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Model Reactions for the Biosynthesis of Thyroxine. XII. The Nature of a Thyroxine Precursor Formed in the Synthesis of Thyroxine from Diiodotyrosine and Its Keto Acid Analog*

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ABSTRACT: The oxidative coupling of 4-hydroxy-3,5-diiodophenylpyruvic acid and 3,5-diiodotyrosine is a nonenzymic model for the biosynthesis of thyroxine. In the pH range of 7.2–7.6 the reaction proceeds with great ease, even near 0°. Thyroxine is formed in yields of up to 0.4 mole/mole of keto acid. The reaction takes place in two distinct phases, an aerobic and an anaerobic one. In the first phase the keto acid, in its enol form, is oxidized to a thyroxine precursor which then reacts with diiodotyrosine to form thyroxine. For this second phase oxygen is not required. In the solid state, the thyroxine precursor is extremely unstable. Its structure was, however, determined through its chemical and spectral properties in solution. It is a hydroperoxide which differs from 4-hydroxy-3,5-diiodophenylpyruvic acid by having a hydroperoxy group, instead of a hydrogen, attached to the carbon atom which is adjacent to the aromatic ring.

Although it has been known for a long time that thyroxine (T_4)[1] is formed in the thyroid from its precursor diiodotyrosine (DIT), nearly nothing is known about the mechanism of this conversion. Various nonenzymic model reactions have been investigated in the past in the hope that an understanding of these model reactions might be of help in the elucidation of the mechanism of the biosynthetic conversion.

The first of these model reactions was published nearly 30 years ago by von Mutzenbecher (1939) who showed that DIT can undergo, in the presence of oxygen, self-coupling, and thus form a small amount of T_4. Hillmann (1956) suggested that the first step in the biosynthetic conversion of DIT to T_4 might be an oxidation of DIT to its keto acid analog 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA) which would then couple with DIT to form T_4. While he believed oxygen to be detrimental in this coupling reaction, Meltzer and Stanaback (1961) clearly showed that oxygen is required. When they mixed DIT and DIHPPA in the presence of oxygen at pH 7.6 and at room temperature, T_4 was formed in over 20% yield[2] within less than 1 hr.

In view of the excellent yield obtained under mild conditions this reaction has been studied in this and other laboratories in recent years.[3] The present work is an attempt to throw light on the mechanism by which this coupling reaction proceeds. It was found that a neutral solution of DIHPPA in 0.2 M boric acid–sodium borate takes up oxygen rapidly at or below room temperature, while a solution of DIT, under similar conditions, consumes oxygen only extremely slowly. Consequently, the reaction between DIHPPA and oxygen was investigated in detail. The major reaction product, referred to below

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[1] Abbreviations used: T_4, thyroxine; DIT, 3,5-diiodotyrosine; DIHPPA, 4-hydroxy-3,5-diiodophenylpyruvic acid; DIHBA, 4-hydroxy-3,5-diiodobenzaldehyde; DISQ, 2,6-diiodobenzosemiquinone.

[2] All yields are expressed in per cent of the theoretical yield.

[3] Compare previous papers in this series and references given therein. Paper XI: Nishinaga *et al.* (1968).

as T_4 precursor, was identified as a hydroperoxide which is capable of reacting with DIT in the *absence* of oxygen to form T_4 in yields of up to 38%.

Experimental Section

Materials. DIHPPA was purchased from the Osaka Laboratory of Synthetic Organic Chemicals, Nishinomiya, Japan. For certain experiments this material was recrystallized from glacial acetic acid and/or from aqueous alcohol. The purity of the material was checked by infrared spectroscopy and/or gas-liquid partition chromatography. Nitrogen was a highly purified grade; its oxygen content was less than 0.0015%.

Spectra. Ultraviolet absorption spectra were determined with a Cary Model 14 recording spectrophotometer. Since neutral and alkaline solutions of DIHPPA are sensitive to oxygen, spectra of such solutions were recorded with cells connected to a small vessel in which the solvent was freed of oxygen by repeated freezing and thawing *in vacuo*. The solvent was then transferred to the cell which contained a measured amount of DIHPPA.

Oxygen-free solutions for the determination of nuclear magnetic resonance spectra were prepared in a similar manner. The nuclear magnetic resonance spectra were determined with a Varian A-60 recording spectrometer. Tetramethylsilane or sodium 3-(trimethylsilyl)-propanesulfonate was used as an internal standard.

Infrared spectra (Nujol mulls) were recorded with a Perkin-Elmer Infracord recording spectrophotometer. Mass spectra were obtained with an AEI MS-9 double-focussing spectrometer. Samples were admitted *via* direct introduction probe and were run at 70 ev.

Chromatograms. For gas-liquid partition chromatograms, columns (6 ft, 4.3-mm i.d.) packed with 1% OV-1 or 1% OV-17, 80-100 mesh on Chromosorb W HP (Supelco), and a hydrogen flame detector were used. The carrier gas was argon. The substances to be analyzed were converted to their trimethylsilyl derivatives by means of *O,N*-bis(trimethylsilyl)acetamide. A detailed description of the gas chromatographic analysis of iodoamino acids and related iodinated substances will be given in a forthcoming publication.

In thin-layer, preparative-layer, and column chromatography, silica gel was used as the adsorbent. In the first two cases a fluorescent mineral material was incorporated into the silica gel.

Oxygen Uptake Experiments. Oxygen uptake was measured with a manometer similar to the one described by Joshel (1943) for the measurement of hydrogen consumption in catalytic hydrogenations. The reaction flask containing 100 ml of the magnetically stirred solvent, either 0.2 M sodium phosphate buffer or 0.2 M boric acid-sodium borate, was kept in a bath through which water of constant temperature (usually 2°) was circulated. In some cases the oxygen uptake was measured at 12 or 24°. The magnetic stirring was always done at the same speed since the rate of oxygen uptake increases with the speed of stirring. The pH of the solvent ranged from 5.5 to 12.5. After the solvent was saturated with oxygen, a thin-walled glass vial containing 0.5 mmole of DIHPPA was dropped into the solution by turning a glass rod from which the vial was suspended. The glass rod was fused on to a glass stopper so that it could be turned by turning the stopper, without disturbing the closed system.

