Model Reactions for the Biosynthesis of Thyroxine. VII. Free Radicals Generated from 4-Hydroxy-3,5-diiodophenylpyruvic Acid and Their Possible Role in the Synthesis of Thyroxine[1]

TERUO MATSUURA,[2] HIDEO KON, AND H. J. CAHNMANN

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received May 6, 1964

Free radicals generated in the presence of oxygen from aqueous solutions of 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) or structurally related substances have been studied by electron spin resonance (e.s.r.) spectrometry. The formation and the stability of these radicals are dependent on pH, concentration, or other conditions. One of the observed radicals has been tentatively identified as the phenoxy radical of DIHPPA. It reacts with 3,5-diiodotyrosine (DIT) and may therefore be an intermediate in the synthesis of thyroxine from DIHPPA and DIT in the presence of oxygen.

More than 20 years ago a free-radical mechanism was postulated[3,4] for the coupling of two molecules of DIT[5] in the presence of oxygen to form thyroxine.[6] The hypothesis that free radicals are involved in the synthesis of thyroxine was supported by a series of model reactions.[7-9] The coupling of two molecules of DIT or its analogs[6,10] yields thyroxine in poor yield and only very slowly. However, thyroxine is formed rapidly and in good yield when 1 mole of DIT is permitted to react in the presence of oxygen with 1 mole of DIHPPA,[5,11] a compound closely related to DIT. In view of the ease with which thyroxine is formed in this reaction, it has been suggested[12] that DIHPPA may play a role in the biosynthesis of thyroxine.

The present investigation was carried out in order to determine whether some evidence could be found for the participation of free radicals in this reaction. Oxygen was bubbled through a solution of DIHPPA under various conditions (pH, concentration, etc.). The formation of free radicals was observed by circulating the reaction mixture through an e.s.r. cell. Some of the characteristics of the free radicals formed, including their behavior upon addition of DIT, were investigated. Solutions of substances related to DIHPPA were also treated with oxygen. In those cases in which free radicals were observed, their characteristics were compared with those of the free radicals obtained with DIHPPA.

Results and Discussion

When oxygen was bubbled through a solution of DIHPPA, one or several of four types of signals were observed (I to IV, Fig. 1), depending on the pH, concentration, or reaction time. Most frequently the shapes of the signals were intermediary between I and II. These signals were caused mainly by superimposition of signals I and II. Their shapes varied, depending on which one of the two radicals was preponderant in the mixture. In dilute solution (~1 m*M*) and within a pH range of about 6.5–9, an only slightly deformed signal I was observed. With increasing reaction time, the signal became more and more deformed, finally assuming shape II. At a higher pH (~8–9), this deformation and conversion to signal II took place more rapidly than at a lower pH (~6.5–8). When the pH was lowered below 6.5, the signal II disappeared, but reappeared again upon raising the pH. Above pH 11 the signal disappeared irreversibly. Signals III and IV were only observed at a very high pH (above 12) and in more concentrated solutions (0.03 and 0.05 *M*). They are due to alkaline decomposition products of DIHPPA. The fact that the *g*-value of signal III is considerably lower than those of signals I and II indicates that at least part of the iodine in DIHPPA has been lost.[13]

The radical giving rise to signal II is not the phenoxy radical of 4-hydroxy-3,5-diiodobenzaldehyde or of 4-hydroxy-3,5-diiodobenzoic acid. When this aldehyde or acid was treated with oxygen in the same manner as DIHPPA, no signals appeared between pH 6.5 and 11. The nature of the free radical II remains to be elucidated. It is possible that it is a polymer radical. In any event, the *g*-value of 2.0064 suggests that radical II still contains iodine.[13]

When in a solution containing radical II, oxygen was replaced with nitrogen, the signal decayed rapidly. In contrast, when nitrogen was bubbled at about pH 8 through a solution that gave rise to a slightly deformed signal I, the signal became sharper and lost its deformation. The pure signal I thus formed did not lose intensity after 40 min. of nitrogen bubbling. The sharpening of the signal seems to be due partly to the elimination of the signal-broadening effect of oxygen and partly to the rapid decay of the small amount of radical II that was originally present. The signal I generated from a solution of DIHPPA at about pH 8 by the successive bubbling of oxygen and nitrogen became gradually weaker when the pH was lowered and stronger when it was raised. Below about pH 6.5 it almost disappeared and above about pH 9 it became deformed.

