Transport and Metabolism of Thiamine in Ehrlich Ascites-Carcinoma Cells

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1. Aerobic or anaerobic incubation at 37° of Ehrlich ascites-carcinoma cells in Krebs-Ringer bicarbonate medium containing glucose and labelled thiamine results in accumulation in the cell of labelled thiamine, so that the concentration of total labelled thiamine in the cells greatly exceeds (by a factor 7) that in the medium. This concentration ratio is approximately constant for small initial external concentrations of labelled thiamine but diminishes when the latter exceed 0.4 μ M. 2. All the labelled thiamine in the tumour cells is present as thiamine phosphates. 3. The uptake of labelled thiamine is markedly diminished by decrease of temperature. At 9° concentration ratio (cells/medium) 0.5 is observed whereas at 37° the concentration ratio is 8.6. 4. The extent of phosphorylation of labelled thiamine depends on the period of incubation. 5. The influx of labelled thiamine is diminished by the presence of its analogues, pyrithiamine and Amprol, and also by the presence of thiamine monophosphate and thiamine diphosphate, which are potent inhibitors of thiamine phosphorylation in Ehrlich ascites cells. 6. Labelled thiamine phosphates leak from the cell into the medium, so that eventually all the labelled thiamine, both in the cell and medium, is converted into thiamine phosphates. However, in the presence of 2,4-dinitrophenol (0.1 mM) and iodoacetate (1 mM) thiamine phosphorylation is diminished, the concentration ratio for labelled thiamine (cells/medium) falls to half its normal value and little or no labelled thiamine phosphates leaks into the medium. 7. In the presence of thiamine phosphates, free labelled thiamine accumulates in Ehrlich ascites cells against a concentration gradient, concentration ratios (cells/medium) greater than unity being evident. 8. The evidence supports the conclusion that thiamine is transferred into the Ehrlich ascites cell by a carrier-mediated energy-assisted process.

It has been shown (Sharma & Quastel, 1965) that carrier-mediated transport of thiamine takes place in rat brain-cortex slices incubated in a physiological Ringer medium and that such transport is energy-dependent. We demonstrate, in the present paper, that a similar process occurs in Ehrlich ascites-tumour cells *in vitro*.

MATERIALS AND METHODS

Chemicals. [14 C]Thiamine hydrochloride labelled in C-2 of the thiazole ring (specific activity 0.1 mc/1.26 mg.) was purchased from The Radiochemical Centre, Amersham, Bucks. It was dissolved in water and kept in frozen condition. Amprol [1-(4-amino-2-*n*-propylpyrimidin-5-ylmethyl)- β -picolinium chloride hydrochloride] was a gift from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A. Pyrithiamine and oxythiamine were purchased 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 ascites-carcinoma cells. Ehrlich ascites tumour was maintained in Swiss white mice by intraperitoneal injection. The tumour-bearing mice were killed by cervical dislocation 7 or 8 days after the injection and the ascitic fluid was withdrawn. The ascites-carcinoma cells were separated by centrifuging and repeated washing as described by Tenenhouse & Quastel (1960). Krebs-Ringer phosphate medium of the following composition was used for washing: NaCl, 134 mM; KCl, 5.2 mM; CaCl₂, 2.8 mM; KH₂PO₄, 1.3 mM; MgSO₄, 1.3 mM; sodium phosphate buffer, pH 7.4, 10 mM. The packed cells were finally suspended in Krebs-Ringer bicarbonate medium.

Determination of the volume of cell water in ascites cells. The amount of intracellular water in the ascites cells was estimated from the dry and wet weights of the cells, which were measured as follows: 1.0 ml. of the final cell suspension was placed in a weighed polypropylene centrifuge tube and spun at 300g for 30 min. The tube was well drained, traces of water on the sides of the tube were removed by wiping with filter paper and the tube together with the cells was weighed again. The difference between the weights was taken as the wet weight of the cells. The dry weight of the cells was estimated by heating 1.0 ml. of the suspension at

110° to constant weight. It was consistently found that the dry weight represented about 15% of the wet weight. Hence, as a routine, wet weights were determined and 85% of these were taken as the weights of cell water.

