ). Perutz<sup>9</sup> provides an ingenious description of the conformational changes which occur in the  $\alpha$  and  $\beta$  subunits of hemoglobin when a ligand attacks each of the four heme groups. The kinetics of oxygen uptake by hemoglobin under suitably restricted conditions have been examined by Gibson and co-workers<sup>10</sup>,11,12 by using a stopped-flow technique developed for this purpose.

The actual study of the heme iron to oxygen interaction is complicated by the size (over 64,000 mw units) and complexity of the hemoglobin molecule and suggests that a model of a reversible oxygen carrying system less complex than hemoglobin might be useful. Since it is the iron porphyrin segment of the hemoglobin molecule which is the actual oxygen carrier, isolation of this unit might well serve as the model sought and, indeed, a number of workers have isolated<sup>13,14</sup> synthesized<sup>15</sup>, analyzed<sup>16</sup>, and studied the kinetics<sup>17,18,19</sup> of reactions of the iron porphyrin unit. One basic problem inherent in this approach is the need to maintain the iron in the iron(II) state throughout the reaction. In most cases iron has successfully complexed with oxygen but oxidation to the iron(III) state has also occurred and rendered the reaction irreversible.

In 1956 the first reversible oxygen reaction with protoheme was described. Corwin and Reyes<sup>20</sup> reported a crystalline diimidazole protohemochrome which combined reversibly with oxygen in the absence of water. In 1958 Corwin and Bruck<sup>21</sup> reported first, that this same protohemochrome reversibly took up oxygen when placed in pyridine solution saturated with imidazole and, second, that a spectral change also accompanied oxygenation. However, no subsequent research has been reported with this system. This work has been an attempt to describe the protohemochrome system more fully by studying the kinetics of the oxygen reaction. For the sake of convenience one small

alteration has been made, the protoheme used by Corwin and Bruck is protoporphyrin IX iron(II), which takes up oxygen almost instantaneously in most systems<sup>17</sup>, thus making it difficult to study. By esterifying the two carboxylic acid groups on the porphyrin ring the rate of reaction with oxygen by 6,7-dimethylester protoporphyrin IX iron(II) becomes slow enough to study by conventional techniques. By using the spectral change in the conversion of the de-oxy to oxyhemochrome, time versus absorbance data are collected and rate constants calculated.

The kinetics reported by Cohen and Caughey<sup>19</sup> were reexamined. They also used 6,7-dimethylester protoporphyrin IX iron(II) (hereafter to be identified as heme diester) in their study, but used a different solvent system. Their results show the conventional iron(II) to iron(III) oxidation upon oxygenation of the complex. However, upon repeating their work certain differences in the rate of reaction were noted which greatly affected the rate constants. The experiments have been repeated in this work, new rate constants calculated, and the reasons for the differences are discussed.

#### EXPERIMENTAL

#### General Procedure

All visible spectra were recorded by use of a Unicam SP800 Ultraviolet Spectrophotometer. Infrared spectra were recorded by a Perkin-Elmer 457 Grating Infrared Spectrophotometer. All solvents were reagent grade and were purified as specified below or were used as received. Solid chemicals were used as received. Glassware was cleaned by immersion for several hours in a potassium hydroxide/ethanol solution, then by several hours immersion in a dilute hydrochloric acid bath, followed by thoroughly rinsing in water, and, finally, by drying in an oven at about 130°.

### Purification of Solvents

<u>Methanol</u>:<sup>22</sup> Clean, dry Mg turnings (10 g) and  $I_2$ (1 g) were placed in a 3  $\ell$  flask fitted with a reflux condenser. Methanol (100 ml) was added through the condenser and the mixture was warmed on a water bath until all the Mg was converted to magnesium methoxide. Methanol (2  $\ell$ ) was next added into the flask and the mixture refluxed for 30 minutes. The methanol was then distilled, moisture was excluded, and the 65-66° fraction was collected.

<u>Chloroform</u>: This solvent was washed three times with water in a seperatory funnel, dried over CaCl<sub>2</sub> and filtered before use.

<u>Benzene</u>: The solvent was washed twice with concentrated sulfuric acid, three times with water, and refluxed over CaO before use.

<u>Pyridine</u>: The pyridine was passed through an alumina column and refluxed over BaO before use.

Acetone: Acetone was dried over CaCl<sub>2</sub> and filtered before use.

Trifluoroacetic acid: The acid was used as received.

### Purification of Gases

Oxygen: Oxygen (received from Liquid Carbonic, industrial grade) was passed through a column (4 cm by 45 cm) of potassium hydroxide before use.

