Transport and Metabolism of Thiamine in Rat Brain Cortex *in vitro*

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(Received 31 July 1964)

1. Aerobic incubation at 37° of rat brain-cortex slices in Krebs-Ringer phosphate medium containing glucose and labelled thiamine results in accumulation in the tissue of labelled thiamine and labelled thiamine phosphates. The concentration of the labelled thiamine in the tissue cell water increases with increase of external labelled thiamine concentration in an approximately linear manner, the concentration ratio for labelled thiamine (tissue:medium) exceeding unity with low external thiamine concentrations (e.g. 0.2 μM) and diminishing to about unity as the external thiamine concentration is increased to 1 μM . The concentration of labelled phosphorylated thiamine in the tissue is at least double that of the labelled thiamine present and its amount increases with increase of external thiamine concentration. Labelled phosphorylated thiamine appears in the medium, its amount being about one-fifteenth of that in the tissue. Phosphorylation of thiamine in the tissue proceeds during incubation for 3 hr. and, with an external labelled thiamine concentration of 0.2 μM , about 48% conversion of thiamine takes place. 2. In the presence of ouabain (0.1 mM), which does not inhibit thiamine phosphorylation in rat brain extract, there is a fall in the uptake of labelled thiamine by brain-cortex slices and the concentration ratio for the labelled thiamine (tissue:medium) falls to below unity. Anaerobiosis, lack of Na^+ or the presence of Amprol (0.01 mM) leads to marked inhibition of thiamine phosphorylation, and the concentration ratio for labelled thiamine (tissue:medium) falls to about unity. The facts lead to the conclusion that thiamine is conveyed into the brain cell against a concentration gradient by an energy-assisted process mediated by a membrane carrier. Pyri-thiamine is a marked inhibitor of thiamine phosphorylation in brain extract. 3. Thiamine monophosphate and thiamine diphosphate inhibit thiamine phosphorylation in brain extract. They diminish 'total' thiamine (free and phosphorylated) uptake into brain-cortex slices and inhibit the transport of thiamine into the brain cell, possibly by competition for the carrier. 4. Phosphorylation of labelled thiamine in brain extract is brought about not only by adenosine triphosphate (in the presence of Mg^{2+}) but apparently by adenosine diphosphate and uridine triphosphate.

It is proposed to demonstrate, by the results given in the present paper, that carrier-mediated transport of thiamine occurs in rat brain-cortex slices incubated in a physiological Ringer medium and that such transport is energy-dependent. It has already been suggested (Polin, Wynosky & Porter, 1963) that absorption of thiamine from the intestine takes place by a carrier mechanism rather than by simple diffusion. The evidence for this suggestion was the fact that administration to laying hens of a thiamine analogue 1-(4-amino-2-*n*-propylpyrimidin-5-ylmethyl)- β -picolinium chloride hydrochloride (amprolium, Amprol), which has marked anticoccidial activity (Cuckler, Cobb, McManus & Ott, 1961), inhibits absorption of thiamine from ligated duodenal loops of hens. It also diminishes

the rate of appearance of thiamine in the yolk of eggs. Some evidence of competition between thiamine and Amprol was obtained. Possibly a similar inhibition of absorption may account for the anticoccidial action of Amprol (Rogers, 1962). This evidence, though suggestive, does not indicate that active transport of thiamine at the cell membrane occurs. To show that such transport takes place it is necessary to demonstrate that the carrier mechanism is energy-assisted and that thiamine may accumulate in the cell against a concentration gradient. The present work was carried out entirely with rat brain-cortex slices and rat brain extracts. A preliminary note indicating that active transport of thiamine takes place in rat brain-cortex slices has been published (Sharma & Quastel, 1964).

MATERIALS AND METHODS

Materials. Thiamine labelled at C-2 of the thiazole ring was used. It was purchased from The Radiochemical Centre, Amersham, Bucks. Its radioactivity was 0.1 mc/1.26 mg. It was dissolved in water and appropriate quantities were added to the incubation vessels. It was stored frozen.

Amprol was obtained, as a generous gift, from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A. Pyrithiamine was obtained from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Thiamine monophosphate and thiamine diphosphate were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Preparation of brain-cortex slices and brain extracts. Rats weighing 100–150 g. were killed by stunning and the brains were removed and placed in crushed ice. The brain-cortex slices were quickly prepared and placed in Warburg manometric vessels containing the appropriate incubation media.

For the preparation of cell-free extracts the cerebral hemispheres from eight rats were homogenized in 30 ml. of 0.1 M-glycylglycine buffer, pH 6.6, in a loose-fitting Potter-Elvehjem homogenizer for 40 sec. Each brain weighed about 1 g. The homogenate was centrifuged at 40 000 rev./min. for 20 min. in a no. 50 rotor of the Spinco model L centrifuge. The supernatant was carefully removed by a Pasteur pipette and definite amounts were pipetted into ice-chilled flasks. The procedure was carried out at 0°.

Incubation procedure with brain-cortex slices. Brain-cortex slices (60–100 mg. fresh wt.) were incubated in Krebs–Ringer phosphate medium, pH 7.4 (containing 10 mM-glucose), at 37°. The amount of tissue was used when the incubation volume was 1 ml. When larger volumes were used, proportionately more tissue was used, e.g. about 300 mg. for 3 ml. The incubation volume is noted in the Tables below. The final concentrations of salts were: NaCl, 134 mM; KCl, 5.2 mM; CaCl₂, 2.8 mM; KH₂PO₄, 1.3 mM; MgSO₄, 1.3 mM; sodium phosphate buffer, 10 mM. In experiments with Na⁺-free medium glycylglycine buffer (10 mM) was used and the NaCl was replaced by choline chloride (134 mM) or sucrose (0.30 M). The incubations were carried out in an atmosphere of O₂. Thiamine was usually tipped into the main compartment of the manometer vessel after about 10 min. equilibration.

