Enzymatic Iodination of Tyrosine and Thyroglobulin with Chloroperoxidase*

Alvin Taurog‡ and Earl M. Howells§

From the Department of Pharmacology, The University of Texas Southwestern Medical School, Dallas, Texas 75235

SUMMARY

1. Crystalline chloroperoxidase is effective in catalyzing the iodination of tyrosine or thyroglobulin when supplemented with H₂O₂, or with the H₂O₂-generating system, glucose-glucose oxidase. The iodination reaction is very rapid at low concentrations of iodide (5 × 10⁻⁵ M), and at low concentrations of tyrosine (1 × 10⁻⁴ M) or thyroglobulin (0.33 mg per ml).

2. Iodide is rapidly bound as 3-iodotyrosine, 3,5-diiodotyrosine, and thyroxine during the chloroperoxidase-catalyzed iodination. After 60 min of incubation with thyroglobulin as acceptor, chromatography of a Pronase digest showed approximately 45 to 50 % of the added I⁻ in the form of 3,5-diiodotyrosine, about 20 to 25% as 3-iodotyrosine, and 4 to 5% as thyroxine.

3. Since iodination of thyroglobulin occurred very effectively in the presence of only a single crystalline enzyme, the results suggest that it may not be necessary to postulate the existence of a separate “tyrosine iodinase” in the thyroid. Moreover, the formation of appreciable thyroxine in the chloroperoxidase-catalyzed iodination of thyroglobulin suggests that a peroxidase may also be involved in the coupling reaction in the thyroid.

4. The chloroperoxidase-catalyzed iodination of tyrosine and thyroglobulin is greatly accelerated by 0.1 M chloride and bromide. The mechanism of this stimulatory effect is not yet known, although several possibilities are discussed.

5. Iodination of thyroglobulin and tyrosine in the presence of chloroperoxidase is readily inhibited by low concentrations of antithyroid drugs and by naturally occurring reducing agents such as cysteine, reduced glutathione, and ascorbic acid. Results obtained with these inhibitors support the view that antithyroid compounds act as competitive inhibitors in the formation of organic iodine in the thyroid.

6. Excess iodide inhibits the chloroperoxidase-catalyzed iodination of tyrosine and thyroglobulin. These results suggest that the antithyroid effect of iodide, observed under certain conditions, may be partly due to inhibition of thyroid peroxidase.

7. Chloroperoxidase is very effective in catalyzing iodination of insulin and serum albumin. 3,5-Diiodotyrosine formation was at least as rapid in the case of these acceptors as it was with thyroglobulin, but thyroxine formation was not as marked. These results suggest that steric arrangement of newly formed 3,5-diiodotyrosine residues in thyroglobulin was more favorable than in the other proteins for promoting the coupling reaction.

8. No evidence was obtained that 3,5-diiodo-4-hydroxyphenylpyruvic acid (DHP) is an intermediate in the formation of thyroxine during the iodination of thyroglobulin with chloroperoxidase. However, when free tyrosine was iodinated with chloroperoxidase, evidence was obtained that DHP was involved as an intermediate. To the extent that the chloroperoxidase-catalyzed iodination of thyroglobulin serves as a model for the thyroid coupling reaction, these results suggest that DHP is not an intermediate in thyroxine formation in the thyroid.

Iodination of tyrosine residues in thyroglobulin, or in its subunits, is one of the essential steps in the formation of thyroxine in the thyroid gland. By analogy with iodination reactions carried out in the laboratory, it is generally believed that iodide ion (the form in which iodine enters the gland) must first be oxidized to a higher oxidation state before it can iodinate tyrosine. Of the known biological oxidizing agents, only H₂O₂ and O₂ are involved in oxidation-reduction couples that possess, at pH 7, a higher oxidation-reduction potential than the couple 2I⁻ → I₂ (⁺ 0.535 volt). It is hardly surprising, therefore, that H₂O₂ has long been implicated as the agent responsible for oxidizing iodide in the thyroid.

Histochemical evidence for the presence of a peroxidase in thyroid tissue was reported by Dempsey in 1944 (1), and this was confirmed and extended by others (2–4). However, early attempts to demonstrate peroxidase activity in thyroid extracts were unsuccessful (5). More recently, primarily because of the work of Alexander (6) and of Serif and Kirkwood (7), there has been a renewed interest in the occurrence of a thyroid peroxidase.
Alexander reported that rat thyroid and salivary homogenates catalyzed the iodination of added tyrosine in the presence of glucose and glucose oxidase as a hydrogen peroxide-generating system, and he concluded that a tissue peroxidase was involved in the reaction. The existence of a thyroid peroxidase has been confirmed by other investigators, and attempts have been made to purify the enzyme (8-14). However, isolation of a pure thyroid peroxidase has proved difficult because of the particulate nature of the enzyme, and also because of its apparent lability.

Evidence that a peroxidase may be involved in biological iodination was reported by Koston (15) in 1944. He demonstrated organic iodine formation in a model milk system, containing added xanthine and radiiodide. He postulated that the xanthine oxidase in milk catalyzed the formation of H₂O₂ by xanthine oxidation, and that the resultant H₂O₂, in the presence of milk peroxidase, promoted iodination of tyrosine residues in casein. Klebanoff, Yip, and Kessler (10) tested purified peroxidase for activity in iodination reactions. They reported that purified preparations of myelo- and lactoperoxidase catalyze the iodination of tyrosine in the presence of H₂O₂-generating systems.

