MECHANISM OF THE CATALYTIC OXIDATION OF ADRENALINE BY FERRITIN*

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The oxidation of adrenaline to the N-methylindolequinone, adrenochrome, is catalyzed by ferritin iron in the presence of \( \text{H}_2\text{O}_2 \) at acid pH (1). The mechanism of this reaction, which involves the intermediate formation of hydroxyl radicals and which does not require molecular oxygen, differs from that involved at neutral or slightly alkaline pH. Our present studies show that ferritin contains a portion of its total iron in a reactive state capable of catalyzing the aerobic oxidation of adrenaline to adrenochrome at pH 7.4 in the absence of added \( \text{H}_2\text{O}_2 \). Adrenochrome, at this pH, is rapidly converted to brown melanin-like pigments, a reaction which takes place very slowly at more acid pH.

The reaction of ferritin iron with adrenaline at pH 7.4 may be of physiological significance since the constrictor response of the mesenteric precapillary blood vessels to topically applied adrenaline is inhibited (2) when ferritin is present in the circulation, necessitating the addition of higher concentrations of adrenaline to bring about a constrictor response. Our findings indicate that the oxidation of adrenaline at pH 7.4 is accompanied by a loss of its vasoconstrictor activity, suggesting that the inhibitory action of ferritin on the response of the smooth muscle cells of the mesenteric precapillaries to topical adrenaline may be a result of ferritin-catalyzed adrenaline oxidation.

EXPERIMENTAL

The preparation of reagents and the method of washing glass-ware to reduce contamination with iron are described in the preceding paper (1). Reaction mixtures were incubated in 50 ml Erlenmeyer flasks connected in series by glass and rubber tubing to allow for gassing with oxygen or nitrogen. The flasks were incubated in a constant temperature bath at 37° with shaking to maintain proper gas equilibration. The course of the

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reaction was followed by reading optical densities of the reaction mixtures in a Beckman spectrophotometer at suitable time intervals.

**Formation of Iron-Adrenaline Complexes**—The reaction of Fe"++ with o-dihydroxy phenols to yield colored chelates is well known. The effect of pH on these complexes has also been studied and the variation in color was found to be due to variable ratios of Fe"++-phenol in the complex (3). Similar colored complexes are formed when adrenaline is treated with Fe"++. A number of solutions were prepared containing a final concentration of 0.5 mg. per ml. of adrenaline in buffers at different pH values, to which was added 0.5 mg. per ml. of a solution of Fe"++. At pH 4.0, a light green color forms which quickly fades to gray. At pH 5.0, a blue-green color is formed with an absorption maximum at 495 m\(\mu\). At pH 6.0, one gets a dark purple color with an absorption maximum at 570 m\(\mu\), and at pH 7.4 the color is red with a maximum at 575 m\(\mu\).

When Fe" was substituted for Fe"++, the colors appeared more slowly; the higher the pH, the more rapid was the color development. Fe" is able to produce colored complexes with adrenaline as a result of its autoxidation to Fe"++. The colors disappeared on addition of reduced glutathione or hydrosulfite to the solutions and reappeared on subsequent shaking with air.

**Oxidation of Adrenaline in Presence of Fe"++**—Although the colored complex, formed at pH 7.4 when Fe"++ is added to adrenaline, appears to be stable, actually the adrenaline undergoes oxidation, producing compounds whose color is masked during the initial period by the color of the Fe"++-adrenaline complex. At low concentrations of Fe"++ the color of the complex is very faint, and in the presence of 100 per cent oxygen the formation of colored oxidation products can be observed.

Incubation mixtures were prepared which contained 6.4 mg. of adrenaline, 3.0 ml. of 0.075 M potassium phosphate buffer, pH 7.4, 10 \(\gamma\) of Fe"++, and water to make a total volume of 15 ml. The Fe"++ solution was added immediately before the start of gas flow through the flasks. Individual flasks were prepared for each time interval, as well as control flasks which contained all the ingredients except Fe"++. The solutions were equilibrated with gas by shaking for 5 minutes at 37° before the tubing outlets of the end flasks were clamped. The flasks were shaken during the entire experiment. For the optical density readings at zero time, the iron solution was added to the mixtures just before they were placed in the spectrophotometer.

