DETERMINATION OF THIAMINE AND ITS PHOSPHATE ESTERS BY ELECTROPHORESIS AND FLUOROMETRY AND PROPERTIES OF SOLUBLE THIAMINE TRIPHOSPHATASE IN RAT TISSUES

BY

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Academic dissertation

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This thesis is based on the following publications:

I Penttinen, H.K. (1976) Synthesis of thiamine triphosphate, Finn. Chem. Lett. 1, 1-2

II Penttinen, H.K. (1976) Differences in thiochrome fluorescence produced by thiamine and its mono-, di-, and triphosphate esters, Acta Chem. Scand. B 30, 659-663

III Penttinen, H.K. (1978) Determination of thiamine and its phosphate esters by electrophoresis and fluorometry, Acta Chem. Scand. B 32, 609-612

IV Penttinen, H.K. (1980) Determination of inorganic phosphate. A method for the determination of phosphatase activities by a continuous flow system, Anal. Biochem. 102, 353-357

V Penttinen, H.K. and Uotila, L.J. (1981) The relation of the soluble thiamine triphosphatase activity of various rat tissues to nonspecific phosphatases, Med. Biol., in press

These papers will be referred in the text by their Roman numerals.

CONTENTS

INTRODUCTION

A phosphorylated derivative of vitamin B_1, thiamine triphosphate, was found in rat liver in 1952. It is reported to occur in other mammalian tissues, bacteria, yeast, and plants. Thiamine triphosphate is not active as a coenzyme in reactions where thiamine diphosphate participates. It has been suggested that thiamine triphosphate might have some role in nerve conduction. Furthermore, a defective metabolism of thiamine triphosphate is suggested to be implicated in a fatal disease in children (subacute necrotizing encephalomyelopathy or Leigh's disease).

Attempts to demonstrate the content and formation of thiamine triphosphate in biological material were unsuccessful. On closer examination it was discovered that the methods were unreliable and insufficiently established. This paper now presents improved methods for the investigation of the metabolism of thiamine phosphate esters.

Because the existence of thiamine triphosphate in biological material could not be established with reasonable certainty, and no formation of thiamine triphosphate could be detected in tissue extracts, the soluble thiamine triphosphatase from various rat tissues was characterized in order to obtain some information about the formation, degradation and the biological significance of thiamine triphosphate.

SUMMARY

Methods for investigating of the metabolism of thiamine phosphate esters are presented.

In contrast to the prevailing view, the intensities of thiochrome fluorescence produced by thiamine and its mono-, di-, and triphosphate esters were found to be unequal.

A new electrophoretic technique for the separation of thiamine phosphate esters is presented. This technique was found to be suitable for the assessment of enzyme activities forming thiamine phosphate esters as well as for the determination of thiamine compounds in various tissue extracts.

For the measurement of activities responsible for the hydrolysis of thiamine phosphate ester, a modification of the procedure for the determination of inorganic phosphate is presented. This technique avoids the precipitation of protein and formation of a complex between ammonium molybdate and thiamine phosphate. It is also adaptable to the continuous flow system.

The soluble thiamine triphosphatase (E.C. 3.6.1.28) of rat brain and liver was found to be different from alkaline and acid phosphatases. This was in contrast to the activity present in the intestine preparation which resembled that of alkaline phosphatase. The apparent molecular weight of the specific thiamine triphosphatase was about 30 000 and the isoelectric point (p I) 4.6.

The present state of knowledge forming the basis for the existence of thiamine triphosphate in biological material is critically discussed.

REVIEW OF LITERATURE

Determination of thiamine

Thiamine (vitamin B_1) (Fig. 1) was isolated and chrystallized from rice by Janssen and Donath in 1926 (1). The first chemical method for its determination, presented by Kinnersley and Peters in 1934, (2) required highly purified samples. The method of Jansen (3) was based on the fluorescence of the thiochrome derivative (Fig. 1) of thiamine produced by oxidation with potassium ferricyanide in alkaline medium. Fujiwara and Matsui (4) have used cyanogen bromide as the oxidising agent. In addition to these thiochrome methods which are more commonly used, some other chemical methods have been presented such as coupling with diazotized 6-aminothymol (5) and the method using bromothymol blue (6).

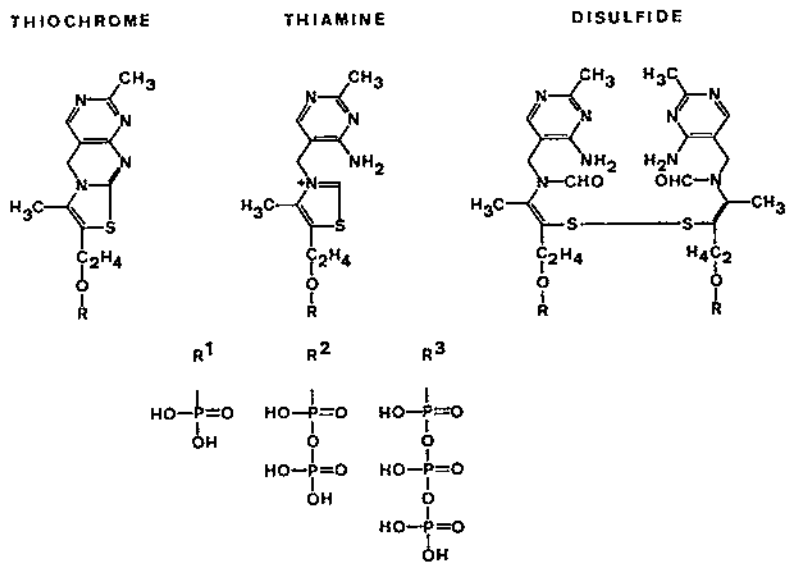

Fig. 1. Structures of thiamine, thiochrome and thiamine disulfide. R^1 = thiamine monophosphate, R^2 = thiamine diphosphate and R^3 = thiamine triphosphate.

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

Microbiological methods have also been used for the determination of thiamine (7,8). These methods are based on the measurement of growth response of organisms (e.g. Streptococcus salivarius or Lactobacillus fermenti) which are dependent on this vitamin.

Attempts to determine thiamine by gas-liquid chromatography (GLC) have not been successful because thiamine is not volatile and decomposes at high temperatures. However, Dwivedi and Arnold (9) have succeeded to estimate thiamine by GLC after cleaving it into pyrimidine and thiazole moieties. The latter compound was chromatographed as a volatile trimethylsilyl derivative.

Field desorption mass spectrometry (10) and high-pressure liquid chromatography (11) have also been used in the determination of thiamine.

Determination of thiamine phosphate esters

The function of thiamine diphosphate as a coenzyme of pyruvate decarboxylase (E.C. 4.1.1.1) has been employed for its quantitation. These methods involve either determination of carbon dioxide (12-14) or acetaldehyde (15,16) formed in the reaction. Determination of the activity of transketolase (E.C. 2.2.1.1), another thiamine diphosphate requiring enzyme, has also been used as an indicator of the amount of this compound (17).

The separation of thiamine from its phosphate esters (Fig. 1) was originally accomplished by extracting the thiochrome into isobutanol, the thiamine phosphate esters remained in the aqueous phase (18). This method did not allow the separation of thiamine mono-, di-, and triphosphate esters from each other. For this purpose paper chromatography (19-23) and paper electrophoresis (22,24) have been widely employed. Different ion-exchange columns, e.g. Amberlite IRC 50 (22), Dowex 1X-8 (25), Dowex 1X-4 (26), and DEAE-Sephadex A-25 (27), have also been used in the separation of these compounds. High-performance liquid chromatography (28-30) and gel filtration on Sephadex G-10 (31) have been presented for the same purpose. Oxidation of thiamine compounds to thiochrome derivatives are required before the separation.

Function of thiamine diphosphate in animal tissues

Thiamine is physiologically active as its phosphorylated form, thiamine diphosphate (32). This compound has been shown to be a coenzyme for pyruvate dehydrogenase (E.C. 1.2.4.1), α-ketoglutarate dehydrogenase (E.C. 1.2.4.2) (33,34), and transketolase (E.C. 2.2.1.1) (35). Thiamine diphosphate may also participate in the oxidative decarboxylation of glyoxylic acid (36). It has been suggested that this coenzyme may be involved in the catabolism of other α-keto acids, especially in the degradation of the branched-chain keto derivatives of leucine, isoleucine, and valine (37).

Mechanism of action of thiamine diphosphate as a coenzyme has been clarified by Reed (38) and Koike et al. (39).

Metabolism of thiamine phosphate esters in animal tissues

Thiamine is absorbed in the small intestine by two mechanisms: at high concentrations thiamine is transported by passive diffusion (40,41) and at low concentrations by an active process (40,42,43). In the mucosal cells thiamine is enzymatically phosphorylated and dephosphorylated (44). Thiamine leaves the cells in the nonphosphorylated form by an active Na^+-dependent mechanism (45).

The phosphorylation of thiamine

(thiamine + ATP → thiamine diphosphate + AMP)

is catalyzed by thiamine pyrophosphokinase (thiamine kinase, E.C. 2.7.6.2). This soluble enzyme has been isolated from pig brain (46), rat brain (47), and rat liver (48). Artsukevich et al. (49) have recently reported a 3000-fold purification of this enzyme from rat liver.

The phosphorylation of thiamine diphosphate to thiamine triphosphate has been reported by Eckert and Möbus (50) to occur in an extract of pig spinal cold. Similar activity was detected in rat heart after the administration of [^{35}S]thiamine (51). Itokawa and Cooper (52,53) found most of the activity of thiamine diphosphate kinase (ATP: thiamine diphosphate phosphotransferase, E.C. 2.7.4.15) catalysing the reaction:

(thiamine diphosphate + ATP → thiamine triphosphate + ADP)

to be located in the mitochondrial fraction of rat brain. In addition to brain, this activity has been reported in the kidney, heart, intestine,

muscle, blood and liver of the rat (54). The formation of thiamine triphosphate was also observed in the soluble fraction of rat liver (55). In this reaction, the substrate however, would be an endogenous protein-bound thiamine diphosphate (56). In contrast, Schrijver et al. (57), using rat and calf brain homogenates as the source of enzyme could not demonstrate any definite thiamine triphosphate-forming activity. On the other hand, Voskoboev and Luchko (58) have presented a 70-fold purification of thiamine diphosphate kinase from rat liver.

Thiamine triphosphate has been found to be hydrolyzed by a soluble and membrane-associated thiamine triphosphatase (59). The partially purified soluble thiamine triphosphatase (E.C. 3.6.1.28)

(thiamine triphosphate → thiamine diphosphate + P_i)

was claimed to be specific for thiamine triphosphate and was inhibited by Ca^{2+} (60,61). The membrane-associated thiamine triphosphatase activity has been indicated to be distinct from the activity of adenosine triphosphatase and nucleoside triphosphatase (62). This thiamine triphosphatase activity had an absolute requirement for divalent cations, and the substrate for this activity was therefore suggested to be the Mg^{2+} thiamine triphosphate complex (63,64). Both the soluble and the membrane-associated thiamine triphosphatase activities have been found in rat intestine, kidney, spleen, brain, liver, heart and skeletal muscle (62).

The hydrolysis of thiamine diphosphate to monophosphate

(thiamine diphosphate → thiamine monophosphate + P_i)

in bovine liver microsomes was demonstrated to be catalyzed by nucleoside diphosphatase that was activated by ATP (65). Similar nonspecific thiamine diphosphatase was found in rat brain microsomes, although this enzyme was not activated by ATP (66). The activity of thiamine diphosphatase in rat brain has been found to be influenced by lipids, monovalent cations, and ATP (67-69). A soluble thiamine diphosphatase activity of low pH optimum was reported in the bovine brain by Castner and Evans (70). Intestinal thiamine diphosphatase activity has been suggested to be identical with the activity of alkaline phosphatase (71). The activities of thiamine diphosphatase and nucleoside diphosphatase in different mice tissues have been compared with each other by Allen (72) using a gel electrophoretic technique.

The information on the dephosphorylation of thiamine monophosphate, i.e.

thiamine monophosphate → thiamine + P_i

is very poor. It is assumed that this reaction is catalyzed by nonspecific phosphatases. Thiamine metabolites have been shown to be excreted finally in urine (73).

Biochemical defects of thiamine metabolism

The classical syndrome of thiamine deficiency in human is called beriberi (74). The clinical manifestations are peripheral polyneuropathy and cardiac enlargement (75,76). Experimental thiamine deficiency has been shown to cause severe neurological disturbances leading finally to death (77,78). Neurological signs were found in thiamine deficient rats before any significant reduction could be shown in the activities of thiamine dependent enzymes (79). The pathophysiology of thiamine deficiency has been described by Gubler (80), Dreufys (81) and Sturman and Rivlin (82) in more detail.

Another disease associated with thiamine deficiency in human is Wernicke's encephalopathy characterized by neurological and mental disturbances. This syndrome is frequently seen among chronic alcoholics, and the significance of thiamine deficiency as an etiological factor has been established (83-86). The finding of an abnormality of thiamine-dependent transketolase in the patients with Wernicke-Korsakoff syndrome suggests an inherited etiology of this disease (87).

An inherited defect in the thiamine-dependent pyruvate dehydrogenase enzyme has been shown to be responsible for cerebellar ataxia and lactic acidosis (88,89). Low activity of another thiamine-requiring enzyme, α-ketoglutarate dehydrogenase, in white cells has been described in a patient with spinocerebellar disease and cortical atrophy (88). An impairment in the catabolism of branched-chain α-keto metabolites of leucine, isoleucine and valine (Maple syrup urine disease) is suggested to be due to a defect in thiamine-dependent decarboxylation of these compounds (90).

Subacute necrotizing encephalomyelopathy (Leigh's disease), a fatal inherited disease in children, has been suggested to be associated with defective thiamine metabolism as well. Cooper et al. (91) found that in

this disease extracts of blood, urine, and spinal fluid contained a factor that inhibited the synthesis of thiamine triphosphate. The brain tissue of the patient was deficient in thiamine triphosphate. Since then several papers have appeared supporting these findings (92-99). The molecular weight of the inhibitor is about 37 000, and it inhibits only the brain enzyme with no effect on the liver enzyme.

Role of thiamine in the nervous tissue

As early as 1938, Minz (100) found that electrical stimulation of a nerve released thiamine into the medium. This observation was confirmed later by other investigators (101-105). The observation that thiamine triphosphate was associated with membrane fractions of nervous tissue led to the suggestion that this might be the neurophysiologically active form of thiamine (106). However, Goldberg (107) could not demonstrate any function for thiamine and its phosphate esters in the nerve conduction. This opinion was supported by Bergman and Fishman (108). On the other hand, Eder et al.have suggested that thiamine may be involved in acetylcholine release (109), and Fox and Duppel (110) presented evidence that thiamine phosphates may stabilize the negative surface charges at the inner side of the nerve membrane. Furthermore, the electrophysiological studies of Waldenlind (111, 112) support the hypothesis that thiamine may play some role in neuromuscular transmission. Although the data suggesting a non-coenzyme role for thiamine seem to be conflicting, the majority of investigators still hold the view that it has some specific function in the nervous tissue (82, 112-114).

OUTLINES OF THE PRESENT STUDY

Since Rossi-Fanelli et al. (115) in 1952 found thiamine triphosphate in rat liver, many papers on its physiological function have been published but its biochemical role is still unclear. Evidence has been presented that it might be involved in nerve conduction (82,100-106, 109-111,113,114).

All methods used so far for the determination of thiamine triphosphate in biological material have employed different chromatographic

and electrophoretic techniques (24-26,30,115-119). The electrophoretic separation described by Itokawa and Cooper (24) has been used most frequently in studies concerning thiamine triphosphate (52,57,60,61,91, 92,95-98,104-106,120). This method, however, was found to be suitable only for aqueous samples of pure thiamine phosphate esters.

The aim of the present study was to obtain more reliable information about the occurrence of thiamine triphosphate and its metabolism in animal tissues. For this purpose it was found necessary to:

- improve methods for the preparation of thiamine triphosphate
- examine the formation and properties of thiochrome derivatives from various thiamine phosphate esters in order to make reliable quantitative determinations possible
- develop a reliable electrophoretic method for the separation of thiamine phosphate esters from each other in suspensions of different chemical composition
- characterize enzymes metabolizing thiamine phosphate derivatives, and determine their substrate specificity and organ distribution.

