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Transport of B-Vitamins in Microorganisms

III. Chromatographic Studies on the Radioactivity Extracted from Non-proliferating Cells of *Lactobacillus fermenti* after Exposure to Labelled Thiamine

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Chromatographic studies were carried out on extracts from thiamine deficient non-proliferating cells of *L. fermenti* after short exposure of the cells to ^{14}C-thiamine in the presence of glucose, Mg^{2+}, various buffers, and certain other additions. The chromatograms were evaluated quantitatively and the relationship between the proportion of free thiamine and the total uptake was studied using different kinds of cells.

It was found that most of the labelled thiamine is rapidly converted to a phosphorylated derivative (presumably thiamine pyrophosphate). The extent of this conversion is considerably decreased, however, under conditions limiting the phosphorylation of thiamine without a simultaneous decrease of the total uptake. In extracts from severely phosphate depleted cells the proportion of free thiamine was about 70 % of the total radioactivity.

Estimations of the intracellular concentrations of free thiamine indicated accumulation of the vitamin against considerable concentration gradients.

It was reported previously that the uptake of labelled thiamine by non-proliferating thiamine deficient cells of *L. fermenti* was stimulated by exogenously supplied glucose and also exhibited several characteristics of enzyme mediated reactions.[1] This indicated that the accumulation of the vitamin may involve active transport. A prerequisite for this type of uptake process is that the compound in question should be accumulated against a concentration gradient. This implies that the compound being taken up occurs, at least to a certain degree, in the same form intracellularly as it does extracellularly. The operative form of thiamine in biochemical reactions is thiamine pyrophosphate and this compound is also found to be the predominant form of thiamine in most biological materials. Since thiamine pyrophosphate is intimately involved in the utilization of glucose and glucose was

required for maximum uptake of labelled thiamine in the non-proliferating cells of *L. fermenti* it could be expected that thiamine pyrophosphate will be the main derivative of thiamine in extracts from such cells. On the other hand it was found earlier that thiamine is taken up more readily when supplied as the free compound than when supplied in a phosphorylated form.[1] These observations indicated that phosphorylation of the vitamin prior to its transport into the cell does not take place.

The purpose of the present investigation was to elucidate if and to what extent the thiamine taken up by the non-proliferating cells occurs free intracellularly or is converted to other derivative(s), *i.e.* whether or not the accumulation process represents merely a passive diffusion across the cell membrane. This aim was approached by comparative chromatographic studies on extracts from thiamine deficient cells grown under otherwise normal conditions and on similar extracts from cells grown under conditions limiting the phosphorylation of thiamine. The variations in the free thiamine fraction of the respective extracts were then compared with the total uptake of the vitamin. In one type of experiment cells depleted of phosphate were employed, and in another selected studies were carried out on the effect on the intracellular phosphorylation of thiamine of certain additions and of the influence of time and pH during the incubation with labelled thiamine.

MATERIALS AND METHODS

In most experiments ^{14}C-thiamine (thiazole-2-^{14}C) from the Radiochemical Centre, Amersham, England, was employed. In certain preliminary experiments ^{35}S-thiamine from Hoffmann-La Roche was used.

Oxythiamine (OT), thiamine monophosphate chloride (TP), and thiamine pyrophosphate chloride (TPP) were preparations obtained from California Biochemical Corporation. All chemicals used for the preparation of the incubation media, and for the permeability experiments were of reagent grade. Glass distilled water was used throughout the investigation.

