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Mechanism of Folate Transport in *Lactobacillus casei*: Evidence for a Component Shared with the Thiamine and Biotin Transport Systems

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Lactobacillus casei cells have been shown previously to utilize two separate binding proteins for the transport of folate and thiamine. Folate transport, however, was found to be strongly inhibited by thiamine in spite of the fact that the folate-binding protein has no measurable affinity for thiamine. This inhibition, which did not fluctuate with intracellular adenosine triphosphate levels, occurred only in cells containing functional transport systems for both vitamins and was noncompetitive with folate but competitive with respect to the level of folate-binding protein. Folate uptake in cells containing optimally induced transport systems for both vitamins was inhibited by thiamine (1 to 10 μM) to a maximum of 45%; the latter value increased to 77% in cells that contained a progressively diminished folate transport system and a normal thiamine system. Cells preloaded with thiamine could transport folate at a normal rate, indicating that the inhibition resulted from the entry of thiamine rather than from its presence in the cell. In a similar fashion, folate (1 to 10 μM) did not interfere with the binding of thiamine to its transport protein, but inhibited thiamine transport (to a maximum of 25%). Competition also extended to biotin, whose transport was strongly inhibited (58% and 73%, respectively) by the simultaneous uptake of either folate or thiamine; biotin, however, had only a minimal effect on either folate or thiamine transport. The nicotinate transport system was unaffected by co-transport with folate, thiamine, or biotin. These results are consistent with the hypothesis that the folate, thiamine, and biotin transport systems of *L. casei* each function via a specific binding protein, and that they require, in addition, a common component present in limiting amounts per cell. The latter may be a protein required for the coupling of energy to these transport processes.

Transport of folate (9, 11, 13) and thiamine (10, 13) into *Lactobacillus casei* is mediated by two separate and specific binding proteins that have been solubilized from *L. casei* membranes, purified to homogeneity, and characterized (12, 13). Both proteins are extremely hydrophobic, contain no carbohydrate, have relatively low molecular weights (25,000 and 29,000, respectively), and bind equimolar quantities of the respective vitamins. The amount of each protein is regulated by the concentration of vitamin in the medium used to propagate the cells. The folate-binding protein is maximally induced at growth-limiting levels (1 nM) of folate, is repressed by 50% at 55 nM folate, and is not detectable at vitamin concentrations above 1 μM. The amount of thiamine-binding protein can be induced and repressed similarly (50% reduction at 17 nM thiamine) by varying the concentration of thiamine in the growth medium. Variations in thiamine concentration have no effect upon the amount of folate-binding protein, and vice versa.

The similarities between these two proteins suggested that they might also utilize a similar mechanism for transport of the vitamins and that, if an additional component were involved in the mechanism, it might be shared by the two systems. Accordingly, co-transport of these (and two other) vitamins into *L. casei* was examined under various conditions, and the results of the experiments have provided evidence for the existence of such a common component. The latter appears to be functionally rate-limiting in the transport process, since it cannot accommodate simultaneously optimal operation of both folate and thiamine transport.

MATERIALS AND METHODS

Radiolabeled vitamins. [3',5',9(*n*)-^{3}H]folate (500 mCi/mmol), [*thiazole*-2-^{14}C]thiamine (14 mCi/mmol), D-[*carbonyl*-^{14}C]biotin (39 mCi/mmol), and [carboxyl-

^{14}C]nicotinate (61 mCi/mmol) were purchased from Amersham/Searle. [^{3}H]folate was diluted with unlabeled folate to a final specific activity of 150 mCi/mmol. Radioactivity was measured in Beckman HP scintillation fluid.

Growth of cells. *L. casei* subsp. *rhamnosis* (ATCC 7469) was grown from a 1% inoculum for 16 h at 30°C in the basic medium described by Flynn et al. (6), except that the prescribed amounts of folate, thiamine, biotin, and nicotinate were varied. Transport systems were induced by growth of the cells in the presence of the following concentrations (9–11, 13) of vitamin: folate transport, 5 nM folate; thiamine transport, 0.5 nM thiamine (endogenous level in medium); biotin transport, 0.5 nM biotin; and nicotinate transport, 2 μM nicotinate. Repression of individual transport systems was achieved by addition of 5 μM folate, 5 μM thiamine, 1 μM biotin, or 50 μM nicotinate, respectively. Cells having the capacity to transport a single vitamin or any combination of the four vitamins could be obtained via these growth conditions.

