

# Enzymatic Iodination of Tyrosine and Thyroglobulin with Chloroperoxidase\*

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## SUMMARY

1. Crystalline chloroperoxidase is effective in catalyzing the iodination of tyrosine or thyroglobulin when supplemented with  $H_2O_2$ , or with the  $H_2O_2$ -generating system, glucose-glucose oxidase. The iodination reaction is very rapid at low concentrations of iodide ( $5 \times 10^{-6}$  M), and at low concentrations of tyrosine ( $1 \times 10^{-4}$  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  $^{131}I$  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.

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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  $H_2O_2$  and  $O_2$  are involved in oxidation-reduction couples that possess, at pH 7, a higher oxidation-reduction potential than the couple  $2I^- \rightarrow I_2$  (+ 0.535 volt). It is hardly surprising, therefore, that  $H_2O_2$  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.

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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 Keston (15) in 1944. He demonstrated organic iodine formation in a model milk system, containing added xanthine and radioiodide. He postulated that the xanthine oxidase in milk catalyzed the formation of  $H_2O_2$  by xanthine oxidation, and that the resultant  $H_2O_2$ , 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 myeloperoxidase and lactoperoxidase catalyze the iodination of tyrosine in the presence of  $H_2O_2$ -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 proposed 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 heme content) was diluted with acetate buffer, pH 4.9, or with water to provide a solution containing 1.6  $\mu$ g per ml. Portions of the latter (1 or 2 ml) were kept frozen at  $-20^\circ$ , 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.  $^{131}I$ -Diiodotyrosine (3.6 mC per  $\mu$ mole) was purchased from Abbott, and horseradish peroxidase (Boehringer, RZ 2.9) from Calbiochem.

**Incubation Procedure**—Incubation was performed without shaking in test tubes (15  $\times$  125 mm) in an aluminum block maintained at  $37^\circ$ . All components of the system, except the glucose oxidase and the peroxidase, were first mixed and prewarmed. Glucose oxidase was then added, followed 3 to 5 min later by chloroperoxidase. The standard system for tyrosine

iodination contained 300 m $\mu$ moles of tyrosine, 236 m $\mu$ moles of  $I^-$  (containing 10 to 20  $\mu$ C of  $^{131}I^-$ ), 3 mg of glucose, 12.5  $\mu$ g of glucose oxidase, 0.3 mmole of  $Cl^-$ , 0.04  $\mu$ g of chloroperoxidase, and 0.1 N 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  $\mu$ g), 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  $\mu$ l 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 NH<sub>4</sub>OH (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  $\mu$ l of 1 N NaOH, and 10  $\mu$ l 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  $37^\circ$ .

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  $^{127}I$** —Stable iodine was measured by the CrO<sub>3</sub> digestion procedure of Chaney (19), as modified in this laboratory.

**Presentation of Results**—Each figure and table presents results of a single experiment, but all experiments were performed at least twice, and only reproducible findings are reported.

#### 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  $8 \times 10^{-6}$  M  $I^-$ . Tyrosine was added at a somewhat higher concentration, either as the free amino acid ( $1 \times 10^{-4}$  M) or as an amino acid residue in thyroglobulin (approximately  $1 \times 10^{-4}$  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  $H_2O_2$  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  $H_2O_2$ . The requirement for  $H_2O_2$  could be met by the addition of  $H_2O_2$ , as well as by the  $H_2O_2$ -generating system, as described below. The observation that  $Cl^-$  was required for optimal iodination was unexpected.

$\text{Cl}^-$  was first tested on the theory that it might inhibit the iodination, but, as indicated in Table I, the presence of  $\text{Cl}^-$  greatly enhanced the iodination. Further studies on the effect of  $\text{Cl}^-$  are described in the following section.

**Effect of  $\text{Cl}^-$  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  $\text{Cl}^-$ . The stimulatory effect of  $\text{Cl}^-$  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

TABLE I

Requirements for iodination of tyrosine and thyroglobulin with chloroperoxidase

Complete system for iodination of tyrosine contained 0.30  $\mu\text{mole}$  of tyrosine; 0.24  $\mu\text{mole}$  of  $\text{I}^-$ ; 0.04  $\mu\text{g}$  of crystalline chloroperoxidase; 3 mg of glucose; 12.5  $\mu\text{g}$  of glucose oxidase; 0.30 mmole of NaCl; and 0.1 N sodium acetate buffer, pH 4.9; total volume, 3.0 ml. Incubation interval, 60 min. Complete system for iodination of thyroglobulin contained 1.4 mg of thyroglobulin; 0.24  $\mu\text{mole}$  of  $\text{I}^-$ ; 0.16  $\mu\text{g}$  of crystalline chloroperoxidase; 3 mg of glucose; 12.5  $\mu\text{g}$  of glucose oxidase; 0.30 mmole of NaCl; and 0.1 N sodium acetate buffer, pH 4.9; total volume 3.0 ml. Incubation interval, 15 min.