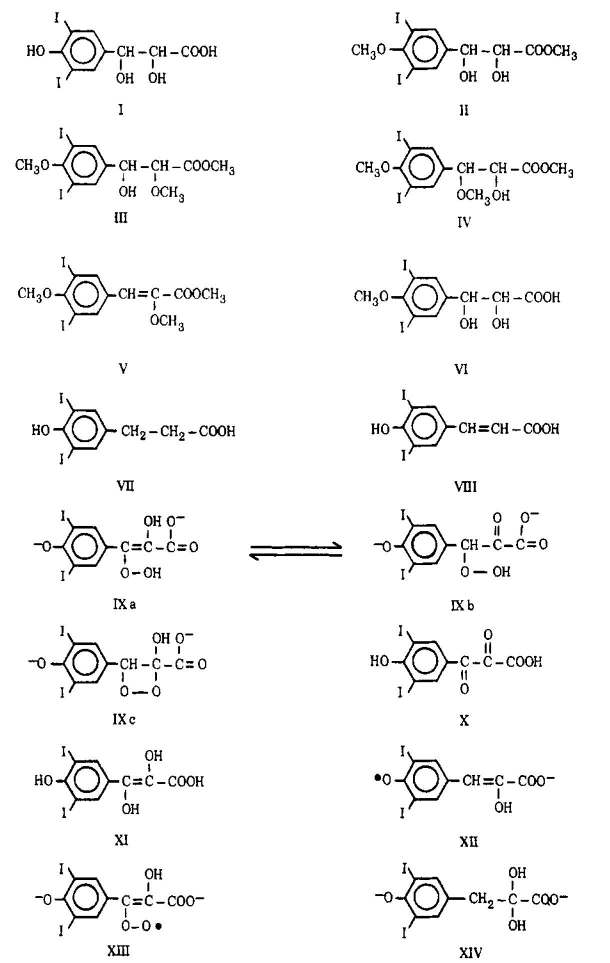
Whenever the relative yields of T_4 precursor were to be determined, the oxygen uptake experiments were carried out on a larger scale (1 mmole of DIHPPA). After the oxygen consumption ceased, DIT (6 mmoles) was added to the reaction mixture anaerobically and the amount of T_4 formed was determined by weighing in the same manner as described below.

Formation of the T_4 Precursor. In experiments whose purpose was to determine optimal conditions for the formation of the T_4 precursor, and consequently also for the synthesis of T_4, the oxidation of DIHPPA was carried out by bubbling oxygen through a 10^{-3}–10^{-2} M solution of DIHPPA in 0.2 M sodium phosphate buffer or in 0.2 M boric acid-sodium borate. The pH was adjusted to the desired value with NaOH or HCl. Reaction temperatures ranged from 0 to 24°, pH values from 6.5 to 8.5, and oxygen-bubbling periods from 5 to 40 min. A typical oxidation experiment will be described in detail because yield and reproducibility depend on the strict adherence to well-defined standard conditions.

DIHPPA (432 mg) was suspended in 5 ml of distilled water. A few lumps were crushed with a spatula. The suspension was then rinsed quantitatively with a small amount of distilled water into 245 ml of a magnetically stirred 0.2 M boric acid-NaOH mixture (pH 7.5) which was kept at ~2° by means of an ice bath, and through which nitrogen had been bubbled for at least 3 min. After a few minutes, when the DIHPPA was completely dissolved, the pH was adjusted to 7.4. The nitrogen was then replaced with oxygen and vigorous oxygen bubbling was continued at ~2° for 20 min after which time the oxygen was again replaced with nitrogen. (The oxidation was carried out in subdued light as a matter of precaution although it has not been determined with certainty that bright light is detrimental.) At least 75-80% of the reaction products consisted of the T_4 precursor (see Results). This solution was used for the determination of the physical and chemical properties of the T_4 precursor as well as for its conversion to T_4.

Conversion of the T_4 Precursor to T_4. After 3 min of nitrogen bubbling, a solution of 2.6 g of DIT in 50 ml of 0.2 M borate buffer, prepared by slight warming, if necessary, and brought to pH 8.0, was added dropwise within 15 min to the solution of the T_4 precursor. After the addition, the pH was readjusted to 8.0 and the reaction mixture was kept in the ice bath for another 15 min, then brought to room temperature by replacing the ice bath with a lukewarm water bath. Stirring and nitrogen bubbling were then continued for another 30 min. (In experiments whose purpose was to prepare T_4 rather than to study the properties of the T_4 precursor, the use of nitrogen during the addition and the reaction of DIT was omitted.) Then the pH was raised to 12 with about 5 ml of 50% NaOH and stirring and nitrogen bubbling were stopped. The solution was transferred quantitatively with a few water rinses to a 500-ml separatory

CHART I



funnel and extracted with 75, 50, and 50 ml of 1-butanol, using 3-min shaking periods. The butanol layers were transferred to a 300-ml flask and evaporated under reduced pressure in a water bath of 45°. After the addition of 5 ml of distilled water to the residue, the solution was again evaporated in order to remove all butanol. The residue was transferred quantitatively to a 25-ml flask by means of 20 ml of distilled water added in several portions. The solution was cooled in an ice bath, 1 ml of concentrated HCl was added, and the mixture was heated on a steam bath for 1–2 min in order to dissolve DIT which contaminates the precipitate of T_4. The mixture was again cooled in a cold water bath for about 1 min and the precipitate was collected by filtration or centrifugation, washed with distilled water, and dried in a vacuum dessicator. The crude T_4 was transferred with 5 ml of ethyl acetate to a 25-ml flask, the mixture was boiled for a few minutes, then filtered, and washed with ethyl acetate and with a small amount of petroleum ether (bp 30–60°). The ethyl acetate treatment removes some 4-hydroxy-3,5-diiodobenzaldehyde (DIHBA) and other impurities. The T_4 thus obtained was pure as judged by thin-layer chromatography, gas–liquid partition chromatography, and elemental analysis.

