Model Reactions for the Biosynthesis of Thyroxine. Nonenzymic Formation of 3,5,3′-Triiodothyronine from 4-Hydroxy-3-iodophenylpyruvic Acid, 3,5-Diiodotyrosine, and Oxygen*

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ABSTRACT: The keto acid analogs of 3-iodo- and 3,5-diiodotyrosine react with oxygen at or below room temperature to form precursors of the thyroid hormones 3,5,3′-triiodothyronine and thyroxine, respectively. The 3,5,3′-triiodothyronine precursor is a hydroperoxide like the previously described thyroxine precursor. It differs from the latter by having no iodine in the 5 position. Both hormone precursors react with 3,5-diiodotyrosine at pH 8 at room temperature to form the corresponding hormones. This "coupling reaction" may be a nonenzymic model for the biosynthesis of the thyroid hormones. The optimal pH for the conversion of the keto acid analog of 3-iodotyrosine into the 3,5,3′-triiodothyronine precursor is 8.4; that for the conversion of the keto acid analog of 3,5-diiodotyrosine into the thyroxine precursor is 7.4. While at the optimal pH nearly 1 mole of thyroxine precursor is formed per mole of keto acid, the formation of the 3,5,3′-triiodothyronine precursor ceases after only about 0.2 mole per mole of keto acid has been formed. As a consequence the yield of 3,5,3′-triiodothyronine after coupling with diiodotyrosine is only one-fifth that of thyroxine. The difference in oxygen consumption by the two keto acids is due to the fact that the formation of the hormone precursors from the corresponding keto acids and oxygen are reversible processes.

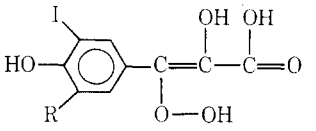
The steady-state equilibrium favors the keto acid in the case of the 3,5,3′-triiodothyronine precursor and the hydroperoxide in the case of the thyroxine precursor. Consequently, when oxygen is applied under pressure, the yield of the thyroxine precursor is improved slightly, that of the 3,5,3′-triiodothyronine precursor considerably.

Paper XII in this series (Nishinaga *et al.*, 1968) dealt with the mechanism by which DIHPPA,[1] the keto acid analog of I_2Tyr, is converted nonenzymically into T_4 in excellent yield. This conversion takes place in two distinct steps: the formation of the hydroperoxide intermediate **1b** (T_4 precursor) from DIHPPA and 1 mole of oxygen and the coupling of 1 mol of the hydroperoxide with 1 mol of I_2Tyr. Oxygen is not required for the second step.

It has been reported (Shiba and Cahnmann, 1964) that MIHPPA also can react with I_2Tyr in the presence of oxygen to form, although in poor yield, another thyroid hormone, T_3. The work described in the present paper was undertaken in order to gain insight into the mechanism by which T_3 is formed from MIHPPA and I_2Tyr and to determine the factors which are responsible for the considerable difference in the yield of the two thyroid hormones.

The formation of T_3, like that of T_4, takes place in two distinct steps. In the first step, oxygen converts MIHPPA into a T_3 precursor; in the second step, the T_3 precursor reacts with I_2Tyr to form T_3. The oxygen uptake in the first step was measured by manometry, the reaction products obtained in the first step were analyzed by gas–liquid partition chromatography, and the amount of T_3 formed in the second step was determined by isolation of T_3 in pure form from the reaction mixture.

The T_3 precursor is a hydroperoxide of structure **1a**. The conversion of MIHPPA into the T_3 precursor as well as that of DIHPPA into the T_4 precursor are at least partly reversible



1a, R = H
b, R = I

processes. The steady-state equilibrium position is dependent on the oxygen pressure. At an oxygen pressure of about 760 mm Hg the equilibrium lies far toward the keto acid in the case of MIHPPA and far toward the hydroperoxide in the case of DIHPPA. This explains the considerably lower yield of T_3 (8% at the optimal pH of 8.4) than that of T_4 (38% at the optimal pH of 7.4) in the coupling reaction between keto acid and I_2Tyr. At increased oxygen pressure the equilibrium is shifted toward the hydroperoxide and consequently the yield of T_4 rises slightly and that of T_3 considerably. The reactions of MIHPPA and DIHPPA with I_2Tyr to form T_3 and T_4, respectively, take place at or below room temperature and within a physiological pH range. They are therefore possible models for the biosynthesis of the thyroid hormones.

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[1] Abbreviations used are: I_2Tyr, 3,5-diiodotyrosine; T_3, 3,5,3′-triiodothyronine; T_4, thyroxine; MIHPPA, 4-hydroxy-3-iodophenylpyruvic acid; DIHPPA, 4-hydroxy-3,5-diiodophenylpyruvic acid.

Experimental Section

4-Hydroxy-3-iodobenzaldehyde. This compound was prepared according to Barnes *et al.* (1950) except that diethylamine was used instead of dimethylamine. The product was crystallized from water and from aqueous ethanol (40% yield[2]); mp 112–115°, lit. (Barnes *et al.*, 1950) 113–115°.[3]

2-Methyl-4-(4-acetoxy-3-iodobenzal)-5-oxazolone. This azlactone was prepared by a modification of the method of Nakano and Danowski (1959). A mixture of 4.96 g (0.02 mol) of 4-hydroxy-3-iodobenzaldehyde, 2.81 g (0.024 mol) of acetylglycine, 1.97 g (0.024 mol) of freshly fused sodium acetate, and 20 ml of acetic anhydride was heated on a steam bath for 2 hr. After cooling, 5 ml of petroleum ether and 5 ml of water was added to the reaction mixture which was then broken up with a glass rod. The crystals were collected by filtration, washed with water, and dried. Recrystallization from benzene–isooctane with the addition of charcoal gave 4.76 g (64%) of yellow needles, mp 184–187°; lit. (Nakano and Danowski, 1959) 180–184°.

