

Studies of Thiamine Uptake in Growing Cultures and in Cell Fragments of *Lactobacillus fermenti*

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A study was carried out on the uptake of labelled thiamine (thiazole-2- ^{14}C) in cultures of *Lactobacillus fermenti*. For comparison some experiments included also a strain of *Staphylococcus aureus* and a strain of *Escherichia coli*.

L. fermenti grown in nutrient medium with 6×10^{-3} mM thiamine accumulated 0.4 % of the vitamin intracellularly. With lower levels of exogenous thiamine an increased proportion of it was taken up. However, not even at growth-limiting concentrations of thiamine (6×10^{-8} mM), the proportion that accumulated intracellularly during the entire growth period (37 h) exceeded 30 %.

The thiamine content of *L. fermenti* varied between 3 and 300 $\mu\text{g/g}$ dry wt., depending on the concentration in the nutrient medium. The ability to concentrate thiamine was significantly lower in the investigated strains of *E. coli* and *S. aureus*.

At high concentrations of exogenous thiamine the addition of pyrithiamine had no influence on growth, but it decreased the cell content of labelled thiamine to about 60 %. In contrast, in a thiamine deficient medium, pyrithiamine had no influence on the thiamine accumulation by the cells, but it decreased the growth rate and the final cell density.

Paper chromatography of cell extracts gave two major radioactive fractions. One of them had a mobility corresponding to thiamine diphosphate, the other was slower moving. Both fractions were susceptible to Taka-diestase.

Results of preliminary studies on binding of ^{14}C -thiamine to membrane preparations and on the effect of certain cofactors are reported.

Lactobacillus fermenti was described for the first time by Beijerinck¹ and following the proposal of Sarett and Cheldelin² the organism has been in use for thiamine assay. It must therefore be of interest to know the mechanism of thiamine uptake and metabolism in this organism.

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During recent years research on the mechanism of transport of micro-molecules across bacterial membranes has received new stimulus. Several workers have demonstrated the existence and involvement in transport of specific binding proteins. Such binding proteins have been isolated for β -galactoside³ for galactose,⁴ for sulphate^{5,6} and for leucine.⁷⁻⁹ The leucine and sulphate binding proteins have been obtained in crystalline form. The concentrative uptake of proline and glycine in isolated cell membranes of *E. coli* has been studied by Kaback and Stadtman.¹⁰

The transport systems for vitamins have been devoted much less interest than microbial transport systems for other micromolecules. Active transport of labelled thiamine (Tm) by non-proliferating cells of *L. fermenti* has been demonstrated by Neujahr.¹¹ The uptake was much higher in cells that had grown in Tm deficient nutrient media (2–20 μg Tm/l) than in cells grown in vitamin rich media. The reason for this difference was not studied. The bulk of ¹⁴C-thiamine in bacterial extracts occurred as Tm, Tm-diphosphate, and a compound moving slower than Tm-diphosphate presumably a more phosphorylated derivative.¹²

The present paper describes the uptake of labelled thiamine by growing cells of *L. fermenti* in nutrient media with different levels of the vitamin. Results of preliminary experiments on thiamine binding by isolated subcellular fractions are also reported. Certain comparative experiments were carried out with *S. aureus* and a thiamine-less mutant of *E. coli*.

MATERIALS

Bacterial strains. *Lactobacillus fermenti* 36 (ATCC 9338). The culture was maintained as described by Neujahr.¹¹ *Staphylococcus aureus* 111¹³ and *Escherichia coli* W 945 (pro⁻, thi⁻) were kept on Bacto Stock Culture Agar (DIFCO). All three organisms were cultured in the complex medium described earlier.¹¹ This medium shall be designated BT and the thiamine (Tm) concentration indicated separately.

Chemicals. Taka-diaxase, desoxyribonuclease, thiamine.HCl, pyrithiamine.HBr, were from Sigma Chem. Co., St. Louis, USA; thiamine-¹⁴C (2-thiazole), specific radioactivity 25.2 mCi/mmol, was purchased from the Radiochemical Centre, Amersham, England. Other chemicals from commercial sources were of Analar or higher purity grade.

