Thyroxine Degradation during Lipoxidase-catalyzed Peroxidation of Linoleic Acid*

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JAMES WYNN

From the Department of Medicine, University of Arkansas Medical Center, Little Rock, Arkansas 72201

SUMMARY

The possibility has been examined that thyroxine may be degraded by an intermediate free radical in the oxidation of linoleate catalyzed by lipoxidase. Such a reaction has been observed and the lipid radical with which thyroxine reacts is suggested to be enzyme attached $\text{RO}_2$ proposed by Tappel (in W. O. Lundberg (Editor), Lipoxidation and antioxidants, Vol. I, Interscience Publishers, New York, 1961, p. 325). A similar reaction has been inferred to occur during thyroxine degradation by peroxidizing microsomal lecithin. Although thyroxine serves as an antioxidant during autoxidation of lecithin, it was predicted that a similar reaction with enzyme-bound $\text{RO}_2$ might not influence oxidation rate or extent. The results bear out the prediction. The purpose of this and prior studies of thyroxine degradation in systems in vitro has been to investigate the mechanism of reaction of thyroxine with biological materials. In light of recent studies of the mechanism of deiodination of iodoaryl compounds, it is suggested that the lipid radical $\text{RO}_2$ might either initiate degradation by electron abstraction or react with a terminal product of thyroxine after deiodination. The enabling property of thyroxine making these reactions probable is its predicted capacity to take up, transiently hold, and yield electrons.

EXPERIMENTAL PROCEDURE

Materials

Thyroxine ($3',5',3''1'I$) was purchased from Abbott Laboratories. Thyroxine ($1-9-14C$) was synthesized as previously described (5). Linoleic acid and oleic acid were purchased from Applied Science Laboratories. Soybean lipoxidase was purchased from Worthington Enzymes.

Methods

Basic Lipoxidase-Linoleate Reaction—Linoleic acid, $1.44 \times 10^{-6}$ mole, in 0.3 ml of alcohol was added to 11.3 ml of 0.1 M sodium phosphate buffer at pH 7.4. Stock thyroxine solution, $1 \times 10^{-3}$ M in ethanol, was added in amounts from 0 to 0.40 ml. Ethanol was added to bring the final volume of ethanol to 0.7 ml. The reaction was begun by the addition of 0.5 ml of phosphate buffer containing varying amounts of lipoxidase. The thyroxine concentration varied between $8 \times 10^{-7}$ and $3.2 \times 10^{-6}$ M.

Oxygen Uptake—Oxygen uptake was measured polarographically in a 12.5-ml constant temperature chamber at 23° with a Teflon-covered platinum electrode.

Measurement of Iodide Released from $\beta$-Phenyl Ring of Thyroxine—The basic methodology used has been previously described (1).

Chromatographic and Electrophoretic Studies—Thyroxine, 3,5-diiodotyrosine, and inorganic iodide were chromatographed by techniques previously described (5). Electrophoresis of presumed 3,5-diiodotyrosine ($1-9-14C$) derived from thyroxine ($1-9-14C$) was carried out in 0.2 mol ammonium carbonate buffer on Whatman No. 3MM paper at 500 volts at room temperature.

Ultraviolet Light Absorption—Ultraviolet light absorption as a measure of conjugated diene production was measured as follows.

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Fig. 1. Oxygen consumption catalyzed by lipoxidase with and without the addition of thyroxine. A 12.5-nl reaction mixture contained 1.43 \mu M linoleate, 0.5 mg of lipoxidase, and 0.1 nmole of thyroxine if added. The arrow indicates the point of addition of lipoxidase. Reaction temperature was 23 °C. The reaction to which thyroxine was added is indicated by the dashed line. The chamber contained 3.3 \times 10^{-5} mole of oxygen at 100% saturation. Disappearance of 40% of contained oxygen represents an uptake by linoleate of 1.32 \times 10^{-5} mole of oxygen or 92% of the predicted value. There is no effect of thyroxine on the rate or extent of the oxidation.

![Graph showing oxygen consumption](image)

Fig. 2. Rate of oxygen uptake with different concentrations of linoleate with and without thyroxine. Reactions included 20 mg per liter of lipoxidase and 8 \times 10^{-4} M thyroxine if added. Maximal reaction rates were estimated during the first 90 sec of the reaction. Temperature, 23 °C. Reactions to which thyroxine was added are shown by the dashed line. There is no effect of thyroxine on the initial rates of the reaction.

![Graph showing rate of oxygen uptake](image)

Fig. 3. Rate of oxygen uptake with different concentrations of thyroxine in 2.3 \times 10^{-4} M linoleate. Lipoxidase concentration was 8 mg per liter. Temperature, 23 °C. There are no significant differences among the oxygen uptake rates plotted.

