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SHORT COMMUNICATIONS

Injection of sulbutiamine induces an increase in thiamine triphosphate in rat tissues

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Vitamin B_1 (thiamine) is thought to have a specific function in the nervous system (for review see Haas [1]). Thiamine diphosphate (TDP*) acts as a cofactor in the energy metabolism of the cell. A decrease in the activity of TDP-dependent enzymes could result in an impairment of neurotransmitter and especially γ-aminobutyric acid [2, 3] and acetylcholine [4] metabolism. The exact function of thiamine triphosphate (TTP) is presently unknown, but it has been proposed that this compound may play a role in the control of membrane permeability [5–8]. Thiamine monophosphate (TMP) and thiamine represent the forms under which the vitamin is distributed to the different tissues via the blood flow. The kidneys are responsible for the excretion of excess thiamine.

Sulbutiamine (isobutyrylthiamine disulfide) (Fig. 1) is a precursor of thiamine where two thiamine molecules are bound together by a disulfide bridge after opening of their respective thiazole rings [9]. An isobutyryl function is attached to the alcohol group of each ring. These modifications decrease the polarity of the molecule compared to thiamine. Sulbutiamine is a psychotropic agent and is prescribed for the symptomatic treatment of functional asthenias [10]. Effects on long term memory formation [11] and on the waking state [12] have also been shown. The mechanism of its action however remains totally unknown. It is thought that because of its hydrophobic character it more easily crosses membrane barriers than thiamine.

In this work, we show the effect of a sulbutiamine treatment on the distribution of thiamine derivatives in rat plasma, brain and kidney.

Materials and Methods

Plasma kinetics of sulbutiamine and thiamine. Female Wistar rats (250 g) were anaesthetized (Nembutal 40 mg/kg) and treated with heparin in order to avoid coagulation of the blood in the catheter which was introduced in the jugular vein. A tracheal cannula was systematically used. Five hundred microlitres of blood were sampled. We set up three groups of three animals. The first group contained the control animals which were injected intraperitoneally with 1 mL of saline. The second group received an injection of sulbutiamine (16 mg/kg) while the third group received an injection of thiamine (15.4 mg/kg). Respective doses were calculated in order to account for the differences in molecular weight and for the fact that sulbutiamine is a dimer. Blood (500 μL) was sampled 5, 15, 30, 45 and 60 min after injection. The blood was centrifuged (1000 *g*, 10 min) and, to 100 μL of plasma, we added 500 μL of 6% trichloroacetic acid. After mixing, the protein precipitate was sedimented by centrifugation (5000 *g*, 15 min) and the supernatant extracted with 3 × 1.5 mL diethyl ether and frozen at −70° until HPLC analysis.

Treatment of rats with sulbutiamine or thiamine and tissue sampling. Three groups of six female Wistar rats were formed. These animals were injected daily, for 14 days, intraperitoneally with 1 mL of saline (group C), 52 mg/kg of sulbutiamine (group SBT) or 50 mg/kg of thiamine (group T). The 15th day, at the time of the injection, the rats were decapitated. We sampled 1 mL of blood and removed the left kidney and the brain.

The blood was treated as described above. About 100 mg of the cortex of the kidney were rapidly frozen in liquid nitrogen. The brain was dissected as described by Glowinski and Iversen [13] and the medulla oblongata (including the pons), the cortex (telencephalon), the cerebellum and the hippocampus were rapidly frozen in liquid nitrogen. In each case the tissues were frozen not later than 5 min after the death of the animal. We have previously shown [14] that thiamine phosphate derivatives are, in contrast to ATP, relatively stable compounds and are not hydrolysed to a significant degree if freezing occurs within a reasonably short delay.

The frozen tissues were homogenized in 6% trichloroacetic acid using a glass–glass homogenizer as described previously [15]. Briefly, the homogenate was centrifuged (5000 *g*, 15 min) and the supernatant extracted with 3 × 1.5 mL diethyl ether and frozen at −70° until HPLC analysis.