METHODS

Quantitation of the amount of cells used in uptake experiments. Cells were washed once with saline and twice with distilled water. The washed cell suspensions were adjusted to a certain optical density (OD) at 625 nm. In introductory experiments the OD values were correlated with dry weight of bacteria and with their volume. 1 ml of washed cell suspension of OD = 1.0 corresponded to 0.49 mg dry weight and 2 μl of cell volume.

Thiamine uptake by growing cells of *L. fermenti* in the absence and in the presence of pyrithiamine. A 30 h culture of *L. fermenti* in BT with 20 μg Tm/ml was used as inoculum. The culture was centrifuged, the bacteria were washed once in fresh Tm-free medium, suspended in the same medium to OD 0.3, diluted 1 : 10 with the same medium and used as inoculum (0.5 ml) for tubes containing 10 ml BT. The tubes were fit for direct reading in a Coleman Junior Spectrophotometer. The cultures were incubated statically at 37°, and OD examined periodically at 625 nm. The BT medium contained varying amounts of ¹⁴C-Tm using duplicate cultures for each level. Simultaneously, cultures were grown with equimolar concentrations of Tm and pyrithiamine. A comparative study was carried out on ¹⁴C-Tm (2.5 \times 10⁴ dpm/ml medium) uptake in *S. aureus* and *E. coli*, grown in BT with 3.0 \times 10⁻³ mM Tm.

The radioactivity of the cultures was determined on 1 ml samples taken after incubation for 11.5, 15.5, 19.5, 24.5, and 37 h. The samples were immediately diluted in 50 ml cold saline and filtered through type HA Millipore filters (mean pore size 0.45 μ). The filters were washed once with 50 ml saline and once with 50 ml H₂O, and transferred to scintillation vials containing Bray's dioxane solution.¹⁴ Three control samples, with identical amounts of ¹⁴C-Tm, but without bacteria, served as filter controls. All values were corrected for filter absorption and for quenching. Filter absorption accounted for 0.18 \pm 0.5 % of the radioactivity input.

Extraction of ¹⁴C-thiamine from bacteria. Bacteria from 50 ml of the culture (30 h at 37°) were collected by centrifugation, and washed once with 15 ml saline, and twice with 10 ml H₂O. 0.5 ml 5 % butanol was added to the packed bacteria.¹⁵ The suspension was centrifuged and the supernatant was used for chromatography. Extracts prepared in this way contained 70–85 % of the total radioactivity of the bacteria. In certain experiments the extracts were treated with Taka-diastase. In such cases a water extract was prepared by heating the packed cells with 0.5 ml distilled H₂O for 2 min at 100° instead of treatment with butanol.

Chromatography of bacterial extracts. The extracts were examined by descending paper chromatography in the following three systems:

1. Paper: Munktell 301. Solvent: propanol : Na acetate (1 M, pH 5.0) : H₂O (7 : 2 : 1).
2. Paper: Whatman No. 1, treated with a solution of ethylene diamine tetraacetic acid (EDTA). Solvent: *sec.*-butanol : pyridine : H₂O : acetic acid (30 : 30 : 30 : 1).
3. Paper as 2. Solvent: isobutyric acid : ammonia : EDTA 0.1 M (100 : 60 : 1.6).¹⁶

Control samples of non-labelled Tm, Tm monophosphate, and Tm diphosphate were always chromatographed alongside with the bacterial extracts. In some of the experiments the control solutions were mixed with non-labelled bacterial extracts,¹⁸ but no significant influence on the R_F values was observed. The spots of non-labelled compounds were revealed under a UV-lamp after spraying with alkaline potassium ferricyanide. Chromatogram strips of the bacterial extracts were cut into fragments of 0.5 or 1.0 cm. Each fragment was soaked in 0.2 ml H₂O in a scintillation vial, and 15 ml of Bray's scintillator solution¹⁴ was added. Radioactivity was measured in a Tricarb Liquid Scintillation Spectrometer. Some of the chromatograms were also developed as autoradiograms on Kodak X-ray films at the X-Ray Department of Asaf Harofe Government Hospital, Zrifin, Israel.

