Ferrilegoglobin as an Oxidizing Agent for Hydrazine

Donald B. Larson
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FERRILEGOGLOBIN AS AN OXIDIZING AGENT FOR HYDRAZINE

by

Donald B. Larson

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE in

Chemistry

UTAH STATE UNIVERSITY
Logan, Utah
1959
ACKNOWLEDGMENT

I wish to express my appreciation to my wife, Mary, and our two lovely children, Doug and Susan, for the inspiration they have given me throughout this study. I am also indebted to Dr. Norman Bauer for his continual interest and help and to the Herman Frasch Foundation for financing this study.

Donald B. Larson
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INTRODUCTION

The system in which atmospheric nitrogen succumbs to enter Nature's cycle has a yet proven evasive with respect to the actual chemical reactions. At present several types of systems are proposed but their chemical makeup is practically unknown.

The actual number of pathways by which nitrogen is converted into usable compounds is in itself not determined, but one very interesting system has drawn the attention of this research group. The system under study is that occurring in the nodules of field grown soybeans.

The nodules of field grown soybeans have a very interesting chemical constituent. This constituent is a heme protein, legoglobin, which Virtanen showed to be always associated with nitrogen-fixing activity in the nodules. The efforts of this research are directed towards arriving at a partial answer relative to the function of this heme protein in the fixation process.

The presence of a heme protein in a biological system leads to speculation on three possible functions, (1) an oxygen carrier, (2) an oxygen storer, or (3) an enzymatic role in an oxidation-reduction system. This reasoning is stimulated by the analogous functions carried on by other heme proteins in other types of systems.

It is likely that legoglobin causes reduction of nitrogen and that the first stable intermediate is hydrazine.
This thesis contributes a study of the effect of heme proteins upon hydrazine, with particular emphasis on the effect of hemoglobin.

This study has been based on the kinetics of the nitrogen-liberating reaction involved in hydrazine oxidation, using a Warburg apparatus to follow changes of gas pressure with time. That is, some reverse steps of a possible nitrogen fixing reaction have been examined. Comparisons with the role of non-heme iron in this reaction have also been made.
LITERATURE SURVEY

The Function of Legoglobin

The function of legoglobin in the process of nitrogen fixation at present is only speculative. No one has satisfactorily explained its role, but a number of ideas have been presented which will be discussed here.

Virtanen\(^{(31)}\) in 1945 found that in sufficient concentration of oxygen the nodules formed by effective bacterial strains always have a red pigment. Nodules from the ineffective strain never contain red pigment and do not fix nitrogen. In oxygen-free conditions no red pigment and no nitrogen fixation occurs. In the dark, active nodules turn green and do not fix nitrogen until the plant is left in the light long enough to turn the nodules red. All the observations indicate that the red pigment is essentially associated with nitrogen-fixation and is necessary to this phenomenon. These results were confirmed by Smith\(^{(27)}\) in 1949.

Keilin and Smith\(^{(19)}\) in 1947 found that within the root nodules of leguminous plants legoglobin displays its usual property of oxygenation. On spectroscopic examination, the nodules showed only the mixture of ferrolegoglobin and oxylegoglobin. No ferrilegoglobin was detected even in the plants kept in the dark for several days. No evidence was found to support the view that nitrogen fixation involves a cyclical change of the valency of legoglobin iron.
Burris and Wilson\textsuperscript{(10)} in 1952 felt that although the participation of legoglobin in the nitrogen-fixation reaction appears to be an attractive and plausible function, examination of the evidence to that date suggested that other alternatives had not been eliminated.

Finally in 1957 Hamilton, Shug and Wilson\textsuperscript{(17)} reported that extracts of soybean nodules exposed to various gases showed spectral changes indicating a conversion of ferrolegoglobin to ferrilegoglobin in the presence of nitrogen. In the presence of hydrogen the opposite reaction was reported. They felt that their data supported the suggestion that legoglobin may act as a oxidation-reduction catalyst in symbiotic nitrogen fixation.

If this information is coupled with the theory proposed by Bauer\textsuperscript{(15)} in 1959, there exists an elegant and plausible system for symbiotic nitrogen-fixation. This system contains the heme protein, legoglobin, as a very essential entity, the oxidation-reduction catalyst. Hydrazine would be the first isolable reduction product.

\textbf{The Oxidation and Decomposition Chemistry of Hydrazine}

Reactions involving nitrogen produced from hydrazine are of interest in the investigation of the catalysis of fixation. Those reactions involving ferric ion will be of special interest.

\textbf{Decomposition of hydrazine}

The change in free energy for the reaction

$$3\text{N}_2\text{H}_4 = \text{N}_2 + 4\text{NH}_3$$

is $\Delta F^\circ = -114,700 \text{ cal.}$.\textsuperscript{(3)} According to this the reaction should go; however, under ordinary conditions it does not take place.
Tanatar (28) in 1903 and Gutbier (16) in 1913 found that, in the presence of active platinum, hydrazine did decompose in acid solution according to the following equation:

$$2N_2H_5^+ = 2NH_4^+ + H_2 + N_2$$

and in basic solution according to this equation:

$$3N_2H_4 = 2NH_3 + 2N_2 + 3H_2.$$  

Abel (1) in 1953 proposed a mechanism for this decomposition process.

The limited number of experimental results now in the literature on the decomposition of hydrazine shed little light upon the subject. Adequate interpretations of the results are so difficult to attain that very little work has actually been done.

The information that Tanatar and Gutbier have furnished on this decomposition leads to speculation that where chemical oxidation of hydrazine by various metallic salts, such as ferric salts, has been studied, the non-stoichiometric nature of the reaction can be explained in part by the catalytic effect of ions.

**Oxidation of hydrazine**

Hydrazine exhibits some unique and interesting characteristics. In addition to hydrazine being capable, given the proper environment, of both oxidation and reduction, it has the property of yielding a variety of oxidation products.

With Zn, Sn$^{++}$, and Ti$^{+++}$ reduction to NH$_3$ is observed. Oxidation yields $N_2$ only, in one case, in another case $N_2$ and NH$_3$, and in still another case NH$_3$ and HN$_3$. (18)

The currently accepted scheme for the mechanisms of oxidation of hydrazine is that proposed first by Kirk and Browne (9) and later modified by Higginson and Wright (18). The modified scheme is as follows.
The oxidation of hydrazine by 2-electron transfer reagents yields nitrogen:

\[ \text{N}_2\text{H}_4 \quad -2e^- \quad \text{N}_2\text{H}_2 \quad -2e^- \quad \text{N}_2 \]

If a one electron transfer reagent such as ferric ion is used, there are two possible outcomes for hydrazine oxidation in aqueous solution. The relative importance of each depends on the pH and the oxidizing agent.

\[ \frac{k_2}{k_1} = \text{NH}_3 \]

Oxidation via the intermediate tetrazane, \( \text{N}_4\text{H}_6 \), yields \( \text{NH}_3 \) as a product. This sequence of reactions was proposed by Higginson and Wright in 1953 and substantiated by Powell and Cahn (11) in 1954 and Rosseinsky (25) in 1957. Rosseinsky reports that for ferric ion the ratio of rate constants \( k_2/k_1 \) is 0.15 at pH 1. The reaction through \( k_1 \) yields 0.5 moles of nitrogen per mole of \( \text{Fe}^{+3} \) reduced, while the reaction through \( k_2 \) yields 0.25 moles of nitrogen per mole of \( \text{Fe}^{+3} \) reduced.

The actual reaction path for the one electron transfer reagents depends on the oxidizing agent and also upon the pH. In extremely alkaline solutions the tetrazane intermediate is not formed and thus no ammonia is produced. As the pH is lowered there appears to be a competition between the two reactions. The limiting reaction as the pH is lowered is probably the reaction through the tetrazane intermediate to produce two ammonia molecules per nitrogen molecule. This limiting reaction has as yet not been achieved.
In a great number of these reactions hydrazoic acid is produced; in most cases however only as a minor constituent. In the case of ferric ion, it is only a minor product in the reaction with hydrazine.

**Effect of oxygen on the oxidation of hydrazine**

Cuy and Bray in 1924 and Gilbert in 1926 and many others since have reported that acid solutions of hydrazine, those containing the hydrazonium ion, $N_2H_5^+$, are stable and not affected by oxygen. In alkaline solutions, however, hydrazine is subject to direct oxidation to $N_2$ and $H_2O_2$ by oxygen. The following data were collected by Cuy and Bray on the decomposition of hydrazine sulfate solutions at various acid and alkaline concentrations.

<table>
<thead>
<tr>
<th>Conc. of $N_2H_4$ M.</th>
<th>Acidity or alkalinity</th>
<th>Time</th>
<th>Decomposed %</th>
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</thead>
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<tr>
<td>0.11</td>
<td>$H^+$ = 0.17</td>
<td>2 months</td>
<td>0</td>
</tr>
<tr>
<td>0.008</td>
<td>$H^+$ = $10^{-7}$</td>
<td>2 hours</td>
<td>0.9</td>
</tr>
<tr>
<td>0.015</td>
<td>$H^+$ = $10^{-7}$</td>
<td>48 hours</td>
<td>8.0</td>
</tr>
<tr>
<td>0.05</td>
<td>$OH^-$ = 0.5</td>
<td>5 minutes</td>
<td>0.9</td>
</tr>
<tr>
<td>0.05</td>
<td>$OH^-$ = 0.5</td>
<td>16 hours</td>
<td>20.0</td>
</tr>
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</table>

To substantiate that their results were due to oxygen oxidation rather than just decomposition of the hydrazine they performed the experiments in a nitrogen atmosphere and found that even in 0.5 M. $OH^-$ the samples after 26 hours showed no decrease in hydrazine concentration.

