Horseradish Peroxidase-catalyzed Conversion of Iodine to Iodide in Presence of EDTA and H₂O₂*

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EDTA (4 mM) blocks the oxidation of iodide to I₅ (increase of extinction at 353 nm) by H₂O₂ catalyzed by horseradish peroxidase, which is reversed by the addition of an equimolar concentration of Zn²⁺. Addition of suboptimal concentration of EDTA (2 mM) not only decreases the rate of forward reaction of I₅ formation but also causes loss of extinction of the same when I₃ is generated. The loss of extinction of I₃ is proportional to the enzyme concentration and is blocked by azide, the inhibitor of the peroxidase. EDTA also causes bleaching of nonenzymatically formed I₃ (from iodide and H₂O₂) only in the presence of horseradish peroxidase, and the effect is reversed by the equimolar concentration of Zn²⁺. Both the bleaching of I₃ by EDTA and reversal of EDTA effect by Zn²⁺ are sensitive to azide. The decrease of extinction of I₃ (formed by dissolving iodine in KI solution) is dependent on EDTA, H₂O₂, and horseradish peroxidase. Molecular iodine is also bleached but at a slower rate than I₃. Evidence is presented to show that this bleaching of I₃ is due to enzymatic conversion of I₃ to iodide in presence of EDTA and H₂O₂ and this involves pseudocatalatic degradation of H₂O₂ to O₂.

During our experiments with gastric peroxidase isolated from the mitochondrial fraction of mouse gastric mucosa (1, 2), we observed that triiodide (I₃) formation from iodide catalyzed by this enzyme was blocked by EDTA (3). This effect of EDTA was reversed by the addition of an equimolar concentration of Zn²⁺, whereas Ca²⁺ or Mg²⁺ was less effective. EDTA also blocked the triiodide formation catalyzed by chloroperoxidase, horseradish peroxidase, and lactoperoxidase. This led us to investigate more critically the effect of EDTA on this reaction using horseradish peroxidase as the model enzyme.

In this paper, we are presenting evidence indicating that EDTA not only interfered with the enzymatic formation of I₃, but also led to the formation of iodide catalyzed by the same enzyme. Using triiodide as initial reactant, we have further shown that horseradish peroxidase could catalyze the formation of iodide from triiodide only in the presence of EDTA and H₂O₂. This reaction is associated with concomitant pseudocatalatic degradation of H₂O₂ leading to the evolution of O₂.

RESULTS

While studying the peroxidation of iodide by horseradish peroxidase, an interesting observation was made in connection with the effect of EDTA on triiodide formation as shown in Fig. 1. The formation of I₃ was found to be completely blocked in the presence of 4 mM EDTA, whereas the suboptimal concentration of EDTA (2 mM) not only decreased the initial rate of I₃ formation by about 50% but it also caused a linear decrease of extinction of I₃ after attainment of the plateau at about the 8th min. Addition of 3 mM N₃⁻ at that time prevented the loss of the extinction. Similar phenomena were also observed when chloroperoxidase or lactoperoxidase was used instead of horseradish peroxidase. However, the concentration of the enzyme should be so chosen that I₃ formation may attain equilibrium as early as possible. Fig. 2 indicates the effect of addition of 4 mM EDTA on the enzymatically generated I₃ (when the reaction tends to saturate) using two different concentrations of horseradish peroxidase. It is clear from the figure that not only the initial rate of I₃ formation was dependent on horseradish peroxidase concentration, but the initial rate of loss of extinction of I₃ on addition of EDTA was also proportional to the enzyme concentration. Addition of 3 mM N₃⁻ prior to the addition of EDTA (not shown) blocked the EDTA effect, indicating that the effect was mediated through the peroxidase. A similar experiment was also

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1 The abbreviation used is: EGTA, [ethylenebis(oxyethylene-nitri1o)]l4etraacetic acid.
EDTA Effect on Enzymatic Formation and Disappearance of Triiodide

FIG. 1. Effect of EDTA on horseradish peroxidase-catalyzed I$_3^-$ formation. Formation of I$_3^-$ was monitored at 353 nm using 2 $\mu$g of horseradish peroxidase in the standard assay system described in the text in the presence and absence of EDTA. ○, control; ●, +2 mM EDTA; ▲, +4 mM EDTA.