Binding of ¹⁴C-Tm to subcellular fractions of *L. fermenti*

Preparation of membranes and other "binding" fractions. Some experiments were carried out with membranes prepared with subtilisin and lysozyme.¹⁷ These will be designated "enz.-membranes". Other experiments involved particulate fractions obtained from cells disrupted in "X-press" (BIOX, Nacka, Sweden).¹⁸ They will be designated as "wall-membranes". For preparation of the wall-membranes, *L. fermenti* was grown in large-scale culture in BT containing 20 μ g Tm/l. The bacteria were harvested and washed twice in saline and the pellet was frozen at -20° until disrupted. Portions of these bacteria were pressed in the X-press. The viable count of the pressed material was less than one pro mille. Processing was continued by a modification of the method described by Kabaek and Stadtman.¹⁹

The disrupted bacteria were suspended in 0.1 M potassium phosphate buffer, (pH 6.6), 20 μ g DNase/ml was added, and the suspension was incubated at 37° for 10 min with vigorous swirling. Thereafter, potassium EDTA (pH 6.6) was added to a final concentration of 5 mM, the incubation was continued for 5 min, and at last MgSO₄ was added to a final concentration of 10 mM. The suspension was centrifuged at 37 000 *g* for 20 min, resuspended in 0.1 M potassium phosphate (pH 6.6) and 10 mM potassium EDTA, centrifuged again at 37 000 *g*, the pellet was resuspended, centrifuged at 1200 *g* for 15 min, and the supernatant fluid centrifuged again at 37 000 *g*. The final pellet was suspended in a solution of 0.5 M potassium phosphate (pH 6.6). This suspension was adjusted to 0.5–5.0 mg of particle proteins per ml using various buffers.

Determination of binding was carried out by filtration or by dialysis. After incubation with labelled Tm the membranes were collected on Millipore filters and the radioactivity of the material retained on the filter as well as that of the filtrate was determined. The

completeness of the separation was tested in separate experiments by determination of the protein content of the filtrate using the method of Warburg and Christian.¹⁹

Binding of Tm by membrane preparations was also examined after dialysis in cellophane bags \varnothing 10 mm.

RESULTS

In introductory experiments the accumulation of ¹⁴C-thiamine was studied in non-proliferating cells of *L. fermenti* using the technique described earlier¹¹ and also by a modified technique involving membrane filters. With both methods, results similar to those reported earlier were obtained.¹¹ The kinetics of thiamine uptake in non-proliferating *S. aureus* 111 were similar to those in *L. fermenti*. However, the maximum amount of accumulated Tm in *S. aureus* was significantly lower than in *L. fermenti*.

Thiamine uptake by growing cultures of L. fermenti. Fig. 1 shows growth curves of cultures in BT with 6×10^{-2} , 6×10^{-5} , and 6×10^{-6} mM Tm. Variations in Tm content in the range 6×10^{-5} – 6×10^{-2} mM had very little influence on growth rate or final turbidity. However, the final turbidity decreased when the Tm concentration in the growth medium was 6×10^{-6} mM only.

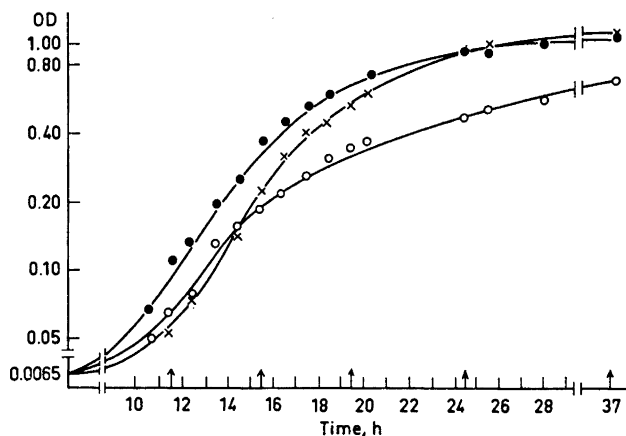


Fig. 1. Growth of *L. fermenti* in nutrient medium with 6×10^{-2} mM (\times), 6×10^{-5} mM (\bullet), and 6×10^{-6} mM (\circ) thiamine.