MATERIALS AND METHODS

Synthesis of thiamine triphosphate (I, 121)

Orthophosphoric acid was heated to 320 °C and allowed to cool to 100 °C; thereafter a mixture of thiamine hydrochloride and phosphorus pentoxide (1:1, w/w) was added in small amounts, and the temperature was maintained at about 100 - 105 °C for a further 20 min. After cooling, the mixture was dissolved in water and poured slowly into a cold (4 °C) ethanol-acetone mixture (1:1, v/v). The precipitate was collected and dissolved in water. Precipitation was repeated three times and the final volume of the aqueous mixture was adjusted to 150 ml after the removal of acetone and ethanol under reduced pressure. The mixture was divided into 15-ml portions, which were stored at - 18 °C. One portion was applied to a Dowex 50 W column (4x16 cm, H^+ form) and eluted at room temperature

with water at a rate of 10 ml/min. The effluent between 300-800 ml was collected and lyophilized. The white powder obtained was dissolved in 1 ml of water. Ethanol was added until the solution became slightly turbid. The mixture was kept at 4 ^{o}C for 3 h and thereafter at - 18 ^{o}C overnight. The precipitate was collected and redissolved in water and the precipitation repeated twice. The clystals were washed successively with cold ethanol and ether, dried at room temperature and stored at - 70 ^{o}C. The column was regenerated with 5 litres of 4 M HCl and washed with water. The crystals from 10 chromatograms were pooled and then recrystallized as described above.

Fluorometric determination of thiamine and its phosphate esters (II, 122)

A 5-µl sample of thiamine or its phosphate ester was added to 3 ml of 50 % ethanol and shaken. After a few minutes, 0.5 ml of alkaline ferricyanide (15 ml of 15 % NaOH and 1 ml of 2 % potassium ferricyanide) was added, and the mixture was agitated for 2 min. Then 10 µl of 30 % H_2O_2 was added to destroy the yellow color of ferricyanide, and the fluorescence was measured. Fluorometric measurements were conducted in a Farrand fluorometer A4 with PC Corning Filters numbers 7-37 as primary, and 3-73 and 5-60 as secondary filters, respectively, or in a Zeiss PMQ II spectrophotometer fitted with a ZFM 4 fluorometer attachment. The excitation wavelength was 365 nm, and the emission maximum was 430 nm.

The background fluorescence was determined by omitting the thiamine compound. It was also estimated by dissolving the sample in 2.8 ml of 50 % ethanol, to which 0.2 ml of benzenesulfonyl chloride (diluted with ethanol, 1:6, v/v) was added and the mixture was stirred. Determination was then continued as described before.

Electrophoretic separation of thiamine and its phosphate esters (III, 123)

A sample of 5 µl was applied on a paper strip (2,5 x 48 cm, Munktells S311) that had been soaked in the sodium citrate buffer (50 mM, pH 5.6, containing 0.025 part, v/v, of a mixture of methanol, ethanol and propanol, 1:1:1, v/v/v) and blotted. A similar sample containing 5 nmol each of thiamine and its phosphate esters was applied on

another strip that was used later to locate the fluorescent 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 50 mM sodium acetate buffer, pH 3.8. The reference strip containing added thiamine compounds was sprayed with alkaline ferricyanide reagent (76 ml of 50 % ethanol, v/v, 15 ml of 15 % NaOH, and 1 ml of 2 % potassium ferricyanide), and the fluorescent bands were visualized with UV light. The thiamine compounds were eluted from the cut out pieces of paper with 3 ml of 50 % ethanol (v/v) for 45 min. After removal of the paper, fluorometric determination were carried out as described before.

When thiamine compounds were determined from biological material, the protein was first precipitated with perchloric acid. The sample was neutralized with K_2CO_3 and then lyophilized to concentrate it. Blanks were determined using benzenesulfonyl chloride to prevent the oxidation of thiamine to thiochrome as described before.

Determination of inorganic phosphate (IV)

To a sample of 1 ml was added 0.2 ml of a mixture consisting of one part of sodium dodecyl sulfate (8 %, w/v), one part of perchloric acid (7 %, v/v) and two parts of ascorbic acid (2 %, w/v). Thereafter, 0.6 ml of ammonium molybdate (1 %, w/v) was added, and the reaction mixture was shaken. After 2 min 0.6 ml of a mixture containing 10 % (w/v) disodium hydrogen arsenite, and 10 % (w/v) sodium acetate was added, and shaking was continued. The absorbance was measured at 730 nm with a Gilford photometer. When the absorbance was measured with a FP-9 Analyzer System (Labsystems Oy, Finland) the final volume of the assay medium was 720 µl. The determination of phosphatase activities using a continuous flow system is described in the original paper IV.
The final volume of the assay medium was 10 ml. The reaction was started by the addition of the substrate, mixed, and immediately introduced into the continuous flow system of Gilford spectrophotometer (Fig. 1, IV). The linear portion of the slope recorded was taken for the determination of the activity.

Assays of enzyme activities

Thiamine pyrophosphokinase was assayed in 110 mM Tris-HCl pH 8.5, 13 mM $MgCl_2$, 33 mM ATP and 2 μM{^{14}C}thiamine in a total volume of 100 μl. After incubation for 60 min at 37 °C the reaction was stopped by adding 0.3 volume of 1.13 M $HClO_4$ containing 2 mM thiamine and 2 mM thiamine diphosphate. The {^{14}C}thiamine diphosphate formed was then separated from the substrate by paper electrophoresis. The radioactivity in the cut out pieces of electrophoretic paper was determined using a Packard Tri-Carb liquid scintillation spectrometer (model 2002). The scintillation medium contained 0.4 % (w/v) PPO and 0.01 % (w/v) POPOP in toluene.

Phosphatase assays were carried out as described in the original paper V.

Other methods

Gel electrophoresis, gel chromatography, isoelectric focusing, and the determination of molecular weight and Stokes radius were carried out as described in the original paper V.

Protein was determined either by the biuret method (124) or a fluormetric method (125).

RESULTS

Synthesis of thiamine triphosphate (I)

The chemical synthesis of thiamine triphosphate from thiamine hydrochloride, phosphorus pentoxide and orthophosphoric acid was performed essentially according to Yusa (126). The strong cation ion-exchange resin Dowex 50 W was used instead of the weak Amberlite IRC 50 used by Yusa (126). With this technique thiamine triphosphate was retained in the column and eluted slowly with water, whereas higher phosphorylated thiamines and inorganic phosphate were eluted in the void volume, and lower phosphorylated thiamine esters were bound in the column (Fig. 1,I). When Amberlite

IRC 50 was used, thiamine triphosphate was eluted in the void volume together with impurities. The crystallization of the compound gave pure thiamine triphosphate as estimated by elemental analysis.

Fluorometric determination of thiamine and its phosphate esters (II)

Fluorescent thiochrome derivatives were formed from thiamine, thiamine mono-, di-, and triphosphate esters in an alkaline medium by the oxidation with potassium ferricyanide. The structures of these thiamine compounds are given in Fig. 1. Ethanol enhanced the formation of fluorescence of all these compounds, which was maximum with thiamine (3.6 fold) and minimum with TTP (2.8 fold).

Thiamine mono-, di-, and triphosphate esters produced unequal molar thiochrome fluorescence (Table 2, II), probably due to the phosphoric acid chains. Thus the fluorescent values for TDP and TTP had to be multiplied by 0.87 and 0.80, respectively, if thiamine was used as the standard; the value of TMP needed no correction. The identity of the UV absorption spectra of thiamine phosphates together with the information given in Table 2 (II) justifies the conclusion that the light-absorption properties of the different thiamine phosphate esters are identical and not influenced by the phosphate group.

Electrophoretic separation of thiamine and its phosphate esters (III)

Sodium citrate buffer was shown to give the best electrophoretic separation of thiamine and its phosphate esters. This buffer was satisfactory even if the sample contained perchloric acid, another buffer or tissue extract as shown in Fig. 1 (III), whereas no acceptable separation was achieved by the method of Itokawa and Cooper (Fig. 2). In experiments depicted in Fig. 1 (III) different mobilities of thiamine compounds on different strips were due to unequal times used for electrophoresis.

Addition of methanol, ethanol and propanol to the electrophoretic buffer improved the elution of thiamine compounds from the paper strip (Table 1, III). 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, which gave a recovery of only 66 %. Table 2 (III) shows the recovery after electrophoretic separation and elution.

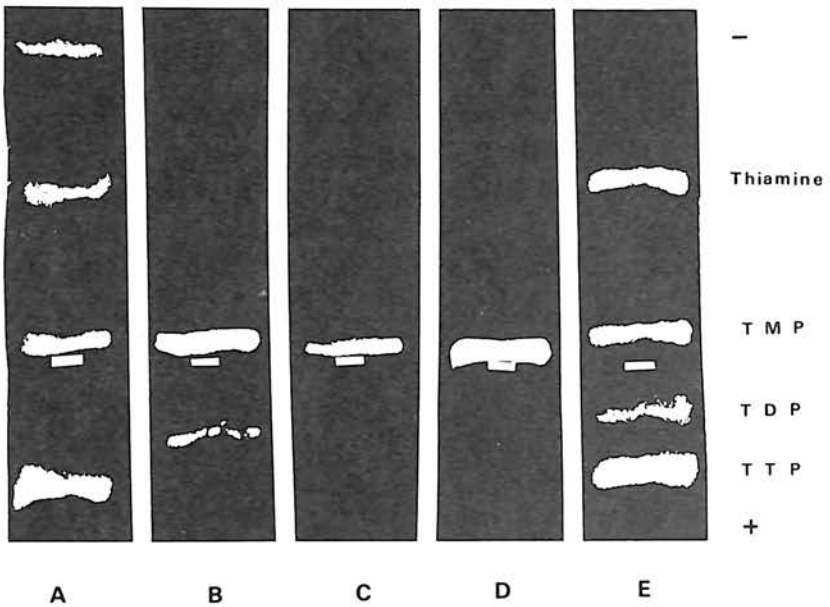

Fig. 2. Comparison of the electrophoretic separation of thiamine and its phosphate esters between the present method and that of Itokawa and Cooper (24). The strips A, B, C and D were treated according to the method of Itokawa and Cooper, and the strip E according the present method. A sample of 5 µl of 0.5 mM thiamine and its mono-, di-, and triphosphate esters was applied in the middle of the strip (white lines) and subjected to electrophoresis, and treated with alkaline ferricyanide as described in Materials and Methods. The solvents for thiamine compounds in the samples applied to each strip were as follows: strip A, water; strip B, 0.05 M potassium phosphate buffer, pH 7.0; strip C, rat brain extract as in Fig. 1 (III), diluted with water 1:2, v/v; and strip D, 0.4 M $HClO_4$. Strip E demonstrates the separation of a sample containing 0.4 M $HClO_4$ with the present method. Similar separations were achieved with this method also when the solvent of the sample was as for strips A, B and C.

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 tissues (III)

The content of thiamine and its phosphate esters in rat brain was determined using the present electrophoretic and fluorometric method. The distribution of the different thiamine compounds were as follows: thiamine 0.70 nmol/g (6.3 %), TMP 2.3 nmol/g (20.6 %), TDP 7.2 nmol/g (64.6 %), and TTP 0.95 nmol/g (8.5 %). These values are calculated from Table 3 (III). Thiamine and its phosphate esters could be determined also from other rat tissues.

Thiamine pyrophosphokinase of rat tissues

Among the enzymes catalyzing the metabolism of thiamine phosphate esters thiamine pyrophosphokinase (E.C. 2.7.6.2) has been perhaps most thoroughly studied. This enzyme has been purified from rat liver (48), rat brain (47), and pig brain (46). Subcellular fractionation has indicated that the enzyme is soluble in rat intestine and liver (44,55). The present study of thiamine pyrophosphokinase was carried out to test the presented electrophoretic method and to obtain information about its distribution in various rat tissues. The results indicated that the activity of thiamine pyrophosphokinase was found in all rat tissues studied: in brain, heart, intestine, kidney, liver, lung, muscle, and spleen (Table I). Kidney and liver were found to be the richest sources of the enzyme, – perhaps an indication of the active metabolism of these tissues. The specific activity of rat liver enzyme (1.6 nmol of TDP/mg protein/h) was comparable to the previously published values (0.8 - 1.0 nmol of TDP/mg protein/h) (127). The present electrophoretic method was proved to be suitable for the determination of the activity of thiamine pyrophosphokinase.

Table I. Specific activities of thiamine pyrophosphokinase in rat tissues. The tissues were prepared as described for gel electrophoresis in paper V, and the activity was determined as described in Materials and Methods. The activity in kidney was chosen as 100 %.

	n mol of TDP/mg protein/h	%
Brain	0.34	13
Heart	0.44	17
Intestine	0.22	8
Kidney	2.66	100
Liver	1.58	59
Lung	0.28	11
Muscle	0.10	4
Spleen	0.38	14

Specificity of thiamine triphosphatase of rat tissues (IV,V)

Because attempts to determine the activity of the enzyme synthesizing thiamine triphosphate were unsuccessful, an attempt was made to investigate thiamine triphosphatase. This soluble enzyme (E.C. 3.6.1.28), isolated from rat brain (60) and claimed to be specific for thiamine triphosphate, has not been shown to be distinct from alkaline and acid phosphatase. To clarify the relationship of these activities, eight tissue extracts of the rat were subjected to gel electrophoresis. Table 1 (V) indicates that thiamine triphosphatase of brain, heart, kidney, liver, lung, muscle, and spleen have rather identical electrophoretic mobility and differed clearly from alkaline and acid phosphatase, whereas the activity of thiamine triphosphatase of intestine was detected at the location of alkaline phosphatase. To obtain more information on the specificity of thiamine triphosphatase, rat brain, liver, and intestine were further fractionated by gel filtration. Because phosphatase assays were complicated by the formation of a precipitable complex between ammonium molybdate and thiamine phosphate, a new modification was developed and used in this study. The characteristics of the method are presented in the paper IV.

The relation between the absorbance of the phosphomolybdate complex and the phosphate concentration of the sample is shown in Fig. 2 (IV). The response was linear up to a phosphate concentration of 1 mM, which corresponds to an absorbance of about 1.8 units. The variation coefficients for 10 replicates for samples containing 10, 100 and 250 nmol of phosphate were 3.6, 0.5 and 1.1 %, respectively. The continuous flow system also gave reproducible results (Fig. 3, IV).

Because the citrate-arsenite solution prevents the formation of phosphomolybdate complex, it was essential to investigate the rate of formation of the colored complex. It was shown that the formation of the phosphomolybdate complex was very rapid, and the most suitable time for the addition of citrate-arsenite reagent was 1-3 min after the addition of molybdate (Fig. 5, IV). The reaction mixture must be acidic; otherwise the formation of color is disturbed.

The citrate-arsenite reagent was found to prevent precipitation of the ammonium molybdate-thiamine phosphate complex. Although some turbidity was produced in the presence of these two compounds, the reaction mixture cleared up upon the addition of citrate-arsenite reagent. Except for thiamine triphosphatase, the activities of alkaline and acid phosphatase could also be determined using this method.

Different preparations from rat liver, brain,and small intestine were fractionated on Sephadex G-100 for the activities of various phosphatases (Fig. 1, V). The thiamine triphosphatase activity from both liver and brain was clearly separated from alkaline phosphatase (i.e. using p-nitrophenyl phosphate in alkaline pH) and acid phosphatase (employing p-nitrophenyl phosphate in acid pH). The intestine, however, gave quite different results. The intestinal thiamine triphosphatase activity was eluted as two separate peaks which exactly corresponded to the peaks of alkaline phosphatase (determined with p-nitrophenyl phosphate as the substrate) (Fig. 1, V). The intestinal preparations contained no thiamine triphosphatase activity corresponding to that present in the liver and brain. Acid phosphatase assay gave a single activity peak for the intestine which was located at the second alkaline phosphatase peak (Fig. 1, V).