Reduction and Methylation of the T_4 Precursor. To a solution of the T_4 precursor, prepared from 1.0 g of DIHPPA as described in the preceeding paragraph, was added portionwise an excess (3 g) of sodium borohydride. During the addition the temperature was kept at ~2° and the pH at 7.0–7.5. The mixture was stirred for 20 min at room temperature, then acidified to pH 1.5, saturated with ammonium chloride, and extracted once with 150 ml and twice with 100 ml of ether. The combined ether extracts, after washing with a small amount of water and drying over sodium sulfate, were evaporated to give 1.02 g of residue. Gas chromatographic analysis showed that at least 75% of the residue consisted of a substance which was obtained in pure form (colorless needles, mp 109–111° with foaming) by recrystallizations from carbon tetrachloride and from water. Elemental analysis, nuclear magnetic resonance, and infrared spectroscopy showed that the substance is the glycol I.

Anal.[4] Calcd for $C_9H_8I_2O_5 \cdot H_2O$: C, 23.10; H, 2.15; I, 54.24. Found: C, 23.02; H, 2.02; I, 54.45.

The nuclear magnetic resonance spectrum (CD_3SOCD_3) shows an AB-type quartet (J = 6 Hz), centered at δ = 4.23 ppm (methine protons) and a singlet at δ = 7.60 ppm (aromatic protons). The infrared spectrum shows a broad band at 3100–3400 cm^{-1} (hydroxyls) and another broad band at about 1700 cm^{-1} (carboxyl).

In other experiments the crude glycol I (Chart I) was treated with diazomethane solutions in ether or in various ether–methanol mixtures, the latter being stronger methylating agents. In all these experiments at least eight reaction products were obtained, three of which, the glycol II and the more highly methylated compounds III and IV, amounted to 70–75% of the total. The ratio of II to III plus IV varied with the methylating conditions. The following is an example of a methylation experiment.

The crude glycol I (1.1 g) was dissolved in a mixture of 30 ml of ether and 15 ml of methanol. An excess of a solution of diazomethane in ether was added slowly at 5°. The mixture was allowed to stand at room temperature for 20 min and was then evaporated to dryness. The residue was chromatographed on three preparative-layer plates (20 × 20 cm), using ether–benzene (1:4) as the developing solvent. The developed chromatograms showed five distinct bands, clearly visible in short-wave ultraviolet light. Three of the bands, one at the origin (A) and two near the solvent front (D and E), contained minor reaction products.

From band E a substance was isolated in 7% yield. It was identified by comparison with an authentic sample as trimethylated DIHPPA (V). The authentic sample was synthesized by the addition at ~2° of an excess of a solution of diazomethane in ether to a solution of DIHPPA (100 mg) in 20 ml of ether–methanol (1:1). The solution was permitted to stand for 2 hr at ~2°, then evaporated. Recrystallization of the residue from

[4] The elemental analyses were done by Schwarzkopf Microanalytical Laboratories, Woodside, N. Y.

carbon tetrachloride–petroleum ether gave colorless needles, mp 115–116°.

Anal. Calcd for $C_{12}H_{12}I_2O_4$: C, 30.40; H, 2.55; I, 53.54. Found: C, 30.34; H, 2.69; I, 53.67.

The nuclear magnetic resonance spectrum (CCl_4) shows singlets at δ = 3.74, 3.78, and 3.82 ppm (methoxy protons), at δ = 6.63 ppm (methine proton), and at δ = 8.03 ppm (aromatic protons). Band B contained 403 mg (36%) of compound II. An analytical sample was recrystallized from benzene; colorless plates, mp 142–144°.

Anal. Calcd for $C_{11}H_{12}I_2O_5$: C, 27.64; H, 2.53: I, 53.10. Found: C, 27.53; H, 2.34; I, 53.28.

The nuclear magnetic resonance spectrum (CD_3-CO_2D) shows singlets at δ = 3.71 and 3.84 ppm (methoxy protons), an AB-type quartet (J = 5 Hz) centered at δ = 4.74 ppm (methine protons), and a singlet at δ = 7.81 ppm (aromatic protons). The infrared spectrum shows a carbonyl band at 1750 cm^{-1} and hydroxyl bands at 3400 and 3500 cm^{-1}. The mass spectrum shows a molecular peak at m/e 478 and a fragment peak at m/e 389 owing to fragmentation between carbon atoms 2 and 3 of the side chain. Hydrolysis of compound II with 1 M NaOH in 50% aqueous methanol (1 hr at room temperature) gave VI which, after recrystallization from chloroform, formed colorless prisms, mp 151–153° with foaming at about 103°.

Anal. Calcd for $C_{10}H_{10}I_2O_5 \cdot H_2O$: C, 24.92; 2.51; I, 52.66. Found: C, 25.04; H, 2.47; I, 52.78.

After drying for 3 hr at 100° the anhydrous acid was obtained. Band C contained 540 mg of a crude mixture of III and IV from which 430 mg (38%) of a viscous material was obtained after a second preparative-layer chromatography. An analytical sample was obtained by high vacuum distillation of the viscous material (bath temperature 140–150°).

Anal. Calcd for $C_{12}H_{14}I_2O_5$: C, 29.29; H, 2.87; I, 51.58. Found: C, 29.27; H, 2.90; I, 51.44.

The two isomers could not be separated by gas–liquid partition or thin-layer chromatography. It was not determined whether they were present in only one or in both of the theoretically possible diastereoisomeric forms.

The mass spectrum shows a molecular peak at m/e 492 and fragment peaks at m/e 389 and 403, owing to splitting between carbon atoms 2 and 3 of the side chain. The mass spectrum determined after trimethylsilylation of the mixture of III and IV with *O,N*-bis(trimethylsilyl)-acetamide (1 hr at 60°) shows fragment peaks at m/e 461 and 161 which shows that the product eluted from band C was indeed a mixture of III and IV and that isomerization did not occur in the course of the mass spectrometric procedure. The mass spectra show that the ratio of III to IV in the mixture was roughly 3:1.

