STUDIES ON HEMIN AND COBALT
CORRINOIDS IN AQUEOUS SOLUTION

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A Thesis submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg
for the Degree of Doctor of Philosophy

December 1980
Declaration

I hereby declare that the work reported in this thesis was carried out exclusively by myself and that this thesis has not been submitted for a degree at any other university.

Vivien Mary Campbell

18th day of December 1980
ACKNOWLEDGEMENTS

I would like to sincerely thank the following people for their assistance:

Professor J.H. Pratt, my supervisor, for his guidance and enthusiasm;

Dr T.A. Baldwin for his advice and encouragement;

Professor R. Hasty for his assistance with the simplex optimization;

Mr A.P. Domleo of Glaxo-Allenbury's (S.A.) (Pty) Limited for samples of vitamin B₁₂a;

The technical staff in the Department of Chemistry for their helpfulness and cooperation;

Mr E. Betterton for proofreading this thesis;

Mrs I. Warner for typing this thesis;

My colleagues for their advice, friendship and tolerance of trials.
To my mother and father
ABSTRACT

Reactions of hemin and cobalt corrinoids have been studied in aqueous solution. The equilibria of hemin in aqueous alkaline solution, in the absence of added ligands, showed five distinct types of complexes (monomers, dimers and polymers) whose spectra fell into two types. These equilibria were independent of pH but dependent on the hemin concentration and ionic strength. The dimerization constant ($\mu = 0.1$) was found to be $> 10^9 \text{M}^{-1}$. The formation of the monomeric hemin-caffeine adduct was confirmed and the complex shown to contain one OH$^-$ ligand. Some detergents were found to form adducts with the dimer, well below the critical micellar concentration ($K \approx 10^5 \text{M}^{-1}$ per mole detergent bound).

Unstable monomeric and dimeric forms of the aquo complex of hemin in aqueous acid were formed by rapid dilution from pH 8 and the equilibrium between these studied (the dimerization constant was $1.1 \times 10^5 \text{M}^{-1}$). Comparison of the spectrum of the monomer with that of hemin in acidic aqueous ethanol, indicates that it is probably a six-coordinate high spin bis-aquo complex ($\lambda_{max} 397 \text{nm}; \varepsilon = 120 \pm 3 \text{mM} \cdot \text{cm}^{-1}$). Since the same spectrum was also observed in very dilute solutions of low ionic strength at $\approx$ pH 7 the pKa for the coordinated water is $> 3$.

The equilibria between hemin and imidazole analogues included adduct formation and aggregation, in addition to that leading to the formation of the bis-ligand complex, but no significant concentration of any monomeric monoligand complex was observed. Quantitative studies with histidine, histamine and pilocarpine, showed the initial formation of an adduct with the dimer, with one
base bound per dimer. At higher concentrations of ligand, the monomeric bis-ligand complexes were obtained. The variation with pH of the overall equilibria from the alkaline hemin dimer indicated that on coordination, the pKa of the pendant -OH of pilocarpine is reduced from 15 to 10 (with a corresponding change, though slight, in the spectrum above and below pH 10) and that of the pendant -NH₂ of histidine and histamine from 7.5 to < 8, which was ascribed to relative stabilisation of the conjugate base by the residual positive charge on the iron.

The reduction of both B₁₂α and bis-histidine hemin by dithiothreitol occurred via an inner sphere electron transfer. The marked difference in rates between the dithiol, dithiothreitol and the monothiols, mercaptoethanol and cysteine where reduction rather than coordination was rate limiting, together with the requirement of a second thiol for the reduction of B₁₂α by cysteine, was ascribed to a one electron reduction of the metal, assisted by the second thiol to give the disulphide radical anion or its conjugate acid.

In the oxidation of thiols by O₂ catalysed by cobalt corrinoids and bis-histidine hemin it was shown that reduction of the metal was rate determining, the rate of O₂ uptake was faster than the rate of reduction of B₁₂α and bis-histidine hemin by the thiol under N₂, and that the disulphide radical anion is a likely reducing agent in the presence of O₂ (where additional paths for its formation are present). The cobalt corrinoids were found to model the suppression of H₂O₂ formation as well as the high turnover number characteristic of cytochrome c oxidase.
The kinetics of the bis-histidine hemin catalysed reactions were complex.

The relevance of these results to the hemoproteins were discussed.
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Hemin (Figure 1.1) is the main, but not only, prosthetic group in hemoproteins\textsuperscript{1-3} and hence, in order to understand the role of the protein in modulating the reactions of the iron porphyrin, the reactions of the protein-free species must be understood.

Figure 1.1. Hemin chloride (Fe(III)) protoporphyrin IX chloride.

The hemoproteins are involved in a variety of reactions\textsuperscript{1-3}. Myoglobin and hemoglobin reversibly bind $O_2$ to store or transport it. The cytochromes transfer electrons. The terminal oxidases
such as cytochrome c oxidase reduce $O_2$ to $H_2O$. The mono-
oxigenases, such as cytochrome P-450, hydroxylate hydrocarbons
(to aid their excretion) by activating $O_2$. The dioxygenases also
do this but add two oxygen atoms. Peroxidase and catalase reduce
and disproportionate $H_2O_2$ respectively. Such diversity and
specificity of the reactions carried out by hemoproteins,
emphasizes the important role of the protein in controlling and
modifying the reactions.

Axial ligands to the iron in hemoproteins so far established
include: two histidines (cytochrome $b_2$)$^4$, one histidine and one
methionine (cytochrome $c$)$^5$, one histidine and one vacant site (or
occupied by $H_2O/OH^-$) (hemoglobin, myoglobin,$^6$ cytochrome $c$
peroxidase)$^7$. It is also generally agreed that cytochrome P-450
has one cysteine coordinated and probably one $H_2O$ (or a vacant
site)$^8$.

Although a particular hemoprotein may contain more than one
iron porphyrin (e.g. four in hemoglobin, catalase) there is no
evidence that they are ever present except as discrete monomeric
complexes.

Although the porphyrin ring is apparently always held in a
hydrophobic cleft in the protein$^4-7$, at least the "vacant"
coordination site (i.e. unoccupied by a protein amino acid residue)
is accessible to solvent and to hydrophilic reagents, i.e. at least
the iron functions in a protic environment while the protein as a
whole is found in an aqueous environment (unless membrane bound).

Hence the mechanism of action of the hemin and the role of
the protein, can only be understood with reference to the coordina-
tion chemistry of monomeric hemin complexes with ligands such as
H$_2$O, OH$^-$, histidine, cysteine and methionine in an aqueous or protic environment.

Yet in spite of the importance of hemin as a co-factor, relatively little is known about its simple coordination chemistry in aqueous solution, in contrast to the extensive study of the coordination chemistry of vitamin B$_{12}$.

Most studies with hemin have been done in non-aqueous solvents, as have many studies on the synthetic iron porphyrins such as iron, α, β, γ, δ-tetraphenyl porphin (FeTPP), to avoid the problems of aggregation which occur in aqueous solution.

The major problems in studying hemin in aqueous solution are:

1) Hemin will only dissolve in aqueous solution at high pH.

2) At pH > 8, it exists mainly as a dimer corresponding to (FeOH)$_2$

3) Hemin forms aggregates and precipitates on reducing the pH below 8. The net result of this is that the coordination chemistry of hemin is limited to pHs above 8. At high pH, OH$^-$ competes with added ligands for the coordination site and the dimeric hydroxo complex is the product. For the ligands studied, this limited the study to pH 8–11.

4) Hemin readily forms adducts through hydrogen bonding, hydrophobic interactions or charge transfer interactions, so that observed changes in the spectrum on adding a potential ligand do not automatically denote coordination of that
A classic case of this is pyridine.

Figure 1.2 Cobalamin: $X = H_2O$ aquocobalamin ($B_{12a}$);

$X = OH^-$ hydroxocobalamin; $X = RS^-$ thiolatocobalamin;

$X = CN^-$ cyanocobalamin (vitamin $B_{12}$).
5) The vinyl side chains are fairly readily oxidised which results in changes in the aggregation properties and hence in non-reproducibility of results. This could be overcome by using fresh solutions or by the storing of the solution at \(-18^\circ C\) under \(N_2\) (see chapter 2).

1.2 Aims
The broad aim of this thesis is to increase our knowledge of the coordination chemistry of monomeric hemin complexes in aqueous solution with particular emphasis on the complexes containing the ligands \(H_2O, NO^-, imidazole, histidine\) and analogues; cysteine and other thiols. The structures, equilibria, redox and catalytic reactions will be investigated with the aim of providing possible answers to the questions concerning the role of the protein in controlling and modifying the equilibria and kinetic (especially redox) properties of these hemin complexes.

Our approach to overcoming some of the above problems are based on three strategies:

1) to establish the nature of the monomeric caffeine adduct, and then to use this as a "stock" monomer in aqueous solution,

2) to investigate the region below \(pH 8\) by stopped flow spectrophotometry.

3) to use \(B_{12a}\) as a model for the mono-histidine complexes present in some hemoproteins.

The existence of some interesting parallels between the iron and cobalt complexes is emphasized by the formation of a functionally active \(O_2\)-carrying "coboglobin" by substituting the \(Fe(II)\) porphyrin by the \(Co(II)\) analogue in hemoglobin, which retains
the cooperative interaction between the four polypeptide subunits. It is known that the Co(II) derivative of B_{12a} reversibly binds O_2 at low temperatures. The use of B_{12a} and other cobalt corrinoids allows the reactions to be studied in an aqueous environment from pH < 0 to pH > 13, and may provide useful pointers to reactions which are experimentally difficult or impossible to study with the protein-free hemin complexes.

The more specific aims are thus to build up our knowledge of the coordination chemistry of hemin (and to a lesser extent the cobalt corrinoids) in aqueous solution by studies in the following four areas which form a progression from equilibria through redox reactions to catalytic activity:

1) the structures and equilibria shown by hemin in the absence of added ligands, with particular emphasis on identifying monomeric complexes with H_2O and OH^- as the only axial ligands (chapters 3 and 4).

2) the structures and equilibria shown by hemin with imidazole, histidine and analogues (chapter 5).

3) the reduction of B_{12a} (chapter 6) and monomeric hemin complexes (chapter 7) by thiols.

4) the catalytic activity of B_{12a} and other corrinoids (chapter 8) and of the bis-histidine hemin complex (chapter 9) in the reduction of O_2 by thiols.

All studies have been done in aqueous solution and most have been done on the Fe(III) complexes. The main techniques used were UV-visible spectrophotometry (ordinary and stopped flow) and kinetic measurements on a Clark type oxygen electrode.
The results will be summarised in chapter 10 and their relevance to the equilibria and reactions of hemoproteins discussed.

1.3 Nomenclature

Hemin consists of iron(III) coordinated to the porphyrin protoporphyrin IX. The proto-prefix indicates that the side chains in the 2,4 positions are vinyl groups (other so-called natural porphyrins have other groups in these positions), while IX indicates the particular structural isomer. Figure 1.1 shows the structure of hemin or iron(III) protoporphyrin IX.

If the oxidation state of the iron is not specified, iron(III) is implied. Heme will be used to denote the Fe(II) protoporphyrin IX.

Ligands can only coordinate in the axial positions and the iron porphyrin can be five or six coordinate. The name reflects the number and nature of the ligands but not the position as the two are not distinguishable, e.g. bis-imidazole iron(III) protoporphyrin IX. However, if the solvent provides the ligand, the ligand is often not included in the name.

Certain cobalt corrinoids are used in this study. The major one used is B_{12a} or aquocyanocobalamín (figure 1.2). Also used is diaquocobinamide. Cobinamides differ from the cobalamins in that the bensimidazole base has been removed by hydrolysis of the phosphate linkage.

1.4 Literature Survey

1.4.1 Spin state and coordination numbers

Iron(III) has five d electrons which can either give a high spin complex in which case all five are unpaired or a low spin complex.
in which case only one is unpaired. Many iron(III) complexes are high spin except those coordinated to strong field ligands such as \( \text{ON}^- \). However, the coordination of porphyrin to iron(III), enables both high and low spin complexes to be formed depending on the axial ligands. Ligands such as pyridine, imidazole and of course cyanide, give low spin complexes while weaker field ligands such as \( \text{OH}^- \), \( \text{Cl}^- \) give high spin complexes.

Until recently it was believed that all the high spin complexes were five coordinate with the iron out of the ring plane while the low spin complexes were six coordinate with the iron in the ring plane. However, a crystal structure of a six coordinate high spin complex in which iron is in the ring plane has been reported, where the ligands are weak field ones such as \( \text{H}_2\text{O} \), dimethylsulphoxide (DMSO) and DMF. This was also shown to be the case by NMR. Hence although the low spin complexes are six coordinate, the high spin ones can be five or six coordinate.

The cobalt corrinoids have six d electrons in Co(III) and five in Co(II). Both oxidation states are low spin. The Co(III) corrinoids are generally six coordinate while the Co(II) ones are generally five coordinate.

1.4.2 Aqueous solution chemistry of iron porphyrins

As mentioned previously, aggregation is a major problem in studying the aqueous solution chemistry of hemin.

Before discussing the factors affecting the aggregation of hemin in aqueous solution, the forces holding the iron porphyrins in dimers in aggregates will be briefly discussed.

The interactions responsible can be broken into two groups:
a) coordination to the iron
b) interactions between the rings to give ring-ring dimers.

Two types of dimers held together by coordination to the iron have been proposed. It was suggested that one propionate side chain of one hemin may coordinate to the iron of another but no evidence supporting this has been found.

The other coordination dimer, the μ-oxo dimer in which O²⁻ bridges two high spin Fe(III) porphyrins, resulting in anti-ferromagnetic coupling has been well established but UV-visible, Mössbauer, IR and EPR spectra as well as the magnetic susceptibility indicate that hemin in aqueous alkaline solutions where it is dimeric, does not have a μ-oxo linkage.

The ring-ring dimers may be hydrophobically bonded or may form μ-μ donor-acceptor complexes and both may be present simultaneously.

Hydrophobic bonding has been found with both the 2,4 disubstituted deuterohemins and the 2,4-disubstituted deuteroporphyrins in aqueous solution. Evidence for μ-μ donor-acceptor interactions between the rings of the 2,4 disubstituted deuterohemins has been found at low temperatures in non-aqueous solvents by NMR.

Various factors are known to affect the aggregation of hemin in aqueous solution. Decreasing the pH to less than 8, results in aggregation. High concentrations of electrolytes are known to favour aggregation. In a 1.2M NaCl alkaline solution, the aggregates apparently contain an average of forty-eight hemin units. Adding electrolytes decreased the Soret intensity (by lifting degeneracies or possibly because of optical
heterogeneity) and the magnetic susceptibility (ascribed to the formation of \( \mu \)-oxo linkages).

Micellar detergents have been shown to dissociate the dimers of natural iron porphyrins \(^{13-15}\), but not the synthetic ones \(^{28}\) (which have \( \mu \)-oxo linkages).

The donor, caffeine, has been shown to split the dimer \(^{12}\) and other donors and acceptors have been shown to interact with porphyrins \(^{37-39}\) and iron porphyrins \(^{39-41}\).

1.4.3 **Imidazole complexes (and analogues) of hemin**

Histidine residues, which coordinate through the imidazole ring, are found coordinated to heme in several hemoproteins (see earlier).

Equilibrium studies have been carried out in non-aqueous solvents \(^{42,43}\) as well as in aqueous, mixed aqueous and detergent solutions, mainly using UV-visible spectrophotometry, but NMR has also been used. \(^{43}\) Except at low ligand concentrations \(^{42}\) and with sterically hindered imidazoles, \(^{42}\) where coordination has not been established, the bis-imidazole complex was obtained with no sign of the mono-imidazole complex \(^{42-49}\), i.e. the binding constant for the first imidazole \((K_1)\) is less than that for the second \((K_2)\), because of a spin change on binding the second.

X-ray studies \(^{50}\) provide further evidence in addition to the EPR \(^{51}\) and UV-visible \(^{42,44-49}\) spectra for the bis-imidazole product. Tailed porphyrins, i.e. those in which imidazole is covalently linked to the porphyrin ring via an organic chain of a suitable length to permit coordination of the imidazole, have been synthesized \(^{52}\) to overcome this problem, but in most cases there is no clear-cut evidence for the imidazole being coordinated to
Fe(III) in the absence of other ligands.

Hydrogen bonding is important in these complexes both in solution and in the solid state and evidence for a $\Pi$ complex between imidazole and the porphyrin has been presented. 56

1.4.4 Reduction of heme and cobalt corrinoids by thiols

Reduction of Fe(III) to Fe(II) occurs in the reaction pathway of many hemoproteins, in particular the cytochromes and hence an understanding of the reduction of heme is necessary to understand these hemoproteins.

Reduction studies have been carried out with various reducing agents with iron porphyrins but little work has been done with thiols. Thiols have been studied with heme but largely in trying to model the spectra of cytochrome P450 and in studying the hydroxylation of aniline. The kinetics of the reduction of FeTPPCl in toluene solution in the presence of pyridine by alkyl thiols has been studied and two paramagnetic intermediates were found.

A kinetic study of the reaction between $B_{12}$ and cysteine in aqueous solution has been reported, but was largely concerned with coordination. However, it was reported that the reduction step required a second thiol group and that Co(II) cobalamin was the product.

In the literature are reports of pKa's of thiols and intermediates found on oxidising them.

Thiols may lose a proton with a pKa of 9 to give the thiolate, i.e.

$$\text{RSH} \quad \rightarrow \quad \text{RS}^- + \text{H}^+$$
Table 1.1 lists the pKas of the thiols used.

Table 1.1: pKas of the thiols used in this study

<table>
<thead>
<tr>
<th>Thiols</th>
<th>pKa</th>
<th>Reference</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ethanethiol</td>
<td>10.6</td>
<td>65</td>
<td>CH₃CH₂SH</td>
</tr>
<tr>
<td>cysteine</td>
<td>8.54</td>
<td>65</td>
<td>NH₃⁺(COOH)CHCH₂SH</td>
</tr>
<tr>
<td>mercaptoethanol</td>
<td>9.43</td>
<td>65</td>
<td>HOCH₂CH₂SH</td>
</tr>
<tr>
<td>penicillamine</td>
<td>8.17</td>
<td>65</td>
<td>NH₃⁺(COOH)HC(CH₃)₂</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>9.12</td>
<td>66</td>
<td>SR</td>
</tr>
<tr>
<td></td>
<td>10.15</td>
<td></td>
<td>HOCH₂CH₂SH</td>
</tr>
</tbody>
</table>

E° values are important to reduction and oxidation reactions. However, there is a paucity of information of the E° values of thiols. Thiols in any case tend to undergo one electron transfers to give the thiol radicals, and the E° value for the reaction:

\[ \text{RS}^- \quad \rightarrow \quad \text{RS}^+ + e^- \]

would be of greater relevance.

Evidence has been presented showing that the thiol radicals are actually found as RS⁻SR which has a three electron two centred bond because the rate of the reaction RS' + RS⁻

\[ \rightarrow \text{RS}^- \quad \text{SR} \quad \text{is rapid} \quad (k > 10^9 \text{ M}^{-1} \text{s}^{-1}) \]

This is apparently more stable than the simple radical.

With a dithiol such as dithiothreitol, this species can be formed intramolecularly. In a dithiothreitol this would result in a six membered ring which would confer additional stability.

The radical species also has a pKa. It has been reported as being 5.5 and refers to the reaction (1 - 1)
These radical species have different second order decay rate constants. For dithiothreitol the neutral radical has a rate constant of $1.7 \times 10^9 \text{ M}^{-1} \text{s}^{-1}$ while that for the anionic radical is $1.7 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$. No values have been reported for the other thiols.

1.4.5 Reactions of iron porphyrins and cobalt corrinoids with $O_2$

Hemoproteins, as mentioned earlier, are involved in a variety of reactions with $O_2$ and its reactions with the prosthetic group, $\text{H}eme$ and models is of interest.

Some studies on the oxidation of Fe(II) porphyrins by $O_2$ have been reported. The reactions are generally first order in $O_2$ and apparently both inner and outer sphere mechanisms occur.

Similarly, $O_2$ is known to oxidise cobalt(II) corrinoids. Reducing agents such as quinols and thiols have been shown to accelerate this reaction, by facilitating the more favourable two electron reduction of bound $O_2$.

A few studies have been carried out on the catalysed autoxidations. These include aquocyanocobinamide with various thiols, a Co(II) phthalocyanine with cysteine, and Fe(III) with cysteine.

Molecular oxygen is reduced to water by the transfer of four electrons Figure 1.3 shows the reduction potentials for $O_2$ reduction. The four and two electron transfers are thermodynamically...
callily favourable but the one electron reduction of $O_2$ is not. This may no longer be the case when the oxygen species are bound to a metal, but at least for the uncoordinated species, a mechanism which involves two electron transfers would be favoured over one involving one electron transfer.

\begin{align*}
O_2 & \quad -0.45V \\
H_2O_2 & \quad +0.98V \\
H_2O & \quad +0.38V \\
& \quad +2.32V
\end{align*}

Figure 1.3 The reduction potentials for the one and two electron reductions of $O_2$ at pH 7.

In addition to the free superoxide, peroxide and hydroxyl radicals, the coordinated species may be present but the competition between the metal complex and $H^+$ may destabilize some (the pKas of superoxide and peroxide are 4.9 and 11.62 respectively). Iron porphyrin species found in non-aqueous solvents include the μ-peroxo dimer and the Fe(IV)$O^{2-}$ species, the latter being stabilized by the coordination of a base. The cobalt corrinoids may form μ-peroxo dimers but steric hindrance makes it unlikely.
CHAPTER 2 - MATERIALS AND METHODS

2.1 Materials

Protohaem was supplied by BDH; mesohaem was prepared by a literature method\textsuperscript{20}; cytochrome $b_3$, prepared by a literature method\textsuperscript{80}, was kindly supplied by Dr D. Baldwin; $b_{12a}$ was supplied by Mr A.P. Donleo of Glaxo-Allamba (Pty) Limited; diaquocobinamide was kindly supplied by Mr E. Betterton.

Water was purified by the Millipore Millirol 40 H$_2$O system.

Other solvents used were 95% ethanol (National Chemical Products), methanol (Merck AR), DMSO (Merck AR) (only from freshly opened bottles), formamide (Merck).

The following AR grade reagents were used: CH$_3$COOH (Merck), NaHCO$_3$ (Hopkins and Williams), borax (Merck), glycine (Merck), potassium hydrogen phthalate (Merck), HNO$_3$ (nitric acid), NaH$_2$PO$_4$ (Merck), Na$_2$HPO$_4$ (Merck), NaNO$_3$ (Merck), KNO$_3$ (Merck), NaCl (Proteas), KCN (BDH), L-arginine (Merck), L-tyrosine (Merck), 1,10 phenanthroline (Merck), methyl violagen (Merck), urea (Merck), sucrose (Merck), CuSO$_4$ (Merck), imidazole (BDH), N-methyl imidazole (Merck), 2-methyl imidazole (Merck), L-histidine, histamine dihydrochloride (BDH), pilocarpinium nitrate (Merck), L-cysteine hydrochloride (BDH), D(-)-penicillamine (Aldrich).

Other reagents used were NaOH (Proteas, > 97%), lithium nitrate (Merck), cetyl trimethyl ammonium bromide (BDH), triton X-100 (BDH), sodium laurel sulphate (BDH), caffeine (Merck), adamine (Merck), theophylline (Merck), guanidine hydrochloride (BDH), sodium silicate (BDH), dithiothreitol (BDH, > 95%), ethanethiol (Merck), 2-mercaptoethanol (Schuchardt; > 95%, redistilled under vacuum just prior to use), sodium dithionite (Hopkins and Williams), superoxide dismutase
from beef erythrocytes (supplied as a powder by Miles Research Laboratories), catalase from bovine liver (supplied by Miles as a crystalline suspension in a phosphate buffer at pH 5.7 containing 565109 units per millilitre (one unit of activity is that amount of enzyme catalysing the decomposition of 1 μ mole of H₂O₂ per minute at 25°C), N₂ was deoxygenated by passing it through a v2+ solution.

2.2 Instrumentation

Ultraviolet-visible absorption spectra were recorded on a Jasco Uv10 spectrophotometer, which was calibrated with holmium glass as a standard. Unless otherwise stated, quartz cells of path length 10 mm were used and thermostatted at 25°C (± 0.5°C).

The kinetics of faster reactions and the determination of certain pKas (see later) were studied using a Durrum D-110 stopped flow system connected to a Datalab DL 901 Transient recorder and an Apple microcomputer which was programmed to accept the data points, subtract out the value at infinite time, plot the semilog plot and carry out a least squares fit. If required, the program converted the voltage output into absorbance. The solutions were thermostatted at 25°C (± 0.2). The rate of O₂ uptake was studied using a Clark type oxygen electrode made by Rank Bros, Cambridge, England (Figure 2.1) connected to a Servogor recorder. The maximum recorder deflection was set manually using O₂ saturated or air saturated water (where applicable) as standards. The zero setting was set using water depleted of O₂ by the addition of sodium dithionite. Sodium dithionite was also used to check the membrane; a good membrane shows depletion of most of the O₂ (i.e. > 80%) in
Figure 2.1. Diagram of Anode-Reduction Oxygen Electrode
solution within ten seconds after adding dithionite, while a leaky one does not and was discarded. The \( O_2 \) content in air and \( O_2 \) saturated water was determined by titrating the \( O_2 \) with glucose in the presence of the enzyme glucose oxidase, using the oxygen electrode. The \( O_2 \) saturated solution (obtained by passing pure \( O_2 \) through the solution for at least ten minutes) was found to have \( 8.7 \times 10^{-4} \text{M} \) \( O_2 \) while the air saturated solution was found to have \( 2.2 \times 10^{-4} \text{M} \) \( O_2 \).

The oxygen electrode consists of three sections (see figure 2.1), the test solution compartment (and capillary cap), the base section containing the \( \text{Pt}/O_2 \) and \( \text{Ag}/\text{AgCl} \) half cells connected by a saturated KCl solution and the stirrer base. The dissolved \( O_2 \) in the test solution diffuses through the teflon film (300 microns thick) onto the polarised Pt electrode where it becomes reduced (equations (2.1); (2.2)) consuming four Faradays per mole of \( O_2 \):

\[
\begin{align*}
\text{O}_2 + 2\text{e}^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 \quad (2.1) \\
\text{H}_2\text{O}_2 + 2\text{e}^- + 2\text{H}^+ & \rightarrow 2\text{H}_2\text{O} \quad (2.2)
\end{align*}
\]

The current produced causes the recorder to deflect and is proportional to the oxygen concentration in the solution (as is the deflection).

The test compartment was sealed from the atmosphere by the capillary cap which, however, allows injection of samples into the system. It was surrounded by a water jacket through which water at 25\(^\circ\)C was pumped. The solution was kept well mixed by a small magnetic stirrer.

In all cases, the reaction was initiated by injecting the thiol solution, through the capillary opening in the cap. In some cases the cobalt corrinoid or iron porphyrin was added before the thiol, while in others it was added after (this enabled the uncatalysed
rate to be determined). Care was taken to exclude all air bubbles from the test compartment when the cap was inserted as these cause erratic results and to ensure that the capillary tube was filled with solution to avoid $O_2$ from the atmosphere entering. The stirring speed was made sufficient to avoid problems with rate limiting mass transport. If injection of reagents was done too rapidly or too near the base, a sudden decrease in the readout occurred due to a rapid depletion of $O_2$ at the base of the cell and this caused problems in measuring the initial slope. Hence care was taken in injection of the reagents.

The pH of solutions was measured with a Metrohm digital E 532 pH meter and a Metrohm glass electrode or for recording of small samples, a Metrohm glass microelectrode. The reference electrolyte was 3M potassium chloride. The pH meter was standardized using BDH standard buffers at pH 7,0 ± 0,1 (phosphate) and pH 4,0 ± 0,1 (phthalate).

2.3 Methods

2.3.1 Preparation of solutions

Hemin in solution is slowly attacked by air. To minimize this, hemin was dissolved in a 0,1M sodium hydroxide solution which had been thoroughly flushed with $N_2$ and the solution kept under $N_2$. It was found that hemin stored under $N_2$ and kept at $-18^\circ C$ was stable (i.e. < 3% change in absorbance) for at least two weeks. The hemin solutions were calibrated by measuring the absorbance at 385 nm in 0,1M NaOH (the position of the Soret band of the dimeric alkaline hemin) and using the extinction coefficient of 58 mm$^{-1}$ Fe.

Buffers were prepared according to the instructions in "Biochemist's Handbook". In all cases, the ionic strength con-
tribution from the buffer was 0.1. In general $\text{HNO}_3 + \text{NaNO}_3$ was used for pHs 1 - 2; glycine + HNO$_3$ for pHs 2.2 - 3.4; acetic acid + NaOH for pHs 3.6 - 5.8; Na$_2$HPO$_4$ + NaOH for pHs 6.0 - 7.8 and NaHCO$_3$ + NaOH for pHs 8.0 - 11.0; Na$_2$HPO$_4$ + NaOH for pHs 11.0-12.0; NaOH for pHs $>12.0$. Halate buffers gave unreliable results when used in the determination of the pKa's of hemin as they decreased the Soret intensity, perhaps due to aggregation or donor-acceptor complex formation; hence they were avoided.

Caffeine is hydrolysed in basic solution. By following the decrease in the band at 273 nm, the rate of decomposition could be followed. At pH 13, 8% of the caffeine had decomposed after two hours while at pH $<12.3$, less than 1% decomposition had occurred after three hours. Hence to avoid problems with hydrolysis, only freshly made up solutions with pH $<12.3$ were used and used within three hours.

Fairly high concentrations of histidine, histamine and pilocarpine were necessary for complete formation of the bis ligand hemin complex. As these species are charged, they contribute to the ionic strength (it was assumed, however, that the zwitterion does not), and for this reason these solutions had fairly high ionic strengths. For consistency, they were all made 0.5 by adding NaNO$_3$ where necessary.

Histidine goes yellow with age, presumably undergoing a photo-oxidation reaction. To minimise this, solutions were used within two hours when the yellow colour was negligible or were well-protected from light. This yellow species did not affect the equilibrium constant determinations but resulted in scattered reduction and $O_2$ uptake kinetic results.
The lactone ring of pilocarpine can be hydrolysed within a few minutes in 0.1M NaOH. The solutions of pilocarpine were therefore made up by dissolving pilocarpine in 0.1M NaOH and leaving to stand for at least an hour before the pH was adjusted to the required value.

As thiols are air-sensitive, they were made up in solutions which had been deaerated by thorough flushing with N₂ and were kept under N₂.

2.3.2 Determination of equilibrium constants

With the exception of the determination of pKₐs of hemin, equilibrium constants were determined by following the change in absorbance with a change in ligand concentration or with a change in the hemin concentration (Shack and Clark dilution studies).

When a sufficiently concentrated stock solution of ligand could be made up, the concentration of ligand was increased by adding aliquots of the stock solution, as in titrations with caffeine, detergent and pilocarpine. This method could not be used, however, if side reactions occurred (as with histamine) or if the ligand had limited solubility (as with histidine); in these cases, each ligand solution required was made up separately.

In most cases the hemin concentration was varied by adding aliquots of the stock hemin solution to the ligand solution, taking care not to change the total volume and hence the ligand concentration by more than 3%. With histamine this was not possible, and each hemin solution required was made up separately.

The results were analysed using the equations derived in appendix 1.

The titration at low ligand concentrations (where the ligands
were histidine, histamine and pilocarpine) were carried out at 390 nm (the position of an isosbestic point between the intermediate and bis-ligand complex) by adding aliquots of the stock ligand solution (in this case the intermediate hemin-histamine complex is stable). Fairly high hemin concentrations (\(1\times10^{-4}\)M) were used to give sufficiently large absorbance changes.

2.3.3 Determination of the pKas of hemin and hemin-caffeine by stopped-flow UV-visible spectrophotometry

Hemin and the hemin-caffeine adduct aggregate rapidly on acidification and stopped flow UV-visible spectrophotometry was used to determine the absorbance at a particular pH after acidification (proton transfers are rapid) but before aggregation.

One barrel of the stopped flow contained approximately 5\(\times10^{-6}\) M hemin (plus caffeine where relevant) (unbuffered, pH was \(\approx 9\)) while the other contained buffer such that when it was diluted twofold, it gave the required pH and ionic strength (0.1) (i.e. used double concentrations of the buffer components).

Aggregation was evident by a decrease in the Soret band (all runs were done at or near the Soret maximum) and the absorbance was taken prior to the decrease. Only in slightly acid solutions did the decrease start shortly after the mixing time (1 ms). (At other pHs the decrease, where it occurred, occurred more slowly.)

In the absence of caffeine, hemin tended to adsorb onto the syringe surfaces. Certain runs were repeated throughout the experiment to enable the correction of absorbances for the change in concentration.

Absorbances were determined at a fixed wavelength (402 nm in the presence of caffeine; 397 nm in the absence of caffeine) at
varying pHs and the results treated using the equations derived in appendix 1 to give the pKas.

The spectrum of the transient intermediates was built up by determining the absorbance by stopped flow spectrophotometry at various wavelengths (re-zeroing at each) in the Soret region.
CHAPTER 3 - STUDIES OF HEMIN IN AQUEOUS ALKALINE SOLUTION

3.1 Introduction
As hemoproteins operate in aqueous solution it would be useful to study the prosthetic group, hemin, in aqueous solution, particularly as pH effects for instance are only meaningful in aqueous solution.

Most aqueous solution studies of hemin have been done at pH > 8 because of aggregation and precipitation below this pH. It is generally agreed that the main species in aqueous alkali is a dimeric species in which all the propionic acid side chains are ionised and in which each iron has one OH\(^-\) (or its equivalent) coordinated.

There is evidence for the formation of high molecular weight aggregates at high hemin concentrations\(^{10,36}\) and in solutions of high ionic strength.\(^{36}\) Blauer and Zvilichovsky\(^{36}\) found that \(4 \times 10^{-4}\) M hemin in 1.2 M sodium chloride at pH 11-12, aggregates and the species formed contained as many as fifty hemin units. However, in the absence of NaCl, hemin did not sediment in the ultra centrifuge\(^{36}\) and hence it will be assumed that at fairly low concentrations of hemin and in solutions of moderate ionic strength, hemin is dimeric. Certainly it behaves as a dimer.\(^{10}\)

It is well known that aqueous alkaline solutions of hemin may show surprising variations in the UV-visible spectra and magnetic susceptibilities, and monomers,\(^{12,15-15}\) dimers and\(^{10}\) polymers\(^{10,36}\) have all been identified.

However, there are gaps in our knowledge. Caffeine is known to split the alkaline hemin dimer to give the monomer\(^{12}\) but the pH dependence has not been studied and hence the product has not
beaten fully identified.

Sucrose is known to affect the magnetic susceptibility\textsuperscript{85} but the effect of sucrose on the spectrum has not been studied.

It is known that salts affect the aggregation of hemin\textsuperscript{34-36} but the effectiveness of different anions and cations compared to their ability to order water is not known.

The aims of this chapter are to improve our understanding of the nature of the complexes and equilibria observed in aqueous alkali as well as to investigate the conditions required for monomer formation in aqueous alkaline solution, in order to model hemoproteins which usually contain isolated hemin. To this end quantitative studies on caffeine and detergents as well as qualitative studies on salts, sucrose and donors/acceptors have been carried out.

Studies in aqueous acid will be reported in Chapter 4.

The following abbreviations will be used in this chapter:

\begin{itemize}
  \item[$M$] for monomer
  \item[$D$] for dimer
  \item[$P$] for polymer
\end{itemize}

Each of these may have a subscript $A$ or $B$. These refer to spectral types, the $A$ type spectrum being the typical high spin spectra\textsuperscript{87} while the $B$ type being analogous to the $\mu$-oxo spectrum.\textsuperscript{26}

3.2 Results

3.2.1 Beer's law plot of hemin

The Soret band for hemin in 0.1M NaOH is at 385 nm. Hence this wavelength was chosen to see if any deviations from Beer's law at low hemin concentrations occur, as this would indicate the formation of the monomer.
A plot of absorbance versus hemin concentration (in 0.1M NaOH to eliminate any ionizations of groups on the hemin) was linear down to $1 \times 10^{-7}$ M hemin (on a per mole iron basis) (figure 3.1)

![Absorbance plot](image)

Figure 3.1 Beer's law plot of hemin in 0.1M NaOH; $\ell = 10$ cm, 25°C

The slope was $5.8 \times 10^{-6}$ M$^{-1}$. As the pathlength was 10 cm, the extinction coefficient was $58 \times 10^{3}$ M$^{-1}$ cm$^{-1}$ (per mole Fe). This is in agreement with earlier results $34$ ($62 \times 10^{3}$ M$^{-1}$ cm$^{-1}$ at 385 nm) at low ionic strength.

The linearity of the plot indicates that monomerization is not occurring to any extent down to at least $1 \times 10^{-7}$ M. At $1.2 \times 10^{-7}$ M hemin the absorbance at 385 nm was $0.069 \pm 0.003$. Taking this error into account, the monomer concentration is $< 7 \times 10^{-9}$ M.
Hence the equilibrium constant for the equation \((3 - 1)\),

\[
2M \rightleftharpoons D
\]  

\(K_D\) is equal to \(1 \times 10^9 \text{ M}^{-1}\). It must be pointed out that \(K_D\) will vary with ionic strength.

It is difficult to compare this value with that previously reported \((4,5)\) as the latter equilibrium involved the loss of a proton on dimerization.

However, using the value reported for the \(pK_a\) of the dimer \((7,5)\), \(K_D\) is calculated to be \(1.4 \times 10^8 \text{ M}^{-1}\). The discrepancy between these values may be a consequence of this latter study being largely carried out at pHs near the precipitation point where higher aggregates are probably present, and hence may be the equilibrium constant for polymerization.

These values are greater than that of \(3.1 \times 10^6 \text{ M}^{-1}\) reported for protoporphyrin, showing that the presence of Fe(III) in the ring enhances dimerization.

1.2.2 **Effect of salts and sucrose**

It has been reported that the addition of electrolytes to hemin results in a decrease in the Soret band as well as the development of a band around 530 nm. These changes have been correlated with aggregation of hemin and are \(pH\) independent between \(pH\) 9 and 13.

Figure 3.2 shows the change in spectrum quite clearly (i.e., the A-type spectrum is converted to a B-type) and confirms the effect of ionic strength, in causing these changes.
Figure 3.2 Effect of the ionic strength on the spectrum of hemin at pH12; 2.26 x 10^-5 M hemin; 25°C; λ = 1 nm.

The formation of the 580 nm band increases with hemin concentration (figure 3.3) which supports the proposal that aggregation occurs.
Figure 3.3 Effect of the hemin concentration on the visible spectrum at pH12; 0,5M NaNO₃; 25°C; l = 1 cm.

--- 22,6 μM hemin (2 x scale); ····· 90,4 μM hemin (0,5 x scale); --- 181 μM hemin (0,25 x scale).

Different salts affect the spectrum to different extents.

This is shown in figure 3.4.
Figure 3.4 Effect of different cations on the spectrum of hemin at pH12; 2.26 x 10^{-5} M hemin; 25°C; λ = 1 cm.

Li⁺ is more effective than K⁺ in decreasing the Soret and visible region.

Table 3.1 shows the effect of different salts and different ionic strengths on the Soret intensity.
Table 3.1: Effect of different salts on the Soret band of hemin in alkaline aqueous solution

<table>
<thead>
<tr>
<th>System</th>
<th>Soret  ( \lambda_{\text{max}}/\text{nm} )</th>
<th>( \varepsilon/(\text{mMFe}^{-1}\text{cm}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 10^{-4} \text{ M NaOH} )</td>
<td>387</td>
<td>(61.3)</td>
</tr>
<tr>
<td>( 10^{-2} \text{ M NaOH} )</td>
<td>385</td>
<td>(59.6)</td>
</tr>
<tr>
<td>( 10^{-1} \text{ M NaOH} )</td>
<td>385</td>
<td>(59.0)</td>
</tr>
<tr>
<td>( 0.5\text{ M LiNO}_3; 10^{-2}\text{ M NaOH} )</td>
<td>385</td>
<td>(52.1)</td>
</tr>
<tr>
<td>( 0.5\text{ M NaNO}_3; 10^{-2}\text{ M NaOH} )</td>
<td>385</td>
<td>(56.8)</td>
</tr>
<tr>
<td>( 0.5\text{ M KNO}_3; 10^{-2}\text{ M NaOH} )</td>
<td>385</td>
<td>(57.4)</td>
</tr>
<tr>
<td>( 2\text{ M NaNO}_3; 10^{-2}\text{ M NaOH} )</td>
<td>385</td>
<td>(45.0)</td>
</tr>
<tr>
<td>( 0.5\text{ M NaCl} )</td>
<td>385</td>
<td>(55.2)</td>
</tr>
</tbody>
</table>

In agreement with figure 3.2, the decrease in the Soret intensity (but not position) increases with ionic strength. There is a shift in the Soret position at very low ionic strength which is reproducible and may be the consequence of a change in the dimer structure because the carboxylates are no longer shielded from each other. It can also be seen that the order of effectiveness of ions in decreasing the Soret is Li\(^+\) > Na\(^+\) > K\(^+\) and NO\(_3\)\(^-\) > Cl\(^-\). This is also the order of the abilities of these ions to increase the structure of water\(^8\) and hence the order of increasing hydrophobic bonding to minimise the unfavourable entropy of solvation. This suggests that the further aggregation shown earlier,\(^{24-26}\) results at least in part, from hydrophobic interactions.

Sucrose has been shown to increase the magnetic moment of high concentrations of hemin in alkaline solution from 3.6 to
5,6 B N, but its effect on the spectrum has not been reported. It was found that 30% sucrose added to hemin in 2M NaNO₃ at pH 12, converted the B type spectrum to an A type, i.e. a µ-oxo type to a typical high spin type. Hence sucrose counteracts the electrolytes and displaces the equilibrium in favour of the dimer (equation 3-2)

\[ \text{electrolytes} \xrightarrow{n} \text{D}_{A} \xleftarrow{\text{sucrose}} \text{P}_{B} \]  

This shift in equilibrium explains the effect of sucrose on magnetic susceptibility particularly as A type species have high magnetic moments while B type species have low magnetic moments. (See later.)

The effect of electrolytes in promoting aggregation can be attributed to their shielding like charges from each other in the formation of the aggregate. The effect of sucrose may reverse the formation of the aggregate by disrupting the structure of water and thus disfavouring hydrophobic interactions.

3.2.3 Effect of caffeine

Caffeine is the heterocyclic compound shown in figure 3.5. It has no pKₐ's in the alkaline region (it has one of <1).

![Figure 3.5 Caffeine](image_url)
Figure 3.6 shows the spectral changes occurring on adding caffeine to hemin at pH 12.3.

The major changes are shifts in the Soret band to 402 nm and the visible band to 506 nm, with isosbestic points at 392 nm and 642 nm, which indicates an equilibrium between two species. Both initial and final spectra are A types. The same spectral changes were observed by Gallagher and Elliott.
3.2.3.1 Quantitative studies

The quantitative studies were limited to pHs between 8 and 12.3. Below pH 8, hemin aggregates, while above 12.3 caffeine is hydrolysed (see Chapter 2). Changes in the absorbance at 400 nm were monitored when the caffeine concentration and hemin concentration were independently changed. All experiments were carried out at 25°C.

Experiments in which the caffeine concentration was varied (at a constant hemin concentration), were performed at pH 8.50 and pH 12.00. Galloway and Elliott showed that reaction (3 - 3) was occurring.

\[ D + 2 \text{cafeine} \rightarrow 2 \text{M-cafeine (K_C)} \]  

(3 - 3)

It can be shown (see appendix 1) that if this reaction is occurring then a plot of

\[ \log \left( \frac{A - A_0}{A} \right)^2 = \frac{2 \sum [\text{cafeine} \text{free}] \text{TOT}}{A_0 - A} \]

versus log [cafeine free] should be linear with a slope of two

\( A, A_0, A_m \) are absorbances at a particular caffeine concentration; in the absence of caffeine; for the fully formed hemin-caffeine adduct respectively; \( [\text{cafeine} \text{free}] \text{TOT} \) is the total hemin concentration (on a per mole iron basis).

This was found to be the case at both pHs (see figures 3.7a and b; tables 1a, b in appendix 2).
Figure 3.7a Analysis of the titration of hemin with caffeine results at pH 8.50 and 12.0; 1 x 10^-5 M hemin;  
\( u = 0.1; 25^\circ C \)
The equilibrium constant, $K_c$, for this reaction was found to be $5.4 \ M^{-1}$ and $6.1 \ M^{-1}$ at pH 8.50 and pH 12.00 respectively.

Experiments in which the hemin concentration was varied (at a constant caffeine concentration) were carried out at pH 8.50; pH 10.00 and pH 12.00. If reaction (3-3) is occurring then a plot of $\log \alpha \ [Fe]_{TOT}$ versus $\log (1 - \alpha) \ [Fe]_{TOT}$ should be linear with a slope of 0.5 (see derivation in appendix 1) (where $\alpha$ is the fraction of the hemin which has reacted; $[Fe]_{TOT}$ is the total...
hemin concentration (on a per mole iron basis)). This was in fact true for all pHs as long as the hemin concentration did not exceed $2 \times 10^{-5}$ M. (See table 2a, b, c in appendix 2; Figures 3, 8 a, b, c.) The equilibrium constant for this reaction, $K_c$, was found to be $5.1 \text{ M}^{-1}$; $5.8 \text{ M}^{-1}$; $5.7 \text{ M}^{-1}$ at pH 8.50; pH 10.00; pH 12.30 respectively.

\[ \log[\text{Fe}]_{\text{TOT}} \]

![Graph a) pH 8.50](image)

![Graph b) pH 10.00](image)
Figure 3.8 Analysis of data obtained by varying the hemin concentration at a constant caffeine concentration, at pH 8.50; 10.00 and 12.30; µ = 0.1; 25°C.

It can be seen that the equilibrium constant, $K_0$, calculated from experiments where the caffeine concentration and hemin concentration are varied, are the same within experimental error.

More interesting, the equilibrium constant doesn't vary with pH between pH 8.5 and 12.3. As the starting species contained one OH$^-$ → iron, the final monomeric hemin-caffeine adduct must...
Gallagher and Elliott ascribed the relatively low \( \Delta G \) and high \( \Delta G' \) as they found for the dissociation of the hemin-caffeine adduct to hydrophobic bonding between the hemin and caffeine.\(^{12}\) As caffeine has been shown to form a donor-acceptor complex with hemin in CDCl\(_3\),\(^{41}\) it is probably fair to assume that this type of interaction occurs in aqueous solution as well.