Organisms and growth media. The maintainance, storage and cultivation of *Lactobacillus fermenti 36* (ATCC 9833) was carried out essentially as previously described.[1] Thiamine sufficient cells were obtained from media containing 1 mg thiamine per litre and thiamine deficient cells from media containing 0.02 mg thiamine per litre. Two types of medium were employed in the preparation of phosphate depleted thiamine deficient cells. One of them (CA) contained Vitaminfree Casaminoacids (Difco), 10 g/l, and levels of potassium monohydrogen phosphate (K_2HPO_4) varying between 0 to 1 g per litre. When the K_2HPO_4 level was decreased below the normally employed 1 g per litre (*cf.* Ref. 1, Table 1), the potassium content of the growth media was correspondingly adjusted by means of potassium chloride (KCl). The other type of medium used for obtaining phosphate depleted cells consisted of an entirely synthetic medium (AA) in which the Casaminoacids and single aminoacids employed in earlier experiments were replaced by a mixture of aminoacids consisting of the following amounts (in g per litre medium): glycine 0.2, DL-alanine 0.8, DL-valine 0.5, DL-leucine 0.5, DL-isoleucine 0.5, DL-aspartic acid 0.4, L-glutamic acid 0.6, L-lysine.2 HCl 0.5, L-arginine HCl 0.5, DL-serine 0.1, DL-threonine 0.4, DL-methionine 0.2, DL-cysteine 0.2, L-cystine 0.3, L-tyrosine 0.2, DL-phenylalanine 0.2, L-histidine·2 HCl 0.2, DL-tryptophan 0.5, L-proline 0.2, L-asparagine 1.0.

The phosphate content of such a medium was varied between 0 and 6.5×10^{-3} M, and the potassium content was kept constant by means of KCl at 13×10^{-3} M. Growth in the various synthetic media was usually much slower than in the corresponding media containing Casaminoacids.

Chromatography of radioactive cell extracts. Washed cell suspensions were prepared and incubated with labelled thiamine under conditions similar to those employed in the previously described uptake experiments.[1] Unless otherwise stated the incubation was carried out at 37°C for 5 min. The incubation mixture was prepared in 5 ml 0.9 % NaCl per tube and usually contained 0.02 M buffer pH 6.8, 0.06 M glucose, 0.02 M $MgCl_2$, 0.03 M ascorbic acid, 4×10^8 cells, and 2×10^{-6} M ^{14}C-thiamine corresponding to 0.26 μC. After the incubation was completed the cells were washed twice with saline and once with water, packed by centrifugation and frozen in the centrifuge tubes, usually overnight. The frozen cells were immersed for 2 min in boiling water, a few ml of water and 100 μg unlabelled carriers (T, TP, TPP) were then added and the mixture was ground in the tube for 5—10 min with glass powder. The water extract was separated by centrifugation. It usually contained approximately 80 % of the total radioactivity taken up by the cells. The residue was washed with small volumes (2—3 ml) of water until the radioactivity of the washing represented no more than 1 % of the total radioactivity extracted. This usually required 2—3 washings. The washed residues were digested with formamide as described elsewhere [1,2] and aliquot portions were counted for radioactivity. The radioactivity which remained in the washed residues was less than 1 % of the total radioactivity taken up by the cells. Breaking of the cells in the Ultrasonic Disintegrator (MSE) was also tried, but for the purpose of subsequent chromatography of the cell contents this proved inferior to the procedure described above. The combined extracts and washings were freeze-dried and then re-dissolved in water (0.2—0.5 ml). Aliquot portions were applied as streaks to Whatman No. 1 paper sheets alongside solutions of crystalline labelled thiamine and subjected to chromatography for 18—24 h in three different solvent systems, *viz.*

I butanol:acetic acid:water, 4:1:5
II sec. butanol:pyridine:water:acetic acid, 30:30:30:1
III propanol:Na-acetate 1 M pH 5:water, 7:2:1

It was found advantageous to mix the solutions of labelled thiamine with some unlabelled boiled cell extract. This eliminated confusion arising sometimes from the influence of the "impurities" in the cell extract on the mobilities of the thiamine metabolites.

The R_F-values of reference bands of TPP, TP, and T were determined using unlabelled substances. The fluorescent spots were detected under UV-light after spraying the chromatograms with alkaline potassium ferricyanide. The dried chromatograms of radioactive extracts were evaluated by scanning in Nuclear Chicago Actigraph II, Model C-100, B, with a Nuclear Chicago Scaler Model 1620 B and/or by autoradiography. Autoradiography involved apposition of the chromatograms against X-ray film, Structurix Gevaert, for 4 weeks. For quantitative determinations the strips were cut into 1 cm fragments and each fragment was soaked with 0.2 ml H_2O in a scintillation vial. Ethyl alcohol (8 ml) and a phosphor solution consisting of 0.3 % PPO in toluene (10 ml) were then added and the counting carried out as described elsewhere.[2] The radioactivity present in each 1 cm fragment was calculated as percent of the total strip counts and the area under each separated band was integrated. The ratios between the areas corresponding to individual bands were then calculated. All other radioactivity measurements were also carried out by the liquid scintillation method. Each determination was carried out in duplicate and each experiment was repeated at least once.