The nuclear magnetic resonance spectrum (C_6D_6) shows bands at δ = 2.97 and 2.94 ppm (aliphatic methyl ether protons), at δ = 3.32 and 3.29 ppm (methyl ester protons), at δ = 3.55 ppm (aromatic methyl ether protons), and at δ = 7.84 and 7.72 ppm (aromatic protons). A pair of doublets (J = 6.5 Hz) centered at δ = 3.61 and 4.67 ppm, the former being partly buried under the aromatic methoxy proton band, is being tentatively assigned to the methine protons. A broad hydroxy proton band is at $\delta \simeq 2.6$ ppm. The infrared spectrum shows a carbonyl band at 1750 cm^{-1} and a hydroxyl band at 3500 cm^{-1}.

By chromatographing the crude methylation product of glycol I (see above) on a column of silica gel and carrying out two successive preparative-layer plate separations of the fractions eluted with dichloromethane, a small amount of the fully methylated glycol I, containing no free hydroxyls, could be isolated. The nuclear magnetic resonance spectrum (CCl_4 + $CDCl_3$) shows singlets at δ = 1.46, 1.76, 3.33, and 3.83 ppm (methoxy protons), an AB-type quartet (J = 6.5 Hz) centered at δ = 4.97 ppm (methine protons), and a singlet at δ = 7.66 ppm (aromatic protons). The infrared spectrum shows a carboxyl band at 1740 cm^{-1}, but no hydroxyl band.

The T_4 precursor can be reduced not only by borohydride but also by milder reducing agents, *e.g.*, iodide. A few milliliters of starch indicator solution were added to an acidified solution (pH ~4) of the T_4 precursor prepared in the usual manner from 500 mg (1.16 mmoles) of DIHPPA. The solution was kept at ~2° and oxygen was excluded by a slow stream of nitrogen. Sodium iodide (3 g) was added and the slowly liberated iodine was titrated, as soon as it was formed, with 0.1 N sodium thiosulfate. The liberation of iodine ceased after 2 hr. The total amount of iodine liberated corresponded to 1.13 mequiv of iodide. The reaction mixture was further acidified to pH 1, saturated with sodium chloride, and extracted with ether. The ether extract was concentrated to a small volume, then treated with an excess of diazomethane in ether. The methylated products (360 mg) isolated from the reaction mixture consisted of at least eight different compounds as shown by thin-layer chromatography. These compounds were not further investigated.

Behavior of DIHPPA and of the T_4 Precursor toward Strong Alkali. In a catalytic hydrogenation flask, 4.32 g (10 mmoles) of DIHPPA was added to 50 ml of 2 M NaOH and a few milliliters of 1-butanol (antifoaming agent). Then the mixture was shaken in an atmosphere of oxygen. Oxygen absorption was nearly completed after 30 min and ceased entirely after 1 hr when 9.7 mmoles of oxygen had been consumed. After concentration and acidification of the reaction mixture 3.53 g (95%) of a white precipitate (mp 197–200°) was obtained. This product was identified as DIHBA through its mixture melting point and infrared spectrum.

The mother liquor was evaporated to dryness and the residue was extracted with ether. Evaporation of the ether extract gave 0.94 g (75%) of crystals (mp 98–101°) which were identified as oxalic acid dihydrate (mixture melting point and infrared spectrum). When the desiodo analog of DIHPPA, *p*-hydroxyphenylpyruvic acid, was oxidized in the same manner, *p*-hydroxybenzaldehyde and oxalic acid were obtained in 89 and 62% yield, respectively.

The following two experiments were carried out in the absence of oxygen. Nitrogen was bubbled through 20

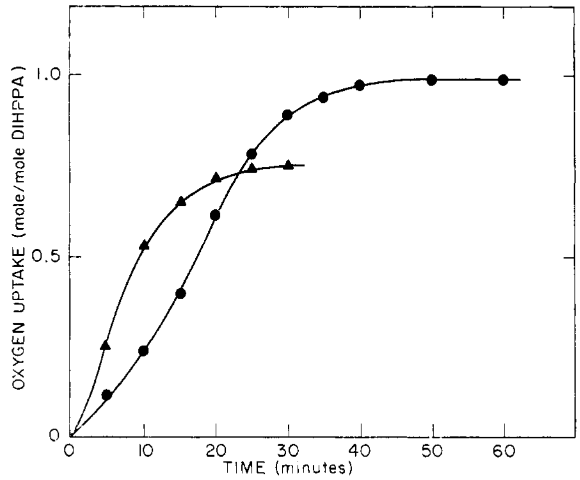


FIGURE 1: Oxygen uptake by DIHPPA at 2° (—●●●●—) and 24° (—▲▲▲▲—).

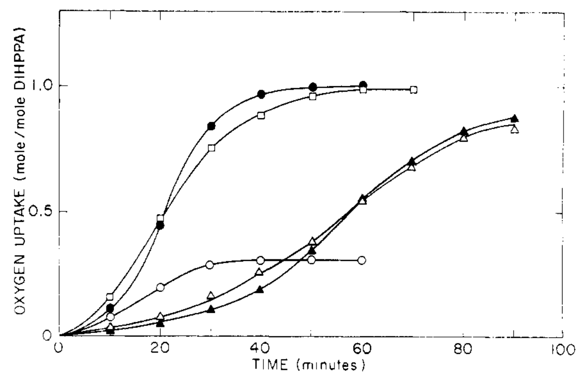


FIGURE 2: Oxygen uptake by DIHPPA in various solvents at 2°. Solvents: 0.2 M boric acid–sodium borate (pH 7.4) (—●●●●—), 0.2 M sodium phosphate buffer (pH 7.4) (—○○○○—), 0.2 M boric acid–sodium borate (pH 6.5) (—△△△△—), 0.2 M boric acid–sodium borate (pH 8.5) (—▲▲▲▲—), and 0.2 M sodium borate–sodium hydroxide (pH 12) (—□□□□—).

ml of 2 M NaOH covered with a few milliliters of 1-butanol. After the addition of 432 mg (1 mmole) of DIHPPA, carried out with continued nitrogen bubbling, the mixture was allowed to stand for 24 hr at room temperature under slight nitrogen pressure. Upon acidification of the mixture, 423 mg (98%) of pure DIHPPA was recovered.

