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Transport of B-Vitamins in Microorganisms

II. Factors Affecting the Uptake of Labelled Thiamine by Non-proliferating Cells of *Lactobacillus fermenti*

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The results of studies on the conditions affecting the uptake of labelled thiamine by non-proliferating cells of *L. fermenti* are presented.

The uptake process was found to exhibit several features of enzyme mediated reactions: it showed pronounced temperature and pH maxima, saturation kinetics and stereospecificity.

It is demonstrated that the uptake reaction requires an exogenous source of energy and that the accumulation process is reversible.

Both the uptake and the exit reactions are stimulated by potassium ions.

The uptake process is stimulated by ascorbic acid and by magnesium ions. The existence of a stoichiometric relationship between the influence of energy and the influence of magnesium is demonstrated.

Both the uptake and the exit processes are inhibited by phosphate, the latter to a much greater extent than the former.

The uptake is inhibited by sodium pyruvate and by high levels of glucose.

The ability of the non-proliferating cells to take up thiamine exhibits an inverse relationship to the thiamine content of the growth medium.

The transport of macro-nutrients into microbial cells has been the subject of extensive studies during recent years. The brilliant works of Monod and his associates [1] have revealed the occurrence in bacterial cells of specific *permeases*, which exhibit many characteristics of enzymic systems. These workers demonstrated also that the inductive character of those systems concerned with the uptake of carbohydrates represents a means of regulating the metabolism of these nutrients. Comparatively little is known about the conditions of vitamin uptake. These substances, owing to their catalytic character, are usually considered to be overproduced and/or present in excess in bacterial cells. Since the concentration in the cell of different vitamin containing coenzymes is known to control the rate of many enzymic reactions it is tempting to assume, however, that a physiological control of

the vitamin supply may provide a means of regulating the cell metabolism. The fact that many micro-organisms have the ability to take up and concentrate vitamins against high concentration gradients indicates that active transport may be involved in these phenomena. Further indications that this may be the case come from the reported studies on the permeability of certain bacteria to vitamin B_{12},[2] folic acid,[3] and biotin.[4,5]

It was previously reported from this laboratory [6] that the accumulation of ^{35}S-thiamine by resting cells of *Lactobacillus fermenti* was stimulated by glucose and Mg^{2+}. The effect of glucose was inhibited by iodoacetate. The purpose of the present work was to carry out a detailed study of this permeability process with respect to its kinetics, stereospecificity, and the exchange of the accumulated vitamin with an exogenous source.

MATERIALS AND METHODS

A part of the investigation was carried out with ^{35}S-thiamine which was a gift from Prof. Dr. O. Wiss, Hoffmann-La Roche. When this material was exhausted the study was continued with ^{14}C-thiamine (thiazole-2-^{14}C) from The Radiochemical Centre, Amersham, England. Pyrithiamine (neopyrithiamine) hydrobromide (PT), oxythiamine chloride hydrochloride (OT), thiamine monophosphate chloride (TP), and thiamine pyrophosphate chloride (TPP) were purchased from California Biochemical Corporation. Sodium iodoacetate was obtained from Fluka, Buchs SG, Switzerland. All chemicals used for the preparation of media for the growth of bacteria and for the permeability experiments were of reagent grade. Glass distilled water was used throughout the investigation.

Organisms and growth media. The stock cultures of *Lactobacillus fermenti 36* (ATCC 9833) were maintained in Micro Assay Culture Agar (Difco) and stored in a refrigerator after growth for 24 h at 37°C. They were transferred to fresh agar media monthly. Working cultures of the organism were grown in two types of media in order to obtain *thiamine sufficient* (medium I) and *thiamine deficient* (media II a and II b) cells, respectively. The compositions of the media employed are given in Table 1. The growth of *L. fermenti* in medium I was rapid, whereas the growth in media IIa and IIb was slow. The preparation of the *thiamine deficient* cells was carried out in two steps. In the first step, 5 ml portions of medium IIa were inoculated with a loop of the stock culture. After growth at 37°C for 30 h, the cells were harvested, washed with saline and transferred to 25 ml portions of medium IIb. Using a heavy inoculum, satisfactory growth in this latter medium was obtained after 18-22 h. In certain experiments the cells from medium IIa were washed and transferred to 25 ml portions of the same medium.

Cell suspensions. The cells grown in media I and IIb, respectively, were washed three times with saline, re-suspended in saline and the density was adjusted to a desired value by means of turbidity determinations using a Coleman Spectrophotometer Model 11. Using the method of tenfold dilution series in liquid media described by Taylor [7] for the estimation of numbers of bacteria, a curve was constructed correlating the % transmission readings and the number of cells/ml. Certain points on this curve were later verified by direct counting of cells under the microscope. With thiamine deficient cells the density was usually adjusted to 10^8-10^9 cells/ml. With thiamine sufficient cells, which accumulated considerably less of the labelled vitamin,[6] heavier suspensions were used in order to obtain reliable radioactivity counts.