RESULTS

Up to ten radioactive bands were usually found on the chromatograms of the cell extracts. Of these, however, only four bands represented appreciable amounts of the radioactivity, the remaining six being present as traces. These latter bands were usually so faint on the autoradiograms that they did not appear on the reproductions (photographs) shown in Figs. 2a—2d. The ^{14}C-thiamine preparation contained trace amounts of other compounds moving both faster and slower than the thiamine band. These impurities did not

Table 1. R_F-values of radioactive bands found on chromatograms of extracts from non-proliferating thiamine deficient cells of *L. fermenti* exposed to ^{14}C-thiamine. Incubation: 5 min at 37°C. Incubation mixture: buffer 0.02 M pH 6.8, NaCl 0.15 M, glucose 0.06 M, $MgCl_2$ 0.02 M, ascorbic acid 0.03 M, 4×10^8 cells, labelled thiamine 2×10^{-6} M, total volume 5 ml. Numbers in brackets indicate trace amounts; those in italics were the dominant bands. T, thiamine; TP, thiamine monophosphate; TPP, thiamine pyrophosphate.

	Solvent I	Solvent II	Solvent III	Identification
Cell extract				
Band *1*	*00.4*	*0.17*	*0.06*	}
2	0.06	0.21	0.09	} TPP
3	0.09	0.30	0.20	TP
(4)	0.21			
(5)			0.32	
6	*0.25*	*0.55*	*0.44*	T
(7)	0.40			
(8)	0.43	0.70	0.50	
(9)	0.65	0.78	0.80	
(10)	0.87	0.87	0.87	
Reference substances				
TPP	0.04	0.22	0.09	
TP	0.09	0.30	0.20	
T	0.25	0.55	0.44	

interfere with the estimation of the four main radioactive bands found in the extracts (*cf.* Figs. 2a—2c). The R_F-values of all the bands detected on chromatography in the three solvent systems are listed in Table 1 along with R_F-values of certain reference substances. It can be seen that among the four main radioactive bands two were quantitatively dominant, *viz.* band 1 and band 6. Band 6 was identified as thiamine. Band 1 was very close to band 2. In solvent I band 1 was indistinguishable from thiamine pyrophosphate, whereas in solvents II and III it moved slightly slower than thiamine pyro-

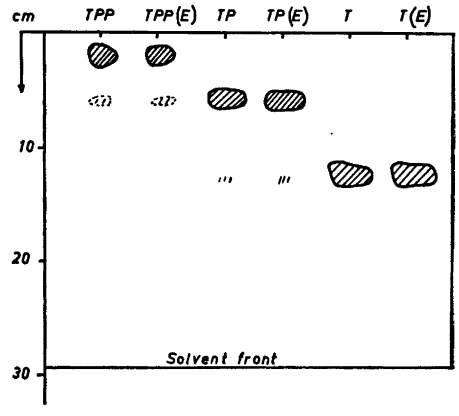

Fig. 1. Diagram of a chromatogram demonstrating the stability of thiamine and certain phosphorylated derivatives during the extraction procedure. Descending chromatography on Whatman No. 1 in solvent III. TPP, thiamine pyrophosphate; TP, thiamine monophosphate; T, thiamine. (E) designates that the solution of the compound was subjected to the same treatment as the cells during extraction. Unlabelled compounds.

