Summary  It was observed, in vitro, that the water extract of the fermented-tea customarily chewed by Thai people has a similar thermostable thiamine-inactivating factor to that found in the water extract of fern. It was also observed that the percentage of thiamine disulfide formed from thiamine with some flavones, catechol, pyrogallol, caffeic acid, dihydroxophenylalanine, and hemin is greater at pH 7.5 than at pH 7.0. With some flavonoids, such as quercetin, rutin, and 6,7,4'-trihydroxyisoflavone, and pyrogallol, hemin, catechol and caffeic acid at pH 7.5, around 30-100% of thiamine is changed into thiamine disulfide. Water extract of shiitake, okra, coffee, black tea and fukinoto have only weak activities of thermostable thiamine-inactivating factors as a large percentage of thiamine disulfide is formed from thiamine even at pH 7.0.

2-Methyl-4-amino-5-aminomethylpyrimidine was isolated from the reaction mixture of 1 g thiamine with 20 mg catechol (1:0.5 mole) at pH 7.0, 45°C, and identified with the synthesized pyrimidine.

It has been nearly 30 years since results of studies on the thermostable thiamine-inactivating activity in ferns and brackens were reported by Weswig et al. (1) in America, Somogyi et al. (2) in Europe, and Miura et al. (3) in Japan. Cows and horses on ranches of areas where ferns and brackens were numerous, showed signs of thiamine deficiency. Since Japanese living in rural mountain areas have the custom of eating Zenmai (Royal ferns, Osmunda japonica Thunb) Warabi (Bracken, Pteridium aquilinum Kuhn) and Tsukushi (Horsetail, Equisetum arvense L.), Fujiwara et al. (4) studied whether the thermostable factors in Zenmai and Warabi are lost in the cooking water. It was concluded that cooked Zenmai and Warabi in the daily diet was acceptable.

Nakabayashi (5) isolated flavonoid pigments, astragalin and isoquercitrin from bracken and also demonstrated the presence of rutin, which they considered the thermostable antithiamine factor. On the other hand Fujita and his group (6-8) studied the mechanisms of thiamine inactivation with the thermostable factors, and reported that isolated crystals from the reaction mixtures of thiamine with some flavonoids were identical with thiamine disulfide, which is an active form of thiamine.

The above results on studies of thermostable thiamine-inactivating factors led to the study of factors in fern and bracken etc. and the reaction mechanisms of thiamine with flavonoids. In the meantime Berutter and Somogyi (9) reported isolation of 3,4-dihydroxycinnamic acid (caffeic acid) from the fern as one of the thermostable antithiamine factors. Davis and Somogyi (10) attempted to clarify the mechanism of the inactivation of thiamine by caffeic acid and observed that the reaction was made partially reversible by the addition of cysteine to the assay mixture, indicating that some thiamine disulfide might be formed. However, the remaining thiamine-inactivating mechanism with caffeic acid had not been clarified.

Since there are many o-dihydroxy compounds beside caffeic acid in food, reaction mechanisms of thiamine with caffeic acid, catechol,
and other dihydroxy compounds should be studied not only for the basic science involved but also for practical dietary considerations. Vimokesant et al. (11) observed that people in Thailand chew fermented-tea leaves showed signs of thiamine deficiency even though they ingested the normal daily requirements of thiamine.

We attempted to determine the reaction mechanisms of thiamine with some diphenol compounds including caffeic acid and flavonoids. Part of the results had already been reported (12) and data from further experiments is presented herein.

**EXPERIMENTAL**

1. Test for the thermostable thiamine-inactivating factors in fermented-tea and fermented-fish. Factors from fermented-tea obtained in Thailand and fern grown in Japan were extracted with 10–20 volumes of distilled water and reaction of thiamine with these extracts was the same as in the previous experiment (12). The activity of the thermostable thiamine-inactivating factors was calculated in dry base and compared with that of fern or shellfish.