Gilbert in 1926 found $H_2O_2$ present whenever oxygen reacted with hydrazine and he also determined the rate equation for the decomposition of hydrazine in the presence of oxygen:

$$-\frac{dC}{dt} = (k)(P_{O_2})^{\frac{1}{n}}$$

where $C$ is the original concentration of the hydrazine and $n$ is a constant equal to 2 at 25° C.
Brown (8) in 1947 verified Gilbert's results and also found that small traces of metallic ions, notably copper, exert a tremendous catalytic effect upon the oxidation of hydrazine by oxygen. The assumption was that the catalytic effect is due to a cuprous ion-cupric ion equilibrium, reduction of the latter being caused by hydrazine and oxidation of the former by oxygen. Here too, $H_2O_2$ was found in all experiments.

Abel (1) has proposed two possible mechanisms through which the oxidation of hydrazine can take place involving oxygen as an oxidizing agent. In each mechanism the first step is an interaction between hydroxide ion and oxygen

$$O_2 + OH^- \rightarrow O_2^- + OH$$

The two possible schemes which Abel presented are:

**I**

<table>
<thead>
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<th>Reaction</th>
<th>I</th>
<th>Reaction</th>
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<tr>
<td>$O_2 + OH^- \rightarrow O_2^- + OH$</td>
<td></td>
<td>$2O_2 + 2OH \rightarrow 2O_2 + 2OH$</td>
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<tr>
<td>$N_2H_4 + OH = NH_2^+ + NH_2 + OH^-$</td>
<td>$N_2H_4 + O_2^- = NH_2^+ + NH_2 + O_2^-$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$NH_2 + O_2^- \rightarrow NH_2^+ + 2O^-$</td>
<td>$NH_2 + O_2^- \rightarrow NH_2^+ + O_2^-$</td>
<td>$2O_2^= + 4H^+ \rightarrow 2H_2O_2$</td>
<td></td>
</tr>
<tr>
<td>$2O^+ + 2H^+ \rightarrow 2OH$</td>
<td></td>
<td>$2NH_2^+ = 2NH + 2H^+$</td>
<td></td>
</tr>
<tr>
<td>$2NH + 2OH = 2HNOH$</td>
<td>$2NH + 2OH = 2HNOH$</td>
<td>$2HNOH = HNNOH + HOH$</td>
<td></td>
</tr>
<tr>
<td>$2HNOH = HNNOH + HOH$</td>
<td>$2HNOH = HNNOH + HOH$</td>
<td>$HNNOH = N_2 + HOH$</td>
<td></td>
</tr>
<tr>
<td>$HNNOH = N_2 + HOH$</td>
<td>$HNNOH = N_2 + HOH$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

with the overall reactions being

$N_2H_4 + O_2 = N_2 + 2HOH$ $N_2H_4 + 2O_2 = N_2 + 2H_2O_2$
Abel justifies scheme I by pointing out that a radical such as OH would give the same tests as $\text{H}_2\text{O}_2$. He feels, as do Rostorfer and Totter\(^{(26)}\), that radical formation rather than $\text{H}_2\text{O}_2$ formation in the oxidation of hydrazine by oxygen is certainly a strong possibility and cannot be ignored.

**The Role Hydrazine Plays in the Nitrogen-Fixation Picture**

It has for many years been postulated that hydrazine may play a part in the nitrogen-fixation picture. However, since hydrazine is relatively unstable and extremely toxic the view that predominated was that the existence of such a component in a biological system was unlikely.

Once in a while through the years articles would appear in the literature which claimed experimental evidence for the involvement of hydrazine in the fixation process. For example, Fedorov\(^{(13)}\) in 1952 reported that he found hydrazine present in the *Azotobacter agile* nitrogen-fixation system. But progress in removing all doubt in regard to these reports was slow.

Recently the results of the research in this field has shifted attention more and more toward the possible involvement of hydrazine as an intermediate in the nitrogen-fixation process.

Bach\(^{(4)}\) in 1957 found that hydrazine disappeared from washed suspensions of *Azotobacter vinelandii* (a well known nitrogen-fixation system) and that organic azines were formed in the suspensions. He also found these azines in the nodules of field grown soybeans. From this information he proposed a theory involving hydrazine as an intermediate in the process of nitrogen-fixation.
More recently, in 1959, Bauer(5) has proposed a theory of symbiotic nitrogen-fixation which involves the formation of hydrazine as the first stable intermediate.

**Reactions of Hydrazine and Heme Proteins**

At the present time there is very little literature available on the kinetics of reaction of hydrazine with heme proteins. However, Rostorfer and Totter(26) have investigated the reaction of hydrazine, phenylhydrazine, phenylhydroxylamine and hydroxylamine with oxyhemoglobin and have determined that some type of radical, or possibly H$_2$O$_2$ is formed. The only other information available on the reactions of hydrazine with heme proteins is that hydrazine in small concentration forms a complex with oxymyoglobin which has spectral absorption maxima at 564, 552, and 528 nm. (21)
EXPERIMENTAL METHODS

Principles of Warburg Manometry

Most of the research that has been done in support of this thesis has utilized the Warburg apparatus; therefore, this section will begin with a discussion of the principles of Warburg manometry. (30)

The manometer operates on the principle that at constant temperature and constant gas volume any changes in the amount of a gas can be measured by changes in its pressure.

The apparatus consists of a detachable flask with one sidearm. This flask is used to contain initially the separated reactants, and later the actual reaction mixture. The flask is immersed in the water bath at a controlled temperature (20.00 ± 0.02° C.) and the system is shaken to promote a rapid gas exchange between the liquid and the gas phase. It is assumed that the temperature of the manometer, which is not immersed, does not differ greatly from that of the flask. This assumption is valid in this experimental sequence since the maximum error from this effect is 0.009cc per 5° C temperature difference which is less than the total error in calculating the total volume of the system.

The scale of the manometer is graduated in centimeters and in millimeters. The readings are recorded in millimeters.

The manometer used has an open and a closed end. A reference point on the closed side of the manometer is chosen, and the liquid
in the closed arm of the manometer is always adjusted to this point before the pressure changes are recorded.

The important assumptions in this method are (1) that only one gas is being evolved or taken up, (2) that the concentration of dissolved gas is directly proportional to the pressure above the liquid, and (3) that the solubility of individual gases in a mixture is independent of the pressure of other gases. Using these assumptions as a starting point, it is possible to derive a useful quantity called the flask constant. (30)

In the gas phase one has a volume of gas, \( V_g \), at a temperature, \( T \), and a pressure \( P - R \), where \( R \) is the vapor pressure of the liquid. This gas volume can be changed to standard conditions \( (P_o, V', T') \), assuming the ideal gas law:

\[
\frac{PV}{T} = \frac{P_o V'}{T'}
\]  

In the flask

\[
\frac{(P - R)V}{T} = \frac{P_o V'}{273^o}
\]  

(2)

The amount of gas in the liquid is

\[
\frac{V_1 \propto (P - R)}{P_o}
\]  

where \( \propto \) is the solubility of the gas being evolved or taken up at a partial pressure of 1 atmosphere and \( V_1 \) is the volume of the liquid present. The gas present at the start was that in the gas phase plus that in the liquid phase or

\[
V_{\text{initial}} = \frac{V_g 273 (P - R)}{T P_o} = \frac{V_1 \propto (P - R)}{P_o}
\]  

(4)
After a change, \( x \), in gas volume the pressure change corresponds to an amount \( h \) mm of manometer fluid. The new pressure is \( (P - R)' + h \), where \( (P - R)' \) is the new pressure of gas due to changes in room pressure and the temperature of the water bath. The gas volume will be

\[
V_{\text{initial}} - x = \frac{V_g}{T} \frac{273}{P_o} ((P - R)' + h) + \frac{V_1}{P_o} ((P - R)' + h).
\]

(5)

To solve for \( x \) subtract equation 5 from equation 4 and the result is

\[
x = \left( \frac{V_g}{TP_o} + \frac{V_1 \alpha}{P_o} \right) (P - R)'.
\]

(6)

providing that one corrects for the difference between \( (P - R) \) and \( (P - R)' \). This correction is made by using a hydrobarometer to measure the fluctuations; and then the appropriate correction is applied to \( (P - R)' \) so that

\[
(P - R)' + \text{correction} = (P - R).
\]

(7)

Since \( V_g, T, \alpha, V_1 \), and \( P_o \) can be calculated for a given experiment and are constant

\[
x = \frac{h}{k}.
\]

(8)

where

\[
k = \frac{V_g}{TP_o} + \frac{V_1 \alpha}{P_o}.
\]

(9)

and is defined as the flask constant, with \( P_o \) expressed in the same units (mm.) as the scale readings. The flask constant can then be used to convert manometer reading in millimeters directly to microliters of gas volume change, reduced to the arbitrary standard of \( T = 273.2^\circ K \). and \( P = P_o = 1 \) atm.
Using a proper manometric fluid, such as Brodie’s solution, one can adjust the manometer readings to a convenient value such that 10,000 mm. equals 1 atm. Such manometer solutions have density 1.033 compared to 13.60 for Hg so that

\[ p_o = \frac{(760)(13.60)}{1.033} = 10,000 \]

**Methods Used to Obtain the Flask Constants**

In order to apply the principles described above it is necessary to check the sensitivity of the constant temperature bath and to calculate the flask constants.

A detailed check of the sensitivity of the Aminco No. BT-1 Warburg constant temperature control was carried out at various temperatures with a Beckman thermometer. The results indicated variations of less than 0.02 degrees over 90 min. intervals. The magnitude of this variation is so small that no significant deviation in the level of the manometer fluid can be attributed to fluctuations in the temperature of the bath.