Incubation of resting cells with labelled thiamine. The final suspensions (2 ml) were added to 16 × 110 mm sterile pyrex tubes containing buffer and variable components in 3 ml of saline. Certain components of the incubation medium, *e.g.* buffer, saline, glucose, Mg^{2+} (as $MgCl_2$), were sterilized in advance and added aseptically to the sterile tubes in order to diminish infection risks during the incubation and subsequent centrifugations and washings. Freshly prepared solutions of ascorbic acid in sterile distilled water were used for every experiment. The reaction was started by the addition of the

Table 1. Composition of the growth media for *L. fermenti*. Prepared in double strength and stored at −20°C for several months. Sterilized (5 min at 121°C) portions of the single strength media stored at +4°C for 2−4 weeks.

	Single strength medium
Casaminoacids Vitamin-free Difco	10 g/l
Sodium acetate.$3H_2O$	20 »
Glucose	40 »
Potassium mono hydrogen phosphate	1 »
Sodium chloride	1 »
Ammonium sulphate	1 »
DL-Alanine	400 mg/l
L-Asparagine	200 »
L-Cysteine	200 »
L-Cystine	200 »
DL-Tryptophan	400 »
Adenine sulphate	20 »
Guanine (hydrochloride or sulphate)	20 »
Uracil	20 »
Xantine	20 »
Magnesium sulphate·$3H_2O$	200 »
Manganese sulphate·$2H_2O$	50 »
Ferrous sulphate·$7H_2O$	20 »
Riboflavin	1 »
Calcium pantothenate	1 »
Niacinamide	1 »
Pyridoxine hydrochloride	1 »
Pyridoxal hydrochloride	1 »
Pyridoxamine	1 »
p-Aminobenzoic acid	1 »
Folic acid	20 μg/l
Folinic acid (Leucovorin, Lederle)	1 »
Vitamin B_{12}	0.1 »
Biotin	10 »
Tween 80	0.5 ml/l
Thiamine: Medium I	2 mg/l
Medium II a	0.02 »
Medium II b	0.002 »
pH adjusted with sodium hydroxide solution to	6.8

labelled thiamine in 0.1−0.2 ml H_2O. Unless otherwise stated the mixtures were incubated at 37°C for 20 min, with gentle shaking, to secure good mixing of the cells with the medium. In certain experiments the incubations were carried out in a nitrogen atmosphere. All incubations were carried out in duplicate and the experiments were repeated at least once.

Measurements of the radioactivity retained by the cells. When the incubation had been completed, the cells were cooled and spun down immediately. The supernatant was

withdrawn by means of a capillary tube under suction and the cells were washed three times with saline, great care being taken to obtain a quantitative recovery of the cells from the suspension. It was found advantageous in later experiments to carry out the last washing with H_2O instead of saline. Very little radioactivity was removed by the washing procedure. Taking the total radioactivity of the unwashed cells as 100 %, the three washings contained 5, 4, and 2 %, respectively, and the washed cells retained 89 %. This was taken as an indication that the washing procedure was necessary to remove the radioactivity present in the intercellular liquid but that none of the radioactivity which had been retained by the cells was removed during the washing. The washed cells were frozen overnight, heated for 5 min in boiling water and digested for 2 h at 60°C with formamide (0.3—0.5 ml).[6,8] Aliquot portions (0.2—0.4 ml) of the digests were counted for radioactivity in a Packard automatic Tri-Carb® Liquid Scintillation Spectrometer by a method described elsewhere.[8] The overall counting efficiency under the conditions used was 56 %. The background varied between 160—200 cpm and the probable counting error was < 5 %. When the difference between the incubation duplicates was greater than 25 %, as happened only occasionally, the results of the experiment were rejected.

Desorption studies. Deficient cells were exposed to labelled thiamine in buffered isotonic solutions containing glucose, Mg^{2+} and ascorbic acid as described above. After incubation at 37°C for 5 min the cells were centrifuged and washed once with 3 ml of saline. The cells were then re-suspended in 5 ml of a desorption medium containing variable components in 0.15 M NaCl and incubated at 37°C for 20 min with gentle shaking. After the incubation was finished the cells were centrifuged and the supernatants were removed carefully and retained. The cells were then washed once with 3 ml saline, once with 3 ml of water and frozen. The frozen cells were digested with formamide as described above. The radioactivity of the digests, that of the desorption supernatants and that of the washings was determined by liquid scintillation counting. The balance of the radioactivity was calculated using, in each experiment, control tubes with the same cell suspension exposed to labelled thiamine, but not subjected to a subsequent desorption. The radioactivity retained by the control cells was taken as 100 % and that retained by the cells subjected to desorption was calculated as a fraction hereof. When the balance calculations did not agree with the radioactivity of the controls within the range of ±25 % or/and when the washings showed unusually high counts, thus indicating possible damage to the cells, the results of the experiment were rejected.