A solution of the T_4 precursor, prepared from 432 mg (1 mmole) of DIHPPA, was brought to pH 13 under exclusion of oxygen (nitrogen bubbling). The solution was permitted to stand for 15 min at ~2° under slight nitrogen pressure. Upon acidification followed by washing and drying of the precipitate formed, 270 mg (72%) of DIHBA, identified through its mixture melting point and infrared spectrum, was obtained.

Results

Oxygen Uptake by DIHPPA. As was to be expected the rate of oxygen uptake is faster at 24° than at 2° (Figure 1). At the higher temperature oxygen uptake was almost completed after 20 min, while at the lower temperature it was only 60% of the maximal consumption which was reached after 40 min under the conditions of the experiment. However, at 24° the total oxygen consumption was only 0.75 mole/mole of DIHPPA while at 2° a full mole of oxygen was taken up, which indicates that at room temperature side reactions occur that do not take place at 2°.

The rate of oxygen consumption (Figure 2) is faster in the pH range 7.2–7.6 than at either a somewhat lower or higher pH. In strong alkali, however, the rate of oxygen uptake becomes again very fast. If DIT were added anaerobically to the reaction mixture after oxygen consumption had ceased at pH 7.4, T_4 was formed in 34% yield which shows that DIHPPA had been converted to the T_4 precursor in a minimum yield of 34% and possibly in a considerably higher one. If the oxygen uptake experiment were carried out in strong alkali, and then DIT were added to the reaction mixture under exactly the same conditions as in the experiment at pH 7.4, not even a trace of T_4 was formed. In this case DIHBA and oxalic acid were isolated from the reaction mixture in excellent yield, which shows that oxygenation of DIHPPA at pH >12 leads to a fission of the molecule between carbons 2 and 3 of the aliphatic side chain. In the oxygen uptake experiments at pH 6.8 and 8.5 the yields of T_4, after addition of DIT, were 25 and 23%, respectively. Practically no oxygen was consumed below pH 5.5–6 which is certainly due to the fact that, as in the case of other phenols, DIHPPA is more easily oxidized when it is present as the phenolate anion. The pK_a of DIHPPA has not been determined, but is should be in the neighborhood of pH 6–6.5 in analogy with that of other di-*o*-iodophenols (Tata, 1959).

All oxygen uptake experiments described above have been carried out in the presence of borate ions. In the absence of borate ions, *e.g.*, in phosphate buffer, only little oxygen was consummed, even at pH 7.4. Oxygen consumption ceased after 0.3 mole of oxygen had been taken up.

Keto–Enol Tautomerism of DIHPPA. It is well known that α-keto acids can exist in the keto or enol form and that in the presence of borate ions the equilibrium position is shifted toward the enol form, owing to the formation of an enol–borate complex (Knox and Pitt, 1957; Lin *et al.*, 1958). It was therefore suspected that the greater oxygen consumption in the presence of borate is due to a preponderance of the enol tautomer. The ratio of keto and enol tautomer present under various conditions (pH, presence or absence of borate ions, and aqueous or anhydrous media) was therefore studied by means of nuclear magnetic resonance and ultraviolet and infrared spectroscopy (Table I). A detailed account of these investigations will be presented elsewhere. In the present paper only those aspects will be discussed

TABLE I: Spectral Data for the Keto and Enol Tautomers of DIHPPA.[a]

		Keto Tautomer	Enol Tautomer
Ultraviolet	λ_{max} (mμ)	210–220, 312	251, 338
	Log ϵ	4.43, 3.88	4.16, 4.46
Nuclear magnetic resonance, δ (ppm)	Aromatic protons	7.6	8.1
	Methylene protons	3.9	
	Methine proton		5.9
Infrared, ν (cm^{-1})	Carbonyl stretching	1725, 1770	1670

[a] The ultraviolet spectra were determined *in vacuo* or under nitrogen. The extinction coefficients were determined under the conditions given in Figure 3. The nmr spectra were determined *in vacuo;* concentration, 0.22 M. The chemical shifts of the keto and enol tautomers were determined in 0.6 M phosphate buffer (pH 7.5) and in 0.9 M boric acid–sodium borate (pH 7.5), respectively. For the infrared spectra Nujol mulls were used.

which are pertinent to the oxidative conversion of DIHPPA to the T_4 precursor.

The nuclear magnetic resonance spectra of DIHPPA in organic solvents (perdeuterated methanol or dioxane) show singlets for the enolic CH= proton at δ = 5.9 and 6.1 ppm, respectively, while a band for the CH_2 protons of the keto tautomer is absent. Crystalline DIHPPA, which cannot undergo rapid tautomerization in anhydrous organic solvents, is therefore entirely in the enol form. This is in agreement with the finding that other crystalline α-keto acids are also the enol tautomers (Schwarz, 1961) and is confirmed by the infrared spectrum of crystalline DIHPPA which shows a single C=O stretching band at 1670 cm^{-1}. When neutral or slightly alkaline solutions of DIHPPA are prepared by dissolving the crystals in phosphate buffer or dilute alkali in the absence of oxygen, the enol is converted largely to the keto tautomer. When such solutions were acidified after 30–60 min, then extracted with ether and the ether extract was evaporated under reduced pressure, a solid residue was obtained, whose infrared spectrum showed, in addition to the band at 1670 cm^{-1}, two other carbonyl stretching bands at 1725 and 1770 cm^{-1} which are due to the carbonyl and carboxyl C=O stretching of the keto tautomer. When the solid keto–enol mixture was recrystallized from glacial acetic acid or aqueous ethanol, the resulting crystals consisted again entirely of the enol form. It was found that the nuclear magnetic resonance band due to the aromatic protons of the enol tautomer is considerably further downfield ($\delta \simeq 8.2$ ppm) than that due to the aromatic protons of the keto tautomer ($\delta \simeq 7.6$ ppm). This downfield shift due to a conjugated double bond in the aliphatic side chain is not a unique property of DIHPPA. Thus, the band for the aromatic protons of the saturated 3,5-diiodophloretic acid (VII), determined in 2 M NaOH, is at δ = 7.58 ppm while that for the aromatic protons of its unsaturated analog, 3,5-diiodo-*p*-cumaric acid (VIII) is at δ = 7.97 ppm.