FIG. 2. Effect of EDTA on the I$_3^-$ formation and disappearance at varying concentration of horseradish peroxidase. I$_3^-$ was generated by two different concentrations of horseradish peroxidase (HRP), 1 $\mu$g (●) and 2 $\mu$g (○), respectively, as described in the text. When the extinction reached about 1.88, 4 mM EDTA was added to follow the loss of extinction at 353 nm.

FIG. 3. Role of peroxidase on the disappearance of I$_3^-$ with EDTA. I$_3^-$ was produced nonenzymatically for 1–2 h in a reaction mixture containing all the components of peroxidase assay except the enzyme. When the extinction at 353 nm reached around 0.6, 4 mM EDTA was added as indicated by the arrow. After 2 min, 2 $\mu$g of horseradish peroxidase was added, and the loss of extinction of I$_3^-$ was monitored. After the extinction dropped to 0.05 at 5 min, 4 mM ZnSO$_4$ was added to follow the reappearance of I$_3^-$. ○ indicates the effect of Zn$^{2+}$ after addition of 3 mM NaN$_3$.

carried out with chloroperoxidase (data not shown). However, chloroperoxidase (0.25 $\mu$g)-mediated loss of extinction of I$_3^-$ required higher concentration of EDTA (6 mM), and the rate of decrease of extinction was also higher than that of the horseradish peroxidase.

In order to understand more about the role of peroxidase on the disappearance of I$_3^-$ by EDTA, I$_3^-$ was prepared nonenzymatically using iodide and H$_2$O$_2$, and the effect of peroxidase was studied. Fig. 3 shows that the extinction of I$_3^-$ did not change significantly on addition of 4 mM EDTA but addition of horseradish peroxidase (2 $\mu$g) caused a sharp decrease of extinction of I$_3^-$. At 5 min, when the extinction reached a value of 0.05, addition of 4 mM ZnSO$_4$ (equimolar to EDTA) to scavenge the EDTA led to the reappearance of I$_3^-$ to the original level. This indicated that horseradish peroxidase was still active and could catalyze the formation of I$_3^-$ from the residual iodide and H$_2$O$_2$ present in the system as well as the iodide formed (shown in later experiment) during the loss of extinction of I$_3^-$. Chloroperoxidase (2 $\mu$g) under an identical condition (data not shown) catalyzed the same reaction but at a relatively higher rate than horseradish peroxidase. However, lactoperoxidase (0.5 $\mu$g) also possesses the same property (data not shown), but requires as high as 8 mM EDTA to catalyze the bleaching of the I$_3^-$; N$_2$ in all the cases could block the reaction when added prior to the addition of peroxidase. When horseradish peroxidase was added before the addition of EDTA, there was no loss of extinction; on the other hand, I$_3^-$ formation increased further due to enzymatic oxidation of iodide present in the system (data not shown).

It may be presumed that the loss of extinction of I$_3^-$ may be due to an adduct of I$_3^-$ with the EDTA in the presence of the enzyme and H$_2$O$_2$. If it were so, the loss of extinction of I$_3^-$ on addition of EDTA as well as reappearance of I$_3^-$ after the addition of Zn$^{2+}$ would have been instantaneous. On the contrary, the results show that both the above-mentioned phenomena were time-dependent peroxidation reactions. The alternate possibility is the backward reaction resulting in the conversion of I$_3^-$ to I$^-$ with EDTA in the presence of the peroxidase. However, requirement of H$_2$O$_2$ for the bleaching of I$_3^-$ color rules out a true back reaction. The disappearance of I$_3^-$ after the addition of EDTA could be further demonstrated by starch solution. Addition of starch solution to the incubation system before the addition of EDTA showed an absorbance of 2.0 at 660 nm, whereas upon addition of the starch solution a few minutes after the addition of EDTA, when I$_3^-$ extinction went down nearly zero, absorbance at 660 nm showed only 0.2. In order to check whether the bleached product contained I$^-$, the experiment was carried out with the limiting amount of iodide. In curve a, 1 $\mu$mol of I$^-$ (instead of 5 $\mu$mol) was allowed to be peroxidized to I$_3^-$ with horseradish peroxidase until there was no further increase of extinction, even after the addition of extra amounts of the

