Journal of Chromatography, 566 (1991) 397–408
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5807

Application of high-performance liquid chromatography to the study of thiamine metabolism and in particular thiamine triphosphatase

LUCIEN BETTENDORFF

Laboratory of General and Comparative Biochemistry, University of Liège, 17 Place Delcour, 4020 Liège (Belgium)

ABSTRACT

Thiamine triphosphate can be found in most tissues at very low levels, but its role is unknown. Organs and muscles that generate electrical impulses are particularly rich in this compound. This paper describes a thiamine triphosphatase from the electrical organ of *Electrophorus electricus*. The activity of this enzyme, as measured by a high-performance liquid chromatographic method, is closely anion-regulated. Furthermore, thiamine triphosphate increases chloride uptake in membrane vesicles prepared from rat brain. Our results suggest that this compound could play an important role in the regulation of chloride permeability.

INTRODUCTION

The first high-performance liquid chromatographic (HPLC) methods used for the determination of thiamine derivatives date back to the end of the 1970s, and gradually more and more powerful methods have become available (for a review see ref. 1). These methods are all based on either a pre-colum or a post-column derivatization of thiamine to yield highly fluorescent derivatives (thiochromes) in the presence of a strong oxidant (ferricyanide or cyanogen) in an alkaline medium. Four thiamine derivatives have been detected in animal tissues: free thiamine, thiamine monophosphate (TMP), thiamine diphosphate (TDP) and thiamine triphosphate (TTP). The first two derivatives represent the forms under which the vitamin is transported in the blood. TDP is well known for its role as cofactor in intermediary metabolism for pyruvate and α-ketoglutarate dehydrogenases and transketolase. The role of TTP is not yet known, but it may play a role in the regulation of ion permeabilities [2–4].

One of the most challenging problems has been the determination of TTP in tissue samples. The level of this compound in most tissues is extremely low, *i.e.* 0.1 nmol/g of fresh tissue. These low concentrations have long been an obstacle to the study of this compound in nervous tissue. Since HPLC methods have become available, reliable determination of TTP in tissue samples has become possible

0378-4347/91/$03.50

[5–10]. The assumption that TTP plays a role in the regulation of membrane permeability is mainly based on the observation that electrical stimulation of nerves produces a release of thiamine [11,12] and concomitantly a dephosphorylation of TTP and TDP [3]. Experimental thiamine deficiency leads to neurological symptoms, and one of the main questions remaining to be clarified is whether these symptoms can be explained by a decrease in the activity of TDP-dependent enzymes [13-15] or whether a specific function of thiamine is involved [3,16–19]. These two points of view, however, do not seem to us to be mutually exclusive.

This paper describes some evidence for a specific neurochemical role for TTP, and the use of HPLC methods in our investigations.

EXPERIMENTAL

Chemicals

TTP was a gift from Dr. M. Yamazaki (Sankyo, Tokyo, Japan). Pyrithiamine, thiamine, TMP and TDP were purchased from Sigma (St. Louis, MO, U.S.A.). N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) came from Calbiochem-Behring (La Jolla, CA, U.S.A.). $Na^{36}Cl$ was from Amersham Belgium (Brussels, Belgium).

All other products were from Merck (Darmstadt, Germany) and were of analytical grade. All the solutions were prepared with Milli-Q water (Millipore, Bedford, MA, U.S.A.).

Assay of thiamine phosphate derivatives

The method was as previously described [20]. The instrument was an Altex Model 334-50 equipped with an LS-4 fluorescence spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The wavelengths were set at 365 nm for excitation and 433 nm for emission. The analytical column was a PRP-1 from Hamilton (Reno, NY, U.S.A.) protected by a guard column (32 mm × 4.2 mm I.D.) dry-packed with Vydac-201 RP (30–44 μm) from Macherey-Nagel (Düren, Germany). The mobile phase was phosphate buffer (15 m*M*, pH 8.5), containing 1% tetrahydrofuran for detection of the three phosphate esters or 10% tetrahydrofuran for thiamine. Before injection into the column, thiamine derivatives were transformed into fluorescent thiochromes [21] by addition of 50 μl of oxidant ($4.3 \cdot 10^{-3}$ *M* $K_3Fe(CN)_6$ in 15% NaOH) to 80 μl of sample. The sample loop was 20 μl, and the solvent flow-rate was 0.5 ml/min.

