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THE HORSERADISH PEROXIDASE-CATALYZED OXIDATION OF IODIDE PRODUCTS FORMED AND IODINATION OF TYROSINE BY THE PRODUCTS

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SUMMARY

1. In the system peroxidase + H_2O_2 + I^- an equilibrium mixture of I_2 and I_3^- together with H_2O are formed as major products. In addition one or more minor unidentified products are formed.

2. In the system peroxidase + H_2O_2 + I^- + oxalate considerable oxalate oxidation occurs. A study of the reaction showed that atomic iodine probably is formed as an intermediate, which can dimerize to I_2 or react with oxalate. The I^- oxidation mechanism is discussed.

3. In the system peroxidase + H_2O_2 + I^- + tyrosine, I_2 is formed as an intermediate, which iodinates tyrosine to 3-monoiodotyrosine. The iodination step can occur spontaneously; possibly it is also catalyzed by peroxidase. 3-Monoiodotyrosine can similarly be iodinated to 3,5-diiodotyrosine.

4. Under suitable conditions about 50% of the I^- present can be utilized for tyrosine iodination.

INTRODUCTION

In the preceding paper[1] it was shown that the main pathway for the horseradish peroxidase-catalyzed oxidation of I^- can be characterized as

$$\text{Compound I} + I^- \rightarrow \text{ferriperoxidase–iodine} \quad (1)$$

$$\text{Ferriperoxidase–iodine} + H_2O_2 \rightarrow \text{Compound I} + \text{product(s)} \quad (2)$$

A side pathway identical with the peroxidase mechanism according to CHANCE[2,3] and GEORGE[4–6] includes the reaction steps

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$$\text{Ferriperoxidase} + H_2O_2 \rightarrow \text{Compound I} \quad (3)$$

$$\text{Compound I} + I^- \rightarrow \text{Compound II} + \text{product(s)} \quad (4)$$

$$\text{Compound II} + I^- \rightarrow \text{ferriperoxidase} + \text{product(s)} \quad (5)$$

In Eqns. 1–5 the unmodified enzyme is called ferriperoxidase. Additional evidence in favor of the mechanism of Eqns. 1 and 2 is given in the present paper.

Atomic iodine ($I\cdot$) is probably the primary product formed from I^- along the side pathway[7], and, as will be shown here, it is also the primary product of the main pathway. In the presence of excess I^- the final product is an equilibrium mixture of I_2 and I_3^- when I^- is oxidized by horseradish peroxidase and by other peroxidases (for references see ref. 1).

In the system peroxidase + H_2O_2 + I^- + tyrosine (or 3-monoiodotyrosine) iodination of the tyrosine or 3-monoiodotyrosine present will occur. Also protein tyrosyl and 3-monoiodotyrosyl residues are iodinated. Several iron-porphyrin peroxidases, including chloroperoxidase[8], horseradish peroxidase[9–13,32] lactoperoxidase[9,14], myeloperoxidase[10,14,15] and thyroid peroxidase[16–18], are known to catalyze the reaction. It is thought that thyroid peroxidase catalyzes the physiologically important iodination of thyroglobulin tyrosine residues. The mechanism of the horseradish peroxidase-catalyzed reaction was studied in the present investigation. Some of the results have been reported in an abstract[19].

MATERIALS AND METHODS

Reagents and equipment

The reagents and their handling were as described in the preceding paper[1], with the addition that a ^{131}I-preparation (Radiochemical Centre) described as "iodide, carrier-free, in aqueous solution free from reducing agent" was also used. KI solutions were labeled by adding some of the $^{131}I^-$. A labeled stock solution containing 600 μM I_2 was prepared immediately before use by adding under intermittent mixing to a small test tube 100 μl of diluted $^{131}I^-$ preparation, 10 μl of 50 mM KI, 10 μl of 250 mM H_2SO_4, 10 μl of 10 mM KIO_3 and 370 μl of water.

The equipment and its handling were as previously described[1], with the addition that a chromatogram strip scanner (Isotope Developments Ltd.) was used for radioactivity measurements.