RESULTS

The deficient cells accumulated considerably larger amounts of radioactivity than the sufficient ones[6] and were therefore more convenient for studies of the influence of varying conditions on the uptake rate. Unless otherwise stated, the data reported here are concerned with deficient cells. Experiments with sufficient cells were conducted only occasionally for the sake of comparison on certain points.

Studies on the uptake rate. The specific factors studied were the influence of temperature, incubation time, pH, and cell and substrate concentrations. The effect of temperature is shown in Fig. 1. It can be seen that the largest uptake takes place in the region 45-60°C with a maximum at 50°C. The curve falls off steeply on both sides of the maximum. In comparison with the maximum the uptake is rather small at temperatures below 33°C, but it increases rapidly above this temperature. The uptake at 60°C was still high as observed in all three experiments carried out at that temperature. There was very little accumulation of radioactivity at temperatures only a few degrees higher than 60°C. This was probably caused by thermal destruction of the cells as indicated by time-uptake studies (*cf.* Fig. 3) and micro-

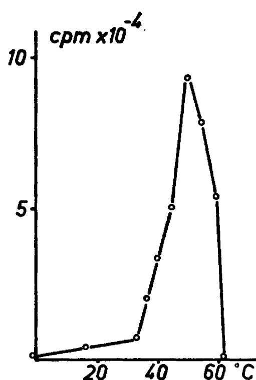

Fig. 1. The influence of temperature on the uptake of labelled thiamine by thiamine deficient non-proliferating cells of *L. fermenti.* Incubation mixture: potassium phosphate 0.02 M, pH 6.8, sodium chloride 0.15 M, glucose 60 mM, Mg^{2+} 20 mM, ascorbic acid 6 mM, ^{14}C-thiamine 2×10^{-3} mM, cells 4×10^{8}, total volume 5 ml. Incubation time 20 min.

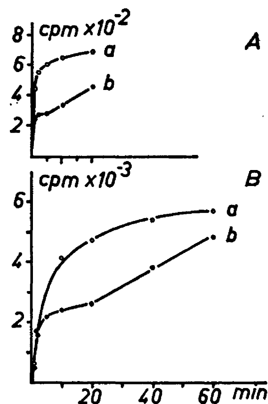

Fig. 2. The influence of incubation time on the uptake of labelled thiamine by non-proliferating cells of *L. fermenti.* ^{35}S-Thiamine 2×10^{-3} mM. Incubation (except time) as in Fig. 1. but at 37°C. A, thiamine sufficient cells; B, thiamine deficient cells; a, uptake in air; b, uptake in nitrogen.

scopic examinations. The absolute value of the maximum uptake rate varied from experiment to experiment, whereas the uptakes at temperatures below 40°C were not subject to such variations and, under carefully standardized conditions, gave reproducible values in a large number of experiments. Because of this and also for practical reasons 37°C was selected as the standard temperature in all subsequent experiments reported here.

The influence of incubation time on the uptake by deficient and sufficient cells is shown in Fig. 2. The incubations were carried out in both air and nitrogen, using aliquot portions of the same cell suspensions. It can be seen that the uptake in air is very rapid. Of the total radioactivity which accumulated after 60 min in the deficient cells, 50 and 80 % were taken up during the first 5 and 20 min, respectively. For subsequent experiments a 20 min incubation time was selected as a standard procedure.

The uptake in sufficient cells seems to be even more rapid, 80 % of the total radioactivity which accumulated after 20 min being taken up during the first 2 min. The shapes of the uptake curves indicate that the sufficient cells become "saturated" with thiamine much more rapidly than the deficient ones.

When the incubations were carried out under a nitrogen atmosphere, but without special precautions being taken to remove the air dissolved in the medium, the time-uptake curves with both deficient nad sufficient cells were different from those obtained in air. The uptake under nitrogen was lower than in air, although the initial uptake was very rapid, and again, the

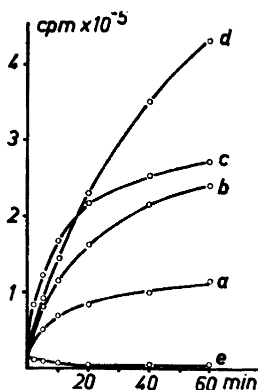

Fig. 3. The influence of different incubation conditions on the uptake of labelled thiamine by thiamine deficient, non-proliferating cells of *L. fermenti*. Incubation mixture (except buffer) as in Fig. 1. a, potassium phosphate 0.02 M, pH 6.8, in small centrifuge tubes removed one by one, 37°C; b, as a, but in one conical flask from which 5 ml samples are removed successively; c, as a, but at 50°C; d, as a, but with Tris-HCl 0.02 M, pH 6.8, containing 0.02 M KCl instead of potassium phosphate; e, as a, but at 62°C.