In order to calculate the flask constants one must know the volume of the gas, the volume of the liquid, the temperature, the solubility of the gas involved and the standard pressure.

The volume of the liquid was fixed in all experiments at 1.10±0.01 ml. The temperature for all experiments was 20.00 ± 0.02° C. The solubility of the gas at 1 atm. was that of nitrogen at 20° C. which is 0.0154 ml/ml. Brodie’s solution was used as the manometer fluid, the density being checked to ± 0.005 g/ml with a pycnometer. This solution gives a standard pressure of 10,000 mm. for 1 atm. of pressure.
In order to find the volume of the gas it was necessary to find the volume of the closed system. The small 5 ml. Warburg flasks used in the experiments were calibrated by weighing the flask and then re-weighing the flask filled with mercury. The temperature was measured to \( \pm 0.5^\circ\). The mercury level was determined by placing the flask firmly on the manometer sidearm and adjusting the level to an etch mark on the side-arm. The known density of mercury at the recorded temperature was then used to calculate the volume of the flask in question. The remaining volume, that volume of capillary from the sidearm etch mark to the reference point on the right hand side of the manometer, was determined by marking two readings, one at the zero level and another at the 30 cm. level, using an Aminco Warburg Manometer Calibrator. With these two values one can, assuming a constant bore for the capillary between these two points, calculate the volume to all possible reference points. The total volume calculated in this manner minus 1.10 \( \pm 0.01 \) ml. will represent the volume of the gas in the system. The following flask constants illustrate the errors involved:

<table>
<thead>
<tr>
<th>Flask number</th>
<th>Vg (cc.)</th>
<th>k (at 15 cm. mark)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>4.30\pm0.01</td>
<td>0.425\pm0.001</td>
</tr>
<tr>
<td>W</td>
<td>4.79\pm0.01</td>
<td>0.453\pm0.001</td>
</tr>
<tr>
<td>K</td>
<td>4.82\pm0.01</td>
<td>0.455\pm0.001</td>
</tr>
</tbody>
</table>

**Procedures for Handling the Oxygen Problem**

**Method used to eliminate oxygen from the Warburg system**

The elimination of the dissolved oxygen from the liquid phase was tedious but very critical. The importance of a careful elimination was
substantiated by the results of analysis for oxygen present in the system. Only where there was a rigorous elimination procedure carried out was it possible to eliminate oxygen down to a level which would leave the ferromyoglobin-oxymyoglobin ratio near 25.

The oxygen was removed from the system by allowing the vessel to shake under a pressure of approximately 30 mm of Hg. During this shaking period the manometer fluid was continually disturbed so that the bubbles forming in the liquid would join the gas phase. The system was then returned to 1 atm. of pressure by allowing nitrogen, which had passed through a vanadium sulfate-amalgamated zinc purification train, to pass into the system. This procedure was repeated 6 times during a 90 min. interval. At the completion of this procedure the reactants, now under 1 atm. of nitrogen, were allowed to equilibrate with the gas phase for a 1 hour period.

Procedure used to determine oxygen in the Warburg system

The method chosen to test for oxygen was that of Bradshaw and Silverman. The reactions are as follows:

\[
\begin{align*}
KI + 2NaOH + MnCl_2 &= Mn(OH)_2 + KI + 2NaCl \\
2HOH + 4Mn(OH)_2 + KI + O_2 &= 4Mn(OH)_3 + KI \\
2Mn(OH)_3 + 2KI &= I_2 + 2Mn(OH)_2 + 2KOH \\
I_2 + Organic Solvent &= I_2 - Organic Solvent \\
Mn(OH)_2 + 2HCl &= MnCl_2 + 2HOH
\end{align*}
\]

The I_2 in the organic solvent was analysed for colorimetrically in the Beckman DU Spectrophotometer and compared with a standard curve. Toluene was chosen as the solvent for the iodine and I_2 in this solvent.
was found, by use of the Perkin-Elmer Model 4000 Spectrophotometer, to give a maximum absorbance at 495 \( \mu \)m.

The following modified procedure was followed in testing for oxygen.

All reagents were boiled and purged with oxygen free nitrogen for five minutes. The reagents were then stoppered while hot to prevent oxygen from redissolving. A two sidearm Warburg flask was used as the reaction vessel. In one sidearm was placed the NaOH and in the other the KI. The MnCl\(_2\) was placed in the bottom of the flask and a mixture of HCl and toluene was introduced into a compartment above one sidearm which would allow mixing with the other reagents later on. The system was then joined to the Warburg manometer and elimination of the oxygen present in the system was carried out under conditions duplicating those prevailing during a measurement of gas evolution. At the completion of this procedure the KI, NaOH and MnCl\(_2\) were mixed and allowed to shake with the purified nitrogen at 1 atm. for 1 hour. At the completion of this shaking period the HCl and toluene were introduced through one sidearm and allowed to mix with the other reagents. This mixing was followed by a 15 min. shaking period. At this point the reaction had been stopped by the HCl and the Toluene had dissolved the iodine formed.

Colorimetric determination of the amount of iodine was made by comparing the reading of this solution on the Beckman DU Spectrophotometer at 495 \( \mu \)m with that of a standard solution.

The most successful runs of oxygen determination indicated between 0.8 and 1.5 \( \mu \)l of oxygen for a manometer system; this corresponded to 44 and 82 p.p.m. of oxygen. In actual gas-evolution measurements under conditions where about 1 atm. of an inert gas was added after using
the above evacuation procedure to remove air, the oxygen impurity was estimated to be approximately 100 p.p.m. or less, which was low enough to insure 95% un-oxygenated ferromyoglobin.

**Preparation of Reagents**

**Hydrazine**

The solutions of hydrazine were prepared from 'Fisher Certified Reagent' hydrazine hydrochloride crystals Lot No. 770105 certified to be 99.9% pure. The resulting solutions were adjusted to pH 7.30 with NaOH and then diluted, by using a volumetric flask, to a concentration of 1.00 M.

**Potassium ferricyanide**

The potassium ferricyanide solutions were prepared by dissolving 'Baker Analysed' potassium ferricyanide, certified to be 99.97% pure, in phosphate buffer of the desired pH. The solutions were diluted to the desired concentration with the phosphate buffer.

**Buffers**

The buffers were made up according to the following table:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Tonic strength</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethanolamine</td>
<td>7.90</td>
<td>0.05</td>
<td>223.8 g. of Triethanolamine and 600 ml of 1.1 M HCl in 15 liters.</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>6.60</td>
<td>0.20</td>
<td>200.5 g. of Phosphoric Acid and 100 g. of NaOH crystals in 18 liters.</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>7.34</td>
<td>0.20</td>
<td>134 g. of Phosphoric Acid and 97 g. of NaOH crystals in 15 liters.</td>
</tr>
</tbody>
</table>

The reagents described thus far were deemed of adequate purity for this preliminary survey of the reactions.
Preparation of myoglobin

The myoglobin used in the experiments was prepared, with the help of D. O. Edlund, using the method of George and Stratman, by grinding two fresh horse hearts with an electric meat grinder. The red water solution which was obtained, after two days of standing at zero degrees, was drained from the twelve pounds of meat by squeezing the solution through cheese cloth. The solution was then neutralized and treated with 1/5 of its volume of saturated basic lead acetate to remove muscle globulins and most of the other impurities. After centrifugation, the solution was again neutralized and sufficient solid sodium phosphate was added to remove excess lead. The pH was again adjusted to 7 by adding solid NaH$_2$PO$_4$ and the solution was centrifuged. All of these steps were carried out at 0° C. The myoglobin solution was further purified by crystallization induced by saturation of the solution with ammonium sulfate. This treatment separates the myoglobin from any traces of hemoglobin which may be present, since hemoglobin is almost completely insoluble in phosphate buffer of this molarity. After three days the precipitate which formed was filtered through a suction flask. The resulting paste was placed in 3 M. phosphate buffer pH 6.8. A metmyoglobin cyanide determination at 542 μm, using an extinction coefficient 11.3 Mm/l., which is based on a molecular weight of 18,400, indicated a yield of approximately 6 grams.

Electrophoretic, ultracentrifuge and spectrophotometric data were used as criteria for purity. The electrophoresis patterns obtained were very similar to those obtained by Theorell and Akeson. The ultracentrifuge run gave indication of only one component which had a
sedimentation constant 1.80 ± 0.1 which is equal to that found by Theorell and Akeson within experimental error. The spectrum of the ferrimyoglobin gave the characteristic absorption shoulder near 525 μm.

The oxygen complex of ferromyoglobin was obtained by reducing a solution of ferrimyoglobin at the required pH with sodium dithionite in slight excess, then dialyzing the solution in buffer until all of the dithionite was removed. The formation of oxymyoglobin was indicated by the change from brown to pink, and identified by the characteristic absorption bands at 542 and 580 μm. Ferromyoglobin was prepared by deoxygenating oxymyoglobin in the Warburg apparatus. Degassing times of approximately 15 min. under conditions similar to that described for removing O₂ from the system were used. A characteristic color change of the sample in the Warburg flask, from pink to brown, confirmed the conversion of MbFe⁺²·O₂ to MbFe⁺² during the degassing. The ferrimyoglobin was prepared by dialyzing the paste, at the required concentration, against a buffer solution in the presence of potassium ferricyanide. The excess ferricyanide was dialyzed out by changing the buffer solution several times with constant rotation of the dialysis bag.