<u>Nitrogen</u>: It was vitally important to use oxygen-free nitrogen during the preparation and reaction of many of these compounds in order to prevent oxidation. This nitrogen (Central Welding Supply, Lansing, MI, prepurified) was further purified in the following manner; the nitrogen was passed through a purification train<sup>23</sup> which consisted of the following components, connected in series, and schematically illustrated in Figure 1;

(A) a column (4 cm by 40 cm) of BTS catalyst (from BASFCorp.) heated to about 140°,

Figure l

The Nitrogen Purification Train



(B) two identical gas scrubbing towers each containing chromium(II) sulfate solution in 0.15  $\underline{N}$  sulfuric acid and zinc amalgam,

(C) a dry ice/acetone cold trap,

(D) a column (70 cm by 3 cm) of aquasorb (Mallenkrodt Chemical).

Component A is the high capacity oxygen remover, while component B should remove any trace oxygen remaining. Components C and D insure that no water is allowed to enter the reaction areas.

The BTS catalyst was prepared and used as directed by instructions supplied by BASF Corp. The chromium(II) sulfate solution used for oxygen removal was prepared as follows:<sup>27</sup> a solution 0.15 N in sulfuric acid is initially prepared and added to the gas scrubbing towers. The zinc amalgam was prepared by adding 20 g of Zn, 0.6 g HgCl<sub>2</sub>, and 40 ml of 1.0 M HCl in a beaker and stirring for several minutes. The resulting solid was washed several times with distilled water and added to the gas scrubbing towers. Nitrogen was then bubbled through the solution while chromium(II) was prepared. Several pieces of chromium metal (about 10 g) are placed in enough concentrated HCl to cover all the pieces and remain in the HCl until vigorous reaction proceeds. The HCl was then decanted, the metal washed several times with water, and then was added to the gas scrubbing towers. To facilitate the dissolving of the chromium in the acid solution, the solution was heated to

about 40°. The solution was ready for use when a blue color appeared.

## Esterification of Hemin, Process One<sup>24</sup>

Hemin (from Eastman-Kodak, 97+% pure), 0.1 g, was dissolved in 25 ml of methanol containing 0.2 g KOH. Then 10 ml of trifluoroacetic acid was added to this solution and the solution was refluxed for 10-15 hours. This solution was evaporated to dryness and 50 ml of acetone was added. After dissolving as much residue as possible in the acetone, the solution was filtered, and again evaporated to dryness. The residue was then treated with three successive 20 ml portions of chloroform, each was filtered through the same fine-fritted filter. The filtrate was dried overnight at 50° in a desiccator.

## Esterification of Hemin, Process Two<sup>25</sup>

A solution containing 50 ml of cold, absolute methanol and 2.6 g of concentrated sulfuric acid was prepared. In this solution 0.2 g of hemin (Eastman-Kodak, 97+% pure) was dissolved and allowed to stand in the refrigerator (ca. 5°) for 48 hours.

The solution was transferred to a 500 ml separatory funnel containing 150 ml dry chloroform and the contents were mixed well. It was washed twice with 100 ml portions of water, twice with 100 ml portions of 2 <u>N</u> NH<sub>4</sub>OH, twice again with 100 ml portions of water, twice with 100 ml of 1 N HCl, and, finally, three times with 100 ml portions of

water. Then the chloroform layer was separated, placed in a 1000 ml round bottom flask, and taken to dryness in a rotary evaporator. By use of acetone (ca. 50 ml), the solid residue was transferred to a small flask (100 ml) to facilitate product collection and the solution was again taken to dryness. Finally, the product was scraped off the sides of the flask, powdered as much as possible, and dried overnight under vacuum.

# <u>Crystallization</u> of Esterified Product<sup>22</sup>

The hemin ester was allowed to dissolve in a small volume of freshly distilled and dried chloroform (1 ml  $CHCl_3$  for 20-30 mg of ester). To the boiling solution an equal volume of boiling, absolute methanol was added. As boiling continues, chloroform distilled off and the methanol concentration increased. This process is allowed to continue until crystals form on cooling. Crystals were then collected, washed with  $H_2O$ , and dried overnight in a desiccator.

This procedure for crystallization of the ester was used to prepare crystals from both processes one and two for preparation of the ester.

# Fe(II)hemin Diester Stock Solutions<sup>24</sup>

To a 60 ml seperatory funnel 10 ml water, 20 ml pyridine, and 15 ml benzene were added. Then approximately 15 mg purified chlorohemin diester was added and the mixture was shaken well to mix the contents thoroughly. The space in the funnel above the solution was flushed briefly with nitrogen and about 0.15 g  $Na_2S_2O_4$  was added. Then the seperatory funnel was quickly capped, shaken well for about one minute, and a bright orange color appeared. The benzene layer was allowed to separate and the lower aqueous layer was discarded. The benzene phase was delivered into a capped 50 ml flask containing 10 ml pyridine and 10 g anhydrous  $Na_2SO_4$ , which had been previously cooled in an ice bath. The mixture was stirred for about 5 minutes and filtered through a fine-fritted funnel into a receiver cooled in an ice bath. This solution was stored in the refrigerator at about 5°.