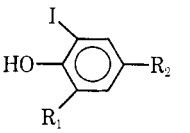
4-Hydroxy-3-iodophenylpyruvic Acid.[4] A suspension of 3.7 g (0.01 mol) of the oxazolone in 30 ml of 2 M HCl was heated on a steam bath for 3 hr. A small amount of undissolved material was removed by filtration and the filtrate kept overnight at 2°. The crystalline precipitate formed was collected by filtration, washed with a small amount of ice-cold water, and dried. A solution in a small amount of ethanol was decolorized by heating with charcoal, then evaporated to dryness. Recrystallization from water containing a few drops of 4 M HCl gave 0.81 g (26.5%) of fine needles, mp 168–170° dec; lit. (Nakano and Danowski, 1959) 169–171° dec. This keto acid as well as the above-mentioned aldehyde and oxazolone were pure as judged by gas–liquid partition chromatography.

Spectra. Ultraviolet absorption spectra were determined with a Cary Model 14 recording spectrophotometer using evacuated cells (light path, 0.2 mm) as described previously (Nishinaga *et al.*, 1968). Nuclear magnetic resonance spectra were determined with a Varian A-60 spectrometer. Tetramethylsilane was used as an internal standard.

Oxygen Uptake Experiments. Oxygen uptake was measured at 2° by the previously described method (Nishinaga *et al.*, 1968).

Gas–Liquid Partition Chromatography. The ether-extractable products obtained in the treatment of MIHPPA with oxygen as well as those obtained after oxygenated solutions of MIHPPA had been treated with I_2Tyr were analyzed by gas–liquid partition chromatography. Similar gas chromatographic analyses were carried out with oxygenated solutions of DIHPPA before and after coupling with I_2Tyr. A 0.5-ml aliquot of the reaction mixture was treated with sodium borohydride. (The hydroperoxides **1a** and **1b** cannot be chromatographed without reduction.) After cooling the aliquot in an ice bath and adjusting the pH to 7.0–7.5 with 1 M HCl, an excess of borohydride (~50 mg) was added in four equal portions to the stirred solution over a period of 45 min. The pH was kept between 7.0 and 7.5 during the first 30 min by the frequent addition of small amounts of 1 M HCl. About 5 min after the addition of the last portion the ice bath was removed and 0.3 ml of acetone was added to the reaction mixture in order to decompose the excess of borohydride. (When sodium borohydride is decomposed by HCl the nascent hydrogen causes some deiodination.) The reaction mixture was concentrated under reduced pressure to about one-half of its volume, then acidified to pH ~1.5 and extracted with ether. In some instances an ether extraction was made first at pH 7–8 (extraction of neutral reaction products) and then at pH 1–2 (extraction of acidic reaction products). Both ether extracts were analyzed by gas–liquid partition chromatography according to Funakoshi and Cahnmann (1969). The residue obtained after evaporation of the dried ether extract (Na_2SO_4) was treated at room temperature with 125 μl of *N,O*-bis(trimethylsilyl)-acetamide. The mixture of trimethylsilylated products was then chromatographed on 3.3 mm × 6 ft columns of 1% OV-1 for products derived from MIHPPA and of 3% OV-17 for those derived from DIHPPA. The latter support gives in most instances better resolution, but does not resolve compounds **2a** and **2h** derived from MIHPPA.

The retention times of the various reaction products were compared with those of authentic reference compounds. Some of these were commercially available. Others were prepared: **2a** from MIHPPA, **2b** from DIHPPA, **2c** from 2,6-diiodobenzoquinone, **2d** from 4-hydroxy-3,5-diiodobenzaldehyde (Matsuura and Cahnmann, 1959), and **2e** from 4-hydroxy-3-iodobenzaldehyde, all by reduction with sodium borohydride; **2f** from **2g**, **2h** from **2i** (Nishinaga *et al.*, 1968), and **2k** from **2c**, all by catalytic hydrogenation at room temperature and slightly above atmospheric pressure. The compounds prepared with sodium borohydride contained traces of the corresponding partly or totally deiodinated analogs; those prepared by catalytic hydrogenation were contaminated with the totally deiodinated analogs and the diiodinated starting materials. These contaminants were not removed. They proved to be useful as additional reference compounds.



2a, $R_1 = H$; $R_2 = CH_2CH(OH)COOH$
b, $R_1 = I$; $R_2 = CH_2CH(OH)COOH$
c, $R_1 = I$; $R_2 = OH$
d, $R_1 = I$; $R_2 = CH_2OH$
e, $R_1 = H$; $R_2 = CH_2OH$
f, $R_1 = H$; $R_2 = CH_2COOH$
g, $R_1 = I$; $R_2 = CH_2COOH$
h, $R_1 = H$; $R_2 = CH(OH)CH(OH)COOH$
i, $R_1 = I$; $R_2 = CH(OH)CH(OH)COOH$
k, $R_1 = H$; $R_2 = OH$

An excess of sodium borohydride was added in portions over a period of 40 min to a stirred neutral or slightly alkaline solution of the starting material. After another 20 min, the solution was acidified and extracted with ether. The ether extract was dried and evaporated.

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

[3] All melting points were taken in capillary tubes and are uncorrected.

[4] For some of the experiments reported in this paper a gas chromatographically pure preparation of MIHPPA kindly supplied by the J. T. Baker Chemical Co., Phillipsburg, N. J., was used

The catalytic hydrogenation of **2g** was done according to Matsuura and Cahnmann (1959). Compounds **2i** and **2c** were hydrogenated in methanol (0.5 mmol in 25 ml) in the presence of 10% Pd on charcoal (50 mg) until 1 mol of H_2/mol of starting material had been taken up (~1 hr for **2i** and ~20 min for **2c**).

Ion-Exchange Chromatography. A cation-exchange resin, AG 50W-X2, 200–400 mesh, 0.7 mequiv/ml of resin bed, was used for the isolation of T_3 from reaction mixtures.[5] This resin was obtained in the hydrogen form from Bio-Rad Laboratories, Richmond, Calif., and was converted with 8 M NH_4OH to the ammonium form. After the NH_4OH washings had become colorless, the resin was washed with water until the pH of the washings dropped below 8. It was then suspended in 0.2 M borate buffer containing 30% (v/v) ethanol (pH 8.0). A jacketed column (2.3-cm internal diameter) kept at 50° by circulating water[6] was packed with the resin to a height of 31 cm. A brief degassing of the suspension of the resin before it is poured into the column prevents the formation of air bubbles in the course of the chromatographic fractionation.