Table I lists optical density readings at 480 m\(\mu\) (absorption maximum for adrenochrome) at 15 minute intervals for three series of flasks incubated, respectively, in 100 per cent oxygen, air, and 100 per cent nitrogen. The results show that the reaction which produces adrenochrome from
adrenaline requires molecular oxygen, and that the reaction velocity is dependent on the oxygen tension. The results also indicate that autoxidation of adrenaline at pH 7.4 is considerable, in spite of precautions taken to eliminate metal contamination from water and glassware. It should be noted that the zero time readings of optical density are the same for all three mixtures and are due to the light absorption at 480 μm of the Fe+++-adrenaline complex. Since only a small fraction of the adrenaline is oxidized in the time during which the experiment is run, the color due to the complex is assumed to remain constant.

In Fig. 1 appears the time-course of the reaction which produces a colored oxidation product of adrenaline in 100 per cent oxygen, at pH 7.4 and 37°, in the presence of Fe+++. A similar experiment with Fe++ is presented for comparison. In both cases, the values plotted were obtained by subtracting the optical densities due to adrenaline oxidation in the absence of iron salts from those obtained in mixtures containing added iron. The curves, therefore, represent the extent of reaction catalyzed by added iron. Fe+++ is more effective for this reaction than Fe++, the activity of the latter being due to its oxidation to Fe+ at this pH. The lag period during the initial stage of the reaction is characteristic of this system.

**Table I**

*Effect of Oxygen Tension on Adrenaline Oxidation in Presence of Fe+++*

The values are given as the optical density at 480 μm.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>100 per cent oxygen</th>
<th>Air</th>
<th>100 per cent nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe+++</td>
<td>Control</td>
<td>Fe+++</td>
</tr>
<tr>
<td>0</td>
<td>0.062</td>
<td>0.003</td>
<td>0.062</td>
</tr>
<tr>
<td>15</td>
<td>0.126</td>
<td>0.032</td>
<td>0.073</td>
</tr>
<tr>
<td>30</td>
<td>0.317</td>
<td>0.078</td>
<td>0.084</td>
</tr>
<tr>
<td>45</td>
<td>0.775</td>
<td>0.280</td>
<td>0.107</td>
</tr>
<tr>
<td>60</td>
<td>1.320*</td>
<td>0.675</td>
<td>0.141</td>
</tr>
</tbody>
</table>

* This solution was diluted before the reading; the optical density reading was multiplied by the dilution factor to give this value.
OXIDATION OF ADRENALINE BY FERRITIN

**Fig. 1.** Oxidation of adrenaline by inorganic Fe$^{+++}$ and Fe$^{++}$ as measured by formation of colored oxidation products absorbing at 480 m$\mu$. Reaction carried out at pH 7.4 and 37° in 100 per cent oxygen.

**Fig. 2.** Oxidation of adrenaline by ferritin and inorganic Fe$^{+++}$. The formation of colored oxidation products measured at 480 m$\mu$, pH 7.4 and 37° in 100 per cent oxygen.
dation of adrenaline were subtracted. As in the case of Fe^{+++}, ferritin also furnished iron for a colored iron-adrenaline complex. The optical densities were read at 480 m\textmu, the absorption maximum for adrenochrome, the only well characterized product formed during adrenaline oxidation. In both curves there is an increasing production of color absorbing at 480 m\textmu for the first 60 minutes. After this time the absorption at 480 m\textmu falls, though to the naked eye the intensity of color increases as it changes from pink to brown.

Nature of Reaction Products—The experiments in Fig. 2 suggest the formation of compounds other than adrenochrome. In experiments identical to those in Fig. 2, the optical densities were read in the spectrophotometer at several time intervals and at several different wave-lengths. These absorption spectra are plotted in Fig. 3 for a reaction involving Fe^{+++}, and show that the product formed during the early part of the reaction has an absorption maximum at 480 m\textmu, but that, as the reaction proceeds, this compound is replaced by a compound or compounds with an increasing absorption in the ultraviolet region.

In an attempt to determine the nature of the reaction more precisely, a mixture containing a small, known quantity of adrenaline was allowed to incubate overnight in oxygen under conditions similar to those noted above. This resulted in the complete transformation of adrenaline to brown melanin-like compounds. The absorption spectra for this brown solution, as
well as for an authentic sample of adrenochrome prepared by silver oxide oxidation of adrenaline (5), appear in Fig. 4. From these data the extinction coefficients for adrenochrome were found to be 4020 (molecules per liter per cm.) at 480 mμ and 1020 at 370 mμ. Although the brown color in solutions of completely oxidized adrenaline represents a mixture of several melanin compounds, the values for "extinction coefficients" at 480

and 370 mμ, 845 and 3741 respectively, were found useful in calculating the relative quantities of adrenochrome and further oxidation products in the reaction mixture.

