1. Keston, A. S. and Brandt, R. *Anal. Biochem.* **6** (1963) 461.
2. Jørgensen, B. B. and Jørgensen, O. B. *Acta Chem. Scand.* **17** (1963) 1765.
3. Dahlqvist, A. *Biochem. J.* **80** (1961) 547.

Received May 17, 1966.

The Peroxidase-Catalyzed Oxidation of Thyroxine

FRED BJÖRKSTÉN

Department of Biochemistry, University of Helsinki, Helsinki 17, Finland

The oxidation of thyroxine (T_4) and related *o*-iodophenols by H_2O_2 is catalyzed by horseradish peroxidase,[1-4] myeloperoxidase,[1,5] and peroxidase-like activity in the livers of various animals.[6] When T_4 is oxidized, I^- and diiodotyrosine have consistently been identified among the products [1,4], but little else is known about this reaction. A further study was therefore undertaken.

Experimental. A commercial (Worthington) crystalline horseradish peroxidase was used. Reactions were carried out at 25° in 0.05 M Tris (pH 9) buffer, except where otherwise stated. The choice of the relatively high pH was due to the extreme insolubility of T_4 at neutral pH. Isosbestic points were observed with the reacting system 10^{-4} M T_4, 1.33×10^{-4} M H_2O_2, and 2×10^{-8} M peroxidase in the sample cell and buffer as the only solute in the reference cell. Consecutive spectra were recorded in the range 260–390 mμ at 2 min intervals for a period of 14 min. In kinetic and titration experiments the oxidation of T_4 and related compounds was followed spectrophotometrically at the wavelengths given in Table 1. The effect of the hydrogen ion concentration on the rate of T_4 oxidation was studied by making rate measurements at the pH values 9.00, 9.49, 10.00, and 10.45 in 0.05 M glycine buffer with 10^{-4} M T_4, 10^{-4} M H_2O_2 and suitable peroxidase concentrations in the reacting solution. When the reaction products were to be analyzed, the oxidation system used contained 10^{-4} M T_4, 10^{-4} M H_2O_2, and 5×10^{-8} M peroxidase. In some cases labeled [3′,5′-^{131}I] thyroxine was used. The reaction was stopped after 10 min by adding catalase to a final concentration of 50 μg/ml. Labeled I^- was separated from other products and remaining T_4 by paper electrophoresis in 0.05 M Tris (pH 9) buffer. Labeled T_4 and diiodotyrosine were separated from other products by electrophoresis in 4 M acetic acid. Formation of non-labeled diiodotyrosine from labeled T_4 was confirmed with previously described [7] chromatography methods. Labeled products on paper strips were quantitated with scanning equipment. A chemical method [8] was also used for the determination of I^- in the reaction mixture. In a spectrophotometric titration experiment graded doses of H_2O_2 were added to a system containing 10^{-4} M T_4 and 10^{-7} M peroxidase.

Table 1. Spectrophotometrically determined rate constants (k) for the oxidation of T_4 and related compounds. The rate constant is defined by eqn. (1). Measurements were performed at the wavelengths (λ) given. The proportionalities between absorbance change and consumption of H_2O_2 ($\Delta A/\Delta[H_2O_2]$) were established by measuring the absorbance changes caused by the addition of graded doses of H_2O_2 to systems containing oxidizable substrate and peroxidase. The proportionalities were used in the calculation of rate constants.

Oxidizable substrate	λ (mμ)	$\Delta A/\Delta[H_2O_2]$ (M^{-1})	k ($M^{-1} \times min^{-1}$)
L-Thyronine	310	−3900	9×10^7
L-Thyroxine (3,5,3′,5′-tetraiodo-L-thyronine)	327	3700	9×10^6
3,5-Diiodo-L-thyronine	320	−3900	4×10^6
3,5,3′-Triiodo-L-thyronine	350	−3100	3×10^6
L-Tyrosine	300	−2400	1×10^5
3,5-Diiodo-L-tyrosine	313	2400	9×10^4
3-Iodo-L-tyrosine	330	−2200	8×10^4

Results and discussion. When consecutive spectra were recorded during the oxidation of T_4, well defined isosbestic points at 308 and 352 mμ were observed. This shows that under the conditions of the experiment the primary reaction(s) were not followed by spectrophotometrically detectable secondary reactions. Some other data, however, indicate that the primary reactions were followed by at least one much slower peroxidase-catalyzed reaction. Thus the absorbance at 327 mμ continued to decrease slowly even after the primary reactions should have reached completion. Oxidation by the H_2O_2-peroxidase system of diiodotyrosine formed in the primary reactions at least partially accounts for the secondary reactions.