The T_3-containing eluate fractions were originally determined either by counting the radioactivity after the addition of a trace of [^{131}I]T_3 to the reaction mixture or by thin-layer chromatography on silica gel containing a fluorescent indicator, after concentration of the eluate fraction to a small volume. A brief development with I-butanol–2 M NH_4OH (upper phase) was sufficient to move the T_3 away from the always fluorescence-quenching origin. Inspection of the developed chromatogram in short-wave ultraviolet light revealed the T_3-containing fractions. The elution pattern with the same batch of resin and with identical resin column dimensions is so reproducible that counting the radioactivity and thin-layer chromatography could be dispensed with in all later experiments. The T_3-containing fractions were determined instead as described in the following paragraph.

Formation of T_3. The oxidative conversion of MIHPPA into the T_3 precursor and the coupling of the latter with I_2Tyr were studied at different pH values. In the following examples the optimal pH of 8.4 was chosen for the oxidation and the probably near-optimal pH of 8.0 for the coupling.

Three procedures were used. In procedure A the keto acid was oxidized and then coupled with I_2Tyr at atmospheric pressure. In procedure B oxidation and coupling were carried out at medium (3.8 atm) or high (122–133 atm) oxygen pressure. In both procedures I_2Tyr was added after the keto acid had been oxidized (two-step method). Procedure C was carried out at high oxygen pressure with I_2Tyr being added at the beginning of the experiment (one-step method). In procedure A, the oxidation was carried out in the apparatus used for oxygen uptake experiments and the coupling in an open container. For the experiments at medium oxygen pressure a Vortex LP Hydrogenator (J. B. Thompson, Cumberland, Md.) and for those at high pressure a stainless steel bomb (American Instrument Co., Silver Spring, Md.) with glass insert (8 × 52 cm) were used. In all procedures, the reaction mixture was continuously stirred by means of a magnetic stirrer.

PROCEDURE A. A 5×10^{-3} M solution of MIHPPA (pH 8.43) prepared by adding 306 mg (1 mmol) of MIHPPA to 200 ml of ice-cold 0.2 M sodium borate buffer (pH 8.60) was treated with oxygen at 2° for 50 min. The pH was then adjusted to 8.0 with 1 M HCl (~1.5 ml) and a solution (pH 8.0) of $I_2Tyr \cdot 2H_2O$ (2.81 g, 6 mmol) in 50 ml of 0.2 M sodium borate buffer was added dropwise (15 min) with ice cooling. The mixture was stirred for 1.5 hr at 2° and for another 1.5 hr at room temperature. Alternatively the solution may be kept at 2° overnight. A small amount of precipitate formed was not removed. It dissolved upon the subsequent addition of ethanol (see below).

PROCEDURE B. The reaction vessel containing the same ingredients as those used in procedure A was flushed with oxygen. Oxygen pressure was then applied and the mixture stirred at 2° (cold room) for 50 min. After release of the pressure, the pH (8.43) was adjusted to 8.0 with 1 M HCl (~1.5 ml). A solution of I_2Tyr (see procedure A) was added at once, and stirring under oxygen pressure continued for 1 hr at 2° and for another 1.5 hr at room temperature. The pressure was again applied and stirring was resumed. The pressure was then released and the reaction mixture degassed by applying reduced pressure (~200 mm Hg) for a few minutes.

PROCEDURE C. MIHPPA (306 mg) and $I_2Tyr \cdot 2H_2O$ (2.81 g) were added to 250 ml of ice-cold 0.2 M sodium borate buffer, pH 9.02. The reaction vessel was inserted into the precooled steel bomb which was then flushed with oxygen. Oxygen pressure was applied for 50 min at 2° (cold room). After release of the pressure the pH of the solution (8.43) was adjusted to 8.0 with 1 M HCl (~2 ml). Then oxygen pressure was worked up as described in procedure B.

Absolute ethanol (120 ml) was added to the reaction mixture obtained in either one of the three procedures (A, B, C). This caused the pH to rise to 8.95. After dilution to 400 ml with water and readjustment of the pH to 8.0 with 1 M HCl, the reaction mixture was filtered through the ion-exchange column described above. The column was then washed with 400 ml of 0.2 M sodium borate buffer containing 30% (v/v) ethanol (pH 8.0). Thin-layer chromatography showed that this is sufficient to elute all I_2Tyr. Then the eluent was changed to a solution containing 50 ml of concentrated NH_4OH (28–30% NH_3) and 45 ml of ethanol in H_2O (total volume 150 ml) and the flow rate reduced to 1.5 ml/min. After 60 ml had been eluted, 3-ml fractions were collected in test tubes which were then kept at 2° overnight. The following morning 2–3 fractions (usually tubes 12–14) were cloudy. The cloudy fractions were combined with the five preceeding and the ten succeeding ones and evaporated to dryness or near-dryness in a bath of 50° and under reduced pressure. A vibrating evaporator (Rotary Evapo-Mix, Büchler Instruments, New York, N. Y.) was found to be convenient for this purpose. The residue was dissolved in a few milliliters of water containing a few drops of concentrated NH_4OH and the solution transferred to a 15-ml centrifuge tube. Occasionally it was necessary to clarify the solution by centrifugation. The clear solution was heated to incipient boiling, then brought to pH 5–6 with 2 M HCl. The precipitate formed was collected by centrifugation and washed with

[5] A resin of smaller mesh size, AG 50W-X4, 30–35 μ, 1.2 mequiv/ml of resin bed (not used in the present investigation), gives better resolution, so that a smaller column can be used (*cf.* Reilly *et al.*, 1961; Block and Mandl, 1962; Lerner, 1963).

[6] Resolution of the iodoamino acids is much better at 50° than at room temperature.

water until the supernatant was free of chloride ion (AgCl test). It was then dried under reduced pressure over P_2O_5 at 100° for 2 hr. The faintly colored T_3 thus obtained was gas chromatographically pure. Treatment of an ammoniacal solution with charcoal, followed by reprecipitation at pH 5–6, yielded a white product giving the correct elemental analysis for T_3.