The experiments in Fig. 2 were then repeated, with the difference that readings were made at each time interval at both 480 and 370 mμ. By applying the standard equations for mixtures of two compounds with different absorption characteristics (6), the content of adrenochrome and melanin compounds in each reaction mixture was calculated from the extinction coefficients at these two wave-lengths. The results in Fig. 5 demonstrate that adrenochrome is formed first, but that even during the
early part of the reaction melanin compounds appear. As the reaction proceeds, the concentration of melanins in the reaction mixture becomes greater than that of adrenochrome. The decrease in optical density at 480 μm observed in earlier experiments is explained by these findings.

Effect of pH on Oxidation of Adrenaline in Presence of Ferritin—The extent of oxidation of adrenaline by ferritin was measured at various pH values by noting the optical densities of the reaction mixtures at 480 μm.

In the results given in Fig. 6, the velocity of color development is greatest at pH 7.4 and falls sharply in more acid solutions. The data used to plot this graph were obtained by subtracting the values for autoxidation of adrenaline in the absence of ferritin; both iron-catalyzed and autoxidation reactions increased in velocity as the pH was made more alkaline. The curve for pH 6.9 is of some interest since it yields the highest optical density reading at 480 μm. It was postulated that differences in rates of adrenochrome disappearance at different pH values might account for this result.

To study the rate of conversion of adrenochrome to the melanin compounds, adrenochrome was incubated at pH 7.4 in the presence and absence of Fe⁺⁺⁺. The conversion to melanins at this pH was very rapid; it was
not affected by iron and occurred in nitrogen as well as in oxygen. The effect of pH on this conversion was studied further by incubating adrenochrome at various pH values in the absence of Fe

\[ \text{Fe}^{+++} \] and reading optical densities at 480 and 370 m\( \mu \) to determine the extent of adrenochrome disappearance. The results are presented in Fig. 7. As the pH decreases, the rate of melanin formation decreases. These data explain the results obtained in Fig. 6, which indicates a higher optical density at 480 m\( \mu \) at
pH 6.9 than at 7.4, since at the latter pH value less adrenochrome is actually present, owing to its rapid conversion to the melanin compound.

The brown solutions, which were formed when adrenochrome was incubated at pH 7.4 in nitrogen as well as in oxygen, were analyzed in the spectrophotometer. In the visible region, the shapes of the absorption curves for these two solutions were identical (see Fig. 4). However, in the ultraviolet region the two solutions were different, each having a series of ill defined maxima but at different wave-lengths. Subsequent incubation of the brown solution which had been prepared in nitrogen, in an oxygen atmosphere, altered the absorption spectrum. It appears from these findings that different chemical compounds are formed, although visibly they appear to be the same, and probably each represents a mixture of several compounds.

**Table II**

*Effect of Inhibitors on Adrenaline Oxidation*

<table>
<thead>
<tr>
<th>Inhibitor, 2.3 μmoles per liter</th>
<th>Adrenochrome formed</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.01 μmol L × 10⁻⁵</td>
<td>100 per cent</td>
</tr>
<tr>
<td>Oxalate</td>
<td>0.49</td>
<td>16 per cent</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>2.02</td>
<td>67 per cent</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.82</td>
<td>94 per cent</td>
</tr>
<tr>
<td>Tartrate</td>
<td>2.96</td>
<td>98 per cent</td>
</tr>
<tr>
<td>EDTA</td>
<td>27.20</td>
<td>904 per cent</td>
</tr>
</tbody>
</table>

*Effect of Iron Inhibitors on Adrenaline Oxidation*—A number of iron-binding compounds were tested for their ability to inhibit the oxidation of adrenaline by ferritin. The inhibitors were added at a final concentration equal to that of adrenaline (2.3 μmoles per ml.) so as to compete effectively with adrenaline for the iron. Table II lists the results. Optical density readings at 480 and 370 mμ were taken after 30 minutes at pH 7.4 and 37° in the presence of 100 per cent oxygen. The concentration of adrenochrome produced was calculated as indicated in a previous section. At equimolar concentrations, oxalate was most effective as an inhibitor, and next most effective was pyrophosphate. In subsequent experiments o-phenanthroline and α,α'-dipyridyl also showed inhibitory properties. Citrate and tartrate were almost without effect at this pH. The outstanding finding was the extraordinary activation of the oxidation reaction produced by ethylenediaminetetraacetate (EDTA).