The present communication describes our results with chloroperoxidase, an enzyme recently isolated by Shaw and Hager (16) from the mold, Caldariomyces fumago. Chloroperoxidase was shown by these workers to be a key enzyme in the formation of a chlorinated hydrocarbon, caldariomycin. The various steps in the biosynthesis of caldariomycin have been elucidated by Shaw and Hager, and the scheme which they propose is basically analogous to the mechanism proposed for iodination in the thyroid. Since chloroperoxidase is involved in a biological halogenation reaction, it seemed of interest to determine whether it would be effective in catalyzing the iodination of tyrosine and thyroglobulin. Results obtained in the present investigation indicate that chloroperoxidase is indeed very active in this respect, and our observations suggest that chloroperoxidase-catalyzed iodination may serve as a useful model for the elucidation of iodination mechanisms in the thyroid gland.

**Materials and Methods**

**Enzymes and Substrates**—Crystalline chloroperoxidase was kindly provided by Dr. Lowell Hager. Purification and crystallization of the enzyme are described elsewhere (17). A solution containing 0.78 mg of protein per ml (estimated from the hemoglobin content) was diluted with acetate buffer, pH 4.9, or with water to provide a solution containing 1.6 μg per ml. Portions of the latter (1 or 2 ml) were kept frozen at −20°C, and generally a fresh tube was used for each experiment. Glucose oxidase (Sigma, type III, 39,000 units per g) was dissolved in acetate buffer, pH 4.9, or in water to provide a solution containing 0.25 mg per ml. Thyroglobulin was isolated from frozen rabbit thyroids (Pel-Freez) by the DEAE-cellulose procedure of Shulman and Armenia (18). The material used for most experiments was at least 95% 19 S protein, and was kept in the refrigerator in dilute phosphate buffer, pH 6.8, under toluene. 131I-Diiodotyrosine (3.0 μC per μmole) was purchased from Abbott, and horse-radish peroxidase (Boehringer, RZ 2.9) from Calbiochem.

**Incubation Procedure**—Incubation was performed without shaking in test tubes (15 × 125 mm) in an aluminum block maintained at 37°C. All components of the system, except the glucose oxidase and the peroxidase, were first mixed and pre-warmed. Glucose oxidase was then added, followed 3 to 5 min later by chloroperoxidase. The standard system for tyrosine iodination contained 300 μmole of tyrosine, 236 μmole of I⁻ (containing 10 to 20 μC of 131I⁻), 3 mg of glucose, 12.5 μg of glucose oxidase, 0.3 mmole of Cl⁻, 0.04 μg of chloroperoxidase, and 0.1 m sodium acetate buffer, pH 4.9, in a total volume of 3 ml. The standard system for thyroglobulin iodination differed only in the amount of chloroperoxidase (0.16 μg), and in containing 1 to 2 mg of thyroglobulin instead of tyrosine. Omissions or additions were made as indicated with the results of particular experiments.

**Chromatography and Digestion**—Portions of the incubation mixture (25 μl each) were applied along a 3.5-cm line near one end of a strip of Whatman No. 3 filter paper, and the paper strips were developed in a solvent containing collidine-3 N NH₄OH (100:33, v/v). The dried chromatograms were exposed to x-ray film for location of the radioactive bands, and then were cut into sections for counting in a well-type scintillation counter.

In the case of the thyroglobulin iodination, chromatography was performed both before and after digestion with Pronase. The digestion procedure was as follows. First, 0.5 ml of the incubation mixture was added to a digestion tube containing 0.1 ml of NaCl-Tris buffer, pH 8.5 (0.66 M NaCl-0.24 M Tris), 20 μl of 1 N NaOH, and 10 μl of 0.75% 1-methyl-2-mercaptoimidazole. Pronase (0.05 ml of a solution containing 35 mg per ml) was then added, followed by 1 drop of toluene, and digestion was allowed to proceed for 8 hours at 37°C.

In early experiments, carrier 3,5-diiodotyrosine, 3-iodotyrosine, and thyroxine were added to identify these components on the chromatograms, but after it became clear that these three products were routinely formed, it was no longer thought necessary to add carriers for identification of these components.

**Measurement of 131I—**Stable iodine was measured by the CrO₃ glucose-glucose oxidase method (20). Omission of either glucose or glucose oxidase reduced iodination practically to zero, indicating that the glucose-glucose oxidase-generating system was effective in catalyzing the iodination of tyrosine and thyroglobulin. Results obtained in the present investigation indicate that chloroperoxidase-catalyzed iodination may serve as a useful model for the elucidation of iodination mechanisms in the thyroid gland.