(1) Paper VI: A. Nishinaga and T. Matsuura, *J. Org. Chem.*, **29**, 1812 (1964).

(2) Guest worker from Kyoto University, Japan; recipient of U. S. Public Health Service Grant AM 07955 from the National Institute of Arthritis and Metabolic Diseases.

(3) T. B. Johnson and L. B. Tewkesbury, Jr., *Proc. Natl. Acad. Sci., U. S.*, **28**, 73 (1942).

(4) C. R. Harington, *J. Chem. Soc.*, 193 (1944).

(5) Abbreviations: DIT, 3,5-diiodotyrosine; DIHPPA, 4-hydroxy-3,5-diiodophenylpyruvic acid.

(6) P. von Mutzenbecher, *Z. Physiol. Chem.*, **261**, 253 (1939).

(7) T. Matsuura and H. J. Cahnmann, *J. Am. Chem. Soc.*, **82**, 2055 (1960).

(8) T. Matsuura and A. Nishinaga, *J. Org. Chem.*, **27**, 3072 (1962).

(9) T. Matsuura, A. Nishinaga, and H. J. Cahnmann, *ibid.*, **27**, 3620 (1962).

(10) T. Matsuura and H. J. Cahnmann, *J. Am. Chem. Soc.*, **81**, 871 (1959).

(11) R. I. Meltzer and R. J. Stanaback, *J. Org. Chem.*, **26**, 1977 (1961).

(12) T. Shiba and H. J. Cahnmann, *Biochim. Biophys. Acta*, **58**, 609 (1962).

(13) M. S. Blois, "Free Radicals in Biological Systems," Academic Press New York, N. Y., 1961, p. 117.

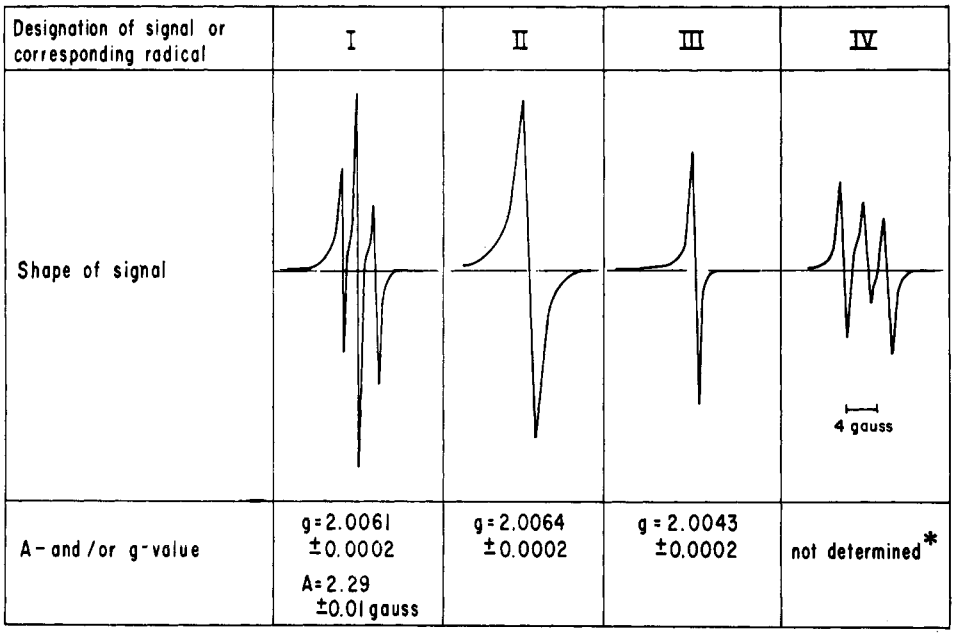
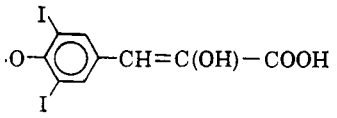


Fig. 1.—E.s.r. signals observed in the reaction of DIHPPA with oxygen; * free radical, very short-lived.