Preparation of electroplax membranes

Crude membranes from the main electrical organ of *Electrophorus electricus* (World Wide Scientific Animals, Apopka, FL, U.S.A.) were prepared as decribed previously [7]. The organ was homogenized in HEPES Tris buffer (10 m*M*, pH 6.8) using a VirTis homogenizer, sieved through a double layer of gauze, and centrifuged for 20 min at 40 000 *g*. The pellet was resuspended in five volumes of

buffer and centrifuged for 1 h at 100 000 *g*. The pellet (10 mg/ml) was stored at −70°C until use. Membranes enriched in TTPase activity were prepared by running two successive discontinuous sucrose gradients [22]. After the second centrifugation, the fraction at the 1.25 *M*–1.5 *M* interface was collected and washed once in HEPES–Tris buffer (10 m*M*, pH 7.8). This fraction is referred to as "purified membranes". In contrast to the crude preparation, it is largely free from both connective fibres and the nucleated microcells that spontaneously form during homogenization of this tissue [23]. Protein concentrations were determined according to the method of Peterson [24].

Assay of TTPase activity

If not otherwise stated, the method was as decribed previously [7]. The reaction medium was composed of 50 μl of sample (membrane preparation), HEPES–Tris buffer (10 m*M*, pH 6.8), $MgCl_2$ (1.5 m*M*), ethylene glycol tetraacetate (EGTA) (1.5 m*M*) and TTP (0.1 m*M*) in a total volume of 100 μl. Incubation was carried out at 25°C for 15 min, and the reaction was stopped by the addition of 500 μl of trichloroacetic acid (6%). The precipitate was centrifuged (15 min, 5000 *g*), and the supernatant extracted with four volumes of diethyl ether. TDP was determined by HPLC as described above. In some cases, inorganic phosphate (Pi) was determined by the method of Baginski *et al.* [25].

Measurement of ^{36}Cl uptake in rat brain membrane vesicles

Membrane vesicles were prepared according to the method described by Booth and Clark [26] with slight modifications. The animal was killed by decapitation, and the brain was washed in 10 ml of a solution containing 0.32 *M* sucrose and 10 m*M* HEPES–Tris (pH 7.5) and homogenized by hand in a glass–PTFE homogenizer. The homogenate was centrifuged at 900 *g* for 10 min. The resulting supernatant was centrifuged at 14 000 *g* for 20 min, and the pellet was suspended (4–5 mg/ml) in 5 ml of incubation buffer (145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 10 m*M* D-glucose, and 10 m*M* HEPES–Tris, pH 7.5). For measurement of $^{36}Cl^-$ uptake, 75 μl of the membrane suspension were preincubated at 37°C. After 5 min, 25 μl of a buffer solution containing $^{36}Cl^-$ (0.5 μCi/ml), $NaHCO_3$ (40 m*M*, pH 7.5) and TTP (4 m*M*) or pyrithiamine (0.4 m*M*) was added. After incubation times varying from 0 to 10 min, uptake was stopped by addition of 4 ml of ice-cold incubation buffer and rapid filtration through 2.4-cm Whatman GF/C filters. The filters were rapidly washed with 8 ml of ice-cold buffer, and the radioactivity of the filters was counted by liquid scintillation spectrometry.

Enzymic synthesis of TTP in rat brain homogenates

A rat brain was homogenized in 12 ml of saline containing 145 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$ and 10 m*M* HEPES–Tris (pH 7.5), and 500 μl of the homogenate were incubated for 15 min at 37°C in the presence of

thiamine (1 m*M*) with or without ATP (2 m*M*) in a total volume of 500 μl. The reaction was stopped by addition of 100 μl of trichloroacetic acid (60%), and thiamine phosphate esters were determined by HPLC.

RESULTS

As electrical stimulation of nerves is accompanied by a dephosphorylation of TTP and TDP, the study of the enzymes involved in these reactions could prove useful. An HPLC method [20] was used as an analytical tool for the determination of the four thiamine derivatives (Fig. 1). As thiamine is much more hydrophobic than its phosphate esters, it is not eluted under the same conditions. At 1% tetrahydrofuran in the mobile phase, TTP is eluted first, followed by TDP and TMP (Fig. 1A). Thiamine is eluted at 10% tetrahydrofuran (Fig. 1B).