**Results**

**Requirements for Iodination of Tyrosine and Thyroglobulin with Chloroperoxidase**—The various components required for the enzymatic iodination of tyrosine and thyroglobulin are indicated in Table I. The complete system contained chloroperoxidase, glucose plus glucose oxidase as a hydrogen peroxide-generating system, tyrosine or thyroglobulin as iodine acceptors, Cl⁻, and I⁻. The standard system, shown in Table I, contained 8 × 10⁻⁹ M I⁻. Tyrosine was added at a somewhat higher concentration, either as the free amino acid (1 × 10⁻⁸ M) or as an amino acid residue in thyroglobulin (approximately 1 × 10⁻⁴ M). Under the standard conditions (Table I), utilization of I⁻ was almost complete after 30 to 60 min of incubation, and almost all of the I⁻ that disappeared was recovered as iodinated amino acid or as iodinated protein. Very little iodination occurred in the absence of chloroperoxidase, although use of higher concentrations of tyrosine and I⁻ might have resulted in more extensive nonenzymatic iodination of tyrosine by the H₂O₂ produced by the glucose-glucose oxidase (20). Omission of either glucose or glucose oxidase reduced iodination practically to zero, indicating the dependence of the reaction on H₂O₂. The requirement for H₂O₂ could be met by the addition of H₂O₂, as well as by the H₂O₂-generating system, as described below. The observation that Cl⁻ was required for optimal iodination was unexpected.
Cl⁻ was first tested on the theory that it might inhibit the iodination, but, as indicated in Table I, the presence of Cl⁻ greatly enhanced the iodination. Further studies on the effect of Cl⁻ are described in the following section.

**Effect of Cl⁻ on Iodination of Tyrosine and Thyroglobulin**—Fig. 1 shows the rate of iodide utilization with tyrosine as acceptor in the presence and absence of 0.1 N Cl⁻. The stimulatory effect of Cl⁻ was apparent as early as 2 min after the initiation of the reaction, and it became progressively greater with time. Similar results were obtained with thyroglobulin as acceptor, as shown in the following section.

![Figure 1](image1.png)

**Figure 1.** Iodination of tyrosine with chloroperoxidase. Time curves showing effect of 0.1 N chloride. The incubation system contained 300 mpmoles of tyrosine, 236 mpmoles of I⁻ (labeled with ¹³¹I⁻), 0.15 μg of chloroperoxidase, 3 mg of glucose, 12.5 μg of glucose oxidase, chloride as indicated, and 0.1 N aceta buffer, pH 4.9, in a total volume of 3 ml.

**TABLE I**

**Requirements for iodination of tyrosine and thyroglobulin with chloroperoxidase**

<table>
<thead>
<tr>
<th>Conditions of incubation</th>
<th>Tyrosine iodination; ¹³¹I⁻ on chromatogram as</th>
<th>Thyroglobulin iodination; ¹³¹I⁻ on chromatogram as</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tyrosine iodination; ¹³¹I⁻ on chromatogram as</td>
<td>Thyroglobulin iodination; ¹³¹I⁻ on chromatogram as</td>
</tr>
<tr>
<td></td>
<td>3,3'-Diiodotyrosine</td>
<td>Iodide</td>
</tr>
<tr>
<td>Complete system</td>
<td>43.2</td>
<td>41.1</td>
</tr>
<tr>
<td>Minus chloroperoxidase</td>
<td>0.16</td>
<td>0.10</td>
</tr>
<tr>
<td>Minus glucose oxidase</td>
<td>0.05</td>
<td>0.07</td>
</tr>
<tr>
<td>Minus acceptor</td>
<td>0.26</td>
<td>0.31</td>
</tr>
<tr>
<td>Minus chloride</td>
<td>1.10</td>
<td>0.6</td>
</tr>
<tr>
<td>Chloroperoxidase heated</td>
<td>100°</td>
<td>for 5 min</td>
</tr>
</tbody>
</table>

**TABLE II**

**Test of not iodination of thyroglobulin**

<table>
<thead>
<tr>
<th>Test of not iodination of thyroglobulin</th>
<th>Total ¹³¹I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per 3 ml of dialyzed incubation mixture</td>
<td>39.3</td>
</tr>
<tr>
<td>In starting thyroglobulin</td>
<td>26.3</td>
</tr>
<tr>
<td>Net increase</td>
<td>26.3</td>
</tr>
<tr>
<td>Expected increase (0.976 x 30.0)</td>
<td>29.3</td>
</tr>
</tbody>
</table>

**Figure 2.** Iodination of thyroglobulin with chloroperoxidase. Time curves showing effects of various concentrations of added chloride. The incubation system contained 2.1 mg of thyroglobulin, 236 mpmoles of I⁻ (labeled with ¹³¹I⁻), 0.15 μg of chloroperoxidase, 3 mg of glucose, 12.5 μg of glucose oxidase, chloride as indicated, and 0.1 N acetate buffer, pH 4.9, in total volume of 3 ml.

![Figure 2](image2.png)

**Fig. 2.** The effects of intermediate concentrations of Cl⁻ are also shown in Fig. 2. There was no appreciable enhancement of the reaction by 0.001 M Cl⁻, even though at this level the concentration of Cl⁻ was still 12.5 times as great as that of I⁻. The presence of 0.01, 0.033, and 0.1 M Cl⁻ produced progressively greater increases in the rate of iodination. Higher concentrations of Cl⁻ were not tested. Sodium bromide also stimulated the iodination reaction, even when present at 0.001 M, but 0.05 M NaF, 0.067 M Na₂SO₄, and 0.1 M NaNO₃ had no stimulatory effect. The mechanism by which Cl⁻ and Br⁻ enhance the iodination reaction has not yet been clarified. Further comment on this point is presented under "Discussion."

**Test of Possible Exchange between ¹³¹I and Thyroglobulin**—Since the thyroglobulin used in these studies was already well iodinated, it was necessary to determine whether incorporation of ¹³¹I into thyroglobulin in the complete system represented net iodination of thyroglobulin or merely exchange with preexisting iodinated amino acid residues. Experiments were performed, therefore, in which stable iodine, as well as ¹³¹I, was measured. Typical results are shown in Table II, which also outlines the
Enzymatic Iodination with Chloroperoxidase

Vol. 241, No. 6

FIG. 3. Iodination of thyroglobulin with chloroperoxidase. Effect of pH. The standard incubation system was used.