Formation of T_4. The conversion of DIHPPA into T_4 was carried out in the same manner as that of MIHPPA into T_3, with the following modifications. The oxidation of DIHPPA (432 mg, 1 mmol) was done at pH 7.4. The reaction mixture after coupling (at pH 8.0) contained much precipitated T_4 which was collected by centrifugation and washed successively with water, 1 M HCl, again water, and finally ether. The supernatant from the original centrifugation and the first wash water were combined. Enough ethanol was added to give an ethanol concentration of 30% (v/v), the pH adjusted to 8.0, and the solution chromatographed on AG 50W-X2. Since T_4 emerges from the column earlier than T_3, the collection of 3-ml fractions was started after 50 ml of eluate had been collected. A precipitate or cloudiness appeared in about three–four fractions (usually tubes 13–17). The T_4-containing fractions were worked up as in the case of T_3 and the T_4 thus obtained was combined with the bulk of T_4. The slightly colored product was gas chromatographically pure but gave a satisfactory elemental analysis only after decolorization with charcoal and reprecipitation from dilute ammonia.

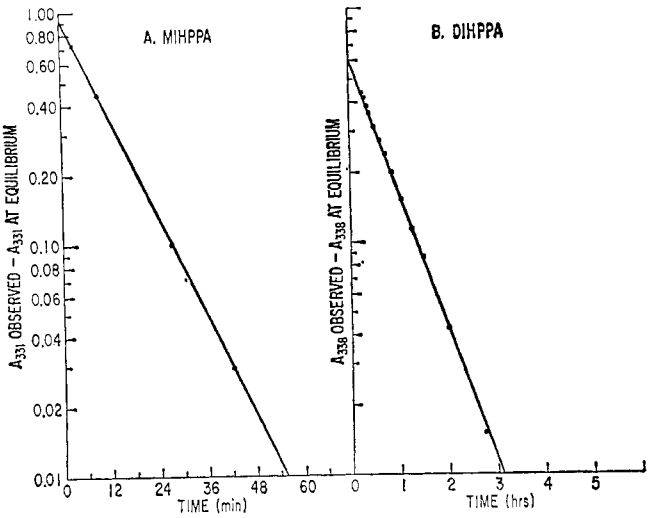


FIGURE 1: Rate of approach to enol–keto equilibrium. (A) MIHPPA (5×10^{-3} M), pH 8.4 in 0.2 M boric acid–NaOH, 24°. (B) DIHPPA (5×10^{-3} M), pH 7.4, in 0.2 M boric acid–NaOH, 24°. Ultraviolet absorption spectra were determined in evacuated cells of 0.2-mm light path at various time intervals after mixing the solvent with the solid keto acid. The absorbance at equilibrium (331 mμ for MIHPPA and 388 mμ for DIHPPA) was deducted from the absorbance (at the same wavelength) at various time intervals and the values obtained were plotted against time on a semilog paper.

Results

Keto–Enol Equilibrium. Crystalline MIHPPA, like crystalline DIHPPA, phenylpyruvic acid, *p*-hydroxyphenylpyruvic acid, and indolyl-3-pyruvic acid, consists entirely of the enol tautomer (Nishinaga *et al.*, 1968; Bücher and Kirberger, 1952; Schwarz, 1961). This is clearly shown by the fact that the nuclear magnetic resonance spectra of MIHPPA in organic solvents, in which enol–keto interconversion does not take place at all or only very slowly, show a sharp methine peak (δ 6.42 ppm in CD_3OD) but no absorption at all in the methylene region. When crystalline MIHPPA is dissolved in borate buffer under optimal conditions for its conversion into the T_3 precursor (see below under oxygen uptake experiments) an interconversion between the enol[7] and keto tautomers takes place and a steady-state equilibrium is approached.

The rate of approach to equilibrium was determined by following the change of the ultraviolet absorption spectrum with time. The enol:keto ratio at equilibrium was calculated from the absorbancies of the enol tautomer, the keto tautomer, and the equilibrium mixture, all at 331 mμ (λ_{max} of the enol). A_{331}(enol) was determined by extrapolating A_{331}(time t) – A_{331}(equilibrium) to time zero (Figure 1A) and then adding A_{331}(equilibrium). A_{331}(keto) was considered to be identical with A_{331} of an equimolar solution of 3-iodotyrosine (a chromophoric analog of the keto tautomer). This assumption is valid on theoretical grounds. A similar assumption was made by Knox and Pitt (1957) in the case of *p*-hydroxyphenylpyruvic acid. A_{331}(equilibrium) was also determined for a solution of MIHPPA in 0.2 M phosphate (in which considerably less T_3 precursor is formed than in borate). From this the enol:keto ratio at equilibrium in phosphate buffer could be calculated.

The corresponding data for DIHPPA were obtained in a similar manner (Figure 1B). The absorbancies were measured at 338 mμ (λ_{max} enol) and I_2Tyr was used instead of 3-iodotyrosine to determine A_{338}(keto).

The spectrophotometric determinations were carried out under conditions (pH, concentration) which are optimal for the conversion of MIHPPA and DIHPPA into the respective hormone precursors, except that oxygen was absent and that the temperature was 24° instead of 2°. The higher temperature increases the rate of approach to equilibrium, but the relative rates for MIHPPA and DIHPPA and the enol:keto ratio at equilibrium should be essentially the same at both temperatures. Table I summarizes the results obtained. As expected, the enol content of both keto acid solutions at equilibrium was found to be considerably higher in the presence than in the absence of borate. The increase in enol content of a MIHPPA solution at pH 8.4 on switching from phosphate to borate which is due to the formation of an enol borate complex is less pronounced than that of a DIHPPA solution at pH 7.4. Although at equilibrium the enol content of the MIHPPA solution in borate is somewhat lower (47%) than that of the DIHPPA solution (66%), the rate of approach to equilibrium is three to four times faster for MIHPPA than for DIHPPA.

Oxygen Uptake Experiments. In order to determine optimal conditions for the oxidative conversion of MIHPPA into the T_3 precursor, the oxygen uptake of 5×10^{-3} M solutions of MIHPPA was measured at various pH values. After the oxygen uptake ceased or became very slow, an excess of I_2Tyr was added and coupling permitted to proceed at pH 8

[7] For the sake of simplicity no distinction is made in this paper between enol and enol borate. This is permissible since the interconversion of enol and enol borate is not rate limiting (*cf.* Knox and Pitt, 1957).