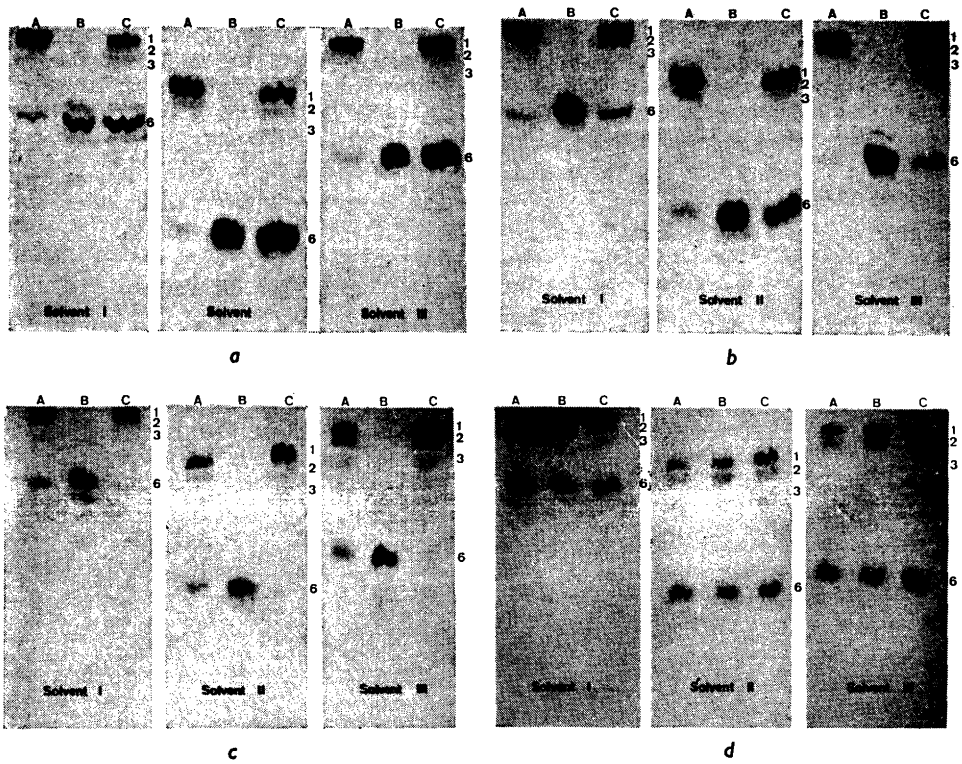

Fig. 2. Chromatograms of extracts from non-proliferating, thiamine deficient cells of *L. fermenti*, exposed to ^{14}C-thiamine in the presence of Tris buffer. When not otherwise stated incubation conditions as in Table 1. Autoradiography

a: A, cell extract; B, thiamine; C, thiamine + cell extract. Front 36—38 cm.
b: A, cell extract after 5 min; B, thiamine; C, cell extract after 60 min. Front 35—38 cm..
c: A, partially phosphate depleted cells; B, thiamine; C, phosphate sufficient cells. Front 32—34 cm.
d: cells grown in the AA-media, containing the following phosphate levels: A, 2×10^{-4} M; B, 6×10^{-4} M; C, 6.5×10^{-3} M. Front 30—33 cm.

phosphate. No attempts were made to identify these two bands as, for the purpose of the present study, only the percentage of free thiamine in the extracts was of interest. In what follows bands 1 and 2 will be referred to together, as band 1-2, containing the main phosphorylated derivatives. Of the remaining bands, band 3 was identified as thiamine monophosphate whereas the bands present in trace amounts (4—5 and 7—10) were not identified.

The stability of TPP, TP, and T during the extraction procedure is demonstrated in Fig. 1, from which it can be concluded that the free thiamine found in cell extracts did not arise from the decomposition of the phosphorylated derivatives.

Table 2. The uptake of ^{14}C-thiamine in non-proliferating thiamine deficient cells of *L. fermenti* in the presence of certain different buffers and the percentage of free thiamine in extracts from such cells. Incubation as in Table 1.

Buffer	Total uptake cpm × 10^{-5}	Fraction of the radioactivity of the extracts found in thiamine %
K-phosphate pH 6.8	0.22	7
Tris-HCl–KCl "	0.28	14
K-phosphate pH 5.5	0.20	3
K-phthalate "	0.18	11

Experiments with thiamine deficient cells obtained from media rich in all other nutrients. Fig. 2a illustrates the chromatographic separation of the radioactive material extracted from the cells. Chromatograms obtained in three solvent systems containing a free and an internal standard of thiamine are given. It can be seen that the fastest moving band (Band 6) is identical with free thiamine. The slow moving band 1-2, corresponding to the phosphorylated derivatives including thiamine pyrophosphate, contains more radioactivity than the thiamine band. In this case Tris-HCl buffer was used in the incubation mixture. It was found that when a phosphate buffer was used instead of Tris, the amount of the phosphorylated derivatives was even higher.