FIG. 4. Effect of varying horseradish peroxidase concentrations on the rate of I$_3^-$ formation, I$_3^-$ disappearance with EDTA, and reappearance of I$_3^-$ on addition of Zn$^{2+}$ using a limiting amount of iodide. In curve a, I$_3^-$ was generated in the reaction mixture containing all the ingredients for peroxidase assay in the concentrations as mentioned in the text except that horseradish peroxidase used was 3.5 $\mu$g and the concentration of KI was 0.33 mM instead of 1.7 mM. EDTA (4 mM) was added when no further oxidation of iodide was observed on further addition of horseradish peroxidase (1 $\mu$g) and H$_2$O$_2$ (0.4 $\mu$mol) as indicated by the arrow. 4 mM ZnSO$_4$ was added when EDTA-induced loss of extinction reached around 0.025. In curve b, the same experiment was repeated on initial addition of 7.0 $\mu$g of horseradish peroxidase.
enzyme and H₂O₂. Addition of 4 mM EDTA at this point brought down the extinction to 0.02, and subsequent addition of an equimolar amount of Zn²⁺ caused about 80% recovery of I⁻, which could be completely prevented by the addition of N₇ prior to the addition of Zn²⁺ (data not shown). This indicates that I₅ was converted to I⁻ after the addition of EDTA, and I⁻ could not be reconverted to I₅ as long as EDTA was present in the system. I⁻ was peroxidized to I₂ only after removal of EDTA by Zn²⁺. The entire process of initial I₅ formation, bleaching with EDTA, and subsequent I₅ formation on addition of Zn²⁺ were dependent on the enzyme concentration. When 7 µg of horseradish peroxidase (curve b) was used instead of 3.5 µg as in curve a, all three rates were found to be increased.

In order to investigate whether I⁻ was really produced as a reaction product after the addition of EDTA, the entire reaction as in Fig. 4, curve a, was allowed to proceed in the presence of ¹³¹I. The reaction mixtures at zero time (before the addition of H₂O₂), at steady state (before the addition of EDTA), and when the extinction dropped almost to zero after the addition of EDTA, were extracted with benzene, which dissolves iodine keeping the free iodide in the aqueous layer. The benzene and aqueous layers were counted for radioactivity, and the results are shown in Table I. It is clear from the table that at zero time almost all the counts of the reaction mixture remained as iodide, since it could not be extracted by benzene. At the steady state before the addition of EDTA, the counts were almost evenly distributed in the aqueous as well as in the benzene layer, suggesting 50% conversion of iodide to iodine. After the addition of EDTA, when the I⁻ extinction dropped to zero, almost all the counts could be recovered as iodide in the aqueous layer, indicating quantitative reconversion to iodide from iodine. Finally, addition of Zn²⁺ re-established the distribution of radioactivity as was observed before the addition of EDTA, i.e. about 50% iodide and 50% iodine. Chromatography and autoradiography of the aqueous layer indicated the presence of only one spot having the same Rₚ value as iodide. No other radioactive spot could be detected on the autoradiogram when an aliquot of aqueous solution was chromatographed on Whatmann No. 3MM paper using a butanol/ethanol/2 NH₃ system (5:1:2) as developer.

