DIFFERENTIAL DETERMINATION OF THIAMINE AND ITS PHOSPHATES, HYDROXYETHYL-
THIAMINE AND PYRITHIAMINE, IN RAT BRAIN

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A reliable and convenient method for the fluorometric determination of free thiamine, the di- and triphosphate esters of thiamine (TDP + TTP), hydroxyethylthiamine (HET) and pyrithiamine (PTH) in individual rat brain tissue is presented. The thiamine phosphate esters, as well as HET and PTH, were extracted from the tissue by homogenizing the rat brain in cold 0.3 M HClO₄. The di- plus triphosphate esters of thiamine were isolated by passing an appropriate aliquot of the extract through a small column of Amberlite CG–50, a weak cation exchange resin. The samples were then dephosphorylated, placed on a Decalso column and eluted with hot 20% KCl in 0.1 N HCl.

Modifications of other methods were used to fluorometrically quantitate the thiamine, HET, and PTH in the brain tissue. There was no interference of HET or PTH in the determination of thiamine since the particular method used in this paper to oxidize thiamine to thiochrome did not cause HET or PTH to be oxidized to fluorescent compounds. There was no appreciable interference of thiamine and HET in the fluorometric determination of PTH until the ratio of µg of thiamine plus HET to µg of PTH in the assay mixture was 6/1. When the above ratio increased to 24/1, the fluorescent determination of PTH was 10% high. The fluorometric determination of HET in the presence of excess thiamine and PTH was 16 and 75% high when the ratio of µg of PTH/µg of HET in the assay solution was 20/1 and 83/1, respectively.

Pyrithiamine administration to rats causes rapid and extensive depletion of the brain thiamine stores(4, 6, 10). However, to date there have been no com-

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prehensive assays designed for the determination of the levels of total thiamine, free thiamine, the mono-, di- and triphosphate esters of thiamine, (TMP, TDP, and TTP, respectively), hydroxyethylthiamine (HET), or pyrithiamine (PTH) in the tissues of rats. MORITA et al. (5) presented a successful assay for total thiamine in the presence of PTH in rat tissue. MORITA et al. (8) reported a successful method for the simultaneous determination of total thiamine as well as total HET in rat tissue. However, the methods developed by MORITA et al. were not designed to determine the levels of the mono-, di- and triphosphate esters of thiamine. The method introduced by RINDI and GUISEPPE (9) is not suited for small samples of tissue, nor are the chromatographic materials readily available.

This report presents an analysis using readily available materials that is designed to determine the total thiamine, free thiamine, di- plus triphosphate esters of thiamine and HET and PTH in individual rat brain tissue.

MATERIALS

Thiamine chloride hydrochloride and pyrithiamine bromide hydrobromide were purchased from Sigma Chemical Company, St. Louis, Missouri. Dr. Morita of Japan donated hydroxyethylthiamine chloride hydrochloride as a gift. Permutit T or Thiochrome Decalso, a silicate cation exchange resin, supplied by Fisher Scientific Company, Fair Lawn, New Jersey, was used to purify the dephosphorylated tissue homogenates. Amberlite CG-50, a polymethylacrylic acid cation exchange resin, chromatographic grade, obtained from Mallinkrodt Chemical, St. Louis, Missouri, was used to separate TDP and TTP from TMP and free thiamine. Taka Diastase, a powdered preparation of Aspergillus oryzae enzymes, was manufactured by Parke Davis and Company, Detroit, Michigan.

Reagent grade isobutyl alcohol, purchased from Matheson, Coleman and Bell, Los Angeles, California, was distilled to reduce the background fluorescence to negligible levels. Deionized water was used in all analytical steps. All other chemicals used in this study were reagent grade quality. The white male Sprague-Dawley strain of rats were obtained from Sprague-Dawley, Inc., Madison, Wisconsin, and from Geneva Mott, Salt Lake City, Utah.

EQUIPMENT

The Turner Fluorometer Model 111, equipped with #7–60, #2A, and #47B filters, G. K. Turner Associates, Palo Alto, California, and the Farrand Spectrofluorometer attached to a Honeywell Elektronic 15 recorder from Farrand Optical Company, New York, were used for all fluorometric measurements.