Incubation procedure. Krebs–Ringer bicarbonate medium of the following composition was usually employed for incubation purposes: NaCl, 134 mm; KCl, 5.2 mm; CaCl₂, 2.8 mm; KH₂PO₄, 1.3 mm; MgSO₄, 1.3 mm; NaHCO₃, 25 mm. Glucose (10 mm) was also added to all flasks. Unless otherwise mentioned, [¹⁴C]thiamine was used without mixing with unlabelled thiamine, and its final concentration was 0.2 or 0.4 μ M as specified. A 1.0 ml. suspension of ascites cells containing 150–200 mg. wet wt. of cells was added to each flask of a Warburg manometric apparatus. The total volume of the incubation mixture was 3.0 ml. Unless otherwise stated, aerobic conditions were employed in all experiments. The flasks were gassed for 10 min. with O₂ + CO₂ (95:5) (for aerobic conditions) or N₂ + CO₂ (95:5) (for anaerobic conditions). After equilibration for 10 min. labelled thiamine was tipped in from the side arm and the flasks were shaken in the Warburg apparatus at 37°. An incubation period of 1 hr. was employed except when otherwise stated.

Determination of radioactivity. After incubation, the ascites cells were separated by centrifugation and washed by suspending in cold Krebs–Ringer phosphate medium and re-centrifuging. The cells were then heated in a water bath at 100° for 5 min. and homogenized in tubes containing 2 ml. of water. The heating step was adopted to facilitate homogenization and thereby complete recovery of the [¹⁴C]thiamine from the cells. Control experiments showed that this procedure had no effect on the quantities of thiamine phosphates and free thiamine present. The homogenate was clarified by centrifugation at 0° and to 1.0 ml. of the supernatant 0.25 ml. of 20% (w/v) trichloroacetic acid was added. A 0.5 ml. sample of the deproteinized extract was added to 10 ml. of the scintillant solution, which had the following composition: toluene (333.3 ml.), dioxan (333.3 ml.), 95% (v/v) ethanol (333.3 ml.), 2,5-diphenyloxazole (5 g.), 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)-benzene (0.05 g.) and naphthalene (80 g.). The radioactivity of the samples was measured with a Packard Tri-Carb liquid-scintillation spectrometer.

Estimations of labelled thiamine and labelled thiamine phosphates. The radioactivity of the deproteinized cellular extract described above gave a measure of the amount of the total labelled thiamine (free and phosphorylated) present in the cells. Determination of the quantities of free thiamine and thiamine phosphates separately was carried out as a routine by column chromatography on the ion-exchange resin Rexyn 102 (H⁺ form). The procedure together with details of the preparation of the resin has been fully described by Sharma & Quastel (1965). By this method thiamine mono-, di- and tri-phosphate were separated from the free thiamine. The different phosphates of thiamine were not separated from each other.

A 1 ml. sample of the supernatant, from the centrifuged 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 10 ml. This contained the labelled phosphorylated thiamine of the cells, 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 and also 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 thiamine phosphates in the medium were estimated in precisely the same manner as that adopted for the supernatant of the cell 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 it was assayed on a sample of the acid eluate of the resin.

Calculations of the amounts of labelled free thiamine and labelled thiamine phosphates. After assay of the radioactivities of the samples of the thiamine and thiamine phosphate fractions of the cells and medium, calculations were made of the radioactivities of the labelled thiamine and phosphorylated thiamine in the tissue cell water, taking into account the weight of tissue taken and the cell water as 85% of the cell weight. These gave values as counts/min. per ml. of tissue cell water or per ml. of medium. These values were converted into $m\mu$ moles/ml. by dividing the observed counts/min. by the specific activity of the thiamine calculated as counts/min./ $m\mu$ mole of thiamine added.

Calculation of concentration ratio. The quantities of total thiamine as well as those of free thiamine and of the thiamine phosphates were calculated from the radioactivities of the fractions obtained by column chromatography. From these results the concentrations of the compounds in cell water as well as in the medium were calculated. The ratio of the concentration for total labelled thiamine in the cell water to that in the medium at the end of the experiment is described as the concentration ratio for total labelled thiamine. Similarly the ratio of the concentration of thiamine phosphates in the cell water to that in the medium is expressed as the concentration ratio for labelled thiamine phosphates. The concentration ratio for free thiamine is expressed in a likewise manner.

Duplication of results. The results of individual experiments are given in the Tables below. Each experiment was performed at least three times and the variation between the results did not exceed $\pm 8\%$.

When the quantities of labelled thiamine or labelled thiamine phosphates were too small to be measured accurately, i.e. when measured counts/min. were less than 50% above the background count, they are referred to as 'Nil' in the Tables below.

RESULTS

Results of preliminary experiments (Menon & Quastel, 1965) showed that [¹⁴C]thiamine accumulates in Ehrlich ascites-carcinoma cells to a concentration several times that present in the medium. It was found that all the thiamine in the cell as well as most of the thiamine in the medium was present in the phosphorylated form. Owing to the

rapid phosphorylation of thiamine, a concentration ratio for free thiamine (tissue/medium) greater than unity could not be obtained; only in the presence of inhibitors of thiamine phosphorylation could a value higher than unity be found.

