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THIAMINE IN NERVE MEMBRANES

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SUMMARY

Membrane fragments isolated from rat brain have been shown to be the site of the drug-induced release of thiamine. As a brain homogenate is purified to yield the membrane fraction, the concentration of thiamine triphosphate rises, suggesting that this may be the neurophysiologically active form of the vitamin. Attempts to find a concurrent release of other compounds along with thiamine were unsuccessful.

INTRODUCTION

In a previous publication[1] we have shown in experiments using rat brain subfractions that thiamine and its phosphate esters, thiamine triphosphate, thiamine pyrophosphate and thiamine monophosphate are localized primarily in the crude mitochondrial fraction of the cell. Sucrose density gradient centrifugation of this fraction revealed the presence of the vitamin in all three subfractions, *i.e.* the membrane–myelin, nerve-ending particles (synaptosomes) and mitochondria. However, when each of these fractions was incubated with neuroactive drugs such as acetylcholine or tetrodotoxin, thiamine was released essentially only from the membrane–myelin fraction. Similar results occurred with subfractions of the spinal cord and the sciatic nerve. In addition, the released vitamin was in the form of either free thiamine or thiamine monophosphate whereas the bulk of the compound in the membrane fraction consisted of thiamine pyrophosphate and thiamine triphosphate suggesting that the release phenomenon involved dephosphorylation. From these experiments it would appear that the binding of thiamine to the membrane fraction was different from its binding to other subfractions of nervous tissue.

In this paper we report further experiments in an attempt to define the release of the vitamin.

METHODS AND MATERIALS

The procedure for injecting [^{35}S]thiamine into rats and subsequent harvesting of the myelin–membrane fraction by sucrose density gradient centrifugation has been described[1]. The separation of this fraction into myelin and membrane was performed using a continuous sucrose gradient of 0.66–1.2 M and centrifuging at 170000 × *g* for 60 min.

The method of Itokawa and Cooper[2] was used for the electrophoretic separation and fluorometric determination of thiamine and its phosphate esters. Protein was determined by the procedure of Lowry *et al*[3].

In the radioactive phosphate experiments, 800 μCi of $^{32}P_i$ was injected intraperitoneally into 200-g rats. 7 days later the rats were sacrificed and the brain subfractionated into myelin–membrane, synaptosomal, and mitochondrial fractions as previously described.

Electron microscopic examination of cell organelles was performed by fixation in 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The pellets were then transferred to 1 % OsO_4 containing 0.1 M phosphate buffer, pH 7.4, dehydrated with ethanol, and embedded in Vestopal-W.

Phospholipase A, phospholipase C, snake venom (*Crotalus atrox*), lipase, and trypsin were purchased from the Sigma Chemical Company. Amersham–Searle was the source of [^{35}S]thiamine. Thiamine triphosphate and tetrodotoxin were generously donated by the Sankyo Company, Japan. Amersham–Searle was the source of [^{32}P]phosphate.

RESULTS

After the injection of [^{35}S]thiamine into rats and subsequent isolation of the membrane–myelin fraction of the brain as previously described, membrane and myelin were separated using a continuous sucrose gradient technique described in Methods and Materials. The separation is shown in Fig. 1. The purity of the fractions was monitored with electron microscopy; that these fractions were relatively pure is shown by the micrographs in Fig. 2.

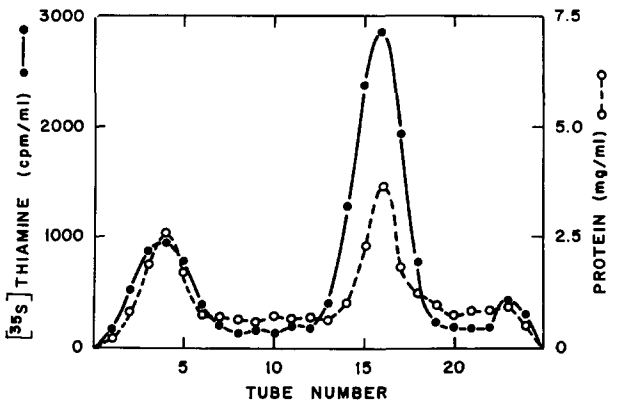

Fig. 1. Separation of membrane and myelin from rat brain. The first peak (Tubes 1–7) represents myelin and the large peak (Tubes 13–18) is the membrane fraction.

With the separation of myelin and membrane fragments it was of interest to determine if the release of thiamine, originally shown to occur primarily from this mixed fraction (as opposed to synaptosomes and mitochondria), was derived specifically from one or the other of these organelles. Accordingly, each fraction was incubated as described earlier[1] with and without the addition of neuroactive drugs to

determine the release of thiamine. As shown in Table I, thiamine was released only from the membrane fraction. Phospholipase A, which discharges the vitamin non-specifically from all cell fractions, released the vitamin from both fractions.