The average value of the equilibrium constant in reaction (3 - 3) is 5.6 \( M^{-1} \) (25°C) which compares well with that found at 28° (6.25 \( M^{-1} \)) by Gallagher and Elliott.\(^{12}\)

Reaction (3 - 3) can be formally separated into two steps, splitting the monomer (3 - 4) and binding caffeine (3 - 5).

\[
\begin{align*}
D & \rightarrow 2M & K_D^{-1} & (3 - 4) \\
M + \text{caffeine} & \rightarrow M-\text{caffeine} & K_{\text{caff}} & (3 - 5)
\end{align*}
\]

\[K_C = K_D^{-1} (K_{\text{caff}})^2\]

Using the values of 5.6 \( M^{-1} \) for \( K_C \) and \( > 10^6 M^{-1} \) for \( K_D \) (3.2.1), \( K_{\text{caff}} \) was found to be \( > 7.5 \times 10^6 M^{-1} \). This value is at least forty times greater than that found for the reaction between caffeine and monomeric hematoporphyrin.\(^{38}\) This difference is similar to that found for \( K_D^1 \) (reaction 3 - 6) for proto- and hemochromin.

\[
2M \rightarrow D + H^+ & & K_D^1 & (3 - 6)
\]

\( K_D^1 \)'s were found to be 4.5\(^{31a}\) and 1.0 \( \times 10^{-2} \)\(^{31f}\) respectively at about pH 7. This suggests that similar types of interactions occur in both the dimer and the caffeine adducts. As discussed above these are likely to include hydrophobic as well as H-H interactions. The more extended \( H \) system as well as the greater
hydrophobicity of vinyl in protohemin compared with hydroxyethyl in hematoporphyrin would then be responsible for the greater binding constant of caffeine to hemin. Hydrogen bonding can be excluded as a major interaction as this would be greater with hematoporphyrin than with hemin.

3.2.3.2 Analogous systems to caffeine
Within experimental error, there was no change in the spectrum of bis-cyanohemin on adding caffeine, showing that if caffeine does bind, the spectral changes observed in figure 3.6 largely arise from splitting the dimer (bis-cyanohemin is monomeric). Only slight changes in the intensities, but not positions of the UV-visible bands, observed on adding caffeine to monomeric hemin in ethanol, formamide and DMSO, also show that the major spectral change results from splitting the dimer.

Similar spectral changes to those observed with hemin (figure 3.6) were found on titrating mesohemin with caffeine, under analogous conditions. Hence, the vinyl group is not essential for this interaction.

Most donors/acceptors added to hemin decreased and shifted the Soret to the red. These include adenine (λmax 400 nm), o-phenanthroline (λmax 406 nm), methyl viologen, arginine at pH 8.3 (λmax 390 nm), theophylline (λmax 408 nm). Tyrosine (at pH 8.5) however, shifted the Soret to 374 nm and increased its intensity slightly while guanidine split the Soret into two bands of roughly equal intensity at 356 nm and 394 nm, and decreased its intensity.

With the exception of phenanthroline and methyl viologen which gave B type spectra, there was little change in the visible region
on adding the above species.

These effects are varied and could be reflecting the relative contributions of hydrophobic, donor-acceptor and possibly hydrogen bonding interactions. The effects of phenanthroline and in the visible region, methyl viologen (which absorbs in the Soret region) are similar to those observed with pyridinium salts.

It must be pointed out that arginine and tyrosine do not bind at pH 13 but do at pH 8.5 which is below the pKₐ of the amino group. This could indicate a coulombic effect as found by Mohr and Scheler and attributed to an interaction between the cation and the propionates.

The effects of caffeine, adenine and theophylline are contrary to the report that these have no effect on hemin.

3.2.3.3 Effect of the hemin concentration on the hemin-caffeine adduct.

It was observed that the reaction (3 - 3) could not explain the results if the hemin concentration exceeded 2 x 10⁻⁵ M, and calculating the extinction coefficient at 402 nm from Gallagher and Elliott's spectra, gave values between 74 and 80 x 10³ M⁻¹ cm⁻¹, with the value decreasing with hemin concentration. Hence, the spectra of hemin in an excess of caffeine, with increasing concentrations of hemin were determined (see figure 3.9).
Figure 3.9 Variation in the spectrum of heroin in 0.1 M caffeine with heroin concentration; pH 10.0, T = 0.1, 25°C.
The changes in the visible region are most marked with the appearance of the band at 575 nm which becomes relatively more intense as the hemin concentration increases. At high concentrations of hemin there are bands at 575 nm, 600 nm and shoulders at 630 nm and 620 nm. These are likely to be vibrational overtones. There is no change in shape or position of the Soret although the extinction coefficient does decrease.

Beer's law at 402 nm was not obeyed above $1.5 \times 10^{-5}$ M hemin (Figure 3.10).

![Beer's Law Plot](image)

To avoid this further reaction, (which is some kind of aggregation or dimerization of the monomeric hemin-caffeine adduct, as it depends on the hemin concentration) experiments were generally done with $< 1.5 \times 10^{-5}$ M hemin. At low hemin concentrations $\varepsilon_{402}$ was found to be $81.1 \times 10^3$ M$^{-1}$ cm$^{-1}$ for hemin caffeine.

The Beer's law plot at 575 nm showed a positive deviation while that at 600 nm showed a negative deviation above...
1.5 \times 10^{-4} \text{ M hemin. The respective extinction coefficients at low hemin concentrations are } 6.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \text{ and } 6.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}.

Unfortunately, a stage is not reached where the extinction coefficients become constant at high hemin concentrations, so this equilibrium cannot be quantitatively studied.

3.2.4 Effect of detergents

3.2.4.1 Qualitative studies

Simplicio and co-workers showed, using equilibrium and kinetic studies, that micellar detergents split the dimer to give monomers intercalated within the detergent micelle.\textsuperscript{11,16} They showed that as the detergent concentration was increased, the absorbance of the detergent monomer Soret increased until a limiting value was reached. This was found to be reproducible using the detergent cetyletrimethyl ammonium bromide (CTMAB) in 0.1 M NaOH at 400 nm. However, following the alkaline hemin dimer Soret (385 nm) a decrease in the absorbance followed by an increase was observed, with signs of a limiting value being approached. (See Figure 3.11.)

The minimum in absorbance occurred at about 1 \times 10^{-4} \text{ M CTMAB}. By comparison, the critical micellar concentration (cmc) of CTMAB in water is 1 \times 10^{-3} \text{ M}.\textsuperscript{20} The fairly similar values suggest that the increase occurs because of incorporation of the hemin monomer into the micelle, bearing in mind that salts generally decrease the cmc and that micelles are found below the cmc which is an average value. The decrease suggests a different kind of interaction with the detergent and has been studied quantitatively. (See later.)
Figure 3.11  Change in the \( A_{385} \) of the alkaline hemin dimer with detergent concentration.

It was found that at low detergent concentrations a limiting spectrum was found, and this enabled the quantitative study to be carried out. The limiting spectra in low and high concentrations of CTMAB are shown in Figure 3.12a.

Both low and high concentrations gave B type spectra with the major differences being in the Soret region.
Figure 13.2a Spectra of hemin in low and high concentrations of CTMAB; 0.1 M NaOH; 25°C; 0.65 x 10^-5 M OH

---- CM CTMAB; --- 3 x 10^-5 M CTMAB;

**** 3% (0.082 M) CTMAB.

The UV-visible bands obtained at high and low CTMAB concentrations are reported in table 3.2 together with those of the μ-oxo dimer in benzene for comparison.
Table 3.2  Extinction coefficients for the UV-visible bands of hemin in a) $30 \times 10^{-6}$ M CTMAB; b) 4% CTMAB (0.11 M) c) $\mu$-oxo dimer

<table>
<thead>
<tr>
<th>Band</th>
<th>$30 \times 10^{-6}$ M CTMAB</th>
<th>4% CTMAB (0.11 M)</th>
<th>$\mu$-oxo dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda_{max}$ (emM $\lambda_{max}$)</td>
<td>$\lambda_{max}$ (emM $\lambda_{max}$)</td>
<td>$\lambda_{max}$ (emM $\lambda_{max}$)</td>
</tr>
<tr>
<td>Soret</td>
<td>390 nm (49.8)</td>
<td>399 nm (66)</td>
<td>397 nm (58)</td>
</tr>
<tr>
<td>$\beta$</td>
<td>568 nm (6.8)</td>
<td>573 nm (8.4)</td>
<td>573 nm (7.0)</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>600 nm (5.9)</td>
<td>597 nm (8.5)</td>
<td>599 nm (6.9)</td>
</tr>
</tbody>
</table>

* expressed on a per mole iron basis.

There are similarities in these spectra. The low detergent species could be a $\mu$-oxo dimer but not the high detergent species as this is monomeric.

Similar spectral changes were observed for triton X-100 (TX) but not sodium lauryl sulphate (SLS) (see figure 3.12b). Hence SLS gives an A type spectrum while CTMAB and TX both give a B type spectrum at both low and high concentrations.

At high detergent concentrations, the absorbance of the Soret band of the detergent monomer decreases with time. This was found to some extent at all pHs with SLS, TX, Tween 80, lauryl pyridinium chloride and mesohemin. It could not be reversed by the ion of KCN which indicates decomposition rather than dimerization. Ways in which this effect could be minimised were by working at low detergent concentrations, high hemin concentrations where the relative
Figure 3.12b Spectra of hemin in low and high concentrations of SLS; 0.1 M NaOH; 25°C; 8.65 x 10^-5 M hemin.

- O M SLS; 1.2 x 10^-4 M SLS; ••• 4% (0.14 M) SLS
change in absorbance was small or running spectra as soon as possible after mixing.

3.2.4.2. Quantitative studies

The titration of hemin with CTMAB, TX and QLS to give the low detergent species, showed isosbestic points (figure 3.13).

\[
D + nL \leftrightarrow DL_n \quad (3 - 7)
\]

\[
D + 2nL \leftrightarrow 2DL_n \quad (3 - 8)
\]
If reaction (3-7) is occurring then a plot of \( \frac{A - A_o}{A_m - A} \) against \( \log [\text{detergent}] \) should be linear with a slope of \( n \). In addition a plot of \( \log [\text{Fe}]_{TOT} \alpha \) versus \( \log (1 - \alpha) [\text{Fe}]_{TOT} \) should be linear with a slope of one. (See appendix 1).

With CTMAB, figure 3.14 a) shows that the former is true, with a slope of two, while figure 3.14 b) shows that the latter is also true. (Data tables in appendix 2, tables 5a, i, ii, iii.)

\[
\log \frac{A - A_o}{A_m - A}
\]

\[0.1 \text{M NaOH} \]
\[\{\text{hemin}\} = 9.72 \times 10^{-6} \text{M} \]

![Graph](image)

Figure 3.14a Analysis of the data for the titration of hemin by CTMAB.

The log \( K \) values were as follows:

At pH 8.5: \( 10.6 \pm 0.3 \) (varying CTMAB) \( (\text{M}^{-2}) \)

In 0.1M NaOH: \( 10.56 \pm 0.03 \) (varying CTMAB) \( (\text{M}^{-2}) \)

\( 10.7 \pm 0.3 \) (varying hemin) \( (\text{M}^{-2}) \)
Figure 3.14b Analysis of the data for the titration of hemin by CTMAB; 0.1M NaOH; 1.5x10^{-5} M CTMAB; 25°C
Hence this reaction is pH independent between pH 8.5 and 13, which implies that each iron still has OH\(^-\) or its equivalent coordinated.

With SLS, only one detergent is bound and \(\log K\) at pH 13 was found to be 5.3 ± 0.2 (M\(^-1\)) (see figure 3.15).

![Figure 3.15](image)

**Figure 3.15** Analysis of the data for the titration of hemin by SLS in 0.1 M NaOH; 25°C; 1,12 x 10\(^{-5}\) M hemin.

The titration with TX showed a curved plot which could be resolved into the binding of one detergent at low concentrations and two detergents at higher concentrations (see figure 3.16a).

The log of the binding constant for one detergent was 4.8 ± 0.2 (M\(^-1\)) while that for two was 9.5 ± 0.1 (M\(^-2\)). TX thus has a lower affinity for hemin than do CTMAB and SLS.

These results may be a consequence of differing hydrophobicities as well as coulombic interactions with the porphyrin core (which has a +1 charge) and the propionate side chains with the different detergents.
In spite of the two overlapping equilibria with TX (Figure 3.16b), isosbestic points were still present, indicating that the first detergent binding causes the major spectral changes.

Figure 3.16a) Analysis of the data for the titration of hemin by TX in 0.1 M NaOH; 10.0 x 10^-6 M hemin; 25°C;

- x---x solved assuming one detergent bound;
- o solved assuming two detergents bound.

Figure 3.16a) Analysis of the data for the titration of hemin by IX in 0.1 M NaOH; 10.0 x 10^-6 M hemin; 25°C;

- x---x solved assuming one detergent bound;
- o solved assuming two detergents bound.

Figure 3.16a) Analysis of the data for the titration of hemin by IX in 0.1 M NaOH; 10.0 x 10^-6 M hemin; 25°C;

- x---x solved assuming one detergent bound;
- o solved assuming two detergents bound.

Figure 3.16a) Analysis of the data for the titration of hemin by IX in 0.1 M NaOH; 10.0 x 10^-6 M hemin; 25°C;

- x---x solved assuming one detergent bound;
- o solved assuming two detergents bound.

Figure 3.16a) Analysis of the data for the titration of hemin by IX in 0.1 M NaOH; 10.0 x 10^-6 M hemin; 25°C;

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- o solved assuming two detergents bound.

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- x---x solved assuming one detergent bound;
- o solved assuming two detergents bound.

Figure 3.16a) Analysis of the data for the titration of hemin by IX in 0.1 M NaOH; 10.0 x 10^-6 M hemin; 25°C;

- x---x solved assuming one detergent bound;
- o solved assuming two detergents bound.

Figure 3.16a) Analysis of the data for the titration of hemin by IX in 0.1 M NaOH; 10.0 x 10^-6 M hemin; 25°C;

- x---x solved assuming one detergent bound;
- o solved assuming two detergents bound.
Figure 3.16 b) Corresponding spectral changes (conditions as in figure 3.16a).

3.2.5 Spectra

UV-visible spectra have been recorded under various conditions. Table 3.3 lists the positions and intensities of the Soret and visible bands of hemin in various alkaline solutions.

There is a reasonable comparison between the results of this study and those of other workers, particularly as many of the quoted figures for the latter have been read off from reported spectra.
Table 3.3 | \( \text{E} \) values for the Soret and visible bands of hemin in alkaline solutions

<table>
<thead>
<tr>
<th>System</th>
<th>Soret ( \text{E} \text{mm}^{-1} \text{cm}^2 \text{mol}^{-1} \text{cm}^2 )</th>
<th>Visible ( \text{E} \text{mm}^{-1} \text{cm}^2 \text{mol}^{-1} \text{cm}^2 )</th>
<th>Type</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Aqueous solution: ( \text{pH} \approx 2 )</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1. low ionic strength</td>
<td>( 1 \times 10^{-4} ) ( \text{pH} 9.5 )</td>
<td>461 (52) ( 340(4,5) ) ( 530(3,6) ) ( 620(4,7) )</td>
<td>A' (B)</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>( \text{pH} 11.0 )</td>
<td>391 (52) ( 491(4,5) ) ( 530(3,6) ) ( 620(4,7) )</td>
<td>A' (B)</td>
<td></td>
</tr>
<tr>
<td>1.2. moderate ionic strength</td>
<td>( \text{pH} 9 - 11 )</td>
<td>345 (52) ( 540(4,5) ) ( 615(4,7) )</td>
<td>A' (B)</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>( \text{pH} 13.3 )</td>
<td>-</td>
<td>-</td>
<td>A' (B)</td>
</tr>
<tr>
<td>1.3. high ionic strength ( 1 \times 10^{-3} ) ( \text{KNO}_3 ) 2.0 ( \mu \text{M} )</td>
<td>385 (47)</td>
<td>575 ( 600(4,7) )</td>
<td>B (B)</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>2.0 ( \text{KNO}_3 ) 1.0 ( \mu \text{M} )</td>
<td>-</td>
<td>570(4,4) ( 600(4,6) )</td>
<td>B (B)</td>
</tr>
<tr>
<td></td>
<td>1.0 ( \text{KNO}_3 ) 0.1 ( \mu \text{M} )</td>
<td>365 (41)</td>
<td>580(4,7) ( 600(4,7) )</td>
<td>B (B)</td>
</tr>
<tr>
<td></td>
<td>0.1 ( \mu \text{M} )</td>
<td>-</td>
<td>580(4,7) ( 600(4,7) )</td>
<td>B (B)</td>
</tr>
<tr>
<td></td>
<td>0.1 ( \mu \text{M} )</td>
<td>-</td>
<td>580(4,7) ( 600(4,7) )</td>
<td>B (B)</td>
</tr>
<tr>
<td>1.4. pils caffeine</td>
<td>0.20 ( \text{M} ) caffeine; ( \text{pH} 8.5; 2 \times 10^{-5} ) ( \text{M} )</td>
<td>402 (78) ( 490(4,1) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>( \text{pH} 8.5 = 3.2 )</td>
<td>-</td>
<td>-</td>
<td>A (B)</td>
</tr>
<tr>
<td></td>
<td>0.1 ( \mu \text{M} ) caffeine; ( 0.1 \mu \text{M} ) ( \text{KNO}_3 )</td>
<td>-</td>
<td>-</td>
<td>B (B)</td>
</tr>
<tr>
<td></td>
<td>0.1 ( \mu \text{M} ) caffeine; 0.1 ( \mu \text{M} ) ( \text{KNO}_3 )</td>
<td>-</td>
<td>-</td>
<td>B (B)</td>
</tr>
<tr>
<td>1.5. Detergents</td>
<td>1.0 ( \text{M} ) ( \text{TX-100} ) ( \text{detergent} )</td>
<td>750 (92) ( 565(4,4) ) ( 600(4,7) )</td>
<td>B (B)</td>
<td>this study</td>
</tr>
<tr>
<td>1.5.1. Low (detergent)</td>
<td>1.0 ( \text{M} ) ( \text{TX-100} ) ( \text{detergent} )</td>
<td>750 (92) ( 565(4,4) ) ( 600(4,7) )</td>
<td>B (B)</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>0.1 ( \text{M} ) 1.0 ( \text{KNO}_3 )</td>
<td>510 (92) ( 415(4,4) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 ( \text{M} ) 0.1 ( \text{KNO}_3 )</td>
<td>510 (92) ( 415(4,4) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
</tr>
<tr>
<td>1.5.2. High (detergent)</td>
<td>1.0 ( \text{M} ) ( \text{TX-100} ) ( \text{detergent} )</td>
<td>400 (65) ( 570(4,7) ) ( 600(4,7) )</td>
<td>B (B)</td>
<td>this study</td>
</tr>
<tr>
<td>1.5.3. SLES; 0.1 ( \text{M} ) ( \text{KNO}_3 )</td>
<td>400 (65) ( 570(4,7) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5.4. SLES; 0.1 ( \text{M} ) ( \text{KNO}_3 )</td>
<td>400 (65) ( 570(4,7) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5.5. SLES; 0.1 ( \text{M} ) ( \text{KNO}_3 )</td>
<td>400 (65) ( 570(4,7) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Mixed aqueous solvents</td>
<td>4.0 ( \text{M} ) 0.1 ( \text{M} ) ( \text{KNO}_3 ) ( \text{NaOH} )</td>
<td>- ( 461(4,1) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td>this study</td>
</tr>
<tr>
<td></td>
<td>4.0 ( \text{M} ) 0.1 ( \text{M} ) ( \text{KNO}_3 ) ( \text{NaOH} )</td>
<td>- ( 461(4,1) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0 ( \text{M} ) 0.1 ( \text{M} ) ( \text{KNO}_3 ) ( \text{NaOH} )</td>
<td>- ( 461(4,1) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.0 ( \text{M} ) 0.1 ( \text{M} ) ( \text{KNO}_3 ) ( \text{NaOH} )</td>
<td>- ( 461(4,1) ) ( 600(4,7) )</td>
<td>A (B)</td>
<td></td>
</tr>
</tbody>
</table>

* in \( \text{cm}^{-1} \) \( \text{mm}^{-1} \text{cm}^2 \text{mol}^{-1} \text{cm}^2 \) c | see text and figure 3.17 | d | see text and figure 3.17 | e | see text and figure 3.17 | f | see text and figure 3.17 | g | see text and figure 3.17 | h | see text and figure 3.17 | i | see text and figure 3.17 | j | see text and figure 3.17 | k | see text and figure 3.17 | l | see text and figure 3.17 | m | see text and figure 3.17 | n | see text and figure 3.17 | o | see text and figure 3.17 | p | see text and figure 3.17 | q | see text and figure 3.17 | r | see text and figure 3.17 | s | see text and figure 3.17 | t | see text and figure 3.17 | u | see text and figure 3.17 | v | see text and figure 3.17 | w | see text and figure 3.17 | x | see text and figure 3.17 | y | see text and figure 3.17 | z | see text and figure 3.17
Figure 3.17 Types of hemin spectra in alkaline solution

(A): $6.55 \times 10^{-5}$ M hemin in 44% aqueous ethanol containing 20 mM NaOH 
(B): $6.55 \times 10^{-5}$ M hemin in 3% (0.082 M) CTMAB containing 0.1 M NaOH.
(a) **Visible region**

There are quite distinctly two types of visible bands as represented in figure 3.17. The A type spectrum is a typical high spin spectrum while the B type spectrum is analogous to that of the μ-oxo dimer. \(^{26}\) (A' is still an A type with the bands shifted slightly to the red.) Curiously, these changes in the visible region are not paralleled in the Soret region.

The B type spectra may in many cases be due to a μ-oxo dimer (e.g. at low (detergent), high (hemin) in caffeine) where dimers have been established or seem likely, or may be due to μ-oxo linkages present in an aggregate (high ionic strengths). There are cases, however, where the hemin is monomeric but still has a B-type spectrum (e.g. micellar CTMA and TX). This suggests that there is some common feature.

It has been proposed \(^{87}\) that although the bands in the UV-visible spectrum of hemin are largely due to \(\pi \rightarrow \pi^*\) transitions, varying amounts of mixing can occur with \(\pi \rightarrow d\) charge transfer transitions, particularly in high spin complexes. If there is little mixing of the low energy \(\pi \rightarrow \pi^*\) transition with the high energy charge transfer transitions, then a relatively weak band at \(<475\) nm and a band at \(\sim 588\) nm (emM-40) (with vibrational progressions) would be expected. A characteristic of the B type spectrum is a band at \(\sim 580\) nm (emM 5-7) with a nearby band at \(\sim 600\) nm. There is also a slight shoulder at \(\sim 475\) nm in all the B type spectra, including the μ-oxo dimer (see for example figures 13a and 16b).

Hence the primary difference between A and B type species could depend on whether there is mixing of the low energy \(\pi \rightarrow \pi^*\)
transition with the high energy $\Pi \rightarrow d$ charge transfer transition or not.

(b) Soret band

The changes in the Soret band do not parallel those found in the visible region, which indicates that different factors determine the positions and intensities of the bands in these two regions.

The Soret intensity decreases as the degree of aggregation increases i.e. the extinction coefficient decreases in the order $M > D > P$. The monomers tend to have their $\lambda_{\text{max}}$ at ~400 nm while the dimers and higher aggregates tend to have their $\lambda_{\text{max}}$ at 385 - 390 nm. The exception to this are the $\mu$-oxo dimers which have their $\lambda_{\text{max}}$ at 397 nm. These changes on dimerization and aggregation must result from interactions between the hemin units. The $\mu$-oxo dimer may differ perhaps because of a different type of interaction or a lack of $\mu - \mu$ interaction.

3.3 Discussion

On the basis that the hemin species in alkaline solution of moderate ionic strength is dimeric, the existence of equilibria between monomers, dimers and higher polymeric forms have been found in aqueous alkaline solution. These equilibria are pH independent ($\text{pH} > 8$).

The visible spectra of the species studied here as well as others in alkaline non-aqueous and mixed aqueous organic solvents show that they all fall into two series characterised as follows: A series with bands at about 610 nm and between 480 and 500 nm as expected for high spin $\text{Fe}^{2+}$.

B series with bands at 570 - 600 nm (most likely being vibrational overtones) with no obvious bands at about 500 nm. These are not
quite those expected for a low spin Fe(III) which have bands between 530 and 580 nm.87,93

The changes in the Soret region showed no correlation with those in the Q region.

A type spectra have been found for monomers and dimers (hence labelled $M_A$ and $D_A$) while B type spectra have been found for monomers, dimers and polymers (hence labelled $M_B$, $D_B$, $P_B$).

Hence the following equilibria exist between the five types of hemin found in aqueous alkaline solution:

<table>
<thead>
<tr>
<th>monomers</th>
<th>dimers</th>
<th>polymers</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_A$</td>
<td>$D_A$</td>
<td>$P_B$</td>
</tr>
<tr>
<td>$M_B$</td>
<td>$D_B$</td>
<td>$P_B$</td>
</tr>
</tbody>
</table>

The equilibria are all pH-independent but obviously depend on the hemin concentration and the different forms are stabilized by conditions as follows:

$M_A$: observed in aqueous alcohols and in aqueous solution in the presence of caffeine and micellar SLS.13

$M_B$: observed in TX, CTMAB detergent micelles in aqueous solution.14

$D_A$: observed in aqueous solution, where it is stabilized by sucrose and at low concentrations of SLS.

$D_B$: observed in DMSO,92 in aqueous solution at low concentrations of TX and CTMAB, with pyridine and pyridinium salts99 and with caffeine.

$P_B$: observed in aqueous solution containing high concentrations of electrolytes.24
Correlations between the spectral types and magnetic susceptibility exist. The species giving A type spectra have magnetic susceptibilities of around 5.9 BM which is what is expected for the high spin species which would have five unpaired electrons. For example, hemin in aqueous alkali gives a value of 5.8 as long as the hemin concentration is not greater than $2 \times 10^{-6}$ M or if 30% sucrose is present.  

The species giving B type spectra which have been measured, give values around two BM which may arise either from the low spin species (with one unpaired electron) or from antiferromagnetic coupling of two high Fe(III)s via an oxo bridge. For example hemin in the presence of high concentrations (~2M) of electrolytes gave values of 2.6-2.9 BM.  

Hemin in 20% pyridine containing 0.2M NaOH gave a value of 1.97 and the μ-oxo dimer gave a value of 2.4 BM at room temperature decreasing with temperature. Unfortunately determinations have not been carried out on $N_A$ and $N_B$ so it is not certain that $N_A$ is high spin and $N_B$ is low spin although the former is probably true. It is not clear that the $B$ type species are necessarily low spin or antiferromagnetically coupled. A high spin species, in which mixing of $\pi - \pi^*$ and charge transfer transitions is unfavourable because of symmetry or energy differences, is a possibility. An equilibrium has been found between high spin and low spin ferrihemoglobin and ferrimyoglobin containing $H_2O$, $HCO_3^-$, $N_3^-$, imidazole, $NCS^-$ and $NO_2$ as ligands (together with the protein histidine). Even cyanocobalamin is 50% high spin. So a mixture of high spin and low spin is also a possibility for the $B$ type species. These equilibrium mixtures demonstrate the ease of high spin to low spin conversion.
Whether or not the conversion of an A species to a B species involves a high spin-low spin equilibrium or a change in mixing of transitions, the metal ligand bond length as well as the distance of the metal from the ring plane are likely to be significant.

In considering possible structures for the species found, the nature and number of ligands as well as the spin state must be considered. In aqueous alkali, possible ligands are H$_2$O, OH$^-$ and bridging O$^{2-}$. The complexes could be five or six coordinate but in practice as the sixth ligand would be H$_2$O these two cannot be distinguished. As discussed above, both high spin and low spin (or even a mixture) are possible. In addition the dimers and polymers may be held together by a 1-oxo bridge, by coordination of carboxylate from another hemin, by hydrophobic interactions or by donor-acceptor interactions. As no evidence has been found for carboxylate dimerization, this will be ignored. Hydrophobic and donor-acceptor interactions give rise to interactions between the porphyrin rings and will result in ring-ring dimers.

As the equilibria were all pH independent, all the species have OH$^-$ or its equivalent coordinated. In each case H$_2$O may be an additional ligand.

Hence the monomeric species can be Fe-OH and H$_2$O-Fe-OH.

The dimeric species may be Fe-O-Fe; H$_2$O-Fe-O-Fe-OH$_2$; (Fe-OH)$_2$; (H$_2$O-Fe-OH)$_2$ (for the μ-oxo dimers and ring-ring dimers respectively).

Coordination of H$_2$O may alter the metal-ligand bond length as well as the distance between the metal and ring plane and enable the transition between A and B to be effected at least in the case of the monomers and the ring-ring dimers. The μ-oxo
dimer is unlikely to change its spin state on coordination of $H_2O$ as antiferromagnetic coupling generally predominates over ferromagnetic coupling in $\mu$-oxo dimers.

Hence $M_A$ is likely to be Fe-OH while $M_B$ could be $H_2O$-Fe-OH. $D_A$ is likely to be (Fe-OH)$_2$ while $D_B$ could be Fe-O-Fe or $(H_2O$-Fe-OH)$_2$. $P_B$ could contain $D_B$ units held together by cations and possibly involving ring-ring interactions between these units, because evidence for hydrophobic bonding was found from the effectiveness of different ions.

The formation of an $X_B$ type species in some detergent micelles is rather puzzling, as $A$ type species are found in their absence. Possibly the low dielectric constant in the micelle interior, by preventing charge delocalisation, induces a short Fe-O bond which enables the transition to occur. SLS may differ perhaps because more solvent is included in the micelle interior.

Hence this study has provided further information on the nature of species and the equilibria exhibited by hemin in alkaline aqueous solution. Five different types of complexes (including monomers, dimers and polymers) were found to be in pH independent equilibria. The species found demonstrate a complex interplay of coulombic, hydrophobic, donor-acceptor and possibly hydrogen bonding interactions.

This provides a framework for further studies on the nature of the equilibria and physical properties of the individual complexes.

Monomeric hemin in a predominantly aqueous environment at pH > 8 can be obtained in the presence of caffeine or in micellar detergents. These are superior to mixed aqueous solvents as pH still has its normal meaning.
CHAPTER 4: HEMIN IN AQUEOUS ACID SOLUTION

4.1 Introduction

The previous chapter dealt with hemin in aqueous alkali (above the pKas of the coordinated hydroxide and propionic acid side chains) where hemin is soluble and hence true thermodynamic equilibria can be studied. Only five types of complexes were found.

By contrast the acid and neutral region (pH<8) is complicated not only by these pKas but also by the insolubility of hemin, with all solutions being metastable (see later).

Hence a study in this region presents the problem of how to get the hemin into solution as well as how to study it.

Three approaches can be used to get hemin dissolved in aqueous acid, dilution into aqueous acid from hemin dissolved in aqueous alkali or organic solvents (e.g. from pure DMSO to 0.1% aqueous DMSO)\(^{92}\), and from hemoproteins (e.g. hemoglobin) dissolved in water, can all be used.

Previous work on equilibria in acid has shown two pKas, one at about 2.92,96 and the other at about 7.5,10,31a,36,92,96 These probably refer to dimers or possibly even higher aggregates in the slightly acid region and the number of protons and the nature of the species has not been identified.

To improve the conditions for study, either the rate of aggregation reactions must be slowed down, as reported earlier in the presence of silicate,\(^{11}\) or else a rapid method of study must be used, such as stopped flow. When using stopped flow pH jumps (i.e. rapidly mixing unbuffered hemin at about pH9 with a buffer of the required pH), it must be borne in mind that any observed pKas may not be a true equilibrium between metastable species but
may include the pH dependence of an irreversible aggregation reaction.

The aims of this chapter are to improve the understanding of the nature of the complexes and equilibria observed in neutral and acid aqueous solution with particular emphasis on identifying the simple monomeric aquo complex and determining at least an approximate pKa for the coordinated water.

4.2 Results

4.2.1 Aqueous solution alone

4.2.1.1 Moderate ionic strength (μ = 0.1)

Preliminary experiments in which equal volumes of hemin in dilute (10^{-4}-10^{-3}N) NaOH and double strength buffers were mixed to give a final concentration of ~2x10^{-6}M hemin gave a fairly stable species (i.e. < 2% decrease in the Soret intensity in one minute) and this enabled it to be studied by ordinary spectrophotometry. The changes in the Soret absorbance were rapid in the pH range 3 - 6 and hence this region was studied by stopped flow spectrophotometry.

Spectra at pH 11 of the Soret region (350 nm to 450 nm) were obtained by adding typically 1μl of a 8.8 x 10^{-3}M hemin stock solution in 0.1M NaOH to 10 ml of water followed by the addition of 10 ml double strength pH 1.1 buffer to give a final hemin concentration of 4.6 x 10^{-7}M and using a 10 cm pathlength cell. (Addition of the hemin stock directly to the buffer resulted in precipitation.) In all cases the new spectrum appeared within five seconds and showed little change with time (i.e. < 2% decrease in the Soret intensity in two minutes). The spectrum showed a reasonably sharp Soret band and the species is possibly monomeric.
The $\lambda_{\text{max}}$ was at 397 nm with an extinction coefficient of $120 \pm 3$, the latter being strong support for a monomer. On standing for five hours, however, $\varepsilon$ dropped to 80.

As the hemin concentration was increased above $2 \times 10^{-5}$ M a decrease in the extinction coefficient was observed as well as a more rapid decrease in the Soret absorbance with time (at $2 \times 10^{-5}$ M hemin a 40% decrease occurred in a minute). Hence a Beer's law study was carried out to investigate these differences. (At higher hemin concentrations the absorbance found 10s after mixing was used.) Figure 4.1 shows the plot of absorbance at 397 nm (the Soret $\lambda_{\text{max}}$) against the hemin concentration. (The data are given in appendix 3 table 1a.)

![Beer's law plot of hemin at pH 1.1; $\mu = 0.1$

$\ell = 1$ cm; 25°C.}
This shows a marked deviation from linearity. It could be shown
(appendix 3 table 1b) that this deviation was due to the equili-
brum (4 - 1)

\[ 2M \xrightarrow{\text{D}} D \quad (4 - 1) \]

and the equilibrium constant was found to be \(1.1 \times 10^5 \text{ M}^{-1}\). This is
considerably less than that found for the alkaline dimer (\(K > 10^9\))
indicating a different type of dimer, with weaker interactions.

The spectra of both the monomer and dimer have the Soret maxi-
mum at 397 nm but the dimer has a lower extinction coefficient as
well as a shoulder at about 360 nm. (See figure 4.2 and table 4.2.)

![Figure 4.2 Spectra of hemin at pH and low concentrations at
pH 1.1 (Soret region) \( \mu = 0.1 \); 25°C
--- 1.4 \times 10^{-5} \text{M hemin}; \ell = 10 \text{ cm}
--- 2.2 \times 10^{-5} \text{M hemin}; \ell = 1 \text{ cm}](image)
In the stopped flow experiments, equal volumes of 4.3 x 10^{-6} M hemin in 10^{-6} M NaOH and double strength buffers from pH 1.1 to 11.0 were mixed to give a final concentration of 2.15 x 10^{-6} M hemin. The change in absorbance (where evident in the time scale used) was monitored at 397 nm.

No changes in absorbance with time were observed between pH 7.8 and 11.0. Below pH 3, the traces showed the end of a rapid rise in absorbance (extending for approximately 1 m s (the mixing time of the instrument is 1 m s)) which then remained steady for at least 100 m s. The absorbance at pH 1 - 2 (0.515) corresponds to an extinction coefficient (at 397 nm) of 120 x 10^{-3} M^{-1} cm^{-1} (l = 2 cm) in agreement with the results of the Beer's law plot at low hemin concentrations.

Between pH 3 and - the traces showed an initial rapid rise to a maximum (which usually occurred at approximately 2 m s after mixing) followed by a slower fall. The maximum values are recorded in table 2 appendix 3 together with the steady values in the other pH regions. The variation of these is shown in figure 4.3a. This shows the occurrence of two apparent pK's at pH 2.8 and pH 6.4. The data were evaluated in terms of various equations involving interconversions of monomers and dimers, dimers and dimers and monomers and monomers with the involvement of one or more protons, but unfortunately the data were not good enough to distinguish between the various possibilities. This is probably due to a significant amount of an aggregated species or dimer in the intermediate pH region which would invalidate the equations used to treat the data.
Figure 4.3 Variation in absorbance of the Soret with pH in the absence and presence of caffeine; 25°C; µ = 0.1
However, further experiments varying the hemin concentrations at pH 6.4 (see Table 4.1) showed that the pKa in this region apparently involves the conversion of a dimer at high pH to a monomer at low pH.

Table 4.1: Variation of [hemin] at pH 6.4. (25°C; µ = 0.1)

<table>
<thead>
<tr>
<th>(Fe)</th>
<th>(A_{397}(pH6.4)) ((\equiv A))</th>
<th>(A_{397}(pH11.0)) ((\equiv A_w))</th>
<th>(A_{397}(pH5.0)) ((\equiv A_o))</th>
<th>(-\log K_a^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16x10^{-6}M</td>
<td>1,720</td>
<td>1,518</td>
<td>3,078</td>
<td>6.59</td>
</tr>
<tr>
<td>8x10^{-6}M</td>
<td>0.85</td>
<td>0.746</td>
<td>1.512</td>
<td>6.69</td>
</tr>
<tr>
<td>4x10^{-6}M</td>
<td>0.478</td>
<td>0.383</td>
<td>0.774</td>
<td>6.59</td>
</tr>
<tr>
<td>2x10^{-6}M</td>
<td>0.267</td>
<td>0.203</td>
<td>0.363</td>
<td>6.69</td>
</tr>
</tbody>
</table>

\(a\) - calculated using \(\varepsilon_{397}(pH5.0) = 96x10^3 \text{ M}^{-1}\) (on a permole Fe basis) (see later).

A plot of \(\log \left(\frac{(A_w-A)}{(A-A_o)(A_o-A_w)}\right)\) vs \(\log [Fe]_{TOT}\) (see derivation in appendix 1b) gave \(R^2 = 0.98; \text{ slope } = -1.08 \text{ (Sd } = 0.10)\)

\[\text{ intercept } = 6.89 \text{ (Sd } = 0.51) \implies \]

\(pK_a^b = 6.84 \pm 0.05\)

\(b\) \(K_a\) refers to the equation \(2M + D + 2H^+\)

The spectra of the intermediate at pH 5 over the range 340 - 450 nm, determined by stopped flow spectrophotometry at several wavelengths, shows a maximum at 397 nm (\(\varepsilon = 96x10^3 \text{ m}^{-1} \text{ cm}^{-1}\)) with a shoulder at about 360 nm. With the exception of the extinction coefficient of the \(\lambda_{\text{max}}\) this is similar to that of the dimer found at pH 1.1 (Figure 4.2b) and may represent a mixture of acid
monomer and acid dimer. The spectrum of the pH 1,1 species is identical to that found by ordinary spectrophotometry at low hemin concentrations (\( \lambda_{\text{max}} \) at 397 nm, \( E_{397} = 120 \text{ M}^{-1} \text{ cm}^{-1} \)) and because of the fairly high extinction coefficient probably represents a monomer.

The similarity in the spectra between the pH 1 dimer and the pH 5 stopped flow species, as well as between the pH 1 monomer and the pH 5,5 - 7 species at very low ionic strength (see later) suggests that these are variants of similar species. Hence the first pKs at 2,8 is likely to correspond to the deprotonation of the carboxylic acids as these would not be expected to have a marked effect on the spectra while the second at 6,6 corresponds to the deprotonation of coordinated water which should have a marked effect on the spectra.

Unsuccessful attempts were also made to pH jump from the monomeric forms at pH 2 and in neutral water (see low ionic strength studies) by stopped flow. In the case of the neutral water the concentrations necessary were too low for meaningful differences to be detected while in the pH2 case, the stock solutions decomposed too fast to enable a sufficient number of quantitative experiments to be made.

4.2.1.2. Low ionic strength

Spectra in the Soret region at low concentrations of hemin (0,8 \( \times 10^{-6} \) M) in very low ionic strength solutions (\( \mu \sim 10^{-5} \)) at pH 7 show a sharp band at 398 with an extinction coefficient of \( (122 \pm 3) \times 10^{3} \text{ M}^{-1} \text{ cm}^{-1} \), which is very similar to that found at pH 1,1 (section 4.2.1.1). This species obeys Beer's law up to \( 4,1 \times 10^{-6} \) M and shows second order decomposition to give a new
species with $\lambda_{\text{max}}$ 398 nm ($c = 73 \text{ M}^{-1} \text{ cm}^{-1}$) (i.e. similar to the pH 1,1 dimer). The addition of buffers or salts transformed this low ionic strength species at pH 7 (before decomposition) into an aggregate (broad flat bands were found).

In this study 1 µL of a 9.18 x 10^{-3} M hemin solution, dissolved in 0.1M NaOH, was added to 25 ml of water to give a final concentration of 0.367 x 10^{-6} M hemin. The pH was varied by adding traces of HNO₃ or NaOH, and was measured with a pH meter immediately after recording the absorbance ($A = 10 \text{ cm}$). Between pH 5.5 and 7, $\varepsilon_{398}$ was found to be $122 \pm 3$, and the absorbance was found to decrease fairly slowly (<4% fall in a minute). Above pH 7, the extinction coefficient was lower, while at pH 10, the alkaline dimer was found ($\lambda_{\text{max}}$ 387 nm and $\varepsilon_{387} = 51.3 x 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) (the slight shift compared to the spectrum in moderate ionic strength alkaline presumably reflecting a slight change in structure because of poor shielding of the carboxylates at low ionic strength). The approximate pKa is ~8. Below pH 5.5 the absorbance at 397 nm rapidly decreased, probably due to the protonation of the carboxylates which would decrease the solubility. Hence this species with a fairly sharp Soret can only be obtained over a fairly narrow range of pH (5.5 - 7) at low ionic strength and is very likely the aquohemin monomer with dissociated carboxylates, which converts slowly to the aquohemin dimer.

4.2.2 Aqueous solution studies in the presence of caffeine

The aqueous solution study of hemin in the presence of caffeine consisted of a stopped flow pH study, analogous to that carried out for the hemin dimer, as well as a qualitative study in the
presence of silicate which slows down aggregation.

The stopped flow pH study in the presence of caffeine was carried out at 402 nm, the Soret maximum of the alkaline monomeric hemin-caffeine adduct (3.2.3) and the final, min concentration was 3.09 x 10^{-6} N. The variation of A_{402} nm with pH is shown in figure 4.3b. (The experimental data are presented in table 3 appendix 3.)

Plots of log A - A_° 
A_{∞} - A
versus pH between pH 2.5 and pH 3.8 for the first pKa and between pH 5.2 and pH 6.0 for the second were linear (figure 4.4) as would be expected for a monomer-monomer conversion (see derivation in appendix 1b). The slopes and intercepts were as follows:

1st pKa: slope = 1.04 (Sd = 0.06)  
intercept = -3.13 (Sd = 0.19)  
R = 0.99

(A_m = 0.700; A_° = 0.915)

2nd pKa: slope = 1.90 (Sd = 0.08)  
intercept = -10.66 (Sd = 0.44)  
R = 0.99

(A_m = 0.530; A_° = 0.700)

(Carbonate buffers were found to decrease A_{402} by 4% relative to phosphate buffers (see table 3 appendix 3 and figure 4.3b) which may be due to some specific interaction of the carbonate. As the absorbances were constant between pH 7.8 and 11.0 using carbonate buffers, the absorbance at pH 7.8 in a phosphate buffer was taken as A_m for the second pKa.)
Figure 4.4 Plot for determining the pKas for hemin—caffeine

$3.09 \times 10^{-6}$ hemin; $0.05M$ caffeine; $25^\circ C$; $\mu = 0.1$
The fact that good linear plots were obtained indicates that unlike in the alkaline dimer system, negligible amounts of dimers and higher aggregates were present up to 20 ms and the equations used were valid.

Hence both pKas corresponded to a monomer-monomer equilibrium. The first pKa had a slope of one which indicates the involvement of one proton (reaction 4-2) while the second had a slope of two indicating two protons (reaction 4-3).

\[
\begin{align*}
M + M' + H^+ (K_{a1}) & \quad (4-2) \\
M' + M'' + 2H^+ (K_{a2}) & \quad (4-3)
\end{align*}
\]

To enable comparisons to be made more readily, the second pKa can be formally split into two reactions both involving one proton loss (reactions 4-4 a & b) and with the same pKa (Ka_2^f):

\[
\begin{align*}
M' & \rightarrow M''' + H^- \\
M''' & \rightarrow M'' + H^+
\end{align*}
\]

It can be seen that \(K_{a2}^f = (K_{a2})^\dagger\).

The derivation (appendix 1b) shows that the pKas for reactions (4-2) and (4-3) are given by (-intercept) of the plot of \(\Delta A_\text{AQ} \) versus pH. Hence

\[
\begin{align*}
pK_{a1} &= 3.1 \\
pK_{a2} &= 10.7 \implies pK_{a2} = 5.3
\end{align*}
\]

Hence the first pKa involves deprotonation of one group with a pKa of 3.1 while the second pKa involves the deprotonation of two groups both with pKas of 5.3. The assignment of pKas to Fe-\(\text{OH}_2\)-Fe-\(\text{OH}\) (one) and -COOH+COO^- (two) can be made in two ways:

a) the two carboxylates have a pKa of 5.3 while the Fe-\(\text{OH}_2\) has a pKa of 3.1 which is much lower than previous pKas reported\(^{10,92,96}\) and the corresponding pKa in the alkaline dimer (\(\text{V}\)) (the
pKas of the carboxylates being normal). b) A cooperative interaction between Fe-OH^ and one carboxylic acid which results in them having a common pKa at 5.3 while the second carboxylic acid has a pKa at 3.1. (Only one pKa (7.6) for hemin in 40-80% KI/SO was found and this was explained as being due to cooperative interactions between the ionizable groups.)

Certainly the hydrophobic character of caffeine would favour the neutral (ignoring side chains) hydroxohemin over the aquohemin which has a +1 charge and hence may account for the pKa of 3.1. Caffeine cannot be acting as a strong donor as this would tend to favour the aquo complex.

The spectra of the acid species at pH 1 and 4.6 over the range 340 - 450 nm were determined by stopped flow spectrophotometry at several wavelengths. Both species had $\lambda_{max}$ at 402 nm while the respective extinction coefficients were 151 and $94 \times 10^3 \, M^{-1} \, cm^{-1}$. Comparisons of the Soret of the corresponding species in the absence of caffeine show that caffeine is bound in both cases (by a shift in the $\lambda_{max}$ and in the case of the pH 1 species by an increase in intensity).

Silicate was added to hemin-caffeine to prevent its aggregation in acid solution. Figure 4.5 shows the effect of pH when 0.74g/100 ml silicate was added to hemin-caffeine and the pH varied.

These changes are reversible and show approximate isosbestic points. The pKas were ~7 and ~3. The sharpness of the Soret and the reversibility shows that silicate has in fact prevented aggregation. This is a kinetic effect as silicate did not reverse the aggregation in a partially aggregated hemin-caffeine solution.
Figure 4.5 Spectra of hemin caffeine in the presence of sodium silicate at various pHs; 7.95x10^-6 M haem; 0.07M caffeine; 0.74g/10L sodium silicate.
In contrast, silicate did not prevent the aggregation of hemin in the absence of caffeine on acidification, as seen by the broad flat Soret band obtained. This could be because the rate of aggregation of hemin in the absence of caffeine is much faster than in the presence of caffeine and hence the viscosity of silicate is less effective in the former case. This ties in well with the stopped flow studies where further reactions after the pH jump of the alkaline dimers prevented pK as from being calculated.