*EDTA Activation of Adrenaline Oxidation*—The degree of activation of the oxidation of adrenaline to adrenochrome by ferritin at increasing con-
Concentrations of EDTA was determined. The reaction mixtures were incubated for 30 minutes and adrenochrome formation measured. The extent of activation increased up to an optimal concentration of EDTA. EDTA also activated the reaction with inorganic iron, and in this case maximal activation occurred at a molar ratio of iron-EDTA of about 1:2. However, inasmuch as the inorganic phosphate which is present as a buffer also binds inorganic iron, as does adrenaline, it appears that enough EDTA was needed to overcome the effect of phosphate and adrenaline and to form a complex of the iron as the well known chelate which contains 1 atom of iron per molecule of EDTA.

**Quantity of Active Iron in Ferritin**—The effectiveness of ferritin in furnishing iron for the catalytic oxidation of adrenaline confirms our previous findings that, despite the chemical inactivity of the bulk of ferritin iron, a small quantity of its iron is capable of taking part in chemical reactions. It was found that 616 \( \gamma \) of total iron as ferritin were required to produce an amount of adrenochrome equal to that formed by 8.5 \( \gamma \) of inorganic Fe\(^{3+}\) under similar conditions. Identical results were obtained when the reactions were repeated in the presence of optimal concentrations of EDTA. Thus, 1.4 per cent of the total iron of ferritin appears to be "available," at pH 7.4, as a catalyst for adrenaline oxidation. Since binding of iron by the protein in ferritin would influence the rate of reaction of ferritin iron as compared with that of inorganic iron, this value cannot be considered quantitative.

**Comparison of Ferritin Activity with That of Other Iron Proteins**—The activities of other iron proteins were compared with those of ferritin and inorganic iron as catalysts for adrenaline oxidation. In all cases the added protein contained total iron equivalent to 8.5 \( \gamma \) of Fe\(^{3+}\). The relative activities are listed in Table III. It may be seen that the plasma iron-binding protein was less active than ferritin, emphasizing the strength of the iron-protein bond. Oxidized cytochrome c, methemoglobin, and oxidized heme were all more active than equivalent amounts of inorganic iron. Ferrocytochrome c was without activity. The relative activities of these compounds were now determined in the presence of EDTA (Table III). Inorganic iron and ferritin activities were increased so that they became the most active; the activities of the others were not affected significantly.

**Participation of H\(_2\)O\(_2\)**—The possibility of intermediate formation of H\(_2\)O\(_2\) during the reaction at pH 7.4 was explored by determining whether the reaction could be inhibited by crystalline catalase. The concentrations of all reagents were the same as in previous experiments except for the addition of 0.1 ml. of a diluted crystalline catalase preparation, the activity of which was found to be equal to 15 units. The quantity of
adrenochrome formed in 30 minutes at 37° in 100 per cent oxygen was determined in the presence and in the absence of added catalase. Estimation of extent of inhibition was determined during the early part of the reaction so as to avoid the period during which melanin formation is great. It should also be pointed out that catalase is easily inactivated in dilute solutions and at 37°, so that any inhibition which is observed is quite significant. Table IV reports the effect of catalase on each iron system. It can be seen that, in increasing order, the activities of Fe⁺⁺⁺-EDTA, ferritin-EDTA, and ferricytochrome c were all inhibited by catalase. In the cases of Fe⁺⁺⁺ (H₂O) and ferritin, an activation by catalase was observed. The reason for this activation will be discussed below.

Since the active chelates of iron were inhibited by catalase, experiments were performed involving addition of H₂O₂ in order to gain information concerning its rôle in these reactions. Table V presents the effects of

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**Table III**

*Relative Activities of Iron Compounds As Adrenaline Oxidants*

<table>
<thead>
<tr>
<th>Iron compound</th>
<th>Relative activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>Ferritin or Fe⁺⁺⁺</td>
<td>100</td>
</tr>
<tr>
<td>Iron-binding globulin of plasma</td>
<td>40</td>
</tr>
<tr>
<td>Heme</td>
<td>170</td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>190</td>
</tr>
<tr>
<td>Ferricytochrome c</td>
<td>290</td>
</tr>
<tr>
<td>Ferrocytochrome c</td>
<td>0</td>
</tr>
</tbody>
</table>

*Activity measured in terms of adrenochrome formation in 30 minutes at 37° in an atmosphere of 100 per cent oxygen; adrenochrome produced by Fe⁺⁺⁺ or ferritin was taken as equal to an activity of 100. All the compounds contained equivalent amounts of total iron, except for ferritin, which was used in amounts equal in activity to the Fe⁺⁺⁺.