Even under these low temperature storage conditions, oxidation of the Fe(II)heme diester became significant after three to four days, after which time the stock solution was discarded and a new stock solution prepared.

# Procedure for Measuring the Rate of Oxygen Uptake by Fe(II)heme Diester in Benzene/Pyridine Solutions

The novel reaction flask for this procedure is shown in Figure 2. With this apparatus the reaction solution can be prepared under vacuum, the required nitrogen and oxygen pressures applied, and transfer to a spectrophotometer cell can all be accomplished. The procedures for preparations of the solution to be used in the kinetics experiments and the rate measurements are described below.

Into Flask A was placed 20.0 ml of a solution containing pyridine in benzene (concentrations are varied by

Reaction Flask for Kinetics Measurements



diluting a stock solution of pyridine in benzene to obtain pyridine concentrations of  $10^{-2}$  to  $10^{-3}$  M). The solution was frozen, the vessel was evacuated, the solid was thawed and re-frozen, the vessel evacuated, then filled with nitrogen, and the solution was thawed again. Next, the solution was shaken vigorously, again it was frozen and the flask was evacuated. This procedure deoxygenated the solution very effectively. Atmospheric pressure was noted on the vacuum gauge attached to the system, a partial pressure of oxygen was applied (oxygen pressures of 150, 300, and 500 torr were used to evaluate the dependence of rates on  $p_{O_2}$ ) and a partial pressure of nitrogen was added to attain a total pressure in the flask of one atmosphere. The solution was next thawed and shaken for at least 5 minutes.

To flask B was next added 2.0 ml of stock Fe(II)heme diester solution. The Fe(II)heme diester solution was evaporated to dryness by application of a vacuum. Flasks A and B were then connected and the space between them was evacuated. Flask B was next filled with partial pressures of nitrogen and oxygen exactly the same as in flask A above.

The entire apparatus (flasks A and B connected to a spectrophotometer cell) was immersed in a temperature bath and allowed to reach thermal equilibrium (about 30 minutes). After this time, the solution and solid were allowed to mix by opening the stopcocks between flasks and swirling the solution for a few seconds. The solution was immediately

transferred to the spectrophotometer cell, placed into the spectrophotometer, and a series of visible spectra taken at about 5 minute intervals (intervals varied depending on the rate of the reaction).

Spectra were measured by means of a Unicam SP800 Ultraviolet Spectrophotometer fitted with a time delay and a temperature controlled cell holder. The time delay allows one to measure a spectrum at any specified time interval and at each specified time interval the spectrum will be recorded indefinitely. The cell holders are jacketed so that water can be circulated through them from a temperature regulated water bath and gives temperature stability during the course of the measurements.

### Procedure for Measuring the Oxygen Uptake by Fe(II)heme Diester in a Pyridine Solution Saturated with Imidazole

The procedure used for these measurements was identical with the procedures described above except that the solvent system used was a pyridine solution saturated in imidazole.

It is crucial that saturation of pyridine with imidazole be obtained because the concentration greatly affects the rate constants. Particular care was taken in preparing these solutions, so that not only was saturation obtained, but also all the solutions were of uniform composition.

Imidazole (recrystallized, from Research Organic/ Inorganic Chemical Corp.) was recrystallized three times from pyridine to insure a pure product. The product from the final crystallization was dried overnight under vacuum. To prepare a saturated solution of imidazole in pyridine enough imidazole was placed into 200 ml of pyridine at about 30° so that the imidazole no longer dissolved in the solution. The solution was cooled to about 20° and allowed to remain at this temperature for several hours. The temperature of this solution was slowly increased and the solution was stirred until 25° was reached. At this point, the stirring was stopped, the solid was allowed to settle while 25° was maintained, and, finally, 10 ml portions were removed and placed in separate vials to be used when needed.

#### RESULTS

## Esterification Procedure

Two methods of preparation of the heme diester from hemin are described in this thesis. The first, refluxing of hemin in trifluoroacetic acid/methanol solution was the method used by Kao and Wang<sup>17</sup> in their initial studies of the oxidation of this complex by molecular oxygen. The second, placing hemin in a sulfuric acid/methanol solution and allowing the reaction to proceed for about 48 hours, is the method suggested by Falk<sup>22</sup>.