The λmax and ε for the Soret in the presence of caffeine with and without silicate are similar at pH ~5 but not at pH 1. This latter discrepancy may result from interactions between the charged hemin species at pH 1 and the silicate/silicic acid (a weak acid).

4.2.3 Comparison with other published spectra

A number of other spectra in neutral and acid aqueous solutions which are similar to those found above have been reported and are collected in table 4.2.

Table 4.2: Spectra of hemin complexes in acid in the Soret region

<table>
<thead>
<tr>
<th>Conditions</th>
<th>λ</th>
<th>ε</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a) Monomers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 1.1-2; &lt;2x10^{-6} M hemin; μ=0.1 (stopped flow and ordinary spectrophotometry)</td>
<td>397</td>
<td>120</td>
<td>this study</td>
</tr>
<tr>
<td>pH 5.5-7; 0.4x10^{-6} M hemin; μ=10^{-5}</td>
<td>398</td>
<td>122±3</td>
<td>this study</td>
</tr>
<tr>
<td>pH 6.6; &lt;1x10^{-6} M hemin (obeys Beer’s law up to 4.1x10^{-6} M) μ=10^{-5}</td>
<td>398</td>
<td>122±3</td>
<td>97</td>
</tr>
<tr>
<td>pH 6.8; 1.23x10^{-6} M hemin, water</td>
<td>398</td>
<td>1.20</td>
<td>95</td>
</tr>
<tr>
<td>Conditions</td>
<td>λ /nm</td>
<td>ε /10^3 M⁻¹ cm⁻¹</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hemin plus albumin, peroxidase, hemoglobin, myoglobin, catalase; pH 7</td>
<td>397</td>
<td>130</td>
<td>99</td>
</tr>
<tr>
<td>Hemoglobin; 3x10⁻⁶ M plus 0.02 M HCl; pH 2.06</td>
<td>398</td>
<td>120</td>
<td>95</td>
</tr>
<tr>
<td>50% aqueous ethanol pH 1</td>
<td>398</td>
<td>134</td>
<td>96</td>
</tr>
<tr>
<td>0.05 M caffeine, pH 1.1</td>
<td>402</td>
<td>151</td>
<td>this study</td>
</tr>
<tr>
<td>ditto plus 0.74 g/100 ml Silicate</td>
<td>399</td>
<td>102</td>
<td>this study</td>
</tr>
<tr>
<td>0.05 M caffeine pH 1.5</td>
<td>402</td>
<td>94</td>
<td>this study</td>
</tr>
<tr>
<td>ditto plus 0.74 g/100 ml Silicate (pH 5.7)</td>
<td>402</td>
<td>94</td>
<td>this study</td>
</tr>
</tbody>
</table>

b) Dimer

| pH 1.1; 3x10⁻⁵ M hemin; u=0.1 | 397 | 78 | this study |
| pH 5; μ=0.1; 2.15x10⁻⁷ M hemin (stopped flow) (probably at least partly dimeric) | 397 | 96 | this study |
| pH 6.8; μ=10⁻⁵; 1x10⁻⁶ M hemin on standing (second order formation) | 398 | 73 | 97 |
| Hemoglobin(3x10⁻⁶ M) at pH 2.06 on standing (second order formation) | 393 | 80 | 95 |
In the absence of caffeine, the monomers obtained under various conditions are remarkably similar, as are most of the dimers. Caffeine shifts the Soret slightly to the red and at pH 1.1 increases its intensity. The presence of sodium silicate has no effect on the spectrum of hemin-caffeine at pH 1.5 but does at pH 1.1.

4.3 Discussion

4.3.1 In the absence of caffeine

Table 4.2 shows that the same spectrum with λmax 397 nm (ε = 120x10^3 M⁻¹ cm⁻¹) can be observed under a variety of conditions from pH 1 - 7. The fairly sharp Soret and relatively high extinction coefficient (figure 4.2) support the proposal that these species are monomeric.

This table also shows that a second type of spectrum, attributed to the dimer, with λmax 397 nm (ε = 80x10^3 M⁻¹ cm⁻¹) can also be observed from pH 1 - 7. This study has shown that these two species are related by a simple monomer-dimer equilibrium at pH 1. (K_D = 1.1 x 10⁵ M⁻¹).
We conclude that, the monomer is the monomeric aquo complex and the dimer is the dimeric aquo complex because of the difference from the alkaline hydroxy dimer. Other species with broad Soret bands at about 365 nm reported in the region pH 2 - 3,\(^{92,96}\) must be higher aggregates.

The ionisation of the carboxylic acid groups, which normally have a pKa of about 5,\(^{98}\) has no significant effect on the spectra of either complex, at least in the Soret region, which is consistent with the negligible effect on the spectrum of esterification.\(^{24}\) Their pKa is about 3. (Previous values reported at \(\sim 1.5\) \(^{92,96}\) probably involve dimers and polymers.) This lowering of the pKa may not be genuine because of the probable significant concentration of the acid dimer in the intermediate pH range, but if it is genuine this may reflect specific hydrogen bonding of water to the carboxylates, as found in the crystal structure,\(^{50}\) which would stabilize the carboxylates relative to the carboxylic acid groups. It is possible that only one carboxylate is ionised at this pKa, although the results do suggest that two are involved.

Although ionisation of the carboxylic acid groups does not affect the spectrum significantly, qualitative observations indicate that it does have a marked effect on the rate of aggregation which is greatest in the region pH 3 - 7 (see stopped flow results). One would expect aggregation to occur most readily with the uncharged complex (e.g. Fe\(\text{-OH}_2\) with one carboxylic acid ionised) whose concentration would probably be at a maximum at \(\sim\) pH 5. It is interesting to note that similar dimer spectra are found at pH1 (\(\mu=0.1\)) and pH7 (\(\mu=10^{-5}\)) where the carboxylates are protonated and ionised respectively. This suggests similar dimer species at
these two pHs which may, of course, be a consequence of the ionic strength as well as ionisation. (At low ionic strength, the repulsion of the carboxylates must be minimised by placing them as far from each other as possible.)

The pKa for the coordinated water involves a monomer-dimer equilibrium at pH 7 (which corrected for the alkaline monomer-dimer equilibrium is pH 8) at moderate ionic strength, but pH (uncorrected) at low ionic strength. By comparison the pKa of hemin in 44% aqueous ethanol (which is a monomer-monomer equilibrium involving one proton) is 6.5 - 6.6. Other pKas reported at pH probably involve acid dimers and polymers. [10,31a,34,92,96]

The monomeric species in 50% aqueous ethanol is high spin (µ = 5.41 BM) and because of the similar spectral characteristics, the monomeric species in the absence of ethanol probably is as well and may be the same species as H₂O is a better ligand than ethanol. It is not certain whether this species is five or six-coordinate (a high spin bis aq iron porphyrin (FeTPP) has been reported) but as five coordinate species appear to have a broader and less intense Soret than the six coordinate species, we may tentatively conclude this is the high spin bis-aquo complex.

It is curious, that in both the stopped flow studies and at low ionic strength, a dimer initially splits to give monomers, which then form new dimers. The different dimerisation constants of the acid and alkaline dimers indicates that they have different structures. Presumably formation of one type from the other proceeds most readily via the monomer.

4.3.2 In the presence of caffeine

It has been shown that caffeine forms an adduct with the aquo and
hydroxide hemin, both of which are monomeric. Hemin-caffeine shows two pKas both involving monomer-monomer conversions. The one at 3.1 involves one proton while the other at 5.3 involves two, thus accounting for all three protons required. There is a problem in assigning these pKas to particular groups. The pKa of coordinated water may be lowered to 3.1 (compared with that in 44% aqueous ethanol which is 6.5-6.6) by the hydrophobicity of the caffeine which would favour the hydroxide species. The two carboxylic acids would then have normal pKas. The other possibility requires cooperative interactions between the coordinated aquo/hydrox and one carboxylic acid/carboxylate for these groups to have the same pKa of 5.3 (this must be the case in 60% DMSO where only one pKa was found) as well as a decrease in the pKa of one carboxylate to 3.1, which was also required in the absence of caffeine and could result from specific hydrogen bonding interactions of water between the carboxylic acid/carboxylate groups.

In the absence of caffeine, ionisation of the carboxylates did not have a marked effect on the spectra, but in the presence of caffeine they do, whatever assignment is chosen. Possibly, caffeine could hydrogen bond to the carboxylic acids, but not carboxylates of course) in such a way as to affect the steric properties of the porphyrin ring and hence its electronic properties, for example by affecting the degree of doming or core expansion.

The fairly sharp Soret band at pH 1.1 may, as in the absence of caffeine, indicate the bis-aquo hemin.

The presence of silicate prevented the aggregation of the hemin-caffeine adduct in acid aqueous solution but not the
hemin dimer in the absence of caffeine. Reversible changes and isosbestic points were found. Approximate pKas were 3 and 7, not too different from those in the absence of silicate. The spectra all showed bands at 610 nm which indicated high spin Fe(III). The Soret spectra were not affected by the presence of silicate except at pH 1, where the charged aquo hemin may be interacting with the silicic acid.
CHAPTER 5 - HEMIN COMPLEXES WITH IMIDAZOLE AND ANALOGUES

5.1 Introduction

The aqueous solution chemistry of the imidazole/histidine monomeric complexes of hemin is of interest because these complexes are models for hemoglobin, myoglobin and peroxidase (if one histidine is coordinated) and cytochrome $b_2$ (if two histidines are coordinated).

A lot of work has been done on imidazole and analogues in non-aqueous solvents, where the iron porphyrins are monomeric. These studies show that $K_1$ (i.e. the equilibrium constant for binding the first ligand) is less than $K_2$ (for the binding of the second ligand) and hence the mono-imidazole complex is not normally observed. Because of the insolubility of histidine in non-aqueous solvents, no work has been done on it, but histamine has been studied.

Some work on imidazole and analogues with hemin in aqueous solution has been done. This includes a quantitative study on the binding of $N$-methyl and $N$-ethyl imidazole, which is reportedly simple, i.e. isosbestic points were found and the monomeric bis-imidazole complex which absorbs at 412 nm was the product, but as will be seen below, this is not the whole story. Imidazole also gives an analogous complex which absorbs at 412 nm, but this decomposes to give a second product which has its Soret at 435 nm, beyond that of other Fe(III) complexes (except those with two Soret bands). The nature of this complex is still in doubt. Histidine gives the 412 nm species but not the 435 nm species. The equilibrium constant has not, however, been quantitatively determined, although some determinations of the
ligand concentration required to half saturate the hemin have been made.

We have therefore undertaken a more detailed study of the coordination of some of its derivatives, in particular histidine, in aqueous solution.

It soon became apparent that several additional minor equilibria, not previously reported, are evident. These may be due to hydrogen bonding or donor-acceptor interactions. Also coordination has interesting effects on the pKas of the side chains and this can be used to model neighbouring acid groups in hemoproteins. The study was therefore extended to histamine and pilocarpine (figure 5.1).

![Figure 5.1 Imidazole and analogues: R = H imidazole; R = -CH₂-COOH histidine; R = -CH₂-CH₂-NH₂ histamine; R = -CH₂-CH⁻ COO⁻ pilocarpine](image)

The aims of this chapter are:

1. To determine the binding constants for histidine, histamine and pilocarpine and to establish the structure of these complexes from the stoichiometry.

2. To study the effect of coordination on the pKas of neigh-
bouring pendant functional groups.

3. To gather further information on the peculiar 435 nm complex of the parent imidazole.

4. To elucidate the nature of the minor equilibria.

5. To test for the occurrence of mono-imidazole hemin in aqueous solution, starting with monomeric hemin-caffeine.

6. To compare the spectra of cytochrome b$_5$ and the bis-imidazole complexes in order to see the similarity in electronic structure between the hemoprotein and model complexes.

5.2 Results

5.2.1 Preliminary experiments

Most experiments were done above pH8, to avoid aggregation which occurs below this pH (Chapter 4). Most of the quantitative studies were done up to pH11, because of the high concentrations of ligand (and hence a high ionic strength, which favours aggregation (Chapter 3)), required to overcome the competition from hydroxide.

The addition of imidazole to hemin resulted in the formation of the 435 nm species (figure 5.2) via the 412 nm species with an isosbestic point for the 412 nm to 435 nm conversion at 430 nm. The formation of this species is relatively slow (at $8 \times 10^{-6}$ M in the presence of 1M imidazole it took about an hour for the equilibrium to be established). High hemin concentrations and low imidazole concentrations, shifted the equilibrium towards the 435 nm species

$$D \leftrightarrow 412 \text{ nm} \leftrightarrow 435 \text{ nm}.$$  

The 435 nm species shows vibrational overtones at 356 nm and 396 nm.
Figure 5.2 Spectrum of the 435 nm species; $7,12 \times 10^{-5}$ M hemin; 1M imidazole; pH12; 25°C; $l = 0.1$ cm.

Gallagher and Elliott reported that "graphical analysis of the data (for the alkaline dimer to 435 nm species conversion) gave a steep titration curve that approximated the ideal curve for a reaction in which four molecules of ligand react with the..."
dimeric hematin without splitting the dimer”.

The effect of the hematin concentration supports the proposal that this 435 nm species is dimeric. The reversible formation of the 412 nm species on adding more imidazole, supports a dimer rather than an aggregate as the formation of the latter tends to be irreversible. However, the shift in equilibrium towards the normal 412 nm species by an increase in imidazole concentration suggests that the dimer contains less than four ligands.

The addition of low concentrations of detergent (4 x 10^{-5} M CTMAB) reversed the formation of this imidazole dimer to give the 412 nm species. This suggests hydrophobic interactions are involved in holding this dimer together, for detergent to have had any effect.

Low concentrations of N-methyl imidazole showed small changes with isosbestic points at 520 nm and 580 nm, while at higher concentrations, the 412 nm species was formed with isosbestic points at 507 and 588 nm (figure 5.3).

The 412 nm species, which has been shown to be the monomeric bis-N-methyl imidazole hemin, slowly converted to the 435 nm species at > 2 x 10^{-5} M hemin with an isosbestic point at 384 nm, but as found with imidazole, could be suppressed by an excess of ligand. It is possible that this conversion is favoured by ionic strength and this may be the reason why Mohr and Scheler who were working in very low ionic strength solutions apparently did not observe it. The equilibria observed with N-methylimidazoles are:

\[
D \leftrightarrow \text{adduct} \leftrightarrow 412 \text{ nm (monomeric bis imidazole hemin)}
\]

\[
435 \text{ nm (dimeric imidazole hemin)}
\]
Figure 5.3 Changes in the spectrum of hemin on adding N-methyl imidazole at pH 11; $5.6 \times 10^{-6}$ M hemin; 25°C;
$\mu = 0.01; \; \kappa = 10 \; \text{cm}$.

--- 0 M; ... 0.025M; 0.038M; ------ 0.063M;
0.10M; 0.14M; --- 0.19M N-methyl-imidazole.

2-methyl-imidazole destabilizes the 412 nm species as shown by its incomplete formation even at concentrations approaching saturation, but forms an adduct at lower concentrations. Figure 5.4 shows the effect on the spectrum in the Soret region at lower concentrations. Little change occurred in the visible region.
Figure 5.4 Changes in the spectrum of hemin on adding 2-methyl-imidazole; $4.5 \times 10^{-6}$ M hemin; pH12
The spectral changes are similar to those observed on adding adenine and guanidine (Chapter 3), which suggests a donor-acceptor, hydrogen bonded or hydrophobic adduct). No 435 nm species was observed. Steric hindrance to coordination can account for the destabilization of the 412 nm species and perhaps also the 435 nm species.

Histidine, histamine and pilocarpine all show small changes in the spectra at low concentrations, analogous to those observed at low 2-methyl imidazole concentrations and at low 2-methyl imidazole concentrations. The isosbestic points were at (400 nm; 610 nm); (401 nm; 502 nm; 614 nm); (392 nm; 498 nm and 590 nm) respectively.

At high ligand concentrations, the 412 nm species was formed with isosbestic points at (392 nm; 500 nm; 585 nm); (394 nm; 502 nm; 582 nm) and (392 nm; 498 nm and 590 nm) respectively. The λmax and ε(λmax) are tabulated in Table 5.1. Histamine shows further changes characterised by an initial increase up to 10 minutes (48% increase in 1 minute) followed by a slow fall (416% decrease in ten hours) in the Soret intensity. No 435 nm species was observed with these ligands, presumably because of steric hindrance.

Imidazole and histidine were both added to hemin-caffeine (in alkaline solution). Figure 5.5 shows the spectral changes observed at low ligand concentrations in the visible region. The absorbance at 612 nm decreased while that at 4370 nm increased. In the Soret region the absorbance at 402 nm decreased. These changes are similar to those observed on forming the hemin-caffeine dimer (Chapter 3). For some reason imidazole and
Figure 5.5 Changes in the spectrum of hemin-caffeine on adding low concentrations of imidazole; $7.3 \times 10^{-6}$M hemin; 0.05M caffeine; $0.00, 0.05, 0.07, 0.08$M imidazole.

Histidine may stabilize this dimer. There is no evidence for significant concentrations of the mono-ligand adduct being present ($\lambda_{max}$ for aquohemoglobin 405 nm; 500 nm; 541 nm (shoulder); 581 nm (shoulder); 629 nm $^{87}$).
Figure 5.6 Spectra of 7,9 μM monomeric bis-histidine heme (---) and cytochrome b₅ (-----) at pH 8,5 and pH 7 respectively (former solution contains 0,2M histidine); 25°C
Figure 5.6 shows the spectra of cytochrome b$_5$ and the monomeric bis-histidine hemin (see later). (Note that the protein solution is slightly turbid.) They are similar which supports the assignment as the bis-histidine complex with coordination occurring through the imidazole (as does the negligible effect of glycine and acetate on the spectrum). There are subtle differences in the position and intensity of the Soret intensity which must reflect the effect of the spin. (Table 5.1 summarises the $\lambda_{\text{max}}$ and $\varepsilon(\lambda_{\text{max}})$ for these systems.

Table 5.1: The $\lambda_{\text{max}}$ and $\varepsilon(\lambda_{\text{max}})$ for the imidazole and analogous complexes with hemin (the 412 nm species)

<table>
<thead>
<tr>
<th>Ligand/Protein</th>
<th>$\lambda_{\text{max}}^a$</th>
<th>$\varepsilon(\lambda_{\text{max}})^b$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>imidazole</td>
<td>412(112); 530(11); 556(9)$^c$</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>N-ethyl imidazole</td>
<td>411(121); 538(12); 560(10)$^c$</td>
<td></td>
<td>44a</td>
</tr>
<tr>
<td>histidine</td>
<td>411(110); 532(10); 557(8)</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td>histamine</td>
<td>409(113); 530(20); 560(9)</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td>pilocarpine</td>
<td>411(111); 554(10,1); 560(8,5)</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td>cytochrome b$_5$</td>
<td>413(108); 532(13,6); 560(11,8)</td>
<td></td>
<td>this study</td>
</tr>
<tr>
<td>imidazole methemoglobin</td>
<td>412(105); 535(14,7); 562(12,5)</td>
<td></td>
<td>87</td>
</tr>
</tbody>
</table>

$^a$ in nm  $^b$ in $10^3 \text{ M}^{-1} \text{ cm}^{-1}$  $^c$ read off spectra

All show similar $\lambda_{\text{max}}$ and extinction coefficients as would be expected for complexes where the iron is coordinated to the same group.

Hence all these bases show a similar pattern in alkaline
solution, viz:

\[
\begin{align*}
\text{FeOH - caffeine} \\
\downarrow \\
\text{adduct} \\
\downarrow \\
D \rightleftharpoons \text{adduct} \rightleftharpoons 412 \text{ nm species} \rightleftharpoons 435 \text{ nm species} \\
\uparrow \ (\text{histamine}) \\
\text{aggregation(?)}
\end{align*}
\]

All equilibria were set up rapidly except those between the 412 nm and 435 nm species and the aggregation with the histamine species.

No evidence was found for any significant concentrations of the mono-imidazole complexes.

It is useful to divide these equilibria into major (which involve changes in the coordination sphere) and minor ones (which may involve hydrogen bonding, donor-acceptor or hydrophobic interactions).

The D \rightleftharpoons \text{adduct} and adduct \rightleftharpoons 412 \text{ nm species} will now be studied more quantitatively with histidine, histamine and pilocarpine as ligands. These were chosen in order to see the effects of the side chains on the equilibria. (Note - the instability of the histamine complex makes the quantitative results unreliable.)

5.2.2 Quantitative determinations of the binding constants

5.2.2.1 Low ligand concentrations

The D \rightleftharpoons \text{adduct} equilibrium was studied at 590 nm, which is the isosbestic point for the next equilibrium.

The results are given in full in the tables in appendix 4A and summarized in table 5.2. The data fitted a D \rightleftharpoons D' equilibrium but not a D \rightleftharpoons N equilibrium. Figure 5.7 shows typical
plots of $\frac{A-A_D}{A_{m-A}}$ versus log [ligand], which are linear as if required if a $D \leftrightarrow D'$ equilibrium holds (appendix 1).

In addition, the slope gives the number of ligands bound per dimer.

In all cases the slope was one, i.e. the equilibrium was

$$D + L \leftrightarrow D \cdots L \quad (K_L)$$

Table 5.2: Summary of the titrations of hemin with low $[L]$.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$pH$</th>
<th>$[\text{Hemin}]_0$</th>
<th>Slope$^a$</th>
<th>$\log K_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>histidine</td>
<td>8.50</td>
<td>$65\times10^{-6}$</td>
<td>1.05</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td></td>
<td>8.50</td>
<td>$136\times10^{-6}$</td>
<td>1.03</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td></td>
<td>10.00</td>
<td>$136\times10^{-6}$</td>
<td>0.95</td>
<td>1.9±0.2</td>
</tr>
<tr>
<td></td>
<td>11.00</td>
<td>$136\times10^{-6}$</td>
<td>1.06</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>Avg:</td>
<td></td>
<td></td>
<td>1.02</td>
<td>1.9±0.2</td>
</tr>
</tbody>
</table>

Figure 5.7 Plots of $\frac{A-A_D}{A_{m-A}}$ versus log [ligand] at pH 11 for histidine, histamine, and pilocarpine at low [ligand].
Within experimental error there is no effect of pH which
indicates no loss of OH\(^-\) or uptake of H\(^+\). Hence the \(K_1\)
equilibrium can be written:

\[
(\text{FeOH})_2 + L \rightleftharpoons (\text{FeOH})_2 \cdots L \quad (K_1)
\]

5.2.2.2 High ligand concentrations

The titration at high ligand concentrations was carried out close
to the isoelectric point of the first equilibrium, and was done at
413 nm, 409 nm and 410 nm for histidine, histamine and pilocarpine
respectively.

In order to obtain the bis-ligand complex, one hydroxide per
iron must be lost and hence the equilibrium \(K_2\) would be expected.

\[
(\text{FeOH})_2 \cdots L + 3L \rightleftharpoons 2\text{FeL}_2 + 2\text{OH}^- \quad (K_2)
\]

The presence of this equilibrium can be tested in two ways,
viz. by varying the ligand concentration and by varying the hemin concentration. The derivations in appendix 1, show that a plot of \( \log \frac{[ML_2]^2}{[L]} \) versus \( \log [L] \) should be linear with a slope of three if this equilibrium holds. This is in fact so, with the exception of histamine which is unstable. (Table 5.3; figure 5.8; Tables in appendix 4Bi).

Table 5.3: Summary of the ligand titration results with hemin at high ligand concentrations; 25°C; \( u = 0.5 \)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>pH</th>
<th>slope (^a)</th>
<th>( \log K_2 )^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>histidine</td>
<td>9.0</td>
<td>3.1 ; 3.1</td>
<td>-11.2 ; -11.2 (±0.1)</td>
</tr>
<tr>
<td>histamine</td>
<td>9.0</td>
<td>2.3</td>
<td>-12.1 (±0.3)</td>
</tr>
<tr>
<td>pilocarpate</td>
<td>9.3</td>
<td>2.95</td>
<td>- 8.4 (±0.3)</td>
</tr>
<tr>
<td>pilocarpate</td>
<td>10.2</td>
<td>3.0</td>
<td>- 8.31 (±0.03)</td>
</tr>
</tbody>
</table>

\(^a\) of plot of \( \log \frac{[ML_2]^2}{[L]} \) versus \( \log [L] \)

\(^b\) \( K_2 \) refers to the equilibrium \((FeOH)_2 \cdots L + 3L \rightleftharpoons 2FeL_2 + 2OH^-\)

\[ \log [L] \quad \frac{[ML_2]^2}{[L]} \]

Figure 8 Plots of \( \log \frac{[ML_2]^2}{[L]} \) versus \( \log [L] \) at high [ligand] for histidine and pilocarpate
If the hemin concentration is varied, then if the proposed equilibrium holds, a plot of log $[Fe]_{TOT}$ vs. log $[Fe]_{TOT}(1-\alpha)$ (where $\alpha$ is the degree of transformation) should be linear with a slope of 0.5 (appendix 1). This is true for all systems except histamine which is known to be unstable (see table 5.4; figure 5.9; tables in appendix 4Bi).
Table 5.4: Summary of the dilution plot results with hemin at high ligand concentrations; 25°C; \( u = 0.5 \)

<table>
<thead>
<tr>
<th>pH</th>
<th>[ligand]/M</th>
<th>slope a</th>
<th>log ( K_2 ) b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) histidine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>0.05</td>
<td>0.4</td>
<td>-11.6</td>
</tr>
<tr>
<td>9.0</td>
<td>0.05</td>
<td>0.5</td>
<td>-11.1</td>
</tr>
<tr>
<td>10.0</td>
<td>0.1</td>
<td>0.5;0.5</td>
<td>-9.4; -9.4</td>
</tr>
<tr>
<td>11.0</td>
<td>0.03</td>
<td>0.5</td>
<td>-8.6</td>
</tr>
<tr>
<td>11.5</td>
<td>0.20</td>
<td>0.5</td>
<td>-8.6</td>
</tr>
<tr>
<td>12.0</td>
<td>0.20</td>
<td>0.5</td>
<td>-8.2</td>
</tr>
<tr>
<td>b) histamine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>0.05</td>
<td>0.2</td>
<td>-12.7</td>
</tr>
<tr>
<td>9.0</td>
<td>0.05</td>
<td>0.3</td>
<td>-12.2</td>
</tr>
<tr>
<td>10.0</td>
<td>0.10</td>
<td>0.3;0.25</td>
<td>-9.7; -9.7</td>
</tr>
<tr>
<td>11.0</td>
<td>0.10</td>
<td>0.5</td>
<td>-8.1</td>
</tr>
<tr>
<td>c) pilocarpine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>0.005</td>
<td>0.4;0.4;0.4</td>
<td>-9.5; -9.5; -9.7</td>
</tr>
<tr>
<td>9.0</td>
<td>0.005</td>
<td>0.5</td>
<td>-9.0</td>
</tr>
<tr>
<td>10.0</td>
<td>0.025</td>
<td>0.5</td>
<td>-8.4</td>
</tr>
<tr>
<td>11.0</td>
<td>0.005</td>
<td>0.5;0.5;0.4</td>
<td>-7.4; -7.5; -7.3</td>
</tr>
</tbody>
</table>

a) Of plot of log [Fe]_{TOT} versus log [Fe]_{TOT}(1-\( \alpha \))

b) refers to the equilibrium \((FeOH)_2\cdot L + 3L \rightleftharpoons 2FeL_2 + 2OH^-\)

Log \( K_2 \) is not independent of pH which means that something is happening to the ligands. However, if corrections are made for the ionisation of the \(-\text{NH}_2\) and \(-\text{OH}\) groups (free and
coordinated respectively), the binding constant becomes pH independent (see Table 5.5) (derivations in appendix 5).

Table 5.5: Correction of the binding constants for the pKas of the ligands

<table>
<thead>
<tr>
<th>pH</th>
<th>log $K_1$</th>
<th>log $K_2$</th>
<th>log $K_1K_2$</th>
<th>log $K_3$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>histidine b</td>
<td>8.5</td>
<td>2.0</td>
<td>-11.8</td>
<td>-9.9</td>
</tr>
<tr>
<td></td>
<td>9.0</td>
<td>-</td>
<td>-11.2</td>
<td>-9.2</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.9</td>
<td>-9.4</td>
<td>-7.5</td>
</tr>
<tr>
<td></td>
<td>11.0</td>
<td>1.8</td>
<td>-8.8</td>
<td>-6.8</td>
</tr>
<tr>
<td></td>
<td>11.5</td>
<td>-</td>
<td>-8.6</td>
<td>-6.9</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>-</td>
<td>-8.2</td>
<td>-6.3</td>
</tr>
<tr>
<td><strong>Average:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| histamine c | 8.5 | 2.6 | -12.7 | -10.3 | -5.2 |
|            | 9.0 | -   | -12.2 | -9.7 | -6.2 |
|            | 10.0 | 2.7 | -9.7 | -7.1 | -6.3 |
|            | 11.0 | 2.4 | -8.1 | -5.5 | -5.4 |
| **Average:** |       |       |       |       | $-5.5\pm0.8$ |

| pilocarpate d | 8.5 | 2.2 | -9.6 | -7.6 | -7.6 |
|              | 9.0 | -   | -9.0 | -7.0 | -7.1 |
|              | 10.0 | 1.8 | -8.4 | -6.4 | -7.0 |
|              | 11.0 | 1.9 | -7.4 | -5.4 | -7.5 |
| **Average:** |       |       |       |       | $-7.2\pm0.4$ |

a $K_3$ refers to the equilibrium $(\text{FeOH})_2 + 4\text{RX} \leftrightarrow 2\text{Fe(RX)}_2 + 2\text{OH}^-$
where $X = \text{NH}_2$ for histidine, histamine; $X = \text{OH}$ for pilocarpate

b pKa of $\text{-NH}_3^+$ (free) used was 9.33
c pKa of $\text{-NH}_3^+$ (free) used was 9.76
d pKa of $\text{-OH}$ (coordinated) used was 10.119
Hence, the conjugate base in all systems is stabilized by the residual positive charge on the iron(III).

A slight difference in the spectrum above and below the pKa of the coordinated pilocarpate was found. Below the pKa at pH 8.5 the bands were found at 411 nm (111 mM$^{-1}$ cm$^{-1}$); 534 nm (10.1 mM$^{-1}$ cm$^{-1}$) and 560 nm (8.5 mM$^{-1}$ cm$^{-1}$) while above the pKa at pH 12 the bands were at 413 nm (113 mM$^{-1}$ cm$^{-1}$); 531 nm (10.7 mM$^{-1}$ cm$^{-1}$) and 559 nm (9.0 mM$^{-1}$ cm$^{-1}$). The latter are more similar to cytochrome $b_5$ than the former (table 5.1). Hence neighbouring group interactions in the cytochrome $b_5$ could account for the difference of the bis-histidine complex spectrum from that of the protein.

The differences in the binding constants are fairly small (and amount to a maximum difference of 1.3 kJ mole$^{-1}$) and are probably due to varying hydrogen bonding and coulombic effects.

Comparisons of the binding constants in other aqueous or mixed aqueous solvents can be made if the binding constant is broken into two parts, i.e. monomerization followed by ligand binding.

\[ (\text{FeOH})_2 ^{\rightarrow} 2\text{FeOH} \quad K_D^{-1} \]

\[ \text{FeOH} + 2L \quad \xrightleftharpoons{\text{FeL}_2} + \text{OH}^- \quad K_L \]

Hence $K_1 K_2 = (K_D^{-1}) (K_L)^2$

$K_L$ is $\geq 1.2; 1.8$ and $0.9$ M$^{-1}$ for histidine, histamine and pilocarpate. These compare well with the values of $K_L$ found in 44% aqueous ethanol (1 M$^{-1}$) and in micellar detergents (10$^{-2}$ to 10$^{-1}$ M$^{-1}$). 48,49
5.3 Discussion

The quantitative study of the equilibrium between dimeric hæmin and histidine, histamine and pilocarpate in alkaline solution has shown that the main product (412 nm) is the monomeric bis-ligand hæmin.

The pH dependence of the equilibrium constants further indicates that the pka of the \(-\text{NH}_2\) of histidine and histamine is reduced from about 9.5 to less than 8 on coordination while the pka of \(-\text{OH}\) of pilocarpate is reduced from about 15 to 10, on coordination. This can be ascribed to the stabilization of the conjugate base by the residual positive charge on the iron.

We have confirmed that imidazole forms the usual 412 nm species which converts to an unusual 435 nm form. This equilibrium has been shown to be shifted to the right by increasing hæmin concentration but to the left by increasing the imidazole concentration, but low concentrations of detergent suppressed it which may be a way of studying the normal bis-imidazole hæmin in aqueous solution. This equilibrium was also observed with N-methyl imidazole and was affected in the same way. However, it was not observed with 2-methyl imidazole, histidine, histamine and pilocarpate suggesting that steric hindrance prevents its formation. We conclude that the 435 nm, species is probably dimeric but has less than four imidazoles per dimer.

Adduct formation of the ligand with the dimer has been observed in the systems tested. Quantitative studies of this equilibrium (from the alkaline hæmin dimer) with histidine, histamine and pilocarpate showed a pH-independent addition of one ligand to the dimer. Because hydroxide was not lost, it was concluded that the ligand was not coordinated but is forming a
hydrogen bonded or donor-acceptor adduct.

The reaction between the monomeric hemin-caffeine and low concentrations of imidazole or histidine shows the formation of additional complex(es) before the normal 412 nm species. These intermediates show spectra similar to that of the dimeric hemin-caffeine. There appears to be no significant amount of the mono-imidazole complex. Hence one of the roles of the protein in peroxidase, for example, is to stabilize the mono-imidazole form, as $K_1 < K_2$ in aqueous solution as well as in organic solvents.\textsuperscript{42,43}

The spectra of the bis-histidine and other bis-ligand complexes (with analogues) are very similar to that of cytochrome $b_5$. The differences may be explained by neighbouring group interactions as shown by the slight spectral changes above and below the pKa of $-\text{OH}$ on coordinated pilocarpate.
CHAPTER 6 - STUDY OF THE REDUCTION OF B_{12a} BY DITHIOETHERITOL

6.1 Introduction

B_{12a} is a useful model for heme proteins in which one imidazole is coordinated. Hence the monomeric hemes and B_{12a} will be studied in parallel. B_{12a} reduction is discussed here while that of the monomeric hemes is discussed in Chapter 7 (as more is known about B_{12a}). An understanding of reduction is necessary to understand the rate of O_2 uptake (Chapters 8, 9).

Thiols have been used as the reducing agents because they are mild and because they are implicated in the mechanism of cytochrome P 450. It is well known that thiols readily coordinate to B_{12a} and that the rate of reduction to Co(II) increases with pH, but no Co(I) has ever been observed. A quantitative study on the reaction of B_{12a} with cysteine has largely focused on coordination, but it was noted that reduction required another cysteine in addition to the one coordinated, and was only observed above pH 7.2.

Dithioetheritol (Figure 6.1) can reduce B_{12a} but no

\[ \text{HO \ OH} \rightarrow \text{HOCH} \]
\[ \text{HSCH}_2\text{CHCHCH}_2\text{SH} \rightarrow \text{HOCH} \]
\[ +2e^- \]

Figure 6.1 Dithioetheritol
quantitative studies have been carried out with this thiol or apparently with any other dithiol. Dithiols are of interest as it is known that some proteins contain two thiol groups close to each other which appear to act in concert, one of the functions of these groups being to transfer electrons.

It was shown that the reduction of FeTPP by thiol in toluene results in the quantitative formation of the disulphide. This was also shown to be the case in the oxidation of thiols by O2 catalysed by aquocyanocobinamide in aqueous solution and hence will be assumed to be the same here.

The aims of this chapter are to study the kinetics of reduction of B12a by dithiothreitol quantitatively and to propose a mechanism. By comparison with the results of Nome and Pendler on cysteine, the effect of the neighbouring thiol group will be examined.

In considering the mechanism, it must be borne in mind that it may be inner or outer sphere and that the homolytic fission of the Co-S bond may be assisted by another thiol group to give the two-centre three-electron bond species (figure 6.1) or may be unassisted to give the thiol radical.

6.2 Results
6.2.1 Qualitative changes
In preliminary experiments, the spectral changes which occur on adding dithiothreitol to B12a under N2, were investigated between pH 1 and 12. In all cases Co(II) corrinoid was obtained. Below pH 3 the product bands were at 315 nm; 400 nm (shoulder); and 470 nm which are the bands of the base of Co(II) corrinoid (315 nm; 403 nm; 470 nm) (i.e. the benzimidazole group becomes
protonated (pKa 2.9) and dissociates from the cobalt). Above pH 3, the product bands were at 312 nm; 405 nm and 474 nm which are the base on Co(II) corrinoid bands (312.5 nm; 405 nm and 473 nm).9

The intermediate spectra differ in acid (pH < 5) and alkaline (pH ≥ 7) solution.

Figure 6.2 Spectral changes occurred on addition dithiothreitol to B12a at pH 5.0 under N2; 6.5x10⁻⁵ M B12a; 1x10⁻³ M dtt; 25°C; i=0.1

--- B12a: ······30s and 3.5 minutes after mixing; --- final product (after 40 minutes)
Figure 6.2 shows typical changes occurring in acid solution, which do not show isosbestic points except in the latter part of the reaction at 332 nm; 392 nm; 498 nm. (Initial bands were found at 350 nm; 410 nm; 500 nm; and 526 nm and are those of the aquocobalamin (351 nm; 411 nm; 500 nm and 527 nm).) Intermediate bands are evident at 370 nm; 532 nm and 552 nm which correspond to those of the thiolocobalamin (370 nm; 532 nm; 552 nm).

Figure 6.3 Spectral changes occurring on adding dithiothreitol to B_{12a} at pH 8.0 under N_2; 3\times10^{-5} M B_{12a}; 2\times10^{-5} M dtt; 25°C; u=0.1

--- B_{12a};...3,5 and 10 minutes respectively after mixing;
---- final product (after 40 minutes)
Figure 6.3 shows typical changes occurring in alkaline solution where isosbestic points are found at 337 nm; 372 nm; 490 nm; and 568 nm throughout. No thiolocobalamin bands are evident. The initial bands at 357 nm; 420 nm; 514 nm and 536 nm at pH > 8 correspond to those of the hydroxocobalamin (358 nm; 421 nm; 516 nm and 537 nm). 102

6.2.2 Quantitative kinetic studies

The kinetic studies were carried out using UV-visible spectrophotometry. The absorbance changes at 474 nm (a 342 band) were followed as a function of time under pseudo first order conditions in H2O, under anaerobic conditions. All the runs gave pseudo first order kinetics.

Most of the runs gave monophasic kinetics over four half lives and in these cases kobs was determined from a plot of ln(Ao - A) vs t (where Ao = absorbance at infinite time, A = absorbance time t, t = time) (kobs = -slope). At pH 4 and 5 the runs were biphasic while at higher dithiothreitol concentrations at pH 6,0 they were triphasic (lower dithiothreitol concentrations at pH 6,0 gave biphasic kinetics). By curve stripping, the observed rate constants can be evaluated for the different phases.

There was an induction period, the length of which was dependent on the oxygen concentration. This was minimised by flushing the solutions with N2 and maintaining the reacting system under N2 and was reduced to < 20% of the reaction time.

The standard deviations within a run were within 2% while the deviation between different runs was within 5% when there was minimal or constant trace metal contamination.
The dependence of the rate on pH and on the dithiothreitol concentration was determined in these studies.

The qualitative studies indicate a difference in spectral changes in acid and alkaline solution.

In acid, the $B_{12}\text{-thiolate}$ was formed rapidly followed by a relatively slow conversion to $B_{12}$. 

In alkaline solution, there were isosbestic points between the initial and final species which implies the absence of any significant amount of intermediate. The reduction could be outer sphere but if it is inner sphere, as at low pH, the coordination of thiolate must be slow and the reduction fast.

Hence to understand this system, both regions must be studied. However, the region below pH 3 is not of much interest. The spectra indicate that in this region, the base of $B_{12}$ was the product and because of the strong trans effect of $RS^{-9}$, the $B_{12}\text{-thiolate}$ may also be base off. The purpose of studying the kinetics of $B_{12}$ was to simplify the system because of the presence of only one coordination site and at pH < 3 this may no longer be the case.

6.2.2.1 pH profile

Table 6.1 and figure 6.4 show the variation of $k_{obs}$ with pH.
Figure 6.4 Effect of pH on $k_{obs}$ for the reduction of $B_{12a}$ by dithiothreitol; $6.5 \times 10^{-5}$ M $B_{12a}$, $1 \times 10^{-3}$ M dtt; 25°C; $\mu = 0.1$

(under $N_2$); $\bigcirc$ $k_{obs}$; $\times$ $k_{obs_1}$; $\Delta$ $k_{obs_2}$
Table 6.1: The effect of pH on the rate of reduction of B$_{12a}$ by dithiothreitol, [B$_{12a}$] = 5.5 x 10$^{-5}$M; [dtt] = 1 x 10$^{-3}$M;
25°C; µ = 0.1

<table>
<thead>
<tr>
<th>pH</th>
<th>10$^2$ k$_{obs}$ s$^{-1}$</th>
<th>a (R)</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.10</td>
<td>1.93</td>
<td>(0.9966)</td>
<td></td>
</tr>
<tr>
<td>1.10</td>
<td>2.17</td>
<td>(0.9988)</td>
<td>A</td>
</tr>
<tr>
<td>2.00</td>
<td>1.93</td>
<td>(0.9967)</td>
<td></td>
</tr>
<tr>
<td>3.04</td>
<td>1.35</td>
<td>(0.9994)</td>
<td></td>
</tr>
<tr>
<td>3.33</td>
<td>1.38</td>
<td>(0.9999)</td>
<td>B</td>
</tr>
<tr>
<td>4.00</td>
<td>1.96; 0.68</td>
<td>(0.9995)</td>
<td></td>
</tr>
<tr>
<td>6.00</td>
<td>14.9; 1.89</td>
<td>(0.999)</td>
<td>b</td>
</tr>
<tr>
<td>7.00</td>
<td>9.10</td>
<td>(0.9992)</td>
<td></td>
</tr>
<tr>
<td>7.00</td>
<td>8.72</td>
<td>(0.9995)</td>
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<td>18.32</td>
<td>(0.9994)</td>
<td></td>
</tr>
<tr>
<td>8.25</td>
<td>22.08</td>
<td>(0.9982)</td>
<td></td>
</tr>
<tr>
<td>8.40</td>
<td>26.66</td>
<td>(0.9990)</td>
<td></td>
</tr>
<tr>
<td>8.50</td>
<td>27.20</td>
<td>(0.9968)</td>
<td></td>
</tr>
<tr>
<td>8.50</td>
<td>28.12</td>
<td>(0.9890)</td>
<td>C</td>
</tr>
<tr>
<td>8.60</td>
<td>24.74</td>
<td>(0.9964)</td>
<td></td>
</tr>
<tr>
<td>8.75</td>
<td>23.35</td>
<td>(0.9933)</td>
<td></td>
</tr>
<tr>
<td>9.00</td>
<td>17.65</td>
<td>(0.9999)</td>
<td></td>
</tr>
<tr>
<td>10.00</td>
<td>5.79</td>
<td>(0.9988)</td>
<td></td>
</tr>
<tr>
<td>10.52</td>
<td>2.01</td>
<td>(0.9933)</td>
<td></td>
</tr>
<tr>
<td>11.00</td>
<td>1.03</td>
<td>(0.9990)</td>
<td></td>
</tr>
<tr>
<td>11.50</td>
<td>0.35</td>
<td>(0.9984)</td>
<td></td>
</tr>
<tr>
<td>12.00</td>
<td>0.11</td>
<td>(0.9987)</td>
<td></td>
</tr>
<tr>
<td>12.88</td>
<td>0.11</td>
<td>(0.9986)</td>
<td></td>
</tr>
<tr>
<td>13.74</td>
<td>0.11</td>
<td>(0.9990)</td>
<td>D</td>
</tr>
</tbody>
</table>

a obtained from -slope of semilog plot

b obtained from curve stripping of semilog plot

c R is the correlation coefficient
As the kinetics are complex between pH 4 and 6, the kinetic study in acid will be focused on the pH 3 region where the kinetics are monophasic (region B). The other region of interest is between pH 7 and pH 12 (region C). At pH > 12, \( k_{\text{obs}} \) becomes pH independent (region D).

### 6.2.2.2 pH 3 region (region B)

A comparison of the \( k_{\text{obs}} \) at pH 3.04 and pH 3.33 (Table 6.1) indicates that in this region, \( k_{\text{obs}} \) is independent of pH, i.e., independent of both \( H^+ \) and \( OH^- \) concentrations. Table 6.2 shows that \( k_{\text{obs}} \) is also independent of the thiol concentration.

**Table 6.2: The effect of the dithiothreitol concentration on the rate of reduction of \( B_{12a} \) by dithiothreitol at pH 3.20; 6.5 \(< 10^{-5} M \) \( B_{12a} \), \( \mu = 0.1 \); 25°C.**

<table>
<thead>
<tr>
<th>( 10^3 \text{[dithiothreitol]} ) M</th>
<th>( 10^2 k_{\text{obs}}, s^{-1} )</th>
<th>( R )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.47</td>
<td>0.9989</td>
</tr>
<tr>
<td>2.5</td>
<td>1.42</td>
<td>0.9990</td>
</tr>
<tr>
<td>5.0</td>
<td>1.42</td>
<td>0.9993</td>
</tr>
<tr>
<td>10.0</td>
<td>1.00</td>
<td>0.9996</td>
</tr>
</tbody>
</table>

Even at \( 1 \times 10^{-4} M \) CuSO\(_4\) and \( 1 \times 10^{-3} M \) EDTA the rate was unaffected (\( k_{\text{obs}} \) was \( 1.35 \times 10^{-2} s^{-1} \) and \( 1.39 \times 10^{-2} s^{-1} \) respectively) unlike the effect of the same concentrations in the alkaline region (see later).

The \( k_{\text{obs}} \) for the reduction of \( B_{12a} \) by mercaptoethanol at pH 5 was found to be \( 3 \times 10^{-5} s^{-1} \) with \( 0.38 M \) mercaptoethanol. The thiolastocobalamin was observed within 20s (bands at 370 nm; 533 nm...
and 553 nm), indicating a slow reduction, but relatively fast coordination.

6.2.2.3 pH 7 - 12 region (region C)

a) Effect of pH

The pH profile of the rate of reduction of \( B_{12a} \) by dtt in this region is bell-shaped implying two opposing pH effects, for example between the conjugate base of one reagent and the conjugate acid of the other. Both \( B_{12a} \) and dtt ionise in this pH region with pK as 7.69 and 9.12; 10.15 respectively. Hence the two possibilities are Co-H\(_2\)O + RS\(^-\) or Co-OH + RSH. As RS\(^-\) is more effective than RSH as a reducing agent and Co-OH\(_2\) more effective than Co-OH as an oxidising agent, the first alternative will be focused on.

