BBA 75392

ION MOVEMENTS AND THIAMINE

II. THE RELEASE OF THE VITAMIN FROM MEMBRANE FRAGMENTS

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(Received August 14th, 1969)

SUMMARY

Rats were injected with [^{35}S]thiamine and subsequently brain, spinal cord and sciatic nerves were removed and homogenized. When particulate fractions of these tissues which contained radioactive thiamine were incubated with a variety of neuroactive drugs, thiamine was released into the medium. After separation of the particulate preparation into membrane, synaptosomes and mitochondria it was found that thiamine was released essentially only from the membrane fragments.

INTRODUCTION

In a continuing study in this laboratory on the role of thiamine in nervous tissue, we have recently shown that when an isolated spinal cord or sciatic nerve from both frogs and rats previously injected with [^{35}S]thiamine were perfused, a variety of neuroactive agents caused the release of the labeled vitamin. Thus, the addition to the perfusion fluid of acetyl choline ($1 \cdot 10^{-6}$ M), tetrodotoxin ($3 \cdot 10^{-8}$ M), ouabain ($1 \cdot 10^{-6}$ M) or lysergic acid diethylamide ($3 \cdot 10^{-7}$ M) resulted in the immediate efflux of radioactivity. In contrast, choline ($1 \cdot 10^{-4}$ M) or NaCl ($5 \cdot 10^{-2}$ M) had no effect. In addition it was found that this released thiamine was primarily in the form of thiamine monophosphate and thiamine whereas in the cell the bulk of the vitamin occurs as thiamine pyrophosphate and some thiamine triphosphate. This latter finding suggested that dephosphorylation of the vitamin was in some way associated with these drugs that effect ion movements in nervous tissue and cause the release of thiamine from the preparations[1,2].

The present report shows that this effect of drugs can also be demonstrated in broken cell preparations, first with a crude mitochondrial fraction of brain and subsequently with membrane fragments isolated by sucrose density gradient centrifugation from this fraction.

MATERIALS AND METHODS

Nuclear Chicago was the source of [^{35}S]thiamine. Tetrodotoxin and thiamine triphosphate were generously donated by the Sankyo Company, Japan. Trypsin was

Abbreviation: LSD, lysergic acid diethylamide.

purchased from Calbiochem. Thiamine deficient diet was obtained from Nutritional Biochemical Corporation. The snake venom (*Crotalus atrox*) was purchased from Sigma.

Preparation of labeled fractions

Rats were placed on a thiamine deficient diet for 5 or 6 weeks and then given two intraperitoneal injections of [^{35}S]thiamine of 0.1 ml each, 18 h apart. The total radioactivity injected was 50 μC and amounted to 150–350 μg of thiamine, depending on the specific activity of the vitamin. In some experiments large bullfrogs (*Rana catesbiana*) were used and the thiamine was injected intrathecally with the same dosage regimen. Animals were sacrificed 15–20 h after the last injection and the brain, spinal cord and sciatic nerves were removed and homogenized with 9 vol. of 0.32 M sucrose containing Tris (0.5 mM) and EDTA (40 μM) at a pH of 7.4. Nuclei were removed by centrifuging at 1000 $\times$ *g* for 11 min. The crude mitochondrial fraction was collected at a centrifugal speed of 16000 $\times$ *g* for 60 min, washed twice, and resuspended in the sucrose medium at a protein concentration of approx. 3 mg/ml. On occasion the supernatant from the mitochondrial centrifugation was combined with the washes and centrifuged at 48000 $\times$ *g* for 90 min to collect the microsomal fraction.

Discontinuous sucrose density gradient centrifugation of the crude mitochondrial fraction was performed essentially as previously described by WHITTAKER[3].

Electron microscopy

Dr. G. Aghajanian kindly provided the electron microscopic examination of the subfractions. The following procedure was employed: pellets were fixed in 5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.4). The blocks were then transferred to 1% OsO_4 containing 0.15 M phosphate buffer (pH 7.4). Blocks were dehydrated with graded ethanol and subsequently embedded in Maraglas.