Determination of thiamine derivatives in rat tissues. HPLC analysis was carried out as previously described [14, 16]. Prior to injection on the column thiamine derivatives were transformed into fluorescent thiochromes. Reference solutions (0.25 μM) of the four thiamine compounds were freshly prepared. Thiamine, TMP and TDP were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). TTP was a gift from Dr M. Yamazaki (Sankyo Co., Tokyo, Japan).

Results and Discussion

Acute experiment. Anaesthetized rats were injected intraperitoneally with sulbutiamine (16 mg/kg), thiamine (15.4 mg/kg) or saline and 500 μL of blood was sampled at different time intervals via a catheter introduced in the jugular vein. As can be seen from Table 1, rat plasma essentially contains thiamine (194 ± 28 nmol/L) and thiamine monophosphate (TMP, 517 ± 56 nmol/L). TDP accounts for only 12.5% of total thiamine and no TTP could be found. These results are in agreement with those of

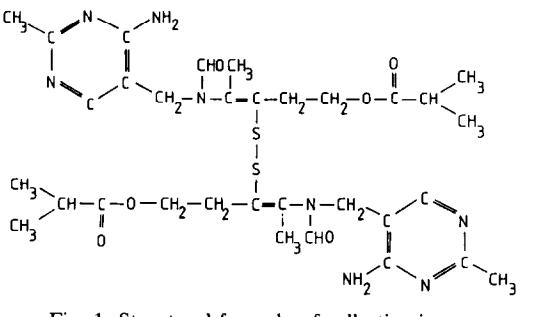

Fig. 1. Structural formula of sulbutiamine.

* Abbreviations: TDP, thiamine diphosphate; TTP, thiamine triphosphate; TMP, thiamine monophosphate.

Table 1. Contents in thiamine and phosphate derivatives of different rat tissues. The animals were injected daily and for 14 days with a saline (1 mL, C), sulbutiamine (52 mg/kg, SBT) or thiamine (50 mg/kg, T)

		TTP	TDP	TMP	T	Total
Serum	C	—	101 ± 28	517 ± 56	194 ± 28	811 ± 66
	SBT	—	236 ± 51†	1549 ± 180†	1244 ± 257†	3030 ± 355†
	T	—	108 ± 41	865 ± 195*	281 ± 107	1253 ± 295*
Brain						
Hippocampus	C	0.054 ± 0.008	11.2 ± 1.2	0.75 ± 0.12	0.54 ± 0.15	12.5 ± 1.3
	SBT	0.083 ± 0.021*	13.8 ± 2.6	0.99 ± 0.13	0.81 ± 0.15	15.7 ± 2.8*
	T	0.050 ± 0.009	15 ± 1*	0.79 ± 0.14	0.61 ± 0.30	16.4 ± 1.3*
Medulla	C	0.034 ± 0.004	13.8 ± 1.3	0.52 ± 0.05	0.34 ± 0.04	14.7 ± 1.3
	SBT	0.047 ± 0.008*	16.9 ± 1.7*	0.69 ± 0.06*	1.11 ± 0.24†	18.8 ± 1.8†
	T	0.039 ± 0.003	16.1 ± 1.8*	0.59 ± 0.09	0.62 ± 0.18*	17.4 ± 2.0*
Cerebellum	C	0.080 ± 0.015	16.4 ± 1.7	1.3 ± 0.2	0.40 ± 0.06	18.2 ± 1.8
	SBT	0.101 ± 0.006*	19.9 ± 1.2*	1.9 ± 0.2*	1.3 ± 0.4†	23.2 ± 1.4†
	T	0.077 ± 0.019	18.9 ± 0.7*	1.9 ± 0.1*	0.76 ± 0.10*	21.6 ± 0.8*
Cortex	C	0.066 ± 0.008	13.9 ± 2.6	0.72 ± 0.09	0.39 ± 0.07	15.1 ± 2.6
	SBT	0.083 ± 0.014*	19.1 ± 1.3*	0.97 ± 0.16*	0.89 ± 0.19†	21.0 ± 1.3†
	T	0.067 ± 0.005	17.8 ± 1.4*	0.92 ± 0.08*	0.61 ± 0.10*	19.4 ± 1.5*
Kidney	C	0.073 ± 0.022	40.8 ± 1.5	1.2 ± 0.2	0.76 ± 0.13	42.9 ± 1.6
	SBT	0.113 ± 0.008†	54 ± 10*	1.6 ± 0.1*	5.5 ± 0.9†	60.9 ± 9.7†
	T	0.087 ± 0.013	47.5 ± 2.7*	1.5 ± 0.3*	1.8 ± 0.4*	50.8 ± 2.9*

