THE OXIDATION-REDUCTION POTENTIALS OF HEMIN AND RELATED SUBSTANCES.

I. THE POTENTIALS OF VARIOUS HEMINS AND HEMATINS IN THE ABSENCE AND PRESENCE OF PYRIDINE.

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As a continuation of our electrochemical study of hemin and related substances (1, 2) we have measured the oxidation-reduction potentials in borax-tartrate buffer solutions by the titration method. The ferric compound was titrated with titanous chloride in the manner previously described; in a few cases the titration of the ferrous compound with ferricyanide was also completed.

At constant hydrogen ion activity at 25° the potential of an oxidation-reduction system involving 1 equivalent change is given by Equation 1.

\[ E = E_0' - 0.06 \log \frac{[\text{Red}]}{[\text{Ox}]} \]

The value of \( E_0' \) (the normal potential at the pH employed) is the value of the potential (referred to the normal hydrogen electrode) at the mid-point of the titration curve. This value, in the case of the hemin and hematin systems, we have found to be fairly reproducible and we feel confident that we have evaluated it within 10 millivolts for the particular systems we have studied. The data summarized in Table I were obtained at 22° ±2° with a buffer solution composed of equal amounts of 0.1 M borax and 0.2 M sodium tartrate; its pH value was 9.15. It is evident that \( E_0' \) for both the hemin and hematin systems is identical within the limits of experimental error. The hemin employed was crys-
talline α-hemin prepared by the usual acetic acid method and recrystallized by means of the chloroform-pyridine treatment (3). We shall refer to this material throughout this paper as hemin. Experiments were also performed with β-hemin ((4), (5) p. 203–205) prepared by the acetone and by the alcohol procedure. Various varieties of hematin were also employed: alkali hematin (6), Küster's hematin ((5) p. 210), Mörner's hemin ((5) p. 203–205), and Verdaung's hematin (7). All these materials gave essentially the same value for $E_0'$ in the borax-tartrate buffer.

**TABLE I.**

* Determination of Normal Potential of Hemin-Reduced Hemin and Hematin-Reduced Hematin Systems.

Concentration = $2 \times 10^{-4}$ molar; temperature = $22^\circ \pm 2^\circ$.

<table>
<thead>
<tr>
<th>pH</th>
<th>Method.</th>
<th>No. of determinations</th>
<th>Highest value $E_0'$</th>
<th>Lowest value $E_0'$</th>
<th>Average value $E_0'$</th>
<th>Mean of $E_0'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.15</td>
<td>TiCl₄</td>
<td>3</td>
<td>-0.224</td>
<td>-0.244</td>
<td>-0.235</td>
<td>-0.230</td>
</tr>
<tr>
<td></td>
<td>K₃Fe(CN)₆</td>
<td>2</td>
<td>-0.224</td>
<td>-0.224</td>
<td>-0.224</td>
<td>-0.224</td>
</tr>
</tbody>
</table>

B. Hemin dissolved at 70° in borate buffer.

<table>
<thead>
<tr>
<th>pH</th>
<th>Method.</th>
<th>No. of determinations</th>
<th>Highest value $E_0'$</th>
<th>Lowest value $E_0'$</th>
<th>Average value $E_0'$</th>
<th>Mean of $E_0'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.15</td>
<td>TiCl₄</td>
<td>4</td>
<td>-0.223</td>
<td>-0.241</td>
<td>-0.237</td>
<td>-0.230</td>
</tr>
<tr>
<td></td>
<td>K₃Fe(CN)₆</td>
<td>1</td>
<td>-0.224</td>
<td>-0.224</td>
<td>-0.224</td>
<td>-0.224</td>
</tr>
</tbody>
</table>

C. Hematin systems; hematin dissolved in borate buffer at 23°.

<table>
<thead>
<tr>
<th>pH</th>
<th>Method.</th>
<th>No. of determinations</th>
<th>Highest value $E_0'$</th>
<th>Lowest value $E_0'$</th>
<th>Average value $E_0'$</th>
<th>Mean of $E_0'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.15</td>
<td>TiCl₄</td>
<td>2</td>
<td>-0.241</td>
<td>-0.244</td>
<td>-0.242</td>
<td>-0.242</td>
</tr>
</tbody>
</table>

A few experiments with a buffer solution of pH 10.26 showed that the potential becomes more negative with increasing pH values. Further experimentation will be necessary to formulate and explain the change of $E_0'$ with pH.