Incubation procedure

To a test tube containing 0.8 ml of Krebs Ringer phosphate buffer (pH 7.2) were added 0.1 ml of the cell fraction in 0.25 M sucrose (3–5 mg protein/ml), the drug to be tested, and 0.25 M sucrose to a final volume of 1.0 ml. Control tubes were identical to the experimental samples except that the drug was omitted. Samples were incubated at 37° for either 20 or 30 min, chilled, and centrifuged in a refrigerated centrifuge at 48000 $\times$ *g* for 60 min in order to deposit the organelle. The radioactive thiamine that was released into the medium during incubation was determined by adding 0.2 ml of the supernatant to 20 ml of Bray's solution and counting the sample in a liquid scintillation spectrometer. An internal standard was used to correct for quenching. Results are expressed as counts/min per standard 0.2-ml aliquot and were obtained from a minimum of 3 experiments.

Protein was determined by the method of LOWRY *et al.*[4]. Thiamine and its phosphate esters were assayed by the electrophoretic and fluorometric procedure of ITOKAWA AND COOPER[5].

RESULTS

The parameters of the incubation system were studied using the crude mitochondrial preparation obtained from rat brain and with acetyl choline as the releasing

agent. When acetyl choline was used, DFP ($1 \cdot 10^{-4}$ M) was also added to inhibit the cholinesterase in the preparation. The requirement for specific cations in the incubation medium (Fig. 1) indicated that Na^+ and Ca^{2+} were necessary for optimal release of the labeled thiamine, K^+ had a slight though consistent effect but no dependency on phosphate or Mg^{2+} could be observed. Nevertheless, these latter two agents were routinely used in the Ringer's solution in which the crude mitochondrial fraction was

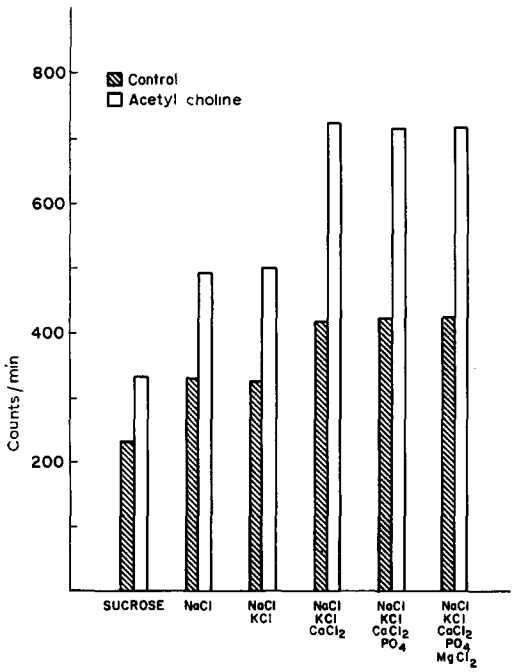


Fig. 1. Composition of incubation medium for release of [^{35}S]thiamine. The complete medium contained 115 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.3 mM K_2HPO_4, 1.3 mM $MgSO_4$ and 25 mM sucrose. The pH was 7.2. When salts were omitted they were replaced by equimolar amounts of sucrose. Incubation conditions were carried out as described in Table I at 37°.

TABLE I

THE EFFECT OF TEMPERATURE ON THE RELEASE OF LABELED THIAMINE

Tubes containing 0.9 ml Krebs–Ringer phosphate medium (pH 7.2), 0.1 ml [^{35}S]thiamine-labeled crude mitochondrial fraction (3 mg protein/ml) in 0.25 M sucrose and 0.02 ml of either acetyl choline–DFP ($1 \cdot 10^{-4}$ M) in sucrose or sucrose (0.25 M) were incubated for 30 min at various temperatures. After incubation the 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 described in MATERIALS AND METHODS.