In order to obtain a quantitative measure of the amount of free thiamine chromatograms were cut in 1 cm pieces and evaluated by means of the liquid scintillation method. Average values from three independent experiments were then employed to calculate the percentage of free thiamine. It was found in this kind of experiment that the extent of the intracellular phosphorylation of thiamine was very much influenced by the type of buffer and by the pH employed during incubation of the washed cells with labelled thiamine. However, there was no correlation between these variations and the total uptake of radioactivity. Results of a representative experiment are shown in Table 2. The potassium content of all the buffers examined in these experiments was maintained at the same level.

It can be seen in Table 2 that the substitution of phosphate with Tris buffer at pH 6.8 results in a considerable increase in the thiamine fraction (*viz.* from 7 to 14 %) with a simultaneous increase in the total uptake of radioactivity. A similar but even more pronounced increase in the percentage of free thiamine (from 3 to 11 %) is observed when phosphate is substituted with phthalate buffer at pH 5.5, but the difference in the total uptake shows an inverse relationship and is much less pronounced.

Experiments were also carried out in order to compare certain other kinetic aspects of the intracellular phosphorylation of thiamine with those of the total uptake. Chromatograms of cell extracts after 5 and 60 min incubation with labelled thiamine in the presence of Tris buffer are shown in Fig.

Table 3. The uptake of ^{14}C-thiamine in non-proliferating thiamine deficient cells of *L. fermenti* and the percentage of free thiamine in extracts from such cells at varying lengths of time. Incubation as in Table 1 employing two different buffers at pH 6.8.

Time of incubation with labelled thiamine	Fraction of the radioactivity of the extracts found in thiamine (% T) and total uptake (cpm)			
	Tris-HCl—KCl		K-phosphate	
min	cpm × 10^{-5}	% T	cpm × 10^{-5}	% T
0.5	0.10	13	0.11	4
5	0.46	13	0.30	7
10	0.59	14	0.35	9
20	1.13	16	0.30	12
40			0.35	12
60	2.15	18	0.43	12

2b. It can be seen that the proportion of free thiamine in the extracts is considerably larger after 60 min than after 5 min.

Some representative results concerning the percentage of free thiamine in relation to the total uptake after varying time intervals and in the presence of two different buffers are shown in Table 3. It can be seen that the increase of the radioactivity uptake with time was followed, with both buffers, by a certain increase in the thiamine fraction. There was, however, no obvious correlation between the extent of the increase in the uptake and the increase in the thiamine fraction. With phosphate buffer the thiamine fraction remained practically constant after 20 min. Shortly (0 to 10 min) after the uptake the proportion of free thiamine is much smaller if phosphate is used in the suspension medium than when Tris is used. This difference becomes less pronounced later on.

Experiments with thiamine deficient cells simultaneously depleted of phosphate. It was shown above (Tables 2 and 3) that the presence of phosphate during incubation with labelled thiamine considerably decreased the amount of the intracellular free thiamine. It was considered therefore to be of interest to study the occurrence of free thiamine in cells depleted of phosphate. The growth media normally employed in this and previous investigations contained, in addition to potassium monohydrogen phosphate and a number of other nutrients, Casaminoacids (Difco).[1] This preparation was found to contain a considerable amount of phosphate. Excellent growth was obtained in the Casaminoacids containing media from which the addition of potassium phosphate was entirely omitted provided that potassium ions were supplied in some other form. The phosphate concentration derived from the Casaminoacids in such media was found to be 8.7×10^{-4} M.

Cells obtained from media in which the addition of crystalline K_2HPO_4 was replaced by an equivalent amount of KCl exhibited a lower degree of intracellular phosphorylation of thiamine than cells obtained from the aforementioned media. A representative chromatogram of cell extracts obtained

in experiments with cells grown in a normal CA-medium and in a corresponding medium without the phosphate addition is shown in Fig. 2c. Both kinds of cells were incubated with the labelled thiamine in the presence of Tris buffer. It can be seen in Fig. 2c that the amount of free thiamine in such phosphate depleted cells was considerably larger than in the cells obtained from the normal CA-medium. A considerable part of the radioactivity, however, was still present in the bands corresponding to the phosphorylated derivatives.