\( k_{\text{obs}} \) can be corrected for both the fraction of aquocobalamin and thiolate present at each pH. Figure 6.5 shows the plot of \( k_{\text{obs}} \) corrected for the fraction of aquocobalamin present (\( k_{\text{corr}} \)) as a function of pH. The midpoint of the curve lies at pH 10.1.

Correcting for the presence of thiolate present at each pH is more tricky because of the two thiol groups. It was found, however, that dividing by either the fraction of "S-R-SH or "S-R-S" present did not give a constant value at all pHs in this region, but that dividing by (the fraction of "S-R-SH + the fraction of "S-R-S") did. The implication of this is that \( B_{12a} \) does not discriminate between "S-R-SH and "S-R-S" in the rate determining step. The \( k_{\text{obs}} \) corrected for both the fraction of aquocobalamin present and the fraction of thiolate (both types) is shown in table 6.3.
Figure 6.5 Variation of $k_{corr}$ (i.e. $k_{obs}$ corrected assuming only aquocobalamin reacts) with pH

(Conditions as in figure 5.3)
Table 6.3: Correction \( k_{\text{corr}} \) for the fraction of aquocobalamin and then for the fraction of thiolate

<table>
<thead>
<tr>
<th>pH</th>
<th>( k_{\text{obs}}^{-1} )</th>
<th>( k_{\text{corr}}^{-1} )</th>
<th>( k'_{\text{corr}}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.00</td>
<td>8.91</td>
<td>0.112</td>
<td>14.85</td>
</tr>
<tr>
<td>8.00</td>
<td>18.32</td>
<td>0.643</td>
<td>9.05</td>
</tr>
<tr>
<td>8.25</td>
<td>22.08</td>
<td>1.211</td>
<td>10.0</td>
</tr>
<tr>
<td>8.40</td>
<td>26.66</td>
<td>1.95</td>
<td>12.0</td>
</tr>
<tr>
<td>8.50</td>
<td>27.66</td>
<td>2.47</td>
<td>12.5</td>
</tr>
<tr>
<td>8.60</td>
<td>24.74</td>
<td>2.72</td>
<td>11.5</td>
</tr>
<tr>
<td>8.75</td>
<td>23.15</td>
<td>3.31</td>
<td>11.4</td>
</tr>
<tr>
<td>9.00</td>
<td>17.65</td>
<td>4.65</td>
<td>10.3</td>
</tr>
<tr>
<td>10.00</td>
<td>5.79</td>
<td>14.48</td>
<td>15.6</td>
</tr>
<tr>
<td>10.32</td>
<td>2.01</td>
<td>16.75</td>
<td>17.0</td>
</tr>
<tr>
<td>11.00</td>
<td>1.03</td>
<td>25.9</td>
<td>25.8</td>
</tr>
<tr>
<td>11.50</td>
<td>0.35</td>
<td>27.0</td>
<td>27.8</td>
</tr>
<tr>
<td>12.00</td>
<td>0.11</td>
<td>27.5</td>
<td>27.5</td>
</tr>
</tbody>
</table>

\( a \quad k_{\text{corr}} = \frac{k_{\text{obs}}}{[H^+] / ([H^+] + K_a)} \)  

where \( K_a = 10^{-7.6} \) and refers to the dissociation of a proton from aquo-cobalamin

\( b \quad k'_{\text{corr}} = \frac{k_{\text{corr}}}{f_{\text{RSH-RS}^-} + \frac{f_{\text{RS-RS}^-}}{f_{\text{RSH-RS}^-}}} \) where \( f_{\text{RSH-RS}^-} = \frac{1}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}} \)

\( f_{\text{RS-RS}^-} = f_{\text{RSH-RS}^-} \frac{K_2}{[H^+]^2} \)

\( K_1 \) and \( K_2 \) are the first and second acid dissociation constants for the thiols on dithiothreitol, \( pK_1 = 9.12 \); \( pK_2 = 10.15 \).
b) Effect of the dithiothreitol concentration on $k_{obs}$

The effect of the dithiothreitol concentration on $k_{obs}$ is shown in Table 6.4.

**Table 6.4:** The effect of dithiothreitol concentration on the rate of reduction of $B_{12a}$ by dithiothreitol at pH 8.6 in the presence of EDTA: $6.5 \times 10^{-5} M$ $B_{12a}$; $2 \times 10^{-3} M$ EDTA; $\mu = 0.1$; $25^\circ C$.

<table>
<thead>
<tr>
<th>$10^2[\text{dithiothreitol}]$, M</th>
<th>$k_{obs}$, s$^{-1}$</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.0169</td>
<td>0.9991</td>
</tr>
<tr>
<td>1.0</td>
<td>0.102</td>
<td>0.9996</td>
</tr>
<tr>
<td>2.5</td>
<td>0.305</td>
<td>0.9996</td>
</tr>
<tr>
<td>5.0</td>
<td>0.519</td>
<td>0.9995</td>
</tr>
<tr>
<td>7.0</td>
<td>0.708</td>
<td>0.9994</td>
</tr>
<tr>
<td>10.0</td>
<td>1.09</td>
<td>0.9993</td>
</tr>
</tbody>
</table>

The plot of $k_{obs}$ against dtt concentration is shown in figure 6.6. It is linear with slope $= 10.5 \ M^{-1} \ s^{-1}$ ($SD = 0.3$).

The slope is a second order rate constant and can be corrected for the fraction of aquocobalamín and both types of thiolate.

- $k_{corr}$ (slope corrected for the fraction of aquocobalamín)
  - $= 115 \ M^{-1} \ s^{-1}$

- $k'_{corr}$ (slope corrected for fraction of $RS^- - RSH + RS^- - RS^-$)
  - $= 489 \ M^{-1} \ s^{-1}$
Figure 6.6 Dependence of the rate of reduction of B$_{12a}$ by dithiothreitol on the dithiothreitol concentration at pH 8.6 (in the presence of 2x10$^{-3}$ M EDTA); 6.5x10$^{-5}$ M B$_{12a}$; 25°C; ν = 0.1 (under S2)

The linear dependence of $k_{obs}$ on the dtt concentration is consistent with both an inner and outer sphere mechanism. However, $k'_{corr}$ falls within the range of second order rate constants $10^4$ reported for the coordination of various ligands (200 - 2000 M$^{-1}$ s$^{-1}$) even when halved because two thiol groups are present and this lends support to an inner sphere mechanism.
c) Effect of CuSO₄ and EDTA on the rate

Table 6.5 shows the effect of CuSO₄ and EDTA on the rate of reduction at pH 8.5. It can be seen that CuSO₄ increases the rate by approximately a factor of two while EDTA decreases it by about the same amount. Both show a limiting effect.

Table 6.5: Effect of CuSO₄ and EDTA on the rate of reduction of B₄₂₈ by dithiothreitol at pH 6.5; 5x10⁻⁵M dtt; 6.5x10⁻⁵M B₄₂₈; 25°C; μ = 0.1 (under N₂)

<table>
<thead>
<tr>
<th>10⁴[CuSO₄] M</th>
<th>kₐₚₛ s⁻¹</th>
<th>10⁴[EDTA] M</th>
<th>kₐₚₛ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.13</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>1.0</td>
<td>0.17</td>
<td>2</td>
<td>0.088</td>
</tr>
<tr>
<td>4.0</td>
<td>0.20</td>
<td>4</td>
<td>0.063</td>
</tr>
<tr>
<td>10.0</td>
<td>0.21</td>
<td>6</td>
<td>0.064</td>
</tr>
</tbody>
</table>

At pH 9, 6x10⁻⁴N cysteamine reduced B₄₂₈ (kₐₚₛ 1x10⁻³ s⁻¹), even in the presence of 5x10⁻⁴M EDTA (kₐₚₛ 9x10⁻⁴ s⁻¹) in conflict with Cavallini's results. As found above Cu(II) increased the rate (kₐₚₛ in the presence of 5x10⁻⁴M CuSO₄ was 2x10⁻³ s⁻¹). Similar results were found with cysteine, mercaptoethanol and penicillamine. These results show that trace metals such as Cu²⁺ do increase the rate, but are not essential as EDTA did not totally inhibit the reaction. Hence there are intrinsic reactions between B₄₂₈ and thiols.
6.3 Discussion

The kinetics of the reduction of \( \text{B}_{12} \) by dithiochreitol differ in acidic and basic solutions.

In acidic solutions, spectral studies show a rapid formation of Co\(^{III}\)-thiolate, followed by a relatively slow formation of Co\(^{II}\). This implies that the rate of coordination is greater than the rate of reduction and that the reaction is most likely inner sphere.

In alkaline solutions, no Co\(^{III}\)-thiolate intermediate is evident which means either that the rate of coordination is slower than the rate of reduction or else the reaction is outer sphere.

In the acid region, the focus will be on the pH 3 region (region B). Above this pH, the kinetics become complex, while below this pH, more than one coordination site may be available (the base of \( \text{B}_{12} \) is formed and the base of Co\(^{III}\)-thiolate may be involved because of the strong trans effect of thiolate) (region A).

In the alkaline region, the focus will be on pHs between 7 and 12 (region C). At pH \( \geq 12 \) (region D), the rate becomes independent of pH.

**pH 3 region** (region B)

At pH 3 spectral studies show that the rate determining step is the reduction of Co\(^{III}\)-dtt to Co\(^{II}\) as thiolocobalamin was formed rapidly but converted to Co(II) slowly. The rapid rate of coordination of dtt requires that \( \text{B}_{12} \) react with the undissociated thiol rather than the thiolate whose concentration is very small at pH 3 (binding of thiolate would require the rate constant for binding to be \( \geq 2 \times 10^5 \) which is one hundred fold larger than other
reported values for coordination to aquocobalamin). The binding of thiol is probably followed by a rapid proton loss to give the Co_{III}-thiolate. Supporting this is the apparent pH independence of the B_{12a}-cysteine spectrum. The quantitative studies showed that the rate was first order in B_{12a}, zero order in dtt and independent of pH, and $k_{obs} = (1.35 \pm 0.1) \times 10^{-2}$ s$^{-1}$. Because of the rapid coordination of dtt, the rate law can be written as:

$$\text{rate} = k_{obs} [\text{Co}^{III}-\text{dtt}]$$

The rate of reduction of B_{12a} by mercaptoethanol is considerably slower (at pH 5 $k_{obs}$ was $3 \times 10^{-5}$ s$^{-1}$ with 0.3M mercaptoethanol). This implies that the second thiol group (presumably undissociated) must be responsible for increasing the rate of reduction. Supporting this is the report by Neme and Fendler that a second thiol group is involved in the reduction of B_{12a} by cysteine and also evidence for RS$^-$ addition radicals which stabilize the radical. (For the equilibrium $RS' + RS^- \rightleftharpoons RS^- - SR$ $k_f = 3 \times 10^9$ M$^{-1}$ s$^{-1}$ $k_r = 8 \times 10^5$ s$^{-1}$ for cysteine.)

Possible mechanisms are:

- Addition of an H atom from SR
group to corrin ring
Mechanism c is unlikely because of the strong donor effect of RS⁻ on the Co and hence a high electron density on the ring.

In mechanisms a and b the further reactions are fast \( k = 1.7 \times 10^9 \text{ M}^{-1} \text{s}^{-1} \) (for reaction of two thiol radicals is 1.7x10⁹ M⁻¹ s⁻¹; 65 proton transfers are usually rapid) and hence the kinetics cannot distinguish between them. However, as the protonated disulphide is not known, mechanism c is the preferred one. (Some of the complications in the pH 4 - 6 region could be due to the pKa of the dtr radical.)

\textbf{pH 7 - 12 region (region C)}

In this pH region, spectral studies showed no intermediate in the reduction of B¹²a by dithiothreitol. This could either be due to an outer sphere mechanism or else an inner sphere mechanism where coordination is slow while reduction is fast.

The pH profile is bell shaped and this can be explained by the major reaction being between aquocobalamin and thiolate, with aquocobalamin not distinguishing between "S —RS⁻" and "S —RS". (The reaction could be inner or outer sphere.)

The rate is first order in B¹²a and thiol and this is consistent with either rate determining coordination in an inner sphere mechanism or an outer sphere mechanism. When the second order rate constant obtained is corrected for the fraction of aquocobalamin and thiolate a value of 489 M⁻¹ s⁻¹ is obtained. This value is within the range of rate constants for coordination (200 - 2000 M⁻¹ s⁻¹) previously reported. ¹⁰⁴ This is support for the inner sphere mechanism but does not rule out the outer sphere mechanism. However, the inner sphere mechanism is preferred because this is the mechanism operating in acid.
The rate of reduction at high pH is considerably faster than at low pH. This implies that a second thiolate group, rather than a second thiol group, results in a lower activation energy in the reduction step. In the alkaline region at pH's where the S—RS⁻ concentration is low, coordination of dtt could well be followed by a rapid proton loss from the second thiol group, resulting in a rapid reduction step. The radical intermediate \( \cdot \) is kinetically more stable than \( \cdot \) (the bimolecular decay rate constants are \( 1.7 \times 10^8 \ M^{-1} \ s^{-1} \) and \( 1.7 \times 10^9 \ M^{-1} \ s^{-1} \) respectively \( ^{\text{68}} \)) and this stability could result in a lower activation energy for the formation reaction of the former.

The effect of Cu²⁺ in this pH region and not in the pH 3 region could well be a consequence of relatively slow coordination in this pH region, which enables the Cu—dtt complex to reduce the B₁₂⁺ by an outer sphere mechanism. In the pH 3 region, the high electron density on the corrin ring in the Co(III)—thiolate complex would inhibit an outer-sphere reduction.

pH 12—14 region (region D)
The rate is pH independent in this region and no thiolocobalamin was observed. This indicates that direct reaction between hydroxocobalamin and thiocysteine (most likely \( \cdot \)) either by outer sphere electron transfer or via coordination, as at other pHs. However, there is insufficient evidence to decide.

It can be concluded that the main redox reaction involves coordination of thiolate to Co(III). The reduction is not simply

\[
RS⁻—Co(III) \rightarrow RS' + Co(II)
\]
but must be assisted by a second RSH, or better R$^-$ to give
RS$^-$SR or RS$^-$RSH.

This emphasises the importance of having two neighbouring
-SH groups (as found in the thioredoxins). 63

Catalysis by copper(II) was also found when the rate of
coordination was slow compared to reduction.
CHAPTER 7 - REDUCTION OF THE HEMIN-CAFFEINE ADDUCT AND THE
BIS-HISTIDINE HEMIN COMPLEX BY THIOLS

7.1 Introduction

As hemin is the actual prosthetic group of hemoproteins, it would obviously be of interest to study its reduction under conditions where it is monomeric. In chapter 3, it was shown that the caffeine adduct of hemin in alkaline solution (hemin-caffeine) is monomeric as long as the hemin concentration does not exceed $1.5 \times 10^{-5}$ M. In chapter 5, it was shown that the complex with histidine at high histidine concentrations was the monomeric bis-histidine hemin (referred to here as hemin-histidine). The reduction of both these complexes will be discussed.

Most studies of the reactions between iron porphyrins and thiols have focused on model studies of oxidised cytochrome P-450, where cysteine coordination in the resting and substrate-bound oxidised forms can account for its observed spectral properties.\textsuperscript{59-61} The other ligand in the resting state could be H\textsubscript{2}O or histidine, with recent model studies supporting the former.\textsuperscript{59} In addition to these model studies, a study of the reduction of FeTPP by thiols in toluene has been reported.\textsuperscript{58}

As was mentioned in chapter 6, reduction could occur via an inner or outer sphere mechanism and if the former, could go via the mono and/or bis chelate complexes and may or may not be assisted by the second thiol functional group in the molecule. In addition, dithiothreitol has a number of hydrogen bonding sites and could hydrogen bond to the propionate side chains of the porphyrin and to the ligand, histidine. Hence dithiothreitol can act as a ligand, as a reducing agent and can hydrogen bond.
It has been shown in our laboratory\textsuperscript{108} that the reduction of the bis-histidine hemin complex by cyclic voltammetry requires the simultaneous uptake of a proton between pH 8 and 10. It was shown in chapter 5, that on coordination of histidine to hemin, the pKa of the -NH$_2$ groups drops from 9.3 to less than 8 because of the stabilization of the conjugate base by the residual positive charge on the Fe(III). No residual charge is present on the Fe(II) and hence the pKa should be close to its normal value. Therefore on reduction between pH 8 and 10, a proton must be taken up by the -NH$_2$. This should also be the case if the bis-histidine complex is reduced by thiols and this will be investigated.

Hence the aims of this study are to elucidate the mechanism of reduction both by quantitative kinetic measurements and from the spectral changes. In addition the effect of different thiols and the presence of copper(II) on the rate of reduction will be examined qualitatively, the former to establish the effectiveness of dithiols as opposed to monothiols, and the latter to see whether copper(II) is necessary for the reduction of hemin, as it is involved in the mechanism of reaction of cytochrome c oxidase.

7.2 Results

7.2.1 Hemin-caffeine

Only qualitative experiments were carried out with hemin-caffeine, as preliminary experiments showed that the kinetics of reduction by dithiothreitol were generally biphasic. Curiously the pseudo first order rate constants increase with hemin concentration, suggesting reduction via a dimeric species whose rate of formation is fast.

The addition of thiols such as dithiothreitol (dtt), mercapto-
ethanol and cysteine to hemin-caffeine, resulted in a final species with bands at 396 nm (shoulder), 414 nm, 428 nm (shoulder), 536 nm and 571 nm, which corresponds well to those reported for heme-caffeine (416 nm, 434 nm (shoulder), 537 nm, 569 nm).\(^{12}\) No bands due to heme c (412 nm, 542 nm, 560 nm) were evident.\(^ {109}\) (Heme c could be formed by addition of the thiol across the vinyl groups on the porphyrin ring.) Isosbestic points were found at 412 nm, 456 nm, 523 nm, 586 nm, 670 nm, but the original hemin-caffeine spectrum did not pass through the last three which indicates the presence of an intermediate. Because of the shoulders of the Fe(III)OH caffeine and the Fe(II) caffeine at 360 nm and 440 nm respectively, the intermediate could not be identified but presumably is the monothiolate hydroxo complex (see later).

It was found that different thiols reduced hemin-caffeine at different rates. At pH 10.0; in the presence of 0.1M caffeine at 25°C, the approximate half lives for the reduction of 1x10\(^{-3}\)M hemin by 4x10\(^{-6}\)M thiol (under N\(_2\)) were 17s (dithiothreitol); 1x10\(^3\)s (mercaptoethanol); 7x10\(^3\)s (cysteine). It can be seen quite clearly that the dithiol, dithiothreitol, reduced hemin-caffeine markedly faster than either of the mono thiols. This was also found to be the case for the reduction of B\(_{12}\) (chapter 6) where it was attributed to a second thiol group which is nearby in the dithiols, assisting the homolytic fission of the Co-S bond, resulting in a fairly stable RS—SR radical.

The addition of CuSO\(_4\) and EDTA had no effect on the rate of reduction by dithiothreitol, cysteine and mercaptoethanol under the conditions outlined above (i.e. rate varied by < 5%). However, no reduction of hemin-caffeine by 1,2x10\(^{-3}\)M penicillamine
Figure 7.1: Spectral changes on adding penicillamine to hemin-caffeine in the presence and absence of CuSO₄. All solutions in pH9 buffer at 25°C; μ=0.1; (caffeine)=0.05M; — no penicillamine; (hemin)=7.5x10⁻⁵M; xxxx plus 5x10⁻⁵M penicillamine 5 hours after addition to hemin-caffeine (no CuSO₄ added); —— 5x10⁻³M penicillamine + 1x10⁻⁴M CuSO₄ between 15s and 3 min after the addition of CuSO₄; ... 1x10⁻⁴M CuSO₄ added to 1x10⁻³M penicillamine between 15s and 3 min after mixing (no hemin)
\((\text{CH}_3)_2\text{C}(\text{SH})\text{CH}(\text{NH}_2)\text{CO}_2\text{H}\) was observed up to an hour after mixing in the absence of Cu(II). When \(1 \times 10^{-4}\) M CuSO\(_4\) was added, the half time was less than two minutes for the formation of the \(a\beta\) bands of heme-caffeine (557 nm, 569 nm) (see figure 7.1). Unfortunately the Cu-penicillamine complex (reportedly \([\text{Cu}^1\text{Cu}^\text{II}L_{12}\text{C}^5\text{L}^2\text{C}^2\text{L}^4]\)\(^{110}\) absorbs in the UV-visible at \(620\) nm, \(380\) nm (shoulder) and \(510\) nm. These bands are evident in the presence and absence of hemin-caffeine when Cu(II) is present. This Cu-penicillamine complex could be reducing heme-caffeine by an outer mechanism, an inner mechanism being unlikely because of steric hindrance.

The bands of heme-caffeine decrease with time, and this could be due to either aggregation and/or decomposition but has not been studied further.

7.2.2 Hemin-histidine

7.2.2.1 Quantitative studies

On adding \(1 \times 10^{-4}\) M thiol (dithiothreitol, cysteine and mercapto-ethanol to \(10^{-5}\) M heme at pH 10, a final spectrum with bands at 421 nm, 526 nm and 556 nm (see figures 7.2a, b) was found. Table 7.1 shows the band positions of the bis-histidine, bis-histamine and bis-pilocarpate complexes reduced by a few grains of dithionite at pH 8.5 (0.2M ligand; \(7.3 \times 10^{-6}\) M heme). (Reduction is complete within 5s. Reoxidation by O\(_2\) is rapid (complete within 1 minute on shaking in air).) The bands of reduced cytochrome b\(_5\) are also included as are those of the monothiomate and bis-thiolate complexes.
Figure 7.20: Spectral changes observed on adding low concentrations of dilithiocarb to homohistidine at 2°C.

---

Final product (after 30 min)
8 min after mixing respectively (4 min per scan)
Homohistidine after dil solution (trans stress at 200, 4 min.
xxxx Homohistidine before dil added
Figure 7-27: Structural changes observed on adding high concentrations of dilithium to hemin-histidine at 2°C.

- Final product (after 35 min)
- After mixing respectively
- 25°C, 4 min
- Hemin-histidine after di added (runs started at
- xxx Hemin-histidine before di added
Figure 2: Spectral changes observed on adding high concentrations of thioacetohydroxylate to hemin-histidine at 2°C.

- [In the diagram, various lines represent different concentrations and conditions.]

- Initial hemin-histidine before dilution.
- Final product after dilution.
Table 7.1: Band positions of species relevant to the reduction of hemin-histidine by thiols

<table>
<thead>
<tr>
<th>System</th>
<th>/nm</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>bis-histidine hemin reduced by thiols; 4x10^-5 M hemin; 0.2 M histidine; pH 10</td>
<td>421;526;556</td>
<td>this study</td>
</tr>
<tr>
<td>bis-histidine hemin reduced by dithionite; 7.3x10^-6 M hemin; 0.2 M histidine; pH 8.5</td>
<td>421;526;556</td>
<td>this study</td>
</tr>
<tr>
<td>bis-histamine hemin reduced by dithionite; 7.3x10^-6 M hemin; 0.2 M histamine; pH 8.5</td>
<td>421;526;556</td>
<td>this study</td>
</tr>
<tr>
<td>bis-pilocarpate hemin reduced by dithionite; 7.3x10^-6 M hemin; 0.2 M pilocarpate; pH 8.5</td>
<td>422;529;556</td>
<td>this study</td>
</tr>
<tr>
<td>reduced cytochrome b^5_5 pH 7.4; reduced by dithionite</td>
<td>422.5;525;556</td>
<td>80</td>
</tr>
<tr>
<td>reduced cytochrome b^3_3 pH 7; reduced by dithionite</td>
<td>423;527;556</td>
<td>this study</td>
</tr>
<tr>
<td>imidazole-thiol-hemin in CH_2Cl_2</td>
<td>360;428;538;568</td>
<td>39</td>
</tr>
<tr>
<td>phenol-thiol-hemin in CH_2Cl_2 + CH_3CH_2OH</td>
<td>360(sh);415;530;561</td>
<td>39</td>
</tr>
<tr>
<td>resting P-450_1M_2 in aqueous solution</td>
<td>350(sh);418;538;568</td>
<td>8</td>
</tr>
<tr>
<td>bis-thiol hemin in CH_2Cl_2</td>
<td>377;475;555</td>
<td>39</td>
</tr>
<tr>
<td>P-450_1M_2 plus 1-octanethiol in aqueous solution</td>
<td>377;467;550</td>
<td>39</td>
</tr>
</tbody>
</table>
It will be clearly seen that the reduced bis-histidine, bis-histamine and bis-pilocarpate heme complexes all have very similar bands to those of reduced cytochrome $b_5$ which indicates that they are Fe(II)$_2$. It makes no difference to the final spectrum whether hemin-histidine is reduced by thiol or dithionite. Certainly the final bands are not those of the oxidised heme with thiol coordinated. It must be noted that at high heme concentrations (1x$10^{-3}$ M) in histidine (reduced by dithionite) the 526 nm and 556 nm bands are only found by themselves in saturated histidine ($\approx 0,62$) and as the histidine concentration is decreased they decrease in intensity while new bands at 558 nm and 580 nm appear. They also decrease with time (3% per min at 0,062; 4% at 0,62 M) at a particular histidine concentration while the bands at 558 nm and 580 nm increase showing that the Fe(II) bis-histidine complex is unstable. At 0,062M histidine only these new bands are evident. These bands correspond well to that reported by Shack and Clark $^{10}$ for the heme dimer (560 - 580 nm). Hence Fe(II)$_2$ tends to aggregate.

In the spectrum of heme-histidine reduced by dithiothreitol, cysteine, ethanethiol and mercaptoethanol, there were no bands due to heme-c bis-histidine (which would be at 414 nm, 520 nm and 549 nm). $^{109}$

Figures 7.2a and b show the spectral changes observed on adding dithiothreitol to heme-histidine at 2°C at pH 10,0 at low ($4x10^{-4}$ M) and high (0,11) dithiothreitol concentrations. Isosbestic points were found both at low dithiothreitol concentrations (at 402 nm, 433 nm, 509 nm, 534 nm, 544 nm and 566 nm) and at high dithiothreitol concentrations (at 402 nm, 430 nm, 509 nm,
535 nm, 544 nm and 566 nm) which were the same within experimental error. The initial spectrum (i.e., before the addition of dithiothreitol) did not pass through these isosbestic points, which indicates the presence of a rapidly formed intermediate. There were no major bands of coordinated thiolate species (see table 7.1) but these could well be masked by those of the reduced species. However, shoulders at 360 nm and 440 nm are present and as these are found in the monothiolate complexes (with imidazole or ethanol coordinated) but not the initial or final species, these are possible intermediates (in this case with histidine or H$_2$O/OH$^-$ coordinated). There is no spectral evidence for the bis-thiolate complex. Similar shoulders at 360 nm as well as isosbestic points between the intermediate and final product but not the starting species were found at room temperature with 4x10$^{-5}$M dithiothreitol (at higher concentrations, the reaction was too fast to follow with the spectrophotometer at 25°C); and 4x10$^{-6}$M mercaptoethanol, cysteine and ethanethiol. Only a small amount of the 360 nm band was found with cysteine, indicating a shift in the equilibrium towards the starting species.

As found with hemin-caffeine, the Fe(II) bands decreased with time and could be due to aggregation and/or decomposition, but has not been studied further. In light of the studies at high hemin concentrations, is is probably aggregation. Care has been taken in the quantitative studies to ensure that this subsequent reaction is negligible during the reduction reaction. Dithiothreitol was chosen because the reduction was much faster than the subsequent reaction. The overlap with the subsequent reaction ruled out quantitative studies with the monothiols.
Different thiols reduced hemin-histidine at different rates.

The reduction of \(1 \times 10^{-5}\)M hemin in the presence of \(0.4\%\) histidine at pH 10; 25°C under \(N_2\) by \(4 \times 10^{-4}\)M thiol occurred with the following half lives: 18s (dithiothreitol); 1 \(\times 10^6\)s (mercaptoethanol); 2.4 \(\times 10^3\) (cysteine). These rates are similar to those found with hemin-caffeine and also show the greater efficiency of the dithiol compared to the monothiol.

The addition of CuSO\(_4\) and EDTA had no effect on the rates of reduction at pH 10 by thiols, including penicillamine. (Rate varied by < 5% in the presence of \(1 \times 10^{-4}\)M CuSO\(_4\) or \(1 \times 10^{-3}\)M EDTA.)

7.2.2.2 Quantitative studies with dithiotreitol as the reducing agent

The kinetics were followed by stopped-flow UV-visible spectro-photometry. The absorbance changes were monitored at 556 nm which is a peak position of Fe(II) bis-histidine protoporphyrin where the absorbance changes are fairly large and are mainly due to the conversion from the monothiolate complex to the product (see figures 7.2a and b). All runs had an induction period, the length of which depended on the oxygen. This was minimised to < 10% of the reaction time by thorough flushing of the solutions with \(N_2\) before mixing and maintaining them under \(N_2\) during the reaction.

Conditions were such that the runs were pseudo first order in hemin and in fact plots of \(\ln(A_0-A_t)\) versus time were linear over at least four half lives. All results will be reported as \(k_{obs}\) which is (-slope) of the above plot. Repeat runs gave results within 8% of each other (the reported values are the average of three repeat runs) while the standard deviations within a run were within 2% and the correlation coefficients > 0.999.
The dependence of the rate on the hemin, thiol and histidine concentrations as well as the pH was determined quantitatively.

$k_{obs}$ at $5.6 \times 10^{-6} \text{M}$ and $3 \times 10^{-5} \text{M}$ hemin with $2 \times 10^{-4} \text{M}$ dtt, in the presence of $0.4 \text{M}$ histidine at pH 10.0 was $2.13 \times 10^{-2} \text{ s}^{-1}$ and $2.15 \times 10^{-2} \text{ s}^{-1}$ respectively. Hence as required by pseudo first order kinetics, $k_{obs}$ is independent of the hemin concentration.

The dependence of $k_{obs}$ on the dithiothreitol concentration was studied over a 500-fold concentration range because of the complex dependence found, as shown in figure 7.3 and table 1 (appendix 6).

![Graph](image)

**Figure 7.3** Effect of the dithiothreitol concentration on the reduction of hemin-histidine by dithioctreitol; $3 \times 10^{-5} \text{M}$ hemin; $0.4 \text{M}$ histidine; pH 10.0; $\nu = 0.5$; $25^\circ \text{C}$. Line is theoretical curve calculated from proposed rate law and calculated parameters.
The histidine concentration dependence was studied at high and low dithiothreitol concentrations. The effect on the rate could only be studied over a two-fold concentration range. The histidine concentration had to be > 0.2M to ensure over 98% formation of the monomeric hemin-bis-histidine complex. This was necessary to avoid complications from reactions of other species (see chapter 5). The solubility of histidine prevented the use of concentrations greater than 0.4M at 25°C. Thus, the study was restricted to concentrations of histidine between 0.2 and 0.4M.

Figure 7.4a shows the effect on \( k_{\text{obs}} \) of the histidine concentration at low dithiothreitol concentration. It can be seen that \( k_{\text{obs}} \) is inversely proportional to the histidine concentration. The zero intercept indicates that no reaction occurs at infinite histidine concentration, which rules out an outer sphere reduction of the bis-histidine as a possible mechanism.

Figure 7.4b shows the effect on \( k_{\text{obs}} \) of the histidine concentration at high dithiothreitol concentration. It is quite clearly independent of the histidine concentration. (Data at both high and low dithiothreitol concentrations are given in tables 2a and b, appendix 6.)

The effect of pH at low dithiothreitol concentrations, where the kinetics are simpler (see later) was determined. Table 3 (appendix 6) gives the experimental data as well as these values corrected for the fraction of bis-histidine present initially. (As the pH increases, ON− competes with histidine as a ligand to Fe(II)).
Figure 7.4 Effect of the histidine concentration on the reduction of hemin-histidine by dithiothreitol;

3x10^{-5} M hemin; pH 10.0; µ = 0.5; 25°C (under \text{N}_2)
Figure 7.5 shows the effect of pH on these corrected values.

The maximum in the pH profile occurred at pH 8.5 and the inflection in the downward limb occurred at pH 10. A bell shaped pH profile is indicative of two opposing pH effects, and was found with B12a where aquocobalamin reacted with the thiolate (chapter 5). In this system, however, the decrease from pH 8.5 to 8 could be a consequence of some aggregation of the starting species.

7.3 Discussion

Both spectral and kinetic results must be considered in deciding on the mechanism. Broad possibilities are inner or outer sphere reduction. Outer sphere reduction of the bis-histidine hemin can be ruled out because no reduction occurs at infinite histidine.
concentration (figure 7.4a). Outer sphere reduction of thiolate complexes is not likely, as their greater electron density compared to that of the bis-histidine complex would make reduction more difficult. Hence reduction must occur via an inner sphere mechanism. The bis-thiolate complex has been reported but can be ruled out as a significant intermediate in this system as none of the expected bands (see table 7.1) were observed. The inhibition by dithiochreitol at high dithiochreitol concentrations, however, requires a species containing two thiolates which reduces more slowly than the species containing one thiolate. If the second thiolate is hydrogen bonded, this would explain the kinetics and would be in accord with the observed spectral changes (hydrogen bonding would not be expected to affect the UV-visible spectrum significantly).

In the inner sphere mechanism, either coordination or reduction can be rate limiting. However, rapid formation of the intermediate followed by relatively slow reduction was observed (see figures 7.2a and b). Hence reduction is rate limiting, which is consistent with the mathematical analysis (see appendix 7). Since good first order kinetics were found even where a mixture of species must be present, a rapid equilibration of the Fe(III) species must occur, together with slow reduction steps.

In deriving a rate law the following assumptions were made:

1) The reverse reactions of the reduction steps are negligible because of the low concentrations of radicals expected (the second order rate constant for the dithiochreitol radical is $1.7 \times 10^8 \text{ M}^{-1} \text{s}^{-1}$).

2) The ligand binding and dissociation reactions of the Fe(II)
complexes are rapid and hence do not appear in the rate law, as they occur after the rate determining step(s) (the first order rate constants for ligand dissociation from Fe(II) porphyrins are of the order of $10^2$ s$^{-1}$).

A mechanism which can account for the observed kinetics and spectra is given in equations (7-1) - (7-4) (ignoring the pHs of the dithiothreitol), where dtt$^+$ is the oxidised radical form.

$$\text{Fe(III)(his)$_2$ + dtt} \xrightarrow{k_{-1}} \text{Fe(III)(his)(dtt) + his} \quad (7-1)$$

$$\text{Fe(III)(his)(dtt) \xrightarrow{k_0} Fe(II)(his) + dtt \text{ fast products}} \quad (7-2)$$

$$\text{Fe(III)(his)(dtt) + dtt} \xrightarrow{k_3} \text{Fe(III)(his)(dtt)...(dtt)} \quad (7-3)$$

$$\text{Fe(III)(his)(dtt)...(dtt) \xrightarrow{k_3} Fe(II)(his)...dtt + slow prod.} \quad (7-4)$$

(The reduction of the hydrogen bonded adduct is not likely to go via an outer sphere mechanism because of the high electron density.)

A rate law was derived (see appendix 7) which predicts three phases. As all the kinetic runs were monophasic, two of these must be rapid and these are the thiol binding steps because the rapid formation of an intermediate is seen. The monophasic rate law can be shown to be:

$$\text{Rate} = k_{obs} \text{[emin-histidine]}$$

where

$$k_{obs} = \frac{x[f(\text{dtt})] + y[f(\text{dtt})]^2}{z + v[f(\text{dtt})] + w[f(\text{dtt})]^2}$$

and

$$x = k_1 k_2 (k_{-3} + k_4)$$

$$y = k_1 k_3 k_4$$

$$z = (k_2 + k_{-1}(\text{his}) + k_{-3} + k_4)$$

$$v = k_3 k_4 + k_1 (k_2 + k_4 + k_{-3})$$

$$w = k_2 k_3$$

(see appendix 7)
Values for $x$, $y$, $z$, $v$, $w$ were found using a simplex optimization routine. Figure 7.6 shows the theoretical curve where $x = 43.9$; $y = 1346$; $z = 0.282$; $v = 432$; $w = 57020$. The theoretical values lay within 5% of all the experimental points.

Values of the microscopic rate constants can be calculated if approximations are made.

If the ligand dissociation steps are rapid compared to the reduction steps then $(k_2 + k_{-1}^{\text{hist}})k_{-3}^{\text{hist}}$; $(k_{-3} + k_4)k_{-3}$; $(k_3k_4 + k_4)k_4k_{-3}$ and the values of $k_2 (\frac{X}{X})$; $k_6 (\frac{X}{X})$; $k_1 (\frac{Y}{Z} [\text{hist}])$ and $k_3 (\frac{Y}{X})$ were $0.102 \text{ s}^{-1}$; $2.36 \times 10^{-2} \text{ s}^{-1}$; 613 and 132 N$^{-1}$ respectively. (If ligand dissociation is slow compared with reduction, the dependence of the rate on the histidine concentration at low thiol concentrations cannot be accounted for.)

These values are reasonable. The binding constant for the second thiol, $K_3$ (132 N$^{-1}$), is comparable with those found for the histidine (and analogous) adduct with the alkaline heme dimer (chapter 5) (79 - 400 N$^{-1}$) and is consistent with a weak interaction such as hydrogen bonding (there is certainly no shortage of suitable sites!).

The dependence on the histidine concentration can also be accounted for with this rate law. At low dithiothreitol concentrations, the $[\text{det}]^2$ terms are unimportant and hence

$$k_{\text{obs}} \approx \frac{k_1k_2k_3[\text{det}]}{k_{-1}^{\text{hist}}k_{-3}^{\text{hist}}k_1k_2[\text{det}]}$$

$$\frac{1}{k_{\text{obs}}} = \frac{k_{-1}^{\text{hist}}}{k_2[\text{det}]} [\text{hist}] + \frac{1}{k_2}$$
Figure 7.6  Plot of $\frac{1}{k_{\text{obs}}}$ versus histidine concentration

$3 \times 10^{-5} \text{M hemin}; 4 \times 10^{-6} \text{M dtt}; \text{pH} 10.0; \nu = 0.4; \ 25^\circ \text{C (under } N_2)\text{.}$

The plot of $\frac{1}{k_{\text{obs}}}$ versus [his] is in fact linear with slope = $37.5 \text{ M s}$ and intercept = $7 \text{ s}^{-1}$ (figure 7.6). This results in $\frac{k_1}{k_{-1}} = K_1 = 467$ and $k_2 = 0.14 \text{ s}^{-1}$ which is in fair agreement with the values found above (613 and 0.10 s$^{-1}$ respectively). This inverse dependence of the rate on the histidine concentration shows that the intermediate is in fact the thiolate-histidine hemin complex as the thiolate-aquo-hydroxo hemin complex would not give this result.

At high diithiothreitol concentrations, the s term becomes negligible and hence

$$k_{\text{obs}} = \frac{k_1 k_2 k_{-3} [\text{dtt}] + k_1 k_2 k_{-4} [\text{dtt}]^2}{k_1 k_{-3} [\text{dtt}] + k_1 k_2 [\text{dtt}]^2}.$$
$k_{obs}$ is independent of the histidine concentration which is what was observed. (The results are not good enough to rule out the loss of histidine in reaction (7 - 3) but the similarity of the isosbestic points at high and low dtt concentrations make this possibility very unlikely.)

Hence the proposed rate law can account for the spectral changes and for the observed dependences on the dithiothreitol concentration and the histidine concentration as well as of course the hemin concentration.

The effect of pH on the rate is too complex to analyse what with two parallel reduction steps and pKas of thiol and histidine ($-\text{NH}_2$) probably being significant, as well as having limited data. We merely note that the apparent pKa of 10 in the downward limb could be associated with the pKa of dithiothreitol or possibly an uptake of a proton on reduction as found by cyclic voltametry.\textsuperscript{108}

7.4 Conclusions

Hemin-histidine like $B_{12a}$ is reduced by thiols via an inner sphere mechanism. Reduction was found to be rate limiting. The simple monothiolate complex reduced with $k = 0.1$ s\(^{-1}\), compared with that of $B_{12a}$ in alkaline solution $k > 0.3$ s\(^{-1}\) (coordination was rate limiting at alkaline pHs). Hence the rate of reduction of $B_{12a}$ is faster than that of hemin-histidine.

Presumably the faster reduction by the dithiol compared to the monothiol, reflects the homolytic fission of Fe-S being assisted by a second thiol group (which is close at hand in the dithiol) as found for the $B_{12a}$ reduction by cysteine.\textsuperscript{54} This suggests a method of preventing the reduction of Fe(III) in the resting P-450 form, by the coordinated cysteine, i.e. by the
protein preventing a second thiol approaching the active site and in doing so, only permitting the apparently unfavourable formation of the RS to occur, which cannot diffuse away being part of the protein and hence can recombine with Fe(II).

Fe(II) bis-histidine has a very similar spectrum to that of reduced cytochrome b₅ and therefore is a good model as far as the electronic structure is concerned. Curiously the reduction of the bis-histidine complex requires proton uptake, which follows from the change in pKa of the -NH₃⁺ of the coordinated histidine, while the reduction of cytochrome b₅ requires Na⁺/K⁺ uptake. But whether a proton or cation is required on reduction of a hemoprotein depends on the pKas of the neighbouring groups in the active site.

A clearcut example of catalysis by copper(II) in the reduction of FeOH-caffeine by penicillamine was found. Some catalysis by Cu(II) was found in the reduction of B₃₈ but the effect in the former case is more dramatic because no reduction occurs in the absence of Cu(II).
CHAPTER 8 - THE CATALYSIS OF THE AUTOXIDATION OF THIOLS BY COBALT CORRINOIDs

8. Introduction

It is well known that cobalt corrinoids such as $B_{12a}$ are good catalysts for the autoxidation of thiols. The only quantitative kinetic studies are those of Peel who used mainly aquocyanocobinamide (factor B) but unfortunately the presence of $CN^-$, which will poison some of the cobalt, complicates the interpretation of the results.

The aims of this study are to establish a general mechanism for the autoxidation of thiols catalysed by cobalt corrinoids through a quantitative study of $B_{12a}$ (as it has effectively only one potential coordination site) and a monothiol. Cysteine was chosen because the reduction of $B_{12a}$ by cysteine has been studied. With less detailed study the effect of changing from mono to a dithiol (viz. dithiothreitol) on the rate will be investigated. (The reduction of $B_{12a}$ by dithiothreitol was discussed in chapter 6.) The effect of replacing the axial benzimidazole by $H_2O$ will be examined by studying the diaquocobinamide catalysed autoxidations of cysteine as well as those of mercaptoethanol and ethanethiol. It is also of interest to see to what extent cobalt corrinoids might serve as models for the reactions of hemoproteins with $O_2$ and to what extent one can model the high turnover number and suppression of $H_2O_2$ formation characteristic of cytochrome c oxidase.

The stoichiometry has been shown by Peel to be

$$2RSH + \frac{1}{2}O_2 \rightarrow RSSR + H_2O$$

and will be assumed here.
8.2 Results

8.2.1 B_{12a} plus cysteine

Figure 8.1 shows a typical plot for the rate of O_2 uptake catalysed by B_{12a} in the presence of cysteine in an O_2 saturated solution.

![Typical experimental plot](image)

Figure 8.1 A typical experimental plot for O_2 uptake in the presence of B_{12a} and cysteine in O_2 saturated buffers; pHi 10; 2x10^{-2} M cysteine; 2.5x10^{-5} M B_{12a}; 25°C.

It shows the uncatalysed as well as the catalysed autoxidation of cysteine. The lag period due to mixing is < 3 seconds, and hence no build up can be seen within this period. There is clearly no effect of the order of addition on either the initial rate or the course of the reaction. In general, the initial rates will be reported.

By contrast, the plots of the diaquocobinamide ca- sed
reactions are autocatalytic (figure 8.2).

Figure 8.2 A typical experimental plot for O₂ uptake by thiols catalysed by dihydroxyquinoline; pH 9.5; 2x10⁻³ M mercaptoethanol; 1x10⁻⁷ M dihydroxyquinoline; O₂ saturated buffer; 25°C

Figure 8.3 shows the pH profile in O₂ saturated buffers, without EDTA, both with and without B₁₂a (data in table 1, appendix 8). The catalysed reaction has a maximum rate at pH 10 and hence further studies will concentrate on pH 10. The uncatalysed rate reaches a plateau in the alkaline region, probably because of complete formation of the thiolate.
Figure 7.3 The pH profile for the autoxidation of cysteine catalysed by: $2 \times 10^{-2}$ M cysteine; $1 \times 10^{-3}$ M $\text{H}_2\text{O}_2$; $25^\circ\text{C}$

Table 8.1 shows the different rates (and order in oxygen) in air and $O_2$ saturated buffers as well as the effects of superoxide dismutase, Cu(II), EDTA on the rate.
Table 8.1: Effects of conditions on the rate of $O_2$ uptake by $\text{B}_{12a}$

<table>
<thead>
<tr>
<th>Added reagent</th>
<th>Air saturated buffer</th>
<th>$O_2$ saturated buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>$2.32$ (n=0 initially)</td>
<td>$3.95$ (n=0.5)</td>
</tr>
<tr>
<td>$2 \times 10^{-6}M$ superoxide</td>
<td>$2.27$ (n=0)</td>
<td>$2.45$ (n=0)</td>
</tr>
<tr>
<td>superoxide dismutase</td>
<td>-</td>
<td>$1.84$</td>
</tr>
<tr>
<td>$1 \times 10^{-3}M$ EDTA</td>
<td>$1.15$ (n=0)</td>
<td>$1.66$</td>
</tr>
<tr>
<td>$2 \times 10^{-3}M$ CuSO$_4$</td>
<td>-</td>
<td>$3.51$</td>
</tr>
</tbody>
</table>

It can be seen that there is an additional reaction in $O_2$ saturated buffers which is suppressed by superoxide dismutase and thus involves superoxide. Hence air saturated and not $O_2$ saturated buffers will be used. EDTA suppresses the reaction by about 50%, as was the case in the reduction of $\text{B}_{12a}$ by cysteine under $N_2$ (chapter 6). Hence $2 \times 10^{-3}M$ EDTA will be used to suppress reactions catalysed by trace metals. Little effect of Cu(II) was found at the concentration used. The half order dependence in $O_2$ found in $O_2$ saturated buffers is similar to that reported earlier for the rate of $O_2$ uptake by Cu(II) plus ascorbate $^{114}$ as well as Fe(III) plus cysteine $^{73}$ but it is not part of the intrinsic reaction in this case as it was suppressed by superoxide dismutase.
Figure 8.4 (and table 2, appendix 8) shows the effect of the $E_{12a}$ concentration on the rate and the rate is quite clearly first order in $E_{12a}$ with only a small amount of uncatalysed rate (given by the intercept).