Temp.	*Counts/min*		
	Control (*1*)	*Acetyl choline* (*2*)	*Difference* (*2—1*)
0°	420	433	13
23°	503	624	121
37°	688	868	180

suspended. Table I illustrates the effect of temperature on the release where it is evident that a 37° incubation results in a much larger acetyl choline mediated release than incubation at 0° or at room temperature (21–23°). The optimum pH for release is illustrated in Fig. 2 and indicates the most effective pH to be in the neighbourhood of 7.0.

In Fig. 3 the amount of labeled thiamine discharged into the medium from the particulate preparation is plotted as a function of time. A linearity of release with time is observed up to 60 min after which the reaction tends to plateau. In most experiments an incubation time of 30 min was employed.

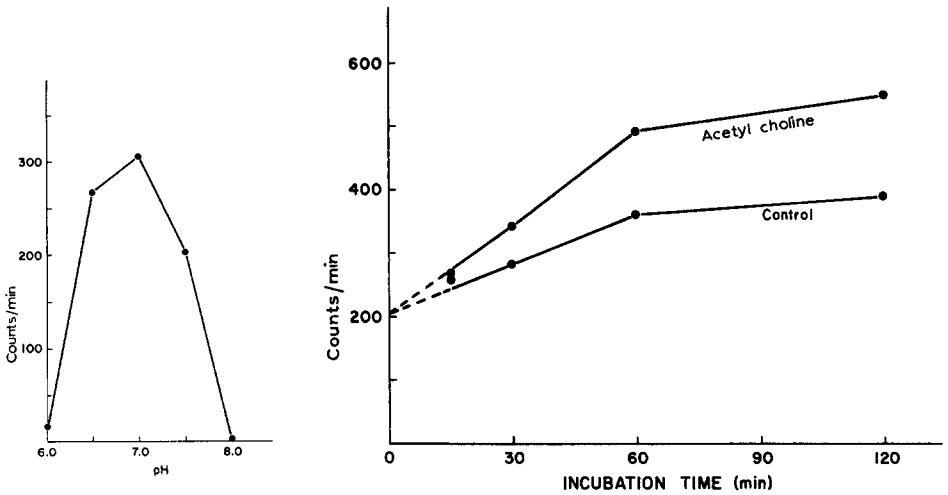


Fig. 2. The relationship between pH and release of labeled thiamine. Incubation as in Table I at 37° with the medium adjusted to various pH values.

Fig. 3. Time-course of release of [^{35}S]thiamine. Incubation conditions as described in Table I.

Finally, in these preliminary experiments it was of interest to determine which cell organelle contained releasable thiamine. Consequently, nuclear, mitochondrial and microsomal fractions of brain were prepared as described in MATERIALS AND METHODS and individually incubated in the presence and absence of acetyl choline. As shown in Table II, the crude mitochondrial fraction contained the largest amount of thiamine and the most releasable.

With the parameters of the system defined, a variety of agents was then tested for their ability to promote the release of labeled thiamine from the rat brain preparation. As shown in Table III, these results were comparable to the data obtained in the intact nerve experiments described in INTRODUCTION. That is, drugs that cause a change in ion movements in nerve such as acetyl choline, ouabain, tetrodotoxin and 5-hydroxytryptamine, also release thiamine from this particulate preparation although higher concentrations were necessary in this system *in vitro*. In contrast, inactive agents such as choline or NaCl had no effect. An additional finding in this system *in vitro* was that trypsin and snake venom (*Crotalus atrox*) also caused a discharge of the radioactive vitamin. Similar results were obtained using the frog brain preparation as shown in Table IV.

TABLE II

THE RELEASE OF THIAMINE FROM CELL ORGANELLES

Fractions were prepared as described in MATERIALS AND METHODS and aliquots were incubated at 37° for 30 min in the presence and absence of acetyl choline and DFP ($1 \cdot 10^{-4}$ M) as described in Table I.