In order to decide which product and method would best suit the needs of the following experiments, a comparison of the properties of these compounds was made. First, the infrared spectrum of each of these products was obtained and compared with that of the initial hemin spectrum (see Figure 3). Since the infrared spectra of both hemin and hemin diester have been reported elsewhere<sup>22,26</sup> simple comparisons could be made with these spectra. The most definitive absorbance, in terms of the ester side group is a shift of the hemin ester absorption at 1685 cm<sup>-1</sup> to about 1740 cm<sup>-1</sup>. This shift is found in spectra of both esterified products. However, in the spectrum of product one an unassigned peak at about 1770 cm<sup>-1</sup> is found. This band is found in no other

# Infrared Spectra of

- A. Initial Commercial Hemin
- B. the Product of the Trifluoroacetic Acid/Methanol Method of Preparation of the Esterified Product
- C. the Product of the Sulfuric Acid/Methanol Method of Preparation of the Esterified Product



reported spectrum of the ester. It has become obvious that the two ester products are not identical and that further observations in the "fingerprint" region  $(750-1250 \text{ cm}^{-1})$  of these spectra substantiate the supposition that these are not identical products. Comparison with the previously reported spectra of the heme diester showed product two was essentially the same and suggests it is the one to be used.

To test the two products further, the reaction of the reduced form of each with oxygen when placed in pyridine was observed. The results of previous studies<sup>17,19,21</sup> suggest that the Fe(II)heme diester is impassive to reaction with oxygen when pyridine is the solvent used. However, in Figure 4 one can see the results of dissolving the reduced forms of products one and two in a pyridine solution in an oxygen atmosphere. Figure 4a shows that over a period of about 35 minutes the visible spectrum of product one showed a drastic change at its 555 nm absorption, indicative of reaction with oxygen. Figure 4b shows that product two showed no such change in its visible spectrum, suggesting product two was impassive to oxygen while in pyridine.

These results implied that product two provided the heme diester which should and was used in all subsequent experiments.

## Kinetics of the Reaction of Fe(II)heme Diester with Oxygen in Benzene/Pyridine Solutions

The experimental procedure described above for kinetics measurements in the benzene/pyridine solution results in a

# Oxidation of $O_2$ of

- A. the Product of Method One in Pyridine
- B. the Product of Method Two in Pyridine



series of spectra (a typical example is found in Figure 5). Over a period of time the absorbance at 555 nm decreases and is a measure of the rate of reaction of the Fe(II)heme diester with oxygen.

In treating the data (time vs. absorbance) one can safely assume the order does not depend on the changing pyridine or oxygen concentrations. By making pyridine >  $10^{-3}$  M and oxygen >  $10^{-3}$  <u>M</u> with the Fe(II) heme diester  $\simeq$  $10^{-5}$  M there is about a one hundredfold excess of pyridine and oxygen over the heme diester concentration and, hence, the pyridine and oxygen concentrations can be considered constant. With this assumption, the data are fitted to pseudo first-order, pseudo second-order, and a mixed pseudo first- and second-order rate equations of the forms  $-dA/dt = k_1(A - A_{\infty})$ ,  $-dA/dt = k_2(A - A_{\infty})^2$ , and -dA/dt = $k_1'(A - A_{\infty}) + (A - A_{\infty})^2$  respectively, where k's are rate constants, A is the observed absorbance, and  $A_{\infty}$  is the absorbance when the complex is completely oxygenated. The correction factor,  $A_{m}$ , is necessary because both the oxygenated and the non-oxygenated complexes absorb at 555 nm<sup>28</sup> (see Appendix B for a more detailed calculation of this correction factor). By using the above first-, second-, and mixed-order forms of the rate equation, rate constants for each were calculated by a general purpose curve fitting program of Dye and Nicely<sup>29</sup>. The computer program adjusts the rate constants and initial absorbance until the calculated constants give results most nearly

Kinetic Spectra of Fe(II)heme Diester in Benzene/Pyridine Solution



equal to the experimental data. If the form of the equation being used in the curve fitting program is of the correct form, then the calculated and experimental data should nearly coincide. In Figures 6, 7, and 8 are shown samples of the computer output for the first-order (Figure 6), second-order (Figure 7), and mixed-order (Figure 8) equations. In every case the data followed the same pattern. Mixed-order rate equations gave the best results.

The final tabulation of the results is found in Table 1. The standard deviation is in parentheses beside the value. For each specified pyridine and oxygen concentration the runs were repeated a minimum of three times to insure the consistency of the results.

### <u>Kinetics of the Reaction of Fe(II)heme Diester with Oxygen</u> <u>in a Pyridine Solution Saturated with Imidazole</u>

By using the experimental procedure described for kinetics measurements in a pyridine solution saturated with imidazole, a series of spectra result (a typical example is found in Figure 9). By following the rate of decrease of the 550 nm absorbance, one has a method of following the rate of oxygenation of this sample. These data are treated in a manner outlined in the previous section. A best fit to either a first-, a second-, or a mixed-order equation was found by using the curve fitting program developed by Dye and Nicely<sup>29</sup>. And, again, the best fit to the data was found by using the mixed-order rate equation (Table 2).