TABLE I: Enol–Keto Equilibrium of MIHPPA and DIHPPA.[a]

	ϵ_{max} (enol) (mμ)	Enol at Equil (%)		Keto at Equil (%)		Approach to Equil (Borate) $T_{1/2}$ (min)	Rate of Approach to Equil (Borate) $\ln 2 \times T_{1/2}^{-1}$ (min^{-1})
		Borate	Phosphate	Borate	Phosphate		
MIHPPA	17600 (331)	47	20	53	80	9	0.076
DIHPPA	22500 (338)	66	9	34	91	33	0.021

[a] Conditions (concentration, pH) were the same as those described in the legend to Figure 1.

as described in the Experimental Section (Formation of T_3, Procedure A).

An optimal yield of T_3 (8.5%; std dev = 0.4%) was obtained when the oxidation of MIHPPA was carried out at pH 8.4. At both lower and higher pH values the T_3 yield fell off rapidly. No T_3 could be isolated from the reaction mixture at either pH 7 or pH 10.

Figure 2 shows the oxygen uptake of MIHPPA in the presence of borate at pH values ranging from 7.8 to 12. At the optimal pH for T_3 formation (8.4) oxygen uptake practically ceased after 17% of the theoretical amount of oxygen required for the conversion of MIHPPA into the hydroperoxide **1a** (1 mol/mol of MIHPPA) had been consumed. At higher pH values more oxygen was consumed, but the plateau changed to a rising curve whose slope increased with increasing pH. At lower pH values less oxygen was consumed. Replacement of borate with phosphate at the optimal pH for T_3 formation resulted in an oxygen uptake of only 5% of the theoretical amount. It becomes clear from this that borate ions which favor the conversion of the keto into the enol tautomer also favor oxidation of MIHPPA to the T_3 precursor. Although oxygen is required for the conversion of MIHPPA into the T_3 precursor, a large oxygen uptake *per se* does not prove the formation of the T_3 precursor. Thus, at pH 12 about 95% of the theoretical amount of oxygen was consumed within 1 hr, but the reaction mixture contained no T_3 precursor. It consisted almost exclusively of 4-hydroxy-3-iodobenzaldehyde and oxalic acid (both determined by gas–liquid partition chromatography). At this high pH the T_3 precursor was probably initially formed but immediately decomposed by side chain fission (*cf.* Nishinaga *et al.*, 1968).

Reversibility of the Conversion of MIHPPA and of DIHPPA into the Respective Hormone Precursors. The most striking feature of the oxygen uptake curve at the optimal pH of 8.4 is the formation of a plateau after only about one-fifth of the theoretical amount of oxygen had been consumed. This is in contrast to the autoxidation of DIHPPA at the optimal pH of 7.4 in which about 95% of the theoretical amount of oxygen is taken up rapidly (Nishinaga *et al.*, 1968). The early formation of a plateau shows that the low yield of T_3 is not due to side chain fission since such a fission also requires

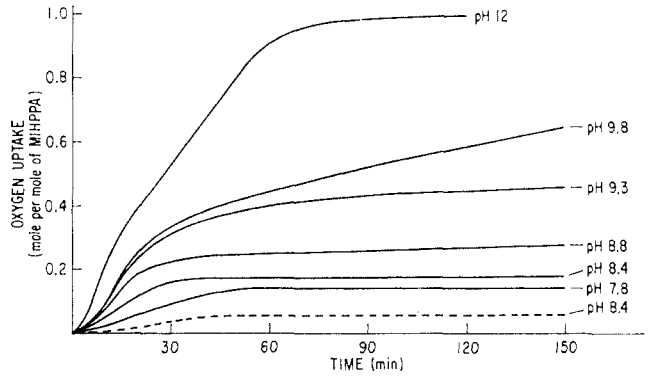


FIGURE 2: Oxygen uptake by MIHPPA (5×10^{-3} M) at 2° in 0.2 M boric acid–NaOH (——) and in 0.2 M phosphate buffer (– – – –). The pH values refer to the pH of the solution, not the solvent.

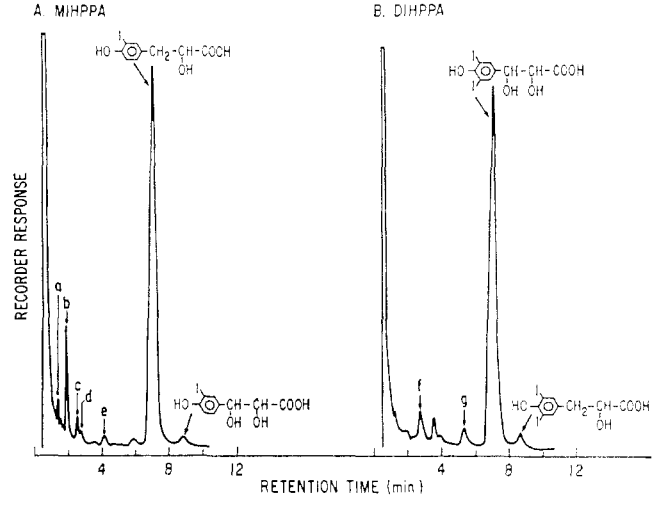


FIGURE 3: Gas chromatograms of oxygenated solutions of MIHPPA (A) and of DIHPPA (B). Solutions of MIHPPA and of DIHPPA (0.5×10^{-3} M) were treated at 2° with oxygen for 50 min at pH 8.4 and 7.4, respectively. After $NaBH_4$ reduction at pH 7.0–7.5, the acidified reaction mixture was extracted with ether and the ether evaporated. The residue was treated with O,*N*-bis(trimethylsilyl)-acetamide. The mixture of trimethylsilylated reaction products was then chromatographed on 3.3 mm × 6 ft columns of 1% OV-1 (A) or 3% OV-17 (B); carrier gas, nitrogen (50 cc/min); hydrogen flame ionization detector; column temperature 175° (A), 240° (B). Some of the minor peaks were tentatively identified by means of reference compounds as: a, monoiodohydroquinone; b, 4-hydroxy-3-iodobenzyl alcohol; c, 3-(*p*-hydroxyphenyl)lactic acid; d, 4-hydroxy-3-iodophenylacetic acid; e, 4-hydroxy-3-iodophenylglycolic acid; f, 3-(4-hydroxy-3-iodophenyl)glyceric acid; g, 4-hydroxy-3,5-diiodophenylglycolic acid.

