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Determination of Thiamine and its Phosphate Esters by Electrophoresis and Fluorometry

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An improved modification for electrophoretic separation of thiamine and its phosphate esters is described. In the modified procedure, electrophoresis is performed in 0.05 M sodium citrate buffer, pH 5.6, containing methanol, ethanol and propanol, instead of the 0.05 M acetate buffer, pH 3.8, used before,[1] in which separation could not be readily obtained. With the citrate buffer for electrophoresis, it was possible to effect the separation even if the samples contained both perchloric acid and either acetate, glycylglycine or phosphate buffer. Thus, the method is convenient for determining the activities of enzymes concerned in the metabolism of thiamine compounds. The method was used for estimating the contents of thiamine, and of thiamine mono-, di- and triphosphate esters in rat brain.

Itokawa and Cooper [1] combined electrophoretic separation [2] and fluorimetric determination [3] in a simple and rapid method that has been used in many studies of thiamine phosphate esters. No data, however, have been presented concerning the validity of the method. After a few experiments, I decided to examine their method in more detail. The unsatisfactory step proved to be the electrophoresis, and a new and better modification was developed, which is described here. The improved procedure has been adapted for the separate quantification of thiamine and its phosphate esters in rat brain.

EXPERIMENTAL

Abbreviations. TMP = thiamine monophosphate, TDP = thiamine diphosphate, TTP = thiamine triphosphate.

Reagents. Thiamine and its mono- and diphosphate esters were obtained from the Sigma Chemical Co., St. Louis, Mo., USA. Before use they were crystallized three times from ethanol. Thiamine triphosphate was prepared as described elsewhere.[4] A standard solution of thiamine was made up from the U.S.P. Reference Standard.[5] {^{14}C}thiamine was produced by the Radiochemical Centre, Amersham, England. Its purity was checked by electrophoresis and found to be over 95 %. Benzenesulfonyl chloride, produced by Koch-Light Laboratories Ltd, Colnbrook, England, was diluted with ethanol (1:6, v/v) just before use. Alkaline reagent A, which is needed for location of the thiamine compounds in the electrophoretic strips, contained 76 ml of 50 % ethanol (v/v), 15 ml of 15 % NaOH, and 1 ml of 2 % potassium hexacyanoferrate(III). Alkaline reagent B, which is used in the oxidation of thiamine compounds, contained 15 ml of 15 % NaOH and 1 ml of 2 % potassium hexacyanoferrate(III). The electrophoresis buffer contained 39 parts of 0.05 M sodium citrate, pH 5.6, and one part of a mixture of methanol, ethanol and propanol (1:1:1, by vol.). Electrophoresis was performed on Munktells S 311 papers, Gryckssbo, Sweden, the strips being 2.5 × 45 cm. To reduce the nonspecific background fluorescence of the papers, they were kept for 3 days in 50 % ethanol (v/v) with daily changes of the medium. Fluorimetric measurements were conducted in a Farrand Fluorometer A 4 with PC Corning Filters numbers 7 − 37 as primary and 3 − 73 and 5 − 60 as secondary filters, respectively. The instrument was standardized with solution containing 0.01 % quinine sulfate in 0.1 M H_2SO_4.

Electrophoretic separation and fluorimetric determination of thiamine and its phosphate esters. A sample (5 μl) was applied in the middle of a paper strip that had been soaked in the buffer and blotted. A similar sample containing 5 nmol each of thiamine and its phosphate esters was applied to another strip; this was

used to locate the compounds. The strips were subjected to electrophoresis for 45 – 75 min at a constant current of 3 mA per strip in a high-voltage apparatus, Analysteknik AB, Vallentuna, Sweden. The voltage ranged from 2 to 4 kV. The electrode vessels contained 0.05 M sodium acetate, pH 3.8. The strip containing the reference thiamine compounds was sprayed with alkaline reagent A, and the fluorescent bands were visualized with UV light and marked. The strips with the samples to be analyzed were cut into pieces corresponding to the marked areas. The thiamine compounds were eluted with 3 ml of 50 % ethanol (v/v) for 45 min. After removal of the paper, 0.5 ml of alkaline reagent B was added and the mixture was agitated for 2 min. Then 10 µl of 30 % H_2O_2 was added and, after the yellow color of hexacyanoferrate(III) had disappeared, the fluorescence was ready to be measured.