In the first place studies were made of the kinetics of thiamine uptake.

Uptake of [^{14}C]thiamine in Ehrlich ascites-carcinoma cells after incubation for different times. Ehrlich ascites cells were incubated with [^{14}C]thiamine for various times under the conditions described above. The results are given in Table 1. The uptake of labelled thiamine in the cells was very rapid, the concentration ratio (cells/medium) for total thiamine (i.e. free and phosphorylated) being 4.4 and 6.7 after 15 min. and 30 min. respectively. The total labelled thiamine content of the cell does not increase significantly when the incubation period is extended up to 2.0 hr. Separation of thiamine phosphates and free thiamine shows that throughout the incubation period studied, namely 15–120 min., all the thiamine present in the cell is in the phosphorylated form.

The main changes in the state of the labelled thiamine, during prolonged periods of incubation, take place in the medium bathing the Ehrlich ascites cells. After incubation for only 15 min. about 11% of the total thiamine in the medium is present as thiamine phosphates and the rest is free thiamine. As the incubation period progresses, the amount of thiamine phosphates in the medium steadily increases. After 2 hr. almost all the thiamine in the medium is phosphorylated.

Uptake of [^{14}C]thiamine in Ehrlich ascites-carcinoma cells at different temperatures. The effects of temperature on the uptake of [^{14}C]thiamine in Ehrlich ascites cells are shown in Table 2. When the temperature of incubation is lowered from 37° to 21°, other conditions being unchanged, the labelled thiamine content in the cell falls from 1.05 to 0.695 m μ mole/ml. of cell water. This corresponds to a fall in the concentration ratio for total labelled thiamine from 8.6 to 4.2. When the temperature is further lowered to 9° the concentration of total labelled thiamine in the cell water is only 0.098 m μ mole/ml. of cell water, giving a

Table 1. *Uptake of [^{14}C]thiamine by Ehrlich ascites-carcinoma cells after incubation for different times*

Ehrlich ascites-carcinoma cells were incubated aerobically in Krebs–Ringer bicarbonate medium at 37° containing glucose (0.01 M) and [^{14}C]thiamine (0.2 μM ; 7000 counts/min./ml.). The total volume was 3.0 ml.

Period of incubation (min.)	Total labelled thiamine			Labelled thiamine phosphates			Free labelled thiamine		
	Concn. (m μ moles/ml.)			Concn. (m μ moles/ml.)			Concn. (m μ moles/ml.)		
	Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio
15	0.710	0.161	4.4	0.685	0.018	38.0	Nil	0.143	Nil
30	0.983	0.147	6.7	0.935	0.045	20.8	Nil	0.102	Nil
60	1.03	0.144	7.2	1.05	0.083	12.7	Nil	0.061	Nil
90	1.01	0.145	7.0	0.963	0.108	8.9	Nil	0.037	Nil
120	1.03	0.144	7.2	0.995	0.135	7.4	Nil	Nil	Nil

Table 2. *Uptake of [^{14}C]thiamine by Ehrlich ascites-carcinoma cells at different temperatures*

Ehrlich ascites-carcinoma cells were incubated under the conditions given in Table 1. The period of incubation was 1 hr.

Temp.	Total labelled thiamine			Labelled thiamine phosphates			Free labelled thiamine		
	Concn. (m μ moles/ml.)			Concn. (m μ moles/ml.)			Concn. (m μ moles/ml.)		
	Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio
37°	1.05	0.122	8.6	1.01	0.122	8.3	Nil	Nil	Nil
21	0.695	0.165	4.2	0.72	0.01		Nil	0.156	Nil
9	0.098	0.195	0.50	Nil	Nil		Nil	0.195	Nil
0	0.063	0.196	0.32	Nil	Nil		Nil	0.196	Nil

concentration ratio 0.50. When the reaction mixture is incubated at 0° the amount of total labelled thiamine in the cell falls still further and a concentration ratio 0.32 is observed. Concentration ratios less than unity, found at the lower temperatures, show the relatively low rate of passive diffusion of thiamine into the ascites cell. In both sets of experiments, at 37° and 21°, all the thiamine present in the cell is phosphorylated. Separate estimations of free thiamine and thiamine phosphate, where the cells were incubated at 9° and 0°, could not be made with any accuracy owing to the very small quantities of thiamine present in the cellular extracts. More marked differences are seen in the values for free and phosphorylated thiamine in the medium. After incubation at 37° much of the thiamine in the medium is present as thiamine phosphates. On the other hand, after incubation at 21°, 9° and 0°, little or no thiamine phosphate is detected in the medium and almost all the thiamine is present in the free form.