Fit of Rate Data to a First-Order Rate Equation



FIT OF RATE DATA TO A FIRST-ORDER BORAFION

Fit of Rate Data to a Second-Order Rate Equation





Fit of Rate Data to a Mixed-Order Rate Equation





## Table 1

Results for Kinetics of Oxygen Uptake By Fe(II)heme Diester in Benzene/Pyridine Solutions<sup>a</sup>

[py] mM	[0 <sub>2</sub> ] <sup>b</sup> mM	$k_{1}(s^{-1}) \times 10^{3}$	$k_2 (A^{-1}s^{-1}) \times 10^3$
2.424	1.79	0.17 (±10%)	0.083 (±12%)
2.424	3.57	0.88 (± 9%)	0.26 (±10%)
2.424	5.95	2.40 (±15%)	2.60 (±10%)
4.857	1.79	0.22 (± 5%)	0.042 (±10%)
4.857	3.57	0.97 (±12%)	0.128 (± 8%)
4.857	5.95	1.28 (± 5%)	1.96 (± 6%)
9.714	3.57	0.30 (± 9%)	0.096 (± 8%)
9.714	5.95	0.61 (± 7%)	1.75 (± 7%)

- a. Temperature = 24.8°C in all cases, total pressure = 760 torr.
- b.  $[O_2] = (11.9 \times 10^{-6} \text{ M/torr}) p_{O_2}$  with this constant derived from the O<sub>2</sub> solubility in benzene.<sup>30</sup>

Spectra of Fe(II) heme Diester in a Pyridine Solution Saturated with Imidazole as a Function of Time



Under these suitably restrictive conditions one has a system with the ability to react reversibly with oxygen and verifies the work of Corwin and Bruck<sup>21</sup>. However, it was necessary to try to repeat this work because it has generally been ignored. Figure 10a is the spectrum of the initial non-oxygenated form of the complex Fe(II)heme diester in pyridine saturated with imidazole, Figure 10b is the completely oxygenated Fe(II) heme diester, and Figure 10c is the spectrum of the oxygenated product which has been under vacuum about 4 hours at 40° and was then redissolved in pyridine/imidazole with the exclusion of oxygen. Each of the spectra correlate well with those of Corwin and Bruck (note especially the shift to higher wavelength and the change in relative intensities of the absorbances in the oxygenated form, and note that in recycling to the original product a spectrum not as intense, but with the same wavelength maxima is obtained). These agreements show there is no need for further substantion of the reversibility of this complex with oxygen.

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Results for Kinetics of Oxygen Uptake by Fe(II)heme Diester in Pyridine Solutions Saturated with Imidazole<sup>a</sup>

PO2 (torr)	$k_1(s^{-1})x10^3$	$k_2 (A^{-1}s^{-1}) \times 10^3$
150	0.49 (± 8%)	1.16 (±8%)
300	2.61 (±10%)	2.42 (±6%)
500	6.92 (± 8%)	4.56 (±9%)

a. Temperature = 24.8°C in all cases, pressure = 760 torr.

Spectra of Initial, Oxygenated, and

Recycled Fe(II)heme Diester

- (a) ----- spectrum of initial Fe(II) complex
- (b) ---- spectrum of completely oxygenated complex
- (c) ——— spectrum of recycled complex





#### DISCUSSION

## Kinetics of the Reaction of Fe(II)heme Diester with Oxygen in Benzene/Pyridine Solutions

It was found that the trifluoroacetic acid/methanol method (product one in the experimental section) of esterifying the hemin provided a poor product for use in the kinetics experiments. This method was used by Kao and Wang<sup>17</sup> to provide them with the heme diester to be used in their kinetics experiments. These results indicate that the work of Kao and Wang and the rate constants are suspect. Cohen and Caughey<sup>19</sup> also repeated some of the work of Kao and Wang and used yet another method to prepare the esterified product<sup>26</sup>. Their results also gave rate constants differing from those of Kao and Wang but, again, there was a discrepancy in their work. The mechanism they described has as its final step;

 $py-N_4Fe-O_2-FeN_4-py + 2H_2O \longrightarrow 2N_4Fe(III)OH + H_2O_2+py$ They report water was always in amounts sufficient to be available for reaction. However, they claim that water was rigorously excluded from solvents and the reaction flask throughout their experimental work, and in no place during their experimental procedure was water added to the reaction solution. How, then, can water enter into this mechanism?

If the amount of water is not a controlled variable, then certainly a variable amount of water, particularly if its concentration is near that of the heme diester concentration, could alter the rate of reaction. One finds up to 50% error in their data, far greater error than what should be considered acceptable.

It was, therefore, necessary to repeat the work of Kao and Wang and Cohen and Caughey for the system Fe(II)heme diester in benzene/pyridine solution. It should be noted that very rigorous procedures for excluding water from the system have been used and that the results differ from either of the previous reports.