Conditions of incubation	Tyrosine iodination; $^{131}\text{I}$ on chromatogram as			Thyroglobulin iodination; $^{131}\text{I}$ on chromatogram as	
	3,5-Diodotyrosine	3-Iodo-tyrosine	Iodide	Origin	Iodide
	%	%	%	%	%
Complete system.....	43.2	41.1	2.5	88.1	11.4
Minus chloroperoxidase.....	0.14	2.2	95.9	0.11	99.7
Minus glucose.....	0.06	0.10	98.3	0.10	99.7
Minus glucose oxidase.....	0.05	0.07	98.2	0.10	99.8
Minus acceptor.....	0.26	0.31	94.8	5.7	93.1
Minus chloride.....	1.0	9.6	86.7	14.4	85.3
Chloroperoxidase heated at 100° for 5 min.....	0.11	2.3	95.8	0.17	98.2

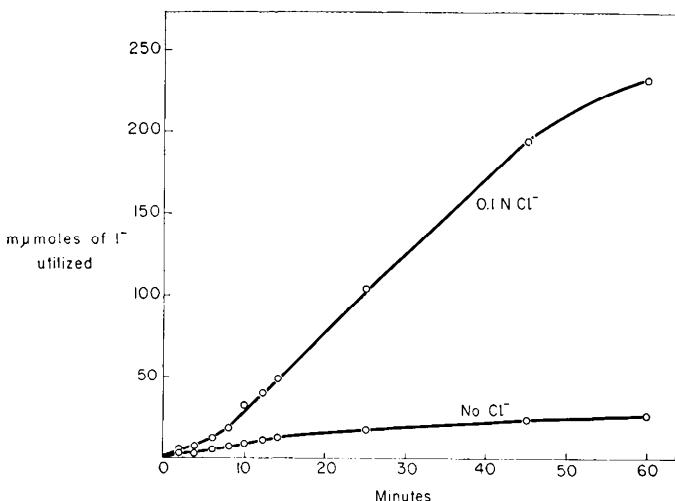


Fig. 1. Iodination of tyrosine with chloroperoxidase. Time curves showing effect of 0.1 N chloride. The incubation system contained 300  $\mu\text{mole}$  of tyrosine, 236  $\mu\text{mole}$  of  $\text{I}^-$  (labeled with  $^{131}\text{I}^-$ ), 0.04  $\mu\text{g}$  of chloroperoxidase, 3 mg of glucose, 12.5  $\mu\text{g}$  of glucose oxidase, chloride as indicated, and 0.1 N acetate buffer, pH 4.9, in a total volume of 3 ml.

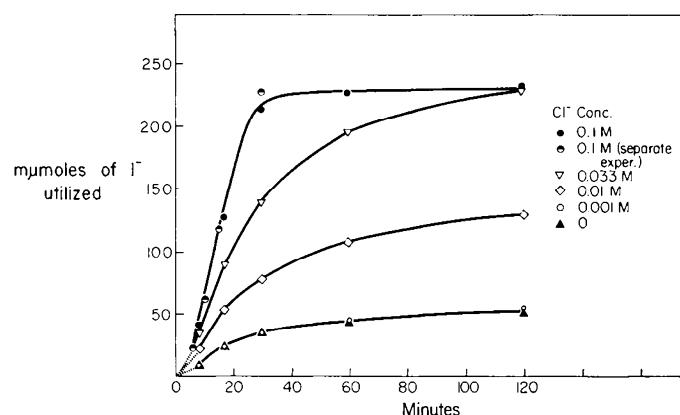


Fig. 2. Iodination of thyroglobulin with chloroperoxidase. Time curves showing effects of various concentrations of added chloride. The incubation system contained 2.1 mg of thyroglobulin, 236  $\mu\text{mole}$  of  $\text{I}^-$  (labeled with  $^{131}\text{I}^-$ ), 0.15  $\mu\text{g}$  of chloroperoxidase, 3 mg of glucose, 12.5  $\mu\text{g}$  of glucose oxidase, chloride as indicated, and 0.1 N acetate buffer, pH 4.9, in total volume of 3 ml.

TABLE II  
Test of net iodination of thyroglobulin

$^{131}\text{I}$ -Labeled iodide (30.0  $\mu\text{g}$ ) was added to an incubation system containing 2.18 mg of purified thyroglobulin, 12.5  $\mu\text{g}$  of glucose oxidase, 3 mg of glucose, 0.15  $\mu\text{g}$  of crystalline chloroperoxidase, 0.1 M NaCl, and acetate buffer, pH 4.9, to make a 3.0-ml final volume. After 45 min of incubation at 37°, a chromatogram of the reaction mixture showed 97.6% of the  $^{131}\text{I}$  remaining at the origin. The reaction mixture (2 ml) was transferred to a dialysis bag and dialyzed to eliminate nonprotein iodine. The contents of the dialysis bag were made to volume for  $^{127}\text{I}$  determination.

	Total $^{127}\text{I}$
Per 3 ml of dialyzed incubation mixture.....	42.3 $\mu\text{g}$
In starting thyroglobulin.....	16.0
Net increase.....	26.3
Expected increase ( $0.976 \times 30.0$ ).....	29.3

Fig. 2. The effects of intermediate concentrations of  $\text{Cl}^-$  are also shown in Fig. 2. There was no appreciable enhancement of the reaction by 0.001 M  $\text{Cl}^-$ , even though at this level the concentration of  $\text{Cl}^-$  was still 12.5 times as great as that of  $\text{I}^-$ . The presence of 0.01, 0.033, and 0.1 M  $\text{Cl}^-$  produced progressively greater increases in the rate of iodination. Higher concentrations of  $\text{Cl}^-$  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  $\text{Na}_2\text{SO}_4$ , and 0.1 M  $\text{NaNO}_3$  had no stimulatory effect. The mechanism by which  $\text{Cl}^-$  and  $\text{Br}^-$  enhance the iodination reaction has not yet been clarified. Further comment on this point is presented under "Discussion."