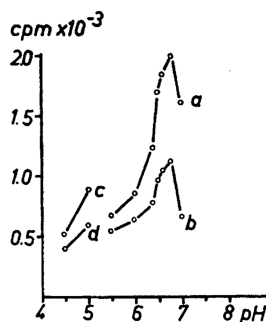

Fig. 4. The influence of pH and buffer on the uptake of labelled thiamine by thiamine deficient non-proliferating cells of *L. fermenti*. ^{35}S-Thiamine 2×10^{-3} mM. Incubation (except buffer) as in Fig. 1; a, potassium phosphate; b, sodium phosphate; c, potassium acetate; d, sodium acetate, all buffers 0.02 M.

curve obtained with sufficient cells levelled off much more rapidly (2 min) than that obtained with deficient cells (10 min). With longer incubation times the uptake curves rose again, but this time according to a linear function. This took place after 5 min with the sufficient and after 20 min with the deficient cells.

Fig 3 shows time-uptake curves in deficient cells using certain different conditions. It can be seen that incubation in small centrifuge tubes, removed one by one after the respective time intervals (curve a), gives considerably smaller uptakes than incubation in one Erlenmeyer-flask from which 5 ml samples are removed successively (curve b). This confirms the observation made above (*cf.* Fig. 2) that aerobic conditions favour the uptake reaction. The uptake in Tris-HCl-KCl buffer is more than three times as large as in potassium phosphate buffer and the time-uptake curve rises constantly and steeply during the 60 min period (curve d). In confirmation of the results shown in Fig. 1 the uptake at 50°C is much larger than at 37°C. The shape of the time-uptake curve (curve c) indicates that this uptake is also more rapid than the corresponding uptakes under other conditions reported here.

The influence of pH (using various buffers) on the thiamine uptake in deficient cells is shown in Fig. 4. A pronounced maximum was obtained at pH 6.8. The uptake, however, was very much influenced by the kind of buffer used. It was considerably larger when potassium was used as opposed to

sodium buffers. This difference was most pronounced at pH values 6.5, 6.8, and 7.0 when the uptake in potassium phosphate buffer was, respectively, 176, 179, and 240 % of the corresponding uptakes in sodium phosphate buffer. The uptakes in the presence of potassium and sodium acetate buffers were substantially higher than could be expected from the extrapolation to the corresponding pH-values of the curves obtained with the phosphate buffers.

Studies on the influence of pH on the thiamine uptake in sufficient cells gave similar results with respect to the effect of the different buffers used. At a given pH the uptake was considerably greater in the presence of potassium than in the presence of sodium buffers. The effects of acetate buffers were similar to those observed in deficient cells. However, an accurate determination of the pH-maximum was not successful. In the two experimental series carried out the maximum was found to be around pH 5.5 with potassium buffer, but around pH 6.5—7.0 with sodium buffer.

The rate of the vitamin uptake was directly proportional to the amount of cells present in the incubation mixture in the range up to 15—20 mg cells (dry weight). This is shown in Fig. 5 where the radioactivity taken up by cells is plotted against the weight of cells.

The relation between the exogenous concentration of labelled thiamine and the radioactivity uptake is shown in Fig. 6. The shape of the curve indicates saturation kinetics as defined by the Michaelis-Menten theory for enzyme mediated reactions when saturating levels of the substrate (here the labelled thiamine) are used. A Lineweaver-Burk plot of reciprocal uptake velocity *versus* the reciprocal mmolar concentration of exogenously supplied thiamine (S) is shown in Fig. 9, curve a. From this plot the K_m for the uptake reaction can be calculated to be $K_m = 4.8 \times 10^{-4}$ mM thiamine.

The influence of energy and magnesium. The influence of varying glucose concentrations on the uptake of the labelled vitamin by a given amount of

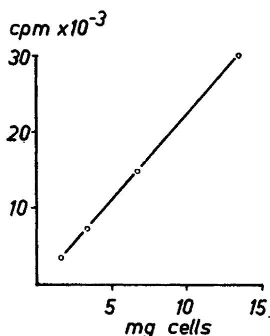

Fig. 5. The relation between the amount of cells and the rate of uptake of labelled thiamine in thiamine deficient non-proliferating cells of *L. fermenti*. ^{35}S-Thiamine 2×10^{-3} mM. Incubation (except amount of cells) as in Fig. 1.