In order to study the requirement of H₂O₂, EDTA, and the enzyme in the peroxidase-catalyzed iodide formation from I₅, the investigations were carried out in I₅ prepared chemically by dissolving iodine in potassium iodide solution. Fig. 5 shows the effect of varied concentration of H₂O₂ on the horseradish peroxidase-catalyzed disappearance of I₅. The inset shows that the rate of disappearance of I₅ was proportional to the H₂O₂ concentration up to 13.2 µM, after which the rate falls. The rate of loss of extinction was insignificant if H₂O₂ was either omitted or added (33 µM) after the addition of 3 mM N₇ (data not shown). The requirement of EDTA in the above reaction is shown in Fig. 6. In the absence of EDTA, I₅ did not disappear to a significant extent after the addition of horseradish peroxidase (2 µg). The reaction was started by the addition of 66 µM H₂O₂, 2 mM EDTA; ○, 3 mM EDTA; ▲, 4 mM EDTA. The inset represents the rate at varying concentrations of EDTA.

### Table I

**Distribution of iodine and iodide before and after the addition of EDTA**

<table>
<thead>
<tr>
<th>System</th>
<th>Organic phase</th>
<th>Aqueous phase</th>
<th>Iodide spot</th>
<th>Other spots</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Reaction mixture at zero time</td>
<td>1,176</td>
<td>137,002</td>
<td>134,440</td>
<td>Nil</td>
</tr>
<tr>
<td>2. Reaction mixture before EDTA</td>
<td>60,219</td>
<td>62,019</td>
<td>57,024</td>
<td>Nil</td>
</tr>
<tr>
<td>3. Reaction mixture after EDTA</td>
<td>5,121</td>
<td>133,836</td>
<td>132,480</td>
<td>Nil</td>
</tr>
<tr>
<td>4. Reaction mixture after Zn²⁺</td>
<td>55,782</td>
<td>70,317</td>
<td>66,650</td>
<td>Nil</td>
</tr>
</tbody>
</table>

Fig. 5. Requirement of H₂O₂ for horseradish peroxidase-catalyzed loss of extinction of I₅ with EDTA. I₅ was prepared by dissolving iodine in KI solution, and 76 µl of this solution were added to the cuvette containing 180 µmol of acetate buffer, pH 5.25, in a final volume of 3 ml so as to get an extinction around 1.3 at 353 nm. EDTA was then added to the cuvette, placed inside the spectrophotometers, to get a final concentration of 4 mM, as indicated by the arrow, followed by the addition of 2 µg of horseradish peroxidase. The fall of extinction was monitored after the addition of H₂O₂ at a final concentration of 0 (Δ), 6.6 (●), 13.2 (○), and 33 µM (▲). The inset represents the rate as a function of varying concentrations of H₂O₂.

Fig. 6. Requirement of EDTA for horseradish peroxidase-catalyzed disappearance of I₅ in presence of H₂O₂. The protocol was the same as in Fig. 5 except that EDTA at varying concentrations was added before the addition of horseradish peroxidase (2 µg). The reaction was started by the addition of 66 µM H₂O₂, 2 mM EDTA; ○, 3 mM EDTA; ▲, 4 mM EDTA. The inset represents the rate at varying concentrations of EDTA.
First, a saturated aqueous solution of iodine was taken in a cuvette containing 50 mM acetate buffer, pH 5.25, to get an extinction around 0.45. EDTA (4 mM) was then added followed by horseradish peroxidase (2 μg). The reaction was started by the addition of 66 mM H$_2$O$_2$. An aliquot of a saturated aqueous solution of iodine was taken in a cuvette containing 50 mM acetate buffer, pH 5.25, to get an extinction around 0.45. EDTA (4 mM) was then added followed by horseradish peroxidase (2 μg). The reaction was started by the addition of 66 mM H$_2$O$_2$.

The reaction was started by adding H$_2$O$_2$ (66 μM). Δ, 0 μg; ○, 0.25 μg; O, 0.5 μg; ▲, 1 μg of horseradish peroxidase. The inset is the rate of the reaction as a function of the enzyme.