Preparation of hemoglobin

The horse hemoglobin used in these experiments was prepared by R. G. Mortimer(24) using a modification of the method of Atschul, Sidwell and Hogness.(2) The fresh horse blood was defibrinated by stirring with a wire loop. After the fibrin was removed the cells were centrifuged out and were removed and washed. Distilled water
and toluene were added and the mixture was centrifuged. A dark red
solution with a clear toluene layer resulted. The water phase was
separated out and ammonium sulfate was added to precipitate the product.
The paste was stored at 0° C. K. Abel dialyzed the resulting paste
against distilled water and then lyophilized the resulting solution.
The lyophilized protein, which was stored at 0° C. and was used in all
hemoglobin experiments, would still contain the more soluble impurities
such as cytochrome, but other differences were not expected.

The various oxidation states were attained under the same
procedure described for myoglobin.

**Preparation of legoglobin**

The following procedure was carried out by K. Abel in preparing
the legoglobin. The nodules from field grown soybeans harvested in
July of 1958 were ground in 1.6 M ammonium sulfate solution. The
resulting solution was centrifuged in a refrigerated centrifuge and
then subjected to a salting-out process which involved raising the
molar concentration of ammonium sulfate in steps of approximately
1 molar units from 1.1 M to 5 M. For each step the pH was adjusted
to 7,2 and then the solution was centrifuged. All of the steps were
carried out of 0° C. Only the most soluble product, that which was
extracted with 5M ammonium sulfate and marked series J, was used
in this experimental sequence. Several days prior to the use of the
legoglobin in March of 1959, the author of this thesis subjected the
series J legoglobin to a refractionation process. Starting with
approximately 1.5 g. of material the fractionation continued until
only the most soluble 1 g. portion was left.
An electrophoretic pattern and a spectrum were run to establish the purity of the resulting material. The electrophoretic pattern exhibited the same two components which Virtanen\(^{(32)}\) observed, but where he noted a ratio 1:1 in the peaks the observed ratio in this pattern was about 3:1. The visible spectrum which was taken at pH 7.9 exhibited no distinguishable difference from a sample of legoglobin having the same electrophoretic pattern as Virtanen described.

The preparation of the various oxidation states of legoglobin was carried out exactly as was described for myoglobin.

**Spectrophotometric Methods to Obtain Difference Spectra**

The Model 4000 Perkin-Elmer Double Beam Spectrophotometer not only proved to be a valuable tool in giving a qualitative picture of the heme protein product in the Warburg runs, but also gave valuable information relative to the rate process for the hydrazine-myoglobin reaction. A technique was used whereby it was possible to run difference spectra as a function of time.

The Perkin-Elmer 1.00 cm. quartz cuvettes were both filled with myoglobin solution and then purged with oxygen free nitrogen gas for several minutes to eliminate as much oxygen as possible from the system. After the purging was completed the cuvettes were sealed by tightening a screw clamp around the Tygon tubing attached to the cuvette opening. Hydrazine was placed in one of the Tygon tubes and purged with nitrogen. A clamp was then placed above the hydrazine level and tightened. A baseline was drawn at 50% transmittance with myoglobin only in each light path. The hydrazine was then allowed to mix with the myoglobin and the reaction was followed by observing the changes in the spectrum as a function of time.
RESULTS

The Effect of Oxygen and pH on Phosphate Buffer-Hydrazine Solutions

In order to establish that all of the gas changes which occur in the proposed reactions are due only to the interaction of hydrazine and the iron-containing compound, it was necessary to check the gas evolution or uptake, if there was any, which was due to the interaction of the buffer with hydrazine. The following interesting results were observed.

At a pH 7.3 the phosphate buffer and the hydrazine did not react to produce any significant amount of gas at any level of oxygen used (levels checked were 0, 5, 15, and 21% with an average barometric pressure equal to 635 mm. of Hg). However in the case of phosphate buffer at pH 6.6, a startling result was observed. At the zero oxygen level no reaction occurred, but as the oxygen level was increased the rate of gas evolved increased up to 0.15 ± 0.02 µl/min. at the 21% level, when hydrazine was 0.033 M. (see Fig. 1). According to Gilbert's rate expression, page 7, there should have been less than 1/10 of this amount of gas produced.

An explanation for this peculiar result has not been found. Some preliminary infra-red spectrophotometric work has been carried out on the gas but at present it has not been identified. Absorption bands at 8230, 8160, 7340, 5740, 4350, and 3450 µm. have been observed. Further work on this problem is needed to elucidate this unique interaction.

Since the pH 6.6 phosphate buffer gives this unexplained interaction with hydrazine, it was decided that all experiments with the heme proteins should be carried out using the pH 7.3 phosphate buffer.
Fig. 1 The Effect of Oxygen and pH on Phosphate Buffer-Hydrazine Solutions

Curve I
- Reactants: Phosphate Buffer and Hydrazine
- Concentration of Buffer: $\mu = 0.20$ M
- Concentration of $N_2H_4$: $3.3 \times 10^{-3}$ M
- $pH$ of Buffer: 6.62
- $pH$ of $N_2H_4$: 7.30
- Atmosphere: Solutions were purged with nitrogen
- Final $pH$: 6.62

Curve II
- Reactants: Phosphate Buffer and Hydrazine
- Concentration of Buffer: $\mu = 0.20$ M
- Concentration of $N_2H_4$: $3.3 \times 10^{-3}$ M
- $pH$ of Buffer: 7.34
- $pH$ of $N_2H_4$: 7.30
- Atmosphere: Solutions were purged with nitrogen
- Final $pH$: 7.33

Rate of Gas Evolved
$\mu l/min$
Experiments with Potassium Ferricyanide

Potassium ferricyanide and hydrazine at various levels of oxygen were reacted in order that the gas evolution rates obtained could be compared with the results obtained from experiments of the same type with heme proteins. This offers comparison of the reactions of ferric type iron in two completely different environments.

All of the experiments with potassium ferricyanide were carried out using $0.91 \times 10^{-3}$ M. potassium ferricyanide and $0.091$ M. hydrazine. The solvent used for phosphate buffer pH 7.34 with an ionic strength of 0.20.

The reactions were carried out at a zero level, a 5%, a 10%, and in air which is a 21% level of oxygen. All experiments exhibited an initially rapid gas evolution over the first 5-8 min. followed by a rapid decrease in rate down to zero at from 10-12 min. (see Fig. 2). The initial rates for the oxygen level were:

<table>
<thead>
<tr>
<th>% Oxygen</th>
<th>Rate (ul/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2-0.1</td>
</tr>
<tr>
<td>5</td>
<td>0.9-0.1</td>
</tr>
<tr>
<td>10</td>
<td>0.9-0.1</td>
</tr>
<tr>
<td>21</td>
<td>0.7-0.1</td>
</tr>
</tbody>
</table>

The reaction involved here is presumably

$$N_2H_4 + Fe(CN)_6^{3-} \rightarrow N_2 \text{ or } N_2H \text{ and } NH_3 + Fe(CN)_6^{4-}$$

The reaction at pH 7.34 will be a composite of these two limiting reactions.

These results indicate that (1) the rate of reaction of hydrazine with potassium ferricyanide is more than sufficiently rapid for measurement in a Warburg system, (2) the rate is decreased when oxygen is present, and (3) the reaction which produces the gas stops, probably
Fig. 2 The Effect of Oxygen on the Evolution of Gas from a Potassium Ferricyanide-Hydrazine Mixture

1. Reactants
   - Hydrazine and K$_2$Fe(CN)$_6$
2. Conc. K$_3$Fe(CN)$_6$
   - 0.91x10$^{-3}$ M or 1x10$^{-4}$ moles in all reactions
3. pH K$_3$Fe(CN)$_6$
   - Dissolved in phosphate buffer pH 7.34 and $\mu = 0.02$
4. Conc. N$_2$H$_4$
   - 0.91 M or 1x10$^{-4}$ moles in all reactions
5. pH N$_2$H$_4$
   - 7.30
6. Final pH
   - 7.34±0.02

- $\bullet$ results at the 21% oxygen level
- $\circ$ results at the 10% oxygen level
- $\Delta$ results at the 5% oxygen level
- $\Box$ results at the 0% oxygen level
due to the complete reduction of the ferricyanide ion, since 0.3 moles of gas was produced per mole of ferricyanide ion and this value lies between the 0.25 and the 0.5 moles for the reaction above.

Experiments with Myoglobin

Myoglobin-hydrazine reactions using difference spectra

Two very interesting difference spectra were obtained from a qualitative look at the rate of an aerobic reaction for hydrazine with ferrimyoglobin and with ferromyoglobin.

Ferrimyoglobin. The first experiment was carried out by reacting 3 ml of a $0.70 \times 10^{-4}$ M. ferrimyoglobin solution with 0.10 ml of 1.00 M. hydrazine. There was a 60 fold molar excess of hydrazine. The solvent was triethanolamine buffer of pH 7.90 and ionic strength 0.05. The experiment was carried out after purging with nitrogen, as described in the experimental procedures, page 22. The temperature change was fairly uniform from 22°C to 31°C over the four hour period recorded. This and all subsequent values for the molar concentration ($M$.) refer to the molarity of the protein not the iron; unless otherwise stated.

One minute after the reactants had been mixed, the initial spectrum was drawn and the absorbance around 432 and 555 mp. began to increase, while at 500 and 390 mp. the absorbance was decreasing (see Fig. 3). These changes continued as an approximately linear function of time until the recording was stopped after four hours.

Since the peaks, absorbance maxima, of ferromyoglobin correspond to 555 and 432 mp., the results of this experiment would certainly justify the conclusion that hydrazine acts as a slow reducing agent in the presence of ferrimyoglobin.
Fig. 3 Difference Spectra for Ferrimyoglobin and Hydrazine at pH 7.90 and 26°C.