In order to reveal the existence of TTP-hydrolysing enzymes, a crude membrane preparation from the electrical organ of *E. electricus* was incubated in the presence of TTP (0.1 m*M*) for different time intervals (Fig. 2). With increasing incubation times we note the disappearance of TTP and the formation of TDP, followed by TMP and finally thiamine. This result demonstrates the superiority of HPLC methods over inorganic phosphate (Pi) determination for the detection of sequential reactions in unknown samples. Indeed, photospectrometric determination of Pi would not have revealed the existence of several reactions. Determination of kinetic parameters (*e.g.* the Michaelis constant, K_M, and the maximum initial velocity, V_{max}) and the effects of inhibitors requires the elimination of side-reactions involving the reaction product.

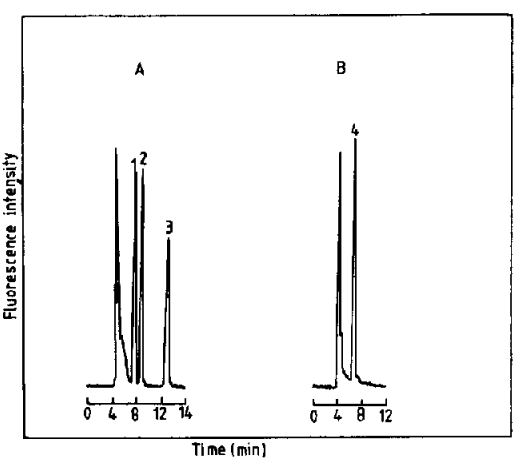

Fig. 1. Chromatograms of standard solutions of thiamine phosphate esters (A) and thiamine (B). The amount of each of the phosphate esters injected was 180 fmol, and that of thiamine was 120 fmol. A 20-μl sample loop was used. Peaks: 1 = TTP; 2 = TDP; 3 = TMP; 4 = thiamine. (Reproduced with permission from ref. 20.)

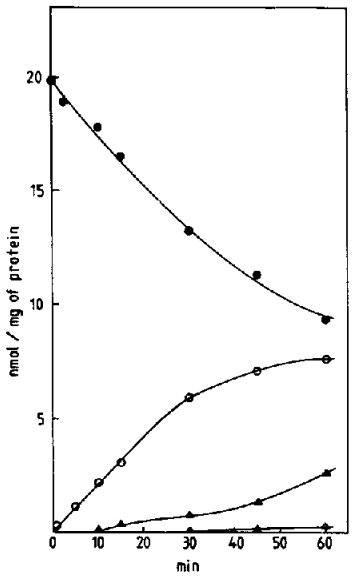

Fig. 2. Hydrolysis of TTP (●) and formation of TDP (○), TMP (▲) and thiamine (△) as a function of time in the electroplax crude membrane preparation. Membranes (4–5 mg of proteins per ml) were incubated in the presence of $MgCl_2$ (1.5 m*M*), EGTA (1.5 m*M*) and TTP (0.1 m*M*) as indicated in Experimental. (Reproduced with permission from ref. 7.)

For the continuation of our studies on TTPase it was thus important to minimize any thiamine diphosphatase (TDPase) activity. This could be achieved by studying the effects of divalent cations on the reaction products. From Fig. 3 we can see that Mg^{2+} mainly activates TTPase but not TDPase. The presence of

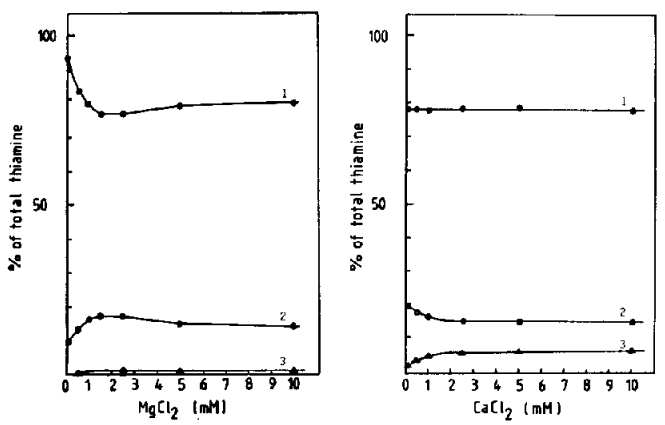

Fig. 3. Percentage of TTP (1), TDP (2) and TMP (3) as a function of $MgCl_2$ and $CaCl_2$ concentration after incubation for 15 min in HEPES–Tris buffer (10 m*M*, pH 6.8) at 25°C. (Reproduced with permission from ref. 7.)