Devices used to mix the aqueous solutions and the isobutyl alcohol include a Rotary Evapo-mix shaking unit, made by Buchler Instruments, Fort Lee, New Jersey, and a Vortex Genie mixing device, supplied by Scientific Industries, Inc., New York.
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METHODS

Extraction and neutralization of homogenate. Immediately after decapitation of the rat, the entire brain was removed, weighed, and placed in 10 ml of ice cold 0.3 M HClO₄ per g of wet tissue, and then homogenized for 3 min with a motorized Teflon pestle that fit snugly into a reinforced glass container immersed in an ice bath. The homogenate was then centrifuged at 5,900×g for 15 min. The pellet was rehomogenized in 2 ml of acid for 30 sec, centrifuged as before, and the supernatant extracts were combined. The tissue extracts were adjusted to pH 5–6 with 30% KOH, centrifuged to remove KClO₄, and then adjusted to pH 4–5 with 4 N pH 4.5 acetate buffer. This solution will be referred to in the following text as the neutralized tissue extract.

Isolation of thiamine di- and triphosphate. Approximately 1/6 of the neutralized tissue extract was placed in a glass column 1/4 inch in diameter with a 3-ml reservoir seated at the top. The column was filled 5 3/4 inches with Amberlite CG–50. This resin is equivalent to the Rexyn 102 used by SHARMA and QUASTEL (12). The resin was washed and prepared for use according to their procedure. After the aliquot had descended into the resin, 4 ml of distilled water was added to the column, which eluted the anionic thiamine di- and triphosphates from the resin while the free thiamine and thiamine monophosphate were retained on the column. The samples were then prepared for dephosphorylation.

Dephosphorylation. One ml of 4 N pH 4.5 acetate buffer and 50 mg of dry Taka Diastase were added to each sample; the tubes were covered with parafilm and incubated at 48–50°C for 3 hr. The samples were then passed through a Decalso column as described below.

Decalso purification. This procedure is a modification of the procedure reported by MORITA et al.(8). The Decalso resin was not washed, rinsed, or sized before use. About 1.5 g of Decalso was allowed to settle into a glass column, 1/4 inch inner diameter, with a 20 ml reservoir on the top. The column was equipped with a glass stopcock. After the column was washed with 25 ml of 0.5% acetic acid and 25 ml of water, the sample was poured into the column and allowed to drain into the resin. About 60 ml of boiling water was then poured through the column, and the eluate was discarded. The thiamine, HET, or PTH was eluted from the column with 25 ml of hot 20% KCl in 0.1 N HCl. The 20% KCl eluent was drained into a 25 ml volumetric flask. The column material was not reused; it was discarded after each run.

Preparation of total thiamine, HET, and PTH samples. About 1/6 of the original neutralized tissue extract was used for these determinations. This aliquot was not passed through the Amberlite CG–50 column but was prepared for dephosphorylation as previously described. This sample contains the free and phosphorylated species of thiamine, HET, and PTH.

Preparation of Free Thiamine Samples. About 2/3 of the volume of neutraliz-
ed tissue extract was used for the determination of free thiamine due to its low concentration in the tissue. This sample was not dephosphorylated and was placed directly onto the Decalso column for purification as previously described.

Calculations of thiamine, HET, and PTH levels. Thiamine, HET, and PTH were calculated as nanomoles of thiamine chloride hydrochloride (mol. wt. 337), HET chloride hydrochloride (mol. wt. 381), and PTH bromide hydrobromide (mol. wt. 420), respectively, per g wet tissue. The formula is defined as follows:

$$\text{nanomoles/g wet tissue} = \frac{A \times B \times C}{D \times E \times F \times G} \times 1000$$

\begin{align*}
A &= \text{fluorescent reading of samples minus blank} \\
B &= \frac{\mu\text{g of pure thiamine, HET, or PTH used as standards divided by the respective fluorescent reading of the standard minus blank}}{25}, \text{which is the volume of the sample eluted from the Decalso column with 20\% KCl in 0.1 N HCl} \\
C &= \text{aliquot of } C \text{ used in the assay} \\
D &= \text{total volume of neutralized tissue extract} \\
E &= \text{aliquot of extract used per assay} \\
F &= \text{appropriate molecular wt.}
\end{align*}

Fluorescent assay. The methods used to determine the levels of thiamine, HET, and PTH in the tissue homogenates are based on the specific oxidation of each to strongly fluorescent compounds, which can be selectively monitored at certain excitation and emission wavelengths. Appropriate aliquots, ranging from 3 to 8 ml of the respective sample purified on the Decalso column, were used so that about 0.1 \(\mu\text{g}\) of thiamine or HET and about 0.3 \(\mu\text{g}\) of PTH were fluorometrically assayed. Pure thiamine, HET, and PTH were dissolved in 20\% KCl in 0.1 N HCl and appropriate aliquots of these standards were oxidized at the same time and in the same manner as in the respective assays of tissue samples.