Apparent molecular weight (30 000) and the Stokes radius (2.5 nm) for brain and liver thiamine triphosphatase were calculated according to the standard curves as presented in paper V.

The brain enzyme was specific for thiamine triphosphate (Table 3, V) whereas the liver preparation exhibited some activity for thiamine diphosphate, CTP, UTP, GTP and ITP indicating that the liver preparation was contaminated with nonspecific phosphatase activity (V). The preparations of brain and liver thiamine triphosphatase were free from alkaline and acid phosphatase activities (with p-nitrophenyl phosphate as substrate). Thiamine triphosphate was not hydrolyzed in acid pH (Table 3, V).

The partially purified thiamine triphosphatase had a pH optimum of pH 9.0. The apparent K_m for thiamine triphosphate was 0.5 mM (determined for the liver enzyme only). The isoelectric point of the brain enzyme, determined by isoelectric focusing in column, was 4.6 (at 4 ^{o}C). The crude cytosolic fraction gave a single symmetrical peak of thiamine triphosphatase. This was partially separated from alkaline phosphatase peaks (pI values 4.4 and 9.4) and totally separated from acid phosphatase (pI values 5.3 and 5.7).

DISCUSSION

The content of thiamine phosphate esters in rat brain obtained by the present method is comparable to the values presented by other investigators (25,118,119,128,129). The fluorescence of thiamine triphosphate was about twice as that of background (Table 3, III). The low amount of TTP in rat brain made it impossible to estimate the thiamine/phosphate ratio of the fluorescent compound electrophoresed at the location of TTP. Furthermore, the possible interference of nucleotides in the estimation of hydrolyzable phosphates of thiamine triphosphate was not excluded in the original study of Rossi-Fanelli et al. (115). At least ADP was found to migrate identically with TTP in the electrophoresis under conditions described in the paper III.

Although TTP has been separated by different chromatographic methods in biological material (24-26,30,115-119,128,129) and estimated by fluorometry, no data have been presented so far on the exact structure of the fluorescent compound. The possibility can not be excluded that different thiamine phosphates may form a complex which has identical chromato-

graphic properties with thiamine triphosphate as suggested by Schrijver et al. (57) before. Thiamine phosphate esters have been found to form complex with Na^+, K^+, Ca^{2+} (130), Tris-HCl (127) and ammonium formate (131). Thus the occurrence of TTP in biological material should not be regarded as established. Similar doubts have been raised by other investigators before (132,133). Furthermore, the verification of the biological synthesis of TTP has been proved very difficult. Moreover, the published methods for the determination of this activity (52-55) have not been reproducible as tested in this laboratory. Schrijver et al. (57) have also concluded that there is no suitable method for the estimation of the activity responsible for the synthesis of TTP. Perhaps one reason for this difficulty is that thiamine triphosphate may be rapidly hydrolyzed by different phosphatases, especially by thiamine triphosphatase, which has been detected both in soluble and membraneous fractions of various cells (59,60,62). However, the recently reported 70-fold purification of thiamine diphosphate kinase in rat liver (58) gives support to the occurrence of TTP.

To obtain information about the metabolism of TTP the soluble thiamine triphosphatase of some rat tissues was characterized in more detail. The present data indicate that the activity of thiamine triphosphatase of rat brain and liver is separable from the activities of alkaline and acid phosphatases (Fig. 1, V). This is in contrast to the intestinal preparation where the activity seems to be related to that of alkaline phosphatase. The last observation is at variance with the view (60,62) that intestine contains the highest activity of the soluble thiamine triphosphatase among rat tissues. In agreement with the present results Iwata et al. (71) have noticed that the activity hydrolyzing TDP in rat small intestine resembles that of alkaline phosphatase. Thiamine triphosphatase of rat brain and liver was found to be specific for thiamine triphosphate, supporting the previous results of Hashitani and Cooper (60).

Gel electrophoretic studies revealed that the soluble thiamine triphosphatase occurs in several rat tissues. In addition to brain and liver, it was found in heart, kidney, lung, muscle, and spleen (Table 1, V). On the basis of electrophoretic mobility of thiamine triphosphatase in these tissues (Table 1, V) it is possible, even likely, that the molecular properties of these enzymes are similar and thus the substrate specificity might be similar to brain enzyme.

Among thiamine phosphate esters only thiamine diphosphate, a cofactor for many enzymes in intermediary metabolism, has an established biochemical function. It is of interest to note that the specific thiamine triphosphatase described in the present study is much more generally distributed than the specific thiamine diphosphatase. According to the electrophoretic studies of Allen (72) the latter enzyme was present in only a few tissues of the mouse (e.g. epididymis, parotid gland and submaxillary gland) and was totally lacking in most of the tissues, for example liver, brain and intestine.

Although the occurrence of TTP in living material is poorly documented, the evidence suggesting a specific thiamine triphosphatase in the soluble fraction of rat brain and liver supports the view that thiamine triphosphate - if it really exists in cells - may have some specific role in metabolism.

Finally, it should be stated that the mystery of TTP is rather difficult to solve at the present time. The first and most important step toward this end would be to ensure the existence of TTP in biological material. Hopefully the methods presented in this study would be of help in obtaining more reliable and unconflicting information on the occurrence and biological significance of thiamine triphosphate.

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REFERENCES

1. Jansen, B.C.P. and Donath, W.F. (1926) Chem. Weekbl. 23, 201-203
2. Kinnersley, H.W. and Peters, R.A. (1934) Biochem. J. 28, 667-670
3. Jansen, B.C.P. (1936) Rec. Trav. Chim. Pays-Bass 55,1046-1052
4. Fujiwara, M. and Matsui, K. (1953) Anal. Chem. 25, 810-812
5. Hayden, K.J. (1957) Analyst 82, 61-66
6. Gupta, V.D. and Cadwallader, D.E. (1968) J. Pharm. Sci. 57, 112-117
7. Snell, E.E. (1950) in Vitamin Methods (György, P., ed.) Vol 1, pp. 372-377, Academic Press, New York
8. Sarett, H.P. and Cheldelin, V.H. (1944) J. Biol. Chem. 155, 153-160
9. Dwivedi, B.K. and Arnold, R.G. (1972) J. Food Sci. 37, 889-891
10. Sammons, M.C., Bursey, M.M. and Brent, D.A. (1974) Biomed. Mass Spectrom. 1, 169-171
11. Roser, R.L., Andrist, A.H., Harrington, W.H., Naito, H.K. and Lonsdale, D. (1978) J. Chromatogr. 146, 43-53
12. Ochoa, S. and Peters, R.A. (1938) Biochem. J. 32, 1501-1515
13. Westenbrink, H.G.K. and Steyne-Parvé, E.P. (1949) Int. Z. Vitaminforsch. 21, 461-479
14. Kay, W.W. and Murfitt, K.C. (1956) Biochem. J. 62, 601-605
15. Kazino, Y. (1957) J. Biochem. 44, 827-838
16. Ullrich, J. (1970) in Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds) Vol 18, Part A, pp. 109-115, Academic Press, New York
17. Gubler, C.J., Johnson, L.R. and Wittorf, J.H. (1970) in Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds) Vol 18, Part A, pp. 120-125, Academic Press, New York

18. Kinnersley, H.W. and Peters, R.A. (1938) Biochem. J. 32, 1516-1520

19. Rossi-Fanelli, A., Siliprandi, N. and Fasella, P. (1952) Science 116, 711-713

20. Bartley, W. (1954) Biochem. J. 56, 379-387

21. Lewin, L.M. and Wei, R. (1966) Anal. Biochem. 16, 29-35

22. Siliprandi, D. and Siliprandi, N. (1954) Biochim. Biophys. Acta 14, 52-61

23. Kiessling, K.H. (1956) Acta Chem. Scand. 10, 1356-1358

24. Itokawa, Y. and Cooper, J.R. (1970) in Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds) Vol 18, Part A, pp. 91-92, Academic Press, New York

25. Rindi, G. and Giuseppe, L. (1961) Biochem. J. 78, 602-606

26. Koike, H., Wada, T. and Manakami, H. (1967) J. Biochem. 62, 472-494

27. Nakamura, A., Sanada, K. and Katsuda, E. (1968) Vitamins 37, 1-11

28. Ishii, K., Sarai, K., Sanemori, H. and Kawasaki, T. (1979) Anal. Biochem. 97, 191-195

29. Kimura, M., Fujita, T., Nishida, S. and Itokawa, Y. (1980) J. Chromatogr. 188, 417-419

30. Sanemori, H., Ueki, H. and Kawasaki, T. (1980) Anal. Biochem. 107, 451-455

31. Nishimune, T., Abe, M. and Hayashi, R. (1972) Biochim. Biophys. Acta 279, 527-534

32. Krampitz, L.O. (1969) Ann. Rev. Biochem. 38, 213-240

33. Green, D.E., Westerfield, W.W., Vennesland, B. and Knox, W.E. (1942) J. Biol. Chem. 145, 69-84

34. Strumpf, P.K., Zarydnaya, K. and Green, D.E. (1947) J. Biol. Chem. 167, 817-825

35. Horecker, B.L., Smyrniatis, P.Z. and Klenow, H. (1953) J. Biol. Chem. 205, 661-682

36. Nakada, H.I. and Sund, L.F. (1958) J. Biol. Chem. 233, 8-13

37. Conelly, J.L. and Johnson, W.A. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 45-55, John Wiley & Sons, New York

38. Reed, L.J. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 19-27, John Wiley & Sons, New York

39. Koike, M., Hamada, M., Koike, K., Hiraoka, T. and Nakaula, Y. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 5-18, John Wiley & Sons, New York

40. Gassmann, B., Proll, J. and Lexow, D. (1967) Int. Z. Vitaminfrosch. 37, 203-212

41. Ventura, U., Giacalone, E. and Rindi, G. (1963) Boll. Soc. Ital. Biol. Sper. 39, 1443-1446

42. Sciorelli, G. and Ventura, U. (1969) Acta Vitaminol. Enzymol. 23, 231-233

43. Ventura, U. and Sciorelli, G. (1965) Experientia 21, 645-646

44. Cusaro, G., Rindi, G. and Scorelli, G. (1977) Int. J. Vitam. Nutr. Res. 47, 99-106

45. Ferrari, G., Ventura, U. and Rindi, G. (1971) Life Sci. 10, 67-75

46. Peterson, J.W., Gubler, C.J. and Kuby, S.A. (1975) Biochim. Biophys. Acta 397, 377-394

47. Johnson, L.R. and Gubler, C.J. (1968) Biochim. Biophys. Acta 156, 85-96

48. Mano, Y. (1960) J. Biochem. 47, 283-289

49. Artsukevich, I.M., Voskoboev, A.I. and Ostrovskii, Y.M. (1977) Vopr. Med. Khim. 23, 203-210

50. Eckert, T. and Möbus, W. (1964) Hoppe-Seylers Z. Physiol. Chem. 338, 286-288

51. Iida, S. (1966) Biochem. Pharmacol. 15, 1139-1145

52. Itokawa, Y. and Cooper, J.R. (1968) Biochim. Biophys. Acta 158, 180-182

53. Itokawa, Y. and Cooper, J.R. (1970) in Methods in Enzymology, (McCormick, D.B. and Wright, L.D., eds) Vol 18, Part A, pp. 226-227, Academic Press, New York

54. Itokawa, Y. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 361-368, John Wiley & Sons, New York

55. Hoekstra, D., Berger, R. and Hommes, F.A. (1974) Nutr. Metab. 16, 317-323

56. Ruenwongsa, P. and Cooper, J.R. (1977) Biochim. Biophys. Acta 482, 64-70

57. Schrijver, J., Dias, T. and Hommes, F.A. (1978) Neurochem. Res. 3, 699-709

58. Voskoboev, A.I. and Luchko, V.S. (1980) Vopr. Med. Khim. 26, 564-568

59. Barchi, R.L. and Braun, P.E. (1972) Biochim. Biophys. Acta 255, 402-405

60. Hashitani, Y. and Cooper, J.R. (1972) J. Biol. Chem. 247, 2117-2119

61. Iwata, H., Baba, A. and Matsuda, T. (1974) Jpn. J. Pharmacol. 24, 817-823

62. Barchi, R.L. and Braun, P.E. (1972) J. Biol. Chem. 247, 7668-7673

63. Barchi, R.L. and Viale, R.O. (1975) J. Biol. Chem. 251, 193-197

64. Barchi, R.L. (1976) J. Neurochem. 26, 715-720

65. Yamazaki, M. and Hayashi, O. (1968) J. Biol. Chem. 243, 2934-2942

66. Barchi, R.L. and Braun, P.E. (1972) J. Neurochem. 19, 1039-1048

67. Baba, A., Matsuda, T. and Iwata, H. (1977) Biochim. Biophys. Acta 482, 71-78

68. Inoue, A., Shim., S. and Iwata, H. (1970) J. Neurochem. 17, 1373-1382

69. Inoue, A. and Iwata, H. (1971) Biochim. Biophys. Acta 242, 459-469

70. Castner, J.F. and Evans, H.J. (1977) Neurosci. Lett. 4, 269-273

71. Iwata, H., Matsuda, T. and Baba, A. (1976) Experientia 32, 1252-1253

72. Allen, J.M. (1963) J. Histochem. Cytochem. 11, 542-552

73. Neal, R.A. and Pearson, W.N. (1964) J. Nutr. 83, 343-350

74. Lonsdale, D. (1975) C.R.C. Crit. Rev. Clin. Lab. Sci. 5, 289-313

75. Platt, B.S. (1958) Fed. Proc. 17, 8-20

76. Weiss, S. (1940) J. Am. Med. Assoc. 115, 823-839

77. Lofland, H.B., Goodman, H.D., Clarkson, T.B. and Prichard, D.W. (1963) J. Nutr. 79, 188-194

78. Gries, C.L. and Scott, M.L. (1972) J. Nutr. 102, 1269-1286

79. Pincus, J.H. and Wells, K. (1972) Exp. Neurol. 37, 495-501

80. Gubler, C.J. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 121-139, John Wiley & Sons, New York

81. Dreyfus, P.M. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 229-239, John Wiley & Sons, New York

82. Sturman, J.A. and Rivlin, R.S. (1975) in Biology of Brain Dysfunction (Gaull, G.E., ed.) Vol 3, pp. 425-436, Plenum Press, New York

83. Jolliffe, N., Wortis, H. and Fein, H.D. (1941) Arch. Neurol. Psychiatr. 46, 569-597

84. Alexander, L. (1940) Am. J. Pathol. 16, 61-69

85. Riggs, H.E. and Boles, R.S. (1944) Quart. J. Stud. Alcohol 5, 361-370

86. Phillips, G.B., Victor, M., Adams, R.D. and Davidson, C.S. (1952) J. Clin. Invest. 31, 859-871

87. Blass, J.B. and Gibson, G.E. (1977) N. Engl. J. Med. 297, 1367-1370

88. Blass, J.P., Gary, E.G. and Kark, R.A.P. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 321-332, John Wiley & Sons, New York

89. Farrell, D.F., Clark, A.F., Scott, C.R. and Wennberg, R.P. (1975) Science 187, 1082-1084

90. Elsas, L.J., Danner, D.J. and Rogers, B.L. (1976) in Thiamine (Gubler, C.J., Fujiwara, M. and Dreyfus, P.M., eds) pp. 335-349, John Wiley & Sons, New York