Oxalate oxidation in the system peroxidase + H_2O_2 + I^- + oxalate

Solutions containing oxalate and $I_2 + I_3^-$ are sensitive to light and were handled only in dim red light. During spectrophotometry slits and exposure times were kept minimal.

In reacting solutions the limiting factor was the H_2O_2 concentration. Absorbance readings at 353 nm were taken when 99% of the H_2O_2 was calculated to have been spent. At least two readings at 1- or 2-min intervals were taken to ascertain that the reaction had gone to completion. The longest reaction time before reading was 15 min.

Parallel measurements were always made on four systems, which were identical except for omissions and replacements as follows: System a, peroxidase + H_2O_2 + I^- + oxalate; System b, peroxidase + I^- + oxalate; System c, peroxidase + H_2O_2 + I^- + acetate; System d, peroxidase + I^- + acetate.

The concentration of I_3^- formed through the enzymic reaction in the presence of oxalate ($\Delta[I_3^-]_{ox}$) or acetate ($\Delta[I_3^-]_{ac}$) was calculated from

$$\Delta[I_3^-]_{ox} = \frac{A_a - A_b}{\varepsilon_{M,I_3^-}} \quad (6)$$

$$\Delta[I_3^-]_{ac} = \frac{A_c - A_d}{\varepsilon_{M,I_3^-}} \quad (7)$$

where A_a, A_b, A_c and A_d are the final absorbances of Systems a, b, c and d, respectively, and $\varepsilon_{M,I_3^-} = 2.64 \cdot 10^4\ cm^{-1} \cdot M^{-1}$ (ref. 20).

A reaction between oxalate and atomic iodine ($I\cdot$), which apparently is formed as an intermediate, can be ignored in the derivation of Eqns. 8–11, since the error introduced is negligible. The overall reaction can then be described as

$$H_2O_2 + 2\ I^- + 2\ H^+ \xrightarrow{\text{peroxidase}} I_2 + 2\ H_2O \quad (8)$$

$$I_3^- \rightleftharpoons I_2 + I^-,\ K = 1.30\ mM\ (\text{ref. } 21) \quad (9)$$

From equations (8) and (9)

$$\Delta[I_2]_{ox} = \frac{K\Delta[I_3^-]_{ox}}{[I^-]} = \frac{K\Delta[I_3^-]_{ox}}{[I^-]_0 - 2[H_2O_2]_0 - \Delta[I_3^-]} \quad (10)$$

$$\Delta[I_2]_{ac} = \frac{K\Delta[I_3^-]_{ac}}{[I^-]} = \frac{K\Delta[I_3^-]_{ac}}{[I^-]_0 - 2[H_2O_2]_0 - \Delta[I_3^-]} \quad (11)$$

where $\Delta[I_2]_{ox}$ and $\Delta[I_2]_{ac}$ are the I_2 concentrations formed in the enzymic reaction in the presence of oxalate and acetate, respectively, and $[I^-]_0$ and $[H_2O_2]_0$ are the initial I^- and H_2O_2 concentrations. The concentration of I^- oxidized in the enzymic reaction is obtained from

$$-\Delta[I^-] = 2(\Delta[I_3^-] + \Delta[I_2]) \quad (12)$$

In System a less $I_2 + I_3^-$ is formed than in System c because of the reaction between $I\cdot$ and oxalate. The overall reaction for the oxidation of oxalate can be characterized as[22]

$$H_2O_2 + \text{oxalate} + 2\ H^+ \xrightarrow{\text{peroxidase} + I^-} 2\ CO_2 + 2\ H_2O \quad (13)$$

The oxalate concentration oxidized ($-\Delta$[oxalate]) can be calculated from

$$-\Delta[\text{Oxalate}] = \Delta[I_3^-]_{ac} + \Delta[I_2]_{ac} - \Delta[I_3^-]_{ox} - \Delta[I_2]_{ox} \quad (14)$$

Chromatography of ^{131}I-labeled reaction products

To the solution to be analyzed the carriers KI, 3-monoiodotyrosine and 3,5-diiodotyrosine were added each to a concentration of 100 μM. Then 20 μl of the solution were applied to a 4-cm-wide strip of Whatman No. 1 paper. Chromatography was carried out using an *n*-butanol–ethanol–ammonia–water solvent[23].