2. Reaction condition of thiamine with thermostable factors. Water solution of thiamine hydrochloride and 1/15 M phosphate buffer (pH 7.0–7.5) was mixed with an equivalent mole of factors in water solution as shown in Table 1. The solution with a total volume of 3–4 ml was placed in a centrifugal tube with a diameter of 3.1±0.1 cm, sealed with a glass stopper, and incubated at 45°C for 24–72 hr. Samples were drawn at suitable intervals for the determination of remaining thiamine by the thiochrome method. Thiamine disulfide was estimated by determination of thiamine after treatment with cysteine as described in the table.

3. Isolation of 2-methyl-4-amino-5-amino-methylpyrimidine from the reaction mixture of thiamine with catechol. Since we previously observed that 0.2–0.5 equivalent moles of catechol with thiamine was sufficient to destroy nearly 100% of the thiamine at pH 7.0, 45°C for 48 hr (12), 1 g of thiamine hydrochloride was incubated with 20 mg catechol and adjusted to pH 7.0 during incubation. When the destruction of thiamine was almost complete, the pH was adjusted to 2.0. The reaction mixture was treated by column chromatography using Amberlite CG-50 buffered with 0.1 M acetate buffer (pH 5.4). After the remaining catechol was washed away with a large volume of water, 2-methyl-4-amino-5-aminomethylpyrimidine was eluted with 0.01 N HCl, tracing with optical density at 245 nm. Isolation of the crystals of the pyrimidine will be described in the results of the experiment.

**RESULTS**

1. Thermostable thiamine-inactivating activities (TIA) of fermented-fish and fermented-tea

Fermented-fish and -tea were purchased from a market in Bangkok and Chaingmai respectively, and brought to our laboratory and stored in a freezer until use. Extraction of the heatstable antithiamine factors in these samples and determination of the activities were carried out as described in **EXPERIMENTAL**. The activities were compared with those of fern and shellfish. The results were shown in Fig. 1. Optimum pH of TIA of the fermented-tea and a fern obtained in Japan was almost the same at pH 7.0. Activities in the fermented-fish and shellfish were rather weak compared with those of the fermented-tea and fern as seen in the figure.
2. Thiamine-decomposing activities with some thermostable factors

Thiochrome negative percentages of thiamine and formation of thiamine disulfide from thiamine with quercetin, rutin, catechol, pyrogallol, caffeic acid, hemin and dihydroxyphenylalanine (DOPA) were estimated at intervals in the condition of both pH 7 and 7.5.
As mentioned in our previous paper (12), some of the plant extracts and diluted tea extract produced large percentages in thiamine disulfide to the thiochrome negative form of thiamine. The experiment was repeated carefully and obtained the results as shown in Table 2. Only small percentages of thiamine were inactivated with these extracts as indicated in the last column of the table.

Table 2. Thiamine disulfide formation and thiochrome negative form of thiamine with thermostable factors in plants (at pH 7.0, 60°C, 1 hr).

| Samples                  | SSB1* / A     | Thiochrome negative form of thiamine (A) (%) | Thiamine decomposed (%) |
|--------------------------|---------------|---------------------------------------------|-------------------------|
| Fresh shiitake (Lentinus edodes Sing) | 86            | 17                                          | 2                       |
| Coffee                   | 72            | 18                                          | 4                       |
| Black tea                | 67            | 34                                          | 11                      |
| Okra (H. esculentus L.)  | 63            | 35                                          | 13                      |
| Butter flower stalk      | 40            | 25                                          | 15                      |
| (Pentastis Japonicus stalk or fukinoto) | 40            | 22                                          | 13                      |
| Carrot (yellowish)       | 30            | 47                                          | 33                      |

*a Thiamine disulfide.

3. Percentages in thiamine disulfide formation to the thiochrome negative form in the reaction mixture of thiamine with plant extracts at 45°C. The results are shown in Figs. 2, 3, 4 and 5, respectively. From these figures it is seen that thiochrome negative percentages of thiamine with caffeic acid are relatively as high as those of catechol, pyrogallol and DOPA and were especially higher at pH 7.5 than at pH 7.0.