91. Cooper, J.R., Itokawa, Y. and Pincus, J.H. (1969) Science 164, 74-75

92. Cooper, J.R., Pincus, J.H., Itokawa, Y. and Piros, K. (1970) N. Engl. J. Med. 283, 793-795

93. Pincus, J.H. (1972) Dev. Med. Child. Neurol. 14, 87-101

94. Murphy, J.V. (1973) Pediatrics 51, 710-715

95. Pincus, J.H., Cooper, J.R., Murphy, J.V., Rabe, E.F., Lonsdale, D. and Dunn, H.G. (1973) Pediatrics 51, 716-721

96. Murphy, J.V., Craig, L.J. and Glew, R.H. (1974) Arch. Neurol. 31, 220-227

97. Murphy, J.V. (1974) Dev. Med. Child. Neurol. 16, 362-364

98. Murphy, J.V. and Craig, L. (1975) J. Neurol. Neurosurg. Psychiatry 38, 1100-1103

99. Pincus, J.H., Solitare, G.B. and Cooper, J.R. (1976) Arch. Neurol. 33, 759-763

100. Mintz, B. (1938) Compt. Rend. Soc. Biol. (Paris) 127, 1251-1253

101. von Muralt, A. (1962) Ann. N.Y. Acad. Sci. 98, 499-507

102. von Muralt, A. (1947) Vitam. Horm. 5, 93-118

103. Gurtner, H.P. (1961) Helv. Physiol. Acta, Suppl. 11, 1-48

104. Itokawa, Y. and Cooper, J.R. (1970) Biochem. Pharmacol. 19, 985-992

105. Itokawa, Y. and Cooper, J.R. (1970) Biochim. Biophys. Acta 196, 274-284

106. Itokawa, Y., Schulz, R.A. and Cooper, J.R. (1972) Biochim. Biophys. Acta 266, 293-299

107. Goldberg, D.J. (1974) Thesis, Yale University, Xerox University Microfilms, Ann Arbor, Michigan 48106

108. Bergman, K. and Fishman, R.A. (1975) J. Neurochem. 24, 457-465

109. Eder, L., Hirt, L. and Dunant, Y. (1976) Nature 264, 186-188

110. Fox, J.M. and Duppel, W. (1975) Brain Res. 89, 287-302

111. Waldenlind, L. (1979) Acta Physiol. Scand. 105, 1-10

112. Waldenlind, L. (1978) Acta Physiol. Scand. Suppl. 459, 1-35

113. Cooper, J.R. and Pincus, J.H. (1979) Neurochem. Res. 4, 223-239

114. Leder, I.G. (1975) in Metab. Pathways (Greenberg, D.M., ed.) Vol 7., pp. 57-85, Academic Press, New York

115. Rossi-Fanelli, A., Siliprandi, N. and Fasella, P. (1952) Science 116, 711-713

116. Greiling, H. and Kiesow, L. (1958) Z. Naturforsch. 13 B, 251-252

117. Kiessling, K.H. (1961) Biochim. Biophys. Acta 46, 603-604

118. Ishii, K., Sarai, K., Sanemori, H. and Kawasaki, T. (1979) J. Nutr. Sci. Vitaminol. (Tokyo) 25, 517-523

119. Patrini, C. and Rindi, G. (1980) Int. J. Vitam. Nutr. Res. 50, 10-18

120. Eliasson, S.G. and Scarpellini, J.D. (1976) Neurochem. Res. 1, 191-200

121. Penttinen, H.K. (1979) in Methods in Enzymology (McCormick D.B. and Wright, L.D., eds) Vol 62, part D, p. 112, Academic Press, New York

122. Penttinen, H.K. (1979) in Methods in Enzymology (McCormick D.B. and Wright, L.D., eds) Vol 62, part D, pp. 58-59, Academic Press, New York

123. Penttinen, H.K. (1979) in Methods in Enzymology (McCormick D.B. and Wright, L.D., eds) Vol 62, part D, pp. 68-69, Academic Press, New York

124. Gornall, A.G., Bardawilli, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751-766

125. Böhlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220

126. Yusa, T. (1959) J. Biochem. (Tokyo) 46, 391-393

127. Deus, B., Blum, H.E.C. and Holzer, H. (1969) Anal. Biochem. 27, 492-501

128. Murdock, D.S. and Gubler, C.J. (1973) J. Nutr. Sci. Vitaminol. (Tokyo) 19, 43-54

129. Dreyfus, P.M. (1959) J. Neurochem. 4, 183-190

130. Hoffman, H., Eckert, T. and Möbus, W. (1964) Hoppe-Seylers Z. Physiol. Chem. 335, 156-160

131. Gounaris, A.D. and Schulman, M. (1980) Anal. Biochem. 102, 145-149

132. Wiss, O. and Brubacher, G. (1962) Ann. N.Y. Acad. Sci. 98, 508-515

133. Rindi, G., Perri, V. and Ventura, U. (1962) Nature 193, 581-582.

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Synthesis of Thiamine Triphosphate

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Thiamine triphosphate is synthesized by heating a mixture of thiamine hydrochloride and phosphorus pentoxide in orthophosphoric acid. The purification procedure has been simplified by using the strong acid cation ion-exchange resin, Dowex 50 W, in the separation of thiamine triphosphate from other phosphorylated thiamines and inorganic phosphate.

Although thiamine triphosphate (TTP) was found as early as 1952 by Rossi-Fanelli [1] in rat liver and later in other biological materials,[2,3] its physiological function is still obscure. It has been supposed to have a specific role in nerve conduction.[4–6]

Thiamine triphosphate was first synthesized by Velluz *et al.*[7] and later by other investigators.[8–10] In this study the synthesis was performed according to Yusa.[11] The purification of TTP was accomplished with the strong acid ion-exchange resin Dowex 50 W, rather than the weak acid resin (Amberlite IRC 50) used by Yusa. The main advantage of the Dowex 50 resin is that TTP, despite some retardation in the column, is slowly eluted by water, whereas thiamine and its mono- and diphosphate esters are tightly bound in the column. Consequently, separation of TTP from other thiamine esters was achieved (Fig. 1). The purification of TTP by Amberlite IRC 50 resin was not adequate because it was eluted in the void volume [12] together with thiamine polyphosphates and inorganic phosphate.

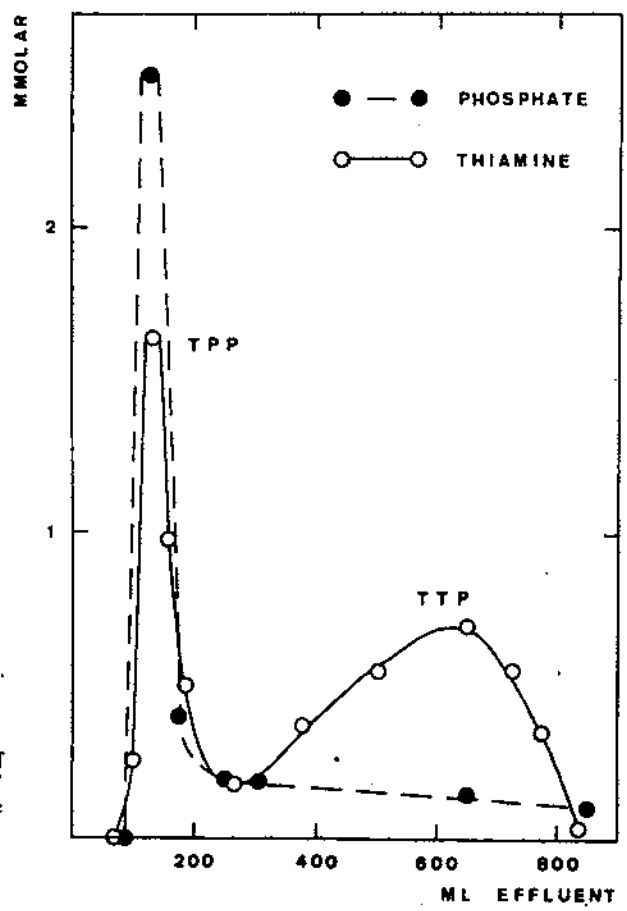

Fig. 1. Purification of a crude mixture of thiamine phosphate esters on a Dowex 50 W column. For details, see text. TPP = thiamine polyphosphate.

EXPERIMENTAL

A mixture of 50 g of thiamine hydrochloride (Sigma Chemical Company, St.Louis, Mo., U.S.A.) and 50 g of phosphorus pentoxide was added in small amounts to phosphoric acid at a constant temperature of 100–105 °C. The phosphoric acid had been prepared from 77 ml of 15 M orthophosphoric acid at 320 °C. The solution was kept a further 20 min at 100–105 °C and then allowed to cool to room temperature. Thereafter, 100 ml of water was added and the resulting solution was poured slowly into two liters of a cold (+4 °C) ethanol – acetone mixture (1:1, v/v) undergoing agitation by a magnetic mixer. The precipitate was collected and dissolved in 100 ml of water. After three successive precipitations the mixture of thiamine and its phosphate esters did not contain significant amounts of inorganic phosphate when tested by the method of Fiske and SubbaRow.[13] By contrast, the final ethanol-acetone mixture used in the precipitation procedure contained 1 mM inorganic phosphate. The final precipitate was dissolved in water and evaporated under reduced pressure at 30 °C for 10 min in order to remove most of the ethanol and acetone. The volume of the mixture was adjusted to 100 ml, and 10 ml (about 3 mmoles) was applied to a Dowex 50 W column (X-8, 200–400 mesh, 4 × 16 cm, H^+-form). The column was eluted at 24 °C with water at a rate of 10 ml per min. Thiamine was located in the effluent by spraying a ferricyanide reagent (76 ml of 50 % ethanol, v/v, 15 ml of 15 % sodium hydroxide and 1 ml of 2 % potassium ferricyanide) onto a drop of effluent on a piece of paper and examining the paper under ultraviolet light. The effluent between 325 and 825 ml was collected and lyophilized. The white powder obtained was dissolved in a minimal amount (*ca.* 0.8 ml) of water and absolute ethanol was added until some turbidity appeared in the solution. Thiamine triphosphate crystallized from this solution after a few hours at +4 °C. The crystallization was repeated twice and the crystals dried overnight in a desiccator. The final yield of analytically pure thiamine triphosphate (see below) was 31 mg.

Thiamine and its mono- and diphosphate esters were removed from the column by washing with four liters of 4 M HCl at +24 °C. Thiamine diphosphate was partly hydrolyzed to thiamine monophosphate during this regeneration. Thiamine and its phosphate esters were determined as described elsewhere.[14]

Identification of thiamine triphosphate. The melting point of the polarizing crystals of thiamine triphosphate was 194 °C (decomp.). Elemental analysis gave the following composition: C 27.27 ± 0.34, H 4.00 ± 0.09, N 10.59 ± 0.16 (N = 4), and P 18.25 ± 0.15 (N = 2). The calculated values from the formula $C_{12}H_{19}N_4O_{10}P_3S \cdot 1/2\ H_2O$ are C 28.08, H 3.93, N 10.92 and P 18.10.[15]

The ultraviolet absorption spectrum of thiamine triphosphate was similar to that of thiamine and its mono- and diphosphate forms in 0.1 M HCl, 0.1 M NaOH and 0.05 M potassium phosphate buffer, pH 7.0. Infrared absorption analysis indicated characteristic differences between thiamine phosphate esters.

For paper chromatography a sample of 5 µl of 0.5 mM thiamine and its phosphate esters was applied to a Whatman No. 1 chromatography paper. The ascending chromatography was conducted at 24 °C for 6 hours. The R_f values for thiamine and its mono-, di- and triphosphate were 0.58, 0.27, 0.17 and 0.12, respectively, with the solvent propanol-water-formic acid (1 M, pH 5.0) (65:20:15, v/v/v). In propanol-water-acetic acid (1 M, pH 5.0) (70:20:10, v/v/v) the corresponding R_f values were 0.56, 0.20, 0.08 and 0.05. These results accord with the R_f values given by other investigators.[11,16]

Stability of thiamine triphosphate. When samples of crystallized thiamine triphosphate were stored for two months at −70, +4 and +24 °C, no destruction was detected, but if they were dissolved in water and kept at −18 C for a similar time, about 18 per cent was hydrolyzed to thiamine diphosphate. In solutions containing 0.4 M $HClO_4$, more than 30 per cent was hydrolyzed after one week at +4 °C. When thiamine triphosphate was incubated in an alkaline medium its ability to form the fluorescent thiochrome derivative was partly or totally lost depending on the degree of alkalinity.

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REFERENCES

1. Rossi-Fanelli, A., Siliprandi, N. and Fasella, P. *Science 116* (1952) 711.
2. Greiling, H. and Kiesow, L. Z. *Naturforsch. Teil B 13* (1958) 251.
3. Kiessling, K. H. *Nature (London) 172* (1953) 1187.
4. Pincus, P. H. *Develop. Med. Child Neurol. 14* (1972) 87.
5. Berman, K. and Fishman, R. A. *J. Neurochem. 24* (1975) 457.
6. Fox, J. M. and Duppel, W. *Brain Research 89* (1975) 287.
7. Velluz, L., Amiard, G. and Bartos, J. *Bull. Soc. Chim. Fr. 15* (1948) 871.
8. Roux, H., Teysseire, Y. and Duchesne, G. *Bull. Soc. Chim. Biol. 30* (1948) 592.
9. Viscontini, M., Bonetti, G. and Karrer, P. *Helv. Chim. Acta 32* (1949) 1478.
10. Rossi-Fanelli, A., Siliprandi, N., Siliprandi, D. and Ciccarone, P. *Arch. Biochem. 58* (1955) 237.
11. Yusa, T. *J. Biochem. (Tokyo) 46* (1959) 391.
12. Siliprandi, D. and Siliprandi, N. *Biochim. Biophys. Acta 14* (1954) 52.
13. Fiske, C. H. and SubbaRow, Y. *J. Biol. Chem. 66* (1925) 375.
14. Itokawa, Y. and Cooper, J. R. *Methods Enzymol. 28 A* (1970) 91.
15. Stecher, P. G., Ed., *The Merck Index*, Merck & Co., Inc., Rahway, N. J., U.S.A. 1968.
16. Lewin, L. M. and Wei, R. *Anal. Biochem. 16* (1966) 29.

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Differences in Thiochrome Fluorescence Produced by Thiamine and Its Mono-, Di-, and Triphosphate Esters

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Equimolar amounts of thiamine and its mono-, di-, and triphosphate esters, when oxidized with potassium hexacyanoferrate(III) in an alkaline medium, were found to produce unequal intensities of thiochrome fluorescence. These differences were less pronounced if the oxidation medium contained ethanol. Assuming that the phosphate groups do not increase the fluorescence, then in 50 % ethanol (v/v); 74 % of thiamine and thiamine monophosphate, 85 % of thiamine diphosphate and 93 % of thiamine triphosphate are converted to thiochrome. The differences observed may be due to the formation of different amounts of nonfluorescent oxidation products, especially the disulfide derivative. This view is supported by the observation that thiamine and its phosphate esters differ in their stability to alkalinity.

In 1936 Jansen [1] found that oxidation of thiamine with potassium hexacyanoferrate(III) in an alkaline medium leads to the formation of the fluorescent thiochrome derivative. This reaction has frequently been used in the quantitative determination of thiamine. Methanol and ethanol, when present in the oxidation medium, favor the production of thiochrome from thiamine,[2,3] but there is no study on their possible effect on the formation of thiochrome from thiamine phosphate esters. In addition to thiamine, its mono-, di-, and triphosphate esters are found in biological materials [4–6] and can also be oxidized to fluorescent thiochrome derivatives. Lewin and Wei [7] claimed that thiamine and its mono- and diphosphate esters in equimolar amounts produce fluorescence of the same intensity. This view seems not to be generally accepted, because most investigators still treat thiamine phosphate esters with phosphatase [8] to liberate the thiamine before oxidation. The aim of the present study was to characterize the formation of thiochrome from thiamine phosphate esters, and to examine some factors influencing this reaction.

EXPERIMENTAL

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

Reagents. Thiamine and its mono- and diphosphate esters were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Thiamine triphosphate was prepared as described elsewhere.[9] All these preparations were crystallized three times and were free from inorganic phosphate. A standard solution of thiamine was made from the U.S.P. Reference Standard.[10] Thiochrome was obtained from Pfalz & Bauer, Inc., New York, U.S.A., and crystallized twice before use.

The quinine standard was 0.01 % (w/v quinine sulfate in 0.1 M H_2SO_4, diluted 1:10 and was taken to have a fluorescence of 100.

Formation of thiochrome. Thiamine and its phosphate esters were oxidized to thiochrome by the method of Lewin and Wei,[7] except that the oxidation medium contained ethanol. A sample (5 μl) of the compound was added to 3.0 ml of 50 % ethanol (v/v) and shaken, and after a few minutes 0.5 ml of alkaline hexacyanoferrate(III) reagent (15 ml of 15 % NaOH and 1 ml of 2 % potassium hexacyanoferrate(III)) was added. The mixture was agitated for 2 min, and 10 μl of 30 % H_2O_2 was added to destroy the yellow color of hexacyanoferrate(III). The fluorescence was then measured with a Zeiss PMQ II spectrophotometer fitted with a ZFM 4 fluorometer attachment (Carl Zeiss, Germany). The excitation wavelength was 365 nm and the emission maximum 430 nm.