TABLE I

STOICHIOMETRY OF TTP HYDROLYSIS BY TTPase

Experimental conditions are as described in legend to Fig. 2, except for the TTP concentration, which is 1 m*M*. The results shown represent three experiments for three different incubation times. Pi is determined by the method of Baginski *et al.* [25] and TTP and TDP were determined by our HPLC method.

Experiment No.	TTP consumed (nmol)	TDP formed (nmol)	Pi formed (nmol)
I	21.3	20.7	22
II	17.3	17.0	18
III	14.5	13.6	15

Ca^{2+} more specifically activates the latter enzyme. Thus working in the presence of Mg^{2+} and EGTA (a Ca^{2+}-chelating agent), no appreciable TDPase activity is observed.

The next step consisted of the determination of the stoichiometry of the reaction under the previously determined conditions (Table I). This table shows that the amount of TTP that disappears is not significantly different from the amount of TDP and Pi formed. From this example, we can deduce a second advantage of HPLC methods over conventional techniques, in that they allow the simultaneous determination of the substrate and the product, *e.g.* TTP and TDP.

At this stage of our studies, we can now be relatively sure that we are dealing with a genuine TTPase reaction of the following stoichiometry:

$$TTP \rightarrow TDP + Pi$$

We have observed that the rate of TTP consumption is slightly greater than the rate of formation of TDP. This can be explained by the fact that a small amount of TMP is formed during the incubation. For the same reason, the rate of Pi formation is slightly greater than the rate of TDP formation. These differences, however, amount to only 5–10%, and TMP formation can be minimized if the incubation times are kept short (less than 15 min).

Fig. 4 shows two chromatograms illustrating the enzymic hydrolysis of TTP. Crude membranes are precipitated immediately after addition of TTP (Fig. 4A). The slight contamination of TTP by TDP is inevitable, owing to spontaneous non-enzymic degradation of TTP. After incubation for 10 min (Fig. 4B), the proportion of TTP has decreased by *ca.* 10% and the peak corresponding to TDP has increased by the same amount. We can notice that, under these conditions (1.5 m*M* $MgCl_2$ and 1.5 m*M* EGTA), no TMP is formed.

Another advantage of HPLC methods in this kind of application is their sensitivity compared with inorganic phosphate determination. The latter method does not permit the use of substrate concentrations significantly below 0.1 m*M* (ATP

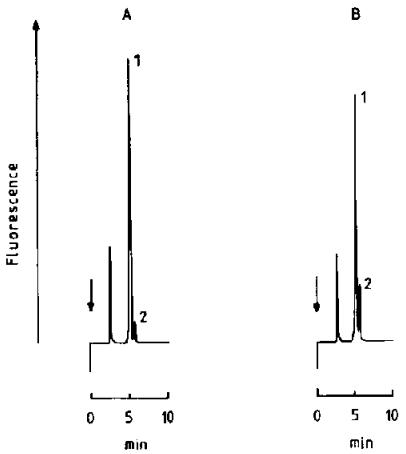

Fig. 4. Chromatograms demonstrating the hydrolysis of TTP by TTPase. Incubation of membranes from the electrical organ was carried out as described in Experimental, in the presence of 10 μM TTP in a total volume of 100 μl. Crude membranes were precipitated by addition of 500 μl of trichloroacetic acid (6%) immediately after addition of TTP (A) or after 10 min incubation at 25°C (B). Chromatographic analysis is as described after derivatization of thiamine compounds into thiochromes. Peaks: 1 = TTP; 2 = TDP.

for example), whereas with our HPLC method nanomolar concentrations of TTP or TDP can be detected [20]. It is thus possible to work with substrate concentrations as low as 10^{-6}–10^{-7} M. This high sensitivity allows us to screen the enzyme