TABLE II: Gas Chromatographically Determined Ratio of Hormone Precursor to Keto Acid after Successive Treatment of the Keto Acid with Oxygen, Nitrogen, and again Oxygen.[a]

	T_3 Precursor[b]	T_4 Precursor[c]
	MIHPPA	DIHPPA
O_2 (20 min)	0.23	46
O_2 (20 min), then N_2 (20 min)	0.15	11
O_2 (20 min), then N_2 (20 min), then O_2 (20 min)	0.20	56

[a] All reactions were carried out at 2° and in the presence of 0.2 M borate. The keto acid solutions were 5×10^{-3} M. The pH was 8.4 for MIHPPA and 7.4 for DIHPPA. [b] Measured as ratio of areas under the peaks derived from the T_3 precursor and from MIHPPA. [c] Measured as ratio of areas under the peaks derived from the T_4 precursor and from DIHPPA.

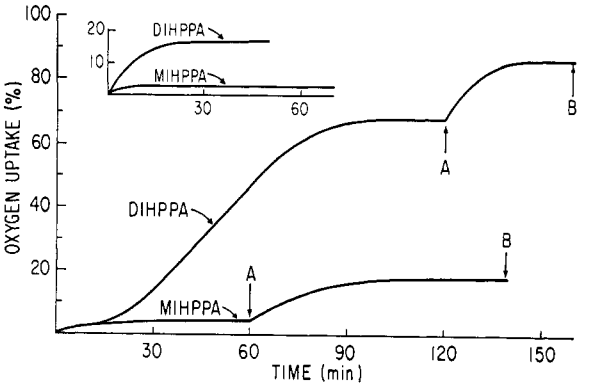


FIGURE 4: Oxygen uptake by MIHPPA and by DIHPPA. A 0.5×10^{-3} M solution of MIHPPA (pH 8.4) in 0.2 M sodium borate buffer was treated at 2° with air. A 0.5×10^{-3} M solution of DIHPPA (pH 7.4) in a mixture of 0.2 M boric acid–NaOH and 0.2 M sodium phosphate buffer (the latter being added to keep the pH constant over extended periods of time) was treated at 2° with air. Oxygen was substituted for air at A and nitrogen was bubbled through the solution for 30 min at B. The solutions were then again treated with oxygen (insert). The oxygen uptake curves shown in the insert are corrected for the amount of dissolved oxygen.

oxygen. It rather suggests that the oxidative conversion of MIHPPA into the T_3 precursor is a reversible process.

If this is so then the reaction mixture after cessation of oxygen consumption at pH 8.4 must consist essentially of about one-fifth of the T_3 precursor and about four-fifths of starting material. The gas chromatographic analysis of the reaction mixture showed that this is indeed the case. The area under the main peak (Figure 3A) of 3-(4-hydroxy-3-iodophenyl) lactic acid (**2a**) derived from MIHPPA by borohydride reduction comprises 81% of the total area. The area under the peak of 3-(4-hydroxy-3-iodophenyl)glyceric acid (**2h**) derived from the T_3 precursor **1a** by borohydride reduction (including a few minor peaks apparently derived from decomposition products of the T_3 precursor, since these products contain an oxygen function in the *para* position or adjacent to the aromatic ring) represents 19% of the total area. Figure 3B shows a gas chromatogram derived from the reaction mixture obtained in the autoxidation of DIHPPA at pH 7.4. The oxygen uptake was 96% of the theoretical amount. Here the major peak is that of 3-(4-hydroxy-3,5-diiodophenyl)glyceric acid (**2i**), derived from the T_4 precursor **1b** (96% of the total area including a few minor peaks apparently derived from decomposition products of the T_4 precursor). The peak of 3-(4-hydroxy-3,5-diiodophenyl)lactic acid (**2b**), derived from DIHPPA, comprises only 2% of the total area.

In a reversible autoxidation the yield of the oxidized product must depend on the oxygen pressure. This was found to be the case, not only in the autoxidation of MIHPPA but also in that of DIHPPA. Figure 4 shows the oxygen uptake curves for both keto acids when oxygen was replaced with air at the same pressure (1 atm). In air whose partial oxygen pressure is only one-fifth that of oxygen, a much lower plateau was reached than in oxygen, *i.e.*, at about 4% and 65–70% of the theoretical amount of oxygen for MIHPPA and DIHPPA, respectively. When oxygen was substituted for air after the plateau had been attained, oxygen was again taken up until a new plateau was reached at about 20% (MIHPPA) and 90% (DIHPPA) oxygen uptake. Then nitrogen (containing less than 0.0015% oxygen) was bubbled through the solution for 30 min, after which time the solution was again capable of consumming oxygen (Figure 4, insert). This shows that the reactions of MIHPPA and of DIHPPA with oxygen are at least partially reversible.

In another set of experiments oxygen was bubbled through a 5×10^{-3} M ice-cooled solution (2°) of MIHPPA (pH 8.4) or of DIHPPA (pH 7.4) in 0.2 M sodium borate. After 20 min, oxygen was replaced with nitrogen and after another 20 min, oxygen bubbling was resumed for 20 min. After each step, an aliquot was analyzed by gas–liquid partition chromatography and the ratio of the areas of the peaks derived from the hormone precursor to those of the peaks derived from the starting keto acid was determined. The data summarized in Table II show not only that DIHPPA is much more efficiently converted into the T_4 precursor than MIHPPA into the T_3 precursor, but also that in both cases the ratio of the hormone precursor to the starting keto acid is altered in favor of the latter after nitrogen bubbling and in favor of the former after renewed oxygen bubbling. It should, however, be pointed out that even after prolonged nitrogen bubbling (3 hr) the amount of oxygen taken up was considerably less than the theoretical amount calculated on the basis of a complete reconversion of the hormone precursor into the starting keto acid.

The oxygen uptake experiments in oxygen and in air indicate that the autoxidative conversion of MIHPPA and of DIHPPA into the respective hormone precursors are reversible reactions and that the steady-state equilibrium under the given experimental conditions lies about four-fifths toward the keto acid in the case of MIHPPA and nearly entirely toward the hormone precursor in the case of DIHPPA. An increase in oxygen pressure should therefore affect the position of the equilibrium only slightly in the latter case but appreciably in the former. This was found to be the case. MIHPPA and DIHPPA were treated with oxygen at 1, 3.8,

TABLE III: Dependence of T_3 and of T_4 Yield on Oxygen Pressure.