Determination of phosphate. Total and hydrolyzable phosphate were determined according to Fiske and SubbaRow.[11] The hydrolyzable phosphate was determined after the specimen had been incubated in 1 M HCl for 10 min in a boiling water bath.

Infrared absorption analysis. Pellets containing 1 mg of thiamine compound in 120 mg of KBr were analyzed with a Beckman IR 10 spectrophotometer.

RESULTS

Effect of different alcohols on thiochrome formation and fluorescence. The effects of methanol, ethanol and propanol on thiochrome formation from a mixture of thiamine and its phosphate esters were compared (Fig. 1). In the light of this experiment, 50 % ethanol was chosen for the oxidation medium. When thiamine compounds were separately oxidized in media containing different proportions of ethanol, it was noted that the increase in thiochrome formation due to the presence of ethanol was greatest with thiamine, and smaller with TMP, TDP, and TTP, in this order. This conclusion can be drawn from the values of the index F/F_w in Table 1 as well.

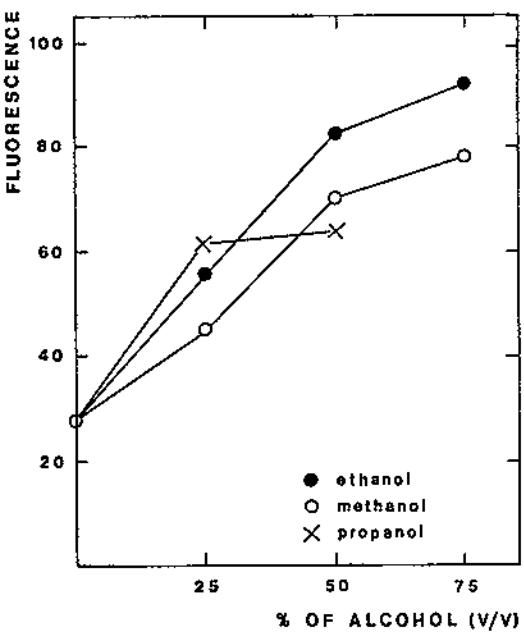

Fig. 1. The effect of different alcohols on formation of thiochrome from a mixture containing the same amounts of thiamine and its mono-, di-, and triphosphate esters.

When thiochrome was dissolved in 50 % ethanol its fluorescence was 2.5 times as intense as in water. Inclusion of potassium hexacyanoferrate(III) and NaOH in the assay medium was found to reduce the fluorescence of thiochrome to 70 % of the maximal. The presence of ethanol did not alter the pH of the assay medium, which was found to be 13.4 under all conditions used.

Differences in absorbance and in thiochrome fluorescence between thiamine and its phosphate esters. In Table 1 the absorbance in 0.1 M HCl is collated with the fluorescence of thiochrome derived from thiamine and its phosphate esters in water and in 50 % ethanol. When oxidized in 50 % ethanol, the thiamine compounds gave equal intensities of fluorescence, but after oxidation in an aqueous medium the intensities were unequal. The absorbances were also unequal. Because of these differences the concentrations were estimated in another way: by determining the total and hydrolyzable phosphate of the thiamine phosphate esters. As the data in Table 2 show, equimolar amounts of thiamine and its phosphate esters have the same absorbance, but do not produce the same

Table 1. Differences in absorbance and in thiochrome fluorescence between thiamine and its phosphate esters oxidized in 50 % ethanol and in water. The preparations were diluted to get equal thiochrome fluorescence in 50 % ethanol. The index F/F_w is the amount of fluorescence when the oxidation was performed in 50 % ethanol compared with that in water. $N = 6$.

	Absorbance $\times 10^{-3} \pm$ S.D. In 0.1 N HCl at 248 nm	Fluorescence $\pm$ S.D. In 50 % ethanol	F/F_w	In water
Thiamine	434 ± 6.34	27.60 ± 0.90	3.60	7.66 ± 0.25
TMP	422 ± 7.23	28.10 ± 0.57	3.34	8.41 ± 0.23
TDP	379 ± 6.47	28.00 ± 1.11	2.88	9.72 ± 0.15
TTP	370 ± 10.23	28.14 ± 0.16	2.84	9.91 ± 0.11

Table 2. The relationship between molarity, absorbance and fluorescence of thiochrome, thiamine and its phosphate esters. The preparations were diluted to get equal absorbance in 0.1 M HCl at 248 nm.

	Molarity ± S.D.[a] according to Weight	Total phosphate	Hydrolyzable phosphate	Relative absorbance ± S.D.[b]	Relative fluorescence ± S.D.[b] in 50 % ethanol Before hydrolysis	After hydrolysis	Correction index
Thiochrome	1.0	–	–	–	100 ± 0.5	–	–
Thiamine	1.0	–	–	100 ± 0.9	74 ± 0.3	74 ± 2.4	1.35
TMP	1.0	1.0 ± 0.01	–	100 ± 0.9	74 ± 0.4	74 ± 3.7	1.35
TDP	1.1	1.9 ± 0.02	1.0 ± 0.02	100 ± 0.6	85 ± 1.6	75 ± 2.5	1.18
TTP	1.0	3.2 ± 0.16	2.0 ± 0.03	100 ± 0.12	93 ± 1.3	79 ± 1.1	1.08

[a] $N = 3$. [b] $N = 4$.

intensity of thiochrome fluorescence. The molar absorption coefficient of thiamine and its mono-, di-, and triphosphate esters at 248 nm in 0.1 M HCl was 13 400.

When the oxidation medium contained 50 % ethanol, the fluorescence intensities produced by thiamine and thiamine monophosphate were equal, but the intensities from thiamine di- and triphosphate were higher (Table 2). To correct for the deficient thiochrome formation, the intensities produced by thiamine, TMP, TDP, and TTP must be multiplied by 1.35, 1.35, 1.18, and 1.08, respectively. On the other hand, if thiamine is taken as the standard, the fluorescence values of TMP need no correction but that of TDP and TTP must be multiplied by 0.87 and 0.80, respectively. The correction indices were found to be constant and independent of the concentration of thiamine compounds.

UV and IR absorption spectra of thiamine and its phosphate esters. The UV absorption spectra of the different thiamine phosphate esters were recorded in 0.1 M HCl, 0.1 M NaOH, and 0.05 M potassium phosphate buffer, pH 7.0. Under the same conditions, all thiamine compounds showed identical spectra. At 234 and 265 nm (the absorption maxima) molar absorption coefficients of thiamine and its phosphate esters in 0.05 M potassium phosphate buffer were found to be 10 900 and 8100 at pH 7.4 and 11 300 and 8250 at pH 7.0, respectively. These results are comparable with the corresponding values reported earlier for thiamine.[10,12–15]

The stabilities of the thiamine compounds were tested under conditions in which the UV spectra were measured. In 0.1 M HCl, absorbance at 248 nm was unchanged, ability to form fluorescent thiochrome was not reduced and no inorganic phosphate was liberated. In 0.1 M NaOH, in contrast, the ability to form fluorescent thiochrome disappeared within 1 min, but returned if the sample was acidified with HCl before oxidation. Neither a decrease in the absorbance at 230 nm nor liberation of inorganic phosphate occurred during the time needed for measurement of the UV spectra.

Characteristic differences, however, were found in the IR spectra of the different thiamine phosphate esters. Thiamine had a unique peak at 1050 cm^{-1}, whereas all three phosphate esters showed peaks at 1160 cm^{-1}. In the spectra of TMP, TDP, and TTP, additional peaks at 940, 1000, and 2560 – 2700 cm^{-1} became amplified as the number of phosphate groups increased. These results are in keeping with the structures of these esters presented in Fig. 2.

Inhibitory effect of alkalinity on formation of thiochrome from thiamine and its phosphate esters. Table 3 shows the effect of changing the pH values of the preincubation medium on formation of thiochrome from thiamine compounds. When the pH of the medium or preincubation time or both were increased, less thiochrome was formed, the effect being greater with thiamine than with its esters. The ester least susceptible to alkali was TTP, followed by TDP and TMP. At pH 9.0 the decrease in ability to form fluorescent thio-

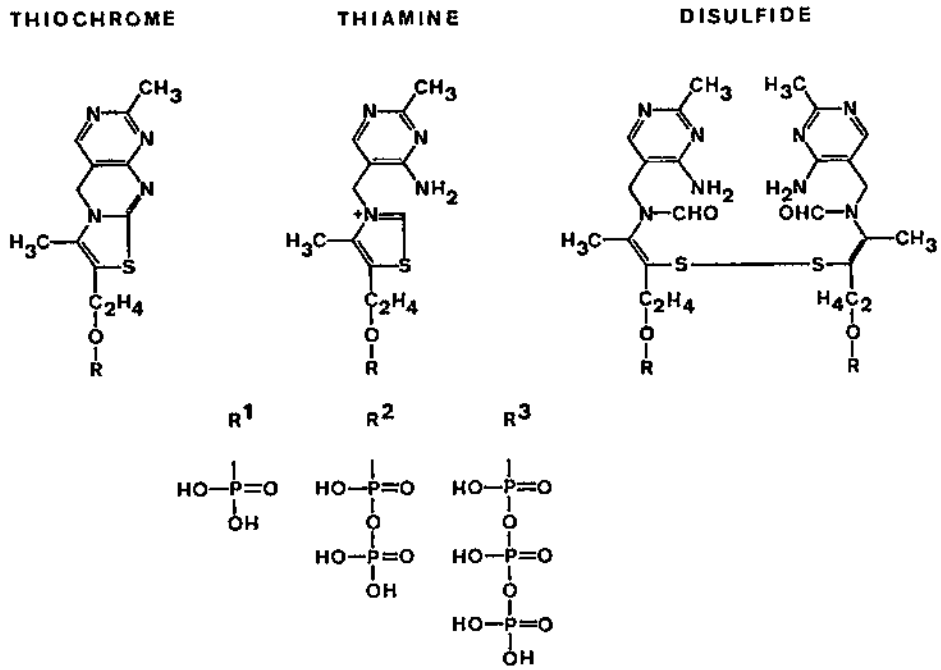

Fig. 2. Structures of thiamine, thiochrome and thiamine disulfide. R^1 = thiamine monophosphate (TMP), R^2 = thiamine diphosphate (TDP) and R^3 = thiamine triphosphate (TTP).

chrome after incubation for 48 h was irreversible, whereas at pH 8.0 it was reversible, provided the sample was acidified with HCl before oxidation.

DISCUSSION

It is not clear why thiamine esters give rise to different intensities of thiochrome fluorescence. They may produce different amounts of thiochrome, or the phosphate groups may intensify the fluorescence, or both mechanisms may be involved. Several observations support the first alternative. The other major oxidation product is the nonfluorescent disulfide (Fig. 2).[2] This is formed when thiamine is exposed to air in an alkaline medium, but can be reduced to thiamine with hydrochloric acid.[16] When incubated in an alkaline buffer, thiamine and its phosphate esters may well show the same tendency to form disulfide (Table 3). The experimental results suggest that on mild oxida-

Table 3. The stability of 1 mM thiamine and its phosphate esters in 0.5 M potassium phosphate buffer of different pH values at 24 °C.

Relative fluorescence										
pH of buffer	9.0				8.0			7.0		
Incubation time	5 min	1 h	24 h	48 h	2 h	24 h	48 h	2 h	24 h	48 h
Thiamine	73	37	36	8[a]	90	93	78[b]	100	97	72
TMP	94	89	79	66[a]	95	91	87[b]	94	91	94
TDP	100	91	60	45[a]	99	96	102	97	93	100
TTP	101	96	79	70[a]	99	97	101	99	99	101

[a] Irreversible change. [b] Reversible change.

tion in an aqueous medium, thiamine is most easily converted to the disulfide, whereas TMP, TDP, and TTP, in this order, show weaker tendencies to form corresponding disulfide derivatives. Thus possibly, the proportions of thiochrome and disulfide may differ. This notion is indirectly supported by observations on the effect of ethanol on thiochrome formation. In the oxidation of thiamine, Risinger and Pell [2] have suggested that solvents of low dielectricity will increase the formation of thiochrome, whereas those with a high dielectric constant will favor the production of disulfide. Comparison of the thiochrome fluorescence values derived from the oxidation of thiamine compounds in media with and without ethanol (Table 1, index F/F_w) showed that in the presence of ethanol, fluorescences of thiamine, TMP, TDP, and TTP were increased 3.6, 3.3, 2.9, and 2.8-fold, respectively, whereas that of thiochrome increased only 2.5-fold. The differences are small, but the tendency is clear: the more phosphorylated the thiamine compound, the smaller the effect of ethanol. Thus ethanol has the greatest effect on thiamine because thiamine has the greatest tendency to form disulfide. Furthermore, the weaker tendency of phosphorylated thiamines to form the disulfides may be due to the electrostatic repulsion between the phosphoric acid chains in the disulfide structure (Fig. 2).

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REFERENCES

1. Jansen, B. C. P. *Recl. Trav. Chim. Pays-Bas 55* (1936) 1046.
2. Risinger, G. E. and Pell, F. E. *Biochim. Biophys. Acta 107* (1965) 374.
3. Wostman, B. S. and Knight, P. T. *Experientia 16* (1960) 500.
4. Rossi-Fanelli, A., Siliprandi, N. and Fasella, P. *Science 116* (1952) 711.
5. Greiling, H. and Kiesow, L. *Z. Naturforsch. B 13* (1958) 251.
6. Kiessling, K. H. *Nature (London) 172* (1953) 1187.
7. Lewin, L. M. and Wei, R. *Anal. Biochem. 16* (1966) 29.
8. Kinnersley, H. W. and Peters, R. A. *Biochem. J. 32* (1938) 1516.
9. Penttinen, H. K. *Finn. Chem. Lett.* (1976) 1.
10. Mickelsen, O. and Yamamoto, R. S. *Methods Biochem. Anal. 6* (1958) 191.
11. Fiske, C. H. and SubbaRow, Y. *J. Biol. Chem. 66* (1925) 375.
12. Beadle, B. W., Greenwood, D. A. and Kraybill, H. R. *J. Biol. Chem. 149* (1943) 339.
13. Yusa, T. *J. Biochem. (Tokyo) 46* (1959) *391.*
14. Vogel, H. and Knobloch, H. *Chemie und Technik der Vitamine,* II Band, 1. Lieferung, Ferdinand Enke Verlag, Stuttgart 1953.
15. Gubler, C. J. and Wittorf, J. H. *Methods Enzymol. 18 A* (1970) 117.
16. Dwivedi, B. K. and Arnold, R. G. *J. Agr. Food. Chem. 21* (1973) 54.

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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, Gryckshbo, 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.).

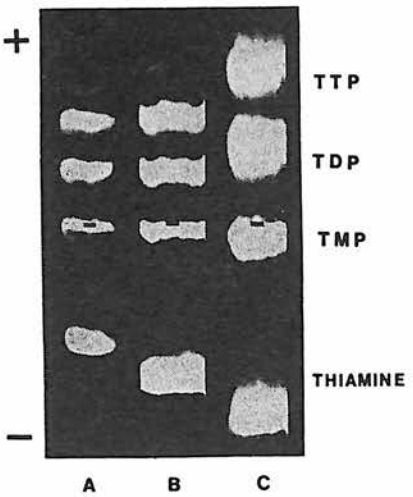

Fig. 1. Electrophoretic separation of thiamine and its phosphate esters. Samples of 5 μl were applied in the middle of electrophoretic paper strips (black lines) and electrophoresis and visualization were carried out as described in Experimental. Thiamine compounds were dissolved in: A, water; B, 0.37 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.