Cell fraction	*Addition*	*Counts/min released*	*Stimulated release (b—a)*	*% Total ((b—a)/c × 100)*
Nuclear	a. None	305		
	b. Acetyl choline–DFP	293	0	0
	c. Trichloroacetic acid*	5928		
Mitochondrial	a. None	1048		
	b. Acetyl choline–DFP	1573	525	2.44
	c. Trichloroacetic acid*	21487		
Microsomal	a. None	41		
	b. Acetyl choline–DFP	109	68	1.45
	c. Trichloroacetic acid*	4688		

* Total radioactivity in the fractions was determined by precipitation of an aliquot with 5% trichloroacetic acid.

TABLE III

EFFECT OF VARIOUS AGENTS ON THE RELEASE OF [^{35}S]THIAMINE FROM RAT BRAIN CRUDE MITOCHONDRIAL FRACTION

The incubation conditions and assay of released radioactivity were carried out as described in MATERIALS AND METHODS.

Agent	*Counts/min*	*Increase (%)*
None	493	0
Choline ($1 \cdot 10^{-4}$ M)	487	0
Choline ($1 \cdot 10^{-4}$ M), DFP ($1 \cdot 10^{-4}$ M)	464	0
Acetyl choline ($1 \cdot 10^{-4}$ M)	547	10.9
Acetyl choline ($1 \cdot 10^{-4}$ M), DFP ($1 \cdot 10^{-4}$ M)	1021	107.1
Ouabain ($1 \cdot 10^{-4}$ M)	824	67.1
Tetrodotoxin ($3 \cdot 10^{-6}$ M)	1075	118.0
ATP ($1 \cdot 10^{-3}$ M)	502	0
Pargyline ($1 \cdot 10^{-4}$ M)	485	0
5-Hydroxytryptamine ($1 \cdot 10^{-4}$ M)	490	0
Pargyline ($1 \cdot 10^{-4}$ M), 5-hydroxytryptamine ($5 \cdot 10^{-5}$ M)	542	10.0
Pargyline ($1 \cdot 10^{-4}$ M), 5-hydroxytryptamine ($5 \cdot 10^{-4}$ M)	567	15.0
Pyrithiamine ($1 \cdot 10^{-4}$ M)	719	45.7
Oxythiamine ($1 \cdot 10^{-3}$ M)	704	42.7
Trypsin (1 mg)	707	43.4
Snake venom (0.5 mg)	1179	139.0
Trichloroacetic acid*	10350	

* Trichloroacetic acid was used to determine the total radioactivity in the sample.

TABLE IV

EFFECT OF AGENTS ON THE RELEASE OF [^{35}S]THIAMINE FROM FROG BRAIN CRUDE MITOCHONDRIAL FRACTION

Incubation procedures were as described in MATERIALS AND METHODS except that frog Ringers medium was used in place of Krebs Ringer.

Agent	*Counts/min*	*Increase* (%)
None	142	
Acetyl choline ($1 \cdot 10^{-4}$ M)	176	24
Acetyl choline ($1 \cdot 10^{-4}$ M), DFP ($1 \cdot 10^{-4}$ M)	290	104.1
KCl ($5 \cdot 10^{-2}$ M)	182	35.0
Tetrodotoxin ($3 \cdot 10^{-6}$ M)	208	47.5
Trypsin (1 mg)	222	56.0
Boiled trypsin (1 mg)	145	0

Sucrose density gradient centrifugation

As a logical extension of the results described above with a crude mitochondrial fraction of tissue, the more discrete localization of the bound thiamine was attempted using the technique as described by WHITTAKER[3]. In Fig. 4, the distribution of radioactivity and protein is illustrated in the brain, spinal cord, and sciatic nerve of the rat. The first peak represents membrane fragments, the second is nerve ending particles