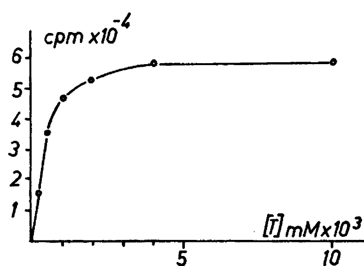

Fig. 6. The relation between the exogenous concentration of ^{14}C-thiamine and the uptake of radioactivity in thiamine deficient, non-proliferating cells of *L. fermenti*. Incubation (except concentration of ^{14}C-thiamine) as in Fig. 1.

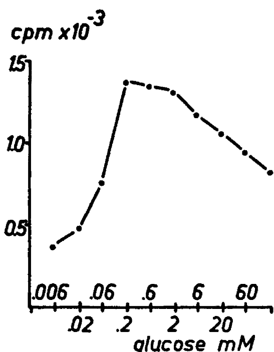

Fig. 7. The influence of varying concentrations of glucose on the uptake of ^{35}S-thiamine by thiamine deficient, non-proliferating cells of *L. fermenti.* Incubation: ^{35}S-thiamine 2.4×10^{-3} mM, other conditions as in Fig. 1.

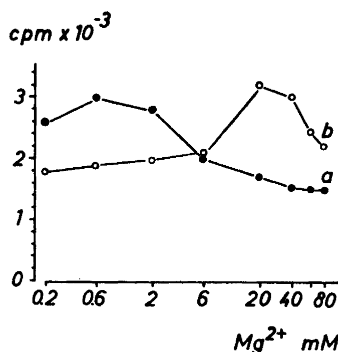

Fig. 8. The relationship between the influence of magnesium and energy on the uptake of labelled thiamine by thiamine deficient non-proliferating cells of *L. fermenti.* ^{35}S-Thiamine 2×10^{-3} mM. Incubation (except glucose and Mg^{2+}) as in Fig. 1; a, in 0.6 mM glucose; b, in 60 mM glucose.

cells is shown in Fig. 7. The initial exogenous concentration of thiamine in these experiments was 2.4×10^{-3} mM, *i.e.* in the range where the rate-substrate concentration curve began to level off (*cf.* Fig. 6). It can be seen in Fig. 7 that the largest uptakes took place in the range 0.2—6.0 mM glucose

Table 2. The influence of certain analogues and derivatives of thiamine on the uptake of ^{35}S-thiamine by thiamine deficient non-proliferating cells of *L. fermenti.*
OT, oxythiamine; PT, pyrithiamine; TP, thiamine monophosphate; TPP, thiamine diphosphate. Incubation conditions as in Fig. 1, but at 37°C and using ^{35}S-thiamine 2×10^{-3} mM.

Addition to the incubation mixture, μmoles		Ratio μmoles analogue / μmoles thiamine	cpm taken up by 4×10^8 cells
None			4669
OT	0.1	10	4522
	1.0	100	4776
PT	0.01	1	2855
	0.1	10	903
	1.0	100	381
	10.0	1000	289
TP	0.1	10	2026
	1.0	100	749
TPP	0.1	10	3530
	1.0	100	1072

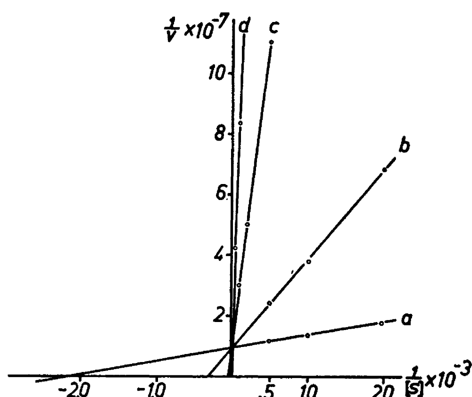

Fig. 9. The Lineweaver-Burk plots of reciprocal uptake velocity (v = μmoles thiamine taken up in 20 min by 10^8-10^9 cells) *versus* reciprocal mmolar concentration of exogenous thiamine (S). Thiamine deficient non-proliferating cells of *L. fermenti* exposed to ^{14}C-thiamine. Incubation conditions as in Fig. 1, but with following levels of pyrithiamine: a, none; b, 2×10^{-3} mM; c, 2×10^{-2} mM; d, 2×10^{-1} mM.

with a possible maximum in 0.2 mM glucose. At glucose levels higher than 6.0 mM the uptake gradually decreased.

Fig. 8 shows the relationship between the influence of magnesium and energy. It can be seen that the optimum magnesium level is much lower using 0.6 mM (curve a) than using 60 mM glucose (curve b). This indicates the occurrence of a stoichiometric relationship between the effects of glucose and magnesium.

In the absence of an exogenous energy source the uptake was approximately 8—10 times lower than in the presence of glucose, irrespective magnesium concentration.