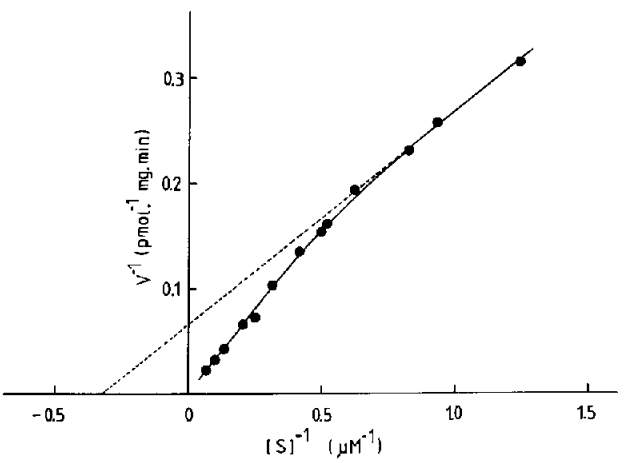

Fig. 5. Lineweaver–Burk plots of TTPase activity at substrate concentrations ranging from 0.8 to 16 μM. Incubation was carried out at 25°C (15 min) in the presence of $MgCl_2$ (3 mM). Protein concentration was 3 mg/ml. The solid line is computer-drawn from a model involving two active sites (α and β) with Michaelis–Menten behaviour ($K_M(\alpha)$ = 0.5 μM, $V_{max}(\alpha)$ = 1 pmol/mg/min; $K_M(\beta)$ = 1.8 mM and $V_{max}(\beta)$ = 5.9 nmol/mg/min). Each point represents the mean for nine to twelve independent determinations. (Reproduced with permission from ref. 27.)

TABLE II

EFFECT OF SODIUM NITRATE ON THE KINETIC PARAMETERS OF TTPase

Purified membranes from the electrical organ were prepared by two successive sucrose gradient steps, as decribed by Bettendorff *et al.* [22] (after Bettendorff *et al.* [27]).

Sample	K_M (mM)	pH optimum	V_{max} (nmol/mg/min)
Control	0.24	5.0	4.7
Added $NaNO_3$ (250 mM)	1.1	7.8	114

activity in the micromolar substrate concentration range (Fig. 5), which can lead to interesting observations. We can see that non-linear Lineweaver–Burk plots are obtained, which could indicate the existence of a high-affinity binding site for TTP. This hypothesis would be in agreement with our finding of a high affinity binding site (K_D = 0.5 μM) for TTP in the electrical organ [27].

Various neurotropic compounds were tested for TTPase activity but without significant effects [7]. The effects of ions, especially anions, proved to be more promising (Table II). Nitate (250 mM) increases the pH optimum, K_M and V_{max} (the third by 24-fold). Furthermore, TTPase is irreversibly inhibited by 4,4′-di-isothiocyanostilbene-2,2′-disulphonic acid (DIDS), and anions are known to protect against inhibition by this compound [22]. On the whole, these results suggest the presence of an anion-binding site on this enzyme, and we propose that TTPase and thus TTP could play a role in the regulation of anion permeability in excitable membranes.

In order to check this possibility, we tested the effects of thiamine compounds on chloride uptake in membrane vesicles (P2 fraction) from rat brain. Fig. 6 shows that TTP (1 mM) and pyrithiamine (0.1 mM), a thiamine anti-metabolite, do indeed increase chloride uptake. The mechanism by which this increase is

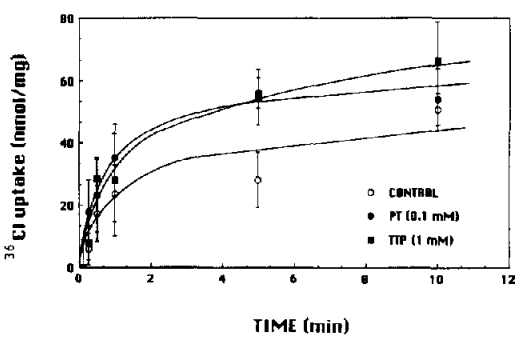

Fig. 6. Membrane vesicles were prepared and chloride uptake was measured as described in Experimental.

TABLE III

SYNTHESIS OF TTP AND TDP IN RAT BRAIN HOMOGENATES

Rat brain homogenates were prepared in incubation buffer as described in Experimental and separated into two aliquots. Thiamine (1 m*M*) was added to both aliquots, but ATP (2 m*M*) was present only in the second. The samples were then incubated for 15 min at 37°C, and thiamine phosphate ester content was measured by our HPLC method. Results are expressed as the mean ± S.D. for three independent experiments. The significance of the differences was tested by Student's *t*-test ($p < 0.05$)*.

Sample	TTP (nmol/ml)	TDP (nmol/ml)	TMP (nmol/ml)
Control	0.016 ± 0.003	2.0 ± 0.1	0.40 ± 0.05
Added ATP (2 m*M*)	0.021 ± 0.002*	2.4 ± 0.1*	0.34 ± 0.03

mediated is, however, not yet known. Oxythiamine (1 m*M*), another thiamine anti-metabolite, decreases chloride uptake [18].

