Isolation and Characterization of a Coupled Compound of Thiamin with Catechol

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Summary Equimolecular amounts of thiamin and catechol incubated under suitable conditions produced an unknown reaction product isolated as a light yellow powder from the reaction mixture by reverse-phase ODS column chromatography.
Elemental analysis of the purified powder showed the molecular formula to be C18H22N4O4S·HCl·H2O in agreement with a molecular ion peak found at m/e 390 in the mass spectrum. These results together with the data of IR and NMR spectrometry possibly show that this material consists of an isomeric mixture of S-3,4- and S-2,3-dihydroxyphenylthiamin (I and II) which probably arise as a result of oxidative addition of the thiol group of thiamin to catechol.

Key Words thiamin, catechol, thioether

In our previous paper (1), high-pressure liquid chromatograms were presented showing that a considerable amount of unknown soluble products was formed by incubation of thiamin with catecol at pH 7.4. These soluble products, which are presumably reaction intermediates, are converted into an insoluble black polymer as final product.

Thiamin couples with several compounds forming sulfide or disulfide linkages. However, the reaction intermediates of thiamin with catechol cannot be converted to thiamin by reduction with cysteine, whereas thin-layer chromatographic studies (2) suggest that the intermediates contain the pyrimidine moiety of thiamin and catechol molecules.

In the present work, we attempted to isolate one of the main intermediates formed during the incubation of thiamin with catechol at pH 7.4, and investigated its chemical structure and the reaction mechanism involed.
METHODS

**HPLC analysis of reaction products.** A reverse-phase partition mode of high-pressure liquid chromatography (HPLC) was used for the detection of reaction intermediates. Instrument: a Waters 45 pump; column, Chemco ODS-7 (4.6 × 150 mm); temperature, ambient; detection wavelength, 245 nm; eluent, 20% methanol adjusted to pH 2.3 with HCl; flow rate, 1.0 ml/min.

**Reverse-phase open column chromatography for isolation.** For isolation of the reaction intermediates, conventional glass columns packed with ODS I-40/64 (Yamamura Chemical Co.) were used. The adsorbed reaction products were eluted with acidic methanol-water (adjusted to pH 2.5 with HCl). Each of the fractions was monitored by HPLC using the same solvent. The fractions containing the intermediates were pooled and lyophilized directly.

**Measurement of physical properties.** NMR spectrum was recorded on a Varian XL-100 spectrometer (δTMS = 0 ppm, solvent DMSO-d₆) using tetramethylsilane as an internal standard. IR spectrum was measured in nujol with a Hitachi 260-10 spectrometer. Mass spectrum was taken on a JEOL JMS-01SC. UV spectrum was measured with a Shimadzu UV-300 spectrometer.

RESULTS

**Isolation of the reaction intermediates**

To obtain a large amount of intermediates, 4 mmol of thiamin and 4 mmol of catechol were dissolved in 1,250 ml of 0.1 M phosphate buffer (pH 7.4) and incubated at 50°C for 32 h. About 9% of the thiamin added was degraded during the incubation. The high-pressure liquid chromatogram for the reaction mixture after 32 h incubation is shown in Fig. 1. It indicates the formation of two intermediates which have tₚ 4.2 and 5.1 respectively. They were tentatively designated compound B for tₚ 4.2 and compound A for tₚ 5.1.

Isolation of compound A was carried out using the procedure shown in Fig. 2. First, the reaction mixture was directly applied to an open ODS column (1.1 × 10.5 cm). As Fig. 3 shows, the column was washed with water to remove unreacted thiamin and catechol (fractions 0–40), and was then eluted with acidic 25% methanol (pH 2.3), which produced a second peak (fractions 50–60). The fractions indicated by the brackets were pooled and lyophilized. A part of the lyophilized light yellow powder was dissolved in 5% methanol and subjected to HPLC as a test. Since 2 peaks, A and B, were well separated as Fig. 6 (I) shows in this test, the rest of the light-yellow powder was applied to a second reverse-phase column (1.1 × 9.9 cm) and subjected to a gradient elution with 5–25% acidic methanol (pH 2.3) (Fig. 4). Fractions numbered 12–18 (indicated by brackets) were collected and lyophilized. HPLC of this pooled fraction showed compound A as the main component (Fig. 6 (II)). To eliminate small amounts of compound B, the pooled fraction (12–18) was subjected to a third session of reverse-phase column chromatography.
THIAMIN DEGRADATION BY DIHYDROXYPHENOL

Fig 1. Profile of HPLC for the reaction mixture. Four mmol of thiamin and 4 mmol of catechol were incubated 50°C for 32 h in 0.1 M phosphate buffer, pH 7.4. Ten μl of reaction mixture was applied on HPLC column (Chemco ODS-7). Details are described in METHODS. A, compound A; B, compound B.

Fig. 2. Procedure for isolation of reaction intermediates. ph, phosphate buffer.

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Fig. 3. Chromatogram of the reaction mixture on ODS column. The reaction mixture described in Fig. 1 was applied to a column (1.2 × 10.5 cm) packed with ODS I-40/64. After washing with H₂O, the column was eluted with 25% methanol (pH 2.3) at flow rate 40 ml/h. Fraction (No. 54-60) was applied to HPLC (Fig. 6 (I)).