When two-dimensional chromatography in some cases was performed, 5 μl of solution were applied to the Whatman No. 1 paper used. The solvent already mentioned was used in the first direction and a water–formic acid (5:1, by vol.) solvent[23] in the second. Radioactive spots were detected through autoradiography.

To detect carrier compounds, papers were sprayed with a $FeCl_3$–$Fe(CN)_6^{3-}$–AsO_2^- reagent[24]. Radioactive spots were identified by comparing their locations with those of carrier compounds.

RESULTS

Products formed from I^-

Atomic iodine is known to oxidize the oxalate ion (refs. 22, 25, 26). We found that oxalate was oxidized in the system peroxidase + H_2O_2 + I^- + oxalate which suggests that $I\cdot$ might be formed as the primary product in the peroxidase-catalyzed oxidation of I^-.

The amounts of oxalate and I^- oxidized were not affected when the addition of I^- or peroxidase was delayed till after a preincubation period (Table I). This shows that little or no reaction took place in the systems peroxidase + H_2O_2 + oxalate and H_2O_2 + I^- + oxalate. In the former system the peroxidase will be present as Compound I, which obviously reacts only slowly if at all with oxalate.

TABLE I

OXALATE OXIDATION IN THE SYSTEM PEROXIDASE + H_2O_2 + I^- + OXALATE

Reagents were added to a spectrophotometer cell in the order given to the following concentrations: 100 mM potassium oxalate (pH 4.0) or 10 mM potassium acetate (pH 4.1), 15 μM I_2, 1 mM KI, 1 nM peroxidase and 60 μM H_2O_2. The I_2 addition was made in order to eliminate I_2-reducing impurities, and the system was left to stand for 4 min to let these react before the enzymic reaction was started with the H_2O_2 addition. Where indicated, one of the components was not added until 10 min after the H_2O_2 to allow for a second preincubation period. The concentrations of I^- and oxalate oxidized when the enzymic reaction was permitted to go to completion are symbolized by $-\Delta[I^-]$ and $-\Delta$[oxalate].

System	*Compound added after second preincubation*	$-\Delta[I^-]$ (μM)	$-\Delta$[Oxalate] (μM)
Peroxidase + H_2O_2 + I^- + oxalate	None	71	16
Peroxidase + H_2O_2 + I^- + oxalate	I^-	70	17
Peroxidase + H_2O_2 + I^- + oxalate	Peroxidase	73	15
Peroxidase + H_2O_2 + I^- + acetate	None	104	—

In the experiment of Table I 30% of the H_2O_2 was spent for oxalate oxidation. Thus the component reacting with oxalate cannot be a product or an intermediate formed along a side pathway with slow turnover. A reaction between oxalate and Compound II, which is formed along such a pathway, cannot explain the observations.

To study whether the ferriperoxidase–iodine complex might react with oxalate, ferriperoxidase–iodine was produced *via* Reactions 3 and 1, and oxalate was then added (Fig. 1A). If the oxalate had reduced ferriperoxidase–iodine to ferriperoxidase, a drop in the absorbance would have been seen[1]. However, no absorbance change and therefore no reaction could be detected.