Estimation of radioactivity. After incubation the slices were rinsed three times in ice-cold Ringer medium and then transferred to, and homogenized in, tubes containing 50% (v/v) ethanol. Occasionally homogenization in water was carried out, with no significant difference in the results. The radioactivity of sample of the supernatant after centrifuging was counted in a thin end-window flow counter after it had been plated on aluminium planchets. Correction was made for self-absorption.

Many of the results recorded in the Table below were obtained with the Packard Tri-Carb liquid-scintillation spectrometer. A mixture consisting of equal volumes of toluene, dioxan and 95% (v/v) ethanol, containing 2,5-diphenyloxazole (5 g./l.), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.05 g./l.) and naphthalene (80 g./l.), was used as the scintillant. The counting efficiency was 57%.

Chromatographic procedure. For chromatography, the ethanol extracts from different samples were pooled and dried in a flash evaporator at 37°, and the residue was dissolved in a small volume of 50% (v/v) ethanol. The extract

was spotted on Whatman no. 3 paper and the chromatograms were run (descending) in the solvent propanol–2 M-ammonium formate (pH 5.5)–water (13:3:5, by vol.) for 18 hr.

The chromatograms were dried at room temperature and the spots were detected by alkaline 5% (w/v) K₃Fe(CN)₆ in ethanol or by radioautographic techniques.

Separation of thiamine from thiamine phosphates. Ion-exchange resins were used for the separation of thiamine phosphates from thiamine.

Preparation of the resins. (a) Acid resin. Rexyn 102 (H⁺ form; 200–400 mesh) (organic weak acid cation-exchanger) was used, its preparation being similar to that described by Hirs (1955). The resin (supplied by Fisher Scientific Co., Montreal, Canada) was stirred in water mechanically for 20 min. and then allowed to settle for 2 hr., and the supernatant and foam were removed. The same procedure was repeated four or five times to remove completely the fine particles. The resin was then filtered and dried by suction with air. The dry resin was stirred in acetone for 3 hr. and the suspension was washed with acetone until the filtrate was clear and colourless. The air-dried resin was washed with water until the last traces of acetone were removed. It was converted into the Na⁺ form by the addition of 40% (w/v) NaOH until the pH of the suspension rose to at least 11. The resin was stirred for 3 hr. and was then washed by decantation several times with water. The Na⁺ form of the resin was converted into the H⁺ form by flowing 3 N-HCl over the resin (in a Buchner funnel) continuously for 4 hr. The product was then washed with water until the filtrate was neutral. The resin prepared in this form was the acid resin, which takes up thiamine monophosphate and thiamine quantitatively from mixtures of thiamine and thiamine phosphates.

(b) Buffered resin. The acid resin (about 500 g.) was stirred in 5 l. of sodium phosphate buffer (33.12 g. of NaH₂PO₄·H₂O + 22.72 g. of Na₂HPO₄ in 2 l. of water), pH 6.5. The pH of the suspension was kept at about 5.5 by the addition of portions of 40% (w/v) NaOH at intervals of a few minutes. The suspension was finally brought to pH 6.4 by the addition of alkali at 15 min. intervals, and when the pH remained stationary at 6.5 the suspension was stirred overnight. The resin was then stored in phosphate buffer, pH 6.5. The resin prepared in this form was the buffered resin, which takes up thiamine quantitatively from mixtures of thiamine and thiamine phosphates. Deionized water was used for all the washings and preparation of solutions. This buffered resin was the one used throughout the experiments reported below.

Estimations of labelled thiamine and labelled thiamine phosphates in tissue and medium. After homogenization of the washed tissue slices and subsequent centrifuging, the supernatant was assayed for radioactivity by counting a sample and correcting for self-absorption. This gave a measure of the 'total' labelled thiamine in the tissue (i.e. the sum of the free and phosphorylated labelled thiamine). A sample of the medium in which the tissue had been incubated was also counted for 'total' labelled thiamine, correction being made for self-absorption.

For estimations of labelled thiamine and labelled thiamine phosphates in the tissue in the scintillation counter the following procedure was adopted. After homogenization of tissue slices in 2 ml. of water, the suspension was centrifuged at 0°. To 0.5 ml. of supernatant 0.2 ml. of trichloroacetic

acid was added to give a final concentration of 5% (w/v). The suspension was centrifuged and the radioactivity of 0.5 ml. of the supernatant was counted. This gave a measure of the sum of labelled free thiamine and labelled phosphorylated thiamine in the tissue.

A 1 ml. sample of the supernatant from the centrifuged tissue homogenate was added to micro-columns of 3 ml. capacity previously packed with 1.5 ml. of buffered resin, which after filtration was washed with water. The filtrate and washings were collected and made up to 8 ml. This contained the labelled phosphorylated thiamine of the tissue, the free thiamine being adsorbed on the resin. Trichloroacetic acid was added to give a final concentration of 5% (w/v) and the mixture was centrifuged. A 0.5 ml. sample of the supernatant was used for assaying the radioactivity of the thiamine phosphates present. The radioactivity of the free thiamine was calculated by difference or by elution from the resin with 0.05 N-HCl with subsequent assay of the radioactivity in the eluate. The two values were usually identical within the experimental error of $\pm 5\%$.

The labelled thiamine and labelled thiamine phosphates in the medium were estimated in the same manner as that adopted for the supernatant of the tissue homogenate, i.e. radioactivity of the 'total' (free and phosphorylated) labelled thiamine was assayed on a sample of the medium, that of the labelled phosphorylated thiamine was assayed after adsorption on the buffered resin and that of the labelled free thiamine was calculated by difference or was assayed on a sample of the acid eluate of the resin.

In every set of experiments a blank experiment was run, in which labelled thiamine was added to the tissue only at the termination of an incubation experiment, and the relatively small value obtained was subtracted from the experimental value.

Incubation procedure with brain extracts. A 1.0 ml. sample of brain extract was added to 3.0 ml. of the incubation mixture consisting of MgSO_4 (1.2 mM), ATP (1.2 mM), labelled thiamine hydrochloride ($4.4 \mu\text{M}$) and other additions as indicated in different Tables below. The volume of each of these solutions when added to the vessels was not more than 10% of the volume of the final incubation mixture. The volume was made up finally to 4.0 ml. by the addition of glycylglycine buffer (0.1 M), pH 6.6. The incubations were carried out for 1 hr. at 37° in air in a shaking incubator. Estimations of the amounts of free labelled thiamine and labelled thiamine phosphate were carried out as described above.