Effects of various concentrations of labelled thiamine on its uptake in Ehrlich ascites-carcinoma cells. The results given in Table 3 show how uptake of labelled thiamine changes when the ascites cells are incubated with different concentrations of this vitamin. When the concentration of labelled thiamine is raised from 0.1 to 0.4 μM the intracellular concentration of total labelled thiamine also increases proportionately and the concentration ratio remains fairly constant. When the concentration of labelled thiamine in the medium is further increased the concentration ratio steadily falls. It reaches a low value of 0.4 when the extracellular concentration is 20 μM .

In these experiments, the whole of intracellular labelled thiamine is found to be present as labelled

thiamine phosphates. The most plausible explanation is that phosphorylation of thiamine takes place within the cell at a rate greater than the diffusion rate into the cell except at high concentrations of thiamine. Thiamine phosphates then leak out into the medium. The results in Table 3 show that when thiamine (20 μM) is added to the medium the concentration ratios observed under the given experimental conditions are always less than unity. The values after 1.0, 3.0 and 5.0 hr. are 0.44, 0.55 and 0.56 respectively (Table 4). This is in agreement with the results given in Table 2 indicating a low rate of passive diffusion of thiamine into the ascites cells. An almost steady state is reached in 3 hr. of incubation. It is noteworthy that after 1 hr. 56% of the total labelled thiamine in the cell is present as thiamine phosphates when the initial external concentration of labelled thiamine is 20 μM . As the period of incubation is increased to 5 hr. almost all the thiamine in the cell becomes phosphorylated (Table 4).

Effects of thiamine analogues on the uptake of labelled thiamine in Ehrlich ascites-carcinoma cells. The large effects of temperature on the uptake of labelled thiamine (Table 2) indicate that the diffusion process, which seems to be rate-limiting, does not take place entirely by passive transfer. It was therefore important to observe whether thiamine analogues inhibit thiamine uptake. The results of experiments carried out with this objective are shown in Table 5. Pyrithiamine at a concentration of 0.5 μM produces about 50% inhibition in the uptake of labelled thiamine and at 1 μM it inhibits the process by about 80%. These concentrations of pyrithiamine correspond to 2.5 and 5 times the concentration of labelled thiamine used. Oxythiamine in concentrations of

Table 3. *Effects of various concentrations of thiamine on its uptake by Ehrlich ascites-carcinoma cells*

Ehrlich ascites-carcinoma cells were incubated aerobically in Krebs-Ringer bicarbonate medium containing glucose (0.01 M). For concentrations of thiamine in the range 0.1–0.4 μM only [^{14}C]thiamine was employed. For higher concentrations, 0.2 μM [^{14}C]thiamine was used and unlabelled thiamine was added to give the required concentrations. The total volume was 3.0 ml. The temperature was 37°. The period of incubation was 1 hr.

Initial concn. of labelled thiamine (μM)	Total labelled thiamine		
	Concn. (m $\mu\text{moles/ml.}$)		Concn. ratio
	Cell water	Medium	
0.1	0.51	0.062	8.2
0.2	1.05	0.122	8.6
0.3	1.65	0.178	9.3
0.4	2.32	0.238	9.7
1.2	2.67	1.00	2.7
2.2	2.75	2.00	1.4
10.2	4.60	9.86	0.5
20.2	7.30	19.7	0.4

Table 4. *Uptake of [¹⁴C]thiamine by Ehrlich ascites-carcinoma cells after incubation for different times*

Ehrlich ascites-carcinoma cells were incubated aerobically with labelled thiamine (20.2 μ M) (0.2 μ M-[¹⁴C]-thiamine mixed with 20 μ M-unlabelled thiamine) in Krebs-Ringer bicarbonate medium. The total volume was 3.0 ml. The temperature was 37°.

Period of incubation (hr.)	Total labelled thiamine			Labelled thiamine phosphates			Free labelled thiamine		
	Concn. (m μ moles/ml.)			Concn. (m μ moles/ml.)			Concn. (m μ moles/ml.)		
			Concn. ratio			Concn. ratio			Concn. ratio
	Cell water	Medium		Cell water	Medium		Cell water	Medium	
1.0	9.6	21.6	0.44	5.4	Nil		4.2	21.6	0.19
3.0	11.8	21.4	0.55	9.8	0.34	28.8	2.0	21.1	0.10
5.0	12.1	21.4	0.56	11.8	0.55	21.4	Nil	20.8	Nil

Table 5. *Effects of thiamine analogues on the uptake of labelled thiamine in Ehrlich ascites-carcinoma cells*

Ehrlich ascites-carcinoma cells were incubated under the conditions given in Table 1. The period of incubation was 1 hr.