Uptake of labelled thiamine in the presence of analogues and derivatives. Certain results of the studies on the influence of analogues and derivatives on the thiamine uptake by deficient cells are given in Table 2. It can be seen that oxythiamine at concentrations 10 and 100 times that of thiamine had a negligible effect on the thiamine uptake. Pyrithiamine, in concentrations one, ten, hundred, and thousand times that of thiamine, decreased the uptake by 39, 81, 92, and 94 %, respectively. It can be further seen in Table 2 that thiamine monophosphate and thiamine diphosphate in concentrations ten times that of thiamine decreased the radioactivity uptake by 55 and 25 %, respectively.

The effect of pyrithiamine was further studied using different levels of this inhibitor at different levels of the labelled thiamine. The results are given in Fig. 9 in the form of Lineweaver-Burk plots of reciprocal uptake velocity *versus* reciprocal concentration of exogenous thiamine. It can be seen that the curves obtained with varying levels of pyrithiamine (curves b, c, and d) and the curve obtained without inhibitor (curve a) all cut the $1/v$ axis in one point. This indicates competitive inhibition by pyrithiamine.

Miscellaneous experiments

Ascorbic acid increased the thiamine uptake by about 10 % in both deficient and sufficient cells. Results of some representative experiments are shown in Table 3.

Table 3. The influence of ascorbic acid on the uptake of ^{35}S-thiamine by non-proliferating cells of *L. fermenti*. Incubation conditions as in Fig. 1, but using ^{35}S-thiamine 0.8×10^{-3} mM.

Addition to the incubation mixture	cpm taken up by			
	Deficient cells 4×10^8		Sufficient cells 4×10^9	
	Expt No.			
	1	2	1	2
None	5473	4325	805	845
Ascorbic acid 30 μmoles	6212	4970	867	941

Table 4. The influence of sodium pyruvate on the uptake of ^{35}S-thiamine by thiamine deficient, non-proliferating cells of *L. fermenti*. Incubation conditions as in Fig. 1, but using ^{35}S-thiamine 2×10^{-3} mM.

Addition to the incubation mixture μmoles		cpm taken up by 4×10^8 cells Expt No.		
		1	2	3
None		7103	5099	4948
Na-pyruvate	1	7112		
Na-pyruvate	170	5185	3840	
Na-pyruvate	430	3078	2125	1892

Table 5. The effect of thiamine content of the growth medium on the ability of the non-proliferating cells of *L. fermenti* to take up labelled thiamine. Incubation conditions as in Fig. 1, ^{35}S-thiamine 2×10^{-3} mM.

Thiamine content in the growth medium	Radioactivity taken up by cells cpm		
	Expt. No. 1		Expt. No. 2
μg/ml	^{35}S-thiamine		^{14}C-thiamine
	without glucose	with glucose	with glucose
0.002	1840	17 520	50 000
0.02	540	4 250	36 000
0.2			31 500
1.0	172	1 135	
2.0			9 600
20	130	550	4 900
200	68	203	593

Table 6. The influence of phosphate concentration on the uptake of ^{14}C-thiamine by non-proliferating cells of *L. fermenti*. Incubation conditions (except potassium phosphate) as in Fig. 1.

Thiamine in the growth medium	cpm taken up by cells in the presence of potassium phosphate	
µg/ml	0.02 M	0.1 M
2.0	2 035	1 232
0.2	20 975	15 740
0.02	24 322	17 150
0.002	33 327	21 568

Sodium pyruvate decreased the thiamine uptake. Some characteristic results are shown in Table 4. It can be seen that when 430 µmoles sodium pyruvate were present in the incubation mixture the uptake of thiamine was only 40—50 % of that in the absence of sodium pyruvate. Only deficient cells were used in these experiments.

The influence of thiamine concentration in the growth medium. It can be seen in Table 5 (*cf.* also Table 6) that with increasing content of thiamine in the growth media the ability of the non-proliferating cells to take up labelled thiamine decreased.

The influence of phosphate. It can be seen in Table 6 that increasing the concentration of phosphate in the incubation medium from 0.02 M to 0.1 M decreased by 25—40 % the uptake of labelled thiamine in cells which had been grown at widely different thiamine concentrations. This correlates with the observation reported in Fig. 3 that the uptake in the presence of potassium phosphate buffer is much smaller than *e.g.* in Tris-HCl-KCl buffer.