**Table II**

Relative efficiency of different hemoproteins on the disappearance rate of I$_3$

<table>
<thead>
<tr>
<th>Hemoprotein</th>
<th>ΔA min$^{-1}$ mg$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroperoxidase</td>
<td>1824</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>311</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>15</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>75</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>28.5</td>
</tr>
<tr>
<td>Albumin</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**Figure 7.** Requirement of enzyme for disappearance of I$_3$ with EDTA and H$_2$O$_2$. The protocol was the same as in Fig. 5 except that varying concentrations of horseradish peroxidase were added after the addition of 4 mM EDTA to the I$_3$ solution. The reaction was started by adding H$_2$O$_2$ (66 μM). Δ, 0 μg; ○, 0.25 μg; O, 0.50 μg; ▲, 1 μg of horseradish peroxidase. The inset is the rate of the reaction as a function of the enzyme.

**Figure 8.** Role of horseradish peroxidase on the loss of extinction of iodine with EDTA and H$_2$O$_2$. An aliquot of a saturated aqueous solution of iodine was taken in a cuvette containing 50 mM acetate buffer, pH 5.25, to get an extinction around 0.45. EDTA (4 mM) was then added followed by horseradish peroxidase (2 μg). The reaction was started by the addition of 66 mM H$_2$O$_2$ and the loss of extinction of I$_3$ was followed at 460 nm. ○ indicates the complete system without EDTA.

**Figure 9.** Oxygen evolution from H$_2$O$_2$ during horseradish peroxidase-catalyzed reduction of iodine in the presence of EDTA. Oxygen evolution was monitored in a Warburg apparatus using the assay system as described in the text. ●, complete system; ○, complete minus H$_2$O$_2$; ▲, complete minus horseradish peroxidase; ▲, complete minus EDTA.

From the foregoing experiments, it appears that EDTA has two significant effects on horseradish peroxidase-catalyzed peroxidation of iodide. It does not only block the conversion of I$^{-}$ to I$_3$ but also bleaches the extinction of I$_3$; generated enzymatically by peroxidation reaction or prepared nonenzymatically. The first effect has been shown in Fig. 1 using 4 mM EDTA, whereas the second effect is evident from Figs. 2-7. Both the effects of EDTA could be observed in Fig. 1, where suboptimal concentration (2 mM) of EDTA was used. Evidence has also been presented showing that both these effects are dependent on the presence of peroxidase. However, all the peroxidases are not equally active in bleaching the extinction of I$_3$; as our results show that chloroperoxidase is more active than horseradish peroxidase and lactoperoxidase. Hemoglobin or cytochrome c also catalyzes the reaction but at a much slower rate, possibly due to their pseudoperoxidase activity. Albumin does not possess the property, indicating that the intact heme part bound to the protein is necessary for the reaction. Sensitivity to azide and requirement of H$_2$O$_2$ are also indicative of the specific involvement of the peroxidase in the reaction. The reaction is specific to EDTA, as similar compounds such as EGTA, Tris, or triethylamine cannot catalyze the reaction. EDTA effect is observed only when iodide is used as an electron donor and not with any other electron donor like O-dianisidine. This indicates that the complete block of forward reaction of I$_3$ formation by 4 mM EDTA is not due to inactivation of the enzyme.