Method[a]	O_2 Pressure (atm)	Yield T_3	Yield T_4
A	1	8.5	
A	1		38
B	3.8	15	
B	119		39
B	127	27	
C	131		42
C	126	27	

[a] See Experimental Section.

and 120–130 atm. The yields of T_3 and of T_4 after coupling with I_2Tyr were determined in each case (Table III). While the yield of T_4 is only a few per cent higher at 120–130 atm than at 1 atm, that of T_3 is doubled at 3.8 atm and more than tripled at 126–127 atm. In each experiment aliquots were analyzed by gas–liquid partition chromatography after treatment with oxygen for 50 min. The size of the peak of the glycol **2h**, which gives a measure of the amount of T_3 precursor formed, increased with increased oxygen pressure as had been anticipated.

Discussion

DIHPPA reacts with I_2Tyr in the presence of oxygen at or below room temperature and near neutrality (pH 7.4) to form T_4 in 40% yield. When, in this nonenzymic model reaction for the biosynthesis of T_4, DIHPPA is replaced with MIHPPA, T_3 is formed instead of T_4. The yield of T_3, however, is only 2% (Shiba and Cahnmann, 1964). The considerable difference in the yield of the two thyroid hormones reflects a similar difference in their abundance in the thyroid. We suspected that the low yield of T_3 might be due to the fact that only the phenolate anion of the keto acid can be converted to the corresponding hormone precursor (Nishinaga *et al.*, 1968) and that at pH 7.4 DIHPPA is probably almost entirely ionized while MIHPPA exists mainly as the un-ionized phenol. It is well known that the p*K* of *o*-monoiodophenols is nearly 2 pH units higher than that of the corresponding *o*-diiodophenols (Herriott, 1947; Gemmill, 1955; Mayberry *et al.*, 1965). In order to check this hypothesis, the oxygen uptake of MIHPPA was measured at various pH values between 7 and 12 and in each case the yield of T_3 determined after treatment of the oxygenated reaction mixture with I_2Tyr under identical conditions.

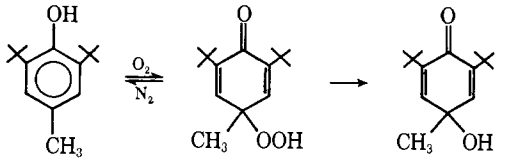
An optimal yield of T_3 was obtained when MIHPPA was treated with oxygen at pH 8.4. This is one pH unit higher than the optimal pH for the formation of the T_4 precursor. The yield of T_3 at pH 8.4, however, is still only 8% as compared with a 40% yield of T_4 under optimal conditions.

The most striking difference between the oxygen uptake curves of DIHPPA and of MIHPPA under optimal conditions for the formation of the corresponding hormone precursor is that in the case of DIHPPA a plateau is reached after nearly 1 mol of oxygen/mol of keto acid has been consumed, while in the case of MIHPPA a plateau is reached after only 0.2 mol of oxygen/mol of keto acid has been taken up. This suggests that the oxidative conversion of MIHPPA into a T_3 precursor is a reversible reaction.

Another remote possibility had to be considered, however, *viz.* that the early plateau formation in the case of MIHPPA and the low yield of T_3 might be due to exhaustion of the enol tautomer which alone is efficiently converted into the T_3 precursor (*cf.* Nishinaga *et al.*, 1968). Such an exhaustion is conceivable only if MIHPPA in a solution of pH 8.4 consists mainly of the keto tautomer, even in the presence of borate ions, and if the enol–keto equilibrium, when disturbed, is reestablished only extremely slowly at that pH. This was found not to be the case. The nuclear magnetic resonance spectra of MIHPPA in organic solvents show that crystalline MIHPPA consists entirely of enol. The enol–keto ratio in the MIHPPA solution used for the oxidation experiments was found to be about 1:1 at equilibrium and the rate with which this equilibrium is approached to be three to four times faster than that with which the enol–keto equilibrium is approached in a solution of DIHPPA at pH 7.4.

That not only the conversion of MIHPPA into the T_3 precursor but also that of DIHPPA into the T_4 precursor are reversible reactions was confirmed in various ways. Gas chromatographic evidence indicated that the reaction mixture obtained after treatment of MIHPPA with oxygen consisted essentially of four-fifths of starting material and one-fifth of the hydroperoxide **1a** (T_3 precursor) and that the reaction mixture obtained in the autoxidation of DIHPPA consisted almost entirely of the hydroperoxide **1b** (T_4 precursor). When oxygen was replaced with air the plateau was much lower (less oxygen uptake). When the oxygen pressure was increased, a somewhat higher plateau was reached in the case of DIHPPA and a considerably higher one in the case of MIHPPA. That the increased oxygen uptake means formation of more hormone precursor was shown by the increased yield of T_4 and particularly of T_3 as well as by direct gas chromatographic evaluation of the amount of hormone precursor formed. Furthermore, when nitrogen was bubbled through a solution containing the maximal amount of hormone precursor obtainable at a given oxygen pressure (plateau region), the reaction mixture was again capable of consuming oxygen. The observation that the reverse reaction, the formation of keto acid from the hydroperoxide, was never complete requires further investigation. Even after prolonged nitrogen bubbling, the amount of oxygen taken up was always considerably less than the amount of oxygen consumed by the pure keto acid.

A literature search revealed that the reversibility of the formation of hydroperoxides is not generally recognized. Only two references were found in which such a reversible reaction is mentioned. One of these refers to a reaction in the crystal lattice, not in solution (Janzen *et al.*, 1967). The other one (Gersmann and Bickel, 1962) describes the partial reversibility of the oxidation of 2,6-di-*t*-butyl-4-methylphenol (**3**) to the hydroperoxide **4**. It was possible to recover 70% of **3** by bubbling nitrogen through a solution of **4**. As a side product the quinol **5** was formed. The irreversible conversion of hydroperoxides into the corresponding hydroxy compounds