Under the conditions used neigher I_3^-, HIO nor IO^- can react with oxalate to an appreciable extent[22,25,26]. Iodinium ion (I^+) or protonated hypoiodous acid (H_2OI^+) could conceivably be formed as primary products and react with oxalate. However,

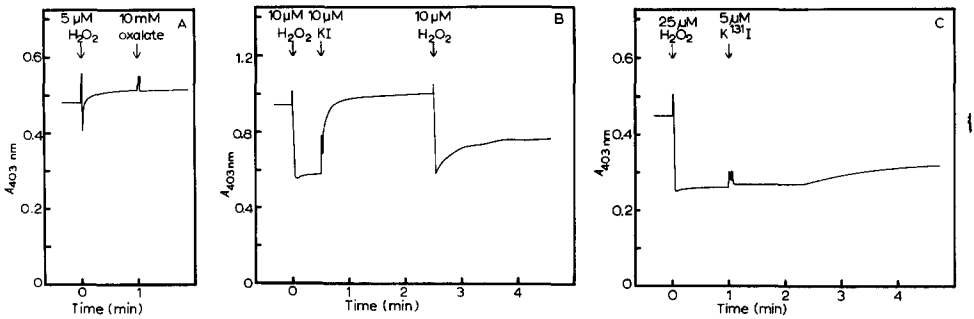

Fig. 1. The interconversion of peroxidase species was followed by recording the absorbance at 403 nm as a function of time. In A the spectrophotometer cell originally contained 8 mM potassium phosphate (pH 6.2), 5.3 μM ferriperoxidase and 50 μM KI. In B there was 30 mM potassium phosphate (pH 6.2) and 10.4 μM ferriperoxidase. In C there was 30 mM potassium phosphate (pH 6.0) and 5.3 μM ferriperoxidase. Other reagents were added as indicated in the figure. The addition of oxalate in A reduced the pH to 4.8.

we are unable to account for the kinetics of oxalate oxidation under this assumption (F. BJÖRKSTÉN, unpublished results).

The most plausible explanation for the observations is that I· is formed as the product of Reaction 2 on the main pathway. Atomic iodine dimerizes rapidly to the final product I_2.

When the stoichiometry of the overall reaction was studied, a ratio for added H_2O_2 to formed $I_2 + I_3^-$ of 1:0.87 was found (Table II). This approaches the 1:1 ratio expected from Eqn. 8 and 9. According to this both electrons for the reduction of H_2O_2 will in most cases eventually come from I^-. The route for one of these electron transfers is apparent from the above results, while the route for the second remains uncertain. If ferriperoxidase–iodine is converted to Compound I by adding an equimolar amount of H_2O_2 in the absence of I^- (Eqn. 2), only one of the two oxidizing equivalents present in ferriperoxidase–iodine can end up in I_2. In this situation the Compound I formed will decay in a complex manner as judged by the slightly oscillating absorbance (Fig. 1B). If the H_2O_2 quantity is larger, Compound I will be more stable (Fig. 1C).

Iodination of tyrosine and 3-monoiodotyrosine

In our hands systems containing peroxidase, H_2O_2 and I^- readily iodinated

TABLE II

STOICHIOMETRY OF THE PEROXIDASE-CATALYZED OXIDATION OF I^-

Reagents were added to a spectrophotometer cell in the order given to the following concentrations: 50 mM potassium acetate (pH 5.5), 32.5 nM peroxidase, 1 mM KI and H_2O_2 as given in the table. The absorbance at 353 nm was measured 2 min after the H_2O_2 addition, at which time the enzymic reaction had gone to completion. The sum of the I_2 and I_3^- concentrations formed was calculated as previously[7].

Added H_2O_2 (μM)	*Formed $I_2 + I_3^-$ (μM)*	*Formed $I_2 + I_3^-$/ added H_2O_2*
21.2	18.7	0.88
42.4	36.3	0.86

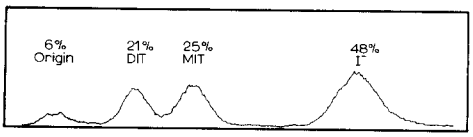

Fig. 2. Simultaneous iodination of tyrosine and 3-monoiodotyrosine. Reagents were added to a small test tube in the order given to the following final concentrations: 30 mM potassium phosphate (pH 6.0), 5.4 μM peroxidase, 5.4 μM KI labeled with ^{131}I, 10.8 μM H_2O_2, 250 μM L-tyrosine and simultaneously with it 250 μM 3-monoiodo-L-tyrosine, 400 μM $Na_2S_2O_3$. A 1-min interval followed the H_2O_2 addition and the addition of the tyrosines. The final volume was 0.5 ml. The solution was analyzed for ^{131}I-containing compounds. The scanner trace and the percentage of the ^{131}I found in different compounds is shown. 3-Monoiodotyrosine is abbreviated MIT and 3,5-diiodotyrosine DIT.