![Graph showing rate dependence on $E_{12a}$ concentration]

Figure 8.5 (and table 3, appendix 8) shows the effect of the cysteine on both the rate of $O_2$ uptake as well as reduction.
Figure 8.5 The rate dependence on the cysteine concentration for both the autoxidation of cysteine catalysed by B_{12a} (in air saturated solutions) as well as for the reduction of B_{12a} by cysteine (under N_{2}) in solutions containing 2x10^{-3} M EDTA; 1.54x10^{-5} M B_{12a}; pH 10; μ = 0.1; 25°C.

The slope for the reduction is 0.38 M^{-1} s^{-1} compared with the tangent at low cysteine concentrations to the O_{2} uptake curve which has a slope of 15.8 M^{-1} s^{-1} giving a ratio of 42 between the rate of O_{2} uptake and reduction. The turnover number (i.e. equivalents L^{-1}/(M B_{12a} x s)) is >0.9 at limiting thiol.
concentrations, Nome and Fandlers results indicate that at pH 10, coordination is rate limiting but reduction involves RS⁻⁺ (RS - Co(III)). Even if the stoichiometry of the reaction is ignored, the rate of O₂ uptake is faster than the rate of reduction (if the stoichiometry is taken into account the asymptotic rate of O₂ uptake increases by a factor of four). Quite clearly, a different dependence on thiol concentration is found for O₂ reduction which is rather surprising.

 Concentrations were run 15 seconds after mixing. The spectrum in the Soret region could be taken within a minute while the complete spectrum took 2 minutes. At low thiol concentrations, the uptake of O₂ under the same conditions was complete within 2.5 minutes while at high thiol concentrations it was complete within 1.5 minutes. In both cases, the Soret band was at 357 nm while at low thiol concentrations, the α,β bands were at 420 nm, 516 nm and 536 nm. These correspond to the bands of the hydroxocobalamin (358 nm, 421 nm, 516 nm, 537 nm). As hydroxocobalamin builds up, it must precede the rate determining step which must involve Co-OH or Co-OH₂ (pKa 7.6). There were no detectable bands due to Co(II) (312 nm, 405 nm, 473 nm) or thiolatocobalamin (371 nm, 425 nm, 524 nm, 554 nm). However, as the O₂ is depleted Co(II) is forced (bands at 312 nm, 470 nm), but with no sign of the thiolatocobalamin bands which is consistent with either coordination being slower than reduction, or an outer sphere mechanism.

 All experiments in air are zero order in O₂ at least initially.
At high cysteine concentrations (>8x10^{-2} M) at 2x10^{-5} M B_{12a}, there was a slight lag period which was, however, within the mixing time of 3 seconds. This was not observed at lower thiol concentrations where a smaller volume of thiol was added. The independence of the rate on O$_2$ agrees with the spectra which indicate that the rate determining step is one of the reduction steps.

The effect of catalase was investigated at high and low cysteine concentrations in an air saturated pH 10.0 buffer containing 2x10^{-3} M EDTA (1.9x10^{-5} M B_{12a}). At low cysteine concentrations (5x10^{-3} M), the rate decreased from 1.22 μNO$_2$ s$^{-1}$ to 0.78 μNO$_2$ s$^{-1}$ (duplicate experiments run) (i.e. a 36% decrease in rate) on addition of 5700 units of catalase. At 0.1M cysteine however, the rate was unaffected by catalase (4.45 μNO$_2$ s$^{-1}$ without catalase; 4.47 μNO$_2$ s$^{-1}$ with catalase).

In neither case was the order in O$_2$ affected. It appears that the amount of H$_2$O$_2$ detected decreases with thiol concentration. Qualitatively the activity of catalase was not affected by the presence of 0.1M cysteine, up to ten minutes.

8.2.2 B$_{12a}$ plus dithiothreitol

Figure 8.6 (and table 4, appendix 8) shows the pH profile for rate of O$_2$ uptake in air saturated buffers.

It shows a maximum like that observed for the B$_{12a}$-cysteine system in O$_2$ saturated buffers as well as for the reduction of B$_{12a}$ by dithiothreitol under N$_2$. However, the maximum occurs at pH 9.2 unlike the B$_{12a}$-cysteine system (pH 10) and the reduction system (pH 8.5). Other experiments were carried out at pH 10.
Figure 8.6 The pH profile for the autoxidation of dithiothreitol catalysed by B$_{12a}$ in air saturated buffers; 1.94x10$^{-5}$M B$_{12a}$; 1x10$^{-3}$M dithiothreitol; $\mu = 0.1$; 25°C.

Figure 8.7 (and table 5 in appendix 8) shows the effect of the dithiothreitol concentration on the rate. The same saturating effect which was observed with cysteine is evident here but has not been taken far enough to level off, because the rates are too great. The rate of reduction by dithiothreitol has been included and as found for B$_{12a}$ plus cysteine, is slower than the rate of O$_2$ uptake. It has a slope of 5.2 M$^{-1}$ s$^{-1}$ compared with that of the tangent to the origin of O$_2$ uptake curve which is
Figure 8.7 The rate dependence on the dithiothreitol (dtt) concentration for both the autoxidation of dtt catalysed by B$_{12a}$ (in air saturated solutions) as well as for the reduction of B$_{12a}$ by dtt (under N$_2$); 1.94x10$^{-3}$M.B$_{12a}$; pH 10; μ = 0.1; 25°C.

$66 \text{ M}^{-1} \text{s}^{-1}$ i.e. a 12 fold difference, which is less than that found for the B$_{12a}$-cysteine system (a 42 fold difference) which is largely a consequence of the difference in the reduction rates (14 fold) rather than O$_2$ uptake results (4 fold) in the two systems (ignoring the fact that dtt has two thiol groups).
The turnover number is > 2.2 equivalents/(M β_{12a} x s) which is larger than that for β_{12a} plus cysteine (> 0.9 equivalents/ (M β_{12a} x s)).

Figures 8.8a and b (and tables 6a and b in appendix 6) show that at both high and low dithiothreitol concentrations, the rate is first order in β_{12a}.

\[
\text{initial rate,} \\
\mu \text{M} \text{s}^{-1} \\
10 \quad 8 \\
6 \\
4 \\
2 \\
0 \\
0 \quad 5 \quad 10 \quad 15 \\
10^5 \beta_{12a} \text{ M}
\]

Figure 8.8a The rate dependence on the β_{12a} concentration for the autoxidation of dtt catalysed by β_{12a} in air saturated solutions at a low dtt concentration (1x10^{-3}M) at pH 10.0; μ = 0.1; 25°C.
As the dithiothreitol concentration increases, the order in \( \text{O}_2 \) changes from 0.5 to zero. Conditions affecting the rate and order at low thiol concentrations were superoxide dismutase and catalase. At 1 mM dithiothreitol (1.94x10^{-3} M \( \text{B}_{12a} \); pH 10; air saturated) 2x10^{-6} M superoxide dismutase reduced the rate from 1.52 \( \mu\text{M} \text{O}_2 \text{s}^{-1} \) to 0.87 \( \mu\text{M} \text{O}_2 \text{s}^{-1} \) (a 42% decrease) and changed the order from 0.5 to zero while 5700 units of catalase reduced it to...
0.60 \ \text{mM}^{-2} \ \text{s}^{-1} \ (a \ \text{60\% decrease}) \ also \ changing \ the \ order \ to \ zero.

In addition $3 \times 10^{-4}$ M CuSO$_4$ increased the rate to 3.37 \ \text{mM}^{-2} \ \text{s}^{-1} \ (i.e. \ doubling \ the \ rate) \ but \ did \ not \ affect \ the \ order. \ At\ 2 \times 10^{-2}$ M dithiothreitol ($3 \times 10^{-4}$ M $\text{B}_{12a}$), $2 \times 10^{-5}$ M superoxide dismutase and 5700 units of catalase decreased the rate by less than 32 in both cases without changing the order. The effect of EDTA was not studied.

The steady state species at both high and low dithiothreitol concentrations is the hydroxocobalamin as shown by bands at 357 nm, 420 nm, 516 nm and 536 nm (literature 358 nm, 421 nm, 516 nm, 537 nm). Hence the rate determining step involves reduction as found with $\text{B}_{12a}$ plus cysteine.

8.2.3 Diaquocobinamide plus monothiols

Qualitative experiments indicated that thiols reduced the diaquocobinamide much faster than $\text{B}_{12a}$ at pH 9.5 at least. Hence at least one step in the reaction has been speeded up. Figure 8.2 shows a typical autocatalytic plot characteristic of diaquocobinamide plus thiols. The maximum rate is always 1.5 - 2.5 times greater than the initial slope (see tables 8 and 9 in appendix 8). Only the initial slopes will be used unless otherwise noted.

Figure 8.9 and table 7 in appendix 8 show the variation in rate with pH in the presence of ethanethiol, mercaptoethanol and cysteine. Ethanethiol reacts about ten times faster than cysteine. We cannot be sure that part of the effect is not due to impurities such as $\text{H}_2\text{S}$ but the results are still of interest in establishing an upper limit to oxidase activity of cobalt corrinoids. All three of the thiols show a maximum at pH 10 (as did $\text{B}_{12a}$ plus
cysteine) in spite of the different pKas of the thiol group (ethanethiol 10.6; mercaptoethanol 9.4; cysteine 8.5).

Figure 8.9 The pH profile for the autoxidation of ethanethiol, mercaptoethanol and cysteine catalysed by diaquocobinamide (1x10⁻⁷ M); 2x10⁻⁷ M thiol; O₂ saturated buffers; 25°C.
Figure 8.10 The rate dependence on the diazocobinamide concentration for the autoxidation of ethanethiol, mercaptoethanol, and cysteine at pH 10; pH 9.5 and pH 10 respectively, catalysed by diazocobinamide; 2x10⁻² M thiol; O₂ saturated buffers; 25°C.

Figure 8.10 and table 8 in appendix 8 show that the reactions are all first order in diazocobinamide and that there is some uncatalysed reaction.
Figure 8.11 The rate dependence on the thiol concentration for the autoxidation of ethanethiol (pH 10), mercaptoethanol (pH 9.5) and cysteine (pH 10) catalysed by diaquocobinamide (1x10^{-7}M) in O_2 saturated solutions at 25°C.
Figure 8.11 and table 9 in appendix 8 shows the effect of the thiol concentration on the rate. All show a saturating effect.

Table 8.2 shows the effect of EDTA, CuSO₄ and catalase on the rate of O₂ uptake in the presence of mercaptoethanol.

Table 6.2: The effect of EDTA, CuSO₄ and catalase on the rate of O₂ uptake in the presence of mercaptoethanol (2×10⁻²M) and 1×10⁻⁷M diaquocobinamide at 25°C; pH 9.5

<table>
<thead>
<tr>
<th>Added reagent</th>
<th>Initial rate /μM O₂ s⁻¹</th>
<th>Maximum rate /μM O₂ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>4.00</td>
<td>15.7</td>
</tr>
<tr>
<td>1×10⁻²M EDTA</td>
<td>4.32</td>
<td>15.2</td>
</tr>
<tr>
<td>1×10⁻³M EDTA</td>
<td>4.00</td>
<td>15.7</td>
</tr>
<tr>
<td>1×10⁻⁵M CuSO₄</td>
<td>8.32</td>
<td>13.7</td>
</tr>
<tr>
<td>1×10⁻⁴M CuSO₄</td>
<td>7.80</td>
<td>10.5</td>
</tr>
<tr>
<td>1×10⁻⁷M CuSO₄</td>
<td>7.40</td>
<td>8.6</td>
</tr>
<tr>
<td>100 units catalase</td>
<td>4.00</td>
<td>4.6</td>
</tr>
</tbody>
</table>

There was no effect of EDTA indicating a genuine reaction. Any catalysis by Cu(II) was small compared to that of diaquocobinamide. In the presence of catalase, the rate was zero order in O₂ at least initially. This is the only evidence for the independence of the rate on O₂ concentration. Hence it is probable that reduction is still the slow step, as found for the B₄₉a system. No spectra were run of the steady state, as the diaquocobinamide concentrations used for the O₂ uptake studies were too low.

8.3 Discussion
All reactions studied share several features in common such as the
sharp pH maximum at \( \text{pH} 10 \) as well as the saturation effect of the thiol, which suggest a common basic mechanism. The pH profile appears similar to that observed for reduction but the two are probably only indirectly related (see below).

Absolute values of catalytic activity observed vary over the range \( \geq 0.9 \) equivalents \( \text{L}^{-1}/(\text{M Co x s}) \) for \( \text{B}_{12a} \) plus cysteine to 920 for diaquocobinamide plus ethanethiol.

All reactions are accompanied by additional reactions as revealed by the effects of superoxide dismutase, EDTA and autocatalysis.

3.1 \( \text{B}_{12a} \)

Experiments at pH 10 (maximum in the pH profile) show that the main features to be explained are:

1. The rate of \( \text{O}_2 \) uptake initially increases with cysteine and dithiothreitol concentrations, then becomes independent (figure 8.5 and figure 8.7).
2. Free \( \text{H}_2\text{O}_2 \) is produced at low but not at high cysteine concentrations.
3. The rate depends linearly on the cobalt concentration, and in the absence of accompanying reactions, is independent (at least initially) of the \( \text{O}_2 \) concentration, while spectra show the presence of hydroxocobalamin during the steady state. Hence the rate determining step involves the reduction of Co(III).
4. The observed rate of \( \text{O}_2 \) uptake is, however, greater than the observed rate of reduction of Co(III) under \( \text{N}_2 \). Even ignoring the stoichiometric requirement for four thiols to reduce \( \text{O}_2 \),
the asymptotes in figures 8, 5 and 8, 7 indicate that the rates of $O_2$ uptake are still 42 and 12 times greater than the rates of reduction for cysteine and dithiothreitol respectively.

We therefore suggest the following mechanism: It is known that Co(II) reversibly forms an adduct with $O_2$ which has only been detected at low temperatures 19 (reaction 8 - 1)

$$\text{Co(II) + } O_2 \leftrightarrow \text{Co(II)O}_2 \quad (8 - 1)$$

The autoxidation of Co(II) is greatly accelerated by reducing agents such as quinols, thiols, Fe(II)(CN)$_6$ through reactions such as reaction (8 - 2). 70

$$\text{Co(II)O}_2 + \text{RSH} \rightarrow \text{Co(III)O}_2H^- + \text{RS}^- \quad (8 - 2)$$

Furthermore, thyl radicals are known to react rapidly 65 according to reaction (8 - 3) (for cysteine, $k_f$ is $3.1 \times 10^9 \, \text{M}^{-1} \, \text{s}^{-1}$; $k_r \, 8 \times 10^5 \, \text{s}^{-1}$) while the hydroperoxide complex can dissociate according to reaction (8 - 4) to give free $\text{H}_2\text{O}_2$, which (together with any subsequent $\text{HO}^-$) can be further reduced by Co(II), RSH, RSSR.

$$\text{RS}^- + \text{RS}^- \rightarrow \text{RSSR} \quad (8 - 3)$$

$$\text{Co(III)O}_2H^- + \text{H}_2\text{O} \rightarrow \text{Co(III)} + \text{H}_2\text{O}_2 \quad (8 - 4)$$

Hence the anion radical RSSR can be formed in the immediate vicinity of the cobalt. This is expected to be a much more powerful reducing agent than RS$^-$ and by analogy with the electron transfer reactions observed in Cu containing electron transfer proteins with RSSR on the protein, 115 will probably reduce Co(III)OH$^-$ or more likely Co(III)OH$_2$ according to reaction (8 - 5) by an outer sphere mechanism.

$$\text{RSSR + Co(III)OH}_2 \rightarrow \text{RSSR + Co(II)} + \text{H}_2\text{O} \quad (8 - 5)$$

It can also undergo rapid disproportionation according to reaction
and could presumably reduce other compounds such as Co(III)O₂, H₂O₂, OH.

\[ 2\text{RSSR} \rightarrow \text{RSSR} + 2\text{RS}^- \]  \hspace{1cm} (8 - 6)

The reaction (8 - 7) does not occur, at least in air saturated as distinct from O₂ saturated buffers as shown by the lack of effect of superoxide dismutase.

\[ \text{RSSR} + O₂ \rightarrow \text{RSSR} + O₂^- \]  \hspace{1cm} (8 - 7)

This scheme immediately explains the main experimental observations:

1. The main reductant of Co(III) is not RS⁻ but more powerful, faster RSSR.

2. As the cysteine concentration increases so will the steady state concentrations of all the abovementioned intermediates so we may expect:

   (a) an increased rate of removal of RSSR by reactions other than (8 - 5), hence a fall-off in the effect of the thiol concentration is expected, as observed.

   (b) an increased rate of reduction of Co(III)O₂H⁻ (and presumably also free H₂O₂) by RSH and RSSR, leading to the suppression of the formation of free H₂O₂, as observed.

We therefore suggest that the observed rate of O₂ uptake can be considered to represent the sum of:

A. the abovementioned reactions which are shown in figure 8,12.
Figure 8.12 Scheme of reactions occurring in the autoxidation of cysteine catalysed by B. th. the further reactions of \( H_2O_2 \) together with smaller contributions from C. the reduction of Co(III) by cysteine (RS\(_2\)) itself D. the uncatalysed reaction of \( O_2 \) with RSH

All four groups of reactions will doubtless interact because of the existence of common intermediates such as \( H_2O_2 \) and RSSR. In particular, reaction (c) is required not only to start reaction (A) but also, since one can never expect all of the RSSR formed to be used in reducing Co(III), it is needed to keep reaction (A) going, i.e. there is bound to be an intimate relationship between reactions (A) and (C) even though the bulk of the "current" is carried by (A). This could explain why both the overall oxidase activity and the rate of reduction show similar sharp pH maxima around pH 10.

These remarks apply to the B\(_{12a}\)-dithiothreitol system as well. However, the RSSR is a cyclic monomer formed by the interaction
between the two -SH groups in the dithiol. The greater difference in the rates of reduction of \( B_{12a} \) by dithiothreitol (\( \nu_4 \)) compared with the rates of \( O_2 \) uptake (\( \nu_4 \)) is support for the importance of the RS\( \tilde{S} \)R in the latter, in carrying the reaction, as this would blur the distinction between mono and dithiols.

8.3.2. Diachiocobinamide

The much more limited data again show a saturation effect with all three thiols (see figure 8.11), a first order dependence on diachio-cobinamide (see figure 8.10) and at least with high mercaptethanol concentrations, the initial rate is independent of the \( O_2 \) concentration and does not produce \( H_2O_2 \).

It is reasonable to assume the same basic mechanism as for \( B_{12a} \) plus cysteine but in this case the much higher turnover number (920 equivalents \( L^{-1}/(MC\times s) \)) for ethane thiol for example can be correlated with the much higher rate of reduction of diachiocobinamide compared with \( B_{12a} \), even by monothiols. This turnover number is greater than that for electron transfer between cytochromes a and \( a^\prime \) (750 equivalents \( L^{-1}/(MC\times a) \)) and between cytochromes c and \( a^\prime \) (450 equivalents \( L^{-1}/(MC\times a) \)).

8.4 Conclusions

All the above evidence is consistent with a basic mechanism which involves the cycle of steps (a) - (d) of figure 8.12 (which in turn contains two different types of reduction \( \cdot \) of Co(III) to Co(II) and of Co(II)\( O_2 \) to Co(III)\( O_2^\prime \)) and with radical anions (RS\( \tilde{S} \)R) playing a key role.

In the case of \( B_{12a} \) and low cysteine concentrations, the reaction produces \( H_2O_2 \) but this formation of \( H_2O_2 \) as "seen" by catalase can be suppressed by increasing the cysteine concentration,
presumably merely by increasing the rate of reduction of coordinated Co-\(\text{O}_2\text{H}^-\) (and also perhaps free \(\text{H}_2\text{O}_2\)), without any dramatic increase in the turnover number.

The turnover number can be dramatically increased by increasing the rate of reduction of Co(III) to Co(II) by changing from a cobalamin to a cobinamide. The following maximum turnover numbers were found:

\[
\begin{align*}
\text{B}_{12\text{a}} + \text{cysteine} & : 0.9 \text{ equivalents } \text{s}^{-1}/(\text{M catalyst } \times \text{s}) \\
\text{B}_{12\text{a}} + \text{dithiothreitol} & : > 2.2 \text{ equivalents } \text{s}^{-1}/(\text{M catalyst } \times \text{s}) \\
\text{diaquocobinamide } - \text{cysteine} & : 78 \text{ equivalents } \text{s}^{-1}/(\text{M catalyst } \times \text{s}) \\
& \text{(initial rate)} \\
\text{diaquocobinamide } + \text{ethanethiol} & : 920 \text{ equivalents } \text{s}^{-1}/(\text{M catalyst } \times \text{s}) \text{ (initial rate)}
\end{align*}
\]

The rate could possibly be increased still further by increasing the rate of supply of reducing equivalents to both Co(III) and Co(II)\(\text{O}_2\), for example, by very efficient outer sphere electron transfer but the rate of \(\text{O}_2\) uptake in the diaquocobinamide plus ethanethiol system is already greater than that of both electron transfer between cytochromes c and a (450) as well as between cytochromes a and \(\alpha_3\) (750). \(^{113}\)

These results provide interesting parallels to the hemoproteins. It has been shown that the \(\text{O}_2\) adduct of hemoglobin which is not an active catalyst, reacts with reagents such as quinols \(^{116}\) and phenylhydrazines \(^{117}\) to give Fe(III) and \(\text{H}_2\text{O}_2\) by steps analogous to (c) and (d), i.e. this can probably be considered to be the basic mechanism common to iron porphyrins which cannot form dimers (as for example when bound in a protein) and cobalt corrinoids which have a coordination site available for coordinating \(\text{O}_2\) to the M(II).
Terminal oxidases such as cytochrome c oxidase also contain iron porphyrins but differ from hemoglobin in their high catalytic activity (maximum rate of electron transfer from cytochrome a to a₃ is 750 equivalents 1⁻¹/mole 1⁻¹ catalyst x seconds) and in not forming free H₂O₂. Although the mechanism of reduction of O₂ by cytochrome a₃ is not known, it is generally agreed that the initial step involves coordination of O₂ to Fe(II) cytochrome a₃ and it is reasonable at least to start, by assuming the occurrence of the basic steps (b), (c), (d). Our results with cobalt corrinoids show that both the high turnover number and the suppression of the formation of F₂O₂ can be achieved by manipulation of this basic mechanism. It is probable, however, that the Fe(III)O₂⁺ complex is converted to the "ferryl" (FeO)³⁺ complex, analogous to compound I of catalase and peroxidase before further reduction.
CHAPTER 9 - THE CATALYSIS OF THE AUTOXIDATION OF DITHIOTHREITOL
BY THE MONOMERIC BIS-HISTIDINE HEMIN COMPLEX

9.1 Introduction
This chapter will extend the study of the catalysis of the
autoxidation of thiols by looking at the catalysis by the bis-
histidine hemin complex in an attempt to elucidate the mechanism.
Catalysis will involve, at least initially, reduction of the
complex by dithiothreitol, the kinetics of which were discussed
in Chapter 7.

9.2 Results
The effect of the d-threitol, O₂, hemin and histidine concen-
trations on the rate of O₂ uptake were investigated. As the pH
studies were not informative in the reduction studies, they have
not been done here.

Figure 9.1 and table 9 in appendix 9 show the effect of the
rate on the dithiothreitol concentration.

This profile is similar to that found for the reduction of
bis-histidine hemin by dithiothreitol (figure 7.3). The
parameters used to fit the latter, do not fit the former, but
there are parallels. To ensure readily measurable rates, the
reduction studies were carried out at a hemin concentration five
times greater than that used in the O₂ uptake studies. The
maximum rate occurs at 2.4x10⁻³ M in dithiothreitol in the
reduction and at 5x10⁻⁴ M in the O₂ uptake studies, i.e., a factor
of five different. In addition there is approximately a two fold
difference in both systems between the maximum and limiting rate.
However, the initial rates of O₂ uptake/concentration of bis-
histidine hemin are all larger (3 - 6 times larger) than $k_{obs}$.
found for the reduction, as found for the $B_{12}$-cystine system.

Figure 9.1 The rate dependence on the DTT concentration for its autoxidation catalysed by bis-histidine hemin,

$6 \times 10^{-6}$M hemin; 0.4M histidine; pH 10.0; $\mu = 0.5$; 25°C

Because of the complex dependence of the dithiothreitol concentrations, other effects were studied both at high and low dithiothreitol concentration.

9.2.1 Low dithiothreitol concentrations

At low dithiothreitol concentrations, the $O_2$ and dithiothreitol
concentrations were very similar. Hence the dependence of the rate on the $O_2$ concentration could not be determined.

Figure 9.2 and table 2a in appendix 9 show that at $3 \times 10^{-4} M$ dithiothreitol at pH 10 the rate is first order in hemin.

[Graph showing the rate dependence on hemin concentration]

Figure 9.2 The rate dependence on the hemin concentration for the autoxidation of dtt catalysed by bis-histidine hemin at a low thiol concentration ($3 \times 10^{-4} M$); 0.4M histidine; pH 10.0; $u = 0.5$; 25°C.

Figure 9.3 and table 3 in appendix 9 show that the rate is independent of the histidine concentration as is the uncatalysed rate.

In 0.4M histidine at pH 10 using 26.3 µM hemin and $3 \times 10^{-4} M$ dithiothreitol, 5000 units of catalase reduced the rate from 2.70 µM O$_2$ s$^{-1}$ to 1.77 µM O$_2$ s$^{-1}$, i.e. a 34% decrease in rate.
Figure 9.3 The rate dependence on the histidine concentration for the autoxidation of dtt catalysed by bis-histidine hemin at a low thiol concentration ($3 \times 10^{-4}$M); 2.6$ \times 10^{-5}$M hemin; pH 10.0; $t_1 = 0.5$; 25°C.

The uncatalysed rate is only 21% that catalysed, which suggests that at least some of the hydrogen is being generated by the catalysed reaction. Unfortunately the range of concentrations of hemin which can be studied is limited by the dimerization of bis-histidine hemin (chapter 5) as well as the range of rates which can be studied by this technique.

The spectrum of $1 \times 10^{-5}$M hemin in the presence of 0.4M histidine and $3 \times 10^{-4}$M dithiothreitol in air saturated pH 10.0 buffer in the steady state (1 minute after mixing) showed shoulders at 360nm and 440nm characteristic of those of monochiolate hemin (see chapter 7) in addition to a band at 412nm, characteristic of the bis-histidine hemin. In addition, reoxidation of the Fe(II) bis-histidine complex was complete within 5 s. Hence reduction is the slow step and coordination of RS$^-$ is fast.

However, at $3 \times 10^{-4}$M dithiothreitol, $k_{obs}$ was 2 times greater for the $O_2$ uptake ($0.08$ s$^{-1}$) (from slope of
figure 9.2) compared with reduction \( (3.4 \times 10^{-2} \text{ s}^{-1}) \) which requires that the reduction is carried out by a faster and presumably more powerful reducing agent, as was the case with the cobalt corrinoid catalysed reactions, but the difference is not as marked.

9.2.2 High dithiothreitol concentrations

Three regions are evident at high dithiothreitol concentrations (figure 9.4). At low hemin concentrations, the rate is half order in oxygen (from a plot of log rate vs log \( (O_2) \)).

![Graph showing the rate dependence on hemin concentration for the autoxidation of dtt catalysed by bis-histidine hemin at a high thiol concentration (5x10^{-3} M); 0.4% histidine; pH 10.0; \( \mu = 0.5 \); 25°C.]

As the hemin concentration is increased, a zero order in \( O_2 \) dependence is found initially. The proportion of the reaction that
is zero order increases with hemin concentration. At high hemin concentrations, the reaction is autocatalytic initially, becoming zero order.

At the hemin concentrations where the rate is half order in oxygen, the rate of the uncatalysed reaction (which is half order in oxygen) is significant, and the half order dependence probably does not reflect the true situation in the hemin catalysed reaction. It is possible that the zero order dependence on the O₂ concentration does reflect the hemin catalysed reaction but too little data is at hand to be certain.

Figure 9.5 shows that the rate is second order in hemin suggesting the involvement of a dimer (data in table 2b, appendix 9).
Because of the complexity of the kinetics under these conditions, no further studies were done.

9.3 Discussion

At low dithiothreitol concentrations rate $\propto [Fe]^{-1} [O_2]^{2} [RSR]^{-1}$ \cite{0.1}. The spectrum of the steady state shows the presence of the monothiolate heme (bands at 360 nm and 440 nm) and reoxidation is rapid, indicating that reduction is the slow step while the coordination of RS is fast. (In contrast coordination of RS to B12 is slow.) The greater rate of O$_2$ uptake compared with reduction (2 fold difference) suggests that a strong reducing agent such as RSRS is largely responsible for reduction, as proposed for the B$_{12a}$-cysteine system. If it reduces heme by an outer sphere mechanism as it must do with B$_{12a}$ to overcome the slow coordination step, the independence of the rate on the histidine concentration, suggests that RSRS does not discriminate between the bis-histidine and monothiolate complexes. The maximum turnover number (corrected for the uncatalysed rate) is 0.3 equivalents $L^{-1}/(M$ heme $\times s)$ seven fold smaller than with B$_{12a}$ and dithiothreitol.

At high dithiothreitol concentrations

rate $\propto [Fe]^{2} [O_2]^{2} [RSR]^{0}$

This change in rate law may suggest either a change in the rate determining step or a change in mechanism but as it was complex and a strong interaction between the catalysed and uncatalysed reaction appeared probable, no further studies were made. However, the complexity as well as the change in the rate law shows the variety of reactions which can occur and correlates with the range of reactions found between O$_2$ and hemoproteins.
CHAPTER 10 - SUMMARY AND CONCLUSIONS

In chapter 3, hemin was studied in aqueous alkaline solution. Five distinct types of complex were found. These were monomers, dimers, and polymers with spectra falling into two types - A (typical high spin) and B (μ-oxo type) but the latter type was even given by monomeric species. These species were related by equilibria which were independent of pH but which depended on the hemin concentration and the ionic strength. The dimerization constant for the usual dimeric hemin (μ = 0;1) was found to be $\gtrsim 10^9 \text{M}^{-1}$. In addition, the formation of the monomeric hemin-caffeine adduct was confirmed and the complex found to contain one OH⁻ ligand. It was also shown that some detergent molecules at least, can form adducts with dimeric alkaline hemin, well below the critical micellar concentration ($K\cdot 10^5 \text{M}^{-1}$ per mole detergent bound).

The large dimerization constant, the stabilization of the monomeric species by caffeine, micellar detergents, and organic solvents as well as adduct formation with single detergent molecules re-emphasizes the known fact that one of the roles of the protein is to stabilize the monomer and that it can do this by hydrophobic and/or donor-acceptor interactions.

Chapter 4 dealt with the study of hemin in aqueous acid, where hemin rapidly aggregates. However, unstable monomeric and dimeric forms of the aquo-complex at pH 1 were formed by rapid dilution from pH 8 and the equilibrium between them was studied (the dimerization constant was $1.1 \times 10^5 \text{M}^{-1}$). Comparison of the spectra of the monomer with that of hemin in acidic aqueous ethanol, indicates that it is probably a six coordinate, high spin bis-aquo complex with the Soret maximum at 397 nm and an extinction
coefficient (at 397 nm) of 120 ± 3 cm⁻¹ cm⁻¹. Since the same spectrum was also observed in very dilute solutions at low ionic strength on rapid dilution to pH 7, the pKa for the coordinated H₂O is > 8 compared with that of 6.5 in 44% aqueous ethanol (organic solvents would be expected to stabilize FeOH⁺ rather than FeOH₂ because of its lower charge (ignoring the side chains)).

In chapter 5, the coordination of imidazole and its analogues to hemin was studied. In addition to the expected equilibria leading to the formation of the bis-ligand complex, additional equilibria involving adduct formation and aggregation were found. However, no significant formation of any monomeric mono-ligand complex was evident, even when the starting complex was the caffeine adduct of hemin in alkaline solution. This was also the case in organic solvents. The pKa is consistent with the binding constant for the first ligand being less than that for the second (K₂) which has been described to the change from high spin to low spin iron(III) on coordinating the second ligand. Hence a second role of the protein in hemoproteins such as peroxidase, which have one histidine coordinated to an Fe(III), is to stabilize the monohistidine complex.

Quantitative determinations of the equilibria involved as a function of pH showed that:

1) dimeric alkaline hemin reacts with histidine, histamine and pilocarpate to first give an adduct (possibility hydrogen bonded) containing one base per dimer and then to give the monomeric bis-ligand complexes.

2) on coordination, the pKa of the pendant -OH of pilocarpate is reduced from 13 to 10 (and a difference in the spectrum
noted above and below pH 10), and that of the pendant -NH\textsubscript{2} of histidine and histamine from \( \alpha \), 3 to \( \alpha \), which was ascribed to the stabilization of the conjugate base and/or the destabilization of the conjugate acid by the residual positive charge on the iron.

This latter finding provides a mechanism for proton or cation uptake on reduction observed with some hemoproteins,\textsuperscript{2} reduction of course eliminating the residual positive charge and allowing the pK\textsubscript{a}s of the neighbouring amino acid residues to revert to their normal values. For example, prior to reduction, the pendant -NH\textsubscript{2} group of histidine should be deprotonated in the pH range 8 - 10, but on reduction at these pHs should become protonated thus picking up a proton. This was in fact found on reducing bis-histidine by cyclic voltammetry in this pH range.\textsuperscript{108}

In chapter 6, the study of the rate of reduction of \( \text{S}_{12a} \) to \( \text{S}_{12c} \) by dithiothreitol over the pH range 1 - 13, revealed an interesting interplay between the rates of coordination of the thiolate to the Co(III) and reduction to Co(II). Significant differences were found in the acid region between the rate of reduction by the dithiols, dithiothreitol and the monothiol cysteine\textsuperscript{64} and mercaptoethanol. It was concluded that over the pH range 3 - 12, coordination always precedes reduction and that the mechanism probably involves a one electron reduction of the cobalt by the coordinated thiol, assisted by the second thiol (which is faster if dissociated (i.e. RS\textsuperscript{-}) than if protonated (i.e. RS\textsubscript{2})), to give the cyclic disulfide radical anion \( \text{S}_{2}^{2-} \) or its conjugate acid (pK\textsubscript{a} 5.5) as the immediate product. No redox potentials are known for the one electron oxidation of thiols but
it would appear that the RS⁻/RSSR couple is a better reducing agent than RS⁻/RS⁺. The absence of a second neighbouring cysteine in cytochrome P-450 may explain why the Fe(III) is not reduced by the coordinated cysteine, as occurs in protein free hemin.

In chapter 7, the reduction of the monomeric bis-histidine complex of hemin by dithiothreitol was shown to proceed via the rapid formation of an intermediate, identified by spectra and analysis of the rate data as the histidine thiolate complex, which then underwent a first order reaction to give Fe(II), i.e. the mechanism was inner sphere analogous to that of B₁₂, and no detectable outer sphere electron transfer was found. In addition, inhibition by dithiothreitol at high concentrations was consistent with a second dithiothreitol forming an adduct (possibly hydrogen bonded) but not coordinating (as UV-visible bands expected for the bis-thiolate complex were not evident) to give a species which reduced more slowly than the histidine-thiolate complex. The much slower reaction with cysteine and mercaptoethanol suggests, by analogy with B₁₂, that a second thiol group is required.

In chapter 8, the detailed study at pH 10 of the oxidation of cysteine by O₂ catalysed by B₁₂, revealed that the rate determining step in the catalytic cycle was the reduction of Co(III) but that this was forty-two times greater than the rate of reduction by cysteine. It was shown that all the results could be explained by a scheme involving a rapid reaction of the transient Co(II)O₂ with the thiol (RH) to give Co(III)O₂⁻ (and hence H₂O₂ which was detected by catalase) and the radical RS which reacts rapidly with a second thiol to give the disulphide radical anion (RSSR) which is the main reductant of Co(III). It was also shown that the
formation of free $H_2O_2$ could be suppressed by increasing the thiol concentration. The catalysed oxidation of dithiothreitol showed very similar features but was not markedly faster. The cobinamides (which lack the benzimidazole ligand of $B_{12a}$) have a much greater catalytic activity, reacting about 520 equivalents $\ell^+/(M$ catalyst x l) which is faster than cytochrome c oxidase due to the faster reduction rate of Co(III). Hence Co corrinoids are able to model both the suppression of $H_2O_2$ formation and the high turnover number, though in cytochrome $a_3Fe(III)O_2H^-$ may convert to $[FeO]^{2+}$. Evidence was also found for Cu(II) catalysis which is of interest as cytochrome c oxidase contains copper.

In chapter 9, the oxidation of dithiothreitol catalysed by bis-histidine hemin was studied. The kinetics were complex which is of interest in light of the variety of reactions between hemo-proteins and $O_2$. The rate dependence on the thiol concentration showed similar features to that found for the reduction, yet once again was faster. As at low thiol concentrations, spectra in the steady state showed the presence of the monothiolate, reduction must be rate determining and hence by analogy to $B_{12a}$ involves reduction by $B_{5SR}$. Bis-histidine hemin is not as good a catalyst as diaquo-cobinamide or even $B_{12a}$, which once again shows the influence of the protein in cytochrome oxidase in increasing the rate.

In this thesis, some of the structures, ligand binding, redox and catalytic reactions of hemin, and to a lesser extent cobalt corrinoids, in aqueous solution have been investigated and some pointers to the role of the protein in modulating these reactions have been given.
APPENDIX 1 : DERIVATION OF EQUATIONS FOR THE EQUILIBRIUM
STUDIES (CHAPTERS 3, 4, 5)

Equations required to analyse the data for the binding of ligands and the loss of protons (pKa) are derived in this appendix in parts (a) and (b) respectively. In each part equations will be derived for monomer-monomer, dimer-dimer and monomer-dimer interconversions.

The following abbreviations have been used here: M for the heme monomer; D for the heme dimer; L for the ligand or a species interacting with the porphyrin ring; n for the number of "ligands" bound; (Fe)_{TOT} for the heme concentration in terms of the concentration of the iron porphyrin units; A for absorbance with A_0 and A referring to the absorbance of the initial and final species respectively.

a) Ligand binding equations

Equations required to treat the data if the ligand concentration, the heme concentration or the pH is varied will be derived.

1) Derivation of equations for binding n ligands to a monomer

\[ M + nL \rightleftharpoons ML_n \]  

\[ K = \frac{[ML_n]}{[M][L]^n} \]

\[ \log K = \log \left( \frac{[ML_n]}{[M][L]^n} \right) = n \log ([L]) \]  

1) Varying \([L]\) \((Fe)_{TOT}\) constant

Now \( A = e_{M}(M) + e_{ML_n}(ML_n) \)

and \((Fe)_{TOT} = (M) + (ML_n)\)

\[ (ML_n) e_{ML_n}(Fe)_{TOT} = (M) \]

\[ A = e_{M}(M) + e_{ML_n}((Fe)_{TOT} - (M)) \]
\[ \text{(M)} = \frac{\text{Fe}^{2+}(ML_n)^+ + \text{Fe}^{2+}(Fe)_{\text{TOT}}}{\text{Fe}^{2+}(ML_n)^+ + \text{Fe}^{2+}(Fe)_{\text{TOT}}} \]

\[ \text{now} \quad A^0 = \text{Fe}^{2+}(Fe)_{\text{TOT}} \rightarrow E_M = \frac{A^0}{\text{Fe}^{2+}(Fe)_{\text{TOT}}} \]

\[ A_m = \text{Fe}^{2+}(ML_n)^+ \rightarrow E_M = \frac{A_m}{\text{Fe}^{2+}(ML_n)^+} \]

\[ \text{(M)} = \frac{A - A_m}{A^0 - A_m} \times \text{Fe}^{2+}(Fe)_{\text{TOT}} \]

\[ \log K = \log \frac{A - A_m}{A^0 - A_m} - n \log (L) \] (2)

\[ \log \frac{A - A_m}{A^0 - A_m} = \log K + n \log (L) \] (3)

plot of \( \frac{A - A_m}{A^0 - A_m} \) vs \( \log (L) \) should be linear with slope = \( n \) and intercept = \( \log K \)

if \( \text{OH}^- \) released (reaction II)

\[ \text{M} + nL \rightleftharpoons ML_n + d \text{OH}^- \] (II)

then equation (3) becomes

\[ \log \frac{A - A_m}{A^0 - A_m} = \log K' + d(14 - \text{pH}) + n \log (L) \] (4)

ii) Varying \( \text{Fe}^{2+}(Fe)_{\text{TOT}} \) (L constant)

let \( \alpha \) be the fraction of metalloporphyrin combined with ligand

\[ \alpha = \frac{\text{Fe}^{2+}(ML_n)^+ + \text{Fe}^{2+}(Fe)_{\text{TOT}}}{\text{Fe}^{2+}(Fe)_{\text{TOT}}} \]

\[ \alpha = \frac{\text{Fe}^{2+}(ML_n)^+}{\text{Fe}^{2+}(Fe)_{\text{TOT}}} \]

\[ \text{(M)} = (1 - \alpha) \text{Fe}^{2+}(Fe)_{\text{TOT}} \]
\[ \log K = \log \frac{\alpha(Fe)_{TOT}}{(1-\alpha)(Fe)_{TOT}} - n \log (L) \]

\[ \therefore \log \alpha(Fe)_{TOT} = \log K + n \log(L) + \log(1-\alpha)(Fe)_{TOT} \]

\[ \therefore \text{A plot of } \log \alpha(Fe)_{TOT} \text{ versus } \log(1-\alpha)(Fe)_{TOT} \text{ should be linear with slope } = 1 \text{ and intercept } = \log K + n \log(L) \]

In general, for dilution experiments where \((L)\) is constant, the slope of \(\log \alpha(Fe)_{TOT} \text{ versus } \log(1-\alpha)(Fe)_{TOT} \) = 

no. FeP units in prod / no. FeP units in react if reaction (II) applies then equation (5) becomes

\[ \log \alpha(Fe)_{TOT} = \log K' + d(14-pH) + n \log (L) + \log(1-\alpha)(Fe)_{TOT} \]

2) Derivation of equations for binding \(n\) ligands to a dimer

\[ \log A = \frac{\log \left( \frac{D_L^m}{D} \right)}{n} \]

\[ \therefore \log K = \log \frac{D_L^m}{D} = n \log(L) \]

1) Varying \((L)\) ((Fe)\(\text{TOT}\) constant)

\[ A = \epsilon_D(D) + \epsilon_{DLn}(DL_n) \]

\[ (Fe)_{TOT} = 2(D) + 2(DL_n) \]

\[ \therefore A = (D)\epsilon_D - \epsilon_{DLn} + \epsilon_{DLn} \frac{(Fe)_{TOT}}{2} \]

\[ (D) = \frac{A - \epsilon_{DLn}}{\epsilon_D - \epsilon_{DLn}} \]

\[ \therefore A = \frac{\epsilon_D}{2} \Rightarrow \epsilon_D = \frac{2A}{(Fe)_{TOT}} \]

\[ A_o = \epsilon_{DLn} \frac{(Fe)_{TOT}}{2} \Rightarrow \epsilon_{DLn} = \frac{A_o}{(Fe)_{TOT}} \]
\[ \text{:. } (D) = \frac{A - A_o}{A_o - A} \times \frac{(Fe)_{TOT}}{2} \]

\[ \text{:. } (DLn) = \frac{A_o - A}{A_o - A_o} \times \frac{(Fe)_{TOT}}{2} \]

\[ \text{:. } \log K = \log \frac{A_o - A}{A_o - A} - n \log (L) \] (7)

\[ \text{:. } \log \frac{A - A_o}{A_o - A} = \log K + n \log (L) \] (8)

I.e. plot of \( \log \frac{A - A_o}{A_o - A} \) vs \( \log (L) \) should be linear with slope = \( n \) intercept = \( \log K \)

If \( OH^- \) is involved in the reaction (i.e. reaction (IV) applies)

\[ D + nL \rightleftharpoons DLn + d OH^- \] (IV)

then equation (8) becomes

\[ \log \frac{A - A_o}{A_o - A} = \log K' + d(14-pH) + n \log (L) \] (9)

ii) Varying \( \frac{(Fe)_{TOT}}{(L) \text{ constant}} \)

let \( \alpha = \frac{2(DLn)}{(Fe)_{TOT}} \)

\[ \text{:. } (DLn) = \alpha \frac{(Fe)_{TOT}}{2} \]

\[ \text{:. } (D) = (1-\alpha) \frac{(Fe)_{TOT}}{2} \]

\[ \log K = \frac{\log \frac{1}{\alpha} \frac{(Fe)_{TOT}}{(1-\alpha) \frac{(Fe)_{TOT}}{2}}} - n \log (L) \]

\[ \text{:. } \log K = \frac{\log \frac{\alpha(Fe)_{TOT}}{(1-\alpha)(Fe)_{TOT}}}{- n \log (L)} \]

\[ \text{:. } \log \alpha (Fe)_{TOT} + \log K + n \log (L) + \log(1-\alpha)(Fe)_{TOT} \] (10)
A plot of log a (Fe)_{TOT} vs log (1-a) (Fe)_{TOT} will therefore be linear with slope = 1 and intercept = log K + n log(L)

If d moles of OH⁻ released then equation (10) becomes

\[ \log a(Fe)_{TOT} = (\log K' + d(14-pH) + n \log(L) + \log(1-a)(Fe)_{TOT}) \]

3) Derivation of equations for binding of n ligands and splitting a dimer to give a monomer:

\[ D + 2nL \rightleftharpoons 2nMLn \]  
\[ K = \frac{(nL)^2}{(D)(L)^n} \]
\[ \therefore \log K = \log \frac{(nL)^2}{(D)} - 2n \log(L) \]  

1) Varying (L) \( (Fe)_{TOT} \) constant

\[ [MLn] = (Fe)_{TOT} - 2(D) \]

\[ [MLn] = \frac{A - MLn}{\varepsilon D} \]  
\[ (Fe)_{TOT} = \frac{(Fe)_{TOT}}{pH} + \frac{\varepsilon D}{2} \]

\[ (MLn) = \frac{(Fe)_{TOT}}{\varepsilon D - 2nMLn} \]

\[ (MLn) = \frac{(Fe)_{TOT}}{\varepsilon D - 2nMLn} \]  
\[ (Fe)_{TOT} = \frac{(Fe)_{TOT}}{2} \times (Fe)_{TOT} \]

\[ A = \frac{e}{A} \]  
\[ A = \frac{e}{2} \]

\[ (MLn) = \frac{(Fe)_{TOT}}{A} \]  
\[ (Fe)_{TOT} = \frac{2A}{(Fe)_{TOT}} \]

\[ (MLn) = \frac{A}{A} \]

\[ (D) = \frac{A - A_{\infty}}{A - A_{\infty}} \times \frac{(Fe)_{TOT}}{2} \]

\[ (MLn) = \frac{A}{A} \times \frac{(Fe)_{TOT}}{1} \]
\[
\log K = \log \left( \frac{A_o-A}{A_o-A_m} \right) \frac{(Fe)_{TOT}}{(Fe)^{2n}} \frac{1}{A^2} - 2n \log(L)
\]

\[
= \log \left( \frac{A_o-A}{A_o-A_m} \right) \frac{2(Fe)_{TOT}}{A^2} - 2n \log(L)
\]

(16)

\[
\log \frac{(A_o-A)^2}{A_o-A_m} \frac{2(Fe)_{TOT}}{A^2} = \log K + 2n \log(L)
\]

(17)

\[
\log \frac{(A_o-A)^2}{A_o-A_m} \frac{2(Fe)_{TOT}}{A^2} = \log K + 2n \log(L)
\]

(18)

If \( K \) is the fraction of metalloporphyrin \((Fe)_{TOT}\) combined with ligand

\[
\alpha = \frac{[MLn]}{(Fe)_{TOT}} = \frac{(Fe)_{TOT} - A}{(Fe)_{TOT} - A_{TOT} - (MLn)}
\]

(19)

\[
(Fe)_{TOT} = (MLn) + 2(D)
\]

then

\[
[MLn] = \alpha (Fe)_{TOT}
\]

and

\[
2(D) = (Fe)_{TOT} - \alpha (Fe)_{TOT}
\]

(20)

\[
\alpha = (1-q) \frac{(Fe)_{TOT}}{2}
\]
\[ \log K = \log \frac{a[(Fe)_{TOT}]}{(1-a)(Fe)_{TOT}^{2}} - 2n \log (L) \]

\[ = 2 \log a(Fe)_{TOT} - \log (1-a)(Fe)_{TOT} - 2n \log (L) \]

\[ = 2 \log a(Fe)_{TOT} - \log (1-a)(Fe)_{TOT} - \log (L) \]

\[ (20) \]

\[ 2 \log a(Fe)_{TOT} = \log K + 2n \log (L) + \log(1-a)(Fe)_{TOT} \]

\[ \log a(Fe)_{TOT} = \frac{1}{2} \log K + 2n \log (L) + \log(1-a)(Fe)_{TOT} \]

\[ (21) \]

\[ \text{a plot of } \log a(Fe)_{TOT} \text{ against } \log (1-a)(Fe)_{TOT} \]

should give slope \( \frac{1}{2} \) and

\[ \text{intercept } = \frac{1}{2} \log K + 2n \log (L) + \log(1-a) \]

\[ \text{iii) Effect of pH} \]

if the reaction involves the release of \( OH^- \) (i.e. reaction (VI))

\[ D + 2nL = 2\text{H}n + d\text{OH}^- \]

\[ (VI) \]

then \( \log K' = \log \frac{(2\text{H}n)^2}{(D)} - 2n \log (L) - d(14-pH) \)

and equation (17) then becomes

\[ \log \frac{(A-A_0)^2}{(A_0-A)} = \frac{2(Fe)_{TOT}}{A_0 - A_0} = \log K' + 2n \log (L) + d(14-pH) \]

\[ (22) \]

and equation (20) becomes

\[ \log a(Fe)_{TOT} = \frac{1}{2} \log K' + 2n \log (L) + \log(1-a)(Fe)_{TOT} + \frac{1}{2} \log(1-a)(Fe)_{TOT} \]

\[ (23) \]

b) Derivation of pKa equations

1) Proton loss involving monomeric species only

\[ N = N' + nh^+ \]

\[ (IV) \]
\[ K_a = \frac{(H^+)(\text{N})}{(\text{M})} \Rightarrow \log K_a = \log \frac{(H^+)}{(\text{M})} + n \log (H^+) \]

\[ \Rightarrow pK_a = -\log \frac{(H^+)}{(\text{M})} + n \text{ pH} \]

\[ \Rightarrow \log \frac{(H^+)}{(\text{M})} = n \text{ pH} - pK_a. \]

Now \( A = \epsilon_M (\text{M}) + \epsilon_{M'} (\text{M}') \)

\( (\text{Fe})_{\text{TOT}} = (\text{M}) + (\text{M}') \Rightarrow (\text{M}') = (\text{Fe})_{\text{TOT}} - (\text{M}) \)

\( . \quad A = \epsilon_M (\text{M}) + \epsilon_{M'} \left[(\text{Fe})_{\text{TOT}} - (\text{M})\right]. \)

\( . \quad A = (\text{M}) (\epsilon_M - \epsilon_{M'}) + \epsilon_{M'} (\text{Fe})_{\text{TOT}} \)

\( . \quad (\text{M}) = \frac{A - \epsilon_{M'} (\text{Fe})_{\text{TOT}}}{\epsilon_M - \epsilon_{M'}} \)

initially \( (\text{M}) = (\text{Fe})_{\text{TOT}} \)

\( . \quad A_o = \epsilon_M (\text{Fe})_{\text{TOT}} \Rightarrow \epsilon_M = \frac{A_o}{(\text{Fe})_{\text{TOT}}} \)

finally \( (\text{M}') = (\text{Fe})_{\text{TOT}} \)

\( . \quad A_o = \epsilon_{M'} (\text{Fe})_{\text{TOT}} \Rightarrow \epsilon_{M'} = \frac{A_o}{(\text{Fe})_{\text{TOT}}} \)

\( . \quad (\text{M}) = \frac{A - A_o}{A_o - A_o} x (\text{Fe})_{\text{TOT}} \)

\( (\text{M}') = (\text{Fe})_{\text{TOT}} - (\text{M}) \)

\( = (\text{Fe})_{\text{TOT}} \left(1 - \frac{A - A_o}{A_o - A_o}\right) \)

\( = (\text{Fe})_{\text{TOT}} \frac{A_o - A}{A_o - A_o} \)

\( . \quad (\text{M}') = \frac{A_o - A}{A_o - A_o} = \frac{A - A_o}{A_o - A} \)

\( . \quad \log \frac{A - A_o}{A_o - A} = n \text{ pH} - pK_a \)

and \( pK_a = -\log \frac{A - A_o}{A_o - A} + n \text{ pH} \)
A plot of $\log \frac{A - A_0}{A_\infty - A}$ versus pH should be linear with slope $= n$ and intercept $= -pK_a$.