Upon comparing the results of this study with those of Cohen and Caughey three notable differences are observed; (1) the mixed-order rate equation provides the best fit to the present data throughout the range of pyridine and oxygen concentration, whereas Cohen and Caughey report a firstorder rate at oxygen concentrations > 3.6 mM, (2) the error within the present results are 15% or less - Cohen and Caughey have error as high as 50%, and (3) the numerical values of the rate constants calculated from this study tend to be about a factor of ten lower than those of Cohen and Caughey. Eliminating water from the system can very possibly account for these differences, and better reproducibility has been obtained. Also, eliminating water prevents the final step in the mechanism of Cohen and Caughey's and suggests a new mechanism must be found.

Since a mixed-order rate equation seems to be followed over the range of concentrations used in this experiment, a mechanism more complex than that proposed by either Kao and Wang or Cohen and Caughey had to be found. This becomes more complicated because no simple relationship between the concentrations of pyridine and oxygen and the rate constants could be found, but as [pyridine] increases the rate constants ( $k_1$  and  $k_2$ ) decrease and as  $p_{O_2}$  increases the rate constants increase. A possible mechanism consistent with the observed results is suggested as follows:

Fe(py)<sub>2</sub> 
$$\xrightarrow{K_p}$$
 Fepy + py fast (1)

$$Fe(py)_2 + O_2 \xleftarrow{K_0} FepyO_2 + py \qquad fast (2)$$

Fe(py)0<sub>2</sub> 
$$\xrightarrow{k_1}$$
 pyFe(III)0<sub>2</sub> slow (3)

FepyO<sub>2</sub> + Fepy 
$$\xrightarrow{k_2}$$
 pyFe(III)O<sub>2</sub><sup>2</sup>Fe(III)py slow (4)

pyFe(III)
$$O_2^-$$
+Fepy  $\xrightarrow{k_3}$  pyFe(III) $O_2^2$ Fe(III)py fast (5)

The mechanism corresponds to a mixed-order rate equation of the form;

$$-d[Fe(II)] /dt = k'_1[Fe(II)] + k'_2[Fe(II)]^2$$

where

$$k'_{1} = \frac{\binom{k_{1}K_{0}[O_{2}]}{[py] + K_{p} + K_{0}[O_{2}]}}{\binom{1+\frac{k_{3}K_{p}[Fe(II)] - k_{-1}([py] + K_{p} + K_{0}[O_{2}])}{k_{3}K_{p}[Fe(II)] + k_{-1}([py] + K_{p} + K_{0}[O_{2}])}}$$

$$k_{2}' = \frac{k_{2}\kappa_{p}\kappa_{o} [O_{2}]}{([py] + \kappa_{p} + \kappa_{o} [O_{2}])^{2}}$$

(see Appendix A for details of the derivation). Thus,  $k'_1$ and  $k'_2$  are pseudo first- and second-order rate constants depending on the experimental [py] and  $p_{O_2}$  and  $k_3 K_p$  [Fe(II)],  $k_{-1}$  [py] and  $k_{-1} K_0 [O_2]$ .

This mechanism provides for a first-order dissociative process (equation 3) followed by second-order dimer formations (equations 4 and 5). Both of these features have also been observed in autoxidations in aqueous systems. George<sup>31</sup> found that the reactions of oxygen with ferrous perchlorate solutions proceeded through an intermediate Fe(II)0<sub>2</sub> complex and eventually to the Fe(III)0<sub>2</sub> product that is analogous to equations 2 and 3 in the above mechanism. Haim and Wilmarth<sup>32</sup> found that the oxidation of pentacyanocobaltate ion by molecular oxygen yielded the bridged peroxo complex (NC)<sub>5</sub>-Co(III)- $O_2^{2-}$ -Co(III)-(CN)<sub>5</sub> and Huffman and Davidson <sup>33</sup> proposed in the reaction of ferrous ammonium sulfate in 1 N sulfuric acid with molecular oxygen that a peroxo diiron(III) bridged complex, (H<sub>2</sub>O)<sub>5</sub>-Fe(III)- $O_2^{2-}$ -Fe(III)-(H<sub>2</sub>O)<sub>5</sub> probably formed. Both are very similar to the bridged complexes in equations 4 and 5. The present mechanism seems to be the best to conform to the data.

### <u>Kinetics of the Reaction of Fe(II)heme Diester with Oxygen</u> in Pyridine Solutions Saturated with Imidazole

Certainly the interest in this system can be traced to the ability of the Fe(II) heme diester to take up molecular oxygen reversibly and only to do so when in a pyridine solution saturated with imidazole. It can be compared with the benzene/pyridine system previously discussed that does not have the reversibility, but rather leads to oxidation of the iron from (II) to (III). It was hoped kinetics experiments with this system would lead to a greater understanding of the mechanism by which reversible oxygenation takes place.