Desorption studies. The results of the desorption studies are summarized in Table 7. It can be seen that in the absence or with low concentrations of exogenous thiamine only insignificant amounts of radioactivity are desorbed. Glucose, magnesium, and 0.02 M potassium phosphate buffer at pH 6.8, do not seem to enhance the exit reaction to any significant extent. On the other hand, calcium or potassium ions at 0.02 M concentrations stimulate the exit reaction even in the absence of exogenous thiamine, the effect of K^+ being to desorb 27 % and that of Ca^{2+} 22 % of the total radioactivity originally present in the cells. With increasing levels of exogenous thiamine the fraction of radioactivity desorbed increases gradually. It is more than 60 % in 0.05 M thiamine and close to 100 % in 0.5 M thiamine. Potassium phosphate was found to have a pronounced inhibiting effect on this exchange reaction, whereas other agents studied seem to be practically without effect.

Table 7. Desorption of the radioactivity retained by thiamine deficient non-proliferating cells of *L. fermenti* after 5 min exposure to labelled thiamine in an isotonic solution containing: 0.02 potassium phosphate pH 6.8, 60 mM glucose, 20 mM Mg^{2+}, 6 mM ascorbic acid. The desorption medium contained varying levels of unlabelled thiamine; desorption time: 20 min; temperature during uptake and desorption 37°C.

Addition to the desorption medium (0.15 M NaCl)	Concentration of unlabelled thiamine in the desorption medium mM					
	0	2×10^{-4}	5×10^{-4}	5×10^{-3}	5×10^{-2}	5×10^{-1}
	% desorbed					
None	0	4	15	27	67	100
Glucose 60 mM	0–16				61	
Glucose 60 mM + Mg 20 mM	5	15	18	28		
Glucose 60 mM + $MgCl_2$ 20 mM + iodoacetate + K-phosphate 0.02 M pH 6.8	0					98
K-phosphate 0.02 M pH 6.8	2–9	4–10	15			22
Tris HCl 0.02 M pH 6.8						99
KCl 0.02 M	27					97
$CaCl_2$ 0.02 M	22					

DISCUSSION

The results of this investigation confirm the earlier observations that thiamine deficient cells take up considerably more thiamine than do the sufficient ones, that the uptake process requires energy and that it is stimulated by magnesium and ascorbic acid.[6] Although the influence of magnesium seems to be related to the energy influence (*cf*. Fig. 8) it was not possible to determine the exact stoichiometric relationship between the two, since nothing is known about the endogenous magnesium level of the thiamine deficient cells and because magnesium participates in so many of the enzymatic reactions that can take place in the non-proliferating cells.

The stimulation of thiamine uptake by ascorbic acid is about 10 % (*cf*. Table 3). Ascorbic acid is known to increase the utilization of a number of B-vitamins in both micro-organisms and animals. Although several explanations have been offered, the mechanisms of these phenomena still remain

obscure. The thiamine sparing action of ascorbic acid has been reported by several authors. Fang and Butts [9] suggested that in their experiments with *L. fermenti* this sparing action might have depended on the stimulation of thiamine synthesis by ascorbic acid. According to Bánhidi the thiamine sparing action of ascorbic acid in *L. fermenti* [10,11] and in the rat [11] depends on its reducing effect on the oxidized (disulfide) forms of thiamine.

The maximum thiamine uptake observed at 50°C (Fig. 1) correlates fairly well with the optimum growth temperature of this organism as listed in the literature.[12] For practical purposes, *e.g.* in the microbiological test for thiamine, the organism is always grown at 37°C. In an earlier preliminary communication [13] the temperature 37°C was erroneously given as the maximum uptake temperature. This was due to the above (*cf.* p. 775) mentioned variations in the absolute value of the maximum uptake and also to the fact that the preliminary investigation was not extended to temperatures above 40°C.

The results of studies on the pH effect (Fig. 4) indicate that not only the hydrogen ion concentration, but also the nature and perhaps concentration of other ions present profoundly influence the rate of thiamine uptake. The stimulating effect of potassium ions is particularly interesting. Although it is generally accepted that potassium is an essential nutrient for bacterial growth, the possibility that K^+ may participate in the transport of other nutrients has been considered only recently. Abrams [14,15] provided evidence that glycolysis in the presence of K^+ promotes the entry of oligosaccharides into protoplasts of *S. faecalis*. The results of the present study indicate that K^+ stimulated the transport of a vitamin into intact bacterial cells. Results of preliminary experiments on the uptake of another vitamin, *viz.* ^{14}C-niacinamide, into intact resting cells of *S. faecalis* indicate that also this transport reaction is stimulated by K^+ (unpublished results from this laboratory).

Further indications of the involvement of K^+ in thiamine transport can be seen in Table 7. It would seem that K^+ stimulates the exit of thiamine from the cells. A similar effect is observed with Ca^{2+}.

The decrease of thiamine uptake in the presence of sodium pyruvate, Table 4, was rather unexpected because of the known function of thiamine in pyruvate metabolism. This phenomenon could not be understood. Thiamine has been reported to undergo non-enzymatic condensation reactions with pyruvate.[19] Such reactions, however, take place in media more alkaline (pH 8.8) than those employed in the present experiments (pH 5.5—6.8).