tyrosine to 3-monoiodotyrosine (Table III) and 3-monoiodotyrosine to 3,5-diiodotyrosine (Fig. 2). The products were identified by one- and two- dimensional paper chromatography (see MATERIALS AND METHODS). Some iodine also became bound to material remaining at the origin during chromatography (Table III, Fig. 2). The nature of this $R_F = 0$ material will be discussed later. Unidentified chromatogram spots containing up to 13% of the total ^{131}I were sometimes seen (Table V).

Only minimal iodinating activity could be detected in a system to which peroxidase, I^- and H_2O_2 had been added in equimolar proportions (Table III). In such a system the ferriperoxidase–iodine complex is formed (Eqns. 3 and 1). It must be concluded that the iodine remains nearly quantitatively bound to the complex, since any I_2 liberated would readily have iodinated tyrosine (Table III). Obviously the complex itself did not iodinate tyrosine, except perhaps at a very slow rate.

In contrast, a highly active iodinating system was formed when equimolar amounts of peroxidase and I^- together with a larger amount of H_2O_2 were used. The system retained its activity for tens of minutes after the I^- oxidation had proceeded to completion and all ferriperoxidase–iodine and $I\cdot$ had decayed (Table IV). Here I_2

TABLE III

IODINATION OF TYROSINE BY I_2 AND BY A PEROXIDASE + H_2O_2 + I^- SYSTEM

All reaction media contained 30 mM potassium phosphate (pH 6.0) and I_2 or KI labeled with ^{131}I. L-Tyrosine to a final concentration of 500 μM was added to the I_2-containing system immediately after the I_2 and to the peroxidase-containing systems 1 min after the H_2O_2 which had started the reaction. All concentrations have been calculated for volumes occurring after the tyrosine addition, which increased them by 11% and brought them to 2 ml and 0.5 ml for the I_2- and peroxidase-containing systems, respectively. The reaction was stopped 1 min after the tyrosine addition by 400 μM $Na_2S_2O_3$. The solution was then analyzed for ^{131}I-containing compounds.

System	*Distribution of ^{131}I (%)*		
	I^-	*3-Monoiodotyrosine*	*$R_F = 0$ material*
30 μM I_2	57	43	
5.2 μM peroxidase + 5.2 μM KI + 5.2 μM H_2O_2	94	1	5
5.2 μM peroxidase + 5.2 μM KI + 21 μM H_2O_2	51	40	9

TABLE IV

IODINATION OF TYROSINE BY A PEROXIDASE + H_2O_2 + I^- SYSTEM

From the reaction solution described in the legend of Fig. 2C, 50-μl aliquotes were withdrawn at times given in the table and mixed with 20 μl of 1.75 mM L-tyrosine solution. 2 min later the iodination reaction was stopped by 5 μl of 5.6 mM $Na_2S_2O_3$ solution. The samples were then analyzed for ^{131}I-containing compounds.

Time after KI addition (min)	*Distribution of ^{131}I (%)*		
	I^-	*3-Monoiodotyrosine*	*$R_F = 0$ material*
0.5	50	43	7
1	45	49	6
2	31	62	7
4	33	57	9
8	38	51	11

must have been formed as an intermediate. Under the present conditions I_2 will readily iodinate tyrosine nonenzymically (Table III).

When tyrosine and 3-monoiodotyrosine were added simultaneously in equimolar proportion to the enzymic iodinating system, nearly equimolar quantitites of iodinated products (3-monoiodotyrosine and 3,5-diiodotyrosine) were formed (Fig. 2). This shows that tyrosine and 3-monoiodotyrosine will react with the iodinating agent at nearly equal rates, if rates are calculated for the total concentration of phenol (phenolate ion *plus* undissociated phenol) present. From the data of MAYBERRY *et al.*[27,28] it can be calculated that this is what should be observed at pH 6 if I_2 is the iodinating agent.