Conc. of Ferrimyoglobin \( 0.7 \times 10^{-3} \text{ M} \), or \( 2.1 \times 10^{-6} \) moles in triethanolamine buffer pH 7.90 and ionic strength 0.05.

Conc. of Hydrazine \( 0.032 \text{ M} \), or \( 1 \times 10^{-4} \) moles at pH 7.30

Curves
1. Base line ferrimyoglobin vs. ferrimyoglobin
2. Reaction mixture vs. myoglobin after 1 min.
3. Reaction mixture vs. myoglobin after 42 min.
4. Reaction mixture vs. myoglobin after 246 min.
Ferromyoglobin. The second experiment involved a reaction of 3 ml. of 0.70x10^{-4} M. ferromyoglobin with 0.10 ml of 1 M. hydrazine. The solvent was phosphate buffer of pH 7.34 and ionic strength 0.20. The solutions were purged with nitrogen. The temperature changed from 24° C to 29° C over the 70 min. interval of the run.

Thirty seconds after mixing the reagents the absorbance at 600 and 560 μm increased. At the end of 2 min. decreases in absorbance at 580, 545, and around 410 μm had begun and increases at 660-700 and 500 μm were prominent (see Fig. 4). These changes continued for 40 min. and then began to reverse very slowly.

The initial spectrum at 30 sec., indicated some initial deoxygenation, giving the peak at 560 μm. This deoxygenation continued for only 5 min.; while the disappearance of oxymyoglobin was maintained, as indicated by the decrease in absorbance at 580 and 545 μm. Thus the de-oxygenation was in competition with oxidation yielding the peaks at 500 and 625 μm, characteristic of ferrimyoglobin.

The above spectra indicate that the purging with nitrogen failed to remove the oxygen bound to the myoglobin and that the oxidation of the oxymyoglobin does not proceed to completion even though the hydrazine is in excess. However, the hydrazine may be used in a reaction with the oxygen that is bound to the myoglobin or the protein itself may be oxidized to ferrimyoglobin by a reaction with the oxygen, catalyzed in some way by the hydrazine. The reversal occurring after 40 min. is probably due to the reaction of the hydrazine with ferrimyoglobin to form ferromyoglobin. A possible reaction scheme:
Fig. 4 Difference Spectra for Ferromyoglobin and Hydrazine at pH 7.34 and 27° C.

Conc. of Ferromyoglobin
0.7×10⁻⁴ M, or 2.1×10⁻⁶ moles in phosphate buffer pH 7.34 and ionic strength 0.20.

Conc. of Hydrazine
0.032 M, or 1×10⁻⁴ moles at pH 7.30

Curve
0 ferromyoglobin vs. ferromyoglobin base line
I reaction mixture vs. MbFe⁺ after 30 sec.
II after 9 min.
III after 25 min.
IV after 40 min.
V after 70 min.
\[
\text{MbFe}^{+2} \cdot \text{O}_2 + \text{N}_2 \text{H}_2 \rightarrow \text{MbFe}^{+2} + \text{X}
\]
\[
\text{X} + \text{MbFe}^{+2} \rightarrow \text{MbFe}^{+3} \quad \text{or} \quad \text{X} + \text{MbFe}^{+2} \cdot \text{O}_2 \rightarrow \text{MbFe}^{+3}
\]
and
\[
\text{MbFe}^{+3} + \text{N}_2 \text{H}_2 \rightarrow \text{MbFe}^{+2} + \text{N}_2(+ \text{NH}_3)
\]

as the reversal reaction.

The results discussed here are only the very beginning of a promising method for studying the rate of reaction for this type of system. Under a controlled temperature and a controlled atmosphere this study could be very enlightening.

**Experiments with myoglobin using the Warburg apparatus**

Using the Warburg system as an experimental tool, the reactions of myoglobin and hydrazine were followed. The rate of gas volume change was used as a measure of the rate of reaction.

**Ferrimyoglobin.** Ferrimyoglobin at a final concentration of $1.00 \times 10^{-3}$ M. was reacted with $0.091$ M. hydrazine at two levels of oxygen, 0% and 21%. One ml. of $1.10 \times 10^{-3}$ M. ferrimyoglobin and 0.10 ml of 1.00 M. hydrazine were mixed, using phosphate buffer, pH 7.34 and ionic strength 0.20, as the solvent. This reaction mixture corresponds to approximately a 90 fold excess of hydrazine over myoglobin, in molar ratios.

At the zero oxygen level the gas evolved in 60 min. was zero within experimental error. However, for the 21% oxygen level the initial rate was $-0.30 \pm 0.04$ ml/min or, in other words, there was an uptake of gas. This uptake tended to level off when it reached $-10.5$ µl of gas (see Fig. 5). A spectrum of the reaction mixture was taken after the Warburg run had been completed and the results indicate that near quantitative reduction of ferrimyoglobin to ferromyoglobin.
Fig. 5 Gas Volume Change in the Reaction of Ferrimyoglobin with Hydrazine at two Oxygen levels

<table>
<thead>
<tr>
<th>Curve I</th>
<th>Curve II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactants</td>
<td>Ferrimyoglobin and Hydrazine</td>
</tr>
<tr>
<td>Concentration</td>
<td>1.6x10^-7 M. or 1.6x10^-6 moles</td>
</tr>
<tr>
<td>pH</td>
<td>7.34</td>
</tr>
<tr>
<td>Concentration</td>
<td>0.091 M. or 1.0x10^-4 moles</td>
</tr>
<tr>
<td>pH</td>
<td>7.30</td>
</tr>
<tr>
<td>Atmosphere</td>
<td>0% oxygen</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.34</td>
</tr>
<tr>
<td>Atmosphere</td>
<td>21% oxygen</td>
</tr>
<tr>
<td>Final pH</td>
<td>7.33</td>
</tr>
</tbody>
</table>
occurred in both cases. For the anaerobic case, however, it cannot be excluded that the completion of the reduction was caused after opening the Warburg vessel to the atmosphere, possibly through some oxygen-induced catalysis. In the case of 21% oxygen level the spectrum indicates that approximately 5% of the myoglobin was converted into a "verdochrome" type heme protein \(^{21}\) by the time the 10.5 µl of gas was taken up (see Fig. 6); and less than 1% "verdochrome" was produced in the "absence" of O\(_2\).

Verdochrome is a result of oxidation of side-chains of the porphyrin ring of a heme protein.

These results indicate that the hydrazine acts as a reducing agent with or without oxygen present. If hydrazine was oxidized to nitrogen, it would yield from 5.5 to 11 µl of gas. For purposes of comparison, note that 10 µl of gas is approximately equal to 0.5x10\(^{-6}\) moles at 20° C. In the experiment where the so called zero level of oxygen was maintained there could be as much as 4 to 5 µl of oxygen present, which would have been taken up as MbFe\(^{+2}\)·O\(_2\). It is possible that nitrogen produced in the "anaerobic" oxidation of hydrazine is nearly counteracted by this 4 to 5 µl of oxygen uptake. But then there still would be a 2 to 5 µl shortage of total gas production, compared to that expected if hydrazine were oxidized to nitrogen. This might be explained by (1) and underestimated oxygen impurity, (2) a reaction in which free radical intermediates such as N\(_2\)H\(_3\), necessary for the conversion of N\(_2\)H\(_4\) to N\(_2\), are consumed by protein and prevented from yielding any gas, or (3) perhaps by a reaction between the nitrogen and the ferrimyoglobin. In the experiment where oxygen
Fig. 6 Spectra of the Reaction Mixture of Ferrimyoglobin after Reaction with Hydrazine in the Warburg

All reactions were in phosphate buffer pH 7.34 and ionic strength 0.20. Hydrazine was present in each reaction at 0.091 M, or as 1.00x10⁻⁴ moles at pH 7.30.

I The original 1.00x10⁻⁵ M, ferrimyoglobin
II After reaction with hydrazine in 0% oxygen
III After reaction with hydrazine at the 21% oxygen level
was at the 21% level the amount of gas taken up corresponded to one-half the amount of oxygen which might combine to form oxymyoglobin. This follows from the fact that the capacity of ferromyoglobin to form oxymyoglobin was approximately 95% unimpaired at the end of the experiment (see Fig. 6). However, if nitrogen was produced the observed 10.5 μl net gas uptake would then correspond very closely to the reaction giving the higher nitrogen production (1-electron mechanism).

If one assumes that no nitrogen was produced during the reduction of the protein, then one must also assume that less than 1 μl of oxygen impurity was present during the reaction at the zero oxygen level, which is possible.

An assumption of zero nitrogen-production at the 21% oxygen level would require a net uptake of 22 μl of oxygen gas, a wide variance from the observed 10.5 μl.

**Ferromyoglobin.** Ferromyoglobin at a concentration of 1.00x10⁻³ M. was reacted with 0.091 M. hydrazine under the same conditions as was described for the reactions with ferrimyoglobin.

At the zero level of oxygen the gas pressure change was essentially zero over the 60 min. period. However, in the case of the 21% oxygen level the initial change was zero for 15 min., then suddenly the rate of change was -0.14 μl/min. The reaction was still going after 60 min., but it was no longer followed (see Fig. 7). Spectra, taken immediately after the Warburg run, indicated no apparent change in the "anaerobic" ferromyoglobin. The aerobic or oxymyoglobin run gave a spectrum which had increased absorbance in the range 660-700 μm. and decreased
Fig. 7  Gas Volume Change in the Reaction of Ferromyoglobin with Hydrazine at two Oxygen levels

Curve I  Curve II

1. Reactants  Ferromyoglobin and Hydrazine
2. Conc. MbFe$^{+2}$  1.0x10$^{-3}$ M. or 1.1x10$^{-6}$ moles
3. pH MbFe$^{+2}$  7.34  7.34
4. Conc. N$_2$H$_4$  0.691 M. or 1.0x10$^{-4}$ moles
5. pH N$_2$H$_4$  7.30  7.30
6. Atmosphere  0% oxygen  21% oxygen
7. Final pH  7.32  7.35
absorbance around 412 mp. (see Fig. 8). The resulting spectrum corresponds to a disappearance of approximately 35% of the ferromyoglobin. These changes in the visible spectra are indicative of formation of a "verdochrome."