**Test of Possible Exchange between  $^{131}\text{I}$  and Thyroglobulin**—Since the thyroglobulin used in these studies was already well iodinated, it was necessary to determine whether incorporation of  $^{131}\text{I}$  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  $^{131}\text{I}$ , was measured. Typical results are shown in Table II, which also outlines the

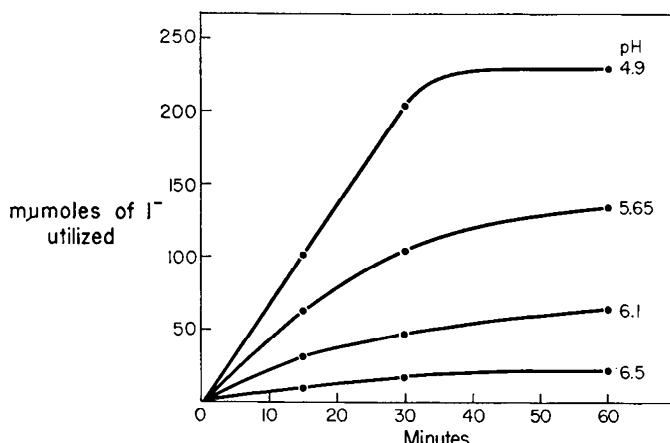


FIG. 3. Iodination of thyroglobulin with chloroperoxidase. Effect of pH. The standard incubation system was used.

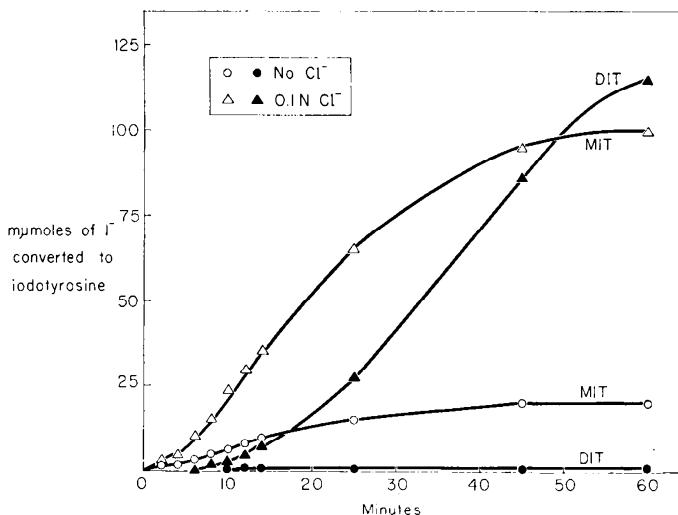


FIG. 4. Iodination of tyrosine with chloroperoxidase. Time curves showing formation of 3-iodotyrosine (MIT) and 3,5-diiodotyrosine (DIT), in the presence and absence of 0.1 N chloride. Incubation conditions were as in Fig. 1.

procedure used. A net increase in the stable iodine content of the thyroglobulin was observed which agreed quite well with that calculated from the  $^{131}\text{I}$  data. Although it is not possible to rule out a small amount of exchange, these results indicate that, under the conditions used here,  $^{131}\text{I}$  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  $\beta$ -ketoadipic 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  $\text{Cl}^-$  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  $\text{Cl}^-$ , 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  $\mu\text{g}$  to 0.16  $\mu\text{g}$ , 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  $^{131}\text{I}$  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  $^{131}\text{I}$  after 60 min of incubation (Table III). The fact that these bands appeared only after the formation of considerable  $^{131}\text{I}$ -3,5-diiodotyrosine and  $^{131}\text{I}$ -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  $^{131}\text{I}$ -3,5-diiodotyrosine, instead of tyrosine, was incubated with the chloroperoxidase system ( $^{131}\text{I}^-$  omitted in this case). In addition, under these conditions, a prominent  $^{131}\text{I}$ -labeled solvent front component was a major product of the reaction. Considerable deiodination of  $^{131}\text{I}$ -3,5-diiodotyrosine was also observed. This probably explains why there was no further decrease in  $^{131}\text{I}$ -iodide after 20 min when 0.16  $\mu\text{g}$  of chloroperoxidase was added in the iodination of tyrosine (Table III). Very likely, both iodination and deiodination were occurring simultaneously. When only 0.04  $\mu\text{g}$  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  $\mu\text{g}$  of chloroperoxidase

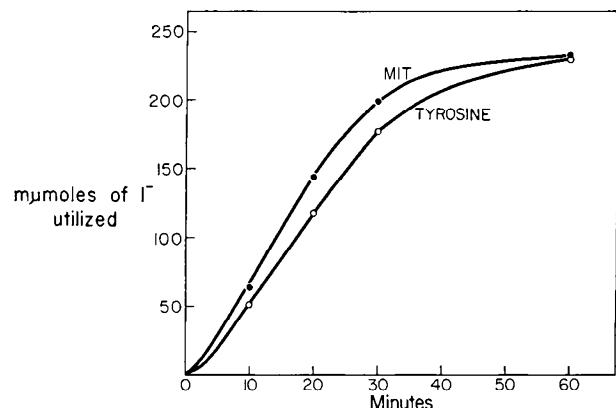


FIG. 5. Comparison of rates of iodination of tyrosine and 3-iodotyrosine (MIT) with chloroperoxidase. The standard incubation system was used, with either tyrosine or 3-iodotyrosine as iodine acceptor.