Analogue added	Concn. of analogue (μ M)	Total labelled thiamine			Labelled thiamine phosphates			Free labelled thiamine		
		Concn. (m μ moles/ml.)			Concn. (m μ moles/ml.)			Concn. (m μ mole/ml.)		
				Concn. ratio			Concn. ratio			Concn. ratio
		Cell water	Medium		Cell water	Medium		Cell water	Medium	
None	—	1.12	0.137	8.2	1.04	0.097	10.7	Nil	0.040	Nil
Pyrithiamine	0.5	0.633	0.164	3.9	0.653	0.019	34.3	Nil	0.145	Nil
	1.0	0.268	0.185	1.5	0.234	0.006	39.0	Nil	0.179	Nil
Oxythiamine	5.0	1.14	0.137	8.3	1.18	0.096	12.3	Nil	0.041	Nil
	20.0	1.13	0.137	8.2	1.06	0.102	10.4	Nil	0.035	Nil
Amprol	5.0	0.652	0.163	4.0	0.733	0.016	45.8	Nil	0.147	Nil
	10.0	0.297	0.183	1.6	0.294	0.010	29.4	Nil	0.173	Nil

5 and 10 μ M (corresponding to 25 and 50 times the concentration of thiamine) has no effect, within experimental error, on thiamine uptake. Amprol, when added in concentrations of 5 and 10 μ M, inhibits the uptake of labelled thiamine by 50% and almost 75% respectively. Amprol thus appears to be approximately 10 times less effective than pyrithiamine in inhibiting the uptake of thiamine by the ascites cells.

Effects of thiamine phosphates on the uptake of thiamine in Ehrlich ascites-carcinoma cells. The effects of thiamine monophosphate and thiamine diphosphate on the uptake of [¹⁴C]thiamine in Ehrlich ascites cells are shown in the results given in Table 6. Concentrations of the monophosphate ranging from 4 to 200 μ M were employed. The 4 μ M-thiamine monophosphate inhibits the uptake of labelled thiamine by 40% whereas 200 μ M-thiamine monophosphate brings about 80% inhibi-

tion of the process. Thiamine diphosphate appears to be 10 times more potent in inhibiting the uptake of labelled thiamine by the ascites cells, 20 μ M of this compound causing 80% inhibition. Analyses of free thiamine and thiamine phosphates show that both thiamine monophosphate and thiamine diphosphate bring about extensive inhibitions of thiamine phosphorylation with the result that accumulation of free thiamine takes place in the cells. Moreover, the accumulations are such that concentration ratios (cell water/medium) for free thiamine are substantially greater than unity occur. Thus in the presence of 20 μ M-thiamine monophosphate the intracellular concentration of free labelled thiamine is 0.407 m μ mole/ml. in the cell water and 0.170 m μ mole/ml. in the medium, giving a concentration ratio 2.4. Similarly a concentration ratio 3.2 is obtained for free labelled thiamine when thiamine diphosphate (1 μ M) is

Table 6. *Effects of thiamine phosphates on the uptake of labelled thiamine in Ehrlich ascites-carcinoma cells*

Ehrlich ascites-carcinoma cells were incubated under the conditions given in Table 1. The period of incubation was 1 hr.

Substance added	Concn. of substance added (μM)	Total labelled thiamine			Labelled thiamine phosphates			Free labelled thiamine		
		Concn. ($\text{m}\mu\text{moles/ml.}$)			Concn. ($\text{m}\mu\text{moles/ml.}$)			Concn. ($\text{m}\mu\text{mole/ml.}$)		
		Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio
None	—	1.15	0.137	8.4	1.20	0.079	15.2	Nil	0.058	Nil
Thiamine monophosphate	4.0	0.698	0.162	4.3	0.298	Nil		0.400	0.162	2.5
	20.0	0.551	0.170	3.2	0.144	Nil; 0.01		0.407	0.170	2.4
	200	0.216	0.190	1.1		Nil			0.190	
Thiamine diphosphate	1.0	0.780	0.158	4.9	0.275	Nil		0.505	0.158	3.2
	4.0	0.557	0.170	3.3	0.244	Nil		0.313	0.170	1.8
	20.0	0.215	0.188	1.1		Nil			0.188	

Table 7. *Effects of the addition of 2,4-dinitrophenol and sodium iodoacetate on the uptake of labelled thiamine in Ehrlich ascites-carcinoma cells*

Ehrlich ascites-carcinoma cells were incubated under the conditions given in Table 1 except that the concentration of [^{14}C]thiamine was $0.4\mu\text{M}$. The period of incubation was 1 hr.