Binding and transport determinations. Cellular binding and transport of folate, thiamine, biotin, and nicotinate were measured essentially as described previously (10). Cells (7×10^8/ml) were suspended in 0.1 M potassium phosphate, pH 7.5, in a final volume of 0.95 ml, and labeled vitamins (0.05 ml) were added to a concentration of 1.0 μM. Suspensions of cells employed for transport determinations contained 5 mM glucose and were preincubated for 5 min at 30°C prior to the addition of labeled substrate. Initial rates of transport (at 30°C) were measured over a 5-min interval for folate and nicotinate, a 2-min interval for thiamine, and a 4-min interval for biotin. Results are expressed in nanomoles per minute per 10^{10} cells (10^{10} cells ≃ 2.2 mg of dry weight).

ATP levels and L-lactate production. Assay mixtures, consisting of 7×10^8 cells in 1.0 ml of 0.1 M potassium phosphate, pH 7.5, containing 5 mM glucose and additions as indicated, were incubated for 5 min at 30°C. ATP was measured by the luciferin-luciferase assay of Cheer et al. (5) in cell extracts prepared as described by Forrest and Walker (7). L-Lactate was measured enzymatically (8) with the cell supernatant, fluids obtained after centrifugation ($12,000 \times g$) for 5 min at 4°C.

RESULTS

Effect of thiamine upon folate transport. *L. casei* cells containing a functional folate transport system and a nonfunctional thiamine transport system (F^+T^- cells) were obtained by growing the cells in the presence of a low level (5 nM) of folate and a high level (5,000 nM) of thiamine (see Materials and Methods). In agreement with previous results (9, 11), these cells displayed optimal folate-binding (0.45 nmol/10^{10} cells) and folate-transporting (0.32 nmol/min per 10^{10} cells) activities. The presence of thiamine (1 μM) in the external medium did not affect either the binding of folate to these cells (0.48 nmol/10^{10} cells) or their ability to transport folate (Fig. 1, curves 1 and 2).

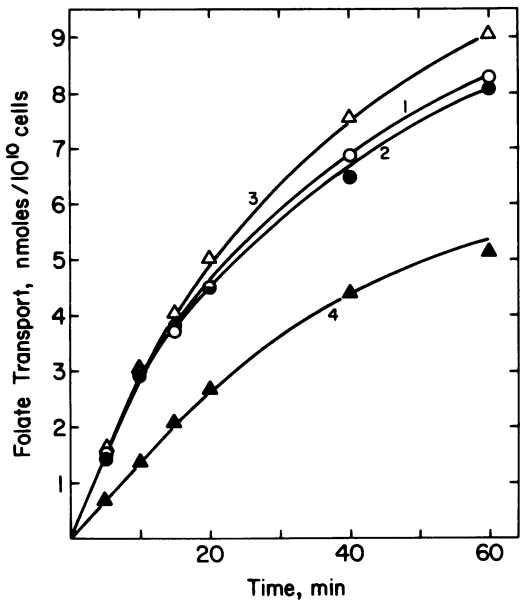

FIG. 1. *Effect of thiamine on folate transport in cells lacking or possessing the ability to transport thiamine. Folate transport was measured as described in Materials and Methods. (1) F^+T^- cells. (2) F^+T^- cells plus 1 μM thiamine. (3) F^+T^+ cells. (4) F^+T^+ cells plus 1 μM thiamine.*

In a parallel experiment, cells were grown on limiting levels of both folate (5 nM) and thiamine (0.5 nM) in order to induce functional transport systems for both vitamins (F^+T^+ cells). The folate-binding and folate-transporting activities of these cells were virtually the same as those of the F^+T^- cells. Folate transport in the F^+T^+ cells, however, was inhibited by the presence of thiamine in the external medium (Fig. 1, curves 3 and 4). In six separate determinations, the inhibition by 1 μM thiamine (a concentration sufficient to saturate the thiamine transport system [10]) averaged 45%, and was not increased by higher levels (10 μM) of the vitamin. Thiamine was also determined to be noncompetitive with folate from an analysis of a double-reciprocal plot of folate transport as a function of folate concentration in the presence and absence of 1 μM thiamine (data not shown).

Since F^+T^+ cells were capable of transporting thiamine at an appreciable rate, it was of interest to ascertain whether inhibition of folate transport by thiamine (see Fig. 1) was due to intracellular accumulation of the latter vitamin. Cells preloaded with thiamine (internal concentration, 40 μM) showed essentially no impairment of folate uptake, whereas 1 μM thiamine added externally inhibited folate transport by 49% (Table 1).