Radical I most likely is the phenoxy radical of DIHPPA of which a number of canonical resonance forms and tautomeric structures can be written. One of these forms is shown.



Signal I is a triplet with an intensity ratio, approximately 1:2:1, which is characteristic of an unpaired electron coupling with two equivalent protons. The hyperfine splitting constant A of 2.29 gauss indicates that these protons are likely to be in *meta* position to the phenolic group.[14] The high g-value of 2.0061, which is virtually identical with that of the 2,6-diiodohydroquinone radical (see below), suggests[13] that two atoms of iodine are present in the molecule. When DIT was added to a solution containing the free radical I, the e.s.r. signal disappeared. Signal II was not affected by the addition of DIT. When DIT was added to a solution that gave rise to a mixed signal, intermediary between signals I and II, the component I of the mixed signal disappeared while the component II remained. The reaction of DIT with the free radical I was carried out at pH 7.8–8.0, which is close to the pH range recommended by Meltzer and Stanaback[11] for the synthesis of thyroxine from DIHPPA and DIT.

The fact that DIHPPA is much more easily oxidized by oxygen to a free radical than DIT and that this free radical is relatively stable may explain at least in part why thyroxine is formed so much more readily by the coupling of one molecule of DIHPPA with one molecule of DIT[11] than by the coupling of two molecules of DIT.[6] Other factors which may contribute to the difference in the yields and reaction rates in these two types of coupling reactions cannot be correlated with the formation or stability of free radicals and will therefore not be discussed here.

It has been suggested that, in the biosynthesis of thyroxine, DIT may couple with 2,6-diiodohydroquinone.[15,16] Therefore, 2,6-diiodohydroquinone was treated with oxygen in the same way as DIHPPA and the behavior of the free radical formed was studied. The shape of the observed signal was almost indistinguishable from that of signal I. Its g-value and hyperfine splitting constant A are 2.0061 ± 0.0002 and 2.30 ± 0.07 gauss, respectively. However, the rate of decay of this radical is considerably faster than rate of decay of this radical is considerably faster than that of radical I. When DIT was added to a solution of the free radical generated from 2,6-diiodohydroquinone, the signal was not affected in contrast to the behavior of signal I. When 4-hydroxy- or 4-hydroxy-3-iodophenylpyruvic acid was treated with oxygen at pH 7.9, no signal was observed. This is in agreement with the finding that these two keto acids cannot effectively replace DIHPPA in the coupling reaction with DIT.[11,17] When on the other hand 3,5-dibromo-4-hydroxyphenylpyruvic acid (which does react with DIT to form 3,5-diiodo-3′,5′-dibromothyronine[18]) was treated similarly, a triplet was observed (g = 2.0058; A = 2.25 gauss). This signal disappeared upon addition of DIT.

In an experiment that was reproduced several times, it was found that signal I was weaker and more deformed when either nitrogen or argon was bubbled through the test solution for some time and was then replaced with oxygen. In view of this unexpected finding, two coupling reactions were carried out with DIHPPA and labeled DIT (I^{125}). In one run, nitrogen was bubbled through the reaction mixture for 15 min. before oxygen was admitted. In the other run, the pretreatment with nitrogen was omitted. In the

(14) P. L. Kolker, T. J. Stone, and W. A. Waters, 6th Internationa Symposium on Free Radicals, University of Cambridge, Cambridge, England, July, 1963, p. Z1.

(15) S. Lissitzky and W. Krotemberg, *Compt. rend.*, **242**, 3002 (1956).

(16) J. G. Ljunggren, *Acta Chem. Scand.*, **15**, 1772 (1961).

(17) T. Shiba and H. J. Cahnmann, *J. Org. Chem.*, **29**, 3063 (1964).

(18) T. Shiba, H. J. Cahnmann, T. Matsuura, A. Nishinaga, and H. Sakamoto, *ibid*; **29**, 3061 (1964).

latter case, the yield of labeled thyroxine was greater than in the former. This again seems to establish a relationship between the formation of radical I and the coupling of DIHPPA with DIT.