REFERENCES

1. Itokawa, Y. and Cooper, J. R. In McCormick, D. B. and Wright, L. D., Eds., *Methods in Enzymology*, Academic, New York 1970, Vol. 28, p. 91. Part A.
2. Siliprandi, D. and Siliprandi, N. *Biochim. Biophys. Acta 14* (1954) 52.
3. Lewin, L. M. and Wei, R. *Anal. Biochem. 16* (1966) 29.
4. Penttinen, H. K. *Finn. Chem. Lett.* (1976) 1.
5. Mickelsen, O. and Yamamoto, R. S. In Glick, D., Ed., *Methods of Biochemical Analysis*, Interscience, New York 1958, Vol. 6, p. 191.
6. Penttinen, H. K. *Acta Chem. Scand. B 30* (1976) 659.
7. Muiruri, K. L., Romsos, D. R. and Kirk, J. R. *Am. J. Clin. Nutr. 27* (1974) 27.
8. Rindi, G. and De Giuseppe, L. *Biochem. J. 78* (1961) 602.
9. Murdock, D. S. and Gubler, C. J. *J. Nutr. Sci. Vitaminol. 19* (1973) 43.
10. Dreyfus, P. M. *J. Neurochem. 4* (1959) 183.

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Determination of Inorganic Phosphate. A Method for the Determination of Phosphatase Activities by a Continuous Flow System

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A sensitive and accurate method for the determination of inorganic phosphate is described. The method enables the estimation of 10 nanomoles of inorganic phosphate with a coefficient of variation of 3.6% for ten replicates. The method is suitable for the estimation of the activities of thiamine triphosphatase, adenosine triphosphatase, and alkaline and acid phosphatase by a continuous flow system.

Determination of inorganic phosphate in the presence of thiamine phosphate esters is complicated by the formation of precipitable complex between ammonium molybdate and thiamine phosphate (1). This difficulty can be overcome by addition of a citrate–arsenite solution which complexes with the molybdate (2). This observation, combined with the use of sodium dodecyl sulfate to solubilize the proteins (3), has led to the development of an improved method for the determination of inorganic phosphate and various phosphatase activities.

MATERIALS AND METHODS

Reagents

Ammonium molybdate, 1% (w/v). Reagent A was a mixture containing one part of 8% (w/v) sodium dodecyl sulfate (SDS),[1] one part of 7% (v/v) perchloric acid and two parts of 2% (w/v) ascorbic acid in water. This mixture is prepared daily from the stock solutions.

Reagent B was a mixture of 10% (w/v) sodium citrate dihydrate, 10% (w/v) disodium hydrogen arsenate and 10% (w/v) sodium acetate in water. Sodium dodecyl sulfate (99%) was obtained from Pierce, Rotterdam, Holland. Thiamine triphosphate (TTP) was prepared as described (4). Alkaline phosphatase from calf intestinal mucosa, type I, and acid phosphatase from potatoes, type IV, were obtained from the Sigma Chemical Company, St. Louis, Missouri. Reagent C for the continuous flow system was the same as reagent A, except that the perchloric acid was 70% (v/v).

Assay Media

The assay mixture for thiamine triphosphatase activity contained 100 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 1 mM TTP, and 200 μg protein per milliliter (final volume) of rat brain supernatant. This was prepared by homogenizing a rat brain in 0.25 M sucrose at 0°C and centrifuging at 100,000g for 60 min. The assay mixture for adenosine triphosphatase activity contained 100 mM

[1] Abbreviations used: SDS, sodium dodecyl sulfate, TTP, thiamine triphosphate; PNPP, *p*-nitrophenyl phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; imid, imidazole.

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Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, and 24 μg protein per milliliter (final volume) of rat kidney mitochondria prepared according to Johnson and Lardy (5). The activity of alkaline phosphatase was assayed in 100 mM glycine–NaOH, pH 10.5, 10 mM $MgCl_2$, and 1 mM *p*-nitrophenyl phosphate (PNPP). The assay medium contained 75 μg protein per milliliter of the calf intestinal enzyme. The activity of acid phosphatase was assayed in 100 mM sodium acetate, pH 5.0, 10 mM $MgCl_2$, and 1 mM PNPP. The assay medium contained 10–20 μg protein of the potato enzyme per milliliter. The protein was determined by the biuret method (6).

Determination of Inorganic Phosphate

Reagent A (0.2 ml) and 1% (w/v) ammonium molybdate (0.6 ml) were added to a sample (1 ml) and shaken. Then, after 2 min reagent B (0.6 ml) was added and the shaking repeated. The absorbance was measured at 730 nm with a Gilford photometer. Figure 1 shows the diagram of the continuous flow system. Immediately behind the pump, the enzyme reaction was stopped with perchloric acid, and the protein was solubilized with SDS (reagent C). Then, after addition of molybdate, the reagents were mixed and air added, and finally the citrate–arsenite solution was added. After passing through a mixing coil the air was removed with an air debubbler and the mixture was passed through a flow cell with a 10-mm light path. The changes in absorbance were recorded with a Honeywell recorder attached to a photometer. After each series of experiments the tube system was rinsed with a solution of 0.1 mM EDTA and 1 mM NaOH.

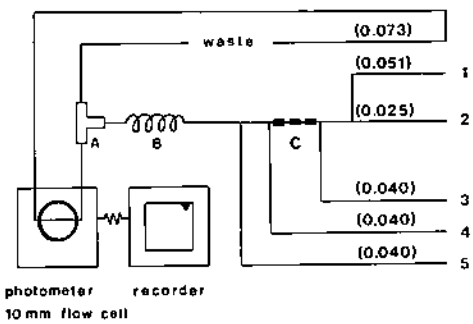

FIG. 1. Scheme of the continuous flow system: (1) sample, (2) SDS/perchloric acid/ascorbic acid (reagent C), (3) 1% (w/v) ammonium molybdate, (4) air, (5) citrate/arsenite/acetate (reagent B), (A) dedubbler, (B) mixing coil, and (C) mixing tubes. The inner diameters of the pump tubings in inches are shown in parentheses.

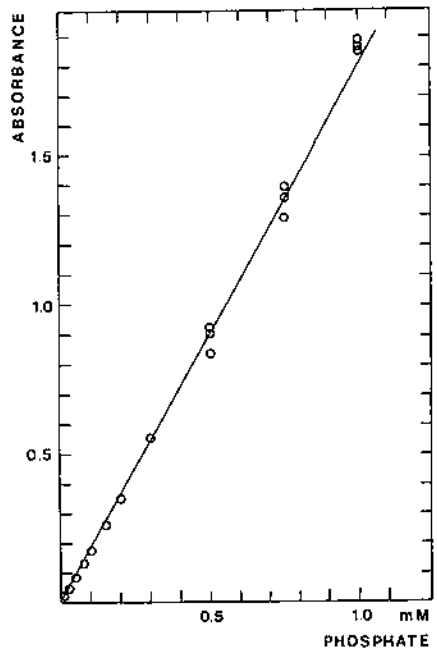

FIG. 2. Dependence of the absorbance of the phosphomolybdate complex on the phosphate concentration of the sample. The determination was as described under Materials and Methods. Each concentration was measured in three replicates.

RESULTS AND DISCUSSION

Linearity and Reproducibility of the Method

The relation between the absorbance of the phosphomolybdate complex and the phosphate concentration of the sample is indicated in Fig. 2. The response was linear up to a phosphate concentration of 1 mM, which corresponds to an absorbance of

about 1.8 units. The repeatability of the method is satisfactory with low concentrations of phosphate. The variation coefficients for 10 replicates each of samples containing 10, 100, and 250 nmol of phosphate were 3.6, 0.5, and 1.1%, respectively. More concentrated samples indicated noticeable variation (Fig. 2). The continuous flow system also gave highly reproducible results (Fig. 3).

Spectrum and Stability of the Phosphomolybdate Complex

The absorption spectrum of the phosphomolybdate complex formed is fairly broad, with a plateau between 700 and 740 nm. The wavelength of 730 was chosen for routine use. Figure 4 shows effect of incubation time on the intensity of the color of the complex at various phosphate concentrations. The color is very stable at low phosphate concentrations (below 0.25 mM), but samples containing more phosphate show a linear increase in absorbance as a function of time.

Some Characteristics of the Method

The blank absorbance of the present method was not significantly different from zero, but when the method of Baginsky *et al.* (2) was used the blank was significant and increased as a function of time. Because the citrate–arsenite solution prevents the formation of the phosphomolybdate complex, it was essential to know how rapidly the colored complex is formed. Figure 5 indicates that the formation of the phosphomolybdate complex is very rapid, the most suitable point for addition of the citrate–arsenite reagent being 1–3 min after the addition of molybdate. It is essential for the reaction mixture to be acidic; otherwise the formation of color is disturbed. When phosphatase activities are determined in alkaline media, it is necessary to ensure that the amount of perchloric

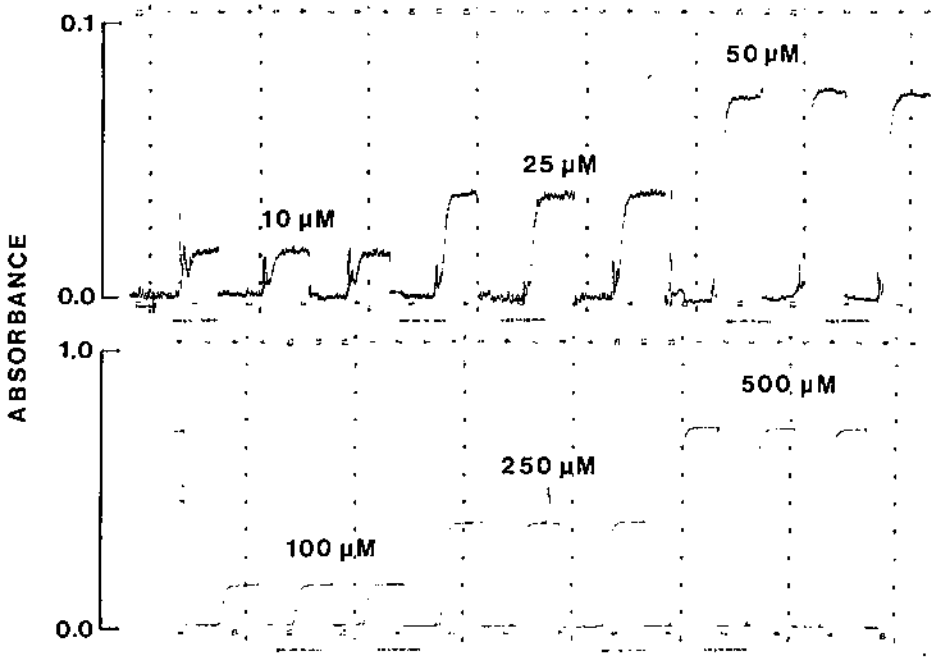

FIG. 3. Repeatability as shown by three replicate determinations on samples containing 10–500 µM phosphate determined by the continuous flow system.

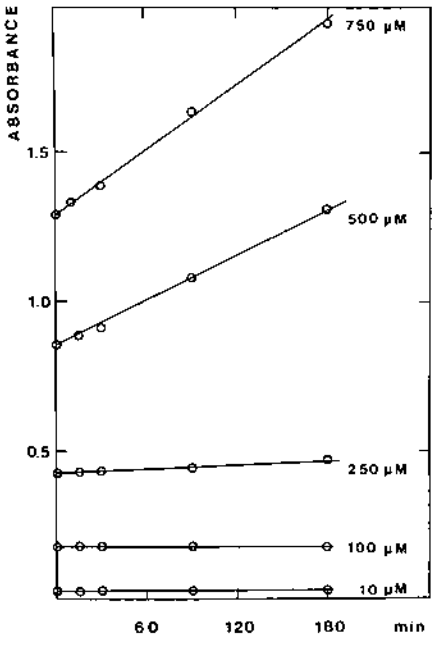

FIG. 4. Effect of incubation time on the intensity of the color of the phosphomolybdate complex. The determination was as described under Materials and Methods. The incubation time means the time from the addition of the last reagent to the point at which the absorbance was measured. The concentration of the phosphate in the sample ranged from 10 to 750 µM.

acid is sufficient to acidify the mixture and therefore a strong concentration of perchloric acid is needed (reagent C). One advantage of the use of the citrate–arsenite solution is that no decomposition of labile phosphate esters can interfere with the estimation (2), but the possibility still remains that these labile compounds may be hydrolyzed by the perchloric acid. That this does not occur was checked by incubating a sample of 1 mM phosphocreatine in reagent C (which contains the strong perchloric acid) for 5 min and continuing the determination as described under Materials and Methods. No liberation of inorganic phosphate could be detected after this treatment.

Determination of Phosphatase Activity by the Continuous Flow System

Figure 6 shows the activities of thiamine triphosphatase, adenosine triphosphatase, and alkaline and acid phosphatase as determined by the continuous flow system. With this method even low phosphatase activities can be determined reliably. When

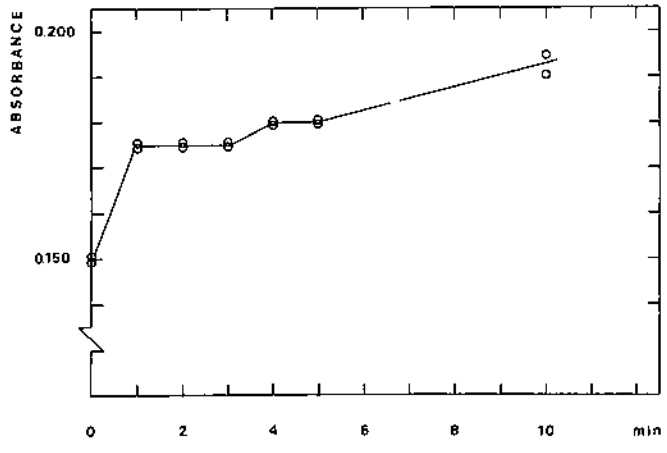

FIG. 5. Effect of the preincubation time on the absorbance of the phosphomolybdate complex. A sample of 100 µM phosphate was processed as described under Materials and Methods to the point of addition of the citrate–arsenite solution. At time zero the citrate–arsenite reagent was added immediately after the molybdate. The minutes indicate the intervals between the times of addition of these two reagents. The absorbances were measured immediately after addition of the latter reagent.

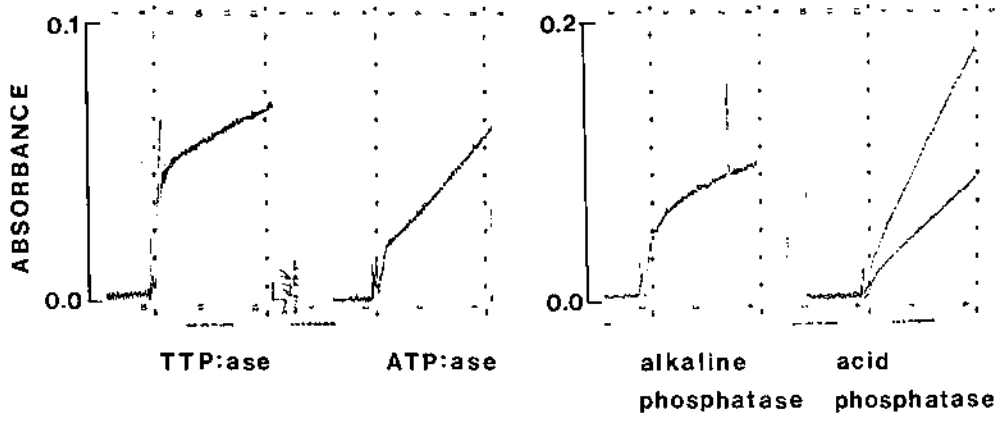

FIG. 6. Activities of thiamine triphosphatase (TTPase), adenosine triphosphatase (ATPase), and alkaline and acid phosphatase determined by the continuous flow system. The units of enzyme and the concentrations of protein used in each assay were 0.34 (200), 0.63 (24), 0.54 (75), and 2.14 (20) $\times 10^{-3}$ IU (μg of protein/ml), respectively. The assay media (10 ml) and the determination were as described under Materials and Methods. The upper line of the activity of acid phosphatase was determined with double the amount of enzyme. The paper speed was 0.4 in. per minute.

the sample contained 50 μM phosphate, color formation was inhibited in the presence of 10 mM ATP, whereas with less than 5 mM ATP there was no inhibition. No inhibitory effect was noted in a medium of 20 mM TTP. The effects of various buffers were tested using 100 μM phosphate as a sample. More than 10 mM sodium citrate and more than 5 mM sodium pyrophosphate prevented the formation of color, whereas 100 mM sodium acetate, glycine–NaOH, Hepes, Imid, Mes, Pipes, and Tris buffers had no effect. Determination of the activity of alkaline phosphatase by another method (7) gave comparable results. The reaction rate estimated from the slope recorded in the continuous flow measurement was a linear function of enzyme concentration. This is shown for acid phosphatase in Fig. 6.