An inspection of the typical titration curves published in our last paper (2) shows that the form is far from regular. In spite of great pains and much work we have been unable to obtain consistent and reproducible slopes of the titration curves either in the absence or presence of pyridine. The irregularities may be best described by noting that the difference in potential between
the 20 per cent reduction point and the mid-point varied from 15 to 120 millivolts, a value of 60 millivolts being most representative. The difference in potential between the mid-point and the point of 80 per cent reduction showed less fluctuation; the extremes were 40 and 70 millivolts, while many results were very near 50 millivolts. The theoretical is 36 millivolts in each case. Thus, the shape of the e.m.f. curves is less consistent than the value of $E'_0$, and the curves on the whole are steeper than would be predicted from Equation 1.

The two experimental difficulties which are reflected in these unsatisfactory results are: (1) extreme sensitivity to oxygen and (2) unsatisfactory electrode equilibria. Probably both difficulties are accentuated by the low molecular concentration which is often necessitated by the insolubility of the compounds. The sensitivity to oxygen of reduced hemin or hematin solutions is very much greater than that of any other compounds which have been studied in this laboratory. Alkaline solutions of anthrahydroquinone derivatives are very sensitive to oxygen. Nevertheless, nitrogen which has been purified by passing through such a solution (Fieser's method) still contains sufficient oxygen to cause a "drift" in potential of partially reduced hemin. Even passing the nitrogen over heated copper was often not entirely satisfactory.

Two bright platinum electrodes were employed in all the experiments. They were cleaned, scoured, and reheated from time to time. In most titrations the electrodes agreed within a few millivolts except at the beginning and the end of the titration, but this agreement was more difficult to obtain than in the measurements of most reversible systems. It is strange that, in spite of the difficulties of obtaining really accurate measurements at a concentration of $10^{-4}$ molar, fairly definite and reproducible measurements can still be obtained at a concentration of only $10^{-6}$ molar. At this same low concentration, many reversible systems no longer impart a definite potential to an inert electrode.

The effect of changing concentration on the hemin or hematin systems appears to be enormous, the potential becoming more positive on dilution. Thus, at a concentration of $1.9 \times 10^{-4}$ molar the value of $E'_0$ for both hemin and hematin is about $-0.230$, while at a concentration of $3.8 \times 10^{-5}$ the value for hemin is $-0.164$, and at a concentration of $1.9 \times 10^{-5}$ is $-0.104$.
Fig. 1. The oxidation-reduction potential of the hemin and hematin systems in the presence of pyridine. Curve AC gives the values of $E'_0$ for hemin hydrolyzed in 0.1 M borax at 70° for 30 minutes = □ and hematin = △, concentration $1.9 \times 10^{-4}$ molar. Curve A' shows the effect of a 5-fold dilution. Curve BC represents data obtained in the experiment noted in Table II. In each case a smooth curve has been drawn through the experimental points.
for hematin. These large changes, if they are real and not due to some undiscovered experimental error, must be due to the fact that either the ferric or ferrous compound (or both) must be considerably associated. This association may well be due to the tendency of the iron atom to draw to itself other basic nitrogenous substances. Since dilution would favor the dissociation of the complex, and since dilution raises the potential, it seems clear that the ferric compound must be more associated than the ferrous compound under comparable conditions.

*Effect of the Addition of Pyridine.*

The addition of small amounts of pyridine to a reduced hemin or hematin solution makes the potential more positive. The change continues with increasing pyridine concentration. This is shown in Column 4 of Table II and in Fig. 1. A comparison of Curves BC and AC shows clearly that hemin is changed by heating with borax at 70° for 30 minutes. This change probably involves the replacement of the chlorine atom of hemin by a hydroxyl group and may involve a rearrangement of the linkages in the porphyrin molecule. If the hemin is heated in the borax solution for shorter periods of time and then titrated with titanous chloride in the presence of pyridine, the normal potential lies between that of the hemin-pyridine and hematin-pyridine systems. These results show that in employing alkaline solutions of hemin one may be dealing with solutions of hemin or a hydrolysis product depending on the alkalinity, the temperature, and the time during which the solution has stood. Hemin heated in borax is practically identical to hematin in its behavior towards pyridine as shown by the points plotted in Fig. 1. As will be shown below, they are not identical substances, however.