TABLE III

*Iodination of tyrosine with chloroperoxidase*

The reaction mixture contained 0.30  $\mu\text{mole}$  of tyrosine, 0.24  $\mu\text{mole}$  of  $\text{I}^-$  (labeled with  $^{131}\text{I}^-$ ), 3 mg of glucose, 12.5  $\mu\text{g}$  of glucose oxidase, 0.30 mmole of NaCl, and 0.1 N acetate buffer, pH 4.9, in a total volume of 3.0 ml.

Quantity of chloroperoxidase $\mu\text{g}$	Incuba-tion time min	Total $^{131}\text{I}$ on chromatogram				
		3,5-Dio-dotyrosine %	3-Iodo-tyrosine %	Iodine %	Thyroxine %	Front %
0.040	10	3.1	16.4	79.0	0.27	
	20	16.3	31.2	50.4	0.37	
	30	33.7	38.6	25.6	0.15	
	60	51.0	39.5	2.6	0.63	3.8
0.16	10	45.7	39.9	12.2	0.25	0.19
	20	37.9	32.3	6.3	0.89	19.5
	30	29.8	26.5	7.6	1.7	30.1
	60	23.5	23.0	8.8	2.2	37.1

(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  $\mu\text{g}$  of chloroperoxidase. The thyroxine band also appeared when  $^{131}\text{I}$ -3,5-diiodotyrosine was incubated with chloroperoxidase (0.16  $\mu\text{g}$ ). 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-NH<sub>4</sub>OH and in butanol-acetic acid-water. As pointed out by Shiba 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<sup>1</sup> 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  $^{131}\text{I}$ -3,5-diiodotyrosine 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 to 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  $^{131}\text{I}$  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  $^{131}\text{I}$  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 thyro-

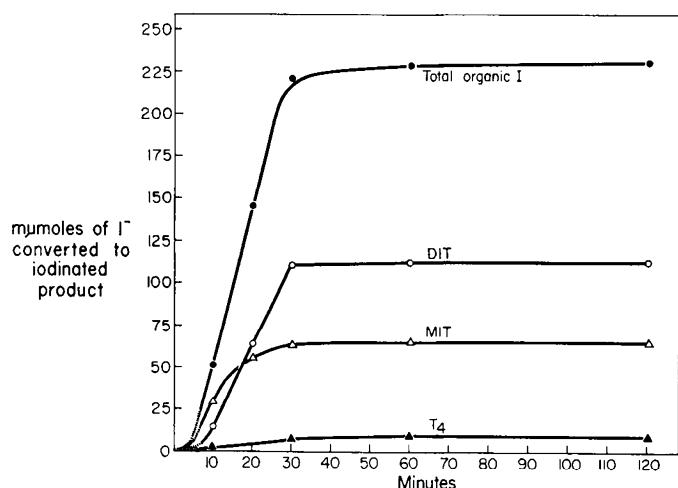


FIG. 6. Iodination of thyroglobulin with chloroperoxidase. Time curves showing formation of 3-iodotyrosine (MIT), 3,5-diiodotyrosine (DIT), and thyroxine. The standard incubation system was used.

globulin 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 tyrosine is the starting acceptor, time is required to build up a sufficient concentration of 3-iodotyrosine so that it too can act as acceptor. Even with thyroglobulin iodination, however, 3-iodotyrosine 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  $^{131}\text{I}$ -thyroxine was formed during thyroglobulin iodination. At 30 min, 3.0% of the total  $^{131}\text{I}$  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  $^{131}\text{I}$ . To prove more conclusively the identity of the  $^{131}\text{I}$ -thyroxine formed during iodination of thyroglobulin, chromatography was also performed in two additional solvents, butanol-ethanol-2 N NH<sub>4</sub>OH (5:1:2) and *tert*-amyl alcohol saturated with 0.5 N NH<sub>4</sub>OH. 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',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.  $^{131}\text{I}$  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,

<sup>1</sup> The abbreviation used is: DHP, 3,5-diiodo-4-hydroxyphenylpyruvic acid.