REFERENCES

1. Yamazaki, M., and Hayaishi, O. (1968) *J. Biol. Chem.* **243**, 2934–2942.
2. Baginski, E. S., Foa, P. P., and Zak, B. (1967) *Clin. Chim. Acta* **15**, 155–158.
3. Arnold, A., Wolf, H. U., Ackerman, B. P., and Bader, H. (1976) *Anal. Biochem.* **71**, 209–213.
4. Penttinen, H. K. (1979) *in* Methods in Ezymology (Colowick, P., and Kaplan, N. O., eds.) Vol. 62, Part. D, p. 112, Academic Press, New York.
5. Johnson, D., and Lardy, H. (1967) *in* Methods in Enzymology (Colowick, P., and Kaplan, N. O., eds.), Vol. 10, p. 94, Academic Press, New York.
6. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766.
7. Komoda, T., and Sakagishi, Y. (1976) *Biochim. Biophys. Acta* **445**, 645–660.

THE RELATION OF THE SOLUBLE THIAMINE TRIPHOSPHATASE ACTIVITY OF VARIOUS RAT TISSUES TO NONSPECIFIC PHOSPHATASES

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ABSTRACT

Polyacrylamide gel electrophoresis was used to investigate the relation of the soluble thiamine triphosphatase activity of various rat tissues to other phosphatases. This technique separated the thiamine triphosphatase of rat brain, heart, kidney, liver, lung, muscle and spleen from alkaline phosphatase (EC 3.1.3.1), acid phosphatase (EC 3.1.3.2) and other nonspecific phosphatase activities. In contrast, the hydrolytic activity for thiamine triphosphate in rat intestine moved identically with alkaline phosphatase in gel electrophoresis. Thiamine triphosphatase from rat liver and brain was also separated from alkaline phosphatase and acid phosphatase by gel chromatography on Sephadex G-100. This gave an apparent molecular weight of about 30 000 and a Stokes radius of 2.5 nanometers for brain and liver thiamine triphosphatase. The intestinal thiamine triphosphatase activity of the rat was eluted from the Sephadex G-100 column as two separate peaks (with apparent molecular weights of over 200 000 and 123 000) which exactly corresponded to the peaks of alkaline phosphatase. The isoelectric point (pI) of the brain thiamine triphosphatase was 4.6 (4 ^{o}C). The partially purified thiamine triphosphatase from brain and liver was highly specific for thiamine triphosphate.

The results suggest that, apart from the intestine, the rat tissues studied contain a specific enzyme, thiamine triphosphatase (EC 3.6.1.28). The specific enzyme is responsible for most of the thiamine triphosphatase activity in these tissues. Rat intestine contains a high thiamine triphosphatase activity but all of it appears to be due to alkaline phosphatase.

INTRODUCTION

A number of reports have appeared claiming the existence of thiamine triphosphate in living cells. This compound was described first from rat liver (32) and later from other animal tissues (16), yeast (21), and bacteria (31). The physiological function of thiamine triphosphate has remained unclear although many papers have been presented supporting the view that thiamine triphosphate might have a specific role in nervous tissue, independent of the coenzyme function of thiamine diphosphate (10). Hashitani and Cooper (18) described a cytosolic enzyme activity from various rat tissues catalyzing the hydrolysis of thiamine triphosphate. They also reported partial purification of the activity from rat brain (18). Rat tissues also contain another thiamine triphosphatase activity which is tightly bound to membranes (4).

Although the partially purified soluble thiamine triphosphatase from brain was inactive with ATP and some other substrates (18), the relation of this activity to alkaline phosphatase (EC 3.1.3.1) and acid phosphatase (EC 3.1.3.2) was not determined. Our preliminary studies showed that calf intestinal alkaline phosphatase and potato acid phosphatase, obtained from commercial sources, used thiamine triphosphate as well as the substrate. Therefore we wanted in this work to find out, by using gel electrophoresis and gel chromatography as the fractionation methods, whether or not various rat tissues contain a specific thiamine triphosphatase. Solving this question may, in part, help in determining whether thiamine triphosphate has a specific biological function.

MATERIALS AND METHODS

Reagents

Thiamine triphosphate was prepared as described earlier (29). The other substrates, Fast Blue RR salt and Fast Garnet GBC salt, were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sephadex G-100 (medium grade) was obtained from Pharmacia, Uppsala, Sweden, and the standard proteins for molecular weight determination from Boehringer (Mannheim,GFR) except alkaline phosphatase from calf intestine which was from Sigma.

Preparation of tissues

Rat tissues were homogenized at 0 to 4 °C in an Ultra-Turrax homogenizer in 2 volumes of 0.25 M sucrose, pH 7.0, containing 1 mM EDTA. The homogenate was centrifuged for 60 min at 100 000 x g in a Beckman ultracentr fuge. The supernatants were dialyzed against 10 mM Tris-HCl, pH 7.6, containing 5 mM 2-mercaptoethanol. Liver and small intestine preparations were further fractionated for gel chromatography with ammonium sulfate. The protein fraction which remained soluble by the addition of 134 g ammonium sulfate per 1 (at 0 °C) was precipitated by adding more ammonium sulfate (395 g per 1) to the supernatant. The precipitate was dialyzed against the Tris-HCl buffer mentioned above, and supplemented with 0.2 M NaCl. The treatment with ammonium sulfate did not remove any of the activity of thiamine triphosphatase.

Gel electrophoresis

Disc electrophoresis was performed at 4 °C in polyacrylamide gel rods prepared according to the gel system 1 of Maurer (25). The samples (up to 100 µl) were layered on the large-pore gel in 13 % (v/v) glycerol or 9 % (w/v) sucrose. A constant current, 0.5 mA per gel during sample penetration and 2.5 mA per gel thereafter, was used. Enzyme mobilities towards the anode were calculated in relation to bromophenol blue.

Determination of the apparent molecular weights by gel chromatography

Partial purification and molecular weight determinations for the phosphatases studied were done on a column of Sephadex G-100 (2.5 x 70 cm). The column was equilibrated and eluted at 4 °C with 10 mM Tris-HCl, pH 7.6, also containing 0.2 M NaCl and 5 mM 2-mercaptoethanol. The void volume (V_o) of the column was determined with blue dextran 2000 and the total volume (V_t) with inorganic phosphate. The column was calibrated with the standard proteins catalase (M_r 240 000), alkaline phosphatase from calf intestine (138 000; ref. 13), bovine albumin (67 000), ovalbumin (45 000), chymotrypsinogen (25 000) and cytochrome c (12 400). The sample containing standards or the unknown was applied to the column in a volume of 2 ml and elution continued at a rate of 10 ml per h. Blue dextran was localized in the effluent by its absorbance at 620 nm, cytochrome c at 410 nm, alkaline phosphatase and catalase (23) by activity assays and the other standards by absorbance at 220 nm. The apparent molecular weights for the unknowns were determined from a plot of V_e/V_o versus log M_r (3), and Stokes radii from a plot of $(-\ln K_{av})^{1/2}$ versus Stokes radius, where is the average distribution coefficient $K_{av} = (V_e - V_o)/(V_t - V_o)$ and V_e the elution volume (23). The Stokes radii of the standard proteins were taken from Ref. 22.

Determination of phosphatase activities

Phosphatase assays were carried out by measuring the amount of inorganic phosphate (28) liberated during 60 min incubation at 37 °C, on a FP-9 Analyzer System (Labsystems Oy, Finland). A continuous flow system was also used in some determinations (28). The assay mixtures for thiamine phosphatase activities contained 165 mM Tris-HCl, pH 9.0, 8 mM $MgCl_2$ and 2 mM thiamine mono-, di- or triphosphate. The assay mixture for alkaline phosphatase contained 165 mM Tris-HCl, pH 9.0, 9 mM $MgCl_2$, and 3 mM p-nitrophenyl phosphate, and the assay mixture for acid phosphatase 165 mM sodium acetate, pH 5.0, 8 mM $MgCl_2$ and 3 mM p-nitrophenyl phosphate. Adenosine triphosphatase (ATPase) activity was determined in 165 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 10 µM $CaCl_2$ and 2 mM ATP.

One unit of enzyme activity catalyzes the formation of one micromole of inorganic phosphate in one hour at 37 °C. Specific activity is defined as number of units of activity per mg of protein.

Localization of enzyme activities in gels

Thiamine triphosphatase. The gels were incubated in 33 mM Tris-HCl, pH 9.5, 15 mM $MgCl_2$, and 3 mM thiamine triphosphate for 60 min at 30 °C. Thereafter, the pericipitate of inorganic phosphate formed at the location of the enzyme was made visible by the lead conversion method of Allen and Hyncik (2).

Alkaline phosphatase. The gels were incubated in 60 mM sodium borate, pH 9.7, 5 mM $MgSO_4$, 0.05 % (w/v) sodium-α-naphthyl phosphate and 0.05 % (w/v) Fast Blue RR salt (17). Another staining method used for alkaline phosphatase was similar to that given above for thiamine triphosphatase, except that the substrate was 3 mM p-nitrophenyl phosphate.

Acid phosphatase. Before staining the gels were incubated twice for 15 min in 0.2 M sodium acetate buffer, pH 5.0, to lower the pH of the gel. The staining mixture contained 0.05 M sodium citrate, pH 4.5, 0.1 % (w/v) sodium α-naphthyl phosphate and 0.1 % (w/v) Fast Garnet GBC salt (5).

Staining for the hydrolytic activities towards a number of other substrates (see Results) was done as the thiamine triphosphatase staining above except that the pH of the medium was 7.2 and thiamine triphosphate was substituted by the substrate in question.

Other methods

Protein was determined by a fluorometric method (7). Bovine serum albumin was used as the standard. Isoelectric focusing was carried out at 4 °C in a 110-ml LKB column (Bromma, Sweden; model 8101) in a sucrose gradient and 1 % Ampholine solution, pH 3.5-10 (LKB). The focusing time was 48 hours and the procedures followed those described by Vesterberg (35).

RESULTS

Polyacrylamide gel electrophoresis

Table 1 summarizes the gel electrophoresis results for thiamine triphosphatase, alkaline phosphatase and acid phosphatase from various tissues of the rat. Seven of the eight tissues studied, viz. brain, heart, kidney, liver, lung, muscle and spleen, contained a thiamine triphosphatase of high mobility (0.79 - 0.84) which clearly separated from all forms of alkaline phosphatase (mobility 0.08 - 0.44) and acid phosphatase (0.15 - 0.43).

The results for the intestine were different, however. No thiamine triphosphatase activity band with high mobility was obtained for the intestine, but the two activity bands obtained with thiamine triphosphate had slow mobilities which exactly corresponded to the alkaline phosphatase bands (Table 1). When 5.6 % polyacrylamide gel was used instead of the usual 7.5 % gel, the intestinal thiamine triphosphatase forms again moved identically with alkaline phosphatase.

Electrophoresis gels for three tissues (brain, liver and intestine) were stained, in addition to the thiamine triphosphatase, alkaline phosphatase and acid phosphatase activities, with the following compounds as the substrate: ATP, CTP, GTP, ITP, UTP, ADP, AMP, thiamine diphosphate and thiamine monophosphate. All gave for the intestine bands of varying intensity corresponding to alkaline phosphatase. For liver and brain thiamine diphosphate, AMP, ADP and ATP gave weak to moderate bands corresponding to alkaline phosphatase. Thiamine diphosphate and ADP gave an additional band for liver with an approximate mobility of 0.55. This band, which was probably due to nucleoside diphosphatase (EC 3.6.1.6) (1), was much stronger with thiamine diphosphate than with ADP. None of these compounds gave any band at the location of the thiamine triphosphatase band of brain and liver.

TABLE 1

Mobilities of alkaline phosphatase, acid phosphatase and thiamine triphosphatase activities of different rat tissues in gel electrophoresis

Tissue	Mobility			
	Alkaline phosphatase (p-nitrophenylphosphate)	Alkaline phosphatase (α-naphthylphosphate)	Acid phosphatase (α-naphthylphosphate)	Thiamine triphosphatase (thiamine triphosphate)
Brain	0.13	0.12	0.37	0.81
Heart	0.14[1)]	0.13[1)]	0.38	0.82
	0.38	0.37		
Intestine	0.11	0.12	0.25	0.11
	0.26	0.26		0.26
Kidney	0.12[1)]	0.11[1)]	0.15	0.81
	0.38		0.28	
	0.43			
Liver	0.09	0.16	0.35	0.82
	0.23			
	0.34			
Lung	0.13[1)]	0.13[1)]	0.39	0.84
	0.38	0.32		
		0.37		
Muscle	0.08	0.08	-	0.79
	0.13	0.21		
	0.18			
	0.24			
Spleen	0.11[1)]	0.11[1)]	0.35	0.82
	0.33	0.34	0.43	
	0.38	0.39		
	0.44	0.44		

1) A very broad band, only the average value is presented.

The substrate used is given in parentheses. The other details are described

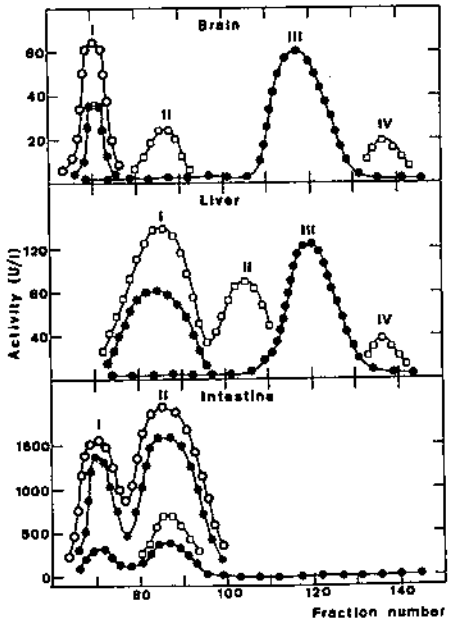

Fig. 1. Fractionation of the phosphatases of rat brain, liver and intestine on Sephadex G-100. The symbols for the activities are : (✱ - ✱) alkaline phosphatase, (□ - □) acid phosphatase, (● - ●) thiamine triphosphatase, and (○-○) adenosine triphosphatase. The fraction volume was 2.0 ml. For other details see Materials and Methods. The height of peak III (Brain) is reduced three fold in the Figure.

Gel chromatography

Fig. 1 shows the activity peaks obtained when preparations from rat liver, brain and intestine were fractionated on Sephadex G-100 and hydrolytic activities determined in the eluate for thiamine triphosphate, p-nitrophenyl phosphate (acid and alkaline pH) and ATP. The thiamine triphosphatase peaks obtained from both liver and brain were clearly separated from the peaks of alkaline phosphatase (activity for p-nitrophenyl phosphate in alkaline pH) and acid phosphatase (activity for p-nitrophenyl phosphate in acid pH) as well as from the activity with ATP (Fig. 1). The intestine, however, gave quite different results.

The intestinal thiamine triphosphatase activity was eluted as two separate peaks which corresponded exactly to the peaks of alkaline phosphatase, determined with p-nitrophenyl phosphate as the substrate (Fig. 1). Two peaks at the same location were further obtained when ATP (Fig. 1), thiamine diphosphate or thiamine monophosphate (not shown) were used as the substrates. The intestinal preparations contained no such thiamine triphosphatase activity as would have been eluted from the column at the location of the thiamine triphosphatase from liver and brain. Acid phosphatase assay gave one activity peak for the intestine. This was located at the second alkaline phosphatase peak (Fig. 1). The standard proteins run in the same Sephadex G-100 column gave linear curves in the plots of V_e/V_o versus log M_r and of $(-\ln K_{av})^{1/2}$ versus Stokes radius. Apparent molecular weights and Stokes radii were calculated from these plots for the components presented in Fig. 1. These results are summarized in Table 2.