Calculations of the amounts of labelled free thiamine and labelled thiamine phosphate. After assay of the radioactivities of the samples of the thiamine and thiamine phosphate fractions of the tissue and medium, calculations were made of the radioactivities of the labelled thiamine and labelled phosphorylated thiamine in the tissue cell water, by taking into account the weight of tissue taken and the cell water as 80% of the tissue weight. These gave values as counts/min./ml. of tissue cell water or counts/min./ml. of medium. These values are converted into $\mu\text{moles/ml.}$ by dividing the observed counts/min. by the specific activity of the thiamine calculated as counts/min./ μmole of thiamine added.

Reproducibility of results. The results of individual experiments are given in the Tables below. Experiments were carried out at least three times and the variation between results did not exceed $\pm 7\%$.

RESULTS

Experiments with rat brain-cortex slices

Uptake of thiamine by rat brain-cortex slices.

Aerobic incubation of rat brain-cortex slices in Krebs-Ringer phosphate medium containing glucose in the presence of labelled thiamine results in the accumulation in the brain tissue, after washing, of labelled thiamine and labelled thiamine phosphates. Homogenization of the brain tissue, after the period of incubation and subsequent washing with Krebs-Ringer phosphate medium, and later extraction with 50% ethanol, yields an extract that on chromatography gives four spots, three of which correspond to those given by thiamine, thiamine monophosphate and thiamine diphosphate. The fourth is possibly due to thiamine triphosphate, but this is uncertain as no authentic sample of thiamine triphosphate was available to us. The thiamine phosphates may be separated quantitatively from thiamine by absorption of the latter on an ion-exchange resin. Another form of the resin (acid resin; see the Materials and Methods section) makes it possible to separate thiamine monophosphate and thiamine from the other thiamine phosphates. The fact that the labelled combined thiamine undergoes conversion by mild hydrolysis with N-hydrochloric acid into a labelled substance that is absorbed on resin indicates transformation, by hydrolysis, into thiamine. That the labelled thiamine compounds are thiamine phosphate esters is indicated, not only by the chromatographic analysis, but by the fact (see below) that their formation *in vitro* is dependent on the presence of ATP and Mg^{2+} .

Results given in Table 1 show the effects of variation of the concentration of labelled thiamine on the accumulation of labelled thiamine and labelled thiamine phosphates in brain tissue when the brain-cortex slices are incubated in Krebs-Ringer phosphate medium containing glucose for 2 hr. at 37° .

The following conclusions may be made from these results. The concentration of free labelled thiamine in the tissue cell water increases with increase of the external concentration of labelled thiamine in an approximately linear fashion, the concentration ratio for labelled thiamine (tissue: medium) exceeding unity with low external concentrations of thiamine (e.g. $0.2 \mu\text{M}$) and diminishing to about unity as the external concentration of thiamine is increased to $1 \mu\text{M}$. The concentration of labelled phosphorylated thiamine in the tissue cell water is at least double that of the labelled thiamine in the tissue cell water when there is an external thiamine concentration of $0.2 \mu\text{M}$ (after aerobic incubation for 2 hr.), and its amount increases with

increase of the external concentration of thiamine, though not in a linear manner.

Labelled phosphorylated thiamine appears in the medium, and the evidence of many experiments (as shown in the Tables below) points to an almost constant ratio between the amount of the labelled

thiamine phosphates in the tissue and that in the medium. The ratio lies between 10 and 20, with a mean value of about 15.

It is clear from the results recorded in Table 1 that aerobic incubation of rat brain-cortex slices in Krebs-Ringer phosphate medium containing glucose in the presence of thiamine leads to an accumulation of thiamine in the tissue against a concentration gradient and to a marked degree of thiamine phosphorylation. The phosphorylated thiamine is held within the tissue, after incubation for 2 hr., at a concentration about 15 times that in the medium.

Experiments were carried out to observe the effects of incubation of brain-cortex slices for various times on the accumulation of labelled thiamine and labelled phosphorylated thiamine in the tissue and medium. Representative results are recorded in Table 2 for an initial external concentration of labelled thiamine of $0.2 \mu\text{M}$.

The phosphorylation of labelled thiamine in the tissue proceeds at an approximately constant rate for the first 2 hr. of incubation and thereafter the rate falls. Labelled thiamine phosphates increase in quantity in the medium as time progresses, the concentration ratio (tissue:medium) being approximately constant after the first hour of incubation. The amount of labelled thiamine in the tissue appears to reach a steady state after 1 hr. of incubation, whereas that in the medium progressively falls. The concentration ratio for labelled thiamine (tissue: medium) increases to values larger than unity after the first $\frac{1}{2}$ hr. of incubation. The amount of conversion of labelled thiamine into labelled thiamine phosphates reaches a value of 48% after

Table 1. *Effects of variation of the thiamine concentration on the accumulation of labelled thiamine and labelled thiamine phosphates by rat brain-cortex slices*

The tissue was incubated aerobically at 37° for 2 hr. in 3 ml. of Krebs-Ringer phosphate medium containing glucose with different initial thiamine concentrations, the radioactivity initially present being constant at 16000 counts/min (efficiency of counting 57%). The values for the concentrations of free and phosphorylated thiamine were calculated from the expression:

$$\frac{\text{m}\mu\text{moles of labelled free or phosphorylated thiamine}}{\text{counts/min./ml. of cell water or medium}} = \frac{\text{specific activity of thiamine in counts/min./m}\mu\text{mole}}{\text{specific activity of thiamine in counts/min./m}\mu\text{mole}}$$