The inhibitory effect of thiamine upon folate transport was not caused by interference with glycolysis (the principal source of ATP in *L. casei* [14]), nor by depletion of the ATP pool as a consequence of the demands made by the simultaneous transport of thiamine (Table 2, experiment A). In addition, a fourfold elevation in the cellular ATP level (by depletion of energy reserves prior to preincubation with glucose) did not alter the ability of thiamine to inhibit folate transport (Table 2, experiment B).

Dependence of the extent of thiamine inhibition upon the level of the folate transport system. Cells maximally induced for thiamine transport were grown on various concentrations of folate (5 to 150 nM) to progressively reduce the level of the folate transport system. As shown in Table 3, these cells exhibited a progressively diminished capacity to bind and transport folate. When folate transport was tested in the presence of the concurrent transport of thiamine, the inhibition by thiamine increased from 42% to 77% as the level of the folate transport system decreased. The folate transport rates observed in the absence and presence of thiamine were also plotted in double-reciprocal form as a function of folate-binding protein concentration (Fig. 2). Two additional points of interest were noted: (i) folate transport did not increase linearly with the level of folate-binding protein, but, instead, reached a maxi-

TABLE 1. *Folate transport in F^+T^+ cells preloaded with thiamine*[a]

Pretreatment	Assay addition	Folate transport[b]	Inhibition (%)
None	None	0.37	—
	Thiamine	0.19	49
Thiamine	None	0.35	5

[a] For the pretreatment, F^+T^+ cells (in 20 ml of 0.1 M potassium phosphate, pH 7.5) were incubated (10 min, 30°C) with glucose and either no addition or 1 μM thiamine. The cells were then centrifuged, washed (at 4°C) with 20 ml of the same buffer, and incubated for 2 min at 30°C (to internalize membrane-bound thiamine) prior to measurement of folate transport. Thiamine added to assay, 1 μM.

[b] Expressed as nanomoles per minute per 10^{10} cells.

TABLE 2. *Effect of thiamine on folate transport, lactate production, and ATP levels in F^+T^+ cells*

Expt[a]	Assay addition	Folate transport[b]	Lactate production[b]	ATP levels[c]
A	None	0.34	960	22.2
	Thiamine	0.18 (47)[d]	880	21.5
B	None	0.37	900	82.9
	Thiamine	0.20 (46)	900	84.1

[a] Experiment A, control cells; experiment B, cells preincubated (in 0.1 M potassium phosphate, pH 7.5) for 1 h at 23°C prior to measurement of the indicated parameters. Thiamine, 1 μM.

[b] Expressed as nanomoles per minute per 10^{10} cells.

[c] Expressed as nanomoles per 10^{10} cells.

[d] Percent inhibition.

TABLE 3. *Effect of thiamine on folate bound and transported by thiamine-transporting cells grown in the presence of various levels of folate*

Folate in growth medium (nM)	Assay addition	Folate-binding protein[a]	Folate transport[b]
5	None	0.45	0.31
	Thiamine[c]	0.48	0.18 (42)
25	None	0.20	0.22
	Thiamine	—[d]	0.11 (50)
60	None	0.03	0.082
	Thiamine	—	0.022 (73)
150	None	0.01	0.026
	Thiamine	—	0.006 (77)

[a] Expressed as nanomoles per 10^{10} cells.

[b] Expressed as nanomoles per minute per 10^{10} cells. Numbers in parentheses show the percent inhibition.

[c] Thiamine, 1 μM.

[d] —, Not determined.

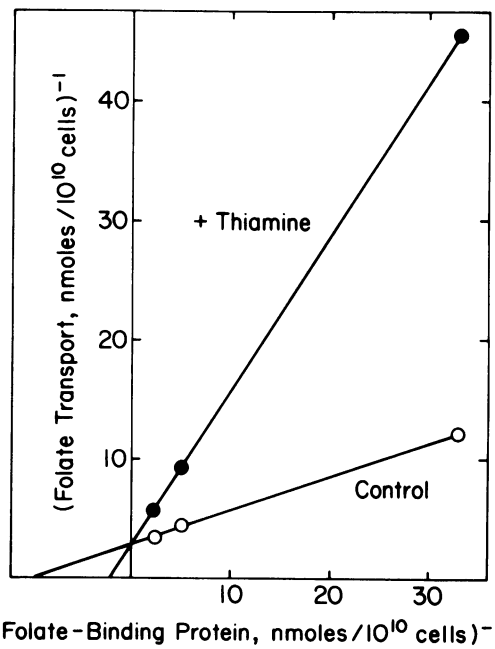

FIG. 2. *Double-reciprocal plot of folate transport in the absence and presence of thiamine as a function of folate-binding protein concentration. For experimental details, see Table 3.*