Gas phase	Substances added	Total labelled thiamine			Labelled thiamine phosphates			Free labelled thiamine		
		Concn. ($\text{m}\mu\text{moles/ml.}$)			Concn. ($\text{m}\mu\text{moles/ml.}$)			Concn. ($\text{m}\mu\text{mole/ml.}$)		
		Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio	Cell water	Medium	Concn. ratio
Aerobic	None	2.21	0.286	7.7	2.14	0.150	14.2	Nil	0.136	Nil
Aerobic	2,4-Dinitrophenol (0.1 mm) + iodoacetate (1 mm)	1.13	0.336	3.4	1.05	Nil		Nil	0.336	Nil
Anaerobic	None	1.93	0.297	6.5	2.05	0.138	14.9	Nil	0.159	Nil
Anaerobic	2,4-Dinitrophenol (0.1 mm) + iodoacetate (1 mm)	1.02	0.342	3.0	0.960	Nil		Nil	0.342	Nil

added to the incubation medium. These results indicate that thiamine can enter the ascites cell against a concentration gradient and points to the conclusion that active transport of thiamine into the tumour cell occurs. The fact that the concentration ratio for total labelled thiamine becomes approximately unity with high concentrations of thiamine phosphates (Table 6) indicates that these phosphates block the active-transport process.

Effects of 2,4-dinitrophenol and sodium iodoacetate on the effects of [^{14}C]thiamine in Ehrlich ascites-carcinoma cells. The uptake and phosphorylation

of thiamine in Ehrlich ascites-carcinoma cells are much affected when the intracellular concentration of ATP is greatly decreased (Tables 7 and 8). In the presence of a mixture of iodoacetate (1 mm) and 2,4-dinitrophenol (0.1 mm), which greatly suppresses ATP formation in Ehrlich ascites cells when incubated aerobically or anaerobically in a glucose medium, the concentration ratio for total labelled thiamine is diminished to 50% (Table 7). In these experiments the ascites cells were pre-incubated with 2,4-dinitrophenol and iodoacetate for 30 min. before adding [^{14}C]thiamine. The

preincubation procedure was adopted to suppress as far as possible the endogenous ATP concentration in the cells. It is apparent that in the absence of any inhibitor the concentration ratio for total labelled thiamine and the amounts of labelled thiamine phosphorylated are almost as high under anaerobic as under aerobic conditions. In the presence of 2,4-dinitrophenol (0.1mM) and iodoacetate (1mM) the phosphorylation of labelled thiamine in the cell is diminished by about 50% but all the labelled thiamine in the cells is present as thiamine phosphates, little or none of which seems to leak into the medium. The concentration of free thiamine in the medium increases markedly (Table 7). It is evident that the diminished rate of phosphorylation is still sufficiently rapid to phosphorylate the thiamine entering the cell. Presumably the influx is much diminished by the fall in the cellular ATP concentration.

The results in Table 8 (obtained with another preparation of Ehrlich ascites cells) show the progress of the intracellular accumulation and phosphorylation of labelled thiamine when the ascites cells are incubated in the presence of 2,4-dinitrophenol (0.1mM) and iodoacetate (1mM). After 30 min. a steady state is obtained and the concentration ratio for total thiamine is constant over a period of 2 hr. All the intracellular thiamine is present as thiamine phosphates. No phosphorylated thiamine is found in the medium.

Effects of omission of Na⁺ on the uptake of [¹⁴C]-thiamine in Ehrlich ascites-carcinoma cells. The uptake of thiamine in Ehrlich ascites cells in the absence of Na⁺ was studied in view of the fact that Na⁺ is now known to be required for the active transport of a number of metabolites into animal tissues (Riklis & Quastel, 1958; Quastel, 1965). In these experiments sodium chloride was omitted

Table 8. *Effects of 2,4-dinitrophenol and sodium iodoacetate on the uptake of [¹⁴C]thiamine by Ehrlich ascites-carcinoma cells incubated for different times*

The experimental conditions were the same as given in Table 7 except that the period of incubation was varied. The gas phase was aerobic. Each flask contained 2,4-dinitrophenol (0.1mM) and sodium iodoacetate (1mM).

Period of incubation (min.)	Total labelled thiamine			Labelled thiamine phosphates		Free labelled thiamine		
	Concn. (mμmole/ml.)		Concn. ratio	concn. (mμmole/ml.)		Concn. (mμmole/ml.)		Concn. ratio
	Cell water	Medium		Cell water	Medium	Cell water	Medium	
30	0.736	0.336	2.0	0.680	Nil	Nil	0.366	Nil
60	0.743	0.366	2.0	0.740	Nil	Nil	0.366	Nil
120	0.812	0.364	2.3	0.740	Nil	Nil	0.364	Nil

Table 9. *Effects of omission of Na⁺ on the uptake of labelled thiamine in Ehrlich ascites-carcinoma cells*

The experimental conditions were the same as in Table 1. The period of incubation was 1 hr.