The results here indicate that (1) the hydrazine does not oxidize the ferromyoglobin to ferrimyoglobin, (2) hydrazine, in the presence of oxygen, in some way promotes a reaction with oxygen in oxymyoglobin to form an entity which can then attack the porphyrin ring and do the damage necessary to form "verdochrome." The 15 min. time lag required for oxygen consumption to begin indicating autocatalysis.

Experiments with Hemoglobin

A sequence of experiments using hemoglobin in both oxidation states and at various levels of oxygen environment was initiated to afford a comparison with the results of the experiments with myoglobin and legoglobin. The experiments were run at the zero oxygen level, the 10% oxygen level, and at the 21% oxygen level.

Ferrihemoglobin

At the zero oxygen level two concentrations were used for ferrihemoglobin, 0.91x10^{-3} and 0.23x10^{-3}M. The solvent was phosphate buffer of pH 7.34 and ionic strength 0.20. The reactions were, as for all experiments, 1.00 ml of 1.00x10^{-3} M. heme protein solution to 0.10 ml. of 1 M. hydrazine, with hydrazine in a 100 fold excess.

The 0.23x10^{-3} M. ferrihemoglobin gave a 1 µl. uptake over the entire time period, while the 0.91x10^{-3} M. ferrihemoglobin gave a 0.15 µl/min evolution over the 60 min. interval (see Fig. 9). The total production at the higher concentration was at least 0.40 moles
Fig. 8  Spectra of the Reaction Mixture of Ferrocyoglobin after Reaction with Hydrazine in the Warburg

All reactions were in phosphate buffer pH 7.34 and ionic strength 0.20.

Hydrazine was present in each reaction at 0.091 M, or as 1.00x10^-3 moles at pH 7.30.

I  The original 1.00x10^-3 M ferrocyoglobin
II  The ferrocyoglobin after reaction with hydrazine at the 0% oxygen level
III  After reaction with hydrazine at the 21% oxygen level
Fig. 9  Gas Volume Change in the Reaction of Ferrihemoglobin and Hydrazine at Various Concentrations and Oxygen levels

The reactions were carried out at pH 7.34 and 20° C.

- • 0.91x10⁻³ M. or 1x10⁻⁶ moles of HbFe⁺³ at the 0% O₂ level
- • 0.23x10⁻³ M. or 0.25x10⁻⁶ moles of HbFe⁺³ at the 0% O₂ level
- △ 0.23x10⁻³ M. or 0.25x10⁻⁶ moles of HbFe⁺³ at the 10% O₂ level
- △ 0.23x10⁻³ M. or 0.25x10⁻⁶ moles of HbFe⁺³ at the 21% O₂ level
- 0 0.91x10⁻³ M. or 1x10⁻⁶ moles of HbFe⁺³ at the 21% O₂ level
of gas per mole of protein, but only 0.10 moles of gas per mole of Fe$^{+3}$, or considerably less than the ratio 0.25 expected from a completed reaction proceeding by the two-electron mechanism, page 6. The spectra obtained after the run indicated nearly quantitative reduction to ferrohemoglobin (see Fig. 10).

These results indicate that (1) the hydrazine at low oxygen levels reduces the ferrihemoglobin, (2) the hydrazine is oxidized to produce a gas, probably nitrogen. The absence of gas evolution at the lower concentration is probably due to oxygen impurities.

At the 10% level the reaction was run at only one concentration of ferrihemoglobin, namely 0.23x10$^{-3}$ M. The gas changes recorded corresponded to an uptake of gas at the rate of 0.18±0.04 µl/min (see Fig. 9). The spectra which was obtained after the run indicated reduction to ferrohemoglobin followed by oxygenation and production of "verdochrome." The production of "verdochrome" was around 10 or 20% of the original hemoglobin (see Fig. 11).

The results at the 10% oxygen level showed once again that the production of some entity through the interaction of oxygen hydrazine and the heme protein is an important point in this study.

At the 21% oxygen level 0.91x10$^{-3}$ and 0.23x10$^{-3}$ M. ferrihemoglobin were used, as before. At the lower concentration the rate of gas uptake was 0.38 µl/min, with a total uptake of at least 4.0 moles of gas per mole of original protein, which turns out to be 8.0 moles per mole of HbFe$^{+2}$ remaining at the end of the reaction time. At the higher concentration the uptake was 0.76 µl/min, with a total uptake of at least 2.0 moles of gas per mole of protein. The spectrum taken after the run indicated reduction followed by oxygenation and production
Fig. 10  Spectrum of Ferrihemoglobin after Reaction with Hydrazine at the 0% Oxygen Level.

The reaction was carried out in phosphate buffer pH 7.34 and ionic strength
Hydrazine was present in the reaction at 0.091 M, or as \(1.00 \times 10^{-4}\) moles at pH 7.30.

I  The original \(0.91 \times 10^{-3}\) M, ferrihemoglobin

II  The ferrihemoglobin after reaction with hydrazine at the 0% oxygen level
Fig. 11 Spectrum of Ferrihemoglobin after Reaction with Hydrazine at the 10% Oxygen Level

The reaction was carried out in phosphate buffer pH 7.34 and ionic strength 0.20. Hydrazine was present in the reaction at 0.091 M, or as 1.00x10^-5 moles at pH 7.30.

I The original 0.91x10^-3 M ferrihemoglobin

II The ferrihemoglobin after reaction with hydrazine at the 10% oxygen level
of about 40 to 50% "verdochrome" at both concentrations of hemoglobin (see Fig. 12).

These results show that an increased oxygen level causes an increased rate of production of "verdochrome." The uptake of gas can be attributed only in part to formation of oxymyoglobin which could amount to 4 moles of gas per mole of intact protein, since hemoglobin has 4 iron sites; but half the hemoglobin had been converted to "verdochrome," incapable of holding oxygen. The lower concentration of protein had already passed its saturation point and was still taking up gas, therefore, one could still postulate possible consumption of oxygen in a reaction with a hydrazine-hemoglobin complex.

Ferrohemoglobin

Ferrohemoglobin at three different levels of oxygen were reacted with hydrazine.

At the zero level of oxygen two concentrations of ferrohemoglobin were run. Both the $0.91 \times 10^{-3}$ and the $0.23 \times 10^{-3}$ M. solutions when reacted with the hydrazine gave essentially a zero rate of gas evolution (see Fig. 13). The spectra taken after the Warburg run indicated that little if any change had occurred. (see Fig. 14, curves 1 and 2.)

The results indicate that in the absence of oxygen hydrazine has little if any effect on ferrohemoglobin.

At the 10% oxygen level ferromyoglobin at a concentration of $0.23 \times 10^{-3}$ M. was used. Gas was taken up at the rate of $0.2 \pm 0.05$ ml/min during a 60 min. interval that was checked. This corresponds to at least 2.0 moles of gas per mole of initial protein (see Fig. 13). The
Fig. 12  Spectrum of Ferrihemoglobin after Reaction with Hydrazine at the 21% Oxygen Level

The reaction was carried out in phosphate buffer pH 7.34 and ionic strength 0.20. Hydrazine was present in the reaction at 0.091 M. or as 1.00x10^-3 moles at pH 7.30.

I  The original 0.91x10^-3 M. ferrihemoglobin

II  The ferrihemoglobin after reaction with hydrazine at the 21% oxygen level
Fig. 13  Gas Volume Change in the Reaction of Ferrohemoglobin and Hydrazine at Various Concentrations and Oxygen levels

The reactions were carried out at pH 7.34 and 20° C.

- 0.91x10^-3 M. or 1.00x10^-6 moles of HbFe^2+ at the 0% O2 level
- 0.23x10^-3 M. or 0.25x10^-6 moles of HbFe^2+ at the 0% O2 level
- 0.23x10^-3 M. or 0.25x10^-6 moles of HbFe^2+ at the 10% O2 level
- 0.47x10^-3 M. or 0.50x10^-6 moles of HbFe^2+ at the 21% O2 level
- 0.91x10^-3 M. or 1.00x10^-6 moles of HbFe^2+ at the 21% O2 level
Fig. 14 Spectra of Ferrohemoglobin after Reaction with Hydrazine at three levels of Oxygen

The reactions were carried out in phosphate buffer pH 7.34 and ionic strength 0.20. Hydrazine was present in each of the reactions at 0.091 M, or as 1.00x10^{-3} moles at pH 7.30.

I The original 0.91x10^{-3} M ferrohemoglobin
II After reaction with hydrazine at the 0% oxygen level
III After reaction with hydrazine at the 10% oxygen level
IV After reaction with hydrazine at the 21% oxygen level
spectrum of the resulting mixture indicated that approximately 25% of the hemoglobin had been converted to "verdochrome."

The reaction that occurred at the 10% oxygen level is apparently a direct result of the presence of oxygen. The reaction between oxygen and the hemoglobin in the presence of hydrazine is responsible for the results obtained.