TABLE V

EFFECT OF REAGENT ADDITION ORDER AND REDUCING AGENTS ON TYROSINE IODINATION

To the "basic" system reagents were added in the order given to the following final concentrations: 30 mM potassium phosphate (pH 6.0), 5.4 μM peroxidase, 5.4 μM KI labeled with ^{131}I, 10.8 μM H_2O_2, 500 μM L-tyrosine and 400 μM $Na_2S_2O_3$. The last three reagents were added at 1-min intervals. To the "tyrosine before H_2O_2" system reagents were added as to the basic system, except that the order of the H_2O_2 and tyrosine additions was reversed. Where indicated, a 10 μM concentration of reducing agent was added 30 sec after the H_2O_2 to a system otherwise identical with the basic system. The final volume was always 0.5 ml.

System	*Distribution of ^{131}I (%)*			
	I^-	*3-Monoiodotyrosine*	*$R_F = 0$ material*	*Unidentified*
Basic	52	35	7	6
Tyrosine before H_2O_2	100	—	—	—
Ascorbic acid added	83	—	4	13
$Na_2S_2O_3$ added	86	—	3	12
1-Methyl-2-mercaptoimidazole added	83	—	4	13
2-Thiouracil added	86	—	4	10

Factors affecting tyrosine iodination

In the iodination experiments of this study, reaction in the peroxidase + H_2O_2 + I^- system was permitted to go nearly to completion before the compound to be iodinated was added. If tyrosine or 3-monoiodotyrosine was added earlier in the comparatively high (500 μM) concentration normally used, no iodination occurred (Table V). This is obviously due to the rapid depletion of the H_2O_2 supply through the peroxidase-catalyzed tyrosine or 3-monoiodotyrosine oxidation[30]. Competitive inhibition of I^- oxidation by tyrosine and 3-monoiodotyrosine might also contribute.

When tyrosine concentrations lower than 500 μM were tried, the yield of 3-monoiodotyrosine was reduced and the amount of ^{131}I in the $R_F = 0$ material was increased (Table VI).

TABLE VI

EFFECT OF THE TYROSINE CONCENTRATION ON THE YIELD OF THE IODINATION REACTION

Reagents were added to a small test tube in the order given to the following final concentrations: 30 mM potassium phosphate (pH 6.0), 4.2 μM KI labeled with ^{131}I, 4.2 μM peroxidase, 8.4 μM H_2O_2, the L-tyrosine concentration given in the table and 400 μM $Na_2S_2O_3$. The tyrosine addition followed 1 min after the H_2O_2 addition and the $Na_2S_2O_3$ addition 15 min after the tyrosine addition. The final volume was 0.5 ml. The solutions obtained were analyzed for ^{131}I-containing compounds.

Tyrosine concn. (μM)	*Distribution of ^{131}I (%)*		
	I^-	*3-Monoiodotyrosine*	*$R_F = 0$ material*
10	51	29	20
30	39	45	16
100	29	57	14
500	23	67	9
1000	26	65	9

Low concentrations of reducing agents such as the antithyroid compounds 1-methyl-2-mercaptoimidazole and 2-thiouracil and also ascorbic acid and $Na_2S_2O_3$ will completely inhibit tyrosine iodination if added before tyrosine to the enzymic system (Table V). The compounds apparently act by reducing I_2.

DISCUSSION

The chemical evidence presented here strongly suggests that atomic iodine is a primary product formed in the horseradish peroxidase-catalyzed oxidation of I^-. In accordance with this, Eqn. 2 can be reformulated as

$$\text{Ferriperoxidase–iodine} + H_2O_2 \rightarrow \text{Compound I} + I\cdot + ? \qquad (15)$$

Eqn. 15 undoubtedly summarizes several still unresolved reaction steps.