mum (0.36 nmol/min per 10^{10} cells), and (ii) inhibition of folate transport by thiamine was competitive with respect to the folate-binding protein. Since thiamine did not compete directly with folate binding (see Table 3), it appeared that the complex formed between thiamine and its binding protein was competing with the function of the corresponding folate-binding protein-folate complex.

Effect of folate upon thiamine transport. Cells containing a thiamine transport system and either a nonfunctional or functional folate transport system (F^-T^+ or F^+T^+ cells) were used to examine the effects of folate upon thiamine transport. Folate in the assay medium had no effect upon either thiamine binding (0.70 nmol/10^{10} cells) or transport (0.35 nmol/min per 10^{10} cells) in F^-T^+ cells. In F^+T^+ cells, however, folate depressed thiamine transport (Fig. 3) without interfering with its binding (data not shown). A maximum inhibition of 25% could be achieved at either 1 or 10 μM folate, concentrations sufficient to saturate the folate transport system (9, 11).

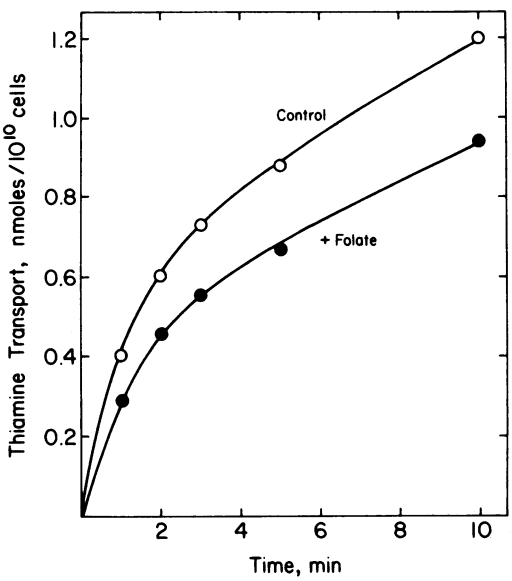

FIG. 3. *Effect of folate on thiamine transport in F^+T^+ cells. Thiamine transport in the absence and presence of folate (1 μM) was measured as described in Materials and Methods.*

Effect of folate and thiamine upon the transport of biotin and nicotinate. Since the inhibition of folate transport by thiamine (and vice versa) appeared to be at the level of the transport systems, the ability of folate and thiamine to interfere with the transport of other vitamins was investigated. Biotin and nicotinate were selected for this purpose, since these compounds had been shown previously to be transported into *L. casei* (G. B. Henderson and E. M. Zevely, Fed. Proc. **35**:1357, 1976). Cells in one case were grown on excess folate and thiamine and limiting levels (0.5 nM) of biotin to induce only the biotin transport system (K_t = 0.05 μM). In these $F^-T^-B^+$ cells, both the binding (data not shown) and transport (Table 4, experiment A) of biotin were unaffected by the presence of folate and thiamine. Conversely, in cells competent to transport all three vitamins ($F^+T^+B^+$ cells), folate and thiamine severely depressed biotin transport (Table 4, experiment B). Biotin, however, had little effect (2 to 10% inhibition) upon the transport of either folate or thiamine. In contrast, nicotinate transport occurred independently of the folate and thiamine uptake processes. In $F^+T^+N^+$ cells, neither folate nor thiamine was able to inhibit nicotinate transport (Table 4, experiment C). Similarly, nicotinate had no effect on folate or thiamine transport.

Relationship between amounts of binding proteins and susceptibility of transport systems to inhibition. Examination of the

TABLE 4. *Biotin and nicotinate transport in the absence and presence of folate or thiamine*

Expt	Cell type	Transport			
		Substrate[a]	Addition[a]	Rate[b]	Inhibition (%)
A	$F^-T^-B^+$	Biotin	None	0.083	—
			Folate	0.080	4
			Thiamine	0.090	−8
B	$F^+T^+B^+$	Biotin	None	0.083	—
			Folate	0.035	58
			Thiamine	0.022	73
C	$F^+T^+N^+$	Nicotinate	None	0.44	—
			Folate	0.44	0
			Thiamine	0.42	5

[a] Substrate and additions, 1 μM.
[b] Nanomoles per minute per 10^{10} cells.