Initial concn. of thiamine in medium ...	$0.2 \mu\text{M}$	$0.6 \mu\text{M}$	$1 \mu\text{M}$
Concn. in tissue ($\text{m}\mu\text{mole/ml. of cell water}$):			
Free thiamine	0.19	0.54	0.78
Phosphorylated thiamine	0.42	0.60	0.96
Concn. in medium ($\text{m}\mu\text{mole/ml.}$):			
Free thiamine	0.12	0.42	0.82
Phosphorylated thiamine	0.023	0.040	0.062
Concentration ratio (tissue:medium):			
Free thiamine	1.6	1.3	0.95
Phosphorylated thiamine	18.2	15.0	15.4

Table 2. *Accumulation of labelled thiamine and labelled thiamine phosphates by rat brain-cortex slices as a function of time*

The tissue was incubated aerobically at 37° in 3 ml. of Krebs-Ringer phosphate medium containing glucose for various times. The initial concentration of labelled thiamine was $0.2 \mu\text{M}$, the radioactivity being 16000 counts/min. (efficiency of counting 57%). Values for the concentrations of free and phosphorylated thiamine were calculated as given in Table 1.

Time of incubation ...	$\frac{1}{2}$ hr.	1 hr.	2 hr.	3 hr.
Concn. in tissue ($\text{m}\mu\text{mole/ml. of cell water}$):				
Free thiamine	0.14	0.21	0.20	0.20
Phosphorylated thiamine	0.13	0.31	0.51	0.74
Concn. in medium ($\text{m}\mu\text{mole/ml.}$):				
Free thiamine	0.16	0.14	0.10	0.09
Phosphorylated thiamine	0.012	0.018	0.03	0.042
Concentration ratio (tissue:medium):				
Free thiamine	0.87	1.5	2.0	2.2
Phosphorylated thiamine	10.5	17.0	17.0	17.8
Total labelled thiamine phosphates formed ($\text{m}\mu\text{mole}$)	0.070	0.125	0.212	0.29
Percentage conversion of labelled thiamine into labelled thiamine phosphates	11.6	20.8	35.3	48.3

Table 3. *Effects of ouabain and anaerobiosis on the accumulation of labelled thiamine and labelled thiamine phosphates by rat brain-cortex slices*

The tissue was incubated aerobically or anaerobically for 2½ hr. at 37° in 3 ml. of Krebs–Ringer phosphate medium containing glucose. The initial concentration of labelled thiamine was 0.2 μM, the radioactivity being 16000 counts/min. (efficiency of counting 57%). Values for the concentrations of free and phosphorylated thiamine were calculated as given in Table 1.

	No addition	Ouabain (0.1 mM)	No addition; N ₂ instead of O ₂
Concn. in tissue (μmole/ml. of cell water):			
Free thiamine	0.23	0.11	0.22
Phosphorylated thiamine	0.56	0.30	0.08
Concn. in medium (μmole/ml.):			
Free thiamine	0.10	0.14	0.18
Phosphorylated thiamine	0.033	0.023	0.006
Concentration ratio (tissue:medium):			
Free thiamine	2.3	0.8	1.2
Phosphorylated thiamine	17	13	13

aerobic incubation for 3 hr. with an initial external concentration of thiamine of 0.2 μM (Table 2).

The results indicate an ability of rat brain-cortex slices to continue to phosphorylate thiamine for at least 3 hr. of aerobic incubation, and the fact that the amount of labelled phosphorylated thiamine in the medium, at any time after 1 hr. of incubation, is a small but approximately constant fraction of that in the tissue.

Effects of anaerobiosis and of 2,4-dinitrophenol on the uptake of thiamine by rat brain-cortex slices. When incubation of rat brain-cortex slices in Krebs–Ringer phosphate medium containing glucose in the presence of thiamine is allowed to occur under anaerobic conditions, oxygen being replaced by nitrogen, the following effects become evident. The amount of phosphorylation in the tissue of labelled thiamine falls by over 80%, and the amount of labelled thiamine phosphate in the medium falls proportionately. The amount of labelled thiamine in the tissue changes but little, whereas that in the medium increases, so that the concentration ratio for labelled thiamine (tissue:medium) falls to about unity. Typical results are shown in Table 3.

It thus appears that development of a concentration gradient, exceeding unity, between tissue thiamine and thiamine in the medium requires energy provided by respiration.

The addition of 2,4-dinitrophenol (50 μM) diminishes by approx. 63% the concentration ratio for 'total' labelled thiamine (i.e. the sum of labelled

free thiamine and labelled thiamine phosphates) (tissue:medium) after aerobic incubation of rat brain-cortex slices in the presence of 0.12 μM-thiamine for 2 hr. A typical result is given in Table 4. The value of 'total' labelled thiamine uptake, under aerobic conditions, by the tissue slices in the presence of 2,4-dinitrophenol is depressed to about the same value as that found under anaerobic conditions in the absence of dinitrophenol (Table 3).

Effects of the addition of ouabain on the accumulation of labelled thiamine and labelled thiamine phosphates by rat brain-cortex slices. The addition of ouabain (0.1 mM) to the medium in which rat brain-cortex slices are incubated aerobically in the presence of thiamine (0.2 μM) at 37° leads to a pronounced fall in the concentration ratio for labelled thiamine (tissue:medium). Typical results, in an incubation for 2½ hr., are shown in Table 3. The concentration ratio for labelled thiamine falls below unity, and it is therefore evident that ouabain diminishes the rate of transfer of thiamine into the brain cells. Phosphorylation of thiamine is also affected, but not to the same extent as is brought about by anaerobiosis (Table 3). Possibly the diminished rate of phosphorylation of thiamine is due, in part, to the diminished influx of thiamine.

The fact that incubation of brain tissue with ouabain leads to a considerable fall in tissue labelled thiamine, and in the concentration ratio between tissue and medium, indicates that there is relatively little binding of labelled thiamine to tissue components, not enough to account for the concentration gradient greater than unity obtained under aerobic conditions in Krebs–Ringer phosphate medium containing glucose.