(Thiamine assay). This procedure is based on the methods reported by Morita et al. (8). An appropriate aliquot of the total thiamine sample, which was purified on the Decalso column, was placed in a 50-ml glass conical tube and mixed for 5 sec on a Genie vortex mixer with 0.3 ml of 1\% HgCl\(_2\). Two milliliters of 30\% NaOH was added to each tube to complete the oxidation of thiamine to thiochrome. The samples were mixed for 5 sec. The blank received only 2 ml of 30\% NaOH. Ten milliliters of distilled isobutyl alcohol was then added to each sample. The tubes were then mixed on a Rotary Evapo-mix shaking unit for 2 min in such a way that the liquid climbed the walls of the container and rotated rapidly around the tube. This mixing technique provided uniform and efficient extraction of the thiochrome from the aqueous layer into the alcohol layer. A similar extraction procedure was performed on the oxidized HET and PTH samples. The samples were centrifuged at 100 \(\times\) \(g\) and about 4 to 5 ml of the alcohol layer was placed in 75 \(\times\) 12 mm unmatched borosilicate or Pyrex tubes. The samples
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were placed in the door compartment of a Model 111 Turner Fluorometer and assayed at an excitation wavelength of 365 nm and emission wavelength of 436 nm. These wavelengths were obtained using a #7-60 filter on the excitation light and #2A and #47B filters on the emission light.

(HET assay). Another 5 to 8 ml aliquot of the total thiamine sample, which was purified on the Decalso column, was used for the HET determination. This oxidation procedure is also based on the assay reported by Morita et al. (8). Two milliliters of 30% NaOH was added to the sample aliquot. The solution was mixed for 5 sec on the Vortex mixer, mixed again for 5 sec with two drops of 0.01% HgCl₂, and then briefly mixed with 0.3 ml of 1% K₃Fe(CN)₆ in water. The blank prepared for the thiamine sample assay served as a blank for the HET determination also. The sample and blank were then extracted with 10 ml of distilled isobutyl alcohol and fluorometrically assayed on the Turner Fluorometer exactly as described previously for the thiamine assay.

(PTH assay). This oxidation procedure is a modification of the procedure reported by Morita et al. (5). Two milliliters of 1% K₃Fe(CN)₆ in 10% NaOH was added to 3 to 8 ml of the total thiamine sample that had been purified on the Decalso column. The solution was mixed for about 5 sec after which the pyrichrome was extracted into 10 ml of distilled isobutyl alcohol as previously described.

Four to five milliliters of the alcohol was transferred to a cuvette, and the fluorescence was monitored on the Farrand Spectrofluorometer using 430 nm excitation wavelength and 460 nm emission wavelength.

RESULTS AND DISCUSSION

Thiamine and HET assay

The assay for thiamine and HET in the presence of each other was previously reported by Morita et al. (8). Although Morita et al. (5) also reported an assay for PTH in the presence of thiamine, which was used with slight modifications in this paper, they did not comment on the feasibility of determining thiamine, HET, and PTH when all are together in the same tissue.

Using the assays presented in this paper, the brain thiamine and HET level, expressed as the mean determination of 10 rats ± standard error is 5.6 ± 0.2 and 0.8 ± 0.03 nanomoles/g wet tissue, respectively, for normal 110–130 g rats. Morita et al. (8) reported that the thiamine and HET levels of the cerebellum of a normal rat, expressed as the mean of five determinations ± the standard error, was 9.0 ± 0.18 and 1.4 ± 0.07 nanomoles/g wet tissue, respectively. Other investigators have reported similar values for total thiamine in normal rat brain (7, 9, 11), but

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1 The fluorescent compound produced when PTH is oxidized with K₃Fe(CN)₆ (see Fujita et al. (1)).
there have been no other successful quantitative determinations of HET in animal tissues.