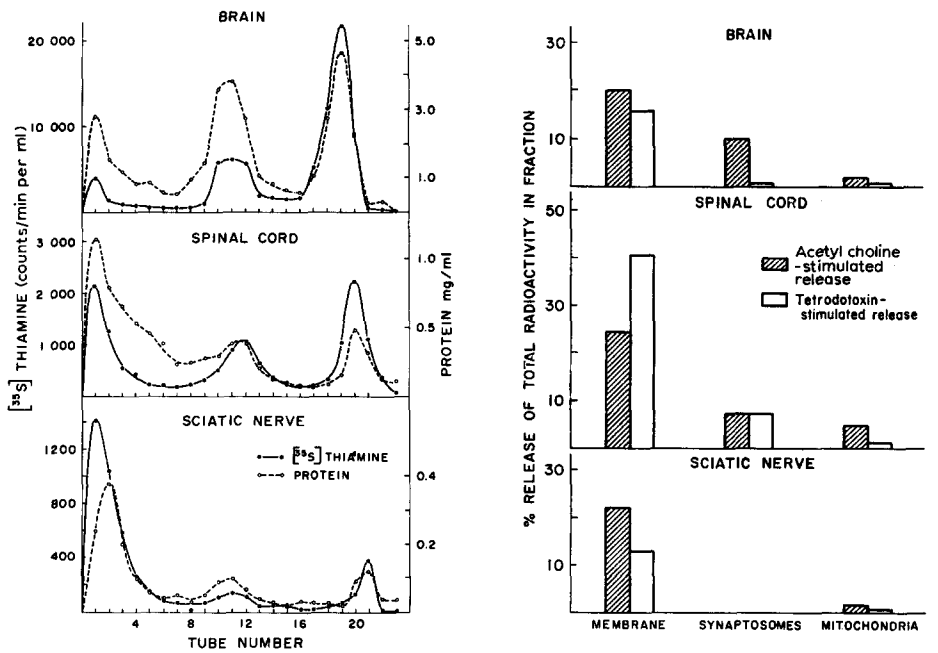
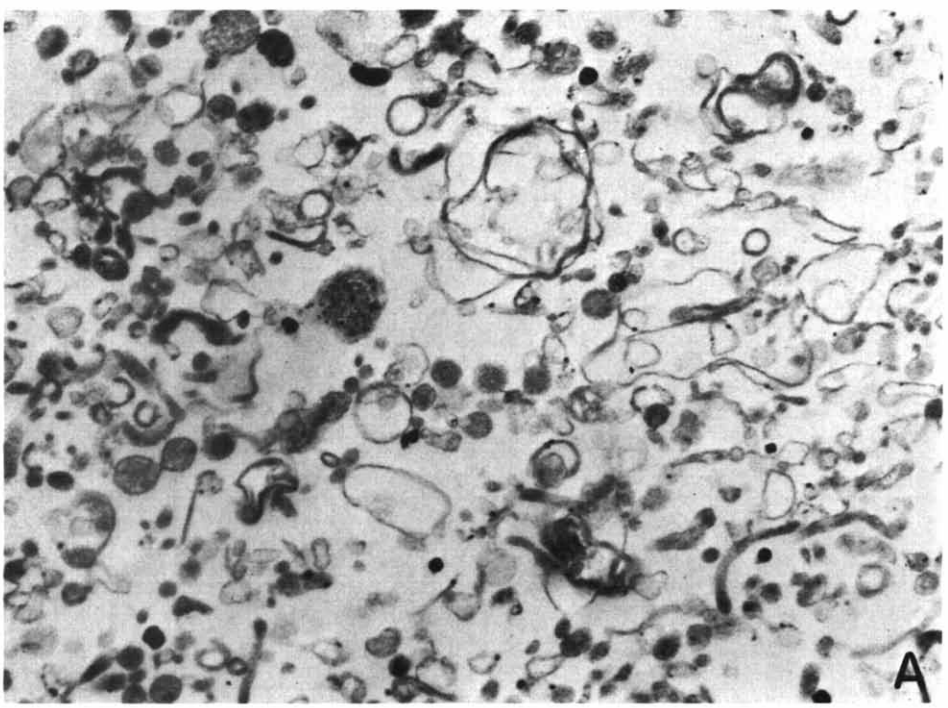


Fig. 4. Distribution of radioactivity and protein in rat brain, spinal cord and sciatic nerve subfractions. Fractions prepared according to WHITTAKER[3]. As validated by electron microscopy the first peak represents membrane fragments, the second is synaptosomes and the third reflects mitochondria.