The difference in the abilities of the deficient and sufficient cells to take up thiamine from the surrounding medium [6] (*cf.* also Fig. 2) probably reflects the difference between the endogenous thiamine levels in these two kinds of cells. This is stressed by the results presented in Table 5, which demonstrate that the rate of thiamine uptake in non-proliferating cells is inversely related to the content of thiamine in the growth medium. No determinations of the thiamine content of the non-proliferating cells were carried out in the present work. In the experiments reported by Fang and Butts [9] the thiamine content of *L. fermenti* cells grown in the presence of 10 μg thiamine/l was approximately 0.010 μg or 0.3×10^{-4} μmoles thiamine per mg dry cells. The deficient cells employed in the present work were grown in the presence of concentrations of thiamine five times smaller, and their endogenous thiamine content was

probably much lower than 0.3×10^{-4} µmoles/ml dry cells (*cf.* Table 5). Judging from the average cell harvest and the concentration of thiamine in the growth medium, the endogenous thiamine content of the deficient cells could be estimated to be at the most 0.4×10^{-6} µmoles of thiamine/mg dry cells, unless some synthesis of thiamine also occurred in such cells, as suggested by Fang and Butts,[9] but never finally confirmed. The results of the present work indicate that the deficient cells were able to take up thiamine in amounts at least two hundred times their own thiamine content.

It appears from the data given in Table 2 that thiamine is taken up more easily when supplied as the free compound than when present as the monophosphate, and more easily in the latter case than when present as the diphosphate. Such a conclusion is based on the fact that the addition of the unlabelled phosphorylated derivatives did not decrease the radioactivity derived from ^{35}S-thiamine to an extent corresponding to their molar ratios. It cannot be concluded on the basis of this investigation, whether the phosphorylated derivatives can be taken up at all as such, or not. The possibility must be considered that such derivatives may become dephosphorylated before uptake, *e.g.* by the action of alkaline phosphatase which has been shown to occur on the surface of certain other bacteria, *e.g. E. coli*. In this connection it is interesting to note a recent report by Cooper *et al.*,[16] which states that it is unchanged thiamine which penetrates the lipid membranes of the spinal cord of rats. Sen and Robinson [17] have found both free and bound thiamine but none of the phosphorylated derivatives in cereals.

It can be seen in Table 2 that of the two powerful inhibitors of thiamine, *viz.* oxythiamine and pyrithiamine, the former, at least at the concentrations studied, has a negligible effect on the thiamine uptake, whereas the latter considerably decreases the radioactivity taken up by the cells. The data given in Fig. 9 demonstrate that the inhibition by pyrithiamine is of a competitive character. It is now well established that both pyrithiamine and oxythiamine act only at the pyrophosphate level as co-carboxylase antagonists in pyruvic decarboxylase and transketolase reactions; the corresponding free compounds do not affect these reactions.[18]

The results of the present investigation indicate that an antagonism between thiamine and pyrithiamine takes place during the uptake of thiamine by non-proliferating cells of *L. fermenti*. It cannot, however, be concluded whether this antagonism represents a competition for the active transport sites or for some intracellular receptors of thiamine, *e.g.* in the phosphorylating reactions. However, as shown in subsequent studies,[20] the phosphorylation of thiamine taking place rapidly after the uptake is not the factor primarily responsible for the accumulation of the vitamin in the cell. It seems therefore probable that pyrithiamine is a true antagonist of thiamine transport.

The results of the desorption studies (Table 7) demonstrate that, at appropriate concentrations, an efficient exchange of the intracellular and extracellular thiamine takes place. This is indicative, but no conclusive proof of the presence of thiamine inside the cell as the free compound.

It appears from the results reported here that the thiamine uptake by non-proliferating cells of *L. fermenti* exhibits several features of enzyme mediated reactions, *i.e.* pronounced optima with respect to pH (Fig. 4) and

temperature (Fig. 1), saturation kinetics (Figs. 5, 6, and 9) as well as stereospecificity (Table 2, Fig. 9). It is also demonstrated that the uptake reaction requires an exogenous source of energy (Figs. 7 and 8) and that the accumulation process is reversible (Table 7). These are several characteristics of an accumulation process called "active transport". However, in order to establish whether active transport of a compound takes place or not it is also necessary to demonstrate that the compound in question occurs in the same form inside the cell as well as outside and thus becomes accumulated against a true concentration gradient. Chromatographic studies to elucidate this point are in progress. On the other hand, the comparatively high sensitivity of the thiamine uptake reaction to various factors, that is reported here, strongly indicates that the uptake is not merely a result of osmotic activity.

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