In order to get an idea about the nature of the reaction product formed after the interaction of I$_3$ with EDTA, we observed no development of color on addition of starch, indicating the absence of iodine as a product. Thus it was thought that the loss of extinction of I$_3$ may be due to the formation of a horseradish peroxidase-EDTA-I$_3$ complex. However, if it were so, the formation of I$_3$; would have been instantaneous after the addition of Zn$^{2+}$ to scavenge the EDTA from the complex. On the contrary, the regeneration of I$_3$; due to addition of Zn$^{2+}$ was a rate reaction and sensitive to azide, indicating that I$_3$; is generated enzymatically from iodide produced by the bleaching of I$_3$ with EDTA. The loss of extinction of I$_3$ by EDTA is comparable to what is observed with the other product besides iodide. Alkaline pyrogallol is known to absorb oxygen quantitatively and for this reaction, evolution of oxygen, if any, was looked for in the presence and absence of alkaline pyrogallol in Warburg's apparatus. Fig. 9 shows the evolution of O$_2$; as a function of time under identical conditions where I$_3$; lost its extinction on addition of EDTA, horseradish peroxidase, and H$_2$O$_2$. The figure also shows that O$_2$ evolution was significantly low in the absence of any one of the three components, indicating dependency on horseradish peroxidase, EDTA, and H$_2$O$_2$.

**DISCUSSION**

From the foregoing experiments, it appears that EDTA has two significant effects on horseradish peroxidase-catalyzed peroxidation of iodide. It does not only block the conversion of I$^{-}$ to I$_3$ but also bleaches the extinction of I$_3$; generated enzymatically by peroxidation reaction or prepared nonenzymatically. The first effect has been shown in Fig. 1 using 4 mM EDTA, whereas the second effect is evident from Figs. 2-7. Both the effects of EDTA could be observed in Fig. 1, where suboptimal concentration (2 mM) of EDTA was used. Evidence has also been presented showing that both these effects are dependent on the presence of peroxidase. However, all the peroxidases are not equally active in bleaching the extinction of I$_3$; as our results show that chloroperoxidase is more active than horseradish peroxidase and lactoperoxidase. Hemoglobin or cytochrome c also catalyzes the reaction but at a much slower rate, possibly due to their pseudoperoxidase activity. Albumin does not possess the property, indicating that the intact heme part bound to the protein is necessary for the reaction. Sensitivity to azide and requirement of H$_2$O$_2$ are also indicative of the specific involvement of the peroxidase in the reaction. The reaction is specific to EDTA, as similar compounds such as EGTA, Tris, or triethylamine cannot catalyze the reaction. EDTA effect is observed only when iodide is used as an electron donor and not with any other electron donor like O-dianisidine. This indicates that the complete block of forward reaction of I$_3$ formation by 4 mM EDTA is not due to inactivation of the enzyme.

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on addition of tyrosine, where the decrease of extinction of I$_2$ is due to formation of iodotyrosine. The possibility of iodination of EDTA is, however, remote. If EDTA was iodinated, addition of Zn$^{2+}$ could not release iodide from the iodinated compound. These perhaps help to postulate an alternate possibility that the loss of extinction of I$_2$ in the presence of EDTA, H$_2$O$_2$, and horseradish peroxidase is due to conversion to iodide. Chromatography of the aqueous phase after benzene extraction of the reaction mixture containing a limiting amount of iodide (15) clearly shows that the product of the bleaching reaction is nothing but iodide.

If the horseradish peroxidase-catalyzed conversion of I$_2$ to I$^-$ involves pseudocatalytic degradation of H$_2$O$_2$, O$_3$ should be the other product. Our results clearly show that disappearance of I$_2$ is associated with the evolution of O$_3$ and the latter is dependent on the presence of EDTA, H$_2$O$_2$, and horseradish peroxidase during the bleaching reaction. The generation of O$_3$ from H$_2$O$_2$ in this horseradish peroxidase-catalyzed reaction in the presence of EDTA may be termed as pseudocatalytic degradation of H$_2$O$_2$, where H$_2$O$_2$ is decomposed to O$_3$ in a catalase-like reaction. It is not a true catalatic reaction where one molecule of H$_2$O$_2$ acts as an electron donor and other molecule acts as an electron acceptor. In this pseudocatalytic degradation, O$_3$ may be formed by a redox reaction between iodinium ion, I$^+$ or I$_2$, and H$_2$O$_2$ according to the following reaction.

$$ I^+ + H_2O_2 \rightarrow O_3 + 2H^+ + 2e^- $$  \(1\)

$$ I_2 + H_2O_2 \rightarrow O_3 + 2I^- + 2H^+ $$  \(2\)

Formation of O$_3$ in such a redox reaction between H$_2$O$_2$ and I$_2$ or I$^+$ has been known for a long time (6-7) and has recently been reported in the peroxidase/H$_2$O$_2$/iodide system (8-11).