Medium used	Total labelled thiamine			Labelled thiamine phosphates			Free labelled thiamine		
	Concn. (mμmoles/ml.)		Concn. ratio	Concn. (mμmoles/ml.)		Concn. ratio	Concn. (mμmole/ml.)		Concn. ratio
	Cell water	Medium		Cell water	Medium		Cell water	Medium	
Normal (containing NaCl)	1.370	0.121	11.3	1.36	0.103	13.2	Nil	0.018	Nil
Choline chloride substituted for NaCl	0.446	0.179	2.5	0.422	Nil		Nil	0.179	Nil
Sucrose substituted for NaCl	1.500	0.134	11.1	1.47	0.092	15.9	Nil	0.042	Nil

from the suspension medium and iso-osmoticity was maintained by adding choline chloride or sucrose. The same medium was used for the washing as well as incubation of the cells. Typical results are given in Table 9. When sodium chloride is replaced by choline chloride the concentration ratio for total labelled thiamine falls from the control value of 11.3 to 2.5. There is a considerable fall in the amount of labelled thiamine phosphorylated. All the labelled thiamine in the cells is present as thiamine phosphates, but the thiamine in the medium is almost entirely in the free form. On the other hand, when sodium chloride is replaced by 0.3M-sucrose no appreciable difference is seen either in the amount of labelled thiamine taken up by the cells or in the amount phosphorylated. The inhibitory effect observed in the choline chloride medium is therefore probably due to a specific action of choline rather than to the absence of Na^+ .

This conclusion is supported by the fact that the addition of ouabain brings about no marked diminution of the total labelled thiamine in the ascites cells or in the amount of labelled thiamine phosphorylated (I. A. Menon & J. H. Quastel, unpublished work).

Effects of thiamine analogues and thiamine phosphates on the phosphorylation of thiamine by extracts of Ehrlich ascites-carcinoma cells. Extracts of Ehrlich ascites-carcinoma cells were prepared as described in the Materials and Methods section. The extract was incubated with [^{14}C]thiamine in

the presence of ATP and Mg^{2+} . The results are shown in Table 10. Pyrithiamine is a potent inhibitor of the phosphorylation process, $0.5\mu\text{M}$ inhibiting the reaction by 45%. Oxythiamine and Amprol at the concentrations tested do not have any significant effects on the activity of thiamine kinase. Thiamine monophosphate and thiamine diphosphate are inhibitory to approximately the same extent. Concentrations of $4.0\mu\text{M}$ of either compound inhibit the enzyme activity by about 70%.

DISCUSSION

The results reported above clearly show that when Ehrlich ascites-carcinoma cells are incubated with [^{14}C]thiamine the concentration of total labelled thiamine in the cell is several times as high as that in the medium. The rate of uptake of thiamine by these cells is far higher than that observed in several normal mammalian tissue slices (Menon & Quastel, 1966). Separate analyses of free thiamine and thiamine phosphates in the cellular extract and the medium show that all the thiamine in the cell, and most of it in the medium, is present in the phosphorylated form. The accumulation of thiamine phosphates in the ascites-tumour cells evidently involves more than transport of the vitamin from the medium against a concentration gradient. The results of experiments in which the uptake of labelled thiamine was studied in the presence of unlabelled thiamine

Table 10. *Effects of thiamine analogues on the phosphorylation of labelled thiamine by extracts of Ehrlich ascites-carcinoma cells*

Packed Ehrlich ascites-carcinoma cells were suspended in 9 vol. of cold water and allowed to stand for 10 min. in ice. The suspension was homogenized in a Potter-Elvehjem homogenizer and centrifuged at 15000g for 30 min. A 1.0 ml. sample of the supernatant was used. The incubation medium contained the following substances: [^{14}C]thiamine ($0.4\mu\text{M}$), ATP (2.0 mM), MgSO_4 (2.0 mM), tris buffer, pH 7.4 (0.01 M). The total volume was 3.0 ml. The temperature was 37° . The period of incubation was 1 hr.