The most significant results given are those obtained with a concentration of $1.9 \times 10^{-5}$ mols per liter. However, in the case of the hemin-pyridine system, there was some precipitation of pyridine-hemochromogen during the titration. There was less precipitate at a concentration of $0.38 \times 10^{-5}$ molar, but the difficulty of obtaining satisfactory end-points at this concentration makes the results less reliable. It will be noted that in both the hemin-pyridine and hematin-pyridine systems the effect of dilution is to make the potentials somewhat more positive;
the effect is relatively slight, however, compared with the enormous effect of dilution in the absence of pyridine.

There is considerable spectroscopic and other evidence which shows that pyridine combines with both hemin and reduced hemin. The combination with the latter substance is known as pyridine hemochromogen. The equilibrium in this reaction has been studied by Hill (8) and more recently by Anson and Mirsky (9) who have questioned the validity of Hill's simple formulation of the reaction. Qualitatively the effect of pyridine on the potential of the hemin or hematin system is just what would have been predicted. The combination between the ferrous compound and

| TABLE II. Effect of Addition of Pyridine on Normal Potential of the Hemin System.* |
|---------------------------------|-----------------|----------------|
| Hemin concentration $0.38 \times 10^{-4}$ molar; temperature $= 22^\circ \pm 2^\circ$. |
| 1.24 M pyridine in 100 cc. | Concentration of pyridine. | Normal potential $E_0'$. |
| cc. | M | volts |
| 3.00 | 0.0361 | +0.001 |
| 5.00 | 0.0590 | +0.011 |
| 10.00 | 0.113 | +0.056 |

The hemin was dissolved at room temperature in 50 cc. of 0.1 M borax to which had been added the amount of 1.24 M pyridine solution shown in the first column; 50 cc. of 0.2 M sodium tartrate were then added and the titration with titanous chloride performed in the usual manner. The value of $E_0'$ was the value of $E$ at the mid-point.

* The data for a similar experiment on hemin of $1.9 \times 10^{-4}$ molar concentration are indicated by Curve BC, Fig. 1.

pyridine being more complete, the potential is raised by the addition of increasing amounts of pyridine. A quantitative treatment is much more difficult, particularly if we attempt to explain by means of association the effect of dilution on the potentials in the absence of pyridine. However, if we neglect association, we may derive an equation for the relationship of $E_0'$ to the concentration of pyridine as follows:

Let $[Py] = \text{concentration of pyridine}$, $[\text{Ferric}]_T$ and $[\text{Ferrous}]_T$ the total concentrations of oxidized and reduced pigment respectively and assume activities equal to concentrations, then we may
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write for the two equilibria the mass law constants $K_r$ and $K_o$ respectively:

\begin{align*}
(2) \quad \frac{[\text{Ferric complex}]}{[\text{Ferric}] [Py]^n} &= K_o \\
(3) \quad \frac{[\text{Ferrous complex}]}{[\text{Ferrous}] [Py]^m} &= K_r
\end{align*}

It thus follows that $[\text{Ferric}]_T = [\text{Ferric}] + K_o [\text{Ferric}] [Py]^n$ and $[\text{Ferrous}]_T = [\text{Ferrous}] + K_r [\text{Ferrous}] [Py]^m$.