Substrate specificity of the partially purified thiamine triphosphatase from liver and brain

The brain enzyme was highly specific for thiamine triphosphate (Table 3) whereas the liver enzyme apparently also had some activity for thiamine diphosphate, CTP, UTP, GTP and ITP. The preparations were free from alkaline and acid phosphatase activities (with p-nitrophenyl phosphate as substrate). Thiamine triphosphate was not used in acid pH.

Thiamine diphosphate actually gave on gel chromatography of the liver preparation a broad activity peak with a maximum of about ten fractions before that of thiamine triphosphatase; only the tailing portion of the former peak was located on the latter peak. Further fractionation of the pooled and concentrated liver thiamine triphosphatase peak (III) by polyacrylamide gel electrophoresis (see Methods) completely separated the activities for thiamine triphosphate and thiamine diphosphate. The relative mobilities in the electrophoresis of the activities for thiamine triphosphate and thiamine diphosphate were 0.79 and 0.57 of the mobility of bromophenol blue, respectively. Thus most, if not all, of the activity for thiamine diphosphate of the liver thiamine triphosphatase (Table 3) was due to a contaminating enzyme, probably nucleoside diphosphatase (1). The active nucleotide substrates of the liver thiamine triphosphatase preparation were also used for activity staining after electrophoresis. However, no activity

TABLE 2

Apparent molecular weights and Stokes radii for the phosphatase peaks obtained from rat liver, brain and intestine

Fraction	Activity (substrate)	Molecular weight	Stokes radius (nm)
Brain I	Alkaline phosphatase (PNPP) (ATP)	> 200 000	> 5.2
Brain II	Acid phosphatase (PNPP)	123 000	4.25
Brain III	Thiamine triphosphatase	30 700	2.54
Brain IV	Acid phosphatase (PNPP)	14 100	1.78
Liver I	Alkaline phosphatase (PNPP) Acid phosphatase (PNPP)	128 000	4.33
Liver II	Acid phosphatase (PNPP)	58 000	3.16
Liver III	Thiamine triphosphatase	29 000	2.48
Liver IV	Acid phosphatase (PNPP)	14 100	1.78
Intestine I	Alkaline phosphatase (PNPP) (ATP) (Thiamine triphosphate)	> 200 000	> 5.2
Intestine II	Alkaline phosphatase (PNPP) Acid phosphatase (PNPP) (ATP) (Thiamine triphosphate)	123 000	4.25

The Roman numerals correspond to those marked in Fig. 1. In cases in which the enzyme(s) responsible for the activity is not quite clear, only the substrate used is given within parentheses. For other details see Materials and Methods. PNPP = p-nitrophenyl phosphate.

TABLE 3

Substrate specificity of thiamine triphosphatase of rat liver and brain

Substrate	Relative activity (%)	
	Liver enzyme	Brain enzyme
Thiamine triphosphate	100	100
Thiamine diphosphate	28	6
Thiamine monophosphate	0	0
ATP	0	0
ADP	0	0
AMP	0	0
CTP	18	0
UTP	29	0
GTP	14	0
ITP	18	0
ATP (pH 7.0)	0	0
p-Nitrophenyl phosphate (pH 9.0)	0	0
p-Nitrophenyl phosphate (pH 5.0)	0	0
Thiamine triphosphate (pH 5.0)	0	0

The fractions with the highest activity of the peaks Liver III and Brain III (see Fig. 1) were pooled and concentrated by ultrafiltration. The activities were determined at the pH of 9.0 as described for thiamine triphosphatase activity in Materials and Methods except in the cases in which a different pH is given. The detection limit was 5 % of the activity for thiamine triphosphate at pH 9.

for these substrates could be seen in the gels.

Some other properties of liver and brain thiamine triphosphatase

The partially purified thiamine triphosphatase had a pH optimum at pH 9.0. The apparent K_m for thiamine triphosphate was 0.5 mM (determined for the liver enzyme only). The isoelectric point of the brain enzyme, determined by isoelectric focusing in column, was 4.6 (at 4 ^{o}C). The crude cytosolic fraction gave one symmetrical thiamine triphosphatase peak. This was partially separated from alkaline phosphatase peaks (pI values 4.4 and 4.9) and totally separated from acid phosphatase (pI values 5.3 and 5.7). The specific activities of the partially purified thiamine triphosphatase preparations were 3.9 and 12.1 units per mg of protein for the liver and brain enzymes, respectively.

DISCUSSION

The present studies have shown that rat liver and brain contain a specific thiamine triphosphatase (EC 3.6.1.28) which has a molecular weight of about 30 000. This enzyme was separated from the various forms of alkaline phosphatase and acid phosphatase by both gel chromatography and gel electrophoresis. The electrophoretic mobility of thiamine triphosphatase towards the anode was higher than that of the nonspecific phosphatase forms. The high electrophoretic mobility of thiamine triphosphatase is explained by its rather low molecular weight and acidic isoelectric point (4.6 as determined for the brain enzyme). For the other five rat tissues studied (heart, kidney, lung, muscle and spleen) only the electrophoretic separation was performed. All seven tissues contained a specific thiamine triphosphatase according to electrophoresis and the mobility of the thiamine triphosphatase from these seven sources was closely similar, if not identical (Table 1). It seems probable that the thiamine triphosphatase from these tissues has closely similar or identical physical properties.

The partially purified thiamine triphosphatase from rat brain was highly specific for thiamine triphosphate. Of the ten other compounds tested as substrates (Table 3) all were at or below the detection limit of the assay. The liver enzyme preparation, in contrast, also had significant activity

for thiamine diphosphate and for some nucleoside triphosphates. Further purification of liver thiamine triphosphatase by electrophoresis removed the activity for thiamine diphosphate. Whether the hydrolysis of the active nucleoside triphosphates was also due to a contaminating enzyme is not clear. The presence of a contaminant is probable because the thiamine triphosphatase preparation achieved was still far from pure and bacause only thiamine triphosphate gave a visible band at the location of this enzyme in electrophoresis.

Our fractionations also separated the specific thiamine triphosphatase from nucleoside diphosphatase (EC 3.6.1.6) and ATPase (EC 3.6.1.3). Nucleoside triphosphatase (EC 3.6.1.15) was also removed, at least from the brain enzyme. The nucleoside diphosphatase, which was eluted close to thiamine triphosphatase in gel chromatography (cf. Results), was probably of microsomal origin (12,33). The cytoplasmic nucleoside diphosphatase of rat liver has a much higher apparent molecular weight (120 000; ref. 20) than that determined for thiamine triphosphatase. The substrates of many of the more specific phosphatases (in the E.C. list under 3.1.3, phosphoric monoester hydrolases, and 3.6, activity on acid anhydrides) were not tested, but none would be expected to have the specificity shown for brain and liver thiamine triphosphatase in Table 3. A large part of the other phosphatases also clearly differ from thiamine triphosphatase in molecular weight and isoelectric point.

Both Hashitani and Cooper (18) and Barchi and Braun (4) reported that rat intestine contained the highest amount of soluble thiamine triphosphatase activity among the rat tissues studied. Our results support these reports (cf. Fig. 1). However, our electrophoretic and chromatographic fractionation strongly suggest that all of the intestinal thiamine triphosphatase activity is actually due to alkaline phosphatase. Both of the intestinal phosphatase forms separated used thiamine triphosphate well, although p-nitrophenyl phosphate and ATP were used more rapidly (Fig. 1). The claim that the intestinal thiamine triphosphatase and alkaline phosphatase activities are the property of the same enzyme(s) can definitely be proven only by purifying the enzyme(s) to homogeneity. The present results show that rat intestine had no detectable amount of a thiamine triphosphatase similar to that of other tissues, based on gel chromatography and electrophoresis.

Although only one thiamine triphosphatase activity peak - that of the specific thiamine triphosphatase - was found in the gel chromatography eluate of brain and liver preparations (Fig. 1), some activity for thiamine triphosphate could also be demonstrated in the alkaline and acid phosphatase peaks after these were pooled and concentrated by ultrafiltration. The activities for thiamine triphosphate varied between 5 and 20 % of the activity for p-nitrophenyl phosphate for the various nonspecific phosphatase forms tested. Acid phosphatases used thiamine triphosphate only in acid pH and thus do not interfere in the thiamine triphosphatase assay of crude preparations of brain and liver when this was conducted at pH 9. Under these conditions 90 to 95 % of the thiamine triphosphatase activity of the brain and liver cytosolic fraction is due to a specific thiamine triphosphatase and the rest to alkaline phosphatase.

The molecular weights determined by us for alkaline and acid phosphatase are in reasonable agreement with earlier data. De Araujo et al. (11) found by gel chromatography on Sephadex G-75 from the cytosolic fractions of rat liver and brain two acid phosphatases, one with M_r over 100 000 and one with M_r under 30 000. Human liver contains three acid phosphatases of different molecular weights, over 200 000, 107 000 and 13 400 (30). A low molecular weight acid phosphatase (M_r 13 000 - 16 000) has also been purified from other sources (9,19). Some investigators (cf. 11) have also found an acid phosphatase with an approximate molecular weight of 60 000, which we found from rat liver. Concerning alkaline phosphatase, a molecular weight of 154 000 has been reported for the enzyme from rat liver (27), of 157 000 for that from rat intestine (24) and values varying from 116 000 to 190 000 for various other mammalian sources (3,6,8,13-15,34). Our alkaline phosphatase peaks Liver I and Intestine II (Table 2) apparently correspond to the enzyme forms referred to above. Some authors have also reported alkaline phosphatase forms which have been excluded or nearly excluded from Sephadex gel columns (14,26,34) in agreement with our alkaline phosphatase forms Brain I and Intestine I (Table 2).

It is of interest to note that although among thiamine phosphate esters only thiamine diphosphate, the cofactor of intermediary metabolism, has an established function in cells, the specific thiamine triphosphatase is much more generally distributed than a specific thiamine diphosphatase. Accord-

ing to the electrophoretic studies of Allen (1) the latter enzyme is present in only a few tissues of the mouse (like epididymis, parotid gland and submaxillary gland) but is totally lacking in most of the tissues, for example liver, brain and intestine (1). In the latter tissues thiamine diphosphate is, however, well hydrolyzed by alkaline phosphatase and nucleoside diphosphatase (1).

Whether thiamine triphosphate has a specific role in nerve function (10,18) or in some other process in the cells remains to be answered. These results, indicating that a specific hydrolase for thiamine triphosphate indeed exists widely in rat tissues, support some specific role for thiamine triphosphate.

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REFERENCES

1. Allen JM: The properties of Golgi-associated nucleoside diphosphatase and thiamine pyrophosphatase: II electrophoretic separation and identification. J Histochem Cytochem 11: 542-552, 1963

2. Allen JM, Hyncik G: Localization of alkaline phosphatases in gel matrices following electrophoresis. J Histochem Cytochem 11: 169-175, 1963

3. Andrews P: The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem J 96: 595-606, 1965

4. Barchi RI, Braun PE: A membrane-associated thiamine triphosphatase from rat brain. J Biol Chem 247: 7668-7673, 1972

5. Barka T: Studies of acid phosphatase. I electrophoretic separation of acid phosphatases of rat liver on polyacrylamide gels. J Histochem Cytochem 9: 542-547, 1961

6. Brunel C, Cathala G, Saintot M: Purification et propriétés de la phosphatase alcaline du cerveau de boeuf. Biochim Biophys Acta 191: 621-635, 1969

7. Böhlen P, Stein S, Dairman W, Udenfriend S: Fluorometric assays of proteins in the nanogram range. Arch Biochem Biophys 155: 213-220, 1973

8. Cathala G, Brunel C, Chappelet-Tordo D, Lazdunski M: Bovine kidney alkaline phosphatase purification, subunit structure, and metalloenzyme properties. J Biol Chem 250: 6040-6045, 1975

9. Chaimovich H, Nome F: Purification and properties of an acid phosphatase from bovine brain. Arch Biochem Biophys 139: 9-16,1970

10. Cooper JR, Pincus JH: The role of thiamine in nervous tissue. Neurochem Res 4: 223-239, 1979

11. De Araujo PS, Mies V, Miranda O: Subcellular distribution of low- and high-molecular weight acid phosphatases. Biochim Biophys Acta 452: 121-130, 1976

12. Ernster L, Jones LC: Study of the nucleoside tri- and diphosphate activities of rat liver microsomes. J Cell Biol 15: 563-378, 1962

13. Fosset M, Chappelet-Tordo D, Lazdunski M: Intestinal alkaline phosphatase. Physical properties and quatenary structure. Biochemistry 13: 1783-1788, 1974

14. Gerbitz KD, Kolb HJ, Wieland OH: Human alkaline phosphatases I. Purification and some structural properties of the enzyme from human liver. Hoppe-Seyler's Z Physiol Chem 358: 435-446, 1977

15. Gottlieb AJ, Sussman HH: Human placental alkaline phosphatase: molecular weight and subunit structure. Biochim Biophys Acta 160: 167-171, 1968

16. Greiling H, Kiesow L: Zur Biochemie der Thiamintriphosphosäure. Z Naturforsch Teil B 13: 251-252, 1958

17. Harris H, Hopkinson DA: Handbook of Enzyme Electrophoresis in Human Genetics, North-Holland, Amsterdam, 1976

18. Hashitani Y, Cooper JR: The partial purification of thiamine triphosphatase from rat brain. J Biol Chem 247, 2117-2119, 1972

19. Heinrikson RL: Purification and characterization of a low molecular weight acid phosphatase from bovine liver. J Biol Chem 244: 299-307, 1969

20. Ishibashi T, Gasa S, Ohkubo I, Makita A: Affinity purification and some properties of nucleoside diphosphatase from rat liver cytosol. Biochim Biophys Acta 525, 265-274, 1978

21. Kiessling KH: Thiamine Triphosphate in Bakers' yeast. Nature (London) 172: 1187-1188, 1953

22. Lindblad B, Lindstedt G, Lindstedt S, Rundgren M: Purification and some properties of human 4-hydroxyphenylpyruvate dioxygenase (I). J Biol Chem 252, 5073-5084, 1977

23. Lück H: Spectrometric method. in Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) pp. 886-888, Verlag Chemie, Weinheim, 1963

24. Malik N, Butterworth PJ: Molecular properties of rat intestinal alkaline phosphatase. Biochim Biophys Acta 446: 105-114, 1976

25. Maurer HR: Disc Electrophoresis, pp. 44-45, Walter de Gruyter, Berlin, 1971

26. Nose K: Purification and characterization of alkaline phosphatase from rat kidney. J Biochem (Tokyo) 79: 283-288, 1976

27. Ohkubo A, Langerman N, Kaplan MM: Rat liver alkaline phosphatase. J Biol Chem 249: 7174-7180, 1974

28. Penttinen HK: Determination of inorganic phosphate. A method for the determination of phosphatase activities by a continuous flow system. Anal Biochem 102, 353-357, 1980

29. Penttinen HK: Preparation of thiamine triphosphate. in Methods in Enzymology (McCormick, D.B. and Wright, L.D., eds.) vol. 62, part D, p. 112, Academic Press, New York, 1979

30. Rehkop DM, Van Etten RL: Human liver acid phosphatases. Hoppe-Seyler's Z Physiol Chem 356: 1775-1782, 1975

31. Rossi-Fanelli A, Ipata PL, Fasella P: On the distribution and transformation of thiamine and its phosphate esters in biological material. Biochem Biophys Res Commun 4: 23-27, 1961

32. Rossi-Fanelli A, Siliprandi N, Fasella P: On the presence of the triphosphothiamine (TPT) in the liver. Science 116: 711-713, 1952

33. Shirasaka T, Arima T, Fujii S: Studies on mitochondrial and microsomal nucleoside diphosphatase on rat liver. J Biochem (Tokyo) 76: 99-106, 1974

34. Trépanier JM, Seargeant LE, Stinson RA: Affinity purification and some molecular properties of human liver alkaline phosphatase. Biochem J 155: 653-660, 1976

35. Vesterberg O: Isoelectric focusing of proteins. in Methods in Enzymology (Jakoby, W.B., ed.), vol. 22, pp. 389-412, Academic Press, New York, 1971

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