Because equimolar amounts of thiamine and its phosphate esters produce unequal thiochrome fluorescence, and thiamine was used as the standard, the fluorescence values derived from thiamine di- and triphosphate were corrected by multiplying by 0.87 and 0.80, respectively.[6]

Determination of thiamine and its phosphate esters in rat brain. The rat was decapitated and the head was immediately placed in liquid nitrogen. The brain was removed and homogenized in the same volume of cold 1 M $HClO_4$ at 0 °C with an Ultra Turrax homogenizer, and centrifuged at 15 000 *g* for 10 min. The supernatant was extracted three times with three volumes of chloroform. After addition of 34.5 mg of K_2CO_3 per ml of brain extract, the mixture was allowed to stand for 3 h in an ice bath. The pH value was adjusted to 5 – 6 with K_2CO_3. After centrifugation the supernatant was extracted once with chloroform as before and divided between at least two small tubes. After lyophilization the residue (about 16.4 mg from 1 ml of supernatant) in one tube was dissolved in water (1/8 of the original volume) and that in the other in a solution containing 10 nmol each of thiamine and its phosphate esters. The latter sample was used for location of the thiamine compounds after electrophoresis. The pH of the preparation was checked and if necessary, adjusted to the same value as before lyophilization. After centrifugation, a 5 µl aliquot was subjected to electrophoresis and fluorimetry as described before. The nonthiochrome fluorescence was estimated using benzenesulfonyl chloride to prevent the oxidation of thiamine to thiochrome.[7] An electrophoresis strip was eluted with 2.8 ml of 50 % ethanol (v/v) instead of 3 ml. After removal of the paper, 0.2 ml of diluted benzenesulfonyl chloride reagent was added, and fluorimetric determination was carried out as described before.

RESULTS AND DISCUSSION

Electrophoretic separation. The best separation of thiamine and its phosphate esters was achieved with the sodium citrate buffer. Fig. 1 shows a representative electrophoretic separation by the modified method presented. The separation was satisfactory in samples containing perchloric acid and extracts of rat brain. Such samples could not have been separated adequately with the 0.05 M sodium acetate buffer recommended by Itokawa and Cooper.[1] Their method was found to give acceptable separation only when thiamine compounds were dissolved in water. A separation compar-

Table 1. Recovery of thiamine and its phosphate esters after elution from electrophoresis paper. A (5 µl) sample containing thiamine, TMP, TDP and TTP was applied to an electrophoresis paper (2.5 × 1,0 cm) soaked in the solution indicated. Elution and fluorimetric determination were as described in Experimental. Percentage recoveries have been calculated as in Table 2.

Munktells S 311 paper soaked in	Total amount of thiamine and its phosphate esters applied, pmol	Recovery/ % ± S.D.[a]
Water	2500	76.8 ± 3.0
0.05 M sodium acetate (pH 3.8)	2500	82.9 ± 4.0
0.05 M sodium citrate (pH 5.6)	2500	88.0 ± 3.5
0.05 M sodium citrate (pH 5.6) containing methanol, ethanol and propanol[b]	2500	98.9 ± 2.5
0.05 M sodium citrate (pH 5.6) containing methanol, ethanol and propanol[b]	25	95.8 ± 4.5

[a] $n = 10$. [b] Thirty-nine parts of sodium citrate and one part of a mixture of methanol, ethanol and propanol (1:1:1, by vol.).

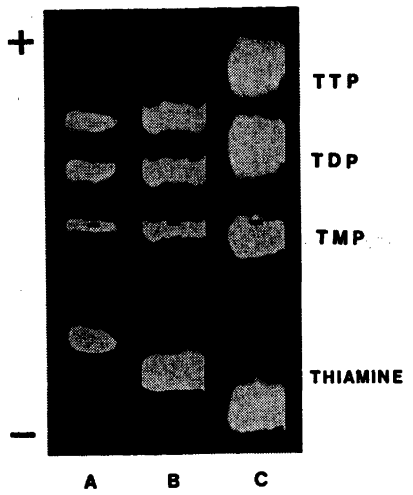

Fig. 1. Electrophoretic separation of thiamine and its phosphate esters. Samples of 5 μl were applied in the middle of electrophoretic paper strips (black lines) and electrophoresis and visualization were carried out as described in Experimental. Thiamine compounds were dissolved in: A, water; B, 0.37 M $HClO_4$ containing 70 mM glycylglycine; C, rat brain prepared as described in Experimental.

able to that shown in Fig. 1 (B) also resulted when the samples contained 0.37 M $HClO_4$ and 70 mM sodium acetate, potassium phosphate or sucrose.