Substituting the values of $[\text{Ferric}]$ and $[\text{Ferrous}]$ for the $[\text{Ox}]$ and $[\text{Red}]$ terms in Equation 1, we have Equation 4:

\begin{equation}
E = E'_0 - 0.06 \log \frac{[\text{Ferrous}]_T}{[\text{Ferric}]_T} - 0.06 \log \frac{1 + K_o [Py]^n}{1 + K_r [Py]^m}
\end{equation}

It is evident that when $[Py] = 0$ Equation 4 reduces to Equation 1. If we assume, further, that $K_r$ is much larger than $K_o$ (an assumption for which there is a good deal of evidence), then for the lower values of $[Py]$ we may write:

\begin{equation}
E = E'_0 - 0.06 \log \frac{[\text{Ferrous}]_T}{[\text{Ferric}]_T} + 0.06 \log K_r [Py]^m
\end{equation}

The last term may be taken as corresponding to the increase of potential with increasing pyridine concentration at least for the first portion of the curve shown in Fig. 1. If we take a value for $K_r$ of $10^5$ and $m = 2$, a very fair agreement with the experimental data is obtained, as illustrated below in the case of the hydrolyzed hemin or hematin data.

<table>
<thead>
<tr>
<th>Concentration of pyridine (Mol per l.)</th>
<th>Difference in $E'$ from $E'_0$ when $[Py] = 0$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found.</td>
</tr>
<tr>
<td>0.01</td>
<td>0.060</td>
</tr>
<tr>
<td>0.02</td>
<td>0.104</td>
</tr>
<tr>
<td>0.03</td>
<td>0.128</td>
</tr>
<tr>
<td>0.05</td>
<td>0.150</td>
</tr>
<tr>
<td>0.10</td>
<td>0.180</td>
</tr>
</tbody>
</table>

Very similar results may be obtained by an application of Equation 5 to the results obtained with hemin dissolved in borax (Table II). Taking $m = 2$, the values of $\log K_r$ found from the
experimental data range from 6.60 to 6.83 with a mean of 6.71. In these calculations no correction has been made for the effect of the added pyridine on the pH value of the buffer solution.

While the fact that by the use of one constant and several reasonable assumptions it is possible to derive a theoretical equation which fits the experimental data is gratifying, it cannot be said to constitute a proof of the correctness of our assumptions. Further work which is now in progress on the effect of dilution on all the systems must be considered before a final answer to the problem may be given.

In conclusion we may mention a few preliminary results of our study of the action of potassium cyanide which is still in progress. It is well known that the cyanide ion combines with hemin and reduced hemin and the resulting products may be characterized spectroscopically. The affinity between cyanide and the iron compounds is much greater than that between pyridine and the same compounds (see Anson and Mirsky (9) and Hill (8, 10)). In keeping with these facts we have found that a concentration of only 0.04 molar of cyanide is sufficient to produce the maximum effect on the potential systems in borax-tartrate solutions. The lower limits of the reaction have not yet been investigated. If hemin is dissolved in the borax buffer in the presence of this amount of cyanide and at room temperature, the resulting value of $E_0'$ is about $-0.160$ (an increase of about 70 millivolt from the value in the absence of cyanide). $\beta$-Hemin (from acetone) gave the same figure, while the various varieties of hematin and $\beta$-hemin (alcohol) gave a slightly lower value ($-0.190$). In all these experiments the concentration of pigment was $1.9 \times 10^{-4}$ molar; at one-tenth this concentration about the same values were obtained. Strangely enough, if hemin or hematin is heated with the borax solution for only a few minutes at $70^\circ$, the sub-

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1 Since this paper was prepared for publication, the recent results of Hill (10) have appeared. In this paper Hill replies to the criticisms of Anson and Mirsky and gives additional data concerning the pyridine-hemochromogen equilibrium. His value for $K = \frac{1}{K_r}$ is $8.9 \times 10^{-6}$. This is probably to be compared with our value for the hematin system of $10^{-8}$ for $\frac{1}{K_r}$. The agreement is all that could be expected.
sequent addition of cyanide has no effect on the value of $E_0'$ although a complex is formed as shown by the color. If cyanide is present during the heating, the usual complex is formed (judged by the effect on the potential). Attention was drawn earlier in this paper to the fact that $\alpha$-hemin hydrolyzed by heating with borax behaved similarly to hematin in regard to the action of pyridine. The experiments with cyanide, however, show that in reality the two compounds are different.

**BIBLIOGRAPHY.**

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