But the experiments undertaken here were not without their, as of yet, unresolved problem and sources of concern. The molecular oxygen concentration in the pyridine/imidazole has not yet been determined (unlike the benzene/pyridine solution in which oxygen solubility was already known). This problem eliminates the possibility of determining the exact dependence of the rate constant on the oxygen concentration but does not prevent one from varying the oxygen concentration, since the partial pressure of oxygen in the reaction flask could be controlled. Also the concentration of imidazole in saturated pyridine has not been accurately determined. It is vitally important that saturation with imidazole is attained, for as the concentration of imidazole falls below saturation the rate becomes slower. However, care was taken to insure a saturated solution even though its concentration was not known.

One may question the method used to prepare the diimidazole heme diester complex and whether, in fact, this is the actual complex used. Although the elaborate procedure of Corwin and Bruck<sup>21</sup> is not employed to isolate this diimidazole complex, the initial visible spectrum is identical with that they reported. Thus it is a good indication that the correct complex was used. Since pyridine is the only other ligand in the system available to complex to the iron heme, it is comforting to note that other reports<sup>34,35</sup> show that the affinity of imidazole for the iron in a heme group is much greater than the affinity of pyridine for the same iron heme and implies that imidazole is the coordinated complex on the heme diester.

The analysis of the results obtained in the reaction of oxygen with Fe(II) heme diester in a pyridine/imidazole solution becomes very difficult due to their small number obtained in this study. However, some general observations can be made: the mixed first- and second-order character of the reaction rate is again found and suggests a complicated mechanism may well be found in this system, too. The rate of reaction is about the same in both of these systems, i.e., the values of the rate constants,  $k_1$  and  $k_2$ , are in the same range as the rate constants of the benzene/pyridine system. Though it seems that there are some comparisons to be made between this system and the

benzene/pyridine system, it must be admitted that much less is known about the way the reversible oxygen system behaves or why the change from benzene/pyridine to pyridine/imidazole solvent causes the reversibility. Unfortunately, the lack of complete data at this time prevents the presentation of a detailed reaction mechanism. It is hoped, however, that the research will be continued until a complete understanding of this system is obtained. BIBLIOGRAPHY

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APPENDICES

#### APPENDIX A

Assuming that the mechanism of the reaction of Fe(II)heme diester with oxygen in benzene/pyridine solvent is that given below, this appendix will show the derivation of the rate equations for the mixed-order equation.

Mechanism

$$Fe(py)_2 \xleftarrow{K_p} Fepy + py$$

$$Fe(py)_2 + O_2 \xrightarrow{K_0} FepyO_2 + py$$

Fepy0<sub>2</sub> 
$$\xrightarrow{k_1}$$
 pyFe(III)0<sub>2</sub>

 $FepyO_2 + Fepy \xrightarrow{k_2} pyFe(III)O_2^{2-}Fe(III)py$ 

 $pyFe(III)O_2^- + Fepy \xrightarrow{k_3} pyFe(III)O_2^{2-}Fe(III)py$ 

## Derivations of Rate Constants

$$K_{p} = \frac{[Fepy][py]}{[Fe(py)_{2}]}$$

$$K_{o} = \frac{[Fe(py)O_{2}][py]}{[Fe(py)_{2}][O_{2}]}$$

$$[Fe(II)] = [Fe(py)_{2}] + [Fepy] + [FepyO_{2}]$$
applying the steady state approximation

$$\frac{d[Fe(III)pyo_{2}^{-}]}{dt} = 0 = k_{1}[Fepyo_{2}] - k_{-1}[Fe(III)pyo_{2}^{-}] - k_{3}[Fe(III)pyo_{2}^{-}] [Fepy]$$

$$[Fe(III)pyo_{2}^{-}] = k_{1}[Fepyo_{2}]/(k_{-1} + k_{3}[Fepy])$$

$$-d[Fe(II)]/dt = k_{1}[Fepyo_{2}] - k_{-1}[pyFe(III)o_{2}^{-}]$$

$$+ k_{2}[Fepyo_{2}][Fepy] + k_{3}[pyFe(III)o_{2}^{-}][Fepy]$$

$$= (k_{1} + k_{2}[Fepy])[Fepyo_{2}]$$

$$+ [pyFe(III)o_{2}^{-}] (k_{3}[Fepy] - k_{-1})$$

= 
$$[FepyO_2](k_1+k_2[Fepy]) + \frac{k_1[FepyO_2]}{k_{-1}+k_3[Fepy]}(k_3[Fepy] - k_{-1})$$

from equilibrium conditions:

$$\begin{split} &[\text{Fepy}] = K_{p}[\text{Fe}(\text{py})_{2}]/[\text{py}] \\ &[\text{Fepy}O_{2}] = K_{o}[\text{Fe}(\text{py})_{2}][O_{2}]/[\text{py}] \\ &[\text{Fe}(\text{II})] = [\text{Fe}(\text{py})_{2}](1 + K_{p}/[\text{py}] + K_{o}[O_{2}]/[\text{py}]) \\ &[\text{Fe}(\text{py})_{2}] = [\text{Fe}(\text{II})]/(1 + K_{p}/[\text{py}] + K_{o}[O_{2}]/[\text{py}]) \\ &[\text{Fe}(\text{py})_{2}] = ([\text{Fe}(\text{II})]/(1 + K_{p}/[\text{py}] + K_{p} + K_{o}[O_{2}]) \\ &[\text{Fe}(\text{py})_{2}] = ([\text{Fe}(\text{II})]/([\text{py}] + K_{p} + K_{o}[O_{2}]) \\ &[\text{Fepy}] = K_{p}[\text{Fe}(\text{II})]/([\text{py}] + K_{p} + K_{o}[O_{2}]) \\ &[\text{te} K_{d} = [\text{py}] + K_{p} + K_{o}[O_{2}] \\ &-d[\text{Fe}(\text{II})]/dt = \frac{k_{1}K_{o}[O_{2}]}{K_{d}} [\text{Fe}(\text{II})] + \frac{k_{2}K_{p}K_{o}[O_{2}][\text{Fe}(\text{II})]^{2}}{K_{d}^{2}} \\ &+ \frac{k_{1}K_{o}[O_{2}]}{K_{d}} [\text{Fe}(\text{II})](\frac{k_{3}K_{p}[\text{Fe}(\text{II})]/K_{d} - k_{-1}}{K_{3}K_{p}[\text{Fe}(\text{II})]/K_{d} + k_{-1}}) \end{split}$$

$$\frac{-d \operatorname{Fe}(II)}{dt} = \frac{k_1 \frac{K_0 [O_2][\operatorname{Fe}(II)]}{K_d}}{k_d} (1 + \frac{k_3 \frac{K_p [\operatorname{Fe}(II)] - k_{-1} K_d}{k_3 \frac{K_p [\operatorname{Fe}(II)] + k_{-1} K_d}}) + \frac{k_2 \frac{K_p K_0 [O_2]}{K_d^2}}{K_d^2} [\operatorname{Fe}(II)]^2$$

Assuming  $k_{3}K_{p}[Fe(II)]$  is much greater than  $k_{-1}K_{d}$  we now have a rate equation both first- and second-order in [Fe(II)].

#### APPENDIX B

The rate constants found in tables 1 and 2 are calculated by fitting the data to a mixed-order rate equation of the form  $-dA/dt = k_1(A - A_{\infty}) + k_2(A - A_{\infty})^2$ where the  $A_{\infty}$  correction is necessary because both the non-oxygenated and oxygenated Fe(II)heme diester absorb at the wavelength at which the absorbances are measured. It is the purpose of this appendix to show how this  $A_{\infty}$  term arises and that the absorbance is actually a measure of the concentration.

The relationship of the absorbance to the concentration can be found from the Beer's Law equation:  $A = \varepsilon cl$  where; A = absorbance,  $\varepsilon = molar absorptivity$ , c = concentration ofthe absorbing species, l = path length in cm of the cell. Having used a l cm path length spectrophotometer cell we find;

 $\mathbf{A} = \varepsilon \mathbf{c} = \varepsilon_{\mathbf{II}} \mathbf{c}_{\mathbf{II}} + \varepsilon_{\mathbf{III}} \mathbf{c}_{\mathbf{III}}$ 

where  $\varepsilon_{II}$  and  $c_{II}$  refer to the non-oxygenated Fe(II)heme diester and  $\varepsilon_{III}$  and  $c_{III}$  refer to the oxygenated Fe(II)heme diester. Since the total concentration ( $c_{T}$ ) is equal to  $c_{II} + c_{III}$ ,  $A = \varepsilon_{II}c_{II} + \varepsilon_{III}(c_{T} - c_{II})$ . But when complete oxygenation has occurred only the oxygenated

Fe(II) heme diester is present, so that  $\varepsilon_{III}c_T = A_{\infty}$  and  $A = (\varepsilon_{II} - \varepsilon_{III})c_{II} + \varepsilon_{III}c_T = \Delta\varepsilon c_{II} + A_{\infty}$ . Rearranging  $(A - A_{\infty})/\Delta\varepsilon = c_{II}$ . Unfortunately,  $\Delta\varepsilon$  has not been calculated for the complexes but it is a constant, so  $A - A_{\infty}$  is directly proportional to  $c_{II}$ . The rate equation is more properly stated as;  $-dc_{II}/dt = k_1c_{II} + k_2c_{II}^2$ , thus if we replace the  $c_{II}$  terms with  $A - A_{\infty}$  and incorporate the  $\Delta\varepsilon$ term into the rate constant one obtains a perfectly valid rate equation of the form;

$$-dA/dt = k_1 (A - A_{\infty}) + k_2 (A - A_{\infty})^2$$