The basic mechanism of iodide oxidation may be represented as follows.

$$ I^- \rightarrow I^+ + 2e^- $$  \(3\)

$$ I^+ + I^- \rightarrow I_2 $$  \(4\)

$$ \text{Sum: } 2I^- \rightarrow I_2 + 2e^- $$  \(5\)

$$ K = [I_2]/[I^-][I^+] $$  \(6\)

In the process of the redox reaction, H$_2$O$_2$ acts as an electron acceptor and iodide as the electron donor which is oxidized to I$^+$ (iodinium ion), as reported by Alexander (4, 12). In the absence of a suitable acceptor such as tyrosine, I$^+$ readily reacts with other nucleophils such as I$^-$, Cl$^-$, or OH$^-$ to form I$_3^-$, ICl, or IOH, respectively. In presence of excess iodide, there is an instantaneous formation of I$_3^-$. Most of the I$_3^-$ remains bound as I$_3^-$ as the equilibrium constant, $K$, for $[I_3^-]/[I^-][I^+]=714$ at 25°C and hydrolysis of I$_3^-$ or polyiodide formation is not significant (13, 14). Equilibrium conditions for the reaction $I_3^- + I^- \rightarrow I_2^-$ have been studied extensively resulting in different K values and have recently been well documented by Huwiler and Kohler (11). It is now generally believed that the first step of iodide oxidation (Equation 4) involves two electron transfers catalyzed by peroxidase-H$_2$O$_2$ complex known as Compound I (12-15), whereas the subsequent reactions (Equations 4 and 6) are spontaneous transformations, neither of which is enzyme-catalyzed. The redox reaction (Equation 3) is affected by oxidizing or reducting agent, whereas Equations 4 and 6 are association equilibrium and can be shifted by many compounds, as discussed by Huwiler and Kohler (11).

The mechanism of peroxidase-catalyzed iodination reaction and the involvement of iodine free radical (16, 17), iodinium ion (15, 18, 19), or hypoiiodite (9, 20) in the process have been proposed. The most extensive studies in this direction have been presented by Hager and co-workers (21, 22) on chloroperoxidase-catalyzed chlorination reaction. They suggested that the possible structure of the halogenating intermediate is an -OCl ligand on the ferric heme of the enzyme. Morrison and Schonbaum (20) proposed the formation of an enzyme-hypoiiodous complex (EOI in the reaction between Compound I and iodide). Very recently, Magnusson et al. (10) have proposed that peroxidase combines with H$_2$O$_2$ to form an activated enzyme, Compound I (represented by EOI), Compound I is proposed to be an Fe(IV)-porphyrin $\pi$-catalysis radical (23). This has been supported by Hager and co-workers (21, 22) who reported that Compound I of chloroperoxidase contains a single oxygen atom derived from H$_2$O$_2$. Morrison and Schonbaum (20) also used E0 to describe Compound I of various peroxidases. According to Magnusson et al. (10), E0 oxidizes I$^-$ to form an intermediate represented as [EOI$^-$], where I is present in an oxidation state equivalent to I$^+$. [EOI$^-$] may give rise to the ferric form of the peroxidase and IO$^-$, which is a free anion, rather than the enzyme-bound hypoiiodite. The scheme (Ref. 10) may be represented as follows.

$$ \text{H}_{2}O \xrightarrow{E + \text{H}_2\text{O}_2} \text{EO} \xrightarrow{[\text{EOI}^-]} \text{E} + \text{IO}^- $$  \(7\)

I$_3^-$ is then formed in the following two successive steps, neither of which is enzyme-catalyzed.