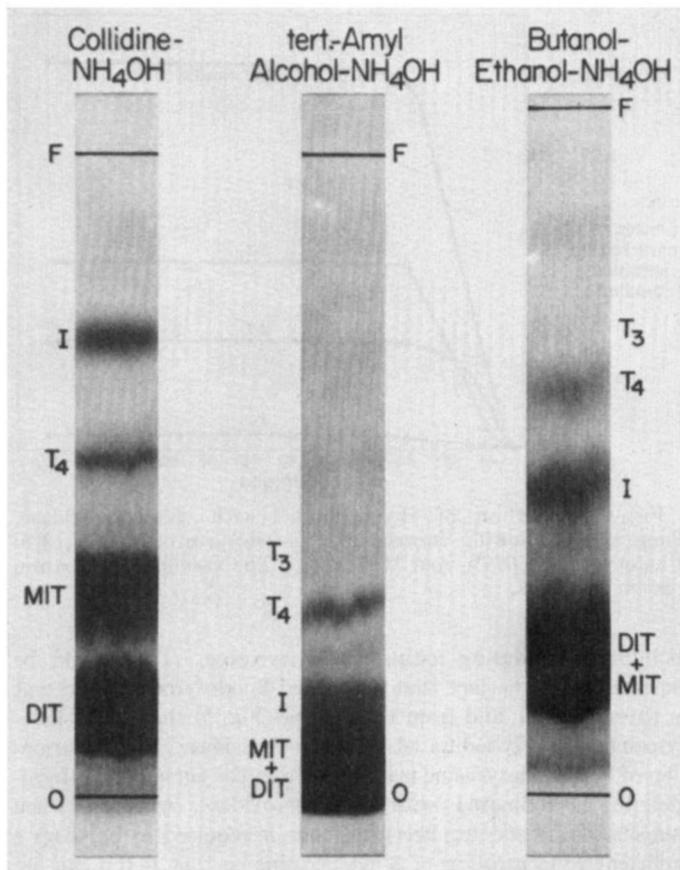


FIG. 7. Radioautograms of paper chromatograms showing  $^{131}\text{I}$  distribution in thyroglobulin iodinated with chloroperoxidase. Thyroglobulin was incubated for 60 min with chloroperoxidase in the standard incubation system, then digested with Pronase. The digest was extracted with butanol, concentrated, and chromatographed in three different solvents with added thyroxine ( $T_4$ ),  $3',3,5\text{-triiodothyronine}$  ( $T_3$ ),  $3,5\text{-diiodotyrosine}$  ( $\text{DIT}$ ), and  $3\text{-iodotyrosine}$  ( $\text{MIT}$ ), carriers. In all solvents, the band labeled thyroxine coincided exactly with the added thyroxine carrier.

although further studies are necessary to completely exclude this possibility. It should be noted also that the yield of  $^{131}\text{I}$ -thyroxine formed during thyroglobulin iodination was about twice that formed during tyrosine iodination, or by the action of chloroperoxidase on  $^{131}\text{I}-3,5\text{-diiodotyrosine}$ .

*Substitution of  $\text{H}_2\text{O}_2$  for Glucose-Glucose Oxidase*—The curves in Fig. 8 show the time course of the chloroperoxidase-catalyzed iodination of thyroglobulin when various concentrations of  $\text{H}_2\text{O}_2$  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  $^{131}\text{I}$  among  $3,5\text{-diiodotyrosine}$ ,  $3\text{-iodotyrosine}$ , and thyroxine was also similar to that observed in the standard system with glucose and glucose oxidase. These results indicate that it is not necessary to generate  $\text{H}_2\text{O}_2$  continuously to obtain marked iodination of thyroglobulin with chloroperoxidase. Neither thyroglobulin nor chloroperoxidase appeared to be rapidly degraded by  $\text{H}_2\text{O}_2$  under the conditions of these experiments. In the absence of chloroperoxidase, iodination of thyroglobulin was negligible, even at the highest concentration of  $\text{H}_2\text{O}_2$  used ( $1 \times 10^{-3} \text{ M}$ ).

The curves in Fig. 8, unlike those in Figs. 2 and 6, do not show

an initial lag period. This suggests that the lag in Fig. 2 is due to some delay in the formation of  $\text{H}_2\text{O}_2$  by the glucose-glucose oxidase under the present conditions of incubation. This view is also supported by experiments in which  $50 \mu\text{g}$  of glucose oxidase, instead of the usual  $12.5 \mu\text{g}$ , were added. In this case also there was no initial time lag.

*Effect of Various Inhibitors on Iodination of Thyroglobulin with Chloroperoxidase*—As shown in Table IV, the chloroperoxidase-catalyzed iodination of thyroglobulin was readily inhibited by a variety of compounds. Included among these were the antithyroid compounds, 1-methyl-2-mercaptoimidazole, thiouracil, and thiourea, and the naturally occurring reducing agents, cysteine, glutathione, and ascorbic acid. It has been suggested that the latter two compounds may 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 antithyroid 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  $5 \times 10^{-5} \text{ M}$ . Perchlorate, on the other hand, which like thiocyanate also blocks  $\text{I}^-$  transport, had very little inhibitory effect on iodination even when tested at  $1 \times 10^{-3} \text{ M}$ . The chelating agent EDTA and the —SH-blocking agent *N*-ethylmaleimide also had little or no inhibitory effect when tested at  $1 \times 10^{-3} \text{ M}$ . 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  $2 \times 10^{-4} \text{ M}$  of the latter was added, the iodination was completely blocked for 60 min. However, when only  $6.7 \times 10^{-5} \text{ M}$  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  $\text{H}_2\text{O}_2$ , and that  $\text{I}^-$  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 thiouracil is a competitive inhibitor in the chlorination of monochlorodimedon with chloroperoxidase, and that thiouracil 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 iodium 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  $\text{I}^-$  Concentration on Iodination of Thyroglobulin and Tyrosine*—Fig. 10 shows results obtained when the