Each group (C, SBT or T) contained six animals. The results are expressed in nmol/L plasma or in nmol/g fresh weight (mean ± SD). Statistical analysis was done by analysis of variance ($P < 0.05$) followed by the Dunnett test for comparing the SBT and T groups to the controls. (*$P < 0.05$; †$P < 0.01$).

Kimura and Itokawa [17]. From Fig. 2 we can see that after injection of thiamine or sulbutiamine, the thiamine concentration in the plasma rapidly increases. An optimum is reached after 10 min when thiamine was injected. Kimura and Itokawa [17], in agreement with our results, have shown that intraperitoneal injection of thiamine induces a rapid increase of its concentration in rat blood followed by a decrease. However, when sulbutiamine is injected, thiamine concentration continues to increase for at least 60 min and higher plasma concentrations are reached than when thiamine was injected. TMP concentration increases in a similar way for more than 60 min after injection of both thiamine and sulbutiamine. No significant variations in TDP plasma concentrations were found after injection of sulbutiamine or thiamine. In control experiments 1 mL of saline was injected intraperitoneally but no time-dependent variations in any of the thiamine derivatives were observed. These experiments prove that, *in vivo*, sulbutiamine can be transformed into thiamine derivatives, which implies the reduction of the disulfide bond and formation of the thiazol rings with generation of two molecules of isobutyryl-thiamine. Indeed, sulbutiamine with its open thiazol ring does not yield any fluorescence after oxidation. Secondly, as the chromatographic behavior of plasma thiamine after injection of sulbutiamine is exactly the same as for genuine thiamine, we conclude that the isobutyryl group has been removed. Indeed, the isobutyryl group increases the hydrophobicity of the molecule and as our HPLC method is based on hydrophobic interactions a higher retention time would be expected for isobutyryl-thiamine compared to thiamine. This conclusion is further supported by the fact that TMP can be formed: indeed, the isobutyryl group would block the alcohol function which serves as phosphate group acceptor for the formation of thiamine phosphate derivatives. In view of these results, we can conclude that sulbutiamine is at least partially transformed into thiamine and can be considered as a precursor of this vitamin.

Chronic experiment. The aim of the acute experiment was to see whether sulbutiamine could be transformed into thiamine and thus in a compound detectable by fluorescence. Once this had been confirmed, it seemed important to check if sulbutiamine has any influence on the levels of thiamine derivatives in the brain. Indeed, several effects of chronic administration of sulbutiamine on the nervous system have been reported [11, 12]. In these cases it had been administered orally at doses of 300 mg/kg daily. For intraperitoneal injections effective doses can be lowered. Sulbutiamine (52 mg/kg), thiamine (50 mg/kg) or saline were injected daily for 14 days. The animals were killed on the 15th day and the contents of thiamine and its phosphate esters were determined in the plasma, the brain and the