4. Ratio in percentages of thiamine disulfide formation to thiochrome negative form of thiamine by treatment with thermostable factors.
As one of thermostable factors, 6,7,4'-trihydroxyisoflavone (Factor 2) with thiamine was tested at pH 7.5 as the isoflavone is not soluble in water at pH 7.0. It was revealed that almost all the thiochrome negative form of thiamine after the 24-hr reaction was reversible with cysteine as shown in Table 3. Also shown here are the data that all factors tested produced larger percentages in thiamine disulfide to the thiochrome negative form at pH 7.5 than those at pH 7.0.

Table 3. Ratio in percentage of thiamine disulfide to thiochrome negative form (at 45°C, 24 hr).

| Samples    | SSB/\text{TNF} \times 100 (%) |
|------------|-------------------------------|
|            | pH 7.5 | pH 7.0 |
| Factor 2   | 100     | —      |
| Futin      | 82.1    | 50.0   |
| Quercetin  | 45.7    | 26.9   |
| Pyrogallol | 48.7    | 48.1   |
| Hemin      | 41.8    | 28.9   |
| Catechol   | 32.5    | 20.0   |
| Caffeic acid| 27.4   | 19.3   |

a Thiamine disulfide.  
b Thiochrome negative form.

5. Isolation of 2-methyl-4-amino-5-aminomethylpyrimidine from the reaction mixture of thiamine with catechol

As shown in Fig. 6, pyrimidine was detected in the eluate of fraction number 720–860. The eluate contained mainly 2-methyl-4-amino-5-aminomethylpyrimidine with trace amounts of thiamine disulfide and thiochrome, which were detected using paper partition chromatography. Fraction number 750–800 was evaporated under a reduced pressure to a small volume, a few drops of ethylalcohol were added and this volume was placed in a refrigerator for 2–3 days. The pyrimidine was crystallized and the melting point of the crystals was 210–219°C. Mixed melting point of the crystals with synthesized 2-methyl-4-amino-5-aminomethylpyrimidine (a gift from Dr. Yurugi, Takeda Research Laboratory) was not decreased. \text{RF} value and UV spectrum of the isolated and synthesized crystals were also identical.

DISCUSSION

Although shellfish contain thiaminase, thermostable thiamine-inactivating activity was found to be lower than that of fern. Fermented-fish which is consumed by Thai people also have an low activity in thermostable thiamine-inactivating factor which is similar to that of shellfish. However, fermented-tea, which is chewed by the people in Thailand has a fairly high activity of thermostable thiamine-inactivating factor as shown in Fig. 1. Factors in the fermented-tea leaves have yet to be deter-
mined. At pH 7.0, thiamine with the water extract of fermented-tea, thiamine disulfide (about 23% of thiochrome negative form of thiamine) was formed from thiamine (data not included in Fig. 1). Factors in the fermented-tea leaves may thus be similar to the caffeic acid isolated from fern or other diphenol compounds such as catechol, some isoflavones, which have been studied as model experiments. In samples of shiitake, coffee, black tea, okra and fukinoto, high activity of thermostable thiamine-inactivating factor could not be demonstrated (Table 2). Fujita et al. (13) suggested that thiamine disulfide may be occurred from thiamine with the shiitake extract. Factors, which accelerate formation of thiamine disulfide from thiamine, have not been elucidated. Hemin reported has antithiamine activity as determined by the thiochrome procedure of Kundig et al. (14, 15). On the other hand, Kuo and Hilker (16) observed that hemin reacts with thiamine to form a fluorescent thiamine derivative, and used the term "thiamine-modifying activity" of hemin. As the derivative can be utilized as thiamine or changed to such in the body. The relationship between such fluorescent thiamine derivatives and the thiochrome negative form of thiamine reacted with hemin is the subject of future studies.

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