In order to obtain cells that were depleted of phosphate even more than was possible by simply omitting K_2HPO_4 from the media containing Casaminoacids (CA-media) the organism was grown in entirely synthetic media (AA-media). Growth in the AA-media was usually much slower than in the CA-media containing a corresponding level of phosphate.

Chromatograms of extracts from such severely phosphate depleted cells are shown in Fig. 2d. It can be seen that the proportion of free thiamine in these extracts was much higher than in the partially phosphate depleted cells grown in medium CA (*cf.* Figs. 2d:A and 2d:B with Fig. 2b:A).

Next, comparative experiments were conducted employing the two kinds of phosphate depleted cells, *viz.* those obtained from the CA-media (the CA-cells) and from the AA-media (the AA-cells), respectively. Table 4 shows the results of two series of such experiments. It can be seen in Table 4 that the total uptake of thiamine in the AA-cells was much lower than in the corresponding CA-cells. The percentage of free thiamine in the AA-cells grown at normal phosphate concentration (6.5×10^{-3} M) was several times larger than the corresponding percentage in extracts from the CA-cells, *viz.* 59 % free thiamine in the extracts from AA-cells as compared with 11 % in the extracts from CA-cells. However, within each series, the amount of free thiamine

Table 4. The relationship between the phosphate content of the growth medium and the ability of non-proliferating, thiamine deficient cells of *L. fermenti* to accumulate free ^{14}C-thiamine. CA-growth medium containing Casaminoacids (Difco); AA-growth medium containing a corresponding mixture of crystalline amino acids. Incubation as in Table 1 employing Tris buffer.

Phosphate content of the growth medium M	CA-cells		AA-cells	
	Total uptake cpm $\times 10^{-5}$	Free thiamine %	Total uptake cpm $\times 10^{-5}$	Free thiamine %
0.5×10^{-4}			insignificant growth	
1.0 "			"	"
2.0 "			0.12	71
4.0 "			0.11	
6.0 "			0.10	62
8.7[a] "	0.33	56		
9.3 "	0.37	50		
1.2×10^{-3}			0.15	
1.5 "	0.30	20		
6.5 "	0.37	11	0.13	59

[a] This value represents the phosphate derived from the Casaminoacids, no crystalline K_2HPO_4 was added to the medium, the potassium content was adjusted with KCl.

in the labelled cell extracts consistently increased with the degree of phosphate depletion of the cells whereas the total uptake was practically unaffected by the depletion.

Since extracts from thiamine deficient cells incubated with labelled thiamine in the presence of phosphate contained less free thiamine than the corresponding extracts obtained from cells incubated in the presence of other buffers (*cf.* Tables 2 and 3) it was considered of interest to study this influence of phosphate in the case of the phosphate depleted cells. The effect of including oxythiamine in the incubation medium was also studied. This thiamine analogue was previously found to have practically no effect on the uptake of thiamine, when supplied at levels ten and hundred times that of thiamine.[1] The results obtained in these experiments are shown in Table 5. It can be seen from the table that, in the presence of phosphate during incubation of the cells with labelled thiamine, both the total uptake of radioactivity and the percentage of free thiamine in the cell extracts decreased. This was observed both in the absence of oxythiamine (expts. 1 and 2) and in the presence of a tenfold excess of this compound (expt. 5). Analogously to what was found previously in experiments with phosphate sufficient cells the addition of oxythiamine at a level ten times that of thiamine did not affect the uptake; in fact the amount of free thiamine in the extracts from phosphate depleted cells even slightly increased upon this addition (*cf.* expts. 1a, 2a, 3a, and 4a with expts. 3b and 5a). However, at a higher concentration of oxythiamine, *i.e.* when a hundredfold excess was employed, both the total uptake of radio-

Table 5. The uptake of ^{14}C-thiamine in non-proliferating thiamine deficient cells of *L. fermenti* simultaneously depleted of phosphate, and the percentage of free thiamine in extracts from such cells when the cells were incubated with the labelled thiamine in the presence and in the absence of oxythiamine (OT) and/or potassium monohydrogen phosphate (KP).
Phosphate level in the growth medium: 8.8×10^{-3} M. Incubation as in Table 1 employing Tris buffer; labelled thiamine 2×10^{-6} M.