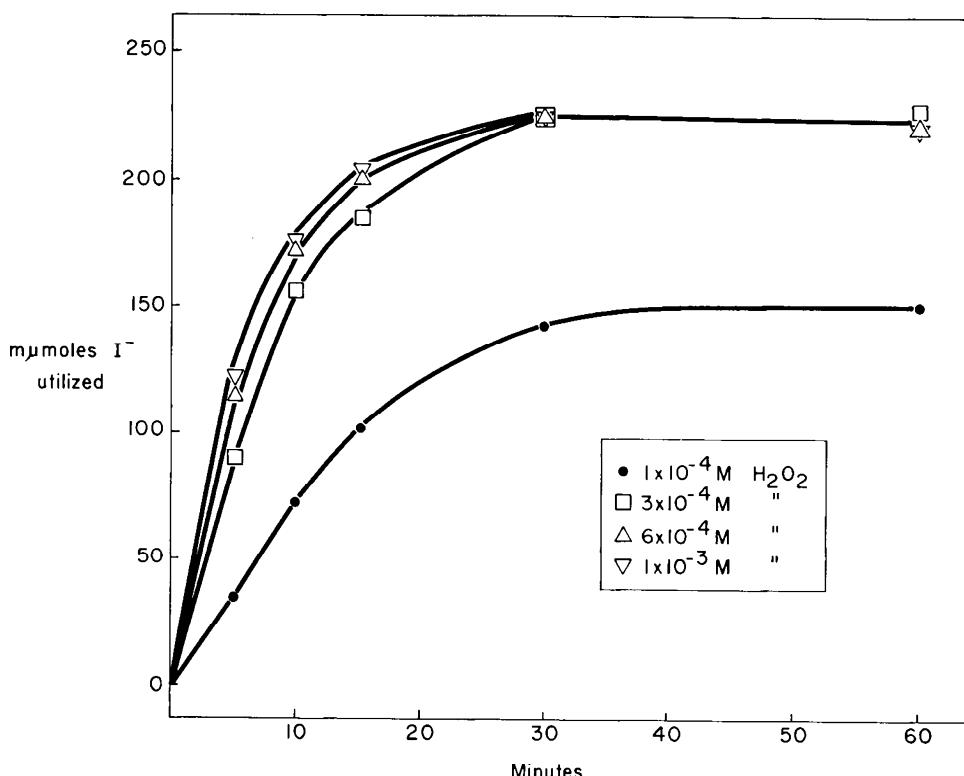


FIG. 8. Iodination of thyroglobulin with chloroperoxidase in the presence of various concentrations of added  $\text{H}_2\text{O}_2$ , instead of glucose-glucose oxidase. Incubation system contained 0.15  $\mu\text{g}$  of chloroperoxidase, 236 m $\mu\text{moles}$  of  $\text{I}^-$  (labeled with  $^{131}\text{I}$ ), 1 mg of thyroglobulin, 0.1 N chloride,  $\text{H}_2\text{O}_2$  as indicated, and 0.1 N acetate buffer, pH 4.9, in total volume of 3 ml.

$\text{I}^-$  concentration was varied from  $1 \times 10^{-5}$  to  $5 \times 10^{-3}$  M in the iodination of thyroglobulin with chloroperoxidase. Except for the  $\text{I}^-$  concentration, conditions were fixed as shown in Table IV. It is apparent that the rate of incorporation of  $\text{I}^-$  into thyroglobulin reached a maximum at approximately  $1 \times 10^{-4}$  M  $\text{I}^-$ , 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  $^{131}\text{I}$ -thyroxine were so low at the highest  $\text{I}^-$  concentrations that it was not possible to determine with any accuracy the effect of excess  $\text{I}^-$  on thyroxine formation.

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

The mechanism of the inhibitory effect of excess  $\text{I}^-$  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  $\text{I}^-$ , and (b) product inhibition, i.e. inhibition of chloroperoxidase by  $\text{I}_2$ , or some other form of oxidized  $\text{I}^-$ . In the latter connection it is of interest that  $\text{I}_2$  was detectable by starch test at those  $\text{I}^-$  concentrations at which iodination of thyroglobulin was inhibited.

Igo, Mahoney, and Mackler (11) reported an inhibitory effect of excess  $\text{I}^-$  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  $\text{I}^-$  could also be demonstrated when  $1 \times 10^{-3}$  M  $\text{H}_2\text{O}_2$  was substituted for the glucose-glucose oxidase system.

TABLE IV

*Effect of various inhibitors on iodination of thyroglobulin*

The test system contained 1.1 mg of thyroglobulin, 0.16  $\mu\text{g}$  of chloroperoxidase, 0.24  $\mu\text{mole}$  of  $\text{I}^-$  (labeled with  $^{131}\text{I}$ ), 3 mg of glucose, 12.5  $\mu\text{g}$  of glucose oxidase, 0.3 mmole of  $\text{Cl}^-$ , 0.1 N acetate buffer, pH 4.9, in a total volume of 3 ml. Incubation time was 15 min.

Inhibitor	Concentration	Inhibition
1-Methyl-2- mercaptoimidazole	$1 \times 10^{-4}$	99.8
	$5 \times 10^{-5}$	99.7
Thiouracil	$1 \times 10^{-4}$	99.9
Thiourea	$1 \times 10^{-4}$	99.9
	$5 \times 10^{-5}$	95.7
KSCN	$1 \times 10^{-4}$	98.6
	$5 \times 10^{-5}$	95.2
KCN	$1 \times 10^{-4}$	95.4
Reduced glutathione	$1 \times 10^{-4}$	98.5
	$5 \times 10^{-5}$	87
Ascorbic acid	$1 \times 10^{-4}$	99.7
	$5 \times 10^{-5}$	64
Cysteine	$1 \times 10^{-4}$	99.5
	$5 \times 10^{-5}$	96.6
NaClO <sub>4</sub>	$1 \times 10^{-3}$	17
EDTA	$1 \times 10^{-3}$	6
N-Ethylmaleimide	$1 \times 10^{-3}$	11

Inhibition by  $\text{I}^-$  of organic iodine formation in the thyroid is known to occur under certain conditions, e.g. in Graves' disease, in  $\text{I}^-$  myxedema, and transiently in normal thyroids. The results obtained here suggest that one of the mechanisms in-