At the 21% oxygen level $0.46 \times 10^{-3}$ and $0.91 \times 10^{-3}$ M. ferrohemoglobin were used. Gas was taken up at the rate of $0.4 \pm 0.04 \mu l/min$ in the first case and at the rate of $0.5 \pm 0.04 \mu l/min$ in the second case. For the entire period of time that was followed, 60 min., there was at least 2.0 and 1.4 moles of gas, respectively, taken up per mole of initial protein (see Fig. 13). The spectrum indicated that approximately 60% of the hemoglobin was converted to "verdochrome." The spectrum gave no indication of oxidation to ferrihemoglobin (see Fig. 14).

Experiments with Legoglobin

This study had as its fundamental goal a series of experiments using legoglobin to react with hydrazine. These experiments, using legoglobin, were carried out as nearly as possible under the same conditions as those experiments with hemoglobin and myoglobin.

Ferrilegoglobin

Using ferrilegoglobin at $1.18 \times 10^{-3}$ and $0.59 \times 10^{-3}$ M. in the absence of oxygen it was found that no gas was evolved or taken up in the time allowed for other studies, 60 min. (see Fig. 15). On observing the spectra it was observed that quantitative reduction to
The reaction was carried out at pH 7.34 and 20°C.

- 0.59x10^{-3} M or 0.65x10^{-6} moles of LbFe^{3+} at the 21% O_2 level
- 1.18x10^{-3} M or 1.30x10^{-6} moles of LbFe^{3+} at the 21% O_2 level
- 0.59x10^{-3} M or 0.65x10^{-6} moles of LbFe^{3+} at the 0% O_2 level
- 1.18x10^{-3} M or 1.30x10^{-6} moles of LbFe^{3+} at the 0% O_2 level

Fig. 15 Gas Volume Change in the Reaction of Ferrilegoglobin and Hydrazine at two Concentrations and two Oxygen levels
ferrolegoglobin had occurred (see Fig. 16). This spectroscopically observed reduction to \( \text{LbFe}^{2+}O_2 \) might have occurred after the removal from the Warburg apparatus, very soon after contact with oxygen. However, this is unlikely since an observed color change from brown to red was noted after the experiment but before air was allowed in the vessel. In the presence of 21% oxygen these two concentrations were again reacted with hydrazine and again no significant gas change was recorded; and the spectrum taken after the Warburg run showed complete conversion to oxylegoglobin, as was found for the "anaerobic" case. No evidence of "verdochrome" formation was observed.

If nitrogen is produced from hydrazine during the reduction of the ferrilegoglobin according to one of the mechanisms on page 6, then from 7 to 14 ul of nitrogen gas should be evolved for the higher concentration of heme protein and from 3.5 to 7 ul for the second concentration. The amounts of ferrolegoglobin represent at the end of the reaction could take up 29 and 14.5 ul of oxygen for the higher and the lower concentration, respectively, to form oxylegoglobin. The spectra of Fig. 16 show that the capacity of the ferrolegoglobin to form oxylegoglobin was unimpaired at the end of the reaction. In the "anaerobic" experiments either or all of three things could contribute: (1) the oxygen impurity could be large enough to compensate for a 7 ul discrepancy, (2) the nitrogen in the system may be taken up in some way, and (3) \( \text{N}_2 \) may not be produced at all inspite of ferrilegoglobin reduction. The first possibility is very unlikely since the maximum value on impurity was set at 5 ul. The third case represents the more likely of the three possibilities, as will be discussed in the conclusions.
Fig. 16  Spectra of Ferrilegoglobin after Reaction with Hydrazine at two levels of Oxygen

The reactions were carried out in phosphate buffer pH 7.34 and ionic strength 0.20. Hydrazine was present in each of the reactions at 0.091 M, or as 1.00x10^-3 moles at pH 7.30.

I  The original 1.18x10^-3 M ferrilegoglobin
II  After reaction with hydrazine at the 0% oxygen level
III  After reaction with hydrazine at the 21% oxygen level
In the aerobic experiments, oxygen is available and would be taken up. There is a discrepancy, lack of gas uptake, of at least 29 µl at the higher concentration of protein, in other words there is one mole of gas produced per apparent mole at Fe\(^{+3}\) reduced. This could indicate the production of nitrogen or some other gas under a completely different mechanism than has been discussed, assuming that the Fe\(^{+3}\) reduction was not caused by the introduction of oxygen after the Warburg run.

**Ferrolegoglobin**

Using ferrolegoglobin at 1.18x10\(^{-3}\) and 0.59x10\(^{-3}\) M. four reactions were carried out. Two reactions at the 21% oxygen level and two reactions at the 0% oxygen level.

The two reactions in air gave no gas change over the entire 60 min. interval (see Fig. 17), while those in the absence of oxygen gave at most one µl. of evolution over the entire 60 min. range. Essentially there was no change in gas volume, within experimental error. The spectra for the legoglobin indicated approximately 35% loss of ferrolegoglobin in the presence of oxygen. The absorption corresponding to the new material was from 630-700 µm. with lowering of the peaks at 410, 570, and 538 µm. which is characteristic of a "verdochrome" type of heme protein. This material, however, to the eye was brown rather than green (see Fig. 18). In the case where the oxygen was absent no significant change was observed.

These results show that at pH 7.34 hydrazine does not oxidize ferrolegoglobin. The formation of a "verdochrome" type substance from
Fig. 17  Gas Volume Change in the Reaction of Ferrolegoglobin and Hydrazine at two Concentrations and two Oxygen levels.

The reactions were carried out at pH 7.34 and 20°C.

- 0.59x10^-3 M, or 0.65x10^-6 moles of LbFe^2+ at the 21% oxygen level
- 1.18x10^-3 M, or 1.30x10^-6 moles of LbFe^2+ at the 21% O_2 level
- 0.59x10^-3 M, or 0.65x10^-6 moles of LbFe^2+ at the 0% O_2 level
- 1.18x10^-3 M, or 1.30x10^-6 moles of LbFe^2+ at the 0% O_2 level
Fig. 18  Spectra of Ferrolegoglobin after Reaction with Hydrazine at two levels of Oxygen

The reactions were carried out in phosphate buffer pH 7.34 and ionic strength 0.20. Hydrazine was present in each of the reactions at 0.091 M, or as 1.00x10^-4 moles at pH 7.30.

I  The original 1.18x10^-3 M ferrolegoglobin
II  After reaction with hydrazine at the 0% oxygen level
III  After reaction with hydrazine at the 21% oxygen level
oxylegoglobin indicates that the reaction observed with ferrilegoglobin to reduce the ferrilegoglobin to ferrolegoglobin must be very slow or proceed through another type of mechanism, perhaps a stable intermediate complex. This could account for the lack of formation of "verdochrome" where oxidation must proceed "verdochrome" formation.
DISCUSSION AND CONCLUSIONS

Comparison of Myoglobin and Hemoglobin

The oxidized forms of hemoglobin and myoglobin in the absence of oxygen are both reduced by hydrazine to their corresponding ferro-type. This was proven spectrophotometrically only for myoglobin; for ferri-hemoglobin the anaerobic reduction of $\text{Fe}^{3+}$ probably occurred too, but the complete reduction observed in the sample withdrawn at the end of the Warburg run may have been induced by oxygen. Note also, that even in the case of anaerobic ferrimyoglobin, the spectral results do not completely justify a conclusion that ferrimyoglobin was entirely reduced in the anaerobic Warburg experiment which lasted 60 min., since the spectrophotometer experiment lasted four times that long without complete reduction. However the concentration and temperature were different in these two experiments. In the presence of oxygen this same kind of reduction occurs, but is followed by the formation of ferriheme proteins and "verdochromes." The ferrihemoglobin appears to be much more susceptible to "verdochrome" formation. From the experiments with ferrihemoglobin it appears that the reaction forming "verdochromes" is dependent upon the level of oxygen present. The production of a gas, probably nitrogen, is a strong possibility during the reduction of the heme protein, this being substantiated by a definite though surprisingly small (0.1 mole gas per mole of a apparent $\text{Fe}^{3+}$ reduction) gas evolution at high concentration of ferrihemoglobin (see Table 1).
Table 1. Results of reactions with hydrazine at pH 7.34, $\mu = 0.20$, and 20° C.

<table>
<thead>
<tr>
<th>Reactant</th>
<th>$% O_2$</th>
<th>Moles of gas evolved per total moles of original iron</th>
<th>Corrected(^{1}) moles of gas evolved per mole of iron</th>
<th>Final state heme protein (spectrum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_3Fe(CN)_6$</td>
<td>0.00</td>
<td>0.31</td>
<td>0.31</td>
<td>--</td>
</tr>
<tr>
<td>HbFe$^+3$</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>reduced</td>
</tr>
<tr>
<td>4(HbFe$^+3$)</td>
<td>0.00</td>
<td>0.1</td>
<td>0.1</td>
<td>reduced</td>
</tr>
<tr>
<td>MbFe$^+3$</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>reduced</td>
</tr>
<tr>
<td>1.3(LbFe$^+3$)</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>reduced</td>
</tr>
<tr>
<td>HbFe$^+3$</td>
<td>21.0</td>
<td>-1.0</td>
<td>-0.5</td>
<td>50% red.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50% verd.</td>
</tr>
<tr>
<td>MbFe$^+3$</td>
<td>21.0</td>
<td>-0.5</td>
<td>0.45</td>
<td>95% red.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5% verd.</td>
</tr>
<tr>
<td>LbFe$^+3$</td>
<td>21.0</td>
<td>0.0</td>
<td>1.0</td>
<td>99%+ % red.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1% verd.</td>
</tr>
<tr>
<td>HbFe$^+2$</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>reduced</td>
</tr>
<tr>
<td>MbFe$^+2$</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>reduced</td>
</tr>
<tr>
<td>LbFe$^+2$</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>reduced</td>
</tr>
<tr>
<td>HbFe$^+2\cdot O_2$</td>
<td>21.0</td>
<td>-0.55</td>
<td>-1.15</td>
<td>40% red.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>60% verd.</td>
</tr>
<tr>
<td>MbFe$^+2\cdot O_2$</td>
<td>21.0</td>
<td>-0.3</td>
<td>-0.65</td>
<td>65% red.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35% verd.</td>
</tr>
<tr>
<td>LbFe$^+2\cdot O_2$</td>
<td>21.0</td>
<td>0</td>
<td>-0.35</td>
<td>65% red.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35% verd.</td>
</tr>
</tbody>
</table>

\(^{1}\)These values have been corrected for $O_2$ uptake in the formation of $HPFe^{+2\cdot O_2}$ based on the amount of $HPFe^{+2}$ found spectrophotometrically at the end of the run.
The reduced form of hemoglobin and myoglobin in the absence of oxygen shows no reaction. However, when oxygen is allowed in the system, uptake of gas occurs and "verdochrome" is produced, with the magnitude of the "verdochrome" production proportional to the oxygen present. These results indicate that the production of some labile entity, probably a free radical, takes place when oxygenated myoglobin or hemoglobin is reacted with hydrazine. It appears that the hydrazine promotes this reaction in such a way that oxygen is used up in forming this labile entity. Possibly the hydrazine is complexed in some way to the heme protein.