**Table IV**

*Effect of Catalase on Adrenaline Oxidation*

<table>
<thead>
<tr>
<th>Form of iron</th>
<th>Activation (+) or inhibition (−) per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe⁺⁺⁺ (H₂O)</td>
<td>+23</td>
</tr>
<tr>
<td>Ferritin</td>
<td>+10</td>
</tr>
<tr>
<td>Fe⁺⁺⁺-EDTA</td>
<td>−16</td>
</tr>
<tr>
<td>Ferritin-EDTA</td>
<td>−24</td>
</tr>
<tr>
<td>Ferrocytochrome c</td>
<td>−43</td>
</tr>
</tbody>
</table>
H$_2$O$_2$; in each case the figures listed were obtained at optimal concentrations of H$_2$O$_2$. Peroxide activated the reaction mixtures containing Fe$^{+++}$-EDTA and ferritin-EDTA by 10 per cent; ferricytochrome $c$ was activated by 157 per cent. Hydrated Fe$^{+++}$ was inhibited, whereas ferritin was activated by about 50 per cent. The reason for these results will be discussed later.

**Table V**

*Effect of Added H$_2$O$_2$ on Adrenaline Oxidation*

<table>
<thead>
<tr>
<th>Form of iron</th>
<th>Activation (+) or inhibition (−) per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{+++}$ (H$_2$O)</td>
<td>−14</td>
</tr>
<tr>
<td>Ferritin</td>
<td>+51</td>
</tr>
<tr>
<td>Fe$^{+++}$-EDTA</td>
<td>+9</td>
</tr>
<tr>
<td>Ferritin-EDTA</td>
<td>+10</td>
</tr>
<tr>
<td>Ferricytochrome $c$</td>
<td>+157</td>
</tr>
</tbody>
</table>

**Table VI**

*Activity of Iron-Chelating Agents Related to EDTA*

<table>
<thead>
<tr>
<th>Form of iron</th>
<th>Adrenochrome formation $\text{moles} \times 10^{-5}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe$^{+++}$</td>
<td>2.58</td>
</tr>
<tr>
<td>Fe$^{+++}$-nitrilo triacetate*</td>
<td>2.78</td>
</tr>
<tr>
<td>Fe$^{+++}$-Versenol†</td>
<td>7.79</td>
</tr>
<tr>
<td>Fe$^{+++}$-EDTA</td>
<td>26.82</td>
</tr>
<tr>
<td>Fe$^{+++}$-isopropylene compound‡</td>
<td>26.96</td>
</tr>
</tbody>
</table>

* Ammonium triacetate.
† N-Hydroxyethyl-ethylenediamine triacetate.
‡ Isopropylendiamine tetraacetate.

Activities of Iron-Chelating Agents Related to EDTA—Because of the unusual activity of EDTA, structurally related compounds were tested for their ability to activate the iron-catalyzed oxidation of adrenaline. The concentrations of all reagents were similar to those of previous experiments. The chelating agents were added at equivalent molar concentrations. The reaction was allowed to proceed for 30 minutes at 37° in the presence of 100 per cent oxygen, and the amount of adrenochrome formed was measured. The results are given in Table VI. Isopropylendiaminetetraacetate had an activity equal to that of EDTA. N-Hydroxyethyl-ethylenediamine triacetate (Versenol) was less active than EDTA, although about three times as active as hydrated Fe$^{+++}$. Nitrilo triacetate (ammonium triacetate) was essentially inactive.
Vasoconstrictor Activities of Adrenaline, Adrenochrome, and Melanins—

The ability of adrenaline and its oxidation products to bring about a vasoconstrictor response when applied topically to the precapillary blood vessels of the rat mesentery (7) was determined. The unit of activity was defined as that concentration which would produce a vasoconstriction lasting for approximately 20 seconds. Such a response required $5 \times 10^{-4}$ $\gamma$ per ml. of adrenaline and $13 \times 10^{-4}$ $\gamma$ per ml. of adrenochrome. The melanin compounds, formed as a result of adrenaline oxidation, were without vasoconstrictor activity. A series of experiments was then performed comparing the vasoconstrictor activities and optical density readings of mixtures containing adrenaline and ferritin incubated for varying intervals of time. The production of melanin was accompanied by a corresponding disappearance of vasoconstrictor activity, in agreement with the above findings.