Figs. 2–4 illustrate various experiments to study the relation between the Tm level in the growth medium and the uptake of ¹⁴C–Tm. Fig. 2 shows the absolute uptake of Tm in relation to Tm concentration in growth medium. It is seen that with Tm levels 6×10^{-6} – 6×10^{-4} mM, the absolute uptake was highly dependent on the exogenous concentration of Tm. At higher Tm levels in the growth medium the uptake was independent. Uptake in *L. fermenti* varied between approximately 3 and 300 $\mu\text{g/g}$ dry wt., corresponding to 4.4×10^3 – 4.8×10^5 molecules per cell. Again, much lower figures were obtained with *S. aureus* and *E. coli*.

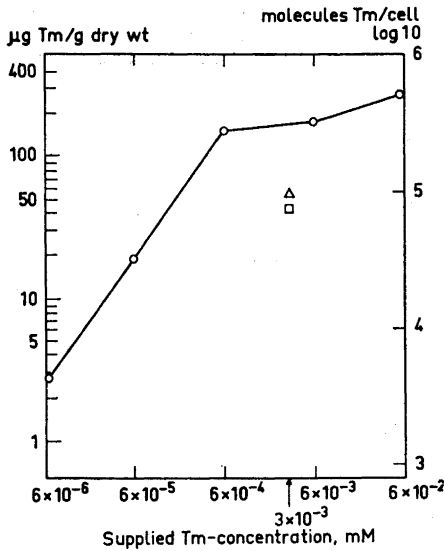


Fig. 2. Uptake of ¹⁴C-thiamine by *L. fermenti*, grown for 19.5 h in BT with the indicated concentrations of thiamine. The uptake is calculated in µg of Tm per g dry wt. and in molecules per cell, taking the dry wt. of one cell as 8×10^{-13} g. The corresponding uptake by *E. coli* W 945 (Δ) and *S. aureus* (□) is indicated for comparison.

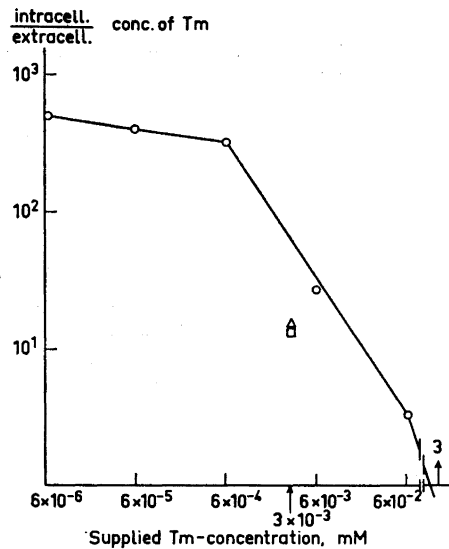


Fig. 3. Ratio intracellular : extracellular concentration of ¹⁴C-thiamine in cultures of *L. fermenti* grown in BT with different levels of thiamine. The corresponding concentrative capacity of *S. aureus* (□) and *E. coli* (Δ) is indicated.

Fig. 3 shows the ratio intracellular : extracellular concentration of Tm (including Tm-derivatives) in relation to the Tm concentration originally supplied. The bacteria grown at low level of Tm concentrated the vitamin intracellularly 500 times, whereas the cultures grown at 6×10^{-2} mM Tm concentrated it only 4 times.

It was of interest to know whether the ratio intracellular : extracellular Tm-conc. would continue to decrease with increasing concentrations of the vitamin in the growth medium and eventually become lower than 1.0. For this purpose a culture was grown in BT with 3 mM Tm. The results indicated that the intracellular vitamin concentration was significantly lower than that of the nutrient medium, but an exact determination was hampered by the necessity to use very high radioactivity inputs, which, in turn, led to high filter controls.

Fig. 4 depicts on a log-log scale the percentage of Tm taken up by the bacteria after growth for 19.5 h in media with different Tm levels. After that time the cultures were well in the stationary phase of growth. It is seen that not more than approximately 38 % of the exogenously supplied Tm can be taken up by *L. fermenti*. Cells grown with the lowest levels of Tm (6×10^{-6} mM) accumulated only 30 % of it. In the range 6×10^{-4} – 6×10^{-2} mM Tm, the percentage of Tm taken up was more or less inversely proportional to the