Enough labeled thiamine was present in both membrane and myelin to allow a determination of the forms of thiamine that were present in the two fractions. Table II lists the percentages of each form of the vitamin; for comparison, the percentages that are found in a whole homogenate[1] are inserted to indicate that as the cell fraction which contains drug-releaseable thiamine is purified, it is thiamine triphosphate that dramatically increases as a percent of total thiamine compounds.

As a first approach in our attempt to determine the manner in which thiamine was bound to cell organelles, a variety of hydrolytic enzymes were incubated with

TABLE I

DIFFERENTIAL RELEASE OF [^{35}S]THIAMINE FROM MEMBRANE AND MYELIN FRACTIONS

Fractions containing [^{35}S]thiamine were incubated in Krebs–Ringer phosphate medium, pH 7.2, at 37 °C for 30 min. After incubation, contents of the tubes were centrifuged at 48000 × *g* for 60 min and the radioactivity of a 0.2-ml aliquot of the supernatant was determined as previously described[1]. The results represent the mean of three experiments.

Fraction	*Radioactivity (cpm)*	% *Increase*
Membrane		
Control	1179	
Acetylcholine–DFP (10^{-4} M)	1536	30.3
Tetrodotoxin (10^{-6} M)	1413	19.8
Phospholipase A (10 units)	1479	25.5
Myelin		
Control	387	
Acetylcholine–DFP (10^{-4} M)	352	−9.0
Tetrodotoxin (10^{-6} M)	388	0
Phospholipase A (10 units)	456	15.1

TABLE II

DISTRIBUTION OF THIAMINE AND ITS PHOSPHATE ESTERS IN BRAIN SUBFRACTIONS

Non-labeled thiamine compounds were added to the [^{35}S]thiamine-labeled fractions. The preparations were deproteinized and subjected to electrophoretic separation and radioactivity determinations as previously described[1].

Thiamine species	*Preparation*		
	Brain homogenate * (%)	*Membrane* (%)	*Myelin* (%)
Thiamine triphosphate	2.3	14.3	5.2
Thiamine pyrophosphate	83.7	58.0	57.8
Thiamine monophosphate	10.2	21.2	22.1
Thiamine	3.8	6.5	14.9

* Itokawa and Cooper[1].