FIG. 4. Iodination of tyrosine with chloroperoxidase. Time curves showing formation of 3-iodotyrosine (MIT) and 3,5-diiodotyrosine (DIT), in the presence and absence of 0.1 N chloride. Incubation conditions were as in Fig. 1.

procedure used. A net increase in the stable iodine content of the thyroglobulin was observed which agreed quite well with that calculated from the 131I data. Although it is not possible to rule out a small amount of exchange, these results indicate that, under the conditions used here, 131I is incorporated into thyroglobulin primarily by a reaction involving net iodination.

Effect of pH on Iodination of Thyroglobulin—As shown in Fig. 3, the rate of iodination of thyroglobulin with chloroperoxidase rapidly diminished as the pH was raised from 4.9. It is unlikely that this was due to an effect on the glucose-glucose oxidase system, since the pH optimum of glucose oxidase is quite broad (21). It seems most likely that the pH effect shown in Fig. 3 reflects a fall in the activity of chloroperoxidase itself. This would be expected from the pH optimum curve for chloroperoxidase-catalyzed chlorination of $\beta$-ketoacidic acid, previously obtained by Shaw and Hager (16).

Experiments were also performed at pH 4.2, but under these conditions thyroglobulin precipitated from solution. Results for this pH, therefore, are not included in Fig. 3.

Formation of 3-Iodotyrosine, 3,5-Diiodotyrosine, and Thyroxine during Iodination of Tyrosine with Chloroperoxidase—The rates of formation of both 3-iodotyrosine and 3,5-diiodotyrosine in the presence and in the absence of 0.1 N Cl⁻ are shown in Fig. 4. In the early stages of the reaction, 3-iodotyrosine formation far exceeded 3,5-diiodotyrosine formation. However, in the presence of Cl⁻, 3,5-diiodotyrosine formation became quite appreciable after 15 min, and by 60 min the quantity of added iodine bound as 3,5-diiodotyrosine exceeded that bound as 3-iodotyrosine. These results indicate that the latter compound is also an acceptor for the chloroperoxidase-catalyzed iodination, and Fig. 5 shows results of a separate experiment comparing rates of iodination of tyrosine and 3-iodotyrosine, under conditions similar to those used to obtain the data shown in Fig. 4. It is evident that iodination of 3-iodotyrosine occurred as readily as iodination of tyrosine.

When the amount of chloroperoxidase added to the reaction mixture was increased from 0.04 $\mu$g to 0.16 $\mu$g, 3,5-diiodotyrosine and 3-iodotyrosine formation reached a peak within the first 10 min, and thereafter began to decline (Table III). The decline in these products was accompanied by the appearance of a large fraction of the 131I just below the solvent front. This activity, which generally consisted of two separate but poorly resolved components, comprised as much as 37% of the total 131I after 60 min of incubation (Table III). The fact that these bands appeared only after the formation of considerable 131I-3,5-diiodotyrosine and 131I-3-iodotyrosine suggested that they were products of the action of chloroperoxidase on the iodotyrosines. This was supported by the results of separate experiments in which 131I-3,5-diiodotyrosine, instead of tyrosine, was incubated with the chloroperoxidase system ($\text{Cl}^-$ omitted in this case). In addition, under these conditions, a prominent 131I-labeled solvent front component was a major product of the reaction. Considerable deiodination of 131I-3,5-diiodotyrosine was also observed. This probably explains why there was no further decrease in 131I-iodide after 20 min when 0.16 $\mu$g of chloroperoxidase was added in the iodination of tyrosine (Table III). Very likely, both iodination and deiodination were occurring simultaneously. When only 0.04 $\mu$g of chloroperoxidase was added, the front running components became noticeable only after about 60 min, and deiodination was much less apparent.

A surprising observation was the appearance of a band that moved identically with thyroxine on chromatograms prepared from reaction mixtures containing 0.16 $\mu$g of chloroperoxidase.

FIG. 5. Comparison of rates of iodination of tyrosine and 3-iodotyrosine (MIT) with chloroperoxidase. The standard incubation system was used, with either tyrosine or 3-iodotyrosine as iodine acceptor.
Digests remained at the origin of the chromatogram. Approximately 10% of the iodine in the Pronase digests were determined by analysis of chromatograms of the origin of the filter paper chromatogram. Rates of formation of the individual iodinated amino acids were determined by analysis of chromatograms of the origin of the filter paper chromatogram. The abbreviation used is: DHP, 3,5-diiodo-4-hydroxyphenylpyruvic acid.