It was found that 99.6 and 98.5% of the total thiamine and HET, respectively, were extracted from the brain tissue by the procedures described above (see Table 1). However, 16% of the HET was destroyed by the 3-hr 48°C incubation. Others have used a 37°C overnight incubation with no loss of HET (7, 8).

Table 1. Characteristic data of thiamine, HET, and PTH analysis.

<table>
<thead>
<tr>
<th></th>
<th>Thiamine</th>
<th>HET</th>
<th>PTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent extracted</td>
<td>99.6</td>
<td>98.5</td>
<td>—</td>
</tr>
<tr>
<td>from brain tissue</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Percent recovered from 48°C 3-hr incubation with Taka Diastase and dephosphorylated, deproteinated brain tissue homogenate. Each value represents the mean of 8 determinations.

97 84 100

* Thiamine, HET, and PTH added to previously dephosphorylated deproteinated brain tissue homogenate.

Table 2. Average percent error encountered in the thiamine, HET and PTH assay

<table>
<thead>
<tr>
<th>Thiamine assay</th>
<th>HET assay</th>
<th>PTH assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± Std Dev</td>
<td>Mean ± Std Dev</td>
<td>Mean ± Std Dev</td>
</tr>
<tr>
<td>% Error</td>
<td>% Error</td>
<td>% Error</td>
</tr>
<tr>
<td>68 ± 0.9</td>
<td>58 ± 2.0</td>
<td>31 ± 0.9</td>
</tr>
<tr>
<td>70 ± 0.9</td>
<td>39 ± 0.6</td>
<td>40 ± 1.6</td>
</tr>
<tr>
<td>67 ± 1.3</td>
<td>40 ± 1.1</td>
<td>47 ± 0.7</td>
</tr>
<tr>
<td>74 ± 0.9</td>
<td>41 ± 0.6</td>
<td>47 ± 1.4</td>
</tr>
<tr>
<td>59 ± 0.7</td>
<td>47 ± 0.7</td>
<td>44 ± 1.0</td>
</tr>
<tr>
<td>64 ± 1.6</td>
<td>54 ± 1.0</td>
<td>65 ± 0.8</td>
</tr>
<tr>
<td>63 ± 1.0</td>
<td>61 ± 0.8</td>
<td>56 ± 0.8</td>
</tr>
<tr>
<td>65 ± 0.8</td>
<td>65 ± 0.8</td>
<td>65 ± 0.8</td>
</tr>
</tbody>
</table>

Average % error 1.8 2.3 3.0

* Six aliquots are used to determine each mean. The mean is the average reading on the Turner Fluorometer scale of 100 units.

Thiamine and HET that had been mixed into dephosphorylated, deproteinated brain homogenate and then added to the Decalso column and subsequently eluted with 20% KCl in 0.1 N HCl were completely recovered (see Table 1). The average percent error associated with the thiamine assay is 1.8 (see Table 2).

Thiamine diphosphate added to crude brain homogenate was completely recovered using these techniques. Also, 10 days of storage at -20°C of the above homogenate did not significantly change the total thiamine or the di- plus triphosphate ester level.
The oxidation of thiamine to thiochrome by 1% HgCl₂ was found to be specific for thiamine in the presence of HET, as reported by Morita et al. (8), and in the presence of excess PTH (see Table 3). The 1% HgCl₂ oxidation procedure does not oxidize the PTH to pyrochrome. The fluorescent scan—with blank subtracted—of the oxidation product of pure thiamine and thiamine that was extracted from rat brains of control and PTH-treated rats are similar (Fig. 1) at high and at low concentrations in the assay.

Table 3. Lack of interference of PTH in the thiamine assay.

<table>
<thead>
<tr>
<th>µg of thiamine added to assay</th>
<th>µg of PTH added to assay</th>
<th>Ratio of µg thiamine/µg PTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>4.96</td>
<td>2.48</td>
<td>0.0</td>
</tr>
<tr>
<td>Fluorometric reading of solutions expressed as percent of reading of pure thiamine. Each value represents the mean of three determinations.</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

a Turner Fluorometer was used.

The selective oxidation of thiamine by the 1% HgCl₂ in the presence of excess PTH seems to be preferable over the cyanogen bromide (CNBr) method (Fujinawa and Matsui (2)), which was evaluated by Morita et al. (5). Although the error in the CNBr method is not significant when the PTH/thiamine ratio is high, the 1% HgCl₂ solution is easier to prepare and need not be replaced during a day’s work. The CNBr solution “… is stable (only) for 3 hr at room temperature (22°C).” (Fujinawa and Matsui (2)).