Summary

It can be concluded from the data presented in this paper that a striking similarity exists between the conditions under which radical I is generated from DIHPPA and reacts with DIT, and those under which thyroxine is formed in the coupling reaction between DIHPPA and DIT. Although these data do not provide an unequivocal proof, it appears likely that radical I is the phenoxy radical of DIHPPA and that it is an intermediate in the synthesis of thyroxine from DIHPPA, DIT, and oxygen.

Experimental

The electron spin resonance was observed with Varian Associates V-4500 spectrometer at modulation frequency of 100 Kc. The magnetic field strength was calibrated by a proton resonance probe coupled with an electronic counter. The sample cell was a flat quartz cell customarily used for aqueous solutions.

Oxygen, and in some instances nitrogen or argon (see Results and Discussion), was bubbled through the reaction mixture in a flask. The mixture was circulated at room temperature by means of a finger pump (Sigma motor, Model T8, Middleport, N. J.) through the sample cell and back to the flask at a rate of about 70 ml./min.

The following is an example of the preparation of a typical reaction mixture. Oxygen was bubbled through 75 ml. of 0.2 *M* sodium phosphate buffer, pH 7.8; then 8 ml. of a solution of DIHPPA in 1-butanol was added. The final concentration of DIHPPA was 1 m*M*.

Other reaction mixtures were prepared in a similar manner with appropriate changes of pH, concentration, etc. Borate or bicarbonate buffer (0.2 *M*) was used in a higher pH range. Ethanol was used in some cases instead of butanol.

DIHPPA[11] was a commercial product.[19] 4-Hydroxy-3,5-diiodobenzaldehyde[10] was recrystallized from aqueous ethanol. 4-Hydroxy-3-iodophenylpyruvic acid was prepared from *p*-hydroxybenzaldehyde.[17] DIT, 4-hydroxyphenylpyruvic acid, 4-hydroxy-3,5-diiodobenzoic acid, and 2,6-diiodohydroquinone were commercial products. A stock solution of DIT-I^{125} was prepared by exchange-labeling of DIT with carrier-free NaI^{125} in the presence of iodine at about pH 3. It was purified by paper chromatography in 1-butanol–ethanol–0.5 *N* ammonia (5:1:2), followed by elution with a 5% solution of concentrated ammonia in methanol, evaporation under reduced pressure, and dissolution of the residue in 0.01 *N* NaOH. Oxygen was 99.96% and argon 99.99% pure. Nitrogen (Seaford grade) contained not more than 0.002% oxygen.

Reaction of DIHPPA with DIT-I^{125}.—To a 5 m*M* solution (pH 8.0) of DIT in 0.05 *M* sodium phosphate buffer was added 20 μl. of the stock solution of DIT-I^{125} (about 10 μc.).

To two 200-μl. aliquots of the thus prepared solution 20 μl. of a freshly prepared 10 m*M* solution of DIHPPA in ethanol was added. Nitrogen was bubbled through one of the two solutions (solution I) for 15 min. Then the solution was shaken for 40 min. under slight oxygen pressure (2 p.s.i.). The other solution (solution II) was shaken under oxygen in the same manner, but the bubbling of nitrogen was omitted. Aliquots of solutions I and II were then chromatographed on paper in 1-butanol–ethanol–0.5 *N* ammonia (5:1:2), together with DIT, thyroxine, and iodide carriers. Further separation of the radioactive components of the two reaction mixtures was achieved by high voltage electrophoresis in 0.05 *M* ammonium carbonate solution. The thyroxine areas, visualized by autoradiography or by short-wave ultraviolet light, were cut out and counted in a scintillation well counter, equipped with a pulse-height analyzer.

Since the molar ratio of DIHPPA–DIT used in the two reactions was 1:5, yields of thyroxine were based on DIHPPA. They were corrected for a small amount of radioactivity found in the thyroxine area in a blank reaction without DIHPPA. The yields of thyroxine were 10 (solution I) and 17% (solution II).

(19) Osaka Laboratory of Synthetic Organic Chemicals, Nishinomiya, Japan.