The rate of disappearance of T_4 and related compounds (and also H_2O_2) was found to be of first order with respect to T_4 and peroxidase and of zero order with respect to H_2O_2 within available concentration ranges. The rate equation can be written as

$$-d[H_2O_2]/dt = k[\text{oxidizable substrate}][\text{peroxidase}] \quad (1)$$

It is seen from Table 1 that k is 30—1000 times higher for thyronine and the iodothyronines than for tyrosine and the iodotyrosines, and that the uniodinated compounds are somewhat more readily attacked than their closely related iodo derivatives. Within the pH range 9.00—10.45 the rate of T_4 oxidation was found to be of first order with respect to hydrogen ion as well. Therefore the value of k for T_4 could be up to 100 times larger at pH 7 than the value given for pH 9. It should be noticed that the solubility of T_4 falls off rapidly below pH 9 and is considerably less than 10^{-5} M at pH 7.

Radioactive I^- was separated from the other products with a rapid electrophoresis system (40 min run) immediately after T_4 oxidation had been stopped by addition of catalase. The electrophoresis buffer used was identical with that in the reaction mixture. The I^- spot was well defined, and there was no indication of further liberation of I^- during electrophoresis. It therefore seems that I^- was liberated in the course of the primary reactions observed spectrophotometrically. Assay of I^- with a rapid chemical method showed that 1.0 mole of I^- was liberated per mole of T_4 oxidized. Work with labeled T_4 showed that 0.7 mole of I^- came from the 3′- and 5′-positions, leaving 0.3 mole to be liberated from the 3- and 5-positions. Diiodotyrosine could also be identified as a major product. It contained no ^{131}I-label and was obviously formed through splitting of the diphenylether bridge of T_4.

Spectrophotometric titrations to determine the molar ratio T_4/H_2O_2 in which the reactants are consumed in the primary reactions gave values in the range 1.3—1.7. Difficulties caused by secondary reactions prevented the determination of an entirely accurate value.

In peroxidase-catalyzed reactions one electron at a time is removed from the oxidizable substrate, and the initial products are generally free radicals.[9] The finding of T_4/H_2O_2 ratios of about 1.5 also indicates an initial reaction of the type

$$2\,T_4 + H_2O_2 \xrightarrow{\text{Peroxidase}} 2\,T_4\cdot + 2\,H_2O \quad (2)$$

where $T_4\cdot$ stands for a free T_4 radical. The radicals initially formed apparently undergo rapid decay with formation of I^-, diiodotyrosine and other products. It was found that per mole of T_4 oxidized, 0.7 mole of I^- is liberated from the 3′- and 5′-positions and 0.3 mole from the 3- and 5-positions. Diiodotyrosine is formed through splitting of the diphenylether bridge in T_4.

1. Björkstén, F., Gräsbeck, R., Karlsson, R. and Lamberg, B.-A. *Ann. Med. Exptl. Biol. Fenniae, (Helsinki)* **41** (1963) 156.
2. Klebanoff, S. J. *Biochim. Biophys. Acta* **44** (1960) 501.
3. Ljunggren, J.-G. *Acta Chem. Scand.* **17** (1963) 567.
4. Mayrargue-Kodja, A., Bouchilloux, S. and Lissitzky, S. *Bull. Soc. Chim. Biol.* **40** (1958) 815.
5. Ljunggren, J.-G. *Acta Chem. Scand.* **11** (1957) 1072.
6. Galton, V. A., Ingbar, S. H. and von der Heyde, S. *Endocrinology* **76** (1965) 479.
7. Björkstén, F., Gräsbeck, R. and Lamberg, B.-A. *Acta Chem. Scand.* **15** (1961) 1165.
8. Jungreis, E. and Gedalia, I. *Mikrochim. Acta* **1960** 145.
9. Yamazaki, I., Mason, H. S. and Piette, L. *J. Biol. Chem.* **235** (1960) 2444.

Received May 26, 1966.