Aqueous solutions of DIHPPA show both types of aromatic proton resonance. From the ratio of the areas under the two bands the keto:enol ratios can be determined. In a 0.2 M solution in 0.8 M borate at pH 7.5 more than 70% of the DIHPPA was present in the enol form. In contrast, a 0.2 M solution in 0.5 M phosphate buffer at the same pH contained more than 90% in the keto form.

When the keto:enol ratio of DIHPPA solutions is determined by means of ultraviolet spectroscopy, which is less accurate than nmr spectroscopy on account of the overlapping of the keto and enol absorption bands, the results are qualitatively similar but quantitatively slightly different, probably because the keto:enol ratio is concentration dependent. When the spectrum is determined at pH 7.4–7.5 where the phenolic hydroxyl is ionized, the keto tautomer has an absorption peak at 312 mμ and the enol–borate has a much higher one at 338 mμ (Figure 3). The molar extinction coefficients vary with concentration and time. The deviation from Beer's law is probably due in part to the concentration dependency of the keto–enol equilibrium and in part to the instability, even in the absence of oxygen, of dilute solutions of DIHPPA. This instability is greater in alkaline than in neutral media. Figure 4 shows the spectra of a 2.4×10^{-5} M solution of DIHPPA in borate buffer (pH 8) recorded 3 min after dissolution and at various time intervals thereafter. The freshly prepared solution had the typical absorption maximum of the enol borate at 338 mμ and another maximum at about 250 mμ. On standing in an atmosphere of nitrogen, the long-wavelength peak decreased and underwent a blue shift toward a final low peak at 218 mμ. The short-wavelength peak disappeared on standing and a new, higher peak appeared at 210–220 mμ. Although the ultraviolet spectrum of the keto tautomer of DIHPPA, determined at pH 8 in phosphate buffer, has a maximum at $\sim$220 mμ and none at 250 mμ, the spectral changes shown in Figure 4 cannot be explained by a conversion of the enol borate to the keto tautomer alone. The extinction coefficient at 318 mμ of the solution after standing for 75 hr is considerably lower than that expected if the enol–borate had been converted completely or almost completely to the keto form. Furthermore, when oxygen

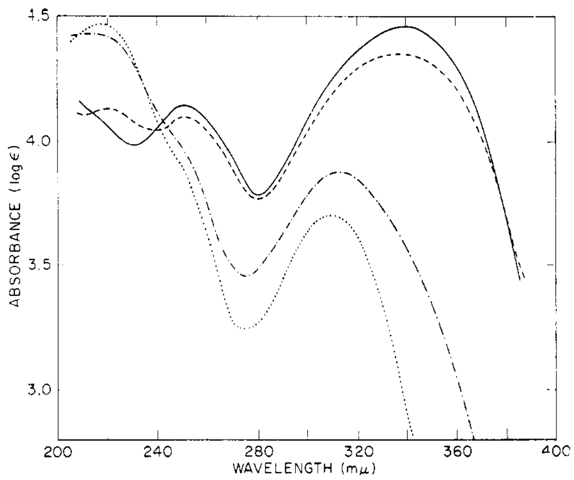


FIGURE 3: Ultraviolet absorption spectra of DIHPPA in 0.2 M boric acid–sodium borate (———), T_4 precursor in the same solvent (— — — —), DIHPPA in 0.2 M sodium phosphate buffer (—·—·—), 4-hydroxy-3,5-diiodophenyllactic acid in the same solvent (.........). The spectra of the DIHPPA solutions were recorded under nitrogen 3 min after the anaerobic mixing of the solid keto acid with the salt solution. The spectrum of the T_4 precursor was determined at 2°. For all determinations the concentration was $\sim 1 \times 10^{-3}$ M, the pH 7.4–7.5, and the light path 0.5 mm.

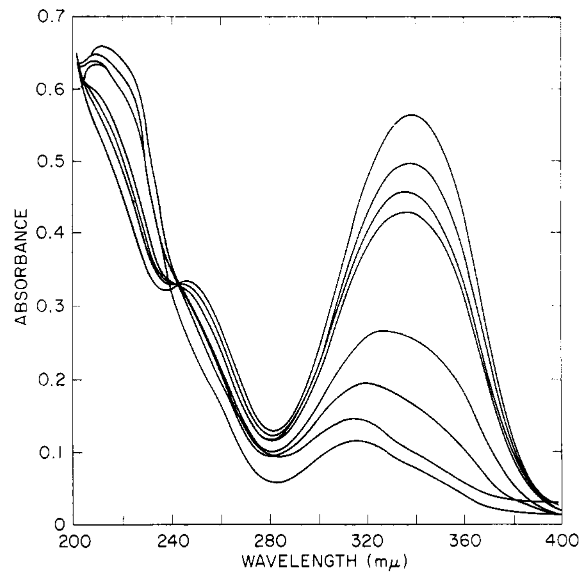


FIGURE 4: Ultraviolet absorption spectra of DIHPPA in 0.2 M boric acid–sodium borate (pH 8) recorded after various time intervals. The spectrum showing the highest absorption at 338 mμ was recorded 3 min after the anaerobic mixing of the solid keto acid with the salt solution. The increasingly lower spectra were recorded 1, 2, 3, 17, 29, 50, and 75 hr after mixing. The recordings were done *in vacuo;* concentration, 2.4×10^{-5} M; light path, 1 cm.