Effects of omission of Na⁺ from the incubating medium on thiamine transport and metabolism by rat brain-cortex slices. Results given in Table 4 show the effects of replacing Na⁺ by choline in the medium in which the rat brain-cortex slices are incubated. There is a considerable fall in the total amount of labelled thiamine (free and phosphorylated) taken up by the tissue, and the concentration ratio for 'total' thiamine (tissue:medium) falls to nearly the anaerobic level. The interpretation of this experimental result is, however, complicated by the fact that choline chloride, which was substituted for sodium chloride to preserve iso-osmolarity, itself undergoes active transport in rat brain-cortex slices (M. Oka & J. H. Quastel, unpublished work) and may therefore act as a competitor of thiamine. Experiments were therefore carried out with rat brain-cortex slices incubated in Krebs–Ringer medium, containing glucose free of Na⁺, to which glycylglycine buffer (10 mM) was added. Sucrose (0.3 M) was added to preserve iso-osmolarity. Typical results are shown in Table 5. The most marked effect is a fall in the amount of labelled phosphorylated

Table 4. *Effects of Na⁺ and of 2,4-dinitrophenol on the accumulation of 'total' labelled thiamine by rat brain-cortex slices*

Rat brain-cortex slices were incubated aerobically for 2 hr. in 3 ml. of Krebs-Ringer phosphate medium containing glucose in the presence of labelled thiamine and other additions as noted. The initial labelled thiamine concentration was 0.12 μ M, the radioactivity being 3200 counts/min. (efficiency of counting 19%). Values for the concentrations of 'total' (free and phosphorylated) thiamine were calculated as given in Table 1.

Concn. of NaCl (mM)	Concn. of choline chloride (mM)	Concn. of dinitrophenol (mM)	Concn. of 'total' thiamine in tissue (m μ mole/ml. of cell water)	Concn. of 'total' thiamine in medium (m μ mole/ml.)	Concentration ratio (tissue:medium)
134	—	—	0.48	0.08	6.0
134	—	0.05	0.23	0.10	2.3
—	134	—	0.26	0.10	2.6

Table 5. *Effects of incubating rat brain-cortex slices in Na⁺-free medium in the presence of labelled thiamine*

The tissue was incubated aerobically for 2 hr. at 37° in 3 ml. of Krebs-Ringer medium containing glucose, devoid of Na⁺ and containing sucrose (0.3 M) and glycylglycine buffer (10 mM) where indicated. The initial labelled thiamine concentration was 0.175 μ M, the radioactivity being 14000 counts/min. (efficiency of counting 57%). Values for the concentrations of free and phosphorylated thiamine were calculated as given in Table 1.

	Krebs-Ringer phosphate medium containing glucose (Na ⁺ present)	Krebs-Ringer glycylglycine medium containing glucose (Na ⁺ present)	Krebs-Ringer glycylglycine medium containing glucose (Na ⁺ absent; 0.3 M-sucrose present)
Concn. in tissue (m μ mole/ml. of cell water)			
Free thiamine	0.21	0.23	0.19
Phosphorylated thiamine	0.50	0.49	0.094
Concn. in medium (m μ mole/ml.)			
Free thiamine	0.10	0.11	0.15
Phosphorylated thiamine	0.026	0.025	0.0106
Concentration ratio (tissue:medium):			
Free thiamine	2.1	2.0	1.2
Phosphorylated thiamine	19	20	9

thiamine in the tissue accompanied by a fall also of that in the medium. At the same time the concentration ratio for labelled thiamine (tissue:medium) diminishes to approximately unity. The effects of Na⁺ lack resemble those due to anaerobiosis, so far as accumulation and metabolism of thiamine are concerned (compare Tables 3 and 5). The results, though indicating a marked dependence of the thiamine-phosphorylation system on Na⁺, show that the thiamine-transport system is also Na⁺-dependent.

Effects of the addition of Amprol on thiamine transfer and phosphorylation by rat brain-cortex slices. The addition of Amprol (10 μ M) to the medium in which rat brain-cortex slices are incubated aerobically in the presence of labelled thiamine causes a pronounced fall in the 'total' labelled thiamine taken up from the medium, the concentration ratio (tissue:medium) falling almost to the

anaerobic level. Even at 1 μ M, Amprol causes a fall outside the experimental error.

Typical results obtained with 10 μ M-Amprol on thiamine transfer and phosphorylation are shown in Table 6. There is a fall in the concentration ratio for labelled thiamine (tissue:medium) to about unity. The concentration of labelled thiamine in the brain tissue falls in presence of Amprol, whereas that in the medium rises. There are parallel falls in the amounts of labelled phosphorylated thiamine in the tissue and in the medium. The results indicate that the thiamine analogue, Amprol, is able to suppress active transport of thiamine, presumably by competition for a membrane receptor site, and in its presence thiamine enters the tissue by simple diffusion. The diminution of phosphorylated thiamine is possibly due to the diminished rate of transfer of thiamine into the brain cells, but a direct effect of Amprol on the thiamine-phosphorylation system

cannot at present be excluded. Results with rat brain extracts show inhibitory effects of Amprol at relatively high concentrations on thiamine phosphorylation.

Effects of thiamine monophosphate and thiamine diphosphate on thiamine transfer and phosphorylation by rat brain-cortex slices. The most marked effects

of the addition of thiamine monophosphate or thiamine diphosphate at a concentration of $1\text{ }\mu\text{M}$ on the transfer of labelled thiamine into rat brain-cortex slices are the inhibitions of the accumulation of labelled thiamine with a fall in the concentration ratio for labelled thiamine (tissue:medium) to less than unity. Typical results are shown in Table 7.

Such inhibitions point to competition between thiamine and thiamine phosphates at a membrane carrier site. On increasing the concentrations of thiamine monophosphate and thiamine diphosphate in the medium bathing the tissue to $5\text{ }\mu\text{M}$, the quantities of labelled thiamine in the brain tissue increase and the concentration ratio for labelled thiamine (tissue:medium) rises to 1.2.