Experiment No.	Additions to the suspension medium OT M	KP 2×10^{-2} M	Total uptake cpm $\times 10^{-5}$	Free thiamine in the extracts %
1 a	—	—	0.23	56
b	—	+	0.20	50
2 a	—	—	0.21	40
b	—	+	0.17	25
3 a	—	—	0.21	50
b	2×10^{-5}	—	0.22	53
4 a	—	—	0.21	50
b	2×10^{-4}	—	0.18	45
5 a	2×10^{-5}	—	0.24	59
b	"	+	0.18	55
6 a	2×10^{-4}	—	0.14	45
b	"	+	0.14	37

activity and the amount of free thiamine in cell extracts decreased (*cf.* expts. 1a, 2a, 3a, and 4a with expts. 4b and 6a) in contrast to the phosphate sufficient cells studied previously.[1]

Estimations of thiamine uptake in relation to concentration gradients. The weight of a single cell of *L. fermenti* was obtained from a series of dry weight determinations using cell suspensions of varying turbidity and by correlation with direct counting under a microscope as well as by reference to a turbidity-cell number curve. The cell number in the latter case was determined by the tenfold dilution series described by Taylor.[3] Determinations with ten cell suspensions obtained on different days during a six month period gave the following values ($\mu g \times 10^7$):

6.85; 7.60; 6.50; 5.00; 11.60; 6.80; 10.00; 7.50; 10.20; 12.00. Average: $(8.4 \pm 2.0) \times 10^{-7}$ μg.

For the subsequent calculations the figure of 8×10^{-7} μg was used for the dry solid weight of one *L. fermenti* cell. No data concerning the water content of lactobacilli could be found in the literature. From the abundance of data concerning related microorganisms [4] it was assumed that the free water in *L. fermenti* constitutes approximately 80 % of the cell mass. Assuming that the specific weight of a microbial cell is close to 1.0, the volume of a *L. fermenti* cell is calculated to be 4.0×10^{-12} ml and the volume of the cell water thus 3.2×10^{-12} ml. In the subsequent calculations the radioactivity retained by the cells is employed as a measure of the intracellular concentration of thiamine and its derivatives (*cf.* Discussion). The calculations of the concentration gradients are summarized in Table 6 using three different uptake cases as examples.

It can be seen in Table 6 that in all the three cases listed there is a considerable concentration gradient between the intracellular and extracellular thiamine. In the thiamine deficient cells, which are normal with respect to phosphate (case 1), the accumulation is 510 times the extracellular concentration as calculated with respect to the intracellular thiamine and its phosphorylated derivatives together, but 57 times when only free intracellular thiamine is taken into consideration. In the thiamine deficient cells simultaneously depleted of phosphate (Table 6, case 2) the accumulation with respect to free thiamine increases to 260 times whereas the total accumulation of thiamine and its phosphorylated derivatives remains unchanged. The corresponding concentration gradients in thiamine sufficient cells, slightly depleted of phosphate (Table 6, case 3) were 50 times on the basis of total uptake and 10 times with respect to free thiamine.

DISCUSSION

The results of this investigation demonstrate that the conversion of thiamine to its phosphorylated derivatives, normally taking place rapidly after the uptake, is not the factor primarily responsible for the accumulation of the vitamin inside the cell. Conditions were designed under which the intracellular phosphorylation of thiamine decreased considerably without a simultaneous decrease of the vitamin uptake. Under such conditions the extent of the

Table 6. Calculations of minimum concentration gradients. Non-proliferating cells of *L. fermenti* grown in the CA medium, exposed for 5 min at 37°C to ^{14}C-thiamine, 2×10^{-6} M, 0.26 μC per 5 ml. Incubation as in Table 1 employing Tris buffer. T, free thiamine; Tph, phosphorylated derivatives of T.