The mechanism whereby citrate exerts its effect is still obscure. As one of the properties of citrate is chelation, this probably plays some role. Perhaps the most useful application of this modified separation method follows from the facility to determine thiamine compounds directly from incubation mixture containing perchloric acid. Thus it can be used for estimating the activities of enzymes metabolizing thiamine phosphate esters.

Table 2. Recovery of thiamine and its phosphate esters after electrophoretic separation. Thiamine compounds were dissolved in water. A 5 μl aliquot was subjected to electrophoresis for 45 min and determined as described in Experimental. One fluorescence unit represents one pmol of thiamine.

	Fluorescence ± S.D.[a]		
	Applied	Recovered	Recovery/ %
Thiamine	113	99 ± 3.8	88
TMP	157	149 ± 6.7	95
TDP	101	102 ± 4.1	101
TTP	72	72 ± 5.8	100

[a] $n = 6$.

Recovery. Addition of methanol, ethanol and propanol to the electrophoretic buffer improved the elution of thiamine compounds from the paper strip, Table 1. Even when the sample contained thiamine compounds in small amounts (25 pmol) recovery was always as high as 96.0 % in contrast to the method of Itokawa and Cooper,[1] which gave a recovery of only 66 %. Table 2 shows the whole recovery after electrophoretic separation and elution. When the amount of thiamine applied was about 100 pmol, recoveries ranged from 88 to 101 %. With even higher sample loads, up to 2.5 nmol, recoveries were similar.

Determination of thiamine and its phosphate esters in rat brain. Table 3 shows the recoveries

Table 3. Recovery of thiamine and its phosphate esters added to a preparation of rat brain just before electrophoresis. Preparation and determination were as described in Experimental. One fluorescence unit represents one pmol of thiamine.

	Fluorescence ± S.D.[a]				
	Background	Brain thiamine and background	Added thiamine, brain thiamine and background	Added thiamine	Recovery/%
Thiamine	27 ± 2.9	41 ± 7.6	118 ± 13.3	113	68
TMP	29 ± 1.5	75 ± 5.9	209 ± 4.4	157	85
TDP	30 ± 1.2	174 ± 17.6	299 ± 15.6	101	124
TTP	28 ± 2.4	47 ± 5.5	115 ± 10.0	72	94

[a] $n = 6$.

of thiamine compounds under the conditions used for determining the thiamine content of rat brain. Recovery of added thiamine ranged from 68 to 124 % in this particular experiment. This large variation is probably due to the extensive concentration of the brain specimen, because in less concentrated samples the electrophoretic mobility was higher and recoveries were better. It was essential, however, to concentrate the sample, as only then was there sufficient difference between the fluorescence values of the sample and the blank. The concentration is especially important when the substance to be measured is thiamine or TTP.

The recovery of thiamine was also checked by adding 17 nmol {^{14}C}thiamine to 1 ml of brain homogenate before precipitation of the proteins. This experiment gave a recovery of 96 – 101 %.

The treatment of rat brain with perchloric acid did not destroy thiamine phosphate esters. The content of thiamine and its phosphate esters in rat brain was 11.2 nmol/g, the distribution of the different thiamine compounds being as follows: thiamine 0.70 nmol/g, (6.3 %); TMP 2.3 nmol/g, (20.6 %); TDP 7.20 nmol/g, (64.6 %); TTP 0.95 nmol/g, (8.5 %). These values, though based on only one brain, are in accord with results of other investigators ranging from 5.6 to 9.0 nmol/g.[8–10] The fluorescence given by thiamine derivatives may be criticised as not absolutely specific, and the reliability of the values obtained by fluorometry may be questioned. Actually, all claims for the existence of TTP in living material are based on fluorometric evidence. Final identification of this compound by a more exact method that would reveal the structure of the fluorophore is therefore highly desirable.

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