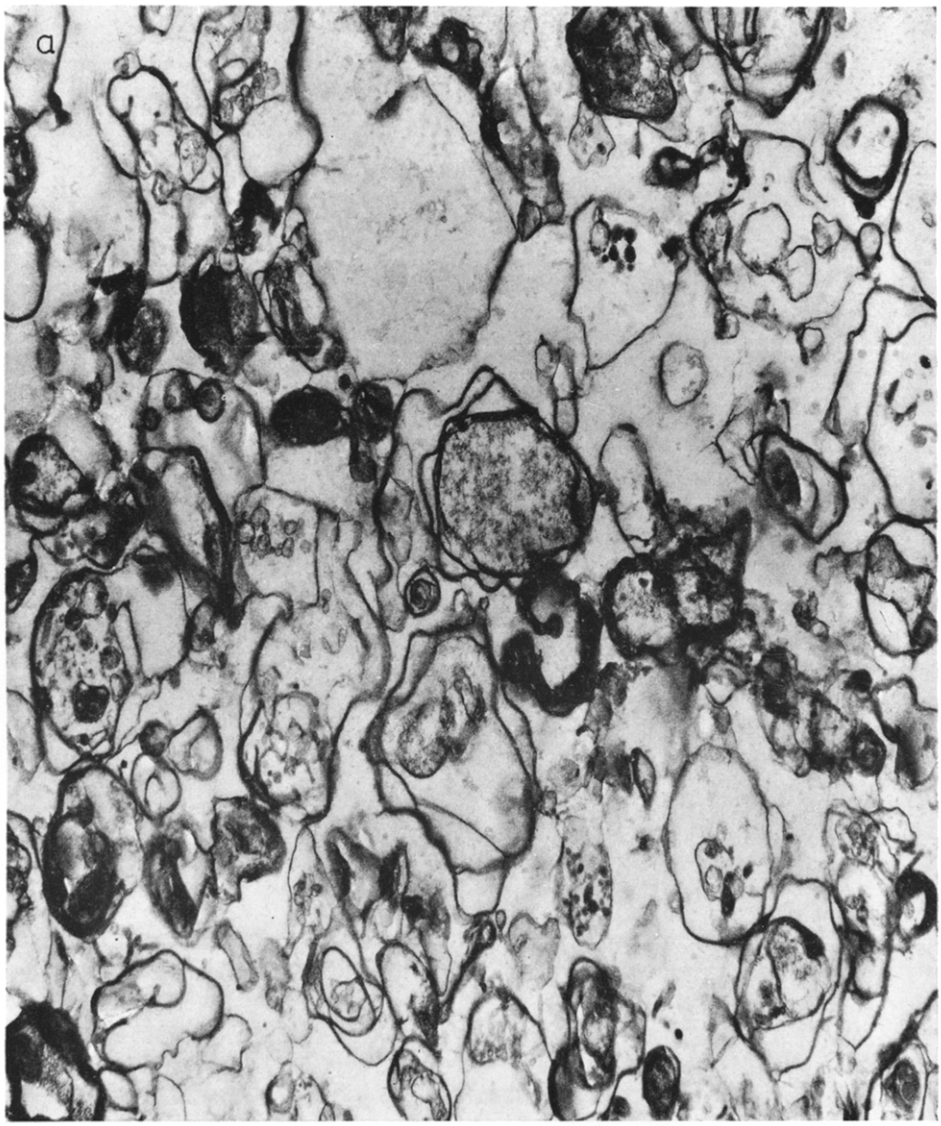

Fig. 2. (a) Myelin fraction derived from rat brain (× 15600).

the [^{35}S]thiamine-labeled crude mitochondrial fraction of rat brain. Snake venom (*Crotalus atrox*) proved to be the most potent agent in releasing thiamine followed by phospholipase A and trypsin. Neither lipase nor phospholipase C showed appreciable activity. However, subsequent experimentation with snake venom and phospholipase A indicated an equivalent release of thiamine from membranes, synaptosomes, and mitochondria (in contrast to the specific release by drugs from membranes). This approach therefore was abandoned as a technique for determining the type of compound to which thiamine is bound in nerve membranes.

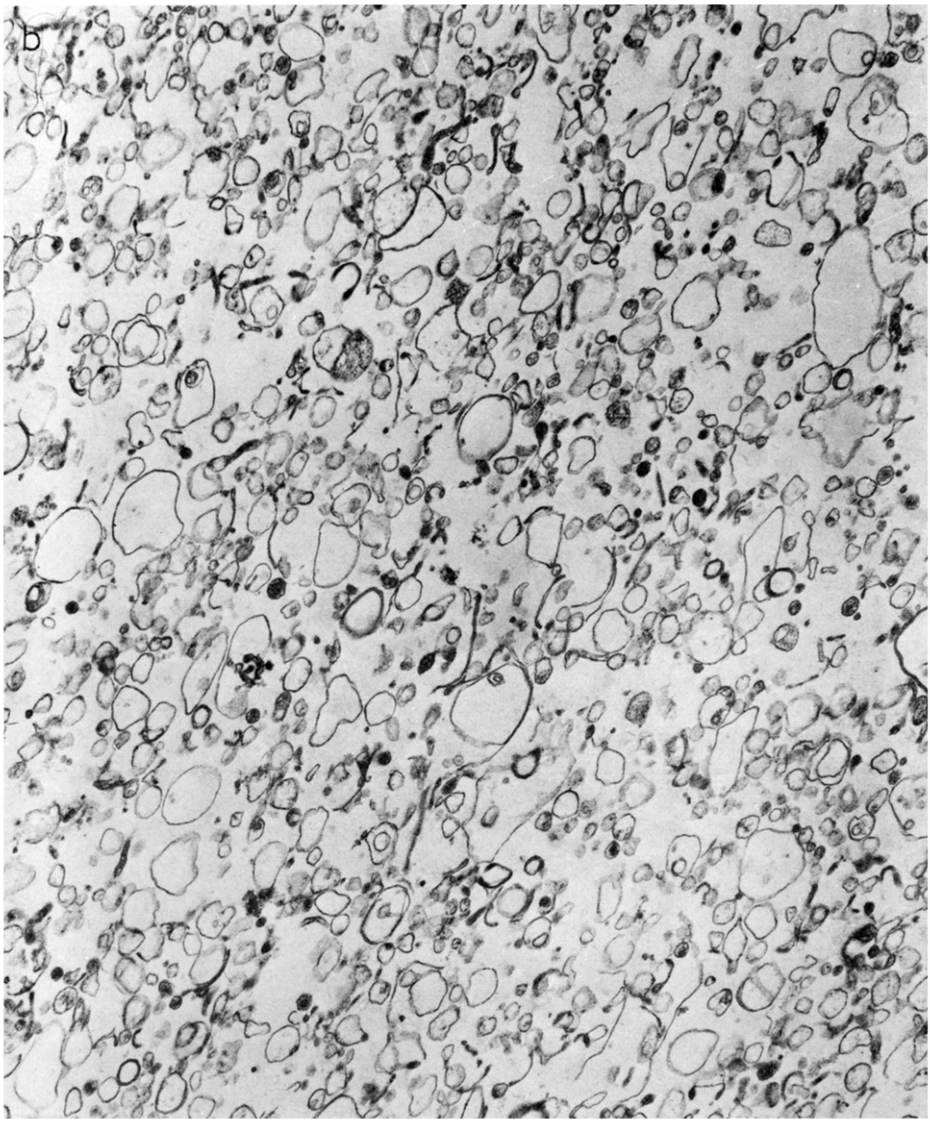

Fig. 2 (b) Membrane fraction (× 24000).