Fig. 5. Drug-induced release of labeled thiamine from neuronal subfractions. Incubation conditions as described in MATERIALS AND METHODS.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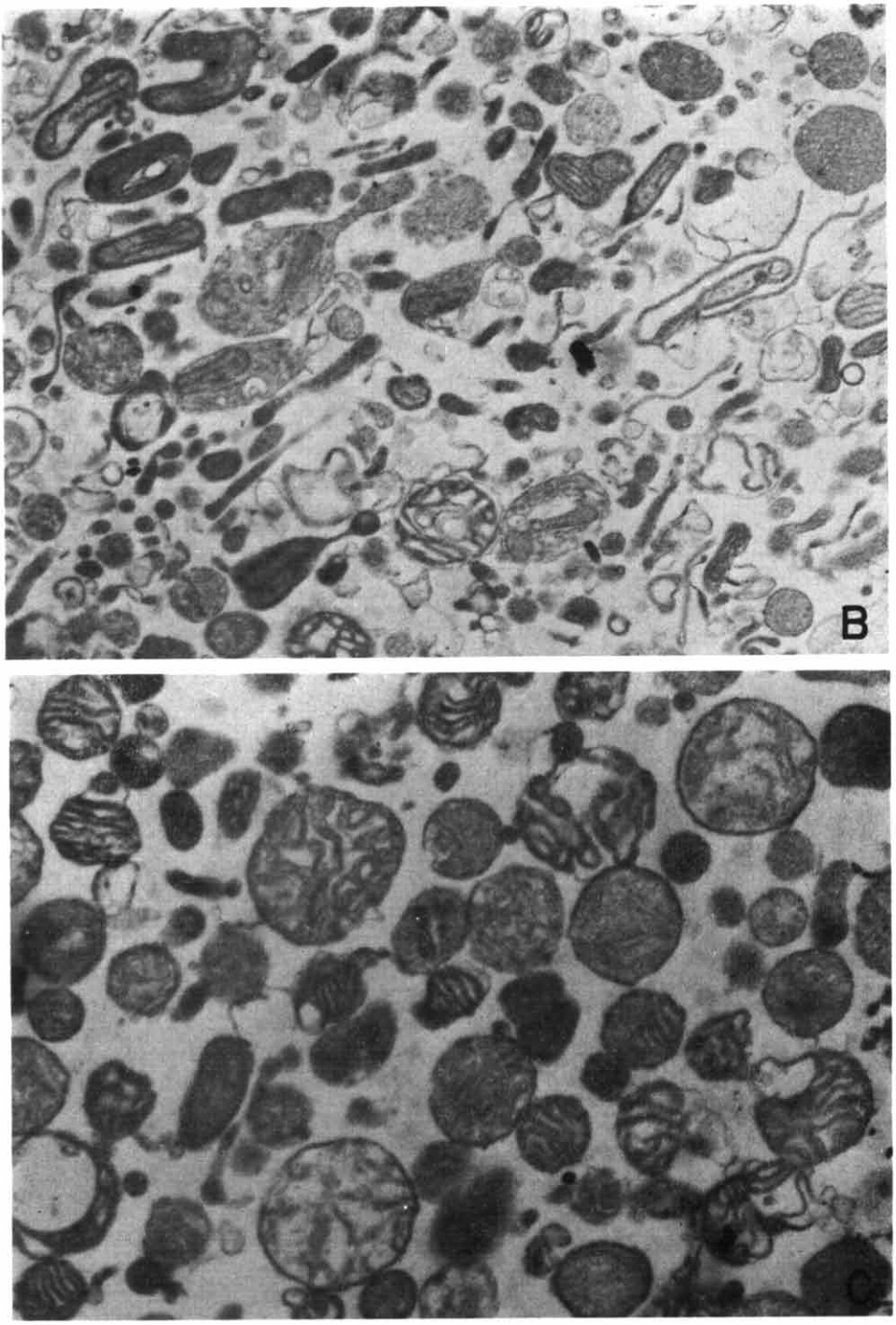