The concentrations of labelled phosphorylated thiamine in the tissue and medium do not fall significantly in the presence of $1\text{ }\mu\text{M}$ -thiamine monophosphate or -thiamine diphosphate. When the concentration of thiamine monophosphate or thiamine diphosphate, added to the medium, is increased to $5\text{ }\mu\text{M}$ there is a marked fall in the accumulation of labelled thiamine phosphates in the brain tissue.

Experiments with rat brain extracts show no significant hydrolysis of thiamine monophosphate in an incubation period of 1 hr. at 37° , so it is evident that the fall in the accumulation of labelled thiamine in rat brain-cortex slices in the presence of $1\text{ }\mu\text{M}$ -thiamine phosphate is unlikely to be due to liberation of free thiamine, which by diminishing the specific activity of the labelled thiamine would give rise to diminished radioactivity of free thiamine

Table 6. *Effects of Amprol on the accumulation of labelled thiamine and labelled thiamine phosphates by rat brain-cortex slices*

The tissue was incubated aerobically for 2 hr. at 37° in 3 ml. of Krebs-Ringer phosphate medium containing glucose. The initial concentration of labelled thiamine was $0.137\text{ }\mu\text{M}$, the radioactivity being 11000 counts/min. (efficiency of counting 57%). Values for the concentrations of free and phosphorylated thiamine were calculated as given in Table 1.

	No addition	Amprol (0.01 mM) added
Concn. in tissue ($\mu\text{mole/ml. of cell water}$):		
Free thiamine	0.17	0.11
Phosphorylated thiamine	0.34	0.17
Concn. in medium ($\mu\text{mole/ml.}$):		
Free thiamine	0.06	0.10
Phosphorylated thiamine	0.029	0.011
Concentration ratio (tissue:medium):		
Free thiamine	2.8	1.1
Phosphorylated thiamine	11.7	15.5

Table 7. *Effects of thiamine phosphates on the accumulation of labelled thiamine and labelled phosphorylated thiamine by rat brain-cortex slices*

Rat brain-cortex slices were incubated aerobically for 2 hr. in 3 ml. of Krebs-Ringer phosphate medium containing glucose in the presence of labelled thiamine and of thiamine monophosphate or thiamine diphosphate. The initial labelled thiamine concentration was $0.187\text{ }\mu\text{M}$, the radioactivity being 15000 counts/min. (efficiency of counting 57%). Values for the concentrations of free and phosphorylated thiamine were calculated as given in Table 1.

	Thiamine (0.187 μM) + thiamine monophosphate (0.187 μM) present	Thiamine (0.187 μM) + thiamine monophosphate (1.0 μM) present	Thiamine (0.187 μM) + thiamine diphosphate (5.0 μM) present	Thiamine (0.187 μM) + thiamine diphosphate (1.0 μM) present	Thiamine (0.187 μM) + thiamine diphosphate (5.0 μM) present
Concn. in tissue ($\mu\text{mole/ml. of cell water}$):					
Free thiamine	0.21	0.096	0.18	0.083	0.175
Phosphorylated thiamine	0.54	0.42	0.18	0.57	0.30
Concn. in medium ($\mu\text{mole/ml.}$):					
Free thiamine	0.11	0.13	0.15	0.12	0.14
Phosphorylated thiamine	0.029	0.024	0.011	0.03	0.02
Concentration ratio (tissue:medium):					
Free thiamine	1.9	0.7	1.2	0.7	1.2
Phosphorylated thiamine	18	17	16	19	15

in the tissue. Moreover, increase of the thiamine monophosphate concentration in the medium to $5\mu\text{M}$ would tend to enlarge this diminishing action, whereas the contrary effect occurs (Table 7). The same consideration applies to thiamine diphosphate, although with a rat brain extract in an incubation period of 1 hr. at 37° a little hydrolysis of thiamine diphosphate ($70\mu\text{M}$) takes place, amounting to 0.7%.

The increased quantity of labelled thiamine in the tissue slices, with thiamine monophosphate or thiamine diphosphate ($5\mu\text{M}$) added to the medium, is doubtless due to the fact that thiamine phosphorylation is inhibited by the thiamine phosphates (Table 7). It is surprising, however, that the amount of labelled phosphorylated thiamine in the tissue does not fall significantly after exposure to $1\mu\text{M}$ -thiamine monophosphate or -thiamine diphosphate in view of the diminished quantity of labelled thiamine present in the tissue (Table 7). No satisfactory explanation of this fact can be offered at present.

Experiments with rat brain extracts

Dependence of thiamine phosphorylation on ATP plus Mg^{2+} . Experiments were carried out with rat brain extracts to obtain information about the effects of ouabain, Amprol etc. on thiamine phosphorylation.

The phosphorylation of thiamine in rat brain extract is dependent, as expected from previous work (Weil-Malherbe, 1939; Steyn-Parvé, 1952), on the presence of Mg^{2+} and ATP.

Preliminary work showed at once the necessity for ATP for thiamine phosphorylation in rat brain

extract. For example, a value of $0.084\text{m}\mu\text{mole}$ of thiamine phosphorylated by rat brain extract in 1 hr. at 37° in the absence of ATP was increased to $0.78\text{m}\mu\text{mole}$ of thiamine phosphorylated in the presence of 0.8mm-ATP under the same experimental conditions. A concentration of 1.2mm-ATP seems to be optimum for thiamine phosphorylation, increased concentrations of ATP having no accelerating effect on the phosphorylation process. Typical results are shown in Table 8. The velocity of phosphorylation by ATP increases with decrease of pH until pH 6.0, and in experiments whose results are recorded in Tables 8, 9, 10 and 11 a pH of 6.6 was adopted for incubation with rat brain extracts.

Effects of variation of thiamine concentration and of the presence of various concentrations of thiamine monophosphate, thiamine diphosphate and ATP on the phosphorylation of labelled thiamine in rat brain extract. It is evident from the representative results given in Table 8 that thiamine monophosphate and thiamine diphosphate inhibit thiamine phosphorylation in rat brain extract. Under the given experimental conditions, no hydrolysis of thiamine phosphate took place and hydrolysis of thiamine diphosphate did not exceed 1%. The thiamine phosphorylation system is saturated at relatively low concentrations of thiamine, a tentative value for K_m being $2.0\mu\text{M}$. This value must be regarded as approximate. An accurate value cannot be given until the content of (unlabelled) thiamine introduced in the rat brain extract (from that originally in the brain) has been estimated. From values of rat-brain thiamine recorded in the literature (see, e.g., Gurtner, 1961) the correction value may not exceed 10%.