DISCUSSION

The catalytic oxidation of adrenaline by metal ions has been extensively studied by Chaix et al. (8), who utilized oxygen consumption measurements as an index of reaction velocity. Our use of the spectrophotometer to determine the nature and concentration of the colored products formed during the oxidation reaction has enabled us to study the mechanism of the reaction in greater detail.

Inorganic ferric iron at pH 7.4 exists for the most part as ferric hydroxide. Formation of a complex at pH 7.4 between Fe$^{+++}$ and adrenaline serves to provide a greater concentration of Fe$^{+++}$ in a soluble state. The purple color of the complex is not intense at low concentrations of Fe$^{+++}$ and, in the presence of oxygen and relatively high concentrations of adrenaline, is replaced by the pink color of adrenochrome. The latter compound is rapidly converted at this pH to brown, melanin-like pigments.

The reaction of ferritin with adrenaline is similar to that of inorganic iron. The colored iron-adrenaline complex is formed and, in the presence of oxygen, adrenaline is oxidized as evidenced by the appearance of adrenochrome, followed by melanin. Although determination of the quantity of iron in ferritin "available" as a catalyst for adrenaline oxidation is only approximate, it does point to the existence of a form of iron in ferritin different from the bulk of its iron, in agreement with results reported previously (4). The active iron is presumed to be at or near the surface of the protein, bound to amino acid groups in a manner which allows it to take part in oxidation-reduction reactions.

Chaix et al. (8) have reported that the iron-adrenaline complex is not autooxidizable. Our finding of a greatly increased activity of Fe$^{+++}$ in the presence of EDTA and our confirmation of the activity of ferricytochrome
c, previously reported by Green and Richter (9), agree with this interpretation and cast doubt on the participation of the iron-adrenaline complex in this reaction. The stability constant of Fe\textsuperscript{+++}·EDTA is large (log $K_2 = 25$ at 20°), representing a highly undissociated state of the iron, although the complex is capable of some hydrolysis at pH 7.4 (10). The quantity of the iron-adrenaline complex is much lower in the presence of EDTA than in its absence. Despite the presence of smaller amounts of the iron-adrenaline complex, the activity of Fe\textsuperscript{+++}·EDTA is some eight to ten times greater than that found in the absence of the chelating agent. The argument against the direct participation of the iron-adrenaline complex is made stronger by the activity of ferricytochrome c, since this iron-chelate is highly undissociated and does not allow for the formation of any iron-adrenaline complex. Indeed, this property of cytochrome c, which makes it impossible to suggest the formation of a complex with its physiologically important substrates, has led Theorell (11) to suggest that electron transfer takes place via the N atoms of the imidazole groups which occupy two of the six coordination bonds of iron in cytochrome c, and results in the reduction of ferricytochrome c to the ferrous state.

Our experimental data do not offer a complete explanation for the greater activity of the Fe\textsuperscript{+++}·EDTA complex or of ferricytochrome c as compared with hydrated Fe\textsuperscript{+++}, except to suggest that formation of iron chelates of these particular types makes the iron available at a higher concentration, and in a form which allows it to undergo rapid oxidation and reduction. Although the two iron chelates which are very active for adrenaline oxidation are quite different chemically, there is some similarity in their structures (Diagram 1). In the case of the EDTA complex, chelation occurs between the iron atom and 2 nitrogen and 2 oxygen atoms, all in the same plane; the remaining bonds, one above and one below this plane, are attached to atoms of the carboxyl groups. In cytochrome c, the iron atom is chelated to the 4 nitrogen atoms of the pyrrole groups and the remaining two bonds, above and below the plane, to 2 nitrogen atoms of histidine. Both EDTA (12) and cytochrome c (13) complexes are paramagnetic.