(Table III). This band was easily detectable in the 30-min sample, but it was not observed even after 60 min in samples incubated with only 0.04 μg of chloroperoxidase. The thyroxine band also appeared when 3,5-diiodotyrosine was incubated with chloroperoxidase (0.16 μg). Thyroxine was never seen unless there was also considerable activity at the solvent front, and it always appeared later in time than the front running bands. One of the front running bands has been identified as 3,5-diiodo-4-hydroxybenzaldehyde by paper chromatography both in collidine-NH₂OH and in butanol-acetic acid-water. As pointed out by Shibata and Cahnmann (22), 3,5-diiodo-4-hydroxybenzaldehyde is readily formed from 3,5-diiodo-4-hydroxyphenylpyruvic acid, and the finding of labeled 3,5-diiodo-4-hydroxybenzaldehyde on the chromatogram suggests that DHP is an intermediate in the formation of thyroxine when tyrosine or 3,5-diiodotyrosine is incubated with the chloroperoxidase system. (See Reference 23 for a discussion of previous studies on the nonenzymatic formation of thyroxine from DHP.) Ljunggren (24) studied the oxidation of 3,5-diiodo-4-hydroxybenzaldehyde by horseradish peroxidase at pH 7.4 and identified DHP and 3,5-diiodo-4-hydroxybenzaldehyde as oxidation products. However, he did not find any appreciable yield of thyroxine under his conditions, and he attributed this rapid degradation of any formed thyroxine by the high concentrations of peroxide and peroxidase employed in his experiments.

Formation of 3-Iodotyrosine, 3,5-Diiodotyrosine, and Thyroxine during Iodination of Thyroglobulin with Chloroperoxidase—Fig. 6 shows results of an experiment in which thyroglobulin was iodinated with the complete system described in Table I. Total organic iodine formation was measured by the percentage of the added 131I that remained at the origin of the filter paper chromatogram. Rates of formation of the individual iodinated amino acids were determined by analysis of chromatograms of Pronase digests. Approximately 10% of the 131I in the Pronase digest remained at the origin of the chromatogram.

Comparison of Fig. 6 with Fig. 4 indicates that 3,5-diiodotyrosine formation was relatively faster during iodination of thyroglobulin than during iodination of tyrosine. This would be expected from the fact that preformed 3-iodotyrosine is present in thyroglobulin, and from the finding (Fig. 5) that free 3-iodotyrosine is readily iodinated by chloroperoxidase. Thus, formation of 3,5-diiodotyrosine may occur from the outset when thyroglobulin is iodinated with chloroperoxidase, whereas, when thyroxine is the starting acceptor, time is required to build up a sufficient concentration of 3,5-diiodotyrosine so that it too can act as acceptor. Even with thyroglobulin iodination, however, 3,5-diiodotyrosine was the major product 10 min after the start of the reaction. This may reflect a greater abundance of tyrosyl residues than 3-iodotyrosine residues in the thyroglobulin, or the relative rates of iodination of the peptide-linked amino acids may not necessarily correlate with the data for the free amino acids shown in Fig. 5.

Of particular interest in Fig. 6 was the observation that a significant amount of 3-iodotyrosine was formed during thyroglobulin iodination. At 30 min, 3.0% of the total 131I was located in the thyroxine area of the chromatogram, and by 60 min this value had risen to 3.9%. No further increase was observed at 120 min. Many similar experiments were performed, and the thyroxine fraction generally contained 4 to 5% of the added 131I. To prove more conclusively the identity of the 3-iodotyrosine formed during iodination of thyroglobulin, chromatography was also performed in two additional solvents, butanol-ethanol-2 N NaOH (5:1:2) and tert-amyl alcohol saturated with 0.5 N NaOH. Radioautograms obtained in such an experiment are shown in Fig. 7. In each case, the added thyroxine marker corresponded exactly with the band on the radioautogram labeled thyroxine. There was no visible band corresponding to the added 3',3,5-triiodothyronine marker.

In contrast to the results obtained with tyrosine iodination, formation of thyroxine during thyroglobulin iodination was not preceded by the appearance of a front running component. 131I in the solvent front region of the chromatogram comprised less than 1% of the total, and this was generally not visible as a distinct band on the radioautogram. It seems likely, therefore, that formation of thyroxine during iodination of thyroglobulin with chloroperoxidase does not involve DHP as an intermediate.

![Fig. 6. Iodination of thyroglobulin with chloroperoxidase. Time curves showing formation of 3-iodotyrosine (MIT), 3,5-diiodotyrosine (DIT), and thyroxine. The standard incubation system was used.](http://www.jbc.org/issue)
although further studies are necessary to completely exclude this possibility. It should be noted also that the yield of 125I-thyroxine formed during thyroglobulin iodination was about twice that formed during tyrosine iodination, or by the action of chloroperoxidase on 125I-3,5-diiodotyrosine.