Table 8. *Effects of variation of the thiamine concentration and of the presence of thiamine monophosphate and thiamine diphosphate on the phosphorylation of labelled thiamine in rat brain extract*

Rat brain extracts were prepared as described in the Materials and Methods section. Sixteen rat brains were homogenized in 60 ml. of 0.1M -glycylglycine buffer, pH 6.6, and the homogenate was centrifuged at 40000 rev./min. for 20 min. To 1 ml. of supernatant were added ATP (1.2mm), MgSO_4 (1.2mm), labelled thiamine ($4.4\mu\text{M}$; $560000\text{ counts/min.}$) and glycylglycine buffer (0.1M) to give a total volume of 4 ml. After aerobic incubation with shaking for 1 hr. at 37° , it was added to 3 ml. micro-columns each packed with 1.5 ml. of buffered resin. The filtrates and washings were collected and trichloroacetic acid (final concn. 5%) was added. The mixture was centrifuged, and a sample of the supernatant was assayed for radioactivity.

Initial concn. of labelled thiamine (μM)	Additions (unlabelled)	Labelled phosphorylated thiamine formed ($\text{m}\mu\text{mole}$)
4.4	None	0.78
4.4	Thiamine ($7\mu\text{M}$)	0.98
4.4	Thiamine monophosphate ($7\mu\text{M}$)	0.50
4.4	Thiamine monophosphate ($70\mu\text{M}$)	0.23
4.4	Thiamine diphosphate ($7\mu\text{M}$)	0.60
4.4	Thiamine diphosphate ($70\mu\text{M}$)	0.28
4.4	ATP (1.2mm) (i.e. total concn. of ATP 2.4mm)	0.79
4.4	ATP ($3.6\mu\text{M}$) (i.e. total concn. of ATP 4.8mm)	0.82

Table 9. *Effects of AMP and ADP on thiamine phosphorylation in rat brain extract*

Conditions were as given in Table 8. Concentrations of ATP etc. are given below. The initial concentration of labelled thiamine was $4.4 \mu\text{M}$ (200 000 counts/min.).

Addition	Labelled phosphorylated thiamine formed (μmole)
None (no nucleotide added)	0.08
ATP (1.2 mM)	0.79
ADP (1.2 mM)	0.31
AMP (1.2 mM)	0.12
ATP (1.2 mM) + ADP (0.6 mM)	0.53
ATP (1.2 mM) + ADP (1.2 mM)	0.37
ATP (1.2 mM) + AMP (0.6 mM)	0.57
ATP (1.2 mM) + AMP (1.2 mM)	0.48
ATP (1.2 mM) + UTP (1.2 mM)	0.65
UTP (1.2 mM)	0.61

Effects of AMP, ADP and UTP on thiamine phosphorylation in rat brain extract. Both AMP and ADP inhibit thiamine phosphorylation by rat brain extract in the presence of ATP. Typical results are given in Table 9. Moreover, ADP alone, in the absence of added ATP, stimulates thiamine phosphorylation under the given experimental conditions. Whether this effect is due to dismutation of ADP to ATP and AMP has not yet been ascertained. UTP also seems to stimulate thiamine phosphorylation, its effect not being additive to the stimulation brought about by ATP (Table 9).

Effects of ouabain and Amprol on thiamine phosphorylation in rat brain extract. Ouabain exercises no inhibition of thiamine phosphorylation in rat brain extract within the experimental error of these investigations. Even at 5 mM, ouabain has no significant inhibitory effect (Table 10).

Amprol has no inhibitory effect on thiamine phosphorylation at 0.01 mM, but it exercises inhibitions at relatively high concentrations. Approx. 50% inhibition, under the given experimental conditions, takes place at 1 mM. Typical results are given in Table 10.

As it is likely that a competition may take place between Amprol and thiamine for a receptor site, the apparent lack of inhibition by 0.01 mM-Amprol on thiamine phosphorylation may be due to the fact that in these phosphorylation studies with brain extracts the thiamine concentration is $4.4 \mu\text{M}$, i.e. about 20 times the concentration present in the medium in which rat brain-cortex slices are usually incubated. Experiments were carried out with 0.4 mM-Amprol (40 times the concentration present in the medium containing rat brain-cortex slices;

Table 10. *Effects of Amprol and ouabain on thiamine phosphorylation in rat brain extract*

Conditions were as given in Table 8, except that the brains were homogenized in 0.25 M-sucrose. The initial concentration of labelled thiamine was $4.4 \mu\text{M}$ (200 000 counts/min.).

Addition	Labelled phosphorylated thiamine formed (μmole)
None	0.56
Amprol (0.1 mM)	0.50
Amprol (1.0 mM)	0.30
Amprol (5.0 mM)	0.19
Ouabain (5 mM)	0.51

Table 11. *Effects of pyrithiamine on thiamine phosphorylation by rat brain extract*

Conditions were as given in Table 8.

Initial concn. of labelled thiamine (μM)	Concn. of pyrithiamine (μM)	Labelled phosphorylated thiamine formed (μmole)
4.4	0	0.78
4.4	2	0.55
4.4	4	0.45
4.4	8	0.32
8.8	0	0.88
8.8	2	0.72
8.8	4	0.62
8.8	8	0.53

Table 6) to observe whether inhibition of thiamine phosphorylation takes place. A value of 30% inhibition was recorded, indicating that, although this antagonist can diminish thiamine phosphorylation, its effect is insufficient to account wholly for its inhibition of thiamine uptake into rat brain-cortex slices.