Analogue added	Concn. of analogue added (μM)	Thiamine phosphate formed (μmole)	Inhibition (%)
None	—	0.182	—
Pyrithiamine	0.5	0.101	45
	1.0	0.050	73
Oxythiamine	5.0	0.150	18
	10.0	0.156	14
Amprol	5.0	0.171	6
	10.0	0.152	16
Thiamine monophosphate	4.0	0.061	67
	20.0	0.030	84
Thiamine diphosphate	4.0	0.062	66
	20.0	0.042	73

monophosphate or thiamine diphosphate showed that when low concentrations of thiamine phosphates are present in the medium the phosphorylation of labelled thiamine is inhibited to a greater extent than its uptake by the cells. Under these conditions free labelled thiamine accumulates in the cell at concentrations 2–3 times that in the medium. This result shows that free thiamine can be transferred into the ascites cells from the medium against a concentration gradient. The observed accumulation of thiamine phosphates in the cell is evidently due to phosphorylation of the vitamin taking place within the cell. Further support for this conclusion is provided by the fact that, when the external concentration of thiamine in the medium is greatly increased, only part of the intracellular thiamine is present in the phosphorylated form (Table 3). Apparently under these conditions the enzyme ATP–thiamine pyrophosphotransferase acts at a lower rate than the diffusion process.

The presence of large amounts of thiamine phosphates in the medium seems to be due to leakage of this compound from the cells. This is borne out by the results in Table 1. The concentration ratio for total thiamine reaches a maximum value after 30 min. and does not appreciably increase when the period of incubation is extended to 120 min. However, the concentration of thiamine phosphates in the medium steadily increases until all the thiamine is phosphorylated. Similarly, when the temperature of incubation is lowered to 21° the concentration ratio for total thiamine falls only to about 50% of that at 37°, but at the lower temperature all the thiamine in the medium is present in the free state whereas at 37° most of it is in the phosphorylated form. It therefore seems that the accumulation of thiamine phosphates in the medium follows the initial transport into the cell of free thiamine with subsequent phosphorylation there. Indeed in the presence of 2,4-dinitrophenol and iodoacetate, when the concentration ratio markedly falls, little or no labelled phosphorylated thiamine appears in the medium although it is present in the cells. As phosphorylation of thiamine seems to take place wholly within the tumour cells a measure of the rate of formation of thiamine phosphates might also be a measure of the rate of thiamine transfer into the cells. However, various circumstances affecting both rates of transport and of phosphorylation may well affect such a direct proportionality.

The uptake and phosphorylation of thiamine in Ehrlich ascites cells seems to be an energy-dependent process. When the ATP content of the cells is depleted by preincubation in the presence of 2,4-dinitrophenol and iodoacetate the concentra-

tion ratio (cell water/medium) for total thiamine falls by about 50%. The inhibition of thiamine phosphorylation greatly exceeds this value.

The accumulation of thiamine by ascites cells, as well as the phosphorylation process, takes place as effectively in the absence of oxygen as under aerobic conditions. This is in contrast with the behaviour of rat brain-cortex slices examined under similar conditions (Sharma & Quastel, 1965). However, Bickis & Quastel (1965) have shown that ascites-tumour cells synthesize ATP to the same extent under anaerobic and aerobic conditions. Hence an ATP-requiring process, like active transport, should be expected to proceed as efficiently under anaerobic as under aerobic conditions. The active transport of several other substances into the ascites cells has been reported to proceed equally efficiently under both these conditions (Quastel, 1964, 1965).

In experiments designed to study the requirements of Na^+ for the transport of thiamine into the tumour cells it was found that when sodium chloride is replaced by choline chloride the process is markedly inhibited. However, when sucrose is used instead of sodium chloride no significant inhibition is observed. As choline is known to be actively transported into brain tissue (M. Oka, T. Nukada & J. H. Quastel, unpublished work cited by Quastel, 1965) and by rat kidney-cortex slices (Sung & Johnstone, 1965), the inhibition of thiamine transport observed in the choline chloride medium might be due to competing choline transport, i.e. to competition for available ATP. In this connexion ouabain ($10 \mu\text{M}$) has very little effect on the uptake of thiamine by the tumour cells.

Among the few analogues of thiamine tested, pyriithiamine and Amprol inhibit the uptake of labelled thiamine into the tumour cell partly perhaps by inhibition of transport and partly by inhibition of thiamine phosphorylation. Oxythiamine at the concentrations tested has little or no effect on these processes. Both thiamine monophosphate and thiamine diphosphate inhibit the uptake of total labelled thiamine in the cells, the decrease being due largely to a diminished rate of thiamine phosphorylation.

The lack of appearance of labelled thiamine phosphates in the medium when incubation experiments are carried out at low temperature (e.g. 21°), or in the presence of 2,4-dinitrophenol, points to the possibility that efflux of thiamine phosphates may not wholly occur by passive diffusion. Whether the transfer of thiamine phosphates into or out of the tumour cell is carrier-mediated has still to be investigated.

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