Production of Diiodohydroxyphenylpyruvic Acid from Diiodotyrosine by Nonenzymatic Reaction*

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Synopsis

The nonenzymatic synthesis of 4-hydroxy-3, 5-diiodophenylpyruvic acid (DIHPPA) from diiodotyrosine is reported in this paper. The reaction was carried out *in vitro* in the presence of pyridoxal and metal ion. The yield of DIHPPA at 37°C for 2 hrs. and at 100°C for 30 mins. were almost the same. Among metal ions used in the present study, Cu^{++} was found to be most effective. Co^{++} was also effective, while other ions, Al^{+++} , Zn^{++} , Mn^{++} , Ni^{++} , Fe^{++} and Fe^{+++} were only very slightly effective. The results obtained in this study suggest that the nonenzymatic reaction possibly takes place also *in vivo*.

It has been suggested that thyroxine (T_4) is synthesized from the reaction between 3,5-diiodotyrosine (DIT) and 4-hydroxy-3,5-diiodophenylpyruvic acid (DIHPPA), which is formed by the action of l-amino acid oxidase (Yip and Klebanoff, 1962) or peroxidase (Harrath, 1962) on DIT (Meltzer and Stanaback, 1961; Toi *et al.*, 1963). The nonenzymatic conversion of DIT to T_4 in the presence of DIHPPA and oxygen was reported by several authors (Toi *et al.*, 1963; Hillmann, 1956; Shiba and Cahnmann, 1962). It is the purpose of the present study to investigate if the conversion of DIT to DIHPPA takes place also in the absence of any enzyme.

Materials and Methods

Enzymatic synthesis of DIHPPA

Experiments were carried out *in vitro* by using a slight modification of the method of Fisher *et al.* (1965). One μCi of ^{131}I -DIT obtained from RCC was added to the incubation medium consisting of 10 mM l-DIT, 1.67 mM pyridoxal phosphate, 3.33 mM MgCl_2 , 10 mM d-glucose, 16.7 mM Tris(hydroxy-methyl)aminomethane, 0.67 mM ethylenediamine tetraacetate, 200 μg of glucose oxidase and 25 mg of bovine thyroid microsomal protein (Fischer *et al.*, 1963). The final volume was 3 ml and the pH was adjusted to 8.5. After incubation at 32°C for 2 hrs., the reaction was stopped by the addition of 1 ml of 2.5 M hydroxylamine hydrochloride. The acidic mixture was thoroughly extracted with n-butanol saturated with H_2O . The butanol extract was evaporated to dryness and the residue was dissolved in 0.2 ml of 30% alcoholic NH_4OH . Samples were chromatographed on Toyo No. 50 filter paper in a solvent system of n-butanol-acetic acid- H_2O (78 : 5 : 17). Spots were detected by staining with Mandl-Block's method for iodide (Mandl and Block, 1959). The paper was then cut into pieces of 0.5 cm in width and the radioactivity of each piece was determined by a

Received for publication December 7, 1968.

*Dedicated to professor Yosoji ITO in commemoration of his unselfish devotion for over fifteen years to the advancement of endocrinological science, through the editorial management of Endocrinologia Japonica.

Nuclear Chicago Scintillation Counter. The paper was then extracted with alkaline solution, and the quantity of each component was determined spectrophotometrically by using a Hitachi-UV Spectrophotometer.

Nonenzymatic synthesis of DIHPPA

One μCi of ^{131}I -DIT was added to the medium consisting of 10 mM DIT, 20 mM pyridoxal hydrochloride and 5 mM metal ion. Metal ions used in these experiments were Cu^{++} ($\text{Cu}(\text{NO}_3)_2$), Co^{++} (CoCl_2), Al^{+++} (AlCl_3), Zn^{++} (ZnSO_4), Mn^{++} (MnCl_2), Ni^{++} (NiCl_2), Fe^{++} (FeSO_4) and Fe^{+++} (FeCl_3). The total volume was 4 ml and the pH adjusted to 5.0, 7.0, 7.5, 8.0 and 10.0. After incubation at 37°C for 2 hrs. or at 100°C for 30 mins., the sample was made alkaline by the addition of 0.04 M KOH. The sample was immediately applied on Toyo No. 50 filter paper and chromatographed in a solvent system of dioxane-n-butanol-2N NH_4OH (1 : 4: 2), of sec. butanol-3.2N NH_4OH (3 : 1) or of n-butanol-acetic acid- H_2O (78 : 5 : 17). Further procedures for the measurement of the radioactivity were the same as used in the enzymic study. The identification of DIHPPA was performed by Rf on paper chromatogram and by the presence of maximal absorption at 340 $\text{m}\mu$.

Results

1) Enzymatic conversion of DIT

On paper chromatography developed with n-butanol-acetic acid- H_2O , DIHPPA migrates very close to the spot of T_4 . These two spots were combined together as a reaction product from DIT.

The yield of the reaction product calculated in terms of the ratio of the radioactivity of the reaction product to the sum of the reaction product and DIT was about 20% in the presence of thyroid microsomal protein. About

the same yields were obtained without thyroid microsomes as well as when the microsomes added were boiled at 100°C for 20 mins. before use. Withdrawal of pyridoxal from the incubation medium markedly decreased the yield of the reaction product (Table 1).