Inhibition of thiamine phosphorylation by pyrithiamine in rat brain extract. Pyrithiamine inhibits markedly the uptake of labelled thiamine into rat brain-cortex slices. Thus $1.0 \mu\text{M}$ -pyrithiamine inhibits uptake of thiamine ($0.2 \mu\text{M}$) by approx. 60%. This inhibition may be largely brought about by its suppression of thiamine phosphorylation. Studies with rat brain extracts show that pyrithiamine exercises an inhibition that is diminished by increased thiamine concentrations. Typical results are given in Table 11. The linear relation that exists between pyrithiamine concentration and the reciprocal of the velocity of phosphorylation (Fig. 1) is consistent with the view that competitive inhibition takes place between thiamine and pyrithiamine.

Fig. 1. Inhibition of thiamine phosphorylation by pyrithiamine in rat brain extract. Conditions were as given in Table 8. The initial concentrations of thiamine were $4.4 \mu\text{M}$ (A) and $8.8 \mu\text{M}$ (B). $1/v$, Reciprocals of amounts of labelled phosphorylated thiamine formed from labelled thiamine in rat brain extract in 1 hr. at 37° .

The fact that pyrithiamine may act as a competitive inhibitor of thiamine in the process of thiamine phosphorylation in chicken blood has been described by Wooley (1951), and its inhibitory effect on thiamine phosphorylation by a liver preparation has been shown by Eich & Cerecedo (1954).

DISCUSSION

The results recorded in the present paper show that labelled thiamine accumulates in rat brain-cortex slices when these are incubated in Krebs-Ringer phosphate medium containing glucose in the presence of labelled thiamine, the concentration of the combined labelled free thiamine and labelled thiamine phosphates in the tissue being far higher (at least fourfold) than the concentration of these substances in the medium bathing the tissue. This is seen only at low external concentrations of thiamine, for with high external concentrations simple diffusion of thiamine into the tissue causes the concentration ratio for 'total' labelled thiamine (tissue:medium) to become approximately unity. A suitable concentration of thiamine for examination of transport reactions into brain-cortex slices is 0.1 – $0.2 \mu\text{M}$.

Analysis of the tissue and medium constituents shows that the concentration ratio for labelled free thiamine in the tissue to that in the medium exceeds unity and has been found to reach a value exceeding 3. It increases with time of incubation and it is thus evident that thiamine may be transferred into the brain cell against a concentration gradient. Conceivably the concentration ratio for free thiamine (tissue:medium) would be considerably greater than those recorded in the present paper if it were experi-

mentally feasible to utilize much smaller concentrations of thiamine than those employed in this work.

Incubation of brain tissue with ouabain (0.1 mM) brings about a considerable fall in the uptake of labelled thiamine, although it has little or no inhibitory effect on thiamine phosphorylation. Analysis of the brain constituents shows that the main effect of ouabain is to depress the transfer of thiamine into the brain-cortex slices, concentration ratios for free thiamine (tissue:medium) less than unity being obtained. The fact that such a ratio can be obtained indicates also that there can be but little 'binding' of thiamine to cell constituents, a phenomenon that might contribute to the establishment of a concentration ratio greater than unity.

The thiamine antagonist, Amprol, is inhibitory to the transfer of labelled thiamine into rat brain-cortex slices, and, although it can bring about some inhibition of thiamine phosphorylation, its effect is insufficient to account wholly for the block of the uptake of labelled thiamine into brain tissue.

Anaerobiosis, or the presence of 2,4-dinitrophenol, suppresses the uptake of labelled thiamine into brain tissue. This suppression might be attributed to the inhibition of thiamine phosphorylation that also takes place. Moreover, in media in which Na^+ has been omitted, phosphorylation of labelled thiamine is suppressed. However, both anaerobiosis and lack of Na^+ lead to a fall in the concentration ratio for labelled thiamine (tissue:medium) to approximately unity.

The results obtained with ouabain, Na^+ -free media, Amprol and anaerobiosis, together with those on labelled thiamine accumulated in the brain cell against a concentration gradient, all lead to the conclusion that thiamine is conveyed into the brain cell by an energy-assisted process mediated by a membrane carrier dependent for its activity on the 'sodium pump'.

It has been found (S. K. Sharma & J. H. Quastel, unpublished work), that the addition of phosphocreatine to a brain extract greatly increases the rate of thiamine phosphorylation, possibly by converting the inhibitory ADP into ATP, and that the accelerating action of phosphocreatine is Na^+ -dependent. The fact that ADP (and AMP) can inhibit thiamine phosphorylation in a liver preparation has been shown by Leuthardt & Nielsen (1952). Inhibition of uptake of labelled thiamine by rat brain-cortex slices by pyrithiamine is doubtless largely connected with its potent effect as a competitor of thiamine in the phosphorylation process, but inhibition of its transport is not excluded.

Another fact emerging from the results recorded in the present paper is the inhibitory effect of thiamine monophosphate and thiamine diphosphate on thiamine phosphorylation in brain slices. Such product inhibition may play an important role in regulating

cocarcboxylase activity in the brain cell. It is also evident that thiamine monophosphate and thiamine diphosphate inhibit the transfer of labelled thiamine into the brain cell (possibly by competition for the carrier), for the concentration ratio for labelled thiamine (tissue:medium) falls to below unity. The uptake of 'total' labelled thiamine (free and phosphorylated) diminishes in the presence of the thiamine phosphates.

The conclusion that thiamine is transferred into the brain cell by a carrier-mediated mechanism depending on the operation of the 'sodium pump' may have important neurological consequences. It is already known that Amprol, which can block such transfer at the brain cell membrane (Table 6), will, if given in the diet of chickens in sufficiently large amounts, produce growth depression and polyneuritis characteristic of thiamine deficiency (Rogers, 1962). It is therefore possible that substances that will block thiamine transfer into the neurone may be formed in, or may enter, the body, producing symptoms of thiamine deficiency under conditions where there may be no actual deficiency of thiamine in the blood.

We acknowledge with gratitude financial assistance from the Medical Research Council of Canada and from the U.S. Public Health Service (Grant MH 05326), which made this work possible.

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