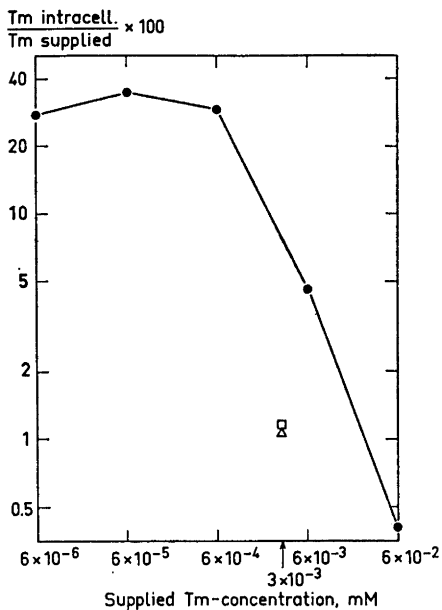


Fig. 4. Proportion of ¹⁴C-thiamine taken up by *L. fermenti*, grown for 19.5 h in BT, with the indicated concentrations of the labelled vitamin. One corresponding value for *S. aureus* 111 (□) and one for *E. coli* W 945 (△) are indicated.

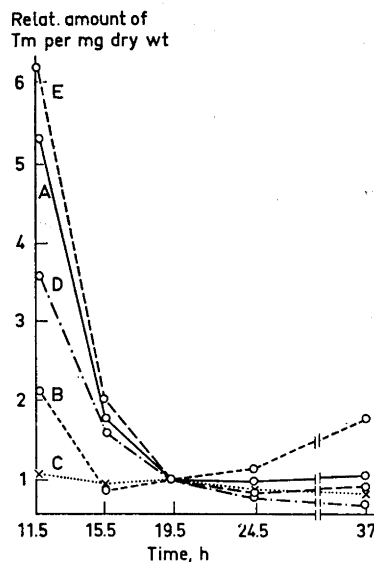


Fig. 5. Proportion of ¹⁴C-thiamine taken up by *L. fermenti* during growth in BT, containing initially following mM levels of thiamine: A, 6×10^{-2} , B, 6×10^{-3} , C, 6×10^{-4} , D, 6×10^{-5} , E, 6×10^{-6} . Uptake after 19.5 h is taken as 1.0.

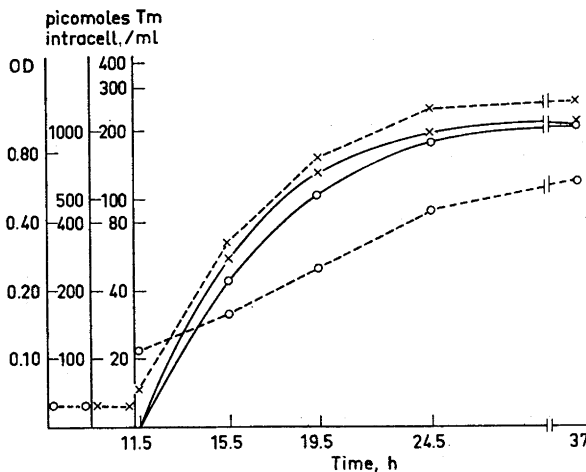


Fig. 6. Growth (solid lines) and thiamine uptake (dashed lines) by *L. fermenti* grown in BT with initial levels of 6×10^{-2} mM (O) and 6×10^{-4} mM (x) thiamine.

Tm-concentration in the growth medium. When this concentration was 6×10^{-2} mM, only about 0.4 % of the exogenous Tm was taken up by *L. fermenti*. Cultures of *S. aureus* and *E. coli* accumulated considerably less ^{14}C -Tm. When grown with 3×10^{-3} mM Tm only about 1 % of the label was taken up by these cultures, *i.e.* approximately six times less than by *L. fermenti*.

The results reported in Figs. 2–4 refer to cultures taken after growth for 19.5 h. Relative uptake at other times of growth taking the uptake after 19.5 h as 1.0 is shown in Fig. 5. It is seen that with highest (6×10^{-2} mM) and lowest (6×10^{-6} mM) concentrations of Tm in the growth medium uptake was particularly high in young cultures (11.5 h).

Fig. 6 depicts variations in the intracellular Tm concentration during growth of the cultures with 6×10^{-2} and 6×10^{-4} mM Tm in the medium. It can be seen that growth and uptake closely follow each other in the culture grown at the lower Tm-level. However, at the higher level of Tm growth considerably exceeds the Tm uptake.