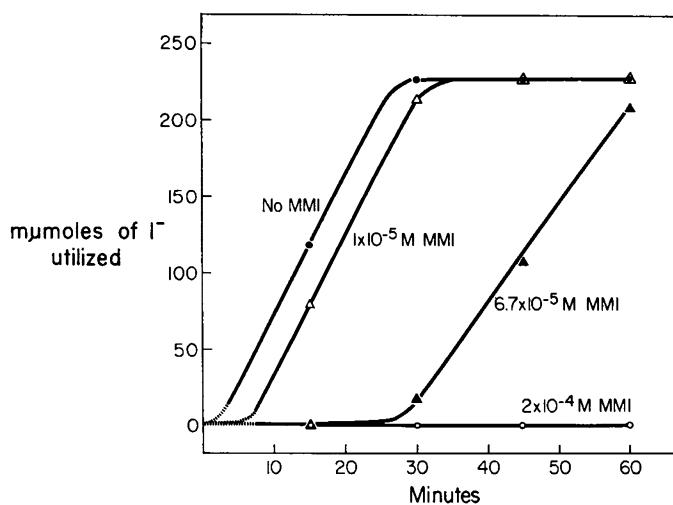


FIG. 9. Iodination of thyroglobulin with chloroperoxidase in the presence of various concentrations of methylmercaptoimidazole (MMI). Time curves showing escape from inhibition at lower concentrations of methylmercaptoimidazole. The standard incubation system was used with methyl mercaptoimidazole added as indicated.

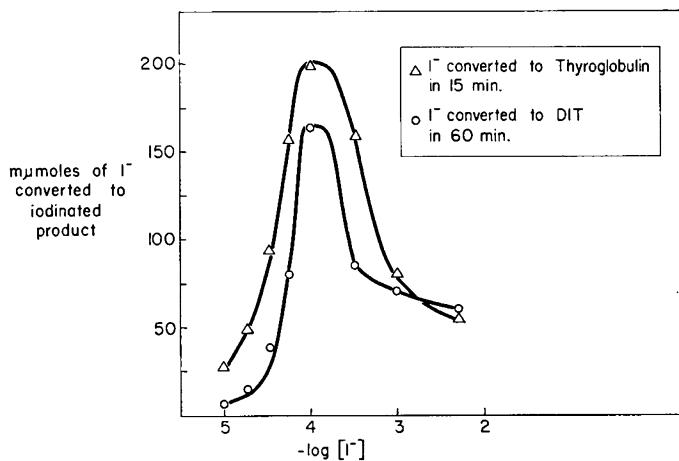


FIG. 10. Effect of varying iodide on rate of iodination of thyroglobulin with chloroperoxidase. The standard incubation system was used with iodide varied as indicated. DIT, 3,5-diiodotyrosine.

volved may be a direct inhibition of thyroid peroxidase by excess I<sup>-</sup>, analogous to the effect on chloroperoxidase. The fact that normal thyroids are only temporarily inhibited by excess I<sup>-</sup> may be explained by the hypothesis recently advanced by Braverman and Ingbar (30) that the thyroid I<sup>-</sup> transport mechanism normally adapts to high I<sup>-</sup> levels by excluding excess I<sup>-</sup> from the cells. However, if excess I<sup>-</sup> does manage to enter the thyroid follicle, then perhaps it exerts its antithyroid action by inhibiting thyroid peroxidase.

*Iodination of Proteins Other than Thyroglobulin with Chloroperoxidase*—As shown in Table V, bovine serum albumin, human serum albumin, and crystalline beef insulin were also very readily iodinated by the standard iodinating system employed for thyroglobulin. The various proteins were compared at equivalent concentrations of tyrosine,  $5 \times 10^{-5}$  M, based on the known percentage of tyrosine in each protein.  $^{131}\text{I}-\text{I}^-$  was also added at a concentration of  $5 \times 10^{-5}$  M.

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

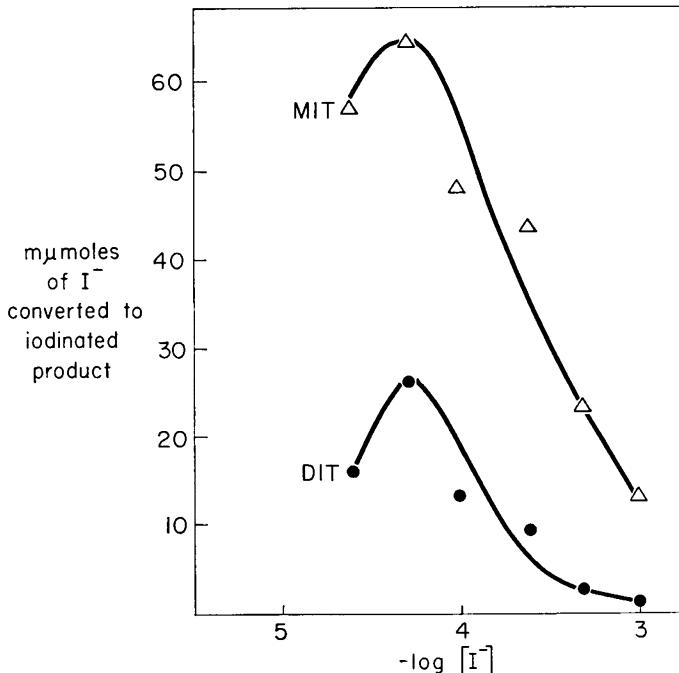


FIG. 11. Effect of varying iodide on rate of iodination of tyrosine with chloroperoxidase. The standard incubation system was used with iodide varied as indicated. DIT, 3,5-diiodotyrosine; MIT, 3-iodotyrosine. Incubation time was 15 min.