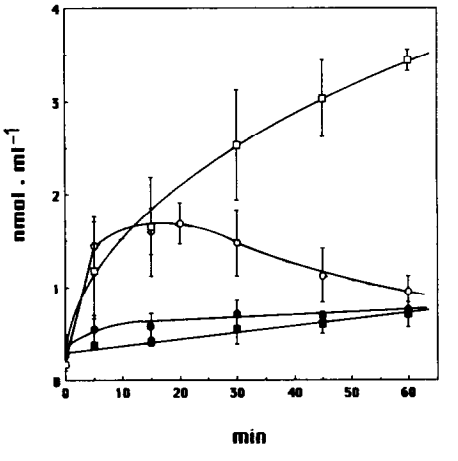

Fig. 2. Time dependence of thiamine and TMP concentrations in plasma following intraperitoneal injection of thiamine or sulbutiamine. Female Wistar rats were injected with thiamine 15.4 mg/kg (○, ●) or sulbutiamine 16 mg/kg (□, ■) as described in Materials and Methods. After different time intervals 500 μL of blood were sampled and thiamine (■, ●) and TMP (□, ○) were measured in the plasma. Points represent the mean ± SD (mmol/mL) for three different rats.

kidney. Four distinct brain regions were dissected: hippocampus, medulla oblongata, cerebellum and cortex. The results are summarized in Table 1. In blood serum, the contents in thiamine, TMP and TDP are significantly increased in rats treated with sulbutiamine when compared to control or thiamine treated animals. As far as group T animals are concerned, only the content in TMP is significantly increased compared to the controls. It is conceivable that free thiamine is much more easily eliminated than TMP. This would explain that in the cortex of the kidney free thiamine undergoes a much more important increase than phosphate esters. Note that in addition to free thiamine, TMP also crosses the blood–brain barrier by a saturable process [18]. Thus in rat plasma, where the TMP concentration is high compared to thiamine (in human the reverse situation is true [16]) TMP transport could be of considerable importance for the supply of the vitamin to nervous tissue. As sulbutiamine cannot be directly transformed into a fluorescent compound, we are unable to tell if it directly crosses the blood–brain barrier, or whether it is totally transformed to thiamine in the blood.

In the four brain regions that we examined, the contents of all four thiamine derivatives are significantly increased in sulbutiamine treated animals compared to the controls. If we compare the thiamine group to the control group we notice that only TDP is significantly increased in the four brain regions, TMP is increased only in the cerebellum and in the cortex. Thiamine contents are increased in three regions but not in the hippocampus. The most important difference however is that thiamine injection does not significantly increase the contents in TTP.

Results obtained in the case of the kidney are qualitatively the same as those obtained for the brain.

The most interesting result is that the injection of sulbutiamine leads to a significant increase of TTP, but injection of thiamine does not. The exact pathway of TTP synthesis *in vivo* has not yet been elucidated. Gaitonde and Evans [19] observed the incorporation of radioactivity in rat brain thiamine phosphate esters after injection of [^{14}C]thiamine. Iwata *et al.* [20] demonstrated the synthesis of TTP *in vivo*. After intracerebro-ventricular injection of [^{35}S]thiamine, they showed the presence of radioactive TMP, TDP and TTP in the cerebral cortex of the rat. Nishino *et al.* [21] reported the isolation and purification of a TTP synthesizing enzyme from bovine brain using ATP and protein-bound TDP as substrates. This TDP-binding protein was isolated from the rat liver supernatant but its nature remains unknown. According to Voskoboev and Chernikevich [22], the only TDP-binding protein in rat liver cytoplasm is transketolase. Recently Shikata *et al.* [23] reported the synthesis of TTP through the reaction TDP + ADP $\rightleftarrows$ TTP + AMP. The reaction is catalysed by adenylate kinase from pig skeletal muscle. However, the affinity of the enzyme for TDP is low and the reaction rate is six orders of magnitude lower than for ADP.