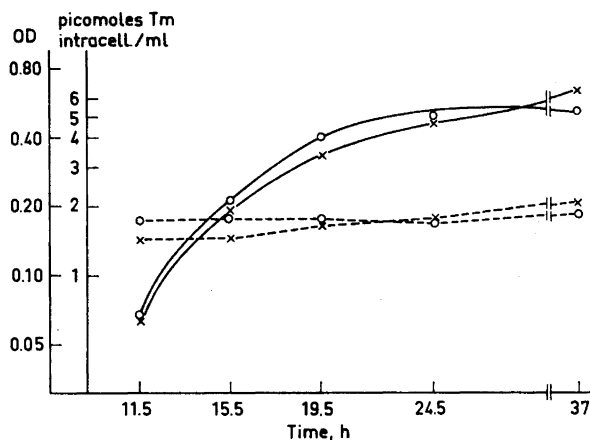


Fig. 7. Growth (solid lines) and thiamine uptake (dashed lines) by two simultaneous cultures of *L. fermenti* grown in BT with an initial level of 6×10^{-4} mM thiamine.

Fig. 7 shows a corresponding relation for the cultures grown at 6×10^{-6} mM. Whereas the graphs in Fig. 6 represent the means of two parallel cultures grown with the same Tm-level, the parallel cultures in Fig. 7 are recorded separately in order to emphasize the similarity between them.

It appears from Fig. 7 that the young culture (11.5 h) has already accumulated a maximum amount of Tm, and during further growth this Tm is diluted until growth eventually is arrested after three generations. This feature is particularly difficult to understand since only 30 % of the vitamin is accumulated intracellularly (Fig. 4).

Table 1 summarizes the influence of pyrithiamine on growth and accumulation of ^{14}C -Tm. Pyrithiamine was added in concentrations equimolar to those of Tm. In Tm rich media (6×10^{-4} – 6×10^{-2} mM Tm) the influence of pyri-

Table 1. Influence of pyrithiamine on growth and thiamine content of *L. fermenti* grown in nutrient medium with different thiamine levels. Pyrithiamine and thiamine at equimolar concentrations.

Thiamine conc. in medium mM	Relative intracell. thiamine conc. (+ pyrithiamine/- pyrithiamine). Mean of five determinations	Influence on growth curve
6×10^{-2}	0.660	None
6×10^{-3}	0.599	None
6×10^{-4}	0.568	None
6×10^{-5}	0.750	None
6×10^{-6}	0.905	Growth inhibition

thiamine on Tm uptake was more or less the same as that one would expect from the addition of an equimolar amount of unlabelled Tm. Under these conditions, growth was unaffected by the antivitamin. As the concentration of Tm in the medium decreased towards growth limiting levels (6×10^{-6} mM Tm), the Tm uptake seemed less and less affected by the antivitamin. However, in the culture grown with 6×10^{-6} mM Tm, both growth rate and final turbidity decreased (the latter by about 25 %).

Chromatography of extracts from cells of L. fermenti grown with ^{14}C -thiamine. Extracts were examined from cells grown with different levels of Tm. The results are summarized in Table 2. Two major radioactive fractions were obtained from extracts of *L. fermenti* grown in BT with 6×10^{-4} , 6×10^{-3} , and 6×10^{-2} mM Tm. In Solvent 1 they overlapped each other, one having R_F about 0.1, and the second being virtually immobile. In Solvent 2 the faster moving fraction was not discernible from Tm-diphosphate. The slower moving fraction may thus represent a higher phosphorylated derivative of Tm.

The extract of the culture grown with 6×10^{-3} mM Tm showed, in addition, a radioactivity peak corresponding to $R_F=0.535$, and a few smaller peaks with higher R_F 's, possibly from Tm-breakdown products.

Extracts from bacteria grown with 6×10^{-5} and 6×10^{-6} mM Tm contained only very low radioactivity and no peaks were obtained. Chromatography of extracts from larger amounts of cells was not successful.

Chromatography in Solvent 3 (isobutyric acid : ammonia : EDTA) gave also one peak co-chromatographing with Tm-diphosphate and a slower moving shoulder.

Treatment of water extracts of the bacteria with Taka-diestase caused, already after 10 min, a disappearance of the radioactivity peak at the application point, and after 2 h the peak with R_F corresponding to Tm-diphosphate also disappeared; new peaks with R_F values very close to that of Tm appeared (Fig. 8).