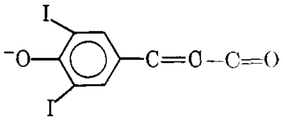
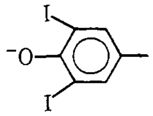
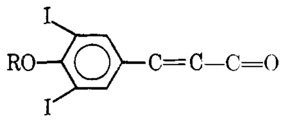
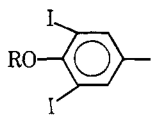
was admitted to the solution after 75 hr, the absorption spectrum remained practically unchanged for many hours, which indicates that DIHPPA had been converted largely to another substance which, in contrast to DIHPPA itself, is not air oxidized at pH 8 to DIHBA which can be easily recognized by its absorption peak at 342 mμ (Shiba and Cahnmann, 1962). Finally, when borate buffer (pH 8) was added to a solution of DIHPPA in phosphate buffer (pH 8), only a transient increase, followed by a decrease, of the extinction coefficient in the region of the enol–borate absorption (338 mμ) was observed. The spectrum recorded after 75 hr looked very much like the one shown in Figure 4 recorded after the same length of time. A possible explanation for the observed increase of transparency in the ultraviolet region with time will be given below (see Discussion). A similar increase in transparency was observed when the spectra of a 1×10^{-3} M solution of the T_4 precursor was recorded after various time intervals and in the absence of oxygen (light path, 0.5 mm).

The ultraviolet spectra of solutions of DIHPPA in various aqueous media, determined in the pH range of 6–8, show that this keto acid, like other α-keto acids, exists in two tautomeric forms, which are in equilibrium, the enol form being preponderant in the presence, and the keto form in the absence of borate ions, and that this equilibrium reaction is superimposed on another reaction which reveals itself by a considerable increase in transparency in the ultraviolet region with time.

Structure Determination of the T_4 Precursor. The T_4 precursor is relatively stable in acid media at or below 0°. It can be extracted into ether from acid solutions (pH <4) in which it is, in contrast to DIHPPA, freely soluble. When a solution of the T_4 precursor was extracted with ether at pH 5.5 in order to remove a small amount of 2,6-diiodobenzosemiquinone (DISQ), traces of which are always formed in neutral or slightly alkaline solutions of the T_4 precursor (Nishinaga *et al.*, 1968), and then DIT was permitted to react with the aqueous layer under the conditions used in the example given in the Experimental Section, T_4 was obtained in 38% yield. (The experimental conditions given in that example are nearly optimal ones which, in a series of runs, led to the formation of T_4 in 32–38% yield. It should be pointed out that oxygen, while not required for the coupling of the T_4 precursor with DIT, is not detrimental to the yield of T_4.) When after the extraction at pH 5.5 a second ether extraction was made at pH 1.8, and that ether extract was dried and evaporated *in vacuo* at −15 to −20°, an almost colorless viscous residue was obtained. This residue still contained much of the originally present T_4 precursor since it reacted with DIT to form T_4 in 21% yield. However, on drying in a vacuum dessicator at 4° the residue turned into a brown powder which did not react anymore with DIT to form T_4. From this powder DIHBA was isolated in 50% yield.

Owing to the extreme instability of the T_4 precursor in the solid state it could not be purified and analyzed by classical methods. Its structure could, however, be

TABLE II: Position of Absorption Peaks of Various Chromophores.

Chromophore ($R = H$ or CH_3)	λ_{max} ($m\mu$)	Examples
	337–338	DIHPPA or T_4 precursor in the presence of borate (pH 7.4)
	312–315	DIHPPA in the absence of borate (pH 7.4), glycol I in the presence or absence of borate (pH 7.4)
	305–306	DIHPPA or T_4 precursor in the presence of borate (pH 4.0)
	278–293 (double peak or peak and shoulder)	DIHPPA in the absence of borate (pH 4.0), glycol I in the presence or absence of borate (pH 4.0), saturated reduction and methylation products in the presence or absence of borate (pH 4.0 or 7.4)

determined through its chemical and spectral properties in solution.

In contrast to DIHPPA and its desiodo analog (*cf.* Pitt, 1962) which in strong alkali underwent fission of the side chain only in the presence of oxygen, the T_4 precursor was converted anaerobically to DIHBA and oxalic acid. This indicates that the T_4 precursor contains two more oxygen atoms than DIHPPA, which is in agreement with the oxygen uptake experiments.

The ultraviolet spectrum above 240 $m\mu$ of a solution of the T_4 precursor determined in the presence of borate at pH 7.4–7.5 is virtually identical (λ_{max} 337 $m\mu$) with that of a solution of DIHPPA determined under the same conditions. It is very different from that of the keto tautomer of DIHPPA (determined in phosphate buffer) or of its chromophoric analog, 4-hydroxy-3,5-diiodophenyllactic acid (Figure 3). The T_4 precursor must, therefore, in the presence of borate ions, have the same chromophoric system as the enol tautomer of DIHPPA, *viz.*, a doubly conjugated unsaturation in the side chain. This alone, in conjunction with the facts that the T_4 precursor is formed from the enol tautomer of DIHPPA by the uptake of 1 mole of oxygen, and that it is converted by alkali to DIHBA in the *absence* of oxygen, indicates that the structure of the T_4 precursor in borate solution must be that of the hydroperoxide IXa.

Further proof for this structure is the behavior of the T_4 precursor on reduction with borohydride. This reduction led to the glycol I in excellent yield. Reduction, followed by methylation, gave the methylated compounds II–IV as the major reaction products. Alkaline hydrolysis of II led to VI, which is further proof for the structure of these glycols. The yield of the reduction or reduction–methylation products derived from the T_4 precursor was 75–80%, not counting V which may also have been formed from the T_4 precursor, but more likely from some residual DIHPPA. This means that DIHPPA had been converted by oxygen to the T_4 precursor in a minimum yield of 75–80%. The ultraviolet absorption peaks above 240 $m\mu$ for DIHPPA and for the T_4 precursor as well as for its reduction and reduction–methylation products are summarized in Table II.