Fig. 1. Paperchromatogram of mixture consisted of DIT, pyridoxal hydrochloride and $\text{Cu}(\text{NO}_3)_2$ developed in a solvent system of dioxane-n-butanol-2N NH_4OH
a: chromatogram of mixture
b: chromatogram after addition of alkaline solution to mixture
c: chromatogram of DIHPPA in ethanol

Table 1. Effect of compositions of medium on yield of T_4 performed by the modified method of Fischer *et al.*

Composition of medium	$\frac{\text{T}_4}{\text{DIT} + \text{T}_4} \%$
presence of thyroid microsome	20.0
absence of thyroid microsome	19.3
presence of thyroid microsome boiled for 20 min.	17.1
without pyridoxal	3.9

2) Nonenzymatic conversion of DIT into DIHPPA.

1. Chromatographic behavior of DIT in the reaction mixture

When the reaction mixture of DIT, pyridoxal and metal ion in neutral or acidic medium was applied on paper chromatography, a spot of Rf 0.5 was detected with iodide staining in addition to the spot of original DIT (Fig. 1a). When the mixture was made alkaline and then chromatographically analyzed, another spot of Rf 0.8 appeared (Fig. 1b). The latter spot of Rf 0.8 was identified as DIHPPA (Fig. 1c). Presumably, the spot of Rf 0.5 was diiodotyrosine-pyridoxal-metal complex, and this complex was easily decomposed into diiodohydroxybenzaldehyde and pyridoxamine in alkaline solution.

2. Conversion of DIT into DIHPPA during incubation

When the mixture of DIT, pyridoxal and Cu^{++} was made alkaline and immediately analyzed on chromatography, 7.7% of DIT was converted into DIHPPA. When the mixture was incubated at 37°C for 2 hrs. at pH 7.5 and then treated with alkaline, 10.2% of the original radioactivity appeared with DIHPPA (Table 2).

Table 2. Effect of conditions of incubation on yield of DIHPPA

Condition of incubation	yield of DIHPPA (%)*
immediately after sampling	7.7
at 37°C for 2 hrs.	10.2
at 100°C for 30 min.	12.2

*yield of DIHPPA

$$= \frac{\text{radioactivity in area of DIHPPA}}{\text{total radioactivity on paper chromatographed}}$$

When the mixture was treated at 100°C for 30 mins. and then made alkaline, 12.2% of the original radioactivity was found with DIHPPA.

3. Effect of pH on the conversion of DIT into DIHPPA during the incubation

At pH between 5.0 and 8.0, the net synthesis of DIHPPA was almost constant (Table 3). At pH higher than 10.0, the yield of DIHPPA was slightly increased.

4. Effect of metal ions

The mixture of DIT and pyridoxal was incubated at 37°C for 2 hrs. at pH 7.5 in the presence of various metal ions. The sample was then made alkaline and analyzed on paper chromatography. The presence of Cu^{++} or Co^{++} were favorable for the conversion of DIT into DIHPPA, while the other metal ions did not show a marked effect on the conversion even in the alkaline medium (Fig. 2).

Table 3. Effect of pH on yield of DIHPPA from DIT

pH	yield of DIHPPA (%)*
5.0	10.1
7.0	10.2
7.5	10.2
8.0	10.2
10.0	11.3

* see Table 2.

Fig. 2. Effect of metal ions on yield of DIHPPA

Discussion

The precise mechanism of the synthesis of thyroxine from diiodotyrosine has not yet been established. The following pathways have been proposed (De Groot, 1966):

- 1) The formation of dityrosine by peroxidase attack (Gross and Sizer, 1959).
- 2) 2,6-diiodobenzoquinone produced by the action of peroxidase on DIT is an intermetabolite of T₄ synthesis (Ljunggren, 1963).
- 3) T₄ is synthesized through the coupling of DIT and DIHPPA which is formed by the action of l-amino acid oxidase (Yip and Klebanoff, 1962) or of peroxidase (Harrath, 1962) on DIT (Fischer et al., 1966).
- 4) The oxidation of tyramine by monoamine oxidase provides hydrogen peroxide required for iodination of tyrosine and iodothyronine synthesis.

Among these hypothesis, the synthesis of T₄ through the coupling of DIT and DIHPPA has been supported by more convincing evidences. DIHPPA was detected in the normal thyroid tissue (Haney and Lissitzky, 1962; Surks et al., 1968). Nonenzymatic synthesis of T₄ from the reaction between DIT and DIHPPA *in vitro* was reported by Hillmann, by Toi and by Shiba independently. The reaction possibly takes place also *in vivo* in physiological conditions. DIHPPA was chemically synthesized by Bergmann et al., (1931) and by Tong et al., (1954). However, the conditions used for the chemical synthesis were not so mild as might be expected for bio-synthesis.

The results of the present investigation reveal that DIHPPA was formed nonenzymatically with a considerably high yield from DIT in a simple medium containing pyridoxal and Cu⁺⁺. Snell and Jenkins (1959) presented a schematical illustration of the catalytic effect of pyridoxal on the conversion of amino acid into the corresponding keto compound. The conversion of DIT into DIHPPA can also be explained by the same mechanism. Present data agree well with the results of Longenecker and

Snell (1957) showing that Cu⁺⁺ demonstrates the most potent action for chelation and catalysis in transamination reaction involving amino acid.

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