The high concentrations needed to obtain these effects suggest the involvement of either permeability barriers or a chemical reaction [18]. Fox and Duppel [16] previously suggested that the site of action of thiamine compounds is located at the internal surface of the membranes.

Another interesting point that we wanted to investigate concerns the synthesis of TTP. A rat brain was homogenized in a saline containing NaCl (145 m*M*), KCl (5 m*M*), $MgCl_2$ (2 m*M*), $CaCl_2$ (2 m*M*) and HEPES–Tris buffer (10 m*M*, pH 7.5) and incubated for 10 min at 37°C in the presence of thiamine (1 m*M*) in the presence or the absence of ATP (2 m*M*). Table III shows that both TTP and TDP levels are increased after incubation in the presence of ATP. TMP levels are not significantly increased.

DISCUSSION

The role of thiamine in the nervous system is an enigma. A specific role, independent of its coenzyme function, has long been suspected, but clear evidence is lacking. Thiamine deficiency induces specific nervous symptoms, and electrical stimulation of nerves results in a dephosphorylation of TTP and TDP. Therefore the discovery of a TTPase whose activity is closely anion-regulated could provide an important clue for improving our knowledge on this subject.

A soluble [28] and a membrane-associated [29,30] TTPase have been detected in rat brain. No soluble enzyme was found in the electrical organ, and the membrane-associated enzyme we describe in this tissue could be equivalent to the membrane-associated enzyme in rat brain. No data about the effects of anions characteristic of our TTPase are, however, available for the rat brain enzyme.

Chloride uptake measurements confirmed our hypothesis that TTP could be involved in the regulation of chloride permeability. The relationship between

TTPase and TTP-mediated chloride uptake, however, remains unknown. Nitrate induces a 24-fold increase in the V_{max} of this enzyme at pH 7.8, but is inhibitory at acidic pH [22,27]. DIDS, an inhibitor of anion exchange in red blood cell membranes, inactivates TTPase, and anions protect against this inactivation [22]. Hydrophobic anions, such as 2,4-dinitrophenolate and tetraphenylboron, inhibit this enzyme [22]. It can be solubilized in a stable form using non-ionic detergents. Sodium dodecylsulphate or desoxycholate proved to be denaturing, whereas cationic detergents were not [31]. Thus anions seem to play a major role in the regulation of this enzyme, and different observations suggest the existence of an anion-binding site.

The metabolism of TTP is complex. Regulatory mechanisms seem to exist, and they oppose to any change in TTP concentration *in vivo*. For instance, in symptomatic thiamine deficiency, a decrease in rat brain levels of thiamine, TMP and TDP is observed but not of TTP [32]. Similar observations were made in lambs [33] where only thiamine, TMP and TDP levels decreased even before the onset of neurological symptoms. On the other hand, we have shown that massive administration of thiamine (50 mg/kg intraperitoneally, daily for fourteen days) does lead to an increase in thiamine, TMP and TDP brain levels but not in those of TTP [34].

Similar observations are made in *in vitro* experiments. Thus, in contrast to ATP for instance, no rapid decrease in TTP is observed after withdrawal [10] or homogenization of rat brains [35]. It thus seems to be a rather stable compound. These observations are in agreement with our findings on TTPase in the electrical organ. The specific activity of this enzyme is *ca.* 100 times smaller than that of (Na^+, K^+)ATPase in crude electrical organ membranes. This could mean either that TTPase has a very low turnover or else that it is an extremely rare enzyme (Bettendorff, unpublished results). Under some conditions, however (*i.e.* in the presence of nitrate), enzyme activity can be strongly enhanced. It is thus possible that TTPase is essentially latent, and full activation only occurs under particular conditions.

This resistance of TTP levels to variations is in apparent contradiction with results from several groups who have shown that the pool of TTP has a higher turnover than the other thiamine derivatives [36, 37], and suggest the existence of powerful regulatory mechanisms. Probably only an extreme stress situation leads to a perturbation of the steady state existing between hydrolysis and synthesis of TTP. For instance, we have shown that intense intermittent light stimulation induces a decrease in TTP levels in the occipital region (visual cortex) of the baboon *Papio papio* [10]. The only case in which a selective decrease in TTP in human brain has been reported is subacute necrotizing encephalomyelopathy [38], a degenerative disease of childhood that is generally lethal. Post-mortem analysis revealed in the body fluids of these patients a factor inhibiting TDP:ATP phosphoryltransferase. This result supports the idea of a predominant role for TTP in the nervous system.