Substitution of H2O2 for Glucose-Glucose Oxidase—The curves in Fig. 8 show the time course of the chloroperoxidase-catalyzed iodination of thyroglobulin when various concentrations of H2O2 were substituted for the glucose-glucose oxidase hydrogen peroxide-generating system. It is evident that iodination proceeded very readily under these conditions. The distribution of 125I among 3,5-diiodotyrosine, 3-iodotyrosine, and 3'-iodothyronine (T3), and 3-iodothyronine (T4), carriers. In all solvents, there was some escape from the block by 30 min, and by 60 min the iodination had proceeded to about 90% completion. Similar findings were observed with many of the other inhibitory compounds shown in Table IV. These results suggest that the inhibitory compounds themselves were oxidized by chloroperoxidase plus H2O2, and that I− was oxidized only after the concentration of inhibitor was reduced to a low level. Similar observations were previously made by Morris, Eberwein, and Hager (27), who showed that thiourea is a competitive inhibitor in the chlorination of monochlorodimedon with chloroperoxidase, and that thiourea itself is oxidized by chloroperoxidase in the presence of a halogen anion. These investigators suggested that thiourea itself is oxidized by chloroperoxidase in the presence of a halogen anion. These investigators suggested that compounds such as thiouracil exert their antithyroid effects by competing with tyrosine residues of thyroglobulin for enzyme-bound iodide ion. A similar conclusion has been reached by Maloof and Soodak (28), although these investigators previously proposed (29) that, in the thyroid, competition occurs between thiocarbamide drugs and iodide for a common oxidizing agent, cysteine, glutathione, and ascorbic acid. It has been suggested that the latter two compounds play a physiological role in regulating iodination of tyrosine in the thyroid (25, 26). Iodination was also blocked by cyanide, a known inhibitor of heme enzymes. Somewhat unexpected was the potent inhibitory effect of KSCN, which is usually considered to exert most of its antimicrobial action by interference with iodide transport. As shown in Table IV, KSCN inhibited iodination about 95%, even when present at the relatively low concentration of 5 × 10−4 M. Perchlorate, on the other hand, which like thiocyanate also blocks I− transport, had very little inhibitory effect on iodination even when tested at 1 × 10−3 M. The chelating agent EDTA and the —SH-blocking agent N-ethylmaleimide also had little or no inhibitory effect when tested at 1 × 10−3 M. Although not shown in Table IV, tyrosine iodination was also blocked by the agents that inhibited thyroglobulin iodination.

The inhibitory effects shown in Table IV were based on a 15-min incubation period. When the reaction was allowed to continue for 60 min, there was a marked escape from inhibition in the case of most of the compounds tested. This is illustrated in Fig. 9, which shows time curves for the iodination of thyroglobulin carried out in the presence of different concentrations of 1-methyl-2-mercaptoimidazole. When 2 × 10−4 M of the latter reagent was added, the iodination was completely blocked for 60 min. However, when only 6.7 × 10−5 M of this reagent was added, there was some escape from the block by 30 min, and by 60 min the iodination had proceeded to about 90% completion. Similar findings were observed with many of the other inhibitory compounds shown in Table IV. These results suggest that the inhibitory compounds themselves were oxidized by chloroperoxidase plus H2O2, and that I− was oxidized only after the concentration of inhibitor was reduced to a low level. Similar observations were previously made by Morris, Eberwein, and Hager (27), who showed that thiourea is a competitive inhibitor in the chlorination of monochlorodimedon with chloroperoxidase, and that thiourea itself is oxidized by chloroperoxidase in the presence of a halogen anion. These investigators suggested that compounds such as thiouracil exert their antithyroid effects by competing with tyrosine residues of thyroglobulin for enzyme-bound iodide ion. A similar conclusion has been reached by Maloof and Soodak (28), although these investigators previously proposed (29) that, in the thyroid, competition occurs between thiocarbamide drugs and iodide for a common oxidizing system. The results shown in Fig. 9 support the view that thiocarbamide drugs act as competitive inhibitors in the iodination of thyroglobulin, but the results obtained here do not define the actual site of the inhibitory effect.

Effect of Varying I− Concentration on Iodination of Thyroglobulin and Tyrosine—Fig. 10 shows results obtained when the
I- concentration was varied from $1 \times 10^{-5}$ to $5 \times 10^{-3}$ M in the iodination of thyroglobulin with chloroperoxidase. Except for the I- concentration, conditions were fixed as shown in Table IV. It is apparent that the rate of incorporation of I- into thyroglobulin reached a maximum at approximately $1 \times 10^{-4}$ M I-, and thereafter began to decline. Essentially similar results were obtained for the 3-iodotyrosine and 3,5-diiodotyrosine components of thyroglobulin (results for the latter are shown in Fig. 10). The values for 131I-thyroxine were so low at the highest I- concentrations that it was not possible to determine with any accuracy the effect of excess I- on thyroxine formation.

An inhibitory effect of excess I- was also observed when tyrosine was iodinated with chloroperoxidase, as illustrated in Fig. 11. The maximum rate of I- utilization occurred at about $5 \times 10^{-4}$ M I-, compared with approximately $1 \times 10^{-5}$ M I- for maximum I- utilization with thyroglobulin as acceptor.

The mechanism of the inhibitory effect of excess I- on thyroglobulin and tyrosine iodination has not yet been established. At least two possibilities deserve consideration: (a) substrate inhibition, i.e. inhibition of chloroperoxidase by excess I-, and (b) product inhibition, i.e. inhibition of chloroperoxidase by I2, or some other form of oxidized I-. In the latter connection it is of interest that I2 was detectable by starch test at those I- concentrations at which iodination of thyroglobulin was inhibited.

Igo, Mahoney, and Mackler (11) reported an inhibitory effect of excess I- with an iodinating enzyme obtained from the thyroid, and they attributed the effect to inhibition of glucose oxidase. However, this does not seem to be the mechanism involved with chloroperoxidase, since the inhibitory effect of excess I- could also be demonstrated when $1 \times 10^{-3}$ M H2O2 was substituted for the glucose-glucose oxidase system.

Inhibition by I- of organic iodine formation in the thyroid is known to occur under certain conditions, e.g. in Graves' disease, in I- myxedema, and transiently in normal thyroids. The results obtained here suggest that one of the mechanisms in-
Enzymatic Iodination with Chloroperoxidase

Vol. 241, No. 6

Of particular interest was the observation that \(^{131}\text{I}\) thyroxine was formed not only with thyroglobulin as acceptor, but also with the other proteins tested. This supports the view that \(^{131}\text{I}\) thyroxine formation in thyroglobulin represented net synthesis, and not merely exchange.