Binding of ^{14}C -thiamine by membrane preparations of *L. fermenti*

Membranes separated by filtration. In preliminary experiments the optimum assay conditions were investigated. Difficulties with filter clogging could be

Table 2. Paper chromatography of extracts from *L. fermenti* after growth with different levels of ^{14}C -thiamine. Solvent 1: propanol : Na-acetate : H_2O . Solvent 2: *sec.*-butanol : pyridine : H_2O : acetic acid.

Level of ^{14}C -Tm in growth medium		Cell extracts	
mM	Peak No.	$R_F(1)$	$R_F(2)$
6×10^{-3}	1	0.0	0.16
	2	0.1	0.23
6×10^{-3}	1	0.0	0.15
	2	0.1	0.24
	3	0.5	Not examined
6×10^{-4}	(4-6)	(> 0.5)	Not examined
	1	0.0	Not examined
6×10^{-4}	2	0.1	Not examined
	6×10^{-4}	Excessive tailing; separation not successful	
Reference substances		$R_F(1)$	$R_F(2)$
TmPP		0.11	0.24
TmP		0.26	0.32
Tm		0.49	0.57

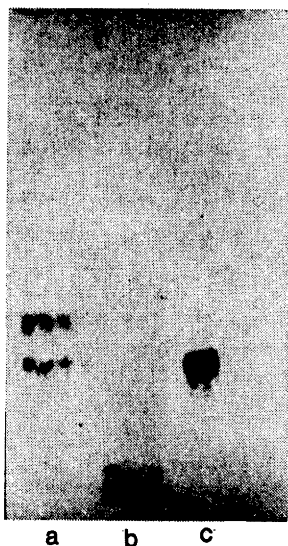


Fig. 8. Autoradiogram of an extract from *L. fermenti* after growth in BT with ^{14}C -thiamine. Solvent system 1 (propanol : Na-acetate : H_2O). Start at the bottom of the figure. a, labelled thiamine (the slower moving band). b, cell extract. c, cell extract after treatment with Taka-diastase.

overcome by the addition of DNase. Filtration of a 4 ml sample without DNase could take up to 15 min. As much as 20–33 % of the total protein then passed into the filtrate. Samples treated with DNase were filtered in less than 1 min; only 10–14 % of the protein passed into the filtrate.

The membrane preparations bound significant amounts of the label from ^{14}C -thiamine. The binding was considerably lower in K-phosphate-Mg buffer than it was in Tris-Mg buffer and it was somewhat lower in membranes prepared enzymatically (from Tm rich bacteria) than it was in membranes prepared by disruption of cells.

Table 3. Binding of ^{14}C -thiamine to wall-membranes from *L. fermenti*.

The membranes were separated on Millipore filters.				
A. Input 82.781 cpm (100 %)		B. Input 19.171 cpm (23 %)		
Addition		Binding cpm/mg protein		$\frac{B}{A} \times 100$
		A	B	
None		413	296	72
Glucose	0.12 M	446	300	67
ATP	0.25 M	197	56	28
PEP	0.25 M	405	Not examined	—

Table 3 summarizes some results of the binding experiments using two different levels of input radioactivity. With no addition or when glucose was added the binding decreased to 72 % when the input radioactivity was reduced to 23 %. ATP, added at a concentration of 0.25 M, significantly decreased the binding. Phosphoenolpyruvate was seemingly without effect.

Membranes separated by dialysis. Also the results obtained in dialysis experiments indicated binding of labelled thiamine to the membranes preparations. In these experiments the binding of ^{14}C -thiamine to wall-membrane was compared with the binding to human serum albumin and to trypsin. The binding to serum albumin was approximately 60 %, and the binding to trypsin 22 % of that obtained with the membrane preparations (data not reported).