2. Proton loss involving dimeric species only

$$D \rightleftharpoons D + nH^+$$

$K = \frac{(D')}{(D)(H^+)^n} \Rightarrow \log \frac{(D')}{(D)} = n \pH - pK_a$

$A = e_D(D) + e_D'(D')$

$$(Fe)_{TOT} = 2 \times (D) + 2(D')$$

$\therefore \quad (D') = \frac{(Fe)_{TOT} - 2(D)}{2}$

$\therefore \quad A = e_D(D) + e_D'(D') \left( \frac{(Fe)_{TOT} - (D)}{2} \right)$

$$= (D) \cdot \left( e_D - e_D' \right) + e_D' \cdot \frac{(Fe)_{TOT}}{2}$$

$$\therefore \quad (D) = \frac{A - e_D'}{e_D - e_D'} \cdot \frac{(Fe)_{TOT}}{2}$$

initially $(D) = \frac{1}{2} (Fe)_{TOT}$

$\therefore \quad A_0 = \frac{(Fe)_{TOT}}{2} \Rightarrow e_D = \frac{2A_0}{(Fe)_{TOT}}$

finally $(D') = \frac{1}{2} (Fe)_{TOT}$

$\therefore \quad A_\infty = \frac{(Fe)_{TOT}}{2} \Rightarrow e_D' = \frac{2A_\infty}{(Fe)_{TOT}}$

$$\therefore \quad (D) = \frac{A - A_\infty}{A_\infty - A_0} \cdot \frac{(Fe)_{TOT}}{2}$$

$$(D') = \frac{(Fe)_{TOT}}{2} - (D) = \frac{A_0 - A}{A_\infty - A_0} \times \frac{(Fe)_{TOT}}{2}$$

$\therefore \quad (D') = \frac{A_D - A}{K - A_\infty} = \frac{A - A_0}{A_\infty - A}$
\[ \log \frac{A - A_0}{A_\infty - A} = n \text{pH} - pK_a \]

plot of \( \frac{A - A_0}{A_\infty - A} \) vs pH should be linear with slope = \( n \) and intercept = \(-pK_a\).

3) Proton loss involving dimerization

\[ 2M \longleftrightarrow D + nH^+ \quad \text{(VI)} \]

\[ K_a = \frac{(\text{D})(n^+)}{\text{NO}^2} \quad \Rightarrow \quad \log \frac{\text{NO}^2}{(\text{D})} = -n \text{pH} + pK_a \]

\[ A = \varepsilon_M (\text{M}) + \varepsilon_D (\text{D}) \]

\[ (\text{Fe})_{\text{TOT}} = (\text{M}) + 2(\text{D}) \quad \Rightarrow \quad (\text{M}) = (\text{Fe})_{\text{TOT}} - 2(\text{D}) \]

\[ A = \varepsilon_M \left[ (\text{Fe})_{\text{TOT}} - 2(\text{D}) + \varepsilon_D (\text{D}) \right] \]

\[ = (\text{D}) \left( \frac{\varepsilon_D - 2\varepsilon_M}{\varepsilon_D} \right) + \varepsilon_M (\text{Fe})_{\text{TOT}} \]

\[ (\text{D}) = \frac{A - \varepsilon_M (\text{Fe})_{\text{TOT}}}{\varepsilon_D - 2\varepsilon_M} \]

initially \((\text{Fe})_{\text{TOT}} = (\text{M})\) initial

\[ A_0 = \varepsilon_M (\text{Fe})_{\text{TOT}} \quad \Rightarrow \quad \varepsilon_M = \frac{A_0}{(\text{Fe})_{\text{TOT}}} \]

initially \((\text{D}) = (\text{Fe})_{\text{TOT}}\)

\[ A_\infty = \varepsilon_D (\text{Fe})_{\text{TOT}} \quad \Rightarrow \quad \varepsilon_D = \frac{A_\infty}{(\text{Fe})_{\text{TOT}}} \]

\[ (\text{D}) = \frac{A - A_0}{A_0 - A_\infty} \times \frac{(\text{Fe})_{\text{TOT}}}{2} \]

\[ (\text{M}) = (\text{Fe})_{\text{TOT}} - 2(\text{D}) = \frac{A - A_\infty}{A_0 - A_\infty} \times (\text{Fe})_{\text{TOT}} \]

\[ \frac{(\text{M})^2}{(\text{D})} = \frac{(A - A_\infty)^2}{(A_0 - A_\infty)^2} \times (\text{Fe})_{\text{TOT}}^2 \times \frac{A_0 - A_\infty}{A - A_0} \times \frac{(\text{Fe})_{\text{TOT}}^2}{2} \]
\[ \frac{(A - A_0)^2}{A_0 - A} \times \frac{(\text{Fe})_{\text{TOT}}}{A_0 - A_0} = \frac{(A - A_0)^2}{A_0 - A} \times \frac{2(\text{Fe})_{\text{TOT}}}{A_0 - A_0} \]

\[ \log \frac{(A - A_0)^2}{(A - A_0)} = -n \text{ pH} + pK_a + \log \frac{2(\text{Fe})_{\text{TOT}}}{A_0 - A_0} \]

\[ \log \frac{(A - A_0)^2}{(A - A_0)} = -n \text{ pH} + pK_a - \log \frac{2(\text{Fe})_{\text{TOT}}}{A_0 - A_0} \]

\[ pK_a = \log \frac{(A - A_0)^2}{(A - A_0)} + n \text{ pH} + \log \frac{2(\text{Fe})_{\text{TOT}}}{A_0 - A_0} \]

b) If the pH is kept constant but (Fe)_{TOT} varied then

\[ \log \frac{(A - A_0)^2}{(A - A_0)(A_0 - A_0)} = -n \text{ pH} + pK_a - \log 2 \]

\[ \log \frac{(A - A_0)^2}{(A - A_0)(A_0 - A_0)} = -n \text{ pH} + pK_a - \log 2 \]

\[ pK_a = n \text{ pH} - \text{ intercept} - \log 2 \] (n determined in a)

\[ pK_a = \log \frac{(A - A_0)^2}{A_0 - A} \times \frac{1}{A_0 - A_0} + n \text{ pH} + \log (\text{Fe})_{\text{TOT}} + \log 2 \]

Note - both reactions (IV) and (V) have no (Fe)_{TOT} term in the final expression and hence \( \log \frac{(P')_D}{(D')} \) and \( \log \frac{(P')_D}{(D')} \) should be invariant with (Fe)_{TOT}.
APPENDIX 2: TABLES OF DATA FOR THE STUDY OF HEMIN IN ALKALINE SOLUTION (ALL EXPERIMENTS DONE IN SOLUTIONS WITH \( \mu = 0.1 \) AT 25\(^{\circ}\)C)

Equations used in analysis of data were:

\[
\log \chi = \log \frac{(A_0 - A)^2}{A - A_\infty} \times \frac{2(Fe)_{TOT}}{A_0 - A_\infty} - 2n \log (L) \quad (1)
\]

\[
\varepsilon_{D/2} (Fe)_{TOT} - \Delta
\]

\[
\varepsilon_{D/2} - \varepsilon_{MLA} (Fe)_{TOT}
\]

\[
\log K = 2 \log \chi (Fe)_{TOT} - \log(1-\varepsilon)(Fe)_{TOT} - \log \frac{1}{2} - 2n \log (L) \quad (3)
\]
Table 1a: Titration of hemin by caffeine at pH 8.50; 10.1x10^-6 M hemin

<table>
<thead>
<tr>
<th>10^3 (caffeine)</th>
<th>A/00</th>
<th>log (caffeine)</th>
<th>( \log \left( \frac{(A-A_o)^2}{2(F(Fe))_TOT} \right) )</th>
<th>( \log k^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL, M</td>
<td></td>
<td>free</td>
<td>( \frac{\lambda_{\infty} - \lambda}{\lambda_{\infty} - \lambda_o} )</td>
<td>N^-1</td>
</tr>
<tr>
<td>0.10</td>
<td>0.344</td>
<td>-4.00</td>
<td>-7.29</td>
<td>0.71</td>
</tr>
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<td>0.363</td>
<td>-3.52</td>
<td>-6.32</td>
<td>0.72</td>
</tr>
<tr>
<td>0.30</td>
<td>0.379</td>
<td>-3.30</td>
<td>-5.90</td>
<td>0.70</td>
</tr>
<tr>
<td>0.50</td>
<td>0.402</td>
<td>-3.10</td>
<td>-5.62</td>
<td>0.78</td>
</tr>
<tr>
<td>1.09</td>
<td>0.420</td>
<td>-2.96</td>
<td>-5.21</td>
<td>0.71</td>
</tr>
<tr>
<td>1.27</td>
<td>0.444</td>
<td>-2.81</td>
<td>-4.90</td>
<td>0.72</td>
</tr>
<tr>
<td>2.24</td>
<td>0.472</td>
<td>-2.63</td>
<td>-4.55</td>
<td>0.71</td>
</tr>
<tr>
<td>3.29</td>
<td>0.494</td>
<td>-2.48</td>
<td>-4.24</td>
<td>0.72</td>
</tr>
<tr>
<td>4.76</td>
<td>0.517</td>
<td>-2.32</td>
<td>-3.80</td>
<td>0.84</td>
</tr>
</tbody>
</table>

\( \lambda_o = 0.334 \)  \( \lambda_{\infty} = 0.538 \)  \( Av: 0.74 \pm 0.1 \)

a calc. using equation (1) with n = 2  \( \lambda_{\infty} = 0.85 \) (Sd = 0.09)

Plot of \( \log \left( \frac{(A-A_o)^2}{2(F(Fe))_TOT} \right) \) vs log (caffeine) free

Table 1b: Titration of hemin by caffeine at pH 12.00; 10.2x10^-6 M hemin

<table>
<thead>
<tr>
<th>10^3 (caffeine)</th>
<th>A/00</th>
<th>log (caffeine)</th>
<th>( \log \left( \frac{(A-A_o)^2}{2(F(Fe))_TOT} \right) )</th>
<th>( \log k^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL, M</td>
<td></td>
<td>free</td>
<td>( \frac{\lambda_{\infty} - \lambda}{\lambda_{\infty} - \lambda_o} )</td>
<td>N^-1</td>
</tr>
<tr>
<td>0.10</td>
<td>0.466</td>
<td>-4.00</td>
<td>-7.18</td>
<td>0.82</td>
</tr>
<tr>
<td>0.20</td>
<td>0.481</td>
<td>-3.70</td>
<td>-6.95</td>
<td>0.85</td>
</tr>
<tr>
<td>0.30</td>
<td>0.492</td>
<td>-3.53</td>
<td>-6.26</td>
<td>0.80</td>
</tr>
<tr>
<td>0.40</td>
<td>0.505</td>
<td>-3.40</td>
<td>-6.00</td>
<td>0.80</td>
</tr>
<tr>
<td>0.60</td>
<td>0.527</td>
<td>-3.22</td>
<td>-5.66</td>
<td>0.78</td>
</tr>
<tr>
<td>0.89</td>
<td>0.553</td>
<td>-3.05</td>
<td>-5.34</td>
<td>0.76</td>
</tr>
<tr>
<td>1.19</td>
<td>0.576</td>
<td>-2.83</td>
<td>-5.00</td>
<td>0.76</td>
</tr>
<tr>
<td>1.67</td>
<td>0.601</td>
<td>-2.78</td>
<td>-4.86</td>
<td>0.70</td>
</tr>
</tbody>
</table>

\( \lambda_o = 0.451 \)  \( \lambda_{\infty} = 0.723 \)  \( Av: 0.79 \pm 0.09 \)

a calc. using equation (1) with n = 2  \( \lambda_{\infty} = 0.46 \) (Sd = 0.07)

Plot of \( \log \left( \frac{(A-A_o)^2}{2(F(Fe))_TOT} \right) \) vs log (caffeine) free
Table 2: Dilution experiments with hemin-caffeine

a) pH 8.50; 1.68x10^{-3} M caffeine

<table>
<thead>
<tr>
<th>10^6(Fe)_{TOT}</th>
<th>%</th>
<th>log(Fe)_{TOT}</th>
<th>log(Fe)_{TOT(l-\alpha)}</th>
<th>log K *</th>
</tr>
</thead>
<tbody>
<tr>
<td>(2,12)</td>
<td>0.147</td>
<td>0.745</td>
<td>-5.80</td>
<td>-6.27</td>
</tr>
<tr>
<td>4.23</td>
<td>0.287</td>
<td>0.701</td>
<td>-5.53</td>
<td>-5.90</td>
</tr>
<tr>
<td>6.35</td>
<td>0.419</td>
<td>0.647</td>
<td>-5.39</td>
<td>-5.55</td>
</tr>
<tr>
<td>8.45</td>
<td>0.565</td>
<td>0.603</td>
<td>-5.29</td>
<td>-5.47</td>
</tr>
<tr>
<td>10.55</td>
<td>0.665</td>
<td>0.560</td>
<td>-5.22</td>
<td>-5.33</td>
</tr>
<tr>
<td>13.80</td>
<td>0.964</td>
<td>0.500</td>
<td>-5.10</td>
<td>-5.10</td>
</tr>
<tr>
<td>21.03</td>
<td>1.242</td>
<td>0.443</td>
<td>-5.03</td>
<td>-4.93</td>
</tr>
</tbody>
</table>

Av: 0.73 ± 0.04

Plot of log(Fe)_{TOT} versus log(Fe)_{TOT(l-\alpha)}

intercept = 2.19 (Sd = 0.13)
slope = 0.57 (Sd = 0.02)

* Calculated using equation (3) (n = 2)

b) pH 10.0; 1.68x10^{-3} M caffeine

<table>
<thead>
<tr>
<th>10^6(Fe)_{TOT}</th>
<th>%</th>
<th>log(Fe)_{TOT}</th>
<th>log(Fe)_{TOT(l-\alpha)}</th>
<th>log K *</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3.79)</td>
<td>0.238</td>
<td>0.708</td>
<td>-5.57</td>
<td>-5.96</td>
</tr>
<tr>
<td>5.68</td>
<td>0.384</td>
<td>0.694</td>
<td>-5.40</td>
<td>-5.76</td>
</tr>
<tr>
<td>7.57</td>
<td>0.502</td>
<td>0.656</td>
<td>-5.30</td>
<td>-5.58</td>
</tr>
<tr>
<td>9.47</td>
<td>0.614</td>
<td>0.613</td>
<td>-5.24</td>
<td>-5.44</td>
</tr>
<tr>
<td>11.36</td>
<td>0.721</td>
<td>0.573</td>
<td>-5.19</td>
<td>-5.31</td>
</tr>
<tr>
<td>15.14</td>
<td>0.922</td>
<td>0.497</td>
<td>-5.12</td>
<td>-5.12</td>
</tr>
<tr>
<td>18.93</td>
<td>1.136</td>
<td>0.471</td>
<td>-5.05</td>
<td>-5.00</td>
</tr>
<tr>
<td>22.72</td>
<td>1.353</td>
<td>0.431</td>
<td>-5.01</td>
<td>-4.99</td>
</tr>
</tbody>
</table>

Av: 0.78 ± 0.05

Plot of log(Fe)_{TOT} versus log(Fe)_{TOT(l-\alpha)}

intercept = -2.58 (Sd = 0.16)
slope = 0.49 (Sd = 0.03)

* Calculated using equation (3) (n = 2)
c) 20 ml NaOH; $2.34 \times 10^{-2} \text{M caffeine}$

\[ \log(K) = 3.90, 7.75, 11.69, 19.48 \]

\begin{tabular}{cccccc}
$10^6(\text{Fe})_{\text{TOT}}$ & $A_{300}$ & $\alpha$ & $\log(\text{Fe})_{\text{TOT}}$ & $\log(\text{Fe})_{\text{TOT}}(1-\alpha)$ & $\log K$ \\
3.90 & 0.281 & 0.825 & -5.49 & -6.17 & 0.75 \\
7.75 & 0.535 & 0.726 & -5.25 & -5.67 & 0.73 \\
11.69 & 0.788 & 0.688 & -5.09 & -5.44 & 0.82 \\
19.48 & 1.237 & 0.574 & -4.95 & -5.08 & 0.74 \\
\end{tabular}

\[ \text{Av: } 0.76 \pm 0.06 \]

Plot of $\log(\text{Fe})_{\text{TOT}}$ versus $\log(\text{Fe})_{\text{TOT}}(1-\alpha)$

- Intercept = -2.37 (Sd = 0.18)
- Slope = 0.51 (Sd = 0.03)

\[ \text{Calculated using equation (3): } \]

Calculations in Table 2a, b, c carried out using

\[ \frac{e_D}{T} = 4.1 \times 10^4 \text{ N}^{-1} \]

\[ \frac{e_{\text{HLN}}}{T} = 7.8 \times 10^4 \text{ N}^{-1} \]

and equations (2) and (3) (where $n = 2$)
### Table 3a: Titration of hemin by cetyl trimethyl ammonium bromide

**i) 0.1M NaOH; 9.72x10^-6M hemin**

<table>
<thead>
<tr>
<th>(detergent), μN</th>
<th>A_385</th>
<th>log(detergent) free a</th>
<th>log ( \frac{A-A_0}{A_{\infty}-A} )</th>
<th>log K b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.532</td>
<td>-6.98</td>
<td>-2.69</td>
<td>12.27</td>
</tr>
<tr>
<td>0.8</td>
<td>0.550</td>
<td>-6.29</td>
<td>-1.38</td>
<td>11.20</td>
</tr>
<tr>
<td>1.8</td>
<td>0.565</td>
<td>-6.06</td>
<td>-1.01</td>
<td>11.08</td>
</tr>
<tr>
<td>3.6</td>
<td>0.535</td>
<td>-5.70</td>
<td>-0.62</td>
<td>10.90</td>
</tr>
<tr>
<td>6.0</td>
<td>0.524</td>
<td>-5.52</td>
<td>-0.36</td>
<td>10.68</td>
</tr>
<tr>
<td>9.0</td>
<td>0.510</td>
<td>-5.33</td>
<td>-0.10</td>
<td>10.56</td>
</tr>
<tr>
<td>12.0</td>
<td>0.495</td>
<td>-5.21</td>
<td>0.17</td>
<td>10.59</td>
</tr>
<tr>
<td>15.0</td>
<td>0.484</td>
<td>-5.09</td>
<td>0.38</td>
<td>10.56</td>
</tr>
<tr>
<td>18.0</td>
<td>0.476</td>
<td>-4.99</td>
<td>0.57</td>
<td>10.55</td>
</tr>
<tr>
<td>24.0</td>
<td>0.466</td>
<td>-4.81</td>
<td>0.90</td>
<td>10.54</td>
</tr>
<tr>
<td>30.0</td>
<td>0.461</td>
<td>-4.68</td>
<td>1.19</td>
<td>10.54</td>
</tr>
</tbody>
</table>

**Av:** 10.56 ± 0.03

\[ a = 2 \text{ using equation (1)} \]

\[ \Delta \theta = 0.35; \ \Delta_\theta = 0.453 \]

\[ n = 2 \text{ (det) 0.9 - 30.0} \]

slope = 1.92 \( (S_d = 0.04) \)

intercept = 10.26 \( (S_d = 0.16) \)

**ii) pH 8.50 (borax); 9.64x10^-6 M hemin**

<table>
<thead>
<tr>
<th>(detergent), μN</th>
<th>A_450</th>
<th>log(detergent) free a</th>
<th>log ( \frac{A-A_0}{A_{\infty}-A} )</th>
<th>log K b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.6</td>
<td>0.087</td>
<td>-6.72</td>
<td>-1.35</td>
<td>12.09</td>
</tr>
<tr>
<td>1.5</td>
<td>0.093</td>
<td>-6.32</td>
<td>-0.92</td>
<td>11.72</td>
</tr>
<tr>
<td>3.0</td>
<td>0.105</td>
<td>-6.13</td>
<td>-0.51</td>
<td>11.75</td>
</tr>
<tr>
<td>6.0</td>
<td>0.114</td>
<td>-5.55</td>
<td>-0.31</td>
<td>10.79</td>
</tr>
<tr>
<td>12.0</td>
<td>0.139</td>
<td>-5.20</td>
<td>0.17</td>
<td>10.57</td>
</tr>
<tr>
<td>18.0</td>
<td>0.152</td>
<td>-4.96</td>
<td>0.44</td>
<td>10.36</td>
</tr>
<tr>
<td>24.0</td>
<td>0.165</td>
<td>-4.81</td>
<td>0.83</td>
<td>10.45</td>
</tr>
<tr>
<td>30.0</td>
<td>0.174</td>
<td>-4.68</td>
<td>1.18</td>
<td>10.84</td>
</tr>
</tbody>
</table>

**Av:** 10.54 ± 0.03

\[ a = 2 \text{ b calculated using equation (1)} \]

\[ \Delta \theta = 0.083; \ \Delta_\theta = 0.177 \]

slope = 1.87 \( (S_d = 0.04) \)

intercept = 9.97 \( (S_d = 1.73) \)

\[ \Delta \theta = 0.083; \ \Delta_\theta = 0.177 \]

Plot of log \( \frac{A-A_0}{A_{\infty}-A} \) versus log (detergent) free
### iii) 0.1M NaOH; 1.5x10^{-5} M CTMAB; 25°C

<table>
<thead>
<tr>
<th>$10^6(\text{Fe})_{\text{TOT}}$</th>
<th>$N$</th>
<th>$A_{450}$</th>
<th>$\alpha$</th>
<th>log(Fe)$_{\text{TOT}}$</th>
<th>log(Fe)$_{\text{TOT}}(1-\alpha)$</th>
<th>$\log K$ $^a$ M$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.44</td>
<td>0.075</td>
<td>0.78</td>
<td>-5.46</td>
<td>-6.00</td>
<td>10.42</td>
<td></td>
</tr>
<tr>
<td>5.66</td>
<td>0.109</td>
<td>0.73</td>
<td>-5.31</td>
<td>-5.74</td>
<td>10.42</td>
<td></td>
</tr>
<tr>
<td>8.68</td>
<td>0.148</td>
<td>0.76</td>
<td>-5.17</td>
<td>-5.66</td>
<td>10.66</td>
<td></td>
</tr>
<tr>
<td>13.29</td>
<td>0.221</td>
<td>0.75</td>
<td>-5.00</td>
<td>-5.48</td>
<td>11.08</td>
<td></td>
</tr>
</tbody>
</table>

Av: 10.7 ± 0.3

$^a$ using equation (3)
Table 3b: Titration of hemein with Triton X-100;  
10.0x10^-6 M hemein; 0,1M NaOH

<table>
<thead>
<tr>
<th>(detergent) µM</th>
<th>log (detergent) free</th>
<th>A-A₀</th>
<th>log K a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,67</td>
<td>0,563</td>
<td>-5,92 (-3,71)</td>
<td>-0,769</td>
</tr>
<tr>
<td>6,67</td>
<td>0,538</td>
<td>-5,37 (-3,26)</td>
<td>-0,509</td>
</tr>
<tr>
<td>11,96</td>
<td>0,553</td>
<td>-5,06 (-4,99)</td>
<td>-0,313</td>
</tr>
<tr>
<td>19,90</td>
<td>0,566</td>
<td>-4,81 (-4,75)</td>
<td>-0,079</td>
</tr>
<tr>
<td>31,75</td>
<td>0,538</td>
<td>-4,59 (-4,54)</td>
<td>0,176</td>
</tr>
<tr>
<td>51,33</td>
<td>0,527</td>
<td>-4,36 (-4,32)</td>
<td>0,603</td>
</tr>
<tr>
<td>70,33</td>
<td>0,520</td>
<td>-4,21 (-4,18)</td>
<td>1,106</td>
</tr>
</tbody>
</table>

Av: 9,45 (4,76)  
± 0,13 ± 0,18

a n = 2 (n = 1)  
b Calculated using equation (1)

A₀ = 0,571  
A₀ = 0,516

slope = (0,79) (Sd = 0,07)  
1,93 (Sd = 0,30)

intercept = (3,71) (Sd = 0,38)  
9,11 (Sd = 1,33)

* Plot of log \( \frac{A-A₀}{A₀-A} \) versus log (detergent) free
Table 3c: Titration of hemin with sodium lauryl sulphate;  
0.1M NaOH; 11.1x10⁻⁶M hemin

<table>
<thead>
<tr>
<th>(detergent), mM</th>
<th>A₃₈₄</th>
<th>log(detergent) free</th>
<th>A - Aₐ</th>
<th>log K a,b</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.629</td>
<td>-5.69</td>
<td>-0.68</td>
<td>5.01</td>
</tr>
<tr>
<td>6</td>
<td>0.623</td>
<td>-5.41</td>
<td>-0.21</td>
<td>5.20</td>
</tr>
<tr>
<td>9</td>
<td>0.621</td>
<td>-5.19</td>
<td>-0.09</td>
<td>5.10</td>
</tr>
<tr>
<td>12</td>
<td>0.619</td>
<td>-5.04</td>
<td>0.03</td>
<td>5.07</td>
</tr>
<tr>
<td>15</td>
<td>0.616</td>
<td>-4.94</td>
<td>0.21</td>
<td>5.15</td>
</tr>
<tr>
<td>18</td>
<td>0.613</td>
<td>-4.85</td>
<td>0.42</td>
<td>5.27</td>
</tr>
<tr>
<td>21</td>
<td>0.610</td>
<td>-4.79</td>
<td>0.50</td>
<td>5.29</td>
</tr>
<tr>
<td>23.9</td>
<td>0.610</td>
<td>-4.71</td>
<td>0.68</td>
<td>5.39</td>
</tr>
<tr>
<td>29.9</td>
<td>0.609</td>
<td>-4.60</td>
<td>0.80</td>
<td>5.40</td>
</tr>
<tr>
<td>41.8</td>
<td>0.609</td>
<td>-4.43</td>
<td>0.50</td>
<td>5.53</td>
</tr>
<tr>
<td>52.7</td>
<td>0.607</td>
<td>-4.31</td>
<td>1.13</td>
<td>5.44</td>
</tr>
<tr>
<td>65.5</td>
<td>0.606</td>
<td>-4.22</td>
<td>1.65</td>
<td>5.67</td>
</tr>
</tbody>
</table>

Av: (5.25) ± 0.2

A₀ = 0.634  A∞ = 0.605

n = 1 (det) 3 → 53.7

slope = 1.27  (Sd = 0.07)

intercept = 6.58  (Sd = 0.34)
APPENDIX 3: TABLES OF DATA FOR THE STUDY OF HEMIN IN AQUEOUS ACID (CHAPTER 4)

Table 1: Variation in absorbance at the Soret maximum (397 nm) at pH 1.1; 25°C; μ = 0.1

<table>
<thead>
<tr>
<th>10^5(hemin), M</th>
<th>A_397</th>
<th>α</th>
<th>log(Fe)_{TOT}</th>
<th>log(Fe)_{TOT}(1-α)</th>
<th>log K</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.71</td>
<td>0.085</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.10</td>
<td>0.136</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.19</td>
<td>0.141</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.27</td>
<td>0.148</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.07</td>
<td>0.252</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.45</td>
<td>0.270</td>
<td>0.767</td>
<td>-5.73</td>
<td>-6.24</td>
<td>-4.92</td>
</tr>
<tr>
<td>2.50</td>
<td>0.269</td>
<td>0.705</td>
<td>-5.75</td>
<td>-6.13</td>
<td>-5.07</td>
</tr>
<tr>
<td>4.31</td>
<td>0.649</td>
<td>0.523</td>
<td>-5.57</td>
<td>-5.79</td>
<td>-5.05</td>
</tr>
<tr>
<td>6.52</td>
<td>0.659</td>
<td>0.549</td>
<td>-5.45</td>
<td>-5.53</td>
<td>-5.07</td>
</tr>
<tr>
<td>7.84</td>
<td>0.776</td>
<td>0.500</td>
<td>-5.41</td>
<td>-5.41</td>
<td>-5.11</td>
</tr>
<tr>
<td>9.83</td>
<td>0.960</td>
<td>0.468</td>
<td>-5.34</td>
<td>-5.28</td>
<td>-5.10</td>
</tr>
<tr>
<td>14.36</td>
<td>1.135</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.03</td>
<td>1.343</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.80</td>
<td>1.677</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average: -5.05 ± 0.13

\( a \)

\[ \varepsilon_{D/2} = 78 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \]

\[ \varepsilon_H = 120 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \]

(from asymptotes of Beer's law plot)

\( b \)

K refers to the reaction \( D \leftrightarrow 2H \)
Table 2: Variation in the absorbance of the Soret as a function of pH for hemin with and without caffeine; 25°C; μ=0.1

<table>
<thead>
<tr>
<th>pH</th>
<th>Absorbance -caffeine (397 nm)</th>
<th>+ caffeine (402 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(2.15x10^-6 M hemin)</td>
<td>(3.09x10^-6 M hemin; 0.05M)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.514</td>
<td>0.915</td>
</tr>
<tr>
<td>1.5</td>
<td>0.514</td>
<td>0.915</td>
</tr>
<tr>
<td>2.0</td>
<td>0.514</td>
<td>0.909</td>
</tr>
<tr>
<td>2.2</td>
<td>0.510</td>
<td>0.905</td>
</tr>
<tr>
<td>2.4</td>
<td>0.480</td>
<td>-</td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
<td>0.865</td>
</tr>
<tr>
<td>2.6</td>
<td>0.467</td>
<td>-</td>
</tr>
<tr>
<td>2.8</td>
<td>0.443</td>
<td>0.832</td>
</tr>
<tr>
<td>3.0</td>
<td>0.427</td>
<td>0.805</td>
</tr>
<tr>
<td>3.2</td>
<td>0.404</td>
<td>-</td>
</tr>
<tr>
<td>3.4</td>
<td>-</td>
<td>0.770</td>
</tr>
<tr>
<td>3.8</td>
<td>0.381</td>
<td>0.725</td>
</tr>
<tr>
<td>4.0</td>
<td>0.380</td>
<td>0.713</td>
</tr>
<tr>
<td>4.5</td>
<td>0.380</td>
<td>0.698</td>
</tr>
<tr>
<td>5.0</td>
<td>0.380</td>
<td>0.698</td>
</tr>
<tr>
<td>5.2</td>
<td>-</td>
<td>0.673</td>
</tr>
<tr>
<td>5.4</td>
<td>-</td>
<td>0.650</td>
</tr>
<tr>
<td>5.6</td>
<td>0.382</td>
<td>0.616</td>
</tr>
<tr>
<td>5.8</td>
<td>-</td>
<td>0.593</td>
</tr>
<tr>
<td>6.0</td>
<td>0.335</td>
<td>0.564</td>
</tr>
<tr>
<td>6.2</td>
<td>0.313</td>
<td>-</td>
</tr>
<tr>
<td>6.4</td>
<td>0.300</td>
<td>0.540</td>
</tr>
<tr>
<td>6.6</td>
<td>0.288</td>
<td>-</td>
</tr>
<tr>
<td>6.8</td>
<td>0.264</td>
<td>-</td>
</tr>
<tr>
<td>7.0</td>
<td>0.245</td>
<td>0.530</td>
</tr>
<tr>
<td>7.8 (phosphate)</td>
<td>0.222</td>
<td>0.522</td>
</tr>
<tr>
<td>7.8 (carbonate)</td>
<td>0.216</td>
<td>0.511</td>
</tr>
<tr>
<td>9.0 (carbonate)</td>
<td>0.215</td>
<td>0.511</td>
</tr>
<tr>
<td>11.0 (carbonate)</td>
<td>0.218</td>
<td>0.510</td>
</tr>
</tbody>
</table>
APPENDIX 4 : TABLES OF DATA FOR THE TITRATION OF HEMIN WITH HISTIDINE, HISTAMINE AND PILOCARPINE

All titrations done at 25°C with μ = 0.5 (NaNO₃)

A. Titrations at low ligand concentrations

1. Histidine

a) pH 8.50; (hemin) = 68x10⁻⁶ M

<table>
<thead>
<tr>
<th>10⁻³ (L)₁₄ M</th>
<th>log(L)² (M)</th>
<th>A₅₉₀</th>
<th>log A - A₀</th>
<th>log K₁ (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.80</td>
<td>-3.10</td>
<td>0.277</td>
<td>-0.98</td>
<td>2.12</td>
</tr>
<tr>
<td>1.59</td>
<td>-2.80</td>
<td>0.277</td>
<td>-0.98</td>
<td>1.82</td>
</tr>
<tr>
<td>2.38</td>
<td>-2.62</td>
<td>0.278</td>
<td>-0.78</td>
<td>1.84</td>
</tr>
<tr>
<td>3.15</td>
<td>-2.40</td>
<td>0.280</td>
<td>-0.51</td>
<td>1.89</td>
</tr>
<tr>
<td>7.05</td>
<td>-2.15</td>
<td>0.282</td>
<td>-0.30</td>
<td>1.85</td>
</tr>
<tr>
<td>10.11</td>
<td>-2.00</td>
<td>0.284</td>
<td>-0.12</td>
<td>1.88</td>
</tr>
<tr>
<td>13.11</td>
<td>-1.88</td>
<td>0.285</td>
<td>-0.04</td>
<td>1.84</td>
</tr>
<tr>
<td>18.99</td>
<td>-1.72</td>
<td>0.288</td>
<td>0.21</td>
<td>1.93</td>
</tr>
<tr>
<td>24.70</td>
<td>-1.61</td>
<td>0.291</td>
<td>0.51</td>
<td>2.12</td>
</tr>
<tr>
<td>35.60</td>
<td>-1.45</td>
<td>0.292</td>
<td>0.63</td>
<td>2.08</td>
</tr>
</tbody>
</table>

Av: 2.0 ± 0.2

A₀ = 0.275  Aₘ = 0.296

Plot of log \( \frac{A - A₀}{Aₘ - A} \) vs log(his)

slope = 1.05  (Sd = 0.08)

intercept = 2.04  (Sd = 0.17)
b) pH 8.50; \((\text{hemin}) = 136 \times 10^{-6} \text{M}\)

<table>
<thead>
<tr>
<th>(10^3 (L)_T, \text{ M} )</th>
<th>( \log (L\text{ (M)})^b )</th>
<th>( A_{590} )</th>
<th>( \log \frac{A-A_0}{A_{\infty}-A} )</th>
<th>( \log K_\alpha^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.79</td>
<td>-3.10</td>
<td>0.547</td>
<td>-0.90</td>
<td>2.20</td>
</tr>
<tr>
<td>2.37</td>
<td>-2.63</td>
<td>0.550</td>
<td>-0.62</td>
<td>2.01</td>
</tr>
<tr>
<td>3.34</td>
<td>-2.40</td>
<td>0.551</td>
<td>-0.54</td>
<td>1.86</td>
</tr>
<tr>
<td>7.03</td>
<td>-2.15</td>
<td>0.557</td>
<td>-0.20</td>
<td>1.95</td>
</tr>
<tr>
<td>13.08</td>
<td>-1.88</td>
<td>0.563</td>
<td>0.10</td>
<td>1.98</td>
</tr>
<tr>
<td>18.94</td>
<td>-1.72</td>
<td>0.567</td>
<td>0.30</td>
<td>2.02</td>
</tr>
<tr>
<td>24.63</td>
<td>-1.61</td>
<td>0.571</td>
<td>0.54</td>
<td>2.15</td>
</tr>
<tr>
<td>30.15</td>
<td>-1.52</td>
<td>0.573</td>
<td>0.70</td>
<td>2.22</td>
</tr>
</tbody>
</table>

Av: 2.1 ± 0.2

\( A_0 = 0.543 \quad A_{\infty} = 0.579 \)

Plot of \( \log \frac{A-A_0}{A_{\infty}-A} \) vs \( \log (\text{hemin}) \):

- Slope = 1.03 (SD = 0.09)
- Intercept = 2.10 (SD = 0.21)
c) pH 10,0; (hemin) = 136x10⁻⁶ M

<table>
<thead>
<tr>
<th>10⁻M</th>
<th>log(L)</th>
<th>b(µ)</th>
<th>h₅₉₀</th>
<th>log $\frac{A - A_0}{A_0 - A}$</th>
<th>log $K_1$ (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,79</td>
<td>-3,10</td>
<td>0,580</td>
<td>-1,20</td>
<td>1,90</td>
<td></td>
</tr>
<tr>
<td>2,7</td>
<td>-2,63</td>
<td>0,582</td>
<td>-0,57</td>
<td>1,96</td>
<td></td>
</tr>
<tr>
<td>3,94</td>
<td>-2,40</td>
<td>0,583</td>
<td>-0,51</td>
<td>1,89</td>
<td></td>
</tr>
<tr>
<td>7,03</td>
<td>-2,25</td>
<td>0,585</td>
<td>-0,26</td>
<td>1,89</td>
<td></td>
</tr>
<tr>
<td>13,08</td>
<td>-1,88</td>
<td>0,587</td>
<td>-0,05</td>
<td>1,83</td>
<td></td>
</tr>
<tr>
<td>18,94</td>
<td>-1,72</td>
<td>0,589</td>
<td>0,15</td>
<td>1,87</td>
<td></td>
</tr>
<tr>
<td>24,63</td>
<td>-1,61</td>
<td>0,590</td>
<td>0,26</td>
<td>1,87</td>
<td></td>
</tr>
</tbody>
</table>

Av: 1,9 ± 0,1

$A_c = 0,579$  $A = 0,596$

Plot of log $\frac{A - A_0}{A_0 - A}$ vs log(his)

slope = 0,95  (Sd = 0,03)

intercept = 1,78  (Sd = 0,06)
d) pH 11,0 ; [hemin] = 136x10^-6 M

<table>
<thead>
<tr>
<th>$10^3$(L)_T, M</th>
<th>log (L)</th>
<th>$A_{590}$</th>
<th>$\log \frac{A - A_o}{A_o - A}$</th>
<th>$\log K_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.79</td>
<td>-3.10</td>
<td>0.578</td>
<td>-1.34</td>
<td>1.76</td>
</tr>
<tr>
<td>2.37</td>
<td>-2.63</td>
<td>0.580</td>
<td>-0.82</td>
<td>1.81</td>
</tr>
<tr>
<td>3.94</td>
<td>-2.40</td>
<td>0.582</td>
<td>-0.56</td>
<td>1.84</td>
</tr>
<tr>
<td>7.03</td>
<td>-2.15</td>
<td>0.584</td>
<td>-0.36</td>
<td>1.79</td>
</tr>
<tr>
<td>13.08</td>
<td>-1.88</td>
<td>0.587</td>
<td>-0.11</td>
<td>1.77</td>
</tr>
<tr>
<td>18.94</td>
<td>-1.72</td>
<td>0.589</td>
<td>0.04</td>
<td>1.76</td>
</tr>
<tr>
<td>24.63</td>
<td>-1.61</td>
<td>0.591</td>
<td>0.19</td>
<td>1.80</td>
</tr>
<tr>
<td>30.15</td>
<td>-1.52</td>
<td>0.593</td>
<td>0.36</td>
<td>1.88</td>
</tr>
<tr>
<td>40.71</td>
<td>-1.39</td>
<td>0.595</td>
<td>0.56</td>
<td>1.95</td>
</tr>
</tbody>
</table>

Av: 1.8 ± 0.1

$A_o = 0.577$ ; $A_\infty = 0.600$

Plot of $\log \frac{A - A_o}{A_\infty - A}$ vs $\log$(his)

slope = 1.06 ($Sd = 0.04$)

intercept = 1.93 ($Sd = 0.08$)
2. Histamine

a) pH 8.50 ; (hemin) = 90.5x10^{-5}M

<table>
<thead>
<tr>
<th>10^{-3}(L)_T, M</th>
<th>log(L) b, (M)</th>
<th>A_{590}</th>
<th>log(A-A_o)/A_o</th>
<th>log K, (1/M) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>-3.00</td>
<td>0.402</td>
<td>-0.37</td>
<td>2.63</td>
</tr>
<tr>
<td>1.99</td>
<td>-2.70</td>
<td>0.410</td>
<td>0.00</td>
<td>2.70</td>
</tr>
<tr>
<td>2.98</td>
<td>-2.53</td>
<td>0.413</td>
<td>0.13</td>
<td>2.66</td>
</tr>
<tr>
<td>3.96</td>
<td>-2.40</td>
<td>0.415</td>
<td>0.22</td>
<td>2.52</td>
</tr>
<tr>
<td>5.91</td>
<td>-2.23</td>
<td>0.417</td>
<td>0.32</td>
<td>2.55</td>
</tr>
<tr>
<td>7.84</td>
<td>-2.11</td>
<td>0.419</td>
<td>0.45</td>
<td>2.56</td>
</tr>
<tr>
<td>11.65</td>
<td>-1.93</td>
<td>0.422</td>
<td>0.60</td>
<td>2.53</td>
</tr>
<tr>
<td>15.38</td>
<td>-1.81</td>
<td>0.424</td>
<td>0.75</td>
<td>2.56</td>
</tr>
<tr>
<td>22.63</td>
<td>-1.65</td>
<td>0.426</td>
<td>0.95</td>
<td>2.60</td>
</tr>
</tbody>
</table>

\( A_o = 0.390 \quad A_w = 0.430 \)

Plot of \( \log \frac{A-A_o}{A_o-A} \) vs \( \log(L) \):

Slope = 0.91 (SD = 0.04)