On the other hand, an increase in TTP levels is also difficult to achieve. From Table III we can calculate that the increase in TTP after incubation of rat brain homogenates with thiamine (1 m*M*) and ATP (2 m*M*) corresponds to only *ca.* $5 \cdot 10^{-4}$% of added thiamine. The yield is thus very low. Two enzymes could be successively involved in the synthesis of TTP from thiamine:thiamine pyrophosphokinase (thiamine + ATP $\rightleftarrows$ TDP + AMP) and TDP:ATP phosphoryltransferase (TDP + ATP $\rightleftarrows$ TTP + ADP). Such a scheme would be compatible with our results shown in Table III, because we observed an increase in TTP and TDP but not in TMP levels. The existence of the first enzyme has been wel documented in rat brain and rat liver [39–41], but the synthesis of TTP from TDP remains controversial [42–45]. Recently Shikata *et al.* [46] have shown that adenylate kinase may catalyse the synthesis of TTP from TDP *in vitro*. That this enzyme is responsible for TTP synthesis *in vivo* has not yet been proven.

In this respect, HPLC methods should prove very helpful. It has been suggested that electrophoretic and low-pressure liquid chromatographic methods could be responsible for artifacts in TTP determination and thus cause overestimates of TTP concentrations [43, 47]. Indeed, it would seem that, for instance, in the case of rat brain, HPLC methods systematically give a TTP content lower than 0.1 nmol/g wet weight or less than 1% of total thiamine [1,5–8,10]. Determinations by conventional analytical techniques [35,48,49], however, give a TTP content five to ten times higher, or closer to 5% of total thiamine, as already pointed out by Kawasaki and Sanemori [1]. Variations observed with different methods could explain discrepancies in results from one group to another, especially where enzymic TTP synthesis is concerned. At present, HPLC methods are the most powerful for studying thiamine metabolism and they should supersede conventional techniques.

ACKNOWLEDGEMENTS

The author thanks Dr. M. Yamazaki from Sankyo for the gift of TTP. Professor E. Schoffeniels and Dr. P. Wins are acknowledged for many valuable and stimulating discussions. This work was supported by the National Funds for Scientific Research (Belgium).

REFERENCES

1 T. Kawasaki and H. Sanemori, in A. P. DeLeenheer, W. E. Lamburt and M. G. M. De Ruyter (Editors), *Modern Chromatographic Analysis of the Vitamins*, Marcel Dekker, New York, 1985, Ch. 8, p. 385.
2 C. J. Arnett and J. R. Cooper, *J. Pharmacol. Exp. Ther.*, 148 (1965) 137.
3 Y. Itokawa and J. R. Cooper, *Biochem. Pharmacol.*, 19 (1970) 985.
4 E. Schoffeniels, *Arch. Int. Physiol. Biochim.*, 97 (1989) 389.
5 K. Ishii, K. Sarai, H. Sanemori and T. Kawasaki, *J. Nutr. Sci. Vitaminol.*, 25 (1979) 517.
6 H. Sanemori, H. Ueki and T. Kawasaki, *Anal. Biochem.*, 107 (1980) 451.