$$ \text{IO}^- + \text{I}^+ + \text{H}^+ \rightarrow \text{I}_2 + \text{OH}^- $$  \(8\)

$$ \text{I}_2 + \text{I}^- \rightarrow \text{I}_3^- $$  \(9\)

In the context of this mechanism of peroxidase-catalyzed triiodide formation, our data may now be explained as follows: EDTA blocks the formation of triiodide in the forward reaction as a result of oxidation of IO$^-$ by H$_2$O$_2$ to form O$_3$ and I$^-$ according to the following reaction.

$$ \text{IO}^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_3 + \text{H}_2\text{O} + \text{I}^- $$  \(10\)

Equation 10, which is possibly favored in the presence of EDTA by a still unknown mechanism, prevents Equation 8 from occurring, blocking the formation of I$_3^-$ (Equation 9). Thus, the inhibition of I$_3^-$ formation with EDTA is really an apparent inhibition and arises because the product of the first enzyme-catalyzed reaction, namely IO$^-$ (Equation 7) is consumed in a second enzyme-catalyzed step (Equation 10), leading to the formation of O$_3$ and I$^-$. This is consistent with our finding that, using a limiting amount of iodide containing $^{131}$I in the reaction mixture (Fig. 4), EDTA caused the liberation of $^{131}$I (Table I, System 3). In fact, Equation 10 which involves the reduction of hypoiiodite by H$_2$O$_2$ has been studied by Liebafska (6, 24), who reported the following reaction: HIO + H$_2$O$_2$ → H$^+$ + I$^- +$ H$_2$O + O$_2$. This reaction may provide the chemical basis for iodide-dependent pseudocatalytic degradation of H$_2$O$_2$ by thyroid peroxidase or lactoperoxidase (9, 11).

Our data also indicate that in the presence of EDTA and H$_2$O$_2$, horseradish peroxidase catalyzes the loss of oxidation of preformed I$_3^-$ with concomitant formation of O$_3$. It may be mentioned here that the extinction of I$_2$ decreases very slowly either in the presence of EDTA or in the presence of H$_2$O$_2$ or by both. This is perhaps the nonenzymatic chemical reaction which becomes very fast on addition of the enzyme. This fast reaction does not occur in the absence of EDTA. It appears that, in presence of EDTA, the conformation of the enzyme may be changed so that it catalyzes the conversion of I$_3^-$ to I$^-$ with concomitant oxidation of H$_2$O$_2$ to O$_3$ as shown in the following
**EDTA Effect on Enzymatic Formation and Disappearance of Triiodide**

reaction:

\[
I_3^- \rightleftharpoons I^- + I_2^- \quad (11)
\]

horseradish peroxidase \( \rightarrow H_2O_2 \)

\[
O_2 + 2I^- + 2H^+ \quad (12)
\]

The evolution of \( O_2 \) as shown in our data (Fig. 9) indicates that Equation 12 is occurring in our system. It has further been shown that the rate of bleaching with EDTA using only \( I_2^- \) is much slower than \( I; \) (Fig. 8). It appears that \( I; \), being a charged molecule, is a better interacting species than nonpolar iodine.

It is thus possible that, in the presence of EDTA, horseradish peroxidase is catalyzing the formation of iodide from iodine in a redox reaction associated with the pseudocatalatic degradation of \( H_2O_2 \) to \( O_2 \). This iodide-dependent pseudocatalatic activity of the peroxidases has been extensively studied by Taurog and co-workers (9, 10) using lactoperoxidase and thyroid peroxidase. Although we have shown that horseradish peroxidase also possesses this activity, the higher rate of bleaching with iodide in our system indicates that this enzyme may possess pseudocatalatic activity much higher than horseradish peroxidase. In fact Thomas et al. (25) have demonstrated that chloroperoxidase possesses significant catalase activity in both the presence and absence of \( Cl^- \). However, the role of EDTA in our system is not clearly understood and it opens up a new field for further investigation.

**REFERENCES**