Fig. 4. Separation of compound A and B by ODS chromatography. Pooled fractions 54–60 in Fig. 3 were lyophilized, dissolved in 10 ml of 5% methanol, then applied on ODS I-40/64 (1.1 × 9.5 cm) column. Compound A was purified by gradient elution of 5–25% methanol (pH 2.3) at flow rate 40 ml/h. Fraction (No. 12–18) was applied to HPLC (Fig. 6 (II)).

Fig. 5. Elution profiles of compound A in the ODS chromatography. Fractions 12–18 in Fig. 4 were applied on an ODS I-40/64 column (1.1 × 9.5 cm), then eluted with 25% methanol (pH 2.3) at flow rate 40 ml/h. Fraction (No. 20–30) was applied to HPLC (Fig. 6 (III)).
Fig. 6. HPLC profiles of compound A.
I: For fraction numbers 54–60 shown in Fig. 3.
II: For fraction numbers 12–18 shown in Fig. 4.
III: For fraction numbers 20–30 shown in Fig. 5.

Fig. 7. UV absorption spectrum of purified compound A.
---, pH 2.35; -- - , pH 5.82; ----, pH 10.87.

Physical properties and chemical composition of compound A
The purified compound $t_R$ 5.1 was hygroscopic and readily soluble in water and methanol. Its UV spectra are shown in Fig. 7. The absorption maximum was 245 nm at pH 2.35.
Elemental analysis:
Calcd. for $C_{18}H_{22}N_4O_4S \cdot HCl \cdot H_2O$: C, 48.59; H, 5.66; N, 12.59
Found: C, 48.23; H, 5.70; N, 12.40
IR, NMR and mass spectrometry of compound A

a) Infra-red spectrophotometric analysis. The infra-red absorption spectrum of compound A is shown in Fig. 8. Absorptions at 1660 cm\(^{-1}\), 1640 cm\(^{-1}\), and 1050 cm\(^{-1}\), which are attributable to N-CHO, pyrimidine-NH\(_2\), and primary OH respectively, suggest the presence of the thiol-form in the thiamin skeleton.

b) NMR spectrometry. In the H-NMR spectrum of compound A shown in Fig. 9, absorptions of two methyl groups, the proton at the 6-position of the pyrimidine ring and the formyl group appear as doublets. This, together with the
chemical shifts of other peaks, suggests that compound A is an isomeric mixture of S-dihydroxyphenylthiamin.

c) Mass spectrometry. Mass spectroscopic studies of compound A showed a molecular ion peak at m/e 390. The base peak at m/e 122, which is characteristic of thiamin derivatives, is assigned to the 4-amino 2-methyl-5-pyrimidinylmethyl ion (Fig. 10).

DISCUSSION

For clarification of the mechanism of thiamin degradation by catechol derivatives, it is essential to purify reaction intermediates and characterize their chemical structures. The initial problem encountered in the purification of the intermediates was that these substances were not stable in the reaction mixture of thiamin and catechol at pH 7.0. This was overcome, in part, by use of reverse-phase column chromatography using a volatile solvent (HCl-methanol-water) as the mobile phase which was easily removed, avoiding the polymerization of unstable intermediates. Isolated compound A (an isomeric mixture) was more than 99.9% pure judging from HPLC (Fig. 6 (III)). We also tried to isolate compound B, but its purity could not be raised above the 90% level by the same method. The mass spectrum (Fig. 10), which showed a molecular ion peak at m/e 390, suggested that compound A might be derived from one molecule of thiamin and one molecule of catechol. Conjugation of thiamin and catechol was also supported by elemental

![Diagram](image)

Fig. 11. The mechanism of the degradation of thiamin by catechol.

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analysis and previous TLC data (1).

These results and the above-mentioned spectral properties (UV, IR, and NMR) may show that compound A is an isomeric mixture of two thiol-thiamin derivatives (I and II in Fig. 11) in which the thiol group is coupled to catechol by a thioether linkage. However, compound A may possibly have another coupled structure.

The degradation of thiamin by catechol was suppressed by anaerobic conditions or reducing reagents (cysteine). From these results, it is postulated that compounds I and II formed as follows: the first step in the degradation of thiamin by catechol is assumed to be the oxidation of catechol into o-quinone. Addition of thiol groups of thiamin to the 4- or 3-position of o-quinone followed by the transfer of one unpaired electron as demonstrated in Fig. 11, would result in the formation of S-3,4- and S-2,3-dihydroxyphenylthiamin (1 and 11), respectively. However, the possibility of an electron mechanism instead of the above radical mechanism, cannot be excluded.

The occurrence of a thioether compound of DOPA (2,5-S,S-dicysteinyl dopa) has been reported by Ito and Nicol (3). They also revealed the formation of 3-S-cysteinyldopachalcone by the oxidative coupling of cysteine with catechol in the presence of tyrosinase and O2 (3). These results give support to the formation of S-thiaminyl catechol in our experiments.

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REFERENCES

1) Hayakawa, F., Ueda, K., and Murata, K. (1983): Detection of reaction products of thiamin degradation by catechol derivatives. J. Nutr. Sci. Vitaminol., 30, 319–326.
2) Hayakawa, F., and Murata, K. (1981): Interaction of thiamin and its decomposition products with dihydroxy compounds. Vitamins (in Japanese), 55, 293–303.
3) Ito, S., and Nicol, J. A. C. (1975): 2,5-S,S-Dicysteinyldopa: A new amino acid in the eye of the gar and its enzymic synthesis. Tetrahedron Lett., 3287–3290.