Fig. 6. Electron micrographs of rat brain subfractions. A, membrane; B, nerve ending particles; C, mitochondria. Magnification 22800 ×.

TABLE V

DISTRIBUTION OF THIAMINE AND ITS PHOSPHATE ESTERS IN VARIOUS BRAIN FRACTIONS

Cold thiamine compounds were added to the [^{35}S]thiamine-labeled fractions. The preparations were deproteinized and subjected to paper electrophoretic separation and radioactivity determination as described in MATERIALS AND METHODS. Figures are averages of at least 3 experiments.

Brain fraction	*Thiamine* (%)	*Thiamine monophosphate* (%)	*Thiamine pyrophosphate* (%)	*Thiamine triphosphate* (%)
Whole homogenate	3.8	10.2	83.7	2.3
Crude mitochondrial fraction	5.9	11.2	75.3	7.6
Membrane	10.9	15.4	63.9	9.8
Synaptosomes	6.4	9.6	81.1	2.9
Mitochondria	6.2	10.2	81.6	2.0

thiamine pyrophosphate, there appears to be a tendency to an increased proportion of thiamine triphosphate as the homogenate is carried through the procedure for obtaining membrane fragments.

Along the same line, it was of interest to determine the forms of thiamine that were released by acetyl choline from the crude mitochondrial fraction and what forms remained in the fraction after the release. The thiamine compounds left in the particulate were separated and assayed as described above. The released compounds in the medium were adsorbed on charcoal, the charcoal was washed to remove salts and the thiamine esters and free thiamine were eluted and concentrated as previously described[2]. The compounds were then electrophoresed and assayed as above. The results of this experiment (Table VI) indicate that the bulk of the released material was primarily thiamine monophosphate and free thiamine whereas the thiamine esters remaining in the particulate fraction were thiamine pyrophosphate and thiamine monophosphate. No thiamine triphosphate was present. These results were similar to those obtained in the perfused spinal cord preparation as noted in INTRODUCTION.

TABLE VI

DISTRIBUTION OF THIAMINE AND ITS PHOSPHATE ESTERS AFTER RELEASE BY ACETYL CHOLINE FROM CRUDE MITOCHONDRIAL FRACTION

[^{35}S]Thiamine-labeled crude mitochondrial fraction was incubated in Krebs–Ringer phosphate buffer containing acetyl choline–DFP ($1 \cdot 10^{-4}$ M) for 30 min at 37° as described in Table I. After incubation and centrifugation radioactive thiamine compounds were determined in the medium and the particulate fraction as described. Figures are average of 3 experiments.

Fraction	*Thiamine* (%)	*Thiamine monophosphate* (%)	*Thiamine pyrophosphate* (%)	*Thiamine triphosphate* (%)
Particulate, before acetyl choline	5.9	11.2	75.3	7.6
Particulate, after acetyl choline	9.0	24.3	64.7	2.0
Medium, after acetyl choline	33.5	47.1	19.4	0

DISCUSSION

The evidence to date that links thiamine to a function in membrane transport, although circumstantial, is moderately compelling. Thus, the effect of antimetabolites of the vitamin on conduction in peripheral nerves[6-8], the localization of thiamine[9] and thiamine pyrophosphatase[10] in peripheral nerve membranes as opposed to axoplasm, the release of thiamine from nerve preparations on electrical stimulation[11-13] and the association of subacute necrotizing encephalomyelopathy with a deficiency of thiamine triphosphate in brain[14,15] all support the original contention of VON MURALT[16] of a specific role of thiamine in neurophysiology that is independent of its coenzyme function. When to this evidence is added our more recent study of the release of the vitamin from perfused nerve preparations by neuroactive drugs at pharmacological concentrations and the present report of a specific drug-induced release from membrane fragments, it is difficult to escape the conclusion that thiamine plays a role in membrane transport.