Also of interest was the observation that thyroxine formation did not correlate with 3,5-diiodotyrosine formation. Iodination of insulin produced the greatest yield of 3,5-diiodotyrosine, yet thyroxine formation was much the lowest in this case. The thyroxine value for insulin in Table V is even relatively too high, since the values shown were not corrected for an appreciable paper background occurring all along the chromatograms. It is

<table>
<thead>
<tr>
<th>Protein</th>
<th>(^{131}\text{I}) converted to (^{131}\text{I})-protein</th>
<th>Pronase digest after 60 min of iodination</th>
<th>(^{131}\text{I}) in Thyroxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroglobulin</td>
<td>0.77 mg</td>
<td>52.0%</td>
<td>95.0%</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.55 mg</td>
<td>81.8%</td>
<td>92.9%</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>0.55 mg</td>
<td>64.2%</td>
<td>88.1%</td>
</tr>
<tr>
<td>Crystalline beef insulin</td>
<td>0.24 mg</td>
<td>90.3%</td>
<td>96.9%</td>
</tr>
</tbody>
</table>
reasonable to conclude, therefore, that 3,5-diiodotyrosine formation in itself does not lead to thyroxine formation. Most likely the 3,5-diiodotyrosine residues must be favorably located within the peptide chain to permit coupling between 2 of these molecules. Of the proteins tested, thyroglobulin showed the highest thyroxine formation, and presumably, therefore, contained 3,5-diiodotyrosine residues in the most favorable steric relationship for coupling.

Iodination of Thyroglobulin with Horseradish Peroxidase—
Purified horseradish peroxidase was tested at levels of 0.5, 1, and 10 pg per incubation tube, compared to 0.16 pg of chloroperoxidase. Conditions of incubation were as described for the complete system in Table I. As shown in Fig. 12, horseradish peroxidase was very effective in catalyzing the iodination of thyroglobulin when it was added in sufficient amounts, although the specific activity of the enzyme was considerably less than that of chloroperoxidase. The distribution of the 13I in Pronase digests of the 60 min-incubated samples is shown in Table VI.

![Graph](image)

**Fig. 12.** Iodination of thyroglobulin with various amounts of horseradish peroxidase, compared with standard amount of chloroperoxidase. The standard incubation system was used, but with horseradish peroxidase (HPX) substituted for chloroperoxidase (CPX) in some samples.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amount (pg)</th>
<th>Chloride concentration</th>
<th>% 3,5-Diiodotyrosine</th>
<th>% 3-Iodothyrosine</th>
<th>% Thyroxine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horseradish peroxidase</td>
<td>0.5</td>
<td>0</td>
<td>7.4</td>
<td>13.9</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>6.2</td>
<td>14.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>15.6</td>
<td>22.3</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>10.1</td>
<td>21.2</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>0.16</td>
<td>0</td>
<td>33.0</td>
<td>24.1</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>34.1</td>
<td>25.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Chloroperoxidase</td>
<td></td>
<td>0</td>
<td>10.3</td>
<td>17.6</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>45.4</td>
<td>27.7</td>
<td>3.9</td>
</tr>
</tbody>
</table>

**Table VI**

*Formation of 3-iodotyrosine, 3,5-diiodotyrosine, and thyroxine during 60-min incubation of thyroglobulin with horseradish peroxidase (standard incubation system)*

Formation of 131I-thyroxine occurred to about the same extent with 10 pg of horseradish peroxidase as with 0.16 pg of chloroperoxidase.

As shown in Fig. 12 and in Table VI, Cl− had no effect on the horseradish peroxidase-catalyzed iodination of thyroglobulin. Stimulation by Cl−, therefore, is not a general property of peroxidases. The pH optimum of horseradish peroxidase, however, did resemble that of chloroperoxidase, since horseradish peroxidase, like chloroperoxidase, was much more active at pH 4.9 than at pH 7.0.

**DISCUSSION**

The observations reported here provide ample indication that chloroperoxidase is very effective in catalyzing the iodination of tyrosine and thyroglobulin when supplemented either with H2O2 or with a glucose-glucose oxidase H2O2-generating system. Chloroperoxidase-catalyzed iodination of tyrosine has also been observed by Hager et al. (31). It is of particular interest that both the oxidation of I− and its transfer to the acceptor occur in the presence of a single, crystalline peroxidase. While it has not yet been determined whether the peroxidase is involved in both these reactions (or only in the oxidation step), it is apparent that iodination of thyroglobulin may occur very effectively in the presence of only a single peroxidase system. Our findings, together with those previously reported by Klebanoff et al. (10), suggest that it may not be necessary to postulate the existence of a separate “tyrosine iodinase” in the thyroid gland. There is no convincing evidence at the present time that two separate enzymes are required for iodination in the thyroid gland (a peroxidase for oxidation of I− and an iodinase for transfer to the acceptor), although this is a prevalent view (32, 33).