With respect to the activity of EDTA, some structural requirements can be seen by comparing its activity with that of related compounds whose structures are given in Diagram 2. Isopropylendiamine tetraacetate is as active as EDTA and contains all of the groups necessary to form six coordinate bonds with the iron atom. N-Hydroxyethylendiamine triacetate is less active than EDTA and contains but five of the six groups required for binding to the iron atom. Nitrilo triacetate (ammonium triacetate) is inactive in this system and can contribute but four groups for chelation with the iron atom. It is presumed in the last two instances that the remaining bonds are attached to water molecules.
In a discussion of amino acid metabolism Stadtman (14) refers to the accelerating effect of EDTA, in the presence of metal ions, on glutathione oxidation. Although no details are given, it is likely that this effect is similar to that which we have found for adrenaline. Pirie and van Heyningen (15) have reported the increased oxidation of glutathione in lens extracts in the presence of EDTA. They consider this activity to be due to the stimulating effect of a metal-EDTA complex on ascorbic acid oxidation, which in turn accelerates the oxidation of reduced glutathione. Chalk and Smith (16) have also reported the enhanced activity of iron salts, on the autoxidation of cyclohexene, in the presence of a metal-chelating agent, disalicylidene. Bonner (17) has demonstrated the activation effect of EDTA on a succinic dehydrogenase-cytochrome system in heart muscle preparations and believes that the chelating agent acts on a series of electron transport reactions between some unknown factor and cytochromes b and c or between the factor and diaphorase. Bowen et al. (18) have reported experimental data which give the activating effect of EDTA on the adenosinetriphosphatase activity of myosin B (natural actomyosin). These reports of the activating effect of EDTA suggest that activation by metal chelation is not an unusual phenomenon among biochemical reac-
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Tions. Together with our findings, they emphasize the need for caution in assigning to EDTA the simple rôle of a chelating agent which removes inhibitory metal ions from solution, thus leading to an activation of enzyme reactions.

Adrenaline is oxidized at a pH more acid than 6 by Fe++ and H2O2 by a mechanism which involves the formation of water radicals, powerful oxidizing agents (1). The participation of H2O2 in the reaction at pH 7.4 was explored because of the reports of its formation during inorganic metal ion catalysis of oxidation reactions (10). The effect of added catalase is often used to demonstrate the presence or absence of H2O2 during enzyme reactions, but the occurrence of a number of intermediate reactions, each with different velocities, may produce negative results with catalase and may lead to the erroneous conclusion that H2O2 is not formed during the course of the reaction.

In our own experiments, the reaction of Fe++ or of ferritin with adrenaline to form adrenochrome is not inhibited by the addition of catalase; on the contrary, there is some acceleration. The addition of catalase does inhibit the reaction of Fe++-EDTA, ferritin-EDTA, or ferricytochrome c with adrenaline. These facts may be interpreted in the following manner: In the initial reaction the iron may be in the form of Fe+++ (H2O), Fe++-EDTA, or ferricytochrome c. All react in a similar fashion and result in the formation of the corresponding ferrous complex, adrenaline o-quinone and H2O2. Hydrogen peroxide brings about the reoxidation of the ferrous to the ferric complex. In the absence of chelating agents, hydrated ferrous ion is oxidized by molecular oxygen at pH 7.4 with such rapidity that H2O2 does not influence the rate of this reaction. This would account for the lack of inhibition of this reaction in the presence of catalase. The accelerating effect of catalase in the absence of chelating agents can be explained in terms of the rapid removal of H2O2, thus increasing the rate of the reaction. The Fe++-EDTA complex is also oxidized by molecular oxygen, but to a lesser extent than is the unchelated metal ion (20). It is also susceptible to oxidation by H2O2. Thus a removal of H2O2 by catalase results in an inhibition of the rate. Ferrocytochrome c cannot react with molecular oxygen at all, but does so with H2O2 to form ferricytochrome c. It would be expected therefore that this reaction, entirely dependent on H2O2 for reoxidation, would be more sensitive to inhibition by catalase. The relative order of inhibition by catalase of the reactions involving these types of iron compounds agrees with this analysis. Ferrocytochrome c would be reoxidized in vivo, by means other than H2O2, e.g. cytochrome oxidase.

The results obtained after adding additional H2O2 are in agreement with this interpretation. The system which contains hydrated Fe+++ is in-
hibited, whereas the systems containing Fe\textsuperscript{+++} and EDTA or cytochrome c are accelerated. The low order of acceleration of the EDTA system as compared with the very marked acceleration of the cytochrome system is in accord with the relative difficulty of oxidation of the corresponding ferrous compounds by molecular oxygen. The accelerating effect of H\textsubscript{2}O\textsubscript{2} on ferritin is accounted for by noting that ferritin contains some of its reactive iron in the Fe\textsuperscript{++} state, a state of iron which is less active than Fe\textsuperscript{+++} for adrenaline oxidation. Fe\textsuperscript{++} in ferritin is, however, stabilized by binding to SH groups. Oxidation of these SH groups by H\textsubscript{2}O\textsubscript{2} results in the oxidation of Fe\textsuperscript{++} to Fe\textsuperscript{+++} in ferritin and accounts for the acceleration of ferritin activity in the presence of H\textsubscript{2}O\textsubscript{2}.