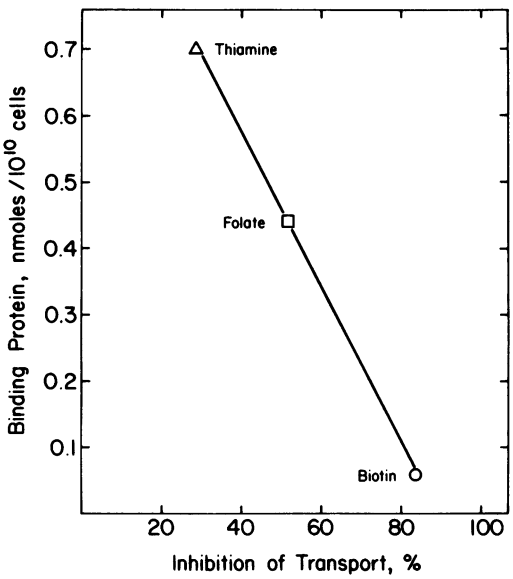

FIG. 4. *Relationship between amounts of binding proteins and inhibition during co-transport of folate, thiamine, and biotin. For each vitamin, the concentration of binding protein for that vitamin is plotted as a function of the observed transport inhibition (combined) by the two remaining vitamins.* (△) *Thiamine-binding activity vs. inhibition of [^{14}C]thiamine transport by folate and biotin.* (□) *Folate-binding activity vs. inhibition of [^{3}H]folate transport by thiamine and biotin.* (○) *Biotin-binding activity vs. inhibition of [^{14}C]biotin transport by folate and thiamine. $F^+T^+B^+$ cells; vitamin concentrations, 1.0 μM.*

above data revealed that the sensitivity of the folate, thiamine, and biotin transport systems to inhibition by the other vitamins was inversely proportional to the amount of binding protein present in the membrane. Thus, a straight line was obtained when the amounts of the thiamine-, folate- and biotin-binding proteins present in $F^+T^+B^+$ cells (0.70, 0.45, and 0.06 nmol/10^{10} cells, respectively) were plotted against percent inhibition of the transport of each vitamin by a combination of the other two vitamins (Fig. 4).

DISCUSSION

An understanding of membrane transport requires a knowledge of the protein components that comprise specific systems. Progress towards this goal has been achieved for the folate and thiamine transport systems in *L. casei* by the identification, isolation, and characterization of high-affinity binding proteins that mediate folate (11–14) and thiamine (10, 13) transport, respectively. These proteins are integral membrane components and may thus act not only as the receptors but also as the carriers of these vitamins across the membrane. Although the concentrative uptake of folate or thiamine might be achieved solely via these binding proteins, the participation of other membrane components in this presumably complex transport process is suggested by analogous studies in other cells. In *Escherichia coli*, for example, monosaccharide transport via the phosphotransferase system requires the participation of at least three proteins: a binding protein to confer specificity and two other components that are involved in energy coupling (15, 16). Other transport systems in *E. coli* also consist of multiple protein components (1, 18, 19), although the functions of most of these proteins have not yet been elucidated. Membrane components have also been reported to be shared by more than one transport system (15, 17).

Similar properties of transport systems may also be indicative of similar transport mechanisms. It has been suggested (3, 4), although recent conflicting evidence has questioned this hypothesis (21), that periplasmic binding proteins in *E. coli* function in transport systems energized primarily by ATP hydrolysis, and that integral membrane binders function solely in response to the energized membrane state (reviewed in 20). Similarly, it was reasoned that the folate- and thiamine-binding proteins of *L. casei*, which show close structural similarities (12, 13), might function via a common mechanism. Pursuing this possibility, the kinetics of folate and thiamine co-transport into the cells were examined. The results of these experiments suggest that the folate, thiamine, and biotin transport systems of *L. casei*, which are separate and specific entities, share a common, essential component. The observations which support this conclusion are as follows. (i) Folate transport was inhibited by thiamine, but only in cells containing transport systems for both folate and thiamine (Fig. 1). The converse likewise applied to folate inhibition of thiamine transport (Fig. 3). (ii) Maximum inhibition of folate transport occurred at thiamine concentrations sufficient to saturate the thiamine transport system; this was also true for inhibition of thiamine transport by folate. (iii) Competition between folate and thiamine did not occur at the binding sites of the respective transport proteins, but rather at a subsequent step in the transport process. (iv) The passage of thiamine into the cells, not simply the presence of thiamine on either side of the membrane, was necessary to achieve inhibition of folate transport (Table 1). (v) Biotin transport was strongly inhibited by co-transport of either folate or thiamine (Table 4), whereas