TABLE V  
*Iodination of various proteins with chloroperoxidase*

The incubation tube contained 3 mg of glucose,  $12.5 \mu\text{g}$  of glucose oxidase,  $0.1 \text{ M Cl}^-$ ,  $5 \times 10^{-5}$  M  $^{131}\text{I}$ -iodide,  $0.16 \mu\text{g}$  of chloroperoxidase, and protein acceptor in 3 ml of 0.1 M acetate buffer, pH 4.9. The calculated tyrosine concentration was  $5 \times 10^{-5}$  M in each case.

Protein	Amount	$^{131}\text{I}$ converted to $^{131}\text{I}$ -protein		Pronase digest after 60 min of iodination; $^{131}\text{I}$ in		
		10 min	60 min	3,5-Dio-diotyrosine	3-Iodo-tyrosine	Thyroxine
Thyroglobulin	0.77	52.0	95.0	49.1	20.3	4.7
Bovine serum albumin	0.55	81.8	92.9	43.6	24.7	2.2
Human serum albumin	0.55	64.2	88.1	46.3	18.0	2.2
Crystalline beef insulin	0.24	90.3	96.9	64.8	22.0	0.9

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 µg per incubation tube, compared to 0.16 µg 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  $^{131}\text{I}$  in Pronase digests of the 60 min-incubated samples is shown in Table VI.

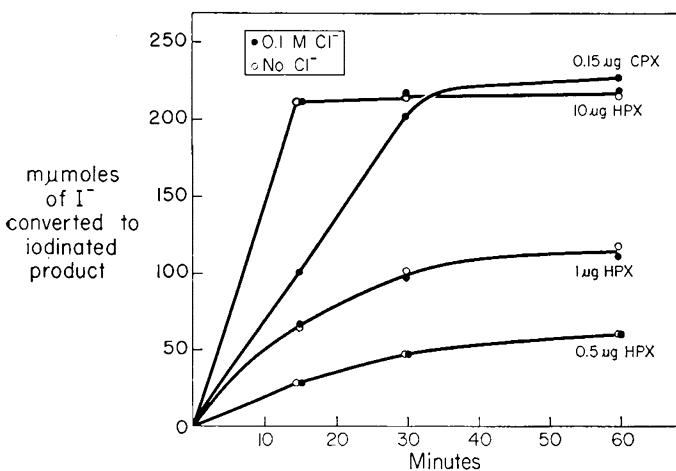


FIG. 12. Iodination of thyroglobulin with various amounts of horseradish peroxidase, compared with standard amount of chloroperoxidase. The standard incubation system was used, but with horseradish peroxidase (HPX) substituted for chloroperoxidase (CPX) in some samples.

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

Enzyme	Amount	Chloride concentration	$^{131}\text{I}$ in Pronase digest		
			3,5-Diiodotyrosine	3-Iodo-tyrosine	Thyroxine
Horseradish peroxidase	0.5	0	7.4	13.9	1.1
		0.1	6.2	14.5	1.0
	1.0	0	15.6	22.3	2.2
		0.1	16.1	21.2	2.0
Chloroperoxidase	10	0	33.0	24.1	3.6
		0.1	34.1	25.9	4.3
	0.16	0	10.3	17.6	1.6
		0.1	45.4	27.7	3.9

Formation of  $^{131}\text{I}$ -thyroxine occurred to about the same extent with 10 µg of horseradish peroxidase as with 0.16 µg of chloroperoxidase.

As shown in Fig. 12 and in Table VI,  $\text{Cl}^-$  had no effect on the horseradish peroxidase-catalyzed iodination of thyroglobulin. Stimulation by  $\text{Cl}^-$ , 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  $\text{H}_2\text{O}_2$  or with a glucose-glucose oxidase  $\text{H}_2\text{O}_2$ -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  $\text{I}^-$  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 iodonase" 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  $\text{I}^-$  and an iodonase 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 thyroids, contained 0.74% total iodine, and this increased to almost 2% upon iodination with chloroperoxidase in the presence of  $8 \times 10^{-5}$  M  $\text{I}^-$ . 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  $\text{I}^-$  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*. (e) 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 Hager<sup>2</sup> that  $Cl^-$  might stabilize the enzyme during the incubation and prevent its deterioration during exposure to excess  $H_2O_2$ . 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 mM  $Cl^-$ . 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  $H_2O_2$  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 ICI 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 ICI 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 determination of small amounts of iodine. The mechanism of this stimulation is also unknown, and whether or not it possesses features in common with the  $Cl^-$ -stimulated chloroperoxidase iodination remains to be determined.

The observation that appreciable thyroxine formation occurred during the iodination of thyroglobulin with chloroperoxidase suggests that a peroxidase enzyme may be involved in the coupling reaction as well as in the iodination reaction. Possible involvement of a peroxidase enzyme in the coupling of 2 molecules of 3,5-diiodotyrosine to form thyroxine was suggested in 1942 by Westerfeld and Lowe (41), based on studies of peroxidase oxidation of *p*-cresol. Evidence for such a mechanism was reported by Yip and Klebanoff (42), who obtained a very small yield of  $^{131}I$ -thyroxine when they incubated  $^{131}I$ -3,5-diiodotyrosine with myeloperoxidase or with a thyroid particulate preparation. Klebanoff *et al.* (10) had previously studied the iodination of tyrosine with purified myelo- and lactoperoxidase, and these workers suggested that a single peroxidase enzyme might be involved not only in the iodination step, but also in the generation of  $H_2O_2$  via the peroxidation of reduced pyridine nucleotides. While no attempt was made in the present study to use peroxidase

itself to generate  $H_2O_2$ , 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.

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