3 4 5

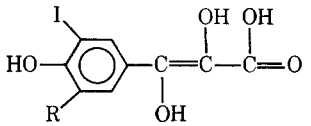
is a well-known reaction (*e.g.*, Kharasch and Joshi, 1957; Ley, 1958; Musso and Maassen, 1965; Musso *et al.*, 1965; Musso and Zunker, 1968; Allara *et al.*, 1968). Apparently the hydroperoxide first undergoes homolytic fission (eq 1). The

$$\mathrm{ROOH} \xrightarrow{-\mathrm{H}\cdot} \mathrm{ROO}\cdot \qquad (1)$$

$$\mathrm{ROO}\cdot \xrightarrow[+\mathrm{H}\cdot]{-\mathrm{O_2}} \mathrm{RH} \qquad (2)$$

$$2\mathrm{ROO}\cdot \xrightarrow[+2\mathrm{H}\cdot]{-\mathrm{O_2}} 2\mathrm{ROH} \qquad (3)$$

hydroperoxy radical thus formed then loses oxygen and forms either the starting compound (eq 2) or a hydroxy compound (eq 3). The hydroxy compounds that correspond to the hydroperoxides **1a** and **1b** are the enediols **6a** and **6b**, respectively. These enediols cannot be distinguished from

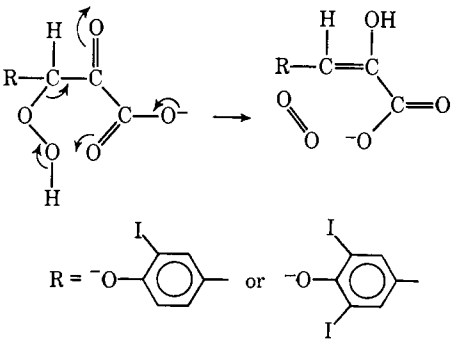


6a, R = H
b, R = I

the corresponding hydroperoxides by gas–liquid partition chromatography, since sodium borohydride reduces both compounds to the same glycols **2h** and **2i**, respectively. In this connection it should be pointed out that contrary to expectations a glycol peak (**2h** or **2i**) was always present in chromatograms of the reaction mixtures obtained by coupling the hydroperoxides **1a** or **1b** with I_2Tyr. It seems therefore possible that the coupling reaction is an oxido–reductive process in which the hydroperoxide is only partly converted into T_3 or T_4 and partly reduced to the enediol **6a** or **6b**. It is of interest to note that under optimal conditions the coupling yield approaches but never exceeds 50% based on the hydroperoxide **1a** or **1b**. As had been pointed out previously (Nishinaga *et al.*, 1968), oxygen is only required for the conversion of the keto into the hydroperoxide but not for the coupling of the latter with I_2Tyr. Nevertheless, yields of T_3 and T_4 were always slightly lower when oxygen was replaced with nitrogen prior to the coupling with I_2Tyr. This is no doubt due to the reversibility of the conversion of the keto acid into the hydroperoxide.

The finding that the conversion of MIHPPA and DIHPPA into the corresponding hormone precursors are reversible processes is of practical as well as theoretical interest. The practical interest lies in the fact that application of pressure permits the synthesis of T_3 in reasonably good yield. While the conventional synthesis by partial iodination of 3,5-diiodothyronine is still preferable for the preparation of unlabeled T_3 or of T_3 labeled with iodine in the phenolic ring, the coupling procedure described in this paper is superior for the synthesis of T_3 labeled with iodine in the nonphenolic ring and particularly for the synthesis of T_3 labeled with carbon either in the phenolic or in the nonphenolic ring or in both.

The only reference to a reversible peroxidation in solution that we found in the literature (Gersmann and Bickel, 1962) deals with the solution of an aromatic hydroperoxide in an organic solvent. The reversible peroxidation of MIHPPA and of DIHPPA takes place in the aliphatic side chain and in aqueous solution. Whether or not the reversibility of the formation of hydroperoxides is a general phenomenon remains to be investigated. It may in many cases have escaped attention because the reverse reaction is superimposed by other decomposition reactions such as the formation of hydroxy compounds (*e.g.*, quinols). Dr. R. Criegee (personal communication, 1968) has suggested that the regeneration of MIHPPA and of DIHPPA from their hydroperoxides might be a cyclic process:



It seems difficult to explain the reversibility of the hydroperoxide formation by this interesting mechanism. A free radical mechanism appears more likely. In such a mechanism the hydroperoxide undergoes homolytic fission with formation of the hydroperoxy radical which reversibly forms the phenoxyl radical and oxygen. Conversely the phenoxyl radical is the first step in the conversion of the keto acid into the hydroperoxide.

Two possible mechanisms for the biosynthesis of the thyroid hormones are at present under consideration. One is the coupling of a I_2Tyr residue with a 3-iodotyrosyl residue or with another I_2Tyr residue in thyroglobulin. The other one is the coupling of a I_2Tyr residue in thyroglobulin with MIHPPA or DIHPPA. The reactions described in the present paper are nonenzymic models for the second mechanism. This mechanism requires the formation of these keto acids in the thyroid either from *p*-hydroxyphenylpyruvic acid by iodination or from mono- or diiodotyrosine by oxidative deamination or transamination. The thyroid contains an amino transferase which, in the presence of α-ketoglutarate, catalyzes the transamination of tyrosine and of monoiodotyrosine and, less efficiently, that of I_2Tyr (Igo *et al.*, 1968). A keto–enol tautomerase has been isolated from thyroid tissue (Blasi *et al.*, 1969a). Such a tautomerase is required to catalyze the interconversion of the keto and enol tautomers of *p*-hydroxyphenylpyruvic acid, MIHPPA, and DIHPPA. Only the enol is efficiently converted into the T_3 and T_4 precursors. Enzymes that catalyze the formation of hydroperoxides by

fixation of oxygen to unsaturated compounds (lipoxidases) are widely distributed in nature (Mason, 1957; Tappel, 1963). Enzymes that catalyze the transfer of a hydroperoxy group from hydrogen peroxide to organic acceptors have not been described although nonenzymic "transperoxylations," catalyzed by copper salts, have been carried out (Paul, 1963). Recent work by Blasi *et al.* (1969b), however, provides circumstantial evidence that peroxidases such as horseradish or thyroid peroxidase, in the presence of a hydrogen peroxide generating system, catalyze the conversion of DIHPPA into a T_4 precursor (presumably the hydroperoxide described by us) which is capable of coupling with I_2Tyr anaerobically to form T_4. It appears then that the thyroid contains all the enzymes required for the conversion of mono- and diiodotyrosine into the respective keto acids, the keto–enol tautomerization of these keto acids, and the peroxidation of the enol tautomers to the T_3 and T_4 precursors.

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