![Graph showing rate of oxygen uptake](image)

**Table I**

| Thyroxine concentration | Total oxygen uptake per reaction flask |
|-------------------------|---------------------------------------|
| X 10^{-6} M             | X 10^{-5} mole                        |
| 0                       | 1.30, 1.34, 1.30                     |
| 1.6                     | 1.27, 1.27                           |
| 3.2                     | 1.37, 1.37                           |

![Graph showing optical density](image)

Fig. 4. Time course of the development of optical density at 236 nm during linoleate oxidation. Reactions contained 5.85 \times 10^{-5} M linoleate, 40 mg of lipoxidase per liter, and 1.6 \times 10^{-4} M thyroxine when added. Temperature, 23 °C. Each reaction was read against an appropriate blank containing no linoleate, but with all other reactants. The lower dashed curve is that reaction to which thyroxine was added at the outset. The arrow indicating addition of thyroxine indicates an amount added to replace thyroxine degraded during the reaction. This amounted to 30% of the starting material. Since the blank for this reaction contained the same initial amount of thyroxine as the experimental reaction at the outset, optical density could not attain the value of the non-thyroxine control until degraded thyroxine in the experimental flask was replaced. The early lag in optical density of the thyroxine-supplemented reaction is due to rapid degradation of thyroxine during this period. When replaced later as shown, extinctions were equal in thyroxine- and non-thyroxine-treated reactions.

The basic reaction mixture was modified in that the concentration of linoleate was only half that indicated in the above description. The final concentration of phosphate buffer remained 0.1 M at pH 7.4. Light absorption was measured in a 1-cm cuvette at 236 nm. The signal was recorded continuously.

Continuous absorption spectra were assessed after allowing the basic reaction to run to completion. The reaction mixture was then diluted 1:5 with water, phosphate buffer, or phosphate buffer containing 5.6% alcohol. The diluent made no measurable difference in the absorption spectra. Continuous absorption spectra were determined with the Cary model 14 spectrophotometer.

**Measurement of Radioactive Isotopes**—Solutions containing \(^{131}\)I were assayed in an autogamma spectrometer at the 264 kev peak. Chromatograms on which \(^{131}\)I- and \(^{14}C\)-labeled substances had been separated were assessed as in a Geiger-Mueller continuous gas flow strip counter.
RESULTS

Influence of Thyroxine on Oxygen Uptake—As shown in Figs. 1, 2, and 3 and Table I, thyroxine neither influences the initial rate nor the extent of oxygen uptake during linoleate oxidation catalyzed by lipoxidase.

Influence of Thyroxine on Hydroperoxide Production—The development of absorbance at 236 nm is interpreted as an index of the quantity of conjugated diene monohydroperoxide produced during linoleate oxidation. This is based on observations described by Bergstrom (6) and Privett (7). As is shown in Figs. 4 and 5, thyroxine alters neither the rate of development of extinction at 236 nm nor the quality of the extinction spectrum between 230 and 360 nm. On this basis, it is presumed that systems with and without thyroxine produce the same conjugated diene structure in equal amounts.

Thyroxine Degradation during Linoleate Oxidation—Although there is no effect of thyroxine on the rate of oxygen uptake by linoleate or on the rate of hydroperoxide formation, thyroxine is deiodinated during the oxidation. In Fig. 6 is shown the direct relationship between thyroxine degraded and oxygen uptake. In Fig. 7 is shown a plot of $1/v$ for thyroxine degraded against $1/s$ for thyroxine added. The apparent $V_{\text{max}}$ for thyroxine with a $1.15 \times 10^{-4}$ molar concentration of linoleate and 8 mg per liter of enzyme is $0.665 \times 10^{-6}$ mole per liter per min. Comparing the measured oxygen uptake rate in Fig. 3 with the estimated thyroxine $V_{\text{max}}$ under the same conditions, there is approximately 1 mole of thyroxine degraded for each 11 moles of linoleate oxidized.

Chromatographic and Electrophoretic Studies—Chromatograms of the 131-labeled products formed during linoleate oxidation in the presence of thyroxine (3', 5'-131) reveal that inorganic iodide and thyroxine are the only labeled substances present following completion of the reaction. Chromatographic and electrophoretic studies of reactions containing thyroxine (1-9-13C) show only thyroxine and 3,5-diiodotyrosine following the reaction.

Control Studies—Thyroxine is degraded only during the active peroxidation of linoleate by lipoxidase. Linoleate, lipoxidase, oxygen, and hydroperoxide end products of linoleate are incapable of effecting such degradation by themselves. Linoleate and lipoxidase incubated anaerobically with thyroxine cause no

**Fig. 6.** Uptake of oxygen and degradation of thyroxine. Oxygen uptake is shown by the dashed line. Thyroxine degraded is shown by the solid upper line. The amount of thyroxine degraded parallels the amount of oxygen uptake. Reactants included $3.3 \times 10^{-6} \text{M}$ linoleate, $1 \times 10^{-4} \text{M}$ thyroxine, and 8 mg per liter of lipoxidase.
degradation. Oleate (which is not oxidized by lipoxidase) incubated with lipoxidase and thyroxine results in neither oxygen uptake nor thyroxine degradation.