It is also of great interest that thyroglobulin, as well as tyrosine, acts as acceptor in the chloroperoxidase-catalyzed iodination, since there is little doubt that, in the thyroid gland, thyroglobulin, and not free tyrosine, is the physiological substrate for iodination (34–38). The starting thyroglobulin, obtained from rabbit thyroid, contained 0.74% total iodine, and this increased to almost 2% upon iodination with chloroperoxidase in the presence of 8 × 10−3 M I−. Experiments were also performed with rat thyroglobulin, purified on DEAE-cellulose, and in this case, too, enzymatic iodination with chloroperoxidase proceeded readily. Most investigators who have attempted to isolate an iodide peroxidase from the thyroid have assayed their preparations with tyrosine as acceptor (9–14). Only Hosaya, Kondo, and Ui (8) have reported studies with a thyroid peroxidase in which thyroglobulin was used as acceptor. It is reasonable to expect that a thyroid peroxidase that is physiologically involved in iodination should readily iodinate thyroglobulin, especially since it has now been demonstrated that chloroperoxidase displays such marked activity in this respect.

To what extent iodination of thyroglobulin with chloroperoxidase may serve as a model for iodination mechanisms in the thyroid gland is open to question, but the following observations may be cited as evidence in support of this view. (a) Thyroglobulin, as well as tyrosine, is a substrate for the iodination reaction. (b) The iodination reaction is very rapid at concentrations of I− and thyroglobulin that may be considered physiological. (c) The reaction products are similar, qualitatively and quantitatively, to those observed in experiments with thyroid tissue. (d) The activity of the enzyme is completely inhibited by antithyroid compounds at concentrations that may be at-
tained in vivo. (c) The inhibitory effect of excess iodide on the chloroperoxidase-catalyzed reaction resembles the inhibitory effect of excess I⁻ on iodination in thyroid tissue, observed under certain conditions (30). On the other hand, the observation that iodination with chloroperoxidase is almost negligible at pH 7.0 suggests that, in this respect, the chloroperoxidase system differs from the thyroid system. Measurements of thyroid intracellular pH have given values near 7 (39). The marked enhancement of the chloroperoxidase system by Cl⁻ also suggests a difference from the thyroid iodinating system.

The mechanism by which Cl⁻ increases the iodinating activity of chloroperoxidase remains unknown, but experiments have been performed which eliminate some of the possibilities. It was suggested by Hager⁴ that Cl⁻ might stabilize the enzyme during the incubation and prevent its deterioration during exposure to excess H₂O₂. However, when chloroperoxidase was preincubated with the glucose-glucose oxidase system for 15 min in the absence of Cl⁻, it was still as effective in catalyzing iodination of thyroglobulin as a control sample preincubated in the presence of 0.1 M Cl⁻. Results were the same whether or not thyroglobulin was present during the preincubation. These observations indicate that Cl⁻ does not exert its effect by maintaining the stability of the chloroperoxidase. Another possibility is that Cl⁻ acts as an intermediate in the reaction, by reacting first with H₂O₂ in the presence of chloroperoxidase to form an oxidized chlorine intermediate. The latter might then be the immediate agent which oxidizes I⁻ to its iodinating form. Against this view is the observation of Shaw and Hager that at pH 4.9 Cl⁻ is not very effectively oxidized by chloroperoxidase (16). Experiments were performed to determine whether labeled ICl might be formed in the presence of Cl⁻. For this purpose, chromatography was performed in butanol-acetic acid water. While no definite conclusions could be drawn, it appeared from these results that ICl formation was not a significant factor in the Cl⁻ enhancement of iodination. At present, therefore, the mechanism of the Cl⁻ stimulation remains unknown. It is of interest that Cl⁻ also stimulates the I⁻-catalyzed reduction of ceric ion by arsenite (40), a reaction used routinely in the quantitative supply of H₂O₂, the other observations of Klebanoff et al. were confirmed and extended. We have been able to demonstrate in a single experiment, with thyroglobulin as acceptor and with a crystalline peroxidase, both the iodination of tyrosine residues and the conversion of some of those residues to thyroxine. Since no definite intermediates were observed on the chromatograms, this suggests that coupling of two diiodotyrosines occurred within the matrix of the thyroglobulin molecule. No involvement of free DHP was suggested, although when free tyrosine was iodinated, the chromatographic results suggested that DHP was an intermediate. A recent report by Toi, Salvatore, and Cahnmann (43) demonstrated that free DHP may couple with 3,5-diiodotyrosine residues in thyroglobulin to form thyroxine. It remains to be determined whether the model suggested by Toi et al., involving free DHP, or the model suggested by our experiments, in which coupling appears to occur without any free intermediate, most nearly parallels thyroxine formation within the thyroid gland.

Acknowledgments—We are greatly indebted to Dr. Lowell P. Hager (Department of Chemistry, University of Illinois) for supplying the crystalline chloroperoxidase used in this study. We also thank Dr. J. Lospalluto (Department of Biochemistry, Southwestern Medical School) for providing the purified thyroglobulin and Dr. Hans Cahnmann (Laboratory of Clinical Endocrinology, National Institute for Arthritis and Metabolic Diseases) for a sample of 3,5-diido-4-hydroxybenzaldehyde. The technical assistance of Margit Moberly is gratefully acknowledged.

REFERENCES
1. DEMPSEY, E. W., Endocrinology, 34, 27 (1944).
2. DEROBERTIS, E., AND GRASSO, R., Endocrinology, 38, 137 (1946).

2 L. P. Hager, personal communication.
Enzymatic Iodination of Tyrosine and Thyroglobulin with Chloroperoxidase
Alvin Taurog and Earl M. Howells


Access the most updated version of this article at http://www.jbc.org/content/241/6/1329

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/241/6/1329.full.html#ref-list-1