In the current study the fact that the release phenomenon has an optimal temperature, pH and ionic composition of the medium, suggests that the binding of thiamine to some membrane component is rather specific. This view is supported by the fact that agents that promote ion movements will release the vitamin whereas compounds such as choline or NaCl had no effect. The most striking aspect of this specificity is associated with brain subfractions. As shown in Fig. 5, the vast majority of labeled thiamine is found in the mitochondrial fraction of the brain with the membrane fraction representing only about 10%. However, acetyl choline and particularly tetrodotoxin release the thiamine essentially only from the membrane fraction and have virtually no effect on mitochondrial-bound vitamin. In contrast, trypsin and snake venom which would be considered non-specific agents were found to release about 15% of the labeled thiamine from each subfraction regardless of whether it was membrane, synaptosomes or mitochondria.

With regard to the neurophysiologically active form of the vitamin in the membrane it is still not clear whether this is thiamine pyrophosphate or thiamine triphosphate since the two esters are rapidly interconvertible[18]. From the results shown in Table V, it may be tentatively suggested that thiamine triphosphate is the active form. Although the free thiamine content also rises with the isolation of the membrane this is presumably artifactual due to the dephosphorylation that is occurring during isolation. This is evident from the fact that the 3 subfractions have a higher thiamine content than the crude mitochondrial preparation from which they were derived.

It should be pointed out that in the release experiments using perfused preparations as discussed in INTRODUCTION, about 0.1–1% of the total thiamine content is lost to the medium; with the cell subfractions as reported here 2–15% of the total thiamine compounds are released. In both types of experiments, the situation is in a sense non-physiological since one is working with cut surfaces of nerves or subcellular fractions. In intact nerves very little thiamine would be expected to be lost to the medium. GURTNER[17] found that electrical stimulation of a peripheral nerve caused the same dephosphorylative shift of the thiamine phosphates as we found with electrical stimulation and drug application, but no loss of total thiamine compounds.

What is currently envisaged with respect to thiamine in nervous tissue is a cyclic dephosphorylation and rephosphorylation as ions cross the nerve membrane.

From a perusal of the variety of drugs that stimulate the release of the vitamin, although different mechanisms are apparently operative, *e.g.*, tetrodotoxin which blocks Na^+ entrance and KCl or acetyl choline which depolarize, the common factor seems to be the movement of Na^+. This view relating thiamine and Na^+ has been suggested by KUNZ[6]. He found that when pyrithiamine, an antimetabolite of thiamine, was applied to a single node of Ranvier, the action potential was blocked: this inhibition appeared to relate to a partial inactivation of the sodium transport system. Our current view of the situation is that thiamine triphosphate (or thiamine pyrophosphate) occupies a site on the membrane that is either a sodium channel or very close to it. The initiation of a nerve impulse activates the dephosphorylation of the thiamine phosphate and perhaps causes a displacement so that Na^+ can freely cross the membrane. The action of tetrodotoxin may be explained by assuming that the poison displaces thiamine but occupies the site so that Na^+ cannot enter. With the other agents it would be inferred that they displace thiamine also but do not occupy the fixed site so that the early inward current of Na^+ is observed.

The nature of the binding of thiamine in the membrane is currently under investigation.

ACKNOWLEDGMENT

This investigation was supported by U.S. Public Health Service Grant No. NB01512I and NB08666.

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