Comparison of Potassium Ferricyanide with the Heme Proteins

Potassium ferricyanide reacted very rapidly with hydrazine to produce a gas which was probably nitrogen, since this reaction has been observed by many other people to produce nitrogen at other pH values. Ferrihemoglobin at high concentration (0.91x10^{-3} M.) was the only reaction with heme proteins which gave a net gas evolution. The rates per unit ferric ion concentration for these two reactions are

\[ K_3Fe(CN)_6 \rightarrow 1.2 \mu l/min \quad \text{HbFe}^3 \rightarrow 0.04 \mu l/min \]

or a 30 times greater affect for potassium ferricyanide if the oxygen impurity was negligible or 20 times if the oxygen impurity was 5 µl. These heme protein rates might actually be still greater if the level of oxygen impurity has been underestimated. However, because one would expect the protein side chains to exert an inhibiting steric effect on the rate at which Fe^{3+} if ferri-heme protein could react with hydrazine the above 30 fold decrease on going from ferricyanide to ferri-heme protein is reasonable. It seems quite logical that the reaction
between the heme proteins and hydrazine would yield nitrogen as a product. This result has not been verified and is thus only a speculation based upon circumstantial evidence.

**Legoglobin as Compared to the Other Heme Proteins**

Ferrilegoglobin, like the other heme proteins and potassium ferricyanide in the oxidized or ferri-state, reacts with hydrazine in some way to produce the corresponding ferro-compound, which in the case of the heme proteins is indicated by the oxy-heme protein type spectrum found after the Warburg run.

The reduction of these heme proteins at the 0% oxygen level gave no net gas evolution or uptake, except for the ferrihemoglobin at the 3.64×10⁻³ M. ferric ion concentration. This result indicates that either the nitrogen evolution was masked by oxygen impurity uptake, or that nitrogen was taken up or that there was no gas evolved. The first two possibilities are very unlikely, however, the possibility of a reaction mechanism which does not produce a gas is a very good possibility. Such a reaction mechanism could and with the oxidation of hydrazine to \( \text{N}_2\text{H}_3 \). The \( \text{N}_2\text{H}_3 \) radical could then attach to the protein in some way and form a stable complex. Ferrihemoglobin with the four closely spaced \( \text{Fe}^{+3} \) ions would have a better probability for further oxidation of the \( \text{N}_2\text{H}_3 \) radical to \( \text{N}_2 \).

At the 21% oxygen level there is no common mechanism for the three ferri-heme proteins; but definite conclusions about the ferrilegoglobin mechanism can be drawn. The large amount of gas that was shown to be produced, per mole of \( \text{Fe}^{+3} \) reduced (1.0 moles of gas per 1.0 moles \( \text{Fe}^{+3} \) found), shows that neither of the Cahn-Powell mechanisms
apply here. The oxygen probably acts as a catalyst for a reaction with 
\( N_2H_3 \), liberating as much nitrogen (and oxygen, finally) as \( Fe^{+2} \) formed. 
The following reaction scheme is proposed:

\[
\begin{align*}
LbFe^{+3} + N_2H_4 &= LbFe^{+2} + N_2H_2 + H^+ \\
N_2H_2 + O_2 &= N_2H_2 + HO_2 \\
HO_2 + LbFe^{+2} &= O_2 + H^*LbFe^{+2} \\
O_2 + N_2H_2 &= HO_2 + N_2H \\
LbFe^{+2} + HO_2 &= O_2 + H^*LbFe^{+2} \\
N_2H + O_2 &= HO_2 + N_2 \\
HO_2 + LbFe^{+2} &= O_2 + H^*LbFe^{+2},
\end{align*}
\]

or a net reaction

\[
N_2H_3 + 3LbFe^{++} (O_2) N_2 + 3H^*LbFe^{+2}.
\]

The mechanisms for ferrihemoglobin and ferrimyoglobin may include still further complications, such as more "verdochrome" and more "normal" oxidation. This final conclusion is partly born out by the fact that ferrihemoglobin showed 50\% conversion to a green pigment with an absorption maximum around 660 mp. (Fig. 10). Ferrimyoglobin gave a similar type pigment with an absorption maximum in the interval 660-700 mp. (Fig. 4); however, the conversion in an equal length of time was only approximately 5\%. In the case of ferrilegoglobin over the same 60 min. time interval, less than 1\% of the material was converted (Fig. 16). Therefore, one must conclude that ferrilegoglobin has a structure which is very much less susceptible to attack by the radicals produced in the reduction of the \( Fe^{+3} \) to \( Fe^{+2} \) or that the reduction process in the case of legoglobin produces radicals which are not capable of attacking the porphyrin ring.
**Ferro-types** of heme protein in the absence of oxygen are non-reactive in the presence of hydrazine. However, in the presence of oxygen these reduced heme proteins take up oxygen to form oxy-heme proteins types and in the presence of hydrazine they are attacked by a labile entity, probably a free radical, which produces a "verdochrome" type of heme protein.\(^{(21)}\)

Oxyhemoglobin at the 21% oxygen level reacted in the presence of hydrazine to produce approximately 60% conversion to a new component which had an absorption maximum at 660 mp, indicating the production of a "verdochrome" type of heme protein (see Fig. 12). Oxymyoglobin under the same conditions as oxyhemoglobin gave approximately 35% of a new component which had the broad absorption interval 630-700mp. (see Fig. 18). The colors of the solution after the reactions were green for hemoglobin and myoglobin and brown for the legoglobin.

The observation that no "verdochrome" production when ferrilegoglobin was reacted with hydrazine in the presence of oxygen may be explained by assuming that the reduction itself is quite slow, requiring the full 60 min. If the reaction had been followed longer the production of this component would probably have occurred.

A comparison of the gas evolution rates of ferrilegoglobin and ferrihemoglobin would indicate, at first, that no gas was produced in ferrilegoglobin reduction. However, the ferrihemoglobin which gave gas evolution when reacted with hydrazine in the absence of oxygen was at a 3-fold higher iron ion concentration than the ferrilegoglobin. The production of gas in the ferrihemoglobin reaction was 9 μl., and if the reactions were to correspond, then only 3 μl. of gas would be
expected for the ferrilegoglobin reaction. Experimental error, the time interval (10-15 min.) until the spectra was recorded, and oxygen impurity taken up could certainly account for the lack of evolution. It can also be concluded that hydrazine when reacted with ferri-myoglobin and ferrilegoglobin is oxidized not to nitrogen but to some other form, such as \( \text{N}_2\text{H}_3 \), which can form a stable complex with the ferro-heme protein. This complex could be broken up by oxygen, or as a function of time with the formation of nitrogen. A second reaction of this type could occur in which the final products are not gases. Experiments designed so that ferrilegoglobin would be at an iron concentration equivalent to the level used for the ferrihemoglobin experiment should be carried out to resolve some of these problems. Also, anaerobic reactions with all of these proteins using the Spectrophotometer at a controlled temperature would certainly aid in resolving these problems.
This study gives a comparison of the reactions of ferricyanide ion, myoglobin, hemoglobin, and legoglobin with hydrazine. The reaction rates were measured using a Warburg apparatus to measure gas volume change. The heme protein reaction mixtures were also examined spectrophotometrically.

All anaerobic reactions using the ferric state plus hydrazine gave nearly complete reduction to the ferrous state. Only ferricyanide gave gas evolution when the oxidizing agents were at the same concentrations of Fe$^{3+}$. However, ferrihemoglobin at a 4-fold increase in the concentration of Fe$^{3+}$ did evolve gas when reacted with hydrazine, but it was a surprisingly slow rate, relative to that of ferricyanide. These results indicate that the protein environment has a large inhibiting effect on this reaction.

Aerobic reactions using the ferric state plus hydrazine gave complete reduction in the case of ferrilegoglobin, but in the case of ferrihemoglobin and ferrimyoglobin the production of a "verdochrome" was observed. The lack of production of a "verdochrome" in the case of ferrilegoglobin plus the absence of any change in gas pressure during the reaction has necessitated the assumption of a completely different mechanism for the oxidation of hydrazine in the presence of ferrilegoglobin.

No interaction was observed when ferro-hemo proteins were reacted with hydrazine anaerobically. However, aerobically "verdochromes" were produced in all cases, indicating that some type of free radical mechanism involving oxygen causes "verdochrome" formation in the heme proteins studied.
LITERATURE CITED

1. Abel, E., Monatsheft für Chemie, 84, 527 (1953).