Intercept = 2.40 (SD = 0.08)
b) pH 10,0 ; (hemin) = 90,5x10⁻⁶ M

<table>
<thead>
<tr>
<th>10³ (L)</th>
<th>M</th>
<th>log(L)</th>
<th>A₅₉₀</th>
<th>log (A₀ - A) / A₀</th>
<th>log Kₐ (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,00</td>
<td>-3,00</td>
<td>0,419</td>
<td>-0,36</td>
<td>2,65</td>
<td></td>
</tr>
<tr>
<td>1,99</td>
<td>-2,70</td>
<td>0,423</td>
<td>-0,03</td>
<td>2,67</td>
<td></td>
</tr>
<tr>
<td>3,96</td>
<td>-2,60</td>
<td>0,427</td>
<td>0,23</td>
<td>2,63</td>
<td></td>
</tr>
<tr>
<td>5,91</td>
<td>-2,23</td>
<td>0,431</td>
<td>0,54</td>
<td>2,77</td>
<td></td>
</tr>
<tr>
<td>7,84</td>
<td>-2,11</td>
<td>0,432</td>
<td>0,64</td>
<td>2,75</td>
<td></td>
</tr>
<tr>
<td>9,76</td>
<td>-2,01</td>
<td>0,433</td>
<td>0,75</td>
<td>2,77</td>
<td></td>
</tr>
<tr>
<td>11,65</td>
<td>-1,93</td>
<td>0,434</td>
<td>0,90</td>
<td>2,83</td>
<td></td>
</tr>
</tbody>
</table>

Av: 2,7 ± 0,1

A₀ = 0,410 Aₐ = 0,437

Plot of \(\frac{A₀ - A}{A₀ - Aₐ}\) vs log(L):

- slope = 1,16 (Sd = 0,05)
- intercept = 3,11 (Sd = 0,11)
c) pH 11,0 ; \( \text{[hemin]} = 90,3 \times 10^{-6} \text{M} \)

| \(10^3 (L)_{11} \text{M} \) | \( \log (L) \text{b} \langle M \rangle \) | \( A_{590} \) | \( \log \frac{A - A_0}{A_m - A} \) | \( \log K_c \text{M}^{-1} \) \\
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1,00</td>
<td>-3,00</td>
<td>0,416</td>
<td>-0,54</td>
<td>2,46</td>
</tr>
<tr>
<td>1,99</td>
<td>-2,70</td>
<td>0,419</td>
<td>-0,30</td>
<td>2,40</td>
</tr>
<tr>
<td>3,96</td>
<td>-2,40</td>
<td>0,423</td>
<td>-0,03</td>
<td>2,40</td>
</tr>
<tr>
<td>5,91</td>
<td>-2,23</td>
<td>0,425</td>
<td>0,10</td>
<td>2,33</td>
</tr>
<tr>
<td>7,84</td>
<td>-2,11</td>
<td>0,427</td>
<td>0,23</td>
<td>2,34</td>
</tr>
<tr>
<td>10,85</td>
<td>-1,93</td>
<td>0,429</td>
<td>0,23</td>
<td>2,26</td>
</tr>
<tr>
<td>15,38</td>
<td>-1,81</td>
<td>0,430</td>
<td>0,46</td>
<td>2,20</td>
</tr>
<tr>
<td>22,64</td>
<td>-1,63</td>
<td>0,432</td>
<td>0,64</td>
<td>2,29</td>
</tr>
</tbody>
</table>

\[ \text{AV: } 2,4 \pm 0,1 \]

\[ A_0 = 0,410 \quad A_m = 0,437 \]

Plot of \( \log \frac{A - A_0}{A_m - A} \) vs \( \log (L) \):

- slope = 0,86 (8d = 0,02)
- intercept = 2,02 (8d = 0,04)
3. **Pilocarpate**

a) pH 8.50 ; (hemin) = 90.5 x 10^{-6} g

<table>
<thead>
<tr>
<th>$10^3 (L)_T$, N</th>
<th>$\text{log} (L)$ (M) b</th>
<th>$A_{590}$</th>
<th>$\text{log} \frac{A-A_0}{A_m-A}$</th>
<th>$\text{log} K_2 (M^{-1})$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>-3.30</td>
<td>0.400</td>
<td>-1.10</td>
<td>2.20</td>
</tr>
<tr>
<td>1.00</td>
<td>-3.00</td>
<td>0.404</td>
<td>-0.76</td>
<td>2.24</td>
</tr>
<tr>
<td>1.98</td>
<td>-2.70</td>
<td>0.410</td>
<td>-0.46</td>
<td>2.24</td>
</tr>
<tr>
<td>2.56</td>
<td>-2.53</td>
<td>0.414</td>
<td>-0.30</td>
<td>2.23</td>
</tr>
<tr>
<td>3.92</td>
<td>-2.41</td>
<td>0.418</td>
<td>-0.16</td>
<td>2.25</td>
</tr>
</tbody>
</table>

**Av:** 2.23 ± 0.03

$A_0 = 0.396 \quad A_m = 0.450$

Plot of $\text{log} \frac{A-A_0}{A_m-A}$ vs $\text{log} (L)$:

- **slope** = 1.04 \ ($S_d = 0.02$)
- **intercept** = 2.35 \ ($S_d = 0.05$)
b) pH 10.0 ; \( \text{hemin} = 90.5 \times 10^{-6} \) M

<table>
<thead>
<tr>
<th>( 10^3(L) )_N</th>
<th>N</th>
<th>log(L) (N)</th>
<th>( A_{590} )</th>
<th>( \log \frac{A - A_0}{A_0 - A} )</th>
<th>( \log K_1 ) (M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>-3.30</td>
<td>0.412</td>
<td>-1.46</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
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<tr>
<td>7.69</td>
<td>-2.11</td>
<td>0.432</td>
<td>-0.24</td>
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<tr>
<td>9.52</td>
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<td>0.434</td>
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</tr>
<tr>
<td>13.08</td>
<td>-1.88</td>
<td>0.438</td>
<td>-0.06</td>
<td>1.82</td>
<td></td>
</tr>
<tr>
<td>16.51</td>
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<td>0.440</td>
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<td>1.78</td>
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<tr>
<td>19.82</td>
<td>-1.70</td>
<td>0.442</td>
<td>0.04</td>
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<td>-1.64</td>
<td>0.443</td>
<td>0.09</td>
<td>1.73</td>
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</tbody>
</table>

\[ A_0 = 0.10 \quad A_\infty = 0.470 \]

Plot of \( \log \frac{A - A_0}{A_0 - A} \) vs log(L):

- Slope = 0.94 \( (sd = 0.02) \)
- Intercept = 1.68 \( (sd = 0.05) \)
c) pH 11.0; (hemin) = 90.5x10^{-16} M

<table>
<thead>
<tr>
<th>10^3(L)_T, M</th>
<th>log(L) (M)</th>
<th>A_590</th>
<th>log A - A_0</th>
<th>log K_1 (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>-3.30</td>
<td>0.428</td>
<td>-1.39</td>
<td>1.91</td>
</tr>
<tr>
<td>1.00</td>
<td>-3.00</td>
<td>0.430</td>
<td>-1.07</td>
<td>1.93</td>
</tr>
<tr>
<td>1.93</td>
<td>-2.70</td>
<td>0.422</td>
<td>-0.82</td>
<td>1.88</td>
</tr>
<tr>
<td>3.00</td>
<td>-2.41</td>
<td>0.436</td>
<td>-0.51</td>
<td>1.90</td>
</tr>
<tr>
<td>5.00</td>
<td>-2.23</td>
<td>0.439</td>
<td>-0.34</td>
<td>1.89</td>
</tr>
<tr>
<td>7.69</td>
<td>-2.11</td>
<td>0.441</td>
<td>-0.23</td>
<td>1.88</td>
</tr>
<tr>
<td>11.32</td>
<td>-1.95</td>
<td>0.446</td>
<td>0.00</td>
<td>1.95</td>
</tr>
<tr>
<td>14.81</td>
<td>-1.83</td>
<td>0.449</td>
<td>0.14</td>
<td>1.97</td>
</tr>
<tr>
<td>18.16</td>
<td>-1.75</td>
<td>0.451</td>
<td>0.23</td>
<td>1.98</td>
</tr>
</tbody>
</table>

\[ A_0 = 0.427 \quad A_\infty = 0.465 \]

Plot of \( \log \frac{A - A_0}{A_\infty - A} \) vs \( \log(L) \) :

- slope = 1.03 (Sd = 0.02)
- intercept = 2.00 (Sd = 0.06)

\[ a \quad K_1 \text{ refers to the reaction } D + XL \rightarrow D \cdots L_x \]

\[ b \quad \log(L)_{\text{TOTAL}} \neq \log(L)_{\text{free}} \text{ so the former is used.} \]
B. **Titration at high ligand concentrations**

i) **Varying the ligand concentration**

1. Histidine; pH 9.0 (two duplicate experiments)

   a) \((\text{Fe})_{\text{TOT}} = 7.03 \times 10^{-6} \text{M}\)  
   b) \((\text{Fe})_{\text{TOT}} = 5.34 \times 10^{-6} \text{M}\)

<table>
<thead>
<tr>
<th>((L)_{\text{T}}, M)</th>
<th>(A_{410})</th>
<th>(\log(L)_{\text{T}}, M)</th>
<th>(10^{6}(D), M)</th>
<th>(10^{6}(\text{ML}_{2}), M)</th>
<th>(\frac{(\text{ML}_{2})^{2}}{(D)})</th>
<th>(\log K_{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) 0.001</td>
<td>0.246</td>
<td>-2.00</td>
<td>3.292</td>
<td>0.446</td>
<td>-7.22</td>
<td>-1.22</td>
</tr>
<tr>
<td>0.02</td>
<td>0.302</td>
<td>-1.70</td>
<td>2.963</td>
<td>1.103</td>
<td>-6.39</td>
<td>-1.29</td>
</tr>
<tr>
<td>0.03</td>
<td>0.369</td>
<td>-1.52</td>
<td>2.570</td>
<td>1.890</td>
<td>-5.86</td>
<td>-1.30</td>
</tr>
<tr>
<td>0.04</td>
<td>0.452</td>
<td>-1.40</td>
<td>2.083</td>
<td>2.864</td>
<td>-5.40</td>
<td>-1.20</td>
</tr>
<tr>
<td>0.06</td>
<td>0.596</td>
<td>-1.22</td>
<td>1.338</td>
<td>4.554</td>
<td>-4.78</td>
<td>-1.12</td>
</tr>
<tr>
<td>0.08</td>
<td>0.669</td>
<td>-1.10</td>
<td>0.810</td>
<td>5.410</td>
<td>-4.14</td>
<td>-1.14</td>
</tr>
<tr>
<td>0.10</td>
<td>0.795</td>
<td>-1.00</td>
<td>0.455</td>
<td>6.115</td>
<td>-4.09</td>
<td>-1.09</td>
</tr>
<tr>
<td>0.15</td>
<td>0.915</td>
<td>-0.72</td>
<td>0.182</td>
<td>6.864</td>
<td>-3.61</td>
<td>-1.15</td>
</tr>
</tbody>
</table>

\(A_{0} = 0.206\)  
\(A_{1} = 0.907\) (Plot of \(\frac{(\text{ML}_{2})^{2}}{(D)}\) vs \(\log(L)\))

\(n = 3.14\) (Sd = 0.06)  
\(I = -1.90\) (Sd = 0.08)

\(A_{413}\)

| b) 0.02        | 0.246   | -1.70           | 2.567           | 1.006           | -6.42          | -1.32          |
| 0.04           | 0.409   | -1.40           | 1.800           | 2.740           | -5.38          | -1.18          |
| 0.06           | 0.516   | -1.22           | 1.152           | 4.037           | -4.85          | -1.19          |
| 0.08           | 0.606   | -1.10           | 0.594           | 5.152           | -4.35          | -1.05          |
| 0.10           | 0.634   | -1.00           | 0.436           | 5.467           | -4.16          | -1.16          |
| 0.20           | 0.692   | -0.70           | 0.085           | 6.170           | -3.33          | -1.25          |

\(A_{1} = -1.18 \pm 0.14\)
\[ A_0 = 0.133 \quad A_w = 0.706 \ (\text{Plot of } \log \frac{(ML_2)^2}{(D)} \ vs \ \log(L)) \]

\[ n = 3.09 \ (Sd = 0.12) \]

\[ I = -1.08 \ (Sd = 0.15) \]

Overall average of both experiments: \(-1.2 \pm 0.1\)

2. Histamine; pH 9.0 \( [(Fe)]_{TOT} = 7.16 \times 10^{-6} M \)

| \((L)_T\) | \(M\) | \(A_{408,5nm} \) | \(\log(L)_T\) | \(10^6(D)\) | \(M \times 10^5 ML_2.M \) | \(\log \left( \frac{(ML_2)^2}{(D)} \right) \) | \(\log(ML_2) \)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>0.257</td>
<td>-2.00</td>
<td>3.399</td>
<td>0.362</td>
<td>-7.41</td>
<td>-1.41</td>
<td>0.3</td>
</tr>
<tr>
<td>0.02</td>
<td>0.277</td>
<td>-1.70</td>
<td>3.274</td>
<td>0.611</td>
<td>-6.94</td>
<td>-1.84</td>
<td>0.3</td>
</tr>
<tr>
<td>0.03</td>
<td>0.301</td>
<td>-1.52</td>
<td>3.125</td>
<td>0.911</td>
<td>-6.58</td>
<td>-2.02</td>
<td>0.3</td>
</tr>
<tr>
<td>0.04</td>
<td>0.330</td>
<td>-1.40</td>
<td>2.944</td>
<td>1.272</td>
<td>-6.26</td>
<td>-2.06</td>
<td>0.3</td>
</tr>
<tr>
<td>0.06</td>
<td>0.382</td>
<td>-1.22</td>
<td>2.620</td>
<td>1.921</td>
<td>-5.85</td>
<td>-2.19</td>
<td>0.3</td>
</tr>
<tr>
<td>0.08</td>
<td>0.448</td>
<td>-1.10</td>
<td>2.208</td>
<td>2.744</td>
<td>-5.47</td>
<td>-2.17</td>
<td>0.3</td>
</tr>
<tr>
<td>0.10</td>
<td>0.526</td>
<td>-1.00</td>
<td>1.721</td>
<td>3.717</td>
<td>-5.10</td>
<td>-2.10</td>
<td>0.3</td>
</tr>
<tr>
<td>0.15</td>
<td>0.586</td>
<td>-0.82</td>
<td>1.360</td>
<td>4.441</td>
<td>-4.84</td>
<td>-2.38</td>
<td>0.3</td>
</tr>
<tr>
<td>0.20</td>
<td>0.651</td>
<td>-0.70</td>
<td>0.942</td>
<td>5.278</td>
<td>-4.53</td>
<td>-2.43</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Av: \(-2.11 \pm 0.3\)

\[ A_0 = 0.228 \quad A_w = 0.802 \ (\text{Plot of } \log \frac{(ML_2)^2}{(D)} \ vs \ \log(L)) \]

\[ n = 2.32 \ (Sd = 0.09) \]

\[ I = -2.93 \ (Sd = 0.12) \]
3. Pilocarpate; pH 9.3 \[ [\text{Fe}]_{30} = 7.25 \times 10^{-6} M \]

<table>
<thead>
<tr>
<th>(10^2 (\text{L})_N)</th>
<th>(A_{410})</th>
<th>(\log(\text{L})_N)</th>
<th>(10^6(\text{D})_N)</th>
<th>(10^6(\text{M}_2)_N)</th>
<th>(\frac{(\text{M}_2)^2}{\text{D}})</th>
<th>(\log K_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.40</td>
<td>0.258</td>
<td>-2.62</td>
<td>3,358</td>
<td>0.534</td>
<td>-7.07</td>
<td>0.79</td>
</tr>
<tr>
<td>4.79</td>
<td>0.334</td>
<td>-2.32</td>
<td>2,096</td>
<td>1,457</td>
<td>-6.13</td>
<td>0.83</td>
</tr>
<tr>
<td>7.17</td>
<td>0.507</td>
<td>-2.14</td>
<td>1,846</td>
<td>3,558</td>
<td>-5.16</td>
<td>1.26</td>
</tr>
<tr>
<td>11.53</td>
<td>0.632</td>
<td>-1.92</td>
<td>1,087</td>
<td>5,076</td>
<td>-4.63</td>
<td>1.13</td>
</tr>
<tr>
<td>16.66</td>
<td>0.699</td>
<td>-1.78</td>
<td>0,680</td>
<td>5,890</td>
<td>-4.29</td>
<td>1.05</td>
</tr>
<tr>
<td>21.37</td>
<td>0.734</td>
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<td>0,680</td>
<td>6,291</td>
<td>-4.08</td>
<td>0.93</td>
</tr>
<tr>
<td>26.06</td>
<td>0.754</td>
<td>-1.58</td>
<td>0,346</td>
<td>6,558</td>
<td>-3.91</td>
<td>0.83</td>
</tr>
<tr>
<td>30.72</td>
<td>0.770</td>
<td>-1.51</td>
<td>0,249</td>
<td>6,752</td>
<td>-3.74</td>
<td>0.79</td>
</tr>
<tr>
<td>35.36</td>
<td>0.785</td>
<td>-1.45</td>
<td>0,158</td>
<td>6,934</td>
<td>-3.52</td>
<td>0.83</td>
</tr>
</tbody>
</table>

\(A_0 = 0.214, A_\infty = 0.811\) (Plot of \(\frac{(\text{M}_2)^2}{\text{D}}\) vs \(\log(\text{L})\))

\(n = 2.95, (Sd = 0.16)\)

\(I = 0.85, (Sd = 0.31)\)
b) *Pilocarpine*: pH 10,2  \( [\text{Ca}^2+]_{\text{TOT}} = 7.46 \times 10^{-6} \text{M} \)

<table>
<thead>
<tr>
<th>(10^3(L)_T), (\text{M})</th>
<th>(A_{410})</th>
<th>(\log(L)_T), (\text{m})</th>
<th>(10^6(D)), (\text{M})</th>
<th>(10^6(ML)_2), (\text{M})</th>
<th>(\log \left( \frac{(ML)_2}{D} \right))</th>
<th>(\log K_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7,87</td>
<td>0,272</td>
<td>-2,10</td>
<td>3,436</td>
<td>0,587</td>
<td>-6,99</td>
<td>-0,69</td>
</tr>
<tr>
<td>15,94</td>
<td>0,346</td>
<td>-1,80</td>
<td>2,984</td>
<td>1,492</td>
<td>-6,13</td>
<td>-0,73</td>
</tr>
<tr>
<td>23,72</td>
<td>0,432</td>
<td>-1,62</td>
<td>2,458</td>
<td>2,544</td>
<td>-5,58</td>
<td>-0,72</td>
</tr>
<tr>
<td>31,50</td>
<td>0,518</td>
<td>-1,50</td>
<td>1,944</td>
<td>3,571</td>
<td>-5,18</td>
<td>-0,68</td>
</tr>
<tr>
<td>39,22</td>
<td>0,577</td>
<td>-1,41</td>
<td>1,571</td>
<td>4,317</td>
<td>-4,93</td>
<td>-0,70</td>
</tr>
<tr>
<td>46,88</td>
<td>0,624</td>
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<td>1,284</td>
<td>4,892</td>
<td>-4,73</td>
<td>-0,74</td>
</tr>
</tbody>
</table>

\[ Av: \quad -0,71 \pm 0,03 \]

\[ A_0 = 0,224 \quad A_m = 0,834 \]

Plot of \( \log \left( \frac{(ML)_2}{D} \right) \) vs \( \log(L) \)

\[ n = 2,98 \quad (Sd = 0,04) \]

\[ t = -0,75 \quad (Sd = 0,07) \]
(ii) Varying the hemin concentration (Shack and Clark dilution plots)

1. Histidine

used $E_{D, \text{Hem}} = 28.4$ ; $E_{L} = 10.5$ (both in cm$^{-1}$ cm$^{-1}$)

2. pH 8.5 ; (histidine)$_{\text{TOTAL}} = 0.05 M$

<table>
<thead>
<tr>
<th>$10^6$ Fe$_{\text{TOTAL}}$</th>
<th>$A_{413}$</th>
<th>$a$</th>
<th>log(Fe)$_{\text{TOT}}$</th>
<th>log(Fe)$_{\text{TOT}}(1-a)$</th>
<th>log $K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.37</td>
<td>0.290</td>
<td>0.824</td>
<td>-5.61</td>
<td>-6.28</td>
<td>-0.74</td>
</tr>
<tr>
<td>3.34</td>
<td>0.325</td>
<td>0.717</td>
<td>-5.37</td>
<td>-5.77</td>
<td>-0.77</td>
</tr>
<tr>
<td>3.91</td>
<td>0.729</td>
<td>0.641</td>
<td>-5.24</td>
<td>-5.49</td>
<td>-0.79</td>
</tr>
<tr>
<td>4.87</td>
<td>0.914</td>
<td>0.585</td>
<td>-5.16</td>
<td>-5.31</td>
<td>-0.81</td>
</tr>
<tr>
<td>5.83</td>
<td>1.030</td>
<td>0.537</td>
<td>-5.10</td>
<td>-5.16</td>
<td>-0.84</td>
</tr>
<tr>
<td>6.85</td>
<td>1.226</td>
<td>0.492</td>
<td>-5.06</td>
<td>-5.04</td>
<td>-0.88</td>
</tr>
<tr>
<td>7.78</td>
<td>1.366</td>
<td>0.456</td>
<td>-5.02</td>
<td>-4.95</td>
<td>-0.89</td>
</tr>
<tr>
<td>8.70</td>
<td>1.507</td>
<td>0.430</td>
<td>-4.99</td>
<td>-4.87</td>
<td>-0.91</td>
</tr>
</tbody>
</table>

$\text{Av: } -0.82 \pm 0.09$

Plot of log (Fe)$_{\text{TOT}}$ vs log (Fe)$_{\text{TOT}}(1-a)$

$n = 0.44 \quad (SD = 0.01)$

$I = 2.24 \quad (SD = 0.05)$
b) pH 9.0; \((\text{histidine})_{\text{TOTAL}} = 0.05\) M

<table>
<thead>
<tr>
<th>(10^6 (\text{Fe})_{\text{TOTAL}}) M</th>
<th>(A_{413})</th>
<th>(\chi)</th>
<th>(\log(\text{Fe})_{\text{TOTAL}})</th>
<th>(\log(\text{Fe})_{\text{TOTAL}} (1-\alpha))</th>
<th>(\log K_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.56</td>
<td>0.237</td>
<td>0.765</td>
<td>-5.71</td>
<td>-6.22</td>
<td>-1.00</td>
</tr>
<tr>
<td>5.12</td>
<td>0.421</td>
<td>0.645</td>
<td>-5.48</td>
<td>-5.74</td>
<td>-1.02</td>
</tr>
<tr>
<td>7.67</td>
<td>0.575</td>
<td>0.361</td>
<td>-5.37</td>
<td>-5.47</td>
<td>-1.07</td>
</tr>
<tr>
<td>10.22</td>
<td>0.725</td>
<td>0.515</td>
<td>-5.28</td>
<td>-5.30</td>
<td>-1.06</td>
</tr>
<tr>
<td>12.76</td>
<td>0.860</td>
<td>0.474</td>
<td>-5.22</td>
<td>-5.17</td>
<td>-1.07</td>
</tr>
<tr>
<td>15.30</td>
<td>0.982</td>
<td>0.437</td>
<td>-5.18</td>
<td>-5.06</td>
<td>-1.10</td>
</tr>
<tr>
<td>20.37</td>
<td>1.206</td>
<td>0.375</td>
<td>-5.11</td>
<td>-4.90</td>
<td>-1.12</td>
</tr>
</tbody>
</table>

Av: \(-1.06 \pm 0.05\)

Plot of \(\log(\text{Fe})_{\text{TOTAL}}\) vs \(\log(\text{Fe})_{\text{TOT}} (1-\alpha)\)
\(n = 0.46\) (Sd = 0.01)
\(r = -2.87\) (Sd = 0.03)
c) pH 10.0; (histidine)$_{\text{TOTAL}} = 0.1 \text{ M}$

<table>
<thead>
<tr>
<th>$\log^6(\text{Fe})_{\text{TOTAL},N}$</th>
<th>$A_{413}$</th>
<th>$\alpha$</th>
<th>$\log(\text{Fe})_{\text{TOT}}$</th>
<th>$\log(\text{Fe})_{\text{TOT}(1-\alpha)}$</th>
<th>$\log K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.60</td>
<td>0.169</td>
<td>0.916</td>
<td>-5.33</td>
<td>-6.87</td>
<td>-1.49</td>
</tr>
<tr>
<td>3.21</td>
<td>0.221</td>
<td>0.881</td>
<td>-5.56</td>
<td>-6.32</td>
<td>-1.50</td>
</tr>
<tr>
<td>4.81</td>
<td>0.467</td>
<td>0.817</td>
<td>-5.41</td>
<td>-6.06</td>
<td>-1.46</td>
</tr>
<tr>
<td>6.40</td>
<td>0.612</td>
<td>0.800</td>
<td>-5.39</td>
<td>-5.89</td>
<td>-1.39</td>
</tr>
<tr>
<td>8.00</td>
<td>0.746</td>
<td>0.773</td>
<td>-5.24</td>
<td>-5.74</td>
<td>-1.38</td>
</tr>
<tr>
<td>9.59</td>
<td>0.873</td>
<td>0.747</td>
<td>-5.11</td>
<td>-5.62</td>
<td>-1.36</td>
</tr>
<tr>
<td>11.19</td>
<td>0.991</td>
<td>0.713</td>
<td>-5.03</td>
<td>-5.50</td>
<td>-1.35</td>
</tr>
<tr>
<td>12.78</td>
<td>1.101</td>
<td>0.697</td>
<td>-5.05</td>
<td>-5.41</td>
<td>-1.39</td>
</tr>
<tr>
<td>14.36</td>
<td>1.222</td>
<td>0.677</td>
<td>-5.01</td>
<td>-5.33</td>
<td>-1.39</td>
</tr>
<tr>
<td>15.97</td>
<td>1.333</td>
<td>0.660</td>
<td>-4.98</td>
<td>-5.26</td>
<td>-1.00</td>
</tr>
</tbody>
</table>

Av: $-1.41 \pm 0.05$

Plot $- \log^6(\text{Fe})_{\text{TOT}} - \alpha$ vs $\log(\text{Fe})_{\text{TOT}(1-\alpha)}$

$n = 0.54$ (Sd = 0.01)

$I = -2.12$ (Sd = 0.06)
d) pH 10,0; (Histidine)$_{\text{TOTAL}}$ = 0.1M

<table>
<thead>
<tr>
<th>$10^6$(Fe)$_{\text{TOTAL}}$ M</th>
<th>$A_{413}$</th>
<th>log(Fe)$_{\text{TOT}}$</th>
<th>log(Fe)$_{\text{TOT}}$(1-a)</th>
<th>logK$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.60</td>
<td>0.175</td>
<td>-5.81</td>
<td>-7.19</td>
<td>-1.13</td>
</tr>
<tr>
<td>3.21</td>
<td>0.328</td>
<td>-5.53</td>
<td>-6.40</td>
<td>-1.40</td>
</tr>
<tr>
<td>4.81</td>
<td>0.468</td>
<td>-5.40</td>
<td>-6.06</td>
<td>-1.44</td>
</tr>
<tr>
<td>6.40</td>
<td>0.605</td>
<td>-5.30</td>
<td>-5.87</td>
<td>-1.43</td>
</tr>
<tr>
<td>8.00</td>
<td>0.729</td>
<td>-5.22</td>
<td>-5.70</td>
<td>-1.44</td>
</tr>
<tr>
<td>9.59</td>
<td>0.846</td>
<td>-5.16</td>
<td>-5.56</td>
<td>-1.46</td>
</tr>
<tr>
<td>11.19</td>
<td>0.965</td>
<td>-5.11</td>
<td>-5.46</td>
<td>-1.46</td>
</tr>
<tr>
<td>12.78</td>
<td>1.076</td>
<td>-5.07</td>
<td>-5.37</td>
<td>-1.47</td>
</tr>
<tr>
<td>14.35</td>
<td>1.185</td>
<td>-5.03</td>
<td>-5.30</td>
<td>-1.46</td>
</tr>
<tr>
<td>15.97</td>
<td>1.291</td>
<td>-5.00</td>
<td>-5.23</td>
<td>-1.47</td>
</tr>
</tbody>
</table>

Av: -1.45 ± 0.05

Plot of log(Fe)$_{\text{TOT}}$ vs log(Fe)$_{\text{TOT}}$(1-a)

\[ n = 0.47 \ (Sd = 0.004) \]

\[ I = -2.53 \ (Sd = 0.02) \]

Av log K$_2$ for pH 10.0 = 1.43 ± 0.07
e) pH 11.0; (histidine)$_{\text{TOTAL}}$ = 0.05M

<table>
<thead>
<tr>
<th>$10^6$(Fe)$_{\text{TOTAL},M}$</th>
<th>$A_{413}$</th>
<th>$\alpha$</th>
<th>$\log$(Fe)$_{\text{TOT}} \alpha$</th>
<th>$\log$(Fe)$_{\text{TOT}}(1-\alpha)$</th>
<th>$\log K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.52</td>
<td>0.103</td>
<td>0.167</td>
<td>-6.38</td>
<td>-5.68</td>
<td>-2.88</td>
</tr>
<tr>
<td>3.78</td>
<td>0.149</td>
<td>0.150</td>
<td>-6.24</td>
<td>-5.49</td>
<td>-2.79</td>
</tr>
<tr>
<td>5.03</td>
<td>0.203</td>
<td>0.161</td>
<td>-6.09</td>
<td>-5.37</td>
<td>-2.61</td>
</tr>
<tr>
<td>7.53</td>
<td>0.284</td>
<td>0.131</td>
<td>-6.01</td>
<td>-5.18</td>
<td>-2.64</td>
</tr>
<tr>
<td>10.04</td>
<td>0.361</td>
<td>0.110</td>
<td>-5.95</td>
<td>-5.05</td>
<td>-2.65</td>
</tr>
<tr>
<td>12.53</td>
<td>0.431</td>
<td>0.094</td>
<td>-5.94</td>
<td>-4.94</td>
<td>-2.74</td>
</tr>
<tr>
<td>17.52</td>
<td>0.578</td>
<td>0.076</td>
<td>-5.87</td>
<td>-4.79</td>
<td>-2.74</td>
</tr>
<tr>
<td>22.49</td>
<td>0.718</td>
<td>0.064</td>
<td>-5.84</td>
<td>-4.68</td>
<td>-2.60</td>
</tr>
<tr>
<td>27.44</td>
<td>0.861</td>
<td>0.058</td>
<td>-5.80</td>
<td>-4.59</td>
<td>-2.81</td>
</tr>
<tr>
<td>32.38</td>
<td>1.001</td>
<td>0.052</td>
<td>-5.77</td>
<td>-4.51</td>
<td>-2.83</td>
</tr>
<tr>
<td>37.31</td>
<td>1.143</td>
<td>0.049</td>
<td>-5.74</td>
<td>-4.45</td>
<td>-2.83</td>
</tr>
</tbody>
</table>

$\Delta v$: $-2.75 \pm 0.14$

Plot of $\log (\text{Fe})_2 \alpha$ vs $\log (\text{Fe})_2(1-\alpha)$

\[ n = 0.47 \quad (Sd = 0.03) \]

\[ I = -3.61 \quad (Sd = 0.17) \]
f) pH 11.5: (histidine)$_{\text{TOTAL}} = 0.2 M$

<table>
<thead>
<tr>
<th>$10^6(\text{Fe})_{\text{TOTAL}}$</th>
<th>$A_{413}$</th>
<th>$a$</th>
<th>$\log(\text{Fe})_{\text{TOT}}$</th>
<th>$\log(\text{Fe})_{\text{TOT}}(1-\alpha)$</th>
<th>$\log K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.66</td>
<td>0.179</td>
<td>0.473</td>
<td>-5.90</td>
<td>-5.85</td>
<td>-3.55</td>
</tr>
<tr>
<td>5.32</td>
<td>0.307</td>
<td>0.362</td>
<td>-5.72</td>
<td>-5.47</td>
<td>-3.57</td>
</tr>
<tr>
<td>7.97</td>
<td>0.421</td>
<td>0.305</td>
<td>-5.61</td>
<td>-5.26</td>
<td>-3.56</td>
</tr>
<tr>
<td>10.62</td>
<td>0.522</td>
<td>0.263</td>
<td>-5.53</td>
<td>-5.11</td>
<td>-3.59</td>
</tr>
<tr>
<td>13.26</td>
<td>0.622</td>
<td>0.237</td>
<td>-5.50</td>
<td>-4.99</td>
<td>-3.61</td>
</tr>
<tr>
<td>13.90</td>
<td>0.722</td>
<td>0.220</td>
<td>-5.47</td>
<td>-4.91</td>
<td>-3.63</td>
</tr>
<tr>
<td>18.54</td>
<td>0.817</td>
<td>0.204</td>
<td>-5.42</td>
<td>-4.83</td>
<td>-3.61</td>
</tr>
<tr>
<td>21.17</td>
<td>0.911</td>
<td>0.192</td>
<td>-5.39</td>
<td>-4.77</td>
<td>-3.61</td>
</tr>
<tr>
<td>23.80</td>
<td>1.015</td>
<td>0.188</td>
<td>-5.35</td>
<td>-4.71</td>
<td>-3.59</td>
</tr>
<tr>
<td>26.62</td>
<td>1.108</td>
<td>0.180</td>
<td>-5.32</td>
<td>-4.66</td>
<td>-3.58</td>
</tr>
</tbody>
</table>

$\text{Av: } -3.59 \pm 0.04$

Plot of $\log (\text{Fe})_\alpha$ vs $\log (\text{Fe})_{\text{TOT}}(1-\alpha)$

$n = 0.48 \quad (Sd = 0.01)$

$I = -3.11 \quad (Sd = 0.04)$
\[ \text{pH 12.0 - (histidine)\textsubscript{TOTAL} = 0.2M} \]

<table>
<thead>
<tr>
<th>(10^6(\text{Fe})_{\text{TOTAL},M})</th>
<th>(A_{413})</th>
<th>(\alpha)</th>
<th>(\log(\text{Fe})_{\text{TOT}})</th>
<th>(\log(\text{Fe})_{\text{TOT} (1-\alpha)})</th>
<th>(\log \alpha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.24)</td>
<td>0.060</td>
<td>0.254</td>
<td>-6.50</td>
<td>-6.03</td>
<td>-4.57</td>
</tr>
<tr>
<td>2.47</td>
<td>0.119</td>
<td>0.252</td>
<td>-6.21</td>
<td>-5.72</td>
<td>-4.29</td>
</tr>
<tr>
<td>4.94</td>
<td>0.210</td>
<td>0.186</td>
<td>-6.04</td>
<td>-5.40</td>
<td>-4.28</td>
</tr>
<tr>
<td>7.40</td>
<td>0.295</td>
<td>0.156</td>
<td>-5.94</td>
<td>-5.20</td>
<td>-4.28</td>
</tr>
<tr>
<td>9.86</td>
<td>0.382</td>
<td>0.143</td>
<td>-5.85</td>
<td>-5.07</td>
<td>-4.23</td>
</tr>
<tr>
<td>12.31</td>
<td>0.485</td>
<td>0.131</td>
<td>-5.79</td>
<td>-4.97</td>
<td>-4.21</td>
</tr>
<tr>
<td>14.76</td>
<td>0.547</td>
<td>0.123</td>
<td>-5.74</td>
<td>-4.89</td>
<td>-4.19</td>
</tr>
<tr>
<td>17.21</td>
<td>0.627</td>
<td>0.116</td>
<td>-5.70</td>
<td>-4.82</td>
<td>-4.18</td>
</tr>
<tr>
<td>19.65</td>
<td>0.710</td>
<td>0.113</td>
<td>-5.66</td>
<td>-4.76</td>
<td>-4.16</td>
</tr>
<tr>
<td>22.09</td>
<td>0.789</td>
<td>0.108</td>
<td>-5.62</td>
<td>-4.71</td>
<td>-4.13</td>
</tr>
<tr>
<td>24.52</td>
<td>0.858</td>
<td>0.104</td>
<td>-5.59</td>
<td>-4.66</td>
<td>-4.12</td>
</tr>
</tbody>
</table>

**Av:** \(-4.20 \pm 0.09\)

**Plot of \(\log(\text{Fe})_{\alpha}\) vs \(\log(\text{Fe})_{\text{TOT} (1-\alpha)}\)**

\[ n = 0.58 \quad (Sd = 0.01) \]

\[ I = -2.88 \quad (Sd = 0.05) \]
2. Histamine

used $e_{\text{H}_{2}L} = 31.9 \text{ M}^{-1} \text{ cm}^{-1}$; $e_{\text{H}_{2}L} = 112 \text{ M}^{-1} \text{ cm}^{-1}$

a) pH 8.5; (histamine)$_{\text{TOTAL}} = 0.05 \text{M}$

<table>
<thead>
<tr>
<th>$10^6(\text{Fe})_{\text{TOTAL}}$</th>
<th>$\lambda_{408.5}$</th>
<th>$\alpha$</th>
<th>log(Fe)$_{\text{TOTAL}}$</th>
<th>log(Fe)$_{\text{TOTAL}}$</th>
<th>log(Fe)$_{\text{TOTAL}} (1-\alpha)$</th>
<th>log $K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.58</td>
<td>0.135</td>
<td>0.670</td>
<td>-5.98</td>
<td>-6.28</td>
<td>-1.48</td>
<td></td>
</tr>
<tr>
<td>3.16</td>
<td>0.211</td>
<td>0.437</td>
<td>-5.86</td>
<td>-5.75</td>
<td>-1.77</td>
<td></td>
</tr>
<tr>
<td>4.74</td>
<td>0.277</td>
<td>0.334</td>
<td>-5.80</td>
<td>-5.50</td>
<td>-1.90</td>
<td></td>
</tr>
<tr>
<td>6.31</td>
<td>0.337</td>
<td>0.271</td>
<td>-5.77</td>
<td>-5.34</td>
<td>-2.00</td>
<td></td>
</tr>
<tr>
<td>7.89</td>
<td>0.403</td>
<td>0.242</td>
<td>-5.72</td>
<td>-5.22</td>
<td>-2.02</td>
<td></td>
</tr>
<tr>
<td>9.46</td>
<td>0.461</td>
<td>0.213</td>
<td>-5.70</td>
<td>-5.13</td>
<td>-2.07</td>
<td></td>
</tr>
<tr>
<td>1.58</td>
<td>0.139</td>
<td>0.701</td>
<td>-5.96</td>
<td>-6.32</td>
<td>-1.40</td>
<td></td>
</tr>
</tbody>
</table>

$\text{Av: } -1.73 \pm 0.34$

Plot of log(Fe)$_{\alpha}$ vs log(Fe)$_{\text{TOTAL}} (1-\alpha)$

$n = 0.23 \ (S_d = 0.01)$

$I = -4.55 \ (S_d = 0.07)$
b) pH 9.0 : (histamine)$_{TOT,L} = 0.05\%$

<table>
<thead>
<tr>
<th>$\log^{6}(Fe)_{TOTAL}^{\times}$</th>
<th>$A_{d08.5}$</th>
<th>$\alpha$</th>
<th>$\log^{5}(Fe)_{TOT\alpha}$</th>
<th>$\log^{5}(Fe)_{TOT(1-\alpha)}$</th>
<th>$\log K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.03</td>
<td>0.119</td>
<td>0.457</td>
<td>-6.03</td>
<td>-5.96</td>
<td>-1.90</td>
</tr>
<tr>
<td>4.06</td>
<td>0.224</td>
<td>0.291</td>
<td>-5.93</td>
<td>-5.54</td>
<td>-2.12</td>
</tr>
<tr>
<td>6.08</td>
<td>0.310</td>
<td>0.238</td>
<td>-5.86</td>
<td>-5.33</td>
<td>-2.15</td>
</tr>
<tr>
<td>8.10</td>
<td>0.377</td>
<td>0.183</td>
<td>-5.83</td>
<td>-5.18</td>
<td>-2.28</td>
</tr>
<tr>
<td>10.12</td>
<td>0.454</td>
<td>0.162</td>
<td>-5.79</td>
<td>-5.07</td>
<td>-2.31</td>
</tr>
<tr>
<td>12.14</td>
<td>0.531</td>
<td>0.150</td>
<td>-5.75</td>
<td>-4.99</td>
<td>-2.29</td>
</tr>
</tbody>
</table>

$\Delta v: -2.15 \pm 0.25$

Plot of $\log^{5}(Fe)_{\alpha}$ vs $\log^{5}(Fe)_{(1-\alpha)}$

$n = 0.29$ (Sd = 0.02)

$I = -4.33$ (Sd = 0.12)
c) pH 10,0 ; \( (\text{histamine})_{\text{TOTAL}} = 0,1 \text{M} \)

\[
\begin{array}{ccccccc}
\log(\text{Fe})_{\text{TOT}}, \text{M} & A_{408.5} & \alpha & \log(\text{Fe})_{\text{TOT}} & \log(\text{Fe})_{\text{TOT}}(1-\alpha) & \log K_2 \\
2,08 & 0,218 & 0,910 & -5,77 & -6,73 & -1,41 \\
4,15 & 0,405 & 0,820 & -5,47 & -6,13 & -1,51 \\
5,19 & 0,461 & 0,711 & -5,43 & -5,82 & -1,74 \\
6,23 & 0,540 & 0,684 & -5,37 & -5,71 & -1,73 \\
6,23 & 0,526 & 0,656 & -5,39 & -5,67 & -1,81 \\
8,29 & 0,651 & 0,582 & -5,32 & -5,46 & -1,88 \\
9,39 & 0,701 & 0,534 & -5,30 & -5,36 & -1,94 \\
12,42 & 0,842 & 0,448 & -5,25 & -5,16 & -2,04 \\
\end{array}
\]

Av: \(-1,71 \pm 0,33\)

Plot of \(\log(\text{Fe})_{\text{Fe}}\) vs \(\log(\text{Fe})_{\text{TOT}}(1-\alpha)\)

\[
\begin{align*}
\alpha &= 0,29 \quad (Sd = 0,01) \\
I &= -3,73 \quad (Sd = 0,11)
\end{align*}
\]
d) pH 10,0 ; (histamine)_{TOTAL} = 0,1M

<table>
<thead>
<tr>
<th>$10^6(\text{Fe})_{TOT}$</th>
<th>M $\text{H}_4\text{O}8,5$</th>
<th>$\alpha$</th>
<th>log($\text{Fe})_{TOT}$</th>
<th>log($\text{Fe})_{TOT}(1-\alpha)$</th>
<th>log $K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1,57)</td>
<td>0,173</td>
<td>0,977</td>
<td>-5,61</td>
<td>-7,45</td>
<td>-0,87</td>
</tr>
<tr>
<td>(3,14)</td>
<td>0,319</td>
<td>0,870</td>
<td>-5,56</td>
<td>-5,40</td>
<td>-1,42</td>
</tr>
<tr>
<td>(4,71)</td>
<td>0,443</td>
<td>0,776</td>
<td>-5,44</td>
<td>-5,98</td>
<td>-1,60</td>
</tr>
<tr>
<td>(6,26)</td>
<td>0,547</td>
<td>0,693</td>
<td>-5,36</td>
<td>-5,72</td>
<td>-1,70</td>
</tr>
<tr>
<td>(7,83)</td>
<td>0,622</td>
<td>0,593</td>
<td>-5,33</td>
<td>-5,50</td>
<td>-1,86</td>
</tr>
<tr>
<td>(9,39)</td>
<td>0,711</td>
<td>0,547</td>
<td>-5,29</td>
<td>-5,37</td>
<td>-1,91</td>
</tr>
</tbody>
</table>

Av: -1,66 ± 0,25

Plot of ; $(\text{Fe})^2$ vs log ($\text{Fe})_{1-\alpha}$

$\text{n} = 0,25$ (Std = 0,01)

$I = -3,93$ (Std = 0,04)

Av log $K_2$ for pH 10,0 = -1,69 ± n. s.
a) pH 11.0; (histamine)_{TOTAL} = 0.1 M; \mu = 0.50

\begin{table}
<table>
<thead>
<tr>
<th>$10^6(P%)_\text{TOTAL}$</th>
<th>$A_{408.5}$</th>
<th>$\alpha$</th>
<th>$\log(\text{Fe})_{\text{TOT}}$</th>
<th>$\log(\text{Fe})_{\text{TOT}(1-\alpha)}$</th>
<th>$\log K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.58</td>
<td>0.146</td>
<td>0.756</td>
<td>-5.92</td>
<td>-5.41</td>
<td>-2.13</td>
</tr>
<tr>
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<td>0.665</td>
<td>-5.66</td>
<td>-6.00</td>
<td>-2.02</td>
</tr>
<tr>
<td>4.74</td>
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</tr>
<tr>
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<td>0.474</td>
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<tr>
<td>7.89</td>
<td>0.561</td>
<td>0.491</td>
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<td>-5.40</td>
<td>-2.12</td>
</tr>
<tr>
<td>9.46</td>
<td>0.627</td>
<td>0.431</td>
<td>-5.39</td>
<td>-5.27</td>
<td>-2.21</td>
</tr>
</tbody>
</table>

Av: $-2.10 \pm 0.11$

Plot of $\log (\text{Fe})_{\alpha}$ vs $\log (\text{Fe})_{\text{TOT}(1-\alpha)}$

$n = 0.47$ \hspace{1cm} (Sd = 0.04)

$I = -2.89$ \hspace{1cm} (Sd = 0.21)
5. Pilocarpine

\[ \text{used } \frac{D_{1,2}}{2} = 23.5 \text{ mN cm}^{-1} \quad \text{and } \frac{D_{1,2}}{2} = 111.8 \text{ mN cm}^{-1} \]

a) pH 8.5; (pilocarpine) = 0.002M

<table>
<thead>
<tr>
<th>( \log^\alpha(Fe)_{TOT} )</th>
<th>( N )</th>
<th>( \alpha )</th>
<th>( \log(Fe)_{TOT} )</th>
<th>( \log(Fe)_{TOT}(1-\alpha) )</th>
<th>( \log K_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1.5)</td>
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<tr>
<td>4.64</td>
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<tr>
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<td>0.400</td>
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<td>-5.33</td>
<td>1.51</td>
</tr>
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<td>1.49</td>
</tr>
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<tr>
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<td>-4.86</td>
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</tr>
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<td>14.93</td>
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<td>0.257</td>
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<td>-4.78</td>
<td>1.40</td>
</tr>
</tbody>
</table>

\[ n = 0.37 \quad (Sd = 0.01) \]

\[ I = -3.53 \quad (Sd = 0.04) \]
b) pH 8.5; (pilocarpate) = 0.005M

<table>
<thead>
<tr>
<th>(10^6(\text{Fe})_{\text{TOT}})</th>
<th>N</th>
<th>(\lambda_{410})</th>
<th>(\alpha)</th>
<th>(\log(\text{Fe})_{\text{TOT}})</th>
<th>(\log(\text{Fe})_{\text{TOT}})((1-\alpha))</th>
<th>(\log K_2)</th>
</tr>
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<tr>
<td>1.57</td>
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<tr>
<td>3.14</td>
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</tr>
<tr>
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<tr>
<td>15.66</td>
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<tr>
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</tr>
<tr>
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<td>-4.77</td>
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</tr>
<tr>
<td>24.99</td>
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<td>-4.70</td>
<td>1.34</td>
<td></td>
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</tbody>
</table>

\[\text{Av: 1.47 } \pm 0.13\]