The diketo acid X and the endiol XI are the only compounds, other than the hydroperoxide IXa, which could give rise to the above-mentioned reduction–methylation products. However, X and XI cannot be present in a solution of the T_4 precursor in more than small amounts. The ultraviolet spectrum is not compatible with the presence of much diketo acid X, and the endiol XI should, like other endiols such as, *e.g.*, ascorbic acid, be a strong reducing agent and consume iodine rather than liberate it from iodide. Furthermore, the formation of XI from DIHPPA would require only 1.5 mole rather than 1 full mole of oxygen.

Discussion

The investigations presented in this paper clearly show that a keto–enol equilibrium exists in aqueous solutions of DIHPPA, but that only the enol tautomer is capable of consuming 1 mole of oxygen, being thereby converted to a T_4 precursor which can react with DIT in the absence of oxygen to form T_4. The keto–enol equilibrium reaction is superimposed by another probably also reversible reaction which is more pronounced in alkaline than in neutral solutions and which can be best explained as a hydration reaction.

$$\text{DIHPPA (enol)} \rightleftharpoons \text{DIHPPA (keto)} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \text{XIV}$$

Such hydration reactions are known to occur in the case of α,β-diketo acids (Doll, 1917). The slower rate of oxygen uptake by DIHPPA at pH 8.5 than at pH 7.4 can perhaps also be explained by such a competing hydration reaction. The spectral changes of DIHPPA with time take place only in protic solvents (water–ethanol–methanol) but not in aprotic ones (cyclohexane–ether). This also supports the hydration hypothesis.

The slower oxygen uptake at pH 8.5 than at pH 7.4 could be due to a faster hydration reaction at the higher pH or possibly also to a decrease of the equilibrium concentration of the enol borate with increasing pH. Such a decrease would be analogous to the one observed in the case of the desiodo analog of DIHPPA (Knox and Pitt, 1957).

The hydroperoxide IXa must be expected to be in equilibrium, even in borate solution, with its keto tautomer IXb. Certain reactions of the hydroperoxide, *e.g.*, its conversion to DIHBA and to DISQ, can indeed be explained only if one assumes that the keto tautomer is the reactive species. The existence of another (cyclic) tautomer (IXc) is conceivable but not likely since as far as we know no four-membered ring structures for peroxides are known. The reaction of the T_4 precursor with iodide is a complex one as evidenced by the large number of reaction products formed. The fact that only one-half of the theoretical amount of iodine was liberated can perhaps be explained by a reduction of IXa to XI which forms part of a redox system $XI + I_2 \rightleftharpoons X + I^-$.

Since the T_4 precursor has been identified as the hydroperoxide IXa, it is evident that a free-radical coupling mechanism for the reaction between DIHPPA and DIT which is based on Johnson and Tewkesbury's (1942) hypothesis is incorrect. According to this hypothetical mechanism, the phenoxyl radical (XII) of DIHPPA, formed from DIHPPA and oxygen, reacts with DIT to form a quinol ether intermediate which then loses a three-carbon side chain to form T_4. The phenoxyl radical of DIHPPA has been prepared in our laboratory (Nishinaga *et al.*, 1968). It is very short-lived and therefore not present in a solution of the T_4 precursor. This does not mean, however, that the phenoxyl radical cannot be an intermediate in the oxidative conversion of DIHPPA to the hydroperoxide IXa. Such a free-radical intermediate is indeed likely. A plausible mechanism for the formation of the hydroperoxide is shown in the following chain reaction.

$$\text{DIHPPA}^- \text{ (anion)} + O_2 \longrightarrow \text{XII} + O_2^-$$

$$\text{XII} + O_2^- + H^+ \longrightarrow \text{IXa}$$

$$\text{XII} + O_2 \longrightarrow \text{XIII}$$

$$\text{XIII} + \text{DIHPPA}^- \text{ (anion)} + H^+ \longrightarrow \text{IXa} + \text{XII}$$

Hardly any coupling takes place between the hydroperoxide IXa and DIT at a pH below the pK_a of the phenolic group of DIT (6.5). With increasing pH the yield of T_4 increases first, then decreases again with the concomitant formation of increasing amounts of DIHBA and DISQ. Apparently both the phenolate anion of DIT and hydroxyl ions react with IXa competitively, forming either T_4 or side-chain fission products.

There are good reasons to believe that the coupling reaction between DIHPPA and DIT may be a fairly accurate model for the biosynthetic formation of T_4 in the thyroid. The biosynthesis of T_4 takes place within the polypeptide chain of thyroglobulin. We do not know whether it involves the coupling of two DIT residues or of a DIT residue with free DIT or, more likely, with DIHPPA. Nonenzymic models for the self-coupling of DIT are the von Mutzenbecher (1939) reaction and the formation of T_4 residues in thyroglobulin caused by the addition of relatively large amounts of iodine (Edelhoch, 1962). An enzymic model involving a peroxidase has been described by Taurog and Howells (1966). However, the ease with which T_4 is formed from DIHPPA and DIT, even near 0°, speaks for a participation of DIHPPA in the biosynthesis of T_4. DIHPPA could be formed in the thyroid from DIT by transamination or oxidative deamination or from *p*-hydroxyphenylpyruvic acid by iodination. The presence of DIHPPA in the thyroid has been claimed (Haney and Lissitzky, 1962; Surks *et al.*, 1967), although rigorous proof is still lacking. The presence in the thyroid, of a tyrosine transaminase has been demonstrated (Rivlin *et al.*, 1962) and that of a transaminase catalyzing the conversion of DIT to DIHPPA has also been reported (Horvath, 1962). Furthermore, DIHPPA reacts nonenzymically not only with DIT but also with DIT residues in thyroglobulin to form T_4 residues (Toi *et al.*, 1963, 1964).

After this work had been completed we were informed that F. Blasi, F. Fragomele, and I. Covelli (personal communication) have also found that the coupling reaction between DIHPPA and DIT takes place in two steps, an aerobic and an anaerobic one.

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