Type of cells and % free T in the extracts	Uptake				Intracellular concentration [a]		Extracellular concentration	Ratio	
	Total		per cell	per cell water	$[T]_i$	$[Tph]_i$	$[T]_e$	$\frac{[T]_i + [Tph]_i}{[T]_e}$	$\frac{[T]_i}{[T]_e}$
	cpm $\times 10^{-4}$	μmoles $\times 10^3$	μmoles $\times 10^{12}$	μmoles/ml	μmoles/ml	μmoles/ml	μmoles/ml $\times 10^3$		
1. thiamine deficient, 11 % free T	3.7	1.2	3.0	0.9	0.10	0.80	1.76	510	57
2. thiamine deficient simultaneously depleted of phosphate, 50 % free T	3.7	1.2	3.0	0.9	0.45	0.45	1.76	510	260
3. thiamine sufficient slightly depleted of phosphate, 20 % free T	0.42	0.14	0.33	0.10	0.02	0.08	1.97	50	10

[a] derived from the uptake

thiamine phosphorylation seemed to be correlated to the magnitude of the "phosphate pool". The percentage of free thiamine was largest, *i.e.* about 60, in phosphate depleted cells and smallest, *i.e.* about 10 in extracts from cells grown in the presence of an excess of phosphate (*cf.* Table 4, CA-cells).

The estimations of thiamine concentrations in the intracellular and the extracellular space indicate that the vitamin can be accumulated against a considerable concentration gradient. In the thiamine deficient cells simultaneously depleted of phosphate the concentration of thiamine after 5 min of incubation with the labelled compound is at least 260 times higher intracellularly than extracellularly. It should be recorded in this connection that 5 min incubation does not give a maximum uptake, which is reached only after 60 min (*cf.* Ref. 1, Fig. 2) with a simultaneous increase in the proportion of free thiamine (*cf.* Table 3). This gives much higher concentration gradients than those shown in Table 6 for the case of 5 min incubation. There was a corresponding concentration gradient also in the case of the thiamine sufficient cells. The concentration of the labelled thiamine in such cells was ten times higher intracellularly than extracellularly. In the calculations summarized in Table 6 the labelled thiamine extracted from the cells was taken as a measure of the intracellular thiamine concentration. It should be stressed, however, that the true intracellular concentration was higher than that of the labelled vitamin owing to the endogenous thiamine level before exposure to the labelled vitamin. It follows that the difference between the calculated and the true concentration gradients was much more pronounced in the thiamine sufficient than in the thiamine deficient cells.[1]

Another point worth consideration is the estimation of the intracellular water content. A considerable part of this water is, of course, bound to different macro-molecules. This gives again higher concentrations of the intracellular thiamine than those calculated. It can thus be concluded that all the three points discussed above indicate that the true concentration gradients between the intracellular and the extracellular thiamine were even higher than those which could be calculated from the available data.

The ability to concentrate thiamine against a concentration gradient as well as the other characteristics of this transport system, *viz.* pH and temperature dependence, stereospecificity and saturation kinetics,[1] indicate that the accumulation of thiamine is not the result of osmotic activity only, *i.e.* a "passive diffusion" through the cell evelope. Rather, it seems that there exists in *L. fermenti* a specific transport system for thiamine and that this system operates more efficiently in thiamine deficient than in thiamine sufficient cells.

Studies on the specificity of this difference with respect to other nutrients and also comparative studies on thiamine uptake by certain organisms normally not requiring exogenous thiamine are in progress, as are attempts to obtain mutants of *L. fermenti* impaired in their ability to accumulate thiamine.

The difference between the thiamine deficient cells grown in the CA-medium and those grown in the AA-medium with respect to the uptake capacity and the proportion of free thiamine at higher phosphate concentrations in either medium (*cf.* Table 4, bottom line) could not be understood. Possibly, the Casaminoacids preparation contains some substances which stimulate both the thiamine transport system and the phosphorylation of thiamine.

It is interesting to note that the proportion of free thiamine increased with the incubation time (*cf.* Table 3). This may be a result of the action of phosphatases which are widely distributed and have been found, *e.g.*, in yeasts and in *E. coli*.

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