We did not observe any qualitative differences between the four brain regions: injection of thiamine or sulbutiamine led to a uniform increase in the contents of thiamine and its derivatives in the brain. Conversely, Dreyfus [24] has shown that in the case of experimental vitamin B_1 deficiency the decrease in total thiamine is relatively uniform from one region to another. More recently, Thornber *et al.* [25] measured the concentration of thiamine and its phosphate esters in the brain of lambs fed on a thiamine-free synthetic diet. After 4 weeks, the content of TDP had fallen to 22% of the control values, while no decrease in TTP was observed. Our observation that in spite of massive vitamin B_1 injection the content in TTP does not increase suggests, like the results of Thornber, that special control mechanisms must exist which oppose a variation of TTP in one direction or the other. This conclusion is in agreement with our observations of a specific thiamine triphosphatase in the electric organ. This activity is partially latent but can be strongly increased under different conditions [26].

In conclusion, we have shown that sulbutiamine can act as a precursor of thiamine and its phosphate derivatives, and injection of pharmacologic doses of sulbutiamine can lead to an increase of TTP in the brain and kidney, an increase not observed when thiamine was injected. This increase in TTP is probably due to the fact that thiamine levels in blood and tissues are higher after sulbutiamine treatment than after thiamine treatment. If, as proposed previously [5–8], TTP plays a role in the regulation of membrane permeability, this could explain the psychotropic properties of sulbutiamine.

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The *in vivo* site of formation of a carcinogen–serum albumin adduct

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The macromolecular binding of xenobiotics in humans is of interest, because of its possible involvement in the production of a toxic response and also as a means of monitoring exposure to potentially harmful substances. Such interaction may take place by the direct covalent binding of a chemically reactive compound to circulating serum albumin, but many environmental toxins and carcinogens will react only after metabolic activation (usually mediated by cytochrome P450 mixed function oxidases). Aflatoxin B_1 (AFB_1) is an hepatotoxic and carcinogenic mycotoxin [1] which, after activation by liver parenchymal cells to AFB_1-8,9-epoxide [2, 3] (or its dihydrodiol derivative [4]), reacts with albumin. The AFB_1-albumin adduct thus formed would appear to offer a useful index of exposure to the toxin [5, 6] in those developing countries where it is believed to be associated with a high level of primary liver cancer [7]. The release of the highly reactive electrophilic AFB_1-8,9-epoxide from the cells in any significant amount appears unlikely and it is possible a large proportion of the reaction with albumin could occur (intracellulary) within the hepatocytes, near the endoplasmic reticulum, at sites adjacent to both the activation of the toxin and synthesis of proalbumin or hepatocyte albumin (formed by cleavage of an N-terminal hexapeptide from proalbumin [8, 9]). We have examined this possibility using a rat model system.

The time courses of the synthesis of albumin and AFB_1-adduct formation in the various albumin fractions were determined (Fig. 1a and b). The time course of synthesis of proalbumin, hepatocyte albumin and serum albumin is in close agreement with that reported by Dorling *et al.* [10]. The proalbumin plus hepatocyte albumin fraction was rapidly labelled with ^{14}C, the level of labelling increasing over the 20 min period following [^{14}C]leucine injection, and with little ^{14}C label being detected in the serum albumin at this time. Subsequently, ^{14}C label declined in the proalbumin plus hepatocyte albumin fraction and entered the serum albumin fraction as the newly-synthesized protein was released from the hepatocytes. The time course of ^3H labelling of the fractions demonstrated a rapid labelling of the serum albumin fraction, over the period (0–20 min following injection) when little newly synthesized ^{14}C labelled albumin had entered this fraction. Extraction of these fractions with chloroform demonstrated that >90% of the ^3H label was soluble in this solvent, and HPLC analysis [4] showed that 80–85% of the label was associated with AFB_1. This result is in agreement with the findings of Dirr and Schabort [11] who reported a tight binding of AFB_1 to rat serum albumin *in vivo*. There was a close parallel between the ^3H and ^{14}C labelling of the proalbumin plus hepatocyte albumin in all samples, the ratio being approximately 1. In the serum albumin sample the $^3H/^{14}C$