Thiamine does not interfere with the HET assay because it is destroyed prior to the alkaline potassium ferricyanide oxidation step (see also (8)). When thiamine and HET are rapidly mixed on a vortex mixer in a solution containing 5 ml of 20% KCl in 0.1 N HCl, 2 ml of 30% NaOH, and 2 drops of 0.01% HgCl₂, the thiamine is completely destroyed in a few seconds, and only 6% of the HET is destroyed. If the 0.01% HgCl₂ reagent is not added to the above solution, 1-4% of the thiamine is not destroyed. These findings are not in complete agreement with those of Morita et al. (8), who incubated the above solution for 5 min in order to destroy all the thiamine but also destroyed 20% of the HET. When attempting to duplicate their 5 min incubation technique, a 40% loss of HET was observed (see Fig. 2). It should be noted from Fig. 2 that the standard deviation of the HET assay increases several fold as the incubation time is extended from 5 sec to 5 min.

Since PTH is sensitive to alkaline K₃Fe(CN)₆, when the PTH concentration is greater than the HET concentration in the HET assay, a significant amount of PTH is assayed as HET. However, until the ratio of µg PTH/µg HET is greater

1 Control and PTH-treated rats were prepared according to Gubler (3).
2 Rats had been treated for 21 days.
Fig. 1. Fluorescence spectra of thiochrome formed from pure thiamine standard and from thiamine in brain extracts. Fluorescent scan of thiochrome formed in the thiamine assay using (1) 5 ml of purified brain extract from control* rat, (2) 5 ml of purified brain extract from PTH-treated rat, (3) 0.12 µg of pure thiamine per assay, (4) 0.05 µg of pure thiamine per assay, (5) 0.02 µg of pure thiamine per assay. The thiochrome produced in these assays was scanned on the Farrand Fluorometer using 360 nanometer excitation wavelength. The blank reading was subtracted from each sample reading.

* Control and PTH-treated rats were prepared according to GUBLER (3).

Table 4. Interference of PTH in the HET assay.

<table>
<thead>
<tr>
<th>µg of HET added to assay</th>
<th>0.06</th>
<th>0.06</th>
<th>0.06</th>
<th>0.06</th>
<th>0.06</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg of PTH added to assay</td>
<td>4.96</td>
<td>1.24</td>
<td>0.62</td>
<td>0.37</td>
<td>0.06</td>
</tr>
<tr>
<td>Ratio of µg HET/µg PTH</td>
<td>1/83</td>
<td>1/20</td>
<td>1/10</td>
<td>1/6</td>
<td>1/1</td>
</tr>
</tbody>
</table>

Fluorometric* reading of solutions, expressed as percent of reading of pure HET. Each value represents the mean of three determinations.

| 175 | 116 | 108 | 103 | 100 |

* Turner Fluorometer was used.

than 20/1, the error in the HET assay is less than 16% (see Table 4).

The fluorescent scans of the oxidation product of pure HET and of HET extracted from control rat brain1 are similar (see Fig. 3) at high and low concentrations.

1 Rats treated according to GUBLER (3).
Fig. 2. Rate of breakdown of HET during incubation. Rate of breakdown of 0.12 µg of HET in 5 ml of 20% KCl in 0.1 N HCl, 2 ml of 30% NaOH plus 0.1 ml of 0.01% HgCl₂, for up to 5 min of incubation. Each point represents the mean of 4 samples ± standard deviation. The HET was then oxidized to thiochrome, extracted into isobutyl alcohol, and the fluorescence was read on the Turner Fluorometer.

Fig. 3. Fluorescence spectra of thiochrome formed during HET assay. Fluorometric scan of thiochrome in the HET assay, using (1) 0.12 µg of pure HET per assay, (2) 5 ml of purified extract from control rat brain, and (3) 0.05 µg of pure HET per assay. The thiochrome produced in these assays was scanned on the Farrand Fluorometer using 360 nanometer excitation wavelength. The blank reading was subtracted from each sample reading.