Plot of \(\log (\text{Fe})_{\alpha}\) vs \(\log (\text{Fe})_{\text{T}}(1-\alpha)\)

\[n = 0.43 \quad (Sd = 0.01)\]

\[I = -3.23 \quad (Sd = 0.06)\]
c) pH 8.5; (pilocarpate) = 0.005 M

<table>
<thead>
<tr>
<th>$10^6(Fe)_{TOT}$</th>
<th>$N$</th>
<th>$A_{410}$</th>
<th>$\alpha$</th>
<th>$\log(Fe)_{TOT}$</th>
<th>$\log(Fe)_{TOT(1-\alpha)}$</th>
<th>$\log K_2$</th>
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</thead>
<tbody>
<tr>
<td>1.02</td>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<tr>
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<td>-4.65</td>
<td>1.17</td>
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</tr>
</tbody>
</table>

$\text{Av: } 1.31 \pm 0.14$

Plot of $\log (Fe)_{1-\alpha}$ vs $\log (Fe)_{\alpha}(1-\alpha)$

$n = 0.43$ ($Sd = 0.01$)

$I = -3.32$ ($Sd = 0.05$)

$\text{Av } \log K_2 \text{ for pH 8.5 } = 4.43 \pm 0.12$
d) pH 9.0; (pilocarpine) = 0.005M

<table>
<thead>
<tr>
<th>$10^6(Fe)_{TOT}$</th>
<th>M</th>
<th>$A_{410}$</th>
<th>$\alpha$</th>
<th>log(Fe)$_{TOT}$</th>
<th>log(Fe)$_{TOT(1-\alpha)}$</th>
<th>log $K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.55</td>
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<td>0.449</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>4.64</td>
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<td>0.94</td>
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</tr>
<tr>
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<td>0.99</td>
<td></td>
</tr>
<tr>
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<td>-5.23</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>9.25</td>
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<td>0.225</td>
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<td>-5.14</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
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<td>0.504</td>
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<td>-5.07</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
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<td>-5.01</td>
<td>0.99</td>
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</tr>
<tr>
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<td>-4.95</td>
<td>0.95</td>
<td></td>
</tr>
<tr>
<td>15.40</td>
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<td>-4.90</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>18.47</td>
<td>0.793</td>
<td>0.163</td>
<td>-5.50</td>
<td>-4.81</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>21.53</td>
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<td>0.156</td>
<td>-5.47</td>
<td>-4.74</td>
<td>1.00</td>
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</tbody>
</table>

Avg: 0.97 ± 0.03

Plot of log (Fe)$_{TOT}$ vs log (Fe)$_{TOT(1-\alpha)}$

$n = 0.51$ (Sd = 0.01)

$\tau = 3.05$ (Sd = 0.03)
e) pH 10.0; (pilocarpate) = 0.025M

<table>
<thead>
<tr>
<th>$\log \beta(\text{Fe})_{\text{TOT}} %$</th>
<th>$A_{410}$</th>
<th>$\alpha$</th>
<th>$\log(\text{Fe})_{\text{TOT}}$</th>
<th>$\log(\text{Fe})_{\text{TOT}(1-\alpha)}$</th>
<th>$\log K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.55</td>
<td>0.136</td>
<td>0.708</td>
<td>-5.96</td>
<td>-6.34</td>
<td>-0.47</td>
</tr>
<tr>
<td>3.09</td>
<td>0.243</td>
<td>0.597</td>
<td>-5.73</td>
<td>-5.90</td>
<td>-0.45</td>
</tr>
<tr>
<td>4.64</td>
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<td>-0.42</td>
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<td>-5.51</td>
<td>-0.40</td>
</tr>
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<td>-5.38</td>
<td>-0.41</td>
</tr>
<tr>
<td>9.25</td>
<td>0.601</td>
<td>0.431</td>
<td>-5.40</td>
<td>-5.28</td>
<td>-0.41</td>
</tr>
<tr>
<td>10.79</td>
<td>0.677</td>
<td>0.404</td>
<td>-5.36</td>
<td>-5.19</td>
<td>-0.42</td>
</tr>
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<td>-5.33</td>
<td>-5.12</td>
<td>-0.43</td>
</tr>
<tr>
<td>13.87</td>
<td>0.826</td>
<td>0.365</td>
<td>-5.30</td>
<td>-5.06</td>
<td>-0.43</td>
</tr>
<tr>
<td>15.40</td>
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<td>-5.00</td>
<td>-0.42</td>
</tr>
<tr>
<td>18.47</td>
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<td>0.320</td>
<td>-5.23</td>
<td>-4.97</td>
<td>-0.42</td>
</tr>
<tr>
<td>21.53</td>
<td>1.163</td>
<td>0.298</td>
<td>-5.19</td>
<td>-4.82</td>
<td>-0.45</td>
</tr>
</tbody>
</table>

Avg: $-0.43 \pm 0.04$

Plot of $\log (\text{Fe})_{\alpha}$ vs $\log (\text{Fe})_{1-\alpha}$

$n = 0.51$  \( (SD = 0.01) \)

$m = -2.74$  \( (SD = 0.04) \)
f) pH 11,0 (pilocarpate) - 0,05M

<table>
<thead>
<tr>
<th>$\log^{6}(\text{Fe})_{\text{TOT}} \times$</th>
<th>$A_{410}$</th>
<th>$\alpha$</th>
<th>$\log(\text{Fe})_{\text{TOT} - \alpha}$</th>
<th>$\log(\text{Fe})_{\text{TOT}}(1-\alpha)$</th>
<th>$\log X_2$</th>
</tr>
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<tbody>
<tr>
<td>1,55</td>
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<td>0,661</td>
<td>-5,99</td>
<td>-6,28</td>
<td>-1,50</td>
</tr>
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<td>3,09</td>
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<td>0,593</td>
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<td>4,64</td>
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<td>-1,36</td>
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<td>-1,37</td>
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<td>-5,36</td>
<td>-1,40</td>
</tr>
<tr>
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<td>0,405</td>
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<td>-5,26</td>
<td>-1,40</td>
</tr>
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<td>-5,17</td>
<td>-1,41</td>
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<tr>
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<td>-1,42</td>
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<td>-4,80</td>
<td>-1,46</td>
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</table>

$\text{Av: } -1,41 \pm 0,09$

Plot of $\log(\text{Fe})_{\text{TOT} - \alpha}$ vs $\log(\text{Fe})_{\text{TOT}}(1-\alpha)$

$n = 0,50 \quad (sd = 0,01)$

$I = -2,80 \quad (sd = 0,07)$
g) pH 11,0 ;  (pilocarpate) = 0,05M

<table>
<thead>
<tr>
<th>$10^6(\text{Fe}_{\text{TOT}})$</th>
<th>$\alpha$</th>
<th>$A_{410}$</th>
<th>$\log(\text{Fe}_{\text{TOT}})$</th>
<th>$\log(\text{Fe}_{\text{TOT}}(1-\alpha))$</th>
<th>$\log K_2$</th>
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<tbody>
<tr>
<td>2,72</td>
<td>0,207</td>
<td>0,566</td>
<td>-5,81</td>
<td>-5,93</td>
<td>-1,49</td>
</tr>
<tr>
<td>5,44</td>
<td>0,350</td>
<td>0,446</td>
<td>-5,62</td>
<td>-5,52</td>
<td>-1,52</td>
</tr>
<tr>
<td>8,16</td>
<td>0,495</td>
<td>0,379</td>
<td>-5,51</td>
<td>-5,29</td>
<td>-1,53</td>
</tr>
<tr>
<td>10,89</td>
<td>0,624</td>
<td>0,338</td>
<td>-5,43</td>
<td>-5,14</td>
<td>-1,52</td>
</tr>
<tr>
<td>13,56</td>
<td>0,744</td>
<td>0,308</td>
<td>-5,38</td>
<td>-5,03</td>
<td>-1,53</td>
</tr>
<tr>
<td>16,27</td>
<td>0,866</td>
<td>0,288</td>
<td>-5,33</td>
<td>-4,94</td>
<td>-1,52</td>
</tr>
<tr>
<td>18,98</td>
<td>0,985</td>
<td>0,272</td>
<td>-5,29</td>
<td>-4,86</td>
<td>-1,52</td>
</tr>
<tr>
<td>21,68</td>
<td>1,099</td>
<td>0,257</td>
<td>-5,25</td>
<td>-4,79</td>
<td>-1,51</td>
</tr>
<tr>
<td>24,38</td>
<td>1,214</td>
<td>0,247</td>
<td>-5,22</td>
<td>-4,78</td>
<td>-1,50</td>
</tr>
<tr>
<td>27,08</td>
<td>1,324</td>
<td>0,236</td>
<td>-5,20</td>
<td>-4,68</td>
<td>-1,52</td>
</tr>
</tbody>
</table>

Av: $-1,52 \pm 0,05$

Plot of $\log(\text{Fe},\alpha)$ vs $\log(\text{Fe},\alpha(1-\alpha))$

$n = 0,50$  
$m = -2,88$  
$(Sd = 0,01)$  
$(Sd = 0,03)$
1) pH 11,0: (pilocarpate) = 0,02M

<table>
<thead>
<tr>
<th>$10^6(\text{Fe})_{\text{TOTAL}}$</th>
<th>$M$</th>
<th>$A_{210}$</th>
<th>$\alpha$</th>
<th>$\log(\text{Fe})_{\text{TOT}}$</th>
<th>$\log(\text{Fe})_{\text{TOT}}(1-\alpha)$</th>
<th>$\log K_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,72</td>
<td>0,234</td>
<td>0,687</td>
<td>-5,73</td>
<td>-6,07</td>
<td>-1,19</td>
<td></td>
</tr>
<tr>
<td>5,44</td>
<td>0,408</td>
<td>0,553</td>
<td>-5,32</td>
<td>-5,61</td>
<td>-1,23</td>
<td></td>
</tr>
<tr>
<td>8,16</td>
<td>0,569</td>
<td>0,489</td>
<td>-5,40</td>
<td>-5,38</td>
<td>-1,22</td>
<td></td>
</tr>
<tr>
<td>10,89</td>
<td>0,697</td>
<td>0,419</td>
<td>-5,34</td>
<td>-5,20</td>
<td>-1,28</td>
<td></td>
</tr>
<tr>
<td>13,56</td>
<td>0,828</td>
<td>0,383</td>
<td>-5,28</td>
<td>-5,08</td>
<td>-1,28</td>
<td></td>
</tr>
<tr>
<td>16,27</td>
<td>0,957</td>
<td>0,356</td>
<td>-5,24</td>
<td>-4,98</td>
<td>-1,30</td>
<td></td>
</tr>
<tr>
<td>18,98</td>
<td>1,079</td>
<td>0,332</td>
<td>-5,20</td>
<td>-4,90</td>
<td>-1,30</td>
<td></td>
</tr>
<tr>
<td>21,68</td>
<td>1,198</td>
<td>0,313</td>
<td>-5,17</td>
<td>-4,83</td>
<td>-1,31</td>
<td></td>
</tr>
<tr>
<td>24,38</td>
<td>1,311</td>
<td>0,295</td>
<td>-5,14</td>
<td>-4,76</td>
<td>-1,32</td>
<td></td>
</tr>
<tr>
<td>27,08</td>
<td>1,421</td>
<td>0,279</td>
<td>-5,12</td>
<td>-4,71</td>
<td>-1,33</td>
<td></td>
</tr>
</tbody>
</table>

$Av: -1,27 \pm 0,08$

Plot of $\log (\text{Fe})_{\text{TOT}}$ vs $\log (\text{Fe})_{1}(1-\alpha)$

$n = 0,45$ \hspace{1cm} (Sd = 0,01)

$I = -3,01$ \hspace{1cm} (Sd = 0,03)

Overall $Av$ $\log K_2$ for pH 11,0 = $-1,6 \pm 0,1$
APPENDIX 5: CORRECTION OF THE BINDING CONSTANTS OF HISTIDINE,
HISTAMINE AND PILOCARPATE FOR THE PKAS OF THE FREE
AND COORDINATED LIGANDS

(Chapter 5)

It is known that coordination of histamine to B_{12a} reduced the
pKa of NH_{2} from 9.7 to 4.7 on coordination. In this analysis,
it will be assumed that the pKa of NH_{2} in histamine and
histidine is reduced to less than 8 on coordination, while the pKa
of OH in pilocarpate is reduced to about 10 (from ~15).

In all cases, the overall binding constant will be used

\[(K_1 K_2),\] which refers to equation (1).

\[(FeOH)_{2} + 4L \rightleftharpoons 2FeL_{2} + 2OH^{-}\] (1)

The corrected binding constants are given in table 5.5.

a) Histidine, histamine

\[(OH)_{2} Fe + 4L \rightleftharpoons 2FeL_{2} + 2OH^{-}\]

\[K_{\text{obs}} = \frac{[FeL_{2}][OH^{-}]}{[(OH)Fe]_{2}[L]}\] (L)^{2}

now

\[Fe(RNH_{2})_{2} \rightleftharpoons Fe(RNH_{3}^{+})_{2} + 2H^{+}\]

where Fe(RNH_{3}^{+})_{2} and Fe(RNH_{2})_{2} refers to the coordinated ligands
with the amine protonated and unprotonated respectively

\[K_{a}(Fe-L) = \frac{[Fe(RNH_{3}^{+})_{2}][H^{+}]^{2}}{[Fe(RNH_{2})_{2}]}\]

and

\[K_{a}(Fe-L)_{2} = \frac{[Fe(RNH_{3}^{+})_{2}]}{[Fe(RNH_{2})_{2}]^{2}}\]

\[= Fe(RNH_{2})_{2} + \frac{(H^{+})^{2}}{K_{a}(Fe-L)}\]
\( \text{Fe(Fe-L)_{2}} \) if \( (\text{H}^+)^2 \ll K_a(\text{Fe-L})_2 \)

also

\[ \text{RNH}_3^+ \leftrightarrow \text{RNH}_2^- + \text{H}^+ \quad K_{a} \]

where \( \text{RNH}_3^+ \) and \( \text{RNH}_2^- \) refer to the free ligands with the amine protonated and unprotonated respectively.

\[
K_{a} = \frac{[\text{RNH}_2^-][\text{H}^+]}{[\text{RNH}_3^+]}
\]

and

\[
(L) = (\text{RNH}_2^-) + (\text{RNH}_3^+)
\]

\[ = (\text{RNH}_2^-) \left( 1 + \frac{[\text{H}^+]}{K_{a}} \right) \]

\[ \therefore K_{\text{obs}} = \frac{(\text{Fe(Fe-L)}_2)^2 [\text{OH}^-]^2}{(\text{H}^+)[\text{RNH}_2^-]^5(1 + \frac{[\text{H}^+]}{K_{a}})^5} \]

If \( K_3 \) refers to the reaction

\[ (\text{FeOH})_2 + 4\text{RNH}_2^- \leftrightarrow 2\text{Fe(Fe-NH)}_2 + 2\text{OH}^- \]

then \( K_{\text{obs}} = K_3 \frac{1}{1 + (\text{H}^+)/K_{a}} \)

\[ \therefore K_3 = K_{\text{obs}} \left( 1 + \frac{[\text{H}^+]}{K_{a}} \right)^4 \]

\[ \therefore \log K_3 = \log K_{\text{obs}} + 4 \log \left( 1 + \frac{[\text{H}^+]}{K_{a}} \right) \]

b) Pilocarpate

The \( pK_a \) of -OH of uncoordinated pilocarpate would be about 15 *

and will thus be ignored.

* which is the approximate \( pK_a \) expected for an alcohol.
\[(\text{FeOH})_2 + 4L \rightleftharpoons 2\text{FeL}_2 + 2\text{OH}^- \quad K_1 K_2 = K_{\text{obs}}\]

\[
K_{\text{obs}} = \frac{(\text{FeL}_2)^2 (\text{OH}^-)^2}{((\text{FeOH})_2) (L)^4}
\]

where \(\text{ROH}\) refers to pilocarpate with an undissociated hydroxyl group.

\[
K_{a(\text{Fe})} = \frac{(\text{Fe}(\text{ROH}))(\text{RO}^-))}{(\text{Fe}(\text{ROH}))^2}
\]

and \((\text{FeL}_2) = (\text{Fe}(\text{ROH})) + (\text{Fe}(\text{ROH}))(\text{NO}^-))
\[
= (\text{Fe}(\text{ROH})) \left(1 + \frac{K_{a(\text{Fe-L})}}{(\text{H}^+)}\right)
\]

\[
K_{\text{obs}} = \frac{(\text{Fe}(\text{ROH}))^2 (1 + \frac{K_{a(\text{Fe-L})}}{(\text{H}^+)}^2}{((\text{OHFe})_2) (L)^4}
\]

If \(K_3\) refers to the equilibrium

\[(\text{OHFe})_2 + 4L \rightleftharpoons 2\text{Fe}(\text{ROH})_2 + 2\text{OH}^- \]

then \(K_{\text{obs}} = K_3 \left(1 + \frac{K_{a(\text{Fe-L})}}{(\text{H}^+)}\right)^2
\]

\[
\log K_3 = \log K_{\text{obs}} - 2 \log \left(1 + \frac{K_{a(\text{Fe-L})}}{(\text{H}^+)}\right)
\]
## APPENDIX 6: TABLES OF DATA FOR THE KINETIC STUDY OF THE REDUCTION OF BIS-HISTIDINE HEMIN BY DITHIOTHREITOL (Chapter 7)

### Table 1: Variation of $k_{obs}$ with dithiothreitol concentration; pH 10.0; 0.4 M histidine; $30 \times 10^{-6}$ M hemin; $\mu = 0.5$

<table>
<thead>
<tr>
<th>(dtt), M</th>
<th>$10^2 k_{obs}$, s$^{-1}$</th>
<th>(dtt), M</th>
<th>$10^2 k_{obs}$, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$2 \times 10^{-4}$</td>
<td>2.19; 2.12</td>
<td>$6.4 \times 10^{-3}$</td>
<td>6.30</td>
</tr>
<tr>
<td>$3 \times 10^{-4}$</td>
<td>3.40; 3.41</td>
<td>$1.0 \times 10^{-2}$</td>
<td>5.86</td>
</tr>
<tr>
<td>$3.5 \times 10^{-4}$</td>
<td>3.76</td>
<td>$2.0 \times 10^{-2}$</td>
<td>5.15</td>
</tr>
<tr>
<td>$4.0 \times 10^{-4}$</td>
<td>3.86</td>
<td>$3.0 \times 10^{-2}$</td>
<td>3.77</td>
</tr>
<tr>
<td>$8.0 \times 10^{-4}$</td>
<td>5.40</td>
<td>$5.0 \times 10^{-2}$</td>
<td>3.21</td>
</tr>
<tr>
<td>$1.6 \times 10^{-3}$</td>
<td>6.72</td>
<td>$7.5 \times 10^{-2}$</td>
<td>3.03</td>
</tr>
<tr>
<td>$2.4 \times 10^{-3}$</td>
<td>6.75</td>
<td>$1.0 \times 10^{-1}$</td>
<td>3.01</td>
</tr>
<tr>
<td>$3.2 \times 10^{-3}$</td>
<td>6.72</td>
<td>$1.0 \times 10^{-1}$</td>
<td>2.91</td>
</tr>
</tbody>
</table>

### Table 2: Variation of $k_{obs}$ with histidine concentration; at pH 10.0; $30 \times 10^{-6}$ M hemin, $\mu = 0.5$

<table>
<thead>
<tr>
<th>Histidine, M</th>
<th>$10^2 k_{obs}$, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>low (dithiothreitol)</td>
</tr>
<tr>
<td></td>
<td>($3 \times 10^{-6}$ M)</td>
</tr>
<tr>
<td>0.205</td>
<td>7.70</td>
</tr>
<tr>
<td>0.250</td>
<td>6.77</td>
</tr>
<tr>
<td>0.300</td>
<td>5.36</td>
</tr>
<tr>
<td>0.350</td>
<td>5.32</td>
</tr>
<tr>
<td>0.380</td>
<td>4.60</td>
</tr>
<tr>
<td>0.400</td>
<td>4.16</td>
</tr>
</tbody>
</table>
Table 1: Variation of $k_{obs}$ with pH; $3 \times 10^{-5}$M hemin; $0.4\%$ histidine; $4 \times 10^{-6}$M dtt; $\mu = 0.5$; 25°C

<table>
<thead>
<tr>
<th>pH</th>
<th>$10^2 k_{obs}$, s$^{-1}$</th>
<th>$f_{Fe(III)(histidine)}^a$</th>
<th>$10^2 k_{corr}$, s$^{-1}$</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>6.16</td>
<td>0.959</td>
<td>6.15</td>
<td></td>
</tr>
<tr>
<td>8.5</td>
<td>6.96±7.23</td>
<td>0.994</td>
<td>7.00±7.27</td>
<td></td>
</tr>
<tr>
<td>9.0</td>
<td>6.78</td>
<td>0.992</td>
<td>6.83</td>
<td></td>
</tr>
<tr>
<td>9.5</td>
<td>6.19</td>
<td>0.983</td>
<td>6.30</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>4.73</td>
<td>0.975</td>
<td>4.85</td>
<td></td>
</tr>
<tr>
<td>11.0</td>
<td>2.10</td>
<td>0.721</td>
<td>2.91</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $Fe(III)(histidine)_2$ is the fraction the bis-histidine remain present (calculated from data in chapter 5)

$^b$ $k_{corr} = k_{obs}/f_{Fe(III)(histidine)}$
APPENDIX 7 : DERIVATION OF THE RATE EQUATION FOR THE REDUCTION OF BIS-HISTIDINE HEMIN BY DITHIOREITHOL
(DISCUSSED IN CHAPTER 7)

The kinetic results for the above reaction were fitted to a rate law derived by Flock's method. The derivation is given below.

\[ a + b \xrightarrow{k_{-1}} c + d \] (where \( a = \text{dtt} \); \( b = \text{Fe}^{III}L_2 \); \( c = \text{Fe}^{III}(L)(RS) \))

\[ c \xrightarrow{k_2} p + f \quad d = L; \quad p = \text{Fe}^{II}_2 \; ; \; f = \text{dtt} \]

\[ c + a \xrightarrow{k_{-3}} g \quad g = \text{Fe}^{III}(L)(RS) \cdots (RS) \]

Assuming that both (a) and (d) are large compared to (b) such that

\( (a) = (a)_0 \) and \( (d) = (d)_0 \) \( (c) \text{ initial concentrations} \)

i.e. pseudo first order conditions. Then

\[ \frac{d}{dt} (b) = k_{-1} (d)(d)_0 - k_1 (a)_0 b \]

\[ \frac{d}{dt} (c) = k_1 (a)_0 (b) + k_{-3} (g) - c (k_2 + k_{-1} (d)_0 + k_3 (a)_0) \]

\[ \frac{d}{dt} (g) = k_2 (c) (a)_0 - (g)(k_{-3} + k_4) \]

\[ \frac{d}{dt} (p) = k_2 (c) + k_4 (g) \]

assuming that \( (b) = b e^{-mt} \) \( \Rightarrow \frac{d}{dt} (b) = -mb e^{-mt} \)

\( (c) = ce^{-mt} \) \( \Rightarrow \frac{d}{dt} (c) = -mc e^{-mt} \)

\( (g) = ge^{-mt} \) \( \Rightarrow \frac{d}{dt} (g) = -mg e^{-mt} \)

\( (p) = pe^{-mt} \) \( \Rightarrow \frac{d}{dt} (p) = -mp e^{-mt} \)

Then

\[ -mb e^{-mt} = k_{-1} (d)_0 ce^{-mt} - k_1 (a)_0 be^{-mt} \]

\[ \Rightarrow c = k_{-1} (d)_0 c + (m-k_1) b \] (7)

\[ -mc e^{-mt} = k_1 (a)_0 be^{-mt} + k_{-3} ge^{-mt} - ce^{-mt} (k_2 + k_{-1} (d)_0 + k_3 (a)_0) \]
\[ o(t) = \left[ m - (k_2 + k_{-1} d) \right] o + k_3(t_0) \quad c + k_1(t_0) \quad o + k_{-3} \quad g \quad (2) \]

\[ -m \alpha e^{-mt} = k_3(t_0) c e^{-mt} - (k_{-3} + k_4) g e^{-mt} \]

\[ \Rightarrow o = \left[ m - (k_{-3} + k_4) \right] g + k_3(t_0) c \quad (3) \]

\[ -m \alpha e^{-mt} = k_2 c e^{-mt} + k_4 g e^{-mt} \]

\[ \Rightarrow o = m p + k_2 c + k_4 g \quad (4) \]

\[
\begin{bmatrix}
    k_{-1}(a_0) & k_2 & 0 & 0 \\
    k_1(a_0) & m - (k_{-1} + k_2) & k_3 & 0 \\
    0 & k_3(a_0) & m - (k_{-3} + k_4) & 0 \\
    0 & k_2 & k_4 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    m - k_1(a_0) & m - (k_{-1} + k_2) & k_3 & 0 \\
    k_3(a_0) & m - (k_{-3} + k_4) & 0 \\
    k_2 & k_4 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    -k_{-1}(d_0) & k_1(a_0) & k_{-3} & 0 \\
    0 & m - (k_{-3} + k_4) & 0 \\
    0 & k_4 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    (m - k_1(a_0)) & m - (k_{-1} + k_2) & k_3(a_0) & 0 \\
    k_3(a_0) & m - (k_{-3} + k_4) & 0 \\
    k_2 & k_4 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    (m - k_1(a_0)) & (m - k_{-1} + k_2) & k_3(a_0) & 0 \\
    k_3(a_0) & m - (k_{-3} + k_4) & 0 \\
    k_2 & k_4 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    -(m - k_1(a_0)) & k_3(a_0) & 0 \\
    k_2 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    -(m - k_1(a_0)) & k_3(a_0) & 0 \\
    k_2 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    -(m - k_1(a_0)) & k_3(a_0) & 0 \\
    k_2 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    -(m - k_1(a_0)) & k_3(a_0) & 0 \\
    k_2 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    -(m - k_1(a_0)) & k_3(a_0) & 0 \\
    k_2 & m
\end{bmatrix}
= 0
\]

\[
\begin{bmatrix}
    -(m - k_1(a_0)) & k_3(a_0) & 0 \\
    k_2 & m
\end{bmatrix}
= 0
\]
\[
\begin{bmatrix}
-\kappa_1(a) - \kappa_2\kappa_1(a) + \kappa_3(a) + \kappa_4(a) \\
-\kappa_2\kappa_1(a) + \kappa_3(a)
\end{bmatrix} \begin{bmatrix}
\kappa_3(a) \\
\kappa_4(a)
\end{bmatrix} = 0
\]

(Note: Pick does not cancel these \(a\)'s but instead finds that one of the roots is zero; cancelling the \(a\)'s gives the same results with all roots non-zero.)

\[
\begin{bmatrix}
-\kappa_1(a) - \kappa_2\kappa_1(a) + \kappa_3(a) + \kappa_4(a) \\
-\kappa_2\kappa_1(a) + \kappa_3(a)
\end{bmatrix} \begin{bmatrix}
\kappa_3(a) \\
\kappa_4(a)
\end{bmatrix} = 0
\]

\[
\begin{align*}
\alpha^3 - \& \ (k_2 + k_1)^2 + k_3(a) + k_4(a) \\
\alpha^2 - \& \ (k_2 + k_1)d + k_3(a)
\end{align*}
\]

i.e. coefficient of \(a^3 = 1
\]

\[
\begin{align*}
\alpha^2 &= -(k_1(a) + k_2 + k_1(d) + k_3(a) + k_4(a)) \\
\alpha &= -(k_2^2 + k_1^2 + k_2 + k_1(d) + k_3(a) + k_4(a))
\end{align*}
\]

\[
\begin{align*}
k_2k_3k_4 + k_1k_2 + k_1k_3 + k_4^2 \\
k_2k_4 + k_1k_2 + k_1k_3(a) + k_4^2 \\
k_2k_4 + k_1k_3(a) - k_2k_3(a)
\end{align*}
\]
If the three roots of the cubic equation are \(m_0, m_1, m_2\) then

\[
\begin{align*}
&\left[m - m_0\right] \left[m - m_1\right] \left[m - m_2\right] = 0 \\
\Rightarrow &\left[m - m_0\right] \left[m^2 - (m_1+m_2)m + m_1m_2\right] = 0 \\
\Rightarrow &m^3 - (m_1 + m_2)m^2 + (m_1m_2)m - (m_1+m_2)m_o m = m_0m_1m_2 = 0 \\
\Rightarrow &m^3 - m^2(m_1 + m_2) + m(m_1 + m_2)m_0 m - m_0m_1m_2 = 0
\end{align*}
\]

Equating coefficients of \(m^3, m^2, m, m^0\):

\[
\begin{align*}
m_o + m_1 + m_2 &= k_1(a) o + k_2 + k_{-1}(d) o + k_3(a) o + k_{-3} + k_4 \\
(m_0 + m_1 + m_2) &= k_1(a) o \left[k_3\left(k_3 + k_4\right) + k_3(a) o k_4\right] \\
m_0m_1 + m_0m_2 + m_1m_2 &= \left[k_2 + k_{-1}(d) o\right] \left[k_3 + k_4\right] + (a) o \left[k_3\left(k_3 + k_4\right) + k_3(a) o k_4\right]
\end{align*}
\]
if \( m_2 \ll m_0, m_1 \) (as reaction is monophasic) (i.e. \( m_0 \) and \( m_1 \) are associated with rapid equilibration)

then \( m_0 m_1 + m_0 m_2 + m_1 m_2 \rightarrow m_0 m_1 \)

\[
(m_0 m_1 \rightarrow \frac{k_1(a)_o + k_{-1}(d)_o + k_3(a)_o + k_{-3}}{k_2 + k_4}
\]

\[
\rightarrow \frac{k_1(a)_o + k_{-1}(d)_o + k_3(a)_o + k_{-3}}{k_2 + k_4}
\]

\[
\text{if } k_2, k_4 \text{ small}
\]

\[
\therefore m_2 = \frac{m_0 m_1 m_2}{m_1 m_2}
\]

\[
x(a)_o + y(a)_o \rightarrow \frac{z + v(a)_o + w(a)_o}{x(a)_o + w(a)_o}
\]

\[
\text{i.e. } m_3 \rightarrow \frac{z + v(a)_o + w(a)_o}{x(a)_o + w(a)_o} \quad (5)
\]

where

\[
x = k_1 k_2 \quad (k_3 ^< 0)
\]

\[
y = z_1 \cdot v_3
\]

\[
z = (k_2 + k_{-1}(d)_o)(k_3 + k_4)
\]

\[
v = (k_3 k_4 + k_1 k_2 + k_1 k_4 + k_1 k_3)
\]

\[
w = k_3 k_4
\]

Equations (5) - (10) with symbols as defined at the start will be used in the text (chapter 7).
All experiments carried out at 25°C in solutions with an ionic strength of 0.1.

Table 1: The effect of pH on the rate of O₂ uptake by B₁₂a + cysteine; 2x10⁻² M cysteine; 1x10⁻⁵ M B₁₂a; O₂ saturated buffers.

<table>
<thead>
<tr>
<th>pH</th>
<th>initial rate - B₁₂a (μM O₂ s⁻¹)</th>
<th>initial rate + B₁₂a (μM O₂ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>6.0</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>8.0</td>
<td>0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>8.5</td>
<td>0.15</td>
<td>0.92</td>
</tr>
<tr>
<td>9.0</td>
<td>0.17</td>
<td>1.52</td>
</tr>
<tr>
<td>9.5</td>
<td>0.14</td>
<td>1.61</td>
</tr>
<tr>
<td>10.0</td>
<td>0.15</td>
<td>1.73</td>
</tr>
<tr>
<td>10.5</td>
<td>0.14</td>
<td>1.52</td>
</tr>
<tr>
<td>11.0</td>
<td>0.20</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Table 2: Effect of the B₁₂a concentration on the rate of O₂ uptake by B₁₂a + cysteine in O₂ saturated solutions containing 2x10⁻³ M EDTA; pH 10.0; 5x10⁻³ M cysteine

<table>
<thead>
<tr>
<th>10⁷[B₁₂a], M</th>
<th>rate, μM O₂ s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.78</td>
<td>0.737</td>
</tr>
<tr>
<td>1.34</td>
<td>1.22</td>
</tr>
<tr>
<td>3.88</td>
<td>2.27</td>
</tr>
<tr>
<td>5.82</td>
<td>3.11</td>
</tr>
<tr>
<td>7.76</td>
<td>4.08</td>
</tr>
</tbody>
</table>
Table 3: Effect of the cysteine concentration on both the rate of reduction of \( B_{12a} \) under \( N_2 \) and the rate of \( O_2 \) uptake in air saturated solutions at pH 10.0; \( 1.94 \times 10^{-5} \) M \( B_{12a} \); 25°C; \( y = 0.1; 2 \times 10^{-2} \) M EDTA

<table>
<thead>
<tr>
<th>10^3 (cysteine)</th>
<th>initial rate of ( O_2 ) uptake, ( \mu \text{M O}_2 \text{s}^{-1} )</th>
<th>( k_{\text{obs}} ) for ( O_2 ) uptake, ( \text{s}^{-1} )</th>
<th>( 10^2 k_{\text{obs}} ) for ( O_2 ) reduction, ( \text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.686</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>1.22</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>1.66</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>1.33</td>
<td>0.99</td>
<td>0.48</td>
</tr>
<tr>
<td>15.0</td>
<td>2.30</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>2.91</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td>30.0</td>
<td>3.07</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>40.0</td>
<td>3.55</td>
<td>1.83</td>
<td></td>
</tr>
<tr>
<td>50.0</td>
<td>3.90</td>
<td>2.01</td>
<td>1.7</td>
</tr>
<tr>
<td>60.0</td>
<td>4.05</td>
<td>2.09</td>
<td></td>
</tr>
<tr>
<td>80.0</td>
<td>4.22</td>
<td>2.18</td>
<td>3.0</td>
</tr>
<tr>
<td>100.0</td>
<td>4.40</td>
<td>2.27</td>
<td>3.6</td>
</tr>
</tbody>
</table>

\[ a \quad k_{\text{obs}} \, \text{for } O_2 \text{ uptake} = \frac{\text{initial rate of } O_2 \text{ uptake}}{(B_{12a})} \]

\[ b \quad \text{derived from the slope of the semilog plot (see chapter 6)} \]
Table 4: Effect of pH on the rate of $O_2$ uptake in the presence of $B_{12a}$ and dithiothreitol; $1.94 \times 10^{-5} \text{M } B_{12a}$; $1 \times 10^{-3} \text{M }$ dithiothreitol.

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial rate/μM O$_2$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.161</td>
</tr>
<tr>
<td>4.0</td>
<td>0.207</td>
</tr>
<tr>
<td>6.0</td>
<td>0.368</td>
</tr>
<tr>
<td>7.0</td>
<td>0.653</td>
</tr>
<tr>
<td>8.0</td>
<td>1.19</td>
</tr>
<tr>
<td>8.5</td>
<td>1.53</td>
</tr>
<tr>
<td>9.0</td>
<td>2.64</td>
</tr>
<tr>
<td>9.5</td>
<td>1.96</td>
</tr>
<tr>
<td>10.0</td>
<td>1.52</td>
</tr>
<tr>
<td>11.0</td>
<td>1.04</td>
</tr>
<tr>
<td>12.0</td>
<td>0.503, 0.575</td>
</tr>
</tbody>
</table>
### Table 5: Effect of the dithiothreitol concentration on the rate of $O_2$ uptake in the presence of $B_{12a}$ and dithiothreitol; $1,94 \times 10^{-5}$M $B_{12a}$, pH 10,0; air saturated solutions.

<table>
<thead>
<tr>
<th>$10^3$ (dithiothreitol), M</th>
<th>Initial rate, $\Delta [O_2]$/s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.667</td>
</tr>
<tr>
<td>1.0</td>
<td>1.29</td>
</tr>
<tr>
<td>2.0</td>
<td>1.95</td>
</tr>
<tr>
<td>4.0</td>
<td>3.35</td>
</tr>
<tr>
<td>6.0</td>
<td>4.33</td>
</tr>
<tr>
<td>10.0</td>
<td>6.03 ; 6.21</td>
</tr>
<tr>
<td>15.0</td>
<td>8.15</td>
</tr>
<tr>
<td>20.0</td>
<td>10.6 ; 10.7</td>
</tr>
</tbody>
</table>

### Table 6: Effect of the $B_{12a}$ concentration on the rate of $O_2$ uptake in the presence of $B_{12a}$ and dithiothreitol in air saturated solutions at pH 10,0

<table>
<thead>
<tr>
<th>$10^3$ ($B_{12a}$), M</th>
<th>Initial rate, $\Delta [O_2]$/s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1 \times 10^{-3}$M dithiothreitol</td>
<td>2$\times 10^{-2}$M dithiothreitol</td>
</tr>
<tr>
<td>0</td>
<td>0.143</td>
</tr>
<tr>
<td>0.388</td>
<td>0.465</td>
</tr>
<tr>
<td>0.776</td>
<td>4.42</td>
</tr>
<tr>
<td>1.94</td>
<td>1.52</td>
</tr>
<tr>
<td>2.91</td>
<td>14.3</td>
</tr>
<tr>
<td>3.88</td>
<td>19.0</td>
</tr>
<tr>
<td>7.76</td>
<td>4.65</td>
</tr>
<tr>
<td>11.6</td>
<td>6.75</td>
</tr>
<tr>
<td>15.5</td>
<td>9.02</td>
</tr>
</tbody>
</table>
Table 7: The effect of pH on the rate of O₂ uptake by
diaquocobinamide + thiols in O₂ saturated buffers;
1x10⁻⁷M diaquocobinamide; 2x10⁻²M thiol; 25°C

<table>
<thead>
<tr>
<th>pH</th>
<th>Ethanethiol</th>
<th>Mercaptoethanol</th>
<th>Cysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Co</td>
<td>+Co</td>
<td>-Co</td>
</tr>
<tr>
<td>4</td>
<td>0.91</td>
<td>0.55</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>0.11</td>
</tr>
<tr>
<td>7</td>
<td>1.16</td>
<td>1.87</td>
<td>0.50</td>
</tr>
<tr>
<td>8</td>
<td>1.65</td>
<td>2.64</td>
<td>0.55</td>
</tr>
<tr>
<td>9.0</td>
<td>0.61</td>
<td>6.11</td>
<td>0.55</td>
</tr>
<tr>
<td>9.5</td>
<td>1.10</td>
<td>12.3</td>
<td>0.55</td>
</tr>
<tr>
<td>10.0</td>
<td>1.38</td>
<td>14.9</td>
<td>0.44</td>
</tr>
<tr>
<td>10.5</td>
<td>1.63</td>
<td>12.1</td>
<td>0.55</td>
</tr>
<tr>
<td>11.0</td>
<td>0.83</td>
<td>6.9</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Initial rate/μM O₂ s⁻¹
Table 8: The effect of the diaziquoxinimide concentration on the rate of O\textsubscript{2} uptake by diaziquoxinimide + thiol in O\textsubscript{2} saturated buffers at the optimum pH; 2x10^{-2} M thiol; 25\degree C

<table>
<thead>
<tr>
<th>(10^{-3}) (DAC), K</th>
<th>Ethanechol (pH 10)</th>
<th>Mercaptoethanol (pH 9.5)</th>
<th>Cysteine (pH 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>initial -Co</td>
<td>initial +Co</td>
<td>initial -Co</td>
</tr>
<tr>
<td>0.11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.14</td>
<td>-</td>
<td>-</td>
<td>0.44</td>
</tr>
<tr>
<td>0.21</td>
<td>0.25</td>
<td>5.54</td>
<td>-</td>
</tr>
<tr>
<td>0.28</td>
<td>-</td>
<td>-</td>
<td>0.44</td>
</tr>
<tr>
<td>0.43</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.57</td>
<td>-</td>
<td>-</td>
<td>0.44; 0.99</td>
</tr>
<tr>
<td>0.64</td>
<td>1.10</td>
<td>11.7</td>
<td>-</td>
</tr>
<tr>
<td>0.85</td>
<td>-</td>
<td>-</td>
<td>0.50</td>
</tr>
<tr>
<td>0.86</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.07</td>
<td>1.38</td>
<td>14.8</td>
<td>-</td>
</tr>
<tr>
<td>1.14</td>
<td>-</td>
<td>-</td>
<td>0.44</td>
</tr>
<tr>
<td>1.22</td>
<td>-</td>
<td>-</td>
<td>0.44</td>
</tr>
<tr>
<td>1.50</td>
<td>1.05</td>
<td>21.4</td>
<td>-</td>
</tr>
<tr>
<td>2.14</td>
<td>0.94; 1.27</td>
<td>29.0; 28.5</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4: The effect of the thiol concentration on the rate of O₂ uptake by diquinocobinamide + thiols at the pH optimum in O₂ saturated buffers; 1×10⁻⁷ M diquinocobinamide

<table>
<thead>
<tr>
<th>10⁻³(thiol) M</th>
<th>Ethenethiol (pH 10)</th>
<th>Mercaptoethanol (pH 9.5)</th>
<th>Cysteine (pH 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial -Co initial +Co max +Co</td>
<td>Initial -Co initial +Co max +Co</td>
<td>Initial -Co initial +Co</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6.0</td>
<td>0.22</td>
<td>7.66, 13.31</td>
<td>-</td>
</tr>
<tr>
<td>7.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10.0</td>
<td>0.55</td>
<td>12.96, 2.20</td>
<td>-</td>
</tr>
<tr>
<td>12.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15.0</td>
<td>-</td>
<td>-</td>
<td>0.32, 3.28, 4.66, 4.81, 7.33</td>
</tr>
<tr>
<td>20.0</td>
<td>1.10</td>
<td>15.97, 0.55, 4.45, 9.66, 0.18, 1.87</td>
<td></td>
</tr>
<tr>
<td>30.0</td>
<td>1.98</td>
<td>21.21, -</td>
<td>-</td>
</tr>
<tr>
<td>40.0</td>
<td>3.30</td>
<td>23.56, -</td>
<td>-</td>
</tr>
<tr>
<td>50.0</td>
<td>2.42</td>
<td>23.09, -</td>
<td>-</td>
</tr>
<tr>
<td>60.0</td>
<td>1.65</td>
<td>22.81, -</td>
<td>-</td>
</tr>
<tr>
<td>69.6</td>
<td>1.80</td>
<td>22.44, 43.0</td>
<td>-</td>
</tr>
<tr>
<td>79.2</td>
<td>1.82</td>
<td>21.5, -</td>
<td>-</td>
</tr>
<tr>
<td>90.0</td>
<td>1.65</td>
<td>23.38, -</td>
<td>-</td>
</tr>
<tr>
<td>100.0</td>
<td>1.76</td>
<td>23.09, -</td>
<td>-</td>
</tr>
</tbody>
</table>
APPENDIX 9 : TABLES OF DATA FOR THE AUTOXIDATION OF
DITHIOTHREITOL CATALYSED BY BIS-HISTIDINE HEMIN
(DISCUSSED IN CHAPTER 9)

All experiments carried out in air saturated solutions with
\( \mu = 0.5 \) at 25°C.

Table 1 : The effect of dithiothreitol concentration on the rate
of \( \mathcal{O}_2 \) uptake catalysed by hemin + histidine;
6x10^-6 M hemin; 0.4M histidine; pH 10.0.

<table>
<thead>
<tr>
<th>( 10^3 ) (dithiothreitol), ( X )</th>
<th>( \mu ) ( \mathcal{O}_2 ) ( \text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.414</td>
</tr>
<tr>
<td>0.1</td>
<td>0.375</td>
</tr>
<tr>
<td>0.25</td>
<td>0.372</td>
</tr>
<tr>
<td>0.5</td>
<td>1.04</td>
</tr>
<tr>
<td>0.75</td>
<td>0.897</td>
</tr>
<tr>
<td>1.00</td>
<td>0.722</td>
</tr>
<tr>
<td>3.05</td>
<td>0.621</td>
</tr>
<tr>
<td>5.00</td>
<td>0.543</td>
</tr>
<tr>
<td>10.0</td>
<td>0.520</td>
</tr>
<tr>
<td>20.0</td>
<td>0.529</td>
</tr>
</tbody>
</table>
Table 2: The effect of the hemin concentration on the rate of \( \text{O}_2 \) uptake catalysed by hemin + histidine at pH 10.0 in 0.4M histidine

<table>
<thead>
<tr>
<th>( 10^6 ) (hemin)</th>
<th>Initial rate, ( \text{\textmu M} \text{O}_2 \text{s}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{M} )</td>
<td>low (dithiothreitol ( (3 \times 10^{-4} \text{M}) ))</td>
</tr>
<tr>
<td>0</td>
<td>0.561</td>
</tr>
<tr>
<td>2.52</td>
<td>1.02</td>
</tr>
<tr>
<td>3.02</td>
<td>1.06</td>
</tr>
<tr>
<td>4.05</td>
<td>-</td>
</tr>
<tr>
<td>6.04</td>
<td>1.12</td>
</tr>
<tr>
<td>8.10</td>
<td>-</td>
</tr>
<tr>
<td>12.1</td>
<td>1.62</td>
</tr>
<tr>
<td>16.2</td>
<td>-</td>
</tr>
<tr>
<td>18.1</td>
<td>2.08</td>
</tr>
<tr>
<td>20.25</td>
<td>-</td>
</tr>
<tr>
<td>20.66</td>
<td>-</td>
</tr>
<tr>
<td>24.16</td>
<td>2.42</td>
</tr>
<tr>
<td>28.36</td>
<td>-</td>
</tr>
<tr>
<td>30.20</td>
<td>3.06</td>
</tr>
<tr>
<td>34.44</td>
<td>-</td>
</tr>
<tr>
<td>40.52</td>
<td>-</td>
</tr>
</tbody>
</table>
The effect of the histidine concentration on the rate of $O_2$ uptake in the presence of hemin + histidine at low dithiothreitol concentration: (3x10$^{-8}$M); 26, 3x10$^{-6}$M hemin; pH 10.0

<table>
<thead>
<tr>
<th>Histidine (M)</th>
<th>Initial rate (with hemin), µM O$_2$ s$^{-1}$</th>
<th>Initial rate (- hemin), µM O$_2$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>2.17</td>
<td>0.515</td>
</tr>
<tr>
<td>0.30</td>
<td>2.34</td>
<td>0.639</td>
</tr>
<tr>
<td>0.40</td>
<td>2.20 ; 2.70</td>
<td>0.453 ; 0.561</td>
</tr>
</tbody>
</table>
REFERENCES


39a J. Cann, Biochem., 5, 3435 (1967).


77b D. Chin, G. La Mar, A. Balch, J. Amer. Chem. Soc., 102, 4344 (1980).