7 L. Bettendorff, C. Michel-Cahay, C. Grandfils, C. De Rycker and E. Schoffeniels, *J. Neurochem.*, 49 (1987) 495.
8 H. Iwata, T. Matsuda and H. Tonomura, *J. Chromatogr.*, 450 (1988) 317.
9 J. W. I. Brunnekreeft, H. Eidhof and J. Gerrits, *J. Chromatogr.*, 491 (1989) 89.
10 L. Bettendorff, E. Schoffeniels, R. Naquet, C. Silva-Barrat, D. Riche and C. Ménini, *J. Neurochem.*, 53 (1989) 80.
11 B. Minz, *C. R. Soc. Biol. (Paris)*, 127 (1938) 1251.
12 A. von Muralt, *Pflügers Arch.*, 245 (1942) 604.
13 R. F. Butterworth, J.-F. Giguère and A.-M. Besnard, *Neurochem. Res.*, 10 (1985) 1417.
14 R. F. Butterworth, J.-F. Giguère and A.-M. Besnard, *Neurochem. Res.*, 11 (1986) 567.
15 J.-F. Giguère and R. F. Butterworth, *Neurochem. Res.*, 12 (1987) 305.
16 J. M. Fox, and W. Duppel, *Brain Res.*, 89 (1975) 287.
17 L. Eder, L. Hirt and Y. Dunant, *Nature*, 264 (1976) 186.
18 L. Bettendorff, P. Wins, E. Schoffeniels, *Biochem. Biophys. Res. Commun.*, 171 (1990) 1137.
19 R. D. Brown, *J. Theor. Biol.*, 143 (1990) 565.
20 L. Bettendorff, C. Grandfils, C. De Rycker and E. Schoffeniels, *J. Chromatogr.*, 382 (1986) 297.
21 J. Bontemps, P. Philippe, L. Bettendorff, J. Lombet, G. Dandrifosse, E. Schoffeniels and J. Crommen, *Chromatographia* 7 (1984) 283.
22 L. Bettendorff, P. Wins and E. Schoffeniels, *Biochem. Biophys. Res. Commun.*, 154 (1988) 942.
23 A. Karlin, *J. Cell. Biol.*, 25 (1965) 159.
24 G. L. Peterson, *Anal. Biochem.*, 83 (1977) 346.
25 E. S. Baginski, P. P. Foa and B. Zak, *Clin. Chim. Acta*, 15 (1967) 155.
26 R. F. G. Booth and J. B. Clark, *Biochem. J.*, 176 (1978) 365.
27 L. Bettendorff, C. Grandfils, P. Wins and E. Schoffeniels, *J. Neurochem.*, 53 (1989) 738.
28 Y. Hashitani and J. R. Cooper, *J. Biol. Chem.*, 247 (1972) 2117.
29 R. L. Barchi and P. E. Braun, *J. Biol. Chem.*, 247 (1972) 7668.
30 R. L. Barchi and R. O. Viale, *J. Biol. Chem.*, 251 (1976) 193.
31 L. Bettendorff, I. Longrée, P. Wins and E. Schoffeniels, *Biochim. Biophys. Acta*, 1073 (1991) 69.
32 J. H. Pincus and I. Grove, *Exp. Neurol.*, 28 (1970) 477.
33 E. J. Thornber, R. H. Dunlop, J. M. Gawthorne, *J. Neurochem.*, 35 (1980) 713.
34 L. Bettendorff, L. Weekers, P. Wins and E. Schoffeniels, *Biochem. Pharmacol.*, 40 (1990) 2557.
35 T. Matsuda and J. R. Cooper, *Anal. Biochem.*, 117 (1981) 203.
36 K. Berman and R. A. Fishman, *J. Neurochem.*, 24 (1975) 457.
37 M. K. Gaitonde and G. M. Evans, *Biochem. Soc. Trans.*, 11 (1983) 695.
38 J. R. Cooper, Y. Itokawa and J. H. Pincus, *Science*, 164 (1969) 74.
39 C. J. Gubler, *Methods Enzymol.*, 18A (1970) 219.
40 B. Deus, H. E. C. Blum and H. Holzer, *Anal. Biochem.*, 27 (1969) 492.
41 T. Matsuda, H. Iwata, and J. R. Cooper, *Biochim. Biophys. Acta*, 817 (1985) 17.
42 P. Ruenwongsa and J. R. Cooper, *Biochim. Biophys. Acta*, 482 (1977) 64.
43 J. Schrijver, T. Dias and F. A. Hommes, *Neurochem. Res.*, 3 (1978) 699.
44 K. Nishino, Y. Itokawa, N. Nishino, K. Piros and J. R. Cooper, *J. Biol. Chem.*, 258 (1983) 11871.
45 A. I. Voskoboev and I. P. Chernikevich, *Biochemistry (U.S.S.R.)*, 50 (1985) 1421.
46 H. Shikata, S. Koyama, Y. Egi, K. Yamada and T. Kawasaki, *Biochem. Int.*, 18 (1989) 933.
47 A. D. Gounaris and M. Schulman, *Anal. Biochem.*, 102 (1980) 145.
48 G. Rindi and L. De Guiseppe, *Biochem. J.*, 78 (1961) 602.
49 C. Patrini and G. Rindi, *Int. J. Vit. Nutr. Res.* 50 (1980) 10.