DISCUSSION

According to our results the content of thiamine (including thiamine derivatives) in *L. fermenti* may vary over a hundredfold scale (3–300 μg thiamine per g dry wt.) depending on the concentration in the medium. Bacteria grown in a thiamine rich medium must therefore be able to multiply without thiamine to 100 times the original cell amount. This implies that an initial inoculum of OD 0.01 can develop to OD 1.00. This emphasizes the fact that bioassay of thiamine with *L. fermenti* is of no value, unless the inoculum culture has been extensively depleted of thiamine. The range of thiamine

content found in this study covers that reported by other authors for *L. fermenti*²⁰ and for *E. coli*.²¹

The intracellular thiamine content of *L. fermenti* varied in some of our cultures with their age, being particularly high during the early growth phase (11.5 h), when OD was 0.1 or less. This was especially pronounced in cultures grown with very high or very low levels of thiamine (Fig. 5). However, some of the observed differences may be due to different cell sizes in young and old cultures and the resulting variations in the relationship between OD and the amount of cytoplasm. Such an explanation, alone, would not suffice in the cases where 5–6 times more intracellular thiamine was found in the 11.5 h culture than at 19.5 h (Fig. 5).

The high vitamin content of young cells in cultures grown in a vitamin rich medium could be understood assuming that the content was related to the function of an energy dependent specific uptake system. The synthesis of this system could possibly be repressed by high concentrations of the vitamin. In this case, the young cells from the inoculum would have more of the uptake system than after adaptation to the vitamin-rich surrounding, and during growth the amount of "uptake material" would be diluted. This would be consistent with the fact that Neujahr¹¹ found uptake by non-proliferating cells grown in vitamin-rich medium manyfold lower than by cells from a thiamine-poor culture. The rate of uptake was higher, however, in the former, a fact contradicting strongly the possibility of this kind of regulation.

The results presented in this report could also be explained in another way. If an energy-dependent "uptake system" is synthesized constitutively, the amount per cell may be lower in an exponentially growing than in a young culture, since synthesis of the concerned proteins probably cannot effectively cope with the dilution due to growth.²² In older cultures, the limiting factor may be the amount of energy reserves for the uptake activity.

The relatively high initial intracellular thiamine level in the culture grown with the lowest thiamine content may reflect a need for a threshold concentration of thiamine for function of the uptake system. As seen in Fig. 7, the cells seem to grow without accumulating more of the vitamin. They only seem to share the amount taken up by the young cells. This in spite of the fact that about 70 % of the vitamin from the medium seems to be left untouched by the bacteria. It is also possible that cells growing under thiamine-limiting conditions become more and more poor in energy sources necessary for concentrative uptake against a steep concentration gradient (Fig. 7).

Observations with cultures grown in thiamine-pyrithiamine media (Table 1) indicate that in Tm-rich media the uptake system does not discriminate seriously between the vitamin and the antimetabolite. At growth limiting thiamine levels, the addition of the analogue inhibited growth, whereas it seemed without measurable effect in all other cultures. This could be explained on the assumption that thiamine and pyrithiamine share a common transport system, which has a different affinity for the two analogues at growth limiting thiamine levels than it has in thiamine rich media. Another explanation would be that in severe thiamine (cocarboxylase) deficiency the synthesis of the

apoenzyme is either impaired or not extensive enough to take care of both, the thiamine and the pyriothiamine containing "coenzymes".

Chromatography of cell extracts showed two major fractions, one corresponding to thiamine pyrophosphate, the other with lower mobility (*cf.* Fig. 8). Both fractions were attacked by Taka-diastase to produce material with the mobility of thiamine or even faster. The mobility of the slowly moving fraction is similar to that found earlier in extracts of non-proliferating cells of *L. fermenti* labelled with ^{14}C -thiamine.¹² This slowly moving fraction may be a higher phosphorylated thiamine derivative, possibly a triphosphate.²³

Our observations on binding of thiamine by subcellular fractions are of a preliminary character. It is interesting, however, that binding by isolated "membranes" was significantly lower in a phosphate buffer than in Tris, a fact reminding of the results with whole cells.¹¹ Low concentrations of glucose, phosphoenolpyruvate and ATP had no influence on binding, whereas high concentrations of ATP interfered negatively (*cf.* Table 3). The latter observation correlates fairly well with the recent report that ATP, at comparatively high concentrations, is bound to membranes from *L. fermenti* and *S. faecalis*.¹⁷ The interference of ATP with the binding of ^{14}C -thiamine to membranes from *L. fermenti* would thus indicate that the bound species is an analogue of ATP, perhaps thiamine triphosphate?

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