Since the possibility existed that the thiamine that was released from membranes by drugs might appear in the medium either attached to some membrane constituent or, alternatively, be released along with another compound, two other procedures were initiated. In the first approach, following the preparation of [^{35}S]-thiamine-labeled membrane fragments as previously described, aliquots were incubated with either acetylcholine or tetrodotoxin in order to effect a release of labeled material. A control tube with no drug was also included. After incubation and centrifugation, aliquots of the supernatant were subjected to paper chromato-

graphy in three different solvent systems. Radioactive areas were located with a strip scanner and in addition, the chromatograms were sprayed with ninhydrin. With all chromatographic systems, there was no difference in either the R_F of released radioactive material or ninhydrin-positive material between control and drug-treated samples.

The second procedure in our attempt to see if other membrane constituents were released in addition to thiamine was designed to explore the possibility that some organic phosphate compound, perhaps a phospholipid, was discharged along with the vitamin phosphate. In these experiments, $^{32}P_i$ was injected into rats and 7 days later, membrane–myelin, synaptosomal, and mitochondrial subfractions were prepared. With the injection of 800 μCi of $^{32}P_i$, $3.3 \cdot 10^6$ cpm were subsequently found in the whole brain and ultimately, $1.4 \cdot 10^6$ cpm in the crude mitochondrial fraction. In the three subfractions from this preparation the radioactivity was approximately the same in each subfraction. In the subsequent incubation of the three ^{32}P-labeled subfractions, using acetylcholine or tetrodotoxin as the releasing agents, no specificity of a release of labeled phosphate compounds into the medium could be observed. That is, the same amount of radioactivity was obtained regardless of whether membrane, synaptosomal, or mitochondrial fractions were used.

DISCUSSION

The premise on which this and our previous studies[1,4–7] is based is that thiamine has a specific function in membrane transport in nerves that is independent of its coenzyme role. To date, evidence in support of this function includes the demonstration that (1) pyrithiamine, an antimetabolite of the vitamin, produces its effect on the action potential of peripheral nerves by displacing thiamine from the nerve, (2) neuroactive drugs, in addition to electrical stimulation, releases the vitamin from nerve preparations whereas drugs that do not effect ion movements are ineffective, (3) this drug-induceable release of the vitamin can be shown in broken cell nerve preparations, and (4) the action potential of ultraviolet irradiated nerves can be restored by the addition of thiamine. In this paper we have shown that the drug-releaseable thiamine is localized in the purified membrane fraction of the cell. It is also clear from this work that as the membrane fraction is purified from a brain homogenate, the concentration of thiamine triphosphate rises. This finding suggests that the triphosphate may be the form of thiamine in the membrane whose binding in this cell fraction is affected by neuro-active agents. It is of interest to note that thiamine triphosphate is involved in Leigh's disease (subacute necrotizing encephalomyelopathy), a fatal genetic neurological disease[8].

As shown previously[1], released material is primarily in the form of free thiamine and thiamine monophosphate. This finding implies that the neuroactive drug may either displace the bound thiamine phosphate ester which is then hydrolyzed and released, or alternatively, the drug may activate thiamine phosphatases directly thus resulting in the release of a dephosphorylated form of the vitamin. Since purified preparations of both thiamine pyrophosphatase and thiamine triphosphatase are not stimulated by either tetrodotoxin or acetylcholine this latter possibility appears unlikely.

In this study, hydrolytic agents showed no specificity in that the vitamin was

released equally well from synaptosomes and mitochondria as it was from the membrane fraction. This lack of specificity coupled with our failure to find (concurrent with the efflux of thiamine, as shown previously) a drug-induced release of either ninhydrin-positive material or ^{32}P-labeled compounds, or any alteration in the R_F of released radioactive thiamine compounds, indicates an unusual type of binding of the vitamin to membrane components. Presumably, a subtle modification of the membrane, induced either by drugs or electrical stimulation permits the efflux only of thiamine compounds. This fine regulation suggests a physiologically significant mechanism.

ACKNOWLEDGEMENT

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