Proof for the intermediate formation of adrenaline o-quinone is indirect, since this compound has not been isolated and is presumed to be unstable. Ball and Chen (21), using dyes, demonstrated the probable formation of this compound during experiments involving determination of the oxidation-reduction potential of adrenaline. They observed a change in potential at a time when the pink color of adrenochrome had not yet been formed, but the dye had been reduced.

The next step in the reaction involves the formation of adrenochrome, an N-methylindole-o-quinone, which can be prepared synthetically and which is stable in acid solution. It can be identified in the reaction mixture at pH 7.4 by means of its characteristic absorption spectrum. The mechanism for the conversion of adrenaline o-quinone to adrenochrome has not been established. The final reaction involves the conversion of adrenochrome to the brown melanin pigment; the chemistry of this reaction is unknown.

The results of these studies and of our previous experiments may be used to suggest a possible mechanism for the inhibitory action of ferritin on the constrictor response of the muscular capillaries in the mesoappendix to topical adrenaline. This vasodepressor activity of ferritin has been demonstrated as being due to the presence of Fe\textsuperscript{++}, which is stabilized against autoxidation by bonding to protein sulfhydryl groups. We have demonstrated that this form of ferritin iron can move across a dialysis membrane (4). On the other hand, the iron of inactive ferric disulfide-ferritin is not able to cross such a membrane. This phenomenon resembles that governing iron absorption in the small intestine; ferric iron must first be reduced to the ferrous state before it can be absorbed. We therefore would postulate that the surface iron of circulating reduced ferritin can be released for transport or attachment to the smooth muscle cells of the precapillary vessels, while that of ferric disulfide-ferritin cannot. The Fe\textsuperscript{++}, now unprotected, would be oxidized to Fe\textsuperscript{+++}. Its conversion to Fe\textsuperscript{+++} in the muscle cell would allow it to catalyze the oxidative inactivation of adrenalin-
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ine by a mechanism similar to that outlined in the present study. As a result, the capillary vessels would no longer respond to the original threshold concentration of adrenaline and would require the topical application of larger amounts to achieve a concentration adequate to elicit the constrictor response.

In view of the absence of any known sympathomimetic action of adrenochrome (22), its constrictor effect on these specific capillaries could be attributed to a musculotropic property. This property of adrenochrome may be related to its reported hemostatic action (23) for which no specific explanation has hitherto been offered.

The biological validity of the hypothesized pathways of adrenaline metabolism, oxidation of the side chain by tissue amine oxidases (24) or metal-catalyzed quinone formation, still remains to be established. The present observations offer a mechanism which involves naturally occurring iron compounds. Some support for the involvement of iron in this process is provided by the observation (25) that cells containing melanin pigment or melanin granules contain amounts of iron larger than those found in surrounding non-pigmented tissue.

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SUMMARY

Spectrophotometric studies demonstrate that the iron-catalyzed oxidation of adrenaline involves molecular oxygen and the production of the N-methylindolequinone, adrenochrome. At pH 7.4, the latter compound is converted to brown melanin-like pigments.

The iron-catalyzed oxidation of adrenaline is increased 10-fold by the presence of the iron-chelating agents, EDTA or its isopropylene analogue. The biochemically important iron-chelate, ferricytochrome c, is also more active than inorganic Fe++. In both instances H2O2 is a product of the reaction and serves to reoxidize the ferrous to the ferric chelate.

Ferritin contains a small portion of its total iron in a state which catalyzes the oxidation of adrenaline by a mechanism similar to that found for inorganic Fe++. Adrenochrome, which lacks the sympathomimetic activity of adrenaline, is still effective as a vasoconstrictor of the smooth muscle cells of the capillary vessels. However, the melanins, formed from adrenochrome, have no constrictor activity. These findings are utilized in an explanation of the biological activity of circulating ferritin in inhibiting the constrictor response of the muscular capillaries to topical adrenaline.

BIBLIOGRAPHY

MECHANISM OF THE CATALYTIC OXIDATION OF ADRENALINE BY FERRITIN
Saul Green, Abraham Mazur and Ephraim Shorr


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