A sample of the HET received from Dr. Morita as a gift could be separated from pure thiamine by paper chromatography, using an ascending n-butanol-ethanol-water solvent system in a ratio of 3.1:1.5:1.5 (see Fig. 4). Morita
et al. (7) reported similar results using thin layer chromatography. The lowest level of thiamine or HET that can be detected is about 20 nanograms/assay. The tissue blank in all the assays is identical to the blank obtained using pure 20% KCl in 0.1 N HCl.

![Chromatographic separation of thiamine and HET](image)

**Fig. 4.** Chromatographic separation of thiamine and HET. Chromatographic separation of thiamine and HET using an ascending *n*-butanol-ethanol-H₂O (3.1:1.5:1.5) solvent system. The *Rf* values for thiamine and HET are 0.51 and 0.59, respectively. Two microliters of solutions containing 200 μg thiamine/ml and 100 μg of HET/ml were spotted on Whatman #1 paper.

**Table 5.** Interference of thiamine and HET in the PTH assay.

<table>
<thead>
<tr>
<th>µg of PTH added to assay</th>
<th>0.37</th>
<th>0.37</th>
<th>0.37</th>
<th>0.37</th>
<th>0.37</th>
<th>0.37</th>
<th>0.37</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg of thiamine added to assay</td>
<td>0.36</td>
<td>0.72</td>
<td>1.8</td>
<td>7.2</td>
<td>12</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>µg of HET added to assay</td>
<td>0.12</td>
<td>0.24</td>
<td>0.36</td>
<td>1.2</td>
<td>12</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>Ratio of µg of thiamine/µg of HET/µg of PTH</td>
<td>3/1/3</td>
<td>3/1/1.5</td>
<td>5/1/1</td>
<td>20/3.2/1</td>
<td>32/32/1</td>
<td>27/0/1</td>
<td>0/27/1</td>
</tr>
<tr>
<td>Fluorometric reading of solutions, expressed as percent of reading of pure PTH solution</td>
<td>100</td>
<td>100</td>
<td>106</td>
<td>110</td>
<td>121</td>
<td>118</td>
<td>119</td>
</tr>
</tbody>
</table>

* Farrand Fluorometer was used.

MORITA et al. (5) previously determined the error in this PTH assay as the ratio of µg thiamine/µg PTH increased to 20/1. Their study did not include HET, but their results are very similar to those reported in this paper. This study showed that there was a 6, 10, and 21% increase in the PTH determination when the ratio of µg of thiamine plus µg of HET/µg of PTH was 6/1, 23/1, and 65/1, respectively (see Table 5). It has been shown (unpublished work in this lab) that the HET levels decreased in the brain of rats when treated with PTH according to the method of GUBLER (3). Other results indicate that the ratio of thiamine/PTH in the brain of PTH-treated rats may not exceed even 6/1 during the PTH treatment period.
Twenty-four hours after a single large intraperitoneal dose of PTH (1 mg/100 g rat) is given to rats fed a commercial rat diet containing thiamine, 33% of the brain total thiamine was replaced by an approximately equal molar amount of PTH(7). When this same dose was administered to 18-day thiamine deficient rats, the PTH had displaced 2/3 of the total thiamine from the brain, and the thiamine/PTH ratio in the brain was 1/3 just 24 hr after treatment.

A daily oral dose of 33 µg of thiamine plus 210 µg of PTH caused the PTH level to increase in the brain (10) to 25–30% of the normal brain thiamine level(9) by the fifth day of treatment.

The fluorometric scans of pure PTH and PTH extracted from the brains of PTH-treated¹ rats are similar (see Fig. 5). The lowest level of PTH required per fluorometric assay is about 60 nanograms.

![Fluorescence spectra](image)

Fig. 5. Fluorescence spectra of pyridochrome formed in PTH assay of pure PTH and PTH in brain extracts of PTH-treated rats. Fluorometric scan of pyridochrome formed in the PTH assay using (1) 0.37 µg of pure PTH per assay, (2) 5 ml of purified extract from brain tissue of PTH-treated* rat. The pyridochrome produced in these assays was scanned on the Farrand Fluorometer using 430 nanometer excitation wavelength. The blank reading was subtracted from each sample reading.

* PTH-treated rats prepared according to GUBLER (3).

REFERENCES

5) MORITA, M., KANAYA, T., and MINESITA, T., J. Vitaminol., 14, 67 (1968).
6) MORITA, M., KANAYA, T., and MINESITA, T., J. Vitaminol., 14, 77 (1968).

¹ Rats were treated according to GUBLER (3).