folate and thiamine transport, for reasons discussed below, were relatively insensitive to inhibition by biotin. Competition among vitamin transport systems is not universal, since nicotinate, which is rapidly transported into *L. casei*, did not interfere with either folate or thiamine transport (Table 4). Similarly, nicotinate transport was not affected by either folate or thiamine. This latter result could signify that nicotinate is transported in *L. casei* via a different mechanism than that operative for folate, thiamine, and biotin.

The identity and function of the postulated component common to the folate, thiamine, and biotin transport systems has not been delineated. The present results, however, can be explained provided that each transport system contains, in addition to a specific (inducible) binding protein, a second common protein component that is synthesized constitutively by the cells and is required for the coupling of energy to the transport of folate, thiamine, and biotin. This energy-coupling factor (ECF) interacts directly with each of the binding proteins, but is present in insufficient amounts to permit maximum transport rates for combinations of folate, thiamine, and biotin. Thus, when folate and thiamine are transported simultaneously by cells (cf. Fig. 1 and 3), a partial inhibition of both processes results. Complete inhibition does not occur since each transport system has access to a finite amount of ECF. The fact that inhibition of folate transport by thiamine is greater than inhibition of thiamine transport by folate can be explained by the relative levels of the thiamine- and folate-binding proteins (0.70 and 0.45 nmol/10^{10} cells, respectively). Similarly, when the amount of thiamine-binding protein remains constant and the level of folate binder is repressed by changes in growth conditions (Table 3), the ability of thiamine to inhibit folate transport is enhanced, since the relative proportion of ECF available for interaction with the thiamine binder is increased by the diminishing amount of the folate binder. Thiamine is competitive, not with folate, but with the folate-binding protein–folate complex (Fig. 2), since the thiamine-binding protein–thiamine complex formed under these conditions competes with the corresponding folate-protein complex for a common site on ECF. It is of interest that folate transport is not inhibited merely by the presence of the thiamine transport system (Fig. 1), and this suggests that ECF interacts only with *functioning* transport systems. Folate and thiamine strongly inhibit biotin transport (Table 4), since the latter proceeds via a binding protein that is present in relatively small amounts (0.06 nmol/10^{10} cells). Conversely, the amount of biotin-binding activity is insufficient to significantly deplete the pool of ECF, and thus biotin has little effect on folate or thiamine transport. These conclusions are strongly supported by the linear relationship obtained by plotting the amount of binding activity for folate, thiamine, and biotin versus the sensitivity of each transport system to inhibition by co-transport of the other vitamins (Fig. 4).

It is possible, although less likely, that ECF is an energy source utilized by the folate, thiamine, and biotin transport systems. ATP levels and lactate production, however, are not affected in *L. casei* by the co-transport of folate and thiamine (Table 2). Likewise, since ATP (the probable energy source for folate transport [14]) is produced rapidly ($t_{1/2} < 30$ s) from glucose metabolism (unpublished data), it should be readily replenished during the operation of a presumably minor, energy-consuming process such as vitamin transport. This conclusion is consistent with the fact that the transport rates for these vitamins are less than 0.1% of the glycolytic rate (800 nmol of lactate produced/min per 10^{10} cells). Finally, if an energy source were limiting in transport, then the ability of folate to inhibit thiamine transport (and vice versa) would be expected to coincide with initial rates of transport, and not, as was observed, with the level of the binding proteins (Fig. 2).

ECF may be similar to hisP, a membrane protein component of the histidine transport system of *Salmonella typhimurium* (1), which is also required for the transport of other metabolites (2, 17). Genetic evidence has been obtained that hisP forms a complex with hisJ, a periplasmic, histidine-binding protein, and that this interaction is an obligatory step in histidine transport (2). Presumably, hisP also interacts with components of the other transport systems noted above, although this has not yet been verified experimentally.

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