In the course of the reaction cycle (Eqns. 1 and 15) Compound I is known to react with one molar equivalent of I^-. One of the two electrons needed for the reduction of the H_2O_2 will come from this I^-. If a second equivalent of I^- is available, the second electron will also come from I^-, but the details of this process remain unknown. The cycle can be completed also in the presence of only one molar equivalent of I^-. In this case the Compound I present at the end will eventually undergo additional complex

reactions as judged by the slight oscillations in the changing absorbance (Fig. 1B).

Atomic iodine is a short-lived intermediate, which dimerizes to form the final products I_2 and I_3^-. In the presence of excess I^- the stoichiometric ratio for added H_2O_2 to formed $I_2 + I_3^-$ was found to be 1:0.87. This is significantly different from the 1:1 ratio expected if I_2, I_3^- and H_2O were the only products (*cf.* Eqns. 8 and 9). Thus side reactions must occur.

It has been suggested that I· is the iodinating species in peroxidase-catalyzed iodinations[14,15]. We found that tyrosine iodination occurred even when tyrosine was added after all I· had decayed to I_2. Protein sulfenyl iodide (–SI) groups have also been considered as a possible iodinating agent[30,31]. We have been unable to detect the formation of such groups in the horseradish peroxidase system[1]. A third suggestion involves tyrosine iodination through a reaction in which tyrosine interacts with a peroxidase–iodine complex[9,12]. We found that such a complex is indeed formed in the horseradish peroxidase system[1], but that it will not iodinate tyrosine at pH 6. An iodination reaction might, however, occur under other conditions and with complexes formed by other peroxidases.

When the products from iodination experiments in which peroxidase had been used were analyzed, a minor amount of iodine-containing material ($R_F = 0$ material) remaining at the origin during paper chromatography was usually found. Treatment with H_2O_2 or reducing agents will liberate the iodine from the ferriperoxidase-iodine complex (Table III and ref. 1), but not the iodine from the $R_F = 0$ material (Tables III and V). The amount of $R_F = 0$ material formed was increased if the amount of tyrosine added was decreased (Table VI) or if the tyrosine addition was delayed (Table IV). These conditions would be expected to favor the iodination of enzyme protein tyrosyl residues at the cost of the iodination of free tyrosine. The observations suggest that the $R_F = 0$ material may be peroxidase containing a 3-iodotyrosyl residue(s). This iodinated peroxidase is a minor product, which must not be confused with the ferriperoxidase–iodine complex. The complex contains an iodine atom probably bound to the heme and definitely no iodotyrosyl residue[1].

Molecular iodine was certainly formed as an intermediate in the iodinating peroxidase + H_2O_2 + I^- + tyrosine (or 3-monoiodotyrosine) system investigated. At the pH of this study (pH 6) nonenzymic iodination by I_2 was found to occur readily if no I^- was present.

THOMAS AND HAGER[8] have shown that tyrosine iodination in the system chloroperoxidase + H_2O_2 + I_2 + tyrosine is enzyme-catalyzed at pH 2.7. From the work of COVAL AND TAUROG[16] it can be concluded that iodination in their thyroid peroxidase + H_2O_2 + I^- + tyrosine system at pH 7 is not due to a nonenzymic reaction between I_2 and tyrosine. This is based on the observation that excess I^- does not inhibit (*cf.* ref. 27) and that 3-monoiodotyrosine is iodinated faster than tyrosine (*cf.* refs. 27, 28). In our horseradish peroxidase system tyrosine and 3-monoiodotyrosine were iodinated at nearly equal rates, if rates were calculated for the total phenol concentration (phenolate ion *plus* undissociated phenol). The reaction between the I_2 formed as intermediate and the I_2 acceptor could therefore have been spontaneous, but horseradish peroxidase catalysis is not ruled out.

Finally it should be stressed that only horseradish peroxidase has been used in the present and preceding[1] studies and that the mechanisms described may not be applicable to other peroxidases.

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