**DISCUSSION**

Although these studies indicate that thyroxine is degraded during lipoxidase-catalyzed oxidation of linoleate, no physiological implications are inferred since thyroxine and lipoxidase do not naturally occur together. The purpose of this and prior studies of thyroxine degradation has been to define the reactivity of thyroxine with biological materials. For this purpose the plant enzyme has certain advantages. Although the autooxidation studies suggested that thyroxine reacts with the lipid radical \( RO_2 \), the radical has not been isolated and the thesis is difficult to test. Because the lipoxidase-linoleate reaction generates the same radicals by a different mechanism, predictions can be made that, if supported, would circumstantially strengthen the thesis that thyroxine reacts with \( RO_2 \). Evidence that thyroxine participates during the enzyme-catalyzed reaction is shown by its degradation. The oxidation rate is not measurably altered. Thus, these results are consistent with a mechanistic expectation of what might happen were thyroxine to react with enzyme-bound \( RO_2 \).

Several questions may be raised in regard to alternative explanations. Does thyroxine react with any of the initial or terminal products? May it react with a transient non-enzyme-bound intermediate? May it react with an intermediate other than \( RO_2 \) and, finally, are the reactions during the enzyme-catalyzed oxidation similar to those that occur during chain-propagated autoxidation?

Because the initial and terminal enzyme products will not effect thyroxine degradation and because degradation takes place only during the course of oxidation, the view is supported that intermediate lipid products are responsible for the reactions. The apparent conformity of the degradation reaction to enzyme kinetics implies a reaction of thyroxine with an enzyme-lipid complex. This latter speculation depends upon the assumption that thyroxine does not react with a small amount of \( RO_2 \) or related radical that may have been first released from the enzyme. Lipoxidase ordinarily catalyzes the production of the cis,trans-13-monohydroperoxide-conjugated diene from linoleate (6–9). If any significant quantity of \( RO_2 \) were released from the enzyme, nonenzymatic autoxidation at \( 23^\circ \) would proceed rapidly forming the cis,trans- and trans,trans-9- and 13-monohydroperoxide-conjugated dienes (9). The influence of thyroxine would be to terminate the chain reaction and slow the rate of oxidation if it were to react in a fashion similar to that in which it reacts with autoxidizing lecithin. Small amounts of thyroxine would cause large decreases in such nonenzymatic oxygen uptake. The fact that oxygen uptake is not influenced at all during this enzymatic reaction supports the view that thyroxine reacts with an enzyme-bound radical to cause its degradation and that there is essentially no parallel, nonenzymatic autoxidation of lipid.

If the enzyme-bound intermediates of the lipoxidase-linoleate reaction may be accurately depicted as Tappel has inferred (4), it is unlikely that thyroxine reacts with any radical other than \( RO_2 \). The proposed enzyme-bound radical intermediates are \( R^{'}, H^{'}, \) and \( RO_2 \). If thyroxine were to react with \( R^{'}, \) oxygen uptake would be inhibited and reaction rate would be slowed. If it were to react with \( H^{'}, \) the reaction rate might be slowed or the end product altered. Neither situation occurs. Unless the lipoxidase-catalyzed reaction involves more complex intermediate products than Tappel proposed, it seems most likely that thyroxine reacts with \( RO_2 \).

No matter with which of the radical intermediates thyroxine may react initially, it seems probable that the mechanism of degradation is similar in the microsomal, autoxidizing lecithin, and lipoxidase-linoleate systems. The identity of the products of the \( \beta \)-phenyl ring iodine and \( \alpha \)-phenyl ring-alanine side chain following thyroxine degradation by all three systems supports this view (2, 5). Although the reaction mechanism is unknown, several insights have been developed. Degradation of thyroxine depends in part upon its predicted free radical stability (10) and Borg has demonstrated the occurrence of a relatively stable thyroxine-free radical signal (11). Complete removal of \( \beta \)-phenyl ring iodine atoms without occurrence of partially iodinated intermediates and net reduction of the deiodinated positions has been shown (2, 5, 12). Heretofore, there has been no model reaction from which a rational scheme could be devised to account for these observations. Two recent reports have been helpful in this regard. Anbar, studying the effect of hydrated electrons on iodobenzene, showed that this compound reacts with such electrons by ejecting inorganic iodide (13). Bunnett studied the deiodination of iodobenzene in alkaline alcoholic solution containing a free radical capable of electron abstraction (14). He demonstrated that iodine is ejected from the aryl structure as iodide and that the position vacated is reduced. He devised a stoichiometrically satisfactory theoretical reaction mechanism assuming that the initial reaction involved acquisition by iodobenzene of a solvated electron or an electron transferred from a negatively charged free radical in solution. The reaction was only observed with iodine-substituted benzene. Chlorine and bromine substitution could not be similarly removed.

Both of these studies suggest that the degradation reactions involving thyroxine may be initiated by either acquisition of an electron or by molecular rearrangement following one electron abstraction. In either case, a negatively charged free radical would be formed followed by ejection of inorganic iodide, further rearrangement, formation of a second negatively charged radical, and ejection of the second iodide ion. The predicted reaction with lipid radical \( RO_2 \) may then be an initial reaction causing electron abstraction or a terminal reaction involving the deiodinated, electron-depleted thyroxine remnant. In either instance, the reactivity of biological importance may relate to an unusual capacity of thyroxine to take up, transiently hold, and then yield unpaired electrons rather than a specific affinity for reaction with particular biological free radicals.

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